OPEN ACCESS

African Journal of Microbiology Research

December 2011
ISSN 1996-0808
DOI: 10.5897/AJMR
www.academicjournals.org

ACADEMIC JOURNALS
expand your knowledge

About AJMR

The African Journal of Microbiology Research (AJMR) is a peer reviewed open access journal. The journal commenced publication in May 2007. The journal covers all areas of microbiology such as environmental microbiology, clinical microbiology, immunology, virology, bacteriology, phycology, molecular and cellular biology, molecular microbiology, food microbiology, mycology and parasitology, microbial ecology, probiotics and prebiotics and industrial microbiology.

Indexing

CAB Abstracts, CABI's Global Health Database, Chemical Abstracts (CAS Source Index) Dimensions Database, Google Scholar, Matrix of Information for The Analysis of Journals (MIAR), Microsoft Academic, Research Gate

Open Access Policy

Open Access is a publication model that enables the dissemination of research articles to the global community without restriction through the internet. All articles published under open access can be accessed by anyone with internet connection.

The African Journal of Microbiology Research is an Open Access journal. Abstracts and full texts of all articles published in this journal are freely accessible to everyone immediately after publication without any form of restriction.

Article License

Article Copyright

When an article is published by in the African Journal of Microbiology Research, the author(s) of the article retain the copyright of article. Author(s) may republish the article as part of a book or other materials. When reusing a published article, author(s) should; Cite the original source of the publication when reusing the article. i.e. cite that the article was originally published in the African Journal of Microbiology Research. Include the article DOI, Accept that the article remains published by the African Journal of Microbiology Research (except in occasion of a retraction of the article).

A copyright statement is stated in the abstract page of each article. The following statement is an example of a copyright statement on an abstract page.

Self-Archiving Policy

The African Journal of Microbiology Research is a RoMEO green journal. This permits authors to archive any version of their article they find most suitable, including the published version on their institutional repository and any other suitable website.

Digital Archiving Policy

The African Journal of Microbiology Research is committed to the long-term preservation of its content. All articles published by the journal are preserved by Portico. In addition, the journal encourages authors to archive the published version of their articles on their institutional repositories and as well as other appropriate websites.
https://www.portico.org/publishers/ajournals/

Metadata Harvesting

The African Journal of Microbiology Research encourages metadata harvesting of all its content. The journal fully supports and implement the OAI version 2.0, which comes in a standard XML format. See Harvesting Parameter

Memberships and Standards

Academic Journals strongly supports the Open Access initiative. Abstracts and full texts of all articles published by Academic Journals are freely accessible to everyone immediately after publication.

All articles published by Academic Journals are licensed under the Creative Commons Attribution 4.0 International License (CC BY 4.0). This permits anyone to copy, redistribute, remix, transmit and adapt the work provided the original work and source is appropriately cited.

Crossref is an association of scholarly publishers that developed Digital Object Identification (DOI) system for the unique identification published materials. Academic Journals is a member of Crossref and uses the DOI system. All articles published by Academic Journals are issued DOI.

Similarity Check powered by iThenticate is an initiative started by CrossRef to help its members actively engage in efforts to prevent scholarly and professional plagiarism. Academic Journals is a member of Similarity Check.

CrossRef Cited-by Linking (formerly Forward Linking) is a service that allows you to discover how your publications are being cited and to incorporate that information into your online publication platform. Academic Journals is a member of CrossRef Cited-by.

Academic Journals is a member of the International Digital Publishing Forum (IDPF).
The IDPF is the global trade and standards organization dedicated to the development and promotion of electronic publishing and content consumption.

Contact

Editorial Office: ajmr@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: http://www.academicjournals.org/journal/AJMR

Submit manuscript online http://ms.academicjournals.org

Academic Journals
73023 Victoria Island, Lagos, Nigeria
ICEA Building, 17th Floor,
Kenyatta Avenue, Nairobi, Kenya.

Editors

Editors

Table of Content

African Journal of Microbiology Research Vol. 5(32), pp. 5796-5805, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.610

Review

Utility and importance of walnut, *Juglans regia* Linn: A review

Nael Abu Taha and Mohammed A. Al-wadaan

Chair of Advanced Proteomics and Cytomics Research, Faculty of Science, King Saud University, Riyadh 11415, Saudi Arabia.

Accepted 9 September, 2011

Juglans regia **Linn is a medicinal plant that has been widely used in traditional medicine for a wide array of ailments that include helminthiasis, diarrhea, sinusitis, stomachache, arthritis, asthma, eczema, scrofula, skin disorders, and various endocrine diseases such as diabetes mellitus, anorexia, thyroid dysfunctions, cancer and infectious diseases. The present review, attempts to provide comprehensive information on the ethnobotanical use, pharmacology, nutritional value, preclinical and clinical studies, toxicity, other uses and current research prospects of the *Juglans regia* L. Currently, there is a renewed interest in walnut, and several investigations aimed at scientific validation of its traditional uses and a humble scientific investigation aimed at isolation and identification of active constituents of crude extracts.**

Key words: *Juglans regia*, nutritional value, bioactivity, clinical trial, traditional use, toxicity.

INTRODUCTION

Origin and distribution

Walnut (*Juglans regia* L.) is the most widespread tree nut in the world. The tree is commonly called as the Persian walnut, white walnut, English walnut or common walnut. It belongs to juglandaceae and has the scientific name *Juglans regia*. The walnut tree species is native to the old world. It is native in a region stretching from the Balkans eastward to the western Himalayan chain (Fernandez-Lopez et al., 2000) and was cultivated in Europe as early as 1000 BC. At present, walnut is cultivated commercially throughout southern Europe, northern Africa, eastern Asia, the USA and western South America. World production of whole walnut was around 1.5 × 106 t in 2008 (FAO, 2008). China is the leading world producer, followed by the USA, Iran, Turkey, Ukraine, Romania, France and India, but production in other countries such as Chile and Argentina has increased rapidly in recent years (Martinez et al., 2010).

Walnut composition and nutritional value

Walnut has been used globally in human nutrition since ancient times. The high protein and oil contents of the kernels of *Juglans regia* L. (Juglandacea) make this fruit indispensable for human nutrition. Therefore, the walnut is classified as a strategic species for human nutrition and is included in the FAO list of priority plants (Gandev, 2007). The seed part of the fruit (kernel) is consumed fresh, toasted, or mixed with other confectionaries. In the Middle East walnuts are added alone or along with almonds, date, and raisin as a special pastry preparation called Ma'moul. Walnuts are nutrient-rich food due to high contents of fats, proteins, vitamins and minerals. They are also good source of flavonoids, sterols, pectic

*Corresponding author. E-mail: nabutaha@ksu.edu.sa.

Abbreviations: FAs, Fatty acids; **PUFAs,** polyunsaturated fatty acids; **AA,** amino acid; **MICs,** minimum inhibitory concentrations; **IC_{50},** half maximal inhibitory concentration; **TMV,** tobacco mosaic virus; **DPPH,** 2,2-diphenyl-1-picrylhydrazyl; **LDL,** low-density lipoprotein; **CCl_4,** carbon tetrachloride; **LDH,** lactate dehydrogenase; **GSH,** glutathione; **GR,** glutathione reductase; **GOT,** glutamyl oxaloacetic transaminase; **GPT,** glutamyl pyruvic transaminase; **TG,** triglycerides; **EDV,** endothelium-dependent vasodilation; **CP,** cyclophosphamide.

Table 1. Nutritional value of *Juglans regia* L.

Principle	Value per 100 g
Vitamins (USDA, 2010)	
Folates	98 mcg
Niacin	1.125 mg
Pantothenic acid	o.570 mg
Pyridoxine	0.537mg
Riboflavin	0.150 mg
Thiamin	0.541 mg
Vitamin A	20 IU
Vitamin C	1.3 mg
Vitamin E-y	20.83 mg
Vitamin K	207 mcg
Minerals	
Potassium	441 mg
Phosphorus	346 mg
Calcium	98 mg
Magnesium	158 mg
sodium	2 mg
Iron	2.9 mg
Copper	1.5 mg
Manganese	3.8 mg
zinc	3.09 mg
Aluminum	0.58 mg
Fatty acids (Muradoglu et al., 2010)	
Unsaturated fatty acids	
Palmitoleic acid C16:1	0.77
Oleic acid C18:1	25.26
Gadoleic acid C20:1	0.05
Total MUFA	22.37
Linoliec acid C18:2	57.10
Lineliec acid C18:3	10.34
Total PUFA	4.29
Saturated fatty acid	
Myristic acid C14:0	0.24
Palmitic acid C16:0	4.28
Stearci acid C18:0	1.85
Archidic acid C20:0	0.19
Total SFA	7.21
PUFA/SFA	9.91

substances, phenolic acids and related polyphenols. The nutritional contents differs from a cultivar to another which can be influenced by genotype, cultivator, different ecology and different soil (Caglarirmak, 2003; Crews et al., 2005; Martinez et al., 2010; Muradoglu et al., 2010). The major components of walnut oil are triacylglycerols (980 g/kg oil), in which monounsaturated fatty acids (FAs) (mainly oleic acid) and polyunsaturated FAs (PUFAs; linoleic and α-linolenic acids) are present in high amounts in all genotypes (Table 1). Oil contents reported by Pereira et al. (2008) (78.83 to 82.4%) were higher than those reported by other researchers (Savage, 2001; Muradoglu et al., 2010). In general, the FA composition of walnut oil resembles that of soybean oil, but walnut oil contains a greater concentration of linolenic acid. In fact, among vegetable oils, walnut oil has one of the highest amounts of PUFAs (up to 78% of the total FA content).

Walnuts have high amount of omega-6 and omega-3 PUFA, which are essential dietary fatty acids. Clinical studies suggest that omega-3 PUFA have significant role in prevention of coronary heart disease (Davis et al., 2007). Oil rich in oleic acid displays greater oxidative stability therefore; it could be widely used as frying oil. According to an investigation conducted by several researchers, It was found that the average value for protein was 18.1% (Amaral et al., 2003); Muradolu, 2005; Mitrovic et al., 1997; Muradoglu, 2010; Savage, 2001). They are mainly composed of glutelins (about 70% of the total seed proteins) together with lesser amounts of globulins (18%), albumins (7%) and prolamins (5%) (Martinez et al., 2010). The amino acid (AA) composition of walnut flour is dominated by the acidic AA residues of aspartate and glutamate together with relatively high levels of arginine. Walnut proteins contain all essential AAs required for the needs of a human adult. The lysine/arginine ratio in walnut proteins is lower than those observed in other common vegetable proteins, and this fact has been identified as a positive feature in the reduction of atherosclerosis development (Sza-Tao et al., 2000; Venkatachakm and Sathe; 2006; Martinez et al., 2010). Walnut cultivars analyzed have recorded rich mineral composition, especially potassium, magnesium, and calcium. The minimum and maximum macro and micro nutrient contents of walnut are presented in Table 1 (Ravai, 1992; Payne, 1985; Souci et al., 1994; Cosmulescu et al., 2009). Walnuts contain high levels of potassium, phosphorus and magnesium and lower sodium. These elements play an important role for many enzymes activity especially as cofactor.

Ethnobotanical use

Juglans regia leaves have been used mostly in worldwide traditional medicines as antimicrobial, antihelmintic, astringent, keratolytic, antidiarrhoeal, hypoglycaemic, depurative, tonic, carminative, and for the treatment of sinusitis, cold and stomach ache (Girzu et al., 1998; Mouhajir et al., 2001; Vaidyaratnam, 2005). In Turkish folk medicine, fresh leaves applied on the naked body or forehead to reduce fever or on swelled joint to alleviate the rheumatic pain (Fujita et al., 1995; Yesilada, 2002). The kernel of *J. regia* has been used for the treatment of inflammatory bowel disease in Iranian traditional medicine (Kim et al., 2006). In Palestine, it is used for treatment of diabetes and asthma (Jaradat, 2005; Kaileh et al., 2007) and to treat prostate and vascular disturbance (Spaccarotella et al., 2008). The plant is

used as a topical remedy for dermal inflammation and excessive perspiration of the hands and feet. It is also a common home remedy for the treatment of chronic eczema and scrofula. The leaves of this plant is used topically to treat scalp itching and dandruff, sunburn and superficial burns as well as an adjunctive emollient in skin disorders (Gruenwald et al., 2001; Robbers et al., 1999; Ali-Shtayeh and Abu Ghdeib, 1999; Blumenthal, 2000; Baytop, 1999). It also has high anti-atherogenic potential and a remarkable osteoblastic activity that adds to the beneficial effect of a walnut enriched diet on cardioprotection and bone loss (Papoutsi et al., 2008). The bark, branches and exocarp of the immature green fruit of this medicinal plant have been used to treat gastric, liver and lung cancer a long time in China (Liu et al., 2004; Baytop, 1999). It is used by traditional healer in northeastern region of Mexico to protect against liver damage (Torres-gonzalea et al., 2011). The bark is used as miswaks for teeth cleaning (Ibrar et al., 2007). In Nepal the bark paste is useful in arthritis, skin diseases, toothache, and hair growth. Seed coat is used for healing wounds (Kunwar and Adhikari, 2005). The shell of *Juglans regia* is used in Calabria folk medicine to heal malaria **(**Tagarelli et al., 2010).

Antibacterial activity

Hot and cold solvent and aqueous extract of leaves, barks, fruits and green husks of *J. regia* from different countries revealed broad spectrum antibacterial activity against gram-positive and gram-negative bacteria viz. *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli*, *Klebsiella pneumoniae, Staphylococcus epidermidis, Micrococcus luteus, Salmonella typhimurium, Enterococcus faecalis, Bacillus thuringiensis, Protomonas extroquens,* and Proteus sp. using agar streak method, disc diffusion method and microplate alalmar blue assay (Deshpande et al., 2011; Poyrazolu et al., 2010; Pereira et al., 2008; Oliveira et al., 2008; Pereira et al., 2007; Qa'dan et al., 2005a b; Citoglu and Altanlar, 2003; Upadhyay et al., 2010b). The antimicrobial activity against gram-negative bacteria were selective since not all the fruit extract of *J. regia* cultivator inhibited the growth of *Pseudomonas aeruginosa* and *E. coli*. cv. Lara inhibited the growth of *K. pneumoniae* (MIC of 100 mg/mL), cv. Mayette inhibited the development of *P. aeruginosa* and *E. coli* with minimum inhibitory concentrations (MICs) of 50 and 10 mg/mL, respectively, and cv. Mellanaise inhibited the growth of *E. coli* and *K. pneumoniae* at concentration of 100 mg/mL (Ali-Shtayah et al., 1999). Mexican aqueous bark and leaves extract exhibited no antimycobacterial activity. Only the hexane and methanol extract showed antimycobacterial activity with MIC of 100 and 125 mg/ml, respectively using Soxhlet extractor (Cruz-Vega et al., 2008). Over 45% of Iranian clinical isolates of *Helicobacter pylori* strain were inhibited by *J. regia* aqueous and equal mixture of methanol, diethyl ether and petroleum benzene extract (Nariman et al., 2004). In a recent study, juglone was shown to potently inhibit the three key enzymes from *Helicobacter pylori,* cystathionine γ-synthase (HpCGS), malonyl-CoAacyl carrier protein transacylase (HpFabD), and β-hydroxyacyl-ACP dehydratase (HpFabZ) with the half maximal inhibitory concentration (IC_{50}) values of 7.0±0.7, 20±1, and 30±4 µmol/L, respectively. Therefore, HpCGS, HpFabD, and HpFabZ are considered to be the potential targets of juglone (Kong et al., 2008). The antibacterial activity of Jordanian *J. regia* leaves extract to acne developing organism revealed that 12.5% *S. epidermidis* isolates were resistant to the leaf extract where as all *Propionibacterium acnes* isolates were sensitive even to 10% of the extract (Qa'dan et al., 2005b).

Antifungal activity

J. regia fruits, leaves and bark aqueous and solvents extract exhibited antifungal activity against wide range of fungi using disc diffusion method, agar dilution method, agar streak dilution and Raddish method. Pereira et al. (2008) reported that all the walnut varieties exhibited antifungal activity against *Candida albicans* and *Cryptococcus neoformans* when soxhleted with light petroleum ether (b.p. 40-60°C). The higher inhibition was observed with cv. Lara extract (MIC of 1 mg/mL). However, *C. albicans* and *C. neoformans* were only resistant to cv Mallanaise extract. Cold extraction of fruit, leaves and bark inhibited the growth of *Microsporum canis, Trichophyton mentagrophytes*, *and Trichophyton violaceum* (Ali-Shtayah et al., 1999). On the other hand, the aqueous extract of green husks showed no antifungal activity against *C. albicans* and *C. neoformans* (Oliveira et al., 2008). Methanol, acetone, chloroform and ethyl acetate bark extract revealed antifungal activity against *A. niger*, *Alternaria alternata*, *Trihoderma viresn*, *fusarium solani*, *Pichia guiliermondii, Pichia jadinii* and all Candida speices tested (Upadhyay et al., 2010c; Ahmad et al., 1973).

Antiviral activity

Mei-zhi et al. (2007) reported that 95% ethanol and ethyl acetate leaves extract of *J. regia*, inhibited tobacco mosaic virus (TMV). The methanol extract of *J. regia* inhibited *Sindbis* virus at a minimum concentration of 1.5 µg/ml (Mouhajir et al., 2001).

Antioxidant activity

The antioxidant potential of ethyl acetate, butanol, meta-

nol, ether and aqueous methanol extract of walnut kernels, husks and leaves were measured by different methods such as reducing power, scavenging activity on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and lipid oxidation inhibition by β-carotene linoleate system. All the extracts showed strong antioxidant activity (Qamar and Sultana, 2011; Carvalho et al., 2010; Abbasi et al., 2010; Rahimipanah et al., 2010; Zhang et al., 2009b; Almeida et al., 2008; Oliveira et al., 2008; Pereira et al., 2008; Pereira et al., 2007; Fukuda et al., 2003). Bullo et al. (2010) reported a decrease in the antioxidant burden observed in enzymatic and non-enzymatic antioxidant systems after the consumption of a whole-walnut or a walnut-skin diet in C57BL/6 mice. The same author also reported that consumption of walnuts and walnut skins have no deleterious effect on low-density lipoprotein (LDL) oxidizing capability, despite their higher contents of omega-6 PUFAs. Several phenolic compounds isolated from *J. regia* such as pyrogallol, p-hydroxybenzoic acid, vanillic acid, ethyl gallate, protocatechuic acid, gallic acid, 3,4,8,9,10-pentahydroxydibenzo pyran-6-one, tannins, glansrins, adenosine, adenine, etc, could provide a chemical basis for some of the health benefits claimed for *J. regia* in foods and folk medicine (Zhang et al., 2009a; Fukuda et al., 2003).

Antidiabetic activity

Fukuda et al. (2004) demonstrated a strong inhibitory activity of walnut polyphenols and the polyphenolic components like Casuarictin, tellimagradin II and Tellimagradin I on different enzymes like glycosidase, sucrose, maltase and amylase. In addition to the above findings, researchers also noticed that walnut polyphenol-rich fraction has triglyceride lowering effect and urine peroxide lowering effect in genetically inherited Type II diabetes mellitus (*db*/*db*) mice at the dose of 200mg/kg/day. The consumption of walnut leaf pellets in alloxan induced diabetic rats at the dose of 185 mg/kg reduced fasting blood sugar significantly and the histomorphometric study of pancreas showed a sign of regeneration of β-cells in the treated group (Jelodar et al., 2007). *J. regia* leaves methanolic extract at dose of 250 mg/kg decreases the postprandinal plasma blood glucose levels in both short and long term models. The plant extract significantly inhibited α-glucosidase activity *in vitro* for both maltase and sucrase enzymes and showed no changes in the insulin and glut-4 genes expression. The author attributed the inhibitory action of the plant extract to gallic acid and caffeoylquinic acid in the leaves (Teimori et al., 2010).

Anthelmintic activity

Kale et al. (2011) reported that stem park of *J. regia* acetone extract exhibited significant activity at all dilution tested when compared to the Albendazole standard against *Eicinia feotida*. The benzene, methanol and ethanol soxhlet extracts of *J. regia* stem bark on adult Indian earthworm, *Pheretima posthuma* exhibited significant anthelmintic activity as comparable to that of standard drug Piperazine citrate (Upadhyay et al., 2010a). The 95% ethanol, petroleum ether and ethyl acetate extract of green walnut hull have obvious anti-feeding effect on armyworm and the small vegetable-moth. The research group indicated that anti-feeding rate, death rates as well as growth inhibition rate of armyworm have correspondingly changed in dose dependant manner (Me-zhi et al., 2006).

Anti-inflammatory activity

The ethanolic extracts of *J. regia* leaves exhibited potent anti-inflammatory activity as potent as indomethacin against carrageenan-induced hind paw edema model in mice without inducing any gastric damage (Erdemoglu et al., 2003). Mokhtari et al. (2008) stated that the alcohol extract of walnut leaves in dose of 1.5 mg/kg caused a significant nociception decrease in acute phase of formalin test where as the aqueous (2.87 and 1.64 g/kg) and ethanolic (2.044 and 1.17 g/kg) extracts of leaves showed antinociceptive activity in hotplate test suggesting a promising analgesic and anti-inflammatory agents against diseases such as rheumatoid arthritis. On the basis of Qamar and Sultana (2011) result, a protective role of methanolic *J. regia* extract against CSE-induced acute lung toxicity in Wistar rats was suggested. The extract significantly decreased the levels of Lactate dehydrogenase (LDH), total cell count, total protein and increased the glutathione (GSH) level in bronchoalveolar lavage fluid. It also significantly restored the levels of Glutathione reductase (GR), catalase and reduced the xanthine oxidase (XO) activity in lung tissue.

Antidepressant activity

The macerated hexane extract of *J. regia* fruit produced significant antidepressant activity at both doses of 100 and 150 mg/kg body weight when compared with standard drug fluoxetine on male Wistar rats. The antidepressant activity was evaluated by forced swimming and tail suspension test (Rath and Pradhan, 2009).

Antityrosinase activity

Ozer et al. (2007) suggested that gel formulation containing ellagic acid and plant leaves extract of *J. regia* is effective in treating uneven skin pigmentation. The ethanolic leaves extract could be suggested as new

sources of skin-whitening agents. Aitani and Shimoda (2005) reported that melanin formation was inhibited at concentration 1 to 30 µg/ml in Pre-cultured B16 melanoma cells incubated with medium containing walnut polyphenols and their result indicated that walnut polyphenols is more superior to the popular skin-lightening agent, ascorbic acid and arbutin upon data comparison.

Hepatoprotective activity

Orally fed Walnut polyphenols prepared from the kernelpellicle demonstrated a dose dependent lowering effect in glutamyl oxaloacetic transaminase (GOT) and glutamyl pyruvic transaminase (GPT) in carbon tetrachloride (CCl_4) induced liver damage in mice model after a single oral administration (200 g/kg). Result indicated that walnut polyphenols is more superior to Curcumin, a commonly used hepatoprotective agent. The effect of each active component of *in vitro* evaluation of walnut polyphenols on CCl_4-induced cytotoxicity in primary cultured rat hepatocytes showed that tellimagrandin I, casuarictin, tellimagrandin II, and rugosin C (Figure 1) are inhibitory on CCl_4-induced cytotoxicity in primary cultured rat hepatocytes however, tellimagrandin I of walnut polyphenols is believed to be the most important active compound responsible for hepatoprotective effect (Hiroshi et al., 2008). The same author, Hiroshi et al. (2006) reported that 50% EtOH extract from endocarps of walnuts on mice liver injury models induced by carbon tetrachloride at the dose of 100 and 200 mg/kg significantly suppressed GOT and GPT deviations. Polyphenolic constituents, tellimagrandins I and II, rugosin C and casuarictin were found to be principal constituents with hepatoprotective activity against oxidative damage.

Hypotriglyceridemic activity

Oral administration of a polyphenol-rich extract (WP) from walnuts (100 and 200 mg/kg) in high fat diet fed mice significantly reduced liver weight and serum triglycerides (TG) where as hepatic β-oxidation in cytosol, including peroxisome, was enhanced by WP (50-200 mg/kg). A polyphenol-rich extract was found to possess hypotriglyceridemic activity via enhancement of peroxisomal fatty acid β-oxidation in the liver. These results suggest that tellimagrandin I is involved in the hypotriglyceridemic mechanism (Shimoda et al., 2009).

Anticancer activity

Juglone has been reported to inhibit intestinal carcinogenesis induced by azoxymethane in rats and might be a promising chemopreventive agent in human intestinal neoplasia (Sugie et al., 1998). Juglone was also proven to be a potent cytotoxic agent *in vitro* in human tumor cell lines, including human colon carcinoma (HCT-15) cells, human leukemia (HL-60) cells and doxorubicin-resistant human leukemia (HL-60R) cells (Kamei et al., 1998; Segura-Aguilaretal, 1992). In a recent study, Juglone inhibited the growth and induce apoptosis of sarcoma and 180 SGC-7901 cells *in vivo*. The mechanism is mediated by the activation of the mitochondrial death pathway, which requires the generation of reactive oxygen species (ROS), down-regulation of Bcl-2 protein expression and up-regulation of Bax protein expression (Ji et al., 2011). Walnut methanolic extracts obtained from *J. regia* seed, green husk and leaf showed concentration dependent growth inhibition against human renal cancer cell lines A-498, 769-P and the colon cancer cell line Caco-2. Concerning A-498 renal cancer cells, all extracts exhibited similar growth inhibition activity (IC_{50} values between 0.226 and 0.291 mg/mL), while 769-P renal and Caco-2 colon cancer cells, walnut leaf extract showed a higher antiproliferative efficiency (IC_{50} values of 0.352 and 0.229 mg/mL, respectively) than green husk or seed extracts (Carvalho et al., 2010). The tested dried fine powder of *J. regia* light petroleum seed extract in cancer induced in Swiss albino mice with the help of 7,12-Dimethylbenz(a)anthracene (DMBA) and croton oil showed the petroleum extract was significant in reducing the cancer cells (Kumudhavalli et al., 2010).

Other medicinal uses

Willis et al. (2009) examined the effects of walnut diet on motor and cognitive ability in aged rats for 8 weeks. The three treated groups (2, 6 and 9%) revealed that the 2% walnut diet improved performance on rod walking, while the 6% walnut diet improved performance on the medium plank walk; the higher dose of the 9% walnut diet impaired reference memory, however the researcher attributed this to the number of polyphenolic compounds that could be negatively effecting reference memory at a higher dose. A 2004 study by the NYS Institute for Basic Research in Developmental Disabilities (OMRDD) revealed that methanolic extract of walnut was able to inhibit and defibrillize fibrillar amyloid β- protein (the principal component of amyloid plaques in the brains of patients with Alzheimer's). It is proposed that polyphenolic compounds present in walnuts may be responsible for its anti-amyloidogenic activity (Chauhan et al., 2004). Similarly, it was found that two of its major components in walnuts, gallic and ellagic acid, act as "dual-inhibitors" of the enzyme acetylcholinesterase which, in association with amyloid inhibits protein aggregation, and inhibit the site of acetylcholinesterase responsible for the breakdown of acetylcholine. These results suggest that walnuts may reduce the risk or delay

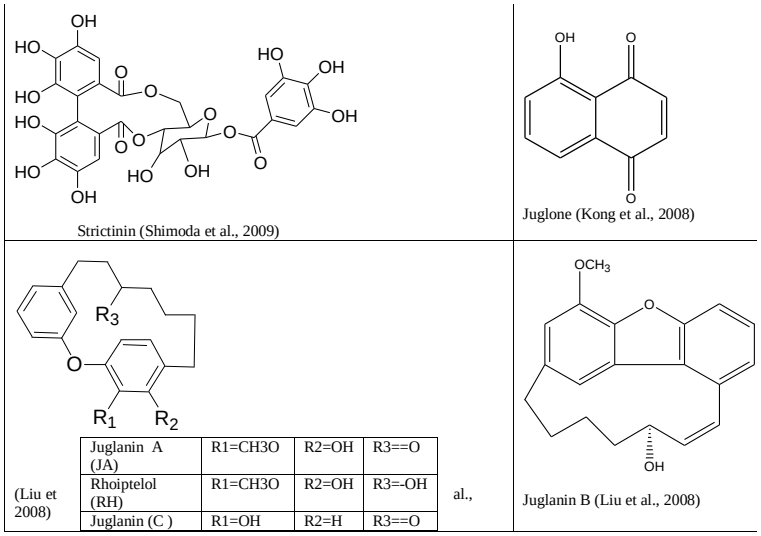


Figure 1. Chemical structures of compounds isolated from *Juglans regia* L.

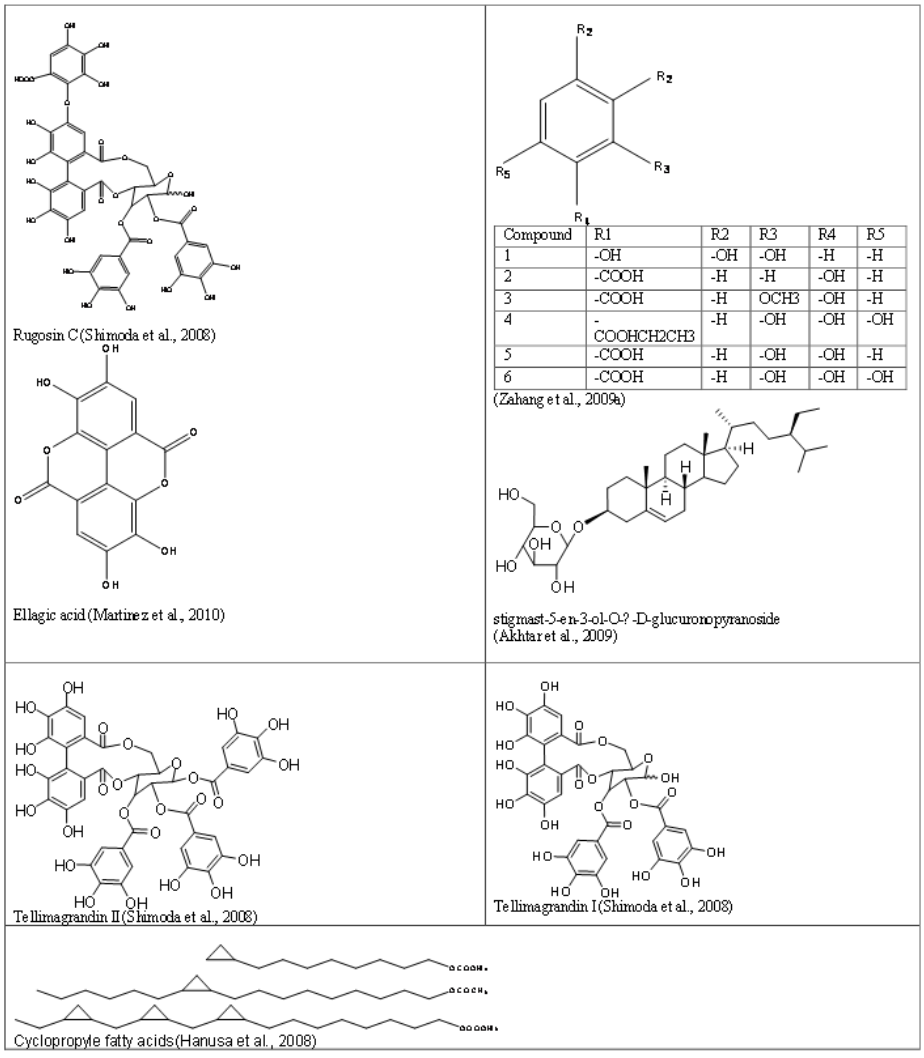


Figure 1 contd.

the onset of Alzheimer's disease by maintaining amyloid-protein in the soluble form and prevent the breakdown of acetylcholine (Society for Neuroscience, 2007).

Clinical study

A daily intake of 43 to 57g of walnuts incorporated into Japanese diet for 4 weeks to 40 healthy Japanese men and women lowered blood cholesterol, particularly in women (Iwamoto et al., 2000). In double-blind case with either plasma triglyceride (TG) concentration more that 350 mg/dl or total cholesterol concentration more that 250 mg/dl were randomized into two groups, group A subject were administered 6 capsules, each filled with 500 mg of the extracted walnut oil, per day for 45 days, group B individual serve as control and received placebo for 45 days. The result of this lowered plasma triglyceride level by 19 to 33% (Zibaeenezhad et al., 2003). Ros et al. (2004) reported that substituting walnuts for monounsaturated fat in a Mediterranean diet improves endothelium-dependent vasodilation (EDV) in hypercholesterolemic subjects. A daily intake of 8-13 walnuts for 4 weeks significantly improves the EDV of 21 hypercholesterolemic males and females.

On the other hand, walnut-enriched meals effectively prevented post prandial lipidemia where triacylglycerol was significantly reduced (Bellido et al., 2004). Spaccarotella et al. (2008) assessed the effect of walnuts on markers of prostate cancer between 45 and 75 years of age. Results suggest that walnuts improved serum γ-T and α-T: γ-T, two biomarkers that are important in prostate and vascular health. Total bilirubin, total protein, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), leucine aminopeptidase (LAP), gamma-glutamyltranspeptidase (γ-GTP), cholinesterase, amylase, lipase, Lecithin: cholesterol acyltransferase (L-CAT), LDL-cholesterol, total cholesterol, triglyceride, phospholipid, free fatty acid (FFA), high-density lipoprotein (HDL)-cholesterol, Na, K, serum Fe, total iron binding capacity (TIBC), unsaturated iron binding capacity (UIBC), urea nitrogen, uric acid, glucose, hemocytes revealed no abnormal reading for four male volunteers were given oral walnut polyphenols at the dose of 50 mg/day for 4 weeks (Oryza, 2007).

Toxicity

A review of the literature showed that juglone can cause irritant reactions as well as skin hyper pigmentation but, although it has been found to be a strong sensitizer in guinea pigs, contact allergy is considered a very rare event in man (Wood and Calnan, 1976; Hausen, 1981). However, a case report of 65- year-old woman complaints of skin hyper pigmentation and large tense blisters involving the palms and fingers caused by the cumulative effect of 15 kilos of walnuts shelled in the 3 days was reported by Bonamonte et al. (2001). Haque et al. (2003) investigated the modulatory effects of walnut aqueous extract on the toxicity of an anticancer drug, cyclophosphamide (CP) with special reference to protection against disruption of drug metabolizing and antioxidant enzymes during the chemotherapy. The extract showed a significant increase in the activity and level of glutathione and glutathione peroxidase in both liver and kidney tissues and catalase in liver only. While the extract CP treated group showed a significant decrease in the lipid peroxidation in liver and kidneys when compared with the CP-treated group. Aqueous extract from *J. regia* leaves reduced 3-(4,5-Dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) formation by about 60% at concentration of 500 µl/ml on HepG2 cell. Additionally, the co-culture of HepG2 with THP1 revealed no sign of any negative effect at all concentration tested after exposure to the extract. The investigator also reported no significant changes of LDH and albumin levels on the culture medium after 24 h of exposure to the extract (Saad et al., 2006). Hosseinzadeh et al. (2011) calculated the half-maximal lethal dose (LD_{50}) values of intraperitoneal injection of *J. regia* aqueous and ethanolic leaves extract and found it to be 5.5 and 3.3 g/kg, respectively. Acute dermal toxicity studies showed that petroleum ether extract of *J. regia* gives lethal effect at 2000 mg/kg (Kumudhavalli et al., 2010).

Other uses

The seeds contain unusual fatty acids which are industrially important, as they are used in protective coatings, dispersants, pharmaceuticals, cosmetics, soaps and a variety of synthetic intermediates as stabilizers in plastic formulations (Hosamani et al., 2000; Eganathan et al., 2006). The wood is of very high quality, and is used to make furniture, and gunstocks. The dye is used as a coloring and tonic for dark hair (Brwon, 1995). The unripe fruits are pickled in vinegar (Facciola, 1990).

Conclusions

The present review article documents the publications on walnut and its constituents in the recent and last few years. The paper highlights the traditional use of this plant and some scientific validation of the claimed biological activity *in vivo* as well as *in vitro*. To best of our knowledge and internet survey only one case of contact dermatitis was reported after shelling 15 kilos of walnuts. The toxicological studies of various secondary metabolites which contribute to its medicinal value are still in its infancy and are becoming an important limiting factor for utilizing the metabolites as therapeutic agent.

Besides, isolation and characterization of active

secondary metabolites responsible for various biological activities have not yet been structurally elucidated, mode of action, target organ of toxicity and molecular mechanism also need to be investigated. Further trials in humans are required to determine the efficacy of walnut extract or one or more of its constituents and to establish what, if any, adverse effects are observed.

ACKNOWLEDGEMENT

The authors extend their appreciation to the Deanship of Scientific Research at king Saud Univer-sity for funding the work through the research group pro-ject No. RGP-VPP-028

REFERENCES

Abbasi MA, Raza A, Riaz T, Shahzadi T, Aziz-ur-Rehman, Jahangir M, Shahwar D, Siddiqui SZ, Chaudhary AR, Ahmad N (2010). Investigation on the volatile constituents of *Juglans regia* and their *in vitro* antioxidant potential. Pakistan Acad. Sci., 47:137-141.

Ahmad S, Mukhtar, Wahid A, Bukhari AQS (1973). Fungistatic Action of Juglans. Antimicrob. Agents Chemother., 3:436-438.

Aitani M, Shimoda H (2005).The Effect of Ascorbic Acid and Arbutin on B16 Melanoma cells. Japan Food Sci.,44: 58-63.

Akhtar N, Ali M, Alam MS (2009). New phytoconstituents from the stem bark of *Juglans regia* L. J. Saudi. Chem. Soc., 13: 111-118.

Ali-Shtayeh MS, Abu Ghdeib SI (1999). Antifungal activity of plant extracts against dermatophytes. Mycoses., 42: 665-772.

Almeida IF, Fernandes E, Lima JLFC, Costa PC, Bahia MF (2008). Walnut (*Juglans regia*) leaf extracts are strong scavengers of pro-oxidant reactive species. Food Chem., 106:1014-1020.

Amaral JS, Casal S, Pereira J, Seabra R, Oliveira B (2003). Determination of sterol and fatty acid compositions, oxidative stability, and nutritional value of six walnut (*Juglans regia* L.) cultivars grown in Portugal. J. Agric. Food Chem., 51: 7698-7702.

Baytop T (1999). Therapy with Medicinal Plants in Turkey (Past and Present), 2nd Ed., Nobel Medicine Publisher, Turkey.

Bellido C, Lopez-Miranda J, Blanco-Colio LM, Perez-Martinez P, Muriana FJ, Martin-Ventura JL, Marin C, Gomez P, Fuentes F, Egido, Perez-Jimenez F (2004). Butter and walnuts, but not olive oil, elicit postprandial activation of nuclear transcription factor κB in peripheral blood mononuclear cells from healthy men. Am. J. Clin. Nutr*.,* 80: 1487-1491.

Blumenthal M (2000). Herbal Medicine (Expanded Commision E Monographs), Integrative Medicine Communications, Newton Publisher, England.

Bonamonte D, Foti C, Angelini G (2001). Hyperpigmentation and contact dermatitis due to *Juglans regia.* Contact Dermatitis., 44: 101.

Brwon D (1995). Encyclopedia of herbs and their uses. Dorling Kindersley publishers, London.

Bullo M, Nogues MR, Lopez-Uriarte P, Salas-Salvado J, Romeu M (2010). Effect of whole walnuts and walnut-skin extracts on oxidant status in mice. J. Nutr., 26: 823-828.

Caglarirmak N (2003). Biochemical and physical properties of some walnut genotypes *(Juglans regia L*). Nahrung Food 47:28–32.

Carvalho M, Ferreira PJ, Mendes VS, Silva R, Pereira JA, Jenimo C. Silva BM (2010). Human cancer cell antiproliferative and antioxidant activities of *Juglans regia* L. Food Chem. Toxicol., 48: 441-447.

Chauhan N, Wang KC, Wegiel J, Malik MN (2004). Walnut extract inhibits the fibrillization of amyloid beta-protein, and also defibrillizes its preformed fibrils. Cur. Alzheimer Res., 1:183-188.

Citoglu GS, Altanlar N (2003). Antimicrobial activity of some plants used in folk medicine. J. Fac. Pharm. Ankara., 32:159-163.

Company, New York.

Cosmulescu S, Baciu A, Achim G, Botu M, Trandafir I (2009). Mineral composition of fruits in different walnut (*Juglans regia* L.) Cultivars. Not. Bot. Hort. Agrobot. Cluj., 37:156-160.

Crews C, Hough P, Godward J, Brereton P, Lees M, Guiet S (2005). Study of the main constituents of some authentic walnut oils. J. Agric. Food. Chem., 53:4853-4860.

Cruz-Vega DE, Verde-Star MJ, Salinas-Gonzalez N, Rosales-Hernandez B, Estrada-Garcia I, Mendez-Aragon P, Carranza-Rosales P, Gonzalez-Garza MT, Castro-Garza J (2008). Antimycobacterial activity of *Juglans regia*, *Juglans mollis*, *Carya illinoensis* and *Bocconia frutescens.* Phytother. Res., 22:557-559.

Davis L, Stonehouse W, Loots DT, Mukuddem-Petersen J, Van Der Westhuizen F, Hanekom SJ, Jerling JC (2007). The effects of high walnut and cashew nut diets on the antioxidant status of subjects with metabolic syndrome. Eur. J. Nutr., 46: 155-164.

Deshpande RR, Kale AR, Ruikar AD, Panvalkar PS. Kulkarni AA, Deshpande NR, Salvekar JP (2011). Antimicrobial Activity Of different extracts of *Juglans Regia* L. against Oral Microflora. Int. J. Pharm. Pharm. Sci., 3:200-201.

Eganathan P, Subramanian HMSR, Latha R, Srinivasa Rao, C (2006). Oil Analysis in Seeds of *Salicornia Brachiata*, Ind. Crops Prod., 23:177.

Erdemoglu N, Kupeli E, Yesilada E (2003). Anti-inflammatory and antinociceptive activity assessment of plants used as remedy in Turkish folk medicine. J. Ethnopharmacol., 89: 123-129.

Facciola S (1990). Cornucopoia . A source book of edible plants. kampong Publisher,USA.

FAO (2008). FAOSTAT Data. Food and Agriculture Organisation, Rome.

Fernandez-Lopez J, Aleta N, Alıas R (2000). Forest Genetic Resources Conservation of *Juglans regia* L. IPGRI Publishers, Rome.

Fujita T, Sezik E, Tabata M, Yesilada E, Honda G, Takeda Y, Tanaka T, Takaishi Y (1995). Traditional medicine in Turkey VII. Folk medicine in Middle and West Black Sea regions. Econ. Bot., 49: 406-422.

Fukuda T, Ito H, Yoshida T (2004). Effect of the walnut polyphenol fraction on oxidative stress in type 33 2 diabetes mice. Biofactors., 2: 251-253.

Fukuda T, Ito H, Yoshida Y (2003). Antioxidative polyphenols from walnuts (*Juglans regia* L.) Phytochem., 63: 795-801.

Gandev, S (2007). Budding and grafting of the walnut (*Juglans regia* L.) and their effectiveness in Bulgaria (Review). Bulgar. J. Agri. Sci., 13:683-689.

Girzu M, Carnat A, Privat AM, Fialip J, Carnat AP, Lamaison JL (1998). Sedative effect of walnut leaf extract and juglone, an isolated constituents. Pharm. Biol., 36: 280-286.

Gruenwald J, Brendler T, Jaenjke C (2001). PDR for Herbal Medicines, Medicinal Economic

Hanusa LJ, Goldshlag P, Dembitskya VM (2008). Identification of cyclopropyl fatty acids in walnut (*Juglans regia* L.) OIL Biomed Pap. Med. Fac. Univ. Palacky Olomouc. Czech. Repub., 152:41-45.

Haque R, Bin-Hafeez B, Parvez S, Pandey S, Sayeed I, Ali M, Raisuddin S (2003). Aqueous extract of walnut (*Juglans regia* L.) protects mice against cyclophosphamide-induced biochemical toxicity. Hum. Exp. Toxicol., 22:473-80.

Hausen B (1981). Woods injurious to human health. Berlin: Publisher, Walter de Gruyter, pp 119–121.

Hiroshi S, Junji T, Toshiyuki F, Hideyuki I, Tsuyoshi H, Takashi Y(2006). Hepatoprotective constituents in endocarps of walnut. J. Pharm. Soc. Japan 126:108-109.

Hiroshi S, Tanaka J, Kikuchi M, Fukuda T, Ito H, Hatano T, Yoshida T (2008). Walnut polyphenols prevent liver damage induced by carbon tetrachloride and d-galactosamine: hepatoprotective hydrolyzable tannins in the kernel pellicles of walnut. J. Agric. Food Chem., 56: 4444-4449.

Hosamani, KM, Sattigeri RM (2000). Industrial utilization of *Rivea Ornata* seed oil: A moderate source of vernolic acid. Ind. Crops Prod., 12: 93.

Hosseinzadeh H, Zarei H, Taghiabadi E (2011). Antinociceptive, anti-Inflammatory and acute toxicity effects of *juglans regia* L. Leaves in mice. Iran Red Crescent Med. J., 13:27-33.

Ibrar MFH, Sultan A (2007). Ethnobotanical studies on plant resources of Ranyal Hill, District Shangla, Pakistan. Pak.J.Bot., 39:329-337.

Iwamoto M, Sato M, Kono M, Hirooka Y, Saka K, Takeshita A, Imaizumi K (2000).Walnuts lower serum cholesterol in Japanese men and women. J. Nutr., 130: 171-176.
Jaradat NA (2005). Medical plants utilized in Palestinian folk medicine for treatment of diabetes mellitus and cardiac diseases. J. Al-Aqsa Unv., 19:1-28
Jelodar G, Mohsen M, Shahram S (2007). Effect of walnut leaf, coriander and pomegranate on blood glucose and histopathology of pancreas of alloxan induced diabetic rats. Afr. J. Trad. CAM., 43: 299-305.
Ji Y, hong-YuanQua Z, XiangZou (2011). Juglone induced apoptosis in human gastric cancer SGC-7901cells via the mitochondrial pathway. Exp. Toxicol. Pathol., 63: 69-78.
Kaileh Mb, Berghea WV, Boonec E, Essawib T, Haegemana G (2007). Screening of indigenous Palestinian medicinal plants for potential anti-inflammatory and cytotoxic activity J. Ethnopharmacol., 113: 510-516
Kale AA, Sucheta A, Gaikwada, Gayatri S, Kamblea NR, Deshpandea, Salvekara JP (2011). *In vitro* anthelmintic activity of stem bark of *Juglans regia* L. J. Chem. Pharm. Res., 3:298-302.
Kamei H, Koide T, Kojima T, Hashimoto Y, Hasegawa M (1998). Inhibition of cell growth in culture by quinones. Cancer Biother Radiopharm., 13:185–8.
Kim HG, Cho JH, Jeong EY, Lim JH, Lee SH, Lee HS (2006). Growth-inhibiting activity of active component isolated from *Terminalia chebula* fruits against intestinal bacteria. J. Food Prot., 69:2205-2209.
Kong Y, Zhang L, Yang Z, Han C, Li-hong (2008). Natural product Juglone targets three key enzymes from *Helicobacter pylori*: inhibition assay with crystal structure characterization. Acta Pharmacologica Sinica., 29: 870-876.
Kumudhavalli MV, Jayakar B, Kumar GA (2010). Phytochemical and pharmacological evaluation of the dried fruit of the plant *Juglans regia* linn. Oil Drug Invent. Today 2: 362-365.
Kunwar RM, Adhikari N (2005). Ethnomedicine of Dolpa district, Nepal: the plants, their vernacular names and uses. J. Ecol. App., 8:43-49
Liu J, Meng M, Li C, Huang X, Di D (2008). Simultaneous determination of three diarylheptanoids and an alpha-tetralone derivative in the green walnut husks (*Juglans regia* L.) by high-performance liquid chromatography with photodiode array detector. J. Chromatogr. A., 1190: 80-85.
Liu L, LiW, KoikeK, Zhang S, Nikaido T (2004). Newalpha-tetralonylglucosides from the fruit of *Juglans mandshurica*. Chem.Pharm.Bull. Tokyo 52:566-569.
Martinez ML, Labuckas DO, Lamarque AL, Maestri DM (2010). Walnut (*Juglans regia* L.): genetic resources, chemistry, by-products. J. Sci. Food. Agric., 90: 1959-1967.
Mei-zhi Z, Bing-nian J, Cai-xia J, Chao-bin L (2007). Study on Extraction Conditions of Active Antiviral Substance from Walnut Leaves. Chemistry and Industry of Forest Products. 02 [Abstract].
Mei-zhi Z, Feng-yun Z , Hua W, Wei W (2006). A Study on the Bioactivity of Secondary Metabolites from Walnut Green Gull University. Journal of Northwest Forestry University-01 [Abstract].
Mitrovic M, Stanisavljevic M, Danjanovic JG (1997). Biochemical composition of fruits of some important walnut cultivars and selections. Proceeding of the third International walnut held at Alcobaca, Portugal, Congress. Acta. Hortcult 442: 205- 207.
Mokhtari M, Shariati M, Sadeghi N (2008). Effect of alcohol extract from leave *Juglans regia* on antinociceptive induced by morphine in formalin test. Med. Sci. J. Islam. Azad. Uni., 18: 85-90.
Mouhajir F, Hudson JB, Rejdali M, Towers GHN (2001). Multiple antiviral activities of endemic medicinal plants used by Berber people of Morocco. Pharm. Biol., 39: 364-374.
Muradoglu FH, Oguz I, Yildiz K, Yilmaz H (2010). Some chemical composition of walnut (*Juglans regia* L.) selections from Eastern Turkey. Afr. J. Agric. Res., 5: 2379-2385.
Muradolu F (2005). Selection of promosing genotypes in native walnut (*Juglans regia* L.) populations of Hakkari central and Ahlat (Bitlis) districht, and genetic diversty. PhD dissertation, University of Yuzuncu Yil, Turkey.
Nariman F, Eftekhar F, Habibi Z, Falsafi T (2004). Anti-*Helicobacter pylori* activities of six Iranian Plants. Helicobacter., 9:2.
Oliveira I, Sousa A, Ferreira ICFR, Bento A, stevinhol LE, Pereira JA (2008). Total phenols, antioxidant potential and antimicrobial activity of walnut (*Juglans regia* L.) green husks. Food Chem. Toxicol., 46: 2326-2331.
Oryza (2007). Hepatoprotective and anti-oxidative extract for metabolic syndrome walnut polyphenol. ver.1.0 HS Oryza Oil and Fat Chemical Co., Ltd (http://www.oryza.co.jp/html/english/pdf/Walnut_polyphenol1.0.pdf).
Ozer B, Kivc MB (2007). Antityrosinase activity of some plant extracts and formulations containing ellagic acid. Pharm. Biol., 45: 519-524.
Papoutsi Z, Kassi E, Chinou I, Halabalaki M, Skaltsounis LA, Moutsatsou P (2008). Walnut extract (*Juglans regia* L.) and its component ellagic acid exhibit anti-inflammatory activity in human aorta endothelial cells and osteoblastic activity in the cell line KS483. British J. Nutr., 99:715-722.
Payne T (1985). California walnuts and light food. Cereal Foods World 30: 215-218.
Pereira JA, Oliveira I, Sousa A, Ferreira ICFR, Bento A, Estevinho L (2008). Bioactive properties and chemical composition of six walnut (*Juglans regia* L.) cultivars. Food Chem. Toxicol., 46: 2103-2111.
Poyrazolu EC, Biyik H (2010). Antimicrobial activity of the ethanol extracts of some plants natural growing in Aydin, Turkey. Afr. J. Microbiol. Res., 4: 2318-2323.
Qa'dan F, Al-Adham1 IS, Nahrstedt A. (2005a). Characterization of antimicrobial polymeric procyanidins from *Juglans regia* leaf extract. Eur. J. Sci. Res., 11:438-443.
Qa'dan F, Thewaini A, Ali D, Afifi R, Elkhawad A, Matalka K (2005b).The Antimicrobial Activities of *Psidium guajava* and *Juglans regia* Leaf Extracts to acne-developing organisms. Am. J. Chin. Med., 33: 197–204.
Qamar W, Sultana S (2011). Polyphenols from *Juglans regia* L. (Walnut) kernel modulate cigarette smoke extract induced acute inflammation, oxidative stress and lung injury in Wistar rats. Hum. Exp. Toxicol., 30:499-506.
Rahimipanah M, Hamedi M, Mirzapour M (2010). Antioxidant activity and phenolic contents of Persian walnut (*Juglans regia* L.) green husk extract. Afr. J. Food Sci. Technol., 1:105-111.
Rath BP, Pradhan D (2009). Antidepressant Activity of *Juglans regia* L. fruit extract. Int. J. Toxicol. Pharmacol. Res., 1: 24-26.
Ravai M (1992). Quality characteristics of califonia walnuts. Cereal Foods World 37: 362-366.
Robbers JE, Tyler VE (1999). Tyler's Herbs of Choise: The therapeutic use of phytomedicinals, The Havvorth Herbal Press, New York.
Ros E, Nnez I, Perez-Heras A, Merce S, Gilabert R, Casals E, Deulofeu R (2004). Walnut diet improves endothelial functions in hypercholesterolemic subject. Circulation 109: 1609-1614.
Saad B, Dakwar S, Said O, Abu-Hijleh G, Al-Battah F, Kmeel A, Aziazeh H (2006). Evaluation of medicinal plant hepatotoxicity in co-cultures of hepatocytes and monocytes. eCAM., 3:93-98.
Salicornia Brachiata, Ind. Crops Prod., 23:177.
Savage GP (2001). Chemical composition of walnuts (*Juglans regia* L.) grown in New Zealand. Plant Foods Hum. Nutr., 56: 75-82.
Segura-Aguilar J, Jonsson K, Tidefelt U, Paul C (1992). The cytotoxic effects of 5-OH-1, 4-naphthoquinone and 5, 8-diOH-1,4-naphthoquinone on doxorubicin-resistant human leukemia cells (HL-60). Leuk Res., 16: 631–637.
Shimoda H, Tanaka J, Kikuchi M, Fukuda T, Ito H, Hatano T, Yoshida T (2009). Effect of polyphenol-rich extract from walnut on diet-induced hypertriglyceridemia in mice via enhancement of fatty acid oxidation in the liver. J. Agric. Food Chem., 57:1786-92.
Society for Neuroscience (2007). "News Release: Diet of walnuts, blueberries improve cognition; may help maintain brain function", Society for Neuroscience, 5 November 2007 [http://www.sfn.org/index.aspx?pagename=news_110507b].
Souci SW, Fachmann W, Kraut H (1994). Food composition and nutrition tables. Medpharm, CRC Press, Stuttgart.
Spaccarotella KJ, Kris-Etherton PM, Stone WL, Bagshaw DM, Fishell VK, West SG, Lawrence FR, Hartman TJ (2008). The effect of walnut intake on factors related to prostate and vascular health in older men. Nutr. J., 7:13.
Sugie S, Okamoto K, Rahman KM, Tanaka T, Kawai K, Yamahara J (1998). Inhibitory effects of plumbagin and juglone on azoxymethane-induced intestinal carcinogenesis in rats. Cancer Lett., 127:177–183.

Sze-Tao KWC, Sathe SK (2000).Walnut (*Juglans regia* L): proximate composition, protein solubility, protein amino acid composition and protein in vitro digestibility. J. Sci. Food Agric., 80:1393–1401.
Tagarelli G, Tagarelli A, Piro A (2010). Folk medicine used to heal malaria in Calabria (southern Italy). J. Ethnobiol. Ethnomed., 6:27
Teimori M, Montasser KS, Ghafarzadegan R, Hajiaghaee R (2010). Study of Hypoglycemic Effect of *Juglans regia* leaves and its Mechanism, J. Med. Plants 9:57-65.
Torres-Gonzalez (2011). Protective effect of four Mexican plants against CCl4 –induced damage in the hyh7 human hepatoma cell. Annals hematology., 10:73-79.
Upadhyay V, Kambhoja S, Harshaleena K (2010c). Antifungal activity and preliminary phytochemical analysis of stem bark extracts of *Juglans regia* linn. IJPBA., 1:442-447
Upadhyay V, Kambhoja S, Harshaleena K, Veeresh, Dhruva K (2010a) Anthelmintic activity of the stem bark of *Juglans regia* Linn. Res J. Pharm. Phytochem. (RJPP) 2: 465-467
Upadhyay V, Kambhoja S, Leena HK (2010b). Antibacterial activity and preliminary phytochemical analysis of stem bark extract of *juglans regia* linn. *Pharmacologyonline* 3: 274-279 Newsletter.
USDA (2010). National Nutrient Database for Standard Reference, Release 23.
Vaidyaratnam PSV (2005). Indian Medicinal Plants a Compendium of 500 species. Orient Longman Private Limited, Chennai 3: 264-65.
Venkatachakm M, Sathe SK (2006) Chemical composition of selected edible nut seeds. J. Agric. Food. Chem., 54:4705–4714.
Willis L, Shukitt-Hale B, Cheng V, Joseph J (2009). Dose-dependent effects of walnuts on motor and cognitive function in aged rats. Br. J. Nutr., 101: 1140-1144.
Woods B, Calnan CD (1976). Toxic woods. Br. J. Dermatol., 94 (suppl, 13): 17.
Yesilada E (2002). Biodiversity in Turkish Folk Medicine. In: Sener, B. (Ed.), Biodiversity: Biomolecular Aspects of Biodiversity and Innovative Utilization. Kluwer Academic/Plenum Publishers, London, pp. 119–135.
Zhang J, Jun-xi L, Fei Z, Duo-long D (2009a). Chemical constituents in green walnut husks of *Juglans regia.* Chinese Traditional and Herbal Drugs. 06 [Abstract].
Zhang Z, Liao L, Moore J, Wua T, Wang Z (2009b). Antioxidant phenolic compounds from walnut kernels (*Juglans regia* L.). Food Chem., 113: 160-165.
Zibaeenezhad MJ, Rezaiezadeh M, Mowla A, Ayatollahi SMT, Panjehshahin MR (2003). Antihypertriglyceridemic effect of walnut oil. Angiology., 54: 4.

African Journal of Microbiology Research Vol. 5(32), pp. 5806-5811, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.949

Review

The use of gamma irradiation in agriculture

Issa. Piri[1], Mehdi. Babayan[2*], Abolfazl. Tavassoli[2] and Mehdi. Javaheri[2]

[1]Departmant of Agriculture, Payame noor University, PO Box 19395-4697, IR. Of Iran.
[2]Department of Agriculture, Esfarayen Branch, Islamic Azad University, Esfarayen, Iran.

Accepted 9 November, 2011

Lately, radiation technology is widely used to produce changes in the product characteristics leading to the development of new products. Gamma irradiation is capable of hydrolyzing chemical bonds, thereby cleaving large molecules of starch into smaller fragments of dextrin that may be either electrically charged or uncharged as free radicals. These changes may affect the physical and rheological properties of irradiated foods, resulting in increased solubility of starch, decreased swelling power, and decreased viscosity of starch paste. Irradiation of gamma rays on bud wood can produce higher frequencies of mutation, leading to the creation of new variants compared to the control. Macronutrients (carbohydrates, proteins and lipids) content are relatively stable against irradiation doses up to 10 kGy, on the other hand, gamma irradiation affects proteins by causing conformational changes, oxidation of amino acids, rupturing of covalent bonds and formation of protein free radicals. Radiation mediated morphological, structural and functional changes in a plant are governed by the intensity and duration of the gamma irradiation.

Key words: Irradiation, Foods sterilized, Mutation, Biochemical change, Agriculture.

INTRODUCTION

Irradiation is an ionic, no-heat process that continues to receive attention as a preservation and functional modification agent in polymer research and application (Abu et al., 2006). It was considered as one of the physical modification methods of nature polysaccharide (Hai et al., 2003; Relleve et al., 2005; Rombo et al., 2004). In comparison with other physical modification methods, such as microwave, UV, ultrahigh hydrostatic pressure and hydrothermal treatment, irradiation treatment is rapid, convenient and more extensive because ionizing energy penetrates through the polysaccharide granule rapidly (Bao et al., 2005). This process is useful in solving various agricultural problems: reduction of post-harvest losses through suppressing sprouting and contamination, eradication or control of insect pests, reduction of food-borne diseases and in extension of shelf life, and breeding of high performance well adapted and disease resistant agricultural crop varieties (Andress, 1994; Emovon, 1996).

*Corresponding author. E-mail: M_ATS57@yahoo.com.

Mutation

Nuclear techniques, in contrast to conventional breeding techniques, are widely applied in agriculture for improving genetically diversity. Unlike conventional breeding procedures which involve the production of new genetic combinations from already existing parental genes, nuclear technology causes exclusively new gene combinations with high mutation frequency. Basic tool of nuclear technology for crop improvement is the use of ionizing radiation which causes induced mutations in plants. These mutations might be beneficial and have higher economical values (Abdul et al., 2010).

Mutagenesis has already been used to improve many useful traits affecting plant size, flowering time and fruit ripening, fruit color, self compatibility, self thinning, and resistance to pathogens. Nowadays, the number of cultivars derived from mutation induction increases constantly (Hearn, 2001; Maluszynski et al., 1995). Inducing mutations by gamma rays has been effectively used with several species of Citrus. Irradiation of gamma rays on bud wood can produce higher frequencies of mutation leading to the creation of new variants

compared to the control. Radio sensitivity (LD 50) of acute exposure of Citrus ranges from 40 to 100 Gy (Sanada and Amano, 1998; Sparow, 1968) depending on species and varieties. Scion (bud wood), seeds, foral stage embryos, and *in vitro* material of Citrus were exposed to gamma rays. Citrus sunki was irradiated with 20 or 40 Gy of gamma rays at three different foral stages (Spiegel-Roy and Padova, 1973) and nucellar seedling (Ikeda, 1976). A spine free mutant was selected from irradiated nuclear. In Citrus sinensis, immature seeds were exposed to gamma rays at the doses 80-100Gy (Sparow, 1968), while those polyembryonic seeds were exposed to gamma rays of 100Gy (Kukimura, 1976). Two seedless mutants were selected, leading to release of a new cultivar “Hongju 418 and Hongju 420” (Kukimura, 1976). Citrus paradisi cv Hudson were exposed with thermal neutron, leading to release of a seedless mutant ‘Star Ruby’ (Chen, 1991). Another fve nearly seedless mutants of Citrus paradisi cv Foster were also selected from irradiated bud wood at the dose 50 gray of gamma rays (Micke, 1985). Citrus limon cv Eureka and Israeli Villafranca were irradiated by 60 and 50 Gy of gamma rays respectively, and completely new seedless varieties were released (Hearn, 1985). A red color of flesh and juice mutant derived from 80 Gy gamma irradiation of Citrus paradisi cv Ruby Red was released as cultivar Rio Red in 1984 (Spiegel-Roy, 1985). Bud woods of pummel, mandarin and Navel Orange irradiated by gamma-rays at doses of 30 to 75 Gy showed high sensitivity at higher dose, while Valencia and grapefruit produced more seedless fruits from those at the higher doses (Wu, 1986). Khatri et al. (2005) collected three high grain yielding and early maturing mutants by treating seeds of *Brassica juncea L*. cv. S-9 with gamma rays (750 to 1000KGy) and EMS.

Shah et al. (2001) developed a new oil seed *Brassica napus* L cv. ABASIN-95 by induced mutation. They exposed seeds of B. napus L. cv. Tower to 1.0, 1.2 and 1.4 KGy gamma rays and the resulting new variety was high yielding, resistant to Alternaria blight and white rust.

Irradiation of gamma rays (10-60 Gy) on calli in vitro proliferation stage resulted in high mortality at the dose of 60 Gy (Predieri, 2001) When nucellus and embryonic mases of Citrus sinensis cv. Pera were exposed to gamma irradiation at the dose 0–160 Gy and 0–189 Gy respectively, normal growth of plantlets was obtained from irradiated nucellus exposed to 20 – 80 Gy (Froneman, 1996).

Physiological changes in crop

Gamma irradiation induced physiological changes in crop although, gamma radiation is a technology with immense applications in agriculture, industry and medicine, its potential exploitation in agriculture is limited mainly because of lack of information awareness on optimal dose of irradiation which differs from one crop to another crop and from one application to another application. Radiation mediated morphological, structural and/or functional changes in a plant are governed by the intensity and duration of the gamma irradiation. In wheat, particularly, research efforts are needed to develop plant types with reduced height, which would enable them to tolerate gusty wind and contain losses due to lodging and subsequently grain yield. Mashev et al. (1995) used high irradiation dose of 5000–15 000 R to achieve a decrease in plant height and an increase in yield and suggested that even higher irradiation dose could be used to develop yield efficient wheat plant types. Wheat grains from irradiated plants were also rich in proteins and essential amino acids (Mashev et al., 1995). Din et al. (2003) studied the effect of gamma irradiation on different wheat varieties at seed irradiation dose of 10, 20, 30 and 35 krad. A higher dose of 30 and 35 krad created some abnormalities in plant types for example, a tiller having two ears attached with each and/or prevalence of sterile ears etc. Mashev et al. (1995), observed a significant decline in grain yield of wheat at doses above 0.10 kGy, however, lower doses of 0.01 and 0.025 kGy increased grain yields. Spielmeyer et al. (2007) had used a high vigour breeding line vigour 18 to identify a QTL on chromosome 6A that accounted for up to 8% of the variation for coleoptile length, 14% of seedling leaf width and was associated with increased plant height. They found a SSR marker, NW 3106, nearest to the 6A QTL that was associated with greater leaf width in a breeding population. The Vigour 18 allele of the QTL on chromosome 6A promoted coleoptile length and leaf width not only during early plant growth but was also found to be associated with increased plant height at maturity (Spielmeyer et al., 2007).

Biochemical change in crop

Extensive research showed that the macronutrients (carbohydrates, proteins and lipids) content are relatively stable against irradiation doses up to 10 kGy (WHO, 1994). However, Lee et al. (2005) reported that gamma irradiation affects proteins by causing conformational changes, oxidation of amino acids, rupturing of covalent bonds and formation of protein free radicals. Also, chemi-cal changes in the proteins caused by gamma irradiation include fragmentation, cross-linking, aggregation and oxidation by oxygen radicals that are generated in the radiolysis of water. Gamma irradiation has a slight effect on the amino acid profile at recommended doses to foods (WHO, 1999). This effect could be related to the structure of each amino acid as revealed by many authors (Simic, 1983; Urbain, 1986; Elias and Cohen, 1997; Matloubi et al., 2004; Erkan and Ozden, 2007). The previous authors concluded that simple amino acids increased upon irradiation, such as glycine, which undergo reductive

deamination and decarboxylation. In addition, aliphatic amino acids with increasing chain length, provide additional C–H bonds for interaction with OH radicals which reduces the extent of oxidative deamination. Wang and von Sonntage (1991) reported that sulfur containing as well as aromatic amino acids are, in general, the most sensitive to irradiation, while simple amino acids could be formed by destruction of other amino acids. Diehl (1995) and Matloubi et al. (2004) reported that there is a mutual protection exerted when different substances are irradiated together.

The results of irradiating multiple compounds together will, generally, not cause much chemical change in any one of the compounds, when irradiated alone. The effects of gamma irradiation on physicochemical changes in proteins have been described in previous studies, where chemical trans-formations of amino acids, breakdown of peptide bonds, hydrogen and disulphide bridges were observed (Ambe et al., 1961; Bernofsky et al., 1959; Nisizawa, 1988; Puchala et al., 1979; Zabielski et al., 1984).

Delincee and Pushpa (1981) observed cross-linking of the chain influences of the tertiary structure of proteins and their physicochemical properties. Decomposition and denaturation were detected in irradiated proteins (Ciesla et al., 2000). The decrease of apparent amylose content was possibly originated from the breakage or cleavage of long chains in amylopectin caused by gamma irradiation (Wu et al., 2002). It also agreed with Descherider and Grant's observation (Descherider, 1960; Grant and D'Apponlonia, 1991) that the decreasing apparent amylose content results from the shortening of polysaccharide chains. Ciesla et al. (2000), stated that indicating modification of proteins occurring after gamma irradiation similar to the transformations taking place under heating.

Kanemaru, et al. (2005), reported that protein content for the irradiated semolina and semolina obtained from irradiated wheat grains was not affected with gamma irradiation and ranged around 10.6-10.9%. Similarly, these results also agreed with the findings of Marathe et al. (2002), Agundez-Arvizu et al. (2006) and Azzeh and Amr (2009). Kwon et al. (1988) concluded from their studies on a Korean garlic cultivar that immediately after gamma irradiation with 100 Gy there are no differences in the levels of linoleic, palmitic, oleic and linolenic acids, the predominant fatty acids of bulbs.

The low radiation dose used could have produced its long-term effects in part by means of the stimulation of lipid degradation, possibly mediated through the action of free radicals that are known to be generated after irradiation (Katsaras et al., 1986; Voisine et al., 1991). In plant tissues subject to different forms of stress, polar lipids are degraded to generate free fatty acids and diacylglycerols, resulting in an eventual accumulation of TG as a defense mechanism (Olsson, 1995; Navari-Izzo et al., 1990).

Effect of irradiation on crop growth and seed germination

When ionizing radiation is absorbed in biological materials, it acts directly on critical cell targets or indirectly through the generation of metabolites that can modify important cell components. Low doses of gamma irradiation have been used to advantage in order to control the degree of ripeness and extend the shelf life of fruits and vegetables. The use of ionizing radiation depends on a country as food irradiation is illegal in some countries. Detection methods of ionizing radiation are also required for enforcing good control. Methods such as inhibition of seed germination and elongation of roots and shoots from germinating seeds have been reported for the detection of irradiated seeds of crop species (Qiongying et al., 1993; Zhu et al., 1993; Selvan and Thomas, 1999; Barros et al., 2000). Chaudhuri (2002) reported a simple and reliable method to detect gamma irradiated lentil seeds by germination efficiency and seedling growth test.

There is a difference in the detection of irradiated seeds of kabuli -type chickpeas and wild Cicer species. Toker and Cagirgan (2004) reported that the shoot length of kabulitype chickpeas was induced by 100Gy irradiation as compared to the controls. For this reason, only 200, 300 and 400 Gy doses were taken into account for irradiation. Chaudhuri (2002) found similar results with lentil. Villavicencio et al. (1998) showed that root growth and the almost totally retarded shoot elongation of irradiated common bean and mung bean were markedly reduced as compared to the non-irradiated checks. They stated that the critical dose that prevented shoot and root elongation varied among species and also ranged from genotype to genotype within species. The kabuli types were more affected than the desi ones. This is a certain and reliable way to identify the effects of gamma rays on Cicer seeds in a very short time. These effects might in time be developed into a method for irradiation detection. Higher doses inhibit germination.

Chromatographic analysis of some herbal extracts indicated that changes in total yield and constituents of volatile oil following irradiation were ranged from none to slight depending upon dose-based irradiation in variety of herbs (IAEA, 1992; Venskutonis et al., 1996; Chatterjee et al., 2000). It can be assumed, therefore, that the dose which can be applied and hence extent to the microbial kill may be limited by undesirable changes in volatile oil constituents, their yield and flavor quality. Farag et al. (1995) that reported terpenes were converted to monoterpe-nesalcohols. b-Eudesmol, an oxygenated monoterpenes, was the major compound in this group, while verbenol, a-eudesmol, verbenone, and (E)-r-2-menthen-1-ol were also detected. The a-and b-eudesmol were increased to 9.52% from 6.91%, with no major variation between the different irradiation doses. The remaining oxygenated terpene levels also did not vary

significantly during irradiation. Quantity of paeoniflorin in Paeoniae radix, i.e. no change with irradiation as cited by Yu et al. (2004). In addition, Owczarczyk et al. (2000) have reported that the content of biologically active substances, including the essential oils, flavonoids, glycosides, anthocyanins, and plants mucus did not change significantly after irradiation. Irradiation can also increase the alkaloids percentage in the different organs of plant, particularly the leaves (Abo Elseud, 1983; El-Kholy, 1987; Habba, 1989). The increase or decrease in the germination percentage was found to attributed to gamma rays treatments. The stimulating effects of gamma ray on germination may be attributed to the activation of RNA synthesis (Kuzin at al., 1975) on coster bean, or protein synthesis (Kuzin et al., 1976) which occurred during the early stage of germination after seeds irradiated with 4 K-rad. These results are in agreement with the findings of Grover and Dhanju, (1980) on *Papaver somniferum* and Donge et al. (1986) on tea seeds. Habba (1989) who reported that increasing the dose of gamma rays up to 100 Gy, gradually increased the germination percentage, and then decreased gradually with increasing the gamma ray dose in the second season in *Hyoscyamus muticus*. Hell et al., (1974) stated that on *Phaseolus vulgaris*, treating seeds with high rates of gamma radiation reduced germination. Abo Elsauod and Omran (1976) indicated that irradiation snap bean seeds with 50, 100 and 150 Gy resulted in greater percentage of germination than the control. Regarding the effect of GA on seed germination an increase in germination percentage was observed by 3 increasing GA concentration was in confirmity with Ruminska et al. (1978) Who reported that the seed 3 soaking, preceding the sowing, in solutions 500, 1000, 1500 and 2000 ppm of GA improved germination ability of seven species of seeds, particularly good effects were achieved with *Lavandula vera* and *Atropa belladonna* where not only germination ability was not only increased but also accelerated and even shooting was obtained. Increase in higher germination percentage at higher doses might be due to their stimulating effects on activating RNA synthesis or protein synthesis (Kuzin et al., 1975; 1976) or it could be due to the elimination of germinating bacterial populations, their spores and mould fungi (Gruner et al., 1992)

Food irradiation

Research on food irradiation dates back to the turn of the twentieth century with the first United States of America and British patents being issued in 1905. It allowed the use of ionising radiation to kill bacteria in food (ICGFI 1999). The United States have since amended their drug regulations to allow the irradiation of certain food products to control food-borne pathogens (USEPA 2002). Food irradiation is a process in which products are exposed to ionizing energy, such as gamma rays, electron beams and X-rays for a specified time (FDA, 1986). A food is irradiated to utilize the destructive power of ionization radiation on the microorganisms with minimum changes in food constituents (Zenthen and Sorensen, 2003). Nowadays, irradiation of food is permitted in over than 60 countries for the purpose of food preservation by destruction of microbes, worms, insects and parasites, as well as for the inhibition of sprouting of potatoes and onions (IAEA, 2007). According to international health and safety authorities; Joint FAO/IAEA/WHO Expert Committee on the Wholesomeness of Irradiated Foods (JECFI), foods irradiated up to 10 kGy are considered safe and present no toxicological hazard and no special nutritional or microbiological problems in food (Anonymous, 1981). Gamma radiation of 30–1000 Gy has been applied to achieve a delay in the ripening of some fruits and vegetables (WHO 1988). A reduction in the amount of visible and total mould present in bread during a storage period of up to 20 weeks was reportedly achieved by applying a gamma radiation dose of 150 Gy to the flour (Adejumo 1998). Bansa and Appiah (2003) have also reported the successful use of gamma radiation dose of 120 Gy to effectively inhibit sprouting in yams for six months under tropical ambient conditions. Furthermore, higher gamma radiation doses of 2–4 kGy have been used to successfully reduce the infection rate in sugar beet seeds (Rizk and Moussa 2003)while a dose of approximately 500 Gy has been employed to disinfect and also reduce microbial populations in cocoa beans (Adesuyi 1996).

Foods sterilized

During the last decade interest has increased in the methods of food sterilization and modification applying medium (1–10 kGy) and high doses (10–70 kGy) of irradiation as well as radiation processing of industrial products that contain starch. Foods sterilized at high doses may be consumed by immunologically depressed patients and can be stored at room temperature (for example bakery products, readily prepared meals). Radiation modification enables, moreover, removal of ant nutritional factors and inhibition of food allergies Doses of several dozen kGy are used for sterilization of pharmaceuticals and medical devices and for starch modification. Accordingly, it appears desirable to acquire knowledge about the functional and structural properties of foods and starch alone irradiated using medium and high doses and in the development of appropriate physicochemical testing methods.

CONCLUSION

Many investigate on effect of gamma radiation on crop were carried out and their results showed usage of

gamma radiation have different effect on crop such as Biochemical and physiological change, growth and germination inhibition. Inhibition of seed germination and elongation of roots and shoots from germinating seeds have been reported for the detection of irradiated seeds of crop species. Gamma irradiation affects proteins by causing conformational changes, oxidation of amino acids, rupturing of covalent bonds and formation of protein free radicals. Also, chemical changes in the proteins caused by gamma irradiation include fragmentation, cross-linking, aggregation and oxidation by oxygen radicals that are generated in the radiolysis of water. Irradiation is effective method for microbiological decontamination of them, and the content of essential biologically active substances and pharmacological activity of medicinal herbs not change significantly with irradiation.

REFERENCES

Abdul Majeed, Asif Ur Rehman Khan, Habib Ahmad, Zahir Muhammad (2010). Gamma irradiation effect on some growth parameters of *Lepidium sativum* L. ARPN, J. Agric. Biol. Sci., 5(1): 39-42.

Abo Elseud MA (1983). Biochemical Studies on Some Medicinal Plants As Affected By Radiation And Protective Agents. M.Sc. Thesis, Fac. Agric. Menoufia Univ., 65-69.

Abu JO, Muller K, Duodu KG, Minnaar A (2005). Functional properties of cowpea (*Vigna unguiculata* L. Walp) flours and pastes as affected by c-irradiation. Food Chem., 93: 103–111.

Adejumo J (1998). Food preservation by gamma irradiation BSc Project Department of Physics, Obafemi Awolowo University, Ile-Ife, Nigeria.

Adesuyi SA (1996). Use of irradiation for post-harvest preservation of crops with special reference to Nigeria, J. Irradiation National Dev., 11: 46–9.

Agundez-Arvizu Z, Fernandez-Ramirez M, Arce-Corrales M, Cruz-Zaragoza E, Melendrez R, Chenov V, Barboza Flores M (2006). Gamma irradiation effects on commercial Mexican bread making wheat flour. Nucl. Instrum. Methods Phys. Res. B, 245: 455–458.

Ambe KS, Kumta US, Tappel AI (1961). Radiation damage to cytochrome C and hemoglobin. Radiat. Res., 15: 709–719.

Andress EL, Delaplane KS, Schuler GA (1994). Food Irradiation. Fact sheet HE 8467 (Institute of Food and Agricultural Sciences University of Florida, USA).

Anonymous (1981). Wholesomeness of irradiated food report of a joint FAO/IAEA/ WHO expert committee. Technical Report Series: 659. WHO, Geneva.

Azzeh F, Amr A (2009). Effect of gamma irradiation on physical characteristics of *Jordanian durum* wheat and quality of semolina and lasagna products. Radiat. Phys. Chem., 78: 818–822.

Bansa D, Appiah V (2003). Preservation of yams by gamma radiation J. Ghana Sci. Assoc., 1: 3.

Bao JS, Ao ZH, Jane JL (2005). Characterization of physical properties of flour and starch obtained from gamma-irradiated white rice. Starch, 57: 480–487.

Barros AC, Freund MTL, Villavicencio ALCH, Delin-cee H, Arthur V (2000). Identification of irradiated wheat by germination test, DNA comet assay and electron spin resonance. Radiat. Phys. Chem., 63: 423–426.

Bernofsky C, Fox Jr. JB, Schweigert BS (1959). Biochemistry of myoglobin VI: the effect of low dosage gamma irradiation on beef myoglobin. Arch. Biochem. Biophys., 80: 9–21.

Chatterjee S, Variyar PS, Gholap AS, Pudwal-Desai SR, Bongir- war DR (2000). Effect of g-irradiation on the volatile oil constituents of turmeric (*Curcuma longa*). Food Res. Int., 33: 103–106.

Chaudhuri SK (2002). A simple and reliable method to detect gamma irradiated lentil (*Lens culinaris*.) seeds by germination efficiency and seedling growth test. Radiat. Phys. Chem., 64: 131–136.

Chen S (1991). Studies on the seedless character of Citrus induced by irradiation. Mutat. Breeding Newslett., 37: 8-9.

Ciesla K, Roos Y, Gluszewski W (2000). Denaturation processes in gamma irradiated proteins studied by differential scanning calorimetry. Radiat. Phys. Chem., 58: 233–243.

Descherider AR (1960). Changes in starch and its degradation products on irradiating wheat flour with gamma rays. Starch/Staerke 12: 197.

Diehl J (1995). Safety of Irradiated Foods, second ed. Marcel Dekker, New York, USA. pp. 89-95.

Din R, Ahmed QK, Jehan S (2003). Studies for days taken to earing initiation and earing completion in M1 generation of different wheat genotypes irradiated with various doses of gamma radiation. Asian J. Plant Sci., 2: 894–896.

Elias P, Cohen A (1997). Radiation Chemistry of Major Food Components. Elsevier Scientific, Amsterdam, Netherlands. 159-163.

Emovon EU (1996). Keynote Address: Symposium Irradiation for National Development (Shelda Science and Technology Complex, SHESTCO, Abuja, Nigeria). pp. 156-164.

Erkan N, Ozden O (2007). The changes of fatty acid and amino acid compositions in sea bream (Sparus aurata) during irradiation process. Radiat. Phys. Chem., 76: 1636–1641.

Farag SED, Aziz Attia ESA (1995). Effect of irradiation on the microbiological status and flavouring materials of selected spices. Eur. Food Res. Technol., 201: 283–288.

FDA (1986). Irradiation in the production, processing and handling of food. Food and Drug Administration (Federal Register), 51(75): 13376–13399.

Froneman IJ (1996). Producing seedless *Citrus* cultivars with gamma irradiation. Proc Int. Soc. Citriculture, pp.159-163.

Grant LA, D' Aponlonia BL (1991). Effect of low-level gamma radiation on water-soluble non-starchy polysaccharides isolated from hard red spring wheat flour and bran. Cereal Chem., 68: 651.

Grover IS, Dhanju MS (1980). Effect of gamma radiation on the germination of *papaver somniferum* and *papaver. rhoeas*. Indian J. Plant Physiol., (1979), 22(1): 75-77. Hort. Abst., 1980, 50: 6595.

Habba IE (1989). Physiological Effect of Gamma Rays on Growth And Productivity Of *Hyoscyamus muticus* L. and Atropa belladonna L. Ph.D. Thesis, Fac. Agric. Cairo Univ., Cairo, Egypt. 65-73.

Hai L, Diep TB, Nagasawa N, Yoshii F, Kume T (2003). Radiation depolymerization of chitosan to prepare oligomers. Nucl. Instrum. Methods Phys. Res. B, 208: 466–470.

Hearn CJ (1985). Development of seedless grapefruit cultivars through budwood irradiation. Hortscience, 20: 84.

Hearn CJ (2001). Development of seedless grapefruit cultivars through budwood irradiation. Hortscience, 20: 84.

Hell KG, Silveira M (1974). Imbibition and germination of gamma irradiation *Phaseolus vulgaris* seeds. Field Crop Abst., 38(6): 300.

IAEA (1992). Irradiation of spices, herbs and other vegetable seasonings. IAEA-TECDOC-639.

IAEA (2007). Food Irradiation: A Powerful Nuclear Tool for Food Safety. Retrieved from: http://www.IAEA-food-irrad-tool0807S.

ICGFI (International Consultative Group of Food Irradiation) (1999). Facts about food irradiation. Series of fact sheets (Vienna: ICGFI).

Ikeda F (1976). Induced bud sports in Citrus sunski from Nucellar seedlings. In: Improvement of vegetatively propagated plants and tree crops through induced mutation, 95-96: 12.

Katsaras J, Stinson RH, Kendal EJ, McKersie BD (1986). Structural simulation of free radical damage in amodelmembrane system: a smallangle X-ray diffraction study. Biochim. Biophys. Acta 861: 243–250.

Kukimura HF (1976). Brief description of mutation in vegetatively propagated and tree crops. Gamma Field Symp., 15: 79-82.

Kuzin AM, Vagabova ME Revin AF (1976). Molecular mechanisms of the stimulating action of ionizing radiation on seeds. 2. Activation of protein and high molecular RNA synthesis. Radiobiologiya, 16: 259-261.

Kuzin AM, Vagabova ME, Prinak-Mirolyubov VN (1975). Molecular mechanisms of the stimulating effect of ionizing radiation on seed. Activation of RNA synthesis. Radiobiologiya., 15: 747-750.

Kwon JH, Yoon HS, Byun MW, Cho HO (1988). Chemical changes in garlic bulbs resulting from ionizing energy treatment at sprout-inhibition dose. J. Kor. Agric. Chem. Soc., 31: 147–153.

Lee S, Lee M, Song K (2005). Effect of gamma-irradiation on the physicochemical properties of gluten films. Food Chem., 92: 621–925.
Maluszynski M (1995). Application of in vivo and in vitro mutation techniques for crop improvement. Euphytica, 85: 303–315.
Marathe S, Machaiah J, Rao B, Pednekar M, Sudha Rao V (2002). Extension of shelf life of whole-wheat flour by gamma radiation. Int. J. Food Sci. Technol., 37: 163–168.
Mashev N, Vassilev G, Ivanov K (1995). A study of N-allyl N-2 pyridyl thiourea and gamma radiation treatment on growth and quality of peas and wheat. Bulgerian J. Plant Physiol., 21(4): 56–63.
Matloubi H, Aflaki F, Hadjiesadegan M (2004). Effect of gamma irradiation on amino acids content of baby food proteins. J. Food Compos. Anal., 17: 133–139.
Micke A (1985). Plant cultivars derived from mutation induction or the use of induced mutants in crop breeding. Mutation Breeding Rev., 3: 1-92.
Navari-Izzo F, Vangioni N, Quartacci MF (1990). Lipids of soybean and sunflowers seedlings grown under drought conditions. Phytochemistry, 29: 2119–2123.
Nisizawa M (1988). Radiation induced sol–gel transition of protein: effect of radiation on amino-acid composition and viscosity. J. Appl. Polym. Sci., 36: 979–981.
Olsson M (1995). Alterations in lipid composition, lipid peroxidation and anti-oxidative protection during senescence in drought stressed plants and non-drought stressed plants of Pisum sativum. Plant Physiol. Biochem., 33: 547–553.
Owczarczyk HB, Migdal W, Kedzia B (2000). The pharmacological activity of medical herbs after microbiological decontamination by irradiation. Radiat. Phys. Chem., 57: 331–335.
Predieri S (2001). Mutation induction and tissue culture in improving fruits. Plant Cell. Tissue and Organ Culture 64: 185-210.
Puchala M, Szweda-Lewandowska Z, Leyko W (1979). g-Irradiation of aqueous solutions of human hemoglobin in atmosphers of air and argon. Radiat. Res., 78: 379–389.
Qiongying L, Yanhua K, Yuemei Z (1993). Studies on the method of identification of irradiated food. I. Seedling growth test. Radiat. Phys. Chem., 42: 387–389.
Relleve L, Nagasawa N, Luan LQ Yagi T, Aranilla C, Abad L (2005). Degradation of carrageenan by radiation. Polymer Degradation and Stability, 87: 403–410.
Rizk MA, Moussa TAA (2003). Impact of gamma irradiation stresses I. Response of gamma-irradiated sugarbeat seeds to infection by soil-borne fungal pathogens Pak. J. Plant Pathol., 2: 28–38.
Rombo GO, Taylor JRN, Minnaar A (2004). Irradiation of maize and bean flours: Effects on starch physicochemical properties. J. Sci. Food Agric., 84: 350–356.
Ruminska A, Suchorska K, Weglarz Z (1978). Effect of gibberellic acid on seeds germination of some vegetable and medicinal plants. International Symposium on Spices and Medicinal plants, ISHS Acta Horticulturae 73: I .
Sanada T, Amano E (1998). Induced mutation in fruit trees. In JAIN, Somaclonal variation and induced mutations in crop improvement. Kluwer Academic Publishers, England, pp. 401- 419.
Selvan E, Thomas P (1999). A simple method to detect gamma-irradiated onions and shallots by root morphology. Radiat. Phys. Chem., 55: 423–427.
Simic M (1983). Radiation chemistry of water-soluble food components.
In: first ed. In: Josephon, E., Peterson, M. (Eds.), Preservation of Food by Ionizing Radiation, CRC Press, London, 2: 1–73.
Sparow AH (1968). Radiosensitivity studies with woody plants. I. Acute gamma irradiation survival data for 28 species and predictions for 190 species. Radiat. Bot., 8: 149-186.
Spiegel-Roy P (1985). Seedless induced mutant in highly seeded lemon (*Citrus limon*). Mutation Breeding Newslett., 26: 1-2.
Spiegel-Roy P, Padova R (1973). Radiosensitivity of Shamouti orange (*Citrus sinensis*) seeds and buds. Radiation Bot., 13: 105-110.
Spielmeyer W, Hyles J, Joaquim P, Azanza F, Bonnett D, Ellis ME, Moore C, Richards RA (2007). A QTL on chromosome 6A in bread wheat (*Triticum aestivum*) is associated with longer coleoptiles, greater seedling vigour and final plant height. TAG Theoretical Appl. Gene., 115(1): 59–66.
Toker C, Cagirgan MI (2004). Spectrum and frequency of induced mutations in chickpea. Int. Chickpea Pigeonpea Newslett. 11: 8–10.
Urbain W (1986). Food Irradiation. Academic Press, Orlando, USA. 88-94.
USEPA (United States Environmental Protection Agency) (2002). The history of food irradiation Bull. USEPA. 96-100.
Venskutonis R, Poll L, Larsen M (1996). Effect of irradiation and storage on the composition of volatile compounds in Basil (*Ocimum basilicum L.*). Flavour Fragr. J., 11: 117–121.
Villavicencio ALCH, Mancini-Filho J, Delinciee H (1998). Application of different techniques to identify the effects of irradiation on brazilian beans after six months storage. Radiat. Phys. Chem., 52: 161–166.
Voisine R, V´ ezina LP, Willemont C (1991). Induction of senescence-like deterioration of micro small membranes from cauliflower by free radicals generated during gamma irradiation. Plant Physiol., 97: 545–550.
Wang D, von Sonntage C (1991). Radiation industrial oxidation of phenylalanine. In: Leonardi, M., Raffi, J., Belliardo, J. (Eds.), Proceedings of the Workshop on Recent Advances on Detection of Irradiated Foods, BRC Information, Chemical Analysis Commission of the European Communities, Brussels, pp. 212–217 (Report EUR 13331 En).
WHO (World Health Organization) (1988). Food Irradiation: A Technique for Preserving and Improving the Safety of Food (WHO Publication in Collaboration with FAO). pp. 144-149.
WHO (1994). Safety and Nutritional Adequacy of Irradiated Food. WHO, Geneva. WHO, 1999. High-dose Irradiation: Wholesomeness of Food Irradiated with Doses above 10 kGy. WHO, Geneva (WHO Technical Report Series no: 890). 120-124.
Wu S (1986). Using Gamma-rays to induce mutations for seedlessness in Citrus. Mutat. Breeding Newslett., 27: 14.
Yu YB, Jeong IY, Park HR, Oh H, Jung U, Jo SK (2004). Toxicological safety and stability of the components of an irradiated Korean medicinal herb, Paeoniae Radix. Radiat. Phys. Chem., 71: 115–119.
Zabielski J, Kijowski J, Fiser W, Niewiarowicz A (1984). The effect of irradiation on technological properties and protein solubility of broiler chicken meat. J. Sci. Food Agric., 35: 662–670.
Zenthen P, Sorensen L (2003). Food Preservation Technique. Wood head Publishing Limited, Cambridge. pp. 89-96.
Zhu S, Kume T, Ishigaki I (1993). Detection of irradiated wheat by germination. Radiat. Phys. Chem., 42: 421–427.
Kuzin AM, Vagabova ME, Prinak Mirolyubov VN (1975). Molecular mechanisms of the stimulating effect of ionizing radiation on seed. Activation of RNA synthesis. Radiobiologiya, 15: 747-750.
Kuzin AM, Vagabova ME, Revin AF (1976). Molecular mechanisms of the stimulating action of ionizing radiation on seeds. 2. Activation of protein and high molecular RNA synthesis. Radiobiologiya, 16: 259-261.
Gruner MM, Horvatic D, Kujundzic, Magdalenic B (1992). Effect of gamma irradiation on the lipid components of soy protein products. Nahrung, 36: 443-450.
Shah SA, Ali I, Rahman K (2001). Abasin 95 a new oilseed rape cultivar developed through induced mutations. Mut. Breed. Newslett., 45: 3-4.
Khatri A, Khan IA, Siddiqui MA, Raza S, Nizamani GS (2005). Evaluation of high yielding mutants of *Brassica Juncea* cv. S-9 developed through Gamma rays and EMS. Pak. J. Bot., 37(2): 279-284.

African Journal of Microbiology Research Vol. 5(32), pp. 5812-5818, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR10.104

Full Length Research Paper

In vitro antiviral activities of Jrani caprifig latex and its related terpenes

Houda LAZREG AREF[1]*, Mahjoub AOUNI[2], Jean Pierre CHAUMON[3], Khaled SAID[1] and Abdelwaheb FEKIH[4]

[1]Laboratoire de Génétique, Biodiversité et Valorisation des Bio ressources (UR 03ES09), Institut Supérieur de Biotechnologie, 5000 Monastir, Tunisie.
[2]Laboratoire des Maladies Transmissibles et Substances Biologiquement Actives, Faculté de Pharmacie 5000 Monastir, Tunisie.
[3]Laboratoire de Botanique et cryptogamie, Faculté de Pharmacie Besançon 25000 cedex, France.
[4]Laboratoire de Chimie, 03/UR/1202, Faculté de Médecine Dentaire, 5000 Monastir, Tunisie.

Accepted 29 November, 2011

The aim of this study was to search for new antiviral agents from Tunisian Jrani caprifig latex (*Ficus carica* L., Moraceae). Terpenes and coumarins were identified by gas chromatography–mass spectrometry (GC-MS) analysis in hexane and hexane ethyl-acetate (1:1, v/v) extracts, and used in experiments to test their influence on a series of viruses, namely *Herpes simplex* (HSV-1), *Adenovirus* (ADV) and *Echovirus* type 11 (ECV-11). To evaluate the capacity of the extracts inhibition of viruses replication cycles by preventing their adsorption and their penetrations in the cells (by interaction with the cellular receivers designed for these viruses) or by inhibition of their intracellular replication or by virus inhibition after a direct contact under microscopic observation of cytopathic effect (CPE). Extracts inhibited virus multiplication in tested techniques at the concentrations of 19.5 and 39 µg/ml, respectively. All extracts had no cytotoxic effect on Vero cells at all tested concentrations. In conclusion, some compounds of Jrani latex which possess antiviral activities may be due to the high level of Triterpens. Their mode of action against the tested viruses was found to be at all stages of multiplication, suggesting the potential use of this compound for treatment of the infection caused by these viruses.

Key words: *Ficus carica*, caprifig, latex, HSV-1, HSV-2, ECV-11, ADV, coumarins, terpens.

INTRODUCTION

The use of herbs and medicinal plant as the first medicines is a universal phenomenon. Every culture on the earth, through written or oral tradition, has relied on the vast variety of natural chemistries found in plants for their therapeutic properties. All drugs from the plant are substances with a particular therapeutic action extracted from plants (Serrentino, 1991). The usage of herbal plants as traditional health remedies is the most popular for 80% of the world population in Asia, Latin America and Africa and is reported to have minimal side effect (Doughari, 2006). In this study Jrani caprifig variety which belongs to the genus *Ficus carica* L. (Moraceae) has been selected. Unlike common figs, the caprifig is a fig tree which produces both male and female flowers and is used to fertilize the female trees of the species. The name caprifig is derived from caprificus (or “goat fig” in Italian) (Storey et al., 1977) produces three crops of syconia. These are known by their Italian terms, profichi,

*Corresponding author. E-mail: ibrahimhoudarf@yahoo.fr. Tel: +216 97 654 133. Fax: 00216 73 568 900.

Abbreviations: CPE, Cytopathic effect; **PBS,** phosphate buffered saline; **MEM,** minimum essential medium; **FBS,** fetal bovine serum; **HSV-1,** herpes simplex virus type 1; **CPE,** cytopathic effect.

mammoni and mamme. The fig is a deciduous tree, which probably originated in Western Asia, and spread to the Mediterranean is commonly known as fig tree (Tous and Ferguson, 1996). Its products are widely used both as a food and in traditional medicine in the Mediterranean; the roots are used in treatment of leucoderma and ringworms and its fruits which are sweet, have antipyretic, purgative, aphrodisiac properties and have shown to be useful in inflammations and paralysis (Kirthikar and Basu, 1996). *F. carica* is claimed to be useful in liver and spleen disorders, to cure piles and in treatment of gout. The leaf decoction affected lipid catabolism in hyperglyceridemic rats (Perez et al., 1999). Several phytochemical investigations of *F. carica* leaves have been published, but with no biological data. Athnasios et al. (1962) have isolated prosalen, β-sitosterol and Bergapten. Others have isolated triterpenoids (Abu-Mustafa et al., 1964). *F. carica* latex released on picking the fruits is used to treat skin tumors and warts (Ghazanfar, 1994). The first scientific investigation of the activity of fig latex was done by Ullman et al. (1945, 1952). The dialysate of the latex contained the active ingredient. Although, isolation of the active components was not pursued further, some pharmacological work was reported by Ullman (1952) and Ullman et al. (1952). Fig latex has also been tested for its anthelmitic activity (De-Amorin et al., 1999).

In this modern age it is very important to provide scientific proof to justify the various medicinal uses of herbs. Herbal drugs are prescribed widely even when their biologically active components are unknown because of their effectiveness, fewer side effects and relatively low cost (Valiathan, 1998). However, we are not aware of a satisfactory remedy for serious viral diseases and search for effective and safe drugs.

In this paper we describe the identification of a potent antiviral terpenes and coumarins from Tunisian caprifig latex, Jrani variety.

MATERIALS AND METHODS

Latex collection

The Jrani caprifig latex was collected from unripe inedible fig fruit growing in Mesjed Aissa agricultural field located in the central cost of Tunisia. The latex was held in ice during the period of collection. The identification of this variety was established by Pr. Massoud MARS, professor of arboriculture at the high school of horticulture of Chott Meriam Sousse, Tunisia, Department of Agriculture, and Arboriculture, the code collection of the tree is JR1. This fig fruit was cat open from its top then slightly squeezed to collect few drops of latex directly into polyethylene centrifuge tubes, frozen immediately in Dry-Ice and maintained in frozen state at –30°C until the analyses were performed.

Extracts preparation

The gum, approximately 30% by weight in *F. carica* latex was removed from the aqueous solution by centrifugation in a refrigerated centrifuge (Bakemann Avanti TM30) at 15 000 rpm for 60 min at 0°C. The clear, straw-colored aqueous solution that was designated as a soluble material was frozen and stored at –30°C until required for analysis.

100 g of defrosted *F. carica* latex were macerated, (on cold) in 300 ml of methanol, over night, and repeated three times (Sarang et al., 2005), then evaporated under reduced pressure to afford (15 g) of yellow brown solid extract product 1 (P1).

The resulted residue was subjected to silica gel flash column chromatography eluted with hexane, hexane-ethyl acetate (1:1 v/v), and finally with ethyl acetate to obtain product 2 (P2), 3 (P3), and 4 (P4) of 4.10 g, 2.10 g and 0.60 g, respectively.

Furthermore, methanol was added to elute the polar compounds, which concentrated under reduced pressure, and then was re-dissolved in H_2O, and re-extracted with chloroform to afford product 5 (P5) of 3 g. All solvent were purchased from Merck, Germany.

GC-MS analysis

The analysis of Jrani caprifig latex hexane and ethyl-acetate (1/1, v/v) extracts was performed on a GC-MS HP model 1909S-433 inert MSD (Agilent Technologies, J and W Scientific Products, Palo Alto, CA, USA), equipped with an Agilent Technologies capillary DB-5MS column (30 m in length; 0.25 mm i.d.; 0.25 mm film thickness), and coupled to a mass selective detector (MSD1909S-433, ionization voltage 70 eV; all Agilent, Santa Clara, CA). The carrier gas was He and was used at 1 ml/min flow rate. The oven temperature program was as follows: 2 min at 150°C ramped from 150 to 240°C at 10°C/min and 1 min at 240°C then ramped from 240 to 280°C at 5°C/min and 15 min at 280°C. The chromatograph was equipped with a split/splitless injector used in the split mode. The split ratio was 1:100. Bis-(trimethylsilyl)-acetamide (BSTFA) (100 ml) was added to 100 ml of extract. The control of the GC/MS system and the data peak processing were carried out by means of MSDCHEM software. Identification of components was assigned by matching their mass spectra with Wiley and NIST library data, standards of the main components.

Cell and virus culture

The cell line used was *Cercopithecus aethiops* African green monkey kidney cells (Vero cell line ATCC CCL-81). The cellular lines maintained in the laboratory of transmissible diseases and bioactive substance (Faculté de Pharmacie de Monastir).

Briefly, the protocol used to obtain primary cell culture from the biopsy was as follows, each biopsy was washed three times with phosphate buffered saline (PBS) containing 200 units/ml of penicillin, 200 µg/ml of streptomycin and 0.5 µg/ml of amphotericin B. The skin was discarded the cartilage and the subcutaneous tissue were minced. Finely, the pieces of tissue were phased in 25 cm^2 cell culture flasks with just enough growth medium, that is eagle minimum essential medium (MEM) with 2 mM L-glutamine, 1% vitamins, 1% non essential amino acids, 100 units/ml of penicillin, 100 µg/ml of streptomycin, 0.25 µg/ml of amphotericin B, and 10% of fetal bovine serum (FBS) to cover the pieces of tissue when the fibroblasts had proliferated to 30 or 40% confluence, the pieces of tissue were discarded by gently shaking with PBS and again the cells were fed with 50% of used medium and 50% of fresh medium. When 80% confluence was reached, the cells were trypsinized and cultured in 150 cm^2 flasks. Once the cells had covered about 80% of the surface, they were trypsinized, centrifuged and cryopreserved. All cells were grown in MEM supplemented with 10% (FBS), 100 units/ml of penicillin, 100 µg/ml of streptomycin (4 ml), 2 mM L-glutamine (2 ml), 0.07% $NaHCO_3$,

1% non essential amino acids and vitamin solution. The cultures were maintained at 37°C in humidified 5% CO_2 atmosphere.

Herpes Simplex (herpes Viridae family) virus Type 1 (HSV-1) was obtained from the Laboratoire des Maladies Transmissibles et Substances Biologiquement actives, Faculté de Pharmacie 5000 Monastir Tunisia. *Echovirus* (ECV-11): Type 11 (Picornaviridae family) clinical strains. *Adenovirus* (ADV): non serotyped (Adenoviridae family) clinical stain.

Titration of viral strains

The virus strains were prepared from HSV-1 infected Vero cell cultures. The infected cultures were subjected to three cycles of freezing-thawing and centrifuged at 2000 rpm for 10 min. The supernatant was collected, titrated, and stored at – 80°C in 1 ml aliquots. Viral strain was carried out on 96-well flat-bottomed plate containing confluent cells into monolayer; decimal dilutions of the initial viral suspension were made then inoculated in to deprived cells from their growth medium. Each dilution was divided up in to a column of 8 wells at a density of 100 µl/well. The last column was reserved for control cells not infected by the virus and containing only the survival medium; then the plaque was incubated at 37°C in humidified 5% CO_2 atmosphere and controlled every 24 h by taking note of each time the number of well showed a CPE, when the CPE ceased to progress (5 to 7 days).

We evaluated the infectious title of the virus according to the method of Reed and Muench (1938) (Watanabe et al., 1994): the title of a virus is expressed by the $TCID_{50}$ (infectious amount on cultured tissue), it shows the dilution of the viral suspension of which 50% of the cells degenerate. The exploitation of dilution DL_{50} or $TCID_{50}$ (in absolute value) corresponds to the dilution which is giving a degeneration immediately above 50%, increased product of the proportional distance X by the logarithm of dilution factor. The proportional distance X between two critical dilutions at which the $TCID_{50}$ is located and obtained by spearman Kàber formula: X = (D > 50% - 50)/(D > 50% to D 50%). D > 50%: degeneration immediately grater than 50%. D < 50%: degeneration immediately lesser than 50% (Lorenz and Bögel, 1973).

To titer the virus suspension, confluent monolayer Vero cells were grown in 96-well flat-bottomed plates, and were infected with 0.1 ml of serial 12-fold dilutions of the virus suspension by 0.1 ml quadruplicated for a period of 48 h. The virus titer was $10^{3.5}$ (the dilution of the virus required to $TCID_{50/0.1ml}$ lytic effect, 50% of the inoculated cultures).

Solvent cytotoxicity assay

The solutions of extracts were already prepared with 100 mg/ml, a serial dilutions (1, 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640, 1/1280, 1/2560 and 1/5120 mg/ml) were done in a medium, made up of RPMI 1640 supplemented with 1% L. glutamine, 2% of antibiotic and 10% (FBS) (serum of be worth fetal) (Chiang et al., 2002). Cells monolayer were trypsinized, washed with culture medium and plated in 96-well flat-bottomed plate with 2. 10^4 cells per well. After 24 h of incubation, each diluted extract was added to the appropriate wells, some wells containing only cells with the medium of dilution [Cat 2% (FBS)] were taken as a cellular control and the plate were incubated for a further 48 h at 37°C in humidified incubator with 5% CO_2. The supernatants were removed from the cells and cells viability during 5 days was evaluated by supervising the state of the cells until the appearance of cytopathic effect (CPE). That effect generally appears when cells become round and fall with repression of the cores in the cellular membranes, phenomenon observed during the cells bursting by a serial of freezing and defrosting. The results are obtained from triplicate assays with at least five extract concentrations.

Antiviral activity assay

Viral reproduction inhibitory, extract can act on the reproduction itself, or on the adsorption, or during the penetration of the virus in the cell. Three tests were carried out.

Contact between the virus and the extract before inoculation with the cells. This test allowed us to know, if the extract is able to deteriorate the integrity of the virus and so to prevent it from carrying out its infectious process. Contact between cells and extract before virus attaching, and so, the extract has the possibility of acting on the cellular receivers and can therefore prevent the adsorption, thus the penetration of the virus in the cell. In both cases, extracts will prevent the adsorption and so the penetration of virus in the cell.

Contact between cells and viral suspension then we add the extract. In this case, the virus has enough time to penetrate in the cell and to begin the replication process. The inhibition of viral multiplication after adding the extract was due to the blocking effect of the viral replication by the extract. These three tests were applied to the viruses and the two extracts (P2 and P3) of which their cytotoxicity has already being studied.

Incubation of the extract with the viral suspension

The various concentrations of the extract were contacted with the viral suspension from line 2 to 11 and from column B to G in 96-well flat-bottomed plate at a density of 10 $TCID_{50}$ /ml (v/v) equalized volumes (Grazìa et al., 2003). In line 1, non infected cells were held in their growth medium used as a negative control, line 12, columns A and H were reserved to infected cells as a positive control. The plate was incubated at 37°C for 1 h, and then inoculated on cultured Vero cells, presenting a confluent layer at a density of 200 µl/well. The cells were again incubated at 37°C and observed for 5 to 7 days until the appearance of a total CPE by the viral control (Ooi et al., 2004; Tshikalange et al., 2005).

Incubation of the cells with the extract

The incubation of the cells, which were separated from their growth medium were contacted with various concentrations of the extract at a density of 100 µl/well for 1 h at 37°C. Then, 100 µl of the viral suspension were added in each well of the plate which was again incubated at 37°C and observed for 5 to 7 days until the appearance of the viral CPE on the control as explained above (Beloin et al., 2005).

Incubation of the cells with the viral suspension

The cells were infected by the viral suspension at a density of 100 µl/well incubated for 1 h at 37°C, then 100 µl/well of various extract concentrations were added, and incubated for 5 to 7 days until the appearance of total CPE on the viral control (Cos et al., 2002). These tests were performed in triplicate for the three viral strains, each test performed used as a negative control of none infected cells held in their growth medium, and as a positive control of the cells infected by the viruses at equal concentration used for all tests (Tshikalange et al., 2005; Beloin et al., 2005). The absence of the CPE at a concentration level of the extract when the viral control expresses a CPE indicates that the extract has an antiviral activity with a specific concentration (Meyer et al., 1996).

RESULTS

GC-MS analysis

The average percentage of individual compounds of caprifig latex (Jrani variety) hexanic and hexane ethyl acetate (v/v) extracts were presented in Tables 1 and 2, respectively. GC-MS analysis of extracts resulted in the identification of 36 and 17 compounds representing 96.12 and 77.46%, respectively. Among the identified compounds, were sesquiterpens, triterpens, monoterpene (bornanone-3), coumarins and alcans, Furthermore, the most abundant compounds (> 8%) of extracts were lanosta-8 (13.17 and 30.82%), urs-12-en-24-oic acid (21.52 and 22.36%), aristolone (15.63 and 10.30%), olean-12-en-3-ol, (23.47, 3.66%), maragenin I acetate (8.78%) and A'-Neogammacer-22(29)-en-3-ol (22.06%).

Extracts cytotoxicity assay

The optical densities obtained after spectrophotometric measurement at 540 nm, were useful to determine the percentage of cellular viability. Only the concentrations having a little or no cytotoxic effect on the cells (cellular viability from 90 to 100%) were tested for antiviral activity.

Effect of hexanic extract (P2)

The observation of the cells state under a microscope after 48 h of contact with the extract and incubated at 37°C did not show any CPE. We note that the percentage of cellular viability is higher than 90% at all concentrations of the extract. We can deduce that P2 does not have any cytotoxic effect on the cells. All tested concentrations were used thereafter for antiviral activity.

Effects of ethyl acetate-hexane (v/v) extract (P3)

We note that this extract does not have any cytotoxic effect on the Vero cells. Thus, all the concentrations were tested for antiviral activity.

We could demonstrate for these tested extracts that all concentration range of the pure extract and the different dilutions were tolerable by the cells and gave a percentage of viability higher than 90%. From the cytotoxicity results of the tested extracts towards the Vero cells, we understand that a cellular fall, in the presence of an extract and of a virus was really due to the cytopathic effect CPE of the virus and not to the cytotoxic effect of the extract.

Activity against HSV-1, ECV-11 and ADV

The results of the three antiviral tests which were carried out against HSV-1, ECV-11 and ADV are presented in Table 3 and proved that P2 and P3 extracts had an antiviral activity by deteriorating the integrity of the virus and to prevent it, from carrying out its infectious course. These extracts acted on the cellular receivers and prevented the adsorption and the virus penetration in the cell and blocked the viral replication in the infected cell. It is noticed, that these positive tests which were obtained in all concentration range starting from the pure extracts to the highest dilution (1/5120).

DISCUSSION

Infectious diseases are leading cause of death world wide due to multidrug resistant strains of viruses. Many medicinal plants remain unexplored; screening of antiviral resistance modifying compounds from plants sources are expected to provide the basis for identifying leads for the isolation of therapeutically useful compounds. This study will definitely open scope for future utilization of the waste products for therapeutic purpose.

The contact type of the first antiviral activity test showed the competition between extracts and viruses on the cellular receivers. If an extract is able to stick to the cellular receivers, it can prevent the adsorption and thus the penetration of the virus on the cells. In this case, the viral CPE will be absent and the antiviral activity of the extract in question will be proved. Among the studied latex extracts P2 and P3 proved to be active against three viral strains (HSV-1, ECV-11 and ADV). The sensitivity of HSV-1 against natural substances was also showed by Beloin et al. (2005) by testing various extracts of *Momordica charantia* (Cucurbitaceae).

In the second test the virus had sufficient time to be adsorbed and penetrated in the cell. Thus, the absence of the viral CPE proved that the extract acted to prevent viral replication progress within the cell therefore, proving the antiviral activity of this extract. Both extracts P2 and P3 expressed an antiviral activity against these three tested strains. Thereafter, these extracts were able to inhibit simultaneously, the DNA viruses HSV-1 and ADV and ARN viruses ECV-11 replication. Cos et al. (2002) studied the antiviral activity of *Colenrs kilimandschari* and *Leonotis neptaeflia* extracts witch belongs to Lamiacées family. Only *C. kilimandschari* leaves extract showed an anti-HSV-1 capacity. The type contact of the third test showed no viral CPE, means that viruses were already inhibited, and showed the antiviral activity.

These tests demonstrate that, ECV-11, HSV-1 and ADV were sensitive to both extracts. Grazia et al. (2003) studied the antiviral activity of essential oils from eight (Lamiaceae) plants of which *Hyptes mutabilis* did not detect any anti-HSV-1 activity. Therefore the studied extracts P2 and P3, were able to stop the replication cycles of HSV-1, ECV-11 and ADV by preventing their adsorption and their penetrations in the cells (by interaction with the cellular receivers designed for these viruses), or by inhibition of their intracellular replication, or by virus inhibition after a direct contact. These results

Table 1. Chemical composition of hexane extract obtained by GC/MS.

Retention time (min)	Area (%)	Constituent	Wiley library Reference No	CAS No	Quality
4.70	0.06	GERMACRENE-D	121792	023986-74-5	98
5.04	0.01	Delta-Cadinene	121465	000483-76-1	97
7.03	0.33	2, 6, 10-Dodecatrien-1-ol	148283	004602-84-0	83
8.88	0.05	Dodecan-2-on	93122	006175-49-1	72
9.09	0.38	Hexadecanoic acid	213894	000112-39-0	98
10.73	0.33	CIS-LINOLEIC ACID METHYL ESTER	243137	000112-63-0	99
11.69	0.08	Pentadecane	134011	000629-62-9	96
11.88	0.04	1, E-8, z-10-Hexadecatriene	145418	080625-33-8	87
12.76	0.03	Tricosane	275685	000638-67-5	96
13.90	0.13	Triacontane	34922	000638-68-6	91
14.51	0.02	8-dimethoxynaphthalene	120716	105372-17-6	90
15.76	0.51	Bis (2-ethylhexyl) phthalate	326908	000117-81-7	91
16.30	0.05	Hexacosane	311168	000630-01-3	94
16.72	0.06	7-Pentadecyne	128099	022089-89-0	64
17.55	0.13	Heptacosane	32679	000593-49-7	95
18.79	0.10	Octacosane	329269	000630-02-4	96
19.23	0.33	Squalene	337959	007683-64-9	93
20.05	0.13	Nanocosane	337002	000630-03-5	97
20.61	0.37	Oxirane	345735	007200-26-2	89
21.73	0.29	1-ethyl-3-acetyl-5	182541	112482-88-9	86
22.51	0.72	4-methoxycarbonyl-2, 6-diphenylpyridine	237065	069209-39-8	74
22.98	1.04	Bornanone-3	49880	013854-85-8	70
23.38	0.19	1-ethyl-3-acetyl-5	182541	112482-88-9	78
23.54	0.15	1, 6, 10, 14, 18, 22-Tetracosahexaen-3-ol	345737	054159-46-5	64
23.67	1.02	[3.2] metacyclophane-10-ene	145464	121733-15-1	91
24.79	0.77	5-HYDROXY-6	236998	063955-63-5	78
27.96	0.98	9, 19-Cyclolanost-24-en-3-ol	345634	000469-38-5	93
28.96	0.94	Beta.-Amyrin	345611	000559-70-6	83
29.47	13.17	Lanosta-8	360770	002671-68-3	96
30.20	0.24	6-Aza-B-homo-5.alpha.-cholestane	345497	066233-39-4	92
30.25	0.37	Lupeol	345599	000545-47-1	87
32.33	21.52	Urs-12-en-24-oic acid	360707	020475-86-9	91
32.75	15.63	Aristolone	141923	006831-17-0	86
32.95	23.47	Olean-12-en-3-ol	360750	001616-93-9	93
33.38	8.78	Maragenin I acetate	360711	071545-16-9	89
33.62	2.32	alpha.-amyrenyl acetate	360746	000863-76-3	64

Table 2. Chemical composition of ethyl acetate extract obtained by GC/MS.

Retention time (min)	Area (%)	Constituent	Wiley library Reference No	CAS No	Quality
8.424	0.10	(+)-Aromadendrene	121608	000489-39-4	96
8.767	0.19	delta.-Cadinene	121454	000483-76-1	99
9.174	1.08	1H-Cycloprop[e]azulen-7-ol	145016	006750-60-3	95
10.398	0.72	Tetradecanal	133817	000124-25-4	81
10.867	0.30	Hexadecanoic acid	213911	000112-39-0	93
11.297	1.24	1H-Naphtho[2,1-b]pyran	238701	001227-93-6	94
11.531	1.06	Heptadecene-(8)-carbonic acid-(1)	228686	000000-00-0	90

Table 2. Contd.

19.616	30.75	Lanosta-8	360770	002671-68-3	95
19.879	1.17	Silicone grease	392047	000000-00-0	58
20.331	10.30	Olean-12-en-3-ol	360750	001616-93-9	93
21.024	19.80	alpha.-Amyrenyl acetate	360746	000863-76-3	76
22.597	10.82	A'-Neogammacer-22(29)-en-3-ol	360754	002085-25-8	86

Total area: 77.5%.

Table 3. Antiviral activity of *Ficus carica* extracts.

Extracts		Antiviral activity (%)	
		P2	P3
Extracts last dilution		**1/5120**	**1/2560**
	HSV	100	100
(E+V) + C	ECV	100	100
	ADV	100	100
	HSV	100	100
(C+E) + V	ECV	100	100
	ADV	100	100
	HSV	100	100
(C+V) + E	ECV	100	100
	ADV	100	100

P2: hexanic extract; P3: (v/v) ethyl acetate-hexane extract; (E+V) + C: extract effect against virus; (C+E) + V: extract effect against Vero cells; (C+V) + E: extract effect against viral cycle after adsorption.

confirmed the evidence that, P2 and P3 extracts had an interesting antiviral activity.

ACKNOWLEDGEMENTS

The authors are grateful to Pr. BEN OUADA Hafed Directeur de l'Institut Supérieur des Sciences Appliquées et de Technologie de MAHDIA.

REFERENCES

Abu-Mustafa E A, El Tawil BAH, Fayez MBE (1964). Constituents of local plants IV. Phytochemistry., 3: 701-703.

Athnasios AK, El Kholy, Soliman G, Shaban MAM (1962). Constituents of the leaves of *Ficus carica* L. part I. Isolation of psoralen, Bergapten,i-taraxastorol and -sitosterof. Int. J. Chem. Soc., 62: 4253-4254.

