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African Journal of Microbiology Research

Table of Contents: Volume 7 Number 41, October 11, 2013

ARTICLES

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Table of Contents: Volume 7 Number 41, October 11, 2013

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Table of Contents: Volume 7 Number 41, October 11, 2013

Review

Biocontrol of *Fusarium* wilt of banana: Key influence factors and strategies

Guo Gang[1,2,3#]**, Wang Bizun**[1,2#]**, Ma Weihong**[1,2]**, Li Xiaofen**[1,2]**, Yang Xiaolin**[1,4]**, Zhu Chaohua**[4]**, Ming Jianhong**[1,2]*** and Zeng Huicai**[1,2,3*]

[1]Haikou Experimental Station/Hainan Key Laboratory of Banana Genetic Improvement, Chinese Academy of Tropical Agricultural Science (CATAS), Haikou 570102, Hainan, China.
[2]Banana Research and Development Department, National Center of Important Tropical Crops Engineering and Technology Research, Haikou 570102, Hainan, China.
[3]Institute of Tropical Bioscience and Biotechnology, CATAS, Haikou 571101, Hainan, China.
[4]College of Environment and Plant Protection, Hainan University, Haikou 570228, Hainan, China.

Fusarium wilt of banana, caused by *