Beloin N, Gbassor M, Akpagana K, Hudson J, de Soussa K, Koumagolo K, Arnason JT (2005). Ethnomedicinal uses of *Momordica charantia* (Cucurbitaceae) in Togo and relation to its phytochemistry and biological activity. J. Ethnopharmacol., 96: 49-55.

Chiang LC, Chiang W, Chang MY, Ng LT, Lin CC (2002). Antiviral activity of *Plantago major* extracts and related compounds *in vitro*. Antiviral Res., 55: 53-62.

Cos P, Hermans N, De Bruyne T, Apers S, Sindambiwe JB, Vanden Berghe D, Pieters L, Vlietinck AJ (2002). Further evaluation of Rwandan medicinal plant extracts for their antimicrobial and antiviral activities. J. Ethnophamacol., 79: 155-163.

De-Amorin A, Borba HR, Carauta J P, Lopes D, Kaplan MA (1999). Anthelmintic activity of the latex of *Ficus* species. J. Ethnopharmacol., 64: 255-258.

Doughari JH (2006). Antibacterial activity of *Tamarindus indica* Linn. Trop. J. Pharm. Res., 5: 597-603.

Ghazanfar S (1994). A. Handbook of Arabian Medicinal Plants; CRC Press: Boca Raton., pp. 148.

Grazìa CC, Talarico L, Almeida N, Colombres S, Duschatzky C, Damonte EB (2003). Virucidal activity of essential oils from aromatic plants of San Luis, Argentina. Phytother. Res., 17: 1073-1075.

Kirthikar KR, Basu BD (1996). Indian medicinal plants. Vol. 3, 2nd ed, India: International Book Distributors, pp. 2329-2331.

Lorenz RJ, Bögel K (1973). Laboratory techniques in rabies. In Kaplan MM, Koprowski H (eds), Laboratory Techniques and Virology, World health Organization, Genova, pp. 321.

Meyer JJM, Afoloyan AJ, Taylor MB, Enelbrecht L (1996). Inhibition of herpes simplex virus type 1 by aqueous extracts from shoots of *Helichrysum aureonitens* (Asteracae). J. Ethnophamacol., 52: 41-43.

Ooi LSM, Wang H, Luk C-W, Ooi VEC (2004). Anticancer and antiviral activities of Youngia japonica (L.) DC (Asteraceae, Compositae). J. Ethnopharmacol., 94: 117-122.

Perez C, Canal JR, Campillo JE (1999). Hypotriglyceridaemic of *Ficus carica* leaves in experimental hypertriglyceridaemic rats. Phytother. Res., 13: 188-191.

Sarang B, Anpurna K, Beenish K, Sheikh FA, Suri KA, Satti NK,

Musarat A, Qazi, GN (2005). Immunosuppressive properties of an ethyl acetate fraction from *Euphorbia royleana*. J. Ethnopharmacol., 99: 185-192.
Serrentino J (1991). How Natural Remedies Work. Harley and Marks Publishers, Point Robert, WA. pp. 20-22.
Storey WB, Enderund JE, Saleeb WS, Nauer EM (1977). The Fig *(Ficus carica* Linnaeus). Its Biology, History, Culture and Utilisation. Jurupa Mountains Cultural Centre, California USA.
Tous J, Ferguson L (1996). Mediterranean fruits. In J. Janick (Ed.), Progress in new crops. Arlington, VA: ASHS Press. pp. 416–430.
Tshikalange TE, Meyer JJM, Hussein AA (2005). Antimicrobial activity, toxicity and the isolation of a bioactive compound from plants used to treat sexually transmitted diseases. J. Ethnopharmacol., 69: 515-519.
Ullman SB (1952). The inhibitory and necrosis- inducing effects of the latex of *Ficus carica* L. on transplanted and spontaneous tumours. Exp. Med. Surg., 10: 26-49.
Ullman SB, Clark GM, Roan KM (1952). The effects of the fraction R3 of the latex of *Ficus carica* L. on the tissues of mice bearing spontaneous mammary tumors. Exp. Med. Surg., 10:287-305.
Ullman SB, Halberstaedter L, Leibowitz J (1945). Some Pharmacological and Biological Effects of the Latex of *Ficus carica* L. Exp. Med. Surg., 3: 11-23.
Valiathan MS (1998). Healing plants. Curr. Sci., 75: 1122-1127.
Watanabe W, Konno K, Ijichi K, Inonue H, Yokota T, Shigeta S (1994). MTT colorimetric system for the screening of anti-orthomyxo- and anti-paramyxoviral agents. J. Virol. Methods., 48: 257-265.

African Journal of Microbiology Research Vol. 5(32) pp. 5819-5825, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 © 2011 Academic Journals
DOI: 10.5897/AJMR10.684

Full Length Research Paper

Role of the quorum-sensing system in biofilm formation and virulence of *Aeromonas hydrophila*

Weihua Chu[1*], Yan Jiang[2], Liu Yongwang[3] and Wei Zhu[1]

[1]Department of microbiology, School of Life Science and Technology, China Pharmaceutical University, Nanjing, P. R. China, 210009.
[2]Jiangsu Entry-Exit Inspection and Quarantine Buearu, Nanjing P. R. China, 210001.
[3]College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, P. R. China, 210095.

Accepted 28 March, 2011

Aeromonas hydrophila **is a pathogen that causes disease in a wide range of homeothermic and poikilothermic hosts due to its multifactorial virulence. The production of many of these virulence determinants is associated with high cell density, a phenomenon that might be regulated by quorum sensing. The quorum sensing system regulates the expression of several virulence factors in a wide variety of pathogenic bacteria. To investigate the pathogenic role of quorum sensing system in *A. hydrophila*, We constructed an *ahyI* mutant strain of a fish-clinical isolate YJ-1, named** YJ-1∆**AhyI. Compared with the wild-type strain, the *ahyI* mutant strain exhibited a significant decrease of total extracellular virulent activity, and decreased in biofilm formation, intraperitoneal LD_{50} of** YJ-1∆**AhyI were more than 10^9 CFU, about 10^4 times higher than the parent strain. These results suggest that *A. hydrophila* is able to regulate its extracellular virulent factors and biofilm formation by quorum sensing systems, and indicate that disruption of quorum sensing could be a good alternative strategy to combat infections caused by *A. hydrophila*.**

Key words: *Aeromonas hydrophila*, quorum sensing, biofilm, virulence factors.

INTRODUCTION

Aeromonas hydrophila is a ubiquitous Gram-negative bacterium of aquatic environments, which has been implicated as a causative agent of motile aeromonad septicemia in a variety of aquatic animals especially freshwater fish species (Hänninen et al., 1997). It causes gastrointestinal and extraintestinal infections in humans, including septicemia, wound infections, gastroenteritis and peritonitis (Daskalov, 2006). A number of virulence factors have been identified in *A. hydrophila*, such as, adhesins (e.g. pili), S-layers, exotoxins such as hemolysins and enterotoxin, and a repertoire of exoenzymes which digest cellular components such as proteases, amylases, and lipases (Cahill, 1990; Pemberton, 1997).

Quorum sensing (QS) (Fuqua et al., 1994) is a mechanism for controlling gene expression in response to an expanding bacterial population. In many Gram-negative bacteria, the diffusible quorum sensing signal molecule is a member of the N-acylhomoserine lactone (AHL) family (Fuqua et al., 1994; March and Bentley, 2004). Several virulence-associated phenotypes in pathogens have been shown to be controlled by their quorum sensing systems (Winzer and Williams, 2001). These phenotypes include biofilm formation (Croxatto et al., 2002), the production of virulence factors such as proteases (Swift et al., 1997, 1999; Croxatto et al., 2002), haemolysin (Kim et al., 2003), a type III secretion system (Henke and Bassler, 2004), extracellular toxin (Manefield et al., 2000) and a siderophore (Lilley and Bassler, 2000). *A. hydrophila* has been found to have homologues of the *Vibrio fischeri* quorum sensing genes *luxI* and *luxR*, designated *ahyI* and *ahyR* (Swift et al., 1997). In this study, we explored the role of the *ahyRI* dependent QS system of *A. hydrophila* by construction an analysis of the *ahyI* mutant.

We evaluated whether deletion of the *ahyI*

*Corresponding author. E-mail: chuweihua2002@yahoo.com.cn.

Table 1. Characteristics of bacterial strains and plasmids used in this study.

Strains or plasmid	Relevant characteristic(s)	Source or reference
A. hydrophila		
YJ-1	Virulent	Chu, 2001
YJ-1ΔahyI	ahyI mutant strain of YJ-1	This study
YJ-1ΔahyIRC	Complement strain of YJ-1ΔahyI	This study
E. coli		
DH5α	recA gyrA	Laboratory stock
CC118	λpir lysogen of CC118 (Δ(ara-leu) araD ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE(Am) recA1)	Dennis et al., 1998
S17-1	Smr Spr hdsR RP4-2 kan : : Tn7 tet : : Mu, integrated in the chromosome	Simon et al., 1983
C. violaceum		
CV026	double mini-Tn5 mutant derived from *C. violaceum* ATCC31532, Hg^r cviI::Tn5 xylE Km^r, plus spontaneous Sm^r	McClean et al., 1997
Plasmids		
pGEMT-Easy	Cloning vector, Amp^r resistant	Promega
pFS100	Km^r, Pgp704 suicide vector	Rubires et al., 1997
p*ahyI*$^-$	pFS100 harboring with an internal fragment of ahyI gene	This study
pGEMT-ahyIR	harboring a 1972bp DNA fragment containing the ahyIR gene	This study

gene affected biofilm formation, motility, extracellular virulence and the pathogenicity in a fish model of infection.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

The bacteria and plasmids used are listed in Table 1. *Escherichia coli* $DH_{5\alpha}$, plasmid-containing *E. coli* strains, *A. hydrophila* and its derivative strains were grown in LB medium, *E. coli* strains were grown at 37°C, while *A. hydrophila* strains were routinely grown at 28°C, *Chromobacterium violaceum* CV026 was kindly provided by Dr. McLean (Texas State University) and was grown in LB medium at 30°C. Media were solidified with 1.5% (wt/vol) agar as needed. Antibiotics were added as required at the following final concentrations: ampicillin, 100 μg ml^{-1}; kanamycin, 50 μg ml^{-1}.

DNA manipulation

Genomic DNA of *A. hydrophila* YJ-1 was prepared as previously described (Sambrook et al., 1989). Plasmid DNA from *E. coli* was extracted using a plasmid purification kit (Shanghai Shenggong Co. Shanghai) according to the manufacturer's instructions. Taq DNA polymerase and restriction enzymes were obtained from Takara (Takara Bio. Inc., Dalian, China); and incubation conditions were as recommended by the suppliers.

Construction of *A. hydrophila ahyI* mutant

To obtain single defined insertion mutant in gene *ahyI*, we used a method based on the suicide pFS100 (Rubires et al., 1997). Briefle, an internal fragment of the selected gene was amplified by polymerase chain reaction (PCR) using *A. hydrophila* YJ-1 chromosome, ligated into pGEM-Teasy (Promega). Oligonucleotides ahyI-F (5′ -CACGGGCAAAACGTTCATC-3') and ahyI-R (5′ -ACGAGCTTTATCGCTTCCG-3') were used to amplify the internal fragment of *ahyI* gene from *A. hydrophila* YJ-1 by PCR. The PCR product was ligated to pGEM-T vector (Promega) and transformed into *E. coli* DH5α. The internal fragment was recovered by EcoRI restriction digestion, and finally ligated into EcoRI digested suicide plasmid pFS100 plasmid vector. The ligation product was transformed into *E. coli* CC118 (λpir) and selected for kanamycin resistance. The recombinant plasmid was isolated and transformed into the *A. hydrophila* YJ-1 strain to obtain the *ahyI* insertion mutant. The insertion of plasmid on the chromosomes of the mutant was confirmed by PCR with appropriate primers.

Complementation of the *A. hydrophila* YJ-1 *ahyI* mutant

To complement the *ahyI* mutant strain of *A. hydrophila*, a 1972 bp fragment containing *ahyRI* open-reading frame (ORF), including its promoter, was amplified from *A. hydrophila* genomic DNA by using two primers *ahyIR*-F/*SalI* 5'-GGGGTCGACAGCAGCTTGTAATCCAACGC-3' and *ahyIR*-R/*EcolR*I 5'-GGGGAATTCATGAACCGTCCAGCAGAGTGA-3'. The amplified product was ligated into pGMT-Easy vector creating pGEMT-*ahyIR*. pGEMT-*ahyIR* was then introduced into the YJ-1ΔAhyI strain by electroporation. Clones exhibiting resistance to ampicillin (100 μg ml^{-1}) were chosen for further study. The presence of luxS on pGEMT-*ahyI* was confirmed by sequencing. To exclude the possible influence from the vector, the empty vector was electroporated into *A. hydrophila* YJ-1ΔAhyI as a control strain.

AHL bioassays

Chromobacterium violaceum CV026 was used as a biosensor to detect AHL. The AHL detection was applied by cross-streaking test

strains against *C. violaceum* CV026 on nutrient agar plate, in which the purple pigment violacein can be restored in response to the presence of AHL molecules. Briefly, strain CV026 was streaked at the center of the nutrient agar plate, the target bacteria were streaked on the same plate against CV026 line, if the target bacteria have AHL-producing ability, diffusible AHL produced by the target bacteria induces strain CV026 to produce a purple pigment (McClean et al., 1997). *C. violaceum* CV026 (a mini-Tn5 mutant) was used as an indicator strain for the detection of C4 and C6-HSLs.

Motility assay

LB medium containing 0.3% (wt/vol) agar was used to characterize the motility phenotype of wild type (wt) *A. hydrophila* YJ-1 and its *ahyI* mutant strain. The plates were then wrapped with Saran Wrap to prevent dehydration and incubated at 30°C for 12 to 14 h, and the motility was assessed by examining migration of bacteria through the agar from the center towards the periphery of the plate.

Detection of extracellular virulence factors

Some extracellular virulence factors activities were detected by patching bacteria on LB agar plates supplemented with different substrates (Swift et al., 1999). All strains were tested in duplicate, and when results were different, a third experiment was carried out to resolve the discrepancies.

Hemolytic activity was tested on agar base (Oxoid) supplemented with 5% sheep erythrocytes. The culture was streaked onto the plates and incubated at 27℃ for 24 to 36 h, The presence of a clear colourless zone surrounding the colonies indicated β-hemolytic activity. Protease production and proteolytic activity was detected on 1.2% agar plates supplemented with 10% (v/v) sterile skimmed milk (105℃ for 30 min). The cultures were streaked on the skim milk agar plates and incubated at 27°C for 24 to 36 h. Proteolytic strains caused a clearing zone around the colonies. Lipase activity was assayed on 0.5% tributyrin (Panreac,Barcelona, Spain) agar emulsified with 0.2% Triton X-100 and incubated at 27°C for 24 to 36 h. The presence of a transparent zone around the colonies indicated lipase activity. Extracellular nucleases (DNases) were determined on Dnase agar plates (Difco) with 0.005% methyl green. The culture was streaked onto the plates and incubated at 27°C for 24 to 36 h, a pink halo around the colonies indicated nuclease activity.

SDS-PAGE analysis of extracellular proteins

To prepare extracellular proteins, *A. hydrophila* YJ-1 and YJ-1ΔluxS were grown for 15 h and inoculated into 8 ml of fresh LB (1% inoculum). After incubation for 24 h, the cells were removed by centrifugation at 12,000 x g for 5 min and 4 ml of the separated culture supernatant was combined with 800 µl of 10% trichloroacetic acid. After 10 min at room temperature, the mixture was centrifuged and residues were solubilized in sample buffer composed of 62.5 mM Tris hydrochloride (pH 6.8), 10% glycerol, 5% 2-mercaptoethanol, and 2% SDS. The protein samples were analyzed by SDS-PAGE using 8% gel and stained with Coomassie Brilliant Blue G-250.

Morphological changes in epithelioma papillosum cyprini (EPC) cells induced by *A. hydrophila*

Cytotoxicity of *A. hydrophila* strains was assayed with EPC cells. The EPC cells were grown as a monolayer at 25°C in Eagle's minimum essential medium (MEM; Sigma) supplemented with 10% fetal calf serum in a 5% CO_2 atmosphere incubator, and harvested with trypsin ethylenediaminetetraacetic acid. A 900 µl aliquot of the cell suspension was inoculated to each well in a 24 well culture plate. After incubation for 24 h, EPC monolayers were infected with *A. hydrophila* cells (wt and QS mutant) suspended in phosphate-buffered saline (PBS) at a multiplicity of infection (MOI) (number of bacteria per cultured cell) of 1 and incubated for 30 min, after infection, the EPC cells were washed three times with PBS. The cell morphology were examined using an Axiover 25CFL phase-contrast inverted microscope (Carl-Zeiss) at 200 magnifications.

Animal experiments

50±3 g (mean ±SD) *Carassius auratus gibelio* were obtained from a aquaculture farm in Nanjing, Jiangsu Province, P. R. China. The *C. auratus gibelio* were kept in 100 L tanks supplied with aerated fresh water and fed with commercial pelleted diet twice a day. The water temperature was kept at (25±1)°C. Before manipulation, the fish were anesthetized with 1:15,000 tricaine methane sulfonate MS-222 (Sigma) in water. For 50% lethal dose (LD50) determinations, six groups of 10 fish were intraperitoneally (i.p.) injected with 0.1 ml of washed culture of *A. hydrophila* YJ-1 and of *A. hydrophila ahyI* mutant, emulsified in sterile phosphate-buffered saline containing 10^3 to 10^9 CFU. The fish were observed for 7 days, and any dead specimen was removed for routine bacteriological examination. The experiment was carried out three times in duplicate, and the LD_{50} was calculated by the statistical approach of Reed and Muench (1938).

Biofilm assay

A quantitative biofilm formation experiment was performed in a microtiter plate as described previously (O'Toole and Kolter, 1998), with minor modification. Briefly, bacteria were grown on LB agar, and several colonies were gently re-suspended in LB (with or without the appropriate antibiotic); 100 µl aliquots were placed in a microtiter plate (polystyrene) and incubated 48 h at 28°C without shaking. After the bacterial cultures were poured out, the plate was washed extensively with water, fixed with 2.5% glutaraldehyde, washed once with water, and stained with a 0.4% crystal violet solution. After solubilization of the crystal violet with ethanol-acetone (80:20, vol/vol) the absorbance at 570 nm was determined using a microplate reader (Bio-Rad, Hercules, Calif.).

Statistical analysis

For animal studies, statistical analyses were performed using Fisher's exact test. For all other studies, Student's t test was used.

RESULTS

Characterization of *ahyI* mutant strain of *A. hydrophila* YJ-1

An *ahyI* mutant strain YJ-1ΔAhyI was constructed with a deletion of 147 bp of *ahyI* (GenBank accession no.X89469). The successful mutant of the *ahyI* gene was confirmed by PCR and DNA sequencing (data not shown). The CV026 bioassay revealed that the YJ-

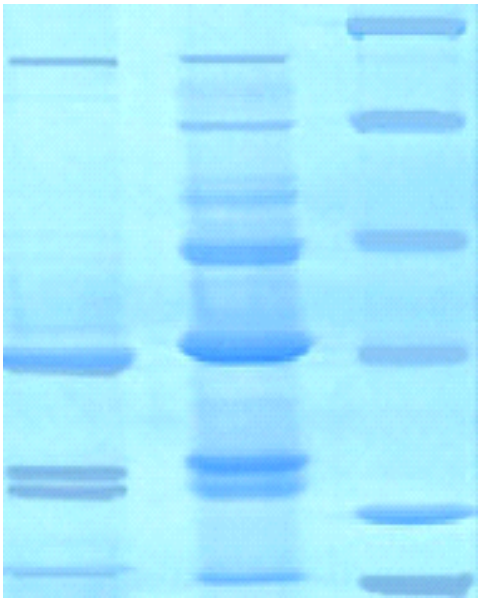


Figure 1. SDS–PAGE analysis of extracellular proteins of *A. hydrophila* strains. Lane 1, YJ-1ΔAhyI; lane 2, YJ-1, lane 3 Molecular weight markers.

1ΔAhyI does not produce AHL signal molecules (data not shown). The motility of YJ-1ΔAhyI was significantly less compared to that of the WT or the complemented strain. These results suggested that the quorum sensing system played an important role in *A. hydrophila* motility. The deletion of the *ahyI* gene did not alter bacterial growth kinetics over a tested period of 24 h. The analysis of extracellular enzyme activities revealed that the *ahyI* mutant could not produce the detectable extracellular proteases, haemolysin, amylase and Dnase, while *A. hydrohila* YJ-1 had a high level of extracellular enzyme activities. The extracellular protein profiles of YJ-1 and YJ-1ΔAhyI were surveyed by SDS–PAGE analysis (Figure 1). Compared with the case of YJ-1, many protein bands were decreased clearly in YJ-1ΔAhyI.

Morphological changes of EPC cells induced by *A. hydrophila* ECP

The cytotoxicity of *A. hydrophila* strains against *A. hydrophila* were EPC cells was further assessed, upon incubated with YJ-1, the ECP cells underwent a series of morphological changes. An monolayer EPC cells incubated with YJ-1ΔAhyI appeared as a smooth sheet with the cells adhere tightly to the neighbors, while incubated with YJ-1, the cells first became slightly detached from one another, the smooth appearance was lost and then large holes separated cells, and last the cells became rounded and the spindle connections were lost (Figure 2).

Role of quroum sensing in biofilm formation of *A. hydrophila*

Biofilm formation of *A. hydrophila* wild-type and *ahyI* mutant strain, YJ-1ΔAhyI was monitored in microtiter plates. As shown in Figure 3, biofilm formation of YJ-1ΔAhyI was significantly decreased, compared with that of wild-type strain YJ-1, while the complemented strain of YJ-1ΔAhyI, YJ-1ΔAhyIC can form biofilm. Thus, quorum sensing has a distinct influence on biofilm formation in *A. hydrophila.*

Fish infection

To ascertain the role of quorum sensing system in the pathogenesis of *A. hydrophila*, the LD50 was determined for *A. hydrophila* YJ-1 and YJ-1ΔAhyI by intraperitoneal challenge of *C. auratus gibelio*. As showed in Table 2, the LD50 values were more than 1.0 x 10^9 CFU bacteria for YJ-1ΔAhyI and 6 x 10^5 CFU bacteria for wild-type respectively. Fish injected with the parental strain died more rapidly than those injected with YJ-1ΔAhyI. All recorded deaths occurred within 4 days when the fish were injected with the wild type; however, deaths were recorded up to 6 days following injection when the fish were injected with YJ-1ΔAhyI. The *ahyI* mutation led to a significant decrease in strain virulence, indicating that quorum sensing system has a role in the pathogenic mechanism of *A. hydrophila*.

Examination of mortality showed typical clinical signs of hemorrhagic septicemia, mainly external lesions (abdominal distension at the injection site) and internal hemorrhages. To confirm stability of the insertional inactivated *ahyI* mutant gene, bacteria were isolated from dead fish inoculated with YJ-1ΔAhyI, all conferring a Kan phenotype.

DISCUSSION

In animal and plant pathogens, such as *Agrobacterium tumefaciens*, *Erwinia chrysanthemi*, *Pseudomonas aeruginosa*, and *Vibrio anguillarum*, AHL systems control the expression of a number of exported products that are proven or putative virulence factors. For example, quorum sensing through AHLs has been shown to be involved in biofilm formation (Kjelleberg and Molinm, 2002), competitive or cooperative bacterial interactions (Keller and Surette 2006) and virulence factors secretion. As described earlier, *A. hydrophila* produces a wide range of virulence factors. These virulence factors are expressed differently, depending on environmental and metabolic aspects of its current habitat. The regulation of many of these virulence factors is based on cell density-dependent cell-to-cell signaling, termed quorum sensing (Lynch et al., 2002; Bi et al., 2007; Khajanchi et al.,

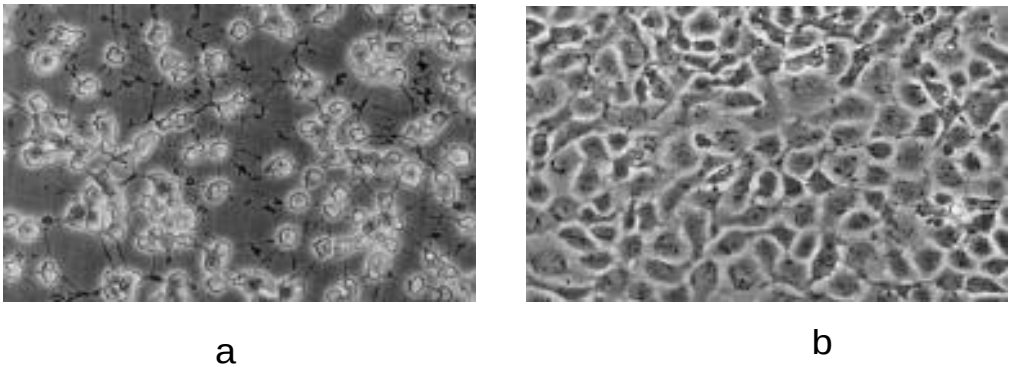


Figure 2. Micrographs of EPC cells infected with *A. hydrophila* YJ-1 (a) and YJ-1ΔAhyI (b) at 5 h post infection.

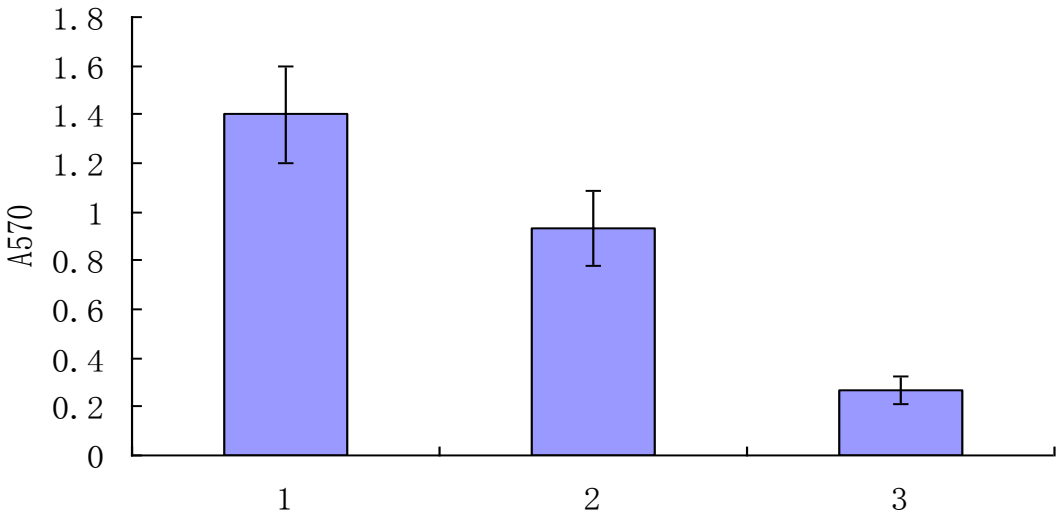


Figure 3. The amount of biofilm formation for each strain was quantified by solubilizing the stained biofilm with ethanol : acetone and measuring the OD_{570}. Each strain was tested in quadruplicate at each time point. Error bars indicate standard deviation. Lane 1, wide type Ah YJ-1, lane 2, YJ-1ΔAhyIRC, lane 3, YJ-1ΔAhyI. The results are representative of three experiments.

Table 2. Calculations of LD_{50} strain YJ-1 and the *ahyI* mutant.

Bacteria CFU/0.1 ml	Mortality (no. dead/no. total)		Day of death (no. dead/no. total)	
	YJ-1	*AhyI* mutant	YJ-1	*AhyI* mutant
10^9	10/10	1/10	1(5/10) 2(8/10) 3(10/10)	6(1/10)
10^8	10/10	1/10	1(5/10) 2(7/10) 4(10/10)	6(1/10)
10^7	8/10	0/10	1(4/10) 2(7/10) 4(8/10)	NA*
10^6	6/10	0/10	1(3/10) 3(5/10) 4(6/10)	NA*
10^5	3/10	0/10	3(4/10) 4(6/10)	NA*
10^4	0/10	0/10	NA*	NA*
10^3	0/10	0/10	NA*	NA*
control	0/10		NA*	
LD value(CFU/ml)	6 x10^5	>10^9		

*NA, not applicable: no death due to *A. hydrophila* infection during the experiment.

2009). To explore the role of quorum sensing system in regulating the extracellular virulent factors secretion and biofilm formation, we constructed an *ahyI* mutant strain of a fish-clinical isolate YJ-1, named YJ-1ΔAhyI. Inactivation of the *ahyI* gene of *A. hydrophila* did not result in noticeable changes in growth patterns compared with those of the wild-type strain. This finding indicates that *ahyi* has no significant effect on basic cellular metabolic processes required for growth of *A. hydrophila in vitro*. This is in contrast to some other bacteria in which *luxS* had an effect on growth, Lyon et al. (2001) reported that disruptions of *Streptococcus pyogenes, s luxS* shown a media-dependent growth defect, and the effect of quorum sensing on *Vibrio harveyi* growth rate can be either positive or negative (Nackerdien et al., 2008).

Decreased virulence has been seen in ΔluxS mutants of several pathogenic bacteria (Winzer and Williams, 2001). A *Vibrio cholerae luxO* mutant is severely defective in colonization of the small intestine in an infant mouse model, inactivation of the *rhlA* gene in *P. aeruginosa* prevents rhamnolipid production, disabling the protection against polymorphonuclear leukocytes (Van et al., 2009), and quorum sensing is necessary for the virulence of *P. aeruginosa* during urinary tract infection (Kumar et al., 2009), and *Vibrio alginolyticus* luxO-luxRval regulatory system control the expression of alkaline serine protease (Rui et al., 2009). In contrast, the *S. epidermidis luxS* mutant shows increased virulence in a model of catheter-associated infection. Most likely, the increased virulence may be partly attributed to the increased synthesis of PIA and more-intense biofilm formation. In this study, virulence factors were detected by patching bacteria on LB agar plates supplemented with different substrates, and the results shown that the virulence factors were decreased in the *ahyI* mutant strain, and on the PAGE, many proteins bands were lost or decreased, this phenomena suggest that QS control the extracellular proteins production, these band not only include the virulence factors, this results is consistent with the report on the protease.

The biofilm formation has been documented as survival strategy of pathogens, regulation of biofilm formation by quorum sensing systems has been shown in a number of bacteria. *In vitro* biofilm formation in *A. hydrophila* has been demonstrated using crystal violet staining assays as well as SEM. We could detect the difference between the parent strain and the QS mutant. Our data showed that the *ahyI* mutant strain was unable to develop a complete biofilm. This effect on biofilm formation by *luxS in vitro* was also observed in *Streptococcal* (Cvitkovitch et al., 2003), *Streptococcus* (Kong et al., 2006) and *V. cholerae* (Waters et al., 2008), while the luxS mutant of *Edwardsiella tarda* (Xiao et al., 2009) and *Streptococcus mutans* (Huang et al., 2009) were considerably increased biofilm formation. Thus, the quorum sensing signaling molecules have contrasting effects on biofilm formation in different strains.

In conclusion, we show quorum sensing system in *A. hydrophila* is functional for the secretion of extracellular virulence factors, the formation of mature biofilm and its pathogenicity, and these findings indicate that disrupt quorum sensing systems of pathogenic bacteria is a promising alternative for antibiotics in fighting bacterial infections.

ACKNOWLEDGEMENTS

This work was supported by State Administration for Entry and Exit Inspection and Quarantine of P. R. China. The authors are much indebted to Dr. RJC McLean, Department of Biology, Texas State University, for generous provision of the AHL bioassay strain and for his helpful comments on the manuscript.

REFERENCES

Bi ZX, Liu YJ, Lu CP (2007). Contribution of AhyR to virulence of *Aeromonas hydrophila* J-1. Res. Vet. Sci., 83: 150-156.

Cahill MM (1990). Virulence factors in motile *Aeromonas* species: a review. J. Appl. Bacteriol., 69: 1-16.

Chu WH (2001). Studies on the pathology and control of the bacterial hemorrhage disease in a hybrid crussian carp. Reservoir Fisheries, 21: 40.

Croxatto A, Chalker VJ, Lauritz J, Jass J, Hardman A, Williams P, Camara M, Milton DL (2002). VanT, a homologue of *Vibrio harveyi* LuxR, regulates serine, metalloprotease, pigment, and biofilm production in *Vibrio anguillarum*. J. Bacteriol., 184: 1617–1629.

Cvitkovitch DG, Li YH, Ellen RP (2003). Quorum sensing and biofilm formation in *Streptococcal* infections. J. Clin. Invest., 112: 1626-1632.

Daskalov H (2006). The importance of *Aeromonas hydrophila* in food safety. Food Control, 17: 474-483.

Dennis JJ, Zylstra GJ (1998). Plasposons: Modular self-cloning minitransposon derivatives for rapid genetic analysis of Gram-negative bacterial genomes. Appl. Environ. Microbiol., 64: 2710-2715.

Fuqua WC, Winans SC, Greenberg EP (1994). Quorum sensing in bacteria-the LuxR-LuxI family of cell density-responsive transcriptional regulators. J. Bacteriol., 176: 269–275.

Hänninen ML, Oivanen P, Hirvelä-Koski V (1997). *Aeromonas* species in fish, fish-eggs, shrimp and freshwater. Int. J. Food Microbiol., 34: 17-26.

Henke JM, Bassler BL (2004). Quorum sensing regulates type III secretion in *Vibrio harveyi* and *Vibrio parahaemolyticus*. J. Bacteriol., 186: 3794–3805.

Huang Z, Meric G, Liu Z, Ma R, Tang Z, Lejeune P (2009). luxS-based quorum-sensing signaling affects Biofilm formation in *Streptococcus mutans*. J. Mol. Microbiol. Biotechnol., 17: 12-19.

Keller L, Surette MG (2006). Communication in bacteria: an ecological and evolutionary perspective. Nat. Rev. Microbiol., 4: 249–258.

Khajanchi BK, Sha J, Kozlova EV, Erova TE, Suarez G, Sierra JC, Popov VL, Horneman AJ, Chopra AK (2009). N-Acylhomoserine lactones involved in quorum sensing control the type VI secretion system, biofilm formation, protease production, and in vivo virulence in a clinical isolate of Aeromonas hydrophila. Microbiol. 155: 3518-3531.

Kim SY, Lee SE, Kim YR, Kim CH, Ryu PY, Choy HE, Chung SS, Rhee JH (2003). Regulation of *Vibrio vulnificus* virulence by the LuxS quorum-sensing system. Mol. Microbiol., 48: 1647–1664.

Kjelleberg S, Molin S (2002). Is there a role for quorum sensing signals in bacterial biofilms? Curr. Opin. Microbiol., 5: 254–258.

Kong KF, Cuong V, Michael O (2006). *Staphylococcus* quorum sensing in biofilm formation and infection. Int. J. Med. Microbiol., 296: 133-139.

Kumar R, Chhibber S, Harjai K (2009). Quorum sensing is necessary for the virulence of *Pseudomonas aeruginosa* during urinary tract infection. Kidney Int., 76: 286-292.

Lilley BN, Bassler BL (2000). Regulation of quorum sensing in *Vibrio harveyi* by LuxO and sigma-54. Mol. Microbiol., 36: 940–954.

Lynch MJ, Swift S, Kirke DF, Keevil CW, Dodd CER, Williams P (2002). The regulation of biofilm development by quorum sensing in *Aeromonas hydrophila*. Environ. Microbiol., 4: 18-28.

Lyon WR, Madden JC, Levin JC, Stein JL, Caparon MG (2001). Mutation of luxS affects growth and virulence factor expression in *Streptococcus pyogenes*. Mol. Microbiol., 42: 145–157.

Manefield M, Harris L, Rice SA, de Nys R, Kjelleberg S (2000). Inhibition of luminescence and virulence in the black tiger prawn (Penaeus monodon) pathogen *Vibrio harveyi* by intercellular signal antagonists. Appl .Environ. Microbiol., 66: 2079–2084.

March JC, Bentley WE (2004). Quorum sensing and bacterial cross-talk in biotechnology. Cur. Opin. Biotechnol., 15: 495–502.

McClean KH, Winson MK, Fish L, Taylor A, Chhabra SR, Camara M, Daykin M, Lamb JH, Swift S, Bycroft BW, Stewart GS, Williams P (1997). Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. Microbiol., 143: 3703–3711.

Nackerdien ZE, Keynan A, Bassler BL, Lederberg J, Thaler DS (2008). Quorum Sensing Influences *Vibrio harveyi* Growth Rates in a Manner Not Fully Accounted For by the Marker Effect of Bioluminescence. PLoS ONE 3(2): e1671. doi:10.1371/journal.pone.0001671.

O'Toole GA, Kolter R (1998). Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol. Microbiol., 30: 295-304.

Pemberton JM, Kidd SP, Schmidt R (1997). Secreted enzymes of Aeromonas. FEMS Microbiol Lett. 152: 1-10.

Penfold RJ, Pemberton JM (1992). An improved suicide vector for construction of chromosomal insertion mutations in bacteria. Gene., 118: 145–146.

Reed LJ, Muench H (1938). A simple method of estimating fifty percent endpoints. Am. J. Hyg., 27: 493–497.

Rubires X, Saigí F, Pigué N, Climent N, Merino S, Albertí S, Tomás JM, Regué M (1997). A gene (*wbbL*) from *Serratia marcescens* N28b (O4) complements the *rfb-50* mutation of *Escherichia coli* K-12 derivates. J. Bacteriol., 179: 7581-7586.

Rui HP, Liu Q, Wang QY, Ma Y, Liu H, Shi CB, Zhang YX (2009). Role of alkaline serine protease, Asp, in *Vibrio alginolyticus* virulence and regulation of its expression by LuxO-LuxR regulatory system. J. Microbiol. Biotechnol., 19: 431-438.

Sambrook J, Fritsch E, Maniatis T (1989). Molecular Cloning: A Laboratory Manual, 2nd Ed, Cold Spring Harbor Laboratory, Press Cold Spring Harbor, New York, U.S.A.

Simon R, Priefer U, Pühler A (1983). Vector plasmids for *in vivo* and *in vitro* manipulations of Gram-negative bacteria. In *Molecular Genetics of the Bacteria–Plant Interaction*, pp. 98–106. Edited by A. Pühler. Heidelberg: Springer.

Swift S, Karlyshev AV, Fish L, Durant EL, Winson MK, Chhabra SR, Williams P, Macintyre S, Stewart GS (1997). Quorum sensing in *Aeromonas hydrophila* and *Aeromonas salmonicida*: identification of the LuxRI homologs AhyRI and AsaRI and their cognate N-acylhomoserine lactone signal molecules. J. Bacteriol., 179: 5271-5281.

Swift S, Lynch MJ, Fish L, Kirke DF, Tomas JM, Stewart GSAB, Williams P (1999). Quorum sensing-dependent regulation and blockade of exoprotease production in *Aeromonas hydrophila*. Infect Immun., 67: 5192–5199.

Van GM, Christensen LD, Alhede M, Phipps R, Jensen PØ, Christophersen L, Pamp SJ, Moser C, Mikkelsen PJ, Koh AY, Tolker-Nielsen T, Pier GB, Høiby N, Givskov M, Bjarnsholt T (2009). Inactivation of the rhlA gene in *Pseudomonas aeruginosa* prevents rhamnolipid production, disabling the protection against polymorphonuclear leukocytes. APMIS., 117: 537-546.

Waters CM, Lu W, Rabinowitz JD, Bassler BL (2008). Quorum sensing controls biofilm formation in *Vibrio cholerae* through modulation of cyclic di-GMP levels and repression of vpsT. J. Bacteriol., 190: 2527-2536.

Winzer K, Williams P (2001). Quorum sensing and the regulation of virulence gene expression in pathogenic bacteria. Int. J. Med. Microbiol., 291: 131-143.

Xiao J, Wang Q, Liu Q, Xu L, Wang X, Wu H, Zhang Y (2009). Characterization of *Edwardsiella tarda* rpoS: effect on serum resistance, chondroitinase activity, biofilm formation, and autoinducer synthetases expression. Appl. Microbiol. Biotechnol., 83: 151-60.

African Journal of Microbiology Research Vol. 5(32), pp. 5826-5830, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.214

Full Length Research Paper

Detection of H9N2 avian influenza virus in various organs of experimentally infected chickens

Somayeh Asadzadeh Manjili[1], Iradj sohrabi Haghdoost[1], Pejman Mortazavi[1], Hamid Habibi[2], Hadi Iashini[3] and Esmaeil Saberfar[3]*

[1]Department of veterinary pathology, Faculty of Specialized Veterinary Sciences, Islamic Azad University, Science and Researches Branch, Tehran, Iran.
[2]Department of Veterinary, Saveh, Markazi Province, Iran.
[3]Research Center for applied virology, Baqiyatallah University of Medical Sciences Tehran, IR Iran.

Accepted 30 September, 2011

H9N2 Avian influenza virus (AVI) infection is a major cause of economic losses in poultry industry. Therefore further study to explain the virus pathogenesis is necessary. In this study tissue tropism and dissemination of A/chicken/Iran/11T/99(H9N2) virus in various organs of specific pathogen free (SPF) chickens were investigated. Fifty 2-week-old chickens hatched from SPF eggs were divided randomly into two groups. Forty chicks in the experimental and ten chicks in the control group. Experimental chicks were inoculated intranasally-intraorally with the virus. Samples of lung, trachea, pancreas, thymus, spleen, brain, bursa of fabricius, proventriclus, cloaca and kidney were aseptically collected at 1, 3, 5, 7, 9 and 10 day post inoculation (DPI). A reverse transcriptase polymerase chain Reaction (RT-PCR) test was performed for virus detection. Viral RNA was detected in the respiratory system on days 3, 5 and 7 PI. The virus was also found in the kidney on days 3,5,7,9 PI and in the pancreas on days 3 and 5 PI. Viral RNA was observed only on day 5 PI in cloaca. The virus was not detected in the blood, brain and immune system. The virus was not found from any organs on day 10 PI. These results suggest that H9N2 AIV has tropism for respiratory, digestive and urinary system following intranasal/intraoral inoculation.

Key words: Avian influenza, H9N2, SPF, RT-PCR.

INTRODUCTION

Avian influenza viruses (AIVs) belong to the family orthomyxoviridae and to the type A influenza virus. These viruses are classified into subtypes based on their surface haemagglutinin (H) and neuraminidase (N) glycoproteins. So far, 16 different H subtypes (H1-H16) and 9 different N subtypes have been indentified (Fouchier et al., 2005). According to the pathogenicity of AIV to domestic and wild bird species these viruses are categorized into two pathotype groups including Highly Pathogenic Avian Influenza (HPAI) viruses and non-Highly Pathogenic Avian influenza (nHPAI) viruses (Capua and Alexander, 2006). The H9N2 AIV outbreaks occurred in domestic poultry in Asia and the Middle East since the 1990s, and have caused severe economic losses in many countries. In 1998 an AI outbreak in Iran caused great economic losses in poultry industry and an non-highly pathogenic avian influenza virus (H9N2) has been reported as causative agent (Pourbakhsh et al., 2000). H9N2 AIVs induce significant troubles for the poultry industry in Iran due to decreased production, increased mortality and cost of vaccination. Avian influenza disease due to H9N2 subtype has been markedly common during 1994 to 1999 in many parts of the world (Vasfi Marandi and Bozorgmehrifard, 2002). H9N2 influenza viruses are also discussed to be one of the potential candidate for the next human widespread epidemic disease (Butt et al., 2005). Experimental infection

*Corresponding author. E-mail: saberfar@yahoo.com. Tel: +98-21-66949644.

in specific pathogen-free (SPF) chickens announced that the H9N2 AIV is not capable to cause pathological lesions, severe clinical signs and mortality by itself (Lee et al., 2007; Pourbakhsh et al., 2000). During outbreaks of non-highly pathogenic AIVs co-infection with other pathogens especially in severe stress conditions may complicate the syndrome and induce sings of respiratory disease and even mortality in field.

Because of widespread incident of the disease and ambiguous behavior of the H9N2 AIV further study to explain the virus pathogenesis is necessary. In a characteristic manner non-highly pathogenic AIVs have been isolated from respiratory exudate and feces of infected birds, and AIV nucleoprotein has been demonstrated in epithelial cells of the intestine, trachea, lungs and air sacs (Shalaby et al., 1994; Swayne et al., 1994). LPAI viruses often need trypsin like enzyme activity to cleave the Hemagglutinin into HA1 and HA2 proteins in order to make the infectious virus particle (Klenk et al., 1975). Hence respiratory and gastrointestinal epithelia that contain these types of enzyme and organs containing epithelial cells like pancreas and kidney are principal places for non-highly pathogenic AIV replication and lesion formation (Klenk et al., 1975; Shalaby et al., 1994). Anyway the pathway of virus distribution into these organs remains ambiguous and it needs more studies to be investigated well. Virus isolation in SPF chickens for identification of AI viruses is time consuming and require specific facilities. Molecular tests like reverse transcription PCR (RT-PCR) are being introduced in order to detection of AIV due to their premium such as rapidity, delicacy and sensitivity (Saberfar et al., 2008). The aim of this study was assessment of the H9N2 virus spreading in various organs of the infected SPF chickens at different days after inoculation. RT-PCR test was performed to diagnose the presence of the virus in different body tissues. It may further help us to investigate the virus pathogenesis.

MATERIALS AND METHODS

Virus strain

The influenza virus A/chicken/Iran/11T/99 H9N2 that was isolated from outbreak among poultry in Iran,was provided by Razi Vaccine and Serum Research Institute (Karaj, Iran). The virus was propagated two times in the allantoic cavity of 9 to 11-day-old embryonated chicken specific pathogen free eggs. Hemagglutination (HA) titers of the viruses ranged from 512 to 1024 HA unit, when tested according to the methods as described previously (Burleson et al., 1992).

SPF chickens

Fifty 2-week-old chickens hatched from SPF eggs were randomly divided in two groups (forty chicks in experimental group and ten chicks in control group). Both groups were housed in same condition in two separate isolated rooms. Feed and water were available ad libitum.

Experimental design

All birds were bled and serologically tested using Hemagglutination inhibition test (HI) (Burleson et al., 1992). They were negative for antibodies to H9N2 influenza virus antigens. Five chickens from treated group were sacrificed and their organs were investigated from virus detection. All of these samples were also negative for virus detection. Subsequently, chickens of the experimental group were inoculated via intranasal/intraoral routes with 120 µl of infectious allantoic fluid containing $10^{7.5}$ EID 50 of the applied virus strain diluted in sterile PBS solution. The control group was received sterile PBS with the same manner. All the birds were monitored daily for 15 days to investigate the changes of antibody titre to H9N2 and mortality. Five chickens from the experimental group and one chicken from the control group were randomly selected on days 1, 3, 5, 7, 9 and 10 post inoculation (PI). They were bled and sacrificed. During this period, all chickens were observed if they have clinical signs of disease or not and observations were recorded. Necropsy was done on sacrificed chickens and all gross lesions were recorded. Samples of lung, trachea, pancreas, thymus, spleen, brain, bursa of fabricius, proventriclus, cloaca and kidney were aseptically collected for virus detection and RT-PCR assay. Blood samples were collected in EDTA tubes. Sera of the birds were also collected at the same days for HI test. All tissue samples were immediately stored at -70º until used.

Serology

Serum samples were collected on the pre-inoculation, first to fifteenth days post inoculation from all chickens and were tested against specific antibodies to H9 antigen by using Haemagglutination Inhibition (HI) test, according to the manual of standards for diagnostic test (OIE, 2008).

Extraction of viral RNA

RNA of blood and tissue samples was extracted using the RNX TM (-Plus) kit (CinnaGen Inc.) according to the manufacturer's protocol. 50 to 100 mg of tissue or 100 µl of blood sample was mixed with 1ml RNX and incubated at room temperature for 5 minutes. After addition of 200 µl chloroform and mixing, the liquid was clarified by centrifugation at 12,000 rpm at 4º for 15 min. The supernatant was transferred into a new tube and mixed with an equal volume of isopropanol followed by centrifugation at 12000 rpm at 4º for 15 min. The pellet was washed with 1ml of 75% ethanol. Finally, the pellet was dissolved in 50 µl of DEPC treated water.

RT-PCR

The cDNA was synthesized using AccuPower RT-Premix kit (BioNeer corporation, South Korea) according to the manufacturer's protocol. The primer sequences are shown in Table 1. 1 µg of total RNA and 20 pmol of each primer were used for cDNA preparation.
PCR was performed to amplify 510 bp fragment of matrix protein gene of influenza virus using the AccuPower PCR PreMix kit (BioNeer Corporation, South Korea).The reaction mixture contained 5 µl cDNA in a final volume 20 µl was subjected to 94ºC for 5 min an 35 cycles of 94°C for 30 s, 49°C for 30 s, 72ºC for 40 s and followed by final extension at 72°C for 5 min. The PCR products were separated by electrophoresis using a 1.5% agarose gel in 1xTBE buffer.

Table 1. RT-PCR Primer Sequences.

Specificity	primers	sequences	size
Influenza A virus	MF	GGC TCT CAT GGA ATG GCT AA	510
Influenza A virus	MR	CTG GCC TGA CTA GCA ACC TC	510

Table 2. H9N2 serum antibody titration (Mean titer) of the test and control groups of chickens experimentally infected with H9N2 AI virus.

DPI	Day 0	Day 1	Day 3	Day 5	Day 7	Day 9	Day 10	Day 13	Day 15
test Group	0	0	0.4	1.4	2.4	3.6	4	6.6	7.4
control Group	0	0	0	0	0	0	0	0	0

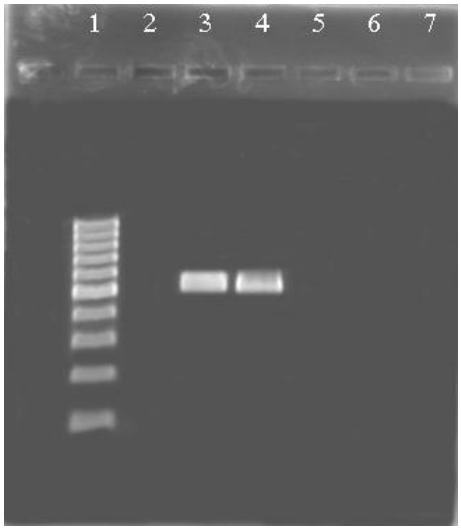


Figure 1. Results of the RT-PCR assay. Amplifying 510-bp segment of M gene of AIV. Lane 1: DNA marker (100-bp), Lane 2: negative control, lane 5,6 and 7: negative samples, lane 3: positive control, lane 4: positive sample.

Amplified products were visualized under ultraviolet light after staining with 0.1 µg /ml ethidium bromide. A 100 base pair ladder was used as a molecular weight marker.

RESULTS

Clinical signs

Daily monitoring did not show any sign of illness in the chickens from control group. The clinical signs observed in the inoculated chickens were depression, facial edema, conjunctivitis, ruffled feather, decrease feed consumption and diarrhoea. Clinical signs were observed from third day post inoculation. On day 7 PI the number of chickens showing clinical signs reduced. The clinical signs disappeared at 12 DPI. No mortality was recorded from each isolate.

Gross Lesions

No gross lesions were observed in the uninfected control group. In experimental chickens the lesions such as mild congestion of the trachea and lungs, hemorrhage in small intestine and pancreas and swollen kidneys were observed.

HI test

There was no evidence of any change in specific antibodies against AIV in pre and post inoculation of control chickens. As shown in Table 2 the mean of antibody titer was increased at 5 DPI and reached to 2^{-8} at 15 DPI in the experimental group.

RT-PCR

The presence of the virus in various organs obtained from the inoculated and control birds at different DPI was determined by RT-PCR test. Tissue samples from 5 different birds that had been taken before inoculation and samples from control group were all negative for virus detection. First positive samples were seen on day 3 PI and the last positive sample was detected on day 9 PI (Figure 1). The virus was detected in the trachea, lungs, pancreas, cloaca and kidney of infected birds during the experiment course. The results of the virus detection are shown in Table 3. The results show that most positive samples were detected on days 5 PI. All brain, blood, thymus, spleen, proventriclus and bursa of fabricius,

Table 3. The results of virus detection from various organs of SPF chickens at different days post inoculation with H9N2 AI virus.

Day PI	Group	Tr	L	Th	Sp	Pr	Cl	Bu	P	K	Br	Bl
1	T	0/5*	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	C	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
3	T	2/5	3/5	0/5	0/5	0/5	0/5	0/5	1/5	2/5	0/5	0/5
	C	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
5	T	2/5	4/5	0/5	0/5	0/5	2/5	0/5	4/5	3/5	0/5	0/5
	C	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
7	T	0/5	2/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5
	C	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
9	T	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5
	C	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
10	T	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	C	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1

T= test group C= control group Tr= Trachea, L= Lung, Th= Thymus, Sp= Spleen, Pr= Proventriclus, Cl= Cloaca, Bu= Bursa of Fabricius, P= Pancreas, K= Kidney, Br= Brain, Bl= Blood. *= No. of positive samples/total samples taken.

samples were negative for virus detection.

DISCUSSION

In this study tissue tropism and dissemination of H9N2 virus throughout the various organs were evaluated following intranasal/intraoral inoculation. The clinical signs and gross lesions found at post mortem examination were alike and milder than lesions reported in naturally infected birds with H9N2 (Pourbakhsh et al., 2000). Although In Some researches (Shalaby et al., 1994; Swayne et al., 1994) as well as present study, inoculation of low pathogenicity AIVs in SPF or broiler chickens have produced absence of mortality, but mortality between 20 and 60% in natural outbreaks have been reported (Naeem et al., 2003; Nili et al., 2003; Vasfi Marandi and Bozorgmehrifard, 2002). Previous studies have shown that the M gene RT-PCR is sensitive and specific method for the detection of influenza A viruses of human and avian origin (Saberfar et al., 2009).

In this study predominant infection in the respiratory organs was observed between days 3 and 7 PI. Detection of the virus from the trachea and lungs indicates that H9N2 AI virus is pneumotropic following intranasal/intraoral inoculation. Repetition of virus recovery in respiratory system was mostly higher for lung tissues. Viral RNA was identified in lung tissue on days 3, 5 and 7 PI. Previous studies (Kwon et al., 2008) have reported that H9N2 viral antigen were detected in the trachea, lungs, thymus, spleen, bursa, cecal tonsils and kidneys of SPF chickens on day 5 PI. We observed infections that are localized to the GI tract at days 3 and 5 PI. The H9N2 virus were detected in the cloaca at day 5 PI and in the pancrease at day 3 and 5 PI. Detect of the H9N2 virus in cloaca only on day 5 PI perhaps originated in temporary replication of the virus in GI tract. Swayne and Halverson reported that LPAI viruses produce infections in respiratory and GI tracts of chickens. AIV nucleoprotein has been identified in epithelial cells of the trachea, lungs, and intestine (Shalaby et al., 1994; Slemons and Swayne, 1995; Swayne et al., 1994). Hablolvarid et al. (2004) detected nucleoproteins of the H9N2 virus in the trachea, lungs and cecal tonsils of experimentally infected 5-week-old SPF chickens using immunoperoxidase assay. We detected H9N2 virus in the pancreas on day 3 and 5 post inoculation. Shinya et al. (1995) reported positive immunoreaction to H5N3 virus antigen in the pancreas of inoculated chicks. Hablolvarid et al. (2003) detected H9N2 virus nucleoproteins in the pancreas of experimentally infected 5-week-old chickens using immunoperoxidase assay following intravenous inoculation. Sometimes LPAIV can spread further than the respiratory and GI tracts, replicate and cause lesions in primarily visceral organs including epithelial cells such as pancreas and kidney (Shalaby et al., 1994; Vasfi Marandi and Bozorgmehrifard, 2002).

Mosleh et al. (2009) showed that A/Chicken/Iran/772/1998 (H9N2) had tissue tropism and pathogenicity for the respiratory system (lung and trachea), immune system (spleen), urinary system (kidneys) and digestive system of commercial broiler

chicks following IN inoculation. We observed predominant infection in the kidney at days 3, 5, 7, and 9 PI. Virus detection in the kidney could indicate virus tropism for the urinary system as previously reported (Shalaby et al., 1994; Swayne and Slemons, 1995; Vasfi Marandi and Bozorgmehrifard, 2002). Swayne and Slemons (1994) reported that LPAIVs were nephrotropic following IV inoculation and pneumotropic following intranasal inoculation, but they did not detect the virus antigens in kidney using immunohistochemistry assay. In other study LPAI virus was not detected in parenchymal cells of the kidneys following IN inoculation (Swayne and Beck, 2005). In previous studies the H9N2 AIV detected from trachea, kidney and lung tissues using indirect immunoperoxidase test (Shamsedini et al., 2002). H9N2 viral antigen was detected from different tissues of experimental infected three-week-old SPF chickens such as spleen, kidney, lung, trachea, thymus, bursa and cecal tonsil (Kwon et al., 2008). However In this study we did not detect the viral RNA in the lymphoid tissues. Viral RNA was not detected in the blood of the chickens in experimental group. Mosleh et al, (2009) did not also detect the virus from blood samples.

Conclusion

Most of the Non Highly Pathogenic AIVs have two basic amino acids at the proteolytic cleavage site of the hemagglutinin protein (Wood et al., 1993) and require cleavage by a trypsin-like enzyme to be infectious and perform multiple virus replication cycles (Klenk et al., 1975). Respiratory and gastrointestinal tracts have this enzyme activity in some cells or lumenal contents and it is responsible for the GI and respiratory tracts being primary sites for LPAI virus replication and lesion production. Therefore more studies must be investigated to realise the ability of replication of the virus in the organs with no trypsin-like enzyme such as lymphoid tissues. The virus was not detected from any organs on day 10 PI. It might be resulted from increasing of the specific AIV antibody titer in chicken's blood. Current study showed that H9N2 AIV has tropism for the respiratory organs, urinary system and digestive system.

REFERENCES

Burleson FG, Chambers TM, Wiedbrauk DL (1992). Nucleic Acids Research 25: 3389-3402. A Laboratory Manual Academic Press. Inc. London.

Butt KM, Smith GJ, chen H (2005). Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. J. Clin. Microbiol., 43: 5760-5767.

Capua I, Alexander DJ (2006). The challenge of avian influenza to the veterinary community Avian Pathol., 3: 189-205.

Fouchier R, Munster V, Wallensten A, Bestebroer T, Herfst S, Smith D, Rimmelzwaan G, Olsen B, Osterhaus A (2005). characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from Black-Headed gulls. J. Virol., 79: 2814-2822.

Hablolvarid MH, Sohrabi HI, Pourbakhsh SA, Gholami MR (2003). A study on histopathologic changes in chicken following intravenous inoculation with avian influenza virus A/Iran/259/1998 (H9N2). Arch. Razi Inst., 55: 41-54.

Hablolvarid MH, Sohraby Haghdost I, Pourbakhsh SA, Gholami MR (2004). Histopathological study of intranasally inoculated A/chicken/Iran/259/1998 (H9N2) influenza virus in chicken. Arch. Razi Inst., 58: 51-62.

Klenk HD, Rott R, Orlich M, Blodorn J (1975). Activation of influenza A viruses by trypsin treatment. Virology, 168: 426-439.

Kwon JS, Lee HJ, Lee DH, Lee YJ, Mo IP, Nahm SS, Kim MJ, Lee JB, Park SY (2008). Immune response and pathogenesis in immunocompromised chickens in response to infection with H9N2 Low phathogenic avian influenza virus. Virus Res., 133: 187-194.

Lee YJ, Shin JY, Song HW, Kim JH, kwon YK, kwon JH, kim CJ, Webby RJ, Webster RG, Choi YK (2007). continuing evoluation of H9 influenza viruses in korean poultry. Virology. 359: 313-323.

Mosleh N, Dadras H, Mohammadi A (2009). Evaluation of H9N2 avian influenza virus dissemination in various organs of experimentally infected broiler chickens using RT-PCR. Iran. J. Vet. Res., 10: 12-20.

Naeem K, Naurin M, Rashid S (2003). Seroprevalence of avian influenza virus and its relationship with increased mortality and decreased egg production. Avian Pathol., 32: 285-289.

Nili H, Asasi K (2003). Avian influenza (H9N2) Outbreak in Iran. Avian Dis., 47: 828-837.

Office International Des Epizooties (OIE) (2008) Chapter 2.3.4 Avian influenza In: MANUAL OF DIAGNOSTIC TESTS AND VACCINES FOR TERRESTRIAL ANIMALS (mammals, birds and bees).

Pourbakhsh SA, khodashenas M, kianizadeh M, Goodarzi H (2000). Isolation and identification of avian influenza virus H9N2 subtype. Arch. Razi Inst., 51: 27-38.

Saberfar E, Najafi A, Goodarzi Z, Lashini H (2009). Multiplex Reverse Transcription-PCR Assay for Detection of Type A Influenza Virus plus Differentiation of Avian H7 and H9 Hemagglutinin Subtypes in Iran. Iranian J Pub Health., 38: 29-34.

Shalaby AA, Slemons RD Swayne DE (1994). pathological studies of A/chicken/Alabama/7395/75 (H4N8) influenza virus in specific pathogen free laying hens. Avian Dis., 38: 22-32.

Shamsedini M, Bahmani-Nejad M Khazraee-Nia P (2002). The use of indirect immunoperoxidase assay in diagrosis of type A (H9N2) avian influenza virus antigen on frozen tissue sections. Arch. Razi Inst., 53: 11-21.

Shinya K, Awakura A, Shimada A, Silvano FD, Umemura T, Otsuka K (1995). Pathogenesis of pancreatic atrophy by avian influenza A virus infection. Avian Pathol., 24:623-632.

Slemons RD, Swayne DE (1995). Tissue tropism and replicative properties of waterfowl-origin influenza viruses in chickens. Avian Dis., 39: 521-527.

Swayne DE, Beck JR (2005). Experimental study to determine if low-pathogenicity and high-pathogenicity avian influenza viruses can be present in chicken breast and thigh meat following intranasal virus inoculation. Avian Dis., 49: 81-85.

Swayne DE, Slemons RD (1994). comparative pathology of a chicken-origin and two duck-origin influenza virus isolates in chickens the effect of route of inoculation. Vet. Pathol., 31: 237-245.

Swayne DE, Slemons RD (1995). comparative pathology of intravenously inoculated wild duck- and turkey-origin type A influenza Virus in chickens. Avian Dis., 39: 74-84.

Vasfi MM, Bozorgmehrifard MH (2002). Isolation of H9N2 subtype of avian influenza viruses during an outbreak in chickens in Iran. Iran. Biomed. J., 6: 13-17.

Wood GW, Mccauley JW, Bashiruddin JB, Alexander DJ (1993). Deduced acid sequences at the haemagglutinin cleavage site of avian influenza A viruses of H5 and H7 subtypes. Arch. Virol., 130: 209-217.

African Journal of Microbiology Research Vol. 5(32), pp. 5831-5840, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.307

Full Length Research Paper

Biological wastewater treatment: Microbiology, chemistry, and diversity measurement of ammonia oxidizing bacteria

AYANDA Olushola Sunday* and AKINSOJI Olatunbosun Seun

Department of Chemistry, Faculty of Applied Sciences, Cape Peninsula University of Technology, Cape Town, South Africa.

Accepted 22 November, 2011

Nitrification is an important biological process in nitrogen cycling and has a significant effect on effluent quality in wastewater treatment. Nitrification occurs in two steps by two types of chemoautotrophic bacteria, the ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). The ammonia oxidizing bacteria is responsible for the oxidation of ammonia with oxygen into nitrite and is often the rate-limiting step in nitrification in wastewater treatment plants. Hence, a better understanding of the ecology, microbiology and chemistry of ammonia oxidizing bacteria in biological wastewater treatment systems is necessary in order to enhance treatment performance and control. A detailed review of various biological wastewater treatment processes, ammonia oxidizing bacteria and archaea; economic importance, problems, various molecular techniques for the investigation of the diversity and community structure, as well as the isolation of ammonia oxidizing bacteria were discussed.

Key words: Wastewater, activated sludge process, nitrification, ammonia oxidizing bacteria, ammonia oxidizing archaea, nitrite oxidizing bacteria, PCR primers.

INTRODUCTION

Wastewater treatment is the process of taking wastewater and making it suitable for discharge back into the environment. Wastewater results from a number of different activities, including industrial activities, rainwater runoff and domestic activities (Van der Hoek, 2004; Thomas, 2005). No matter where the wastewater comes from, this water is full of bacteria, chemicals, and other contaminants. Wastewater treatment therefore reduces the contaminants to acceptable levels so as to be safe for discharge into the environment. There are two types of wastewater treatment systems: a biological treatment plant and a physical/chemical treatment plant. Either of the treatment plants are utilized depending on the nature and components of pollution but some other pollution will require a combination of both wastewater treatment systems. When considering either a chemical or biological wastewater treatment for a particular application, it is very important to understand the sources of the wastewater generated, typical wastewater composition, discharge requirements, events and practices within a facility that can affect the quantity and quality of the wastewater, and pretreatment ramifications. Consideration of these factors will allow for maximization of the benefits the treatment plant will gain from effective wastewater treatment.

The various wastewater treatment processes have the following objectives: To confer and preserve the inherent physical, chemical and biological qualities of water of different origins which make it suitable for specific uses such as water for drinking and for use in productive processes, to protect the public from health risks without causing any damage to the environment and to confer and preserve those characteristics of water in its natural

*Corresponding author. E-mail: osayanda@gmail.com. Tel.: +27784417935 or +2348054642362.

environment which are necessary for the conservation and development of fauna and aquatic vegetation, and for provision of drinking water for cattle and wild animals or for recreational and aesthetic purposes (Boari et al., 1997).

Ammonia wastewater treatments

Nitrogen appears in wastewater as ammonia, nitrite, nitrate and organic nitrogen (Sotirakou, 1999). With the advancement in technology, there have been various biological and chemical wastewater treatments meant to address the problems of ammonia in wastewater (Jorgensen and Weatherley, 2003). The various treatment processes for treating ammonia include; lagoon systems, membrane bioreactor, fixed film treatment processes etc.

Lagoon systems are not expensive and are much easier to operate than mechanical wastewater treatment systems. Its components use little concrete and built through excavation. However, the effluent quality can become substandard because it is hard to control the wastewater's temperature, return rate, and oxygen level inside the system (Middlebrooks et al., 1999).

Membrane bioreactor (MBR) technology which combines biological-activated sludge process and membrane filtration has become more popular, abundant, and accepted in recent years for the treatment of many types of wastewaters, whereas the conventional activated sludge (CAS) process cannot cope with either composition of wastewater or fluctuations of wastewater flow rate (Jelena et al., 2007). The MBR has three essential components - the anoxic basin, the pre-aeration basins, and the MBR basin. The raw wastewater is poured into a fine screen for filtration, before placing it inside the anoxic basin. In the anoxic basin, the mixed liquor gushes into the pre-aeration basins until it reach the MBR basins. The fluids will then pass through the membranes of the MBR basins, wherein the membrane that pushes the effluent for disinfection is connected. The effluent must be sent first to the disinfection stage to ensure the quality of wastewater prior to release. The membranes lessen the repeat clarification process, making wastewater treatment more practical and convenient (Churchouse, 1997; Maryam et al., 2009). The MBR treatment process is known for its high effluent quality. Unlike other treatments, MBR treatment does not need additional filtration or clarification because the membranes are designed to clear the impurities in wastewater, and removes the total nitrogen from wastewater. The MBR machine is also less cumbersome and fits to small areas compared to other wastewater treatment machines. The costs involved in operating MBR are thus much higher compared to other wastewater systems.

BOD removal and biological nitrification can also be achieved using fixed film treatment (Park et al., 1996). This technique involves the use of microorganisms such as the ammonia oxidizing bacteria (AOB) to treat wastewater. It uses a trickling filter/activated sludge treatment process wherein a plastic media contains microorganisms, which will grow inside a tower where the wastewater is placed for treatment. It is then followed by activated sludge process. The trickling filters/activated sludge treatment process harness the best quality of wastewater. Trickling filters are more energy efficient and reduce the production of low quality effluent. The disadvantages of fixed film treatment include high solid retention and foul odour.

Activated sludge process

The activated sludge process is a system used for the treatment of sewage and industrial wastewaters that involves the mixture of biological mass and wastewater (Beychok, 1967). It is a complex biological wastewater treatment system that is currently designed for removing carbon (Akpor, 2011), phosphorus (Metcalf and Eddy, 1991; Henze, 1996) and/or nitrogen constituents (Metcalf and Eddy, 1991; Larsdotter, 2006) in the wastewater. In activated sludge process, organic waste is fed to the system and leaves the process depending on the desired treatment efficiency set by the operator. The process begins by mixing the biological waste present in industrial wastewater or sewage with an aerobic bacterial culture in the reactor and air. This mixture is known as the mixed liquor. Once in the reactor, the mixed liquor is aerated for a particular period of time in order to ensure that this solution is fully mixed. This mixture therefore undergoes separation through the gravity clarifier, where the waste activated sludge is removed from the treatment and mixed with primary treated wastewater before it is recycled back to the beginning of the process in order to maintain the desired concentration of organisms and sludge. Lastly, the sludge goes through further treatment and the result of all this process is the treated wastewater that can be safely disposed to nature. A generalize schematic diagram of an activated sludge process is as shown in Figure 1.

There are basically three types of activated sludge processes, they are conventional activated sludge processes (Brucculeri et al., 2005; Marcos, 2007), contact stabilization processes (Gujer and Jenkins, 1975) which uses two separate aeration processes, and the extended aeration processes (Lowe and Gaudy, 1989; Sotirakou et al., 1999). The different activated sludge processes all accomplish the biochemical reduction of organics using aeration basins and the return and waste sludge systems. It is the detention times, mixed liquor suspended solids (MLSS), and food/microorganism ratio (F:M) loadings that are different. Other modifications are oxidation ditch, complete mix activated sludge process,

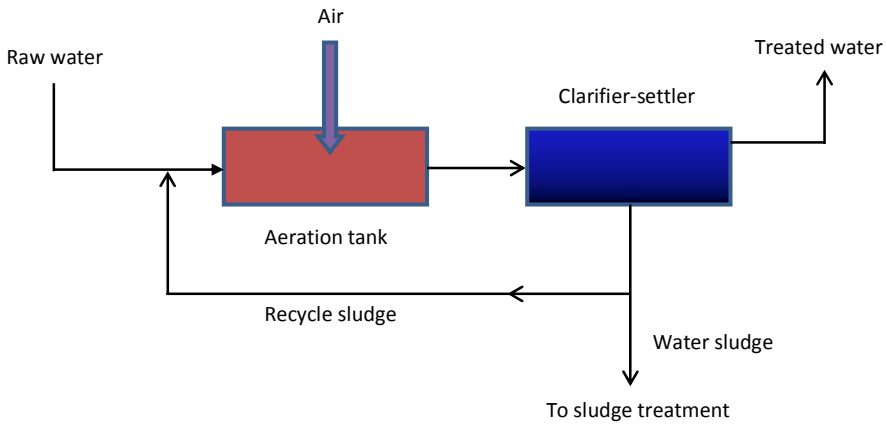


Figure 1. A generalize schematic diagram of an activated sludge process.

step feed, tapered aeration and Kraus process. The different types of activated sludge plants include: Package plants, oxidation ditch, deep Shaft and surface-aerated Basins/Lagoons (Beychok, 1967).

Ammonium oxidizing bacteria and archaea

Ammonia in water environments is toxic to fish and other aquatic life at high concentrations, and also contributes to eutrophication. Biodegradation and elimination of ammonia in wastewater is thus one of the main functions of wastewater treatment plants (WWTPs) and can be achieved by nitrification. Nitrification is the biological oxidation of ammonia with oxygen into nitrite followed by the oxidation of these nitrites into nitrates and can be represented by equation 1 and 2, respectively. A chemolithotrophic nitrification is a two-step process and is carried out by two different groups of organisms, the AOB and the nitrite oxidizing bacteria (NOB) (Bin et al., 2009). AOB are primarily responsible for the first step which is the oxidation of ammonia with oxygen into nitrite and often the rate-limiting step in nitrification. It is this process that is essential for the removal of ammonia from the wastewater.

$$NH_3 + O_2 \rightarrow NO_2^- + 3H^+ + 2e^- \quad (1)$$

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^- \quad (2)$$

It is generally accepted that ammonia (NH_3) and not ammonium (NH_4^+) is used as substrate, and the ammonia/ammonium ratio may therefore affect the growth of AOB. AOB are obligatory chemolithoautotrophs and can be found among the beta-proteobacteria and gamma-proteobacteria (Purkhold, 2000). Most AOB are phylogenetically closely related to other activated sludge bacteria within the beta subdivision class of *Proteobacteria* (Harms, 2003). AOB are generally rod-shaped, spherical, spirillar, or lobular, typically Gram-negative, and flagellation of motile cells is polar to sub-polar or peritrichous. Most species are aerobic but can grow at reduced oxygen partial pressure. Studies suggest that there are physiological and ecological differences between the different AOB genera and lineages and that environmental factors such as salinity, pH, and concentrations of ammonia and suspended particulate matter select for certain species of AOB (Kowalchuk and Steven, 2001; Bin et al., 2009). The physiological activity and abundance of AOB in wastewater processing is important in the design and operation of waste treatment systems, particularly since these organisms display low growth rate and high sensitivity to environmental disturbances and inhibitor (Okabe, 1999).

Ammonia oxidizing archaea

Autotrophic ammonia/ammonium oxidation was initially assumed to be restricted to aerobic AOB and anaerobic ammonium oxidizing (Anammox) bacteria until recent molecular and culture-dependent evidence showed that autotrophic ammonia oxidation also occurs in the domain Archaea. The first strain of ammonia oxidizing archaea (AOA), *Nitrosopumilis maritimus*, was isolated from the rocky substratum of a tropical marine aquarium tank (Erguder et al., 2009). Some evidences showed that AOA are more abundant than AOB in marine, lake waters and soil environments (Sonthiphand and Limpiyakorn, 2010). AOA have also been detected in activated sludge bioreactors by using specific PCR primers targeting archaeal *amo*A gene. However, AOA abundances seemed to be much lower (four orders or more) in most cases than AOB based on analysis of *amoA* gene copy number (Jin et al., 2010; Limpiyakorn et al., 2011). The factors influencing the presence or/and dominance of AOA in different environments are ammonium levels, salinity, temperature, organic carbon, dissolved oxygen (DO) levels, pH, sulphide levels, and phosphate levels.

Ammonia monooxygenase

Ammonia monooxygenase (AMO) is a membrane-bound enzyme in *Nitrosomonas europaea* and other autotrophic AOB of the beta and gamma-subclasses of *Proteobacteria*. The enzyme contains multiple subunits; *amoA, amoB* and *amoC*. All the three AMO genes have been cloned and sequenced from several AOB (McTavish et al., 1993). AMO is responsible for the conversion of ammonia to hydroxylamine. Hydroxylamine is then oxidized to nitrite by hydroxylamine oxidoreductase (HAO) in an endergonic reaction. HAO is an unusual enzyme with a highly complex structure, located as a soluble enzyme in the perplasmic space, but anchored in the cytoplasmic membrane. AMO and HAO enzymes are necessary for energy conversion during the oxidation of ammonia. The initial oxidation of ammonia, which yields hydroxylamine as a reduced product, is an O_2-dependent reaction catalyzed by AMO:

$$NH_3 + O_2 + 2e^- + 2H^+ \rightarrow NH_2OH + H_2O \quad (4)$$

Hydroxylamine is further oxidized to nitrite by HAO:

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^- \quad (5)$$

Two of the four electrons generated from hydroxylamine are used to support the oxidation of additional ammonia molecules; the other two enter the electron transfer chain and are used for CO_2 reduction and ATP biosynthesis (Wood, 1986).

Ammonia monooxygenase subunit A (*amoA*) gene and 16S rRNA genes has been widely used to analyze the diversity and abundance of AOB in various samples. Based on comparative analysis of 16S rRNA and *amoA* gene sequences, it was found that 16S rRNA gene is more conserved than *amoA* gene and the suggested similarity thresholds of 16S rRNA and *amoA* genes to define different AOB species are 97 and 80%, respectively (Ye and Zhang, 2011).

Economic importance of ammonium oxidizing bacteria

Nitrogenous wastes are increasing as a result of the expansion of animal husbandry, nitrogen-producing industries, and human activities and have therefore become a critical factor in environmental management. The removal of nitrogen from wastewater treatment is of extreme environmental importance. This is because the release of untreated waste can result to toxic effect on aquatic animals and can lead to eutrophication of the environment. Even in cases where treatment does not lead to successful denitrification, nitrification helps to avoid environmental contamination with potentially toxic ammonia salts (Painter, 1986). The broad specificity of the AMO complex common to all AOB often permits the co-oxidation of numerous recalcitrant aliphatic, aromatic, and halogenated molecules. AOB may also play a role in methane oxidation and biofilter systems. Biofilter systems have been used for the elimination of odours associated with waste treatment and composting (Bohn, 1992) and also for purposes as providing long-term filtering capacity suitable for manned spacecraft (Joshi et al., 2000). The reduction of ammonia released into the environment reduces the risk of local oxygen depletion.

Problems associated with ammonia oxidizing bacteria

AOB can lead to the production of the ozone-depleting gas NO or the greenhouse gas N_2O either at low or high levels via partial-denitrification processes under reduced oxygen conditions (Cho and Kim, 2000). The process of ammonia oxidation leads to a net acidification of the environment. The acidification of forest soils may thus have a detrimental effect on tree, and high levels of nitrification may intensify problems involving the effects of acid rain. Nitrogen transformations that lead to an increased proton load can lead to the release of metals such as aluminum, which can contribute to root damage and forest decline. AOB may generate elevated levels of nitrous acid and can lead to corrosion of natural stones, historical monuments, and building materials (Meincke et al., 1989).

Diversity, abundance and community structure measurement

A better understanding of the microbial ecology of AOB and AOA in wastewater treatment systems could potentially enhance the treatment performance and control, and would also help engineers to utilize the functional characteristics of the microbial population to model and improve the design and operation of the systems (Wang et al., 2010). The development of culture independent molecular techniques has enhanced the ability of researchers to analyze environmental samples.

Denaturing gradient gel electrophoresis (DGGE)

DGGE is a molecular fingerprinting method that separates polymerase chain reaction (PCR)-generated DNA products. The technique enables sequence diversity of PCR-amplified genes from a large number of samples to be compared in one gel to reveal changes in community structure over time or space. This approach separates DNA sequences and allows 95% of single base sequence differences to be detected. This approach also has the ability to separate genomic sequences differing by more than one base (Muyzer et al., 1993).

DGGE is a useful method for bacterial community

profiling by targeting the 16S rRNA and/or *amoA* genes of AOB (Nicolaisen and Ramsing, 2002). This method is less time consuming for comparing AOB communities than conventional analysis by cloning and sequencing.

Hornek et al. (2006) reported the communities of AOB in activated sludge of a municipal wastewater treatment plant (WWTP) located in Linz (Austria) by the use of DGGE technique. DGGE analyses of PCR products generated by the *amoA* primers; amoA-1F, amoA-2R, amoAf-I, and amoAr-I were performed with the D-gene system. Selected bands were recovered, sequenced and subsequently submitted to BLAST to allocate to available partial *amoA* sequences. Sequences were aligned using the program ClustalX 1.81. The application of the primer set amoA-1F in combination with amoA-2R and amoAr-I was suggested for a rapid PCR-DGGE analysis, because they seem to complement each other to screen for present AOB in the environment.

The microbial community composition and dominant bacterial populations in anoxic-oxic activated sludge from a full-scale WWTP in Liaoning, China were investigated with PCR-DGGE coupled with sequence analysis of 16S rRNA gene fragments from dominant bands by Ding et al (2011). PCR amplification of bacterial 16S rRNA gene fragments was performed using primer 968F-GC (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3') and primer 1401R (5'-CGG TGT GTA CAA GGC CCG GGA ACG-3') for the Domain Bacteria, corresponding to positions 968 and 1401 in the 16S rDNA of *Escherichia coli*, with a 40 bp-GC-rich sequence (5'- CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G - 3') attached to the 5' end of the forward primer to stabilize the melting behavior of the DNA fragments. DGGE of the PCR amplified 16S rDNA was carried out using the DCodeTM Universal Mutation Detection System. The prominent DGGE bands were selected and excised for nucleotide sequence determination. The sequences obtained from the DGGE were then analyzed in comparison with the 16S rDNA sequences in the GenBank database by using the basic local alignment search tool (BLAST). The alignment was calculated by the neighbor-joining method using Clustal X. The phylogenetic affiliation of the sequences was further analyzed and a phylogenetic tree was plotted by Mega 3.1 program.

Because of high similarity of the 16S rDNA sequences that makes it difficult to identify closely related ammonia oxidizing species, some difficulties have been experienced in studying ammonia oxidizing bacterial diversity using DGGE of 16S rDNA gene sequence. Another method based on the same principle is the temperature gradient gel electrophoresis (TGGE).

Real-time PCR

Real-time PCR is a highly sensitive technique that facilitates amplification and quantification of a specific DNA sequence with the detection of the PCR product in real time. Quantification of DNA targets can easily be achieved by determination of the cycle when the PCR product can first be detected. Thus, Real-time PCR technique is reliable and reproducible for AOB and for evaluating correlations between microbial activities, cell numbers and population changes in time and space. Real-time PCR analysis has been applied to numerous environmental samples to reveal the comparative abundance of AOA and AOB. In many cases, according to Caffrey et al. (2007), the archaeal *amoA* gene outnumbered that of AOB.

The abundance of *amoA* genes of AOB and AOA in activated sludge of seven full-scale wastewater treatment plants in Thailand was investigated by Limpiyakorn (2011). Quantitative real-time PCR was performed with duplicate sets of extracted DNA. Each set of extracted DNA was prepared by pooling the DNA extracted in triplicate, then diluted for four different 10-fold dilutions and a quantitative real-time PCR was carried out for each dilution in duplicate with a Brilliant II SYBR Green QPCR Master Mix in an Mx3005P instrument. Archaeal *amoA* genes were quantified using the primers Arch-amoAF (5'-STA ATG GTC TGG CTT AGA CG-3') and Arch-amoAR (5'-GCG GCC ATC CAT CTG TAT GT-3'), the quantification of bacterial *amoA* genes was performed using the primers amoA 1F (5'-GGG GTT TCT ACT GGT GGT-30) and amoA 2R (5'-CCC CTC KGS AAA GCC TTC TTC-3') while AOB 16S rRNA gene was quantified using the primers CTO 189A/Bf (5'-GGA GRA AAG CAG GGG ATC G-3'), CTO189Cf (5'-GGA GGA AAG TAG GGG ATC G-3'), and CTO 654r (50-CTA GCY TTG TAG TTT CAA ACG C-3'). To confirm the single target fragment of the PCR amplified products, dissociation curves were analyzed and plotted at the end of every quantitative real-time PCR reaction and to verify the correct amplification of the target microorganisms' DNA, few clones from the clone libraries constructed from the real-time PCR amplified products were randomly selected for sequencing and the results for every reaction tested verified the correct amplification of the target microorganisms' DNA.

Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP is a molecular biology technique for profiling of microbial communities based on the position of a restriction site closest to a labeled end of an amplified gene. The method is based on the digestion of a mixture of PCR amplified variants of a single gene using one or more restriction enzymes and detecting the size of each of the individual resulting terminal fragments using a DNA sequencer. The result is a graph image where the X axis represents the sizes of the fragment and the Y axis represents their fluorescence intensity.

Wang et al. (2010) investigated the communities of AOB in activated sludge collected from eight wastewater treatment systems in Beijing using polymerase chain reaction (PCR) followed by T-RFLP, cloning, and sequencing of the α-subunit of the *amoA* gene. The primers amoA-1F (5'-GGG GTT TCT ACT GGT GGT-3') and amoA-2R (5'-CCC CTC KGS AAA GCC TTC TTC-3') were used to amplify a 491 base pairs (bp) fragment of the *amoA* gene of AOB. A Clone library was constructed and the software Clustal X 1.81 was used to align sequences of the recovered clones with other published *amoA* sequences and software Mega 4.0 was used to generate a phylogenetic tree using the neighbor joining method. Their results (T-RFLP fingerprint analysis) showed that the different wastewater treatment systems harbored distinct AOB communities and that the source of influent affected the AOB community with the WWTPs treating domestic wastewater containing a higher AOB diversity than those receiving mixed domestic and industrial wastewater. A combination of the results of sequencing the *amoA* gene and the T-RFLP profiles of clones clearly indicated which species each peak represented.

The AOB populations in Marshall WWTP (an aerated-anoxic Orbal process treating 900 ~ 1,300 m^3/day of domestic wastewater) and Nine Springs WWTP (a variation of the UCT process and treats 150,000 ~ 200,000 m^3/day of domestic wastewater) with T-RFLP was investigated by Park et al (2001). Primers amoA-1F and amoA-2R were used to amplify a 491-bp fragment of the *amoA* gene.

The analysis was complemented by cloning and sequencing the *amoA* gene fragment to detect and identify AOB.

TRFLP is one of several molecular methods aimed to generate a fingerprint of an unknown microbial community (Liu et al., 1997). Because of its relatively high resolution and reproducibility, T-RFLP has been widely used to assess the AOB community (Osborn et al., 2000).

Other molecular techniques are fluorescent *in situ* hybridization (FISH), restriction fragment length polymorphism (RFLP), amplified ribosomal DNA restriction analysis (ARDRA), singlestranded-conformation polymorphism (SSCP), randomly amplified polymorphic DNA (RAPD) or DNA amplification fingerprinting (DAF), bisbenzimide-polyethyleneglycol (Bb-PEG) electrophoresis, etc.

Fluorescent in situ Hybridization (FISH)

FISH (fluorescent *in situ* hybridization) is a cytogenetic technique developed by Christoph Lengauer that is used to detect and localize the presence or absence of specific DNA sequences on chromosomes. The technique is one of the methods that have been described for direct visualization of AOB. Hybridization techniques generally use directly extracted DNA as a "probe" for specific detection of various microorganism species. The application of *In situ* Hybridization approaches to AOB has been most effective in detecting AOB that dominate total bacterial community in environment such as the sewage treatment plants (Wagner et al., 1995).

Restriction fragment length polymorphism (RFLP)

RFLP is a technique that exploits variations in homologous DNA sequences. Ribotyping (RFLP of rRNA genes) combines restriction enzyme digestion of the total genomic DNA with a Southern analysis, in which rRNA gene-specific DNA probes are used. The hybridization pattern obtained is a characteristic of each organism. RFLP determines the multiplicity, the arrangement and the relative location of rRNA genes in bacterial genomes and also contain taxonomic information. Aakra et al. (1999) in their study, ribotyped 12 isolates of AOB, and the sequences of the 16s-23s rDNA intergenic spacer region (ISR) were determined and used in a phylogenetic study.

Amplified ribosomal DNA restriction analysis (ARDRA)

ARDRA is a DNA fingerprint technique based on PCR amplification of 16S ribosomal DNA using primers for conserved regions, followed by enzyme digestions and agarose gel electrophoresis (Smit el al., 1997).

T-RFLP is different from ARDRA and RFLP in that only the terminal fragments (i.e. the labeled end or ends of the amplicon) are read and all other fragments ignored whereas all restriction fragments are visualized for ARDRA and RFLP.

Singlestranded-conformation polymorphism (SSCP)

With SSCP, DNA fragments such as PCR products obtained with primers specific for the 16S rRNA gene, are denatured and directly electrophoresed on a non-denaturing gel. Separation is based on differences in the folded conformation of single-stranded DNA, which influences the electrophoretic mobility.

Randomly amplified polymorphic DNA (RAPD) and DNA amplification fingerprinting (DAF)

RAPD and a similar approach termed DAF use short (5-10 nucleotides) random primers, which anneal at different sites of the genomic DNA, generating PCR products of various lengths. The products are separated on agarose

Table 1. PCR primers.

Primers	Sequences (5'→3')	Target	Reference
AmoA-1F-Clamp[a]	GGGGTTTCTACTGGTGGT	amoA	Nicolaisen and Ramsing (2002)
AmoA-2R-Clamp[a and b]	CCCCTCKGSAAAGCCTTCTTC	amoA	Nicolaisen and Ramsing (2002)
AmoA-2R-KS[b]	CCCCTCKGSAAAGCCTTCTTC	amoA	Nicolaisen and Ramsing (2002
AmoA-2R-TS[b]	CCCCTCTGSAAAGCCTTCTTC	amoA	Nicolaisen and Ramsing (2002)
AmoA-2R-GS[b]	CCCCTCGGSAAAGCCTTCTTC	amoA	Nicolaisen and Ramsing (2002)
AmoA-2R-TC	CCCCTCTGCAAAGCCTTCTTC	amoA	Nicolaisen and Ramsing (2002)
AmoA-2R-TG	CCCCTCTGGAAAGCCTTCTTC	amoA	Okano et al. (2004)
AmoA-2R-GC	CCCCTCGGCAAAGCCTTCTTC	amoA	Nicolaisen and Ramsing (2002)
AmoA-2R-GG	CCCCTCGGGAAAGCCTTCTTC	amoA	Nicolaisen and Ramsing (2002)
Cren-amoAF	ATGGTCTGGCTAAGACGMTGTA	amoA	Hallam et al. (2006)
Arch-amoAR	GCGGCCATCCATCTGTATGT	amoA	Francis et al. (2005)
CTO189f-Clamp[a and b]	GGAGRAAAG**Y**AGGGGATCG	16s rDNA	Kowalchuk et al. (1997)
CTO654r[b]	CTAGC**Y**TTGTAGTTTCAAACGC	16s rDNA	Kowalchuk et al. (1997)
6R	AGAAAGGAGGTGATCCAGCC	16s rDNA	Dorsch and Stackebrandt (1992)
7F	GCCTTGTACACACCGC	16s rDNA	Lane et al. (1985)
9F	GAGTTTGATCCTGGCTCAG	Bacteria	Dionisi et al. (2003)
11F	TGGCGAAGGCGGCCCCCTGGA	16s rDNA	Edwards et al. (1989)
13R	GCCAAGGCATCCACCACATG	23s rDNA	Gurtler and Stanisich (1996)
23SF	CCGAATGGGGAAACC	23s rDNA	Gurtler and Stanisich (1996)
23SR	CCTTTCCCTCACGGTA	23s rDNA	Gurtler and Stanisich (1996)
1393R	ACGGGCGGTGTGTAC	Bacteria	Dionisi et al. (2003)
1055F	ATGGCTGTCGTCAGCT	Bacteria	Dionisi et al. (2003)
341F[c]	TACGGGAGGCAGCAG	Bacteria	Lopez-Gutierrez et al. (2004)
518R	ATTACCGCGGCTGCTGG	Bacteria	Lopez-Gutierrez et al. (2004)

[a]5' GC-Clamp (CGCCGCGCGGCGGGCGGGGCGGGGGC); [b]Degeneracies are shown in bold; [c]5' GC-clamp (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG).

or acrylamide gels, and visualized by ethidium bromide or silver staining (Muyzer, 1999).

Bb-PEG Electrophoresis

Electrophoresis is performed in agarose gels containing the DNA ligand bisbenzimide to which long chains of polyethyleneglycol (PEG) are covalently coupled. Bisbenzimide binds to adenine and thymine (A+T) rich sequence motifs in the DNA. Therefore, being loaded with the Bb-PEG conjugate, the A+T-rich DNA molecules are more retarded in the gel than the molecules which are low in A+T, and so separation is achieved (Muyzer, 1999).

PCR primers

Primers are strands of nucleic acid that function as starting point for DNA synthesis. Some other PCR primers used in several other studies are presented in Table 1. Primers are required for DNA replication because the enzymes DNA polymerases, which catalyze the process can only add new nucleotides to an existing strand of DNA. The polymerase therefore starts replication at the 3'-end of the primer, and copies the opposite strand.

Isolation of ammonia oxidizing bacteria

AOB can be isolated in most aerobic environments where ammonia is available through the mineralization of organic matter or anthropogenic nitrogen sources, such as fertilizers and waste. AOB pure cultures are also obtained by picking colonies from a solid medium or by the use of dilution methods in liquid culture (Ford et al., 1980; Schmidt and Belser, 1982). The selective medium used must be free of organic carbon sources and contain inhibitors of heterotrophic organisms, an ammonia source, and essential trace elements (MacDonald and Spokes, 1980). AOB are very difficult to handle as a result of their slow growth and low maximum growth yield, making their isolation and maintenance in pure culture difficult and time-consuming. Culture-dependent techniques such as selective plating and the most probable number (MPN) method have been used for the

enumeration of AOB; however, such techniques are thought to underestimate actual cell numbers. In addition to medium selectivity and bias, MPN underestimation may also stem from inadequate suspension of cells from solid substrates in the environmental sample or dispersal of flocks and microcolonies. Cell damage due to rigorous disruption methods or osmotic shock and the possible dependence on inter- or intraspecies interactions for growth may also generate inaccuracies (Kowalchuk and Steven, 2001).

Conclusion

Ammonia has been found to be very dangerous to water environment especially to fishes and other aquatic life, its high concentration is mostly responsible for this. It is therefore necessary to convert ammonia to other nitrogen compounds that will have little or no effect on water environment. An example of microorganisms widely used for this purpose are the AOB, they are used for the oxidation of ammonia into nitrite in biological WWTPs. AOB are ecologically important, being the only group of organisms that oxidize ammonia to nitrite in significant amounts, and they appear to be present in all environments in which nitrogen is mineralized (Aakra et al., 1999). Because these microorganisms display low growth rate and high sensitivity to environmental disturbances and inhibitor, the physiological activity and abundance of AOB in wastewater processing is important in the design and operation of waste treatment systems. AOB can be isolated in most aerobic environments as well as low-oxygen environments. The different techniques for the investigation of diversity and community structure of AOB are: denaturing gradient gel electrophoresis (DGGE), Real-Time PCR, fluorescent *in situ* hybridization (FISH), terminal restriction fragment length polymorphism (T-RFLP), restriction fragment length polymorphism (RFLP), amplified ribosomal DNA restriction analysis (ARDRA), singlestranded-conformation polymorphism (SSCP), randomly amplified polymorphic DNA (RAPD) or DNA amplification fingerprinting (DAF), and bisbenzimide-polyethyleneglycol (Bb-PEG) electrophoresis. Among the various techniques, DGGE appears to be the most frequently used community fingerprinting method. It allows a rapid comparison of the microbial communities between the samples and is generally used to detect shifts in microbial population under different environmental conditions. DGGE technique based on 16S rDNA gene enables the investigation of the spatial and temporal variability of the population in environment, provide information on the predominant species in a community and analyze multiple samples simultaneously.

However, it should also be noted that none of these methods is absolutely perfect; they all have their advantages and disadvantages (Muyzer, 1999). Hence, only a polyphasic approach combining different molecular biological techniques, microbiological methods, and methods to determine the environmental parameters will lead to an unbiased understanding of the role of microorganisms in their environment.

REFERENCES

Aakra A, Utaker JB, Nes IF (1999). RFLP of rRNA genes and sequencing of the 165-235 rDNA intergenic spacer region of ammonia-oxidizing bacteria: a phylogenetic approach. Int. J. Syst. Bacteriol., 49: 123-130.

Akpor OB (2011). Wastewater effluent discharge: Effects and treatment processes. 3rd International Conference on Chemical, Biological and Environmental Engineering, IACSIT Press, Singapore.

Beychok MR (1967). Aqueous wastes from petroleum and petrochemical plants (1st ed.). John Wiley & Sons Ltd. LCCN 67019834.

Bin Z, Baosheng S, Min J, Huina L (2009). Population dynamic succession and quantification of ammonia-oxidizing bacteria in a membrane bioreactor treating municipal wastewater. J. Hazardous Mater., 165: 796–803.

Boari G, Mancini IM, Trulli E (1997). Technologies for water and waste water treatment. Ciheam-Options mediteraneene, pp 261-287.

Bohn H (1992). Consider biofiltration for decontaminating gases. Chem. Eng. Prog., 88:34–40.

Brucculeri M, Bolzonella D, Battistoni P, Cecchi F (2005). Treatment of mixed municipal and winery wastewaters in a conventional activated sludge process: a case study. Wat. Sci. Technol., 51: 89-98.

Caffrey JM, Bano N, Kalanetra K, Hollibaugh JT (2007). Ammonia oxidation and ammonia oxidizing bacteria and archaea from estuaries with differing histories of hypoxia. ISME J., 1: 660–662.

Cho JC, Kim SJ (2000). Increase in bacterial community diversity in subsurface aquifers receiving livestock wastewater input. Appl. Environ. Microbiol., 66: 956–65.

Churchouse S (1997). Membrane bioreactors for wastewater treatment - operating experiences with the Kubota submerged membrane activated sludge process. Membrane Technol., 83: 5-9.

Ding L, Zhou Q, Wang L, Zhang Q (2011). Dynamics of bacterial community structure in a fullscale wastewater treatment plant with anoxic-oxic configuration using 16S rDNA PCR-DGGE fingerprints. Afri. J. Biotechnol., 10: 589-600.

Dionisi HM, Hamrs G, Layton AC, Gregory IR, Parker J, Hawkins SA, Robinson KG, Sayler GS (2003). Power analysis for real-time PCR quantification of genes in activated sludge and analysis of the variability introduced by DNA extraction. Appl. Environ. Microbiol., 69: 6597-6604.

Dorsch M, Stackebrandt E (1992). Some modifications in the procedure of direct sequencing of PCR amplified 16s rDNA. J. Microbiol. Methods 16: 271-279.

Edwards U, Rogall T, Blocker H, Emde M, Bottger EC (1989). Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16s ribosomal RNA. Nucleic Acids Res., 17: 7843-7853.

Erguder TH, Boon BN, Wittebolle L, Marzorati M, Verstraete W (2009). Environmental factors shaping the ecological niches of ammonia oxidizing archaea. FEMS Microbiol. Rev., 33: 855–869.

Ford DL, Curchwell RL, Kachtick JW (1980). Comprehensive analysis of nitrification of chemical processing wastewaters. J. Water Pollut. Control Fed., 52: 2726–45.

Francis CA, Roberts KJ, Beman JM, Santoro AE, Oakley BB (2005). Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. Proc. Natl. Acad. Sci., 102: 14683–14688.

Gujer W, Jenkins D (1975). The contact stabilization activated sludge process—oxygen utilization, sludge production and efficiency. Water Res., 9: 553-560.

Gurtler V, Stanisich VA (1996). New approaches to typing and identification of bacteria using the 16s-23s rDNA spacer region. Microbiol., 142: 3-16.

Hallam SJ, Mincer TJ, Schleper C, Preston CM, Roberts K, Richardson PM, DeLong EF (2006). Pathways of carbon assimilation and ammonia oxidation suggested by environmental genomic analyses of marine crenarchaeota. PLoS Biol., 4: e95.
Harms G, Layton AC, Dionisi HM, Gregory IR, Garrett VM, Hawkins SA, Robinson KG, Sayler GS (2003). Real-time PCR quantification of nitrifying bacteria in a municipal wastewater treatment plant. Environ. Sci. Technol., 37: 343–351.
Henze M (1996). Biological phosphorus removal from wastewater: processes and technology. Water Quality International, pp 32-36.
Hornek R, Pommerening-Roser A, Koops HP, Farnleitner AH, Kreuzinger N, Kirschner A, Mach RL (2006). Primers containing universal bases reduce multiple amoA gene specific DGGE band patterns when analysing the diversity of beta-ammonia oxidizers in the environment. J. Microbiol. Methods 66: 147–155.
Jelena R, Marin M, Ivan M, Mira P, Damià B (2007). Membrane Bioreactor (MBR) as an Advanced Wastewater Treatment Technology. Hdb. Env. Chem., 5: 37–101.
Jin T, Zhang T, Yan Q (2010). Characterization and quantification of ammonia-oxidizing archaea (AOA) and bacteria (AOB) in a nitrogen-removing reactor using T-RFLP and qPCR. Appl. Microbiol. Biotechnol., 87: 1167–1176.
Jorgensen TC, Weatherley LR (2003). Ammonia removal from wastewater by ion exchange in the presence of organic contaminants. Water Res., 37: 1723-1728.
Joshi JA, Hogan JA, Cowan RM, Strom PF, Finstein MS (2000). Biological removal of gaseous ammonia in biofilters: space travel and earth-based applications. J. Waste Manage. Assoc., 50: 1647–54.
Kowalchuk GA, Stephen JR (2001). Ammonia-oxidizing bacteria: a model for molecular microbial ecology. Annu. Rev. Microbiol., 55: 485–529.
Kowalchuk GA, Stephen JR, De Boer W, Prosser JI, Embley TM, Woldendorp JW (1997). Analysis of ammonia-oxidizing bacteria of the beta subdivision of the class *Proteobacteria* in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Appl. Environ. Microbiol., 63: 1489–1497.
Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR (1985). Rapid determination of 16s ribosomal RNA sequences for phylogenetic analyses. Proc. Natl. Acad. Sci., 82: 6955-6959.
Larsdotter K (2006). Microalgae for phosphorus removal from wastewater in a Nordic climate. Doctoral Thesis from the School of Biotechnology, Royal Institute of Technology, Stockholm, Sweden, ISBN: 91-7178-288-5.
Limpiyakorn T, Sonthiphand P, Rongsayamanont C, Polprasert C (2011). Abundance of amoA genes of ammonia-oxidizing archaea and bacteria in activated sludge of full-scale wastewater treatment plants. Biores. Technol., 102: 3694–3701.
Liu W, Marsh T, Cheng H, Forney L (1997). Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl. Environ. Microbiol., 63: 4516-4522.
Lopez-Gutierrez JC, Henry S, Hallet S, Martin-Laurent F, Catroux G, Philippot L (2004). Quantification of a novel group of nitrate-reducing bacteria in environment by real-time PCR. J. Microbiol. Methods 57: 399-407.
Lowe WL, Gaudy AF (1989). Modified extended aeration process for removal and recovery of cadmium from wastewaters. Biotechnol. Bioeng., 34: 600-608.
MacDonald RM, Spokes JR (1980). A selective and diagnostic medium for ammonia oxidizing bacteria. FEMS Microbiol. Lett., 8: 143–45.
Marcos S (2007). Activated sludge and aerobic biofilm reactors. Biological Wastewater Treatment Series, IWA Publishing, Alliance House, 12 Caxton Street, London SW1H 0QS, UK, pp 1-313.
Maryam TR, Tahereh K, Ali K (2009). Application of membrane separation processes in petrochemical industry: a review. Desalination 235: 199–244.
McTavish H, Fuchs JA, Hooper AB (1993). Sequence of the gene coding for ammonia monooxygenase in Nitrosomonas europaea. J. Bacteriol., 175: 2436-2444.
Meincke M, Kreig E, Bock E (1989). Nitrosovibrio spp., the dominant ammoniaoxidizing bacteria in building stones. Appl. Environ. Microbiol., 55: 2108–10.
Metcalf, Eddy (1991). Wastewater Engineering. Treatment, disposal, reuse. 3rd edition, McGraw-Hill Int. Ed., Singapore.
Middlebrooks EJ, Sherwood CR, Abraham P, Adams VD (1999). Nitrogen removal in wastewater stabilization lagoons, Presented at 6th National Drinking Water and Wastewater Treatment Technology Transfer Workshop Kansas City, Missouri 64105. p. 38.
Muyzer G (1999). Genetic fingerprinting of microbial communities – present status and future perspectives. Proceedings of the 8th International Symposium on Microbial Ecology Bell CR, Brylinsky M, Johnson-Green P (ed) Atlantic Canada Society for Microbial Ecology, Halifax, Canada, 10pp.
Muyzer G, De Waal EC, Uitterlinden AG (1993). Profiling of complex microbial population by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol., 59: 695-700.
Nicolaisen MH, Ramsing NB (2002). Denaturing gradient gel electrophoresis (DGGE) approaches to study the diversity of ammonia-oxidizing bacteria. J. Microbiol. Meth., 50: 189-203.
Okabe S, Satoh H, Watanabe Y (1999). In situ analysis of nitrifying biofilms as determined by in situ hybridization and the use of microsensors. Appl. Environ. Microbiol., 65: 3182–3191.
Okano Y, Hristova KR, Leutenegger CM, Jackson LE, Denison RF, Gebreyesus B, Lebauer D, Scow KM (2004). Application of real-time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil. Appl. Environ. Microbiol., 70: 1008–1016.
Osborn AM, Moore ERB, Timmis KN (2000). An evaluation of terminal-restriction fragment length polymorphism (TRFLP) analysis for the study of microbial community structure and dynamics. Environ. Microbiol., 2: 39–50.
Painter HA (1986). Nitrification in the treatment of sewage and wastewaters. Spec. Publ. Soc. Gen. Microbiol., 20: 185–213.
Park HD, Regan JM, Noguera DR (2001). Molecular analysis of ammonia-oxidizing bacterial populations in aerated-anoxic orbal processes. IWA Conference on microorganisms in activated sludge and biofilm processes, Rome.
Park TJ, Lee HK, Kim SD, Kim WC (1996). Petrochemical wastewater treatment with aerated submerged fixed-film reactors (ASFFR) under high organic loading. Water Sci. Technol., 34: 9-16.
Purkhold U, Pommerening-Roser A, Juretschko S, Schmid MC, Koops HP, Wagner M (2000). Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: implications for molecular diversity surveys. Appl. Environ. Microbiol., 66: 5368–5382.
Schmidt EL, Belser LW (1982). Nitrifying bacteria. in Methods of soil analysis, part 2. Chemical and microbiological properties. ed Page A. (American Society of Agronomy, Inc., Crop Science Society of America, Inc., and Soil Science Society of America, Inc. Madison, Wis), pp 1027–1042.
Smit E, Leeflang P, Wernars K (1997). Detection of shifts in microbial community structure and diversity in soil cause by coper contamination using amplified ribosomal DNA restriction analysis. FEMS Microbiol. Ecol., 23: 249-261.
Sonthiphand P, Limpiyakorn T (2010). Communities of ammonia-oxidizing archaea and bacteria in enriched nitrifying activated sludge. World Acad. Sci. Eng. Technol., 64: 425-428.
Sotirakou E, Kladitis G, Diamantis N, Grigoropoulou H (1999). Ammonia and phosphorus removal in municipal wastewater treatment plant with extended aeration. Global Nest: the Int. J., 1: 47-53.
Thomas ES (2005). Biological wastewater treatment. Chemical engineering magazine, Access Intelligence, LLC, pp 1-6.
Van der Hoek W (2004). A frame work for a global assessment of the extent of wastewater irrigation: The need for a common wastewater typology. In: Scott CA, Faruqui NI, Raschid-Sally L (eds.). Wastewater use in irrigated agriculture: Confronting the livelihood and environmental realities. CABI Publishing, Wallingford, IWMI, Colombo, IDRC, Ottawa, pp 11-24.
Wagner M, Rath G, Amann R, Koops HP, Schleifer K (1995). *In situ* identification of ammonia oxidizing bacteria. Syst. Appl. Microbiol., 18: 251-264.
Wang X, Wen X, Criddle C, Wells G, Zhang J, Zhao Y (2010). Community analysis of ammonia oxidizing bacteria in activated

sludge of eight wastewater treatment systems. J. Environ. Sci., 22: 627–634.
wastewater stabilization lagoons, Presented at 6th National Drinking Water and Wastewater Treatment Technology Transfer Workshop Kansas City, Missouri 64105. 38pp.
Wood PM (1986). Nitrification as a bacterial energy source, Nitrification Spec. Publ. Soc. Gen. Microbiol., IRL Press, Oxford. 20: 39-62.
Ye L, Zhang T (2011). Ammonia-oxidizing bacteria dominates over ammonia-oxidizing archaea in a saline nitrification reactor under low DO and high nitrogen loading. Biotechnol. Bioeng,, 108: 2544-2552.

African Journal of Microbiology Research Vol. 5(32), pp. 5841-5847, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.351

Full Length Research Paper

Purification and Characterization of 56 KDa cold active Protease from *Serratia marcescens*

A. L. TARIQ*, A. L. REYAZ and J. JOHN PRABAKARAN

Department of Biotechnology, K.S.R College of Arts and Science Autonomous, Tiruchengode-637215, Tamilnadu, India.

Accepted 30 July, 2011

The extracellular cold active protease produced from *Serratia marcescens* TS1. The protease was purified to homogeneity from the production medium by ammonium sulphateation then followed by acetone precipitation with 80% saturation. The cold active protease was fractionized by diethylaminoethyl (DEAE) cellulose column chromotography. The molecular weight of protease was approximately 56 KDa. The isoelectric point was close to 6.4. The maximal activity towards casein was found at 40°C and its pH activity was at 8.0. The protease was strongly inactivated by $HgCl_2$ metal ion and reactivated by $FeSO_4$ thus indicated as metalloprotease. The protease was inhibited by Na_2 ethylenediaminetetraacetic acid (EDTA). The protease of *S. marcescens* TS1 showed a potential application in the laundry industry by removeing the blood, chocolate and egg youlk stains from the white cotton cloths in a short period without changing texture of cloths.

Key words: Cold active Protease, *S. marcescens* TS1, extracellular protease, metalloprotease, laundry application.

INTRODUCTION

Serratia marcescens is a gram-negative bacteria belonging to the genus *Serratia* and family enterobacteriaceae (Grimont and Grimont, 2006). The protease secreted by *Serratia marcescen* was purified by thin layer chromatography (Matsuyama et al*.,* 1986). The advance in biotechnological techniques and enzyme engineering paves way for industrial application of protease (White et al*.,* 1973)**.** Proteolytic enzymes from microorganisms may be located within the cell or excreted into the media (Kohlman et al*.,* 1991). Proteases added to laundry detergent enable to release the proteinaceious materials from stained cloths (Masse and Tilburg, 1983). In addition it improves washing efficiency allows shorter period of agitation, often after a preliminary period of soaking (Nielsen et al*.,* 1981; Demidyuk et al., 2008). Many other keratinolytic alkaline proteases were used in feed technology for the production of peptides for degrading waste keratinous materials in bathtub and drains in public places (Takami et al., 1992). It is now firmly established that enzymes in organic solvents can expand the applications of biocatalysts in synthetic chemistry (Zaks and Kilbanov, 1984; Zaks, 1991). Some studies have demonstrated the possibility of using alkaline protease to catalyse peptide synthesis in organic solvents (Golobov et al., 1994). The mechanism in each case is the ability of an enzyme to cleave or cut proten target in to two or more pieces usually at a very specific cleavage sites (Mazzone et al., 1990).

*Corresponding author. E-mail: tariqtasin@gmail.com.

MATERIALS AND METHODS

Bacterial strains

The psychrotrophic bacterial strain *S. marcescens* TS1 screened from the soil of dense apple garden around Badran Magam in Ksahmir at altitude of 1630 meters above the sea level. The bacterial strain was grown at 15°C for 24 h in casein enzyme hydrolysate medium (Rifaat et al., 2007). The strain TS1 has the high proteolytic activity was identified by morphological and biochemical test. Then confirmed based on 16s ribosomal deoxyribonucleic acid (rDNA) gene sequence which were submitted to the Gene Bank and compared with other bacteria by

phylogenetic analysis.

Enzyme production

The cold active protease was produced by following a method of Salamone and Wodzinski (1997) using the enzyme production medium Tryptone-yeast extract glucose broth containing: Tryptone 5 g/L, Yeast extract 2.5 g/L, Glucose 1 g/L and pH 7.2. For the study of protease production 250 ml of medium was poured into 1000 ml of Erlenmeyer flask capacity, were sterilized at 121°C for 15 min.

After cooling 0.5 ml of stationary phase culture of strain TS1 was inoculated and incubated on shaker at 28°C for 48 h.

Enzyme purification

The cold active protease was purified by following method of Matsumato et al. (1984) from culture broth by centrifuged at 8,944 ×g for 20 min at 4°C. The supernatant was collected and filtered through membrane filter having porosity of 0.022 µm at 4°C. To the supernatants ammonium sulphate was added slowly with continuous string to the final concentration of 80% saturation. The enzyme solution was allowed to stand for 24 h at 4°C and centrifuged at 8,944 ×g for 20 min. The precipitate was resuspended in 50 mM Tris HCl having pH 8.0 and further precipitated with acetone by adding slowly to the final concentration 80% saturation and left for 1 h at 4°C. The pellet was obtained by centrifugation at 8,944 ×g for 20 min at 4°C and resuspended in 20 mM Tris HCl pH 8.0 then dialyzed against 500 ml of 5 mM Tris-HCl pH 8.0 containing 1 mM $MgCl_2$ over night at 4°C with stirring conditions. The dialyzate was centrifuged at 5,724 ×g for 20 min at 4°C and supernatant were subjected to diethylaminoethyl (DEAE) - cellulose anion exchange column chromatography equilibrated with 10 mM Tris-HCl buffer pH 8.3. The 15 ml of dialysate eluted with 10 mM Tris-HCl buffer pH 8.3 at the flow rate of 20 ml/h. A linear gradient consisting of 50 ml of 10 mM Tris-HCl buffer pH 8.3 and 50 ml of the same buffer with 0.3 M NaCl. The 5ml of fractions elute was collected and absorbance measure at 280 nm and enzyme activity was determined.

Determination of protein content and assay of proteolytic activity

The protein concentration of strain TS1 was determined by the method of Lowry et al. (1951) by taking bovine serum albumin as standard. The proteolytic activity was determined by following a method of Kunitz (1947) using casein as substrate. The substrate contained 3.75 ml of 1.0% casein in 100 mM Tris-HCl and 1 mM $MgCl_2$ at pH 8.0. The 0.5 ml of protease sample was added to the substrate and incubated for half an hour at 30°C. After incubation, the reaction was quenched with 0.5 ml of 10% trichloro acetic acid. The quenched reaction mixture was centrifuged at 10000 rpm for 10 min to pellet precipitated protein and absorbance for the supernatant was determined at 280nm. One unit of proteolytic was defined as the amount of enzyme that produced an increase of absorbance at 280 nm of 0.1 under the conditions of the assay.

Molecular weight determination by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight of the protein in strain TS1 was determined by the method of Laemmli et al. (1970) staining the protein with 10% methanol, 7% acetic acid and 0.2% coomassie brilliant blue for 4 h and destained with 10% methanol, 25% acetic acid solution for 12 h. The molecular weight analysed by calculated the distance travelled by the protein marker and distance travelled by the sample.

Isoelectric focusing of protein

The isoelecric focusing of purified protease of strain TS1 was determined using mini-gel system (Robertson et al., 1987)**.** The gel was placed in staining solution for 30 min and destained for one hour. The bands were observed in white light transilluminator.

Effect of temperature on protease activity

A 0.2 ml of cold acive protease of strain TS1 was added to the substrate mixture containing 1.5 ml of 1.0% (w/v) casein in 100 mM Tris-HCl in 1 mM $MgCl_2$ at pH 8.0 and incubated at 25, 30, 35, 40, 45, 50, 55, 60°C for 1 h. After the incubation, the proteolytic activity was determined by the protease assay, an optical density was measured at 280 nm.

Effect of pH on protease activity

A 0.2ml of cold acive protease of strain TS1 was added to the substrate mixture containing 1.5ml of 1% (w/v) casein, and 0.1 mM $MgCl_2$ in various buffers. Such as Glycine-HCl buffer having pH 2.0, 2.5, 3.0, 3.5, Acetate buffer having pH 4.0, 4.5, 5.0, 5.5, phosphate buffer having pH 6.0, 6.5, 7.0, Tris- HCl buffer having pH 7.5, 8.0, 8.5, 9.0 and carbonate bicarbonate buffer having pH 9.5, 10.0, 10.5, 11.0 and incubated at 37°C for 60 min. After incubation the proteolytic activity was determined by the protease assay.

Effect of metal ions on protease activity

A 0.2 ml of cold acive protease of strain TS1 was added to the 1.5 ml of 0.1 M Tris-HCl pH 7.5 and to the same buffer supplemented with 100 µl of 8.3 mM of metal ions viz $MgSO_4$, $MnCl_2$, $CaCl_2$, $CuSO_4$, $FeSO_4$, $HgCl_2$ and $ZnCl_2$ and mixtutes were incubated at room temperature 25°C for 30 min and proteolytic activity was determind by protease assay. In addition the purified protease sample preparation (200 µl/ml) was incubated for 30 min at 25°C in 0.1 M acetate buffer having pH5 5.0 supplemented with (100 µl/ml) Na_2EDTA and protease activity was determined by protease assay.

Effect of inhibitors on protease activity

A 0.2 ml of cold acive protease of strain TS1 preparation was added in to 1.5 ml of 0.1 M tris- hydrochloride buffer having pH 7.5 and to the same buffer supplemented with 100 µl of various inhibitors 20 mM Na_2EDTA, 8.3 mM iodioacetic acid, 8.3mM dithiothreitol, 8.3 mM leupeptin, 1% of 2 β-mercaptoethanol, 1% of tween-20 and 3% of ethanol and mixtutes were incubated at 25°C for 30 min and proteolytic activity was determind by protease assay.

Assessment of detegent additive role of protease in laundry industry

The application of cold active protease strain TS1 as detergent addivites in the laundry industry (Masse and Tillburg, 1983) was carried out by taking 100µl/ml of protease strain TS1 and 200mg/ml of wheel detergent on white cotton cloth pieces (10 ×10 cm) stained with human blood, chocolate and egg yolk. The stained clothes incubated at room temperature for 4 h and washed with water

Table1. Purification of Protease from *S. marcescens* TS1 in the supernatants of tryptone yeast extract glucose medium.

S/N	Purification Stage	Volume (ml)	Protein conc. protein (mg/ml)	Total (mg)	Activity (U/ml)	Specific activity (U/mg)	Total Recover activity (U)	Purification fold	(%)
1	Cell free culture supernatant	2500	0.9	2250	864	960	2160000	1	100
2	Ammonium Sulphate fraction	200	4.5	900	7890	1753.3	1578000	1.9	92
3	Acetone fraction	50	5.9	295	23800	4033.8	1190000	4.2	70
4.	Dialysis	100	0.6	60	3950	6583.3	395000	6.9	60
5.	DEAE cellulose Fraction	50	0.3	15	2840	9466.6	142000	9.9	51

then result was noted.

RESULTS

Bacterial soil isolate

The total 211 strains were isolated from the soil and the potential cold actine proteolytic strain TS1 was found gram negative rod shaped bacterium, non-flagellated, non motile, non endospore former. The colony morphological appreance found red pigmented convex, transparent in nature. The biochemical tests showed indole negative, methyl red negative, vogues proskauer positive and citrate positive, bacteria does not produce hydrogen sulphide gas. 16s rDNA gene sequences confirmed that it belongs to *S. marcescens* therefore this bacterium named as *S. marcescens* TS1 under Gene Bank ACC. No. GU046543.

Enzyme purification

The purification process showed that 80% ammonium sulphate saturation had precipitated the protease in the solution by salt out mechanism and further recovered with 80% acetone saturation. The dialysed cold active precipitated protease of *S. marcescens* strain TS1 fractionalised by DEAE Cellulose anion exchanged chromatography with 10 mM Tris HCl buffer pH 8.3 were shown in Table 1.

Determination of protein content and proteolytic activity of protease enzyme

The proteolytic activity was 83.84 IU/ml in casein as substrate. The molecular weight of protease *S. marcescens* strain TS1 was found approximately 56 KDa protein band when observed under white transilluminator (Figure 1) and isoelectric point was 6.4 in an ampholyte buffer having pH ranges from 2.0 to 11.0 (Figure 2).

Effect of temperature on protease activity

The maximium temperature for the cold active protease of *S. marcescens* TS1 was 20°C in 100 mM Tris HCl buffer as shown in Figure 3. The activity declined rapidly above 25°C and was negligible above 50°C. The enzyme retained its 82% activity at 25°C when temperature increased the enzyme activity decreases rapidly and lost at 50°C.

Effect of pH on protease activity

The hydrogen ion concentration of cold active protease *S. marcescens* strain TS1 was 8.5 with a sharp decrease in activity above pH 9.0. The protease had half maximal activity near pH 7.5 and exhibited a little activity below pH 3.5. The protease retained its maximum activity from pH 6.5 to 9.0 (Figure 4).

Effect of metal ions on protease activity

The metal ions have altered the protease activity of *S. marcescens* strain TS1. The $HgCl_2$ and Na_2EDTA have inactivated the protease at both pH 8.5 and pH 6.5. The protease have retained maximum activity in $FeSO_4$, $MgSO_4$, $ZnCl_2$ and minimum activity in $MnCl_2$, $CaCl_2$, $CuSO_4$ and lost its activity in $HgCl_2$ and Na_2EDTA (Table 2). The data indicates that cold active proteases of the *S.*

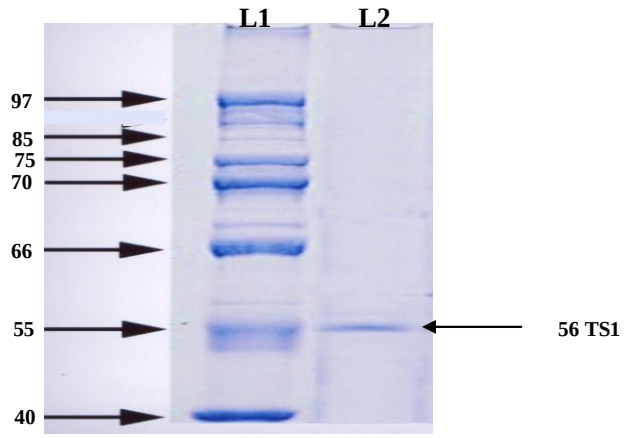


Figure 1. Determination of molecular weight of proteases by sodium dodecyl sulphate agarose gel electrophoresis**. L1—** Molecular marker mass standards: phosphorylase b (97 kDa), tyrosine (85 kDa), acid phosphate (75 kDa), bovine serum albumin (66 kDa), glutamic dehydrogenase (55 kDa) and aldolase (40 kDa), **L2**--Protease sample of *S. marcescens* TS1 (56 kDa).

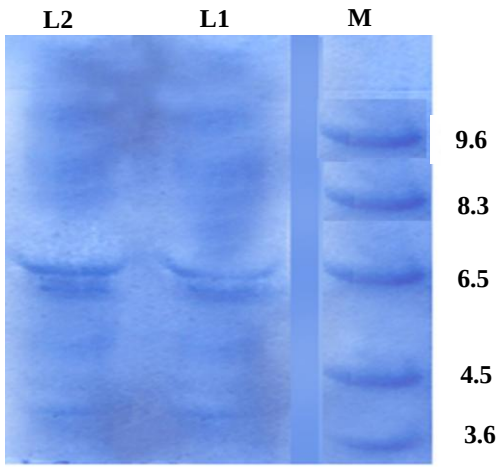


Figure 2. Isoelectric focusing electrophoretogram, pH 2.0 to 11.0 stained with coomassie blue. **M**—Isoelectric focusing standards: amyloglucosidase (pI 3.6), trypsin inhibitor (pI 4.5), carbonic anhydrase II (pI 6.5), lentil lectin (pI 8.3) and ribonuclease A (pI 9.6), **L1 and L2--***Serratia marcescens* TS1 showing pI 6.4.

marcescens strain TS1was to be a metalloprotease because inactivated by the Na_2EDTA and reactivated by the Mg^{2+}, Fe^{2+}, Zn^{2+} is due to increase in the absorbance value at 280 nm and their residual enzyme activity.

Effect of inhibitors on protease activity

The cold active protease of *S. marcescens* strain TS1

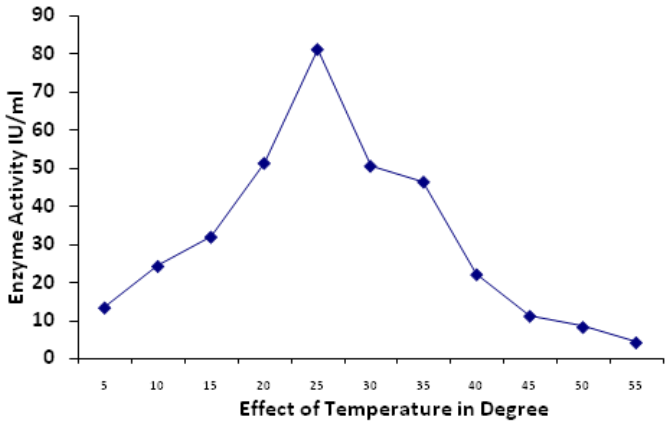


Figure 3. Effect of Temperature on protease activity was examined in 100 mM Tris-HCl buffer having pH 8.0 at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 and 55°C for 30 min. The *Serratia marcescens* strain TS1 showed maximum activity at optimum 25°C.

showed the resistant against the all inhibitors except 20 mM EDTA. The protease retained 80% activity in iodioatic acid, 83% activity in 2-mercaptoethanol, 90% activity in tween 20, 87% activity in 3% ethanol, 73% activity in leupeptin but lost its activity in 20 mM EDTA (Table 3).

Detergent application

There was the litte blood stain in the white cotton cloth which was treated with detergent only but the blood stain was completely removed from the white cotton cloths which were treated with both detergent and protease of *S.marcescens* strain TS1. In case of chocklate and egg yolk there was a stains even when treated with detergent but completely removed when treated with both detergent and protease of *S. marcescens* strain TS1. Thus indicated that protease in presence of detergent removed the stains completely from white cotton cloth pieces (Figure 5).

DISCUSSION

The *S. marcescens* strain TSI secreates large extracellular enzyme protease in the surrounding medium (Yanagida et al., 1988). The production was stoped at early stationnary phase at that time maximium protease was produced (Henriette et al., 1993). The 80% ammonium sulphate saturation leads the precipitation of the protease at 4°C and fractional precipitation with acetone (Salamone and Wodzinski, 1997). The excess salt removed from protease by means of a dialysis (Morita et al., 1997). The dialyzate of *S. marcescens* strain TS1 purified by ion exchange chromatography

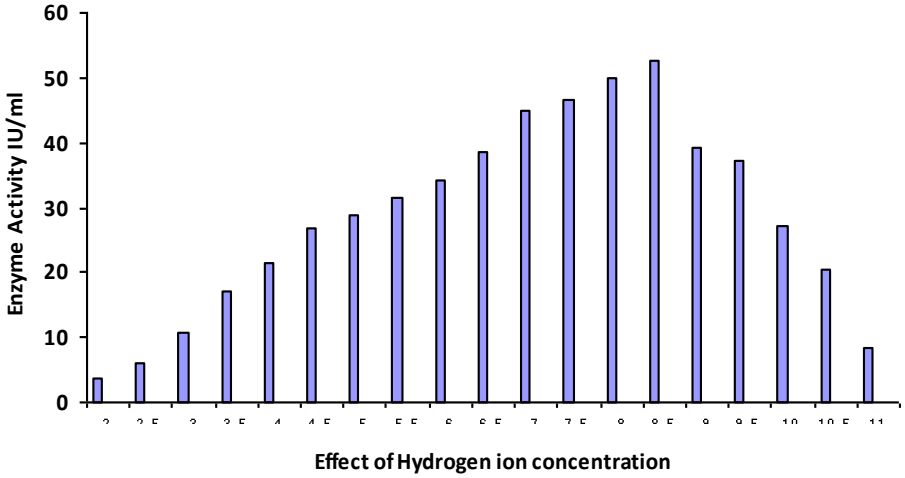


Figure 4. Effect of pH on protease activity was examined in various buffers such as Glycine-HCl buffer having pH 2.0 to 3.5, Acetate buffer having pH 4.0 to 5.5, Phosphate buffer having pH 6.0 to7.0, Tris-HCl buffer having pH 7.5 to 9.0 and Carbonate-Bicarbonate buffer having pH 9.5 to 11.0 at 30°C for 30 min. The pH optimum of *Serratia marcescens* strain TS1 was at pH 8.5 Tris-HCl buffer.

Table 2. Effect of metal ions on protease activity was examined in 8.3 mM of $MgSO_4$, $MnCl_2$, $CaCl_2$, $CuSO_4$, $FeSO_4$, $HgCl_2$ and $ZnCl_2$ in 0.1 M Tris-HCl buffer having pH 7.5 at 25°C for 30 min. The metal ions $HgCl_2$ and 20 mM Na_2EDTA have inactivated the protease of *Serratia marcescens* TS1.

S/n	Metal ion	Residual protease activity (%)
1	Native protease	100
2	$FeSO_4$	81
3	$MnCl_2$	22
4	$CaCl_2$	15
5	$CoSO_4$	11
6	$ZnCl_2$	70
7	$MgSO_4$	78
8	$HgCl_2$	00
9	Na_2EDTA	00

Table 3. Effect of inhibitors on protease activity was examined in 20 mM EDTA, 8.3 mM Iodioacetic acid, 8.3 mM Dithiothreitol, 8.3 mM Leupeptin, 1% of 2- β Mercaptoethanol, 1% of Tween 20 and 3% of ethanol in 0.1 M Tris-HCl having pH 7.5 at 25°C for 30 min. The protease of *Seratia marcescens* strain TS1 was inactivated completely by 20 mM EDTA.

S/N	Inhibitor	Residual protease activity (%)
1	Native protease	100
2	Iodioacetic acid	80
3	2- Mercaptoethanol	83
4	Tween 20	90
5	3% Ethanol	8
6	Dithiothreitol	73
7	Leupeptin	82
8	20 mM EDTA	00

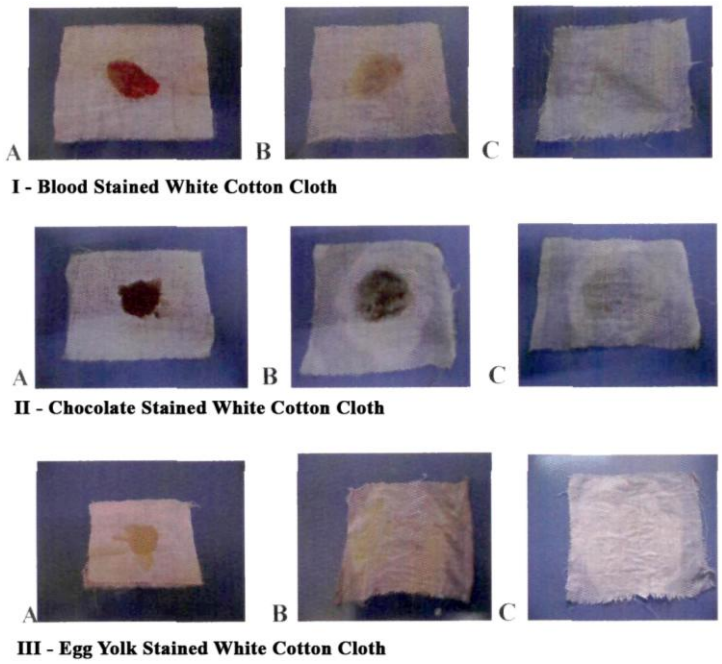


Figure 5. Washing test of protease *Serratia marcescens* strain TS1.
1. A. Blood stained cotton cloth, **B.** Washed with detergent wheel only, **C.** Washed with both wheel detergent and protease of *Serratia marcescens* TS1
II. A. Chocolate stained cotton cloth, **B**. Washed with wheel detergent only, **C.** Washed with both protease of *Serratia marcescens* TS1 and wheel detergent.
III. A. Egg yolk stained cotton cloth, **B**. Washed with wheel detergent only, **C.** Washed with both protease of *Serratia marcescens* TS1 and detergent.

relied on the attraction between oppositely charged particles. The net charge exhibited by these compounds was depends on their pka and pH of the solution. The proteolytic activity was determined by using casein as substrate (Kunitz, 1947) in Tris HCl buffer pH 8.0 showed the protease activity of 83.84 IU/ml. The purified exocellular protease turned out to be one polypeptide chain with a molecular weight of 56 KDa averages of the values obtained by SDS-PAGE (Laemmli, 1970). The isoelectric point of protease *S. marcescens* strain TS1 was 6.4 (Robertson et al., 1987). As proteins are differing in the composition each and every protein has its own characteristic p^{I} value. The optimal timperature of protease *S. marcescens* strain TS1was 40°C in Tris HCl buffer containing $MgCl_2$ having pH 8.0. The protease activity lost when temperature increased at 60°C there was neglible activity. The protease activity was neglible when temperature increased from mesophilic bacteria is around 60°C (Boguslawski et al., 1983). A psychrotrophic *Pseudomonas fluorescens* 114 produced a protease with an optimal temperature of 35°C (Hamamoto, 1994), 37°C an antarctic yeast (Ray et al., 1992) and 40°C by psychrophilic *vibrio* sp. strain 5709 (Hamamoto et al., 1995). The pH characteristics of cold proteases *S. marcescens* strain TS1 showed high enzyme activity between 6.5 to 9.0 and maximum at 8.5 in Tris HCl buffer (Lyerly and Kreger, 1979). The metals ions $HgCl_2$ and Na_2EDTA completely inactivated the protease activity (Matsumoto et al., 1984) and reactivated by Mg^{2+}, Fe^{2+}, Zn^{2+} and Mn^{2+} are essential for the enzyme activity so named as metallprotease (Aiyappa and Haris, 1976). The 20 mM EDTA inhibited the enzyme activity completely while as other inhibiters did not showed much impact on enzyme activity. The potease of *S. marcescens* TS1 acted on the stains and degraded the protein bonds among the proteins present in the blood, chocolate, and egg yolk (Masse and Tilburg, 1983). The increased usage of these proteases as detergent additives is mainly due to the cleaning capabilities of these enzymes, environmentally acceptable and non phosphate detergents.

Conclusion

The cold active protease of *Serrratia marcescens* strain TS1found to more active to remove the dirts and stains from the clothes at low temperature in a short time

without damaging the nature of the cloth. So the economic values and enhencer actions will be boom to the detergent industry as detergent additives.

REFERENCES

Aiyappa PS, Harris JO (1976). The extracellular metalloprotease of *Serratia marcescens*: I. Purification and characterization. Mole Cellular Biochem., 13(2): 95-100.

Boguslawski G, Shults JL, Yehle CO (1983). Purification and characterization of an extracellular protease from *flavobacterium arborescens*. Anal. Biochem., 132: 41-49.

Demidyuk IV, Gasanov EV, Safina DR, Kostrov SV (2008). Structural organization of precursors of Thermolysin-like proteinases. J. Protein., 27: 343–354.

Golobov MY, Stepanov VM, Voyushina TL, Morozoea IP, Adlvereutz P (1994). Side reactions in enzymatic peptide synthesis in organic media; Effect of enzyme, solvent and substrate concentration. Enzy. Micrbial Technol., 16:522-528.

Grimont F, Grimont PAD (2006). The Genus *Serratia.* J. Prokaryotes., 6:219-244.

Hamamoto T, Kaneda M, Horikoshi K, Kudo T (1994). Characterization of a protease from psychrotroph, *Pseudomonas fluroescens* 114. Appl. Envinron. Microbiol., 60: 3878-3880.

Hamamoto T, Kaneda M, Kudo T, Horikoshi K (1995). Characterization of protease from psychrophilic *Vibrio* sp. strain 5709. J. Mar. Biotechnol., 2: 219 -222.

Henriette C, Zinebi S, Aumaitre MF, Petitdemange E, Petitdemange H (1993). Protease and lipase production by a strain of *S. marcescens* (532 S). J. Indstrl. Microbiol., 12:129-135.

Kunitz M (1974). Crystalline soybean trypsin inhibitor.II.General properties. J. Gen. Physiol., 30:291-297.

Laemmli UK (1970). Clearage of structural proteins during the assembly of the head of bacteriophage. J. T. Nat., 227: 680– 685.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265-275.

Lyerly D, Kreger A (1979). Purification and characterization of a *S. marcescens* metalloprotease. J. Infect. Immunity., 24(2): 411-421.

Masse FWJ, Tilburg RV (1983). The benfit of detergents enzymes under changing washind conditions. J. Oil Chem. Soci., 60: 1672-1675.

Matsumoto KMH, Takata K, Kamata R, Okamura R (1984). Purification and characterization of four proteases from clinical isolates of *S. marcescens* kums 3958. J. Bacteriol., 157(11): 225-232.

Matsuyama T, Murakami T, Fujita M, Fujita S, Yano I (1986). Extracellular vesicle formation and biosurfactant production by *Serratia marcescens.* J. Genrl. Microbiol., 132: 865-875.

Mazzone M, Catalani M, Costanzo D (1990). Evaluation of Serratia peptidase in acute or chronic inflamation of otorhinolaryngology pathology: a multicentre, double-blind, randomized trial verses placebo. J. Int. Med. Res., 18(5): 379-388.

Morita Y, Kondoh K, Quamaral H, Sakaguchi T, Murakami Y, Yokoyama K, Tamiya E (1997). Purification and characterization of a cold-active protease from psychrotrophic *S. marcescens* AP3801. J.A.O.C.S., 74: 1377-1383.

Nielsen MH, Jenson SJ, Outtrup H (1981). Enzymes for a low temperature washing. J. Amr. oil Chem. Soci., 58:644-649.

Ray MK, Devi KU, Kumar GS, Shivaji S (1992). Extracelluar protease from the Antarctic yeast *candida humicola*, Ibid., 58: 1918 – 1923.

Rifaat HM, Said OHE, Hassanein SM, Selim MSM (2007). Protease activity of some mesophilic *Streptomycetes* isolated from Egyptian habitats. J. Cultu. Collection., 5:16-24.

Robertson EF, Dannelly HK, Malloy PJ, Reeves HC (1987). Rapid isoelectric focusing in a vertical polyacrylamide mini gel system. Anal. Biochem., 167: 290-294

Salamone PR, Wodzinski RJ (1997). Production, purification and characterization of a 50-KDa extracellular metalloprotease from *Serratia marcescens*. Appl. Microbiol. Biotechnol., 48: 317-324.

Takami HT, Akiba A, Horikoshi K (1992) Substrate specificity of Thermostable Alkaline Protease from *Bacillus* spp. No . AH – 101. Biosci. Biotechnol. Biochem., 56: 333-341.

White A, Handler P, Smith E L (1973) Principles of Biochemistry. 5th edition 1296. Williams and Wilkins. 787.

Yanagida N, Uozumi T, Beppu T (1988). Specific excretion of *S. marcescens* protease through the outer membrane of *Escherichia coli*. J. Bacteriol., 166: 937-944.

Zaks A (1991). Enzyme in organic solvent. In: Dordisk. J. S., ed Biocatalyst for industry. New York: Plenum Press. 166-180.

Zaks A, Kilbanov AM (1984). Enzymatic catalysis in organic media at 100°C. Enzy. Microb. Technol., 224:1145-1152.

African Journal of Microbiology Research Vol. 5(32), pp. 5848-5853, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.703

Full Length Research Paper

Overlap effects of cyromazine concentration, treatment method and rearing temperature on the Southern cowpea weevil (*Callosobruchus maculatus* F.) reared on cowpea

Fahd Abdu Al-Mekhlafi[1], Ashraf Mohamed Ali Mashaly[1]*, Ahmed A. Mahmoud Abdel Mageed[2], Mohamed Ahmed Wadaan[1] and Nazar M. Al-Mallah[3]

[1]Chair of Advanced Proteomics and Cytomics Research, Department of Zoology, College of Science, King Saud University, Riyadh 11451, Kingdom of Saudi Arabia.
[2]Department of Botany and Microbiology, King Saud University, Riyadh 11451, Kingdom of Saudi Arabia.
[3]Plant Protection Department, College of Agriculture and Forestry, Mosul University, Mosul, Iraq.

Accepted 31 October, 2011

Overlap of insect growth regulator (cyromazine) concentration, treatment method and rearing temperature was studied on the southern cowpea weevil, *Callosobruchus maculatus* F. (Bruchidae: Coleoptera), under laboratory conditions. A concentration of 5% cyromazine and dipping treatment at 30°C showed a significant reduction in the reproductive rate by 0.5%. Moreover, increasing the cyromazine concentration led to a decrease in food consumption. Hence, treatment of cowpea seeds via dipping method provided more protection compared to spraying method. Also, the increase of cyromazine concentration led to an increase of generation's lifespan. The longest generation lifespan recorded was 33.33 days when treated with 5% cyromazine by dipping at 30°C. On the other hand, the shortest generation lifespan recorded was 25.33 days when treated with 1% cyromazine concentration by dipping at 30°C. This study showed that there is no effect of the overlap between studied factors, neither in the disparity of sex ratio nor in the disparity of males and females body weights.

Key words: IGR, Insect pest, reproductive rate, sex ratio, Trigard, cyromazine.

INTRODUCTION

Legumes are considered as important crops in the world, as they are used for feeding human and animals in the form of green crops or dry seeds. Legumes are attacked by many insect pests in the field and in stores. The southern cowpeas weevil, *Callosobruchus maculates*, is one of the most common pests, with more than 35 kinds (in the field and stores) of which the most important of them is the cowpea weevil. This insect inflicts heavy losses to seeds up to 62%, since a single larva consumes about 50% of single seed weight during its development (Elazawe and Mahadi, 1983). Saplina (1980) reported that this insect is spread over more than 30 countries around the world. Its ability to fly facilitates the operation of transferring from store to field and reverse easily. The damage is caused as a result of the larvae feeding on the growing seeds, and this damage increases as larvae continue to grow due to increasing consumption of seeds contents (Howe and Currie, 1964; Pajni, 1965; Elazawe et al., 1990). Bastos (1973) found out when testing 241 samples of cowpea seeds that the rate of hit with southern cowpea was 37.8% of the tested samples and after storage for 56 days the damage percentage reached 68.6%, which subsequently led to decrease of the trade value of cowpea seeds by 56%.

The presence of residues in food, resistance develop-

*Corresponding author. E-mail: mmashely@ksu.edu.sa. Tel: +966552574903. Fax: +96614678514.

ment by pest species, health risks (Arthur, 1996), increased cost (Hagstrum and Subramanyam, 2006) and toxicity to non-target organisms (Fields, 1992) has created strong concerns with the use of synthetic chemicals in controlling stored-product insects. Thus, insect growth regulators (IGR) are considered one of the most suitable alternative natural pesticides as they may adversely affect insects by regulating or inhibiting specific biochemical pathways or processes essential for insect growth and development. Some insects exposed to such compounds may die due to abnormal regulation of hormone-mediated cell or organ development. Other insects may die either from a prolonged exposure at the developmental stage to other mortality factors (susceptibility to natural enemies, environmental conditions, etc) or from an abnormal termination of a developmental stage itself (Tunaz, 2004). Miller et al. (1981) pointed out that the use of insect growth regulator (cyromazine) 0.25, 0.5 and 0.1% concentration against the larvae of *Musca domestica* Linn caused 95% mortality, recording rate of success of 3 to 10 over the growth regulator diflubenzuron. In addition, Saito (1988) reported that adding 75% of cyromazine to water resulted in 88% mortality amongst larvae of *Liriomyza bryoniae* Kalt. In an experiment involving the use of cyromazine at the rate of 0.1, 0.5 and 1.1% g/ kg weight of animal in the field of milk cows, Miller et al. (1996) found that its residue in cows' waste discouraged the development of *M. domestica* larvae. Levot and Sates (1998) also found that the use of cyromazine and dezinion each individually against *M. domestica* by concentration 0.4 g/L led to a reduction in the number of *M. domestica* at rate of 69% after one day of treatment and 99.97% in three days of treatment. Vazirianzadeh et al. (2007) concluded that cyromazine (Trigard) should be used in a larvicidal program to control house-fly. More importantly, insect growth regulators were considered to be better used in an integrated pest management program, rather than being used alone (Oberlander et al., 1997).

The objectives of the current study were to determine the impact of overlapping between the different concentrations of growth regulator (cyromazine), treatment methods and the temperatures in the biological activity of southern cowpea weevils.

MATERIALS AND METHODS

Experimental insects

The southern cowpea weevil, *Callosobruchus maculatus* F. (Bruchidae: Coleoptera), was obtained from an entomological research laboratory in the College of Agriculture and Forestry, University of Mosul. All cowpeas (*Vigna unguiculata*) seeds were put in glass jar (1/2 kg each). The southern cowpea weevils were added (20 weevil/jar), covered with a piece of cloth, bond with rubber firmly and then incubated at 30 ± 2°C or 50 ± 5% (Ishimoto et al., 1996). Cultures were renewed after each generation by taking the newly emerged insects for construction of a new culture to conduct further studies.

Insect growth regulator (cyromazine)

This pesticide works as a growth regulator and is used to control larvae of Diptera and Coleoptera. It is being used as a powder in water containing 750 g /kg effective cyromazine (N-cyclopropyl 1, 3, 5-triazine-2, 4, 6-triamine) and sold under the names of various commercial (Larvadex, Premix) classifies toxically within the Class III according to the classification of the World Health Organization (WHO). Solutions of cyromazine were freshly prepared immediately prior to the experiments (Awad and Mulla, 1984).

Pesticide bioassays

Three concentrations of cyromazine were applied (1, 3 and 5%) in the treatment of chickpea seeds. Seeds (25 g per time) were treated twelve times and six times treated in a spraying manner using the Potter Tower at 5 lbs/inches pressure and 2.5 ml of cyromazine solution, ensuring coverage of the surface of seeds. The remaining six were treated by dipping in the cyromazine solution for 1 min. For the control, seeds were treated with water only. Drought seeds have been placed in plastic pots (7 × 7 cm) then five pairs (males and females) of newly emerged adults were transferred into each pot and covered with a piece of cloth sealed with a rubber bond. Pots were then incubated at 25 ± 1 or 30 ± 1°C and 50 ± 5% RH. Treatments were followed up to two successive generations to specify the overlap between different concentrations of cyromazine, treatment method and rearing temperature on the following: Reproductive rate of the southern cowpea weevils is calculated for two successive generations using the formula of Krebs (1978):

$$r = \frac{dn / dt}{n}$$

Where r is the reproductive rate; n is the number of colony individuals; dn is the change in the number of colony individual; and dt is the change in time.

The rate of food consumption was measured by weighing the treated seeds after the end of experiment and deducted from the original weight (25 g). Generation lifespan was calculated from the new adult emergence (from the pupae) until the advent of insects in the second-generation. Sex ratio and weight of males and females were tested by taking a random group of full complete insects each in pot and calculating the number of males and females and their weight.

Data analysis

For conducting the test and analyzing its data, the factorial completely randomized design and Duncan's multiple range tests to change the averages of endurable level 5% (Daoud and Elyass, 1990) were used.

RESULTS AND DISCUSSION

The results presented in Table 1 indicate that increasing in the cyromazine concentration resulted in significant reduction in the reproductive rate of the treated southern cowpea weevils compared to control ones. Cyromazine-treated insects (with a concentration of 5%) showed reproductive rate of 2.58% compared to 36.1% in control ones. House et al. (1978) stated that when they used

Table 1. Summarized results of studied parameters on the southern cowpea weevil, *C. maculatus*, reared on cowpeas with the different concentrations of cyromazine, treatment methods and temperatures.

		Concentration (%)				Treatment methods		Temperatures	
		1	3	5	Control	Dipping	Spraying	25°C	30°C
Reproductive rate		23.3^{C}	8.74^{B}	2.58^{A}	36.11^{D}	14.41^{A}	21^{B}	18.50^{B}	16.9^{A}
Food consumption		8.87^{C}	5.61^{B}	2.89^{A}	18.28^{D}	6.89^{A}	10.94^{B}	9.29^{B}	8.54^{A}
Generation lifespan		28.7^{C}	29.79^{B}	30.67^{A}	27.71^{D}	28.81^{B}	29.63^{A}	31.79^{A}	26.65^{B}
Sex ratio	M	1.05^{A}	1.18^{A}	1.19^{A}	1.11^{A}	1.16^{A}	1.11^{A}	1.10^{A}	1.17^{A}
	F	1.14^{A}	1.05^{A}	1.05^{A}	1.04^{A}	1.07^{A}	1.07^{A}	1.07^{A}	1.07^{A}
Average weights	M	0.96^{A}	1.07^{B}	1.05^{B}	0.95^{A}	0.99^{A}	1.02^{A}	1.02^{A}	0.99^{A}
	F	1.36^{A}	1.59^{B}	1.42^{A}	1.44^{A}	1.42^{A}	1.49^{A}	1.45^{A}	1.46^{A}

Averages of similar characters refer to the existence of significant differences at the 0.05% level of probability. M, Male; F, female.

diflubenzuron to control the boll weevils, *Anthonomus grandis* (Boheman), at a rate of 35, 70 and 140 g/ha, there was an effective reduction in the total percentage of adult emergence from pupae (37.7, 22.21 and 15.8%, respectively). The use of 4 growth regulator, hydroprene, methoprene, diflubenzuron, and MV-678 to control *Ephestia cautella* (Walker) on the peanut, with increased concentration resulted to a decrease of adult emergence (Nickle, 1979). These growth regulators led to down-regulation of the rate of the fertility among treated insects. In oblique-banded leafroller *Choristoneura rosaceana* (Harris), the pupation and adult emergence was significantly delayed at pyriproxyfen concentrations higher than 1 ppm (Sial and Brunner, 2010).

Considering the effect of treatment methods on the reproductive rate, Table 1 shows a decrease in the southern cowpea weevils' number when treated via dipping method (14.41%), compared with the spraying methods (21%). The production rate was monitored with temperature since it was 16.90 at 30°C, while at 25°C it was 18.50. Buholzer et al. (1992) also tested the efficiency of growth regulator (Match) at three different degrees of temperature 18, 24, 30°C in controlling cotton leaf worm *Spodoptera littoralis* (Boisd.) and reported a positive relation between temperature and the efficiency of the Match. The mortality rate increase accordingly with increase in temperature, perhaps that relevant to increasing of insect activity at high temperature leading to increase of growth regulator pick up.

The data from overlap experiment indicated that dipping-treated cowpea seeds (in 5% cyromazine solution at 30°C) showed significant decrease of the production rate of the southern cowpea weevil, recording 0.5, where it was 36.43 in spraying methods (Table 2). The obtained results as shown in Table 1 revealed that the rate of food consumption (8.87, 5.61, and 2.89 g) decreased when cyromazine concentration (1, 3, and 5%) increased compared to control (18.28 g). A significant influence of treatment methods, preferring the dipping method over spraying one where the dipping method resulted in decrease in the rate of food consumption (6.89 g) compared to that of the spraying methods (10.94 g). Temperature also had a significant impact, where at 25°C the rate of food consumption was high (2.29 g), compared to that at 30°C (3.30 g). Gabouri (2000) mentioned that Southern cowpea weevils at 25°C consumed 17.17 g of the food during a complete generation in contrast with that raised at 30-35°C which consumed 12.38 g, and 10.88 g, respectively. Data from overlap experiment indicated that cowpea seeds treated via dipping method in 5% cyromazine solution at 30°C led to significant decrease of the food consumption rate of the southern cowpea weevil, recording 0.5, whereas it was 4.04% in spraying methods (Table 2).

The generation lifespan reached 28.71, 29.79, and 30.67 days at the concentration of 1, 3, and 5% respectively, in contrast with water treated control. The generation lifespan clearly decreased when the dipping method was applied at 28.81 days, while in spraying method was 29.63 days. Temperature plays important role; at 30°C the average of the generation lifespan was 26.65 days, in contrast with generation lifespan at 25°C recorded 31.79 days (Table 1). The highest generation lifespan recorded was 33.33 days in spraying treatment at 5% concentration and 25°C, and the lowest generation lifespan was 26.33 days in dipping treatment at 1% concentration and 30°C (Table 2). Furthermore, Tables 1 and 3 showed no significant differences observed for the sex ratio in any test concentration, treatment method and the temperature compared to the control. Similarly, in *Chironomus riparius,* no significant differences to solvent control were observed in any test concentration, although an exclusive production of males by Daphnia was observed with pyriproxyfen at 100 ng/L (Wang et al., 2005; Tatarazako et al., 2003). Other studies with *C. riparius* which reported sex-related effects at sensitive (molecular) level also exist (Hahn et al., 2001; Hahn and Schulz, 2002). For example Hahn and Schulz (2002)

Table 2. Overlap effect of different concentrations of cyromazine, treatment methods and temperatures on the reproductive rate, food consumption rate and generation lifespan of the southern cowpea weevil, *C. maculates* reared on cowpeas.

Concentration (%)	Treatment method	Temperature	Reproductive rate	Food consumption rate	Generation lifespan
1	Dipping	25	20.60 ± 0.26^{F}	7.23 ± 0.16^{F}	31.17 ± 0.17^{C}
3			3.27 ± 0.15^{C}	4.93 ± 0.23^{E}	32.33 ± 0.17^{B}
5			1.53 ± 0.15^{B}	2.56 ± 0.09^{B}	32.5 ± 0.29^{AB}
Control			36.07 ± 0.23^{J}	12.72 ± 0.14^{K}	30.17 ± 0.17^{D}
1	Spraying		30 ± 0.29^{I}	11.18 ± 0.16^{G}	32 ± 0.50^{B}
3			15.47 ± 0.15^{E}	7.75 ± 0.25^{I}	32.67 ± 0.33^{AD}
5			4.9 ± 0.06^{D}	4.46 ± 0.07^{DE}	33.33 ± 0.33^{A}
Control			36.2 ± 0.12^{J}	23.45 ± 0.21^{N}	30.17 ± 0.17^{D}
1	Dipping	30	15.5 ± 0.29^{E}	7.02 ± 0.18^{FH}	25.33 ± 0.17^{HI}
3			1.4 ± 0.21^{B}	3.19 ± 0.04^{C}	26.67 ± 0.33^{H}
5			0.05 ± 0.06^{A}	0.50 ± 0.06^{A}	27.67 ± 0.17^{F}
Control			36.43 ± 35^{J}	16.94 ± 0.24^{L}	24.67 ± 0.17^{J}
1	Spraying		27.43 ± 0.07^{H}	10.04 ± 0.28^{J}	26.33 ± 0.17^{H}
3			14.83 ± 0.44^{E}	6.55 ± 0.16^{F}	27.5 ± 0.50^{F}
5			3.4 ± 0.21^{C}	4.04 ± 0.18^{D}	29.17 ± 0.17^{E}
Control			35.73 ± 0.15^{J}	20.02 ± 0.13^{M}	25.83 ± 0.17^{HI}

Averages of similar characters refer to the existence of significant differences at the 0.05% level of probability.

Table 3. Overlap effect of different concentrations of cyromazine, treatment methods and temperatures on the sex ratio of the southern cowpea weevil, *C. maculates* reared on cowpeas.

Concentration (%)	Treatment method	Temperature	Mean of the sex ratio ± S.E	
			Male	Female
1	Dipping	25	1.05 ± 0.05^{A}	1.12 ± 0.12^{AB}
3			1.13 ± 0.13^{A}	$1.O3 \pm 0.02^{AB}$
5			1.09 ± 09^{A}	1.06 ± 0.06^{AB}
Control			1.11 ± 0.08^{A}	1.06 ± 0.06^{AB}
1	Spraying		1.07 ± 0.07^{A}	1.04 ± 0.03^{AB}
3			1.15 ± 0.10^{A}	1.09 ± 0.09^{AB}
5			1.09 ± 0.09^{A}	1.12 ± 0.08^{AB}
Control			$1.O7 \pm 0.07^{A}$	1.04 ± 0.04^{AB}
1	Dipping	30	1 ± 0^{A}	1.32 ± 0.27^{B}
3			1.25 ± 0.09^{AB}	1 ± 0^{A}
5			1.47 ± 0.14^{B}	1 ± 0^{A}
Control			1.17 ± 0.17^{A}	1 ± 0^{A}
1	Spraying		1.07 ± 0.04^{A}	$1.O7 \pm 0.07^{AB}$
3			1.19 ± 0.10^{AB}	1.06 ± 0.06^{AB}
5			1.11 ± 0.08^{A}	1.04 ± 0.04^{AB}
Control			1.07 ± 0.07^{A}	1.07 ± 0.07^{AB}

Averages of similar characters refer to the existence of significant differences at the 0.05% level of probability.

Table 4. Overlap effect of different concentrations of cyromazine, treatment methods and temperatures on the average weights of the southern cowpea weevil, C. maculates reared on cowpeas.

Concentration (%)	Treatment method	Temperature	Mean of the average weights ± S.E	
			Male	Female
1	Dipping	25	1.07 ± 0.7^{CDE}	1.37 ± 0.02^{AB}
3			1.05 ± 0.05^{BCDE}	1.52 ± 0.02^{BC}
5			1 ± 0.03^{BCDE}	1.53 ± 0.15^{BC}
Control			1 ± 0.03^{BCDE}	1.45 ± 0.1^{AB}
1	Spraying		0.95 ± 0.03^{ABCD}	1.27 ± 0.07^{A}
3			1.08 ± 0.04^{CDE}	1.83 ± 0.14^{D}
5			1.02 ± 0.11^{BCDE}	1.30 ± 0.03^{AB}
Control			1.02 ± 0.02^{BCDE}	1.32 ± 0.03^{AB}
1	Dipping	30	0.82 ± 0.04^{A}	1.30 ± 0.09^{AB}
3			1.02 ± 0.03^{BCDE}	1.32 ± 0.06^{AB}
5			1.07 ± 0.04^{CDE}	1.37 ± 0.03^{AB}
Control			0.92 ± 0.04^{ABC}	$1.5 \pm 0.06A^{BC}$
1	Spraying		1 ± 0.05^{BCDE}	1.52 ± 0.06^{BC}
3			1.12 ± 0.02^{DE}	1.70 ± 0.06^{CD}
5			1.13 ± 0.0^{7E}	1.48 ± 0.02^{ABC}
Control			0.88 ± 0.05^{AB}	1.50 ± 0.05^{ABC}

Averages of similar characters refer to the existence of significant differences at the 0.05% level of probability.

observed a sex-related effect of tributyltin on the ecdysteroid synthesis and the imaginal disk development by *C. riparius*. In *Leptomastix dactylopii* (Howard), when kinoprene was applied, Rothwangl et al. (2004) found that the sex ratio was equivalent in the petri dish experiment, whereas in the cage experiment the sex ratio was biased toward males.

Regarding the rate of male weight, the increase of cyromazine concentration led to increase of male weight recording 0.96, 1.5 and 1.7 mg at concentrations of 1, 3 and 5, respectively (Table 1), in contrast with control treatment (0.95 mg). Also the study concluded a non significant effect of concentration, temperature and treatment method in the average of male weight recording 1.13 mg at 5% concentration using the spraying method at 30°C as the highest weight. However the lowest male weight recorded was 0.82 mg at 1% concentration via dipping method and at 30°C. For the female average weight, the weight recorded was 1.59 mg at concentration 3% and recorded 1.36, 1.42 mg at 1 and 5% concentration, respectively. Taking together, these results showed no significant differences in the average weight of female between the two concentrations (1 and 5%) and the control, but there was a little difference in female weight at 3% concentration. Also there were no differences in the female weight at 25 to 30°C and the two different methods of treatment (Table 1). The highest female average weight recorded was 1.83 mg at 3% concentration via spraying method at 25°C and the lowest weight recorded was 1.27 mg at 1% concentration via spraying method at 25°C (Table 4). Sial and Brunner (2010) studied the effect of pyriproxyfen, on oblique-banded leafroller *C. rosaceana* (Harris) and found that the weights of adults were significantly increased.

In conclusion, the study of the overlap between cyromazine concentration, treatment method and temperature on the southern cowpea weevil for two successive generations indicated the existence of a difference in the reproductive rate, the rate of food consumption and average of generation lifespan. However, there was no effect neither in the disparity of sex ratio nor in the disparity in the weight of males and females.

ACKNOWLEDGEMENT

The authors extend their appreciation to the Deanship of Scientific Research at king Saud University for funding the work through the research group project no. RGP-VPP-028.

REFERENCES

Arthur FH (1996). Grain protectants: current status and prospects for the future. J. Stored Prod. Res. 32: 293-302.

Awad TI, Mulla MS (1984). Morphogenetic and histo pathological effects induced by the insect growth regulator Cyromazine in *Musca domistica* L Diptera: Muscidae. J. Med. Entomol. 21(4): 419-426.

Bastos JAM (1973). Evaluations of the damage caused by the weevil *C. maculatus* (F.) in samples of coupea *vigna sinensis* (L) collected at Fortaleza ceara. Pesquisa Agropecualia Brasileira Seric Agronomia 8(7):131-132, Cited by The Rev. Appl. Entomol., 64(2): 936.

Buholzer F, Draber J, Bourgeois F, Guyer W (1992). CGA, 184; 699 anew acylurea insecticide Med. Fac. Landbouw. Univ Gent. 57(3): 790.

Daoud KM, Elyass ZA (1990). Statistical methods for agricultural research, National Library of Printing and Publishing, University of Mosul, Iraq.

Elazawe AF, Mahadi MT (1983). Stored products insect. Directorate of University Press, Mosul, Iraq.

Elazawe AF, Quado IQ, Al Haydary HS (1990). Economic insects. Dar Al-Hekma Press, University of Baghdad, Iraq.

Fields PG (1992). The control of stored-product insects and mites with extreme temperatures. J. Stored Prod. Res., 28: 89-118.

Gabouri IAH (2000). Food preference of the Southern cowpea weevil, *Callosobruchus maculatus* (F.) (Bruchidae: Coleoptera) and the effect of different temperatures on the biology, Master thesis, Faculty of Agriculture and Forestry, University of Mosul, Iraq.

Hagstrum DW, Subramanyam BH (2006). Fundamentals of Stored-Product Entomology. AACC International, St. Paul.

Hahn T, Liess M, Schulz R (2001). Effects of the hormone mimetic insecticide tebufenozide on *Chironomus riparius* larvae in two different exposure setups. Ecotoxicol. Environ. Saf. 49(2): 171–178.

Hahn T, Schulz R (2002). Ecdysteroid synthesis and imaginal disc development in the midge *Chironomus riparius* as biomarkers for endocrine effects of tributyltin. Environ. Toxicol. Chem. 21(5): 1052–1057.

House VS, Ables JR, Jones SL, Bull DL (1978). Diflubenzuron for control of the Boll weevil in unisalated isolated cotton fields. J. Econ. Entomol. 71: 797-800.

Howe RW, Currie JE (1964). Some laboratory observations on the rates of development mortality and oviposition of several species of Bruchidae breeding in stored pulses. Bull. Entomol. Res. 55(3): 437-477

Ishimoto M, Sato T, Chrispeels MJ, kitamura K (1996). Bruchid resistance of transgenic azuki bean expressing seed amylase inhibitor of common bean. Entomolgia Experimentalis et Applicata, 79: 309-315

Krebs J (1978). The experimental analysis of distribution and abundance. Harper and Row Publishers. New York, U.S.A.

Levot GW, Sastes N (1998). Effectiveness of amixture of Cyromazine and Diazinon for controlling fly strike on sheep, Aust. Vet. J. 76(5): 343-344.

Miller RW, Corlex C, Cohen CF (1981). (GA-19255 and CGA-72662: Efficacy against flies and possible mode of action and metabolism. South Western Entomol. 6(2): 272-278.

Miller RW, Schmidtmann ET, Wauchope RD, Clegg CM, Herner AE, Weber H (1996). Urine delivery of Cyromazine for suppressing house and stable flies in outdoor dairy calf hutches. J. Econ. Entomol. 89(3): 689-694.

Nickle DA (1979). Insect Growth Regulators: New protectants against the almond moth in stored in shell peanuts. J. Econ. Entomol. 72: 816-819

Oberlander H, Silhacek DL, Shaaya E, Ishaaya I (1997). Current status and future perspectives of the use of insect growth regulators for the control of stored product insects. J. Stored Prod. Res. 33: 1-6

Pajni HR (1965). Bioassay of insecticides relative toxicity of films of different insecticides against the adults of *C. maculatus* (F.). Res. Bull Bonjab Univ. 16(4): 559-341.

Rothwangl KB, Cloyd RA, Wiedenmann RN (2004). Effects of insect growth regulators on citrus mealybug parasitoid *Leptomastix dactylopii* (Hymenoptera: Encyrtidae). J. Econ. Entomol. 97(4): 1239-44.

Saito T (1988). Insecticide activity of several chemicals to the bryony Leafminer *Liriomyza bryoniae* and their application methods. Plant Protect. 35: 168-171

Saplina GS (1980). *Callosobruchus maculates.* Zashchita Rastenii, 7: 41.

Sial AA, Brunner JF (2010). Lethal and Sublethal Effects of an Insect Growth Regulator, Pyriproxyfen, on Obliquebanded Leafroller (Lepidoptera: Tortricidae). J. Econ. Entomol. 103(2): 340-347.

Tatarazako N, Oda S, Watanabe H, Morita M, Iguchi T (2003). Juvenile hormone agonists affect the occurrence of male Daphnia. Chemosphere, 53(8): 827–833

Tunaz H (2004). Insect Growth Regulators for Insect Pest Control. Turkish J. Agric. Forest. 28: 377-387.

Vazirianzadeh B, Jervis MA, Kidd NAC (2007). The Effects of Oral Application of Cyromazine and Triflumuron on House-Fly Larvae. Iranian J. Arthropod-Borne Dis. 1(2): 7-13.

Wang HY, Olmstead AW, Li H, LeBlanc GA (2005). The screening of chemicals for juvenoid-related endocrine activity using the water flea Daphnia magna. Aquatic Toxicol. 74(3): 193–204.

African Journal of Microbiology Research Vol. 5(32), pp. 5854-5862, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.707

Full Length Research Paper

Antioxidant and antibacterial activities of *Camptotheca acuminate* D. seed oil

Lin Wang[1, 2], Zhiwei Yang[1*], Sicen Wang[3], Shuqiu Wang[1] and Junxing Liu[1*]

[1]School of Basic Medical Sciences, Jiamusi University, Jiamusi 15400, P. R. China.
[2] The First Affiliated Hospital of Jiamusi University, Jiamusi 154003, P. R. China.
[3]Research and Engineering Center for Natural Medicine, Xi'an Jiaotong University, Xi'an 710061, P. R. China.

Accepted 11 November, 2011

This study was designed to explore the *in vitro* antioxidant and antibacterial activities of *Camptotheca acuminate* D. seed oil, which were extracted by supercritical fluid extraction (SFE) or petroleum ether extraction methods. The major constituent of the oil were described as (Z,Z,Z)-9,12,15-Octadecatrien-1-ol (54.92%) and 2-[(trimethylsilyl)oxy]-3-[4-[(trimethylsilyl)oxy]phenyl]-trimethylsilyl ester (26.53%) in supercritical fluid and petroleum ether extracts. The oil and the components were subjected to screen for their possible antioxidant activity by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay and β-carotene bleaching test. In the DPPH test system, free radical scavenging activities of supercritical fluid extracts and petroleum ether extracts were determined to be 7.55 ± 0.11% and 4.38 ± 0.08% (v/v), respectively. As to the β-carotene bleaching test system, the two values were 15.93 ± 0.11% and 6.87 ± 0.15% (v/v), respectively. The activities of antioxidant and antibacterial in components of petroleum ether were more efficient than in components of supercritical fluid extraction. As to the antimicrobial activities of the essential oil against 8 species bacterium, *C. acuminate* D. seed oil had remarkable antibacterial activity, especially to *staphylococcus aureus* (ATCC 6538). Thus, *C. acuminate* D. seed oil could be judged as a kind of patent drug which has antioxidant and antibacterial activity effectively.

Key words: *Camptotheca acuminate* D. seed oil, antioxidant activities, antibacterial activities.

INTRODUCTION

Camptotheca acuminata Decaisne belongs to the family of Nyssaceae. It is a kind of deciduous and medicinal tree from south China and listed as an endangered species in China (Cheng et al., 2008; Li et al., 2002; (Yang et al, 2011) DOI: 10.1002/qua.23046). The main active ingredients of *C. acuminate* D. extracts are quinoline and indole alkaloids (Wall et al., 1966). The camptothecin (CPT) (a terpenoid quinoline alkaloid) and its analogues are the potent topoisomerase I inhibitors, which have been used as the anticancer drugs to treat ovarian, lung and colorectal cancers or the antiviral agents (Li and Adair, 1994; Oberlies and Kroll, 2004).

Some studies on anticancer activities of *C. acuminata* D. have been reported. However, the antioxidant activity of its seed oil has not yet been studied. The seed oil of *C. acuminata* D. possesses the potential as high-quality edible oil that is beneficial to health and valuable natural antioxidants in cosmetic and pharmaceutical industries.

Industrial seed oils are generally obtained with the aid of mechanical process and organic solvent extraction (mainly hexane). The oil prepared by mechanical separation is of high quality, but in most cases the yield is low. Hexane extraction can achieve almost complete recovery of the oil, but the solvent is quite harmful to human health and environment, embarrassing the use in food, cosmetic and pharmaceutical industries. Supercritical fluid extraction (SFE) with supercritical carbon dioxide (SC-CO_2) is an alternative method to

*Corresponding author. E-mail: yzws-123@163.com. iujunxing0982@163.com, Tel: +086 0454 6166452. Fax: +086 0454 8618355.

extract the oils from natural products and has received considerable attention (Gomes et al., 2007; Lu et al., 2007; SalgIn, 2007). The oil obtained by SC-CO_2 extraction is of high quality, and the yield is comparable with those of organic solvent extractions (Friedrich et al., 1982; Molero Gómez et al., 1996). In fact, CO_2 extracts have been generally considered as safety in food applications (Gerard and May, 2002), and SFE has been served as a very potential technology in food and pharmaceutical operations (King, 2000).

To the best of our knowledge, the antioxidant and antibacterial activities of *C. acuminate* D. seed oil *in vitro* have not yet been evaluated. Therefore, in this work, the oil will be separately extracted by SFE and petroleum ether extraction methods, and the activities will be evaluated by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay, β-carotene bleaching test, as well as minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) determinations. We anticipate that the investigation will be of value in the development of antioxidant and antibacterial agents.

MATERIALS AND METHODS

Preparation of extract

Isolation of C. acuminata seed oil by petroleum ether

C. acuminate D. seeds were air dried at room temperature and ground, and 100 g were subjected to the distillation with a British-type clevenger apparatus at 100℃ for 3 h. Then, the extracts were filtered and concentrated in vacuum at 45℃, yieldin g the seed soil in yellow.

Supercritical carbon dioxide (SC-CO_2) extraction

C. acuminate D. seeds were air dried at room temperature and ground in a grinder with a mesh of 2 mm in diameter. Then, they were passed through a 0.5 mm sieve to obtain a fine powder. The extraction temperature was 45℃ and the pressure was 5 .5 MPa. The flow rate was determined using a watch. The flow rate of CO_2 was 10 kg/h; and the extraction time was 2 h. Liquid CO_2 was supplied from a gas cylinder. Before passing into the extraction vessel filled with the samples by pump, the liquid CO_2 was adjusted to the desired pressure and heated to a specified temperature to reach the supercritical state. Finally, the extracts were lyophilized and kept in dark at 4℃ until the next step.

Gas chromatography

The sample was diluted by ethyl acetate (1:100) and mixed. The analysis of the essential oil was performed using a VG platform II Gas chromatography-Mass spectroscopy (GC–MS) system equipped with an Rtx-5MS capillary column (30 × 0.25 mm; film thickness 0.25 mm). For the Rtx-5MS detection, the injector temperature was set at 280℃; split injection with a ratio of 100: 1; and the injection volume was 1 µl with a flow controlled by a linear model. Helium was the carrier gas at a flow rate of 1.6 ml/min. Starting from 60℃, the temperature was raised to 280 a t 10℃ /min and held for 5 min. Injector and detector MS transfer line temperatures were set at 200 and 280℃, respecti vely, and the sample collection time was 3 min (m/z = 40 to 500).

Antioxidant activity

General description

Antioxidant activity was assessed by DPPH assay and β-carotene bleaching test. All data collected for each assay were the average of the measurements of three independent experiments.

DPPH radical scavenging assay and the oil obtained by SC-CO_2 extraction

We measured the bleaching of purple-colored ethanol solution of DPPH. This spectrophotometric assay uses the stable radical DPPH as a reagent (Wu et al., 2010). An aliquot of the sample (100 µl) was mixed with 1.4 ml of ethanol and then added to 1 ml of 0.004% DPPH (Sigma–Aldrich) in ethanol. The mixture was vigorously shaken and then immediately placed in a UV–Vis spectrophotometer (AWARENESS) to monitor the decrease in absorbance at 517 nm. Monitoring was continued for 70 min until the reaction reached a plateau. Ascorbic acid (Sigma–Aldrich), a stable antioxidant, was used as a synthetic reference. The radical scavenging activities of samples were calculated as the inhibition percentage of DPPH according to the formula:

Inhibition percentage (Ip) = [(AB-AA)/AB] ×100 (Yen and Duh, 1994)

where AB and AA are the absorbance values of the blank sample and the tested samples examined after 70 min, respectively.

β-Carotene-linoleic acid bleaching assay

Antioxidant activity of the samples was determined with β-carotene bleaching test (Wu et al., 2010). Approximately 10 mg of β-carotene (type I synthetic, Sigma–Aldrich) was dissolved in 10 ml chloroform. The carotene–chloroform solution of 0.2 ml was pipetted into a boiling flask containing 20 mg linoleic acid (Sigma–Aldrich) and 200 mg Tween 40 (Sigma–Aldrich). Chloroform was removed by a rotary evaporator (RE-52AA) at 40℃ for 5 min, and to th e residue, 50 mL of distilled water was added, slowly with vigorous agitation, to form an emulsion. The emulsion (5 ml) was added to a tube containing 0.2 ml of the sample solution and the absorbance was immediately measured at 470 nm against a blank consisting of an emulsion without β-carotene. The tubes were placed in a water bath at 50℃, and the oxidation of the emulsion was moni tored by a spectrophotometer at 470 nm over a period of 60 min. Control samples contained 10 ml of water instead. Butylated hydroxytoluene (BHT) (Sigma–Aldrich), a stable antioxidant, was used as a synthetic reference. The antioxidant activity was expressed as the inhibition percentage relative to the control after 60 min incubation with the following equation:

AA = 100(DRC - RS)/DRC

Where AA, antioxidant activity; DRC, degradation rate of the control [ln(a/b)/60]; DRS, degradation rate in the presence of the sample [ln(a/b)/60]; a, absorbance at time 0; b, absorbance at 60 min.

Antimicrobial activity

The microorganisms used for testing antimicrobial sensitivity included *Bacillus subtilis* 6633, *Staphylococcus aureus* ATCC 6538

and *Staphylococcus epidermidis* ATCC 49134, *Escherichia coli* ATCC 11229, *Proteus vulgaris, Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillus niger V. Tiegh*. They were obtained from the Center for Microbiology Research, Jiamusi Medical Research Institute.

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) determination of *Camptotheca acuminate* D. seed oil

The MIC and MBC were measured by the broth micro-dilution method (NCCLS, 2002). The essential oils were individually dissolved in sterilized physiological saline solution (0.9% w/v) supplemented with Tween 80 (Sigma) at a final concentration of 0.5% (v/v). Serial doubling dilutions of the oils were prepared in a 96-well microtiter (µL) plate in the range of 0.156 to 4.000% (v/v). Each essential oil dilution (100 µL) was dispensed into the wells of a microtiter plate, and each well was then inoculated with 100 µL of the suspension. The obtained suspensions were mixed with a micro-pipette. The final concentration of each strain was adjusted to 10^5 to 10^6 CFU/mL. All microtiter plates against all microorganisms were incubated at 37℃ for 24 h, except for *A. niger* that was incubated at 25℃ for 5 days. After incubation, the wells were examined for the microorganism growth, and the MICs were determined. The MIC was defined as the lowest concentration of the essential oil at which the microorganism did not show visible growth. The MBCs were confirmed by reinoculating on agar plates with 10 µL of each culture medium from the microplates. The MBCs were defined as the lowest concentration of the essential oil at which incubated microorganisms were completely killed. *Streptomycin* and *Amphotericin B* were served as the positive controls. Each experiment was repeated for three times.

RESULTS AND DISCUSSION

Chemical composition

We identified 50 components in the *C. acuminate* D. seed oil obtained by SC-CO_2 extraction (Table 1). Fifty-three (53) components were identified in the oil obtained by petroleum ether (Table 2). The major components were (Z,Z,Z) - 9, 12, 15 - Octadecatrien- 1- oil (54.92%), octadecanoic acid (13.46%), n-hexadecanoic acid (11.63%) in the seed oil (Table 1) obtained by SC-CO_2. While the major components were [4-[(trimethylsilyl)oxy]phenyl]-2-[(trimethylsilyl)oxy]-3-2-propenoicacid trimethylsilyl ester (26.53%), gamma-sitosterol (23.49%), (Z,Z,Z)-9,12,15-octadecatrienoic acid, and methyl ester (19.19%) in the seed oil obtained by petroleum ether.

Antioxidant activity

The antioxidant activities of the essential *C. acuminate* D. seed oil obtained by SC-CO_2 extraction or petroleum ether were determined by two complementary test systems: DPPH assay and β-carotene bleaching tests. The results of antioxidant activity in these test systems were collected and shown in Figures 1 to 3. In the DPPH test system, free radical - scavenging activity of *C. acuminate* D. seed oil obtained by SC-CO_2 extraction was determined to be 81.39 ± 0.92%; whereas the oil obtained by petroleum ether was 87.13 ± 1.81% (Figure 1). As for the lipid peroxidation inhibitory activity of the essential oil by the β-carotene bleaching test, the results were consistent with the data obtained from the DPPH test (Figure 2). Compared with BHT, the effects of *C. acuminate* D. seed oil obtained by SC-CO_2 extraction or petroleum ether were 80.82 ± 0.32% and 85.47 ± 0.54%, respectively. The concentration of 50% inhibition (IC_{50}) values of BHT, *C. acuminate* D. seed oil obtained by SC-CO_2 or petroleum ether were 3.24 ± 0.12%, 7.55 ± 0.11% and 4.20 ± 0.08%, respectively (Figure 3). It seemed that the antioxidant activities of all the tested samples were mostly related to their concentrations, and the IC_{50} values of these two types of seed oil were both higher than that of the synthetic antioxidant BHT (Figures 2 and 3).

Antimicrobial activity

Minimal inhibitory concentration (MICs) and minimal bactericidal concentration (MBCs) of Camptotheca acuminate D. seed oil

As shown in Tables 3 and 4, the essential oils exhibited inhibitory effects of all the testing organisms. The oil obtained by SC-CO_2 exhibited somewhat higher antimicrobial activity on *S. epidermidis* ATCC 49134 rather than other microorganisms; whereas the oil obtained by petroleum ether showed more potent on *S. aureus* ATCC 6538, *P. aeruginosa* and *C. albicans*. The antimicrobial activities of the seed oil obtained by SC-CO_2 against *B. subtilis* 6633, *P. vulgaris* and *A. niger V. Tiegh* were less than those of other microorganisms; whereas the oil obtained by petroleum ether showed less inhibitory effects on *B. subtilis* 6633 and *P. vulgaris.* The MICs of the oil obtained by SC-CO_2 extraction ranged from 0.625% (v/v) to more than 5.000% (v/v) for all testing microorganisms; while as to petroleum ether, the values ranged from 0.625% (v/v) to more than 5.000% (v/v). The MBCs were similar or even higher than the corresponding MIC values.

The activity components of Camptotheca acuminate D. seed oil

The essential oil of *C. sativum* obtained on hydro-distillation was analyzed by GC–MS. We identified 24 components, representing 92.7% of the total oil. Table 1 summarized the constituents identified by GC–MS analysis, their retention indices and area percentages (concentrations). The oil was dominated by aldehydes and alcohols, which accounted for 55.5 and 36.3%, respectively. The major aldehydes were 2E-decenal (15.9%) and decanal (14.3%), while the alcohols mainly consisted of 2E-decen-1-ol (14.2%) and n-decanol

Table 1. The chemical compositions of *C. acuminate* D. seed oil obtained by supercritical carbon dioxide extraction (SC-CO_2).

No.	RT	Compounds	Molecular formula	MW	Relative
1.	3.246	Nonane	128	C_9H_{20}	0.25
2.	3.652	propyl-cyclohexane,	126	C_9H_{18}	0.10
3.	4.097	Hexanoic acid	116	$C_6H_{12}O_2$	0.19
4.	4.443	(E,E)-2,4-Heptadienal,	110	$C_7H_{10}O$	0.14
5.	4.627	1-ethyl-Cyclohexene,	110	C_8H_{14}	0.05
6.	6.079	Phenylethyl Alcohol	122	$C_8H_{10}O$	0.06
7.	8.510	8-Methylene-3 oxatricyclo[5.2.0.0(2,4)]nonane	136	$C_9H_{12}O$	0.13
8.	8.552	(E,E)-2,4-Decadienal,	152	$C_{10}H_{16}O$	0.09
9.	8.872	2,7-Dimethyl-1,3,7-Octatriene,	136	$C_{10}H_{16}$	0.33
10.	12.737	7-Bromomethyl-Pentadec-7-ene,	302	$C_{16}H_{31}Br$	0.06
11.	14.169	Tetradecanoic acid	228	$C_{14}H_{28}O_2$	0.07
12.	14.574	Heneicosane	296	$C_{21}H_{44}$	0.09
13.	15.015	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	296	$C_{20}H_{40}O$	0.07
14.	15.090	6,10,14-Trimethyl-2-Pentadecanone,	268	$C_{18}H_{36}O$	0.09
15.	15.225	Pentadecanoic acid	242	$C_{15}H_{30}O_2$	0.06
16.	15.417	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	278	$C_{16}H_{22}O_4$	0.79
17.	15.888	1,2-Benzenedicarboxylic acid, butyl octyl ester	334	$C_{20}H_{30}O_4$	0.09
18.	16.077	9-Hexadecenoic acid	254	$C_{16}H_{30}O_2$	0.14
19.	16.329	n-Hexadecanoic acid	256	$C_{16}H_{32}O_2$	11.63
20.	16.595	Heneicosane	296	$C_{21}H_{44}$	0.28
21.	17.217	Heptadecanoic acid	270	$C_{17}H_{34}O_2$	0.07
22.	17.424	1-Octadecanol	270	$C_{18}H_{38}O$	0.14
23.	17.537	2,6,10,15-Tetramethyl-Heptadecane,	296	$C_{21}H_{44}$	0.12
24.	17.618	(Z,Z,Z)-9,12,15-Octadecatrienoic acid, methyl ester,	292	$C_{19}H_{32}O_2$	0.10
25.	17.714	Phytol	296	$C_{20}H_{40}O$	0.15
26.	18.195	(Z,Z,Z)-9,12,15-Octadecatrien-1-ol,	264	$C_{18}H_{32}O$	54.92
27.	18.265	Octadecanoic acid	284	$C_{18}H_{36}O_2$	13.46
28.	18.917	cis,cis,cis-7,10,13-Hexadecatrienal	234	$C_{16}H_{26}O$	0.55
29.	19.297	Pentacosane	352	$C_{25}H_{52}$	0.15
30.	19.542	11,14,17-Eicosatrienoic acid, methyl ester	320	$C_{21}H_{36}O_2$	0.29
31.	19.882	E-8-Methyl-7-dodecen-1-ol acetate	240	$C_{15}H_{28}O_2$	0.24
32.	20.912	Tetratetracontane	618	$C_{44}H_{90}$	0.56
33.	21.084	2-Mono-Palmitin,	330	$C_{19}H_{38}O_4$	0.99
34.	21.672	Pentatriacontane	492	$C_{35}H_{72}$	0.16
35.	21.975	Octadecanal	268	$C_{18}H_{36}O$	0.07
36.	22.281	(Z,Z)-9,12-Octadecadienoic acid, trimethylsilyl ester	352	$C_{21}H_{40}O_2Si$	0.18
37.	22.350	(all-Z)-4,7,10,13,16,19-Docosahexaenoic acid, methyl ester,	342	$C_{23}H_{34}O_2$	0.14
38.	22.502	E,Z-1,3,12-Nonadecatriene	262	$C_{19}H_{34}$	3.94
39.	22.585	Methyl(Z)-5,11,14,17-eicosatetraenoate	318	$C_{21}H_{34}O_2$	4.30
40.	22.683	1-Mono-Stearin,	358	$C_{21}H_{42}O_4$	0.63
41.	23.251	1-Hentetracontanol	592	$C_{41}H_{84}O$	0.66
42.	23.667	(all-E)- 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22 Tetracosahexaene,	410	$C_{30}H_{50}$	1.39
43.	24.183	6,6-Dimethyl-Bicyclo[3.1.1] hept-2-ene-2-ethanol,	166	$C_{11}H_{18}O$	0.06
44.	24.268	Triacontane	422	$C_{30}H_{62}$	0.66
45.	24.331	17-Pentatriacontene	490	$C_{35}H_{70}$	0.51
46.	24.540	2-Nonadecanone	282	$C_{19}H_{38}O$	0.14
47.	25.074	8-Methyltocol	402	$C_{27}H_{46}O_2$	0.18
48.	26.091	Octadecanal	268	$C_{18}H_{36}O$	0.10
49.	26.519	β-Tocopherol	416	$C_{28}H_{48}O_2$	0.28
50.	26.862	Tetratriacontane	478	$C_{34}H_{70}$	0.15

Table 2. The chemical compositions of *C. acuminate* D. seed oil obtained by petroleum ether.

No.	RT	Compounds	Molecular formula	MW	Relative
1.	3.235	1,3,5,7-Cyclooctatetraene	104	C_8H_8	0.10
2.	5.199	3,6-dimethyl-Decane,	170	$C_{12}H_{26}$	0.06
3.	6.183	1,2,4,5-tetramethyl-Benzene,	134	$C_{10}H_{14}$	0.07
4.	8.071	Pentadecane	212	$C_{15}H_{32}$	0.07
5.	8.301	4,6-dimethyl-Dodecane,	198	$C_{14}H_{30}$	0.15
6.	8.923	Heptadecane	240	$C_{17}H_{36}$	0.04
7.	11.008	Acetamidocyclohexane	141	$C_8H_{15}NO$	0.07
8.	11.120	Cetyl iodide	352	$C_{16}H_{33}I$	0.12
9.	11.158	Heptadecane	240	$C_{17}H_{36}$	0.06
10.	11.393	2,4-bis(1,1-dimethylethyl)-Phenol,	206	$C_{14}H_{22}O$	0.07
11.	11.668	Heneicosane	296	$C_{21}H_{44}$	0.04
12.	13.629	Eicosane	282	$C_{20}H_{42}$	0.08
13.	14.103	Hexadecane	226	$C_{16}H_{34}$	0.04
14.	14.177	Tetradecanoic acid	228	$C_{14}H_{28}O_2$	0.11
15.	15.008	5-Isopropyl-1-methyl-1-cyclohexene	138	$C_{10}H_{18}$	0.07
16.	15.095	6,10,14-trimethyl-2-Pentadecanone,	268	$C_{18}H_{36}O$	0.06
17.	15.419	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	278	$C_{16}H_{22}O_4$	0.16
18.	15.600	diethyl-Borinic acid,	86	$C_4H_{11}BO$	0.05
19.	15.878	2,6,10,14-tetramethyl-Hexadecane,	282	$C_{20}H_{42}$	0.06
20.	16.270	n-Hexadecanoic acid	256	$C_{16}H_{32}O_2$	3.19
21.	17.424	1-Octadecanol	270	$C_{18}H_{38}O$	0.06
22.	17.538	Heneicosane	296	$C_{21}H_{44}$	0.08
23.	17.714	Phytol	296	$C_{20}H_{40}O$	0.13
24.	18.037	(Z,Z,Z)-9,12,15-Octadecatrienoic acid, methyl ester,	292	$C_{19}H_{32}O_2$	19.19
25.	18.162	(Z,Z)-9,12-Octadecadienoic acid	280	$C_{18}H_{32}O_2$	3.35
26.	18.442	1,54-Dibromotetrapentacontane	914	$C_{54}H_{108}Br_2$	0.60
27.	19.292	Pentacosane	352	$C_{25}H_{52}$	0.11
28.	20.118	Tetracosane	338	$C_{24}H_{50}$	0.08
29.	20.792	trans-9-Octadecen-1-ol	268	$C_{18}H_{36}O$	0.09
30.	20.910	Tetratetracontane	618	$C_{44}H_{90}$	0.37
31.	21.078	2-mono-Palmitin,	330	$C_{19}H_{38}O_4$	0.14
32.	21.673	Pentatriacontane	492	$C_{35}H_{72}$	0.07
33.	21.906	(3.beta.)-Ergost-5-en-3-ol,	400	$C_{28}H_{48}O$	1.98
34.	22.142	3-Fluorobenzoic acid, 4-hexadecyl ester	364	$C_{23}H_{37}FO_2$	0.23
35.	22.342	1-Pent-3-ynylcyclopenta-1,3-diene	132	$C_{10}H_{12}$	0.10
36.	22.441	1,54-dibromo-Tetrapentacontane,	914	$C_{54}H_{108}Br_2$	0.46
37.	22.489	9-Octadecenoic acid, (E,E,E)-1,2,3-propanetriyl ester,	884	$C_{57}H_{104}O_6$	0.31
38.	22.569	(Z,Z,Z)-9,12,15-Octadecatrienoic acid, ethyl ester,	306	$C_{20}H_{34}O_2$	0.34
39.	22.608	2-Nonadecanone	282	$C_{19}H_{38}O$	0.29
40.	22.675	Stigmastane-3,6-dione	428	$C_{29}H_{48}O_2$	0.18
41.	23.250	Pentafluoropropionic acid, heptadecyl ester	402	$C_{20}H_{35}F_5O_2$	0.28
42.	23.483	1-Hexadecanesulfonyl chloride	324	$C_{16}H_{33}C_lO_2S$	0.46
43.	23.664	All-trans-Squalene	410	$C_{30}H_{50}$	0.85
44.	24.167	[4-[(trimethylsilyl)oxy]phenyl]-2-[(trimethylsilyl)oxy]-3-2-Propenoic acid, trimethylsilyl ester	396	$C_{18}H_{32}O_4Si_3$	26.53
45.	24.265	.gamma.-Sitosterol	414	$C_{29}H_{50}O$	23.49
46.	24.578	Fucosterol	412	$C_{29}H_{48}O$	1.38
47.	25.034	[3S-(3.alpha.,5a.alpha.,7a.alpha.,11a.beta.,11b.alpha.)]-dodecahydro -3,8,8,11a-tetramethyl-5H-3,5a-Epoxynaphth[2,1-c]oxepin, ,	278	$C_{18}H_{30}O_2$	1.39
48.	25.318	Betulin	442	$C_{30}H_{50}O_2$	0.43
49.	25.560	Cedran-8-yl acetate	264	$C_{17}H_{28}O_2$	3.32
50.	25.725	Lup-20(29)-en-3-one	424	$C_{30}H_{48}O$	4.30
51.	26.091	cis-1-Chloro-9-octadecene	286	$C_{18}H_{35}Cl$	0.52
52.	26.323	Lupenyl acetate	468	$C_{32}H_{52}O_2$	3.79
53.	26.514	.beta.-Tocopherol	416	$C_{28}H_{48}O_2$	0.41

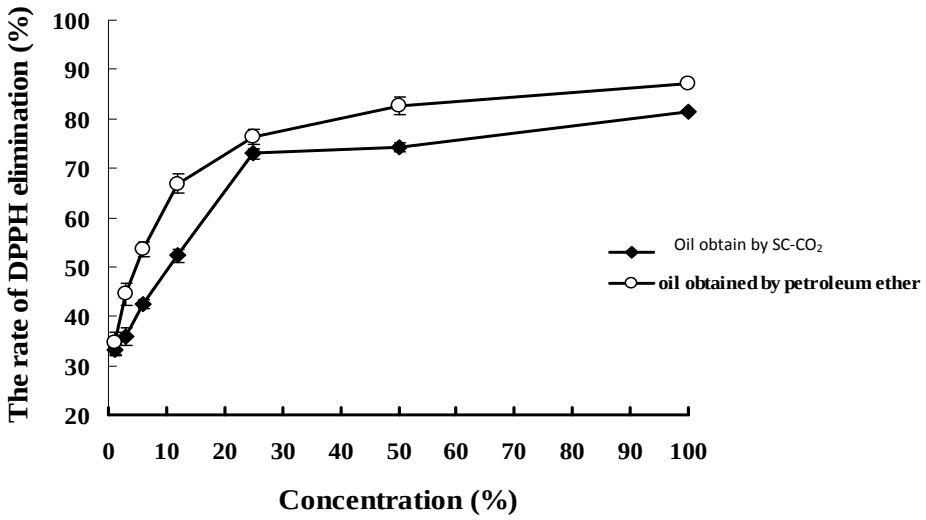


Figure 1. The rate of DPPH elimination. Values of each curve are means ± SD (n, 3). $p < 0.01$.

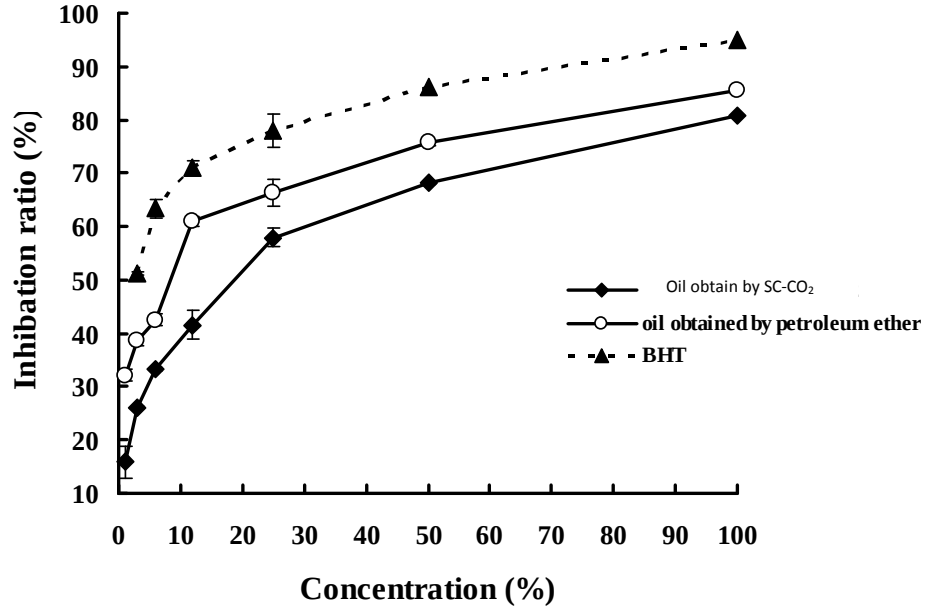


Figure 2. β-Carotene bleaching test. Values of each curve are means ± SD (n, 3). $p < 0.01$.

(13.6%). Other aldehydes in appreciable amounts were 2E-tridecen-1-al (6.75%), 2E-dodecenal (6.23%), dodecanal (4.36%) and undecanal (3.23%). The alcohol undecanol (3.37%) was also in fairly good amount. The monoterpenes apinene (0.04%) and linalool (0.32%) were in trace amounts. However, the chemical composition of the essential oil was different from that observed from Tunisian plant materials (Msaada et al., 2007). Indeed, in the Tunisia study, the predominant aldehyde was 2E-dodecenal, while in our study, it was 2E-decenal. The essential oil was evaluated for antimicrobial activity against pathogenic strains of Gram positive (*S. aureus* and *Bacillus* spp.) and Gram negative (*E. coli*, *P. aeruginosae*, *S. typhi*, *Klebsiella pneumoniae*, and *Proteus mirabilis*) bacteria. It was active against all the bacterial strains except *P. aeruginasae*.

The oil also showed an obvious antifungal activity against *C. albicans* and *P. aeruginosae*, which also been

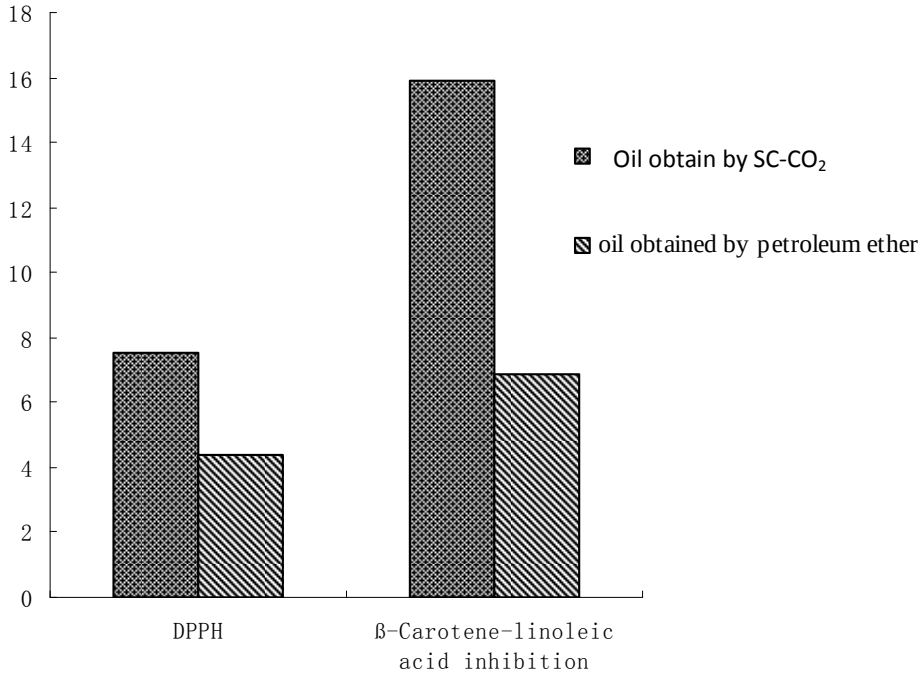


Figure 3. The concentration of 50% inhibition (IC_{50}) values of *C. acuminate* D. seed oil obtained by SC-CO_2 or petroleum ether.

Table 3. The inhibitory effects of C. acuminate D. seed oil obtained by SC-CO2 against all the testing organisms.

Bacterial strain	MIC (%)	MBC (%)
B. subtilis 6633	>5	>5
S. aureus ATCC 6538	1.25	1.25
S. epidermidis ATCC 49134	0.625	2.5
E. coli ATCC 11229	2.5	>5
P. vulgaris	>5	>5
P. aeruginosa	2.5	>5
C. albicans	1.25	5
A. niger V. Tiegh	>5	>5

Table 4. The inhibitory effects of C. acuminate D. seed oil obtained by petroleum ether against all the testing organisms.

Bacterial strain	MIC (%)	MBC (%)
B. subtilis 6633	>5	>5
S. aureus ATCC 6538	0.625	1.25
S. epidermidis ATCC 49134	2.5	2.5
E. coli ATCC 11229	5	>5
P. vulgaris	>5	>5
P. aeruginosa	0.625	1.25
C. albicans	0.625	2.5
A. niger V. Tiegh	1.25	5

observed to be resistant to the essential oils from other plants, such as *Achillea holosericea* (Magiatis et al., 1999) and Stachys species (Skaltsa et al., 2003). This microorganism is less susceptible to the anti-microbial properties of essential oils than others, and its tolerance is thought to result from its outer membrane (Cox and Radolf, 2001). And the ability of essential oil to disrupt the permeability barrier of cell membrane structures and the accompanying loss of chemiosmotic control are the most likely reason for its lethal action (Cox and Radolf, 2001). This antimicrobial activity against bacteria and fungi has also been demonstrated in essential oils extracted from *C. sativum* seeds (Lo Cantore et al., 2004). Although the concentrations of the oil were generally about 100 times more than those of the standard antibiotics (chloramphenicol), they showed marked antibacterial and antifungal activities, as demonstrated by their zones of inhibition (Tables 3 to 6). This concentration difference between the essential oil and the standard antibiotic can be explained by the fact that the active components in the oil comprise only a fraction of the oil. Therefore, the concentration of the active components could be much lower than the standard antibiotics we used. Importantly, if the active components were isolated and purified, they would probably show higher antimicrobial activities than those observed here. Among the Gram negative bacteria, the oil was very active against *K. pneumoniae* and *P. mirabilis.* The best activity was observed for the Gram positive bacteria. In general, the oil showed greater

Table 5. The antimicrobial activity curve of oil obtained by SC-CO_2.

Concentration/Time	MIC/2	MIC (MBC)	2MIC	Control
0	5600	7000	8400	6200
1	4000	4400	600	10600
2	4400	1700	0	18800
4	2400	600	0	23800
8	6300	0	0	28600
12	9500	0	0	42400
24	20100	0	0	61800
30	21300	0	0	64600

Table 6. The antimicrobial activity curve of oil obtained by petroleum ether.

Concentration/Time	MIC/2	MIC (MBC)	2MIC	Control
0	9300	11100	10400	11500
1	5700	4400	1100	14000
2	5000	3800	300	19300
4	3100	2200	200	24000
8	400	1200	0	28800
12	1000	200	0	53400
24	8800	6900	0	68800
30	10600	11300	0	74800

antibacterial activity than antifungal activity (Tables 3 to 6). Aldehydes and alcohols are known to be active but with different specificity and activity levels, which is related not only to the functional group but also to hydrogen bonding parameters (Skaltsa et al., 2003). As a minor component in this study, linalool has been found to have antimicrobial activity against various microbes, except for *P. aeruginosae* (Carson and Riley, 1995), which is also known to inhibit spore germination and fungal growth. The inhibition of sporelation appeared to arise from respiratory suppression of aerial mycelia (Lahlou and Berrada, 2001).

Conclusions

Our study showed that *C. acuminate D.* seed oils had extraordinary antioxidant and antibacterial activity *in vitro*. Due to its virulence, this seed oil can work as natural antioxidants and antimicrobial, which is a promising alternative to the use of synthetic antioxidants in food supplement or in pharmaceutical and cosmetic industry. But there have been few studies on the activity of *C. acuminate* D. seed oil. In this study, we evaluated its inhibitory activity in several common bacteria and estimated its antioxidant effectiveness by β-carotene bleaching and DPPH tests. Our results further demonstrated that *C. acuminate* D. seed oil had remarkable antioxidant and antibacterial activity, especially *S. aureus* ATCC 6538. The seed oil possesses various biological functions, notably antibacterial and Antioxidant properties that can be widely used as alternative to synthetic antioxidant or antibacterial. Therefore, we hope our study provides a foundation for future research of extracting ingredients from plants or herbs as natural antioxidant and antibacterial.

ACKNOWLEDGEMENT

We are grateful for the financial supports from the Key Research Subject of Jiamusi University (No. Szj2008-016).

REFERENCES

Carson CF, Riley TV (1995). Antimicrobial activity of the major components of the essential oil of Melaleuca alternifolia. J. Appl. Bacteriol., 78(3): 264-269.

Cheng Y, Li M, Xu T (2008). Potential of poly (amidoamine) dendrimers as drug carriers of camptothecin based on encapsulation studies. Eur. J. Med. Chem., 43(8): 1791-1795.

Cox DL, Radolf JD (2001). Insertion of fluorescent fatty acid probes into the outer membranes of the pathogenic spirochaetes Treponema pallidum and Borrelia burgdorferi. Microbiology, 147(Pt 5): 1161-1169.

Friedrich J, List G, Heakin A (1982). Petroleum-free extraction of oil from soybeans with supercritical CO2 J. Am. Oil Chem. Soc., 59(7): 288-292.

Gerard D, May P (2002). Herb and spice carbon dioxide extracts-versatile, safe ingredients for premium food and health food. Food Tech. p 1-5.
Gomes PB, Mata VG, Rodrigues AE (2007). Production of rose geranium oil using supercritical fluid extraction. J. Supercrit. Fluids,
41(1): 50-60.
King JW (2000). Advances in critical fluid technology for food processing. Food Sci. Tech. Today, 14(4): 186-191.
Lahlou M, Berrada R (2001). Composition and niticidal activity of essential oils of three chemotypes of Rosmarinas officinalis L. Pharmaceutical Biol., I41: 207-210.
Li S, Adair KT (1994). Camptotheca acuminata Decaisne. Xi Shu, a promising anti-tumor and anti-viral tree for the 21st century. Nacogdoches: Stephen F. Austin State University Press.
Li S, Yi Y, Wang Y, Zhang Z, Beasley RS (2002). Camptothecin accumulation and variations in camptotheca. Planta Med., 68(11): 1010-1016.
Lo Cantore P, Iacobellis NS, De Marco A, Capasso F, Senatore F (2004). Antibacterial Activity of Coriandrum sativum L. and Foeniculum vulgare Miller Var. vulgare (Miller) Essential Oils. J. Agric. Food Chem., 52(26): 7862-7866.
Lu ZG, Zheng GC, Yu SM (2007). Composition analysis of groundcherry seed oil by supercritical CO_2 extraction. Food & Machinery, 2: 88-89+113.
Magiatis P, Melliou E, Skaltsounis AL, Chinou IB, Mitaku S (1999). Chemical composition and antimicrobial activity of the essential oils of Pistacia lentiscus var. chia. Planta Med., 65(8): 749-752.
Molero Gómez A, Pereyra López C, Martinez de la Ossa E (1996). Recovery of grape seed oil by liquid and supercritical carbon dioxide extraction: a comparison with conventional solvent extraction. Chem. Eng. J. Biochem. Eng. J., 61(3): 227-231.
Msaada K, Hosni K, Taarit MB, Chahed T, Marzouk B (2007). Variations in the essential oil composition from different parts of Coriandrum sativum L. cultivated in Tunisia. Ital. J. Biochem., 56(1): 47-52.
NCCLS (2002). In National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of yeasts: proposed standard. P 1-25.
Oberlies NH, Kroll DJ (2004). Camptothecin and Taxol: Historic Achievements in Natural Products Research. J. Nat. Prod., 67(2): 129-135.
SalgIn U (2007). Extraction of jojoba seed oil using supercritical CO2+ethanol mixture in green and high-tech separation process. J. Supercrit. Fluids, 39(3): 330-337.
Skaltsa HD, Demetzos C, Lazari D, Sokovic M (2003). Essential oil analysis and antimicrobial activity of eight Stachys species from Greece. Phytochemistry, 64(3): 743-752.
Wall ME, Wani MC, Cook CE, Palmer KH, McPhail AT, Sim GA (1966). Plant Antitumor Agents. I. The Isolation and Structure of Camptothecin, a Novel Alkaloidal Leukemia and Tumor Inhibitor from Camptotheca acuminata1, 2. J. Am. Chem. Soc., 88(16): 3888-3890.
Wu N, Zu Y, Fu Y, Kong Y, Zhao J, Li X, Li J, Wink M, Efferth T (2010). Antioxidant Activities and Xanthine Oxidase Inhibitory Effects of Extracts and Main Polyphenolic Compounds Obtained from Geranium sibiricum L. J. Agric. Food Chem., 58(8): 4737-4743.
Yang ZW, Wu XM, Zu YG, Yang G, ZHou LJ (DOI: 10.1002/qua.23046). Understanding the chiral recognitions between neuraminidases and inhibitors: studies with DFT, docking and MD methods Int. J. Quantum. Chem.,
Yen GC, Duh PD (1994). Scavenging Effect of Methanolic Extracts of Peanut Hulls on Free-Radical and Active-Oxygen Species. J. Agric. Food Chem., 42(3): 629-632.

African Journal of Microbiology Research Vol. 5(32), pp. 5863-5871, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.729

Full Length Research Paper

Preparation, characterization and *in vitro* antimicrobial activity of compound sustained-release periodontal suppository of ornidazole and pefloxacin mesylate

Rui Liu, Yan Jiang, Yan-hua Duan, Nan Li, Guo-dong Zhang, Xin Nie and Lu-chuan Liu*

Department of Stomatology, Research Institute of Surgery and Daping Hospital, the Third Military Medical University, Chongqing 400042, China.

Accepted 26 August, 2011

Local delivery of sustained-release drugs in periodontal pocket is an effective approach for the treatment of periodontitis. The present study aimed to optimize and characterize the sustained-release periodontal suppository of compound ornidazole and pefloxacin mesylate (O&P) *in vitro*. Before suppository was prepared, the combined effect of O&P against dominant anaerobe and facultative anaerobe in periodontitis was investigated and then the optimal ratio of each drug was determined. After the compound suppository of O&P was prepared by cold compression, the optimal content of the suppository was determined through evaluating the bacteriostatic effect and then the sustained-release level of optimized suppository was investigated. There was no incompatibility between ornidazole and pefloxacin mesylate. When being applied in combination, they could exert synergistic bacteriostatic effect against 'porphyromonas gingivalid' and 'fusobacterium nucleatum', and additive effect against 'prevotella intermedia' and 'peptostreptococcus spp' and inhibit anaerobe growth at a low concentration. The optimal ratio of O&P is 2:3 and the optimal content of suppository 20% *in vitro*; this suppository could release both drugs in a sustained manner for 12 h. The compound suppository of O&P can serve as one of the potential candidates for the treatment of periodontitis and has favorable sustained-release ability which can meet the requirement of clinical treatment of periodontitis.

Key words: Ornidazole, pefloxacin mesylate, periodontitis, sustained-release delivery system.

INTRODUCTION

Periodontitis is a group of dentoalveolar infections and remains one of the major causes of adult tooth loss. These infections involve a variety of bacteria in local periodontal tissues. Plaques and their products are the original cause of parodontopathy (Zambon, 1996). To date, numerous investigations have been confirmed that anaerobe is a dominant type of pathogenic bacteria, including *Porphyromonas gingivalid* (Pg), *Fusobacterium nucleatum* (Fn), *Prevotella intermedia* (Pi) and *Peptostreptococcus* spp (Ps). Additionally, these facultative anaerobe such as *Staphylococcus aureus* (Sa), *Staphylococcus epidermidis* (Se), etc (Takahashi, 1998; Paju et al., 2009), also have synergistic action in the process of pathogenesis. Although, the use of systemic antibiotic treatment of periodontitis has shown some benefits, multiple systemic doses of antibiotics have several drawbacks including inadequate antibiotic concentration (subtherapeutic level) at the site of periodontal pocket and high plasma concentration. Inadequate antibiotic concentration is not able to continuously inhibit or kill the pathogenic microorganisms, and fail to control the periodontal inflammation which may facilitate the reconstruction of damaged tissues. High plasma concentration may be associated with bacterial resistance and occurrence of side effects (Bidault et al., 2007a, b). These disadvantages have evoked an interest in the development of novel local drug delivery systems for the treatment of periodontal diseases (Greenstein, 2006; Hussein et al., 2007).

*Corresponding author. E-mail: liuvery001@163.com. Fax: 86-23-68715568.

With the development of modern pharmacy, sustained-release drug has been used in the treatment of parodontopathy (Vandekerckhove et al., 1997; Vyas et al., 2000). If a therapeutic effective concentration can be maintained in the periodontal pocket for a desired period, local sustained-release drug may significantly improve the therapeutic efficacy of periodontitis. Moreover, side-effects can also be greatly decreased due to avoidance of high plasma concentration (Vyas et al., 2000). Ornidazole is a nitroimidazole antiprotozoal agent and has better anti-anaerobic activity than quinolones (Quirynen et al., 2002). Although, anaerobe is the dominant population involving in periodontitis, facultative anaerobe also plays a part role in the periodontal destruction. Pefloxacin mesylate is a quinolone antibiotic and has better activity against facultative anaerobes than nitroimidazoles (Wang et al., 2007). In the present study, the optimal ratio and optimal content of ornidazole (O) and pefloxacin mesylate (P) were determined to prepare the compound O&P sustained-release periodontal suppository for the treatment of periodontitis.

MATERIALS AND METHODS

Dominant pathogenic bacteria of periodontitis

Pg, Fn, Pi, Ps, Mutans streptococcu (Ms), Sa and Se are standard strain purchased from the R&D Department of P&G company.

Instruments for experiments

Agar medium (Shanghai Li Chen Biotechnology Co., Ltd.), drug susceptibility papers (Shanghai Wufeng Scientific Instruments Co., Ltd.), high performance liquid chromatograph (HPLC) (Shanghai Wufeng Scientific Instruments Co., Ltd.), O&P standard substance and chromatography-pure methanol (Shanghai Shengke Biotechnology Co., Ltd.) were used. SPSS13.0 was used for statistical analysis.

In vitro study on the anti-microbic characteristic of O&P

To investigate whether O&P have synergic anti-microbic action, the Kirby-Bauer test was applied. Round arid anti-microbic filter papers measuring 6 mm in diameter were prepared and soaked with 5 μg of ornidazole or pefloxacin mesylate, respectively. Then, these papers were put onto the agar plate containing Pg, Fn, Pi, Ps, Sa or Se. There were one ornidazole paper and one pefloxacin mesylate paper on each agar plate. Then, the synergic action was preliminarily estimated by the shape of intersecting angles between two inhibition rings. The interaction was further assessed by using agar dilution method and the effects evaluated with fractional inhibitory concentration (FIC) and minimun inhibitory concentration (MIC). In brief, Pg, Fn, Pi, Pa, Sa, Se (2 × 107 ml) were inoculated into a Mueller-Hinton broth and dispensed at 0.1 ml/well in 96-well plates. MICs were determined by a serial twofold dilution of ornidazole and/or pefloxacin mesylate from 2 to 1/32 of MIC alone.

After 24 h of incubation at 37°C, the minimal compound concentration at which the ornidazole/pefloxacin mesylate prevented the growth of a given organism was determined and defined as the MIC of the compound. The MIC was determined in three independent assays.

Screen on the optimal ratio of O&P

In the agar plates of Pg, Fn, Pi, Ps and Fn, the ditch plate method of agar diffusion was employed to detect anti-anaerobic effects of O&P at different ratios. L36 (62) orthogonal table was designed to select the optimal ratio of O&P on the 4 strains of anaerobes. In the orthogonal table, O&P are two factors and each factor is classified into 6 levels. In the orthogonal experiment, there were 36 groups in which the ratio of O&P varied from 1:6 to 6:1 and only 23 groups were finally subjected to analysis because sever groups were integrated into one (for example ratios at 1:2, 2:4 and 3:6 were the same to 1:2) (Table 1). According to findings in the orthogonal experiment, the range of each factor was calculated: the larger the range, the higher the antibacterial effect. Single-factor analysis of variance was used to identify the critical point of ratio for each anaerobe.

In order to get the optimal ratio, the MIC and MIC50 of dominant periodontal pathogenic bacteria were determined according to each critical point.

Preparation of compound O&P sustained-release periodontal suppository

A special mold (Figure 1) of suppository was designed according to anatomic shape and the depths of different periodontal pockets. Cold compression was employed for the preparation of suppository. The carrier which can reduce the application of antibacterial agents was composed of ethyl cellulose (EC) and hydroxypropyl methyl cellulose (HPMC). Firstly, EC was swollen with 95% alcohol, and then with sufficient distilled water. The glycerine, pefloxacin mesylate and ornidazole were added in a proper order. When all these materials were mixed sufficiently, HPMC was then added to the mixture. After agitation, alcohol was volatilized, the mixture was swollen to doughing time and then it was taken into the mold.

Screening optimal content of compound O&P periodontal suppository

Different contents (1, 2, 5, 10 and 20%) of O&P periodontal suppository were prepared. In every content group, three suppositories with same content were completely dissolved in 3 ml of purified water. After being filtrated, 5 μl of drug-filtrate was put into the drug -sensitive paper plates measuring 6 mm in diameter. So, in the paper plate of each group, the drug content was 0.5, 1, 2.5, 5 and 10 μg; which corresponded to the contents of suppositories. Then, Kirby-Bauer method was used to estimate the bacteriostatic effect on the 4 strains of anaerobic bacteria (Pg, Fn, Pi and Ps), and 3 strains of facultative anaerobe (Ms, Sa and Se). The optimized content of suppository was determined by comparing the size of inhibition rings.

Medicine release level of compound sustained-release periodontal suppository *in vitro*

Cumulative release ratio of drugs was detected through HPLC. The conditions for HPLC were chromatographic column: C18 (250 × 4.6 mm, 5 μm); mobile phase: 0.1 mol/L monopotassium phosphate solution-methanol (60:40); detecting wavelength: 277 nm; flow velocity: 1.0 ml/min; sampling size: 20 μl; column temperature: 30°C. Firstly, the standard curves of O&P were delineated and the peak areas were corresponding to the drug concentration. Then, the suppositories with 20% drug were soaked into a beaker containing 5 ml of purified water and stirred every 10 min (n = 5). At the designed time points (0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 15, 18 and 24 h), 200 μl of fluid were collected for the detection and

Table 1. Ratio of ornidazole and pefloxacin mesylate for testing.

Group	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Ornidazole	6	5	4	3	5	2	5	3	4	5	6	1	5	4	3	2	3	1	2	1	1	1	1
Pefloxacin	1	1	1	1	2	1	3	2	3	4	5	1	6	5	4	3	5	2	5	3	4	5	6

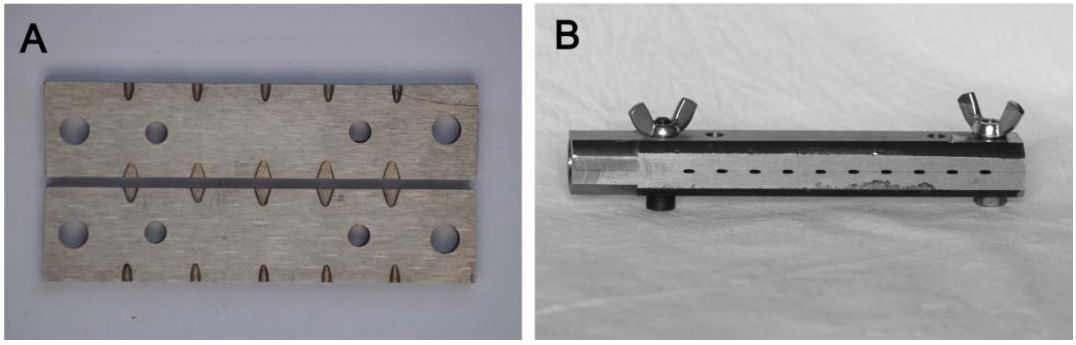


Figure 1. Mold for sustained-release periodontal suppository. The mould is opened (A) and the mould is closed (B). The mold of suppository is made of stainless steel. According to anatomic shape and the depth of different periodontal pockets, the die hole of anterior teeth is 3 ~ 4 mm in length, 2 mm in width and 350 to 500 μm in thickness. The die hole of posterior teeth is 3 to 4 mm in length, 4 mm in width, 350 to 500 μm in thickness. All the die holes are shuttle-shaped with obtuse margin.

additional 200 μl of purified water was added to the beaker. The peak areas at different time points were measured by HPLC. According to the standard curve, average concentrations at different time points were calculated. Based on the average concentrations, the discharge amounts at different time points were obtained.

Finally, the release curves of two drugs were delineated according to the time and corresponding release rate.

RESULTS

Anti-microbic characteristic of O&P

On the anaerobe flat plate, the intersecting angles of two inhibition rings of two drugs displayed straightened or even evaginated which suggests they may have synergistic action against anaerobe (Figure 2A). On the facultative anaerobe flat plate, there was no inhibition rings of ornidazole (Figure 2B); which indicates that they have independent effect against facultative anaerobe and only pefloxacin mesylate exerts anti-anaerobic effect. FIC of two drugs against anaerobe were lower than 1, further demonstrating that both drugs can confer synergetic or additive effect against anaerobes when being used together; and they can inhibit the growth of anaerobes at a low concentration (Table 2). For the facultative anaerobe, when two drugs were used together, the concentration of pefloxacin was equal to or higher than its own MIC and the FIC was 1.0625, which suggests that, the activities of two drugs are independent and the bactriostatic effect depends on pefloxacin mesylate alone (Table 2).

Optimization on the ratio of O&P

The results of orthogonal test are shown in Table 3. Analysis showing the range of ornidazole and pefloxacin could be calculated (Table 4). The range of ornidazole was larger than that of pefloxacin which provides the evidence that ornidazole has high anti-anaerobic activity than pefloxacin mesylate. In each group, the length of bacteriostatic area at each ratio was analyzed by single-factor analysis of variance. For the Pg, Fn, Pi and Ps, the critical point of the ratio of two drugs was 1:2, 2:3, 2:3 and 1:1, respectively. According to the 3 critical points, the MIC and MIC50 of dominant periodontal pathogenic bacteria were determined (Table 5). The MIC of ornidazole against Pg, Fn, Pi, Ps was 0.062, 0.062, 0.125 and 0.25 mg/L, respectively; showing that all these 4 kinds of bacteria are very sensitive to ornidazole. The MIC of pefloxacin mesylate against these bacteria was 0.25, 0.25, 0.5 and 4 mg/L, respectively; showing that pefloxacin mesylate has bacteriostatic effect on all the 4 kinds of bacteria. The MIC of pefloxacin mesylate against Sa and Se was 0.062 and 0.125 mg/L, respectively; also showing a bacteriostatic effect.

When ornidazole was combined with pefloxacin mesylate, the MIC of ornidazole or pefloxacin mesylate against anaerobes was lower than their own MIC. The results demonstrated that two drugs have synergic action

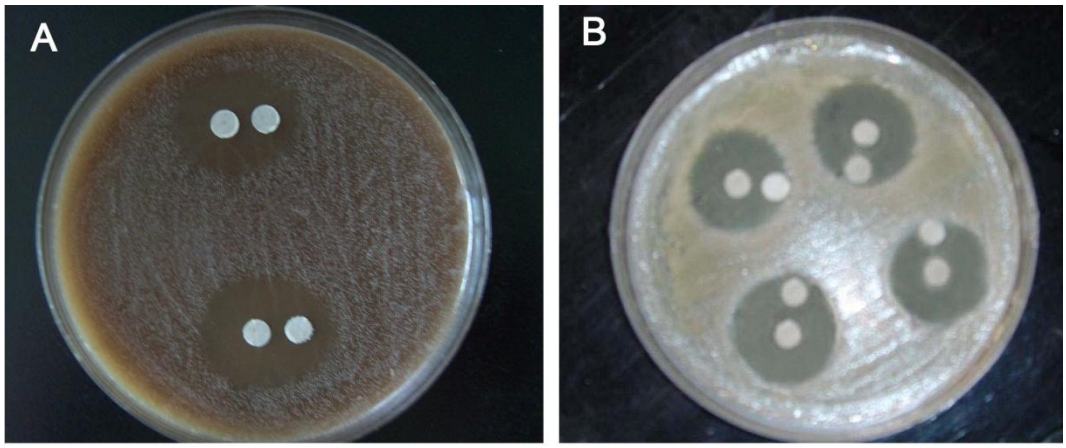


Figure 2. Compound synergic anti-microbic characteristic of ornidazole and pefloxacin mesylate with Kirby-Bauer method. The synergic anti-microbic characteristic of ornidazole and pefloxacin mesylate (A). On the anaerobe flat plate, the intersecting angles of two inhibition rings of two drugs displayed straightened or even evaginated which suggests they have synergistic action against anaerobe (B). The indenpedent anti-microbic characteristic of ornidazole and pefloxacin mesylate. On the facultative anaerobe flat plate, there was no inhibition rings of ornidazole, which suggests that two drugs have indenpedent actions against facultative anaerobe and the bactriostasis depends on pefloxacin mesylate alone.

Table 2. FIC of compound ornidazole and pefloxacin mesylate.

Bacterium		Ornidazole (MIC)	Pefloxacin mesylate (MIC)	FIC
Anaerobe	*Porphyromonas gingivalid*	1/4	1/16	0.3125
	Fusobacterium nucleatum	1/4	1/8	0.375
	Prevotella intermedia	1/4	1/16	0.5625
	Peptostreptococcus spp	1/4	1/4	0.75
Facultative anaerobe	*Staphylococcus aureus*	1/16	1	1.0625
	Staphylococcus epiermidis	1/16	1	1.0625

FIC = ($MIC_{Drug\ A\ in\ combination}/MIC_{Drug\ A\ alone}$) + ($MIC_{Drug\ B\ in\ combination}/MIC_{Drug\ B\ alone}$). FIC ≤ 0.5, 0.5 < FIC≤1, 1 < FIC ≤ 2, and > 2 were defined as synergistic, additive, independent, and antagonism, respectively. All FICs of two drugs against anaerobes were less than 1. For *Pg* and *Fn*, the FIC was less than 0.5 which suggests two drugs have synergistic action. For *Pi* and *Ps*, the FIC was between 0.5 and 1 which suggests two drugs have additive action. All FICs of two drugs against facultative anaerobes were higher than 1 but lower than 2. For *Sa* and *Se*, the FIC was 1.0625, which suggests two drugs have independent action and the bactriostatic effect depends on pefloxacin mesylate alone.

against the anaerobic bacteria. Especially, MIC against Pg was kept constantly at 0.062 mg/L. The MIC at 3 different ratios of O&P showed no significant difference against 4 kinds of anaerobic bacteria. The antiblastic effect was not significantly diminished when reducing the ratio of ornidazole or increasing that of pefloxacin.

Optimal content of each drug in the compound O&P periodontal suppository

The inhibition rings were examined at different drug contents ranging from 1 to 20% and results showed that the anti-bacterial effects of both drugs were dose-dependent against the 7 major pathogenic bacteria (Figure 3A). The 20% drug had the strongest anti-bacterial effect against Sa (inhibition ring was 27 mm in diameter) and 1% drug had the weakest anti-bacterial effect against Ms (inhibition rings was 7.9 mm in diameter). The sizes of bacterial inhibition rings against the 7 different bacteria decreased in the following order: Ms, Ps, Pi, Fn, Pg, Se and Sa (Figure 3B).

In vitro release of sustained-release periodontal suppository containing 20% drug

HPLC showed the retaining times of ornidazole (Bodyguard Pharmaceutical Co. Ltd., China) and pefloxacin mesylate standard substance (North China

Table 3. Anti-anaerobic effect of different ratios of ornidazole and pefloxacin mesylate.

Factor			Anti-anaerobic effects (mm)			
No.	O	P	*Pg*	*Fn*	*Pi*	*Ps*
y_1	6	1	31.2±1.37	31.0±1.17	28.3±1.06	26.4±1.28
y_2	6	2	30.5±1.07	30.9±1.22	28.3±0.87	26.3±0.91
y_3	6	3	30.8±1.05	30.83±1.35	27.91±0.95	25.72±0.87
y_4	6	4	30.5±1.61	30.6±0.93	27.3±0.74	25.1±1.16
y_5	6	5	30.4 ±1.21	30.6±1.07	27.0±1.13	24.8±1.13
y_6	6	6	30.0±0.94	30.4±1.02	26.9±1.02	24.5±0.74
y_7	5	1	31.4±1.53	31.1±1.45	28.3±0.87	26.0±.1.03
y_8	5	2	31.0± 1.32	30.7±1.10	28.1±0.89	26.1±1.01
y_9	5	3	30.2±1.32	30.7±1.01	27.6±0.86	25.1±0.99
y_{10}	5	4	30.5±1.34	30.4±0.95	27.1±0.81	25.1±1.15
y_{11}	5	5	30.0±0.94	30.4±1.02	26.9±1.02	24.5±0.74
y_{12}	5	6	29.9±1.32	30.0±1.55	26.8±1.11	23.4±0.85
y_{13}	4	1	31.2± 1.36	31.1±1.42	28.2±0.94	26.4±0.96
y_{14}	4	2	30.86±1.05	30.83±1.35	27.91±0.95	25.72±0.87
y_{15}	4	3	29.9 ±1.26	30.5±1.09	27.3±0.97	25.0±0.99
y_{16}	4	4	30.0±0.94	30.4±1.02	26.9±1.02	24.5±0.74
y_{17}	4	5	29.9±1.33	29.8±1.07	26.8±1.22	23.2±0.67
y_{18}	4	6	30.1±1.05	29.5±1.06	26.7±1.01	22.8±0.99
y_{19}	3	1	30.5±1.07	30.9±1.22	28.3±0.87	26.3±0.91
y_{20}	3	2	30.5±1.61	30.6±0.93	27.3±0.74	25.1±1.16
y_{21}	3	3	30.0±0.94	30.4±1.02	26.9±1.02	24.5±0.74
y_{22}	3	4	30.2±1.50	30.0±1.02	26.9±0.99	22.9±0.87
y_{23}	3	5	29.3±1.33	29.0±0.94	26.5±0.74	22.8±1.02
y_{24}	3	6	29.2±1.52	28.6±0.72	25.9±1.00	22.6±1.20
y_{25}	2	1	30.4±1.29	30.8±1.37	27.7±1.13	25.5±1.35
y_{26}	2	2	30.0±0.94	30.4±1.02	26.9±1.02	24.5±0.74
y_{27}	2	3	30.1±1.05	29.5±1.06	26.7±1.01	22.8±0.99
y_{28}	2	4	29.2±1.52	28.6±0.72	25.9±1.00	22.6±1.20
y_{29}	2	5	28.3±0.95	27.6±0.96	24.2±1.16	22.4±1.29
y_{30}	2	6	27.1±1.12	26.5±1.16	23.6±1.01	21.8±0.94
y_{31}	1	1	30.0±0.94	30.4±1.02	26.9±1.02	24.5±0.74
y_{32}	1	2	29.2±1.52	28.6±0.72	25.9±1.00	22.6±1.20
y_{33}	1	3	27.1±1.12	26.5±1.16	23.6±1.01	21.8±0.94
y_{34}	1	4	26.7±1.17	26.0±0.81	22.7±0.91	21.5±1.02
y_{35}	1	5	26.0±1.13	25.5±0.89	22.1±1.15	20.2±0.98
y_{36}	1	6	26.0± 0.78	25.1±0.80	21.3±1.59	19.8±0.89

Pharmaceutical Group Co. Ltd., China) were 10.1 and 13.7 min, respectively (Figure 4A). The regression equation of ornidazole was y = 5.7507x + 1.0714, r = 0.9993 (Figure 4B) and that of pefloxacin mesylate was y = 1.1279x + 0.9904, r = 0.9999 (Figure 4C). The peak areas, average concentrations and release amounts of O&P at different time points are shown in Tables 6 and 7. Ornidazole was released by 9.08% at 30 min, 59.50% at 5 h and 98.30% at 13 h, while pefloxacin mesylate was released by 15.26% at 30 min, 45.00% at 5 h and 96.01% at 13 h (Figure 4D).

DISCUSSION

The appearance of periodontal pocket is one of the important signs of periodontitis (Liu et al., 2003). The periodontal pocket provides a natural space for the local delivery system (Mundargi et al., 2007). Meanwhile, the gingival crevicular fluid provides a liquid medium for the release of drug from a carrier and for its distribution throughout the periodontium (Jain et al., 2008). Therefore, a therapeutic effective concentration of an anti-microbial agent could be maintained in the periodontal

Table 4. Antibacterial index of ornidazole and pefloxacin mesylate with range analysis.

Bacterium	Ornidazole	Pefloxacin mesylate
Porphyromonas gingivalid	$R^{(1)} = 7.5955$	$R^{(2)} = 2.6142$
Fusobacterium nucleatum	$R^{(1)} = 3.3219$	$R^{(2)} = 1.8027$
Prevotella intermedia	$R^{(1)} = 8.3327$	$R^{(2)} = 2.7861$
Peptostreptococcus spp	$R^{(1)} = 10.8025$	$R^{(2)} = 4.7611$

The range of ornidazole was larger than that of pefloxacin which provides the evidence that ornidazole has stronger sensitivity than pefloxacin mesylate against anaerobes. The range can be calculated from Table 3. $R^{(1)} = [\bar{k}_1^{(1)} - \mu]^2 + [\bar{k}_2^{(1)} - \mu]^2 + [\bar{k}_3^{(1)} - \mu]^2 + [\bar{k}_4^{(1)} - \mu]^2 + [\bar{k}_5^{(1)} - \mu]^2 + [\bar{k}_6^{(1)} - \mu]^2$; $R^{(2)} = [\bar{k}_1^{(2)} - \mu]^2 + [\bar{k}_2^{(2)} - \mu]^2 + [\bar{k}_3^{(2)} - \mu]^2 + [\bar{k}_4^{(2)} - \mu]^2 + [\bar{k}_5^{(2)} - \mu]^2 + [\bar{k}_6^{(2)} - \mu]^2$; $\mu = (y_1 + y_2 + y_3 + y_4 \ldots\ldots\ldots + y_{36}) \times 1/36$; $k_1^{(1)} = y_1 + y_2 + y_3 + y_4 + y_5 + y_6$: $\bar{k}_1^{(1)} = 1/6\ k_1^{(1)}$: $k_2^{(1)} = y_7 + y_8 + y_9 + y_{10} + y_{11} + y_{12}$: $\bar{k}_2^{(1)} = 1/6\ k_2^{(1)}$; $k_1^{(2)} = y_1 + y_7 + y_{13} + y_{19} + y_{25} + y_{31}$ $\bar{k}_1^{(2)} = 1/6\ k_1^{(2)}$: $k_2^{(2)} = y_2 + y_8 + y_{14} + y_{20} + y_{26} + y_{32}$: $\bar{k}_2^{(2)} = 1/6\ k_2^{(2)}$ and so on.

Table 5. MIC in 4 strains of anaerobes and 3 strains of facultative anaerobes with 3 different critical points in ratio.

Bacterium (strain)	Ornidazole	Pefloxacin mesylate	1:1	2:3	1:2
	MIC/MIC_{50}	MIC/MIC_{50}	MIC/MIC_{50}	MIC/MIC_{50}	MIC/MIC_{50}
Pg	0.062	0.25	0.062	0.062	0.062
Fn	0.062	0.25	0.062	0.062	0.125
Pi	0.125	0.5	0.125	0.25	0.25
Ps	0.25	4	0.25	0.5	1
Sa	>256	0.06~8/0.5	0.125~32/1	0.06~16/0.5	0.06~8/0.5
Se	>256	0.125~16/1	0.25~32/2	0.25~32/1	0.25~16/1
Ms	>256	0.125~64/4	0.25~64/8	0.25~64/4	0.125~64/4

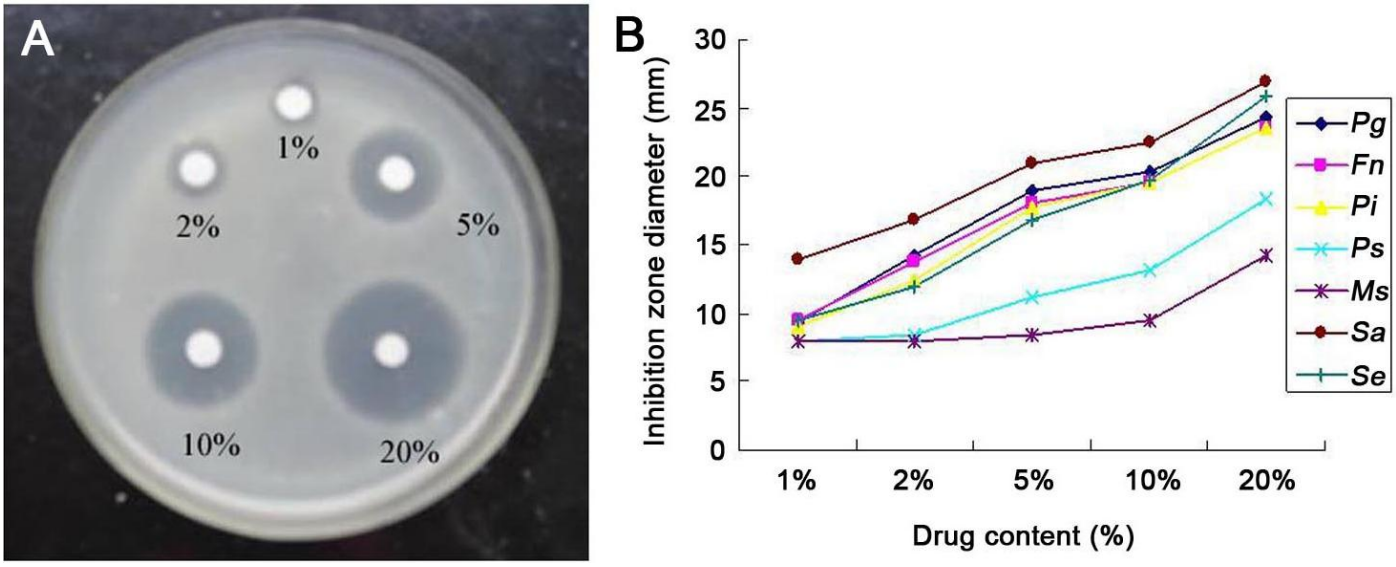


Figure 3. *In vitro* bacteriostatic effect of ornidazole and pefloxacin mesylate periodontal suppository with five different drug contents. Diameters of inhibition zones at five different drug contents against one bacterium (A); bacteriostatic effect against different bacteria at different drug contents (B). An obvious dose-dependent manner was observed in the anti-bacterial effect against the 7 major suspected pathogenic bacteria.

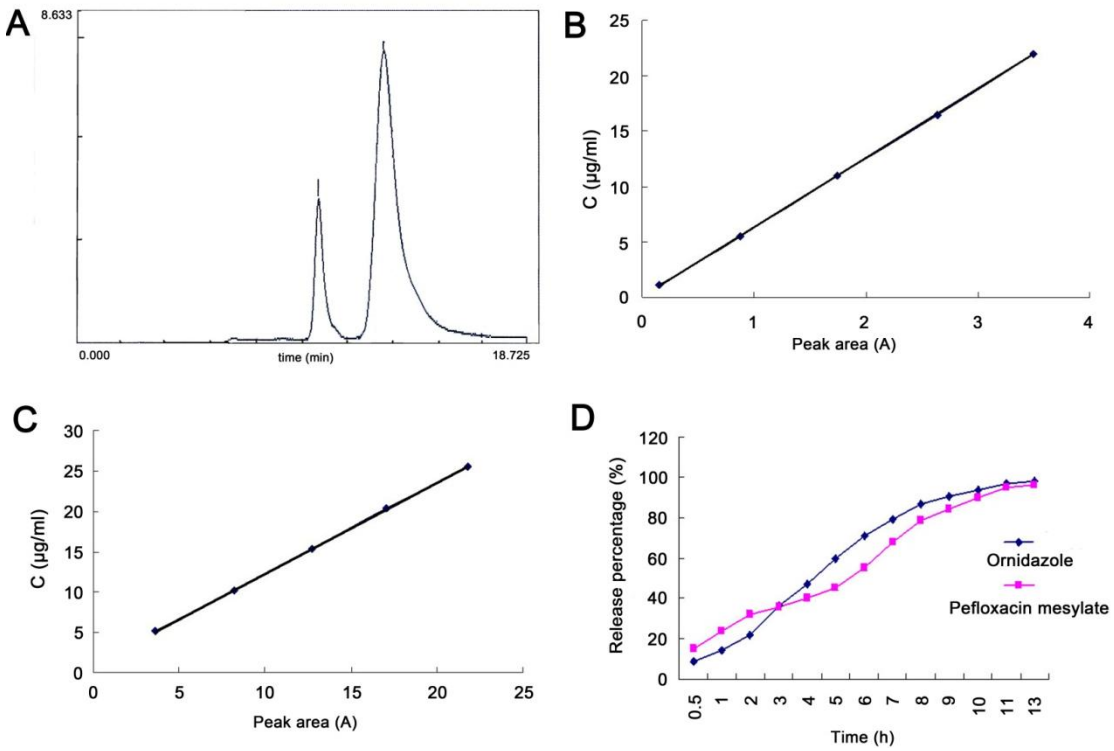


Figure 4. *In vitro* release of sustained-release suppository containing 20% drug. Chromatography of ornidazole and pefloxacin mesylate standard substance (A); A represents ornidazole T_R = 10.166 min and B pefloxacin T_R = 13.722 min; the standard curve of ornidazole (B); the standard curve of pefloxacin mesylate (C) and the release curve of ornidazole and pefloxacin mesylate *in vitro* (D).

Table 6. The peak area and delayed release of ornidazole in the periodontal suppository.

Time (h)	Peak area	Average concentration (mg/ml)	Release amount (mg)	Accumulative release degrees (%)
0.5	5.757	0.036	0.18	9.08
1	8.781	0.055	0.10	14.63
2	12.793	0.080	0.14	22.01
3	20.629	0.129	0.26	36.25
4	26.227	0.164	0.20	47.20
5	32.623	0.204	0.23	59.50
6	37.900	0.237	0.21	70.86
7	41.579	0.260	0.16	79.47
8	44.137	0.276	0.13	86.55
9	47.816	0.299	0.07	90.18
10	47.816	0.299	0.06	93.34
11	48.775	0.305	0.03	96.79
13	47.655	0.298	0.03	98.30

pocket for a desired period, which is the basis of; and ensure the successful treatment of periodontitis. A variety of potentials of sustained-release preparations have been displayed in this treatment (Chen et al., 2006). In this

Table 7. The peak area and delayed release of pefloxacin mesylate in the periodontal suppository.

Time (h)	Peak area	Average concentration (mg/ml)	Release amount (mg)	Accumulative release degrees (%)
0.5	101.470	0.084	0.42	15.26
1	155.154	0.128	0.24	24.10
2	199.423	0.164	0.22	32.11
3	215.410	0.177	0.10	35.80
4	236.315	0.194	0.12	40.23
5	258.449	0.212	0.13	45.00
6	319.935	0.262	0.29	55.45
7	388.110	0.317	0.33	67.62
8	444.136	0.363	0.29	78.33
9	467.500	0.382	0.17	84.48
10	483.486	0.395	0.14	89.64
11	498.243	0.407	0.14	94.57
13	488.405	0.399	0.04	96.01

study, in order to provide a high anti-microbial activity, we designed local compound O&P periodontal suppository which consisted of ornidazole, pefloxacin mesylate and carrier. The carrier was composed of EC and HPMC, materials widely used in controlled release system and its application reduced the content of antibacterial agents.

The Kirby-Bauer test and FIC detection indicated that there was no incompatibility between ornidazole and pefloxacinmesylate, and they could exert synergetic effect against anaerobe and inhibit the anaerobic growth at a low concentration when they were applied together. For facultative anaerobe, however, the anti-bacterial effect depended on pefloxacin mesylate alone. In this study, the ratio of two drugs was for the first time optimized. Orthogonal experiment was performed to determine the optimal ratio against 4 strains of anaerobes. Through single-factor analysis of variance, three critical points of ratio were identified: 1:2, 2:3 and 1:1. According to 3 critical points, the MIC and MIC50 of the dominant periodontal pathogenic bacteria were determined. Among the 3 different critical points, for the anaerobes, there were no significant differences in the anti-bacterial activities and their MIC. The present study also demonstrated that the antibacterial activities of the compound increased with the increase of pefloxacin when the anti--bacterial effect against facultative anaerobes was taken into account. Because two drugs have independent anti-bacterial effect against facultative anaerobe and the bactriostatic effect depends on pefloxacin mesylate alone; that is to say, the ratio of 1:2 is preferred. However, periodontal diseases are predominantly caused by anaerobic infection, and quinolones have been clinically applied for years, there is a possibility of drug resistance.

In addition, ornidazole has first expose effect and its anti-bacterial effect is concentration dependent (Kamma et al., 2000). Moreover, the anti-bacterial activity of pefloxacin mesylate against anaerobes *in vivo* should be further studied (Appelbaum, 1999). Taking the reasons earlier mentioned into account, we speculate that the ratio of 2:3 is a preferred ratio. O&P are freely soluble in gingival crevicular fluid, and hence selection of release-retarding excipient is necessary to achieve a constant input rate in the gingival crevicular (Paquette et al., 2008; Akncbay et al., 2007; Bosco et al., 2009). Because of its flexibility, HPMC (a hydrophilic polymer matrix) was used to obtain a desirable drug release profile and broad regulatory acceptance. HPMC has been well known to retard drug release by swelling in aqueous media (Li et al., 2005). However, for a water soluble drug, application of a hydrophilic matrix system alone is restricted because of rapid diffusion of dissolved drug through the hydrophilic gel network. In such circumstances, EC, one of hydrophobic polymers is required along with the HPMC for developing sustained-release delivery system (Barat et al., 2007).

Incorporation of a high concentration of EC controls the drug release in a better manner, which may be attributed to the decreased penetration of solvent molecules in the presence of hydrophobic polymer, leading to decreased drug diffusion from the matrix (Bromberg et al., 2001). Furthermore, because of the presence of EC which is generally responsible for the hardness of suppository, the suppository is not susceptible to being cracked when it is held by pliers. The sustained-release materials used in this study have certain drug saturation. When the drug content is higher than 20%, the precipitation of drug may occur. So, 5 different contents (1, 2, 5, 10 and 20%) were applied. The optimal content was selected by comparing the size of inhibition rings. Statistical analysis demonstrated that content at 20% has obvious advantages. The *in vitro* experiment on the sustained-release periodontal suppository containing 20% drug revealed that the average release rates of ornidazole and

pefloxacin mesylate were 0.14 and 0.20 mg/h, respectively; and the accumulated release percentages of ornidazole and pefloxacin mesylate at 13 h were 98.3 and 96.01%, respectively. Moreover, the initial burst release was not observed. All the parameters related to sustained-release behavior were within the limits proposed by the pharmacopeia.

The present study indicated that the compound periodontal suppository containing 20% drug had potent sustained-release bacteriostatic effect against the suspected pathogenic bacteria. Especially, for the sensitive bacteria, the sustained-release could be sustained for about 24 h. For the non-sensitive bacteria such as Ps and Ms, the sustained-release could be sustained for about 10 h. These features demonstrated that the suppository can meet the requirement of sustained-release. So, in the treatment of periodontitis, the suppository may be medicated every other day. This study affirmatively provides a better understanding of the synergistic effect of O&P which may be helpful for the periodontal therapies. Our results provide convincing evidence and useful information for future clinical application of sustained-release periodontal suppository containing 20% drug. In future study, we will further optimize the periodontal suppository. For example, the ratio of sustained-release materials should be optimized which may prolong the sustained-release time.

In addition, the therapeutic effect of periodontal suppository will be investigated in rats with ligature-induced periodontitis.

ACKNOWLEDGEMENT

This work was supported by a grant from the Science and Technology foundation of Chongqing China (Project No.2004BB5065 and No.2009AC5019)

REFERENCES

Akncbay H, Senel S, Ay ZY (2007). Application of chitosan gel in the treatment of chronic periodontitis. J. Biomed. Mater. Res. B. Appl. Biomater. 80 (2): 290-296.

Appelbaum PC (1999). Quinolone activity against anaerobes. Drugs 58 Suppl 2: 60-64.

Barat R, Srinatha A, Pandit JK, Mittal N, Anupurba S (2007). Ethylcellulose inserts of an orphan drug for periodontitis: preparation, in vitro, and clinical studies. Drug Deliv. 14 (8): 531-538.

Bidault P, Chandad F, Grenier D (2007a). Risk of bacterial resistance associated with systemic antibiotic therapy in periodontology. J. Can. Dent. Assoc. 73(8): 721-725.

Bidault P, Chandad F, Grenier D (2007b). Systemic antibiotic therapy in the treatment of periodontitis. J. Can. Dent. Assoc. 73(6): 515-520.

Bosco JM, Lopes BM, Bosco AF, Spolidorio DM, Marcantonio RA (2009). Local application of tetracycline solution with a microbrush: an alternative treatment for persistent periodontitis. Quintessence. Int. 40(1): 29-40.

Bromberg LE, Buxton DK, Friden PM (2001). Novel periodontal drug delivery system for treatment of periodontitis. J. Control Release, 71(3): 251-259.

Chen FM, Zhao YM, Wu H, Deng ZH, Wang QT, Zhou W, Liu Q, Dong GY, Li K, Wu ZF, Jin Y (2006). Enhancement of periodontal tissue regeneration by locally controlled delivery of insulin-like growth factor-I from dextran-co-gelatin microspheres. J. Control. Release. 114(2): 209-222.

Greenstein G (2006). Local drug delivery in the treatment of periodontal diseases: assessing the clinical significance of the results. J. Periodontol. 77 (4):565-578.

Hussein I, Ranka M, Gilbert A, Davey K (2007). Locally delivered antimicrobials in the management of periodontitis: a critical review of the evidence for their use in practice. Dent. Update. 34(8): 494-496, 499-502, 505-496.

Jain N, Jain GK, Javed S, Iqbal Z, Talegaonkar S, Ahmad FJ, Khar RK (2008). Recent approaches for the treatment of periodontitis. Drug. Discov. Today. 13 (21-22):932-943.

Kamma JJ, Nakou M, Mitsis FJ (2000). The clinical and microbiological effects of systemic ornidazole in sites with and without subgingival debridement in early-onset periodontitis patients. J. Periodontol. 71(12): 1862-1873.

Li CL, Martini LG, Ford JL, Roberts M (2005). The use of hypromellose in oral drug delivery. J. Pharm. Pharmacol. 57(5): 533-546.

Liu L, Wen X, He H, Shi J, Ji C (2003). Species-specific DNA probe for the detection of Porphyromonas gingivalis from adult Chinese periodontal patients and healthy subjects. J. Periodontol. 74(7): 1000-1006.

Mundargi RC, Srirangarajan S, Agnihotri SA, Patil SA, Ravindra S, Setty SB, Aminabhavi TM (2007). Development and evaluation of novel biodegradable microspheres based on poly(d,l-lactide-co-glycolide) and poly(epsilon-caprolactone) for controlled delivery of doxycycline in the treatment of human periodontal pocket: in vitro and in vivo studies. J. Control. Release, 119 (1):59-68.

Paju S, Pussinen PJ, Suominen-Taipale L, Hyvonen M, Knuuttila M, Kononen E (2009). Detection of multiple pathogenic species in saliva is associated with periodontal infection in adults. J. Clin. Microbiol. 47(1): 235-238.

Paquette DW, Ryan ME, Wilder RS (2008). Locally delivered antimicrobials: clinical evidence and relevance. J. Dent. Hyg. 82 Suppl 3: 10-15.

Quirynen M, Teughels W, De Soete M, van Steenberghe D (2002) Topical antiseptics and antibiotics in the initial therapy of chronic adult periodontitis: Microbiological aspects. Periodontology, 28: 72-90.

Takahashi K (1998). Microbiological, pathological, inflammatory, immunological and molecular biological aspects of periradicular disease. Int. Endod. J. 31(5): 311-325

Vandekerckhove BN, Quirynen M, van Steenberghe D (1997). The use of tetracycline-containing controlled-release fibers in the treatment of refractory periodontitis. J. Periodontol. 68(4): 353-361

Vyas SP, Sihorkar V, Mishra V (2000). Controlled and targeted drug delivery strategies towards intraperiodontal pocket diseases. J. Clin. Pharm. Ther. 25(1): 21-42.

Wang LC, Chen XG, Zhong DY, Xu QC (2007). Study on poly(vinyl alcohol)/carboxymethyl-chitosan blend film as local drug delivery system. J. Mater. Sci. Mater. Med. 18(6): 1125-1133.

Zambon JJ (1996). Periodontal diseases: microbial factors. Ann. Periodontol. 1(1): 879-925.

African Journal of Microbiology Research Vol. 5(32), pp. 5872-5876, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.751

Full Length Research Paper

Emergence of oligoclonal *Acinetobacter baumannii* nosocomial infection in a Hospital in Nepal

Badri Thapa[1], Chanwit Tribuddharat[2] and Sulochana Mahat Basnet[3]

[1]Department of Microbiology, Kathmandu Medical College, Kathmandu, Nepal, Microbiology Section, Genesis Laboratory and Research, Kathmandu, Nepal.
[2]Department of Microbiology, Siriraj Hospital, Mahidol University, Bangkok, Thailand.
[3]Faculty of Health, University of Canberra, Canberra, Australia.

Accepted 13 September, 2011

The molecular epidemiology of fifteen clinical strains of *Acinetobacter baumannii* recovered from various clinical specimens from different wards during January to June, 2010 from a hospital in Nepal was evaluated. Kirby-Bauer disk diffusion test was used for determining *in-vitro* activities of antibiotics. Molecular epidemiology was investigated by polymerase chain reaction-randomly amplified polymorphic DNA (PCR-RAPD) and plasmid profiling. *A. baumannii* recovered were multidrug resistant. Isolates represented three antibiotypes (a, b and c). Isolates in antibiotype c (n=12) were resistant to all antibiotics tested while isolates in antibiotype a (n=2) was susceptible to netilmicin and b (n=1) was susceptible to aminoglycosides and fluoroquinolones tested. Four plasmid profiles (i) 1 isolate; (ii) 1 isolate; (iii) 1 isolate; and (iv) 12 isolates and four PCR-RAPD types (I)1isolate; (II) 8 isolates; (III) 1 isolate; (IV) 5 isolates revealed oligoclonal population of *A. baumannii*. Antibiotypes, plasmid profiles and PCR-RAPD types showed no empirical association. *A. baumannii* isolates were oligoclonal and multi-drug resistant. The emergence of multi-drug resistant oligoclonal population of this pathogen in a hospital warrants for development of appropriate antibiotic policies and immediate implementation of infection prevention and control measures.

Key words: *Acinetobacter baumannii*, multidrug-resistant, oligoclonal, Nepal.

INTRODUCTION

Acinetobacter baumannii is emerging as a nosocomial pathogen around the globe. This pathogen is ubiquitous in the hospital environment, is multidrug, pandrug to extensively drug-resistant, can survive wide range of pH, salinity, humidity, and can thrive on almost all nutrient sources. They frequently colonize respiratory and digestive tract, skin, and throat causing wide array of infections especially in immunocompromised and debilitated patients admitted in intensive care units (ICU) (Montefour et al., 2008; Rosenthal and Tager, 1975; Somerville and Noble, 2008). *Acinetobacter* spp. is responsible for 3 to 4% of ventilator associated pneumonia and crude mortality rate due to *A. baumannii* is 30 to 70% (CDC, 1984).

Since its emergence as nosocomial infection in USA in 1991, *A. baumannii* has been isolated in numerous health care facilities and city, country and continent wide outbreak of this pathogen have been documented (Go et al., 1994; Peleg et al., 2008). This pathogen has successfully overcome therapeutic armament by accumulating its innate and acquired resistance repertoire (Peleg et al., 2008). *A. baumannii* resistant to all beta-lactams has already emerged (Peleg et al., 2008). Polymyxins, tigecycline and rifampin are considered as magic bullets to treat *A. baumannii* infections but resistant strains to these antibiotics are emerging (Ko et al., 2007; Thapa et al., 2009a).

Multidrug resistant strains of *Enterobacteriaceae* like, *Escherichia coli Klebsiella pneumoniae*, *Citrobacter* spp., *Proteus* spp., and *Enterobacter* spp., have been the subject of attention in Nepal but nosocomial infection by

*Corresponding author. E-mail: badri_bishal@yahoo.com. Tel: 977-1-4426059. Fax: 977-1-4426461.

Table 1. Antibiotypes, plasmid profiles and PCR-RAPD types of isolates studied.

Isolates	PCR-RAPD	Plasmid profile	Antibiotypes	AST, sensitive to
104	I	iv	c	-
106, 107, 1011, 1012, 1015, 1016	II	iv	c	-
109				-
1010	II	iv	a	AK, G, K, NT, NX, CF
108	III	iii	c	-
101	IV	i	a	AK, G, K, NT, NX, CF
102	IV	ii	b	NT
105,1013,1014	IV	iv	c	-

AST, Antibiotic susceptibility test; AK, Amikacin; G, Gentamicin; K, Kanamycin; NT, Netilmicin; NX, Norfloxacin; CF, Ciprofloxacin.

non-*Enterobacteriaceae*, like *A. baumannii* is also emerging (Banjara et al., 2003; Gaur et al., 2007; Thapa et al., 2009b). Molecular studies of *A. baumannii* from Nepal are scarce. Here, we studied the molecular epidemiology of nosocomial strains of *A. baumannii* isolated from Nepal.

MATERIALS AND METHODS

Bacterial strains

Out of 36 strains of *A. baumannii* isolated from various specimen sources in Microbiology laboratory of Kathmandu Medical College and Teaching Hospital (KMCTH), Kathmandu, Nepal during 6 months period (January to June, 2010), 15 were studied. The isolates were identified based on the published reports (Malini et al., 2009).

Antibiotic susceptibility test

Antibiotic susceptibilities of these pathogens were tested using Kerby-Bauer disk diffusion assay following CLSI guidelines (CLSI, 2005). The disk containing antibiotics (µg/disk) (HiMedia Pvt. Ltd, India) used were; Amoxicillin (20), Amoxicillin-Clavulanic acid (20+10), Piperacillin (100), Ceftizoxime (30), Ceftriaxone (30), Ceftazidime (30), Cefazolin (30), Cefoxitin (30), Amikacin (30), Gentamicin (30), Kanamycin (30), Netilmicin (30), Norfloxacin(10), and Ciprofloxacin (5).

Genetic analysis

Genomic and plasmid DNA from these isolates were extracted using Genomic DNA extraction Kit (Puregene, Minneapolis, Minnesota, USA) and Plasmid Miniprep (MN, Germany), respectively. polymerase chain reaction-randomly amplified polymorphic DNA (PCR-RAPD) was performed on Genomic DNA extract as described previously (Thapa et al., 2010). Briefly, PCR reaction was carried in 20 µl containing 50 ng of genomic DNA template, 0.2 µM primer (R003, 5' CTTGACGCA 3'), 0.2 mM dNTPs (FINZYMES), 2.5 µl of supplied PCR buffer, and 1.0 U of *Taq* polymerase (FINZYMES). 5% dimethylsulfoxide was added into the reaction. The PCR (PERKIN ELMER) profile used was: initial denaturation at 94°C for 2 min; followed by 40 cycles of 94°C for 10 s, 36°C for 30 s, and 72°C for 1 min; and a final heating at 72°C for 2 min. Amplified products and extracted plasmids were resolved in 1% TAE agarose (Research organics, inc. USA). Plasmid profiles were interpreted on the basis of the number and size of the plasmids. The study was approved by the institutional review board, Kathmandu Medical College, Nepal.

RESULTS

Antibiotic sensitivity test and antibiotype

The *in-vitro* activities of 14 antibiotics were tested against these isolates. All strains were multidrug-resistant (Table 1). Most of the isolates (n=13) were resistant to all antibiotics tested. All isolates were also resistant to beta-lactam antibiotics tested. Aminoglycosides and fluoroquinolones were effective against two isolates (101, 1010) while netilmicin was only effective to an isolate, 102. Based on the antibiotic susceptibility test these isolates were grouped into three antibiotypes, a, b, and c (Table 1). Most isolates (n=12) in antibiotype c were resistant to all antibiotics while antibiotype a (n=2) was sensitive to amino glycosides and fluroquinolones and b (n=1) was sensitive to netilmicin.

Plasmid profile

The size and number of the plasmids were able to categorize isolates into 4 plasmid profiles (i, ii, iii, and iv) (Figure 1 and Table 1). Most isolates (n=12) were grouped into plasmid profile iv while rest of the isolates represented individual plasmid profile type i, ii, and iii (Table 1).

PCR-RAPD

PCR-RAPD analysis of these strains revealed three RAPD types (I, II, and III) (Figure 2 and Table 1). Most of the isolates (n=8) accounted for type II and isolates 104 and 108 accounted for type I and III, respectively. Few

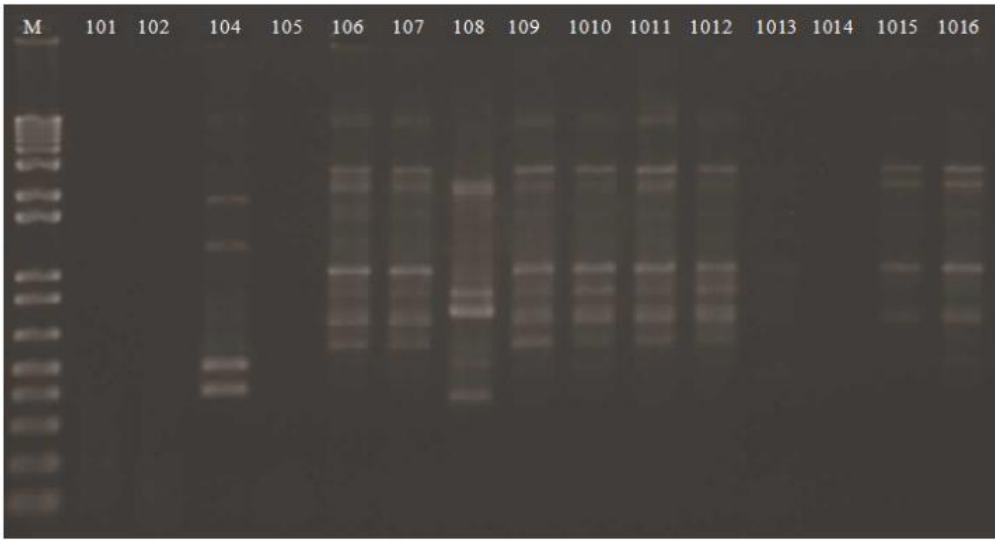


Figure 1. RAPD for *A. baumannii* studied. Lane M, molecular weight marker (1 kb+, Invitrogen); Numbers above lanes (2-17) indicates the isolates.

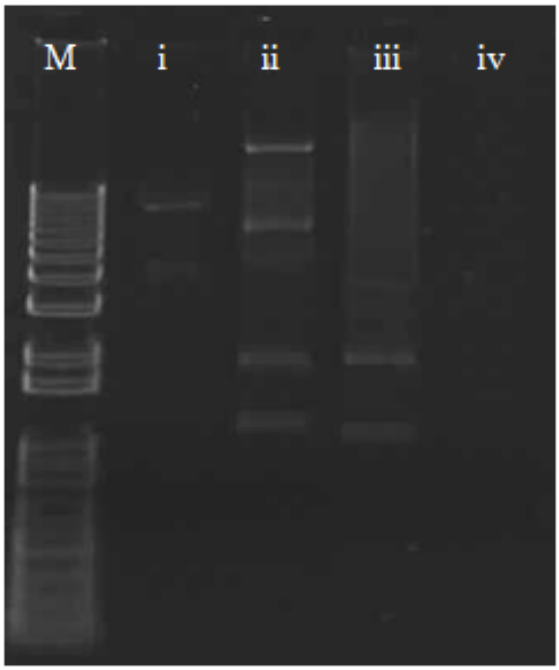


Figure 2. Plasmid profile of *A. baumannii* studied. Lane M, Molecular weight marker (1 kb^{+}, Invitrogen); lanes (2-5) indicates different plasmid profiles.

isolates (n=5) were not amplified and were grouped as type IV.

DISCUSSION

The indiscriminate use of antibiotics has led to the emergence of MDR, PDR and XDR strains of *A. baumannii* which was conventionally considered as less virulent and clinically unimportant. *A. baumannii* infection is a growing concern around the globe but the evidence of the emergence of this pathogen in Nepal is scarce. Out of 195 bacterial isolates obtained from surgical wound infection in Nepal, 13 bacterial species were identified and *Acinetobacter* spp. ranked 5th with the prevalence rate of 7.6%, and 9 strains were MDR (Banjara et al., 2003). In this study, all *A. baumannii* isolates were multidrug resistant. All were resistant to beta-lactam antibiotics tested. Some isolates were resistant to all antibiotics tested (antibiotype c) while others were sensitive to aminoglycosides and fluoroquinolones. Strains resistant to these antibiotics and to carbapenems have already been reported elsewhere (Chaiwarith et al., 2005; Thapa et al., 2010). At the time of conducting this study, carbapenems were just introduced in the clinical practice in Nepal and carbapenem susceptibility was not performed. Carbapenems including polymyxin, tigecycline, and rifampin in combination with other antibiotics which have been recommended for the management of multidrug-resistant *A. baumannii* can be the choices (Kasiakou et al., 2005; Thapa et al., 2009a). There are no current guidelines for treating *A. baumannii* in Nepal and the susceptibility data to these antibiotics should be generated before formulating such guidelines.

The clonality of the isolates was evaluated using antibiotypes, plasmid profiles, and PCR-RAPD. The evaluation of genetic relatedness using PCR-RAPD is an easy, cost effective, and rapid (Thapa et al., 2010). Using this arbitrarily primed PCR, we successfully identified the circulating local oligoclones (I to IV). Similar olioglonal outbreaks of *A. baumannii* have been reported (Thapa et al., 2010; Naas et al., 2005; Jeon et al., 2005). Plasmid

profiling a conventional typing tool grouped these isolates into four types (i to iv), and the isolates were grouped into three antibiotypes (a, b and c). Type II PCR-RAPD clone was most commonly encountered (n=8). These isolates also had similar plasmid profile and antibiotype. Similar plasmid profiles (type iv) and antibiotypes (type a and c) were observed among the isolates in PCR-RAPD types II and IV suggesting transfer of plasmids and resistance genes among different lineages. Two isolates (101 and102) within same PCR-RAPD type IV had independent plasmid profile and antibiotype. This reflected high rate of genetic promiscuity among similar genotypes. The difference of plasmid profiles and antibiotypes among different PCR-RAPD types can be explained by the high transformation capability of *Acinetobacter* spp. to expand its genetic pool of resistance (resistant plasmids and genes) (Metzgar et al., 2004). The same genotype of *A. baumannii* was found circulating in different wards. This suggests that the particular clone is hovering between wards and urge for prompt detection and elimination of the source.

Acinetobacter can be found in normal human skin, nosopharynx and digestive tract of hospitalized patients and infects debilitated and immunocompromised patients (Rosenthal and Tager, 1975). Most of the *A. baumannii* nosocomial outbreaks are also linked to the environmental sources in the hospital like, particles, air, injectable intravenous fluids, hands of medical staffs, and medical equipments (Deitz et al., 1988). These sources must be detected to control the spread of these clones. MDR international *A. baumannii* clones known as European clones I, II, and II have been reported in several European countries and also in United States (Nemec et al., 2004, van Dessel et al., 2004; Wroblewska et al., 2007). The rise in *A. baumanni* in United States has been contributed by the injured military personal returning from war in Iraq and Afghanistan (Scott et al., 2007; Davis et al., 2005). There was also an increase in prevalence of MDR *A. baumannii* between 1997 to 2001 in South American countries like, Argentina, Colombia, Chile, and Brazil (Tognim et al., 2004). Similarly, numerous PDR *A. baumannii* outbreaks have been reported from Asian hospitals (Thapa et al., 2010; Koh et al., 2007; Ying et al., 2006). Multidrug- and pandrug-resistant *A. baumannii* have been reported from almost all continents and is now a global problem.

This study, for the first time showed inter-ward spread of the *A. baumannii* clones and sensitized the need for monitoring of inter-institutional and international clones in Nepal. PCR-RAPD offers a dynamic platform to investigate clones in rapid and cost effective manner, has high sensitivity and high resolution for local epidemiological studies but it lacks reproducibility and produce categorical data that cannot be used to understand global epidemiology (Grundmann et al., 1997). More robust molecular typing tool-multi locus sequence typing-is necessary to establish the spread of these clones outside this institution (Bartual et al., 2005).

In conclusion, oligoclonal multidrug resistant *A. baumannii* has emerged as a successful nosocomial pathogen in this hospital in Nepal and warrants for tracing and elimination of the source. Prudent use of antibiotics, infection control and prevention practices, monitoring of these multidrug oligoclonal *A. baumannii* will help to stop the emergence and spread of the pathogen and its resistance genes across Nepal and internationally.

ACKNOWLEDGEMENT

Authors would like to acknowledge Dr. Chanwit Tribuddharat, Department of Microbiology, Siriraj Hospital, Mahidol University, a coauthor of this study for providing laboratory space and reagents for this study.

REFERENCES

Banjara MR, Sharma AP, Joshi AB, Tuladhar NR, Ghimire P, Bhatta DR (2003). Surgical wound infections in patients of Tribhuvan University Teaching Hospital. Nepal Health Res Counc. 3: 41-45.

Bartual S, Seifert H, Hippler C, Luzon MA, Wisplinghoff H, Rodriguez-Valera F (2005). Development of a multilocus sequence typing scheme for characterization of clinical isolates of *Acinetobacter baumannii*. J Clin Microbiol. 43: 4382-4390.

Centers for Disease Control (CDC) (1984). Nosocomial infection surveillance. CDC Summ. 35:17SS-29SS.

Clinical and Laboratory Standards Institute (CLSI) (2005). Performance standards for antimicrobial susceptibility testing; 15th informational supplement. CLSI document M100-S15. Clinical Laboratory Standards Institute, Wayne, PA.

Chaiwarith R, Mahattanaphak S, Boonchoo M, Supparatpinyo K, Sirisanthana K (2005). Pandrug-resistant *Acinetobacter baumannii* at Maharaj Nakorn Chaingmai Hospital. J. Infect. Dis. Antimicrob Agents, 22:1-8.

Davis KA, Moran KA, McAllister CK, Gray PJ (2005). Multidrug-resistant *Acinetobacter* extremity infections in soldiers. Emerg. Infect. Dis. 11: 1218-1224.

Deitz JW, Goodrich JA, Brown WB (1988). *Acinetobacter calcoaceticus* foot infection following to high pressure injection injury: a case report. Foot Ankle. 8: 216-222.

Naas T, Levy M, Hirschauer C, Marchandin H, Nordmann P (2005). Outbreak of carbapenem-resistant *Acinetobacter baumannii* producing the carbapenemase OXA-23 in a tertiary care hospital of Papeete, French Polynesia. J. Clin. Microbiol. 43: 4826-4829.

Gaur A, Prakash A, Anupurba S, Mohapatra TM (2007). Possible role of integrase gene polymerase chain reaction as an epidemiological marker: study of multidrug-resistant *Acinetobacter baumannii* isolated from nosocomial infections. Int. J. Antimicrob Agents. 4: 446-450.

Go ES, Urban C, Burns J, Kreiswirth B, Eisner W, Mariann N, Mosinka-Snipas K (1994). Clinical and molecular epidemiology of *Acinetobacter* infections sensitive only to polymyxin B and sulbactam. Lancet. 344: 1329-1332.

Grundmann HJ, Towner KJ, Dijkshoorn L, Gerner-Smidt P, Maher M, Seifert H, et al (1997). Multicenter study using standardized protocols and reagents for evaluation of reproducibility of PCR-based fingerprinting of *Acinetobacter* spp. J. Clin. Microbiol. 35: 3071-3077.

Jeon BC, Jeong SH, Bae IK, et al (2005). Investigation of a nosocomial outbreak of imipenemresistant *Acinetobacter baumannii* producing the OXA-23 β-lactamase in Korea. J Clin Microbiol. 43: 2241-2245.

Kasiakou SK, Michalopoulos A, Soteriades ES, Samonis G, Sermaides GJ, Falagas ME (2005). Combination therapy with intravenous colistin for management of infections due to multidrug-resistant Gram-negative bacteria in patients without cystic fibrosis. Antimicrob

Agents Chemother, 49: 3136-3146.
Ko KS, Suh JY, Kwon KT, Jung SI, Park KH, Kang CI, et al (2007). High rates of resistance to colistin and polymyxin B in subgroups of *Acinetobacter baumannii* isolates from Korea. J. Antimicrob Chemother. 60: 1163-1167.
Koh TH, Sng TH, Wang GCY, Hsu LY, Zhao Y (2007). Carbapenemase and efflux pump genes in *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex strains from Singapore. J. Antimicrob Chemother. 5: 1173-1174.
Malini A, Deepa EK, Gokul BN, Prasad SR (2009). Nonfermenting Gram-negative bacilli infections in tertiary care hospital in Kolar, Karnataka. J. Lab. Physician. 1: 62-66.
Metzgar D, Bacher JM, Pezo V, Reader J, Doring V, Schimmel P (2004). *Acinetobacter* spp. ADP1: an ideal model organism for genetic analysis and genome engineering. Nucleic Acids Res. 32:5780-5790.
Montefour K, Frieden J, Hurst S, Helmich C, Headley D, Martin Mary (2008). An emerging multidrug resistant pathogen in critical care. Crit. Care Nurse. 28:15-25.
Nemec A, Dijkshoorn L, van der Reijden TJ (2004). Long-term predominance of two pan-European clones among multi-resistant *Acinetobacter baumannii* strains in the Czech Republic. J. Med. Microbiol. 53:147-153.
Peleg AY, Seifert H, Paterson DL (2008). *Acinetobacter baumannii*: Emergence of a successful pathogen. Clin Micobiol Rev. 21: 538–582.
Rosenthal S, Tager IB (1975). Prevalence of Gram-negative rods in the normal pharyngeal flora. Ann Intern Med. 83: 355–337.
Scott P, Deye G, Srinivasan A, Murray C, Moran K, Hulten E (2007). An Outbreak of Multidrug-resistant *Acinetobacter baumannii-calcoaceticus* complex infection in the US military health care system associated with military operations in Iraq. Clin. Infect. Dis. 44: 1577-1584.
Somerville DA, Noble WC (1970). A note on the gram-negative bacilli of human skin. Eur. J. Clin. Biol. Res. 40: 669-670.
Thapa B, Tribuddharat C, Rugdeekha S, Techachaiwiwat W, Srifuengfung S, Dhiraputra C (2009a). Rifampin resistance in carbapenem-resistant *Acinetobacter baumannii* in Siriraj Hospital, Thailand. Nepal Med. Coll. J. 11: 232-237.
Thapa B, Adhikari P, Mahat K, Chhetri MR, Joshi LN (2009b). Multidrug-resistant nosocomial Citrobacter in a Hospital in Kathmandu. Nepal Med. Coll. J. 11: 195-199.
Thapa B, Tribuddharat C, Srifuengfung S, Dhiraputra C (2010). High prevalence of bla_{OXA-23} in oligoclonal carbapenem resistant *Acinetobacter baumannii* from Siriraj Hospital, Madhidol University, Bangkok, Thailand. South East Asian J. Trop. Med. 4: 625-635.
Tognim MC, Andrade SS, Silbert S, Gales AC, Jones RN, Sader HS (2004). Resistance trends of *Acinetobacter* spp. in Latin America and characterization of international dissemination of multi-drug resistant strains: five-year report of the SENTRY Antimicrobial Surveillance Program. Int. J. Infect. Dis. 8:284-291.
Van Dessel H, Dijkshoorn L, Van der Reijden T, Bakker N, A Paauw, Van den Broek Pl (2004). Identification of a new geographically widespread multiresistant *Acinetobacter baumannii* clone from European hospitals. Res Microbiol. 155: 105-112.
Wroblewska MM, Towner KJ, Marchel H, Luczak M (2007). Emergence and spread of carbapenem-resistant strains of *Acinetobacter baumannii* in a tertiary-care hospital in Poland. Clin Microbiol Infect.13: 490-496.
Ying CM, Ling TK, Lee CC, Ling JM (2006). Characterization of carbapenem-resistant *Acinetobacter baumannii* in Shanghai and Hong Kong. J. Med. Microbiol.55: 799-802.

African Journal of Microbiology Research Vol. 5(32), pp. 5877-5881, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.801

Full Length Research Paper

The effects of *bifidobacterium lactis* and galactooligosaccharide (GOS) on ileum and distal colon motility: *In vitro* study

Nevcihan Gursoy

Department of Food Engineering, Faculty of Engineering, Cumhuriyet University, Sivas, TR-58140, Turkey.
E-mail*:* ngursoy2@gmail.com and ngursoy@cumhuriyet.edu.tr. Tel: + 90-346-219 10 10 ext. 2889.
Fax: + 90-346-219 11 77.

Accepted 13 September, 2011

Twenty one male Wistar albino rats each weighing approximately 280 g were used in this study. Animals were divided into three groups. The first group (n = 7) consisted of sham controls, in the second (n = 7), rats were administrated 0.1 g/1 ml/galactooligosaccharide by by oral gavage for 4 weeks. In the third group (n = 7), rats were administrated 10^9 CFU/1ml/day *Bifidobacterium lactis* by oral gavage for 4 weeks. After 4 weeks, rats were sacrified; ileum and proximal colon segments were removed. The spontaneous contractions of ileum and proximal colon were evaluated by using organ bath. It has been detected that both prebiotics and probiotics increased intestinal motility. While probiotics have effects on both ileum and proximal colon, prebiotics seem to be effective in colon. All data are expressed as mean ± SEM (standard error of mean). Statistical comparisons between groups were performed using general linear models of analysis of variance (ANOVA) followed by the Turkey test.

Key words: *Bifidobacterium lactis,* galactooligosaccharide, ileum, rat, distal colon, *in vitro.*

INTRODUCTION

Epidemiological studies indicate that diet has a major impact on human health: a diet low in fat and high in fruit and vegetables has been correlated with a decreased incidence of so-called Western diseases such as coronary heart disease and colon cancer (Trock et al., 1990). Such a diet contains not only nutrients that are readily absorbed in the small intestine but also coponents that escape digestion by pancreatic and small bowel enzymes. The latter are the principal substrates of the bacteria resident in the human intestinal tract. Since a number of nutritional health effects are mediated by the intestinal microflora, diet is key in influencing their composition and activity. It has been increasingly recognized that the bacterial community in the intestine influences human health and well-being (Cummings and Macfarlane, 1997). Consequently, nutrition may be considered as a tool for influencing the intestinal microbiota in such a way that harmful bacteria are suppressed and beneficial bacteria are stimulated. Dietary strategies that serve to support health-promoting effects of the intestinal microflora include the ingestion of probiotics (Goldin, 1998) and or prebiotics (Gibson and Roberfroid, 1995), as well as a diet rich in fiber (Salminen et al., 1998).

Three approaches exist to increase the number of health-promoting organisms in the gastrointestinal tract. The first is the oral administration of live beneficial microorganisms. At present, these microorganisms, called probiotics, have been selected mostly from lactic acid bacteria and bifidobacteria that form a part of the

Abbreviations: GOS, galactooligosaccharide; **KBS,** Krebsbicarbonate solution; **CFU,** colony forming units; **NDO,** non-digestible oligosaccharides; **NaH_2PO_4,** sodium phosphate; **NaCl,** sodium chloride; **KCl,** potassium chloride; **$CaCl_2$,** calcium chloride; **$MgCl_2$,** magnesium chloride; **$NaHCO_3$,** sodium bicarbonate; **SEM,** standart error of mean; **ANOVA,** analysis of variance.

normal intestinal microflora of humans, these organisms are also indigenous to the colon. These bacteria have been suggested to be useful in the treatment of diarrhea (Rota virus, traveler's diarrhea, and *Clostridium difficile*), constipation, irritable bowel syndrome, and inflammatory bowel disease. They also have putative effects on enhancing the immune system and decreasing lactose intolerance (Bhutto and Morley, 2008).

The mechanisms of probiotic action appear to be multifactorial. Probiotic bacteria can promote fermentation processes that metabolize varying quantities of lactic, acetic, and formic acids; synthesis of vitamins; and the production of antimicrobial bacteriocidins and fatty acids (Bourlioux et al., 2002). Probiotics can also affect innate intestinal host defenses, including strengthening intestinal tight junctions, increasing mucous secretion, enhancing motility, and producing metabolic products (amino acids such as arginine and glutamine and short-chain fatty acids) that secondarily function as protective nutrients. They contribute to microflora diversity, thus helping to establish a normal commensal flora that protect against potential microbial pathogens (Neu and Caicedo, 2005).

The second strategy for increasing their number is to supply those already present in the intestine with selective carbon and energy source that provides them with competitive advantage over other bacteria in this ecosystem, thus selectively modifying the composition of the microflora using dietary supplements. These selective dietary components were named "prebiotics".

A prebiotic has been defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and or activity of one or a limited number of bacteria in the colon" (Gibson and Roberfroid, 1995). Prebiotics are intended to modify the intestinal microbiota in such a way that bacterial activities advantageous to the host are stimulated and bacterial activities adverse to host health are suppressed. The concept of prebiotics arose from the observation that inulin and fructooligosaccharides selectively stimulate the growth of bifidobacteria (Potter et al., 1993; Cummings, 1994) which are considered to be beneficial for human health (Gibson and Roberfroid, 1995). Although most research has been done on inulin and fructooligosaccharides, other non-digestible oligosaccharides (NDO) including xylooligosaccharides, galactooligosaccharides and isomalto oligosaccharides have also been tested for their prebiotic effect (Fuchs et al., 1999). The majority of candidate prebiotics are oligosaccharides but also include polysaccharides.To serve as a bacterial substrate in the colon, a prebiotic may not be hydrolyzed or absorbed in the upper part of the gastrointestinal tract.

And the last approach is a mixture of probiotic and prebiotic "synbiotic" has recently been proposed to characterise health-enhancing food and supplements used as functional food ingredients in human (Kontula et al., 1998).

Like probiotics, the prebiotics belong to a more general class of "colonic foods", that is foods entering the colon and serving as substrates for the endogenous colonic bacteria, thus indirectly providing the host with energy, metabolihc substrate and essential micronutrients (Gibson and Roberfroid, 1995).

Although it has been shown in clinical studies that pre and probiotics have positive effects on gastrointestinal motility, *in vitro* effects of pre and probiotics are not clear.

In this study we aimed to investigate and compare the effects of pre and probiotics on gastrointestinal motility in different segments of gastrointestinal track.

MATERIALS AND METHODS

Animal preparation twenty one male Wistar albino rats each weighing approximately 280 g were used in this study. The study was approved by. Animals were divided into three groups. The first group (n = 7) consisted of sham controls in which rats were administrated 1% ml 0.9 NaCl/ day by oral gavage for 4 weeks. In the second group (n = 7), rats were administrated 0.1 g/1 ml/day galactooligosaccharide (GOS) used as a prebiotic by oral gavage for 4 weeks. In the third group (n = 7), rats were administrated 10^9 CFU/1ml/day *Bifidobacterium lactis* used as a probiotic by oral gavage for 4 weeks. *B. lactis* were grown from frozen stocks (-80 ^{o}C) prepared for ingestion and counted as in Kamiya at al. (2006). At the end of the four weeks, rats were killed by cervical dislocation. The abdomen was opened with a midline incision. Ileum and proximal colon was removed and placed in previously aerated (95% O_2 and 5% CO_2) Krebs-bicarbonate solution (composition in mmol/L: NaCl, 120; KCl, 4.6; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 22; NaH_2PO_4 and glucose 11.5). Whole full-thickness segments of ileum and proximal colon were placed in circular direction in a 10 mL tissue baths, filled with pre-aerated Krebsbicarbonate solution (KBS) at 37°C. The upper end of the preparation was tied to an isometric transducer (Grass FT 03, Quincy, MA, USA) and preloaded with 1 to 1.5 g. Tissues were allowed to equilibrate for 30 min.

In vitro muscle contractility studies

Muscle segments from each group were contracted with 80 mmol/L KCl to ensure that they worked properly at the beginning and end of each experiment.

At the beginning of each experiment, 80 mmol/L KCl was added to the organ bath, and the contraction was considered as reference response. Subsequently, the amplitude of spontaneous contractions of the isolated Ileum and proximal colon muscle segments were calculated as a percentage of the contraction induced by KCl (80 mmol/L) from both control, prebiotic and probiotic groups. Changes in the frequency (number or min.) of spontaneous contractions were expressed as the number of contractions for 10 min intervals. Isometric tensions were recorded on a Grass model 79 E polygraph. All experiments were performed in duplicate.

Data analysis

All data are expressed as mean ± SEM (standard error of mean). Statistical comparisons between groups were performed using general linear models of analysis of variance (ANOVA) followed by the Turkey test and P-values of less than 0.05 were considered to be statistically significant.

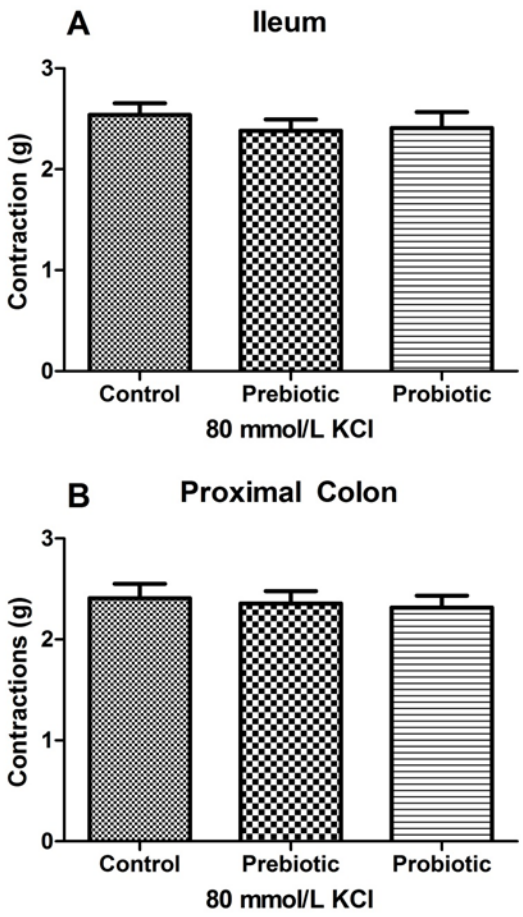


Figure 1. KCl (80 mmol/L) induced contractions of isolated ileum muscle segments in control, prebiotic and probiotic groups. No statistical difference was observed between groups (P > 0.05).

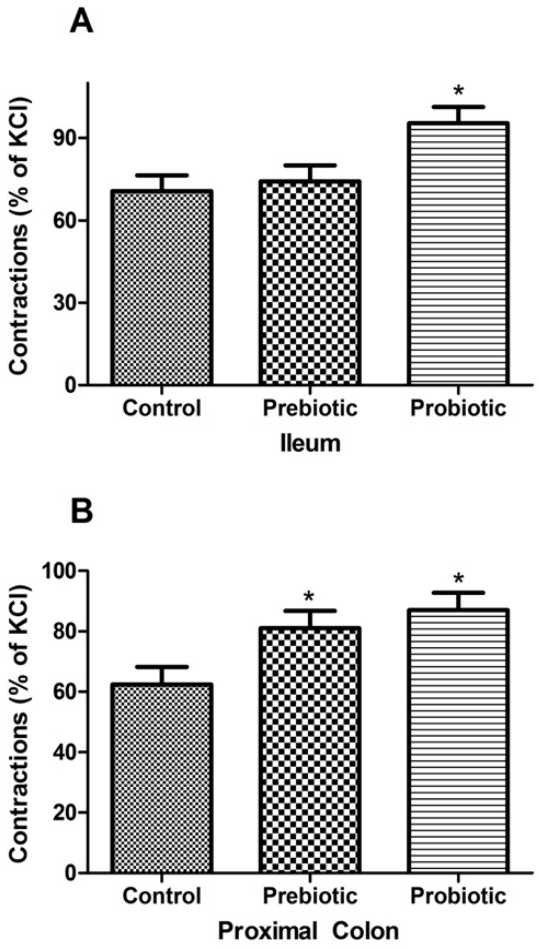


Figure 2. Changes in the spontaneous contraction amplitudes of the isolated smooth muscle segments. A. Ileum B. Proximal Colon.

RESULTS

Contractions induced by 80 mmol/L KCl were not significantly different between control, prebiotic and probiotic groups in isolated ileum smooth muscle segments which indicated that muscle segments from both groups worked properly (Figure 1).

In the smooth muscle segments from ileum, the mean amplitude of the spontaneous contractions was 70.6 ± 4.6 in the control group, 74.2 ± 5.2 in prebiotic group and 95.5 ± 7.1 in probiotic group, respectively. There was no significant difference between the amplitude responses of control and prebiotic groups ($p > 0.05$). But the amplitude of probiotic group was significantly higher than both control and prebiotic groups ($p < 0.05$) (Figure 2A).

In the smooth muscle segments from proximal colon, the mean amplitude of the spontaneous contractions was 62.4 ± 3.5 in the control group, 81.4 ± 4.4 in prebiotic group and 87.5 ± 5.2 in probiotic group, respectively. Both amplitude responses of spontaneous contractions of prebiotic and probiotic groups were significantly high when compared to the control group ($p < 0.05$). There was significant difference between prebiotic and probiotic groups ($p > 0.05$) (Figure 2B).

In the smooth muscle segments from ileum, the mean frequency of the spontaneous contractions was 27.2 ± 1.6 in the control group, 28.8 ± 2.2 in prebiotic group and 35.7 ± 4.1 in probiotic group, respectively. There was no significant difference between the frequency responses of control and prebiotic groups ($p > 0.05$). But the frequency of probiotic group was significantly higher than both control and prebiotic groups ($p < 0.05$) (Figure 3A).

In the smooth muscle segments from proximal colon, the mean frequency of the spontaneous contractions was 12.3 ± 1.5 in the control group, 11.1 ± 1.4 in prebiotic group and 17.5 ± 2.2 in probiotic group, respectively. Although there was no significant difference between the frequency responses of control and prebiotic groups ($p > 0.05$), spontaneous contraction amplitude responses of probiotic group was significantly high when compared to the control and prebiotic groups ($p < 0.05$) (Figure 3B).

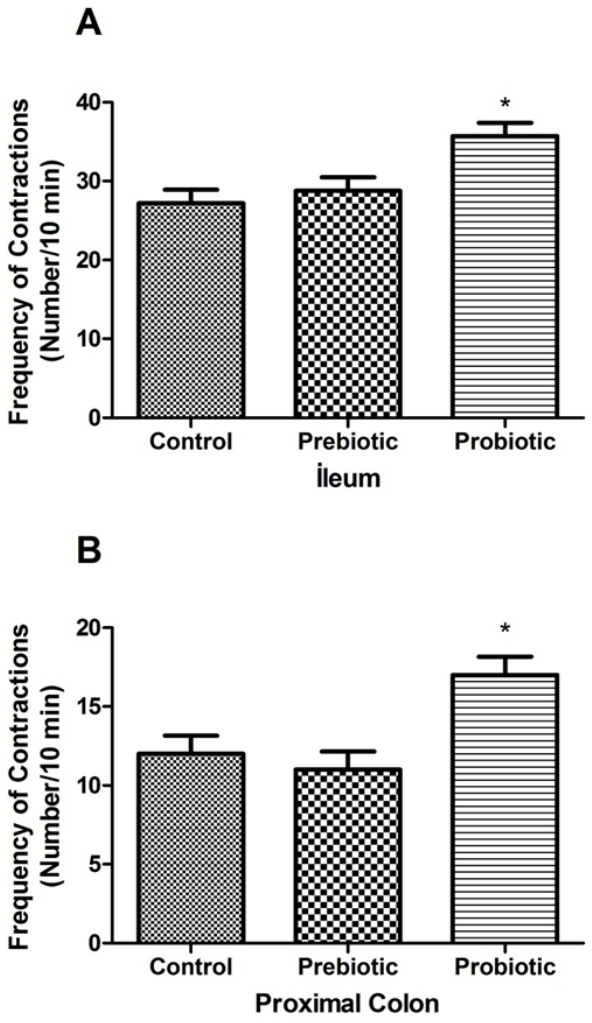


Figure 3. Changes in the spontaneous contraction frequency of the isolated smooth muscle segments. A. Ileum B. Proximal Colon.

DISCUSSION

The gut represents a complex and dynamic microbial ecosystem in which intestinal micro flora has an important and specific metabolic, trophic, and protective function. Normal gut structure and function are the end-point of a complex set of interactions between the host and microorganisms colonizing the gut (Guarner and Malagelada, 2003). Bacteria can be used to improve human health. A bacterium that provides specific health benefits when consumed as a food component or supplement would be called a probiotic. A consensus definition of the term was issued a few years ago and states that oral probiotics are living microorganisms that upon ingestion in specific numbers exert health benefits beyond those of inherent basic nutrition (Guarner and Schaafsma, 1998; Guarner et al., 2005). While probiotics are the live microbial feed supplements that beneficially affect the host animal by improving its intestinal microbial balance (Fuller, 1989); prebiotics are defined as food ingredients that promote the growth or activity of a limited number of bacterial species for the benefit of host health (Gibson and Roberfroid, 1995). Organisms used as probiotics are most frequently of the *Lactobacillus* or *Bifidobacterium* species, and clinically beneficial effects of probiotics have been described in travellers' diarrhea, irritable bowel syndrome and inflammatory bowel disease (Walker and Buckley, 2006; Shanahan, 2007).

There are many conflicting studies about the effects of pro and prebiotics on gastrointestinal motility. While some of these studies suggest that pro and prebiotics increase intestinal motility, others suggest opposite. It has been shown that *Lactobacillus reuteri* ingestion consistently alters the motility of colon segments in an *ex vivo* organ bath recording setup. The effect is a decrease in the amplitudes of contractions at constant luminal filling preasure, and an increase in the threshold luminal pressure required to evoke rhythmic contractions (Wang et al., 2010). On the other hand, an *in vivo* study showed that administration of probiotics induces increased colonic propulsive contractions and defecation rate in pigs (Ohashi et al., 2001). It has been shown in a human clinical study that probiotic supplements may have a positive effect on bowel movements among orthopedic rehabilitation elderly patients (Zaharoni et al., 2011). In addition, Tabbers et al. (2009) suggested that *B. lactis* strain DN-173 010 is effective in increasing stool frequency after 3 weeks of product consumption in children with functional constipation and a defecation frequency less than 3 weeks.

There are little data available related to the influence of prebiotics on gastrointestinal motility in preterm infants. In a study in healthy preterm infants, Boehm et al. (2002) demonstrated that preterm infants fed with mother's milk had lower stool consistency and higher stool frequency than infants fed a preterm bovine milk formula. Supplementation of the same formula with a mixture of scGOS and lcFOS resulted in a reduction in stool consistency and an increase in stool frequency. More recently, Mihatsch et al. (2006) demonstrated a clinically relevant reduction in the gastrointestinal transit time in preterm infants fed a formula supplemented with these prebiotics.

In this study, consistent with these positive studies, we found that probiotics increased spontaneous contraction amplitude and frequency of both ileum and proximal colon. On the other side, while prebiotics increasing spontaneous contraction amplitudes of proximal colon, did not changed spontaneous contraction amplitude of ileum. Also prebiotics did change neither spontaneous contraction frequency of ileum nor spontaneous contraction frequency of proximal colon. The difference between the effect of pro- and prebiotics on ileum spontaneous contraction amplitude may be related to the difference in physiology and bacterial colonization between ileum and proximal colon. It is clear that a complex, resident gut microflora is present in human

subjects. While the transit of residual foodstuffs through the stomach and small intestine is probably too rapid for the microbiota to exert a significant impact, this slows markedly in the colon. Colonic micro-organisms have ample opportunity to degrade available substrates (Cherbut, 2003; Gibson et al., 2004; Flint et al., 2008). Due to the high residence time of colonic contents, as well as a diverse and profuse flora, the colonic microbiota plays a more important role in host health and well-being than is the case in the small intestine. As a result, it has been defined that both prebiotics and probiotics increased intestinal motility. While probiotics have effects on both ileum and proximal colon, prebiotics seem to be effective in colon. The difference possibly related to the microbial flora. It is well known that changes in gastrointestinal micro flora exhibit an intestinal motility response and that such change can be initiated by addition of synbiotics to the diet. According to these findings it seems that food supplemented with probiotic and prebiotics would prevent impared motility seen in lots of gastrointestinal diseases. Further work is necessary in order to identify the underlying mechanisms responsible for diet/bacterial induced changes in gastrointestinal motility.

REFERENCES

Bhutto A, Morley JE (2008). The clinical significance of gastrointestinal changes with aging. Curr. Opin. Clin. Nutr. Metab. Care 11(5):651-660.

Boehm G, Lidestri M, Casetta P, Jelinek J, Negretti F, Stahl B (2002). Supplementation of an oligosaccharide mixture to a bovine milk formula increases counts of faecal bifidobacteria in preterm infants. Arch. Dis. Child., 86:F178–81.

Bourlioux P, Koletzko B, Guarner F, Braesco V (2002). The intestine and its microflora are partners for the protection of the host: report on the Danone Symposium ‘‘The Intelligent Intestine,’’ held in Paris, June 14. Am. J. Clin. Nutr., 78:675-683.

Cherbut C (2003). Motor effects of short-chain fatty acids and lactate in the gastrointestinal tract. Proc. Nutr. Soc., 62: 95–99.

Cummings JH (1994). Anatomy and physiology of the human colon. ILSI Workshop on Colonic Microflora: Nutrition and health, Barcelona, Spain.

Cummings JH, Macfarlane GT (1997). Colonic microflora: Nutr.Health Nutr., 13:476–478

Flint HJ, Bayer EA, Rincon MT, Lamed R, White BA (2008). Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. Nat. Rev. Microbiol., 6:121–131.

Fuchs CS, Giovannucci EL, Colditz GA, Hunter DJ, Stampfer MJ, Rosner B, Speizer FE, Willett WC (1999). Dietary fiber and the risk of colorectal cancer and adenoma in women. Nutr. Engl. J. Med., 340:169–176.

Fuller R (1989). Probiotics in man and animals. J. App. Bacteriol., 66(5): 365–378.

Gibson GR, Probert HM, Van Loo JAE, Rastall RA, Roberfroid MB (2004). Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutr. Res. Rev., 17: 259–275.

Gibson GR, Roberfroid MB (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. Nutr., 125:1401–1412.

Goldin BR (1998). Health benefits of probiotics. Br. J. Nutr., 80:S203–207.

Guarner F, Malagelada JR (2003). Gut flora in health and disease. Lancet 361: 512-519.

Guarner F, Perdigon G, Corthier G, Salminen S, Koletzko B, Morelli L (2005). "Should yoghurt cultures be considered probiotic?", Br. J. Nutr., 93(6):783-786.

Guarner F, Schaafsma G (1989). Probiotics. Int. J. Food Microbiol., 39: 237–238.

Kamiya T, Wang L, Forsythe P, Goettsche G, Mao Y, Wang Y (2006). Inhibitory effects of *Lactobacillus reuteri* on visceral pain induced by colorectal distension in Sprague-Dawley rats. Gut., 55: 191–196.

Kontula P, Jaskary J, Nollet L, De Smet I, Von Wright A, Poutanen K, Mattila-Sandholm T (1998). The colonization of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) by a probiotic strain fed in a fermented oat bran product: Effects on the gastrointestinal microbiota. Appl. Microbiol. Biotechnol., 50: 242–252.

Mihatsch WA, Hoegel J, Pohlandt F (2006). Prebiotic oligosaccharides reduce stool viscosity and accelerate gastrointestinal transport in preterm infants. Acta Paediatr., 95:843–848.

Neu J, Caicedo R (2005). Probiotics: protecting the intestinal ecosystem? J. Pediatr., 147(2):143-146.

Ohashi Y, Inoue R, Tanaka K, Umesaki Y, Ushida K (2001). Strain gauge force transducer and its application in an pig model to evaluate the effect of probiotic on colonic motility. J. Nutr. Sci. Vitaminol., 47: 351–356.

Potter JD, Slattery ML, Bostick RM, Gapstur SM (1993). Colon cancer: a review of the epidemiology. Epidemiol. Rev., 15:499–545.

Salminen S, Bouley C, Boutron-Ruault MC, Cummings JH, Franck A, Gibson GR, Isolauri E, Moreau MC, Roberfroid M, Rowland I (1998). Functional food science and gastrointestinal physiology and function. Br. J. Nutr., 80(1): S147–171.

Shanahan F (2007). Irritable bowel syndrome: shifting the focus toward the gut microbiota. Gastroenterol., 133: 340–342.

Tabbers MM, Chmielewska A, Roseboom MG, Boudet C, Perrin C, Szajewska H, Benninga MA (2009). Effect of the consumption of a fermented dairy product containing *Bifidobacterium lactis* DN-173 010 on constipation in childhood: a multicenter randomised controlled trial (NTRTC: 1571). BMC Pediatr., 18:9-22.

Trock B, Lanza E, Greenwald P (1990). Dietary fiber,vegetables,and colon cancer: critical review and meta-analyses of the epidemiologic evidence. J. Natl. Cancer Inst., 82:650–661.

Walker R, Buckley M (2006). Probiotic microbes: the scientific basis. In A Report of the American Academy of Microbiology. Walker, R & Buckley, M. (Eds.) Washington, D.C.: American Society for Microbiology.

Wang B, Mao YK, Diorio C, Wang L, Huizinga JD, Bienenstock J, Kunze W (2010). *Lactobacillus reuteri* ingestion and IK(Ca) channel blockade have similar effects on rat colon motility and myenteric neurones. Neurogastroenterol. Motil., 22(1):98-107.

Zaharoni H, Rimon E, Vardi H, Friger M, Bolotin A, Shahar DR (2011). Probiotics improve bowel movements in hospitalized elderly patients--the PROAGE study. J. Nutr. Health Aging., 15(3):215-220.

African Journal of Microbiology Research Vol. 5(32) pp. 5882-5888, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.831

Full Length Research Paper

Cloning, expression and characterization of a glucose dehydrogenase from *Bacillus* sp. G3 in *Escherichia coli*

Xuejiao Chen[1,2], Haitao Ding[1], Yiqing Du[1], Hui Lin[1], Zeli Li[1] and Yuhua Zhao[1]*

[1]Institute of Microbiology, College of Life Science, Zhejiang University, Hangzhou 310058, China.
[2]Hangzhou Wahaha Group Co., Ltd, Hangzhou 310000, China.

Accepted 17 October, 2011

The glucose dehydrogenase gene (*gdh*), cloned from *Bacillus* sp. G3, was composed of 786 bp nucleotide and the deduced protein molecular mass of one subunit was 28.1 kDa. The recombinant glucose dehydrogenase (rGDH-G3) was functionally expressed in *Escherichia coli*. The results revealed that expressed rGDH-G3 had a high specific activity of 371.9 U/mg at 25°C and pH 8.0, with oxidized nicotinamide adenine dinucleotide (NAD^+) as the cofactor. The enzyme was optimally active at 40°C and pH 9.0. The enzyme displayed broad specificity for other sugars such as D-galactose or maltose. The catalytic efficiency of the rGDH-G3 would be improved 4 times when oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$) was used as cofactor instead of NAD^+.

Key words: *Bacillus* sp. G3, enzymatic property, glucose dehydrogenase, inverse polymerase chain reaction (IPCR), optimal pH.

INTRODUCTION

Glucose dehydrogenase (GDH, EC 1.1.1.47), a member of the short-chain family of alcohol dehydrogenase, consists of four identical subunits (30 kDa) (Pauly and Pfleiderer, 1975), it catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone in the presence of cofactor oxidized nicotinamide adenine dinucleotide (NAD^+) or oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$), and it posses the property of dual cofactor specificity. It has been shown that the GDH plays an important role in spore germination, and is a marker enzyme synthesized at sporulation stage (Nakatani et al., 1989). In recent years, GDH has been widely studied and used in many fields including biofuel cells (Okuda-Shimazaki et al., 2008), clinical tests (Du et al., 2008), and as a catalyst for coenzyme regeneration in large-scale chiral synthesis (Lin et al., 1999; Wong and Drueckhammer, 1985) .

In order to meet the increasing demands for the above applications, the glucose dehydrogenase gene (*gdh*) of microorganism have been cloned and over-expressed in *Escherichia coli* from sporulating cells of *Bacillus megaterium* (Heilmann et al., 1988; Nagao et al., 1992) and *Bacillus subtilis* (Vasantha et al., 1983). The characteristics of GDH from *B. megaterium* (Makino et al., 1989a; Mitamura et al., 1989; Nagao et al., 1992) and *B. subtilis* (Fujita et al., 1977) have been investigated in detail. In this paper, we reported the results of a study that was aimed at isolating the *gdh* from *Bacillus* sp. G3, expressing in *E. coli* BL21 (DE3), as well as purifying and

*Corresponding author. E-mail: yhzhao225@zju.edu.cn. Tel: 86-571-88208557. Fax: 86-571-88206995.

Abbreviations: ***gdh*,** Glucose dehydrogenase gene; **GDH,** glucose dehydrogenase; **GDH-G3,** glucose dehydrogenase from *Bacillus* sp. G3; **PCR,** polymerase chain reaction; **IPCR,** inverse PCR; **ORF,** open reading frame; **rGDH-G3,** recombinant glucose dehydrogenase; **NAD^+,** oxidized nicotinamide adenine dinucleotide; **$NADP^+$,** oxidized nicotinamide adenine dinucleotide phosphate; **SDS-PAGE,** sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Table 1. Oligonucleotide primers used for *gdh* isolation, DNA amplification and cloning.

Primer	Sequence
dG3F	5'-GAYRTNATGATHAAYAAYGC-3'
dG3R	5'-ATRTANCCCATNGGDATCAT-3'
iG3F	5'-AATCGCTTCACGGCTTCC-3'
iG3R	5'-AGGGCGGATTGAAACTAA-3'
GDH3F	5'-GGAATTCCATATGTATAGTGATTTAGAAGGA-3'
GDH3R	5'-CGGGATCCTATTACCCACGCCCAGC-3'

[a]Underlined bases are restriction sites (*Nde*I in GDH3F and *Bam*HI in GDH3R).

characterizing this new enzyme.

MATERIALS AND METHODS

Bacterial strains, plasmid and chemicals

The strain *E. coli* DH5α and *E. coli* BL21 (DE3) were used, respectively for cloning and expression. *Bacillus* sp.G3 used as the source of *gdh* was cloned and identified by our laboratory. Plasmids pMD19-T (Takara, Dalian, China) and pET28 (a+) (Invitrogen, Shanghai, China) were used as vectors for the cloning and expression of the *gdh*, respectively. Restriction enzymes and other modification enzymes, Taq DNA polymerase, and T4 DNA ligase were purchased from Takara, Dalian, China. Primers were synthesized by Invitrogen, Shanghai, China. Ni-NTA-resin was purchased from Invitrogen, Shanghai, China. DNA gel extraction kit was purchased from Axygen, Shanghai, China. NAD^+ and $NADP^+$ were purchased from Alfa Aesar, Tianjin, China. All other chemicals and solvents used were of analytical grade and available commercially.

Enzyme activity assays

The activity of glucose dehydrogenase from *Bacillus* sp. G3 (GDH-G3) was assayed by measuring the increase in absorbance of nicotinamide adenine dinucleotide (NADH) at 340 nm. The standard reaction mixture contained 100 mM sodium phosphate buffer (pH 8.0), 200 mM glucose, and 1 mM NAD^+ with a final volume of 1 ml. One microlitre (1 µl) diluted enzyme solution was added to the assay mixture and incubated at 25°C for 5 min. The apparent extinction coefficient of NADH was 6220 M^{-1} cm^{-1}. One unit of GDH activity was defined as the amount of enzyme required to release 1 µM of NADH per minute at 25°C and the pH of 8.0. All assays were repeated three times.

Cloning of the glucose dehydrogenase gene (*gdh*)

To clone a fragment of *gdh* from *Bacillus* sp.G3, a polymerase chain reaction (PCR) strategy with degenerate primers were used. Primers dG3F and dG3R (Table 1) were designed based on the conserved amino acid sequence (D V/I MINNA and M V/I PMGYI) of GDHs from different species of *Bacillus*. The PCR was conducted under the following conditions: 94°C for 5 min; 5 cycles of 94°C for 30 s, 45°C for 30 s, 72°C for 30 s; followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s; and a terminal extension at 72°C for 2 min. After separation and purification, a 400 bp PCR product was sequenced. The complete GDH-G3 gene was obtained by using the inverse PCR (IPCR) technique (Ochman et al., 1988). The genomic DNA from *Bacillus* sp.G3 was digested with several endonucleases, and then self-ligated by using T4 DNA ligase at 16°C overnight. The ligation products were used as template for IPCR. A pair of primers iG3F and iG3R (Table 1) used were designed based on the above product using Primer Premier 5.0. And the reaction was conducted for 30 cycles: 94°C for 30 s, 55°C for 30 s and 72°C for 3 min. The resultant fragment was sequenced, and then the complete GDH-G3 gene was assembled according to overlapping sequences from the two fragments.

Construction of expression plasmid

Recombinant DNA techniques were carried out according to standard methods described by Sambrook and Russell (2001). The open reading frame (ORF) of GDH-G3 was amplified by PCR with the genomic DNA of *Bccillus* sp. G3 as the template, and GDH3F and GDH3R (Table 1) as primers, respectively. The purified PCR product was digested with *Nde*I and *Bam*HI, and ligated with the pET-28a (+) that was linearized with the same enzymes, forming a new ORF that encoded an N-terminal His_6-tag. The recombined plasmids were transformed into *E. coli* DH5α competent cells for amplification.

Expression and purification of recombinant glucose dehydrogenase (rGDH-G3)

To express 6his-tagged *gdh*, the recombinant vector was transformed into *E. coli* BL21 (DE3) competent cells using $CaCl_2$-heat shock method (Sambrook and Russell, 2001). The positive transformants were cultured at 37°C in Luria–Bertani (LB) medium containing 50 µg ml^{-1} kanamycin to an OD_{600} = 0.6. After induction with 0.1 mM IPTG at 25°C for 16 h, the culture was harvested. The cell pellet was washed twice with Buffer A (25 mM NaH_2PO_4, 250 mM NaCl, pH 8.0) and lysed by the ultrasonic disruption, followed by centrifugation at 14000 rpm for 30 min at 4°C. The supernatants were loaded onto a Ni-NTA-resin (Invitrogen, Shanghai, China) column pre-equilibrated with Buffer A. After washed with the Buffer B (25 mM NaH_2PO_4, 250 mM NaCl, 20 mM imidazole, pH 8.0), the rGDH-G3 was eluted with 10 ml Buffer C (50 mM NaH_2PO_4, 250 mM NaCl, 250 mM imidazole, pH 8.0), and then supplemented with 20% (v/v) glycerol. The purified enzyme fractions were dialyzed and stored at 4°C. The protein concentration was determined by the method of Bradford with BSA as the standard (Bradford, 1976). The purity and molecular mass of the enzyme was analyzed by 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and stained with 0.05% Coomassie brilliant blue R-250 (Varghese and Diwan, 1983).

Characterization of the recombinant glucose dehydrogenase (rGDH-G3)

The optimal temperature of rGDH-G3 was studied at various

temperatures between 25 and 65°C under standard conditions. To determine the thermostability, the enzyme solution was preincubated in sodium phosphate buffer (25 mM, pH 8.0) at 25–65°C for 60 min, respectively. To investigate the optimum reaction pH range for rGDH-G3, four buffers were used and they include 100 mM citrate buffer (pH 4.0–6.0), 100 mM sodium phosphate buffer (pH 6.0–8.0), 100 mM Tris/HCl (pH 7.0–9.0), and 100 mM Gly/NaOH (pH 8.5–10.5). The pH stability was determined by pre-incubating diluted enzyme in the above buffers at 25°C for 60 min. For kinetic studies, the reaction rates were measured for a variety of substrates concentrations and the kinetic parameters were evaluated by Lineweaver–Burk plots method.

RESULTS AND DISCUSSION

Isolation and sequence analysis of *gdh* from *Bacillus* sp.G3

A BLAST search in the GenBank database showed that the fragment was highly identical to other *gdh* in the database (Table 1). Based on the conserved DNA sequence, a pair of gene-specific primers iG3F and iG3R (Table 1) for IPCR were designed. The digested genomic DNA from *Bacillus sp*.G3 was self-ligated, and was used as template for IPCR to clone the flanking sequence of the GDH-G3 gene. A notable 1500 bp band was amplified only from *NdeI*-digested genomic DNA. It was sequenced and then the complete GDH-G3 gene was assembled according to overlapping sequences of the two fragments. The DNA sequence of GDH-G3 gene showed significant homology (97-78% identities) with other *gdh* sequences present in NCBI.

The nucleotide sequence of the gene and the deduced amino acid sequence were deposited in GenBank (Accession no. GQ402830). The gene contained a 786 bp ORF encoding a subunit of 261 residues with a predicted molecular mass of 28.1 kDa.

The deduced amino acid sequence of GDH-G3 showed 99% (maximum) identity with GDH subunit from *B. cereus* BDRD-ST26 (Accession no. ZP_04269989) and *B. cereus* H3081.97 (Accession no. ZP_03238186). In addition, it showed more than 80% sequence homology with the GDHs from most species of *Bacillus*. Sequence alignment of the deduced amino acid sequence of the GDH-G3 with other GDH sequences in GenBank database revealed the presence of highly conserved regions (Figure 1).

Expression and purification of glucose dehydrogenase (GDH)

After transformation and induction, the recombinant plasmid was successfully expressed in heterologous host strain *E. coli* BL21 (DE3). The recombinant protein was further purified by using Ni^{2+}-chelating affinity chromatography. With the His_6-tag at the N terminus, which facilitated strongly binding of the protein to the Ni-NTA matrix, most unbound proteins were washed away by the wash buffer. As shown in Figure 2, portion of the enzyme was expressed in a highly soluble form and the purified enzyme revealed a single protein band corresponding to approximately 28 kDa on SDS-PAGE, which was agreed with the predicted 28.1 kDa molecular mass. After being purified 20-fold, the specific activity of the enzyme was 371.9 U/mg (25°C, pH 8.0, with NAD^+ as the cofactor).

Effects of pH and temperature on enzyme activity

The rGDH-G3 had optimal activity at 40°C (Figure 3A) and pH 9.0 (100 mM Tris/HCl, Figure 3B), respectively. The enzyme had more than 60% of the maximum activity in a pH range of 7.5–9.5 and a temperature range of 30-50°C. The assays of optimal temperature indicated that the activity increased in an almost linear fashion from 25-40°C, but decreased once the temperature was above 45°C. The activity was hardly detected at temperature higher than 70°C. It was observed that the activity of rGDH-G3 was affected by the buffers used. The recombinant enzyme in Tris/HCl performed much better than in Gly/NaOH buffers, while sodium phosphate buffer was better than Tris/HCl at the same pH.

As shown in Figure 3B, the pH/activity profile of GDH-G3 was similar to GDH-I, GDH-II and GDH-Iwg3 (Mitamura et al., 1989), with the optimal activity shifted a little towards a higher value. Optimal rGDH-G3 activity occurred at pH 9.0 while that of others from *B. megaterium* (Mitamura et al., 1989), *B. thuringiensis* M 15 (Boontim et al., 2004), *B. subtilis* (Fujita et al., 1977) occurred at pH 8.0, but the optimal pH in this study was lower than pH 9.5 for LsGDH from *L. sphaericus* G10 (Ding et al., 2010). Figure 1 showed the amino acid residues in GDH-G3 that are different from the other GDHs. Maybe the surrounding residues near the acid/base catalytic center affect the protonation and then effect a change in optimal pH (Shibuya et al., 2005).

Effects of pH and temperature on enzyme stability

The assays of enzyme thermostability indicated that the enzyme was stable below 40°C. After 60 min incubation, there was 72% of enzyme activity remained with 40°C treatment, whereas only 29% of the activity remained at 45°C (Figure 4A). The thermostability of GDH-G3 was similar to its homologous counterparts, except GDH-III from *B. megaterium* IAM1030. Nagao et al. (1992) have reported that the two alterations, Leu-167 to Gln and Ala-258 to Thr, weakened the intersubunit interaction of the tetramer of GDH-III (Nagao et al., 1992). Therefore, it could be presumed that, maybe, the replacement of Gln-167, Thr-258 of GDH-III with the Leu and Aln could have promoted the thermostability of GDH-G3. And it is

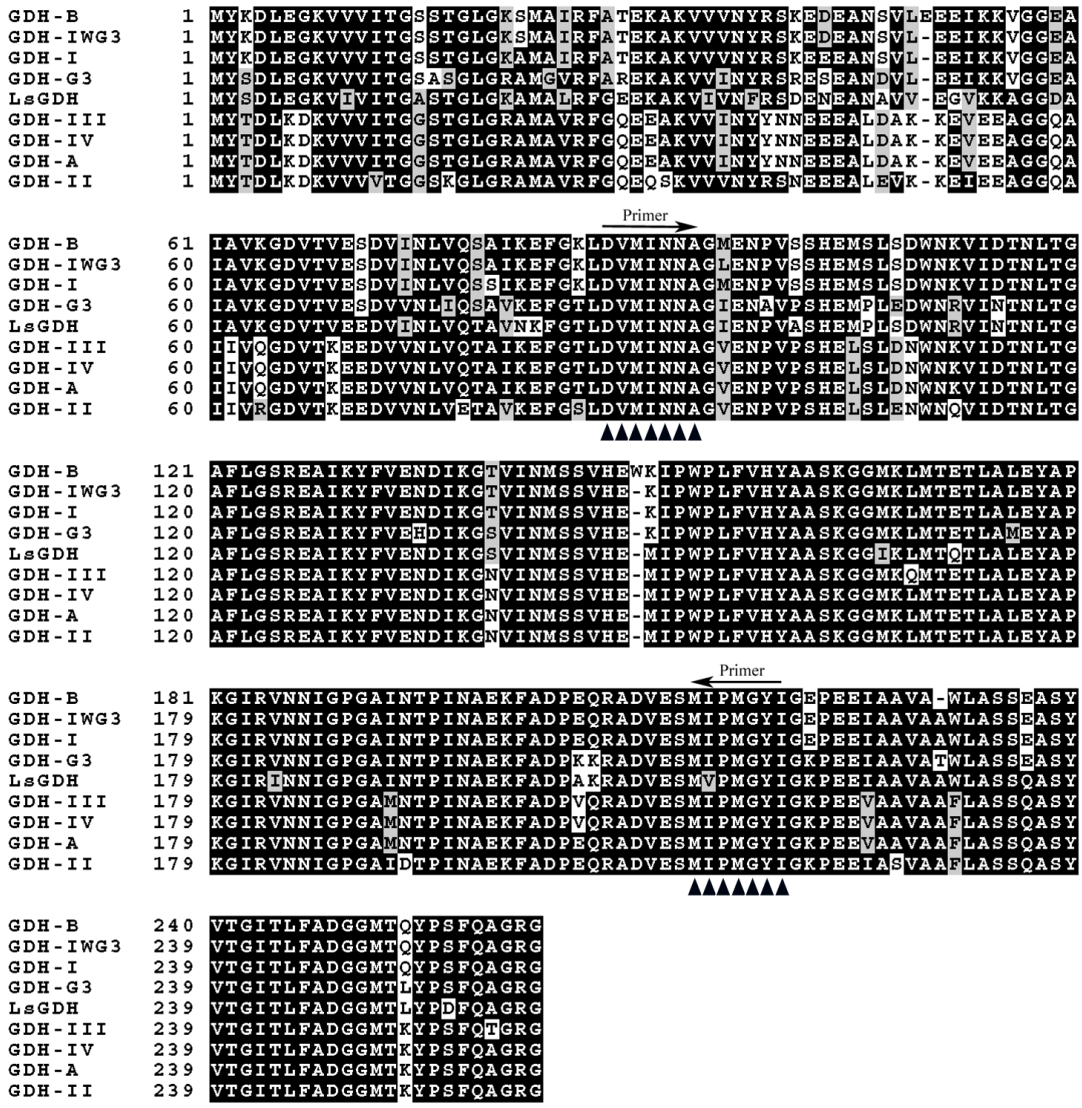


Figure 1. Sequence alignment of glucose dehydrogenases of *Bacillus* sp.G3 and other species of *Bacillus*. Sequences were aligned using Clustalx1.83. Identical residues and conserved substitutions are shaded black and gray by BOXSHADE 3.21 (K. Hofmann and M. Baron), respectively. GDH-I (Accession no. BAA14098.1), GDH-II (Accession no. BAA14100.1), GDH-III (Accession no. BAA01475.1), and GDH-IV (Accession no. BAA01476.1) were cloned from *Bacillus megaterium* IAM1030. GDH-Iwg3 (Accession no. 1RWB_A) was cloned from *Bacillus megaterium* Iwg3. GDH-G3 (Accession no.ACU78107) was cloned from *Bacillus* sp. G3.

possible that the presence of Leu-252 instead of Lys increased the heat resistance of GDH-G3, as described in previous reports (Makino et al., 1989b; Mitamura et al., 1989).

The pH-stability of GDH was examined by incubation at 25°C at various pH for 60 min and measurement of residual activity taken. The rGDH-G3 preserved its activity at the pH range between 4.0 and 9.0 (Figure 4B). Almost all of the GDHs from *Bacillus*, either wide type or mutant, were stable in the range of pH 6.0-7.5, particularly at 6.0 or 6.5, but the rGDH-G3 was more stability at pH 7.0. The GDH-G3 and GDH-Iwg3 are similar in the sequence (88% identity), and stable in the acidic range although GDH-Iwg3's stability was comparatively higher. The observed stability of the clones could be attributed to the effects of replacements as earlier reported by Mitamura et al. (1989).

Substrate specificity and enzyme kinetics

Table 2 illustrated that the rGDH-G3 possessed broad substrate specificity toward aldose sugars and disaccharides than others, particularly for D-galactose,

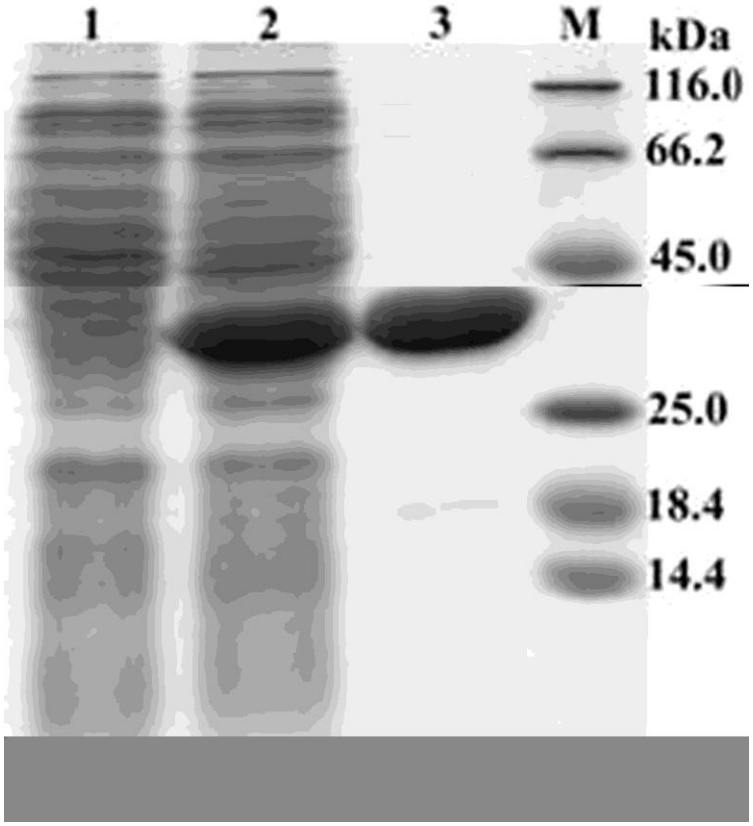


Figure 2. SDS-PAGE analysis of the rGDH-G3 from *E. coli* BL21 (DE3). **Lane 1,** Uninduced cellular extract; **Lane 2,** induced protein sample; **Lane 3,** the recombinant GDH (corresponds to 28 kDa) purified by Ni^{2+}-NTA; **Lane M,** standard protein molecular weight markers.

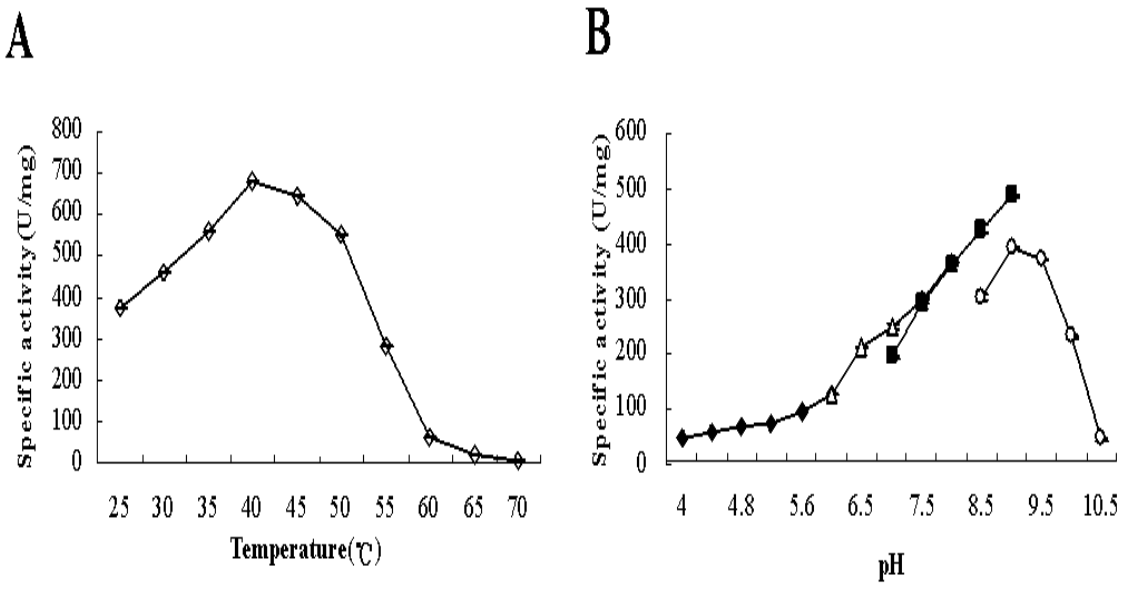


Figure 3. Effects of pH and temperature on enzyme activity. **A,** Effect of temperature on enzyme activity of the rGDH-G3 from 25-65°C in 100 mM sodium phosphate buffer (pH 8.0); **B,** effect of pH on enzyme activity of the rGDH-G3. pH range from 4.0-10.5 was used with the following buffers: ◆**,** 100 mM citrate buffer (pH 4.0–6.0); ▲**,** 100 mM sodium phosphate buffer(pH 6.0–8.0); ■**,** 100 mM Tris/HCl (pH 7.0–9.0); ○**,** 100 mM Gly/NaOH (pH 8.5–10.5).

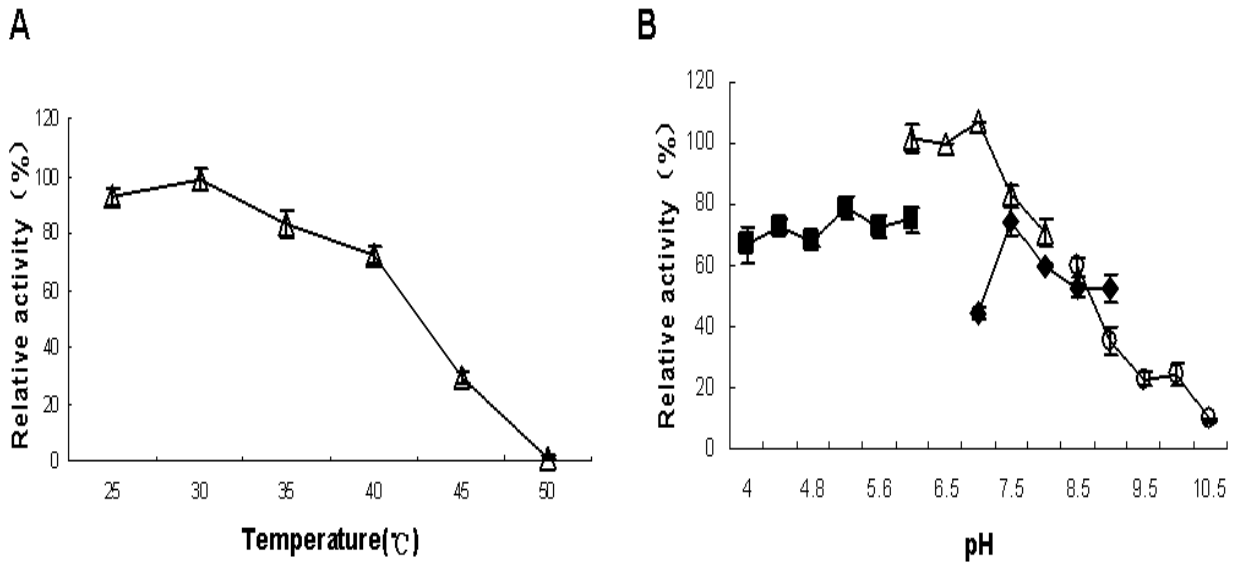


Figure 4. Effects of pH and temperature on enzyme stability. **A,** Thermostability of the rGDH-G3. After exposure for 60 min to the indicated temperature in sodium phosphate buffer (25 mM, pH 8.0). The activity of untreated rGDH-G3 was defined as 100% (319.8 U/mg protein); **B,** effect of pH on the stability of the rGDH-G3. The diluted enzyme pre-incubated in the different buffers (pH 4.0-10.5) at 25°C for 60 min. ■**,** 100 mM citrate buffer (pH 4.0–6.0); ▲**,** 100 mM sodium phosphate buffer(pH 6.0–8.0); ◆**,** 100 mM Tris/HCl (pH 7.0–9.0); ○**,** 100 mM Gly/NaOH (pH 8.5–10.5). The activity at pH 6.5 was defined as 100% (224.9 U/mg protein).

Table 2. Substrate specificity of rGDH-G3.

Substrate (0.2M)	Relative activity (%)[a]								
	GDH-G3	LsGDH	GDH-lwg3	GDH-I	GDH-II	GDH-IV	GDH-III	GDH-A	GDH-B
D-Glucose	100	100	100	100	100	100	100	100	100
D-Mannose	7.1	7.6	13	16	5.4	11	2.6	2	1
D-Galactose	22	17.3	3	5.8	1.8	3.8	0.9	0	0
D-Fructose	0.6	0.5	1.5	1.7	0.4	0.6	0.1	0	0
D-Arabinose	0.2	0	<0.1	<0.1	<0.1	<0.1	<0.1	ND[b]	ND
D-Xylose	6.4	22.5	9.9	12	3.5	7.1	1.8	ND	ND
Sucrose	6.3	6.3	ND	ND	ND	ND	ND	ND	ND
Lactose	2.6	4.2	ND	ND	ND	ND	ND	ND	ND
Maltose	13	22.4	ND	ND	ND	ND	ND	ND	ND
References	This study	(Ding et al., 2010)	(Mitamura et al., 1989)			(Nagao et al., 1992)		(Heilmann et al., 1988)	

[a]Initial rates were determined as described in Materials and Methods, except that D-glucose was replaced with various sugars. The activities were related to that of D-glucose (347 U/mg protein) as 100%. For comparison, the values of other GDHs were also given, and all used NAD^+ as cofactor.
[b] None detected.

the C4 epimer of glucose, which reached 22% of the activity with D-glucose. This property broadens the application fields of the enzyme. It catalyzed a range of saccharide substrates, but almost no activity was detected towards fructose and arabinose. As reported by Bonete et al. (1996), any configuration change of glucose at C2, C3 and C4 positions markedly decrease the enzymes affinity for the substrate.

In order to investigate the kinetic parameters of rGDH-G3, the initial rate of enzyme reaction was measured under different substrates concentrations. The results in Table 3 showed that the K_m, and K_{cat} values for NAD^+ were remarkably larger than that for $NADP^+$, whereas the K_{cat} / K_m values for $NADP^+$ was approximately fourfold higher than that for NAD^+. Due to the different affinity for NAD^+ and $NADP^+$, the catalytic efficiency of the rGDH-G3 was higher when $NADP^+$ was used as coenzyme, which was consistent with GDH-I, GDH-II and GDH-lwg3

Table 3. The kinetic analysis of the recombinant GDH.

	K_m (mM) [a]	V_{max}(μM s^{-1} mg^{-1})	K_{cat} (s^{-1}) [b]	K_{cat}/K_m (mM $^{-1}$ s^{-1})
D-glucose	31.8±0.4	0.82±0.05	23±1	0.73±0.06
NAD^+	0.210±0.001	5.131±0.005	144±0.1	687.5±3.6
$NADP^+$	0.0095±0.0018	1.20±0.02	33.8±0.6	3687±629

[a]The kinetic parameters were determined as described in Materials and Methods. The K_m of glucose was determined in the range of 10-200 mM, with a fixed NAD^+ 1 mM. The K_m for NAD^+ (0.05–1 mM) and $NADP^+$ (0.005–0.1 mM) was determined with a fixed glucose concentration of 200 mM.
[b] The values of K_{cat} were calculated for one subunit.

(Mitamura et al., 1989). However, the amino acid residues involved in the kinetic constants are still unknown. Therefore, further studies based on directed evolution, site-directed mutagenesis and crystallography is necessary to unravel the exact relationship between structure and function of GDH-G3.

ACKNOWLEDGEMENTS

This study was supported by the National Hi-Tech Research and Development Program (863) of China (No. 2007AA06Z329), National Natural Science Foundation of China (31070079), the Science and Technology Project of Zhejiang Province (2008C13014-3), and the International Cooperation Project in Science and Technology of Zhejiang Province (No. 2008C14038).

REFERENCES

Bonete MJ, Pire C, FI LL, Camacho ML (1996). Glucose dehydrogenase from the halophilic archaeon *Haloferax mediterranei*: enzyme purification, characterisation and N-terminal sequence. FEBS Lett., 383(3): 227-229.

Boontim N, Yoshimune K, Lumyong S, Moriguchi M (2004). Purification and characterization of D-glucose dehydrogenase from *Bacillus thuringiensis* M15. Ann Microbiol, 54(4): 481-492.

Bradford MM (1976). Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Ding HT, Du YQ, Liu DF, Li ZL, Chen XJ, Zhao YH (2010). Cloning and expression in E. coli of an organic solvent-tolerant and alkali-resistant glucose 1-dehydrogenase from Lysinibacillus sphaericus G10. Bioresour. Technol., 102(2): 1528-1536.

Du P, Wu P, Cai C (2008). A glucose biosensor based on electrocatalytic oxidation of NADPH at single-walled carbon nanotubes functionalized with poly(nile blue A). J. Electroanal. Chem., 624(1-2): 21-26.

Fujita Y, Ramaley R, Freese E (1977). Location and properties of glucose dehydrogenase in sporulating cells and spores of *Bacillus subtilis*. J. Bacteriol., 132(1): 282-293.

Heilmann HJ, Magert HJ, Gassen HG (1988). Identification and isolation of glucose dehydrogenase genes of *Bacillus megaterium* M1286 and their expression in *Escherichia coli*. FEBS J., 174(3): 485-490.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227(5259): 680-685.

Lin SS, Miyawaki O, Nakamura K (1999). Continuous production of L-carnitine with NADH regeneration by a nanofiltration membrane reactor with coimmobilized L-carnitine dehydrogenase and glucose dehydrogenase. J. Biosci. Bioeng., 87(3): 361-364.

Makino Y, Ding JY, Negoro S, Urabe I, Okada H (1989a). Purification and characterization of a new glucose dehydrogenase from vegetative cells of *Bacillus megaterium*. J. Ferment. Bioeng., 67(6): 374-379.

Makino Y, Negoro S, Urabe I, Okada H (1989b). Stability-increasing mutants of glucose dehydrogenase from *Bacillus megaterium* IWG3. J. Biol. Chem., 264(11): 6381-6385.

Mitamura T, Urabe I, Okada H (1989). Enzymatic properties of isozymes and variants of glucose dehydrogenase from *Bacillus megaterium*. FEBS J., 186(1-2): 389-393.

Nagao T, Mitamura T, Wang XH, Negoro S, Yomo T, Urabe I, Okada H (1992). Cloning, nucleotide sequences, and enzymatic properties of glucose dehydrogenase isozymes from *Bacillus megaterium* IAM1030. J. Bacteriol., 174(15): 5013-5020.

Nakatani Y, Nicholson WL, Neitzke KD, Setlow P, Freese E (1989). Sigma G RNA polymerase controls forespore-specific expression of the glucose dehydrogenase operon in *Bacillus subtilis*. Nucleic Acids Res., 17(3): 999-1017.

Ochman H, Gerber AS, Hartl DL (1988). Genetic applications of an inverse polymerase chain reaction. Genetics, 120(3): 621-623.

Okuda-Shimazaki J, Kakehi N, Yamazaki T, Tomiyama M, Sode K (2008). Biofuel cell system employing thermostable glucose dehydrogenase. Biotechnol. Lett., 30(10): 1753-1758.

Pauly HE, Pfleiderer G (1975). D-Glucose Dehydrogenase from Bacillus-Megaterium M-1286-Purification, Properties and Structure. H.S. Z. Physiol. Chem., 356(10): 1613-1623.

Sambrook J, Russell DW (2001). *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, New York, pp. 1.84-1.88, 1.116-1.119.

Shibuya H, Kaneko S, Hayashi K (2005). A single amino acid substitution enhances the catalytic activity of family 11 xylanase at alkaline pH. Biosci. Biotechnol. Biochem., 69(8): 1492-1497.

Varghese G, Diwan AM (1983). Simultaneous staining of proteins during polyacrylamide-gel electrophoresis in acidic gels by countermigration of coomassie Brilliant blue R-250. Anal. Biochem., 132: 481-483.

Vasantha N, Uratani B, Ramaley RF, Freese E (1983). Isolation of a developmental gene of Bacillus subtilis and its expression in Escherichia coli. Proc. Natl. Acad. Sci. U. S. A., 80(3): 785-789.

Wong CH, Drueckhammer DG (1985). Enzymatic synthesis of chiral hydroxy compounds using immobilized glucose dehydrogenase from *Bacillus cereus* for NAD(P)H regeneration. Bio-Technol, 3(7): 649-651.

African Journal of Microbiology Research Vol. 5(32), pp. 5889-5895, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.837

Full Length Research Paper

Investigation of bioremediation of arsenic by bacteria isolated from contaminated soil

Hadis Ghodsi[1], Mehran Hoodaji[1]*, Arezoo Tahmourespour[2] and Mohammad Mehdi Gheisari[3]

[1]Department of soil science, Khorasgan (Isfahan) branch, Islamic Azad University, Isfahan, Iran.
[2]Department of microbiology, Khorasgan(Isfahan) branch, Islamic Azad University, Isfahan, Iran.
[3]Department of basic science, Khorasgan (Isfahan) branch, Islamic Azad University, Isfahan, Iran.

Accepted 9 November, 2011

The aims of this study are isolating arsenite-resistant bacteria from arsenic contaminated soil and the investigation of arsenite bioremediation efficiency by the most resistant isolates. Isolation of arsenite-resistant bacteria and the minimum inhibitory concentration (MIC) were conducted by spread plate method and the agar dilution method on PHG-II agar plates supplemented with sodium arsenite respectively. The results showed that, 69 and 25% of arsenite resistant isolates were geram positive and negative bacilli, respectively. Its maximum MIC was 128 mM/L, which is related to such bacteria as *Bacillus macerans, Bacillus megaterimand Corynebacterium vitarumen.* There is a significant difference (P< 0.01) between three isolates in arsenite removal potential and arsenite bioaccumulation. The maximum percentage of arsenite removal potential (92%) and arsenite bioaccumulation (36%) were related to *B. macerans*. The removal efficiency of arsenite for *B. macerans, C. (vitaromen)* and *B. megaterim* were 60, 43 and 38% after 48 h of growth, respectively, while after 144 h of *Bacillus macerans, Corynebacterium (vitaromen)* growth and 120 h of *Bacillus megaterim*growth were 92, 80 and 73% respectively. The results also were shown the highest percentage of arsenite in biomass (36%), arsenate from oxidation (27%) were related to *B. macerans, B. megaterium* and *B. megaterium*. These results express the probability of finding more arsenic accumulating bacteria from the contaminated soil environment and can be concluded that arsenic resistant and/or accumulating bacteria, such as *Bacillus* sp., are widespread in the polluted soils and are valuable candidates for bioremediation of arsenic contaminated ecosystems.

Key words: Arsenite, bacteria, bioremediation, MIC.

INTRODUCTION

Human activities over the centuries has contaminated many areas of developing and developed countries (Evangelou et al., 2007). Soil contamination with heavy metals is one of the great problems of modern societies. Heavy metals periodically increase in the environment due to industrial activities and technology development. Increasing of these pollutants in the environment is considered as a serious threat to human and environmental health (Banaa Araghi et al., 2010). Unlike many organic contaminants disintegrated in the soil, heavy metals are kept in the soil storage and according to their nature a group of pollutants are of most interest because of their danger and of course plenty of stability in most environments (Garbisu and Alkorta, 2001). Arsenic has long been an important environmental pollutant and in long term has been as a health risk to humans and other living organisms. In the past, arsenic compounds have been widely used in pesticides, herbicides and soil disinfectors, thus in some soils was in high concentrations (Pais and Jons, 1997). Arsenic is highly toxic metal element that annually threatens the health of millions of people in the world (Chen and Shao, 2009). Inorganic arsenic forms are more dangerous than other forms for human health and in terms of classification fall in cancer-causing ingredient (Andrews,

*Corresponding author. E.mail: m_hoodaji@khuisf.ac.ir. Tel/ Fax: 00983115354045.

2001). In recent decades following increasing environmental pollution by heavy metals, scientists attracted to biological purification methods. In most cases of cleaning the contaminated ecosystems with chemical methods involves heavy costs and irreparable damages (Brooks, 1995; Nwuche and ugoji, 2008). Therefore one appropriate method is using biological method. Generally population and microbial activities in soil and water contaminated with the presence of metal will be reduced and modified (Kelly et al., 1998). On the other hand resistant microorganisms have evolved mechanisms to tolerate the toxicity of heavy metals. Application of microorganisms for heavy metals remediation is considered as a natural, stable and economical solution. Previous researches have described the isolation and characterization of arsenic resistant bacteria from different environments and have indicated that these bacteria are able to grow chemolithotrophically with oxygen as an electron acceptor and As(III) as an electron donor (Duquesne et al., 2008; Santini et al., 2000). Arsenic-resistant bacteria play an important role in controlling the speciation and cycling of arsenic in the ecosystems (Inskeep et al., 2007). The aims of this study are isolating arsenite-resistant bacteria from arsenic contaminated soil and the investigation of arsenite bioremediation efficiency by high resistant isolates.

MATERIALS AND METHODS

Sampling

Soil samples were collected from the three different points of soil surface (0-20 cm) of the Research Farm (in Lavark, Najaf Abad) located in southwest of Isfahan, Iran. This soil was contaminated previously with arsenic through using of urban sewage sludge. The samples were mixed, transferred to the laboratory, passed through 2 mm sieve and used for physical-chemical and microbial analysis (Nwuche and ugoji, 2008).

Arsenic measurement

In this study to measure arsenic the spectrophotometry method was used along with a reagent called Leuco malachite green (LMG). In this method arsenic reacts with Potassium iodate (KIO_3) in the acidic environment and iodine will be released. Released iodine oxidizes LMG to MG and changes the color to the color of malachite green. Detection range of arsenic concentration in this method is 0.09-0.9 micro g/ml. The MG dye shows maximum absorption at 617 nm (Revanasiddappa et al., 2007).

Arsenite measurement (As III)

Initially for the preparation of arsenite stock solution (1,000 µg ml), amount of 0.1734g $NaAsO_2$ (sodium arsenite) resolved in 100 ml deionized distilled water. The standard solutions including 0.9-9.0 µg of arsenite removed and poured in 10 ml volumetric balloons. Then 1 ml of Potassium iodate 1%, 0.5 ml of 1 M hydrochloric acid were added and the reaction mixture was shaked for 2 min. Then 0.5 ml of 0.05 LMG was added with shaking. Finally 2 ml acetat buffer (pH =4.5) was added and heated (40°C) in a water bath for 5 min, cooled and diluted with distilled water. After 5 min, absorbance of the dye was measured at 617 nm against the reagent blank. The concentration of arsenic (III) content was established by reference to the calibration graph (Revanasiddappa et al., 2007).

Arsenate measurement (As V)

After filtering of this sample a certain volume will be removed, then 0.5 ml of 5% KI and 5 M HCL were added to the samples. All of the available arsenate were reduced to arsenite. In order to remove yellow to brown color which is due to the high amount of released iodine, the droplets of ascorbic acid were added (Pillai et al., 2000) then the amount of total arsenic in samples were measured by the method of arsenite measurment.

Soil arsenic measurement

One gram of soil sample was placed in the nickel plate that already covered its bottom with NaOH. Heat the Nickel plate in order to NaOH be fully melted and mixed with soil (alkaline digestion). After cooling, the nickel plate was immersed in HCL (0.5 N) and waited in order to be digested slowly (Almond, 1953) then the amount of arsenite was measured.

Isolation of arsenite-resistant bacteria

One gram of each soil samples was used to provide series. 0.1 ml of each dilution was added to each of PHG-II agar plates (4 g pepton, 1 g yeast extract and 2 g glucose and 15 g agar per liter) supplemented with 0.5 mM sodium arsenite (pH=7) by spread-plate method. The plates were incubated at 30°C for 3-5 days. This experiment was conducted in three replicates. After isolation of resistant colonies, their enrichment, purification and identification were done by the help of Gram Staining and biochemical tests (catalase- Licetinase- citrate – MR- VP -manitol fermentation and acid production - fermentation of glucose, sucrose and galactose - nitrate reduction test, urease and Esculine Hydrolysis) (Sneat et al., 1989; Cappuccino and Sherman, 1996).

Minimum inhibitory concentration (MIC) determination

The (MIC) of arsenite at which no colony growth occurred was determined by the agar dilution method. PHG-II agar plates supplemented with different concentration of arsenite the level of resistance (0.5, 1, 2, 4, ... and 192 mM /L) were inoculatedas aseptically with a culture of bacterial isolates in exponential growth phase. The plates were incubated for 48 h at 35°C. Minimum concentration of arsenite allowing growth of the isolates was an indication of positive tolerance (Hassen et al., 1998).

Growth curve and arsenite removal by bacterial strains

The growth curve of the most resistant bacteria at sub MIC concentration of arsenite were monitored by measuring the optical density (OD) of the cultures at 600 nm using a spectrophotometer. At each intervales a certain volum of medium was removed, after measuring OD at 600 nm. It was centrifuged and filtered. Than, the arsenite and arsenate concentration were measured by the above mentioned method (Chen and Shao, 2008; Revanasiddappa et al., 2007; Pillai et al., 2000). At the end of growth phase arsenite concentration in bacterial cell structure was measured by the method of Takeuchi et al., 2007. Briefly, the culture media were shake at 100 rpm, centrifuged at 5000 ×g at 4°C for 20 min. The

Table 1. Soil chemical and physical properties.

Depth (cm)	Texture	Clay	Silt	Sand	pH	EC	P_{ava}	K_{ava}	As_{Total}	As^{5+}	As^{3+}
		(%)				($dS.m^{-1}$)	($mg.kg^{-1}$)				
0-20	SiCL	35.83	48.83	15.34	7.63	2.6	10	203	60.2	40.3	19.7

Table 2. The bichemical tests and MIC of the greatest arsenite resistant bacteria.

Bacteria test	Strain 4	Strain 8	Strain 10
Spore	+	-	+
Catalase	+	+	+
Manitol	+	ND	+
VP	-	-	-
MR	+	+	+
Nitrate	+	+	+
Citrate	-	-	+
Glucose	+	+	+
Sucrose	ND	+	+
Urea	ND	+	ND
Probable general and species	*B. macerans*	*C. (vitaromen)*	*B. (megaterim)*
MIC	128	128	128

pelletes were washed twice with distilled water and placed in an oven with 100°C temperature for drying. The dried sampels were weighted and digested with nitric asid. Then the arsenite concentration was measured at 617 nm by the spectrophotometric method.

Statistical analysis

Statistical analysis was conducted using the SPSS System software. For comparison of means the Duncan test was used at the 5% probability level.

RESULTS

Soil physical and chemical properties

The physical and chemical properties of soil is presented in Table 1.

Resistance to arsenite

The results showed that, 69 and 25% of arsenite resistant isolates were geram positive and negative bacilli, respectively and 6% of them were gram positive cocci.

According to MIC determination results, the greatest resistance to arsenite has been related to gram positive bacilli. Its maximum MIC and MBC were 128 and 192 mM/L respectively, which is related to such bacteria as *B. macerans, B. megaterim* and *C. vitarumen* (Table 2).

Growth curve and arsenite removal by bacterial strains

In Figure 1 growth curves of *B. macerans, C. vitaromen, B. megaterium* were shown in 128 mM arsenite.

The removal efficiency of arsenite for *Bacillus macerans, Corynebacterium (vitaromen)* and *Bacillus megaterim*were 60, 43 and 38% after 48 h of growth, respectively. While after 144 h of *Bacillus macerans, Corynebacterium (vitaromen)* growth and 120 h of *Bacillus megaterim* growth, the removal efficiency of arsenite were 92, 80 and 73% respectively (Figures 2, 3 and 4).

Finally, the percentage of arsenite in bacterial mass (bioaccumolation), arsenate from oxidation and remained arsenite were determined. The results were shown in Figure 5. The highest percentage of arsenite in biomass (36%), arsenate from oxidation (27%) were related to *B. macerans, B. megaterium* and *B. megaterium*.

There is a significant difference ($P< 0.01$) between three isolates in arsenite removal potential and arsenite bioaccumulation (Figure 6). The maximum percentage of arsenite removal potential (92%) and arsenite bioaccumulation (36%) were related to *B. macerans*.

DISCUSSION

The first step in the identification of bacteria with the ability of bioremediation is isolation of resistant bacteria

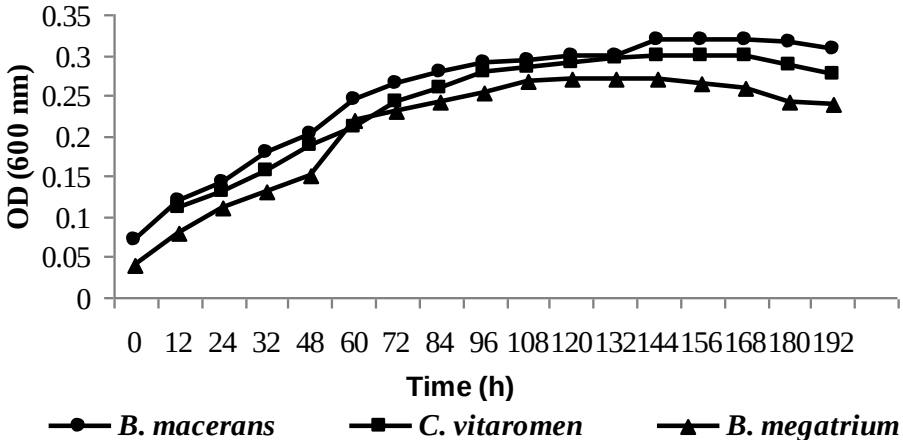


Figure 1. Bacterial growth curves of *Bacillus macerans, Corynebacterium vitaromen and Bacillus megaterium* in 128 mM of arsenite.

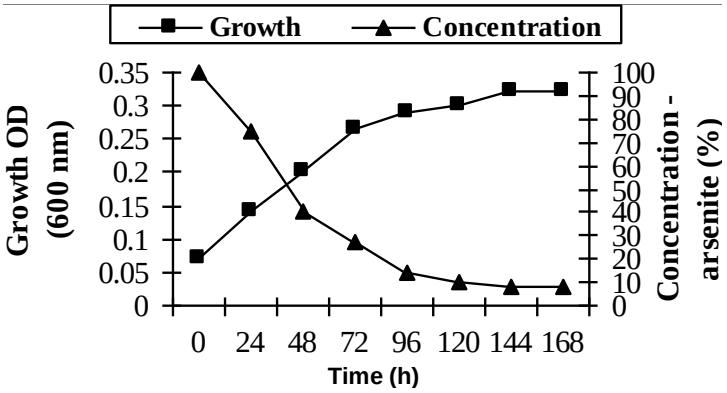


Figure 2. Growth and arsenite removal curvs of *Bacillus macerans.*

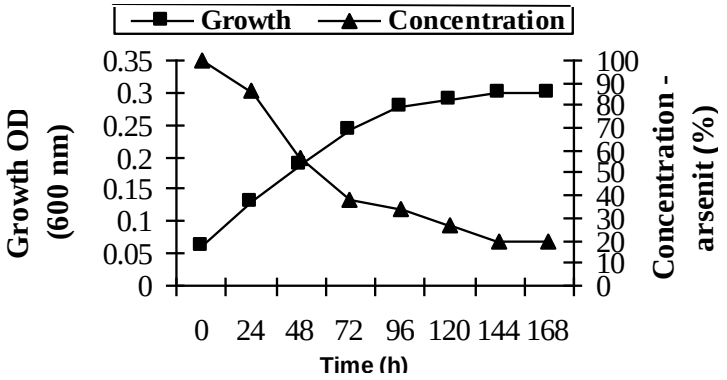


Figure 3. Growth and arsenite removal curvs of *Corynebacterium vitaromen.*

which tolerate high concentrations of heavy metals (Trevors et al., 1985). Most arsenic resistant bacteria are separated from arsenic-rich environments. In natural environments, the number of arsenite resistant bacteria is less than arsenate resistant bacteria. Arsenate is more toxic than arsenite (Jackson et al., 2005). Among the isolated resistant strains from contaminated soil , three strains demonstrated dramatic resistance to arsenite 128

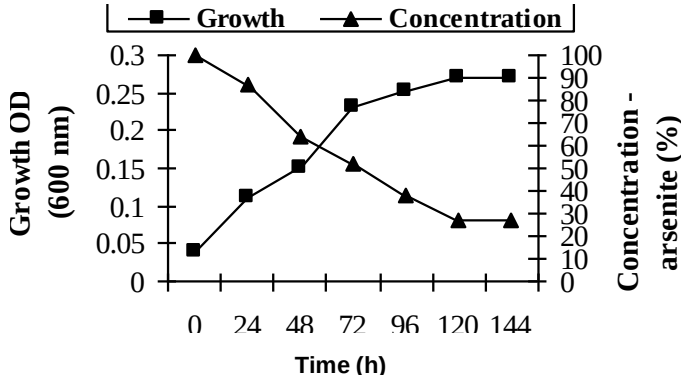


Figure 4. Growth and arsenite removal curvs of *Bacillus megaterium.*

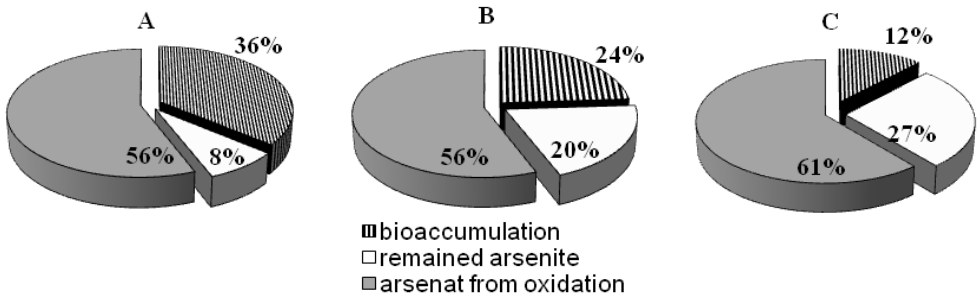


Figure 5. Arsenite in biomass (bioaccumulation), arsenate from oxidation and remaind arsenite percentage of **A:** *Bacillus macerans*, **B:** *Corynebacterium vitaromen* and **C:** *Bacillus megaterium.*

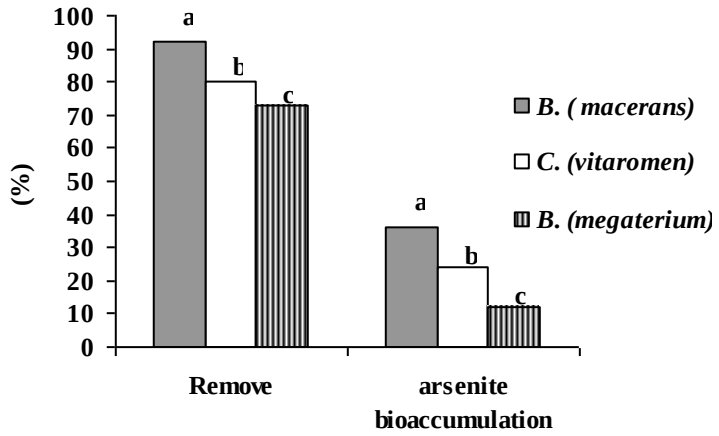


Figure 6. The comparision of arsenite removal potential in 3 isolates.

Mm. These arsenite-resistant strains were probably *B. macerans*, *C. vitaromen* and *B. megaterium.* Concentrations of metals used in this study are also used in the similar studies for bacteria that their medium contains extracted yeast. Abu-shnab et al. (2003) showed that in a contaminated soil the 11.1% of isolated bacteria were resistant to As with the MIC of 20 mM/L. High levels of soil metal concentration can lead to achieving such a high MIC in resistant strains. Also Chitpirom et al. (2009), in Thailand, isolated arsenic-resistant bacteria from tannery effluent and agricultural soils that were belonged to *Klebsiella*, *Pseudomonas*, *Comamonas* and

Enterobacter genera with the MIC of 40 mM (arsenite) and 400 mM (arsenate). Pepi et al. (2007) isolated 3 arsenic resistant genera (*Aeromonas*, *Bacillus* and *Pseudomonas*) from contaminated sediments with the MIC of 16.66 mM (arsenite) and 133.47 mM (arsenate). They also concluded that these bacteria are suitable for arsenic bioremediation in contaminated sediments. In a study by Luis et al. (2006) in Spain with the aim of biological removing of arsenic, *Corynebacterium glutamicum* with over 60 mM arsenite resistance identified as one of the most tolerant species to arsenic. This results are in agreement with our findings but our isolates could tolerate the higher concentration of arsenite that was related to high level of arsenite in soil. In the study after 144 h of *B. macerans*, *C. vitaromen* growth and 120 h of *Bacillus (megaterium)* growth, the removal efficiency of arsenite were 92, 80 and 73% respectively. The highest percentage of arsenite in biomass (36%), arsenate from oxidation (27%) were related to *B. macerans, B. megaterium* and *B. megaterium.* Among resistant isolates, *B. macerans* was able to remove 92% of arsenite in the medium and also store 36% of it in the cell mass which is introduced as superior strain in this regard. Studies by Mondal et al. (2008) on three strains of *Ralstonia eutropha*, *Pseudomonas putida* and *Bacillus indicus* showed that these strains were able to remove (67, 60 and 61% respectively) arsenic from wastewater.

Takeuchi et al. (2007) could isolate a non- genetically engineered potent arsenic accumulating bacterium, *Marinomonas communis*, from marine and non marine environment in Japan which accumulated 2290 µg Asg dw^{-1} of arsenic in presence of 5 mg As/ l of arsenat (45.8%). Our results are in agree with Takeuchi et al. (2007) and although details of such mechanisms are not yet clear, accumulation of arsenic into the cell would be a result of higher uptake and lower efflux. The high effective concentration of As in this study and previous study (Takeuchi et al., 2007) could be related to the presence of arsenic resistance systems such as regulatory protein of the *ars* operon that has a specific binding site available for arsenite. However, presence of *ars* operon in bacteria is known to extrude arsenate from the cell by an efflux system. Consequently, arsenic is not accumulated in bacteria. Furthermore, the other known arsenic-resistant system, the phosphate-specific transport (Pst) system, would also lead to lower uptake of arsenat by the cell. Therefore, the present results obtained in our isolates and previousley isolated *M. communis* with its higher resistance and higher accumulation of arsenic contradict the known arsenic-resistant systems, suggesting existence of an as yet unknown arsenic resistance system for these strains. Cai et al. (1998) also could isolate *Pseudomonas* strains without the *ars* operon with a yet unknown arsenic resistance system.

These results express the probability of finding more arsenic accumulation bacteria from the contaminated soil environment. It can be concluded that arsenic resistant and/or accumulating bacteria are widespread in the polluted soil environment,and that arsenic-accumulating bacteria such as *Bacillus* sp*.* are valuable candidates for arsenic contaminated ecosystems bioremediation.

REFERENCES

Abu-Shanab RAI, Delorme TA, Angle JS, Chaney RL, Ghanem K, Moawad H, Ghozlan HA (2003). Phenotypic characterization of microbes in rhizosphere of Alyssum murale. Int J Phyto 5: 367-380.

Almond H (1953). Field method for determination of trace of arsenic in soils. J. Anal. Chem., 25: 1766-1767.

Andrews JM (2001). Determination of minimum inhibitory Concentration. J. Antimicrob. Chemother., 48:5-16.

Banaa AN, Hoodaji M, Afyuni M (2010). Use of EDTA and EDDS for enhanced zeamays' phytoextraction of heavy metals from a contaminated soil. J. Residual. Sci. Tech., 7(3): 139- 145.

Brookes PC (1995). The use of microbial parameters in monitoring soil pollution by heavy metals. J. Biol. Fertile Soils 19:269-279.

Cai J, Salmon K, Du Bow MS (1998). A chromosomal ars opran homologue of *Pseudomonas aeruginosa* confers increased resistance to arsenic ana antimony in Escherichia coli. J. Microbiol., 144:2705-2713.

Cappuccino J, Sherman N (1996). Microbiology (a laboratory manual) . Benjamin/ Cumming publishing company INC.

Chen Sh, Shao Z (2009). Isolation and diversity analysis of arsenite-resistant bacteria in communities enriched from deep- sea sediments of the South west Indian Ocean Ridge. J. Extremophiles 13:39-48.

Chitpirom K, Akaracharanya A, Tanasupawat S, Leepipatpibooim N, Woong Kim K (2009). Isolation and characterization of arsenic resistant bacteria from tannery wastes and agricultural soils in Thailand. J. Ecol. Environ. Microbiol., 59(4): 649-656.

Duquesne K, Lieutaud A, Ratouchniak J, Muller D, Lett M-C, Bonnefy V (2008). Arsenite oxidation by a chemoautotrophic moderately acidophilic Thiomonas sp.: from the strain isolation to the gene study. Environ. Microbiol., 10: 228–237.

Evangelou MWH, Ebel M, Schaeffer A (2007). Chelate assisted phytoextraction of heavy metals from soil. Effect, mechanism, toxicity and fate of chelating agents. Chemosphere 68: 989-1004.

Garbisu C, Alkorta I (2001). Phytoextraction: a cost- effective plant-based technology for the removal of metals from the environment. J. biores Technol., 77: 229-236.

Hassen A, Saidi N, Cherif M and Boudabous A (1998). Resistance of environmental bacteria to heavy metals. Biores Technol., 64: 7-15.

Inskeep WP, Maser RE, Hamamura N,Warelow TP,Ward SA, Santini JM (2007). Detection, diversity and expression of aerobic bacterial arsenite oxidase genes. Environ. Microbiol., 9:934–943.

Jackson CR, Dugas SL, Harrison KG (2005). Enumeration and characterization of arsenat-resistant bacteria in arsenic free soils. J. Soil Biol. Biochem., 37(12): 2319-2322.

Kelly JJ, Tate RL. (1998). Effects of heavy metals contamination and remediation on soil microbial communities in the vicinity of a zinc smelter. J. Environ. Qual., 27. pp: 1458-1467.

Luis M, Ordonez E, Letek M, Gil J (2006). *Corynebacterium glutamicum* as a model bacterium for bioremediation of arsenic. Int. J. Microbiol., 9: 207-215.

Mondal P, Majumder CB, Mohanty B (2008). Growth of three bacteria in arsenic solution and their application for arsenic removal from wastewater. J. Basic Microbiol., 48(6): 521-525.

Nwuche CO, Ugoji EO (2008). Effects of heavy metal pollution on the soil microbialactivity. J. Environ. Sci. Technol., 5:409-4140.

Pais IJ, Benton Jons JR (1997). The hand book of trace elements. Publishing by: St. Luice press Boca Rrton Florida.

Pepi M, Volterrani M, Renzi M, Marvasi M, Gasperini S, Franchi E, Focardi SE (2007). Arsenic-resistant bacteria isolated from contaminated sediments of the Orbetello Lagoon, Italy, and their characterization. J. Appl. Microbiol., 103(6):2299-308.

Pillai A, Sunitha G, Gupta VK (2000). A new system for the spectrophotometric determination of arsenic in environmental and biological semples. J. Anal. Chem. Acta 408: 111-115.
Revanasiddappa HD, Dayananda BP, Kumar TNK (2007). A sensitive spectrophotometric method for the determination of arsenic in environmental sampeles. J. Environ. Chem. let., 5: 151-155.
Santini JM, Sly LI, Schnagl RD, Macy JM (2000). A new chemolithotrophic arsenite-oxidizing bacterium isolated from a gold mine:phylogenetic, physiological and preliminary biochemical studies. Appl. Environ. Microbiol., 66:92–97.
Sneat PH, Mair ANS, Sharp ME, Holt JG (1989). Bergeys manual of systematic bacteriology. Vol (2). Williams and Wilkins.
Takeuchi M, kawahata H, Prasad Gupta L, Kita N, Morishita Y, Ono Y, Komai T (2007). Arsenic resistance and removal by marine and non-marine bacteria. J Biotechnol 127: 434-442.
Trevors JT, Oddie KM, Belliveau BH (1985). Metal resistance in bacteria. FEMS Microbiol. Lett., 32:39-54.

African Journal of Microbiology Research Vol. 5(32), pp. 5896-5901, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.841

Full Length Research Paper

Effects of temperature on recruitment and phytoplankton community composition

Xiao Tan

College of Environment, Hohai University, Nanjing 210098, China. E-mail: biotan@163.com. Tel: +8613057610908.

Accepted 11 November, 2011

Effects of temperature on phytoplankton recruitment and variations in phytoplankton community were studied by using hiemal sediment from Taihu Lake and performing a simulation experiment. Sediment samples were cultured in filtered lake water with elevated temperatures. Recruitment patterns and photosynthetic capacity of cyanobacteria, chlorophytes and diatoms were recorded, respectively. Results showed that recruitment of chlorophytes and diatoms was observed above 9°C, but recruitment of cyanobacteria was not evidently detected until 12.5°C. Chlorophytes dominated the phytoplankton community at 12.5 and 16°C, subsequently cyanobacteria established dominance above 19.5°C. In this study, algal cells remained weak photochemical vitality at lower temperatures before recruitment, which reactivated and increased gradually with elevated temperatures.

Key words: Recruitment, temperature threshold, cyanobacteria, blooms, Taihu Lake, phytoplankton community.

INTRODUCTION

In winter, some species of phytoplankton are capable of dormancy on lake sediment after autumnal sedimentation (Tsujimura et al., 2000; Brunberg and Blomqvist, 2002). These benthic portions are able to renew growth and return to the pelagic phase with increased temperature in spring (Ståhl-Delbanco and Hansson, 2002; Karlsson-Elfgren and Brunberg, 2004; Verspagen et al., 2005). Especially, in some eutrophic lakes, recruitment is a key process in cyanobacteria life cycle and blooms formation (Oliver and Ganf, 2000; Kong and Gao, 2005). Various versions of migration traps had been designed to study cyanobacteria recruitment in lakes (Hansson et al., 1994; Brunberg and Blomqvist, 2003; Cao et al., 2005). In labs, some simulation experiments were performed to investigate influences of environmental factors on cyanobacteria recruitment (Ståhl-Delbanco and Hansson, 2002; Li et al., 2004; Tao et al., 2005). According to these field studies and simulation experiment, temperature had been confirmed to play an important role in driving cyanobacteria recruitment (Latour et al., 2004a; Li et al., 2004; Tao et al., 2005). However, previous studies mainly focused on the recruitment of some species of cyanobacteria and did not adequately analyze the variations in phytoplankton community composition synchronously. Furthermore, cyanobacteria undergo a series of biomass accumulation and population competitive processes with other algae from recruitment to dominance establishment before blooms formation (Cao et al., 2005).

These processes remained to be further understood. Variable chlorophyll *a* fluorescence yield has become an important tool for studying phytoplankton photosynthesis (Schreiber, 1994; Oliver and Whittington, 1998), because it is sensitive to photon flux density and is reliable as a parameter to offer insight into the immediate past light history of phytoplankton (Zhang et al., 2008). The ratio of maximum variable fluorescence to the maximum yield (F_v/F_m) has been used to estimate changes in the proportion of functional reaction centers and as an indicator of the photosynthetic capacity of phytoplankton (Falkowski and Kolber, 1995). Moreover, PHYTO-PAM fluorometer allows a separate measurement of the fluorescence signal of each algal group in mixed phytoplankton populations. Accordingly, effects of temperature on photosynthetic capacity of different algal groups could be detected synchronously, which is an important physiological index to analyze recruitment and competitive processes (Latour et al., 2004a). In this

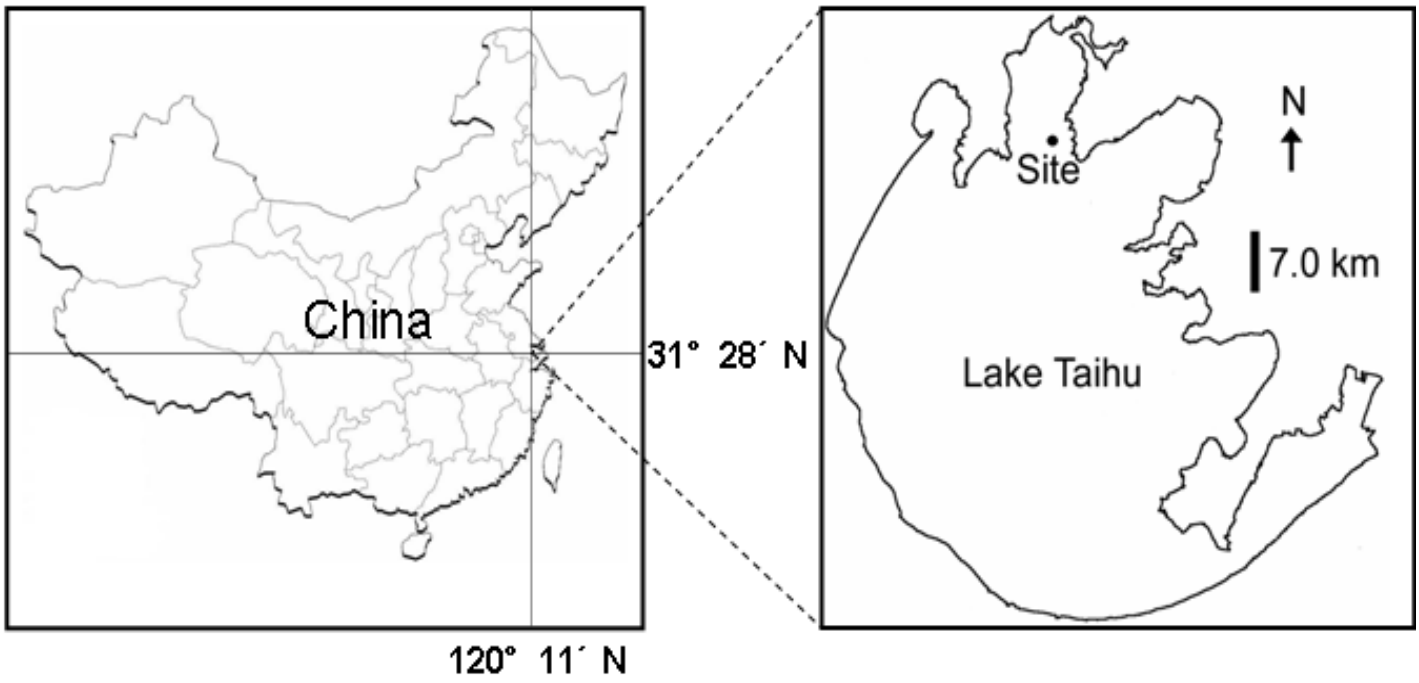


Figure 1. Location of the sampling site.

paper, a simulation experiment was performed by using hiemal sediment samples from Taihu Lake, which were static cultured in light incubator, so as to study the temperature threshold for algae recruitment and effects of temperature changes on algal community composition. Additionally, variations of algal fluorescence were also analyzed to investigate responses of different algal groups to temperature changes.

MATERIALS AND METHODS

Lake and sampling site description

Taihu Lake is a large eutrophic lake in China (with an area of 2, 338 km^2 and the annual average water depth of 1.9 m and maximum of 2.6 m) (Hu et al., 2006). Major cyanobacteria blooms composed of *Microcystis* spp. had appeared annually for decades in this lake (Chen et al., 2003). Meiliang Bay lies in the northern part of Taihu Lake, where serious blooms frequently occurred in summer (Chen et al., 2003; Tan et al., 2009). In the present study, the sampling site (31°28´46´´N, 120°11´34´´E) is located between Meiliang Bay and offshore regions (Figure 1).

Sample collection and treatment

On January 9, 2008, about 300 g of surface sediment (0 to 3 cm) was collected at the sampling site by a columnar sampler (KC-Denmark). During the sampling period, underwater environmental parameters (such as depth, water temperature, density of cyanobacteria cells and chlorophyll *a* concentration) were real-time recorded by using a multi-parameter water quality sonde (YSI 6600V2, USA). Sediment samples were transferred to laboratory immediately and were divided into three equal aliquots approximately. Each portion was laid on the bottom of a beaker. Subsequently, sterilized *in situ* lake water (5 L of 0.22 μm filtrate by Whatman GF/C membrane) were gently added. The three beakers were then incubated under an illumination intensity of 40 μmol photons m^{-2} s^{-1}, provided by cool white fluorescent lamps (36W FSL, China), with a light-dark period of 12/12 h . The incubation temperature increased along eight levels (5.5, 9, 12.5, 16, 19.5, 23, 26.5 and 30°C), with each temperature being maintained for three days.

Microscopic analysis of phytoplankton

At the beginning and the end of each temperature level, 110 mL of the culture liquid were obtained gently from each beaker by a plastic tube (length=20 cm, diameter=2 cm). 10 ml of the culture liquid were used for algal fluorescence analysis immediately; the residual 100 ml were fixed with Lugol's iodine and sedimented for 48 h prior to microscopic analysis. Phytoplankton cell densities were enumerated by using a haemocytometer. And then, their specific growth rates (SGRs) were calculated according to the following equation:

$$SGR = \ln(C_t/C_0)/t$$

Where C_0 is the initial cell density at the beginning of each temperature level, C_t is the cell density at the end and t is the duration of incubation period under each temperature level in days. Moreover, unicells, dividing cells (two connected cells), and colonies were enumerated. Colonies were grouped into consecutive groups: small colonies (cell number per colony between 2 and with a maximum of 10 cells), middle colonies (cell number per colony was up to 10 and maximum of 100 cells), and large colonies (cell number per colony with more than 100).

Table 1. Mean and ranges of real-time recorded environmental parameters at the sampling site.

Environmental parameter	Mean and ranges
Depth (m)	1.95 (1.93 to 1.97)
Water temperature (°C)	5.2 (5.1 to 5.3)
Chlorophyll *a* (μg L^{-1})	0.09 (0.07 to 0.11)
Cyanobacteria cell density (cells mL^{-1})	129 (118 to 139)

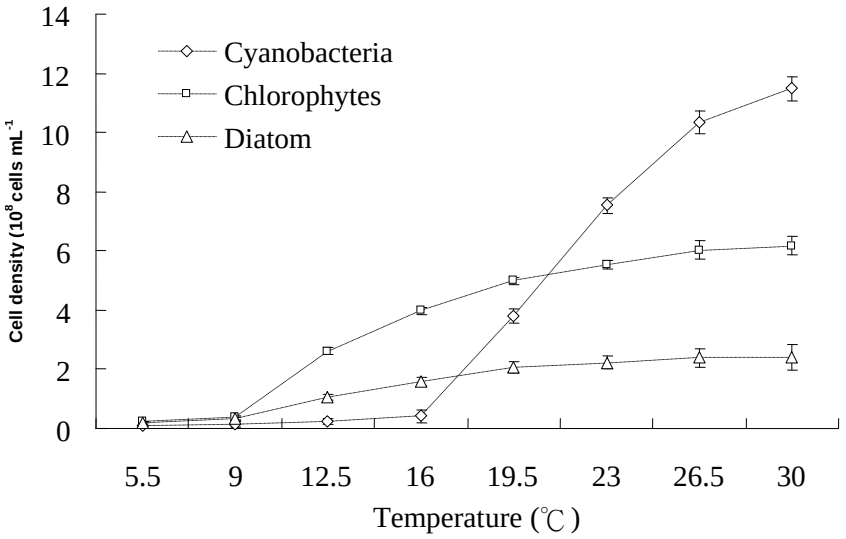


Figure 2. Phytoplankton dynamics during the experiment.

PHYTO-PAM fluorometer analysis

Algal fluorescence was measured by using a multiwavelength phytoplankton pulse-amplitude-modulated fluorometer (Phyto-PAM Walz, Germany) after dark adaption for 15 min. The Phyto-PAM fluorometer equipped with a special emitter-detector unit (Phyto-ED) for distinguishing cyanobacteria, chlorophytes and diatoms/dinoflagellates by means of four excitation wavelengths (665, 645, 520 and 470 nm). For instance, in chorophytes chlorophyll fluorescence is much more effectively excited by blue and red light (470, 645 and 665 nm) than by green light (520 nm). In the case of cyanobacteria, almost no chlorophyll fluorescence is excited by blue light (470 nm), while excitation at 645 nm is particular strong due to phycocyanin and allophycocyanin absorption. As for diatoms and dinoflagellates, excitation by blue (470 nm) and green (520 nm) is relatively high resulted from strong absorption by fucoxanthin, chlorophyll *c* and carotenoids. The fluorescence signals from the four wavelengths excitation were assigned to the three algal groups by using the Phyto-WIN software (version 1.47) and the reference spectra (Zhang et al., 2008). The maximal efficiency of photosystem II photochemistry was determined as F_v/F_m, which was used for an indicator of the photosynthetic capacity of phytoplankton (Falkowski and Kolber, 1995). F_v/F_m was calculated by the following equation:

$$F_v/F_m = (F_m - F_0)/F_m$$

Where F_0 is the fluorescence of dark-adapted algal cells stimulated by a weak probe light immediately after 15 min of darkness and F_m is the maximum fluorescence signal after the closure of all reaction centers by 600 ms pulse of saturating irradiance (Schreiber et al., 2002).

RESULTS

Environmental parameters at the sampling site

Sediment samples were collected in a sunny and windless day. Environmental parameters at the sampling site are displayed in Table 1. During the sampling period, water depth of the sampling site fluctuated between 1.93 and 1.97 m. This range was near to the average depth of Taihu Lake. Water temperature was about 5.2°C, which was very close to the initial culture temperature (5.5°C) and was easier for hiemal algae to accommodate. At the sampling site, chlorophyll *a* concentration and the density of cyanobacteria cells were fairly low in water.

Phytoplankton dynamics

According to the algae growth curves (Figure 2) and the calculated SGRs, recruitment of chlorophytes and diatoms was observed at 9°C, but the recruitment of

cyanobacteria was not evidently detected until 12.5°C. SGR of cyanobacteria peaked at 19.5°C (about 0.256 D^{-1}). As for chlorophytes and diatoms, the maximum of SGRs simultaneously appeared at 12.5°C, reaching to 0.231 D^{-1} and 0.175 D^{-1}, respectively. Particularly, *Microcystis* spp. (such as *Microcystis aeruginosa*, *Microcystis wesenbergii*, and *Microcystis flos-aquae*) constituted the dominant species of cyanobacteria. After recruitment, pelagic *Microcystis* colonies experienced an enlargement process, but unicells and dividing cells occupied the highest proportion all along (Figure 3).

Variations in phytoplankton community composition

Phytoplankton community structure at different temperatures was displayed in Figure 4. At the lower temperatures (5.5 and 9°C), cyanobacteria, chlorophytes, diatoms and some species of euglenophyta or chrysophyta were found in the culture media. However, concentration of euglenophyta and chrysophyta cells did not markedly increase with elevated temperatures. Above 12.5°C, phytoplankton community was overwhelmingly composed of cyanobacteria, chlorophytes, and diatoms. Specifically to say, chlorophytes established dominance at 12.5 and 16°C. Subsequently, cyanobacteria maintained dominant position above 19.5°C.

Algal photosynthetic capacity

The maximal efficiency of photosystem II photochemistry was measured at different temperature levels, and was demonstrated in Figure 5. Algae cells remained weak photochemical vitality before recruitment, their photosynthetic capacity increased gradually after recruitment. As for chlorophytes and diatoms, photosynthetic capacity peaked at 23°C, while that of cyanobacteria reached its peak value at 26.5°C.

DISCUSSION

Influences of environmental factors on algae recruitment had been investigated previously (Barbiero and Kann, 1994; Ståhl-Delbanco and Hansson, 2002; Cao et al., 2005; Tao et al., 2005). Based on most of the literature, temperature, light and sediment resuspension were recognized to be the most important driving factors (Tan et al., 2008). In this paper, a simulation experiment was performed by static culture in light incubator, aiming to study effects of temperature on algae recruitment and populations succession. According to the results, we inferred that the temperature threshold for cyanobacteria recruitment would be between 9 and 12.5°C, which was higher than that for chlorophytes and diatoms. This threshold range was in agreement with field studies in Taihu Lake made by other persons, who reported that benthic cyanobacteria started to grow in March when average temperature was about 10°C (Cao et al., 2005; Zhang et al., 2005). Previous studies indicated the protein synthesis of *Microcystis aeruginosa* accelerated when the temperature rose above 7°C, if below this threshold physiological metabolism of benthic cyanobacteria was bogged down (Càceres and Reynolds, 1984).

It suggested that temperature plays an important role in the recovery of the active form of *M. aeruginosa* in spring (Latour et al., 2004b). Considering facilitated action of resuspension and bioturbation in lakes, migration of overwintering phytoplankton in sediment might be initiated at lower temperature than static incubation (Karlsson-elfgren et al., 2004; Verspagen et al., 2004). In the experiment, sterilized lake water from sampling site was used as culture medium. Therefore, at the beginning of recruitment pelagic algae all originated from sediments. Thereafter, increases in abundances of the pelagic algae could result from two sources: growth of the phytoplankton already present in water and the amounts of recruitment from sediments. These two portions both contributed to the development of phytoplankton in water. Thus, recruitment of phytoplankton played two roles: either as a source of initial pelagic growth or as a supplement to further pelagic development (Cao et al., 2005). In the present study, recruitment of cyanobacteria initiated later than chlorophytes and diatoms, but higher SGR helped cyanobacteria establish and keep dominant position shortly after recruitment. Additionally, colony enlargement phenomena of *Microcystis* were also observed in the absence of zooplankton. Pelagic *Microcystis* mainly existed as unicells or small colonies comprising a couple of cells at lower temperatures and gradually formed large colonies after warming up.

Two mechanisms are involved in the enlarging pattern: one is that colonies are formed when daughter cells of a recently divided cell remain in a regular arrangement during the reproductive process and the other is that formation of colonies is adhesion of already existing single cells (Lürling, 2003). Large colonies consisted of dozens or hundreds of cells, which could be conglutinated together by sheath to form blooms and effectively defend against grazing by zooplankton (Yang et al., 2006). Many *Microcystis* unicells and dividing cells coexisted with colonies after recruitment, the proportion of dividing cells showed a significant correlation to the frequency of dividing cells, which was mainly responsible for algae growth rate and cell viability (Latour et al., 2004b). Thus, rapid proliferation and colony enlargement strategy provided *Microcystis* with an effective competitive power. In contrast, chlorophytes and diatoms, did not maintain high growth rates for occupying ecological space, whose predominance was exceeded by cyanobacteria easily.

Previous studies had successfully detected esterase activities of overwintering *M. aeruginosa* as an indicator for cell viability (Latour et al., 2004a).

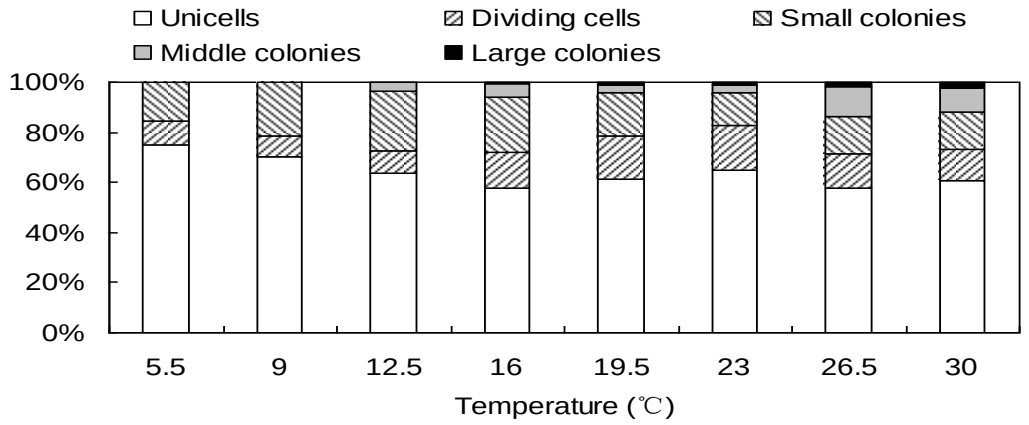
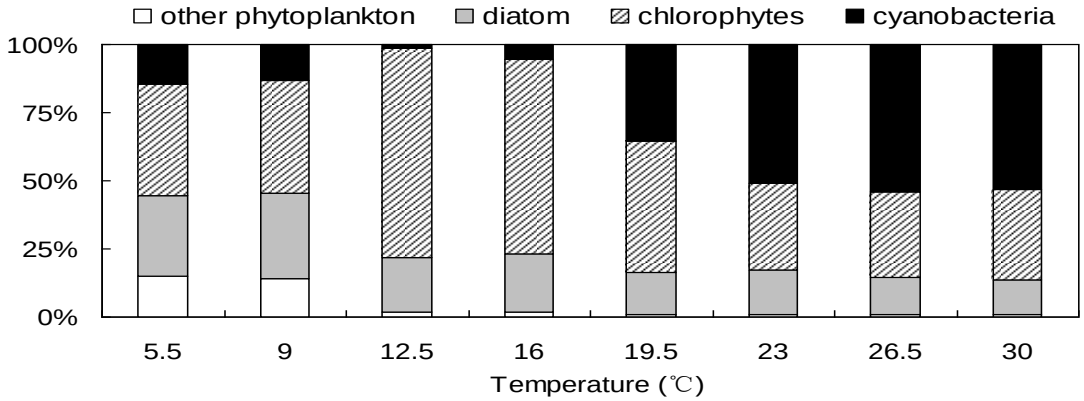


Figure 3. Percentages of *Microcystis* spp. unicells, dividing cells, and colonies. Colonies were grouped into consecutive groups: small colonies (cell number per colony from 3 to 10), middle colonies (cell number per colony between 10 and 100), and large colonies (cell number per colony more than 100).



Fi**gure 4.** Phytoplankton community composition at different temperature levels.

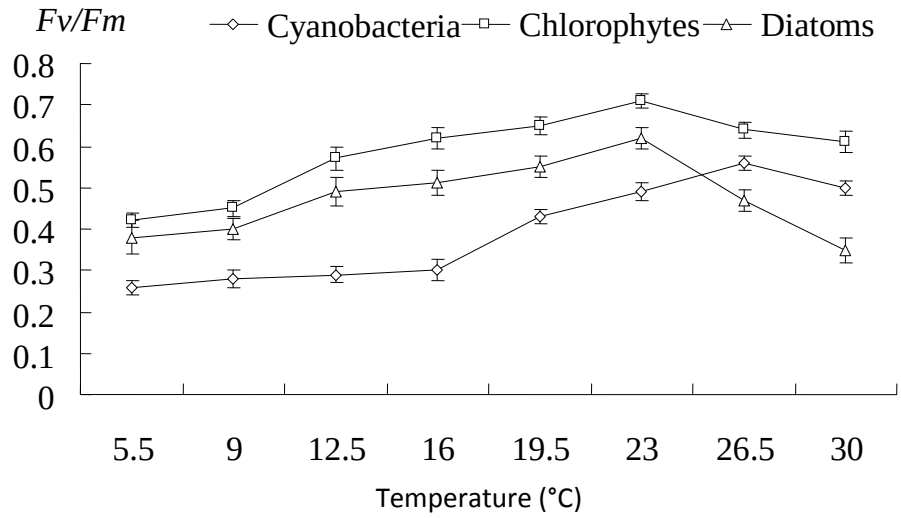


Figure 5. Photosynthetic capacities of cyanobacteria, chlorophytes and diatoms at different temperature levels.

While, esterase activities of different algal groups hardly could be measured separately in mixed samples. In the present study, photosynthetic capacities of cyanobacteria, chlorophytes and diatoms were analyzed by using Phyto-PAM (Walz, Germany) and displayed by F_v/F_m index, respectively, owing to the absence of dinoflagellates all along. Before recruitment, phytoplankton remained weak photochemical vitality, these dormant algae cells could be referred to as 'physiologically resting cells' (Sicko-goad, 1986). They could be reactivated and increased gradually with elevated temperature (Li et al., 2004). Their maximum of photosynthetic capacity usually occurred at the optimum temperature for physiological metabolism (Blanchard et al., 1996). Interestingly, the maximal efficiency of photosystem II photochemistry did not change with growth rate synchronously. Frequency of algal dividing cells might be under the control of an endogenous component (Latour et al., 2004b), which merits further studies.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (31000220) and China Postdoctoral Foundation (20110491341).

REFERENCES

Barbiero RP, Kann J (1994). The importance of benthic recruitment to the population development of *Aphanizomenon flos-aquae* and internal loading in a shallow lake. J. Plankton Res., 16: 1581-1588.

Blanchard G, Guarini JM, Richard P, Gros P, Mornet F (1996). Quantifying the short-term temperature effect on light-saturated photosynthesis of intertidal microphytobenthos. Mar. Ecol. Prog. Ser., 134: 309-313.

Brunberg AK, Blomqvist P (2002). Benthic overwintering of *Microcystis* colonies under different environmental conditions. J. Plankton Res., 24:1247-1252.

Brunberg AK, Blomqvist P (2003). Recruitment of *Microcystis* from lake sediments: the importance of littoral inocula. J. Phycol., 39: 58-63.

Càceres O, Reynolds CS (1984). Some effects of artificiallyenhanced anoxia and the growth of *Microcystis aeruginosa* Kütz Emend, with special reference to the initiation of its annual cycle in lakes. Arch. Hydrobiol., 99: 379-397.

Cao HS, Kong FX, Tan JK, Zhang XF, Tao Y, Yang Z (2005). Recruitment of total phytoplankton, chlorophytes and cyanobacteria from lake sediments recorded by photosynthetic pigments in a large, shallow lake (Lake Taihu, China). Internat. Rev. Hydrobiol., 90: 345-355.

Chen YW, Qin BQ, Teubner K, Dokulil M (2003). Long-term dynamics of phytoplankton assembles: *Microcystis*-domination in Lake Taihu, a large shallow lake in China. J. Plankton Res., 25: 445-453.

Falkowski PG, Kolber Z (1995). Variations in chlorophyll fluorescence yields in phytoplankton in the world oceans. Aust. J. Plant Physiol., 22: 341-355.

Hansson LA, Rudstam LG, Johnson TB, Soranno P, Allen Y (1994). Patterns in algal recruitment from sediment to water in a dimictic, eutrophic lake. Can. J. Fish. Aquat. Sci., 51: 2825-2833.

Hu WP, Jørgensen SE, Zhang FB (2006). A vertical-compressed three-dimensional ecological model in Lake Taihu, China. Ecol. Model., 190: 367-398.

Karlsson-Elfgren I, Brunberg AK (2004). The importance of shallow sediments in the recruitment of *Anabaena* and *Aphanizomenon* (cyanophyceae). J. Phycol., 40: 831-836.

Kong FX, GAO G (2005). Hypothesis on cyanobacteria bloom-forming mechanism in large shallow lakes. Acta Ecol. Sinica, 25: 589-595 (in Chinese with English abstract).

Latour D, Giraudet H, Berthon JL (2004a). Frequency of dividing cells and viability of *Microcystis aeruginosa* in sediment of a eutrophic reservoir. Aquat. Microb. Ecol., 36: 117-122.

Latour D, Sabido O, Salençon MJ, Giraudet H (2004b). Dynamics and metabolic activity of the benthic cyanobacterium *Microcystis aeruginosa* in the Grangent reservoir (France). J. Plankton Res., 26: 1-8.

Li KY, Song LR, Wan N (2004). Studies on recruitment and growth characteristic of *Microcystis* in sediment. Acta Hydrobiol. Sinica, 28: 113-118 (in Chinese with English abstract).

Lürling M (2003). Phenotypic plasticity in the green algae *Desmodesmus* and *Scenedesmus* with special reference to the induction of defensive morphology. Ann. Limnol. – Int. J. Lim., 39: 85 -101.

Oliver RL, Ganf GG (2000). Freshwater blooms. In Whitton BA, Potts M (eds) The Ecology of Cynobacteria, Kluwer Academic Publishier, Netherlands, pp149-194.

Oliver RL, Whittington J (1998). Using measurements of variable chlorophyll-a fluorescence to investigate the influences of water movement on the photochemistry of phytoplankton. Physical Processes in Lakes and Oceans. In Imberger J (ed), Physical Processes in Lake and Oceans. Coastal and Estuarine Studies, American Geophysical Union, Washington, pp 517-534.

Schreiber U (1994). New emitter-detector-cuvette assembly for measuring modulated chlorophyll fluorescence of highly diluted suspensions in conjunctions with the standard PAM fluorometer. Zeitschrift für Naturforschung C – J. Biosci., 49: 646-656.

Schreiber U, Gademann R, Bird P, Ralph PJ, Larkum AWD, Kühl M (2002). Apparent light requirement for activation of photosynthesis upon rehydration of desiccated beachrock microbial mats. J. Phycol., 38: 125-134.

Sicko-Goad L (1986). Rejuvenation of *Melosira granulata* (Bacillariophyceae) resting cells from the anoxic sediments of Douglas Lake, Michigan. II. electron microscopy. J. Phycol., 22: 28-35

Ståhl-Delbanco A, Hansson LA (2002). Effects of bioturbation on recruitment of algal cells from the 'seed bank' of lake sediments. Limnol. Oceanogr., 47: 1836-1843

Tan X, Kong FX, Zeng QF, Cao HS, Qian SQ, Zhang M (2009). Seasonal variation of *Microcystis* in Lake Taihu and its relationships with environmental factors. J. Environ. Sci., 21: 892-897.

Tan X, Kong FX, Cao HS, Yu Y, Zhang M (2008). Recruitment of bloom-forming cyanobacteria and its driving factors. Afr. J. Biotechnol., 7: 4726-4731.

Tao Y, Kong FX, CAO HS, ZHANG XF (2005). Laboratory investigations on recruitment of *Microcystis* in sediment of Taihu Lake. J. Lake Sci., 17: 231-236 (in Chinese with English abstract).

Tsujimura S, Tsukada H, Nakahara H, Nakajima T, Nishino M (2000). Seasonal variations of *Microcystis* populations in sediments of Lake Biwa, Japan. Hydrobiologia, 434: 183-192.

Verspagen JMH, Snelder EOFM, Visser PM, Huisman JEF, Mur LR, Ibelings BW (2004). Recruitment of benthic *Microcystis* (cyanophyceae) to the water column: internal buoyancy changes or resuspension? J. Phycol., 40: 260-270.

Verspagen JMH, Snelder EOFM, Visser PM, Jöhnk KD, Ibelings BW, Mur LR, Huisman JEF (2005). Benthic–pelagic coupling in the population dynamics of the harmful cyanobacterium *Microcystis*. Freshwater Biol., 50: 854-867.

Yang Z, Kong FX, Shi XL, Cao HS (2006). Morphological response of *Microcystis aeruginosa* to grazing by different sorts of zooplankton. Hydrobiologia, 563: 225-230.

Zhang M, Kong FX, Wu XD, Xing P (2008). Different photochemical responses of phytoplankters from the large shallow Taihu Lake of subtropical China in relation to light and mixing. Hydrobiologia, 603: 267-278.

Zhang XF, Kong FX, Cao HS, Tan JK, Tao Y, Wang ML (2005). Research on recruitment dynamics of bloom- forming cyanobacteria in Meiliang Bay, Taihu Lake. Chin. J. Appl. Ecol., 16:1346-1350 (in Chinese with English abstract).

African Journal of Microbiology Research Vol. 5(32), pp. 5902-5909, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.934

Full Length Research Paper

Assessing antibiotic resistance profiles in *Escherichia coli* and *Salmonella* species from groundwater in the Mafikeng area, South Africa

Philemon Thabo Phokela, Collins Njie Ateba* and David Tonderai Kawadza

Department of Biological Sciences, School of Environmental and Health Sciences, Faculty of Agriculture, Science and Technology, North-West University Mafikeng Campus, Private Bag X2046, South Africa.

Accepted 11 November, 2011

***Escherichia coli* and *Salmonella* species occur as normal flora in the gastrointestinal tract of animals and humans. However, pathogenic strains exist that cause disease in humans. Infections may result from the consumption of water and food contaminated with faeces of human and animal origin. In South Africa, residents of most rural communities rely on untreated ground water for survival. This practice results to the transfer of pathogenic micro-organism to humans and thus amplifies the need to identify contaminated water systems. Results obtained may adequately address water quality problems and hence protect public health. The study was conducted to isolate and determine the antibiotic resistance profiles of *E. coli* and *Salmonella* species from the groundwater obtained from two rural communities in the North-West province, S.A. Nineteen ground water samples were analyzed for characters of *E. coli* and *Salmonella* species. Only those isolates that satisfied all the primary (oxidase and the triple sugar iron) and secondary identification criteria (API 20E and rapid slide agglutination test) for *E. coli* and *Salmonella* species were used. A total of 63 *E. coli* and 64 *Salmonella* isolates were identified. The antibiotic susceptibilities of these isolates were evaluated against a panel of 10 antibiotics. A large proportion (56.7 to 57.6%) of the *E. coli* isolated from both Dibate and Verdwall were resistant to vancomycin. Similarly, large proportions (51.5 to 78.8%) of the *E. coli* isolated from Verdwall were resistant to ampicilin and erythromycin when compared to those isolated from Dibate. Furthermore, a higher percentage (90.0 to 100%) of *Salmonella* isolated from both Dibate and Verdwall were resistant to ampicillin, erythromycin and vancomycin. AP-E-VA was the predominant phenotype for *E. coli* isolated from both Dibate and Verdwall in 40 and 20%, respectively, while the phenotypes AP-E-T-VA and AP-E-VA were dominant among the *Salmonella* species isolated in this study. The results indicated that *E. coli* and *Salmonella* could serve as indicator organisms necessary to assess the quality of ground water and their levels are critical parameters that could help to drive management strategies. This will limit the effect of these pathogens on consumers, as some water samples were visibly identified to be contaminated.**

Key words: *Salmonella*, *E. coli*, multiple antibiotic resistant (MAR), phenotype.

INTRODUCTION

Access to drinking water is a fundamental human need and therefore a basic right of every individual, since contaminated water jeopardizes both the physical and social health to consumers (WHO, 2003). In South Africa, residents of most rural communities use untreated groundwater for drinking and household activities. Generally, ground water from boreholes in these rural communities is usually of poor quality that results from chemical and microbial contamination hence considered to be unsafe (Momba et al., 2003, 2005, 2006; Obi et al.,

*Corresponding author. E-mail: atebacollins1@hotmail.com.

2006). Despite this, it is still used for drinking by residents in many communities who do not usually have access to portable water. Residents of Dibate and Verdwall in the Mafikeng area face these same problems. Although the infections such as diarrhoea that are caused by pathogens in water can be self-limiting in some patients, they may also be life-threatening in infants, the elderly and immune-compromised individuals (Momba et al., 2008). The high prevalence of Human immunodeficiency virus/ acquired immune deficiency syndrome (HIV/AIDS) in South Africa amplifies the risks associated with drinking water from these sources (Bessong et al., 2009). Antibiotic resistant faecal coliforms have been isolated from different water sources in the area (Mulamattathil et al., 2000). Multiple antibiotic resistant *E. coli* O157strains, enterococci species and *Staphylococcus aureus* have also been isolated from animal faeces, human stool samples and milk in the area (Ateba et al., 2008; Ateba and Bezuidenhout, 2008; Moneoang and Bezuidenhout, 2009; Ateba et al., 2010).

These isolates could be deposited in water bodies if proper hygiene standards are not implemented and later contaminate ground water (Sorum and L'Abee-Lund, 2002). Consequently, this could contribute in the pool of antibiotic resistant genes among bacteria species in the area and hence negatively affect the treatment of infections caused by these pathogens. Under these circumstances, it is important to determine the prevalence of faecal bacterial contaminants in drinking water so as to reduce mortality caused by these pathogens. Prior to 1994, between 14 to 18 million South Africans were not receiving adequate water supply (Momba et al., 2006). However, as of 2004 about 4 million inhabitants in South Africa still rely on untreated water sources which include rivers, ponds and springs (Kasrils, 2004). Recently, 3.3 million inhabitants of South Africa were identified to be living without access to portable water while about 15.3 million did not have access to adequate sanitation (Council for Scientific and Industrial Research, 2008). Although the government is continually setting up strategies to ensure that residents in rural communities have access to portable water, these facilities are usually faced with maintenance problems. Residents are often left with no option but to revert to use the unprotected water sources like boreholes (WRC, 1993).

These unprotected water sources easily get contaminated with faecal matter of both human and animal origin through rainfall runoffs and human defecations (Ahmed et al., 2005). Bacteria that belong to the family of *Enterobacteriaceae* are a major threat to humans as they frequently contaminate drinking water and hence have the potential of causing water-borne diseases (Young, 1996). However, it is difficult to determine the effect of these pathogens in rural societies due to the fact that individuals that encounter diarrhoea related infections rarely report cases to hospitals (Pascal et al., 2009). This amplifies the need to identify contaminated water systems so as to adequately address water quality problems and hence protect human health. Furthermore, there is a need to regularly determine the bacterial load in these water systems. This might reduce water borne infections in humans that live in these poverty driven communities. This could reduce the health risks associated with the pathogens that are found in ground water. The study was aimed at isolating and determining the antibiotic resistance profiles of *E. coli* and *Salmonella* species from ground water obtained from two rural communities in the North-West province of South Africa.

MATERIALS AND METHODS

Area of the study

This research was conducted in the North-West University, Mafikeng Campus, North-West Province, South Africa. 19 ground water samples were collected from two rural communities in the Mafikeng area. This consisted of 10 from Dibate and 9 from Verdwall. Verdwall is situated approximately 5 km to Itsoseng and Dibate is on the west of Megacity shopping complex in Mmabatho. The samples were collected from both storage tanks and borehole taps. Prior to collecting the samples, the taps were made to run for about 1 min. Water samples were collected into sterile 500 ml Duran Schott bottles and were immediately transported on ice to the laboratory for analysis.

Laboratory analysis

Media utilized in the study

To determine the bacterial load from water samples m-Fc, m-ENDO and plate count agar obtained from Biolab (UK) and supplied by Merck, South Africa were utilized. Plate count agar was used to determine the heterotrophic bacterial count while m-ENDO and m-Fc media were used to determine the total and faecal coliform counts, respectively. Salmonella-shigella agar (SSA) and eosin methylene blue agar (EMBA) were used to selectively isolate *Salmonella* species and *E. coli*, respectively.

Determination of bacterial load

Water samples were analyzed within 2 h upon arrival in the laboratory according to standard methods (APHA, 1998). Each sample was analyzed in triplicates. An aliquot of 100 ml from each sample was filtered using 0.45 µm filter paper (Whatman®Glass Microfiber GS Filterpaper) on a water pump machine (model Sartorius 16824). A sterile forcep was used to remove the membrane filters from the machine. These filter papers were placed on m-FC and m-ENDO agar, respectively. Aliquots of 50 µl of the samples were spread-plated on plate count agar. Plate count agar and m-ENDO agar plates were incubated at 37℃ for 24 h while m-FC plates were incubated at 45℃ for 48 h.

After incubation typical blue colonies on the m-FC plates were regarded as potential faecal coliforms. However, on m-ENDO agar plates, characteristic metallic-sheen colonies were considered potential total coliform bacteria. These isolates were counted using a colony counter. The results were recorded and averages computed. The colonies on the agar were also counted and the results were reported as colony forming units (CFU) per 100 ml of sample plated.

***Selective isolation of E. coli and Salmonella* species**

Aliquots of 50 µl from each water sample was spread-plated onto eosin methylene blue agar (EMBA) and salmonella-shigella agar (SSA) plates for selective isolation of *E. coli* and *Salmonella* species, respectively. The plates were incubated at 37℃ for 24 h. After incubation, characteristic metallic-sheen colonies on EMBA and pale yellow colonies with black spots on their centres on SSA were considered to be presumptive *E. coli* and *Salmonella* species, respectively. These isolates were sub-cultured on EMBA and SSA plates for *E. coli* and *Salmonella* species, respectively and the plates were incubated at 37℃ for 24 h. Pure isolates for *E. coli* and *Salmonella,* respectively were retained for identification using specific biochemical tests.

Control strains

E. coli (ATCC 25922), *Salmonella arizonae* (ATCC 13314) and *Salmonella paratyphi* (ATCC 1950) were used as positive control strains in all experiments.

Bacterial identification

Presumptive isolates were identified using the following criteria.

Cellular morphology

Isolates were gram stained using the method of Cruikshank et al. (1975). All gram-negative isolates from EMBA and SSA plates were subjected to primary and secondary biochemical identification tests.

Preliminary biochemical identification tests for E. coli and Salmonella spp

Triple sugar iron agar test: Triple sugar iron (TSI) agar (Biolab) obtained from Merck, S.A. was used to determine the potential of *E. coli* and *Salmonella* isolates in utilizing three sugars, (glucose, sucrose and lactose) at final concentrations of 0.1, 1.0 and 1.0%, respectively. The test was performed as previously recommended by (United States Pharmacopeial Convention; Incorporated 2001). The results were recorded and data interpreted as previously determined by (Forbes and Weissfeld, 1998).

Oxidase test: The oxidase test was performed using the oxidase test reagent from Pro-Lab Diagnostics, United Kingdom and the test was performed as instructed by the manufacturer (Whatman International Limited, Maidstone, England). All isolates that satisfied the preliminary identification criteria for *E. coli* and *Salmonella,* respectively were subjected to confirmatory biochemical identification test.

Confirmatory identification tests for *E. coli* and *Salmonella* spp

Analytical profile index (API) API 20E

All the presumptive *E. coli* and *Salmonella* spp. were confirmed using the API 20E test which is a standardized test kit designed to facilitate the identification of bacteria that belong to the family *Enterobacteriaceae*. The test was performed as instructed by the manufacturer (BioMerieux®, France). The indices obtained after reading the results were interpreted using the API web software (BioMerieux® S.A).

Antibiotic susceptibility test

Antibiotic susceptibility tests were performed on all *E. coli* and *Salmonella* species to determine their antibiotic resistant profiles using the Kirby-Bauer disc diffusion technique (Kirby et al., 1966). Before antibiotic sensitivity testing, the isolates were revived by culturing onto EMBA and SSA plates for *E. coli* and *Salmonella,* respectively. The plates were incubated aerobically at 37℃ for 24 h. Bacterial suspensions were prepared using these pure isolates and aliquots of 100 µl from these suspensions were spread-plated on Mueller Hinton agar (Biolab, Merck, South Africa). The susceptibilities of the isolates against a panel of ten different antibiotic discs obtained from Mast Diagnostics, United Kingdom were determined. The antibiotic discs were gently pressed onto the inoculated Mueller Hinton agar to ensure intimate contact with the surface and plates were incubated aerobically at 37℃ for 24 h (CLSI,1999) formerly (NCCLS, 1999). The antibiotic inhibition zone diameters were measured and results obtained were used to classify isolates as being resistant, intermediate resistant or susceptible to a particular antibiotic based on standard reference values (CLSI,1999) formerly (NCCLS, 1999). Table 1 indicates the details of antibiotics that were used in the study. The antibiotics tested are those to which resistance have been reported in the area (Ateba and Bezuidenhout, 2008; Bezuidenhout and Moneoang, 2009). Moreover, some antibiotics that are used to treat human infections were also included.

Multiple antibiotic resistant (MAR) phenotypes

MAR phenotypes were generated for isolates that were resistant to 3 and more antibiotics (Rota et al., 1996). Phenotypes were generated using the abbreviations that appear on the antibiotic discs.

Statistical analysis

Statistical analysis was done using SPSS software (version 14.0). Pearson's moment correlation product ($P<0.05$) was used to determine whether the antibiotics tested exhibited similar reactions against the *E. coli* and *Salmonella* spp. isolated from the different sampling stations. Cluster analysis was performed using the Statistica software (version 9.0).

RESULTS

Bacterial load in ground water samples

A total of 19 ground water samples were analyzed to determine the faecal coliform, total coliform and heterotrophic bacterial counts. Results obtained were recorded as number of colony forming units (cfu) per 100 ml of water sample for m-FC and m-ENDO while for heterotrophic counts the results were recorded as number of cfu per 1 ml of water sample. Results obtained are shown in Tables 2 and 3 for samples obtained from Dibate and Vredwall, respectively. The results in Table 2 indicate that a large number (9 of 10) of the water samples obtained from Dibate village were contaminated with microbial pathogens. Generally, the number of colonies per sample was higher for the heterotrophic bacterial count than the total coliform and the faecal coliform count (Table 2). Although one of the samples did

Table 1. Details of antibiotics that were used in the study.

Group	Antibiotic	Disc conc	R	I	S
Aminoglycosides	S	300 µg[d]	≤11	12 to 14	≥15
	Ne	30 µg[c]	≤12	13 to 16	≥17
	K	30 µg[c]	≤13	14 to 17	≥18
Beta –Lactams	Ap	10 µg[a]	≤11	12 to 14	≥15
	GM	10 µg[a]	≤12	-	≥13
Glycopeptides	VA	30 µg[c]	≤9	10 to 11	≥12
Tetracyclines	T	30 µg[c]	≤14	15 to 18	≥19
Quinolones	Nor	10 µg[a]	≤12	13 to 16	≥17
Phenols	C	30 µg[c]	≤12	13 to 17	≥18
Marcrolides	E	15 µg[b]	≤13	14 to 22	≥23

The superscripts [a] to [d] indicate the generally accepted concentrations of the discs according to the standard method stipulated by the manufacturer, Mast Diagnostics, Merseyside, United Kingdom (mention the abbreviation here).

Table 2. The total number of bacterial counts from the ground water samples obtained from Dibate and Verdwall villages, respectively.

Sample no /source	FCC	TCC	HPC	Sample no /source	FCC	TCC	HPC
DW1 (Storage tank)	4	16	224	VW1 (Storage tank)	10	20	40
DW2 (Pump)	8	30	374	VW2 (Storage tank)	1	4	15
DW3 (Pump)	4	10	314	VW3 (Storage tank)	3	12	20
DW4 (Pump)	6	28	172	VW4 (Pump)	5	10	32
DW5 (Storage tank)	2	4	125	VW5 (Pump)	7	19	116
DW6 (Pump)	8	170	250	VW6 (Pump)	4	16	286
DW7 (Storage tank)	0	2	0	VW7 (Pump)	0	22	90
DW8 (Pump)	0	2	28	VW8 (Pump)	1	1	6
DW9 (Pump)	9	12	40	VW9 (Pump)	0	5	100
DW10 (Storage tank)	1	1	4				

DW = Dibate water; VW = Verdwall water; FCC = Faecal coliform count (cfu/100 ml); TCC = Total coliform count (cfu/100 ml); HPC = Heterotrophic plate count (cfu/1ml).

Table 3. The percentages of *E. coli* and *Salmonella* spp isolated from the two sampling sites.

Bacterial species	Sample area	No isolated	Percentage of *E. coli* and *Salmonella* isolated
E. coli	Dibate	30	47.6
	Verdwall	33	52.4
Salmonella spp	Dibate	14	21.9
	Verdwall	50	78.1

not reveal any contamination, it was observed that most water were contaminated with bacteria of faecal origin. However, an interesting observation was that the counts were higher in samples obtained from borehole pumps than in tanks. Table 2 also indicates that faecal coliform bacteria were identified in all but 1 water sample obtained from Vredwall. Despite this, total coliform bacteria and very high levels of heterotrophic bacteria were identified in these samples. It was identified that the number of colony forming units were generally higher for water samples obtained from borehole pumps than those in taps that are linked to storage tanks.

Occurrence of *E. coli and Salmonella* spp. in ground water samples

19 ground water samples that comprised of 10 and 9

Table 4. Percentage antibiotic resistance of *E. coli* and *Salmonella* isolated from the different sampling stations.

Antibiotics	*E. coli* (N=63)		*Salmonella* spp (N=64)	
	Sampling site		Sampling site	
	Dibate (N=30)	Verdwall (N=33)	Dibate (N=14)	Verdwall (N=50)
Ap	33.0	78.8	100	100
E	20.0	51.5	92.9	90.0
T	23.3	12.1	14.3	64.0
GM	0	24.2	0	2.0
VA	56.7	57.6	100	100
Ne	0	30.0	0	4.0
C	3.3	27.3	7.1	26.0
K	0	27.3	0	30.0
S	0	15.2	0	6.0
Nor	0	9.1	0	16.0

Ap (ampicillin), E (erythromycin), T (tetracycline),GM (gentamicin), VA (vancomycin), Ne (neomycin), C (chloramphenicol), K (kanamycin), S (streptomycin), Nor (norfloxacin).

from Dibate and Verdwall villages respectively were collected directly from the pumps and storage tanks. These samples were analyzed for the presence of *Salmonella* spp. and *E. coli,* respectively. Isolates that satisfied both the preliminary and confirmatory identification tests for these pathogens were retained. A total of 63 *E. coli* isolates and 64 *Salmonella arizonae* were positively identified as shown in Table 3. The proportion of both *E. coli* and *Salmonella* spp. isolated were higher (33) and 50, respectively in water samples obtained from Verdwall. Moreover, the proportion of *Salmonella* isolated from Verdwal (50) was higher when compared to those isolated from water obtained in Dibate (14).

Antibiotic resistance of *E. coli and Salmonella spp.* in ground water samples

All the *E. coli* and *Salmonella* isolated were tested to evaluate their susceptibilities to 10 different antibiotics. Results obtained are depicted in Table 4. A large proportion (51.5 to 78.8%) of the *E. coli* isolated from Verdwall was resistant to ampicillin and erythromycin when compared to those isolated from Dibate. Similarly, a large proportion (56.7 to 57.6%) of the *E. coli* isolates from both Dibate and Verdwall were resistant to vancomycin. Despite the fact that a small proportion (9.1 to 30.3%) of the *E. coli* isolates from Verdwall were resistant to gentamicin, neomycin, kanamycin, streptomycin and norfloxacin, none of those isolated from Dibate were resistant to these antimicrobial agents. Small proportions (12.1 to 23.3%) of the *E. coli* isolates from both sample sites were resistant to tetracycline. A very large proportion (90.0 to 100%) of the *Salmonella* isolates from both Dibate and Verdwall were resistant to ampicillin, erythromycin and vancomycin. Despite the fact that only a small proportion (14.3%) of the *Salmonella* isolates from Dibate were resistant to tetracycline, a relatively larger proportion (64.0%) of those isolated from Verdwall were resistant to this drug. *Salmonella* isolates from Verdwall showed little resistance (2.0 to 30.0%) to gentamycin, neomycin, kanamycin, streptomycin and norfloxacin. However, none of those isolated from Dibate were resistant to these antimicrobial agents (Table 4).

MAR phenotypes of *E. coli* isolated

The predominant antibiotic resistant phenotypes that were determined for *E. coli* isolated from ground water were obtained from Dibate and Verdwall, respectively are shown in Table 5. The MAR phenotype AP-E-VA was dominant among 40.0 and 20.0% of the *E. coli* isolates from Dibate and Verdwall, respectively. Furthermore, the phenotype AP-T-VA was also identified in 40.0% of the isolates from Dibate (Table 5). Although none of the *E. coli* isolated from Dibate was resistant to more than four antibiotics, an isolate from Verdwall was resistant to nine of the ten antibiotics tested. However, all of the *E. coli* isolated from both sites sampled was resistant to three or more antibiotics.

MAR phenotypes of *Salmonella* species isolated

The dominant MAR phenotypes amongst *Salmonella* isolated from ground water in the sampled sites were AP-E-VA and AP-E-T-VA in 85.7 and 22% for those isolated from Dibate and Verdwall, respectively. Phenotypes AP-E-VA and AP-E-T-VA-K-C were also identified in 20 and 14%, respectively for the *Salmonella* isolates obtained from Verdwall (Table 5). *Salmonella* species from Dibate were not resistant to more than four of the ten antibiotics tested. An isolate from Verdwall was resistant to eight of

Table 5. The predominant multiple antibiotic resistant (MAR) phenotypes for *E. coli* and *Salmonella arizonae* isolated from the different sampling sites.

Specie	Sampling site	Phenotype	No observed	%
E. coli	Dibate (N=10)	AP-E-VA	4	40.0
		AP-T-VA	4	40.0
	Verdwall (N=20)	AP-E-VA	4	20.0
		AP-GM-E-VA	2	10.0
Salmonella spp	Dibate (N=14)	AP-E-VA	12	85.7
	Verdwall	AP-E-T-VA	11	22.0
	(N=50)	AP-E-VA	10	
		AP-E-T-VA-K-C	7	
		AP-VA-Nor	3	
		E-T-VA	3	

Ap (ampicillin), E (erythromycin), T (tetracycline),GM (gentamicin), VA (vancomycin), Ne (neomycin), C (chloramphenicol), K (kanamycin), S (streptomycin), Nor (norfloxacin).

the antibiotics tested. All the *Salmonella* species isolated from Dibate and a large proportion of those from Verdwall were resistant to three or more antibiotics MAR.

DISCUSSION

The primary objective of this study was to isolate *E. coli* and *Salmonella* species from ground water that is used for drinking by residents of two rural communities in the North-West Province, of South Africa. An analysis of the bacterial load in water samples was also investigated. *E. coli* and *Salmonella* species were successfully isolated and their identities were confirmed. Results indicated that a greater proportion (7 out 9) of the water samples from each site was contaminated with bacteria of faecal origin. Moreover, the levels of total coliforms and heterotrophic bacteria were high (Table 2). Similar studies have been conducted to evaluate the quality of drinking water, by means of determining the bacterial load in water samples (Nevondo and Cloete, 1999; Potgieter, 2007). In these studies it was reported that the proportion of indicator microorganisms was far beyond the standards recommended for potable water in South Africa (Nevondo and Cloete, 1999; Potgieter, 2007). However, the results obtained in the latter studies were higher than those reported herein although both *E. coli* and *Salmonella* species were the predominant coliform bacterial species that were most frequently isolated (Nevondo and Cloete, 1999). *E. coli* isolated in the present study was not identified at strain level. Some of these isolates may belong to the serotype *E. coli* O157:H7 that is highly pathogenic to humans. Thus their presence in drinking water may have severe health implications on consumers.

Another objective of the study was to determine the extent to which *E. coli* and *Salmonella* species isolated from ground water in these two rural communities are resistant to antibiotics. A motivation to this is the fact that previous studies conducted in the area involving *E. coli* and *E. coli* O157 strains from humans, cattle and pigs revealed that a large proportion of the isolates (50 to 100%) were resistant to some antibiotics (Ateba and Bezuidenhout, 2008; Moneoang and Bezuidenhout, 2009). The emergence of multidrug resistant *Salmonella* species in the developing countries has been attributed to the misuse of antibiotics in animals (Threlfall, 2002). This indicates the need to regulate the usage of antimicrobial agents as this may have severe implication on both the veterinary and health care profession (Mulamattathil et al., 2000). Results obtained in the present study revealed that MAR, defined as resistance to three or more antibiotics was observed in 30 (47.6%) and 64 (100%) of *E. coli* and *Salmonella* species, respectively. Several studies have shown that *E. coli* and *Salmonella* species isolated from ground water are resistant to multiple antibiotics (Armstrong et al., 1981; Harakeh et al., 2006). However, in the present study a greater proportion of *Salmonella* species were resistant to most of the antibiotics tested.

A larger proportion of *E. coli* and *Salmonella* species isolated were resistant to ampicillin, erythromycin and vancomycin. Resistance to ampicillin may have resulted from the fact that the drug is frequently used in both veterinary and human medicine in the area. However, resistance to erythromycin and vancomycin was a course for concern since these antibiotics are not used on animals and humans in the study area. Similar observations have been reported (Oguttu, 2007; Ateba

and Bezuidenhout, 2008). *E. coli* and *Salmonella* species from both sites showed little or no resistance to gentamycin, neomycin, kanamycin, streptomycin and norfloxacin. However, both *E. coli* and *Salmonella* species from Verdwall showed high levels of resistant to these drugs as compared to those from Dibate (Table 4). This indicates the difficulty that may arise in treating water-borne infections in humans that are resident in Verdwall if caused by these multiple antibiotic resistant strains. A small proportion (3.3 to 27.3%) of the *E. coli* and *Salmonella* isolates showed resistance to chloramphenicol (Table 4). Chloramphenicol had been banned for inclusion as additives in feeds for animals (Bischoff et al., 2002). Moreover, it is not used in veterinary medicine in the area. The predominant MAR phenotypes for *Salmonella* and *E. coli* isolated was AP-E-VA, AP-E-VA and AP-GM-E-VA. Resistance phenotypes arise from different genetic determinants that present specific epidemiological features (Lanz et al., 2003). Consequently, an assessment of the resistance situation at genetic level would be of great importance in controlling antimicrobial resistance. Although a large proportion of these isolates were resistant to most of the antibiotics that were tested, high levels of resistance were observed against ampicillin. Although nothing is known about the distribution and contribution of antibiotic resistance genes in *Salmonella* species in the area, little has been documented for *E. coli* (Ateba and Bezuidenhout, 2008). This is therefore an aspect that needs further investigation.

ACKNOWLEDGEMENTS

Authors are grateful to the North-West University, Mafikeng Campus for providing the funds and work space needed to conduct the research. And the assistance received from the laboratory technician Mrs. Rika Hyser is highly acknowledged.

REFERENCES

Ahmed W, Neller R, Katouli M (2005). Host spices-specific metabolic fingerprint database for *Enterococci* and *Escherichia coli* and its application to identify source of faecal contamination in surface water. Appl. Environ. Microbiol. 71(8): 4461-4468.

American Public Health Association (APHA) (1998). Standard Methods for the examination of water and wastewater, 19th edition. American Public Health Association, Washington DC.

Armstrong JL, Shigeno DS, Calomiris JJ, Seidler RJ (1981). Antibiotic resistant bacteria in drinking water. Appl. Environ. Microbiol. 42(2): 277-283.

Ateba CN, Bezuidenhout CC (2008). Characterization of *Escherichia coli* O157 strains from humans, cattle and pigs in the North-West Province, South Africa. Int. J. Food Microbiol., 128: 181-188.

Ateba CN, Mbewe M, Bezuidenhout CC (2008). The Prevalence of *Escherichia coli* O157 strains in cattle, pigs and humans in the North-West Province, South Africa. SAJS, 104: 7-8.

Ateba CN, Mbewe M, Moneoang MS, Bezuidenhout CC (2010). Antibiotic resistant *Staphylococcus aureus* from milk in the Mafikeng Area, North West Province, South Africa. SAJS 106: (11/12) 1-6.

Bessong PO, Odiyo JO, Musekene JN, Tessema A (2009). Spatial Distribution of Diarrhoea and Microbial Quality of Domestic Water during an Outbreak of Diarrhoea in the Tshikuwi Community in Vhenda, South Africa. J. Health Pop. Nutri., 27 (5): 652-659.

Bischoff KM, White DG, McDermott PF, Zhao S, Gaines S, Maurer JJ, Nisbet DJ (2002). Characterization of chlororampenicol resistance in beta-haemolytic *Escherichia coli* associated with diarrhoea in neonatal swine. Clin. Microbiol., 40(2): 389-394.

Council for Scientific and Industrial Research (CSIR) (2008). Water Quality and Pollution: Parliamentary Portfolio Committee on Water Affairs and Forestry. Pp. 1-173

Cruikshank R, Duguid JP, Marmoin BP, Swain RH (1975). Medical Microbiology, 12th ed. Longman, New York. (2): 3-4.

Forbes S, Weissfeld (1998). Bailey and Scott's diagnostic microbiology, 10th ed. Mosby, Inc., St. Louis, Mosby.

Harakeh S, Yassine H, El-Faidel M (2006). Antibiotic resistant patterns of *Escherichia coli* and *Salmonella* strains in the aquatic Lebanese environments. Environ. Pollut., 143: 269-277.

Kasrils R (2004). A decade of delivery. Minister of Water Affairs and Forestry.

Kirby WMM, Bauer AW, Sherris JC, Turck M (1966). Antibiotic susceptibility testing by single disc method. Am. J. Clin. Pathol., 45: 4.

Lanz R, Kuhnert P, Boerlin P (2003). Antimicrobial resistance and resistance gene determinants in clinical *Escherichia coli* from different animal species in Switzerland. Vet. Microbiol., 91: 73-84.

Momba MNB, Abongo BO, Mwambakana JN (2008). Prevalence of enterohaemorrhagic *Escherichia coli* O157:H7 in drinking water and its predicted impact on diarrhoeic HIV/AIDS patients in the Amathole District, Eastern Cape Province, South Africa. Water SA., 34(3): 365-372.

Momba MNB, Makala N, Zani B, Brouckaert BM (2005). Key causes of drinking water quality failure in a rural small water supply of South Africa. In: JH Lehr and J Keeley (eds.) Water Encyclopedia-Domestic, Municipal, and Industrial Water Supply and Waste Disposal. John Wiley and Sons, Inc. New Jersey, pp. 221-227.

Momba MNB, Ndaliso S, Binda MA (2003). Effect of a combined chlorine-monochloramine process on the inhibition of biofilm re-growth in potable water systems. Water Supp., 3(1-2): 215-221.

Momba MNB, Tyafa Z, Makala N, Brouekaert BM, Obi CL (2006). Safe drinking water is still a dream in the rural areas of South Africa. Case study: The Eastern Cape Province. Water SA., 32(5): 715-720.

Moneoang MS, Bezuidenhout CC (2009). Characterization of *enterococci* and *E. coli* isolated from commercial and communal pigs from Mafikeng in the North-West Province, South Africa. AJMR, 3(3): 088-096.

Mulamattathil SG, Esterhysen HA, Pretorius PJ (2000). Antibiotic-resistant gram-negative bacteria in a virtually closed water reticulation system. Appl. Microbiol., 88: 30-937.

National Committee for Clinical Laboratory Standards. (NCCLS) (1999). Performance standards for antimicrobial disc and dilution susceptibility test for bacteria isolated from animals. National Committee for Clinical Laboratory Standards, Wayne, Pennsylvania, Approved Standards M, 31-A19 (11).

Nevondo TS, Cloete TE (1999). Bacterial and chemical quality of water supply in the Dertig village settlement. Water SA. 25 (2): 215-220.

Obi CL, Onabulu B, Igumbor EO, Ramalivhana J, Bessong PO, Van Rensburg EJ, Lukoto M, Green E, Ndou S, Mulaudzi TB (2006). The interesting cross-paths of HIV/AIDS and water in Southern Africa with special reference to South Africa. Water SA., 32 (3): 322-344.

Oguttu JW (2007). Antimicrobial drug resistance of enteric bacteria from broilers fed antimicrobial growth enhances and exposed poultry abattoir workers. MSc thesis, University of Pretoria.

Potgieter N (2007). Water storage in rural households: Intervention strategies to prevent water borne diseases. PhD thesis, University of Pretoria.

Rota C, Yanguela J, Blanco D, Carrminana JJ, Arino A, Herrera A (1996). High prevalence of multiple resistance to antibiotics in 144 *Listeria* isolates from Spanish dairy and meat products. Food Protect., 59: 938-943.

Sorum H, L'Abee-Lund TM (2002). Antibiotic resistance in food-related bacteria result of interfering with the global web of bacterial genetics.

Int. J. Food Microbiol., 78: 43-56.
Threlfall J (2002). Antimicrobial drug resistance in *Salmonella*: Problems and perspective in food and water-borne infections. *FEMS* Microbiol. Rev., 26: 141-148.
United States Pharmacopeial Convention, Inc. (2001). The United States Pharmacopeia 25. Rockville, M.D.
Water Research Commission (WRC) (1993). Guidelines on the cost effectiveness of rural water supply and sanitation projects. Water Research Commission Report No. 231/1/93, Pretoria.
World Health Organisation (2003). Emerging Issues in Water and Infectious Disease, World Health Organisation, Geneva, Switzerland.
Young P (1996). Safe drinking water: A call for global action. ASM News, 62: 349-352.

African Journal of Microbiology Research Vol. 5(32), pp. 5910-5915, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.942

Full Length Research Paper

Determination of hepatitis C virus genotypes among HCV positive patients in Shahrekord, Iran

Elahe Tajbakhsh[1*], Abbas Dosti[2], Sara Tajbakhsh[3], Manochehr Momeni[4] and Forough Tajbakhsh[5]

[1]Department of Microbiology, Faculty of Basic Sciences, Islamic Azad University Shahrekord branch, Shahrekord Iran.
[2]Department of Microbiology, Faculty of Basic Sciences, Islamic Azad University Shahrekord branch, Shahrekord Iran.
[3]University of Medical Sciences, Shahre-kord, Iran.
[4]Biotechnology Research Center, Islamic Azad University Shahrekord branch, Shahrekord Iran.
[5]Young Researchers Club, Islamic Azad University, Shahrekord Branch, Shahrekord-Iran.

Accepted 20 October, 2011

Hepatitis C is one of the most common causes of the liver failure and cancer and represents a major public health problem. Recent studies have focused on whether different hepatitis C virus (HCV) genotypes, are associated with different profiles of pathogenicity, infectivity and response to antiviral therapy. Genotyping system based on polymerase chain reaction (PCR) of the core region with genotype-specific PCR primers for the determination of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a was developed. Different genotypes have been reported in different parts of the world. Genotype 1 is difficult to treat, while genotypes 2 and 3 are easy to treat. Therefore, identification of HCV genotype in patients is necessary to begin and follow up the treatment. In this study, viral genomic of 94 patients extracted from sera were detected by nested-real time (RT) PCR. PCR products were digested with proper enzymes and studied by restriction fragment length polymorphism (RFLP). The results of PCR-RFLP were as follows: 1a (54.26%), 1b (11.71%), 3a (27.66%), 2a (2.12%) and 4 (4.25%). This indicates that a high percentage of HCV infected patients.

Key words: Genotyping, Hepatitis C Virus, PCR, RFLP, Iran.

INTRODUCTION

Hepatitis C virus (HCV) is a single stranded ribonucleic acid (RNA) virus; approximately 9.5 kb which belongs to the Flaviviridae family, HCV demonstrates a high degree of sequence variation throughout its genome (Choo et al., 1989). HCV is a causative agent for chronic, acute and fulminant hepatitis (Alavian et al., 2002). The association of HCV among patients with cirrhosis of liver and hepatocellular carcinoma has been reported (Alavian et al., 2006). HCV infection is a global health problem and it is estimated that 200 million people of the world population are infected with HCV (Lee et al., 2008). Serological tests detecting antibody to HCV have shown that HCV is the major etiological agent for both transfusion acquired and sporadic non-A, non-B hepatitis (Alter et al., 1989; Kuo et al., 1989; Mosley et al., 1990). Chronic hepatitis occurs in more than 50% of HCV infected patients and can lead to cirrhosis and liver cancer. HCV causes 20% of acute hepatitis cases, 70% of all chronic hepatitis cases, 40% of all cases of cirrhosis of the liver, 60% of hepatocellular carcinomas, and 30% of liver transplants in Europe.

The study of genetic variability of HCV strains has led the consensus classification into six major genotypes. Some studies suggest that the clinical features of liver

*Corresponding author. E-mail: ee_tajbakhsh@yahoo.com or ee_tajbakhsh@iaushk.ac.ir. Tel/Fax: 0098 3813361060.

Abbreviations; HCV, Hepatitis C virus; **RT PCR,** reverse transcriptation polymerase chain reaction; **5'-UTR,** 5' untranslated region; **DNA,** deoxyribonucleic acid; **RIBA,** recombinant immunoblot assay.

disease depend on HCV genotypes (Farshad et al., 2010). Classification of HCV is based on the diversity of the genome, and the criterion for HCV classification was proposed by Simmonds and colleagues (Simmonds et al., 1993).

The HCV genotypes have been determined primarily based on analysis of partial genome sequences. The most extensive database exists for the 5'-UTR, core, E1, and NS5B (Bukh et al., 1992a; Furione et al., 1999). Whereas the 5'-UTR is highly conserved and therefore preferred for diagnosis, the core, envelope, and NS5B regions are less conserved and therefore highly discriminative and may be preferred for subtyping (Prescott et al., 1997).

Sequence analysis of multiple strains of HCV has demonstrated that the nucleotide sequence can differ by as much as 30%. However, the levels of heterogeneity differ considerably among various regions of the virus. For example, sequence variation ranges from as little as 10% in the 5' untranslated region (5'-UTR) to as much as 50% or more within the E1 region. HCV isolates from around the world can be divided into distinct major groups or genotypes with about 66 to 69% nucleotide similarity, which can in turn be divided further into subtypes with about 77 to 80% nucleotide similarity (Chan et al., 1992; Bukh et al., 1993).

Since the first report of the HCV genome by the Chiron research group numerous complete or partial nucleotide sequences of HCV isolates have been reported worldwide. Comparison of these sequences revealed marked genetic heterogeneity of the HCV genome (Okamato et al., 1990; 1992; Choo et al., 1991; Inchauspe et al., 1991).

Investigators of HCV genotyping have used sequence analysis of HCV NS5, core, E1, and 5'-UTRs. HCV genotyping by Okamoto et al. using type-specific primers was first introduced by using primers specific for the core region. (Okamoto et al., 1996) This method lacked acceptable sensitivity and specificity (McOmish et al., 1993). Several deoxyribonucleic acid (DNA) hybridization assays for HCV genotyping have been described.

A commercial kit for HCV genotyping has been introduced in Europe by Innogenetics (InnoLipa, Belgium) and is based on hybridization of 5'- UTR amplification products with genotype specific probes. Others have used restriction enzymes to determine viral genotype by restriction fragment length polymorphism (RFLP).

In this method, a PCR-amplified DNA fragment is digested into fragments with different lengths by enzymes (restriction endonucleases) that recognize cleavage sites specific for each genotype (Stuyver et al., 1993). Although all these methods are able to identify the major genotypic groups, only direct nucleotide sequencing is efficient in discriminating subtypes (Bukh et al., 1995).

In the current study, we have typed HCV strains with RFLP rapidly and reliably by digesting the amplified DNA from the primary specimens by selected restriction enzymes and some by sequencing.

MATERIALS AND METHODS

Serological data

The sera were collected from 94 HCV infected patients, referred to Al Mahdi Laboratory (Shahrekord, Iran) during 2009-2010. All patients had elevated serum aminotransferases for at least 6 months, a positive test for anti-HCV antibodies (third generation ELISA [ORTHO HCV 3.0 ELISA Test system; Ortho Diagnostics, Raritan, New Jersey, USA], the confirmatory recombinant immunoblot assay (RIBA) test (Inno-LIA TM HCV Score) and HCV RNA in serum by reverse transcription nested PCR for the 5'-UTR of the HCV genome (Simmonds et al., 1993). The average age was varying from 18 to 64 year. while the mean age was 41.

HCV RNA extraction and cDNA synthesis

For detection of HCV RNA in serum and for genotyping studies, RNA was extracted from 50 µl of serum by using STRP^{TM} HCV detection kit (Cinna Gen Inc Company). For extraction of HCV RNA, add 50 µl serum to 450 µl cold RNX^{TM} plus solution and vortex sample to dissolve clamps, then add 100 µl of chloroform and centrifuged at 12000 g and transfer the aqueous phase to a new tube then add equal volume of isopropanol, and stored at -20°C for at least 20 min, and centrifuged at 12000 g. Then discard aqueous phase and to the pellet 200 µl 70% ethanol and centrifuged at 12000 g, and discard aqueous phase and dry the pellet (RNA). At least dissolve RNA in 30 µl DEPC treated water and stored in -70°C. According to the kit protocol, cDNA was synthesis and by use specific primers RT-PCR was done.

PCR genotyping primers

For specific and nested PCR, four oligonucleotide primers form 5'-UTR of HCV were designed using generunr (Hastings software) and synthesized at the Cinna Gene Company (Iran). In the first round of PCR, the primers corresponded to HCV-1 sense oriented nucleotides -268 to -251 F1 (AGCGTCTAGCCATGGCGT), numbered according to Bukh et al., (1992b) and antisense nucleotides -4 to -22 R1 (GCACGGTCTACGAGACCT). For the second round, the primer F2 (GTGGTCTGCGGAACCGG) corresponded to sense-oriented nucleotides -199 to -183 and R2 (GGGCACTCGCAAGCACCC) corresponded to antisense nucleotides -26 to -43.

PCR

The first round was carried out for 30 cycles which consisted of initial denaturation at 94°C for 5 min, denaturation at 94°C for 35 s, annealing at 58°C for 40 s, extension at 72°C for 45 s and, the final extension at 72°C for 5 min. The second round was followed for 25 cycles which consisted of initial denaturation at 94°C for 5 min, denaturation at 94°C for 35 s, annealing at 64°C for 40 s, extension at 72°C for 45 s, and the final extension at 72°C for 5 min. The 174-bp second PCR product was submitted to electrophoresis by using a 1.5% agarose gel in 0.5X TBE buffer, and was visualized by ethidium bromide staining under ultraviolet light (Han et al., 1991).

Genotyping by RFLP

Total volumes of each nested-PCR product (25 µl) were divided into

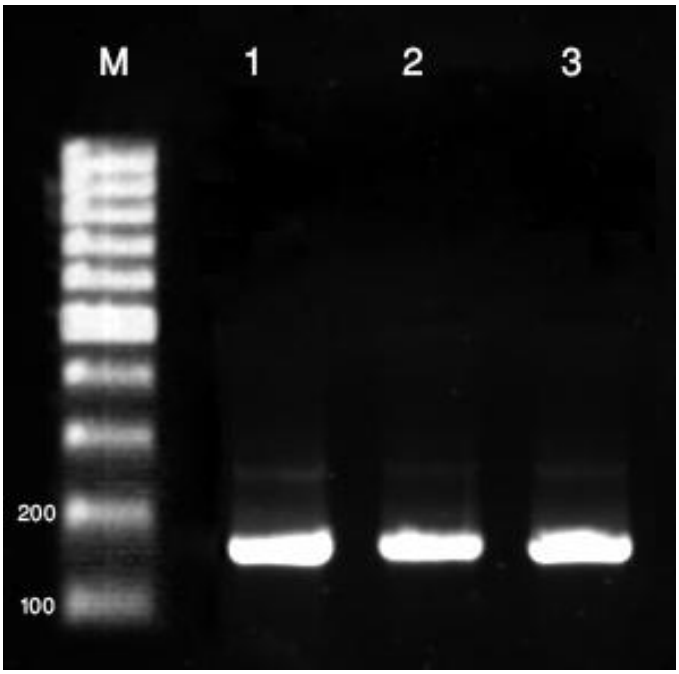


Figure 1. Ethidium bromide stained gel of PCR products amplified with HCV primers. DNA 100 bp markers (lane M), samples positive (lanes 1, 2 and 3).

Table 1. Demonstrates cutting sites of *Hinf* I, *Apa* I, *EcoR* II and *Bsh1236* I restriction enzymes for different strains of HCV as published by Bukh et al. (1992).

Genotype	Segment (bp)		
	Tube A	Tube B	Tube C
1a	97	97	129
1b	97	97	99
2a	97	174	174
2b	174	174	174
3a	129	145	99
3b	97	145	99
4	97	145	129
5	97	174	99
6	97	97	174

three tubes containing appropriate buffers. Restriction enzymes, *Apa* I, *Hinf* I, *EcoR* II and *Bsh*1236 (Fermentas, Co.) used as the following combinations: 1.*Apa* I / *Hinf* I; 2. *EcoR* II/*Hinf* I; 3. *Bsh1236* I. The other enzymes were similar to McOmish et al (1994) method.

The tubes were incubated with 1 U of the enzyme mixture for 3 h at 37°C. The digestion temperature was 37°C. If the samples could not be analyzed immediately after digestion, they were stored at -20°C before the analysis vertical 12% polyacrylamide gel electrophoresis and the digested products were heated for 5 min. After ethidium bromide staining, the DNA fragments were identified under ultraviolet light. Molecular weight 100 bp plus marker (Fermentas, Co.) and undigested PCR products was included in each analysis. The genotypes were deduced from the fragmentation patterns of the amplified DNA.

RESULTS

The 5'- UTR of 94 HCV positive serum samples were amplified and digested by appropriate restriction enzymes for genotype determination. The RFLP results revealed: 1a (54.26%), 1b (11.71%), 3a (27.66%), 2a (2.12%), 4 (4.25%). Figure 1 shows the 174 bp nested RT-PCR amplification of HCV RNA extracted from blood samples. Table 1 show fragments yielded upon restriction

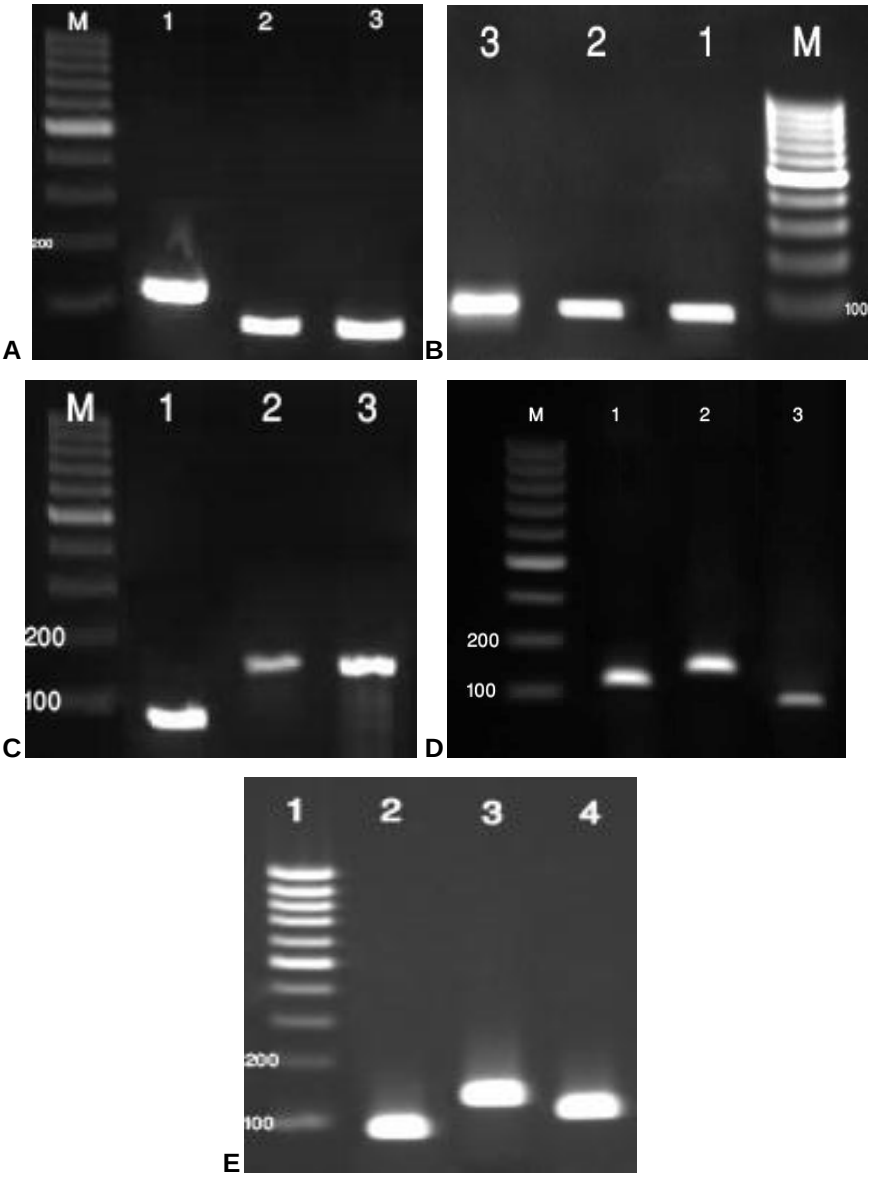


Figure 2. 12% polyacrylamide gel electrophoresis of the digestion products of the amplified DNA from different genotypes. Marker; DNA 100 bp (lane M), A : Genotype 1a (129. 97 and 97 bp). B: Genotype 1b (99, 97 and 97 bp), C: Genotype 2a (97, 174 and 174 bp), D: Genotype 3a (129, 145 and 99 bp) E: Genotype 4 (97, 145 and 129 bp).

enzyme digestion of 5'-UTR region. The cutting sites of restriction enzymes are shown (Tube A) *Apa* I/*Hinf* I; (Tube B) *EcoR* II /*Hinf* I; (Tube C) *Bsh*1236 I.

Figure 2 demonstrates the analytical polyacrylamide gel electrophoresis of HCV types 1a, 1b, 2a, 3a and 4 after digestion of the amplified DNA with the selected restriction enzymes.

Genotyping of 94 sera from patients who were either recently infected by HCV or with history of previous HCV infection and positive PCR results were performed. The results of PCR-RFLP were as follows: 1a (54.26%), 1b (11.71%), 3a (27.66%), 2a (2.12%) and 4 (4.25%). This indicates that a high percentage of HCV infected patients in Iran are infected with 1a or 3a genotypes (Table 2).

Out of 94 patients, 63 patients were male and 32 were female. Analysis of population previously infected with HCV showed that 26 patients (27 %) were less than 30 years of age, 43 patients (45.74%) between 30-50 and 25 patients (26.5%) were above 50 yr of age. The most frequent genotype in patients above 50 was 3a, while 1a genotype was more prevalent in patients under 50 years old. Hemodialysis patients and cases by known history of transfusion were known to be infected by only subtypes 1a and 3a. Also the patients fewer than 30 year old were

Table 2. Hepatitis C virus genotypes in 94 Iranian patients with RFLP method.

Genotype	Number	%
1a	51	54/26
1b	11	11/71
2a	2	2/12
3a	26	27/66
4	4	4/25
Total	94	100

infected by only subtypes 1a and 3a.

DISCUSSION

The study of viral diversity provides a better understanding of the origin and dynamics of viral infections. Genetics variants of HCV are known to be widely spread around the world. Genotypes 1, 2 and 3 are found on all countries, but in some geographical areas, such as Africa and Southeast Asia, viral isolates are highly divergent and particular genotypes or subtypes are predominant (Moller et al., 1995). These data suggest the existence of a long term endemic infection in these areas and some researchers have hypothesized that HCV have originated in such places (Simmonds et al., 1993).

Epidemiological studies in different regions of the world show the virus is distributed worldwide with prevalence varying between different countries from 0.2 up to 40%. It is clearly revealed that the incidence of HCV is higher among less developed nations. In Iran, HCV prevalence in general population is less than 1%.

In our study HCV genotypes were found, 1a (54.26%), 1b (11.73%), 3a (27.66%), 2a (2.12%), 4 (4.25%). HCV is highly variable, leading to the classification of at least six genotypes, each with several subtypes. This heterogeneity is, at least partly, responsible for lack of availability of an effective vaccine (Samimi-Rad et al., 2004). Investigators of HCV genotyping have used sequence analysis of HCV NS5, Core, E1 and 5'-UTRs. However, direct sequencing is not practical on a large scale. RFLP has been used widely for this aim, especially for screening of large number of specimens.

The use of 5'-UTR assay designed for the detection of HCV in clinical specimens provides a sensitive, standardized amplification protocol specifically designed for large-volume testing and rapid turnaround time and is also used widely for HCV genotyping by different investigators. In this report, we have focused on chronically infected group of patients, to determine the most prevalent genotypes in Iran (Ahmadi et al., 2006).

RFLP of HCV PCR positive sera and sequencing of 174 bp fragment of 5'-UTR region was used to achieve this purpose. In our study, HCV genotype 1a was the most frequent (%54/26), followed by genotype 3a (%27/66) and genotype 1b (%11/71). This is compatible with the findings of Zali et al. (2000), Samimi-Rad et al. (2004) and Ahmadi et al. (2006) in Iranian patient.

Ahmad et al. (2006) reported 1a (%52.88), 3a (%27.57) and 1b (14%) genotypes HCV among Iranian patients by RFLP method. Samimi-Rad et al. (2004) found 1a (47%), 3a (36%) and 1b (8%) genotypes HCV among Iranian patients, and revealed that genotype 1a is frequent in the South of Iran (70%), while 3a is more prevalent in the North-West of Iran (83%).

In the present study, our data showed the same result as those demonstrated by Zali et al. (2000), Samimi-Rad et al. (2004) and Ahmadi et al. (2006). Although the frequency of genotype 1a was slightly higher in theses study. Patients infected by blood products more frequently had genotype 1a (57%), while younger drug users had genotype 3a (54%) more frequently (Farshad et al., 2010).

Keyvani et al. (2007), reported HCV genotypes in Iran. In their study, genotype 1a with 39.7% had the highest frequency. Genotype 3a (27.5%) and 1b (12.1%) were the other frequent genotypes (Farshad et al., 2010). The prevalence of HCV among blood donors is less than 1% in Northern European countries (Choo et al., 1989). Higher rates have been reported in South East Asian countries, including India (1.5%), Malaysia (2.3%), and the Philippines (2.3%) (Farshad et al., 2010).

The incidence in Japan is 1.2% (Kato et al., 1990). Alarming rates of 14.5% are reported in Egypt (Farshad et al., 2010). HCV genotype 4 is common in countries such as Yemen, Kuwait, Iraq, and Saudi Arabia (Samimi-Rad et al., 2004).

However, in Turkey genotype 1b, in Pakistan genotypes 3a and 3b, in Uzbekistan 1a, 1b, 2a, 2k and 3a, in Lebanon 1g are reported to be the dominant genotypes (Pavio and Lai, 2003).

Genotype 4 is the main genotype circulating in most Arabic countries. In Bahrein 4a and in Saudi Arabia HCV genotype 4 were detected in 50% of patients and genotype 1b was found in nearly 40% of patients (Elahi et al., 2003).

Genotype information is important when HCV treatment is being considered, since some genotypes respond more favorably to the medications. Genotype also determines the length of therapy, for example, treatment for genotypes 2 and 3 requires only 24 weeks while genotypes 1 and 4 require 48 weeks (44).

In addition to treatment purposes, detection of HCV genotypes in different regions can be used for the purpose of molecular epidemiology (Pavio and Lai, 2003).

REFERENCES

Alavian SM, Gholami B, Masarrat S (2002). Hepatitis C risk factor in Iranian volunteer blood donors: A case-control study. J. Gastroentroll. Hepatol., 17(10): 1092-1097.

Alavian SM, Gholami B, Massarat S (2006). Hepatitis C, Chronic Renal Failure, Control Is Possible! Hepatitis Monthly, 6 (2): 51-52.
Alter HJ, Purcell RH, Shih JW, Melpolder JC, Houghton M, Choo QL, Kuo G (1989). Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. Engl. J. Med., 30 (321): 1494-1500.
Ahmadi pour MH, Keivani H, Sabahi F, Alavian SM (2006). Determination of HCV genotypes, in Iran by PCR-RFLP. Iranian J. Public Health, 35: 54-61.
Bukh J, Puecell RH, Miller RH (1992a). Sequence analysis of The 5' non coding region of hepatitis C virus. Proc Natl. Acad. Sci. USA., 89(11): 4942-4946.
Bukh J, Purcell RH, Miller RH (1993). At least 12 genotypes of hepatitis C virus predicted by sequence analysis of the putative E1 gene of isolates collected worldwide. Proc. Natl. Acad. Sci., USA, 90(17): 8234-8238.
Bukh J, Purcell RH, Miller RH (1992b). Importance of primer selection for the detection of hepatitis C virus RNA with the polymerase chain reaction assay. Proc. Natl. Acad. Sci., USA, 1 89): 187-191.
Bukh J, Miller RH, Purcell RH (1995). Genetic heterogeneity of hepatitis C virus: quasispecies and genotypes. Semin. Liver Dis., 15(1): 41-63.
Chan SW, McOmish, Hlmes EC, Dow B, Peutherer JF, Follet E Yap PL, Simmonds P (1992). Analysis of a new hepatitis C virus type and its phylogenetic relationship to existing variant. J. Gen. Virol., 73: 1131-1141.
Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M (1989). Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science, 244: 359-62.
Choo QL, Richman KH, Han JH, Berger K, Lee C, Dong C, Gallegos C, Coit D, Medina-Selby R , Barr PJ (1991). Genetic organization and diversity of the hepatitis C virus. Proc. Natl. Acad. Sci., USA, 88: 2451–2455.
Elahi E, Pourmand N, Chaung R, Rofoogaran A, Boisver J, Samimi Rad K, Davis RW, Ronaghi M (2003). Determination of hepatitis C virus genotype by pyrosequencing. J. Virol. Methods, 109(2): 171- 176.
Farshad pour F, Makvandi M, Samarbafzadeh AR, Jalalifar MA (2010). Datermination of hepatitis C virus genotypes among blood donors in Ahvaz, Iran. Iranian J. Med. Microbiol., 28(1): 54-56
Furione M, Simoncini L, Gatti M, Baldanti F, Revello MG, Gerna G (1999). HCV genotyping by three methods: analysis by discordant results based on sequencing. J. Clin. Virol., 13(3): 121-130.
Han JH, Shyamala V, Richman KH, Brauer MJ, Irvine B, Urdea MS, Tekamp-Olson P, Kuo G, Choo QL, Houghton M. (1991). Charachterization of the terminal regions hepatitis C viral RNA: Identification of conserved sequence in the 5 untranslated region and poly A tails at the 3 end. Proc. Natl. Acad. Sci., 88 (5): 1711-1715.
Inchauspe G, Zebedee SI, Lee DHH, Sugitani M, Nasoff M, A. M. Prince AM (1991). Genomic structure of the human prototype strain H of hepatitis C virus: comparison with American and Japanese isolates. Proc. Natl. Acad. Sci., USA, 88(22): 10292–10296.
Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugiura T, Shimotohono K (1990). Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Acad. Sci., USA, 87(24): 9524–9528.
Keyvani H, Alizadeh AH, Alavian SM, Ranjbar M, Hatami S (2007). Distribution frequency of hepatitis C virus genotypes in 2231 patients in Iran. Hepatol. Res., 37 (2): 101-103.
Kuo G, Cho QL, Alter HJ, Gitnick GL, Redeker AG, Purcell RH, Miamura T, Finestag JL, Alter M J, Stevence C E, Tegtmeier GE, Bonono F, Colombo M, Lee W S, Kuo C, Berger K, Shuster JR, Overby LR, Bradley DW, Houghton M (1989). An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science, 244(4902): 362-364.
Lee CM, Hung CH, Lu SN, Changchien CS (2008). Hepatitis C virus genotypes: Clinical relevance and therapeutic implications. Chang Gung Med. J., 31(1): 16-25.
McOmish F, Yap PL, Dow BC, Follett EAC, Seed C, Keller AJ, Cobain TJ, Krusius T, Kolho E, Naukkarinen R, Lin C, Lai C, Leong S, Medgyest GA, Hejjas M, Kiyokawa H, Fukada K, Cuypers T, Saeed AA, AL-Rasheed AM, Lin M, Simmonds P (1994). Geographical distribution of hepatitis C virus genotypes in blood donors: an international collaborative survey. J. Clin. Microbiol., 32 (4): 884-892.
McOmish F, Chan SW, Dow BC, Gillon J, Frame WD, Crawford RJ, Yap PL, Follett EA, Simmonds P (1993). Detection of three types of hepatitis C virus in blood donors: investigation of typespecific differences in serologic reactivity and rate of alanine amino transferase abnormalities. Transfusion, 33 (1): 7–13.
Moller J, Holmes EC, Jarvis LM, Yap PL, Simmonds P (1995). Investigation of the pattern of hepatitis C virus sequence diversity in different geographical regions: implications for virus classification. The international HCV collaborative study group. J. Gen. Virol., 76 (10): 2493-2507.
Mosley JW, Aach RD, Hollinger FB, Stevens CE, Barbosa LH, Nemo GJ, Holland PV, Bancroft WH, Zimmerman HJ, Kuo G, Choo QL, Houghton M (1990). Non-A Non-B hepatitis and antibody to hepatitis C virus. JAMA, 263(1): 77-78.
Okamoto H, Kurai K, Okada S, Yamamoto K, Iizuka H, Tanaka T, Fukuda S, Tsuda F, Mishiro S (1992). Full-length sequence of a hepatitis C virus genome having poor homology to reported isolates: comparative study of four distinct genotypes. Virology, 188: 331–341.
Okamato H, Okada S, Sugiyama Y, Yotsumoto S, Tanaka T, Yoshizawa H, Tsuda F, Miyakawa Y, Mayumi M (1990). The 59-terminal sequence of the hepatitis C virus genome. Jpn J. Exp. Med., 60: 167–177.
Okamato H, Kobata S, Tokita H, Inoue T, Woodfield GD, Holland PV, Al-Knawy BA, Uzunalimoglu O, Miyakawa Y, Mayumi M. (1996). A second-generation method of genotyping hepatitis C virus by polymerase chain reaction with sense and antisense primers deduced from the core gene. J Virol Methods, 57(1) 31-45.
Pavio N, Lai M (2003). The hepatitis C virus persistence: how to evade the immune system. J. Biosci., 28: 287-304.
Prescott LE, Berger A, Pawlotsky JM, Conjeevaram P, Simmonds P (1997). Sequence analysis of hepatitis C virus variants producing discrepant results with two different genotyping assays. J. Med. Virol., 53 (3): 237-244.
Samimi-Rad K, Nategh R, Malekzadeh R, Norder H, Magnius L (2004). Molecular pidemiology of hepatitis C virus in Iran as reflected by phylogenetic analysis of the NS5B region. J. Med. Virol., 74 (2): 246-252.
Simmonds P, Holmes EC, Cha TA, Chan SW, McOmish F, Irvine B, Beall E, Yap PL, Kolberg J, Urdea MS (1993). Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. J. Gen. Virol., 74 (11): 2391-2399.
Stuyver L, Rossau R, Wyseur A, Duhamel M, Vanderborght B, Van Heuverswyn H, Maertens G (1993). Typing of hepatitis C virus isolates and characterization of new subtypes using a line probe assay. J. Gen. Virol., 74: 1093-1102.
Zali MR, Mayumi M, Raoufi M, Nowroozi A (2000). Hepatitis C virus genotypes in the Islamic Republic of Iran: a preliminary study. East Mediterr. Health J., 6 (2-3): 372-377.

African Journal of Microbiology Research Vol. 5(32), pp. 5916-5922, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.961

Full Length Research Paper

Analysis of agricultural input-output based on Cobb–Douglas production function in Hebei Province, North China

Zaijian Yuan

School of Economics and Management, Hebei University of Science and Technology, Shijiazhuang, Hebei 050018, PR China. E-mail: selfsurpass@163.com.

Accepted 15 November, 2011

This study is to analyze the temporal and spatial variation of the agricultural input-output and the relation between agricultural output and input factors in Hebei Province by Cobb-Douglas production function in which cultivated area, effective irrigation area, chemical fertilizer usage, agricultural machinery power, rural electricity consumption and manpower are taken as independent variables. It proves that the agricultural output, effective irrigation area, chemical fertilizer usage, agricultural machinery power and rural electricity consumption have an upward trend from 1999 to 2008, but the cultivated area and agricultural manpower have a downward trend. In terms of spatial distribution, the agricultural input and output in the southeastern part of the province are higher than those in northwest. In the 6 input factors, the effective irrigation area has the biggest influence on agricultural output, chemical fertilizer and agricultural machinery power of the second, and other factors have relatively small influence. Therefore, Hebei should pay attention to effective use of water resources and accelerate investment in technology and mechanization to promote agriculture sustainable development.

Key words: Agricultural input-output, temporal and spatial variation, Cobb-Douglas production function.

INTRODUCTION

Agriculture is the human activity in which solar energy is utilized for the production of sugars that are used in the plant to construct carbohydrates, proteins, lipids and other compounds (Van and Rabbinge, 1997). Agricultural activity results in outputs, such as grain, potatoes, nutrient emissions and so on. Thus, agricultural production systems can be characterized by their inputs and outputs, that is, input-output combinations. Agriculture is a significant component of China's economy constitution, accounting for 11.3% it of gross domestic product (GDP) and 23.1% of it employment rate (2008). China now accounts for over 18% of global agricultural production, substantially, more than traditional agricultural production and trade heavyweights such as the European Union, the United States, India and Brazil (Huang et al., 2009).

Some research has been done on the input-output of China's agriculture: Wu et al. (2008) constructed an input-output model for Changde City of Hunan Province using EViews software by choosing the production of agriculture as output factor and labor, fertilizer inputs, dynamic mechanical total, arable land area and the area of effective irrigation as input factor. Dong (2009) analyzed the relationship between input and output of agriculture in China by the partial least squares regression method, and the result showed that: the agricultural output is mainly affected by the expenditure for household management, the financial expenditure for supporting agriculture and the fertilizer input, and is less affected by the input of cultivated area. Jiang and Zhang (2010) established an input-output model about regional agriculture of the southern Xinjiang Province by principal component analysis according to the input and output data in the years 2002 to 2007. Unfortunately, previous studies were based on a single spatial scale (for example, one county, one province or the whole country) in short time series, and less attention has focused on the agricultural input-out of different temporal and spatial scales in China's agriculture.

The main purpose of this paper is to analyze the agricultural input and output in the last 10 years. The objectives of this study are:

1. To analyze temporal and spatial variation of agricultural input-output in Hebei Province;
2. To construct an agricultural input-output potential model based on Cobb-Douglas production function;
3. To analyze the main influence factors to affect the agricultural output in Hebei.

MATERIALS AND METHODS

Hebei Province

Hebei Province (Figure 1) is 190,000 km^2 area with a population of 69 million (2009), and is divided into 11 prefecture level cities (including 138 counties). The topography consists of mountains, hills, and plateaus in the northwest, and a broad plain in the central and southeastern region. A total of 34% of the area is cropland with mainly winter wheat and summer maize double cropping system (winter wheat is cultivated from early October to early June, summer maize is grown from mid-June to late September).

The study area is located in a temperate and continental monsoon climate zone with a mean annual precipitation of 498 mm, 69% of which occurs between June and September (1999 to 2008). Mean annual temperature is 11°C (1999 to 2008). Precipitation and temperature decrease from southeast to northwest.

Data

The economic statistics data for each county from 1999 to 2008, including grain yield, grain price, cultivated area and effective irrigation area, chemical fertilizer usage, agricultural machinery power, rural electricity consumption, and rural manpower, were obtained from Hebei economic statistical yearbooks (2000 to 2009).

Agricultural input-output potential model

Agricultural input factors mainly include labor, irrigation, cultivated land, fertilizer, machinery power and electricity. Accordingly, the agricultural input-output potential model is focused on seven variables: output (Y, 10^4 Yuan), cultivated land area (Ac, ha), effective irrigation area (Ai, ha), rural electricity consumption (Ce, 10^4 kWh), agricultural machinery power (Pm, KW), chemical fertilizer usage (Fc, T) and rural manpower (Mr, person). The relation of agricultural output and input can be expressed as,

$$Y(t) = f(Ac(t), Ai(t), Ce(t), Pm(t), Fc(t), Mr(t)) \quad (1)$$

In economics, the Cobb-Douglas functional form of production functions is widely used to represent the relationship of an output to inputs, and it appears to be a good approximation to actual production (Romer, 2001). So Cobb-Douglas production function is used and it is shown as below:

$$Y(t) = A\,Ac(t)^{\alpha}\,Ai(t)^{\beta}\,Ce(t)^{\gamma}\,Pm(t)^{\delta}\,Fc(t)^{\lambda}\,Mr(t)^{\psi} \quad (2)$$

where α, β, γ, δ, λ, and ψ are the output elasticities of cultivated land area, effective irrigation area, rural electricity consumption, agricultural machinery power, chemical fertilizer usage and rural manpower respectively, and 0<α, β, γ, δ, λ, ψ<1.

If

$$A+\beta+\gamma+\delta+\lambda+\psi=1 \quad (3)$$

The reduction function has constant returns to scale in its six arguments.

However, if

$$A+\beta+\gamma+\delta+\lambda+\psi<1 \quad (4)$$

returns to scale are decreasing, and if

$$A+\beta+\gamma+\delta+\lambda+\psi>1 \quad (5)$$

returns to scale are increasing.

Equation 2 can be changed as below:

$$\ln Y(t) = \ln A + \alpha \ln Ac(t) + \beta \ln Ai(t) + \gamma \ln Ce(t) + \delta \ln Pm(t) + \lambda \ln Fc(t) + \psi \ln Mr(t) \quad (6)$$

RESULTS

Temporal variability of agricultural input-output

Trend analysis shows that the agricultural output of Hebei Province has an upward trend in the last ten years, especially after 2003 the output increased rapidly (Figure 2a); in the six input factors, except cultivated land area (Figure 2b) and rural manpower (Figure 2g) are decreasing, the other five factors are increasing (Figure 2c, d, e and f).

Spatial distribution of agricultural input-output

Based on prefecture level city spatial scale, the distribution of agricultural input and output was analyzed (Figure 3) and the results showed that: in 11 cities of Hebei, Baoding City's agricultural output value is the greatest, followed by Shijiazhuang and the least is Zhangjiakou (Figure 3a); Baoding City's cultivated land area is the biggest, followed by Zhangjiakou and the least is Qinhuangdao (Figure 3b); Baoding City's effective irrigation area is the biggest too, followed by Cangzhou and the least is Chengde (Figure 3c); Tangshan City's rural electricity consumption is the most, followed by Shijiazhuang and Zhangjiakou is the least (Figure 3d); Shijiazhuang City' agricultural machinery power is the greatest, followed by Handan and Chengde is the least (Figure 3e); Shijiazhuang City' chemical fertilizer usage is the most, followed by Handan and Baoding and Zhangjiakou is the least (Figure 3f); Baoding City' rural

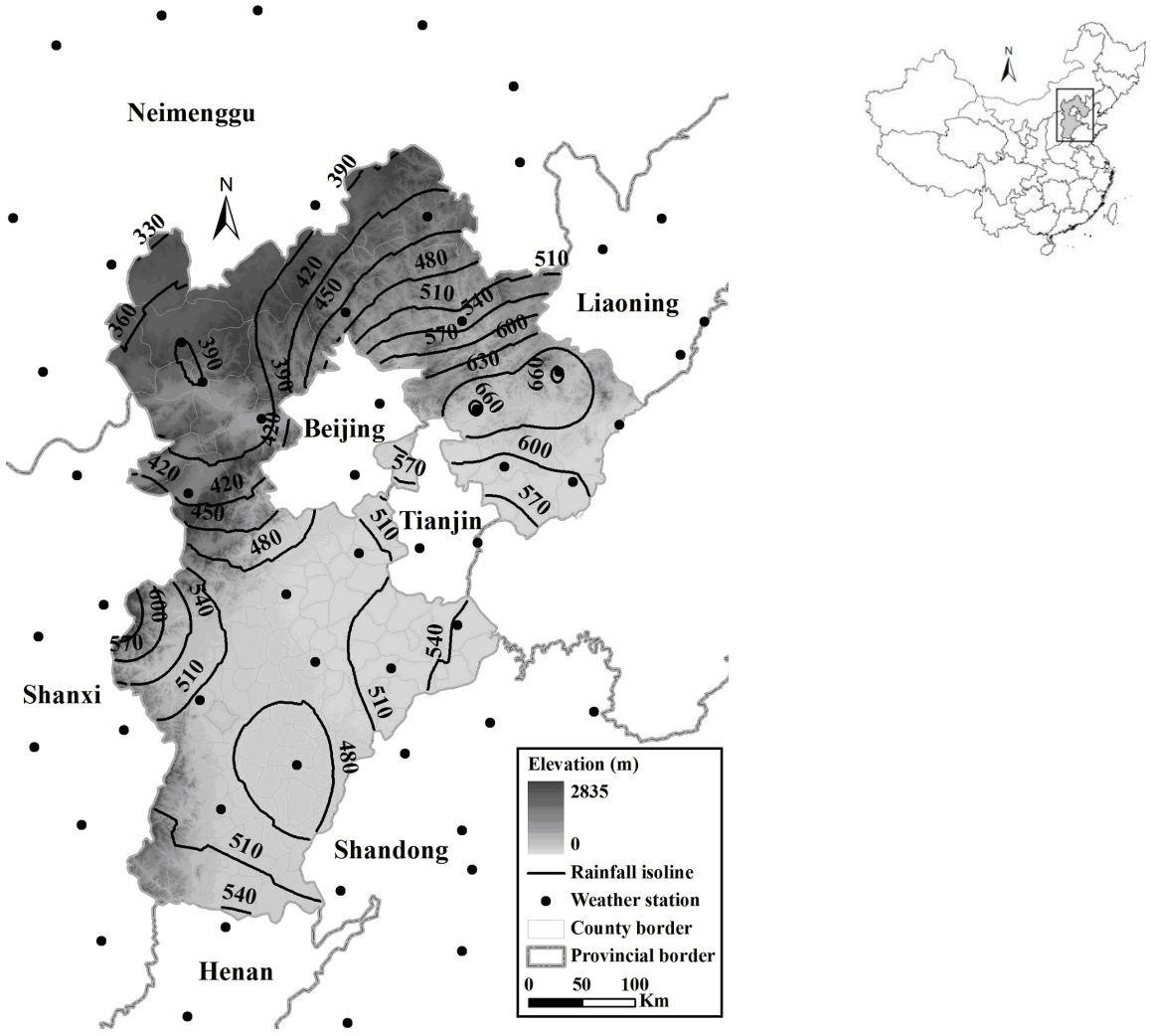


Figure 1. Geographical position of Hebei Province. The contour lines and the points indicate average precipitation (1984 to 2008) and locations of weather stations respectively.

manpower is the most, followed by Handan and Qinhuangdao is the least.

Spatial variability of agricultural input-output

In order to discuss the spatial variability of agricultural input-output, we analyzed the change of agricultural output and the six input factors from the periods of 1999 and 2003 to 2004 and 2008 for 11 cities in Hebei and the results showed that: the agricultural output is increased in all cities and the value of Cangzhou is the largest and with Qinhuangdao is the smallest (Figure 4a); the cultivated land area is decreased except Langfang (Figure 4b); the effective irrigation area is increased except Shijiazhuang, Baoding and Qinhuangdao (Figure 4c); the rural electricity consumption is increased in all cities and the value of Tangshan is the largest (Figure 4d); the agricultural machinery power is increased in all cities and the value of Handan is the largest (Figure 4e); the chemical fertilizer usage is increased except Shijiazhuang and Langfang (Figure 4f); and the rural manpower is decreased except Qinhuangdao and Zhangjiakou (Figure 4g).

Main influence factors to affect the agricultural output

According to Equation 3, an agricultural input-output potential model was constructed by regression analysis based on county spatial scale, and it is shown as follows,

$$\ln Y(t) = 7.87 - 0.14\ln Ac(t) + 0.64\ln Ai(t) - 0.07\ln Ce(t) + 0.01\ln Pm(t) + 0.09\ln Fc(t) + 0.03\ln Mr(t) \qquad F = 122.79 \quad R^2 = 0.85 \quad (n = 138) \tag{7}$$

The elasticity coefficient of effective irrigation area (with0.64) is the highest which indicates that the effective irrigation area is the biggest influent factor on the

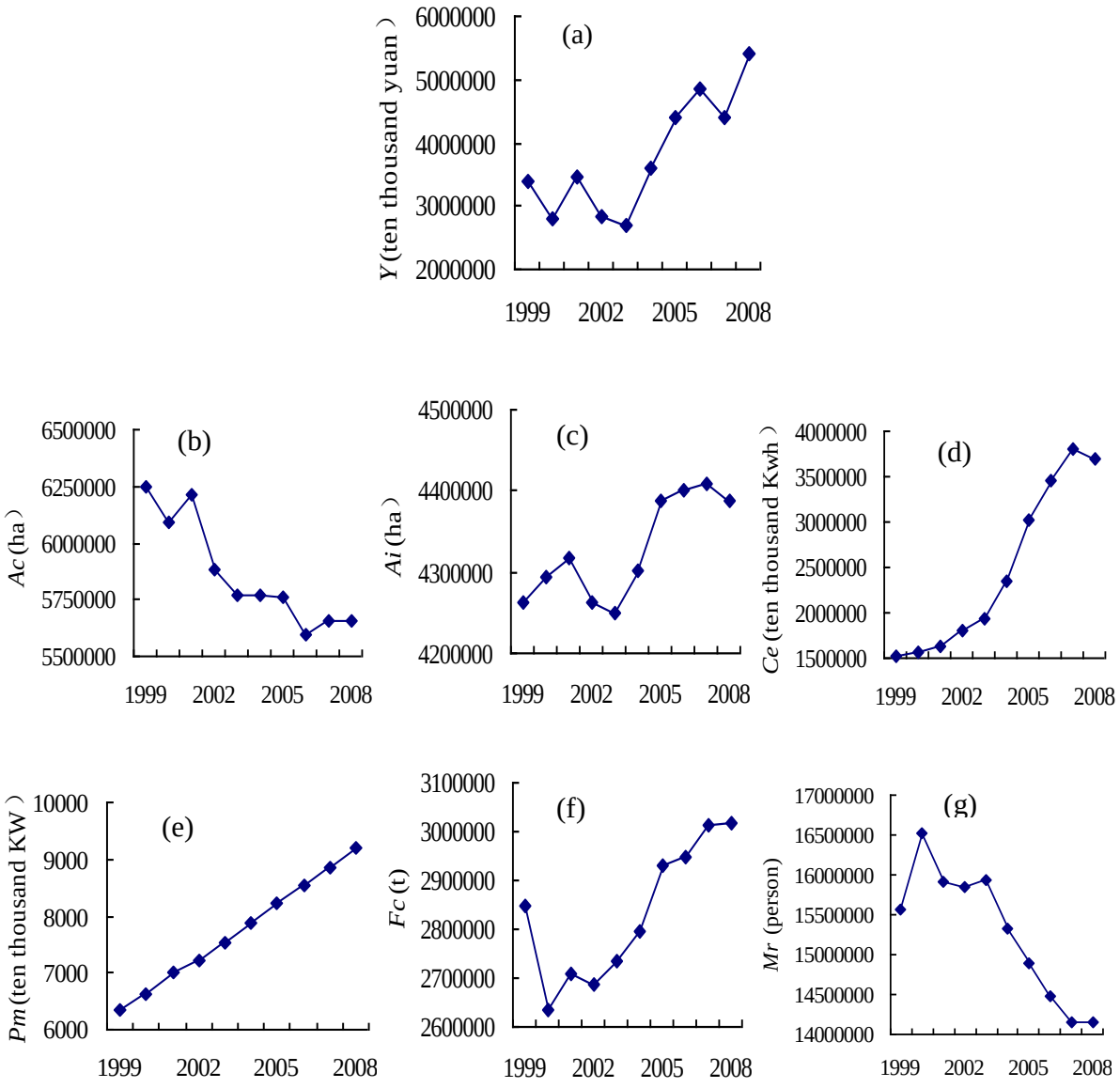


Figure 2. Annual agricultural output (a), cultivated land area (b), effective irrigation area (c), rural electricity consumption (d), agricultural machinery power (e), chemical fertilizer usage (f), and rural manpower (g) from 1999 to 2008 of Hebei Province.

agricultural output in the 6 input factors; followed by chemical fertilizer usage (with 0.09) and rural manpower (with 0.03), while cultivated land area (with -0.14), rural electricity consumption (with -0.07) and agricultural machinery power (with 0.01) have relative small influence on the agricultural output. The sum of elasticity of the six input factors is 0.56 (<1) which showed that the returns to scale is decreasing.

DISCUSSION AND CONCLUSIONS

From 1999 to 2008, the 138 counties in Hebei Province produced a total of 0.26 billion tons of grain which conversed to agricultural output value is 37.84 billion Yuan, and consumed 28.31 million tons chemical fertilizer, 247.76 billion kWh electricity and 0.77 billion KW machinery power. Annual use of cultivated land area is 5.86 million ha with 4.33 million ha is irrigated, and annual human input is 15.28 million persons. In the last ten years, the agricultural output, effective irrigation area, rural electricity consumption, agricultural machinery power and chemical fertilizer usage of Hebei Province have an upward trend, while cultivated land area and rural manpower are decreasing. In terms of spatial distribution, the agricultural input and output in the southeastern part of the province are significantly higher than those in northwest.

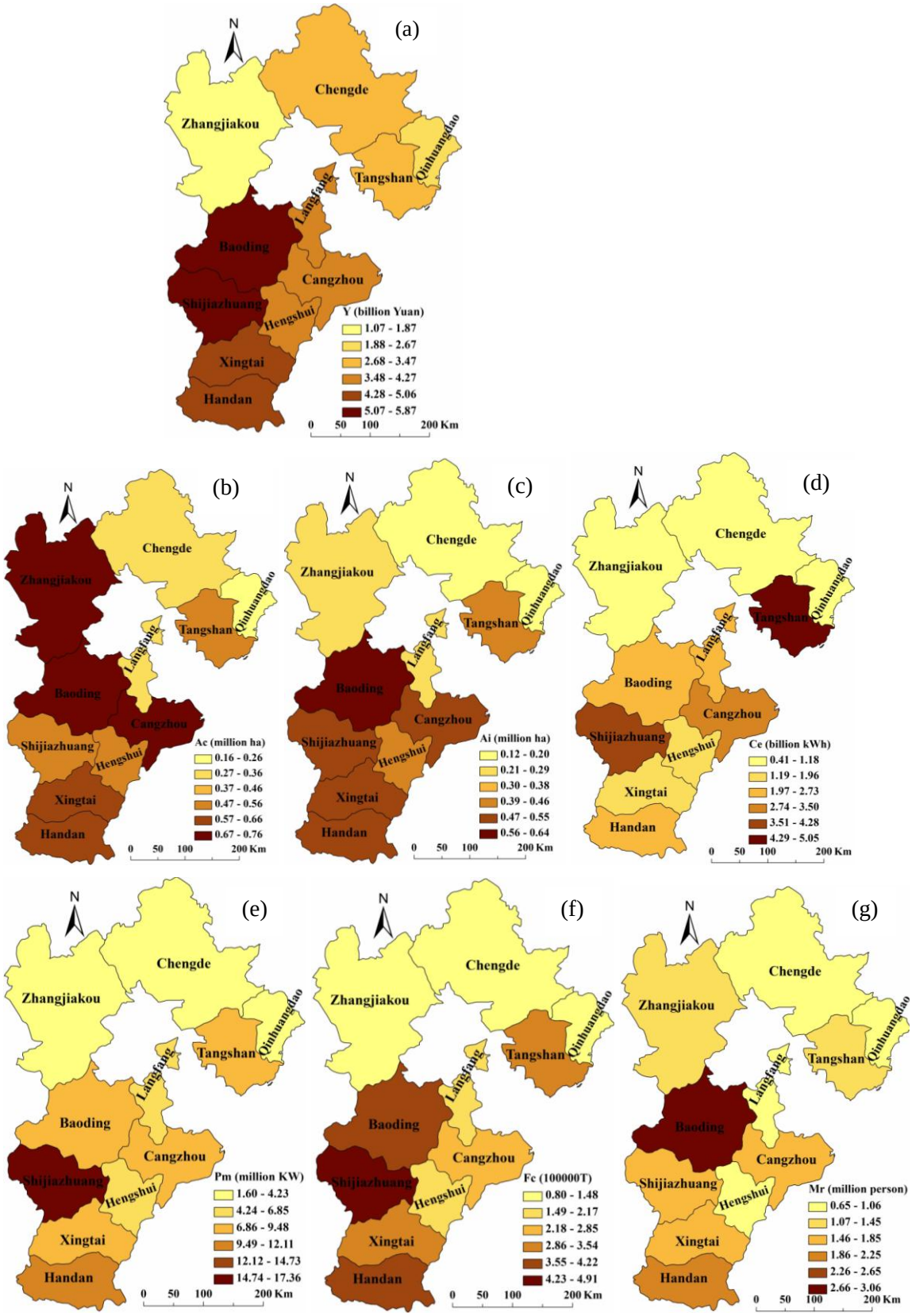


Figure 3. Annual mean agricultural output (a), cultivated land area (b), effective irrigation area (c), rural electricity consumption (d), agricultural machinery power (e), chemical fertilizer usage (f), and rural manpower (g) from 1999 to 2008 of 11 cities in Hebei Province.

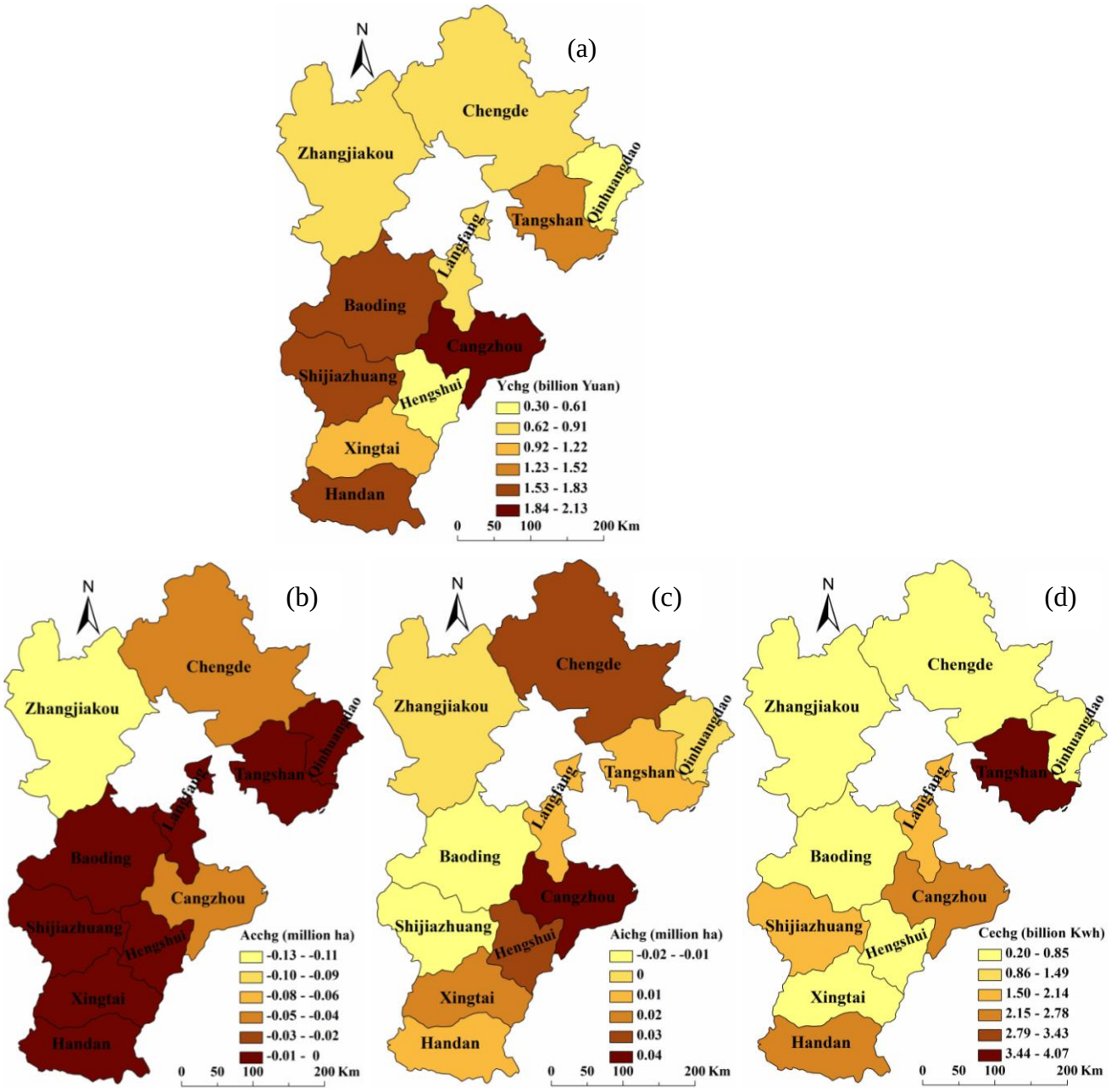


Figure 4. Change of agricultural output (a), cultivated land area (b), effective irrigation area (c), rural electricity consumption (d), agricultural machinery power (e), chemical fertilizer (f) and rural manpower (g) from the periods of 1999 to 2003 and 2004 to 2008 in Hebei Province.

At present, one of the greatest challenges in Hebei is severe water shortage, driven by strong water demands from the huge population, and rapidly expanding irrigated-agriculture, commercial and domestic sectors. Agriculture has been specifically identified as the major water user, accounting for about 70% total water use here. Water-saving especially in agriculture should be promoted by decision makers, irrigation planners and agro-scientists. Water-saving measures such as the adoption of drought-resistant crop varieties, the readjustment of planting patterns, and the use of deficit irrigation and advanced tillage and mulching techniques could reduce water use by limiting soil evaporation and plant transpiration (Zhang et al., 2008). Agricultural water-saving in combination with the long-distance water transfer and optimized water reallocation, are necessary prerequisites for comprehensively redressing the worsening water shortage problems in Hebei.

There are many factors that affect agricultural output except the above six factors, such as temperature, precipitation and crop breed. In this study, we only analyzed the relation of agricultural output and six main input factors and found in the six factors, the effective irrigation area has the biggest influence on agricultural output. This study has provided scientific information for developing efficient irrigation practices to improve crop water productivity and help to maintain sustainable development of agriculture in Hebei.

ACKNOWLEDGEMENTS

The paper was supported by the Natural Science Foundation of China (40901130) and the Instruction Project of Hebei Province Palace of Science (10457205D-2). We are also grateful to the editors and reviewers.

REFERENCES

Dong MS (2009). The relationship between input and output of agriculture in China: analysis based on partial least squares regression model. Technol. Econ., 28(1): 37-41 (in Chinese with English abstract).

Huang JK, Liu Y, Martin W, Rozelle S (2009). Changes in trade and domestic distortions affecting China's agriculture. Food Policy, 34: 407-416.

Jiang QS, Zhang XJ (2010). Regional agricultural input-output model and countermeasure for production and income increase of farmers in southern Xinjiang. J. Anhui Agric. Sci., 38(28): 15932-15935 (in Chinese with English abstract).

Van Ittersum MK, Rabbinge R (1997). Concepts in production ecology for analysis and quantification of agricultural input-output combinations. Field Crops Res., 52, 197-208.

Wu HL, He HB (2008). Research on agricultural production input-output model of Changde City. Econ. Res. guide, 11, 53-56 (in Chinese with English abstract).

Zhang XY, Chen SY, Sun HY, Pei D, Wang YM (2008). Dry matter, harvest index, grain yield and water use efficiency as affected by water supply in winter wheat. Irrig. Sci., 27 (1), 1-10.

African Journal of Microbiology Research Vol. 5(32), pp. 5923-5932, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.966

Full Length Research Paper

Management of viral disease in banana using certified and virus tested plant material

El-Dougdoug, Kh. A.[1] and M. M. El-Shamy[2]

[1]Department of Microbiology, Faculty of Agriculture, Ain Shams University, P. O. Box 68 Hadayek Shobra 11241 Cairo, Egypt.
[2]Department of Botany, Faculty of Science, Menoufia University, Egypt.

Accepted 22 November, 2011

Viruses are major limitations to cultivation. These viruses were detected by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using specific polyclonal antibodies for Banana bunchy top virus (BBTV) and Cauliflower mosaic virus (CMV). Polymerase chain reaction, (PCR) based detection of a 500 bp amplicon from BBTV infected tissues and or a 600 bp amplicon from infection Brome mosaic virus (BMV) infected tissues confirmed the presence of the viruses in these plants. As well as the major deoxyribonucleic acid (DNA) fragments of expected size, 500 bp was amplified from BBTV infected tissues and the size of the major amplified product in BMV infected tissues was 600 bp. The application of banana meristem tip (0.3 mm) is more effective for BBTV and or BMV eradication *in vitro*. Chitosan (0.12%), treatment for infected plants was more effective for BBTV and BMV eradication *in vivo*. The results proved that there is five important precautions for success of the rouging program of banana viral control included: (1) To ensure that the nursery stock is clean and free from latent virus infection via starting tissue culture seedlings virus tested or suckers treated with 0.12% chitosan, (2) Detecting infected plants periodically every month by fortnightly inspection via external symptoms and every season by a DAS-ELISA test for the presence viral diseases (3) Rouging the infected plants after two inspections. The rouged plants were destroyed by burning at the end of growing season, (4) Spraying the plants and weeds with malathion and cilecron every two weeks alternatively to kill the aphid vectors from the first April to end of growing season is December, (5) Eradication of woods and grasses from plantations (secondary virus hosts) by digging up and inherbicide. Dealing with this problem as a community.

Key words: Banana, nursery, orchard, banana bunchy top virus (BBTV), Brome mosaic virus (BMV) *in vitro, in vivo*, eradication, PCR, ELISA.

INTRODUCTION

Banana is one of the most important fruit in Egypt and cultivated in wide areas. Banana production increased to 512.5 thousand metric tons and the average crop was 11.27 to 13.71 feddan (Ministry of Agriculture, ARE, 1996) as a result of cultivation of new varieties high in production. Two viruses, Banana bunchy top virus (BBTV) and banana Cauliflower mosaic virus Bean common mosaic virus (BCMV) are considered able as one of the limiting factors in the production of banana crop. The virus causes serious losses in many countries (about 20 to 30% and occasionally reaching 50 to 80%). They are usually spread from plant to plant in nature by insect vectors, but often are also transmitted over long distances and from one crop cycle to another in vegetative planting material. The use of healthy planting materials and destruction of infected or diseased plants are essential for the control of viruses. The control strategy using pathogen-free stocks is to dilute the effects of disease through the supply of large quantities of healthy planting material. An important feature of this approach is the maintenance of pathogen-free foundation materials, which are protected from re-infection (Ang and

*Corresponding author. E-mail: magdyelshamy@yahoo.com.

Ong, 1998).

Four factors influenced the success of a rouging program for the control of BBTV. These are incubation period of the virus, relative infection rate, detection efficiency and eradication efficiency (Allen, 1978). The control measures of BBTV consist of: (a) Early disease recognition and prompt eradication of infected plants, (b) Control of its insect vector, Pentalonia nigronervasa, (c) use of virus-free planting materials and (d) Quarantine for areas that are free from disease (Nakahara, 2000).

This study aims to eradicate the banana viruses via detection of the virus from naturally infected banana plants and produced virus-free banana plants by applying two programs: Establishment of adeptic culture *in vitro* and continuation of banana plants growth and control the vectors and weeds in open field orchards and nursery.

MATERIALS AND METHODS

Source of plant materials

The banana seedlings and suckers (200 samples with 20 to 30 cm) were collected from mother plants *Musa* spp. cv. Williams, Cavendish subgroup cultivated in Meet El-Attar, Benha, Qualubia Governorate. The mother plants exhibited BBTV and Brome mosaic virus (BMV) distinct external symptoms. The selected plants were investigated depending on serological and molecular detection.

Virus detection

The viruses were detected in naturally infected and treated banana plants, in this study, by their external symptoms. This was also by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and polymerase chain reaction (PCR). The virus isolates (BBTV and BMV) were detected in banana plants by DAS-ELISA according to Clark and Adam (1977) using specific poly clonal IgG BBTV and BMV. Enzyme-Linked Immunosorbent Assays (ELISA) Kits were provided by Sanofi Sante Animal Paris, France. PCR and reverse transcriptase PCR (RT-PCR) techniques were used to detect BBTV and BMV nucleic acids in banana plants. Total DNA of infected banana leaves was extracted using a version of CTAB (Cetyl trimethyl ammonium bromide) according to Dellaporta et al. (1983). Total Ribonucleic acids (RNA) of infected banana leaves were extracted according to Gibbs and Mackenzie (1997).

PCR amplification of BBTV

Oligonucleotide primers (Table 1) for PCR were derived from the published sequences of BBTV- DNA N (Harding et al., 1993). The PCR reactions were performed according to condition and parameters described by Harding et al. (1993). The complementary DNA (cDNA) of BMV-RNA was done using the CMV/CP complementary primer (Table 1) of the conserved ultimate of CMV-RNA-3 and Avion Myeloblastosis virus reverse transcriptase (AMV-RS). The PCR reaction was performed according to conditions and cycling parameters described by Quenmada et al. (1991).

Preparation of chitosan

Chitosan with a degree of deacetylation 72% was obtained from Aldrish Chemical Company. Chitosan was dissolved in 0.05% (W/V) acetic acid and pH was adjusted to 5.5 with NaOH (Mahmoud et al., 2003).

Two programs of virus controlling in banana plants were carried out.

Establishment of an aseptic culture of banana

Micropropagtion of banana in vitro

The infected banana plants cv. Williams confirmed by PCR technique was used as source of meristems for tissue culture.

Meristems tip

The meristems were excised from shoot apices with rhizomatous base (about 2.5 cm^2 × 5 cm length). Individual meristem (the dome with 2 to 4 leaf primordial with rhizomatous base) were then excised with 0.3 mm under the binocular using fragments of a razor blade attached to a scalpel handles. The meristem tip was soaked in ethanol 76% for sec. before transferred to the culture medium. Individual meristem tip was cultured on MS starting medium. The cultured jars were incubated in growth room under incubation conditions at 3 weeks. The meristems were transferred on MS multiplication medium. Monthly subcultured of the plantlets to a fresh multiplication medium was carried on at subculture fourth. After that, the plantlets were transferred on MS rooting medium (Table 2).

Virus indexing

To be sure of virus free banana plantlets resulted by meristem tip culture confirmed using DAS-ELISA.

$$\text{Percentage of virus-free plantlets} = \frac{\text{No. of virus free plantlets}}{\text{Total No. of survived plantlets}} \times 100$$

Acclimatization

Healthy plantlets that showed negative results by DAS-ELISA were removed from the culture jars. The roots were rinsed with tap water and shortened to 3 cm. The roots of plantlets were immersed in penlate solution (1 gL^{-1}) and transferred into steam sterilized soil (peat: sand: vermiculite mixture by 2:1:1 ratio) in pots (12 cm Q) and covered with wet polyethylene for 10 days under greenhouse conditions. The air humidity exceeds 90% during the first days and decrease gradually.

Production of banana seedlings under nursery

Naturally infected banana plants cv. Williams exhibited typical bunchy top and stunting or mosaic symptoms (+ve results with DAS-ELISA) as well as healthy one was used to produce virus free banana seedlings. The corm of these plants was subjected to treatment with 0.5 to 1.0 ml of 0.12% chitosan. The treated seedlings were planted in clay soil at farm (30 × 20 m^{23}) in Meet El-Attar contains 200 lots and designed to produce virus-free banana seedlings. The distance between lots was 1 m^2. The seedlings were fixed to a 25 cm depth in the lots at the first march.

The seedling (healthy and infected) were treated with chitosan by two ways. a- Injected by syringe in the corms and with paraffin wax

Table 1. Oligonucleotide primers for BBTV and CMV.

Virus	Nucleotide sequence
BBTV Reverse	5`GCTAGGTATCCGAAGAATC-3`
Forward	5`-TCAAACATGATATGTAATTC-3`
CMV Reverse	5`-CCCCGGATCCTGGTCTCCTT-3
Forward	5`-CCCCGGATCCACATCAYAGTTTTRAGRTTCAATTC-3

Table 2. Chemical constituents of MS media for different growth stages of banana production *in vitro*.

Constituents	Medium of growth stages		
	Starting	Multiplication	Rooting
* Stock salts gL^{-1}	4.5	4.5	4.5
Sucrose gL^{-1}	30	30	30
6-benzyl amino purine mgL^{-1}	3	5	-
Nphthalene acetic acid mgL^{-1}	-	-	2
pH	5.8	5.8	5.8
Phytagel gL^{-1}	2.2	2.2	-
Agar gL^{-1}	-	-	7
Muo-inositol gL^{-1}	0.1	0.1	0.1
Culture's containers	250 ml	500 ml	500 ml
Size of media/container	30 ml^3	40 ml^3	40 ml^3

* Stock salts

days for 2 months). After 6 months data were recorded on survival before planting. B- Sprayed with chitosen periodically (each 15 percentage, average of shoot length or pant; number and leaf area, diameter of pseudostem, number and thickness of roots. Leaves tissues were obtained from the plants and tested by DAS-ELISA for the presence of BBTV and BMV.

Eradication of banana virus in orchards

The second content program was done in banana groves (about-five feddan including 200 lots) in banana groves, Meet Attar Benha, Qualubia Governorate. Mother banana plants were grown under natural conditions. The control program was based on:

Detecting infected plants periodically every month by fortnightly inspection via external symptoms and DAS-ELISA test for the presence viral diseases. BBTV and BMV rouging the infected plants after two inspections. The rogued plants were destroyed by burning at the end of growing season. The plants and weeds were sprayed with malathion cilecron with 1.5% alternatively to the end of growing season in December. Eradication of woods and grasses from plantations (secondary virus hosts) by digging up and insecticide. The percentage of virus infection was determined four times by DAS-ELISA through this a program.

RESULTS

Virus detection

It is easy to detect the viral infection on banana plants in the nursery and orchards because the external symptoms are clear and distinctive.

Symptoms of BBTV

The symptoms of infected banana with BBTV were dark green streaks on the midrib, reduced size, brittle of the leaves and gather at the top of plant making a resetting shape. Some leaves, veins are dark green colored and form a "hook" shape, as the midrib is approached (Figures 1a and b).

Symptoms of CMV

The symptoms of infected banana with Cauliflower mosaic virus (CMV) are characterized by a conspicuous molting and mosaic of the leaves, green streaks on midrib of leaves and are wavy (Figure 1c). Common observation of infected pants is stunted growth. In severe cases this is accompanied by rotting of the heart and central cylinder.

Using DAS-ELISA, indicate the presence and the percentage of BBTV, BMV and mixing of them naturally infected banana cv. Williams was 73.6; 46.6 and 20%, respectively. It also noticed that banana plants infected with BBTV or and BMV gave less number of suckers compared with corresponding healthy ones. As well as,

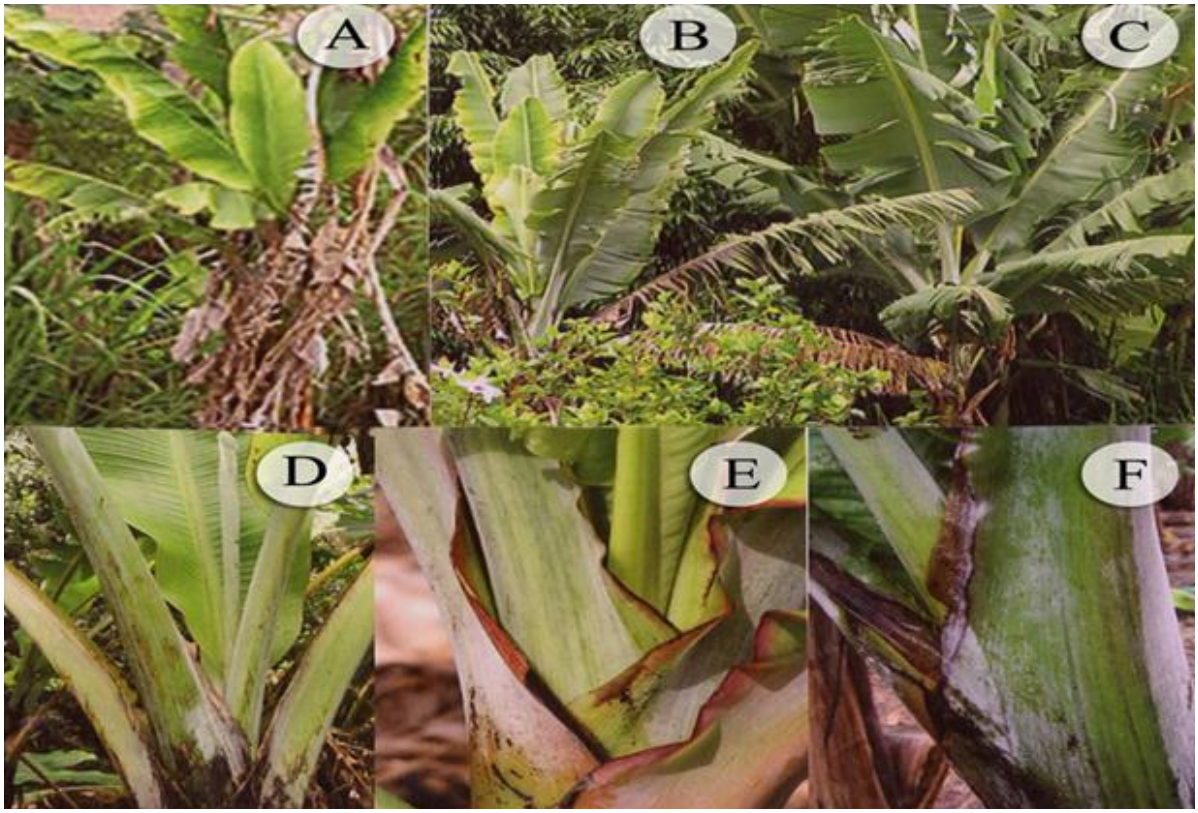


Figure 1. Naturally infected banana plants exhibited different viral symptoms. Leaves are bunched up, narrow, stiff, upright and with yellow and irregular or wavy leaf margin (A, B and E). Petioles & leaf sheaths are mottled, streaked (A, D, and F). Healthy plant (C).

the percentage of healthy suckers 26.5% resulted from infected banana plants.

Molecular detection

The total DNA of BBTV infected banana leaves was determined spectrophotometrically as 240 µg/0.02 g of tissues. The total RNA of infected banana leaves was measured spectrophotometrically as 150 ng/0.02 g of tissues as well as the purity of total DNA and Ribonucleic acid (RNA) as indicated by A_{260}/A_{280} ratio was 1.72 and 1.52, respectively.

PCR as an enzymatic procedure was used successfully to detect very low amounts of nucleic acid belonging to several plant viruses with high sensitivity and specificity. The results showed that BBTV was detected in naturally infected leaves (Figure 2A) as amplicons of expected size, 500 bp were seen in only the infected tissues (Lane 1 and 2). No amplification was obtained with uninfected banana leaves samples (Lane 3).

The CMV-RNA was reverse transcriptased by Moloney Murine Leukemia Virus (MMLV) using the oligo-dt (5`-CCCCGGATCCTGGTCTCCTT-3`) as minus sense primers.

The resulting complementary DNA (cDNA) was amplified by PCR using primers (CM1 and CM2) for coat protein gene. The PCR product was investigated using agarose gel electrophoresis analysis (Figure 2b). The size of the major amplified product in all samples was600 bp (Lane 3 and 4). This product was not detected in uninfected leaves (Lane 3)

Establishment of virus-free banana plants

Production of virus-free banana seedlings in vitro

This experiment aimed to study the meristem tip size related to virus elimination from BBTV and BMV infected banana plants.

Meristem tip sizes of 0.3, 0.5 and 1.0 mm was excised from diseased banana plants BBTV or BMV under steromicroscope. They were cultured on starting MS medium and incubated under convenient conditions. After 6 weeks post-cultivation the meristems were developed to the shoot (Figure 3) and tested against BBTV and CMV virus-using with DAS-ELISA. The

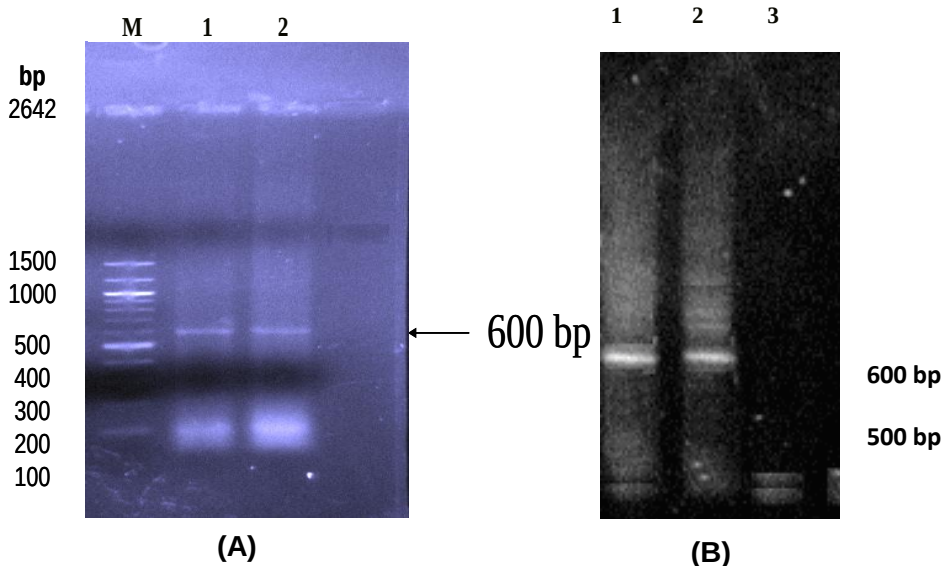


Figure 2. Agrose gel (0.7%) showing PCR products (CP gene amplified) of BBTV and BMV. (A)BBTV-PCR products Lanes 1,2 infected banana plants and Lane 3 healthy ones, (B) BMV-PCR products, Lanes 1,2 infected banana plants and Lane 3 healthy ones. M: DNA Molecular weight marker (XVI, Roche). The arrow indicates the correct size of amplified PCR products.

Figure 3. Different stages of healthy banana production *in vitro* from infected plants using (0.3 min) meristem tip culture.

Table 3. Production of virus-free banana seedlings using meristem tip culture *in vitro*.

Size of Meristem (mm)	Parameter						
	BBTV				BMV		
	Survival (%)	Virus free No.	Virus infected No.	Virus free (%)	Virus free No.	Virus infected No.	Virus free (%)
0.3 mm	75	65	10	86.66	70	5	93.33
0.5 mm	85	45	40	52.94	50	35	58.82
1.0 mm	100	10	90	10	12	88	12.00

smallest size (0.3 mm) gave 75% survival with 86.66 and 93.33% virus free (BBTV and BMV, respectively) plants. But using the size of 0.5 mm, gave 85% survival with 52.94 and 58.82 virus free plants (BBTV and BMV respectively). While using the size 1.0 mm gave 100% survival with 10 and 12% of virus free plants (BBTV and BMV, respectively; Table 3).

The explants (subculture 1) virus tested were transplanted on multiplication medium and incubated under convenient conditions. The explants (sub culture 2) were generated at subculture 6 on multiplication medium. The explants (subculture 6) were transplanted on rooting medium and incubated under convenient conditions at about 3 to 4 weeks until formation of roots.

Acclimatization

The plantlets were adapted into steam-sterilized soil in pots and grown under greenhouse conditions (Figure 3).

Production of virus-free banana seedling in nursery

All banana suckers (200 samples) were tested against BBTV and BMV in nursery through two seasons via external symptoms and confirmed with DAS-ELISA test. The results revealed that, BBTV and BMV were detected in about 75 and 45% (about 200 plants), respectively. The suckers treated by injection and spraying with 0.12% chitosan solution did not have any external viral symptoms. While, the percentage of BBTV infected plants in 1st and 2nd seasons were 9 and 5% as well as BMV infected plants were 5 and 2% respectively by using DAS-ELISA test. So, chitosan showed actively against viral infection and induction of the plants growth: Whereas, the chitosan treatment due to increasing in survival of suckers with 14.5 and 10% compared with BBTV and BMV infected plants respectively. As investigation results of suckers excised from infected banana mother plants, it was found that the BBTV or BMV infection due to reduction in suckers growth whereas reduction in morphological characters (Table 4) compared with suckers excised from healthy mother plants. Data in Table 4 show the effect of chitosan an morphological characters of infected banana plants after 6 months post-chitosan treatment *in vivo*, data revealed that, the increasing of shoot length/plant; no. of leaves/plant, leaf area; diameter of pseudostem; corm diameter; No. of roots and root diameter of infected banana suckers with BBTV or BMV compared with pre-chitosan treatment (Table 4). As well as, increasing in chlorophyll a and b and carotenoids contents of infected banana plants treated with 0.12% chitosan, than un-treated banana ones.

Continuation of growth of banana plants and virus control in orchards

The application of the procedures as described in materials and methods very effective in controlling banana viruses and producing virus free suckers. The viruses were detected via external symptoms and confirmed by DAS-ELISA test. The data in Table 5 showed that, the previous procedures due to reduction of BBTV and BMV infected banana plants were 73.66, 29.2 and 15.5% in the first year to 4.25 and 1.75% in the second year, respectively.

The present investigation also clearly indicated that, BBTV or BMV naturally infected banana mother plants gave lowest number of suckers compared with corresponding healthy plants. It also be noticed the lowest number of healthy suckers (1 and 3 suckers, respectively) compared with 6 sucker per healthy plant based on DAS-ELISA test. The percentage of virus in infection in cv. Williams was decreased season after season. It was 73.3 and 46.6% in first season(start experiment), 10.75 and 2.5% second season and 3.50 and zero% in third season for BBTV and BMV, respectively. In addition, the number of healthy suckers per plant was increased 1, 4, 5 (BBTV); 2, 5, 6 (BMV) and 3.5.6 (BBTV + BMV) at

Table 4. Effect of chitosan solution in growth of banana plants infected with BBTV and BMV*.

Morphological parameters	Treatments				
		BBTV		BMV	
	Healthy	Without chitosan	Post chitosan treatment	Without chitosan	Post chitosan treatment
Survival (%)	100	85.5	100	90	100
Shoot length/plant (cm)	75	34	65	52	70
No. of leaves/plant	5	7	8	5	6
Leaf area (cm)	450	200	350	300	400
Pseudostem diameter (cm)	20	9	15	12	17
No. of roots/plant	10	8	9	8	10
Thickness of roots	0.7	0.3	0.5	0.4	0.6
Corm diameter (cm)	22	15	18	17	19
Chlorophyll a	3.75	1.50	3.15	1.25	3.25
Chlorophyll b	2.25	0.91	2.10	0.75	1.95
Carotenoids	3.25	1.85	2.95	1.65	2.50

* First season.

Table 5. Percentage of BBTV and BMV infection in banana plants and their suckers growing under environmental conditions in orchards.

Growing seasons	Parameters				
	Percentage of infection				
	Mother plants		Suckers per plant		
	BBTV	BMV	BBTV	BMV	BBTV + BMV
1st season	73.66*	46.6	5/6**	4/6** 1/6 0/6	3/6**
2nd season	10.75*	2.5	2/6		1/6
3rd season	3.50*	0.0	1/6		0/6

* Average of sucker plant^{-1} calculated from 100 mother plants. ** No. of infected suckers/No. of total sucker plat, calculated from 100 plants based on DAS-ELISA test.

1st, 2nd season respectively, (Table 5).

On the other hand, the suckers associated of mother plants not appeared viral like symptoms as those of healthy ones in the field. The rate of infection was higher in the winter season than in the summer season. Young plants that were virus infected early after planting showed severe symptoms and never grow more than one meter at the end of growth season. Old plants that were virus infected showed no significant change in the growth. It was also observed that the suckers in the same lot may exhibit infection. But if one of the suckers is in one lot, the lot became infected after 3 month of planting. The mother plant rarely exhibited any symptoms by the end of growth

season.

The eradication of viruses in banana groves in this experiment was based on: 1) periodically detection of BBTV and BMV via external symptoms and yearly by random method using ELISA test. 2) Rouging and destruction of the infected plants outside in the groves and +ve ELISA tested. The rouged plants destroyed by burning at the end of growing season. 3) At the same time control of aphid vectors by spraying with (0.2%) an effective insecticide. 4) Eradication of weeds and grasses by using glyphosate.

DISCUSSION

BBTV and BMV are of the most widespread banana viruses in different countries of the world (Smith et al., 1990) which are concerned with banana cultivation in Egypt (Allam et al., 1988). In Egypt, the most threatening viral diseases are those caused by BBTV and BMV, these viruses are considered as limiting factors in banana production (Allam et al., 1988; El-Dougdoug et al., 2002). Banana plantations are propagated asexually by suckers since almost all of their cultivars are seedless or seed sterile. Banana diseases subjected to many natural caliseases constitute a major problem, virus diseases are serious as insect vectors are abundant and there are many alternate hosts.

We noticed that, the first symptoms of banana infected with BBTV were dark green streaks on the lower portions of the midrib of the leaf; the fresh infected leaves were brittle, reduced in the size and gather at the top of plant making a resetting shape. These symptoms reported also by Allam et al. (1988), El-Sayed (1994), Othman et al. 1996) and El-Dougdoug et al. (2006). The symptoms of BMV are characterized by a conspicuous interveinal chlorosis of the leaves. Common observation of infected (plants stunted growth. In severe cases this is accompanied by rotting of the heart leaf and central cylinder as stated by Nurhadi and Setyobudi (1998), Allam et al. (2000) and El-Dougdoug et al. (2006).

All banana plants used for starting this work were found to be infected with either BBTV or BMV and were detected in leaf samples by different methods as the biological, serological and molecular. DAS-ELISA was used to detect BBTV and BMV because of their sensitivity, specificity and speed (Clark and Adam, 1977; El-Dougdoug et al., 2002, 2006). We obtained the same conclusion.

A polymerase chain reaction (PCR) assay was developed for detection of BBTV and BMV of banana plants as well as single aphid (Xie and Hu, 1995). They added that, dot blot hybridization assay were as sensitive as ELISA, while PCR was 1.000 times more sensitive than dot blot immunoassay and ELISA. Furthermore El-Sayed (1994) found that PCR and dot blot hybridization were more sensitive than other traditional methods for the detection of BBTV and BMV.

The smallest size of meristem tip 0.3 mm more effective for elimination of banana viruses than 0.5 and 1.0 cm meristem size, whereas gave largest number of virus-free plants.

The active growing points of the plant shoot are meristem and nodel cuttings. They contain the truly meristemic cells, which surrounded by leaf primordial and primary leaves. Since more differentiated vascular tissues are found in meristem from a distance. Vascular elements of the leaf primordial are still incipient, and have not yet made contact with main strand system in the stem. Therefore, virus particles can reach the meristematic region of the apex only through cell to cell movement slowly. For this reason, virus concentration decreases in both apical axillary buds of infected plants (Perez et al., 1999).

Recent investigations of tissue culture methods proved that the number of virus free plants produced is inversely proportional to the size of the explants cultured. Thus, in some instances it is possible to excise a meristem tip free of the virus present in the infected parent and regenerate them into a healthy plant (Zilkah et al., 1992). Moreover, they indicated the importance of explants size in the successful elimination of some viruses and the role of certain host virus combination in determining the success of virus elimination (George, 1993). Virus eradication procedure depends partly on the nature of viruses; some of these viruses are more readily eliminated than others. The mechanism of such *in vitro* virus inactivation remains unknown, but whatever the explantation it seems probable that this type of virus eradication is more likely to occur if low, rather than high concentrations of virus particles are present in the tip (Walkey, 1991).

Four factors influence the success of a rouging program for the control of BBTV. These are incubation period of the virus, relative infection rates, detection efficiency and eradication efficiency (Allen, 1978). The virus activity might be prevented by alternation of the charge on the virus particles caused by polybasic substances. Such effects could be produced by chitosan (Mahmoud et al., 2003). Usually chitosan is obtained from decaylation of chitin crusts in crustacea. The chitosan possess a wide biological activity including induction of many plant defense responses such as accumulation of chitinases, production of phytoalexin (Walter-Simmons et al., 1983); synthesis of proteinase inhibitors (Walter-Simmons et al., 1989), lignification (Barber et al., 1984) and callose synthesis (Lienart et al., 1993). Chitosan was found to inhibit plant viral infections such as *alfalfa mosaic virus, tobacco mosaic virus; potato virus X, peaut stunt virus, tobacco necrosis virus; cucumber mosaic virus* and BBTV and BMV (Posppieszny et al., 1991 and Mahmoud et al., 2003). In addition, it was exhibited antiviroid action (Posppieszny, 1997). Mahmoud et al. (2003) postulated that, chitosan may be substitute for the virus particles when attached to

cell receptors. Compared to suckers, the use of plantlets grown by tissue culture has many advantages. Tissue culture plantlets are cheaper and easier to propagate and transport. They have a higher survival rate in the field. They reduce the cost of controlling foliar diseases by 50%. Their uniformity of growth makes it possible to control the time of flowering and harvesting and give a significant increase in yield and fruit quality (Hwang et al., 1984).

Tissue culture is now standard practice in banana propagation to ensure that the nursery stock is clean and free from latent infection of BBTV and BMV (Magnaye and Valnayor, 1995). For banana which is propagated vegetative, production of virus-free starting material (seedling and rhizomes) is very important to reduce yield loss due to over viral infection in field. Espino et al. (1998) reported that a control measure of BBTV consists of a) early disease recognition and prompt eradication of infected plants, b) Control of insect vector *Pentalonia nigonervosa*, c) use of virus-free planting materials and d) quarantine for areas that are free from the disease.

Quarantine laws should be revised and enforced on the import of new suckers and on the movement of infected suckers within the country. With the use of tissue culture techniques disease free planting material (suckers) can be produced and used for new plantation (Leghari, 2002). To control banana viruses removing the infected plants, control woods and aphid vectors during the growing period, use virus-free planting materials and continuous monitoring and inspection of banana plants (Calo, 2005).

It was noticed that for established plantings, effective control of the diseases requires early detection and immediate eradication of infected plants followed by replanting with disease free planting materials. As most banana virus produces character symptoms on the eaves, eradication was commonly done under symptomatology basis as stated by Nurhadi and Steyobudi (1998).

The control program can be summarized as follows: Detecting infected mother plants periodically every month by fortnightly inspection via external symptoms and ELISA test for the presence of viral diseases. Rouging the infected plants after two inspections the rouge plants were destroyed by burning at the end of growing season. Spraying the plants and weeds with malathion to kill the aphid vectors every two weeks from first of April to the end of growing season in December), eradication of weeds and grasses from plantations (secondary virus hosts) by insecticide. Quarantie regulation must be implemented. This control program is similar to that done by Allam et al. (1988).

REFERENCES

Allam EK, Abo El-Aid AF, Abd El-Wahab S, Sowailam HF Ouf RA Omar MA Abo El-Nasr MM, Deiab AE, Mohammed AS, Sadik G (1988). Annual report of pests and diseases of banana in Egypt. Academic of Technology and Scientific Research Ministry of Scientific Research, Cairo, Egypt. (22): 79-85

Allam EK,Othman A, El-Sawy A, Thabet SD (2000). Eradication of banana bunchy top virus (BBTV) and banana mosaic virus (BMV) from diseased banana plants. Annals Agric. Sci., Cairo, 45: 1: 33-48.

Allen RN (1978). The spread of bunchy top disease within a banana plantation to rouging. Plant Dis. Survey, pp. 25-26.

Ang OC, Ong L (1998). Virus and virus like diseases of banana and citrus in Malaysia: Status and Control Strategies. In: Imanaging banana and citrus diseases. Proceedings of a regional workshop on disease management of banana and citrus through the use of disease-free planting materials held in Davao City Philippines, pp. 14-16.

Barber MS, Bertram RF, Ride JP (1989). Chitin oligosaccharides elicit lignification in wounded leaves. Physiol. Molec. Plant Pathol., 34: 2-12.

Calo T (2005). All about banana in Carage. Department of Science and Technology Carage Region. Extension Butuan City, Philippine. pp. 321.

Clark MF, Adams AN (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol., 34: 475-483.

Dellaporta S, Wood J, Hicks JB (1983). A plant DNA mini preparation; Version II. Plant Mol. Biol. Rep., 1: 19-21.

El-Dougdoug KHA, Taha RM, Hazaa AK (2002). Sensitivity of two banana cultivars for banana mosaic and banana bunchy top viruses. Az. J. Microbiol., 9(56): 268-280.

El-Dougdoug KA, Hazaa MM, Hanan HA, Sabah Abo El-Maaty (2006). Eradication of banana virus from naturally infected banana plants. 1. Biological and Molecular detection of cucumber mosaic virus and banana bunchy top virus. J. Appl. Sci. Res., 2(12): 1156-1163.

El-Sayed SA (1994). Studies on viruses affecting banana in Egypt. Ph.D. Thesis, Fac. Agric., Ain Shams Univ., Cairo, pp. 125.

Espino RRC, Ligat BS, Magnaye LV, Espino TM (1998). Rehabitation of BBTV-affected Areas in the Philippines: Experiences and problems. In; managing banana and citrus diseases Proceedings of a regional workshop on disease management of banana and citrus through the use of disease free planting materials held in Davao City, Philippines, pp. 14-16.

George EF (1993). Plant propagation by tissue culture (Part 1 and 2). Exeyetics Lid Edington, pp. 1361.

Gibbs A, Mackenzie A (1997). A primer pair for amplifying part of the genome of all potyvirids by RT-PCR. J. Virological. Methods, 63: 9-16.

Harding RM, Burns tM, Hafner G, Dietzgen RG, Dale JL (1993). Nucleotide sequence of one component of banana bunchy top virus genome contains a putative replicase gene. J. Gen. Virol., 74: 323-328.

Hwang SC, Chen CL, Lin JC. Lin HL (1984). Cultivation of banana using plantlets from meristem culture. Hortic. Sci., 19: 231-233.

Leghari HB (2002). Banana bunchy top virus in Sindi DAWN Business. The Internet Edition http://DAWN.com.

Lienart Y, Gautier C, Dubois-Dauphin D, Domord A (1993). Chitin (chitosan) as elicitors in Rubus protoplasts RAA Mzzarelli ed., Chitin Enzymol., 1: 271-278.

Magnaye LV, Valmayor RV (1995). BBTV, BMV and other viruses affecting banana, in Asia and bacific. Bureau of Plant industry, Davao, Philippines INIBAP Asia and Pacific Network. C/O PCARRD, Los Bafies, Layuna Laguina. Philippines, (19): 532-544.

Mahmoud SYM, El-Dougdoug KhA, Abdel-Ghafar MH, Aly AS (2003). Inhibitory effects of chitosan and its derivatives on some plant viruses. N. Egypt. J. Microbiol., (4): 108-124.

Nakahara L (2000). In: Banana bunchy top virus found in Kohala from net Keation @ Westhawaiitoday.com.

Nurhadi A, Setyobudi L (1998). Status of banana and citrus viral diseases in Indonesia. In: Managing banana and citrus diseases. Proceedings of a regional workshop on disease among of banana and citrus through the use of disease-free planting materials held in Davao City, Philippines, 14-16.

Othman B, El-Dougdoug KhA, Sadik M (1996). Detection of banana bunchy top virus in some banana plantations in Kalubia Governorate. Annals Agric. Sci., Ain Shams Univ., Cairo, 41(2): 627-634.

Posppieszny H (1997). Antiviral activity of chitosan. Crop Protect.,

16(2): 105-106.
Posppieszny H, Chirkov S, Atabekov JG (1991). Induction of antiviral resistance in plant by chitosan Plant Sci., (79): 64-68.
Perez Tornero O, Burgas H, Egea J (1999). Introduction and establishment of apricot in vitro through the regeneration of shoots from meristem tip. *In vitro* cell. Dev. Biol. Plant, (85): 249-253.
Quenmada H, Hostis BL, Gonsalves P, Reardon IM, Heinriksan R, Herbert EL, Sten LC, Slightom JL (1991). The nucleotide sequence of the 3` terminal regions of papaya ring-spot virus strains. W and P. J. Gen. Virol., (71): 203-210.
Smith MK, Drew RA (1990). Current applications of tissue culture in plant propagation and improvement. Australian J. Plant Physiol., (17): 267-289.
Walkey DA (1991). Applied plant virology. Chapman and Hall, London, pp. 338.
Walter-Simmons M, Hadwiger L, Ryan CA (1983). Chitosan and pectic polysaccharides both induce the accumulation of the antifungal phytoalexin in pea pods and antinutrient proteinase inhibitors in tomato leaves. Biochem. Biophys. Res. Commun., (110): 194-199.
Walter-Simmons M, Hadwiger L, Ryan CA (1984). Proteinase inhibitors synthesis in tomato leaves. Plant Physiol., (76): 787-790.
Xie W, Hu J (1995). Molecular cloning sequence analysis and detection of banana bunchy top virus in Hawair, Phytopath., 85(3): 339-347.
Zilkah S, Paingersh E, rotbanum A (1992). In vitro propagation of three M x M (*Prunus avium* X *Prunus mahaleb*) Cherry rootstocks Acta Hortic., 314: 93-97.

African Journal of Microbiology Research Vol. 5(32), pp. 5933-5940, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.992

Full Length Research Paper

Scavenging and anti-fatigue activity of Wu-Wei-Zi aqueous extracts

Chen Xiang* and Zhang Guohai

College of Physical Education, Wenzhou University, Wenzhou 325035, China.

Accepted 11 November, 2011

In this study, the radical scavenging properties and the anti-fatigue activity of Wu-Wei-Zi aqueous extracts (WAE) were evaluated, respectively. Forced swimming exercise of mice was carried out after 4 weeks of WAE administration and biochemical parameters related to fatigue, such as blood lactic acid (BLA), blood urea nitrogen (BUN), hepatic glycogen (HG), superoxide dismutase (SOD) and glutathione peroxidase (GPX) contents were determined. Results showed that WAE had strong scavenging activity to superoxide anion and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. And it had significant anti-fatigue activity, which could not only increase the hepatic glycogen, SOD and GPX contents but also extend the swimming time of the mice. It indicated that WAE is worthy of further study.

Key words: Scavenging, anti-fatigue, Wu-Wei-Zi, mice.

INTRODUCTION

*Schisandra chinensis (*Turcz.) Baill*,* a perennial lignifying liana, is mainly distributed in northeastern China. Fruits from *S. chinensis* (Turcz.) Baill, is called Wu-Wei-Zi in Chinese and is a traditional Chinese herb originally recorded in Shen Nong Ben Cao Jing (over 2000 year old Herbal Pharmacopoeia in China) (Xu et al., 2008; Kim et al., 2010). The main effective constituents of Wu-Wei-Zi are essentially oil and lignans (schisandrin A, deoxyschizandrin, schisandrin B and schisandrin C) (Gao et al., 2003; Ma et al., 2007). Wu-Wei-Zi has been used for nourishing heart and stomach and strengthening immune function in traditional Chinese medicine (Huang et al., 2005; Ma et al., 2007). It is also used as a tonic for the treatment of chronic fatigue, night sweats, wasting disorders, irritability, palpitations and insomnia (Siwicki et al., 2004). Fatigue can be defined as the reversible decline in skeletal muscle contractile performance due to intense muscle activity (Mach et al., 2010). Fatigue can be divided into two categories: physical and mental fatigue. Physical fatigue is caused by such things as forced exercise or swimming, while mental fatigue is caused by sleep deprivation, etc (Chen et al., 2009).

There are several theories about the mechanisms of physical fatigue: ‘‘exhaustion theory”, ‘‘clogging theory”, ‘‘radical theory”, ‘‘homeostasis disturbance theory”, ‘‘protective inhibition theory” and ‘‘mutation theory” (Wang et al., 2008; You et al., 2011). The ‘‘radical theory” suggests that intense exercise can produce an imbalance between the body’s oxidation system and its anti-oxidation system. The accumulation of reactive free radicals will put the body in a state of oxidative stress and bring injury to the body by attacking large molecules and cell organs (Wang et al., 2008). Muscle cells contain several anti-oxidant defense mechanisms to protect themselves from free radical injury, including endogenous antioxidants and antioxidant enzymes. Moreover, many studies have indicated that exogenous dietary antioxidants can decrease the contribution of exercise-induced oxidative stress and improve the animal’s physiological condition. The reason may be that exogenous antioxidants can promote or interact with endogenous antioxidants to form a cooperative network of cellular antioxidants (Morillas-Ruiz et al., 2006; Mizuno et al., 2008; Muñoz et al., 2010). Reports from recent studies demonstrated that a large number of traditional Chinese herbs have been found to act as antioxidants by scavenging free

*Corresponding author. E-mail: tyxycx@gmail.com or tyxycx@wzu.edu.cn. Tel: +8613868551014. Fax: +86057786680835.

radicals/reactive oxygen species (ROS) and some of them have anti-fatigue activity (Yang et al., 2000; Morihara et al., 2006; Yu et al., 2010). In traditional Chinese medicine, Wu-Wei-Zi has been widely used for the treatment of chronic fatigue (Saito et al., 1974). However, this has not been validated by scientific approach. Therefore, the present study was designed to determine the radical scavenging properties of WAE and Further the anti-fatigue activity of WAE was investigated through forced swimming exercise of mice.

MATERIALS AND METHODS

Plant materials

Wu-Wei-Zi was purchased from Dongfeng Medicinal Materials Factory, Wenzhou, China and judged by Chinese Traditional Medicine Research Institute in Zhejiang and fitted for Chinese Pharmacopoeia. The voucher specimen (Number: WU-KO 0231) was deposited in the Herbarium of Wenzhou University.

Chemicals and reagents

Butylated hydroxytoluene (BHT), DPPH and nitro blue tetrazolium (NBT) were purchased from Sigma Chemicals Company (St. Louis, MO). Methionine was purchased from Sangon Biotech Company Limited (Shanghai, China). Riboflavin was purchased from Huamei Biochemical Company (Shanghai, China). BUN reagent kit was purchased from Biosino Biotechnology and Science Incoporated. (Beijing, China). BLA, HG, SOD and GPX reagent kits were purchased from Jianchen Biological Engineering Institute (Nanjing, China). All other chemicals were of analytical grade and were purchased from Zhejiang Chemical Reagent Company Limited (Hang Zhou, China).

Experiment animal

Male Kunming mice (3 month old, weighing 18 to 22 g) were obtained from the Animal Center of the Wenzhou Medical College, Wenzhou, China. To avoid possible individual's differences, only male mice were studied in this study. Because male animals have small individuals differences and there is no obvious physical characteristics when compared with female animals. The study was carried out according to the "Principles of Laboratory Animal Care" World Health Organization (WHO) (Chronicle, 1985). The mice were acclimatized for 1 week before being used for the experiment. Before and during the experiment the mice were housed under controlled environmental conditions of temperature (22 ± 2°C) and a 12 h light and dark cycle and maintained on (unless otherwise stated) standard food pellets and tap water *ad libitum*.

Preparation of Wu-Wei-Zi aqueous extracts

WAE was prepared by boiling the dried Wu-Wei-Zi with distilled water for 5 h. The extract was filtered, freeze-dried and kept at 4°C. The yield of extraction was approximately 11.62% (w/w). The direct extract was dissolved in distilled water before being used.

Superoxide anion radical scavenging assay

Superoxide anion radical scavenging activity of WAE was determined according to the method described by Prasad et al. (2010) with slight modifications. Briefly, all solutions were prepared in 0.2 M phosphate buffer (pH 7.4). The test samples at different concentrations (12.5, 25, 37.5 and 50 ug/ml) were mixed with 3 ml of reaction buffer solution (pH 7.4) containing 1.3 uM riboflavin, 0.02 M methionine and 5.1 uM NBT. The reaction solution was illuminated by exposing them to two 30 W fluorescent lamps for 20 min and the absorbance was measured at 560 nm. BHT was used as positive control. Superoxide anion radical scavenging activity (SRSA) was calculated by the following equation:

$$SRSA(\%) = \frac{1 - A_{sample}}{A_{control}} \times 100$$

Where $A_{control}$ and A_{sample} represent the absorbance of blank control group and sample group under 560 nm.

DPPH radical scavenging assay

DPPH radical scavenging activity of WAE was determined according to the method described by Schlesier et al. (2002) with slight modifications. Briefly, 0.1 ml of the samples at different concentrations (25, 50, 75 and 100 ug/ml) was mixed with 1 ml of 0.2 mM DPPH (dissolved in methanol). The reaction mixture was incubated for 20 min at 28°C under dark. The control contained all reagents without the sample while methanol was used as blank. The DPPH radical scavenging activity was determined by measuring the absorbance at 517 nm. BHT was used as positive control. DPPH radical scavenging activity (DRSA) was calculated by the following equation:

$$DRSA(\%) = \frac{1 - A_{sample}}{A_{control}} \times 100$$

Where $A_{control}$ and A_{sample} represent the absorbance of blank control group and sample group under 510 nm. In this study, scavenging activity of the sample was expressed as 50% effective concentration (EC50), which represented the sample concentration (μg/ml) inhibiting 50% of the DPPH radical activity.

Acute toxicity assay

Acute toxicity test of WAE was carried out on Kunming mice (weighing 18 to 22 g) of the either sex. Animal were randomly divided into five equal groups (n = 10) and were orally administered with the WAE at 12.5, 25, 50, 100 and 200 g/kg body weight, respectively. The following profiles of animals were observed continuously for 2 h (Li et al., 2009). Behavioral profile: Alertness, restlessness, irritability and fearfulness; Neurological profile: Spontaneous activity, reactivity, touch response, pain response and gait; Autonomic profile: Defecation and urination. After a period of 24 and 72 h lethality or death was observed.

Anti-fatigue activity assay

Anti-fatigue activity of WAE was investigated through forced swimming exercise of mice. The model was a reliable measure of anti-fatigue treatment as established in both laboratory animals and humans (Tang et al., 2007; Zhang et al., 2009). WAE was given to mice at concentrations of 0, 5, 10 and 20 g/kg body weight, named as negative control dose group (CD group), low dose treatment group (LD group), middle-dose treatment group (MD group) and

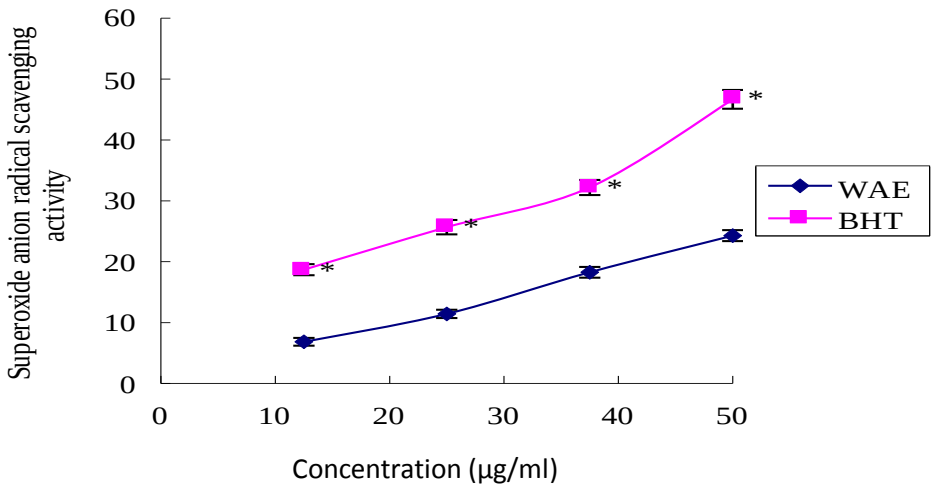


Figure 1. Superoxide anion radical scavenging activity of WAE and BHT.

high-dose treatment group (HD group), respectively. Distilled water was given to mice in CD group. Samples were orally administered into mice using a feeding atraumatic needle, once per day for 4 weeks. The doses of these treatments were chosen from literature references and pilot studies. After the final treatment with WAE, forced swimming exercise of mice was performed in acrylic plastic pool (50 × 50 × 40 cm) filled with water (25 ± 2°C) to a depth of 30 cm (Matsumoto et al., 1996).

The mice were loaded with a steel washer weighing approximately 5% of their body weight attached to the tail, which forced the mice to maintain continuous rapid leg movement (Misra et al., 2009). The mice were determined to be exhausted when they failed to rise to the surface to breathe after 10 s (Jung et al., 2007). This 10 s criterion was considered to correlate with exhaustion and was used as an indication of the maximum swimming capacity of the animal. Mice were removed at this point, before drowning. The exhaustive swimming time were observed. After the forced swimming exercise, the mice were allowed to rest for 30 min. Then they were anesthetized with ether and whole blood samples were collected in tubes by heart puncture to determine BLA, BUN, SOD and GPX contents using commercial kits. In addition, immediately after the blood had been collected, the liver was dissected out quickly from the mice, washed with physiological saline and dried with absorbent paper. Then the contents of HG were analyzed with commercial kits.

Statistical analysis

All experiments were carried out in triplicate and all the data were expressed as means ± SD (standard deviation). The significance of statistics was evaluated using Student's t-test and $P < 0.05$ was taken as being significant.

RESULTS AND DISCUSSION

Superoxide anion radical scavenging activity of WAE

Among different ROS, $O_2^{\bullet}$ is generated first. Although $O_2^{\bullet}$ is a relatively weak oxidant, it may decompose to form stronger ROS, such as singlet oxygen and hydroxyl radical ($OH^{\bullet}$), which initiates peroxidation of lipids. $O_2^{\bullet}$ is also known to initiate indirectly the lipid peroxidation as a result of the formation of H_2O_2, creating precursors of $OH^{\bullet}$ (Qiao et al., 2009). Therefore, $O_2^{\bullet}$ scavenging is extremely important to antioxidant work. Superoxide anion radical scavenging activity of WAE was presented in Figure 1. In this study, WAE exhibited an excellent superoxide anion scavenging activity, and the scavenging effects of WAE were significant stronger than that of BHT ($P < 0.05$). The maximum DPPH radical scavenging activity of WAE was 46.67±1.53%.

DPPH radical scavenging activity of WAE

DPPH free radical is a stable free radical, which has been widely accepted as a tool for estimating the free-radical scavenging activities of antioxidants (Hu et al., 2004). DPPH radical scavenging activity of WAE was presented in Figure 2, the EC50 of WAE and BHT were 21.29 and 23.23 ug/ml, respectively. In this study, WAE showed moderate DPPH radical scavenging activity. The maximum DPPH radical scavenging activity of WAE was 88.13±2.97%. Compared with the BHT, WAE performed higher activity on DPPH. In humans, muscular exercise promotes the production of ROS in the working muscle. Growing evidence indicates that ROS are responsible for exercise-induced protein oxidation and contribute highly to physical fatigue (Tharakan et al., 2005). Thus, treatments that reverse muscle fatigue may be acting through mechanisms that scavenge ROS. The present study established that WAE possessed superoxide anion and DPPH radical scavenging activity, which suggested that WAE may be beneficial to the alleviation of physical fatigue, so the WAE was used for the *in vivo* experiment in

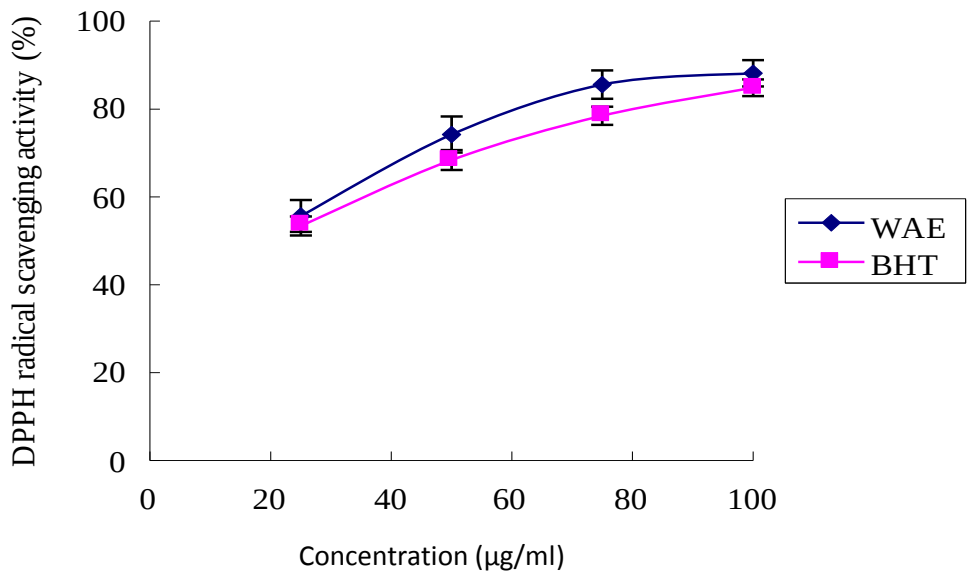


Figure 2. DPPH radical scavenging activity of WAE and BHT.

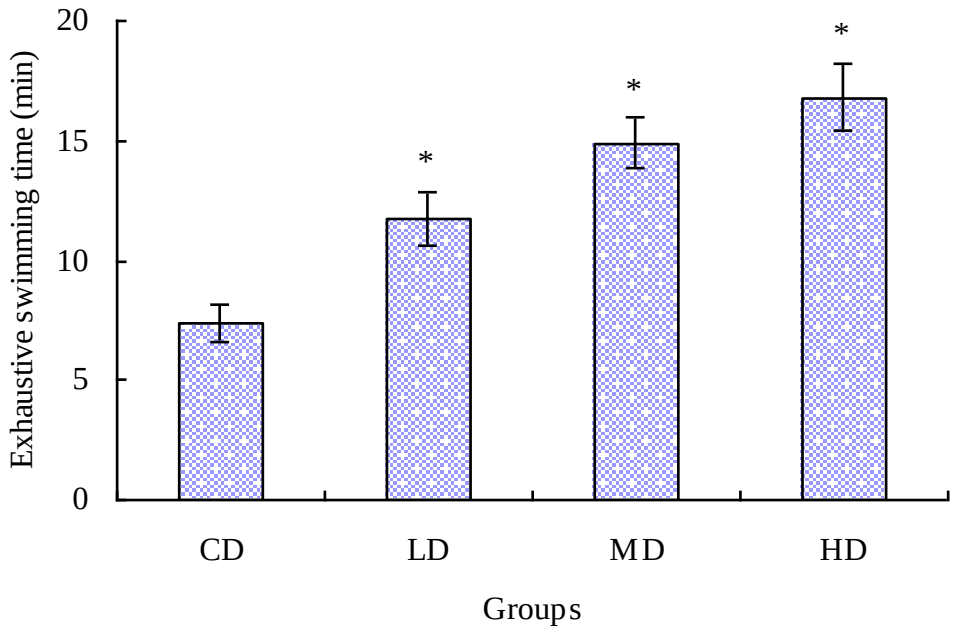


Figure 3. Effect of WAE on the exhaustive swimming time of mice.

mice to estimate the anti-fatigue activity.

Acute toxicity test

Acute toxicity test revealed the non-toxic nature of the WAE. There was no lethality or any toxic reactions found at any of the doses selected until the end of the study period.

Effect of WAE on the exhaustive swimming time of mice

Swimming to exhaustion is an experimental exercise model to evaluate anti-fatigue activity; it works well for evaluating the endurance capacity of mice and gives a high reproducibility (Zhang et al., 2006; Yao and Li, 2010; You et al., 2011). Effects of WAE on the exhaustive swimming time of mice were presented in Figure 3. There

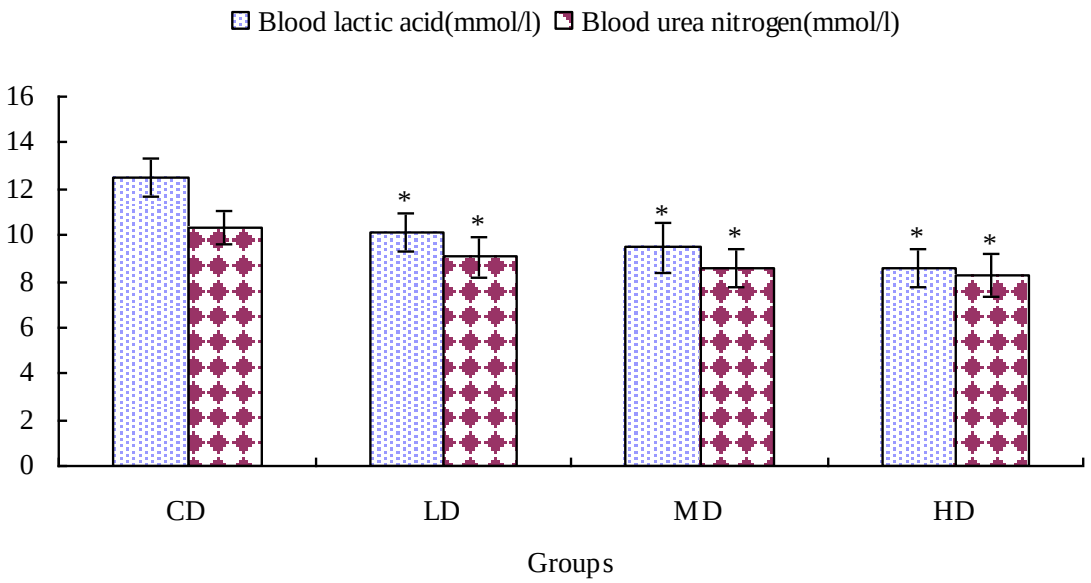


Figure 4. Effect of WAE on the blood lactic acid and blood urea nitrogen contents of mice.

are significant differences in the exhaustive swimming time between the negative control group and each treatment group. The swimming time to exhaustion of the CD, LD, MD and HD groups were 7.4 ± 0.8, 11.7 ± 1.1, 14.9 ± 1.0 and 16.8 ± 1.4 min, respectively. Thus, the exhaustive swimming time of the LD, MD and HD groups were significantly longer than that of the CD group ($P < 0.05$). This result suggested that WAE had significant anti-fatigue activity. Fatigue is one of the most frequent physiological reactions. It often occurred in aging, cancer, depression, Human immunodeficiency virus (HIV) infection, multiple sclerosis and Parkinson's disease (Tharakan et al., 2006). However, there were very few pharmacological drugs or therapies available for the treatment of fatigue (Uthayathas et al., 2007). Natural products not only could improve athletic ability, postpone fatigue and accelerate the elimination of fatigue in human beings, but also had few side effects (Kim et al., 2001). Data from previous investigations indicated that some traditional Chinese herbs extracts have anti-fatigue activity, including *Radix Rehmanniae Preparata* (Tan et al., 2011), *Ganoderma lucidum* (Guo et al., 2011), *Cordyceps sinensis* (Kumar et al., 2011), *Acanthopanax senticosus* (Zhang et al., 2010; Huang et al., 2011), *Ginseng* (Wang et al., 1983; Zhao et al., 2009; Wang et al., 2010), *Eucommia* (Deyama et al., 2001), *Rhodiola rosea* (Panossian et al., 2007; Olsson et al., 2009), *Cynomorium songaricum* (Yu et al., 2010), *Morinda officinalis* (Zhang et al., 2009), etc. In the present study, it has also been shown that WAE enhanced the swimming capacity by lessening of fatigue in mice. To explore the mechanism of anti-fatigue activity, some biochemical parameters such as BLA, BUN, HG, SOD and GPX contents in the mice were determined after they have swam for 30 min.

Effect of WAE on the BLA and BUN contents of mice

BLA and BUN are important blood biochemical parameters related to fatigue (Xu and Luo, 2001). BLA is the glycolysis product of carbohydrate under an anaerobic condition and glycolysis is the main energy source for intense exercise in a short time (Ding et al., 2011). According to the study of Wilber (1959), violent swimming to exhaustion results in a significantly elevated BLA contents and the rate at which BLA accumulates in the blood showed an inverse relation to swimming time. Therefore, blood lactate acid represents the degree of fatigue after exercise and the condition of recovery (Wang et al., 2006). As shown in Figure 4, the BLA contents in the CD, LD, MD and HD groups were 12.48 ± 0.86, 10.13 ± 0.79, 9.47 ± 0.96 and 8.56 ± 0.84 mmol/l, respectively. Thus, the BLA contents in all treatment groups (LD, MD and HD groups) were lower than that in the CD group (P <0.05). This result suggests that WAE can effectively retard and lower the BLA produced and postpone the appearance of fatigue. BUN, which is the metabolism outcome of protein and amino acid, is a sensitive index to evaluate the bearing capability when human bodies suffer from a physical load (Huang et al., 2011). Wu (1999) pointed out that the BUN in the blood rises significantly for a long-run athlete after exercise. In other words, the worse the body is adapted to exercise tolerance, the more significantly the BUN contents increase. Therefore, BUN is another index of fatigue status. As shown in Figure 4, the BUN contents in the CD, LD, MD and HD groups were 10.34 ± 0.71, 9.06 ± 0.86, 8.57 ± 0.85 and 8.25 ± 0.93 mmol/l, respectively. Thus, the BUN contents of the LD, MD and HD groups were significantly lower than that of the CD group ($P < 0.05$). This result suggests that WAE may reduce catabolic decomposition

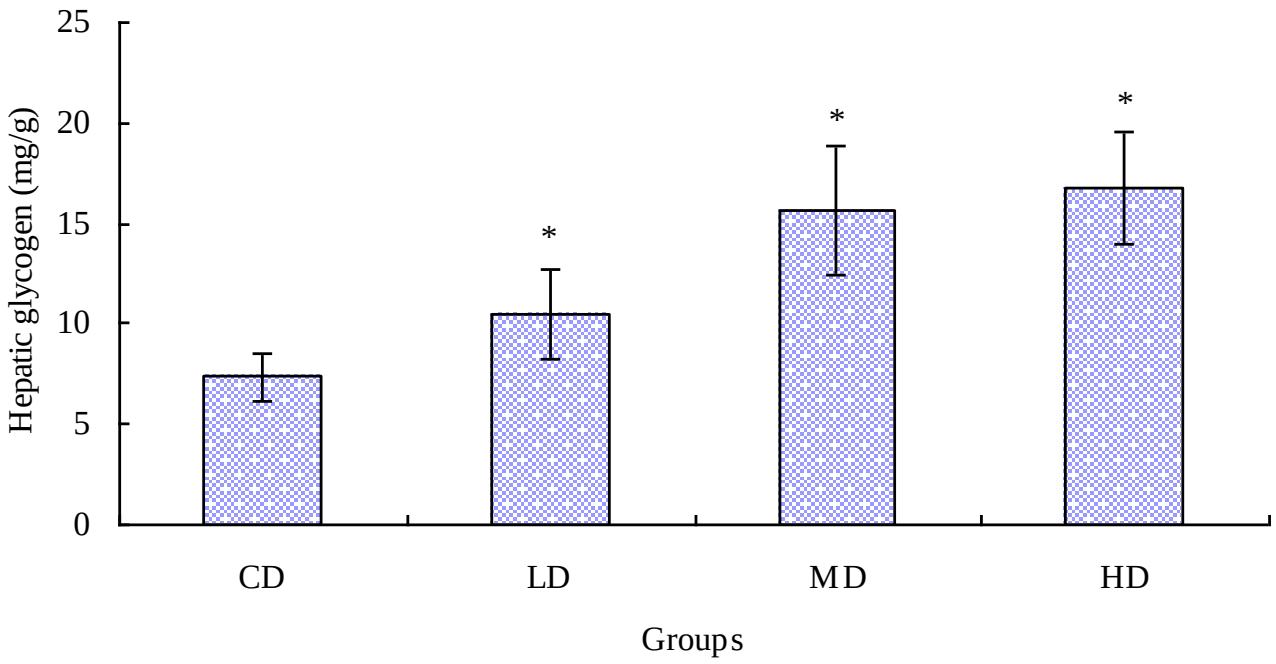


Figure 5. Effect of WAE on the hepatic glycogen contents of mice.

of protein for energy.

Effect of WAE on the hepatic glycogen contents of mice

The liver converts lactate back to glycogen and releases glycogen into the blood. Energy for exercise is derived initially from the breakdown of glycogen and later from circulation glycogen released by the liver and from non-esterified fatty acids (Dorchy, 2002). So increasing the HG storage conduces to enhancing the endurance capacity and locomotory capacity (Tang et al., 2008). HG is a sensitive parameters related to fatigue. Effects of WAE on the HG contents of mice were presented in Figure 5. There are significant differences in the HG contents between the negative control group and each treatment group. The HG contents of the CD, LD, MD and HD groups were 7.35 ± 1.21, 10.48 ±2.17, 15.69±3.19 and 16.74±2.82 mg/g, respectively. Thus, the HG contents of the LD, MD and HD groups were significantly higher than that of the CD group ($P < 0.05$). This result suggests that the anti-fatigue activity of WAE may be related to the improvement in the metabolic control of exercise and the activation of energy metabolism (Wang et al., 2006).

Effect of WAE on the SOD and GPX contents of mice

It has been demonstrated that ROS are responsible for exercise-induced protein oxidation and contribute strongly to muscle fatigue (You et al., 2009). To protect against exercise-induced oxidative injury, muscle cells contain complex endogenous cellular defense mechanisms (enzymatic and non-enzymatic antioxidants) to eliminate ROS (Powers et al., 2004). Antioxidant agents such as reduced glutathione (GSH), vitamin C, E and enzymes such as SOD, catalase (CAT) and GPX, are important factors (Hassan and Schellhorn, 1988). SOD reduces superoxide to hydrogen peroxide; and GPX reduces hydrogen peroxide from the SOD reaction to water. In addition, GPX can reduce lipid peroxides directly (Finaud et al., 2006). Growing evidence indicates that the improvement in the activities of antioxidant enzymes can help to fight against fatigue (You et al., 2011). As shown in Figure 6, the SOD contents of the CD, LD, MD and HD groups were 96.54 ± 7.84, 146.81 ± 8.93, 163.48 ± 11.26 and 171.29 ± 13.21 U/mg.pro, respectively. And the GPX contents of the CD, LD, MD, and HD groups were 4.68 ± 0.94, 8.37 ± 1.23, 10.45 ± 1.17 and 12.67 ± 1.36 U/mg.pro, respectively. Thus, SOD and GPX contents of the LD, MD and HD groups were significantly higher than that of the CD group ($P < 0.05$). This result suggests that WAE can promote increase in the activities of these antioxidant enzymes and again supporting that WAE has anti-fatigue activity.

Conclusions

WAE had strong scavenging activity to superoxide anion and DPPH radical. And it had significant anti-fatigue

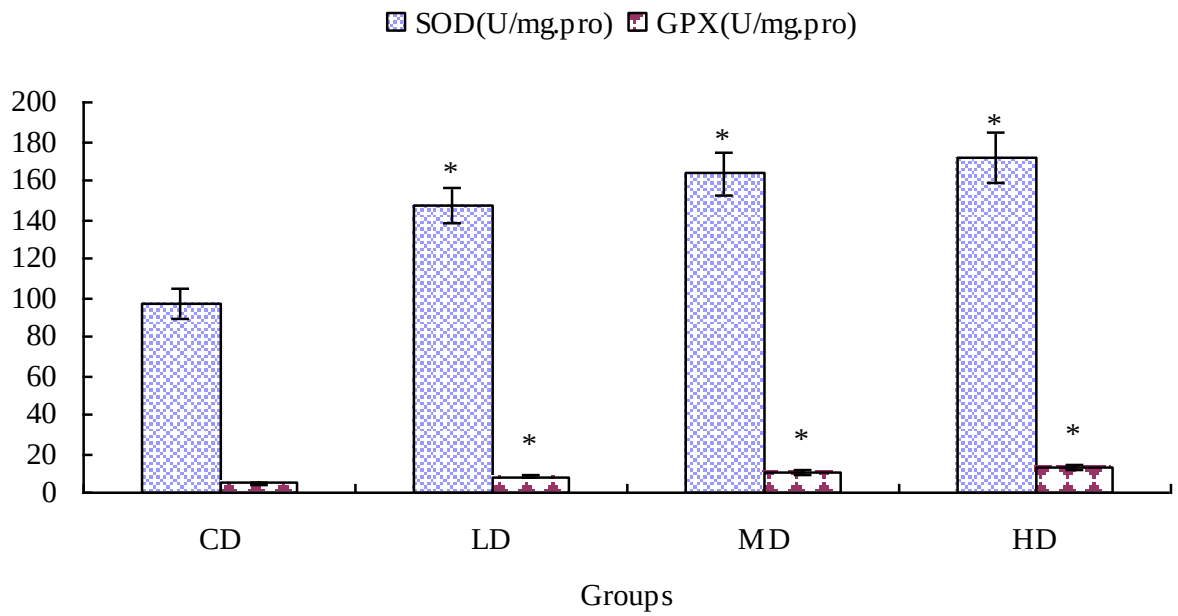


Figure 6. Effect of WAE on the superoxide dismutase and glutathione peroxidase contents of mice.

activity, which could not only extend the swimming time of the mice, increase the hepatic glycogen and antioxidant enzymes (SOD and GPX) contents, but also decrease the BLA and BUN contents. However, further research needs to be carried out to evaluate its antioxidant and anti-fatigue activity at cellular and molecular levels.

REFERENCES

Chen JR, Wang TJ, Huang HY, Chen LJ, Huang YS, Wang YJ, Tseng GF (2009). Fatigue reversibly reduced cortical and hippocampal dendritic spines concurrent with compromise of motor endurance and spatial memory. Neuroscience, 161(4):1104-1113.

Deyama T, Nishibe S, Nakazawa Y (2001). Constituents and pharmacological effects of Eucommia and Siberian ginseng. Acta Pharmacol. Sin., 22(12):1057-1070.

Ding JF, Li YY, Xu JJ, Su XR, GaoX, Yue FP (2011). Study on effect of jellyfish collagen hydrolysate on anti-fatigue and anti-oxidation. Food Hydrocol., 25: 1350-1353.

Dorchy H (2002). Sports and type I diabetes: personal experience. Rev Med. Brux., 23(4):A211-217.

Finaud J, Lac G, Filaire E (2006). Oxidative stress: Relationship with exercise and training. Sport. Med., 36: 327–358.

Gao JP, Yu YQ, Chen DF (2003). Determination of lignans of Schisandra rubriflora by HPLC. Chin. Tradit. Herbal Drug., 34: 950-951.

Guo Y, Luo X, Yu M, Zheng L (2011). Active ingredients and efficacies of *Ganoderma lucidum* cultivated on non-medicinal parts of Chinese medicinal herbs. Wei Sheng Wu Xue Bao, 51(6):764-768

Hassan HM, Schellhorn HE (1988). Superoxide dismutase an antioxidant defense enzyme. In: Cerruti PA, Fridovich I, McCord JM (eds). Oxyradicals in Molecular Biology and Pathology. Alan R. Liss Inc, New York, pp. 183–93.

Hu F, Lu R, Huang B, Liang M (2004). Free radical scavenging activity of extracts prepared from fresh leaves of selected Chinesemedicinal plants. Fitoterapia, 75(1):14-23.

Huang LZ, Huang BK, Ye Q, Qin LP (2011). Bioactivity-guided fractionation for anti-fatigue property of Acanthopanax senticosus. J. Ethnopharmacol., 133(1):213-219.

Huang T, Shen P, Shen Y (2005). Preparative separation and purification of deoxyschisandrin and gamma-schisandrin from Schisandra chinensis (Turcz.) Baill by high-speed counter-current chromatography. J. Chromatogr A., 1066(1-2): 239-242.

Jung KA, Han D, Kwon EK, Lee CH, Kim YE (2007). Antifatigue effect of *Rubus coreanus* Miquel extract in mice. J. Med. Food, 10(4):689-693.

Kim KM, Yu KW, Kang DH, Koh JH, Hong BS, Suh HJ (2001). Anti-stress and anti-fatigue effects of fermented rice bran. Biosci Biotechnol Biochem., 65(10):2294-2296.

Kim MG, Lee CH, Lee HS (2010). Anti-platelet Aggregation Activity of Lignans Isolated from Schisandra chinensis Fruits. J. Korean Soc. Appl. Biol. Chem., 53(6): 740-745.

Kumar R, Negi PS, Singh B, Ilavazhagan G, Bhargava K, Sethy NK (2011). Cordyceps sinensis promotes exercise endurance capacity of rats by activating skeletal muscle metabolic regulators. .J Ethnopharmacol., 136(1):260-266.

Li F, Li Q, Gao D, Peng Y (2009). The optimal extraction parameters and anti-diabetic activity of flavonoids from Ipomoea batatas leaf. Afr J Tradit. Complement. Altern. Med., 6(2):195-202.

Ma DY, Liu YQ, Liu SW, Li QD, Shan AS (2007). Influence of Ligustrum lucidum and Schisandra chinensis Fruits on Antioxidative Metabolism and Immunological Parameters of Layer Chicks. Asian-Aust. J. Anim. Sci., 20(9): 1438-1443.

Mach J, Midgley AW, Dank S, Grant RS, Bentley DJ (2010). The Effect of Antioxidant Supplementation on Fatigue during Exercise: Potential Role for NAD+(H). Nutrients, 2: 319-329.

Misra DS, Maiti R, Ghosh D (2009). Protection of swimming-induced oxidative stress in some vital organs by the treatment of composite extract of Withania somnifera, Ocimum sanctum and Zingiber officinalis in malerat. Afr. J. Tradit. Complement. Altern. Med., 6(4):534-543.

Morihara N, Ushijima M, Kashimoto N, Sumioka I, Nishihama T, Hayama M, Takeda H (2006). Aged garlic extract ameliorates physical fatigue. Biol. Pharm. Bull., 29(5):962-966.

Morillas-Ruiz JM, Villegas García JA, López FJ, Vidal-Guevara ML, Zafrilla P (2006). Effects of polyphenolic antioxidants on exercise-induced oxidative stress. Clin. Nutr., 25(3):444-453.

Muñoz ME, Galan AI, Palacios E, Diez MA, Muguerza B, Cobaleda C, Calvo JI, Aruoma OI, Sanchez-Garcia I, Jimenez R (2010). Effect of an antioxidant functional food beverage on exercise-induced oxidative stress: a long-term and large-scale clinical intervention study.

Toxicology, 278(1):101-111.
Olsson EM, von Schéele B, Panossian AG (2009). A randomised, double-blind, placebo-controlled, parallel-group study of the standardised extract shr-5 of the roots of Rhodiola rosea in the treatment of subjects with stress-related fatigue. Planta Med., 75(2):105-112.
Panossian A, Hambardzumyan M, Hovhanissyan A, Wikman G (2007). The adaptogens rhodiola and schizandra modify the response to immobilization stress in rabbits by suppressing the increase of phosphorylated stress-activated protein kinase, nitric oxide and cortisol. Drug Target Insights, 2:39-54.
Powers SK, DeRuisseau KC, Quindry J, Hamilton KL (2004). Dietary antioxidants and exercise. J. Sports Sci., 22(1):81-94.
Prasad KN, Xie HH, Hao J, Yang B, Qiu SX, Wei XY (2010). Antioxidant and anticancer activities of 8-hydroxypsoralen isolated from wampee [*Clausena lansium* (Lour.) Skeels] peel. Food Chem. Qiao D, Ke C, Hu B, Luo JG, Ye H, Sun Y, Yan XY, Zeng XX (2009). Antioxidant activities of polysaccharides from Hyriopsis cumingii. Carbohyd. Polym., 78: 199–204.
Schlesier K, Harwat M, Böhm V, Bitsch R (2002). Assessment of antioxidant activity by using different in vitro methods. Free Radic. Res., 36(2): 177-87.
Siwicki AK, Skopińska-Różewska E, Nartowska J, Małaczewska J, Wojcik R, Sommer E, Trapkowska S, Filewska M, Skurzak H (2004). Effect of Immunostim plus – a standardized fixed combination of Schizandra chinensis with Eleutherococcus senticosus extracts on granulocyte activity and tumor angiogenesis in mice. Bull. Vet. Inst. Pulawy., 48: 489-492.
Tan W, Yu KQ, Liu YY, Ouyang MZ, Yan MH, Luo R, Zhao XS (2011). Anti-fatigue activity of polysaccharides extract from Radix Rehmanniae Preparata. Int. J. Biol. Macromol., Epub ahead of prin.
Tang J, Hu ZY, Chen XW (2007). Free radical scavenging and antioxidant enzymes activation of polysaccharide extract from Nostoc sphaeroides. Am. J. Chin. Med., 5(5):887-896.
Tang W, Zhang Y, Gao J, Ding X, Gao S (2008). The anti-fatigue effect of 20(R)-ginsenoside Rg3 in mice by intranasally administratio. Biol. Pharm. Bull., 31(11):2024-2027.
Tharakan B, Dhanasekaran M, Brown-Borg HM, Manyam BV (2006). Trichopus zeylanicus combats fatigue without amphetamine-mimetic activity. Phytother. Res., 20(3):165-168.
Tharakan B, Dhanasekaran M, Manyam BV (2005). Antioxidant and DNA protecting properties of anti-fatigue herb Trichopus zeylanicus. Phytother Res., 19: 669-673.
Uthayathas S, Karuppagounder SS, Tamer SI, Parameshwaran K, Degim T, Suppiramaniam V, Dhanasekaran M (2007). Evaluation of neuroprotective and anti-fatigue effects of sildenafil. Life Sci., 81(12):988-992.
Wang BX, Cui JC, Liu AJ, Wu SK (1983). Studies on the anti-fatigue effect of the saponins of stems and leaves of panax ginseng (SSLG). J. Tradit. Chin. Med., 3(2):89-94.
Wang J, Li S, Fan Y, Chen Y, Liu D, Cheng H, Gao X, Zhou Y (2010). Anti-fatigue activity of the water-soluble polysaccharides isolated from Panax ginseng C. A. Meyer. J. Ethnopharmacol., 130(2):421-423
Wang JJ, Shieh MJ, Kuo SL, Lee CL, Pan TM (2006). Effect of red mold rice on antifatigue and exercise-related changes in lipid peroxidation in endurance exercise. Appl. Microbiol. Biotechnol., 70(2):247-253.
Wang L, Zhang HL, Lu R, Zhou YJ, Ma R, Lv JQ, Li XL, Chen LJ, Yao Z (2008). The decapeptide CMS001 enhances swimming endurance in mice. Peptides, 29(7):1176-1182.
Wilber CG (1959). Some factors which are correlated with swimming capacity in guinea pigs. J. Appl. Physiol., 14(2):199-203.
Wu IT (1999). The effects of serum biochemical value with different beverage to replenish and intermittent exercise in high intensity. Tahan Jr. College Engr. Business J., 13: 387-400.
Xu GD, Luo QM (2001). A study on the relationship between blood acid lactate in motion and hemoglobin saturation density-a new hemoglobinmetry. Wuhan Ti Yu Xue Yuan Xue Bao., 35(3): 40–42.
Xu M, Wang G, Xie H, Huang Q, Wang W, Jia Y (2008). Pharmacokinetic comparisons of schizandrin after oral administration of schizandrin monomer, Fructus Schisandrae aqueous extract and Sheng-Mai-San to rats. J. Ethnopharmacol., 115(3): 483-488.
Yang QS, Zhao HL, Chen L (2000). Studies on senility retarding effect of qiangshengye liquid. Zhongguo Zhong Yao Za Zhi. 25(6):367-369.
Yao LQ, Li FL (2010). Lycium barbarum polysaccharides ameliorates physical fatigue. Afr. J. Agric. Res., 5: 2153–2157.
You LJ, Zhao MM, Regenstein JM, Ren JY (2011). In vitro antioxidant activity and in vivo anti-fatigue effect of loach (Misgurnus anguillicaudatus) peptides prepared by papain digestion. Food Chem., 124: 188–194.
You Y, Park J, Yoon HG, Lee YH, Hwang K, Lee J, Kim K, Lee KW, Shim S, Jun W (2009). Stimulatory effects of ferulic acid on endurance exercise capacity in mice. Biosci. Biotechnol. Biochem., 73(6):1392-1397.
Yu FR, Liu Y, Cui YZ, Chan EQ, Xie MR, McGuire PP, Yu FH (2010). Effects of a flavonoid extract from Cynomorium songaricum on the swimming endurance of rats. Am. J. Chin. Med., 38(1): 65-73.
Zhang HL, Li J, Li G, Wang DM, Zhu LP, Yang DP (2009). Structural characterization and anti-fatigue activity of polysaccharides from the roots of Morinda officinalis. Int J Biol Macromol., 44(3): 257-261.
Zhang XL, Ren F, Huang W, Ding RT, Zhou QS, Liu XW (2010). Anti-fatigue activity of extracts of stem bark from Acanthopanax senticosus. Molecules, 16(1):28-37.
Zhang Y, Yao X, Bao B, Zhang Y (2006). Anti-fatigue activity of a triterpenoid-rich extract from Chinese bamboo shavings (Caulisbamfusae in taeniam). Phytother. Res., 20(10): 872-876.
Zhao W, Zhang X, Wang W, Zhang L (2009). Experimental study for the anti-fatigue effect of ginseng general ginsenosides P.E. in vivo. Wei Sheng Yan Jiu., 38(2):184-187.

African Journal of Microbiology Research Vol. 5(32), pp. 5941-5949, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.995

Full Length Research Paper

Proteomic analysis of differentially expressed proteins in intestinal epithelial cell in response to Enteroinvasive *Escherichia coli* infection and *Lactobacillus plantarun* treatment

Zhongwei Zhang and Minghua Mao*

Department of Geriatrics, The 6th People's Hospital Affiliated to Shanghai Jiaotong University, Shanghai 200233, China.

Accepted 27 October, 2011

A proteomic approach was taken to compare the proteomes of Enteroinvasive *Escherichia coli* (EIEC) infection alone, *Lactobacillus plantarun* pre-treatment and control group. Two-dimensional gel electrophoresis (2-DE), coupled with mass spectroscopy and protein database searching, 8 differentially expressed proteins was identified. Of them, Glutathione Transferase (down-regulated), Peroxisomal enoyl-coenzyme (up-regulated) and Peroxiredoxin (up-regulated) in EIEC infection group were compared with control group which are all associated with antioxidant-related proteins. Glyceraldehyde-3-phosphate dehydrogenase (up-regulated) and Triosephosphate Isomerase (up-regulated) were identified related with carbohydrate metabolism in EIEC infection group compared with control group. Keratin 8 (up-regulated) and hnRNP C1/C2 (down-regulated) were linked to antagonize cytoskeleton reorganization and apoptosis in *L. plantarun* pre-treatment group. Identification of these proteins provides insights that may lead to a better understanding of the molecular basis for EIEC infection process and *L. plantarun* protection function.

Key words: *Lactobacillus plantarun*, Enteroinvasive *Escherichia coli*, tight junction.

INTRODUCTION

Enteroinvasive *Escherichia coli* (EIEC) is a human intestinal pathogen responsible for the majority of cases of endemic bacillary dysentery prevalent in developing country (Song et al., 2005). The underlying pathogenesis is proposed as follows (Parsot, 2005; Croxen and Finlay, 2009). In the colonic mucosa, bacteria are supposed to cross the epithelial layer by invading M cells overlaying lymphoid follicles. Entry into epithelial cells involves rearrangements of the cell cytoskeleton (Cossart and Sansonetti, 2004). Bacteria released from M cells or epithelial cells interact with macrophages, escape from the phagocytic vacuole and induce apoptosis of infected cells. Apoptotic macrophages release pro-inflammatory cytokines facilitates further invasion by luminal bacteria (Steiner et al., 2000; Lahouassa et al., 2007). Recently, there are also evident that probiotic bacteria protect and enhance human intestinal epithelial barrier function. For example, enteropathogenic *E. coli* (EPEC)-induced neutrophil migration and EPEC binding to monolayers were inhibited by viable *Lactobacillus plantarum* but only when added to the monolayers before EPEC (Michail and Abernathy, 2003).

Identically, live *Streptococcus thermophilus* (ST)/*Lactobacillus acidophilus* (LA) interact with intestinal epithelial cells to protect them from the deleterious effect of EIEC via mechanisms that include interference with pathogen adhesion and invasion (Resta-Lenert and Barrett, 2003; Resta-Lenert and Barrett, 2006). In

*Corresponding author. E-mail:maominghuamnh@gmail.com. Tel: +86-021-64369181. Fax: +86-021-64701361.

Abbreviations: EIEC, enteroinvasive *Escherichia coli*; **EPEC,** enteropathogenic *Escherichia coli*.

addition, *L. plantarum* have also been demonstrated reduce EIEC adhesion to Caco-2 by reducing intestinal permeability and increasing tight junction proteins (such as ZO-1, occludin and claudin-1 protein) in our previous reports (Qin et al., 2009). However, the detail mechanism was not very clear. Therefore, it is valuable to explore the regulation mechanism between *L. plantarun*, EIEC and intestinal epidermal-barrier function. In this study, we have, for the first time, taken a proteomic approach to identify differential proteins in EIEC infection alone, *L. plantarun* pre-treatment and control group. We anticipate these proteins may provide initial insights into the role of EIEC infection process and *L. plantarun* protection function.

MATERIALS AND METHODS

Preparation of bacteria

L. plantarum strain CGMCC No.1258 collected from Institute of Science Life of Onlly, Shanghai Jiao Tong University, Shanghai, China, a gift from Dr. Hang Xiaomin (Institute of Science Life of Onlly, Shanghai Jiao Tong University, Shanghai, China) was maintained on MRS agar (Difco Laboratories, Detroit, MI, U.S.A.). Enteroinvasive *Escherichia coli* EIEC strain 0124:NM (ATCC 43893, serotype O124:NM) was obtained from the Center of Diseases Prevention and Control of Shanghai, China and maintained in LB medium (Difco Laboratories, Detroit, MI, U.S.A). They were cultivated at 37°C for 16 h to reach stationary phase. The *L. plantarun* and EIEC suspensions were centrifuged for 5 min at 1500 × g. After removing the supernatant, the pellet was re-suspended in sterile PBS buffer to determine the bacterial concentration. Quantification of bacterial suspension was determined using a standard curve for visible absorbance (600 nm; Beckman DU-50 spectrophotometer) and adjusted the final concentration to 1×10^8/ml.

Preparation of monolayer

DMEM supplemented with 10% fetal bovine serum, 1×10^5 U/L of penicillin and 100 mg/L streptomycin was used as a standard medium to cultivate Caco-2 cells (human colonic epithelial-like cancer cell line obtained from the Cell Institute Affiliated China Science Research Institute, Shanghai, China). When cell growing to 80 to 90% fusion cells under 5% CO_2 saturated humidity and 37°C conditions, 0.25% pancreatic enzyme with 0.03% EDTA was performed to digest cells to subculture (1:3). Then, the cells were inoculated to glass slide in six-well culture plate (gelatin treatment) and cell concentration was 5 × 104 cells/cm^2. After 7 to 10 days, monolayer cells were collected and used in later experiment.

Infection of intestinal epithelial monolayer

Caco-2 cells were washed three times in Hank's balanced salt solution (Life Technologies) to remove the antibiotic media. For rapid infection of the monolayer, 100 µl EIEC at 1.0×10^8 /ml was added to the apical side of the cell culture insert, and the insert was placed in a 50 ml tube and centrifuged at 200 g for 4 min. *L. plantarum* (100 µl of 1.0×10^8/ml) was added to the monolayers in different groups for 24 h. Caco-2 cells monolayers were cultured and served as the control group, Caco-2 cells were infected EIEC as the EIEC group, Caco-2 cells infected EIEC were co-incultured with *L. plantarum* as the *L. plantarum* group. The average number of Caco-2 cells in each monolayer was approximately 1×10^6. The inoculation ratio of EIEC to Caco-2 cells was 100:1. The ratio of lactobacillus to EIEC was 10:1.

Protein sample preparation

The Caco-2 cells monolayer cells in each group were harvested by centrifugation, rinsed in phosphate-buffered saline and re-suspended in 300 µl lysis buffer (9.5 M urea, 4% CHAPS, 65 mM DTT, 2% carrier ampholyte and protease inhibitor cocktail). Then, the Caco-2 cells were removed from the surface with a cell scraper and collected to a 1.5 Eppendorf tube. The crude extract solution was obtained by ultrasonic disruption (80 W, 2 min, with a 15 s interval every 10 s) and centrifugation (14000 rpm, 60 min). The resulting supernatant was concentrated on Biomax-5 K ultra-filtration membrane. The protein concentration was determined by a standard Bradford protein assay and stored at -80°C until use for 2-DE analysis.

Gel electrophoresis and analysis

First-dimensional electrophoresis was carried out using an IPGphor II (Amersham Biosciences) isoelectric focusing system. 100 g of total extract were loaded. IPG dry strips (pH 3-10, linear) were rehydrated at 30 V for 12 h. After rehydration, isoelectric focusing was performed under the following conditions: 500 V for 1 h, 1000 V for 1 h, 8000 V for 6 h and 500 V for 4 h. After equilibration of the isoelectric focusing strips, SDS electrophoresis was performed on 12.5% gels. SDS-PAGE was performed using a Hofer SE 600 System (Amersham Biosciences): 15 mA for 30 min and 30 mA until the Bromophenol Blue front reached 0.5 cm of the gel. After two-dimensional gel electrophoresis, proteins were stained with silver for subsequent mass spectrometry. To ensure data reliability, sample preparation and 2-DE were performed in triplicate. Silver-stained gels were scanned with Bio-Rad GS710 scanner. Images were analyzed using the specialized software program Image Master 2D Elite software (Amersham Biosciences).

MALDI-TOF mass spectrometry and protein identification

Proteins of interest were excised and digested in gel using trypsin for 20 h (sequencing grade, Promega, Charbonnie`res, France). Digest products were completely dehydrated in a vacuum centrifuge and resuspended in 10 ul of formic acid (2%), desalted using Zip Tips C18 (Millipore, Bedford, MA), eluted with acidα-cyano-4-hydroxy-trans-cinnamic acid (Sigma, 5 mg/ml in 0.1% TFA) and loaded on the target of a Bruker-Daltonics AutoFlex TOF−TOF LIFT mass spectrometer (Bruker Daltonics, Bremen, Germany). Analysis was performed in reflectron mode with an accelerating voltage of 20 kV. Identification of proteins was performed using both Mascot and PeptIdent software (available at www.matrixscience.com and www.expasy.org/tools/peptident.html, respectively). Search parameters were as follows: database: NCBInr (release date: 20070326); taxonomy: homo sapiens (human); type of search: peptide mass fingerprint; enzyme: trypsin; fixed modifications: carbamidomethyl (C); mass values: monoisotopic; protein mass: unrestricted; peptide mass tolerance: ± 100 ppm; peptide charge state: 1+; and max missed cleavages: 1.

Statistics analysis

All data were analyzed by SPSS13.0 and the results were

Table 1. Differentially expressed proteins between EIEC and normal group.

Group ID	Normal group	EIEC infection group	Group ID	Normal group	EIEC infection group
3780	-6.46544	6.46544	4399	1.94149	-1.94149
4075	-3.90939	3.90939	3676	-1.93011	1.93011
3959	-3.35281	3.35281	4388	1.87136	-1.87136
3969	-3.03349	3.03349	4118	-1.75635	1.75635
4364	3.00393	-3.00393	4448	1.74678	-1.74678
3863	-2.66937	2.66937	4106	-1.74489	1.74489
3792	-2.48479	2.48479	3096	-1.6833	1.6833
4435	2.46231	-2.46231	3410	-1.6324	1.6324
4241	2.37954	-2.37954	3368	1.6012	-1.6012
4445	2.30779	-2.30779	3392	1.56976	-1.56976
4250	-2.30228	2.30228	3777	-1.56414	1.56414
4110	-2.27937	2.27937	3597	-1.56405	1.56405
4070	2.19601	-2.19601	3380	-1.51271	1.51271
3299	-2.05878	2.05878	4050	-1.51244	1.51244
4337	1.96883	-1.96883	2930	-1.50543	1.50543

measured by average ± standard deviation. One-way ANOVA was performed on all experiments with Tukey Kramer post-hoc comparison. $P < 0.05$ was considered as statistically significant.

RESULTS

Differentially expressed proteins analysis

The differentially expressed proteins between different groups were analyzed based on image master software (class report ratio > = 1.5). The results showed that there were 30 spots identified as differentially expressed between normal group and EIEC group. Of this, 19 genes were up-regulated and 11 genes were down-regulated expression compared with normal group (Table 1, Figures 1 and 2). When comparison betweennormal group and *L. plantarum* group, the results indicated 25 differentially expressed proteins (14 genes up-regulated and 11 genes down-regulated) (Table 2, Figures 3 and 4). 15 differentially expressed proteins were identified between *L. plantarum* group and EIEC group. Among them, 5 genes were down-regulated expression, and 10 genes were up-regulated expression compared with EIEC group (Table 3, Figures 5 and 6).

MALDI-TOF mass spectrometry and protein identification

Total of 16 differentially expressed proteins were excised from 2-DE gels, in-gel digested by trypsin and subjected to MALDI-TOF-TOF/MS analysis. Of them, 8 differentially expressed proteins have been identified by NCBInr database searching (Table 4).

DISCUSSION

In this study, we have used 2-DE and MS to establish the proteomic profiles of intestinal epithelial cell in response to EIEC infection and *L. plantarun* pre-treatment. 2-DE was employed as it permits the identification of the alteration of protein isoforms and determination of protein expression levels and post translational modifications. The 2-DE gel in each group was analyzed to screen differentially expressed proteins by Image Master 2D Elite software. Based on this analysis, 16 significantly differential proteins was chosen to identify by MALDI-TOF-TOF/MS, of them, 8 differentially expressed proteins have been identified by NCBInr database searching. Literature searches were conducted for all the proteins identified in the up- and down-expressed lists to find possible links of the change in expression and their implications in intestinal epithelial cell in general. Most proteins identified show homology with antioxidant-related proteins, such as glutathione transferase, peroxisomal enoyl-coenzyme A hydratase-like protein and peroxiredoxin. Glutathione-S-transferases (GSTs) are a family of Phase II detoxification enzymes that catalyse the conjugation of glutathione (GSH) to a wide variety of endogenous and exogenous electrophilic compounds (Townsend and Tew, 2003; Wu et al., 2004). GST was down-regulated expression in EIEC group, indicating decrease in detoxification function. This result led to EIEC invasion and damage intestinal epithelial cell. Peroxiredoxin 1 (PRDX1) is a ubiquitously expressed antioxidant with vital roles in basal metabolic functions. In addition, PRDX1 is involved in cell differentiation and proliferation, apoptosis and innate immunity (Daly et al., 2008). In addition, glyceraldehyde-3-phosphate

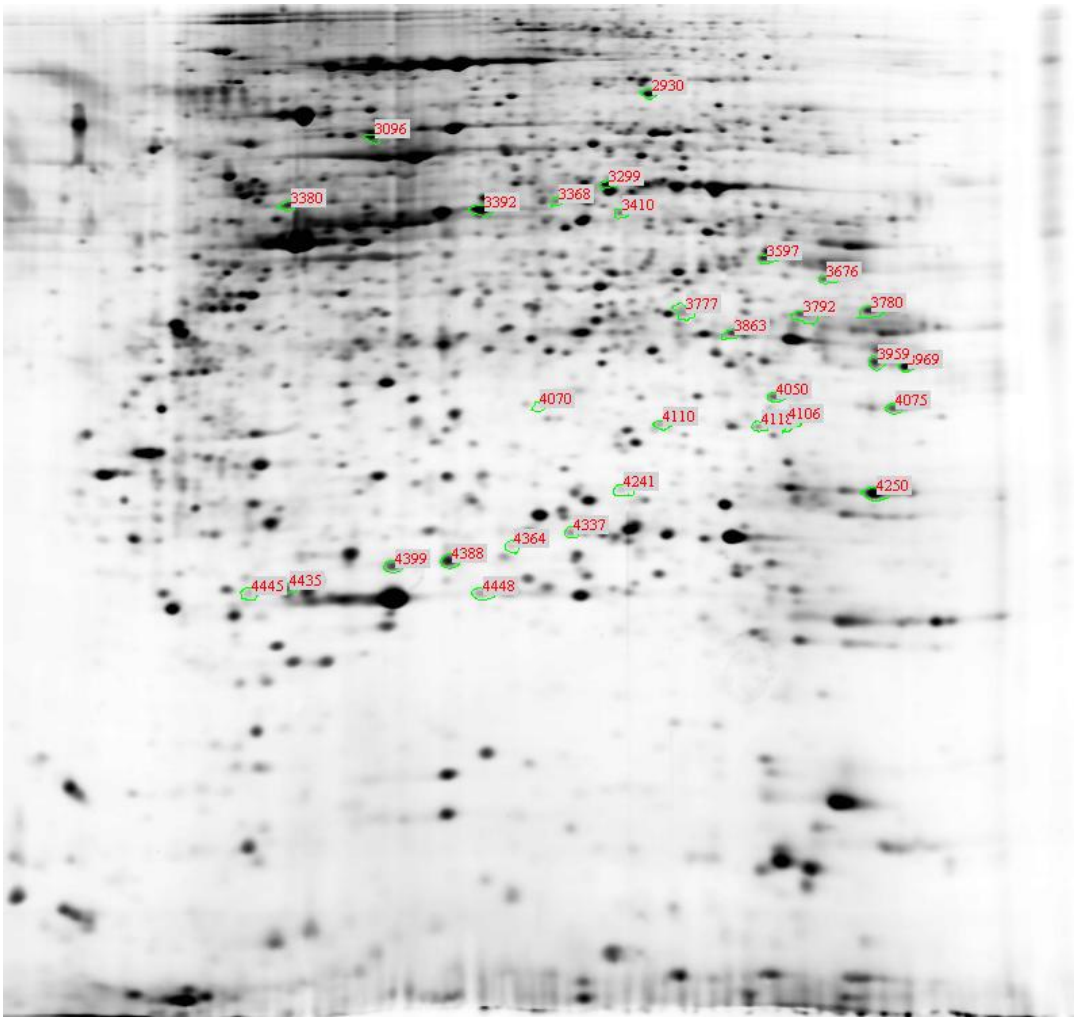


Figure 1. Differently expression spots in EIEC and normal group.

dehydrogenase (GAPDH) and triosephosphate isomerase (Tim) were also identified, which all play an important role in carbohydrate metabolism.

Of them, GAPDH catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate in the glycolytic pathway. As part of the conversion, GAPDH converts NAD^+ to the high-energy electron carrier NADH. GAPDH has been referred to as a "housekeeping" protein based on the view that GAPDH gene expression remains constant under changing cellular conditions. Triosephosphate isomerase (TIM) is also an enzyme with a role in glycolysis and gluco-neogenesis by catalyzing the interconversion between glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (Moraes et al., 2011). Carbohydrate metabolism has been implicated in pathogenesis of enteroinva-sive *E. coli* (EIEC). In a cell culture model, an EIEC mutant defective in both glucose and mannose transport was significantly impaired in adherence and invasion (Gore and Payne, 2010). And Egea et al. (2007) found that GAPDH could be present in the surface of enteropathogenic (EHEC) and enterohaemorrhagic (EPEC) *E. coli* (all as Gram-negative bacteria) and GAPDH could adhere to the cell surface after co-cultivation with Caco-2 cells. This may partially explain the pathogenesis of EHEC and EPEC infection. Therefore, in our study, we found GAPDH and TIM were up-regulated expression in EIEC group contrast to normal group, suggesting EIEC also express the GAPDH and increase its ability of adherence and invasion to Caco-2 cells.

Recently, GAPDH has also been known to contribute to a number of diverse cellular functions unrelated to glycolysis such as cytoskeletal organization, apoptosis and viral pathogenesis (Tatton et al., 2000). These may also be associated with Caco-2 cells damage and apoptosis upon EIEC infection. The clinical study found probiotics can restrain the damage effect of intestinal pathogenic bacteria, and can be used to treat acute diarrhea (Sazawal et al., 2006) and intestinal flora

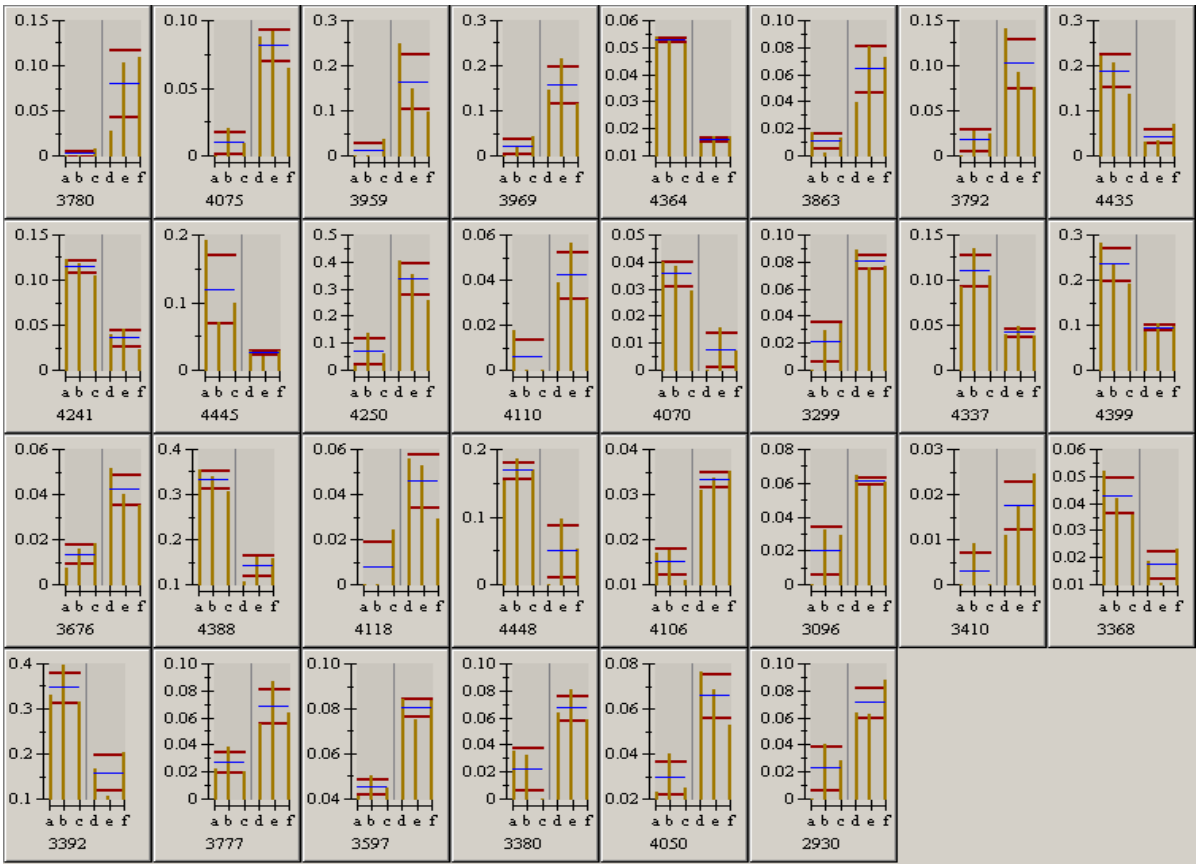


Figure 2. Relative expression profile of differently expression spots in EIEC and normal group.

Table 2. Differentially expressed proteins between *L. plantarum* and normal group.

Group ID	Normal group	*L. plantarum* group	Group ID	Normal group	*L. plantarum* group
1122	-3.01255	3.01255	1118	-1.85844	1.85844
1615	-2.79918	2.79918	1804	-1.78829	1.78829
2007	-2.67766	2.67766	1955	1.75013	-1.75013
900	-2.58965	2.58965	1698	1.72375	-1.72375
1746	-2.56488	2.56488	639	-1.59668	1.59668
1656	2.51169	-2.51169	925	-1.59597	1.59597
1558	-2.49958	2.49958	1842	-1.5682	1.5682
1944	2.46618	-2.46618	641	-1.55929	1.55929
390	-2.04106	2.04106	1684	1.54977	-1.54977
1917	1.99593	-1.99593	1754	1.53794	-1.53794
1914	1.9928	-1.9928	638	-1.52214	1.52214
1883	1.90046	-1.90046	2159	1.51294	-1.51294
1886	1.89765	-1.89765			

(Kuehbacher et al., 2006). Keratin 8 was induced up-regulated expression upon *L. plantarum* treatment. Keratins 8 and 18 belong to the keratin family of intermediate filament proteins and they can be covalently conjugated to constitute a hallmark for all simple epithelia (Gilbert et al., 2001; Magin et al., 2007). Phosphorylation in some sites may affect the structure and function of this protein and even cells signal transduction (Feng et al., 1999; Ridge et al., 2005). Phosphorylation facilitates formation of Keratin 8/18 aggregates, but is not crucial.

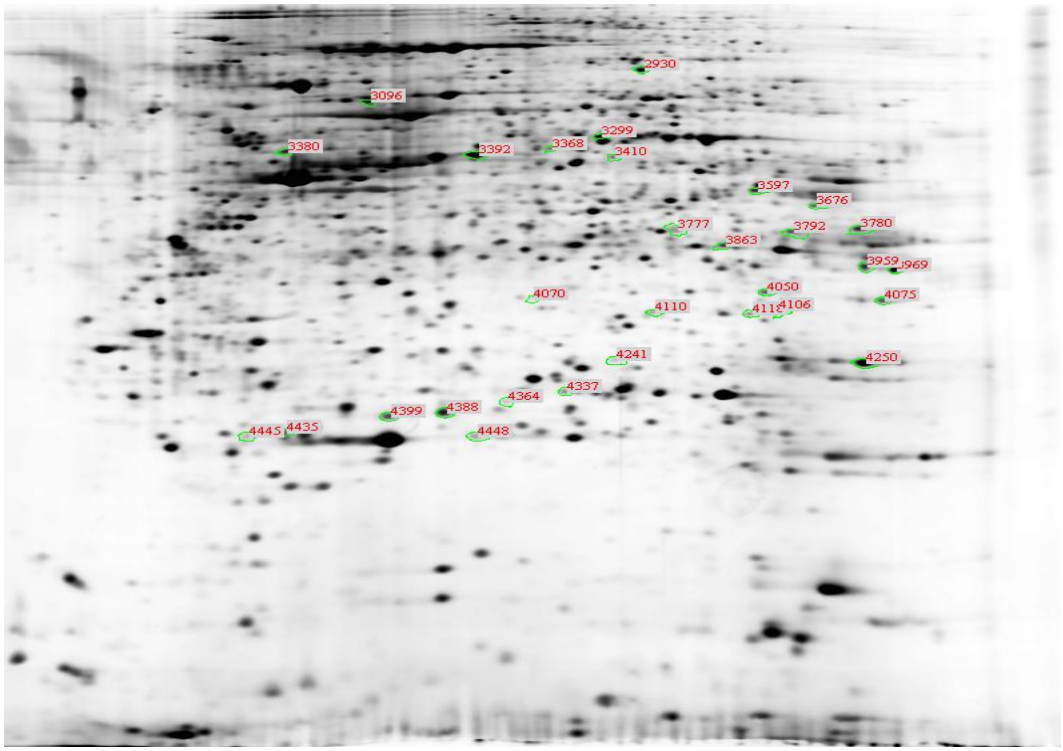


Figure 3. Differently expression spots in *L. plantarum* and normal group.

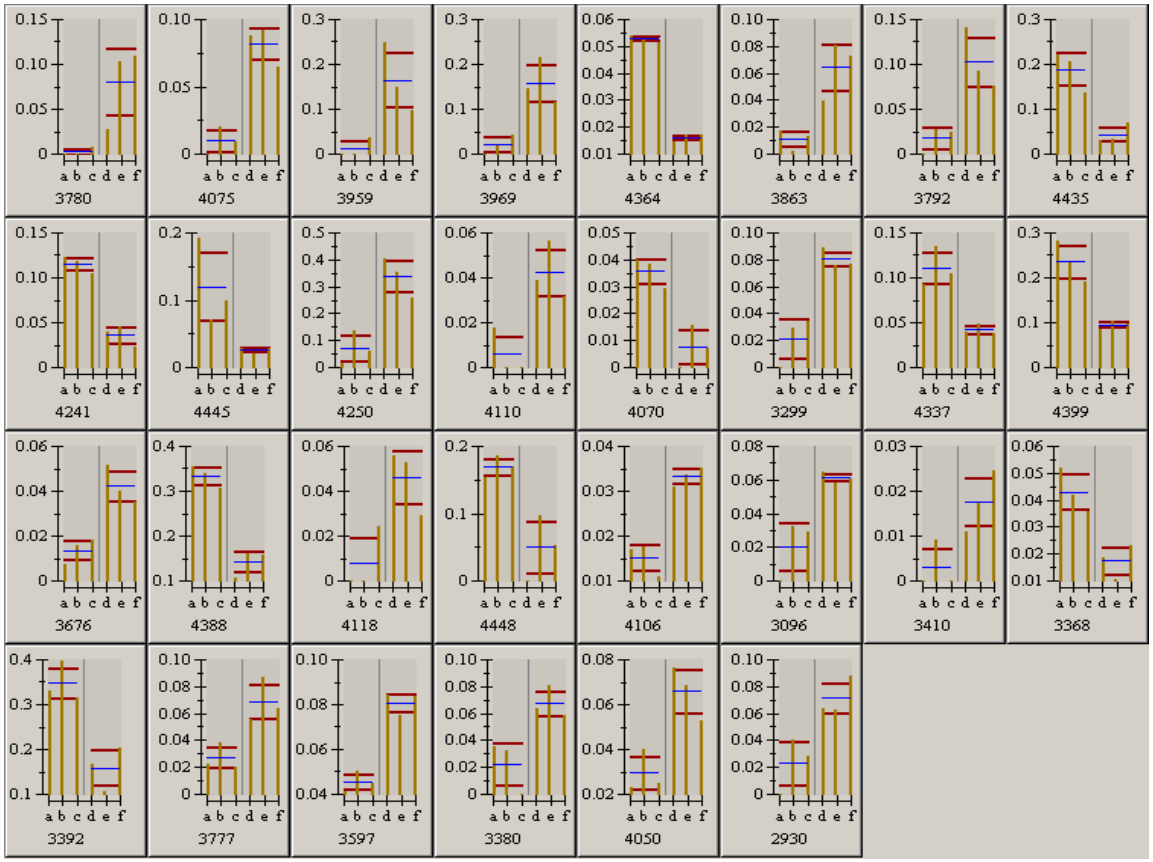


Figure 4. Relative expression profile of differently expression spots in *L. plantarum* and normal group.

Table 3. Differentially expressed proteins between *L. plantarum* and EIEC group.

Group ID	*L. plantarum* group	EIEC group	Group ID	*L. plantarum* group	EIEC group
1313	-3.59685	3.59685	1829	1.90584	-1.90584
1403	-3.56569	3.56569	1549	-1.79476	1.79476
1043	-3.33972	3.33972	613	1.61932	-1.61932
1217	-2.51966	2.51966	680	-1.57435	1.57435
1792	-2.32483	2.32483	1341	1.55353	-1.55353
1710	-2.29915	2.29915	927	-1.5412	1.5412
1664	1.95007	-1.95007	1851	1.50677	-1.50677
1413	-1.93483	1.93483			

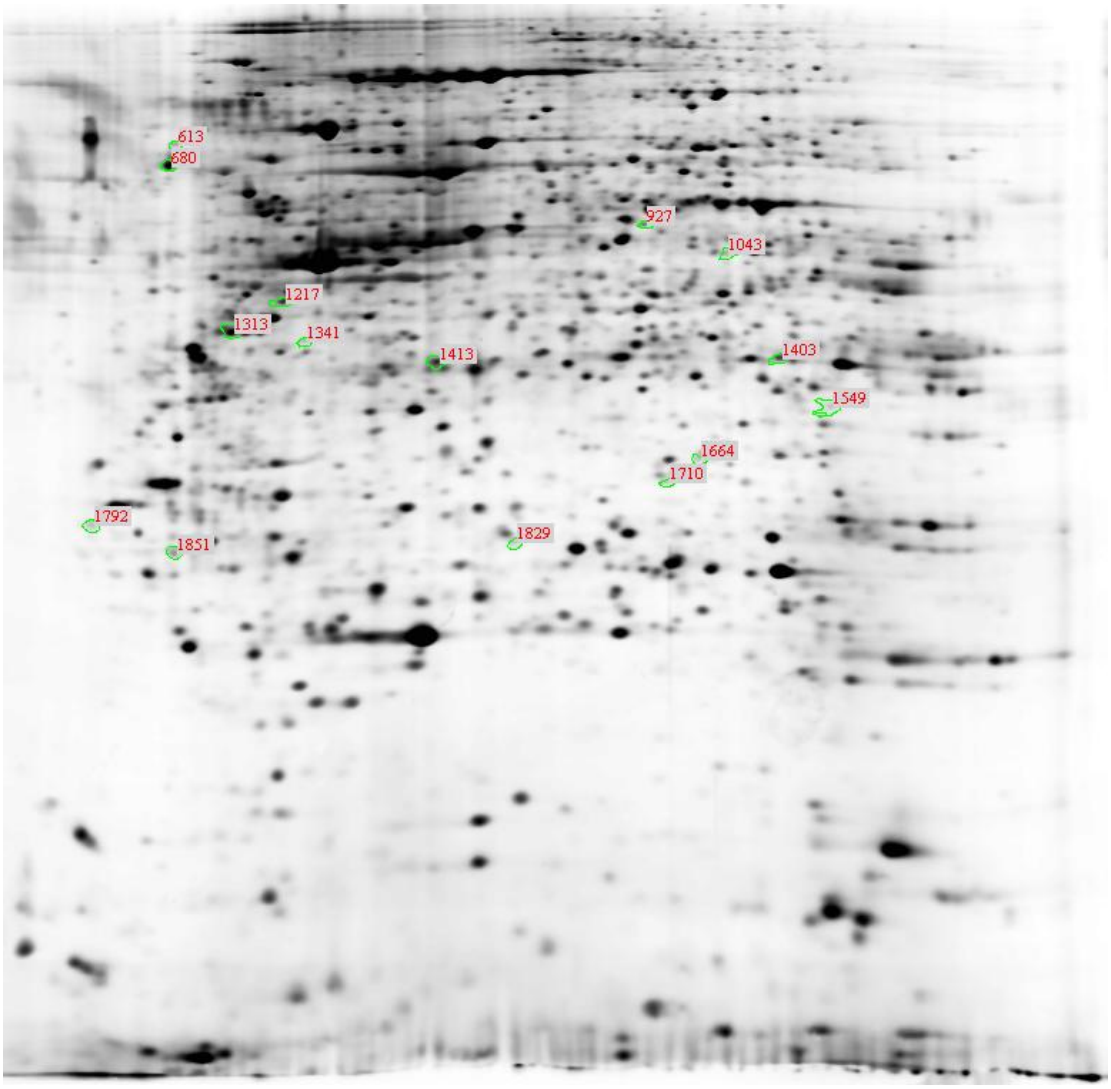


Figure 5. Differently expression spots in *L. plantarum* and normal group.

Keratin 8/18 would breakdown and reorganize during apoptosis. And at later stages of the apoptotic process, that is, when the integrity of the cytoplasmic membrane becomes compromised, keratin aggregates are shed from the cells (Schutte et al., 2004). Our study discovered the keratin-dependent protection of Caco-2 cells from

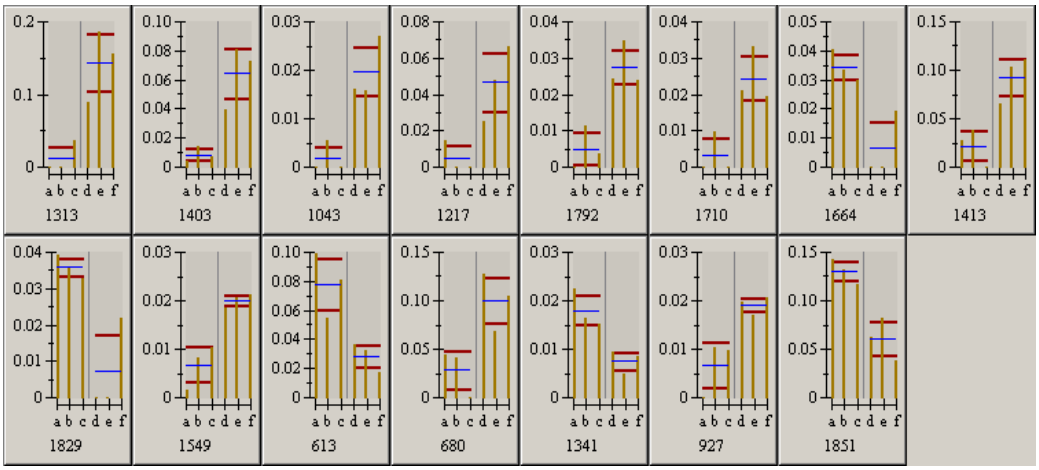


Figure 6. Relative expression profile of differently expression spots in *L. plantarum* and normal group.

Table 4. Identification of differentially expressed proteins by NCBInr database searching.

Spot No	Protein ID	Protein name
1403	Gi|31645	Glyceraldehyde-3-phosphate dehydrogenase.
1313	Gi|109082737	Predicted: heterogeneous nuclear ribonucleoprotein C (C1/C2) isoform 2 (Macaca mulatta).
1217	Gi|62913980	KRT8 protein.
1615	Gi|70995211	Peroxisomal enoyl-coenzyme A hydratase-like protein.
2007	Gi|55959887	Peroxiredoxin 1.
4445	Gi|20664358	Chain A, crystal structure of a recombinant glutathione transferase, created by replacing the last seven residues of each subunit of the human class Pi isoenzyme with the additional C-terminal helix of human class alpha isoenzyme.
4337	Gi|999892	Chain A, triosephosphate isomerase (Tim) (E.C.5.3.1.1) complexed with 2-phosphoglycolic acid.
4435	Gi|20664358	Chain A, crystal structure of a recombinant glutathione transferase, created by replacing the last seven residues of each subunit of the human class Pi isoenzyme with the additional C-terminal helix of human class alpha isoenzyme.

EIEC induced apoptotic challenge may be a key function of simple epithelial keratins (Jaquemar et al., 2003). And intermediate filament proteins might be induced over-expression by *L. plantarum* to antagonize the apoptosis and destructive effect on cytoskeleton by EIEC (Nishizawa et al., 2005).

The heterogeneous nuclear ribonucleoprotein C1/C2 (hnRNP-C1/C2) is one of the most abundant proteins in the nucleus, and shown to have roles in cellular differentiation and proliferation through post-transcriptional regulations of certain mRNA species (Williamson et al., 2000). Many hnRNP-C1/C2 have been found to be phosphorylated in response to extracellular stimulations. These changes have been proposed to regulate splice site selection in pre-mRNA alternative splicing, which is recognized as the cause or the consequence of numerous human diseases such as tumors and inflammatory injuries (Zhu et al., 2003). Translocation of hnRNP C1/C2 from nuclei to cytoplasm in PMA-induced pro-apoptotic cells have been identified dependent on ROCK-mediated cytoskeleton rearrangement (Lee et al., 2004). In our study, down-regulation of hnRNP-C1/C2

upon *L. plantarum* treatment might be as a novel mechanism to enhance the resistance of Caco-2 cells to apoptosis, inflammatory and indirectly decrease the hnRNP C1/C2 translocation.

REFERENCES

Cossart P, Sansonetti PJ (2004). Bacterial invasion: the paradigms of enteroinvasive pathogens. Science, 304(5668): 242-248.

Croxen MA, Finlay BB (2009). Molecular mechanisms of *Escherichia coli* pathogenicity. Nat. Rev. Microbiol., 8(1): 26-38.

Daly KA, Lefévre C, Nicholas K, Deane E, Williamson P (2008). Characterization and expression of Peroxiredoxin 1 in the neonatal tammar wallaby (Macropus eugenii). Comp. Biochem. Physiol. Biochem. Mol. Biol., 149(1): 108-119.

Egea L, Aguilera L, Giménez R, Sorolla M, Aguilar J, Badía J, Baldoma L (2007). Role of secreted glyceraldehyde-3-phosphate dehydrogenase in the infection mechanism of enterohemorrhagic and enteropathogenic *Escherichia coli*: interaction of the extracellular enzyme with human plasminogen and fibrinogen. Int. J. Biochem. Cell Biol., 39(6): 1190-1203.

Feng L, Zhou X, Liao J, Omary MB (1999). Pervanadate-mediated tyrosine phosphorylation of keratins 8 and 19 via a p38 mitogen-activated protein kinase-dependent pathway. J. Cell. Sci., 112(13): 2081-2090.

Gilbert S, Loranger A, Daigle N, Marceau N (2001). Simple epithelium keratins 8 and 18 provide resistance to Fas-mediated apoptosis. The protection occurs through a receptor-targeting modulation. J. Cell Biol., 154(4): 763-73.

Gore AL, Payne SM (2010). CsrA and Cra influence Shigella flexneri pathogenesis. Infect Immun., 78(11): 4674-82.

Jaquemar D, Kupriyanov S, Wankell M, Avis J, Benirschke K, Baribault H, Oshima RG (2003). Keratin 8 protection of placental barrier function. J. Cell Biol., 161(4): 749-56.

Kuehbacher T, Ott SJ, Helwig U, Mimura T, Rizzello F, Kleessen B, Gionchetti P, Blaut M, Campieri M, F lsch UR (2006). Bacterial and fungal microbiota in relation to probiotic therapy (VSL# 3) in pouchitis. Gut., 55(6): 833-41.

Lahouassa H, Moussay E, Rainard P, Riollet C (2007). Differential cytokine and chemokine responses of bovine mammary epithelial cells to *Staphylococcus aureus* and *Escherichia coli*. Cytokine., 38(1): 12-21.

Lee HH, Chien CL, Liao HK, Chen YJ, Chang ZF (2004). Nuclear efflux of heterogeneous nuclear ribonucleoprotein C1/C2 in apoptotic cells: a novel nuclear export dependent on Rho-associated kinase activation. J. Cell. Sci., 117(23): 5579-89.

Magin TM, Vijayaraj P, Leube RE (2007). Structural and regulatory functions of keratins. Exp. Cell Res., 313(10): 2021-2032.

Michail S, Abernathy F (2003). *Lactobacillus plantarum* inhibits the intestinal epithelial migration of neutrophils induced by enteropathogenic *Escherichia coli*. J. Pediatr. Gastroenterol. Nutr., 36(3): 385-91.

Moraes J, Arreola R, Cabrera N, Saramago L, Freitas D, Masuda A (2011). Structural and biochemical characterization of a recombinant triosephosphate isomerase from *Rhipicephalus* (Boophilus) microplus. Insect Biochem. Mol. Biol., 41(6): 400-9.

Nishizawa M, Izawa I, Inoko A, Hayashi Y, Nagata K, Yokoyama T, Usukura J, Inagaki M (2005). Identification of trichoplein, a novel keratin filament-binding protein. J. Cell. Sci., 118(5): 1081-90.

Parsot C (2005). *Shigella* spp. and enteroinvasive *Escherichia coli* pathogenicity factors. FEMS Microbiol. Lett., 252(1): 11-18.

Qin H, Zhang Z, Hang X, Jiang Y (2009). L. plantarum prevents enteroinvasive *Escherichia coli*-induced tight junction proteins changes in intestinal epithelial cells. BMC Microbiol., 9(1): 63.

Resta-Lenert S, Barrett K (2003). Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive *Escherichia coli* (EIEC). Gut., 52(7): 988-97.

Resta-Lenert S, Barrett KE (2006). Probiotics and commensals reverse TNF-[alpha]-and IFN-[gamma]-induced dysfunction in human intestinal epithelial cells. Gastroenterology, 130(3): 731-746.

Ridge KM, Linz L, Flitney FW, Kuczmarski ER, Chou YH, Omary MB, Sznajder JI, Goldman RD (2005). Keratin 8 phosphorylation by protein kinase C delta regulates shear stress-mediated disassembly of keratin intermediate filaments in alveolar epithelial cells. J. Biol. Chem., 280(34): 30400-5.

Sazawal S, Hiremath G, Dhingra U, Malik P, Deb S, Black RE (2006). Efficacy of probiotics in prevention of acute diarrhoea: a meta-analysis of masked, randomised, placebo-controlled trials. Lancet Infect. Dis., 6(6): 374-382.

Schutte B, Henfling M, K lgen W, Bouman M, Meex S, Leers MPG, Nap M, Bj rklund V, Bj rklund P, Bj rklund B (2004). Keratin 8/18 breakdown and reorganization during apoptosis. Exp. Cell Res., 297(1): 11-26.

Song T, Toma C, Nakasone N, Iwanaga M (2005). Sensitive and rapid detection of *Shigella* and enteroinvasive *Escherichia coli* by a loop-mediated isothermal amplification method. FEMS Microbiol. Immunol., 243(1): 259-263.

Steiner TS, Nataro JP, Poteet-Smith CE, Smith JA, Guerrant RL (2000). Enteroaggregative *Escherichia coli* expresses a novel flagellin that causes IL-8 release from intestinal epithelial cells. J. Clin. Invest., 105(12):1769-1778.

Tatton WG, Chalmers-Redman RM, Elstner M, Leesch W, Jagodzinski FB, Stupak DP, Sugrue MM, Tatton NA (2000). Glyceraldehyde-3-phosphate dehydrogenase in neurodegeneration and apoptosis signaling. J. Neural. Transm. Suppl., 60: 77-100.

Townsend DM, Tew KD (2003). The role of glutathione-S-transferase in anti-cancer drug resistance. Oncogene, 22(47): 7369-7375.

Williamson DJ, Banik-Maiti S, DeGregori J, Ruley HE (2000). hnRNP C is required for postimplantation mouse development but is dispensable for cell viability. Mol. Cell. Biol., 20(11): 4094-105.

Wu G, Fang YZ, Yang S, Lupton JR, Turner ND (2004). Glutathione metabolism and its implications for health. J. Nutr., 134(3): 489-492.

Zhu YQ, Lu Y, Tan XD (2003). Monochloramine induces reorganization of nuclear speckles and phosphorylation of SRp30 in human colonic epithelial cells: role of protein kinase C. Am. J. Physiol., Cell Physiol., 285(5): C1294-303.

African Journal of Microbiology Research Vol. 5(32), pp. 5950-5954, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.996

Full Length Research Paper

A survey on the prevalence of poultry salmonellosis and detection of different *Salmonella* serovars isolated from poultry in broiler chicken farms

Jafar Akbarmehr

Department of Microbiology, Islamic Azad University, Sarab Branch, Sarab, Iran. E-mail: ja_mehr@yahoo.com.

Accepted 27 October, 2011

Salmonellosis is an important public health problem and food of poultry origin is one of the most common sources of human salmonellosis. The aim of this study was detection of S*almonella* spp and determination of the prevalence of Salmonellosis in broiler poultry farms of Ardebil province, Iran. *Salmonella* detection by both conventional culture and multiplex PCR methods were performed on 400 samples obtained from poultry. The samples were obtained from poultry farms of five different geographic zones (North, South, West, East and Central zone) of Ardebil province and were examined by standard microbiological tests. m-PCR technique was carried out with four and three pairs of specific primers for *Salmonella typhimurium* and *Salmonella enteritidi*s respectively. Out of a total of 400 samples, 37(9.25%) were positive for *Salmonella* by bacteriological tests. The highest prevalence of *Salmonella* was recorded in Central zone (10.43%) while the lowest prevalence was in South zone of Ardebil province (8%). Based on the m-PCR results among 37 isolated *Salmonella*, 11 serovars were S. *typhimurium* and 21 serovars were identified as *S. enteritidis.* Also, there was no significant difference between the prevalence rate of *Salmonella* in five different selected areas (P>0.05). For control and prevention programs of salmonellosis, the results of this study can be used by agriculture and health organizations in Iran.

Key words: m-PCR, ardebil, salmonellosis, poultry.

INTRODUCTION

Salmonella species are gram negative, flagellated, facultatively anaerobic bacilli which are considered as major zoonotic pathogens for both animals and humans (Giannella et al., 1973). Salmonellosis is common throughout the world. The disease in humans usually takes the form of a self-limiting food poisoning but occasionally manifested as a serious systemic infection or enteric fever. Contaminated food is the major mode of transmission for non typhoidal *Salmonella* because salmonellosis is a zoonosis and has an enormous animal reservoir (Doyle and Beuchat, 2007). The most common animal reservoirs are chickens, turkeys, pigs and cows. Other domestic and wild animals also harbor these organisms. *Salmonella enterica* serovar *enteritidis* is a major cause of food borne disease and during last decade it has been isolated worldwide in increasing numbers. Furthermore *S. enterica* serovar Typhimurium is the most frequently isolated serovar worldwide (Madadgar et al., 2008). Dairy products, vegetables, fruits, shellfish, beef, poultry and eggs are the most common sources of human salmonellosis (Doyle and Beuchat, 2007). Poultry are commonly infected with a wide variety of *S. enterica* serovars. The two serovars that have been of most concern in recent years are *S. enteritidis* and *Salmonella typhimurium* (Madadgar et al., 2008). Since 1987, *Salmonella enteritidis* has been the main cause of *Salmonella* poisoning in humans from poultry products (Doyle and Beuchat, 2007). In order to minimize the risk of human salmonellosis, epidemiological studies and microbiological control of the food chain is being increasingly applied. In recent years various molecular techniques have been used to improve the identification and differentiation of *Salmonella* serovars including: PCR-single-strand conformation

Table 1. Sequences of oligonucleotides used as primers in m- PCR for *S. typhimurium* (a) and *S. enteritidis* (b) (Rahn et al., 1992).

Primer	Sequence	Target gene	Amplicon fragment(bp)
RfbJ-s RfbJ-as (a)	5'-CCAJCACCAGTTCCAACTTGATAC 5'-GGCTTCCGGCTTTATTGGTAAGCA	rfbJ	663
FliC-s FliC –as (a)	5'-ATAGCCATCTTTACCAGTTCCCCC 5'-GCTGCAACTGTTACAGGATATGCC	fjlC	183
FljB-s FljB -as (a)	5'-ACGAATGGTACGGCTTCTGTAACC 5'-TACCGTCGATAGTAACGACTTCGG	fljB	526
ST 139-s ST141-as (a)	5'-GTGAAATTATCGCCACGTTCGGGCAA 5'-TCATCGCACCGTCAAAGGAACC	invA	284
ST11 ST14 (b)	5'-GCCAACCATTGCTAAATTGGCGCA 5'-GGTAGAAATTCCCAGCGGGTACTGG	Random sequence	429
S1 S4 (b)	5'-GCCGTACACGAGCTTATAGA 5'-ACCTACAGGGGCACAATAAC	spv	250
SEFA2 SEFA4 (b)	5'-GCAGCGGTTACTATTGCAGC 5'-TGTGACAGGGACATTTAGCG	sefA	310

polymorphism analysis (Satheesh et al., 2002), genomic and phenotyping evaluation (Madadgar et al., 2008), pulsed field gel electrophoresis (Mhand et al., 1999; Thong, 1998), PCR assay (Hoorfar and Ahrens, 2000; Feeder et al., 2001; Kongmuang et al., 1994; Lin and Tsen, 1999; Malorny et al., 2003), RFLP (Aarts et al., 1998). One of the most important used techniques for identification of *Salmonella* serovars is PCR technique using *Salmonella* genes (Kisiela and Kuczkowki, 2005).

In 2004, Alvarez et al. (2004) have desecribed m- PCR as a method for *Salmonella* diagnosis that is simple, inexpensive and sensitive and enables the quick and precise detection of the most prevalent serotypes of *Salmonella* in human clinical samples. In recent years several studies were carried out by different authors in order to determine the prevalence of poultry salmonellosis in Iran. Based on these studies the prevalence of *Salmonella* in poultry were reported in different areas in Iran (Zahraei et al., 2005; Madadgar et al., 2008; Jamshidi et al., 2008; Akbarmehr, 2010). But until now epidemiological study about poultry salmonellosis in Ardebil province which is located in Northwest of Iran has not been widely studied. Therefore in the present study we investigated the poultry salmonellosis in broiler chicken farms of Ardebil province using conventional culture and mPCR assay.

MATERIALS AND METHODS

Sampling and microbiological tests

This study was carried out in Ardebil province, Iran. The province was divided into five different geographic areas as follows: North, South, west, East and Central zone. A total_of 400 samples were collected from broiler poultry farms in the aforementioned areas from January 2010 to June 2011(60, 50, 85, 90, 115 samples were obtained from North, South, west, East and Central zone respectively). The samples were harvested from yolk sac, spleen, intestine and liver of chickens and examined by standard procedures (enrichment 24 h in selenite F (Merk) at 37°C, plated on XLD agar and incubated at 37°C for 24 h, confirmation of suspected colonies by biochemical tests) as described by Quinn et al. (1994).

PCR amplification

The *Salmonella* isolates were grown overnight at 37°C in brain heart infusion broth. 2 ml of the bacteria culture were centrifuged for 10 min at 16000 rpm. Purified DNA was used as a templet for the PCR assay. For the m- PCR, seven primer pairs were used. Four pairs of primers were used for *S. typhimurium* and three pairs of them for *S. enteritidis* (Table 1). PCR was carried out in a 25 µl amplification mixture consisting of 200 mM dNTPs, 1 µm of each primer, 40 ng of genomic DNA, 1.5 mM $MgCl_2$ and 1U of *Taq* DNA polymerase (fermentase). Amplification was performed in a thermal cycler (Biosystem). The cycling condition was as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 60 s, annealing at 65°C for 30 s, elongation at 72°C for 30 s and final extenstion period for 7 min (Zahraei et al., 2005). Amplified products were subjected to electrophresis at 100 V on a 1.2% agarose gel for 1 h and a 100 bp DNA ladder was used as a size reference. After staining with ethidium bromide, the gel was documented and photographed under ultra violet light. *S. typhimurium* with ATCC- 14025 and *S. enteritidis* with RTCC-1624 were used as positive control.

RESULTS

The prevalence of salmonellosis in poultry in the five

Table 2. Prevalence of salmonellosis in the broiler farms of five selected zones of Ardebil province, Iran.

Zone	No. of examined samples	No. positive samples	Prevalence rate (%)
North	60	5	8.33
South	50	4	8
West	85	8	9.4
East	90	8	8.88
Central	115	12	10.43
Total	400	37	9.25

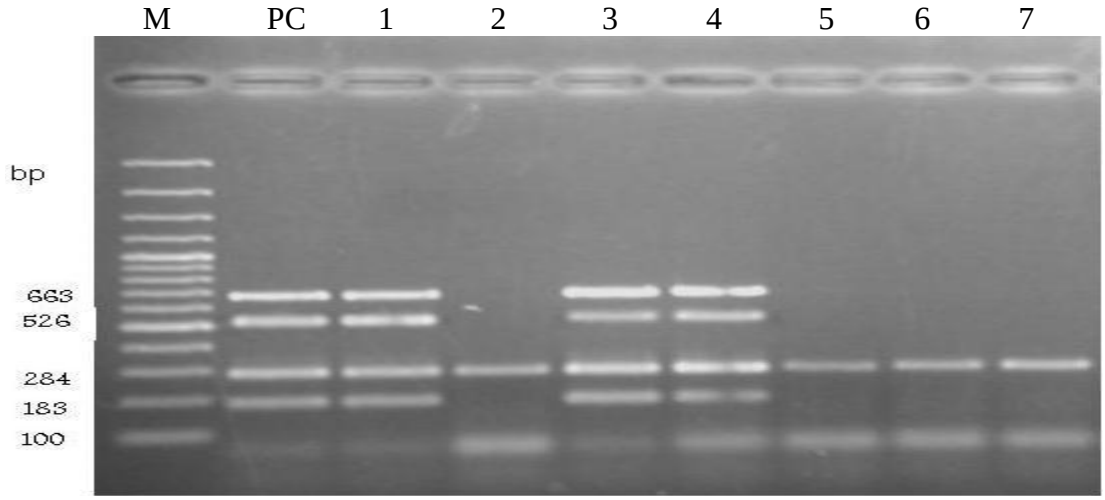


Figure 1. Multiplex PCR with four pairs of primers for *S .typhimurium* isolated from poultry. The 183 bp bands produced by fliC gene (specific for *S .typhimurium*) and the 284 bp bands produced by invA gene (specific for the genus *Salmonella*). The 526 and 663 bp bands produced by fljB and rfbJ genes respectively. M: marker (100 bp). PC: positive control (*S. typhimurium* with ATCC- 14025). Lanes 1, 3 and 4 are positive samples for *S. typhimurium.* Lanes 2, 5, 6 and 7 are positive samples for genus of *Salmonella*.

selected zones of Ardebil province are shown in Table 1. Out of a total 400 samples, 37(9.25%) were positive for *Salmonella* by bacteriological tests. As Table 2 shows the highest, prevalence was recorded in Central zone (10.43%) while the lowest prevalence was in south zone of Ardebil province (8%). MPCR technique which was carried out with specific primers for *S. typhimurium* and *S. enteritidis* (Table1). Among 37 isolated *Salmonella*, 11 serovars of *S. typhimurium* and 21 serovars of *S. enteritidis* were confirmed by MPCR technique. Distribution of *S. typhimurium* and *S. enteritidis* in different geographic area of Ardebil province is shown in Table 2. Also, Figures 1 and 2 shows the MPCR results (Table 3) of *S. typhimurium* and *S. enteritidis* respectively.

DISCUSSION

Salmonella is an important cause of food-borne infections. Most of these infections are caused by *Salmonella* originated from poultry (Doyle and Beuchat, 2007). As Table 1 shows out of 400 samples which was examined by bacteriological tests 37(9.25%) of them had positive results for *Salmonella* strains. This finding is comparable to the reports of previous works from other cities and provinces in Iran. While some previous authors reported higher prevalences of *Salmonella* in poultry, the others reported lower prevalence compared to this study. In a study which was conducted in Fars province, Iran the prevalence of poultry salmonellosis was determined 15.62% (Zahraei et al., 2005). Another survey which was conducted in Mashhad city, Iran; the prevalence of *Salmonella* in poultry carcasses was determined by 8.3% (Jamshidi et al., 2008). In 2010, Akbarmehr et al. (2010) reported the prevalence of poultry salmonellosis in Sarab city, Iran as 7.25%. According to Table 1 although the prevalence of *Salmonella* is widely distributed in all over the five selected geographic area in Ardebil province but the prevalence rates were varied from 8% (South zone) to 10.43% (Central zone). This may be due to confirement system of poultry farms in central zone of Ardebil province which provides the easily distribution of

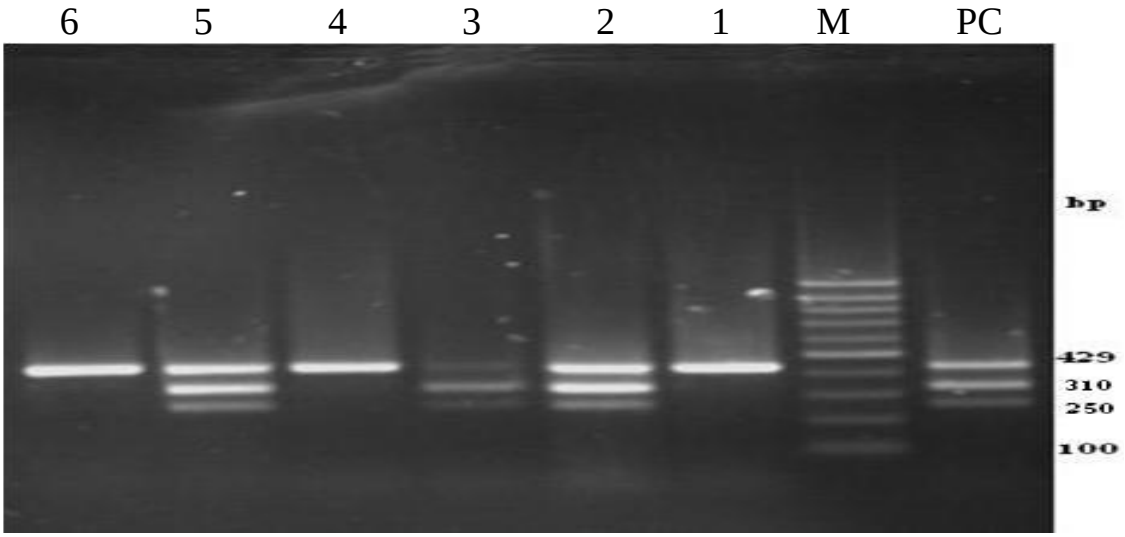


Figure 2. m-PCR with three pairs of primers for *S. enteritidis* isolated from poultry: the 250 bp bands produced by *Salmonella* plasmid virulent gene. The 310 bp bands produced by S.*enteritidis* fimbrial antigen gene and the 429 bp bands produced by randomly cloned sequence which is specific for the genus *Salmonella*. M: marker (100 bp). PC: positive control (*S. enteritidis* with RTCC-1624). Lanes 2, 3 and 5 are positive samples for *S. enteritidis*. Lanes 1, 4 and 6 are positive samples for genus of *Salmonella*.

Table 3. Distribution of *S. typhimurium* and *S. enteritidis* in different geographic area of Ardebil province based on MPCR results.

Zone	No. of positive samples	*S. typhimurium*		*S. enteritidis*	
		No	%	No	%
North	5	1	20	3	60
South	4	1	25	3	75
West	8	2	25	5	62.5
East	8	3	37.5	4	50
Central	12	4	33.33	6	50
Total	37	11	29.72	21	56.75

Salmonella between poultry farms. Although there were no significant differences between different prevalence rates (P>0.05). According to Figure 1, *S. typhimurium* serovars confirmed with four pairs of primers by m-PCR method. As Figure 1 shows the 284 bp bands produced by invA genes which were found in all of the *S. typhimurium* serovars. Primers targeting the rfbJ, fliC and fljB genes were used for specific identification of *S. typhimurium* which produced 663, 183 and 526 bp bands respectively. Olivera reported that the m-PCR technique using invA gene for detection of *Salmonella* and fliC gene for identification of *S. typhimurium* from poultry- related samples was 100% specific (Oliveira et al., 2002). Also, *S. enteritidis* serovars comfirmed with three pairs of primers in this study (Figure 2).

The 429 bp bands were found in all of the *Salmonella* serovars produced by randomly cloned sequenced which were specific for the genus *Salmonella*. *Salmonella* plasmid virulent gene (Spv) and *S. enteritidis* fimbrial antigen gene (sefA) (which produced 250 and 310 bp bands respectively) were used for specific identification of *S. enteritidis* serovars (Madadgar et al., 2008). Based on m-PCR results, *S. enteritidis* with 56.75% frequency and *S. typhimurium* with 29.72% frequency were the most common serovars in five different zones of poultry farms in Ardebil province. Out of 37 *Salmonella* isolates, 5 (13.51%) were other seovars which were not identified in this study. It should be considered that the predominant *Salmonella* serovars differ in different countries. But in Iran many authors showed that *S. enteritidis* and *S. typhimurium* are the most prevalent serotypes of *Salmonella* (Zahraei et al., 2005; Akbarmehr et al., 2010; Madadgar et al., 2008). Because of the ability of *Salmonella* in poultry meat and egg that are not thoroughly cooked, poultry originated products are the main vehicle of transmission (Gianella, 1973). Finally the present research is the first precise study about epidemiology of salmonellosis in poultry farms of Ardebil

province, Iran using conventional culture and m-PCR Assay and our results revealed an important public health and veterinary problem which must be considered by agriculture and public health organizations in Iran.

ACKNOWLEDGEMENTS

This work was supported by the Islamic Azad University of Sarab Branch. We are thankful to Mr. S. S. Taheri for his valuable collaboration.

REFERENCES

Aarts HJ, Vanlith J T, Keljer J (1998). High resolution genotyping of *Salmonella* Strains by AFLP fingerprinting. Lett. Appl. Microbiol., 26:131-135.

Akbarmehr J, zahraei Salehi T, Nikbakht Gh (2010). Identification of *Salmonella* isolated From poultry by MPCR technique and evaluation of their hsp *groEL* gene diversity based on the PCR-RFLP analysis. Afr. J. Microbiol. Res., 4(15):1599-16o4.

Alvarez J, Sota M, Vivanco AB, Perales I, Cisterna R, Rementeria A, Garaizar J (2004). Development of multiplex PCR technique for detection and epidemiological typing of *Salmonella* in human clinical samples. J. Clin. Microbiol., 42:1734-1738.

Doyle MP, Beuchat LR (2007). Food Microbiology.third edition ASM Press. Washington D.C., pp. 187-219.

Feeder I, Nietfeld JC, Galland J, Yeary T, Sargeant JM, Oberst R, Tamplin ML, Luchansky JB(2001). Comparison of Cultivation and PCR hybridization for detection of *Salmonella* in porcine fecal and Water samples. J. Clin. Microbiol., 39:2477_2484

Giannella RA, Formal SB, Dammin GJ (1973). Pathogenesis of salmonellosis :studies of fluid secretion, mucosal invasion, and morphological reaction in the rabit ileum. J. Clin. Invest., 52:441.

Hoorfar JP, Ahrens P (2000).Automated 5' nuclease PCR assay for identification of *Salmonella enterica* .J. Clin. Microbiol., 38:3429-3435.

Jamshidi A, Bassami MR, Afshari-Nic S (2008).Identification of *Salmonella spp* and *Salmonella typhimurium* by multiplex PCR-based assay from poultry caracasses in Mashhad –Iran. Int. J. Vet Res., 3(1):43-48.

Kisiela D, Kuczkowki M (2005). Differentiation of *Salmonella gallinarum* biovar gallinarum from *Salmonella gallinarum* biovar pullorum by PCR-RFLP of the fim H gene. J. Vet. Med., B52:214-218.

Kongmuang U, Luk JMC, Lindberg AA (1994). Comparison of three stool- processing methods for detection of *Salmonella* serogroups B,C2, and D by PCR. J. Clin. Microbiol., 23:3072-3074.

Lin JS, Tsen HY (1999). Development and use of polymerase chain reaction for the detection of *S. typhimurium* in stool and food samples. J. Food Prot., 62:1103-1110.

Madadgar O, Zahraei Salehi T, Tadjbakhsh H, Mahzounieh M, Feizabadi M (2008). Genomic and phenotypic evaluation of *Salmonella typhimurium* and *Salmonella enteritidis* in Iran. Comp. Clin. Pathol., 17:229-235.

Mhand RA, Brahimi N, Moustaoui N, Mdaghri NE, Amarouch H, Grimont F (1999). Characterization of extended spectrum beta lactamase producing *Salmonella typhimurium* by phenotypic and genotypic typing methods. J. Clin. Microbiol., 37:3769-3773.

Malorny B, Hoorfar JC, Bunge C, Helmuth R (2003). Multicenter validation of the analytic accuracy of *Salmonella* PCR:towards an international standard . Appl. Environ. Microbiol., 69:290-296.

Oliveira SD, Santos LRD, Schuch M T, Silva ABC, SalleT P, Canal CW (2002). Detection and identification of *Salmonella* from poultry_related samples by PCR. Vet. Microbiol., 87:25_35.

Quinn PJ, Carter ME, Markey B, Carter GR (1994). Clinical Vet Microbiology .Wolf publishing. pp. 209-236.

Rahn K, DeGrandis S, Clarke R, Mcewen S (1992). Amplification of an *invA* gene seguence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonell*a. Mol. Cell. Probe, 6:271_279.

Satheesh N, Thong KL , Tikki P, Martin A (2002). Characterization of *Salmonella* serovars by PCR-Single-Strand Conformation Polymorphism analysis. J. Clin. Microbiol., pp. 2346-2351.

Thong KL (1998).Molecular analysis of *S.paratyphi* A from outbreak in New Dehli, India. Emerg. Infect. Dis., 4:507-508.

Zahraei Salehi T, Mahzounieh M, Saeedzadeh, A (2005).The isolation of antibiotic-resistant *Salmonella* from intestine and Liver of poultry in Shiraz province of Iran. Int. J. Poult. Sci., 4(5):320-322.

African Journal of Microbiology Research Vol. 5(32), pp. 5955-5959, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.1000

Full Length Research Paper

Isolation and exploitation of *Aspergillus ochraceus* RM82 against human pathogenic bacteria

Riaz Muhammad, Sajid Ali and Bashir Ahmad*

Pharma biotech Research Laboratory, Centre for Biotechnology and Microbiology, University of Peshawar, KPK, Pakistan.

Accepted 11 November, 2011

In the present research work, soil samples were collected from different hospitals of District Peshawar, Khyber Pakhtunkhwa, Pakistan, contaminated with hospital disposable materials. *Aspergillus ochraceus* was isolated from the soil through serial dilution followed by morphological identification. Furthermore, different strains of human pathogenic bacteria were isolated from hospitalized patient's blood, urine and pus samples. The isolated *A. ochraceus* was tested against the isolated pathogenic bacterial strains for antimicrobial activities. Antibacterial results were observed against multi drugs resistant (MDR) human pathogenic bacteria.

Key words: *Aspergillus ochraceus*, pustule, antimicrobial, multi drugs resistant.

INTRODUCTION

Multi drugs resistant bacteria such as methiciline-resistant *Staphylococcus aureus* (MRSA) emerged during the last decade and clinically are the cause of serious problems (Levin and Andreasen, 1999). The last choice for the treatment of MDR is vancomycin. Due to vancomycin-resistant Enterococci (VRE) and vancomycin-intermediate resistant *S. aureusarises* a global health concerns (Gilmore and Hoch, 1999). Accordingly it is the cry of the day to exploit antibiotic producing microorganisms for the extraction of novel antimicrobial compounds against MDR, VRE and VISA (Levy, 1998).

Although very important compounds have been extracted from microorganisms but antibiotics have prominent importance due to therapeutic value (McCarthy and Williams, 1990; Ouhdouch et al., 2001; Saadoun and Gharaibeh, 2003). It has been found that emergence of high antibacterial resistance is the alarming problem throughout the world. It is obvious from the irrational use of antibiotics that the efficacies of present antibiotics are decreasing slowly and steadily due to antibiotic resistance. In the perspective of documented reports it is the need of the hour to search for novel compounds (Barbara and Clewes, 2003; Shahghasi et al., 2004). Search for microorganisms producing novel antimicrobials, is still have prime importance due to the continuous emergence of antibiotic resistance (Zrimec et al., 2004).

It has been found that the divers medium of soil has essential macro and micronutrients, playing a vital role in the metabolic pathways of microorganisms (Luzhetskyy et al., 2007; Thomashow et al., 2008). Since micro biologist believed that only a minute fraction of soil microflora has been identified (Thomashow et al., 2008).

After the discovery of magic bullet penicillin, a lot of different antibiotics have been investigated and searched out largely from soil inhabiting microbes. During the last 20-30 years, researchers interest in the soil inhabiting microorganism have been enhanced, due to the increased possibility of searching novel bioactive compounds, like antibiotics and enzymes, active in diverse environmental parameters (Nedialkova and Naidenova, 2004).

A large numbers of antimicrobial secondary metabolites

*Corresponding author. E-mail: bashirdr2001@yahoo.com. Tel: +92-921-6701, Ext. 3070.

Table 1. Fungal strains isolated from soil samples collected from various hospitals of Peshawar.

Name of Hospital	No of soil samples	fungi isolated
Khyber teaching hospital	9	RM 75, RM76, RM77
Lady Reading hospital	13	RM 78, RM79, RM80, RM81
Hayatabad Medical complex	11	RM 82 (Selected), RM83,

RM: specific code for isolated fungi RM82: Selected *Aspergillusochraceus.*

extracted from microbe rich soil environment, including bacteria, actinomycetes and molds (Luzhetskyy et al., 2007; Thomashow et al., 2008). Although some new bioactive compounds are under evaluation, however there is an emergency demand for the investigation and evaluation of novel bioactive compounds against bacterial and fungal pathogens and the bioactive product against these pathogens is the important key source to control them (Luzhetsky et al., 2007). In the light of above facts the objectives of the present research study were to isolate soil inhabiting fungi and then evaluate antimicrobial activities of isolated fungi against human pathogenic bacteria isolated from patient samples.

Due to the world wide problem of antibiotic resistance our research work have profound importance to isolate and screen out such types of microorganisms which produce novel types of compounds against multi drugs resistant MDR bacteria.

MATERIALS AND METHODS

Collection of soil samples

A total of 33 soil samples were collected from different hospitals of District Peshawar, Khyber Pakhtunkhwa, contaminated with hospitals disposable materials like disposable cottons, syringes and plastics. Soil samples were taken from surface to 10 cm depth from the selected sites in the sterilized polystyrene bags using sterilized gloves. All samples were immediately transferred to the laboratory and stored at 4℃ till further use.

Isolation of fungi

Soil samples were serially diluted up to 10^{-5} in distilled water and spread 1 ml sample on the Potato dextrose agar (PDA) and Sabroud dextrose agar (PDA) media and incubated for 3-5 days at 28℃. Isolated fungi were further sub cultured and purified on PDA and SDA media (Table 1).

Morphological identification of *Aspergillusochraceus*

The isolated fungus was preliminary identified on the basis of fungal colony, color, growth pattern, diffusible pigments, hyphae, conidia and sporulation.

Isolation of multi drug resistant human pathogenic bacteria

Human pathogenic bacteria were isolated from patient's blood, urine and pus samples. Blood and urine were collected from hospitalized patient in sterilized vials and bottles. Immediately brought to the pathology laboratory of Lady Reading Hospital Peshawar and were streaked on the selective media such as McConkey agar, Eosine Methylene Blue Agar, ManitolSalt Agar, Cysteine Lactose Electrolyte Deficient Agar, Bismith Sulphite Agar and Blood Agar. All petri plates were incubated at 37℃ for 24 h.

Identification of pathogenic bacteria

All isolated pathogenic bacteria were identified on the basis of morphological and biochemical characteristics on selective media. After Gram staining, biochemical tests were carried out to identify the pathogenic bacteria up to species level. The following biochemical tests were performed for identification, DNase test, coagulase test, catalase test, haemolysis, Motility test, Urease test, Indole production test, Hydrogen sulphide (H_2S) production fermentation test, Mannitol and Sucrose Triple Sugar Iron test for lactose/glucose fermentation, Oxidase test, Citrate utilization test, Nitrate reduction test, Methyl Red Voges Proskauer (MR-VP)Test (Monica, 2005).

Sensitivity assay

Disc diffusion assay was performed according to the procedure of Kirby bauer (CLSI, 2006), for all isolated pathogenic strains using 10 different antibiotic discs. The turbidity of the indicator pathogenic bacteria was adjusted with McFarland solution and then dipped a sterilized cotton bud in the adjusted culture and prepared a uniform lawn on nutrient agar media for every isolated pathogen. Selected antibiotic discs were gently placed on the prepared lawn with equal distance using forcep. Then the nutrient agar plates were incubated at 37℃ for 24 h. After 24 h the results for sensitivity were observed (Table 3).

Preliminary tests for bioactive compounds production, by *Aspergillusochraceus* against human pathogenic bacteria

Aspegillusochraceous was cultured in the PDA media and incubated for 5 days at 28℃. Human pathogenic strains were inoculated in 50 ml nutrient broth media and incubated in orbital shaker (150 rpm) at 37℃ for 24 h. After 24 h 5 ml of broth culture was taken in sterilized test tubes and the turbidity of culture was adjusted with McFarland solution, normal saline was added for turbidity adjustment. Sterilized cotton bud was dipped in the adjusted indicator strains and prepares lawn of each pathogenic strain on the nutrient agar media.

Pustules of 8 mm diameter was taken from already incubated culture on PDA media using cork borer of the same diameter and gently placed on each prepared lawn using sterilized forcep. All Petri dishes were incubated for 24 h at 37℃. After 24 h zone of inhibition around the pustule was observed (Table 2).

Table 2. Antimicrobial activity of *Aspergillusochraceus* against human pathogenic bacteria.

Pathogenic bacteria	Antimicrobial activity
Staphylococcus aureus /15	+
Klebsiela spp /6	+
Streptococcus spp /4	+
Escherichia Coli/10	+
Pseudomonas aureginosa /6	+
Salmonella typhi/4	+

+= positive. /15, 6, 4, 10, 6, 4=number antibiotic resistant bacteria isolated from patient samples.

RESULTS

Isolation and identification of fungi

Nine Fungal strains were isolated from 33 soil samples collected from different hospitals of Peshawar. Primarily one strain of fungi was selected for antimicrobial activity and identification. All other isolated fungi showed no activity against pathogenic strains and were not processed further. The selected strain was identified using variety of manuals and monographs based upon the morphology and colony characteristics, like growth pattern of colony, colorand exudates produced and sporulating structure like conidial head, types of conidiogenous cells, arrangement of conidia, sporangial head, types of spores, pycnidia, accervuli, sporodochia and ascocarps (Domsch et al., 1980; Salar and Aneja, 2007). The fungus was identified as *A. ochraceus*.

Determination of antimicrobial spectra of isolated fungi

Isolated fungal strain was tested against 45 human pathogenic bacteria. Antimicrobial results were observed against both Gram positive and Gram negative pathogenic bacteria around 8 mm pustules. Significant results were observed against Gram positive bacteria which were15-20 mm zone of inhibition around the pustules. Good results were observed against Gram negative pathogenic bacteria, which were 10-15 mm zone of inhibition around 8 mm pustules except *E. coli* which were less than 10 mm (Table 2).

Identification, sensitivity assay of pathogenic bacterial strains and antimicrobial activity of *Aspergillusochraceus*

The bacterial isolates were identified on the basis of morphological and biochemical characteristics these includes, *S. aureus, P. aeruginosa, S. typhi, streptococcus* spp*, klebsiella* spp and *E. coli*. Majority of the isolated bacterial strains were resistant to two or more than two antibiotics. That is why these all isolated pathogens were considered multi drugs resistant for those antibiotics *A. ochraceus* showed activity against all MDR bacterial strains (Table 3).

DISCUSSION

Diverse groups of fungi exits in soil because of its natural habitat. Fungi are the second largest fraction of soil micro flora. Due to its nutritional requirements they survive as saprophytes in their natural habitat. Physical and chemical conditions of soil increases or decreases the population of fungi (Tariq et al., 2008). Many bioactive compounds have been isolated from soil fungi having structural novelty which become the important source for antibiotics developments. Six prescribed medicines out of 20 recommended medications are from fungal source (Tangjang and Arunachalam, 2009).

We isolated *A. ochraceus* which produced bioactive secondary metabolites against multi drugs resistant bacteria.

Multi drugs resistant bacteria were isolated from patients samples and sensitivity assay was conducted. Isolated *A. ochraceus* showed significant results against both Gram positive and Gram negative MDR bacteria.

In the previous studies *A. ochraceus* CL41582 was isolated which inhibited the growth of MDR *S. aureus*, *S. pyogenes* and *E. faecalis* (Yutaka et al., 2001).

In this study we used 10 different antibiotics for sensitivity tests against isolated pathogenic bacteria (Cefotaxime, Cephradine, Levofloxacin, Sparfloxacin, Linezolid, Vancomycin, Teicoplanin, Fusidic acid, Cotrimaxazole, Chloramphenicol) to confirm antibiotic resistance. Pustules of 8 mm disc having diffused secondary metabolites of *A. ochraceus* were used against all isolated pathogens. 10-15 mm zone of inhibition was observed against Gram negative bacteria except *E. coli* which showed minimum activity. While 15-20 mm zone of inhibition was observed against Gram positive bacteria.

Our results correlates with the previous study conducted on *A. cohraceus* a new compound was extracted from *A. ochraceus* which was active against MDR,

Table 3. Disc diffusion assay for all isolated pathogenic strains and anti-pathogenic activity of *Aspergillus ochraceus*.

Pathogens used	Anti-biotic discs										
	CEF	**CEPH**	**LEVO**	**SPAR**	**LINE**	**VANCO**	**TEICO**	**FUS**	**COT**	**CHLO**	**RM82**
Klebsiella spp	R	R	R	R	S	S	S	S	R	S	+
Klebsiella spp	R	R	S	S	S	S	S	S	R	S	+
Klebsiella spp	R	S	S	R	S	S	S	S	S	S	+
Klebsiella spp	R	S	S	S	S	S	S	S	R	S	+
Klebsiella spp	S	R	S	S	S	S	S	S	S	S	+
Klebsiella spp	S	R	S	S	S	S	S	S	R	S	+
Streptococcus	R	S	S	S	S	S	S	S	R	S	+
Streptococcus	S	S	S	S	S	S	S	S	R	S	++
Streptococcus	R	S	S	S	R	S	S	S	S	S	+
Streptococcus	S	R	S	S	S	S	S	S	R	S	+
P. aeruginosa	R	R	R	R	S	S	S	S	S	S	+
P. aeruginosa	R	R	R	S	S	S	S	S	S	S	+
P. aeruginosa	S	R	I	R	S	S	S	S	S	S	+
P. aeruginosa	S	S	S	S	S	S	S	S	S	S	+
P. aeruginosa	S	R	R	R	S	S	S	S	S	S	+
P. aeruginosa	R	R	I	S	S	S	S	R	S	S	+
S. aureus	S	S	S	S	S	S	S	R	R	S	++
S. aureus	R	S	S	R	S	S	S	S	R	S	++
S. aureus	S	S	S	S	S	S	S	S	S	S	++
S. aureus	R	S	R	R	R	S	R	S	S	S	+
S. aureus	R	R	R	R	S	S	S	R	R	R	+
S. aureus	R	S	R	R	S	S	S	R	R	R	++
S. aureus	R	R	R	R	S	S	S	S	R	R	+
S. aureus	S	R	S	S	S	S	S	S	R	S	++
S. aureus	R	S	S	R	R	S	S	S	S	S	++
S. aureus	R	S	S	R	S	S	S	S	R	S	++
S. aureus	S	R	S	S	S	S	S	S	R	S	++
S. aureus	R	R	S	S	R	S	S	S	R	S	++
S. aureus	R	S	S	S	S	S	S	S	R	R	++
S. aureus	R	S	S	S	R	S	S	S	S	S	++
S. aureus	R	S	S	S	R	S	S	S	S	R	++
E. coli	R	R	S	S	S	S	R	S	S	S	--
E. coli	R	R	R	R	S	S	S	S	S	S	--
E. coli	R	S	S	S	S	I	S	S	R	S	--
E. coli	R	R	S	S	S	S	S	S	R	S	--
E. coli	R	R	S	S	S	S	S	S	R	S	--
E. coli	S	R	S	S	S	S	S	S	S	S	--
E. coli	R	S	R	R	S	S	S	S	S	S	--
E. coli	S	R	R	R	S	S	S	S	S	S	--
E. coli	S	R	S	S	S	S	S	S	S	S	--
E. coli	S	R	S	R	S	S	S	S	S	S	--
S. typhi	S	R	S	S	S	S	S	R	S	R	+
S. typhi	R	R	S	S	S	S	S	S	S	S	+
S. typhi	S	R	S	S	S	S	S	S	S	S	+
S. typhi	S	R	S	S	S	S	S	S	S	S	+

S. aureus (cefr, genf, methr, MLSBr, penr, tetr, cipr and vans) were MDR clinical strain. *S. pyogenes* was MLSBr, kanr and str1". *E. faecalis* were also an MDR clinical strain (cefr, eryr, genf, chlr, kanr, tets and van1). Extracted compound from *A. ochraceus* showed no activity against *E. coli* (Yutaka et al., 2001).

In another study carried out on molds bioactive compounds seven fungal species were isolated and evaluated against clinical isolates, the results revealed the presence of bioactive compounds with antimicrobial activity against 22 clinical bacterial isolates including eleven isolates of Gram positive and remaining were Gram negative. The fractions extracted from molds were thoroughly more active on Gram positive bacteria than Gram negative (Amal and Mekawey, 2010).

We used forty five clinical isolates and isolate nine fungal species, *A. ochraceus* have antibacterial activity. Significant antimicrobial activity was observed against Gram positive bacteria while good and minimum results were observed against Gram negative bacteria. In conclusion we need further evaluation and purification of bioactive fractions produced by *A. ochraceus*, so as to minimize resistance associated with MDR bacteria.

ACKNOWLEDGEMENTS

Author is greatly thankful to the administration of Lady Reading Hospital (LRH), Peshawar especially Sardar Ali senior technician pathology laboratory LRH. We are also thankful for University of Peshawar to support us in this research.

REFERENCES

Amal AI, Mekawey (2010). The Antibiotic Properties of Several Strains of Fungi Australian Journal of Basic and Applied Sciences, 4(8): 3441-3454.

Barbara DJ, Clewes E (2003). Plant pathogenic Verticillium species: How many of them are there?"Molecular Plant Pathology, 4(4): 297-305.

Clinical and Laboratory Standards Institute (CLSI) (2006).CLSI: Performance Standard for Antimicrobial Susceptibility Testing. 16th Informational supplement. CLSI document M100-S16.

Gilmore MS, Hoch JA (1999). A vancomycin surprise. Nature, 399: 524-527.

Levy SB (1998). The challenge of antibiotic resistance. Scientific Am., pp. 46-53.

Levin SA,Andreasen V (1999). Disease transmission dynamics and the evolution of antibiotic resistance inhospitals and communal settings. Proc. Natl. Acad. Sci.USA. pp. 800-801.

Luzhetskyy A, Pelzer S, Bechthold A (2007). The future of natural products as a source of new antibiotics. Curr. Opin. Investigat. Drugs, 8: 608-613.

Shahghasi AGH, Shahidi, Fooladi MH, Mahdavi MJ (2004). Broadspectrim, a novel antibacterial from *Streptomyces* sp. Biotechnology, 3(2): 126-130.

McCarthy AJ, Williams ST (1990). Methods for Studying the Ecology of Actinomycetes. Methods In Microbiology. Ed. by. Grigorova R and Norris JR, Academic Press Limited, London, 22: 533-363.

Monica C (2005). District laboratory practice in tropical countries. 2nd edition: pp. 62-72.

Nedialkova D, Naidenova M (2004). Screening the antimicrobial activity of actinomycetes strains isolated from Antarctica. J. Cult. Collections, 4: 29-35.

Domsch KH, Gams W, Anderson TH (1980). Compendium of Soil Fungi. Academic Press, London.

Ouhdouch Y, Barakate M, Finanse C (2001). Actinomycetes of Moroccan habitats: Isolation and screening for antifungal activities. Eur. J. Soil Biol., 37: 69-74.

Saadoun I, Gharaibeh R (2003).The Streptomyces flora of Badia region of Jordan and its potential as a source of antibiotics active against antibiotic- resistant bacteria. J. Arid Environ., 53: 365 - 371.

Salar RK, Aneja KR (2007).Thermophilic fungi: Taxonomy and biogeography. J. Agri. Technol., 3: 77-107.

Tangjang S, Arunachalam K (2009). Microbial population dynamics of soil under traditional agroforestry system in northern India. Res. J. Soil. Biol. 1: 1-7.

Tariq M, Dawar M, Mehdi FA (2008). Studies on the rhizosphere mycoflora of mangroves. Turk. J. Bot., 32: 97-101.

Thomashow LS, Bonsall RF, David M (2008). Detection of antibiotics produced by soil and rhizosphere microbes *in situ*. In: Karlovsky, P. (Ed.). Secondary Metabolites in Soil Ecology. Springer Berlin, Heidelberg, 23-36.

Yutaka S, Hideo H, Taisuke I, Masaru I, Yoon. Jm, Yasuhiro K, Tatsuo Sa, Shinichi S, Akemi S, Yumiko S, Lori B, Joan D, Liang HH, Joyce S, Nakao K (2001). A New Antibiotic CJ-17,665 from *Aspergillus ochraceus*. J. Antibiotics, 54: 911 – 916.

Zrimec MB, Zrimec AP, Slanc, Kreft S (2004).Screening for antibacterial activity in 72 species of wood-colonizing fungi by the Vibrio fisheri bioluminescence method. J. Basic Microbiol., 44(5): 407-412.

African Journal of Microbiology Research Vol. 5(32), pp. 5960-5963, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.1082

Full Length Research Paper

Seroprevalence of avian origin H3N2 canine influenza virus infection in pet dogs in Shenzhen, China

Fu-Rong Zhao[1,2#], Shou-Jun Li[1#], Dong-Hui Zhou[2], Ning Chen[3], Yan-Zhong Zhang[4], Wen-Bao Qi[1], Pei-Rong Jiao[1], Ming Liao[1], Guang-Zhi Tong[5] and Gui-Hong Zhang[1]*

[1]College of Veterinary Medicine, South China Agricultural University, Guangzhou, Guangdong Province 510642, People's Republic of China.
[2]State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, CAAS, Lanzhou, Gansu Province 730046, People's Republic of China.
[3]Shenzhen Institute for Drug Control, Shenzhen, Guangdong Province 518057, PR China.
[4]Shenzhen Rui-Peng Pet Hospitol, Shenzhen, Guangdong Province 518001, PR China.
[5]Division of Swine Infectious Diseases, Shanghai Veterinary Research Institute, CAAS Shanghai 200241,People's Republic of China.

Accepted 22 November, 2011

Canine influenza virus (CIV) is an emerging pathogen that causes severe and acute respiratory disease in dogs. Canine influenza is caused by two subtypes of influenza. A virus: H3N2 and H3N8. In recent years, surveys of avian origin CIV infection in dogs have been reported worldwide. However, little is known about the prevalence of CIV in pet dogs in China. In the present study, the prevalence of avian origin CIV H3N2 in pet dogs in Shenzhen, Southern China was investigated using the enzyme-linked immunosorbent assay (ELISA) and hemagglutination inhibition (HI) assay. Thirty-one (6.71%) of the 462 serum samples tested were seropositive for avian origin CIV by ELISA. Use of the HI test revealed the presence of anti-H3 antibodies in 28 (6.06%) of 462 serum samples. The prevalence ranged from 4.87% (HI) or 6.19% (ELISA) to 7.41% among dogs of different ages, with high prevalence in pet dogs of 1 to 3 years old, but low prevalence in pet dogs ≤1 year. The seroprevalence in female dogs was 5.21%, and in male dogs it was 7.78% (ELISA) or 6.67% (HI). These findings demonstrated that avian origin canine influenza virus infection is prevalent in pet dogs and can spread rapidly through local dog populations, which indicates its potential for becoming established in pet dogs throughout China.

Key words: Canine influenza virus, seropervalence, pet dog, enzyme-linked immunosorbent assay (ELISA), hemagglutination inhibition (HI) assay.

INTRODUCTION

Canine influenza virus (CIV) is a member of the influenza virus A genus in the family Orthomyxoviridae and an emerging pathogen that causes severe and acute respiratory disease in dogs (Jirjis et al., 2010; Lee et al., 2010). CIV was first identified in racing greyhounds in Florida in January 2004 (Payungporn et al., 2008). Canine influenza is caused by 2 subtypes of influenza A virus: H3N2 and H3N8. In 2005, the H3N8 CIV is known to be an equine-derived H3N8 influenza virus and was first identified in dogs in the United States, and in 2007, the H3N2 CIVs are of avian origin and detected in dogs in Korea and China (Crawford et al., 2005; Payungporn et al., 2008; Song et al., 2011b; Lee et al., 2010; Li et al., 2010).

Regardless of subtype, avian origin H3N8 or H3N2 CIV could infect nascent individuals and causes clinical signs. The most common sign of canine influenza is a mild

*Corresponding author. E-mail: guihongzh@scau.edu.cn.

#These authors contributed equally to this work.

respiratory disease that resembles infectious tracheobronchitis. The experimental reproduction of the disease caused by H3N2 CIV induced clinical signs including coughing, sneezing, nasal discharge, fever, and shedding of the virus in nasal discharge (Song et al., 2011a, 2009; Lee et al., 2010). In previous pathological findings, the infection produced a distinctively severe and persistent bronchopneumonia with neutrophil infiltration and apoptosis in the tracheal epithelium (Jung et al., 2010).

Epidemics of avian origin H3N2 CIV among dogs have been observed in Korea, specifically in contaminated kennels in veterinary clinics. Serologic and virological survey of the avian-origin H3N2 CIV in dogs in South Korea suggest that the epidemiological situation resembles that of equine origin H3N8 CIV currently circulating in the dog populations of the United States (Payungporn et al., 2008; Song et al., 2008; Lee et al., 2009). However, no such serological or etiological studies have been carried out in pet dogs in Shenzhen, Southern China.

The objectives of the present investigation were to examine avian origin H3N2 CIV in pet dogs in five pet hospitals in Shenzhen, Southern China under the present husbandry practice and animal welfare, and to evaluate the risk factors for CIV infection in different ages and genders of pet dogs.

MATERIALS AND METHODS

Study area

Shenzhen is located in the very South of Guangdong province, overlooking HongKong to the South and bordering kowloon. It has an area of 1984.69 square meters, it is east to the Daya and Dapeng Bays, west to Pearl River, North to Dongguan and Huizhou and south to Hong Kong Special Administrative Regions of the People's Republic of China. It consists of 6 districts: Luohu, Futian, Nanshan, Yantian, Bao'an and Longgang which the first two are mainly urban areas. It has a mild subtropical oceanic climate with an annual average temperature of 22.3°C.

Serum preparation

A total of 462 blood samples were collected from 5 different pet hospitals which are distributed in Futian and Luohu districts of Shenzhen city between May and July 2009. 82, 114, 127, 101 and 38 blood samples were obtained from pet dogs in Cuizhu, Futian, Honggui, Meilin and Shangbu pet hospitals, respectively. These samples were put aside for solidification followed by centrifugation at 1,000 × g for 10 min, and supernatants were transferred to new centrifuge tubes and saved at -20℃ until use.

Serological tests

ELISA test

The 462 serum samples described above were analyzed for CIV-specific antibodies by using a commercial ELISA Kit (Animal Genetics Inc., South Korea) that can detect anti-nucleoprotein (NP) antibodies based on competition principles. The use of this ELISA kit for CIV detection has been previously reported (Lee et al., 2009; An et al., 2010). Briefly, ELISA plates coated with the antigen (nucleoprotein) are incubated with an equal mixture of 50 µl serum and 50 µl anti AIV antibody-HRP (Horseradish Peroxidase, 1:100 dilution in the conjugate diluent) for 30 min at 37°C. Then, the wells were washed 6 times with 350 µl of diluted washing solution. Then, 100 µl substrate was added to each well and incubated for 10 minutes at room temperature. Finally, 100 µl of stopping solution was added to each well. The absorbance of the wells was read with a bichromatic spectrophotometer at 450 nm with reference wavelength at 620 nm. Reading must be completed within 1 hour from the end of an assay. Positive and negative control sera were provided by the kit with 2 wells for each.

The mean absorbance of the negative controls was calculated, and then the PI (Percent inhibition) value of each serum was calculated, using the following formula:

PI value = [1-(OD sample/mean OD negative)] × 100

Based on PI value, classification of each sample was as follows: PI<50, negative (-); PI ≥ 50, positive (+).

Hemagglutination inhibition (HI) assay

In the present study, the A/canine/Guangdong/01/2007(H3N2) avian origin canine influenza virus strain was used in the serological tests. Genetically, this strain is highly similar to the avian influenza H3N2 virus and was isolated at the Animal Clinics of South China Agricultural University in 2007 (Li et al., 2010). The HI assay was performed according to procedures recommended by the World Organization of Animal Health (OIE). These samples were also analyzed by the HI test, which measures the ability of the sera to inhibit the hemagglutinating activity of the reference virus. The following antigens were also used for HI tested: H1N2 influenza virus (A/swine/Guang dong/06/2009/GH5) for H1, H3N2 influenza virus (A/Swine/Guangdong/01/2005) for H3, H5N1 influenza virus (A/Swan/Guangdong/197/2004) for H5, H9N2 influenza virus (A/Chicken/Guangdong/HL/2006) for H9. These viruses were isolated by the College of Veterinary Medicine, South China Agricultural University. All serum samples were treated with receptor-destroying enzyme (RDE) before testing in HI assays. Briefly, 25 µl of serial two-fold dilutions of the treated serum samples were mixed with 4 HA units of virus in V-shaped microtiter plates and incubated at room temperature for 30 min. Then, 25 µl of 0.5% (v/v) chicken red blood cells (RBCs) was added to each well and incubated at room temperature for 40 min. The HI titer was expressed as the reciprocal of highest serum dilution that completely inhibited hemagglutination of 4 HA units of the virus.

RESULTS

In this investigation, a total of 462 pet dogs (219 females and 243 males) from Shenzhen, Southern China were examined by ELISA and HI for avian origin CIV antibodies. All serum was subjected to NP-specific ELISAs, anti-influenza virus antibodies were detected in 31 samples (6.71%). Use of the HI test revealed the presence of anti-H3 antibodies in 28 of the 462 samples (6.06%). Five samples were seropositive for avian origin H3N2 CIV by ELISA and HI test. Different levels of seropositivity were detected in different pet hospitals,

Table 1. Seroprevalence of canine influenza virus (CIV) in pet dogs in different pet hospitals in Shenzhen, southern China.

Pet hospitals	Examined number	Seroprevalence (%)	
		ELISA	HI
Cuizhu	82	17.07 (14/82)	14.63 (12/82)
Futian	114	2.63 (3/114)	2.63 (3/114)
Honggui	127	6.30 (8/127)	5.51 (7/127)
Meilin	101	5.94 (6/101)	5.94 (6/101)
Shangbu	38	0 (0/38)	0 (0/38)
Total	462	6.71 (31/462)	6.06 (28/462)

Table 2. Seroprevalence of canine influenza virus (CIV) in pet dogs of different ages and genders in Shenzhen, Southern China using ELISA and HI test.

Variable	Class	Prevalence (%)	
		ELISA	HI
Age (years)	≤1	6.19 (14/226)	4.87 (11/226)
	1-3	7.41 (10/135)	7.41 (10/135)
	> 3	6.93 (7/101)	6.93 (7/101)
Gender	Male	7.78 (21/270)	6.67 (18/270)
	Female	5.21 (10/192)	5.21 (10/192)

namely 0, 2.63, 5.94, 6.30 and 17.07% of the samples from Shangbu, Futian, Meilin, Honggui and Cuizhu were CIV antibody-positive, respectively (Table 1). Among these positive pet dogs, seroprevalence varied in different age groups, ranging from 4.87% (HI) or 6.19% (ELISA) to 7.41% (Table 2). The investigation also showed that the seroprevalence in female animals was 5.21%, and 7.78% (ELISA) or 6.67% (HI) in male animals using ELISA or HI (Table 2). All samples were also tested with other antigens (H1, H3, H5, H9) for HI test, and they were all negative.

DISCUSSION

Canine H3N2 influenza viruses of avian origin were recently isolated and found to induce disease in dogs in Korea (Song et al., 2008). Recently, four genetically similar canine influenza H3N2 viruses of avian origin were isolated in South China with severe respiratory disease (Li et al., 2010). The emergence of these canine influenza cases in China could result also from the ecological changes in China, especially as the changing of socio-economic circumstances, particularly in urban areas where dogs are continuing to be raised for food, in some circumstances. CIV replicates efficiently in the respiratory system of dogs and causes severe respiratory disease. Active replication of CIV in the canine respiratory system results in intense inflammatory responses central to the pathogenesis of H3N2 CIV (Lee et al., 2011). Most natural cases of H3N2 CIV died from associated respiratory diseases and the carcasses were generally quickly discarded by veterinarians for quarantine purposes.

Here, we report for the first time the seroprevalence of avian origin CIV H3N2 infection in dogs in Shenzhen city, southern China. 31 of the 462 tested pet dogs were seropositive for CIV by Ab ELISA (6.71%), which is higher than that in Korea, New Zealand and Japan. The seroprevalence of avian H3N2 influenza in Korean pet dogs in 2007 was 0.48% (2/419) by ELISA (Lee et al., 2009). In 2010, 16 (5.59%) of the 286 serum samples collected from pet dogs were CIV seropositive by ELISA in Korea (An et al., 2010). In New Zealand, the 251 dogs serum samples tested was not positive for CIV antibodies by indirect fluorescent antibody (IFA) (Knesl et al., 2009). In Japan, 12 (2.1%) of the 582 serum samples collected from dogs were HI-positive against human H3 virus, only one serum each from dogs was NI-positive against N2 virus (Said et al, 2011). By comparison, 31 of 74 (42%) dogs were seropositive for antibodies against CIV H3N8 in a metropolitan animal shelter (Holt et al., 2010). In Colorado, CIV H3N8 seroprevalence was 2.9% (4/140) for dogs seen by the Community Practice service and 4.5% (5/110) for dogs seen by other hospital services (P = 0.48) (Barrell et al., 2010). In Italy, CIV H1N1 seroprevalence was 0.7% (7/1061) for canine serum specimens in 2009 (Dundon et al., 2010).

In the present study, the seropositivity rates of avian origin H3N2 CIV in pet dogs differed depending on whether an ELISA or HI assay was performed. For

example, while ELISA text found that 6.30 and 17.07% of the pet dogs from Honggui and Cuizhu were exposed to avian origin CIV, the HI test detected seropositivity rates of 5.51 and 14.63%, respectively. While the HI assay is often used to detect antibodies against viral hemagglutinin (HA) in human and animal serum, it is not very reliable in detecting antibodies to avian influenza viruses in mammalian serum because nonspecific hemagglutination inhibitors in the sera can result in false positives (Lu et al., 1982). In addition, Lee et al. (2009) found that the HI assay detected anti-influenza H3N2 virus antibodies 2 days later than the NP-based ELISA test. These results suggest that the NP-based ELISA is a better method for the serological survey of CIV infections in pet dogs.

Conclusions

In summary, the present survey revealed a relatively low seropositivity of CIVs in pet dogs in Shenzhen, Southern China, which raises the concerns regarding the rapid spread of avian origin CIV in pet dogs in animal hospitals in China. These findings suggest that commercial vaccines against canine influenza virus must be developed and used in pet dog population.

ACKNOWLEDGEMENTS

This project was supported in part by the National Natural Science Foundation of China (grant No. 30972233), the National Key Basic Research Program (Project 973) of China (grant no. 2011CB504700-G), the Natural Science Foundation of Guangdong Province (No. 8251064201000008) and the Basic Conditions for Science and Technology Projects of Guangdong Province (No. 2011B060400015).

REFERENCES

An DJ, Jeoung HY, Jeong W, Chae S, Song DS, Oh JS, Park BK (2010). A serological survey of canine respiratory coronavirus and canine influenza virus in Korean dogs. J. Vet. Med. Sci., 72: 1217-1219.

Barrell EA, Pecoraro HL, Torres-Henderson C, Morley PS, Lunn KF, Landolt GA (2010). Seroprevalence and risk factors for canine H3N8 influenza virus exposure in household dogs in Colorado. J. Vet. Intern. Med., 24: 1524-1527.

Crawford PC, Dubovi EJ, Castleman WL, Stephenson I, Gibbs EP, Chen L, Smith C, Hill RC, Ferro P, Pompey J, Bright RA, Medina MJ, Johnson CM, Olsen CW, Cox NJ, Klimov AI, Katz JM, Donis RO (2005). Transmission of equine influenza virus to dogs. Science, 310: 482-485.

Dundon WG, De Benedictis P, Viale E, Capua I (2010). Serologic evidence of pandemic (H1N1) 2009 infection in dogs, Italy. Emerg. Infect. Dis., 16: 2019-2021.

Holt DE, Mover MR, Brown DC (2010). Serologic prevalence of antibodies against canine influenza virus (H3N8) in dogs in a metropolitan animal shelter. J. Am. Vet. Med. Assoc., 237: 71-73.

Jirjis FF, Deshpande MS, Tubbs AL, Jayappa H, Lakshmanan N, Wasmoen TL (2010). Transmission of canine influenza virus (H3N8) among susceptible dogs. Vet. MicrobioI., 144: 303-309.

Jung K, Lee CS, Kang BK, Park BK, Oh JS, Song DS (2010). Pathology in dogs with experimental canine H3N2 influenza virus infection. Res. Vet. Sci., 88: 523-527.

Knesl O, Allan FJ, Shields S (2009). The seroprevalence of canine respiratory coronavirus and canine influenza virus in dogs in New Zealand. N. Z. Vet. J., 57: 295-298.

Lee C, Jung K, Oh J, Oh T, Han S, Hwang J, Yeom M, Son D, Kim J, Park B, Moon H, Song D, Kang B (2010). Protective efficacy and immunogenicity of an inactivated avian-origin H3N2 canine influenza vaccine in dogs challenged with the virulent virus. Vet. Microbiol., 143: 184-188.

Lee C, Song D, Kang B, Kang D, Yoo J, Jung K, Na G, Lee K, Park B, Oh J (2009). A serological survey of avian origin canine H3N2 influenza virus in dogs in Korea. Vet. Microbiol., 137: 359-362.

Lee YN, Lee HJ, Lee DH, Kim JH, Park HM, Nahm SS, Lee JB, Park SY, Choi IS, Song CS (2011). Severe canine influenza in dogs correlates with hyperchemokinemia and high viral load. Virol., 417: 57-63.

Li S, Shi Z, Jiao P, Zhang G, Zhong Z, Tian W, Long LP, Cai Z, Zhu X, Liao M, Wan XF (2010). Avian-origin H3N2 canine influenza A viruses in Southern China. Infect. Genet. Evol., 10: 1286-1288.

Lu BL, Webster RG, Hinshaw VS (1982). Failure to detect hemagglutination-inhibiting antibodies with intact avian influenza virions. Infect. Immun., 38: 530-535.

Payungporn S, Crawford PC, Kouo TS, Chen LM, Pompey J, Castleman WL, Dubovi EJ, Katz JM, Donis RO (2008). Influenza A virus (H3N8) in dogs with respiratory disease, Florida. Emerg. Infect. Dis., 14: 902-908.

Said AW, Usui T, Shinya K, Ono E, Ito T, Hikasa Y, Matsuu A, Takeuchi T, Sugiyama A, Nishii N, Yamaguchi T (2011). A sero-survey of subtype H3 influenza A virus infection in dogs and cats in Japan. J. Vet. Med. Sci., 73: 541-544.

Song D, Kang B, Lee C, Jung K, Ha G, Kang D, Park S, Park B, Oh J (2008). Transmission of avian influenza virus (H3N2) to dogs. Emerg. Infect. Dis., 14: 741-746.

Song D, Lee C, Kang B, Jung K, Oh T, Kim H, Park B, Oh J (2009). Experimental infection of dogs with avian-origin canine influenza A virus (H3N2). Emerg. Infect. Dis., 15: 56-58.

Song D, Moon H, Jung K, Yeom M, Kim H, Han S, An D, Oh J, Kim J, Park B, Kang B (2011a). Association between nasal shedding and fever that influenza A (H3N2) induces in dogs. Virol. J., 8: 1.

Song DS, An DJ, Moon HJ, Yeom MJ, Jeong HY, Jeong WS, Park SJ, Kim HK, Han SY, Oh JS, Park BK, Kim JK, Poo H, Webster RG, Jung K, Kang BK (2011b). Interspecies transmission of the canine influenza H3N2 virus to domestic cats in South Korea, 2010. J. Gen. Virol., 92: 2350-2355.

African Journal of Microbiology Research Vol. 5(32), pp. 5964-5968, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.1103

Full Length Research Paper

Assessment of inflammatory cytokines and soluble adhesion molecules in patients with systemic inflammatory response syndrome in an intensive care unit of a Saudi tertiary hospital

Obeid E. Obeid* and Manal I. Hassan

Department of Microbiology, College of Medicine, University of Dammam, P.O. Box 2114, Dammam 31451, Saudi Arabia.

Accepted 18 November, 2011

Cytokines are endogenous inflammatory mediators, which play a central role in the pathophysiology of sepsis and in the expression of the adhesion molecules. The aims of this study are to analyze the levels of cytokines and the soluble adhesion in serum of infected (N = 68) and non-infected (N = 41) patients with systemic inflammatory response molecules (SIRS). 109 patients in the intensive care unit (ICU) of a tertiary hospital were included. IL-6, TNF-α, IL-10, IL-13, sICAM-1 and VCAM-1 were measured using enzyme-linked immunosorbent assay (ELISA). Patients with infectious SIRS, the levels of IL-6 varied between 27.65 to 39.6 pg/L(mean = 33.4 pg/L); the levels of sVCAM-1 varied between 543 and 1079 ng/ml (mean = 782 ng/ml) and the levels of sICAM-1 varied between 320 and 664 ng/ml (mean = 458 ng/ml). In patients with non-infectious SIRS the levels of IL-6 varied between 18.2 to 20.3 pg/L(mean 19.2 pg/L); the levels of sVCAM-1 varied between 251 and 635 ng/ml (mean = 286 ng/ml) and the levels of sICAM-1 varied between 98 and 351 ng/ml (mean = 168 ng/ml). The levels of IL-6, sVCAM-1 and s ICAM-1 were significantly higher in septic patients than in non-septic patients (p = 0.002; p = 0.003 and p = 0.0002, respectively). There was no statistically significant difference in the levels of TNF-α, IL-10 and IL-13 between infectious and non-infectious SIRS patients. Measurement of pro-and anti- inflammatory cytokines and soluble adhesion molecules may be useful in the follow up of ICU patients and in providing a point of care tests that will help in decision making and in management of ICU patients.

Key words: Cytokines, adhesion molecules, enzyme-linked immunosorbent assay (ELISA), intensive care unit (ICU) patients.

INTRODUCTION

Systemic inflammation is a highly organized response to infectious and noninfectious threats to homeostasis (Shubin et al., 2011). The main effectors of systemic inflammation are inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6; chemokines and other mediators of inflammation (Shubin et al., 2011; Kibe et al., 2011).

Cytokines (including chemokines) are endogenous inflammatory mediators, which play a central role in the pathophysiology of sepsis (Nagai et al, 2011). TNF-α is a principal pro-inflammatory cytokine that induces systemic inflammatory response against the infectious insult (Nagai et al., 2011). Other pro-inflammatory cytokines include IL-1β, IL6, IL-8, interferon (IFN) γ, and macrophage migration inhibitory factor (MIF) (Eggimann and Pittet, 2001; Martin et al., 1994; Goldie et al., 1995; Pinsky et al., 1993). Excessive production of pro-inflammatory cytokines by immunocompetent cells may induce systemic inflammatory response syndrome (SIRS) (Damas et al., 1992; Oda et al., 2005).

Sepsis also activates the production and release of

*Corresponding author. E-mail: oobeid@yahoo.com. Tel: 00966509929487.

specific anti-inflammatory substances, including the cytokine receptor antagonists, the soluble cytokine receptors and the anti-inflammatory cytokines (Makhija, 2005; Delsesto and Opal, 2011; Tamayo et al., 2011). IL-10, IL-13 and transforming growth factor β (TGF- β) are anti-inflammatory cytokine, which probably have an important down-regulatory function in decreasing the production of various pro-inflammatory cytokines, such as TNF-α and IL-6 (Marchant et al., 1994; Martin et al., 1997).

Nosocomial infections (NIs) are today by far the commonest complications affecting hospitalised patients. Currently, 5 to 10% of patients admitted to acute care hospitals acquire one or more infections, and the risks have steadily increased during recent decades (Esposito and Leone, 2007; Jarvis, 2001). Although representing only 5 to 15% of hospital beds, intensive care units (ICUs) account for 10 to 25% of healthcare costs, corresponding to 1 to 2% of the gross national product of the United States (Esposito and Leone, 2007).

Vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) are mediators of endothelial-leukocyte adhesion in inflammatory states (Shapiro et al., 2010; Gando et al., 2005; Figueras-Aloy et al., 2007). They mediate tight binding and extravasation of leukocytes through endothelial cell junctions (Brenner et al., 2010). Low levels of adhesion molecules are detected in serum of normal individuals and several investigators have recently documented increased levels in patients with sepsis and other critical illnesses (Brenner et al., 2010; Cumming et al., 1997). There is interest in examining serum levels of adhesion molecules in sepsis and other inflammatory conditions and relating their measurement to outcome from critical illness (Newman et al., 1993). Up-regulation of membrane-bound and soluble forms of adhesion molecules and their corresponding ligands on endothelial cells is induced by inflammatory mediators, such as TNF-α and IL-6. Soluble isoforms of adhesion molecules are critical for the early events of leukocyte recruitment (Jaber et al., 2009).

The published data on the relationship between the serum levels of soluble adhesion molecules and SIRS is sparse. Serum levels of cytokines and adhesion molecules could be different in different groups of SIRS patients because of the differences in the underlying cause. The purpose of this study was to assess the level of cytokines and soluble adhesion molecules in different groups of SIRS patients in the ICU of a tertiary hospital.

MATERIALS AND METHODS

Study population

All patients admitted to the ICU with SIRS over a period of 12 months (2007 to 2008) were included (N = 109). The study was conducted in a tertiary hospital in Eastern Saudi Arabia (Alzahrani et al., 2009). Patients were categorized into the following groups: patients with sepsis (N = 68) and those with SIRS without infection (N = 41). Clinically suspected infection was defined by the attending physician including the suspicion of an ongoing infection, combined with the initiation of a diagnostic work-up. Patients with SIRS were defined as having two or more of the following criteria (Rosengart, 2006): fever (body temperature >38°C) or hypothermia (body temperature <35.5°C), tachycardia (heart rate >90 beat s min^{-1}), tachypnea (>20 breaths min^{-1}) and leukocytosis or leukopenia (white blood cell count >12 000 or <4000/mm^3 . The infection was confirmed if the patient has an identifiable site of infection which is confirmed by positive microbiological cultures. Bacterial cultures were obtained from trachea, urine, abdominal or mediastinal drains or from perioperative or percutaneous bacteriological samples. A written consent was obtained.

Measurements

For cytokine assays, peripheral blood samples were collected from the subjects by venepuncture in plain tube, left to coagulate and fresh serum samples were separated and aliquots and stored at −70°C prior to analysis. Aliquots were frozen within 8 of collection. The samples were taken within 24 h of admission to the ICU (day 1). Cytokine and soluble adhesion (IL-4, IL-6, IL-10, IL-13, TNF-α, sVCAM-1 and sICAM-1) analyses were performed with commercially available enzyme-linked immunosorbent assays (Quantikine®, R and D Systems, Minneapolis, Minn., USA; Biosource International, Camarillo; Pelikine Compact™, Central Laboratory of the Netherlands Red Cross Transfusion Service, Amsterdam, the Netherlands) according to the manufacturer's recommendations. In addition, complete blood counts were obtained on all patients including a full white blood cell (WBC) differential. Bacterial, viral and candida cultures/molecular detection of cytomegalovirus, respiratory syncytial virus and adenovirus were done using established assays.

Statistical analysis

Data was entered in a personal computer and statistical analyses were performed using the statistical package for social sciences (SPSS-PC version 16). The descriptive statistics were reported as mean and range values. The patient groups were compared using the unpaired Student's t-test, the chi-squared test and the Friedman's test as indicated. P-values below 0.05 was considered significant in the SPSS software.

RESULTS

The present study used the same study population of ICU patients that we used in previous study (Alzahrani et al., 2009). We evaluated a total of 109 SIRS patients admitted to ICUs in a tertiary hospital over the period of the study. Out of 109 patients, 68 fulfilled the criteria of infectious SIRS. The remaining 41 patients were classified as non-infectious SIRS, with no defined source of infection as proved by negative bacterial cultures. The mean age of the study population (45 males and 64 females) was 49.8 ± 20.9 years. The clinical diagnosis included infection with varying degrees of sepsis (68), trauma (12), respiratory failure (10), post surgical (7), non infective complications requiring ICU (5), heart failure (3), sickle cell with crisis (3) and seizure disorder (1). Among

Table 1. Cytokines levels in infectious and non-infectious SIRS patients.

Cytokine	Level in infectious SIRS	Level in non-infectious SIRS
IL-6	27.65-39.6 pg/L(mean=33.4 pg/L)	18.2-20.3 pg/L(mean 19.2 pg/L)
TNFα	2.5-5.6 pg/ml (mean 2.8 pg/ml)	2.3-4.2 pg/ml (mean 2.6 pg/ml)
IL-10	11.2-14.2 pg/L(mean=13.4 pg/L)	10.7-14.8 pg/L(mean 12.7 pg/L)
IL-13	22.5-39.2 pg/L(mean 31.5 pg/ml)	28.3-41.1 pg/L(mean=32.2 pg/ml)

Table 2. levels of soluble adhesion molecules (SAM) in infectious and non-infectious SIRS patients.

SAM	Level in infectious SIRS	Level in non- infectious SIRS
sVCAM-1	543-1079 ng/ml (mean=782 ng/ml)	251-635 ng/ml (mean=286 ng/ml)
sICAM	320-664 ng/ml (mean=458 ng/ml)	98-351 ng/ml (mean=168 ng/ml)

patients with defined source of infection, blood stream infection, lower respiratory tract infection, soft tissue infection and urinary tract infection constituted the most common infections.

The levels of proinflammatory cytokines were measures using enzyme-linked immunosorbent assay (ELISA) (Table 1). In patients with infectious SIRS, the levels of IL-6 varied between 27.65 to 39.6 pg/L(mean = 33.4 pg/l) and in patients with non-infectious SIRS the levels varied between 18.2 to 20.3 pg/L(mean 19.2 pg/L). In patients with infectious SIRS, the levels of TNF-α varied between 2.5 to 5.6 pg/ml (mean 2.8 pg/ml) and in patients with non-infectious SIRS the levels varied between 2.3 to 4.2 pg/ml (mean = 2.6 pg/ml). The levels of IL-6 was significantly higher in septic patients than in non-septic patients (p = 0.002). There was no statistically significant difference in TNF-α levels between infectious and non-infectious SIRS patients.

The levels of anti-inflammatory cytokines were measures using ELISA (Table 1). In patients with infectious SIRS, the levels of IL-10 varied between 11.2 to 14.2 pg/L(mean = 13.4 pg/l) and in patients with non-infectious SIRS the levels varied between 10.7 to 14.8 pg/L(mean 12.7 pg/l). In patients infectious SIRS, the levels of IL-13 varied between 22.5 to 39.2 pg/L(mean 31.5 pg/ml) and in patients with non- infectious SIRS the levels varied between 28.3 to 41.1 pg/L(mean = 32.2 pg/ml). There was no statistically significant difference in IL-10 and IL-13 levels between infectious and non-infectious SIRS patients.

The levels of sICAM-1 and sVCAM-1 were measured using ELISA (Table 2). The levels of sVCAM-1 in patients with infectious varied between 543 and 1079 ng/ml (mean = 782 ng/ml) and in non-infectious SIRS the levels varied between 251 and 635 ng/ml (mean = 286 ng/ml). The levels of sICAM in infectious SIRS varied between 320 and 664 ng/ml (mean = 458 ng/ml) and the levels in non-infectious SIRS varied between 98 and 351 ng/ml (mean = 168 ng/ml). The levels of sVCAM and sICAM were significantly higher in the infectious SIRS than non-infectious SIRS patients (p = 0.003 and = 0.0002 respectively).

DISCUSSION

Excessive production of pro-inflammatory cytokines by immunocompetent cells can induce SIRS and that these cytokines may play an important role in the development of acute respiratory distress syndrome (ARDS) or multiple organ dysfunction syndromes (MODS) (Oda et al., 2005; Delsesto and Opal, 2011). It has been reported that blood levels of these pro-inflammatory cytokines are elevated in patients with ARDS and septic shock, and that measurement of blood levels of these cytokines is useful in evaluating the severity and in predicting the outcome of the patients with these pathophysiological conditions (Delsesto and Opal, 2011). Among these pro-inflammatory cytokines, IL-6 has a longer half-life than TNF-α and IL-1β and its blood level remains consistently elevated in the presence of various diseases (Tamayo et al., 2011). For these reasons, the measurement of cytokines such as IL-6 blood levels is potentially useful in severity assessment and outcome prediction in patients with septic shock, trauma, severe acute pancreatitis, and cardiogenic shock (Martin et al., 1997). The rapid measurement system that allows blood IL-6 levels to be measured within about 30 min using chemiluminescent enzyme immunoassay (CLEIA) has recently been reported. This system can yield results of IL-6 measurement on approximately real-time basis when incorporated into the clinical laboratory test menu. The longer half-life of IL-6 in comparison with TNF-αmay explains why we can demonstrate a difference in the level of IL-6 and not in the level of TNF-α.

IL-6 levels are significantly elevated in the majority of patients with sepsis and the circulating IL-6 levels have correlated with the severity of sepsis in most studies

(Damas et al., 1992; Oda et al., 2005). The persistently high levels of IL-6 have been associated with the development of multiple organ failure (MOF) and poor prognosis (Tamayo et al., 2011). It has been suggested that elevated IL-6 levels reflect the activation of inflammatory response in sepsis, although the exact role of IL-6 in the pathogenesis of sepsis is not clear.

There was no statistically significant difference in IL-10 and IL-13 levels between infectious and non-infectious SIRS patients. This could be explained by the short half-life that leads to transient increase in their level in the plasma which makes their measurement in the plasma more difficult. Although many cell types express IL-10 mRNA, not all make detectable amounts of protein, and this variation can be accounted for by posttranscriptional mechanisms (Powell et al., 2000). Intra-cellular measurement of cytokines may provide more sensitive mean of cytokine analysis.

Theoretically, these anti-inflammatory substances may have an important regulatory function in controlling and attenuating the systemic inflammatory response in sepsis (Delsesto and Opal 2011). However, patients with an excessive compensatory anti-inflammatory response syndrome may be immunosuppressed and at increased risk of infection or death.

The published data on the relationship between the serum levels of soluble adhesion molecules and SIRS is sparse (Brenner et al., 2010). To assess the serum soluble adhesion molecules in the SIRS ICU patients, the levels of, soluble Inter-cellular adhesion molecule-1 (sICAM-1) and Soluble Form of Vascular Cell Adhesion Molecule 1(sVCAM-1) were measured using ELISA. The levels of sVCAM and sICAM were significantly higher in the infectious SIRS than non-infectious SIRS patients. This could be due to the vital role of adhesion molecules in the process of leukocyte adhesion and migration that occur during inflammation. ICAM-1 participates in the adhesion of leukocytes to the endothelium and may be crucial in regulating leukocyte activation at a very early inflammatory response. Expression of adhesion molecules is regulated by cytokine activation and recent studies examined the role of sICAM-1 in neonatal infections, but with controversial results.

Both mononuclear cells and endothelial cells secrete ICAM-1, and ICAM-l expression on endothelial cells is up-regulated by cytokines such as IL-6. ICAM-1 is a ligand for lymphocyte function-associated antigen-i (LFA-1) and is one of the receptors responsible for adhesion of T lymphocytes, monocytes and granulocytes to endothelium. VCAM-I is expressed on dendritic cells and vascular endothelium activated by TNF, IL-I and IL-4. It plays a major role in the adhesion of leucocytes to the endothelium by interaction with its ligand very late activation antigen-4 (VLA-4), which is expressed by lymphocytes and monocytes (Jaber et al., 2009).

In conclusion, measurement of IL-6, sVCAM-1 and sICAM-1 appear to correlate with the development of infection and sepsis in SIRS patients. Measurement of pro-and anti-inflammatory cytokines and soluble adhesion molecules may be useful in the follow up of ICU patients and in providing a point of care tests that will help in decision making and in management of ICU patients. There is a need for more studies to establish a clear strategy for a diagnostic algorithm for cytokine and adhesion molecules measurements before such tests are eventually used in practice.

ACKNOWLEDGEMENT

Authors gratefully acknowledge the Dean of Scientific Research at the University of Dammam (grant No. 90065) for the financial support.

REFERENCES

Alzahrani AJ, Hassan MI, Obeid OE, Diab AE, Qutub HO, Gupta RK (2009). Rapid detection of procalcitonin as an early marker of sepsis in intensive care unit in a tertiary hospital. Intern. J Med. Med. Sci., 1: 516-522.

Brenner T, Hofer S, Rosenhagen C, Steppan J, Lichtenstern C, Weitz J, Bruckner T, Lukic IK, Martin E, Bierhaus A, Hoffmann U, Weigand MA (2010). Macrophage migration inhibitory factor (MIF) and manganese superoxide dismutase (MnSOD) as early predictors for survival in patients with severe sepsis or septic shock. J. Surg. Res., 164:163-171.

Cumming CJ, Sessler CN, Beal LD, Fisher BJ, Best AM, Fowler III AA (1997). Soluble E-Selectin Levels in Sepsis and Critical Illness Correlation with Infection and Hemodynamic Dysfunction. Am. J. Respir. Crit. Care Med., 156: 431-437.

Damas P, Ledoux D, Nys M, Vrindts Y, De Groote D, Franchimont P, Lamy M (1992). Cytokine serum level during severe sepsis in human IL-6 as a marker of severity. Ann. Surg., 215: 356–362.

Delsesto D, Opal SM (2011). Future perspectives on regulating pro-and anti-inflammatory responses in sepsis. Contrib. Microbiol., 17:137-156.

Eggimann P, Pittet D (2001). Infection control in the ICU. Chest, 120: 2059–2093.

Esposito S, Leone S (2007). Antimicrobial treatment for Intensive Care Unit (ICU) infections including the role of the infectious disease specialist. Intern. J. Antimicrb. Agents, 29: 494-500.

Figueras-Aloy J, Gómez-López L, Rodríguez-Miguélez JM, Salvia-Roiges MD, Jordán-García I, Ferrer-Codina I, Carbonell-Estrany X, Jiménez-González R (2007). Serum soluble ICAM-1, VCAM-1, L-selectin, and P-selectin levels as markers of infection and their relation to clinical severity in neonatal sepsis. Am. J. Perinatol., 24: 331-338.

Gando S, Kameue T, Matsuda N, Hayakawa M, Hoshino H, Kato H (2005). Serial changes in neutrophil-endothelial activation markers during the course of sepsis associated with disseminated intravascular coagulation. Thromb. Res., 116: 91-100.

Goldie AS, Fearon KC, Ross JA, Barclay GR, Jackson RE, Grant IS, Ramsay G, Blyth AS, Howie JC (1995). Natural cytokine antagonist and endogenous antiendotoxin core antibodies in sepsis syndrome. The sepsis intervention group. JAMA., 274: 172–177.

Jaber SM, Hamed EA, Hamed SA (2009). Adhesion molecule levels in serum and cerebrospinal fluid in children with bacterial meningitis and sepsis. J. Pediatr. Neurosci., 4: 76-85.

Jarvis WR (2001). Infection control and changing health-care delivery systems. Emerg. Infect. Dis., 7: 170–173.

Kibe S, Adams K, Barlow G (2011). Diagnostic and prognostic biomarkers of sepsis in critical care. J. Antimicrob. Chemother., 2: 33-40.

Makhija P, Yadav S, Thakur A (2005). Tumor necrosis factor alpha and interleukin 6 in infants with sepsis. Indian Pediatr., 42: 1024-1028.
Marchant A, Devière J, Byl B, De Groote D, Vincent JL, Goldman M (1994). Interleukin-10 production during septicemia. Lancet, 343: 707–708.
Martin C, Boisson C, Haccoun M, Thomachot L, Mege JL (1997). Patterns of cytokine evolution (tumor necrosis factor-α and interleukin-6 after septic shock, hemorrhagic shock, and severe trauma. Crit. Care Med., 25: 1813–1819.
Martin C, Saux P, Mege JL, Perrin G, Papazian L, Gouin F (1994). Prognostic value of serum cytokines in septic shock. Intensive Care Med., 20: 272–277.
Nagai M, Hirayama K, Ebihara I, Higuchi T, Imaizumi M, Maruyama H, Miyamoto Y, Kakita T, Ogawa Y, Fujita S, Shimohata H, Kobayashi M (2011). Serum TNF-Related and Weak Inducer of Apoptosis Levels in Septic Shock Patients. Ther. Apher. Dial., 15: 342-348.
Newman W, Beall LD, Carson CW, Hunder GC, Graben N, Randhawa ZI, Gopal TV, Wiener-Kronish J, Matthay MA (1993). Soluble E-selectin is found in supernatants of activated endothelial cells and is elevated in the serum of patients with septic shock. J. Immunol., 150: 644-654.
Oda S, Hirasawa H, Shiga H, Nakanishi K, Matsuda K and Nakamua M (2005). Sequential measurement of IL-6 blood levels in patients with
systemic inflammatory response syndrome (SIRS)/sepsis. Cytokine, 29: 169-175.
Pinsky MR, Vincent JL, Deviere J, Alegre M, Kahn RJ, Dupont E (1993). Serum cytokine levels in human septic shock. Relation to multiple-system organ failure and mortality. Chest, 103: 565–575.
Powell MJ, Thompson SA, Tone Y, Waldmann H, Tone M (2000). Posttranscriptional regulation of IL-10 gene expression through sequences in the 3'-untranslated region. J. Immunol., 165: 292-296.
Rosengart MR (2006). Critical care medicine: landmarks and legends. Surg. Clin. North. Am., 86: 1305-1321.
Shapiro NI, Schuetz P, Yano K, Sorasaki M, Parikh SM, Jones AE, Trzeciak S, Ngo L, Aird WC (2010). The association of endothelial cell signaling, severity of illness, and organ dysfunction in sepsis. Crit. Care, 4 :182.
Shubin NJ, Monaghan SF, Ayala A (2011). Anti-inflammatory mechanisms of sepsis. Contrib. Microbiol., 17: 108-124.
Tamayo E, Fernández A, Almansa R, Carrasco E, Heredia M, Lajo C, Goncalves L, Gómez-Herreras JI, de Lejarazu RO, Bermejo-Martin JF (2011). Pro- and anti-inflammatory responses are regulated simultaneously from the first moments of septic shock. Eur. Cytokine Netw., 22: 82-87.

African Journal of Microbiology Research Vol. 5(32), pp. 5969-5971, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.1126

Full Length Research Paper

Seroprevalence of hepatitis-A virus among children aged 1-16 years in Eastern Anatolia, Turkey

Uğur DEVECI[1], Cemal USTUN[2]* and Ozlem HAMANCA[3]

[1]Department of Pediatric, Ministry of Health, Tunceli State Hospital, Tunceli –Turkey.
[2]Department of Infectious Diseases and Clinical Microbiology, Ministry of Health, Elazig Harput State Hospital, Elazig-Turkey.
[3]Department of Clinical Microbiology, Ministry of Health, Tunceli State Hospital, Tunceli-Turkey.

Accepted 5 December, 2011

This study aims to determine the seroprevalence of hepatitis A among children aged 1-16 years in eastern region of Turkey. The study was conducted at Tunceli State Hospital in Eastern Anatolia, Turkey. Anti-HAV IgM and Anti-HAV IgG antibodies were evaluated among 351 patients admitted to our pediatric policlinic. Anti-HAV IgM and Anti-HAV IgG serologic markers were determined using the ELISA method. The mean age of 351 pediatric patients was 7.5±4.2; of these, 198 (56.4%) were male and 153 (43.6%) were female. A total of 305 (86.9%) cases in this study were seronegative against hepatitis A. Anti-HAV IgG was positive among 46 (13.1%) patients, of these 22 (47.8%) were male and 24 (52.2%) were female. The mean age of seropositive cases was 8.4±4.8. Anti-HAV IgM seropositivity was not detected in the study. The application of a routine hepatitis A vaccine among children will reduce the potential for the development of severe complications.

Key words: Hepatitis A, seroprevalence, children, vaccination.

INTRODUCTION

Viral hepatitis is a major public health problem in developing and developed countries worldwide (Ustun et al., 2009). Hepatitis A infections spread predominantly by fecal-oral route and occur throughout the world. However the disease is seen most commonly in developing countries, where the prevalence rate approaches 100% in children by 5 years of age (Yazigi, 2007). The prevalence rate of hepatitis A has been reported as 64.4% overall in Turkey. The prevalence rate for western and central regions has been reported as 80% and it was more than 90% for south-eastern and eastern regions of Turkey (Ceyhan et al., 2008).

The clinical spectrum of hepatitis A virus infection ranges from asymptomatic infection to fulminant hepatitis. Clinical manifestations depend on the age of the host: less than 30% of infected young children are symptomatic, while about 80% of infected adults manifest severe hepatitis with remarkably elevated serum aminotransferases (Jeong and Lee, 2010). Hepatitis A may lead to severe clinical manifestations, including fulminant hepatitis, in about 10-15% of adults. Thus, the outbreaks of hepatitis A can cause the severe economic and work force lost (Richardus et al., 2004).

This study aims to determine the seroprevalence of hepatitis A virus among children aged 1-16 years and to observe the changes in the seroprevalence of hepatitis A and, whether the hepatitis A vaccination is necessary in Tunceli Province of Eastern Anatolia of Turkey or not.

MATERIALS AND METHODS

This retrospective study was conducted in Tunceli State Hospital, which is a general hospital that contains 150-beds and is located in Eastern Anatolia of Turkey, between August and December 2010. A total of 351 patients admitted to pediatric policlinic with any reason were included into the study. Patients aged between 1-16 years, who have not any chronic liver disease were screened. The

*Corresponding author. E-mail: drcustun@gmail.com. Tel: +90 532 6964378.

Table 1. The demographic characteristics of 351 patients.

	Patient count (%)	Mean Age ± Sd*	Male (%)	Female (%)
All cases	351 (100)	7.5 ±4.2	198 (56.4)	153 (43.6)
Seropositive cases	46 (13.1)	8.4 ±4.8	22 (47.8)	24 (52.2)
Seronegative cases	305 (86.9)	7.4 ±4.0	176 (57.7)	129 (42.3)

*Sd: Standard deviation.

Table 2. The studies of hepatitis A seroprevalence conducted in Turkey.

Researcher	Location of Turkey	Date	Age	Case number	Seropositivity rate (%)
Kanra et al. (2002)	General	2002	1-4 years	727	42.7
Alabaz et al. (2005)	Southern	2005	12 months	147	36.1
Ozen et al. (2006)	Eastern	2006	3-6 years	286	17.5
Ceyhan et al. (2008)	Southeastern	2006	0-14 years	701	90
Aslan et al. (2001)	Southeastern	1999	2-64 years	400	66.5
Tekay (2006)	Eastern	2004	0-14 years	416	63
Present study	Eastern	2010	1-16 years	351	13.1

data of hepatitis A serological markers were retrospectively collected from patients' files. Anti-HAV IgM and Anti-HAV IgG serological markers were tested by using the ELISA method (Abbott Architect I 2000 SR).

Statistical analysis of the data was done by SPSS for Windows 16.0 software (SPSS Inc, Chicago, USA). *Student's t*-test was used to compare the data of patients.

RESULTS

The demographic characteristics of 351 patients are shown in Table 1. Anti-HAV IgG seropositivity was found among 46 (13.1%) patients. Anti-HAV IgM seropositivity was not detected in any case during study period. There were no statistical significant differences between seropositive and seronagative cases in terms of the mean age of cases (p=0.7).

DISCUSSION

To our knowledge, this is the first study to investigate the seroprevalence of hepatitis A virus in Tunceli Province in Eastern of Turkey. The seroprevalence rates of hepatitis A virus in previous studies conducted in the eastern and south-eastern region of our country is presented in Table 2. According to these results, the seroprevalence rate of hepatitis A virus in our study was lower than the other studies presented in Table 2. This discordance has been considered probably due to the high socioeconomic level of population admitted to our hospital. Also, improved sanitary and hygienic condition of the population included to present study because of the fact that the present government has increased the investment for sewerage, and sanitary and hygienic condition in our region in the last 8 years. In Turkey, socio-economic improvements and drinking water quality have been followed by a decrease in HAV infection. [http://www.saglik.gov.tr]. Our lowest rate of hepatitis A virus seropositivity among children indicates the requirement of vaccination against hepatitis A virus; because hepatitis A leads to severe complication as the child gets older. Nowadays, age of exposure to hepatitis A virus infection is increasing towards puberty worldwide. This is probably because of the epidemiological changes of hepatitis A virus (Jeong and Lee, 2010). Similarly, nowadays, hepatitis A virus is the most common detected cause of fulminant hepatitis among children in our country as well as worldwide (Santos et al., 2009; Aydogdu et al., 2003). In this study, being the 8.4±4.8 years of the mean age of hepatitis A seropositivity may be due to the exposure to hepatitis A during school and nursery school age. Improved sanitary conditions and hygienic practices have reduced the incidence of HAV infection, especially in developed countries. Reduction in the number of new cases is generally accompanied by a shift in the age of first contact with HAV towards older age groups. As a consequence, both the severity of the reported cases and the risk of outbreaks of disease would increase (Ceyhan et al., 2008). In the present study, the reason for not detecting anti HAV IgM seropositivity may be due to the population including to the study and the time of study which was made between August and December 2010.

In our country, Topal et al. (2011) have reported that the seropositivity rate of hepatitis A virus among children aged between 1-6 years is 9.4% in western region. Ince et al. (2011) have reported that the seroprevalence rate of hepatitis A virus among infants aged 12-month old is

23.5% in central region. It is noticed that Turkey has intermediate endemicity of hepatitis A infections, and endemicity may be change by the geographical and socio-economic conditions (Ceyhan et al., 2008). The results of present study have confirmed this condition as the reported previous studies. The lower results from this study have shown that the age of exposure to hepatitis A has increased toward puberty. Thus, the vaccination is necessary for children older than 2 years in order to prevent the severe complications of the disease among adults.

Routine vaccination of young children can prevent infection at a later age which likely would be more serious. Universal vaccination of young children in Israel and Catalonia has resulted in significant reductions in the incidence of hepatitis A disease in these countries (Dagan et al., 2005; Dominguez et al., 2004).

The limitation of this study is that its results do not indicate the general population of our region, because this study is limited with patients admitted to pediatric policlinic.

Conclusion

The present study demonstrated that the age of exposure to hepatitis A infection has been increasing towards puberty in our region. The immunization against hepatitis A is necessary. The application of a routine hepatitis A vaccine among children will reduce the potential for the development of severe complications.

REFERENCES

Alabaz D, Aksaray N, Alhan E, Yaman A (2005). Decline of maternal hepatitis A antibodies during the first two years of life in infants born in Turkey. Am. J. Trop. Med. Hyg., 73: 457-459.

Aslan G, Seyrek A, Iscan A, Sevinc E, Ulukanligil M, Bakir M (2001). Hepatit A seroprevalence in Sanliurfa. J. Viral. Hepat., 7: 270-273.

Aydogdu S, Ozgenc F, Yurtsever S, Akman SA, Tokat Y, Yagcı RV (2003). Our experience with fulminant hepatic failure in Turkish children: etiology and outcome. J. Trop. Pediatr., 49: 367-370.

Ceyhan M, Yıldırım I, Kurt N (2008). Differences in hepatitis A seroprevalence among geographical regions in Turkey: a need for regional vaccination recommendations. J. Viral. Hepat., l5: 69-72.

Dagan R, Leventhal A, Anis E, Slater P, Ashur Y, Shouval D (2005). Incidence of hepatitis A in Israel following universal immunization of toddlers. JAMA, 294(2): 202–210.

Dominguez A, Bruguera M, Plans P, Costa J, Salleras L (2004). Prevalence of hepatitis A antibodies in schoolchildren in Catalonia (Spain) after the introduction of universal hepatitis A immunization. J. Med. Virol., 73(2): 172–176.

Ince TO, Yalcın S, Yurdakok K, Ozmert EN (2011), Hepatitis A seroprevalence among infants aged 12 months in Ankara. Turk J. Pediatr., 53:114-116.

Jeong SH, Lee HS (2010). Hepatitis A: clinical manifestations and management. J. Intervirol., 53(1): 15-19.

Kanra G, Tezcan S, Badur S and Turkish National Study Team (2002). Hepatitis A seroprevalence in a random sample of the Turkish population by simultaneous EPI cluster and comparison with surveys in Turkey. Turk. J. Pediatr., 44: 204-210.

Ozen M, Yologlu S, Isık Y, Tekerekoglu MS (2006). Anti-HAV IgG seropositivity in children aged 2-16 years who were admitted to Turgut Ozal Medical Center. Turk. J. Pediatr. Arch., 41: 36-40.

Richardus JH, Vos D, Veldhuijzen IK, Groen J (2004). Seroprevalence of hepatitis A virus antibodies in Turkish and Moroccan children in Rotterdam. J. Med. Virol., 72:197- 202.

Santos DC, Martinho JM, Pacheco-Moreira LF(2009). Fulminant hepatitis failure in adults and children from a Public Hospital in Rio de Janerio, Brasil. Braz. J. Infect. Dis., 13: 323-329.

Tekay F (2006). Hepatitis A frequency in children aged between 0-14 years who admitted to Hakkâri State Hospital. Dicle Med. J., 33: 245-247.

Topal E, Hatipoglu N, Turel O, Aydogmus C, Hatipoglu H, Erkal S, Siraneci R (2011). Seroprevalence of Hepatitis A and Hepatitis A vaccination rate in preschool age in Istanbul Urban. J. Pediatr. Inf., 5: 12-15.

Ustun C, Basuguy E, Deveci U (2009). Seroprevalence of hepatitis B and hepatitis C in children admitted to pediatric surgery policilinic. Nobel Med., 5 (Sup. 1): 4-9.

Yazigi N (2007). Viral Hepatitis. In: Kliegman virus RM, Behrman RE, Jenson HB, Stanton BF. Nelson textbook of pediatrics. 18th ed. Philadelphia: Saunders, pp. 1680-1690.

African Journal of Microbiology Research Vol. 5(32), pp. 5972-5977, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.1173

Full Length Research Paper

Diversity of *nifH* gene sequences in the sediments of South China Sea

Lixian Wu[1,2#], Yanhua Cui[3#] and Sanfeng Chen[1]*

[1]State Key Laboratory of Agrobiotechnology and College of Biological Sciences, China Agricultural University, Beijing 100193, China.
[2]Department of Pathogen Biology, Hainan Medical University, Haikou 571101, China.
[3]School of Food Science and Engineering, Harbin Institute of Technology, Harbin 150090, China.

Accepted 21 October, 2011

In order to contribute to knowledge about structure of marine diazotrophic communities in the sediments of South China Sea, the molecular diversity of the *nifH* gene, which encodes the Fe protein of the nitrogenase complex, was assessed by polymerase chain reaction (PCR) amplification using PolF/R primers, followed by cloning and sequencing. Sequences of *nifH* genes were amplified from environmental deoxyribonucleic acid (DNA) samples collected during three stations including shallow sea (75 m, station L10), shelf (450 m, station L2) and deep sea (1000 m, station L21), and covering an area between 17 to 19°N and 111 to 119°E. Samples from shallow sea contained β-, and δ-proteobacteria; the shelf contained α-, β-, δ- proteobacteria; the deep sea contained α-, δ-, γ-proteobacteria, firmicutes, and green nonsulfur (GNS) bacterium. These results suggested that diazotroph was significant component potentially contributing to nitrogen fixation in South China Sea.

Key words: *nifH*, diversity, sediment, the South China Sea.

INTRODUCTION

The diazotroph, which is a fundamental component of ecosystems, catalyses the reduction of atmospheric N_2 gas to biologically available ammonium, providing an important source of fixed nitrogen for the biosphere (Moir, 2011). Most microorganisms that perform biological N_2 fixation with the nitrogen fixation (*nif*) gene cluster (Rees et al., 2005). The *nifH* gene, which encodes the iron protein of nitrogenase, is a highly conserved functional gene useful in phylogenetic studies (Zehr et al., 2003). Culture-independent and molecular methods were developed and applied in assessment of diazotroph diversity by amplifying, cloning and sequencing of the *nifH* gene from environmental DNA samples (Falcon et al., 2002; Jenkins et al., 2004; Moisander et al., 2007, 2008; Langlois et al., 2008). The diversity of diazotrophs was accessed in many different habitats by this approach, including soils, freshwater and saltwater lakes, salt marshes, deep-sea vents and so on (Falcon et al., 2002; Jenkins et al., 2004; Moisander et al., 2007, 2008; Langlois et al., 2008).

The South China Sea (SCS) is one of the largest marginal seas in the tropical Pacific that potentially shares microbial community components from coastal and open ocean ecosystems (Moisander et al., 2008). The SCS has a deep basin with a maximum depth of 5000 m and a shelf less than 100 m deep, the conditions such as warm, permanently stratified, oligotrophic, and dust rich. The environment is favorable for nitrogen fixation (Karl et al., 2002; Moisander et al., 2008; Zhang et al., 2011).

In the present study, diazotrophic bacteria associated with sediments were investigated by the diversity analysis of sequences amplified by polymerase chain reaction (PCR) from deoxyribonucleic acid (DNA) extracted from

*Corresponding author. E-mail: chensf@cau.edu.cn. Tel: +86 10 62731551. Fax: +86 10 62731551.

#, The first and the second authors contributed equally to this work.

different depths of the SCS. The amplified *nifH* products were characterized by DNA sequencing and were compared with the sequences of nitrogenase genes available in database from different environment. Investigations of *nifH* diversity and phylogenetic analysis in the SCS sediments may help to understand the distribution of diazotrophic bacteria.

MATERIALS AND METHODS

Sampling and DNA extraction

Samples were collected from the subsurface sediment of the SCS of different depths of water column in the range of 70-1,000 m. Samples from shallow sea (75 m, station L10), shelf (450 m, station L2) and deep sea (1000 m, station L21). Three sediment samples were collected during the month of July 2007; collection was three times in one sample. Undisturbed surface sediments down to 1-5 m depth were sampled using sterile techniques and stored in liquid nitrogen during the cruise and at -80°C after returning to the laboratory. Sediment DNA was extracted by a previously established procedure (Zhou et al., 1997).

Polymerase chain reaction (PCR), cloning and restriction fragment length polymorphism (RFLP) analysis

Bacterial nitrogenase reductase genes were amplified with primers PolF (5'-TGCGAYCCSAARGCBGAC TC-3') and PolR (5'-ATS GCCATCATYTCRCCGGA-3') (Poly et al., 2001). Thermal cycling conditions were 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 53°C for 1 min, 72°C for 40 s, and a final extension step of 72°C for 5 min. The PCR products were then cloned using a TAKARA TA cloning kit (TOYOBO Shanghai, Shanghai, China). PCR amplification products containing the right-size (402 bp) insert were digested with 1 U of restriction enzymes *Mbo* I, *Rsa* I, *Msp* I (MBI) for 4 to 8 h at 37°C. The restriction profiles were evaluated by electrophoresis in 3% agarose gel. Clones that produced the same RFLP pattern were grouped together and considered representatives of the same operational taxonomic unit (OTU). The PCR products showing different RFLP patterns were randomly selected for sequencing. Plasmid DNA was prepared and sequenced, at least twice in both directions, by using an ABI PRISM 377 DNA sequencer (Perkin-Elmer Cetus Instruments, Norwalk, CT).

Sequence alignment and phylogenetic analysis

The product of about 402 bp fragment was obtained by PCR reaction. The sequences of *nifH* gene from the NCBI GenBank database were selected on the basis of sequence similarity to one or more of the marine sequences. These diazotrophs sequences were utilized for phylogenetic reconstructions. Protein sequences for each major sequence cluster were aligned in Clustal W (Thompson et al., 1994). Maximum likelihood phylogenies were constructed in MEGA (ver. 5.0) by the Bootstrap method using pair wise deletion of gaps and missing data with 1000 bootstrap resamplings (Tamura et al., 2011).

Nucleotide sequence accession numbers

The *nifH* sequences determined in this study are available in the GenBank database, accession numbers HM063747-HM063831.

RESULTS AND DISCUSSION

Analysis of all *nifH* clones in three stations

The *nifH* gene has been one of the most important functional genes used when studying diversity in numerous habitats in last few years. In this study, the *nifH* gene was used as a molecular marker for studying the diazotrophic diversity and abundance in the SCS sediments in three stations. A total of 203 *nifH* clones from three sediment samples were obtained (station L10, 43 clones; station L2, 91 clones; station L21, 69 clones). The diversity of *nifH* sequences was analyzed by restriction fragment length polymorphism (RFLP), and 48 restriction profiles were obtained in this study. The deduced amino acid sequences shared 39% to 99% similarity to the closest match GenBank *nifH* and *nifH*-like sequences. Phylogenetic analysis indicated that most *nifH* protein sequences might be obtained from currently uncultured or uncharacterized bacteria, and covered diverse environments. The similarity of sequenced *nifH* genes ranged from 73 to 100% between each other.

nifH diversity in station L10

In shallow sea (75 m, station L10), 9 protein sequences (43 clones) clustered into three major groups, including β-δ- subdivisions of proteobacteria (Figure 1). The most sequences were belonging to δ-proteobacteria, including L10-H10, L10-H66, L10-H134, L10-H192, L10-H244, L10-H290 and L10-H297. These sequences were very similar to sequences previously recovered from various environments, including rhizosphere of plant, soil, corals, oligotrophic tropical sea grass bed communities, salt marsh, beach, marine sediment, and bay (Zhang et al., 2006; Musat et al., 2006; Coelho et al., 2008; Lovell et al., 2008; Teng et al., 2009). These sequences showed high homology with *nifH* genes of the *Desulfovibrio* sp. (*Desulfovibrio magneticus*, *Desulfovibrio aespoeenisis*), *Desulfonatronospira thiodism*, *Sinorhizobium* sp., and *Bradyrhizobium japonicum.* The sequences L10-H134 and L10-H290 had high identity with sequences previously recovered from northern South China Sea (ADT90055.1), Chesapeake Bay (AAZ06761.1), Jiaozhou Bay (ACN77086.1), eastern Mediterranean Sea (ABQ50774.1, Man-Aharonovich et al., 2007). The L10-H10, L10-H244, and L10-H297 showed a high homology with the *nifH* gene of *Sinorhizobium* sp. TJ170 and *Methylococcus capsulatus str*.

The β-proteobacteria cluster contains L10-H200 and L10-H243. The protein sequence L10-H200 had a high identity with with the *nifH* genes of *Dechloromonas* sp. SIUL and *Zoogloea oryzae.* The latter bacterium was isolated from rice paddy soil (Xie and Yokota, 2006). The protein sequence L10-H243 showed a high homology with the *nifH* gene of uncultured bacterium which was

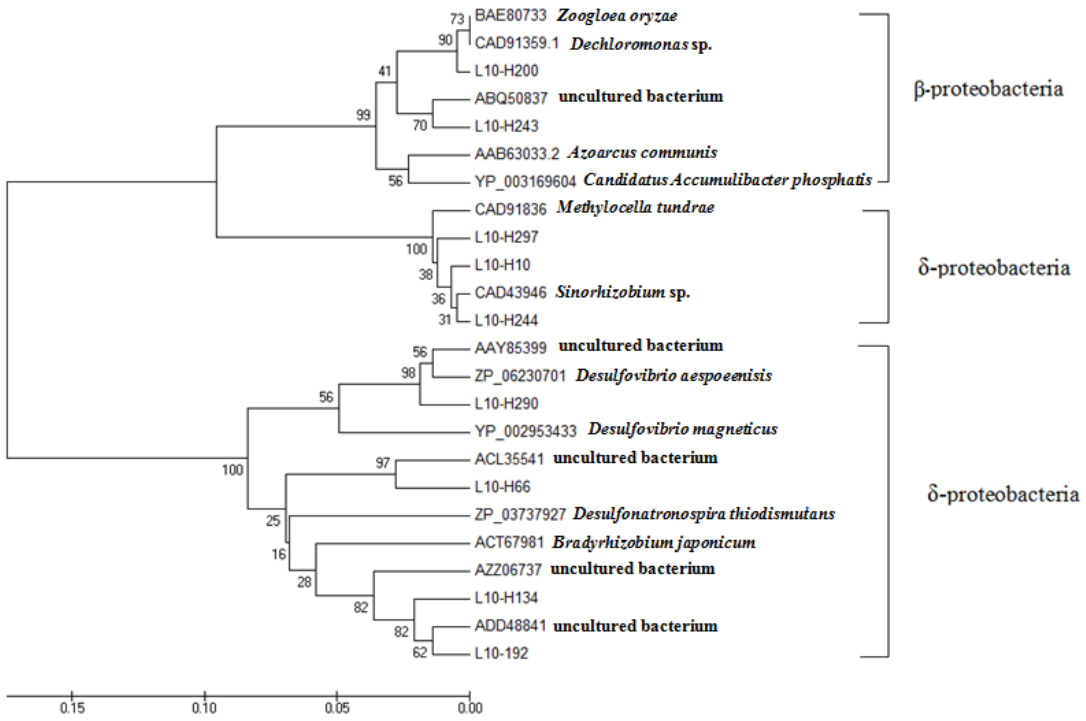


Figure 1. Phylogenetic trees for *nifH* of the station L10 constructed in MEGA (ver. 5.0) based on amino acid sequences using the Bootstrap method. Phylogenetic relationships were bootstrapped 1000 times, and bootstrap values are shown.

found in the eastern Mediterranean Sea (Man-Aharonovich et al., 2007).

nifH diversity in station L2

In shelf site (450 m, station L2), 20 protein sequences (91 clones) clustered into four major groups, including α-, β-, δ- subdivisions of proteobacteria (Figure 2). The α-proteobacteria cluster contained a distinct sequence L2-H81. It had a 98% protein sequence similarity with the *nifH* sequences affiliated with *Azospirillum brasilense*, an aerobic, plant growth-promoting rhizobacteria (PGPR) isolated from cereal root (Umali-Garcia et al., 1980; Steenhoudt and Vanderleyden, 2000; Bashan et al., 2004; Cui et al., 2006).

Among the β-proteobacteria, *Dechloromonas* sp. SIUL was the dominant diazotroph covering 50.5% (46/91) of the clone library. These protein sequences showed high similarity to sequences previously obtained from various environments, including rhizospheres, soil, corals, coast, salt marsh, eastern Mediterranean Sea, and wastewater (Man-Aharonovich et al., 2007; Bowers et al., 2008). The protein sequences L2-H1, L2-H9 and L2-H10 showed high identity with *Dechloromonas* sp. SIUL. The protein sequences L2-H39 and L2-H111 had 92.9% similarity of uncultured marine bacteria (ADT89967 and ADT89983) which were found in the northern SCS (Kong et al., 2011).

The most sequences belonging to δ-proteobacteria, including L2-H3, L2-H4, L2-H6, L2-H15, L2-H18, L2-H19, L2-H22, L2-H37, L2-H66, L2-H75, L2-H90, L2-H95, L2-H106 and L2-H142. The protein sequences of L2-H3, L2-H75, L2-H90 and L2-H106 showed 89%-91% similarity with *nifH* sequences of uncultured bacterium (AAT48890, AAT48897) which were found in the Tibetan plateau (Zhang et al., 2006). L2-H4 and L2-H22 had high protein sequence identity with the *nifH* sequences of *B. japonicum* which is a species of legume-root nodulating, microsymbiotic nitrogen-fixing bacterium species (Dashti et al., 1997). L2-H6, L2-H15, L2-H18, L2-H37, L2-H66 and L2-H142 had high identity of protein sequences with *nifH* sequences of *Desulfuromonas acetoxidans* DSM 684 and *Desulfovibrio dechloracetivorans* (Ju et al., 2007).

nifH diversity in station L21

In deep sea (1000 m, station L21), 19 protein sequences (69 clones) clustered into seven major groups, including α-, δ-, γ-proteobacteria, firmicutes, and green nonsulfur (GNS) bacterium (Figure 3).

The α-proteobacteria cluster contained a single protein sequence L21-H146. It had a 91% protein sequence similarity with the *nifH* sequences of *A. brasilense* which is an important diazotroph isolated from cereal root (Umali-Garcia et al., 1980; Steenhoudt and

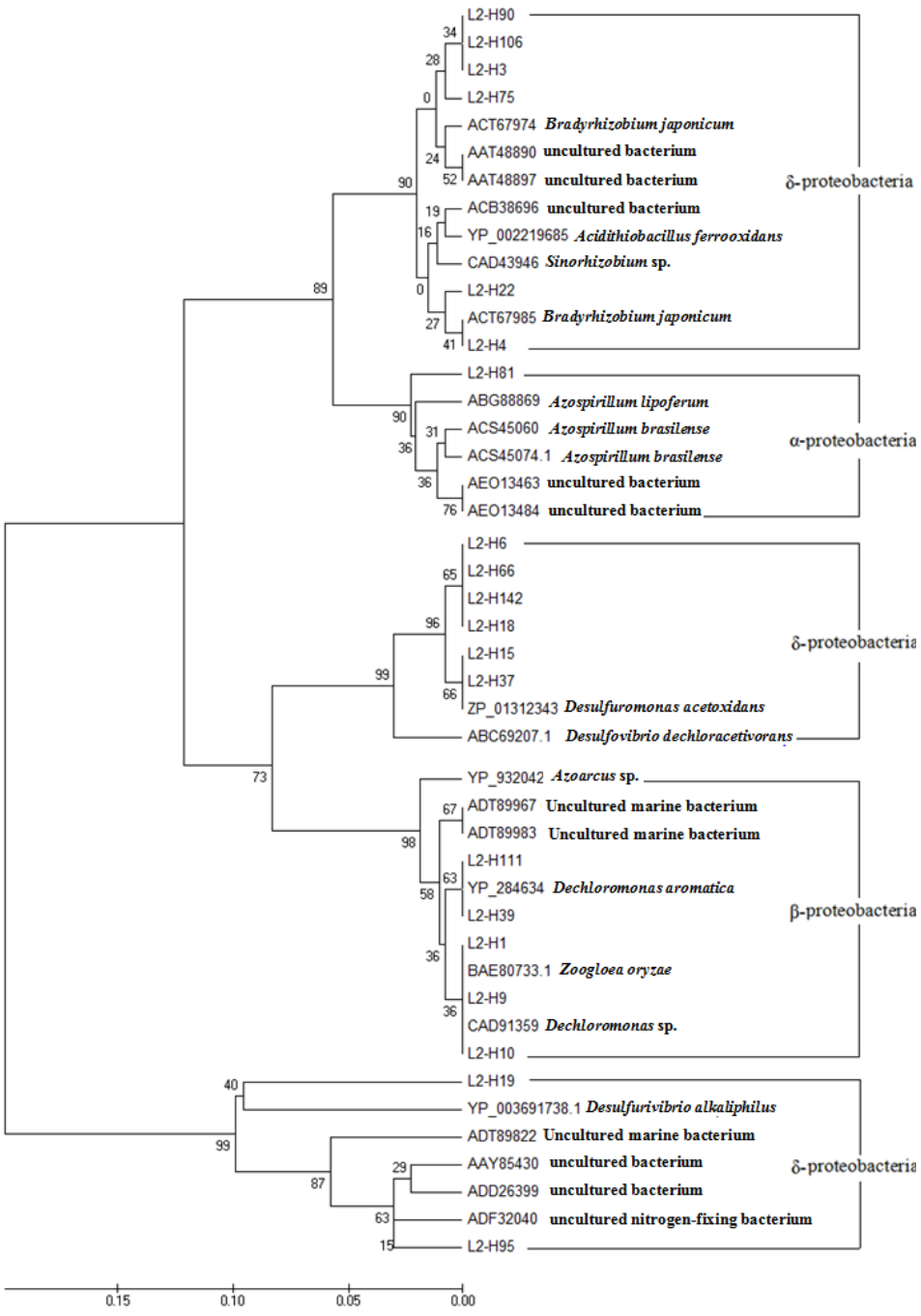


Figure 2. Phylogenetic trees for *nifH* of the station L2 constructed in MEGA (ver. 5.0) based on amino acid sequences using the Bootstrap method. Phylogenetic relationships were bootstrapped 1000 times, and bootstrap values are shown.

Vanderleyden, 2000; Bashan et al., 2004; Cui et al., 2006).

The most sequences belonging to δ-proteobacteria, formed the dominant diazotrophic group, including L21-H23, L21-H45, L21-H89, L21-H151, L21-H187, L21-H209, L21-H212 and L21-H408. The L21-H212 had 90% similarity with *nifH* sequence of uncultured bacterium (ADT89832.1) which was isolated from the northern SCS (Kong et al., 2011). The L21-H151 had high identity with *nifH* sequence of uncultured bacterium (ADT89817.1) which was also isolated from the northern SCS (Kong et al., 2011). The sequence L21-H408 showed 90% similarity with *nifH* sequence of *Pelobacter carbinolicus* DSM 2380. The sequences L21-23, L21-H45, L21-89,

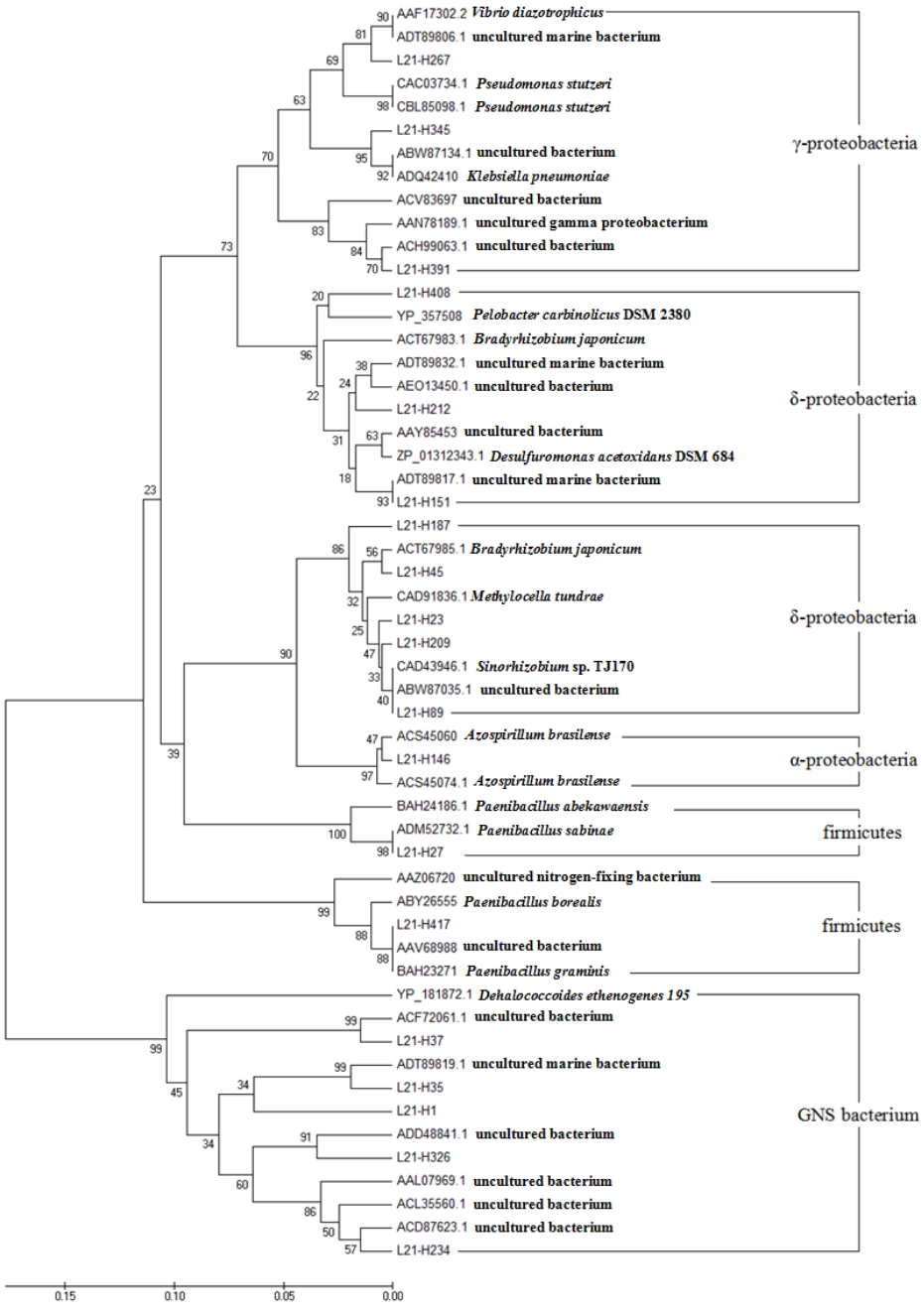


Figure 3. Phylogenetic trees for *nifH* of the station L21 constructed in MEGA (ver. 5.0) based on amino acid sequences using the Bootstrap method. Phylogenetic relationships were bootstrapped 1000 times, and bootstrap values are shown.

L21-H187, and L21-H209 showed high identity with *nifH* sequence of *B. japonicum, Methylocella tundra* and *Sinorhizobium* sp. TJ170.

The protein sequences L21-H267, L21-H345 and L21-H391 belong to the γ-proteobacteria cluster. L21-H267 had 90% similarity of uncultured marine bacterium (ADT89806) which was found in the northern SCS (Kong et al., 2011). L21-H345 showed 90% protein similarity with *Klebsiella pneumoniae* which was isolated from the root surface of rice (Liu et al., 2011). The L21-H391 had a 90% protein sequence similarity with different environmental sequences, including African sweet potato (AAN78189.1; Reiter et al., 2003), Italian white truffle Tuber magnatum (Barbieri et al., 2010), rhizosphere of mangrove, coastal microbial mats.

The L21-H27 and L21-H417 belong to firmicutes

cluster, which had high identity with *nifH* sequences of firmicutes *Paenibacillus* sp. The GNS bacterium cluster contained L21-H1, L21-H35, L21-H37, H21-H234 and L21-H326. These protein sequences showed high similarity with *Dehalococcoides ethenogenes* 195 (Seshadri et al., 2005).

In this study, we examined the phylogenetic diversity and abundance of diazotrophs in the SCS by analysis of *nifH* gene from three different stations. The results indicated that deep sea sediment had higher diversity of diazotrophic bacteria than those of shelf site and shallow sea.

ACKNOWLEDGEMENTS

This work was supported by the National Nature Science Foundation of China (Grant No. 30470028).

REFERENCES

Barbieri E, Ceccaroli P, Saltarelli R, Guidi C, Potenza L, Basaglia M, Fontana F, Baldan E, Casella S, Ryahi O, Zambonelli A, Stocchi V (2010). New evidence for nitrogen fixation within the Italian white truffle,*Tuber magnatum*. Fungal Biol., 114(11-12): 936-942.

Bashan Y, Holguin G, de-Bashan LE (2004). *Azospirillum*-plant relationships: physiological, molecular, agricultural, and environmental advances (1997-2003). Can. J. Microbiol., 50: 521-577.

Bowers TH, Reid NM, Lloyd-Jones G (2008). Composition of *nifH* in a wastewater treatment system reliant on N_2 fixation. Appl. Microbiol. Biotechnol., 79 (5): 811-818.

Coelho MR, de Vos M, Carneiro NP, Marriel IE, Paiva E, Seldin L (2008). Diversity of *nifH* gene pools in the rhizosphere of two cultivars of sorghum (*Sorghum bicolor*) treated with contrasting levels of nitrogen fertilizer. FEMS Microbiol. Lett., 279 (1): 15-22.

Cui YH, Tu R, Guan Y, Chen SF (2006). Cloning, sequencing and characterization of the *Azospirillum brasilense fhuE* gene. Curr. Microbiol., 52(3): 169-177.

Dashti N, Zhang F, Hynes R, Smith D L (1997). Application of plant growth-promoting rhizobacteria to soybean (*Glycine max* [L.] *Merr*.) increases protein and dry matter yield under short-season conditions. Plant Soil, 188 (1): 33-41.

Falcon LI, Cipriano F, Chistoserdov AY, Carpenter EJ (2002). Diversity of diazotrophic unicellular cyanobacteria in the tropical North Atlantic Ocean. Appl. Environ. Microbiol., 68: 5760-5764.

Jenkins BD, Steward GF, Short SM, Ward BB, Zehr JP (2004). Fingerprinting diazotroph communities in the Chesapeake Bay by using a DNA macroarray. Appl. Environ. Microbiol., 70: 1767-1776.

Ju X, Zhao L, Sun B (2007). Nitrogen fixation by reductively dechlorinating bacteria. Environ. Microbiol., 9 (4): 1078-1083.

Karl D, Michaels A, Bergman B, Capone D, Carpenter E, Letelier R, Lipschultz F, Paerl H, Sigman D, Stal L (2002). Dinitrogen fixation in the world's oceans. Biogeochemistry, 57/58: 47-98.

Kong LL, Jing HM, Kataoka T, Sun J, Liu HB (2011). Phylogenetic diversity and spatio-temporal distribution of nitrogenase genes (*nifH*) in the northern South China Sea. Aquat. Microbial. Ecol., 65: 15-27.

Langlois RJ, Hummer D, LaRoche J (2008). Abundances and distributions of the dominant *nifH* phylotypes in the Northern Atlantic Ocean. Appl. Environ. Microbiol., 74: 1922-1931.

Liu Y, Wang H, Sun XL, Yang HL, Wang YS, Song W (2011). Study on mechanisms of colonization of nitrogen-fixing PGPB, *Klebsiella pneumoniae* NG14 on the root surface of rice and the formation of biofilm. Curr. Microbiol., 62(4): 1113-1122.

Lovell CR, Decker PV, Bagwell CE, Thompson S, Matsui GY (2008). Analysis of a diverse assemblage of diazotrophic bacteria from *Spartina alterniflora* using DGGE and clone library screening. J. Microbiol. Methods, 73 (2): 160-171.

Man-Aharonovich D, Kress N, Zeev EB, Berman-Frank I, Beja O (2007). Molecular ecology of nifH genes and transcripts in the eastern Mediterranean Sea. Environ. Microbiol., 9(9): 2354-2363.

Moir JWB (editor) (2011). Nitrogen Cycling in Bacteria: Molecular Analysis. Caister Academic Press. ISBN 978-1-904455-86-8.

Moisander PH, Beinart RA, Voss M, Zehr JP (2008). Diversity and abundance of diazotrophic microorganisms in the South China Sea during intermonsoon. ISME J., 2: 954-967.

Moisander PH, Morrison AE, Ward BB, Jenkins BD, Zehr JP (2007). Spatial-temporal variability in diazotroph assemblages in Chesapeake Bay using an oligonucleotide *nifH* microarray. Environ. Microbiol., 9: 1823-1835.

Musat F, Harder J, Widdel F (2006). Study of nitrogen fixation in microbial communities of oil-contaminated marine sediment microcosms. Environ. Microbiol., 8 (10), 1834-1843.

Poly F, Monrozier LJ, Bally R (2001). Improvement in the RFLP procedure for studying the diversity of nifH genes in communities of nitrogen fixers in soil. Res. Microbiol., 152: 95-103.

Rees DC, Akif Tezcan F, Haynes CA, Walton MY, Andrade S, Einsle O, Howard JB (2005). Structural basis of biological nitrogen fixation. Philos. Transact. A Math. Phys. Eng. Sci., 363: 971-984.

Reiter B, Burgmann H, Burg K, Sessitsch A (2003). Endophytic *nifH* gene diversity in African sweet potato Can. J. Microbiol., 49 (9): 549-555.

Seshadri R, Adrian L, Fouts DE, Eisen JA, Phillippy AM, Methe BA, Ward NL, Nelson WC, Deboy RT, Khouri HM, Kolonay JF, Dodson RJ, Daugherty SC, Brinkac LM, Sullivan SA, Madupu R, Nelson KE, Kang KH, Impraim M, Tran K, Robinson JM, Forberger HA, Fraser CM, Zinder SH, Heidelberg JF (2005). Genome sequence of the PCE-dechlorinating bacterium *Dehalococcoides ethenogenes*. Science, 307 (5706): 105-108.

Steenhoudt O, Vanderleyden J (2000). *Azospirillum*, a freeliving nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS Microbiol. Rev., 24: 487-506.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol., 28: 2731-2739.

Teng Q, Sun B, Fu X, Li S, Cui Z, Cao H (2009). Analysis of nifH gene diversity in red soil amended with manure in Jiangxi, South China. J. Microbiol., 47 (2): 135-141.

Thompson JD, Higgins DG, Gibson TJ (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res., 22(22): 4673-4680.

Umali-Garcia M, Hubbell D, Gaskins M, Dazzo F (1980). Association of *Azospirillum* with grass roots. Appl. Environ. Microbiol., 39: 219-226.

Xie CH, Yokota A (2006). *Zoogloea oryzae* sp. nov., a nitrogen-fixing bacterium isolated from rice paddy soil, and reclassification of the strain ATCC 19623 as *Crabtreella saccharophila* gen. *nov*., sp. Nov. Int. J. Syst. Evol. Microbiol., 56 (PT 3): 619-624.

Zehr JP, Jenkins BD, Short SM, Steward GF (2003). Nitrogenase gene diversity and microbial community structure: a cross-system comparison. Environ. Microbiol., 5: 539-554.

Zhang Y, Zhao ZH, Sun J, Jiao NZ (2011). Diversity and distribution of diazotrophic communities in the South China Sea deep basin with mesoscale cyclonic eddy perturbations. FEMS Microbiol. Ecol., 78(3): 417-427.

Zhang Y, Li D, Wang H, Xiao Q, Liu X (2006). Molecular diversity of nitrogen-fixing bacteria from the Tibetan Plateau, China. FEMS Microbiol. Lett., 260 (2): 134-142.

Zhou J, Davey ME, Figueras JB, Rivkina E, Gilichinsky D, Tiedje JM (1997). Phylogenetic diversity of a bacterial community determined from Siberian tundra soil DNA. Microbiology, 143: 3913-3919.

African Journal of Microbiology Research Vol. 5(32), pp. 5978-5984, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.1216

Full Length Research Paper

Difference in photoinhibition and photoprotection between seedings and saplings leaves of *Taxus cuspidata* under high irradiance

Wei Li[1], Yu-Sen Zhao[1]* and Zhi-Qiang Zhou[2]

[1]College of Forestry, Northeast Forestry University, Harbin 150040, China.
[2]Key Laboratory of Forest Plant Ecology, Northeast Forestry University, Harbin 150040, China.

Accepted 29 November, 2011

The differences in chloroplast pigments, gas exchange and photosystemII (PSII) photochemistry as well as xanthophyll in seeding and sapling leaves of *Taxus cuspidata* grown in full sunlight were examined. Compared with the sapling leaves, the chlorophyll content, photosynthetic capacity and light intensity for saturation of photosynthesis were lower in seeding leaves. The response curves of PSII photochemistry demonstrated that both seeding and sapling leaves occurred a down-regulation of PSII photochemistry at high irradiance, more serious down-regulation being examined in seeding leaves. And the down-regulation of PSII photochemistry occurred significantly when measured at midday, indicating that photoinhibition occurred heavily in seeding leaves when exposed to high light. The actual PSII efficiency (ΦPSII) and the efficiency of excitation capture by open PSII centers drastically decreased with the increase of non-photochemical quenching (NPQ) at midday. The photorespiration rate in seeding leaves was lower than that in sapling leaves under high irradiance. The results indicated that the xanthophlly cycle was activated in both the seeding and sapling leaves at midday and an increase of de-epoxidation were observed, but a little higher level of de-epoxidation was measured in seeding leaves. The xanthophyll cycle may play an important role in the dissipation of excess light energy associated with NPQ to avoid photodamage. Our results suggested that photoinhibition occurred in seeding leaves significantly due to lower capacity of CO_2 assimilation, photorespiration and the light intensity for saturation of photosynthesis, as well as the lower PSII photochemistry at high irradiance; therefore the *T. cuspidata* seeding could not adapt to growing at high irradiance.

Key words: Japanese yew (*Taxus cuspidata Sieb, et Zucc.*), photosynthesis, chlorophyll fluorescence, photorespiration, xanthophyll cycle.

INTRODUCTION

Japanese yew (*Taxus cuspidata Sieb, et Zucc.*) is a rare relic plant of the 'tertiary period', which has a wide geographical distribution (Potenko, 2001). It grows in vegetated mixed forests in mountainous regions. Taxol, an effective anticancer drug extracted from the bark of Japanese yew, receives attention (Kobayashi et al., 1994). Japanese yew is a declining species, but conservation strategies have been developed (Potenko, 2001). As we known, high light may decrease the rate of photosynthesis in plant which may cause the photoinhibition (Müller et al., 2001; Huang et al., 2006). Japanese yew is a shade-tolerant species (Iszkulo and Boratynski, 2006). The saplings can survive in both shady and sunny environments, but the seedlings are always observed under the canopy of mature trees (Iszkulo and Boratynski, 2006). Therefore, when the Japanese yew seedings are exposed to the high light, photoinhibition could occur in Japanese yew seeding leaves. However, plants have developed some photoprotective mechanisms to protect the photosynthesis apparatus against photodamage (Lu et al., 2003; Chow, 1994; Anderson et al., 1997). Dissipation of excess excitation

*Corresponding author. E-mail: ysz_1957@163.com.

energy as heat in order to minimize photodamage to PSII reaction centers is well known to be one of the mechanisms for the protection of the photosynthetic apparatus, which involves the xanthophyll cycle (Guo et al., 2009).

In the xanthophyll cycle, excess light energy absorbed by antennae complexes of photosystem II is converted to heat, which prevent the formation of reactive oxygen. In this process, violaxanthin (V) is converted to zeaxanthin (Z) and antheraxanthin (A) under conditions of excess excitation energy (Demmig-Adams and Adams, 1992; Gilmore, 1997; Horton et al., 1996). And photorespiration pathway is reported as a very important photoprotection mechanism against photooxidation and photoinhibition (Kozaki and Takeba, 1996; Jiang et al., 2006; Niyogi, 1999). Photorespiration could act as a sink for excess excitation energy in photosynthetic apparatus when CO_2 assimilation is reduced (Niyogi, 1999). In this study, we conducted an experiment to determine the differences between *T. cuspidata* seeding and sapling leaves in the CO_2 assimilation capacity, photorespiration capacity and xanthophyll cycle-dependent energy dissipation under high irradiance and whether the *T. cuspidata* seeding could adapt to full sun light.

MATERIALS AND METHODS

Plant material

The research was carried out from March to August, 2010 in the Botanical Garden of North East Forestry University. The 30 *T. cuspidata* seedings of 4 years and the 16 ones of 15 years which grew in plastic pots (25 cm in diameter and 20 cm in height; 80 cm in diameter and 70 cm in height, respectively) were transplanted from 70% PPFD (photosynthetic photon flux density) of full sunlight to 90% PPFD of full sunlight. After 4 weeks under 90% PPFD of full sunlight, they were moved to the full sunlight. Six weeks later when the *T. cuspidata* seedlings and saplings were acclimated to full sun light, the current-year leaves from the mid-crown on the south side of each tree were studied as the experimental materials.

Gas change measurements

Photosynthetic rate-photosynthetic photon flux density (*P*n-PFD) response curves were made at leaf chamber temperature of 30° and at 350 $\mu molmol^{-1}CO_2$ with an open gas exchange system (Li-6400). PFDs were fixed in a sequence of 1800, 1600, 1200, 800, 600, 400, 200, 100, 500 $\mu molmolm^{-2}s^{-1}$. Photosynthetic rate was monitored at two O_2 concentrations: 21% O_2 + 350 $\mu molmol^{-1}CO_2$ and 2% O_2 + 350 $\mu molmol^{-1}CO_2$ under 1400 $\mu molmolm^{-2}s^{-1}$PFD and this was used to calculate photorespiration.

Chlorophyll fluorescence measurements

Chlorophyll fluorescence was measured with a pulse-modulated fluoremeter (FMS-2, Hansatech, UK). Before each measurement, the sample leaf was dark-adapted for 35 min with dark leaf clips. To determine the Fo (initial fluorescence), the low modulated measuring light (<0.1 $m^{-2}s^{-1}$) was turned on and Fo was recorded. Then the sample leaf was exposed to a 0.7 s saturating white light (>3000 $m^{-2}s^{-1}$) to obtain the Fm (maximal chl fluorescence). Fv/Fm (the maximum quantum yield of photosystemII; Fv, the variable Chl fluorescence yield is defined as Fm - Fo) was calculated automatically. Fs (the steady-state fluorescence) and Fm' (the maximum Chl fluorescence level) during exposure to illumination were also measured. The actual PSII efficiency (ΦPSII) was calculated as (Fm'-Fs)/Fm' (Genty et al., 1989). Non-photochemical quenching (NPQ) was calculated as $(Fm/Fm')^{-1}$ according to Bilger and Björkman (1990).

To examine the light response curves for the fluorescence parameters of *T. cuspidata* sapling leaves and seeding leaves, the Fo was measured at first and then a saturating pulse was applied to determine the Fv/Fm. The actinic light was increased in a sequence of 100, 200, 400, 600, 800, 1000 and 1400 in steps. Each PFD was maintained at least 10 min.

Pigment determination

The content of chlorophyll in leaf were extracted with 80% acetone, being analyzed with a UV-2800 system (Hitachi, Japan) according to Lichtenthaler (1987). Leaf samples were taken at morning. The content of carotenoid components of xanthophyll was extracted with 100% acetone under the ice-cold condition. Then the extracts were filtered through a 0.45 μm filter. Leaf samples were taken at predawn and midday. Afterwards, they were immediately frozen into liquid nitrogen. The content of the carotenoid components of xanthophyll were analyzed in the method described by Thayer and Björkman (1990) for 5 times.

Statistical analyses

Data of measurements were analyzed by using SPSS 10.0. The least significant differences between the means were calculated at 95% confidence level. Plots and fit curves were performed by using Sigmaplot10.0. Unless otherwise indicated, the significant differences between seedings and saplings were given at $P<0.05$.

RESULTS

Differences in chlorophyll content

The Chl *a*, Chl *b* and total *a* + *b* content in saplings leaves were significantly higher than those in seedings leaves. The ratios Chl a/Chl b did not show significant differences between saplings leaves and seedings leaves. The result demonstrated that the content of Chl *a* was higher than that of Chl b under the high light (Table 1).

Differences in photosynthesis and photorespiration

There were significant differences between *T. cuspidata* seeding and sapling in CO_2 assimilation capacity and photorespiration. Measurements of light response curves for photosynthesis of *T. cuspidata* seeding and sapling leaves show that the maximum photosynthetic rates were 7.22 ± 0.33 and 9.6 ± 0.21 $\mu molm^{-2}s^{-1}$ in seedlings and saplings leaves, respectively (Figure 1). Sapling leaves exhibited higher saturation light of photosynthetic rate

Table 1. Differences in chlorophll pigments between *T. cuspidata* seeding and sapling leaves. Each value is means of ± S.E. n = 5.

Variable	Chl a (mg g^{-2} FW)	Chl b (mg g^{-2} FW)	Chl a+b (mg g^{-2} FW)	Chl a/b (mg m^{-2})
Seeding	314 ± 10[a]	88 ± 2[a]	443 ± 7[a]	3.56 ± 0.06[a]
Sapling	362 ± 7[b]	98 ± 1[b]	487 ± 4[b]	3.62 ± 0.09[a]

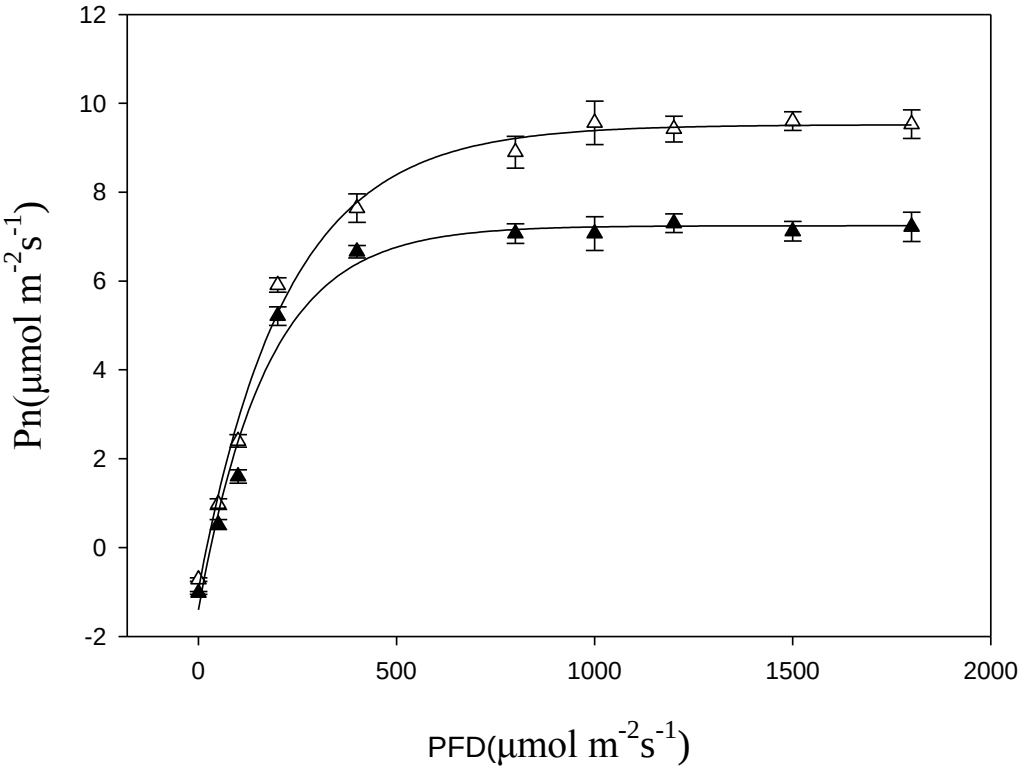


Figure 1. Light response curves for photosynthesis of *T. cuspidata* seedings and saplings leaves measured at 26°C and the 350 μmolmol$^{-1}CO_2$ in the chamber. (△) and (▲) represent sapling leaves and seeding leaves, respectively. Values are means ± S.E., n = 3 – 5.

than that of seeding leaves. Sapling leaves had higher CO_2 assimilation capacity under high irradiance. Similarly, photorespiratory in sapling leaves also showed higher than that in seeding leaves (Figure 2).

Response of Chl fluorescence parameters to changes in irradiance

With the irradiance increasing, the decrease in ΦPSII and Fv'/Fm' and an increase in NPQ were observed in seeding and sapling leaves, but the sapling leaves had higher ΦPSII and Fv'/Fm' than the seeding ones. However, NPQ in seeding leaves was significantly higher than sapling ones. The results showed that a greater down-regulation of PSII efficiency in seeding leaves in high light (Figure 3).

Fluorescence parameters at predawn and midday

A significant decline in Fv/Fm, ΦPSII and Fv'/Fm' were observed at midday in the seeding and sapling leaves, but a considerable increase in NPQ. Compared with seeding leaves, sapling leaves showed higher values for Fv/Fm, ΦPSII and Fv'/Fm' and lower values for NPQ at midday (Table 2).

The xanthophyll cycle under high irradiance

There were significant differences between Japanese yew in the content of xanthophyll. We observed that the relative xanthophyll pool size (A + V + Z)/Chl in seeding leaves was higher than that in sapling leaves (Figure 4A, B and C). Compared with sapling leaves, the de-

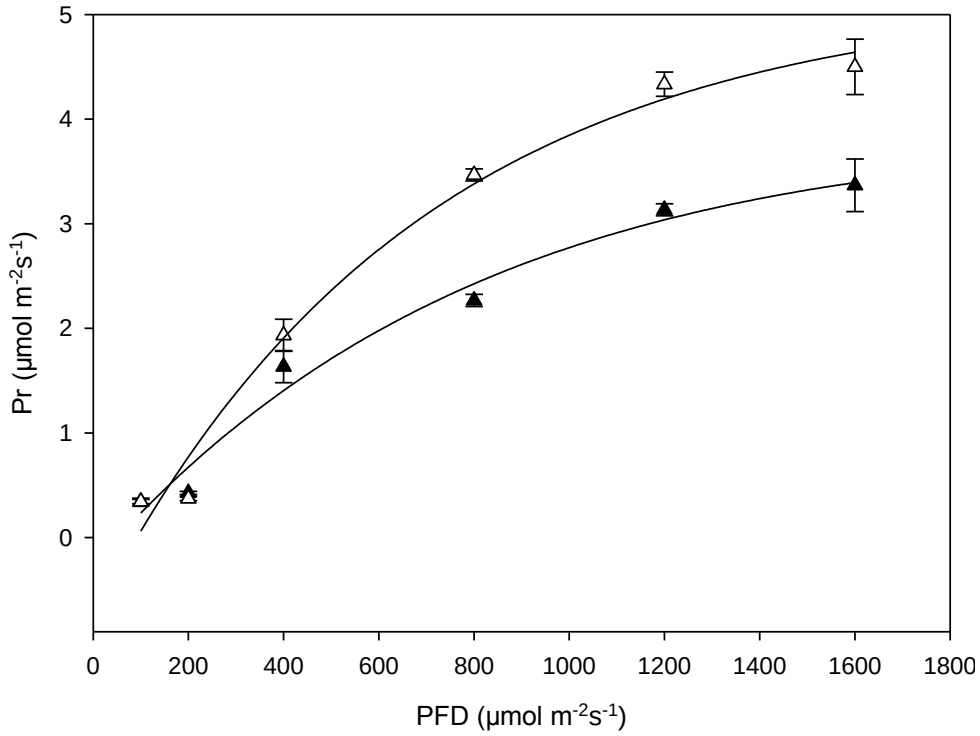


Figure 2. Light response curves for photorespiratory of *T. cuspidata* seedings and saplings leaves measured at 26°C. (△) and (▲) represent sapling leaves and seeding leaves, respectively. Values are means ± S.E., n = 3 – 5.

epoxidation components of the xanthophyll cycle pigments were more increased in seeding leaves at midday. And the results showed an increase in (A + Z)/(A + Z + V) ratio in seeding and sapling leaves at midday.

DISCUSSION

The data that sapling leaves had higher chlorophyll content, Chl*a*/Chl*b* ratio (Table 1) and photosynthetic capacity (Figure 1) indicated that sapling leaves had a more developed photosynthetic apparatus, which more excited energy would be used in CO_2 assimilation rather than dissipated. The long exposure to high irradiance levels is a major source of stress to the photosynthetic apparatus (Genty et al., 1989). When CO_2 assimilation is restricted, photorespiration also acts as a key role in the protection of leaves against high irradiation and uses energy. Sapling leaves had more capacity to allocate excited energy to photorespiration than seeding ones at high irradiance (Figure 2). Increased allocation of excited energy of photorespiration can maintain the utilization of excited energy by allowing metabolism to continue using the products of photosynthetic electron transport. This can mitigate the deleterious effects such as photodamage. The maximal efficiency of PSII photochemistry (Fv/Fm) showed only a slight decrease in seeding leaves when measured at predawn, indicating that seeding leaves had almost the same primary photochemistry as sapling leaves (Table 2), so the activity of PSII may not be the limiting step of photosynthesis in seeding leaves.

With an increasing series of irradiances, the values of ΦPSII and Fv'/Fm' decreased gradually (Figure 3A, B and C). However, decrease in PSII efficiency (ΦPSII) and the efficiency of excitation energy captured by open PSII centers (Fv'/Fm') in seeding leaves revealed a down-regulation of PSII in the light-response curves. The changes in the light response curves of PSII photochemistry in seeding leaves also showed higher stepwise increases in NPQ at high PFDs. This demonstrated that seeding leaves had to dissipate excess excitation energy as more heat when exposed to high light. It has been reported that xanthophyll cycle is an important photoprotection mechanism correlated to energy dissipation in plants to avoid photodamage. The data demonstrated that a 'little more' de-epoxidation components (A + Z) were observed in seeding leaves than that in sapling leaves when measured at midday, which was associated with NPQ. The results showed that an increase in NPQ in both seeding and sapling leaves at midday was associated with an increase in content of (A + Z) and increase in (A + Z)/(V + A + Z) ration. The higher content of (A + Z) and the higher (A + Z)/(V + A + Z) ration

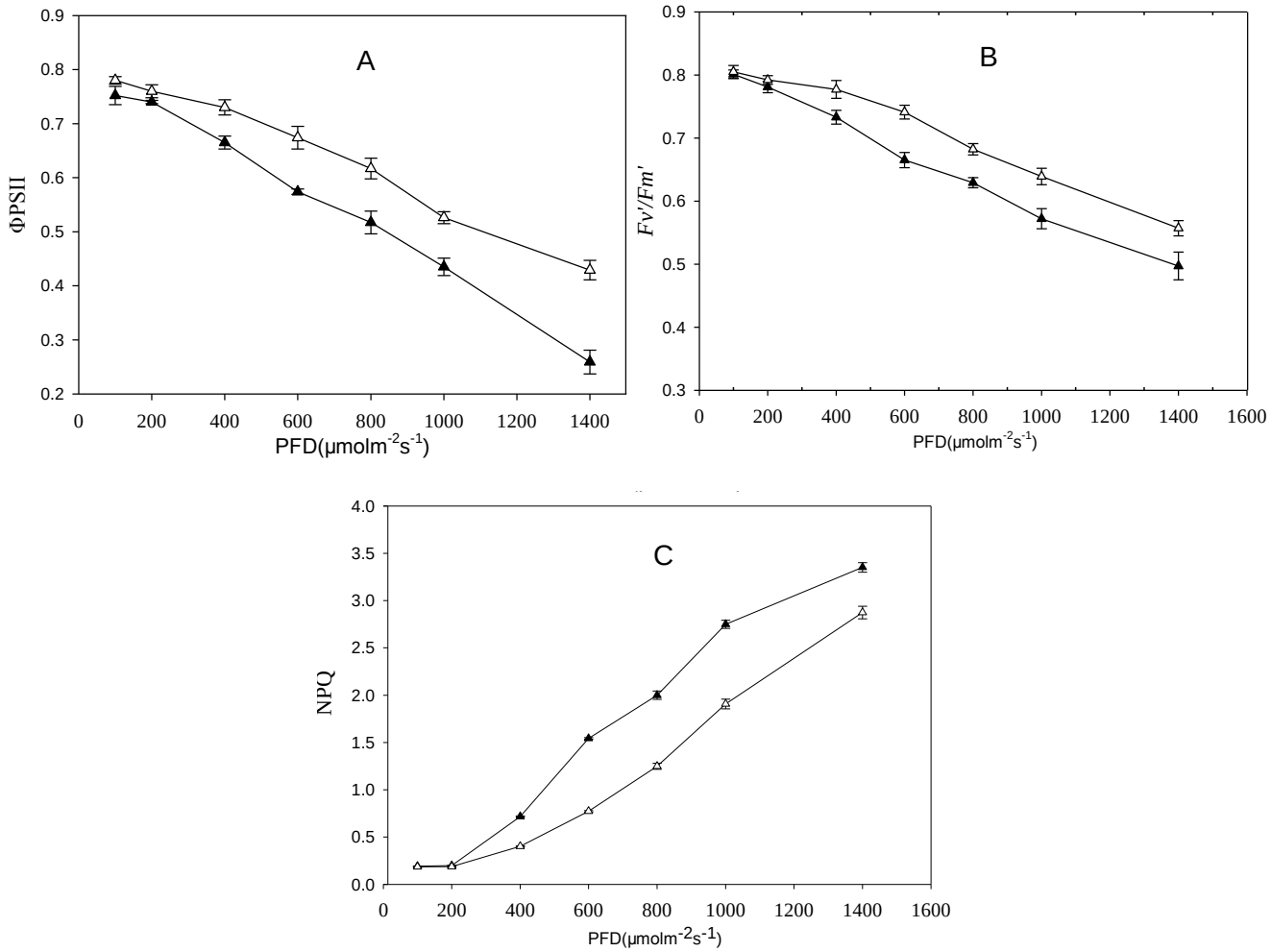


Figure 3. Responses of actual PSII efficiency (Φ_{PSII}, A), the efficiency of excitation capture by open PSII reaction centers (Fv'/Fm', B) and non-photochemical quenching (NPQ, C) to PFDs in *T. cuspidata* seeding (▲) and sapling (△) leaves. Values are means ± S.E., n = 3.

Table 2. Differential changes of chlorophyll fluorescence ratios in the maximal efficiency of PSII photochemistry (Fv/Fm), actual PSII efficiency (ΦPSII), the efficiency of excitation energy capture by open PSII centers (Fv'/Fm') and non-photochemical quenching (NPQ) in *T. cuspidata* seeding and sapling leaves at predawn and midday with PFD 1500 μmol·m^{-2}·s^{-1}. Values are means ± S.E., n = 4.

Variables	Seeding		Sapling	
	Predawn	Midday	Predawn	Midday
Fv/Fm	0.8±0.01	0.655±0.02	0.841±0.04	0.798±0.01
ΦPSII	0.587±0.01	0.344±0.02	0.616±0.01	0.476±0.02
Fv'/Fm'	0.695±0.01	0.483±0.02	0.816±0.154	0.71±0.01
NPQ	1.37±0.04	2.702±0.133	1.147±0.02	2.227±0.07

at high irradiance might act as a strengthened acclimation to cope with excess irradiance.

In conclusion, seeding leaves can dissipate the excess energy by xanthophyll cycle, but photoinhibition occurred in seeding leaves due to lower capacity of CO_2 assimilation and photorespiration and the light intensity for saturation of photosynthesis as well as the lower PSII photochemistry at high irradiance.

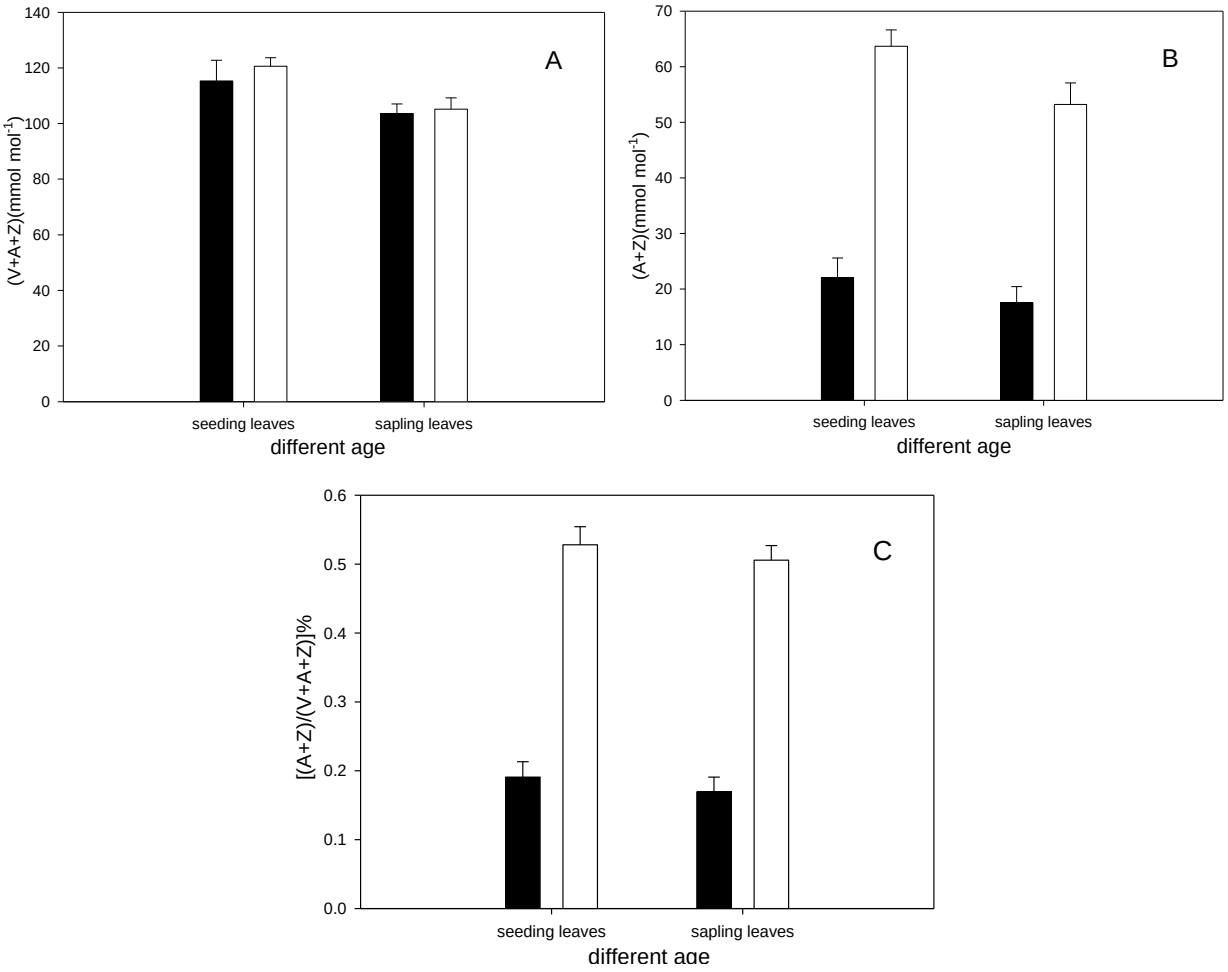


Figure 4. Changes of xanthophyll cycle pigment pool size, the de-epoxidation components per Chl, and conversion state of the xanthophyll cycle (A + Z)/(A + Z + V) in *T. cuspidata* seeding and sapling leaves. Samples were taken at predawn (■black bars) and at midday (□empty bars). Values are means ± S.E., n = 3.

REFERENCES

Anderson JM, Park YI, Chow W (1997). Photoinactivation and photoprotection of photosystem II in nature. Physiol Plant, 100(2): 214-223.

Bilger W, Bj rkman O (1990). Role of the xanthophyll cycle in photoprotection elucidated by measurements of light-induced absorbance changes, fluorescence and photosynthesis in leaves of Hedera canariensis. Photosyn. Res., 25(3): 173-185.

Chow W (1994). Photoprotection and photoinhibitory damage. Adv. Mol. Cell Biol., 10: 151-196.

Demmig-Adams B, Adams Iii W (1992). Photoprotection and other responses of plants to high light stress. Annu. Rev. Plant Biol., 43(1): 599-626.

Genty B, Briantais JM, Baker NR (1989). The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biophys. Acta., 990(1): 87-92.

Gilmore AM (1997). Mechanistic aspects of xanthophyll cycle©\dependent photoprotection in higher plant chloroplasts and leaves. Physiol Plant., 99(1): 197-209.

Guo WD, Guo YP, Liu JR, Mattson N (2009). Midday depression of photosynthesis is related with carboxylation efficiency decrease and D1 degradation in bayberry (Myrica rubra) plants. Sci. Hortic., 123(2): 188-196.

Horton P, Ruban A, Walters R (1996). Regulation of light harvesting in green plants. Annu Rev Plant Biol., 47(1): 655-684.

Huang L, Zheng J, Zhang Y, Hu W, Mao W, Zhou Y, Yu J (2006). Diurnal variations in gas exchange, chlorophyll fluorescence quenching and light allocation in soybean leaves: The cause for midday depression in CO2 assimilation. Sci. Hortic., 110(2): 214-218.

Iszkulo G, Boratynski A (2006). Analysis of the relationship between photosynthetic photon flux density and natural Taxus baccata seedlings occurrence. Acta Oecol., 29(1): 78-84.

Jiang CD, Gao HY, Zou Q, Jiang GM, Li LH (2006). Leaf orientation, photorespiration and xanthophyll cycle protect young soybean leaves against high irradiance in field. Environ. Exp. Bot., 55(1-2): 87-96.

Kobayashi J, Ogiwara A, Hosoyama H, Shigemori H, Yoshida N, Sasaki T, Li Y, Iwasaki S, Naito M, Tsuruo T (1994). Taxuspines A∼C, new taxoids from Japanese yew *Taxus cuspidata* inhibiting drug transport activity of P-glycoprotein in multidrug-resistant cells. Tetrahedron., 50(25): 7401-7416.

Kozaki A, Takeba G (1996). Photorespiration protects C3 plants from photooxidation. nature, 384: 557-560.

Lichtenthaler HK (1987). [34] Chlorophylls and carotenoids: Pigments of

photosynthetic biomembranes. Meth. Enzymol., 148: 350-382.
Lu Q, Wen X, Lu C, Zhang Q, Kuang T (2003). Photoinhibition and photoprotection in senescent leaves of field-grown wheat plants. Plant Physiol. Biochem., 41(8): 749-754.
Müller P, Li XP, Niyogi KK (2001). Non-photochemical quenching. A response to excess light energy. Plant Physiol., 125(4): 1558.
Niyogi KK (1999). Photoprotection revisited: genetic and molecular approaches. Annu Rev Plant Biol., 50(1): 333-359.
Potenko VV (2001). Inheritance of allozymes and genetic variation in natural population of Japanese yew in Petrov Island, Russia. For. Genet., 8(4): 307-314.
Thayer SS, Bj rkman O (1990). Leaf xanthophyll content and composition in sun and shade determined by HPLC. Photosyn. Res., 23(3): 331-343.

African Journal of Microbiology Research Vol. 5(32), pp. 5985-5989, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.1331

Full Length Research Paper

Response of *Cercospora beticola* in sugar beet at different cultivars and fertilization level

Yong-Gang Li*, Li Zhang and Feng-Ming Mang

Department of Plant Protection, Agricultural College, Northeast Agricultural University, Harbin Heilongjiang, 150030, People's Republic of China.

Accepted 12 December, 2011

Cercospora leaf spot (CLS) caused by *Cercospora beticola* is one of the most destructive foliar disease of sugar beets in all sugar beet-growing areas worldwide. In this study, field trials were carried out to determine the effect of CLS at different cultivars and fertilization level. The result showed that level of resistance against *C. beticola* from 20 variables were differed significantly (P<0.05) and sorted KWS0149>BETA356>Hi0940>KWS6167>KWS8138>KWS4121>Hi0166>DVA02234>BETA807> KWS0142> Ma096> KWS9522> IS0436> BSTO2431> Ma097> BETA464> BETA812> KWS9145> Hi0474> Hi0732. But, Strong and weak of same varieties resistance from three locations apart from 100 km away of this trial series were significantly different. Levels of resistance against *C. beticola* from optimized fertilization were significant different (P<0.05). Low nitrogen reduced sugar beet resistant against *C. beticola.* And level of resistance from 20 variables were differd significantly (P<0.05) and were sorted $N_2P_1K_1$> $N_2P_2K_1$> $N_2P_2K_0$ > $N_2P_2K_2$> $N_2P_1K_2$> $N_1P_2K_1$> $N_2P_3K_2$> $N_2P_2K_3$ > $N_2P_0K_2$> $N_1P_2K_2$> $N_3P_2K_2$> $N_1P_1K_2$> $N_0P_0K_0$> $N_0P_2K_2$. So, resistance against *C. beticola* improved after balance fertilizing. It is possible to reduce the pathogen appearance by using varieties resistance and balance fertilizing, which enhanced host resistance to soft rot disease in a way.

Key word: Sugar beet, cercospora leaf spot, varieties, resistance, fertilization level.

INTRODUCTION

Cercospora leaf spot (CLS) caused by *Cercospora beticola* is one of the most destructive foliar disease of sugar beets in all sugar beet-growing areas worldwide (Malandrakis et al., 2006). Control of CLS in Greece and other areas in a warm climate and irrigation is based mainly on frequent fungicide applications (Karaoglanidis and Ioannidis, 2010). However, serious problems have resulted from the extensive appearance of fungicide resistant C. beticola isolates to the intensively used benzimidazoles, organotin fungicides and sterol biosynthesis inhibiting triazoles.

Agricultural scientists are becoming aware of the potential contribution of farmers in developing integrated management of crop diseases in general (Bentley and Thiele, 1999). Much disease management practices such as the applications of fungicides and fumigant; focus on controlling pathogens is often too late to be effective, when disease symptoms are apparent. A more reliable approach is to concentrate on the period before infection occurs and encourage conditions that are unfavorable to the pathogen and favorable to the plant (Wolf and Verreet, 2002; Ghorbani et al., 2008).

Various control strategies, including host-plant resistance, resistant cultivars, integrated control and biological control have been developed. Breeding efforts to generate Cercospora resistance in sugar beet started in the 1920s by Munerati (1920). Historically, resistance was introgressed from the wild sea beet, *Beet vulgaris* L. spp. Maritima (Hecker and Helmerick, 1985). Additional resistant accessions were also found in other subspecies of *B. vulgaris* and in other sections of the genus Beta, namely Corollinae, Nanae and Procumbentes (Asher et

*Corresponding author: E-mail: neaulyg@yahoo.cn.

al., 2001). Resistant against *C. beticola* is a quantitative trait based on the additive effects of at least four to five major resistance genes (Smith and Gaskill, 1970). Therefore, sugar beet lines are selected for resistant against *C. beticola* in the greenhouse using artificial inoculation or in regions where natural infection occurs annually, namely Italy and Greece in southern Europe (Byford, 1996). As the climatic conditions in these countries are different from Germany, resistance of sugar beet varieties is influenced by environmental and cultivation factors (Märländer et al., 2003).

However, the exact number of host genes involved is unknown (Weiland and Koch, 2004). Due to highly variable climatic conditions on a single location, resistant cultivars adapted to the different sugar beet-growing areas worldwide where *C. beticola* occurs regularly are available (Byford, 1996; Mechelke, 2000; Pfleiderer and Schäufele, 2000). Host resistance is not efficient to prevent infection by *C. beticola* entirely but reduces the pathogen's development (Rossi et al., 2000). Therefore, sugar beet lines selected for resistant against *C. beticola* are unreliable in different regions and variable climatic conditions in commercial breeding.

Soil conditions for plant growth can influence the occurrence and severity of plant diseases. Managing and exploiting the suppressive effects of the soil environment as part of an integrated control strategy could make a significant contribution to agricultural sustainability and environmental quality (Quimby et al., 2002*).*

In this study, the impact of different cultivars and fertilization levels on CLS disease severity under natural infection in Heilongjiang, China in 2010. In this study, field trials were carried out to determine the effect of CLS at different cultivars and fertilization level. Secondly, resistant against *C. beticola* in different geographic regions were determined.

MATERIALS AND METHODS

Detection of disease resistant against *C. beticola* for sugar beet varieties

Sugar beet cultivars (KWS0142, KWS0149, KWS9145, KWS8138, KWS6167, KWS9522, KWS4121, BETA807, BETA356, BETA464, BETA812, BSTO2431, Ma096, Ma097, Hi0940, Hi0166, Hi0732, Hi0474, DVA02234, IS0436) differing in the level of resistant against *C. beticola* were used in this study. One location with severe disease occurrence in 2009 was selected to determine cultivars resistant against *C. beticola* under natural infection in Heilongjiang, China in 2010. Three locations apart from 100 km away with severe disease occurrence were selected to determine KWS1049 and KWS4121 cultivars resistant against *C. beticola* of different geographic area.

Field trials were sown between mid and end of April with 70 cm distance between rows. The distances between plants within rows in the natural infection trial was 40 cm, and the trials were manually thinned to a density of 49,500 to 52,500 plants ha^{-1} in seedling trays filled with a standard soil. Weed control were carried out according to local standards.

Response of sugar beet against *C. beticola* at different fertilization level

Field experiments with Sugar beet cultivars KWS0149 were conducted in a location of Heilongjiang province in 2010. Soil nutrients of tested field were obtained under large of 0～15 cm. Organic matter was measured, including the contents of organic matter, available nitrogen, available phosphorus and available potassium etc. for the pre-test. The results showed that the organic matter content is medium rate (20.18～50.20 $g \cdot kg^{-1}$) in tested field soils, ranging from (118.12～204.20 mg $N \cdot kg^{-1}$, 7.18～14.32 mg $P \cdot kg^{-1}$, 44.75～139.57 mg $K \cdot kg^{-1}$). Field trials were sown with 70 cm distance between rows. A 140 cm wide protective belt is left without fertilization by using randomized group (every group mean 5.6 m^2) design with 4 replications.

Traditional fertilization and optimized fertilization were using to analyze effect of sugar beet against *C. beticola* at different fertilization level in this study. Nitrogen (N), phosphors (P) and potassium (K) were replaced respectively by using carbamide (N), diammonium phosphate (P), kalium sulfuricum (K).

Fertilizer application rates of traditional fertilization were designed as the treatments of 600 $kg \cdot ha^{-1}$ (240 kg $N \cdot ha^{-1}$,195 kg $P \cdot ha^{-1}$,165 kg $K \cdot ha^{-1}$), 675 $kg \cdot ha^{-1}$ (270 kg $N \cdot ha^{-1}$, 210 kg $P \cdot ha^{-1}$,195 kg $K \cdot ha^{-1}$), 750 $kg \cdot ha^{-1}$ (300 kg $N \cdot ha^{-1}$,255 kg $P \cdot ha^{-1}$,195 kg $K \cdot ha^{-1}$), 825 $kg \cdot ha^{-1}$ (375 kg $N \cdot ha^{-1}$, 270 kg $P \cdot ha^{-1}$,180 kg $K \cdot ha^{-1}$), and 900 $kg \cdot ha^{-1}$ (420 kg $N \cdot ha^{-1}$, 255 kg $P \cdot ha^{-1}$, 225 kg $K \cdot ha^{-1}$).

Fertilizer application rates of optimized fertilization were $N_0P_0K_0$, $N_0P_2K_2$, $N_1P_2K_2$, $N_2P_0K_2$, $N_2P_1K_2$, $N_2P_2K_2$, $N_2P_3K_2$, $N_2P_2K_0$, $N_2P_2K_1$, $N_2P_2K_3$, $N_3P_2K_2$, $N_1P_1K_2$, $N_1P_2K_1$, and $N_2P_1K_1$ (Detailed data refer to Table 4).

Disease assessment

Disease index severity of all individual sugar beet plants per treatment was assessed according to the modified agronomica disease index severity (Vereijssen et al., 2003; Battilani et al., 1990), which covers a scale from 0 (healthy) to 9 (totally destroyed foliage). Disease index severity in each treatment group was estimated in the middle of August, 2010 using a scale of 0 to 9: 0 = no symptoms on fully leaves; 1= few disease spots of most leaves; 3 = most disease spots of most leaves; 5 =most disease spots of most leaves, dead lateral 1 to 3 leaves; 7 = most disease spots of most leaves, dead lateral 3 to 5 leaves; 9 = most disease spots of most leaves, all leaves and leafstalk dead or whole plant dead.

Statistics

Analysis of variance was carried out with the programme SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Significant differences were indicated with different letters for probabilities ($P<0.05$).

RESULTS

Detection of disease resistant against *C. beticola* for sugar beet varieties

Disease index investigation was carried out in the middle of August. Univariate comparisons showed that level of resistance of 20 cultivars had been differed significantly ($P<0.05$) and were sorted KWS0149> BETA356> Hi0940 >KWS6167> KWS8138>KWS4121> Hi0166> DVA0-2234 > BETA807> KWS0142> Ma096> KWS9522> IS0436>

Table 1. The resistance determination of sugar beet varieties against *C. beticola.*

Cultivars	Disease index	Cultivars	Disease index
KWS0142	17.36±0.54^{abcde}	Ma096	19.01±0.89^{bcdef}
KWS0149	13.08±0.49^{a}	Ma097	20.86±1.01^{cdefg}
KWS9145	23.95±2.85^{fgh}	Hi0940	15.31±0.25^{abc}
KWS8138	16.30±0.86^{abcd}	Hi0166	16.79±0.25^{abcd}
KWS6167	15.81±0.25^{ab}	Hi0732	27.61±1.39^{h}
KWS9522	19.14±0.81^{bcdef}	DVA0-2234	16.79±1.31^{abcd}
BETA807	17.04±1.13^{abcd}	BSTO-2431	19.38±1.60^{bcdef}
BETA356	14.62±4.40^{ab}	IS0436	19.26±1.86^{bcdef}
BETA464	21.73±2.51^{cdef}	Hi0474	25.80±2.50^{gh}
BETA812	22.84±1.39^{efgh}	KWS4121	16.54±1.38^{abcd}

Data are treatment means of pooled data ± standard errors. Values of each column followed by different letters are significantly different at $P < 0.05$ according to Duncan's multiple range tests.

Table 2. The determination of sugar beet resistant against *C. beticola* from different geographic area.

Breeds	Different regions	Disease index
KWS1049	1	13.08±0.49^{a}
	2	27.66±0.25^{c}
	3	17.12±1.72^{b}
KWS4121	1	16.54±1.38^{a}
	2	29.14±1.37^{b}
	3	19.35±3.74^{a}

The distance among the three zones (1,2,3) is 100 km; Data are treatment means of pooled data ± standard errors. Different letters for the same assessment date indicate significant different at $P < 0.05$ according to Duncan's multiple range tests.1, 2, 3 for three locations apart from 100 km away.

BSTO-2431> Ma097> BETA464> BETA812> KWS9145> Hi0474> Hi0732 (Table 1).

Three locations apart from 100 km away with severe disease were selected to analyze relationship between KWS1049, KWS4121 cultivars resistant against *C. beticola* and different geographic area. The result showed that level of same varieties resistance from different geographic area were significant different (Table 2).

Response of sugar beet against *C. beticola* at different fertilization level

Traditional fertilization was designed to analyze cultivars resistant against *C. beticola*. The results showed that level of resistant against *C. beticola* from traditional fertilization were not significant different (Table 3).

Optimized fertilization was designed to analyze cultivars resistant against *C. beticola* from different fertilization level. The results showed that level of resistant against *C. beticola* from optimized fertilization were significant different (Table 4). The results showed low nitrogen reduced sugar beet resistant against *C. beticola* and level of resistance from optimized fertilization were sorted $N_2P_1K_1$> $N_2P_2K_1$> $N_2P_2K_0$ > $N_2P_2K_2$> $N_2P_1K_2$> $N_1P_2K_1$> $N_2P_3K_2$> $N_2P_2K_3$ > $N_2P_0K_2$> $N_1P_2K_2$> $N_3P_2K_2$> $N_1P_1K_2$> $N_0P_0K_0$> $N_0P_2K_2$.

DISCUSSION

In this study, we aimed to estimate effect of different cultivars and fertilization level under natural infection against *C. beticola* in Heilongjiang, China. The 20 cultivars resistant against *C. beticola* were evaluated under natural infection in cultivar trial series. The result showed that KWS series varieties had the character of high resistance to disease in Heilongjiang, such as

Table 3. The determination of resistant against *C. beticola* from traditional fertilization.

Sum (kg·ha^{-1})	Carbamide (kg·ha^{-1})	Diammonium phosphate (kg·ha^{-1})	Potassium sulfate (kg·ha^{-1})	Disease index
600	240	195	165	20.99±1.37^{a}
675	270	210	195	22.47±1.73^{a}
750	300	255	195	21.97±0.99^{a}
825	375	270	180	23.21±1.73^{a}
900	420	255	225	21.48±1.96^{a}

Data are treatment means of pooled data ± standard errors. Different letters for the same assessment date indicate significant different at $P<0.05$ according to Duncan's multiple range tests.

Table 4. The determination of resistant against *C. beticola* from optimized fertilization.

NPK content	Disease index	NPK content	Disease index
$N_0P_0K_0$	22.48±2.52^{bc}	$N_2P_2K_0$	14.79±1.72^{a}
$N_0P_2K_2$	24.68±2.76^{c}	$N_2P_2K_1$	13.16±1.08^{a}
$N_1P_2K_2$	17.69±2.58^{ab}	$N_2P_2K_3$	16.30±1.47^{a}
$N_2P_0K_2$	17.08±2.22^{ab}	$N_3P_2K_2$	18.43±0.40^{ab}
$N_2P_1K_2$	15.55±0.85^{a}	$N_1P_1K_2$	18.56±0.74^{ab}
$N_2P_2K_2$	15.22±1.24^{a}	$N_1P_2K_1$	15.86±1.33^{a}
$N_2P_3K_2$	16.18±2.04^{a}	$N_2P_1K_1$	13.01±1.30^{a}

Data are treatment means of pooled data ± standard errors. Different letters for the same assessment date indicate significant different at $P < 0.05$ according to Duncan's multiple range tests; Carbamide g/ 5.6m^2: N_0 mean 0.0, N_1 mean 51.6, N_2 mean 103.2, N_3 mean 154.8; Diammonium phosphate g/ 5.6 m^2: P_0 mean 0.0; P_1 mean 54.8; P_2 mean 109.6; P_3 mean 164.3; Kalium sulfuricum g/ 5.6m^2: K_0 mean 0.0; K_1 mean 50.4; K_2 mean 100.8; K_3 mean 151.2.

KWS0149, KWS6167, KWS8138, KWS0142 and KWS4121. In addition, others varieties had strong resistance to CLS, such as Hi0166, Hi0940, DVA0-2234, BETA356 and BETA807 to offer basis in preventing CLS and in selecting scientific distribution of resistant variety in Heilongjiang.

In three locations apart from 100 km away, relationship between KWS1049, KWS4121 cultivars resistant against *C. beticola* and different geographic area was analyzed. Same varieties resistance from different geographic areas was significantly different (Table 2). Sugar beet resistant cultivars adapted to the different sugar beet-growing areas worldwide where *C. beticola* regularly occurs were available (Byford, 1996; Mechelke, 2000). But, the suppression of plant defence reactions plays a crucial role in causing plant diseases (Bouarab et al., 2002; Hauck et al., 2003). Schmidt et al. (2004) have shown that inducible plant defences are repressed during the development of CLS. Therefore, host resistance is not efficient to prevent infection by *C. beticola* (Rossi et al., 1999, 2000). So, Field identification of sugar beet resistant from different geographic area cultivars against C. beticola needs to further strengthen and expands the area.

As Walters et al. (2005) pointed out; we need to pay attention to factors that are likely to influence the effectiveness of bio-controls in the field. There are evidences which show both a positive and a negative relationship between available plant nutrients and incidence of certain diseases (Ghorbani et al., 2008). Fertilizer application rates of traditional fertilization and optimized fertilization were designed to analyze cultivars resistant against *C. beticola*. The result showed that strong and weak of resistant against C. beticola from different fertilization level of traditional fertilization were not significantly different (P <0. 05) (Table 3).

However, the results showed significant differences among different fertilization level of optimized fertilization (P <0.05) (Table 4). The results showed low nitrogen could reduce host resistant against *C. beticola.* And balance fertilizing could enhance host resistance to CLS. Fertilizer application rates were designed for enhance sugar beet resistant against *C. beticola* as the treatments of adaptive rate (N:P:K=2:1:1).

Abundant nitrogen encourages succulent growth, a prolonged vegetative period, and delayed maturity of the plant, which increases the period of susceptibility to pathogens. Deficient plants are weaker and slower growing, which are also more susceptible to pathogens (Agrios, 1997). The effect of soil nitrogen level on disease development in different agricultural crops has been shown. For example, Sharma and Kolte (1994)

suggested that the plants in pots or field plots which received NK (N 90 kg ha $^{-1}$) + (K 40 kg ha^{-1}) were more resistant to infection than plants which received N (alone) or P (alone) or NP and PK combinations. Such results provide interesting evidence to support the view that balanced soil fertility could lead to better sugar beet resistant against *C. beticola*.

All in all, a comparative study of resistance determination of sugar beet varieties against *C. beticola* is needed to understand better from different geographic area in order to design comprehensive control on CLS. Accumulation of more knowledge regarding control of CLS should stimulate further conversion of conventional systems of sugar beet production, which incorporate agro-ecological strategies to optimize soil fertilization, sugar beet varieties diversity management and more natural systems of disease regulation without incurring much yield.

ACKNOWLEDGEMENTS

Authors wish to thank to Heilongjing Postdoctoral Science Foundation (LRB09-279), Dr. Start-Up fund research of Northeast Agricultural University (2009RC48) and Ministry of Agriculture Key Laboratory Foundation of Cold Crop Physiology Ecology (Northeast Agricultural University) for financial support.

REFERENCES

Agrios GN (1997). Climate change and plant diseases in Ontario. Plant pathology. Academic Press, San Diego.

Asher MJC, Luterbacher MC, Frese L (2001). Wild Beta species as a source of resistance to sugar-beet pests and diseases. Int. Sugar J., 103: 447–456.

Battilani P, Beltrami G, Meriggini P, Ponti I, Rossi A, Rossi V, Rosso F, Tugnoli V, Zocca A (1990). Nuovo indirizzi, di difesa anticercosporica. L'Informatore Agrario, 46:53–70.

Bentley JW, Thiele G (1999). Bibliography: Farmer knowledge and management of crop disease. Agriculture and Human Values, 16: 75–81.

Bouarab K, Melton R, Peart D, Baulcombe D, Osbourn A (2002). A saponin-detoxifying enzyme mediates suppression of plant defences. Nat., 418: 889–892.

Byford WJ (1996). A survey of foliar diseases of sugar beet and their control in Europe. Paper presented at the 59th IIRB Congress, Brussels.

Ghorbani R, Wilcockson S, Koocheki A, Leifert C (2008). Soil management for sustainable crop disease control: a review. Environ. Chem. Lett., 6(3): 149-162.

Hauck P, Thilmony R, He SY (2003). A Pseudomonas syringae type III effector suppresses cell wall-based extracellular defense in susceptible Arabidopsis plants. Proc. Natl. Acad. Sci. USA., 100(14): 8577–8582.

Hecker RJ, Helmerick RH (1985). Sugar-beet breeding in the Unites States. In Progress in Plant Breeding—1 (Russell GE, ed.). London: Butterworths, pp. 37–61.

Karaoglanidis SG, Ioannidis MP (2010). Fungicide resistance of *Cercospora beticola* in Europe. In Cercospora leaf spot of sugar beet and related species, ed. R.T. Lartey, J.J. Weiland, L. Panella, P.W. Crous, and C.E. Windels, St. Paul, MN: The American Phytopathological Soc., pp. 189–211.

Malandrakis AA, Markoglou AN, Nikou DC, Vontas JG, Ziogas BN (2006). CLS caused by *Cercospora beticola* is one of the most serious diseases of sugar beets with world-wide economic importance. European J. Plant Pathol., 116: 155–166.

Märländer B, Hoffmann C, Koch HJ, Ladewig E, Merkes R, Petersen J, Stockfisch N (2003). Environmental Situation and Yield Performance of the Sugar Beet Crop in Germany: Heading for Sustainable Development. J. Agronomy Crop Sci., 189: 201–226.

Mechelke W (2000). Züchtungs-und Sortenstrategien zur Resistenz bei Zuckerrüben gegenüber *Cercospora beticola*. Zuckerindustrie, 125: 688–692.

Munerati O (1920). Osservazione e Richerche sulla Barbabietola da Zucchero. Observations and Research on Sugar Beet-Part 1. reprint 1999. Tipografica della R. Accademia dei Lincei.

Pfleiderer UE, Schäufele WR (2000). Development of a testing method for resistant against *Cercospora beticola* in sugar beet. In M. J. C. Asher, B. Holtschulte, M. R. Molard, F. Rosso, G. Steinrücken, & R. Beckers (Eds.), *Cercospora beticola* Sacc. Biology, agronomic influence and control measures in sugar beet. Advan. Sugar Beet Res., 2: 147-153.

Quimby PC, King LR, Grey WE (2002). Biological control as a means of enhancing the sustainability of crop/land management systems. Agric. Ecosyst. Environ., 88:147–152.

Rossi V, Battilani P, Chiusa G, Giosuè S, Languasco L, Racca P (2000). Components of rate-reducing resistance to CLS in sugar beet: conidiation length, spore yield. J. Plant Pathol., 82: 125–131.

Schmidt K, Heberle B, Kurrasch J, Nehls R, Stahl DJ (2004). Suppression of phenylalanine ammonia lyase expression in sugar beet by the fungal pathogen *Cercospora beticola* is mediated at the core promoter of the gene. Plant Molecular Biol., 55: 835–852.

Sharma SR, Kolte SJ (1994). Effect of soil-applied NPK fertilizers on severity of black spot disease *(Alternaria brassicae)* and yield of oilseed rape, Plant Soil, 167: 313-320.

Smith GA, Gaskill JO (1970). Inheritance of resistance to CLS in sugarbeet. J. Am. Society of Sugar Beet Technol., 16: 172–180.

Vereijssen J, Schneider JHM, Termorshuizen AJ, Jeger MJ (2003). Comparison of two disease assessment keys to assess *Cercospora beticola* in sugar beet. Crop Protection 1: 201–209.

Walters D, Walsh D, Newton A, Lyon G (2005). Induced resistance for plant disease control: maximizing the efficacy of resistance elicitors. Phytopathol., 95:1368–1373.

Weiland J, Koch G (2004). Sugarbeet leaf spot disease (*Cercospora beticola* Sacc.). Pathogen profile. Molecular Plant Pathol., 5:157–166.

Wolf PFJ, Verreet JA (2002). The IPM sugar beet model, an integrated pest management system in Germany for the control of fungal leaf diseases in sugar beet. Plant Dise., 86: 336-344.

African Journal of Microbiology Research Vol. 5(32), pp. 5990-5993, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.1342

Full Length Research Paper

In vitro antioxidant activities of polysaccharides from endophytic fungus *Fusarium oxysporum* Dzf17

Peiqin Li, Chao Luo, Weibo Sun, Shiqiong Lu, Yan Mou, Youliang Peng and Ligang Zhou*

College of Agronomy and Biotechnology, China Agricultural University, Beijing 100193, China.

Accepted 9 December, 2011

Three polysaccharides, namely exopolysaccharide (EPS), water-extracted mycelial polysaccharide (WPS) and sodium hydroxide-extracted mycelial polysaccharide (SPS), from the endophytic fungus *Fusarium oxysporum* Dzf17 were investigated for their *in vitro* antioxidant activities. Among them, SPS was the most active antioxidant component, and WPS exhibited moderate antioxidant activity. The median effective concentration (EC_{50}) values of the polysaccharides were 162.38 µg/ml (for WPS), 63.37 µg/ml (for SPS) by DPPH radical scavenging activity assay, and 54.54 µg/ml (for WPS) and 44.91 µg/ml (for SPS) by using ferrous ions chelating activity assay. The polysaccharides from *F. oxysporum* Dzf17 could be an alternative source as the antioxidant components.

Key words: Antioxidant activity, polysaccharides, endophytic fungus, *Fusarium oxysporum* Dzf17.

INTRODUCTION

Fungi have been regarded as important resources of natural bioactive compounds with a variety of bioactivities, and have been widely applied in agriculture, medicine and food industry (Greve et al., 2010; Zhong and Xiao, 2009; Zhou et al, 2010). Plant endophytic fungi are a special group of fungi that reside within plant tissues intercellularly or intracellularly without causing any apparent symptoms of disease (Wilson, 1995). In the past two decades, many valuable bioactive compounds with antimicrobial, insecticidal, cytotoxic and anticancer activities have been successfully obtained from the endophytic fungi (Kharwar et al., 2011; Verma et al., 2009; Zhao et al., 2011). These bioactive compounds could be mainly classified as alkaloids, terpenoids, steroids, quinones, isocoumarins, lignans, phenylpropanoids, phenols and polysaccharides (Aly et al., 2010; Yu et al., 2010; Zhang et al., 2006). To the best of our knowledge, the antioxidant activities of the polysaccharides from endophytic fungi have been rarely reported, though there were some reports from other fungi (Liu et al., 1997; Ooi and Liu, 1999).

Fusarium oxysporum Dzf17 is an endophytic fungus isolated from the rhizomes of *Dioscorea zingiberensis*, a well known traditional Chinese medicinal herb indigenous to the south of China (Li and Ni, 2011; Zhang et al., 2009). Three polysaccharides, namely exopolysaccharide (EPS), water-extracted mycelial polysaccharide (WPS) and sodium hydroxide-extracted mycelial polysaccharide (SPS), prepared from *F. oxysporum* Dzf17 were observed in our previous study to have enhancement effects on cell growth and diosgenin accumulation in *D. zingiberensis* cell cultures (Li et al., 2011). The purpose of this study was to investigate the antioxidant activities of three kinds of polysaccharides from the endophytic fungus *F. oxysporum* Dzf17 in order to provide fundamental data for the research and application of the polysaccharides from this fungus.

MATERIALS AND METHODS

General

The microplate spectrophotometer (PowerWave HT, BioTek Instruments, USA) was employed to measure the light absorption value. 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (USA) in Beijing. 3-(2-Pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) was obtained from Johnson Matthey (UK) in Beijing. Butylated hydroxytoluene (BHT), ferrous chloride ($FeCl_2$), and ethylene diamine tetraacetic acid (EDTA) were

*Corresponding author. E-mail: lgzhou@cau.edu.cn.

bought from Beijing Chemical Company. All other chemicals and reagents were of analytical grade.

Endophytic fungus and culture conditions

The endophytic fungus *F. oxysporum* Dzf17 (GenBank accession number EU543260) was isolated from the healthy rhizomes of the medicinal plant *D. zingiberensis* C. H. Wright (Dioscoreaceae) in our previous study (Zhang et al., 2009). The living culture has been deposited in China General Microbiological Culture Collection Center (CGMCC) under the number CGMCC 2472. It was also maintained on potato dextrose agar (PDA) slants at 25℃, and in 40% glycerol at -70℃ at the Herbarium of the College of Agronomy and Biotechnology, China Agricultural University. The mycelia were grown in a 1000 ml Erlenmeryer flask containing 300 ml of liquid medium consisting of glucose (50 g/L), peptone (13 g/L), NaCl (0.6 g/L), K_2HPO_4 (0.6 g/L), and $MgSO_4•7H_2O$ (0.2 g/L). About 500 flasks were used. All flasks were maintained at 25°C on a rotary shaker at 150 rpm for 14 days. A total of 150 L of fermentation broth was harvested. The mycelia were separated from the supernatant by centrifugation at 7,741 ×g for 20 min. Mycelia were washed twice with deionized water, then lyophilized. About 600 g of mycelia in dry weight (dw) was obtained.

Preparation of polysaccharides

The preparation process of exopolysaccharide (EPS) has described in our previous study (Li et al., 2011). Briefly, the supernatant was concentrated under vacuum at 60℃ by a rotary evaporator to a proper volume and mixed with three volumes of 95% ethanol, then kept at 4ºC for 48 h. After that, the solution was centrifuged at 17,418 × g for 15 min, and the precipitate from ethanol dispersion was collected as crude EPS which was further subjected to deproteination with Sevag reagent (chloroform-*n*-butanol at 4:1, v/v), decolorization with H_2O_2, and re-movement of small molecular impurities by dialysis. Polysaccharide mixture with molecular weight greater than 8,000 to 14,000 Da was kept in dialysis tube. The carbohydrate content of EPS was measured by the method of anthrone-sulfuric acid spectrophotography (Wang et al., 2007), which involved sulfuric acid hydrolysis of the sample in the presence of anthrone agent at 100℃. The absorbance at 620 nm was measured and calibrated to carbohydrate content using glucose as a reference. After lyophilization, the purified EPS (31.98 g) was stored in a desiccator at room temperature.

Water-extracted mycelial polysaccharide (WPS) and sodium hydroxide-extracted mycelial polysaccharide (SPS) were also prepared according to our previous research (Li et al., 2011). Briefly, the lyophilized mycelia (600 g) were powdered in a high disintegrator, and then subjected to heat circumfluence extraction at 50℃ by 95% ethanol-petroleum ether at 1:1 (v/v) as the refluxing solvent to remove monosaccharide, disaccharide and lipid. The ratio of mycelia powder (g) to refluxing solvent (ml) was 1:5 (w/v). Defatted mycelial powder was obtained by centrifugation (7,741 × g, 20 min) and drying in an oven at 40℃ for 2 h, and then immersed in hot water at 90℃ for 2 h with the ratio of water (ml) to the material (g) as 30:1 (v/w). After that, centrifugation was carried out at 7,741 × g for 20 min to separate the residue and the supernatant. The supernatant was condensed to a certain volume under vacuum at 60℃, and then mixed with three volumes of 95% ethanol, then kept at 4℃ for 48 h. The following procedure for polysaccharide preparation and purification was the same as the treatments of exopolysaccharide (EPS). The gained polysaccharide (33.24 g) was named as water-extracted mycelial polysaccharide (WPS). The residue not containing WPS was further extracted with 10% sodium hydroxide (NaOH) solution at room temperature for 24 h. The remaining steps were the same as the treatments of EPS. The obtained polysaccharide (35.89 g) was designated as sodium hydroxide-extracted mycelial polysaccharide (SPS).

***In vitro* antioxidant activity assay**

The polysaccharides were subjected to a screening for antioxidant activity by two complementary tests, namely the DPPH radical scavenging assay and ferrous ions chelating assay, The free radical scavenging activity of different antioxidants was measured in terms of hydrogen donating or radical scavenging ability of the stable free radical DPPH (1,1-diphenyl-2-picryhydrazyl) (Ono et al., 2008). The scavenging activity of DPPH was measured according to the method reported by Qiao et al. (2009) with some modifications. Briefly, DPPH dehydrated alcohol solution (0.2 mg/ml, 100 µl) and polysaccharide water solution (100 µl) were added to each well of the microplate and mixed. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance was measured at 517 nm against a blank. Butylated hydroxy toluene (BHT) was used as the positive control. Lower absorbance of the reaction mixture indicates higher free-radical scavenging activity. All the tests were performed in triplicate and the graph was plotted with the mean values and standard deviations. The scavenging activity was calculated by the following equation:

$$\text{Scavenging activity (\%)} = [A_0-(A_1-A_2)]\times 100/A_0.$$

Where A_0 is the absorbance of DPPH solution without tested samples, A_1 is the absorbance of the sample, and A_2 is the absorbance of the sample under identical conditions as A_1 with water instead of DPPH solution.

The median effective concentration (EC_{50}) value was calculated using the linear relation between the effective probability and concentration logarithm according to the method of Sakuma (1998).

Metal ions chelating activity was determined according to the method of Wang et al. (2010) with some modifications. Briefly, polysaccharide solution (50 µl) was mixed with $FeCl_2$ solution (0.2 mg/ml, 30 µl), and shaken vigorously. The ferrozine solution (2 mg/ml, 70 µl) was then added to the reaction solution. The reaction mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture reached equilibrium, the absorbance of the solution was then measured at wavelength 560 nm using a microplate spectrophotometer. EDTA was used as the positive control. Lower absorbance of the reaction mixture indicates higher chelating activity. All the tests were performed in triplicate and the graph was plotted with the mean values and standard deviations. The ferrous ions chelating effect was calculated as the percentage (%) of inhibition of ferrozine-Fe^{2+} complex formation determined as:

$$\text{Chelating activity (\%)} = [B_0-(B_1-B_2)]\times 100/B_0.$$

Where B_0 is the absorbance of reaction solution without tested samples, B_1 is the absorbance of the sample and B_2 is the absorbance of the sample under identical conditions as B_1 with water instead of ferrozine solution.

The EC_{50} value calculation for ferrous ions chelating activity was the same as that for DPPH radical scavenging activity.

RESULTS AND DISCUSSION

DPPH radical scavenging activity

DPPH has been widely adopted as a reference for evaluating the free radical scavenging activities of the

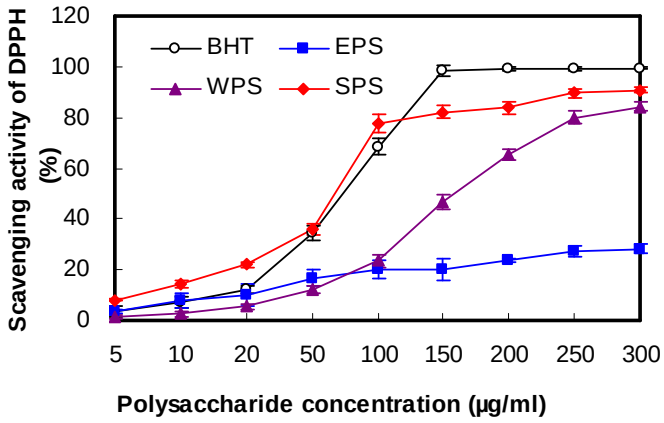


Figure 1. DPPH radical scavenging activity of the polysaccharides.

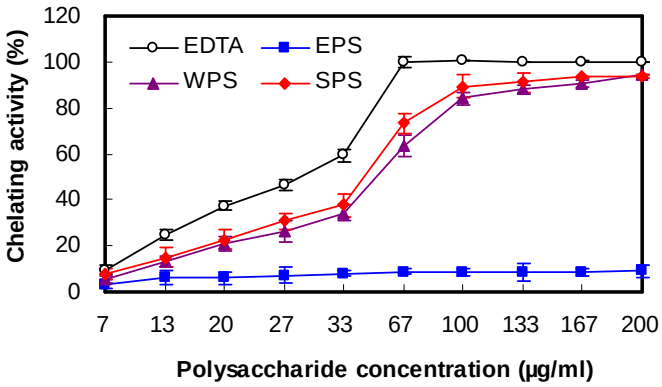


Figure 2. Ferrous ions chelating activity of the polysaccharides.

Table 1. EC_{50} values of the polysaccharides for antioxidant activity.

Assay	EC_{50} (μg/ml)			
	EPS	WPS	SPS	CK^+
DPPH scavenging activity	-	162.38a	63.37c	74.94b
Ferrous ions chelating activity	-	54.54a	44.91b	28.86c

The positive controls (CK^+) for DPPH scavenging and ferrous ions chelating assays are BHT and EDTA, respectively. '-' means that EC_{50} values cannot be obtained at the test concentrations. Different letters in each row indicate significant differences of the antioxidant activity for each assay at p = 0.0.5.

concentrations of 7 to 200 μg/ml, the chelating activity of natural compounds (Amarowicz et al., 2004). The DPPH radical scavenging effects of the polysaccharides EPS, WPS and SPS from *F. oxysporum* Dzf17 were presented in Figure 1. Among them, SPS exhibited the strongest scavenging DPPH activity at concentrations of 5 to 100 μg/ml, showing a good linear dependence between SPS concentration and DPPH scavenging activity. In contrast, WPS showed moderate, and EPS showed non-antioxidant activity. When WPS was at concentration of 300 μg/ml, the scavenging activity was 84.27%. The EC_{50} values (shown in Table 1) of WPS and SPS for antioxidant activity were 162.38 μg/ml and 63.37 μg/ml, respectively.

Ferrous ions chelating activity

Ferrous ions chelating activity was employed as another indicator to assess the quantity of the antioxidants (Lianhe et al., 2011; Xiao et al., 2011). In this research, the chelating activities of the polysaccharides from *F. oxysporum* Dzf17 on ferrous ions (Fe^{2+}) were investigated, which were presented in Figure 2. All the tested samples showed evident Fe^{2+} chelating activity in a concentration-dependent manner except EPS. At EPS varied only from 3.36 to 9.09%, which did not show its antioxidant activity. WPS or SPS showed stronger chelating activity than that of EPS, but slightly weaker than that of the positive control (EDTA). When the concentration of WPS was changed from 7 to 100 μg/ml, the chelating activity was rapidly increased from 5.59 to 84.07% showing a good linearity. With the concentration of WPS varied from 100 to 200 μg/ml, the chelating activity showed a gently increase with the value of 84.07 to 94.59%. The chelating activity of SPS exhibited the same trend but slightly stronger than that of WPS. The EC_{50} values (shown in Table 1) of WPS and SPS for antioxidant activity were 54.54 and 44.91 μg/ml, respectively.

In summary, this is the first report on the antioxidant activities of the polysaccharides from the endophytic fungus *F. oxysporum* Dzf17. Among three polysaccharides, SPS was the most active antioxidant component, WPS showed moderate, and EPS showed non-antioxidant activity. The antioxidant activity results of the polysaccharides obtained by two complementary assays were similar which indicated that they should have similar antioxidant mechanisms. DPPH is a stable free radical with a maximum absorption at 517 nm and can be readily scavenged by the antioxidants (e.g., phenolics, flavonoids, carotenoids and polysaccharides) which have hydrogen donating groups (Muller et al., 2011; Paixao et al., 2007; Qiao et al., 2009). Ferrozine quantitatively forms complexes with Fe^{2+}. In the presence of other chelating agents (e.g., polysaccharide), the complex formation is disrupted with the result that the red color of the complex was decreased (Yamaguchi et al., 2000). As the antioxidant mechanisms of polysaccharides are very complicated, other methods such as OH free radical scavenging and reducing powder assays should be employed in our further investigation (Huang et al., 2005). In our previous study, WPS was found to be the most effective polysaccharide to have enhancement effects on cell growth and diosgenin accumulation in *D. zingiberensis* cell cultures (Li et al., 2011). WPS showed

moderate antioxidant activity in this investigation which means that WPS should be studied in detail for its enhancing effect on secondary metabolite biosynthesis and antioxidant activity. The present study will provide additional data for supporting the utilization and development of the polysaccharides from *F. oxysporum* Dzf17 as the antioxidant components. Further studies to clarify other biological activities (e.g. immunoregulatory and antitumor activities) of the polysaccharides, their preparation on a large scale, composition including protein and carbohydrate percentage, antioxidant mechanisms, as well as the physiological and ecological roles of the polysaccharides on host plant cells are now in progress.

ACKNOWLEDGEMENTS

This work was co-financed by the grants from the Natural Science Foundation of Beijing (6092015), the program for Changjiang Scholars and Innovative Research Team in University of China (IRT1042), and the National Natural Science Foundation of China (30871662 and 31071710).

REFERENCES

Aly AH, Debbab A, Kjer J, Proksch P (2010). Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. Fungal Divers, 41: 1-16.

Amarowicz R, Pegg RB, Rahimi-Moghaddam P, Barl B, Weil JA (2004). Free radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. Food Chem., 84: 551-562.

Greve H, Mohamed IE, Pontius A, Kehraus S, Gross H, Konig GM (2010). Fungal metabolites: structural diversity as incentive for anticancer drug development. Phytochem. Rev., 9: 537-545.

Huang D, Ou B, Prior RL (2005). The chemistry behind antioxidant capacity assays. J. Agric. Food Chem., 53: 1841-1856.

Kharwar RN, Mishra A, Gong SK, Stierle A, Stierle D (2011). Anticancer compounds derived from fungal endophytes: their importance and future challenges. Nat. Prod. Rep., 28: 1208-1228.

Li N, Ni J (2011). Treatment of wastewater from *Dioscorea zingiberensis* tubes used for producing steroid hormones in a microbial fuel cell. Bioresource Technol., 102: 2731-2735.

Li P, Mou Y, Shan T, Xu J, Li Y, Lu S, Zhou L (2011). Effects of polysaccharide elicitors from endophytic *Fusarium oxysporium* Dzf17 on growth and diosgenin production in cell suspension culture of *Dioscorea zingiberensis*. Molecules, 16: 9003-9016.

Lianhe Z, Li W, Xing H, Zhengxing C (2011). Antioxidant activities of seed extracts from *Dalbergia odorifera*. Afr. J. Biotechnol., 10: 11658-11667.

Liu F, Ooi VEC, Chang ST (1997). Free radical scavenging activities of mushroom polysaccharide extracts. Life Sci., 60: 763-771.

Muller L, Frohlich K, Bohm V (2011). Comparative antioxidant activities of carotenoids measured by ferric reducing antioxidant power (FRAP), ABTS bleaching assay (αTEAC), DPPH assay and peroxyl radical scavenging assay. Food Chem., 129: 139-148.

Ono M, Oda E, Tanaka T, Lida Y, Yamasaki T, Masuoka C, Ikeda T, Nohara T (2008). DPPH radical-scavenging effect on some constituents from the aerial parts of *Lippia triphylla*. J. Nat. Med., 62: 101-106.

Ooi VEC, Liu F (1999). A review of pharmacological activities of mushroom polysaccharides. Int. J. Med. Mushrooms, 1: 195-206.

Paixao N, Perestrelo R, Marques JC, Camara JS (2007). Relationship between antioxidant capacity and total phenolic content of red, rose and white wines. Food Chem., 105: 204-214.

Qiao D, Ke C, Hu B, Luo J, Ye H, Sun Y, Yan X, Zeng X (2009). Antioxidant activities of polysaccharides from *Hyriopsis cumingii*. Carbohyd. Polym., 78: 199-204.

Sakuma M (1988). Probit analysis of preference data. Appl. Entomol. Zool., 33: 339-347.

Verma VC, Kharmar RN, Strobel GA (2009). Chemical and functional diversity of natural products from plant associated endophytic fungi. Nat. Prod. Commun., 4: 1511-1532.

Wang J, Zhao J, Liu H, Zhou L, Liu Z, Wang J, Han J, Yu Z, Yang F (2010). Chemical analysis and biological activity of the essential oils of two Valerianaceous species from China: *Nardostachys chinensis* and *Valeriana officinalis*. Molecules, 15: 6411-6422.

Wang Z, Luo D, Ena C (2007). Optimization of polysaccharides extraction from *Gynostemma pentaphyllum* Makino using uniform design. Carbohyd. Polym., 69: 311-317.

Wilson D (1995). Endophyte – the evolution of a term, and clarification of its use and definition. Oikos, 73: 274-276.

Xiao J, Xiao D, Sun Z, Xiao Y, Zhong J (2011). Antioxidative potential of polysaccharide fractions produced from traditional Chinese medicinal macrofungus *Cordyceps jiangxiensis* in vitro. Afr. J. Biotechnol., 10: 6607-6615.

Yamaguchi F, Ariga T, Yoshimira Y, Nakazawa H (2000). Antioxidant and anti-glycation of carcinol from *Garcinia indica* fruit rind. J. Agric. Food Chem., 48: 180-185.

Yu HS, Zhang L, Li L, Zheng CJ, Guo L, Li WC, Sun PX, Qin LP (2010). Recent developments and future prospects of antimicrobial metabolites produced by endophytes. Mycrobiol. Res., 165: 437-449.

Zhang HW, Song YC, Tan RX (2006). Biology and chemistry of endophytes. Nat. Prod. Rep., 23: 753-771.

Zhang R, Li P, Xu L, Chen Y, Sui P, Zhou L, Li J (2009). Enhancement of diosgenin production in *Dioscorea zingiberensis* cell culture by oligosaccharide elicitor from its endophytic fungus *Fusarium oxysporum* Dzf17. Nat. Prod. Commun., 4: 1459-1462.

Zhao J, Shan T, Mou Y, Zhou L (2011). Plant-derived bioactive compounds produced by endophytic fungi. Mini-Rev. Med. Chem., 11: 159-168.

Zhong J-J, Xiao J-H (2009). Secondary metabolites from higher fungi: discovery, bioactivity, and bioproduction. Adv. Biochem. Eng. Biotechnol., 113: 79-150.

Zhou L, Zhou J, Shan T, Cai X, Peng Y (2010). Spirobisnaphthalenes from fungi and their biological activities. Mini-Rev. Med. Chem., 10: 977-989.

African Journal of Microbiology Research Vol. 5(32), pp. 5994-5997, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2012 Academic Journals
DOI: 10.5897/AJMR11.1426

Full Length Research Paper

Production of calcium gluconate from cassava by *Penicillium citrinum* SCG-112

Hai-Yan Sun[1,2], Pingjuan Zhao[1,2], Juanhua Li[1], Enshi Liu[1] and Ming Peng[1,2]*

[1]Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Key Laboratory of Tropical Crop Biotechnology, Ministry of Agriculture, Haikou 571101, China.
[2]School of Agriculture, Hainan University, Haikou 571101, China.

Accepted 14 December, 2011

The feasibility of using cassava powder as the main material for production of calcium gluconate by *Penicillium citrinum* SCG-112 was evaluated in this study. The effect of incubation temperature, initial pH of the medium and inoculum size on production of calcium gluconate was investigated. The maximum yield of calcium gluconate (155 g/L) was obtained after 36 h incubation. The result was both technically competitive and economically attractive.

Key words: Calcium gluconate, cassava, *Penicillium citrinum*.

INTRODUCTION

Calcium gluconate ($C_{12}H_{22}CaO_{14}\cdot H_2O$) finds extensive applications in the pharmaceutical and food industry. Research is ongoing to increase the production of this salt to meet its commercial demand (Bayraktar and Mehmetoglu, 2001). Most studies on calcium gluconate production have focused on the use of pure or easily fermentable substrates such as glucose or sucrose (Mariam et al., 2010; Liang et al., 2010). Due to the high costs of these pure materials, the process is less economic for industrial applications. The production cost of calcium gluconate might be significantly reduced if cheap raw materials could be used, such as starchy and cellulosic materials. Cassava is one of the most efficient crops in terms of carbohydrate production. It is a tropical perennial plant that grows on poor or depleted soils in which the yields of other crops are very low (Peters, 2007). Cassava is very rich in starch. Starch content of cassava root and dry cassava powder reached about 30 and 70%, respectively (Shen et al., 2009). Therefore, cassava has been successfully used as the main material for production of ethanol, lactic acid and sugar etc. (Shanavas et al., 2011; Kostinek et al., 2007; Abdul et al., 2005; Gaouar et al., 1998). Production of calcium gluconate from cassava is a promising strategy, for it will decrease the production cost. However, till now there is still no report regarding adoption of cassava as the substrate for production of calcium gluconate.

In the present study, the starch in cassava was enzymatically hydrolyzed (liquefaction with α-amylase and saccharification with glucoamylase) into glucose, which acted as the main material for production of calcium gluconate by a high-producing calcium gluconate strain, *Penicillium citrinum* SCG-112.

MATERIALS AND METHODS

Strain

P. citrinum SCG-112, a newly isolated calcium gluconate producer, was used in this study for its high yield of calcium gluconate. It was maintained on slants of potato dextrose agar and subcultured every month. The conidial suspension with the spores concentration of 10^8/ml was prepared form 3-4 day old slant of the strain.

Medium and fermentation

Cassava powder with the starch content of about 70% was purchased from local market in China. All other material and

*Corresponding author. E-mail: hysun168@126.com. Tel: +86-898-66963161. Fax: +86-898-66890978.

Table 1. Effect of temperature on production of calcium gluconate from cassava by *Penicillium citrinum* SCG-112.

Temperature (℃)	Maximum yield of calcium gluconate (g/L)	Time consumed to reach the maximum yield (h)
30	100±1.2	64
31	105±1.3	64
32	106±2.0	60
33	108±0.8	60
34	108±0.6	56
35	106±1.5	48
36	108±1.1	44
37	110±0.7	40
38	91±3.4	40
39	82±2.0	36
40	65±1.2	36

chemicals were also commercially available. Cassava powder was mixed with water to prepared cassava slurry with the concentration of 300 g/L. Thermo-stable α-amylase was added into the slurry according to 15 U per g cassava powder. Then the slurry was liquefied by heating it to 110℃ and keeping at 110℃ for 5 min, then cooling it to 90℃ and keep at 90℃ for 2 h. The liquefied cassava was centrifugated and squeezed to remove cassava residues. The pH of obtained clarifying solution was adjusted to 4.6-4.8, added gulucoamylase at the ratio of 200 U per g cassava powder. After saccharification at 60℃ for 24 h, the starch in cassava powder was converted to glucose thoroughly. The final glucose solution diluted to the concentration of 150 g/L with water was used for preparing medium for production of calcium gluconate. The basal medium containing (g/L): glucose 150, $(NH_4)_2SO_4$ 0.5, K_2HPO_4 0.05, $CaCO_3$ 42, natural pH (about pH 5.0). 50 ml medium in 500-ml flasks were autoclaved at 121°C for 15 min. One flask was inoculated with 1 ml spore suspension (10^8 spores/ml) and incubated on a rotary shake with the speed of 250 rpm. When the glucose in the medium reached less than 1 g/L, fermentation was terminated. During the process the sample was withdrawn at regular intervals to determine calcium gluconate yield and glucose concentration. The optimal levels of incubation temperature, initial pH of the medium, inoculum size were determined by varying them in the basal medium.

Analytical methods

Calcium gluconate present in the supernatant sample was determined by disodium ethylene diamine tetra acetic acid (dEDTA) titration. The glucose in the medium was measured by SBA-80C biosensor analyzer (Institute of Biology, Shandong Academy of Sciences, China), which could provide quick measurements of glucose based on technology of the immobilized oxidases. All the experiments were run parallel in a set of triplicates. All values given are means of three determinations ± standard deviation.

RESULTS AND DISCUSSION

Effect of incubation temperature on production of calcium gluconate from cassava by *P. citrinum* SCG-112

As shown in Table 1, 37℃ proved to be the best temperature for calcium gluconate in the present study. Incubation at lower temperature resulted in longer time to reach the maximum yield, though the maximum yield was near to the yield at 37℃. Meantime, temperature higher 37℃ is not conducive to the production of calcium gluconate, neither. The possible reason for the observation is that higher temperature affected the fungus harmfully, and then decreased the calcium gluconate production.

Effect of initial pH on production of calcium gluconate from cassava by *P. citrinum* SCG-112

Table 2 indicated that the optimum pH of calcium gluconate production from cassava by *P. citrinum* was pH 6.5. pH less or more than 6.5 both decreased calcium gluconate synthesis. The possible reason may be that at pH 6.5, the strain grown best and its mycelia produced maximal enzyme glucose oxidase, then brought highest yield of calcium gluconate. These results are in agreement with the result reported previously by Sheu et al. (2002), Munk and Hanus (2005) and Mariam et al. (2010).

Effect of inoculum size on production of calcium gluconate from cassava by *P. citrinum* SCG-112

The inoculum size also plays a significant role in the fermentation process. As shown in Table 3, maximum yield was obtained when the inoculum size was 2 ml spore suspension (with the count of 10^8/ ml) per flask. A lower level of inoculum size may not be sufficient for initiating growth and enzyme synthesis. An increase in inoculum size ensures a rapid proliferation of biomass and enzyme synthesis. After a certain limit, production could decrease because of depletion of nutrients due to the enhanced biomass, which would result in a decrease

Table 2. Effect of initial pH on production of calcium gluconate from cassava by *Penicillium citrinum* SCG-112.

Initial pH	Maximum yield of calcium gluconate (g/L)	Time consumed to reach the maximum yield (h)
4.0	60±0.3	48
4.5	86±1.5	44
5.0	94±1.1	44
5.5	110±3.1	40
6.0	120±2.0	40
6.5	133±1.3	40
7.0	121±1.2	40
7.5	112±0.9	44
8.0	105±1.0	44

Table 3. Effect of inoculum size on production of calcium gluconate from cassava by *Penicillium citrinum* SCG-112.

Inoculum size (10^8 spores/mL)	Maximum yield of calcium gluconate (g/L)	Time consumed to reach the maximum yield (h)
0.5	126±0.4	60
1.0	133±0.7	40
1.5	141±1.9	40
2.0	155±2.3	36
2.5	132±1.4	36
3.0	128±1.0	32
3.5	113±0.3	32
4.0	102±1.3	32
4.5	100±0.2	32
5.0	96±0.9	32

in metabolic activity (Kashyap et al., 2002). A balance between the proliferating biomass and available substrate material would yield maximum enzyme.

Conclusion

Based on optimization, the fermentation conditions for production of calcium gluconate from cassava by *P. citrinum* SCG-112 and the maximum yield of calcium gluconate (155 g/L) was obtained after 36 h. This result is significantly competitive compared with the recent relevant report, in which the maximum yield was 110.35 g/L after 72 h incubation. The other advantage of our study is: we used a kind of relatively cheaper material (cassava) while other researchers adopted expensive pure chemicals (such as glucose). Therefore, the study not only brings technical advantage, but also it is economically attractive.

ACKNOWLEDGEMENTS

This research was supported by Chinese 973 Project (no. 2010CB126600), National natural science fund (no. 31000029), the Institute Fund of Institute of Tropical Bioscience and Biotechnology in Chinese Academy of Tropical Agricultural Sciences (no. ITBBKF1010, no. ITBBZD0951 and ITTBB110103), Hainan introduction and composition special program (no. YJJC2011004 and 2011 Hainan Province graduate incretion program (no. Hyb2011-4).

REFERENCES

Abdul G, Shingo O, Takao K (2005). Production of L-Lactic Acid from Fresh Cassava Roots Slurried with Tofu Liquid Waste by *Streptococcus bovis*. J. Biosci. Bioeng., 100(6):606–612.

Bayraktar E, Mehmetoglu U (2001). Back mixing and liquid hold-up in a concurrent up-flow packed bed bioreactor. Chem. Eng. Commun., 185: 125–140.

Gaouar O, Zakhia N, Aymard C, Rios GM (1998). Production of maltose syrup by bioconversion of cassava starch in an ultrafiltration reactor. Ind. Crop Prod., 7: 159–167.

Kostinek M, Specht I, Edward VA, Pinto C, Egounlety M, Sossa C, Mbugua S, Dortu C, Thonart P, Taljaard L, Mengu M, Franz CMAP, Holzapfel WH (2007). Characterisation and biochemical properties of predominant lactic acid bacteria from fermenting cassava for selection as starter cultures. Int. J. Food Microbiol., 114: 342–351.

Liang JJ, Li XY, Guan W, Pang CW, Mai ZM (2010). Study on mutagenesis of oxidase-producing *Aspergillus niger* and its fermentation condition of calcium gluconate production. Sci. Technol. Food Ind., 31(12):218-220.

Mariam I, Nagra SA, Haq I, Ali S (2010). Application of 2-factorial design on the enhanced production of calcium gluconate by a mutant strain of *Aspergillus niger*. Bioresour. Technol., 101:4075–4080.

Munk P, Hanus F (2005). Factors influencing glucose oxidase activity and gluconate production in submerged cultivation of *Aspergillus niger*. Folia Microbiol., 83:20–27.

Shanavas S, Padmaja G, Moorthy SN, Sajeev MS, Sheriff JT (2011). Process optimization for bioethanol production from cassava starch using novel eco-friendly enzymes. Biomass Bioenergy, 35:901-909.

Shen NK, Wang QY, Lu Y, Qin Y, Huang RB (2009). Study on the high-concentration mash and high-temperature fermentation of cassava. Liquor Making, 37(5):47-51

Sheu DC, Duan KJ, Cheng CY, Bi JL, Chen JY (2002). Continuous production of high-content fructo-oligosaccharides by a complex cell system. Biotechnol. Prog., 18:1282–1286.

Peters D (2007). Raw materials. Adv. Biochem. Eng. Biotechnol., 105: 1–30.

Related Journals: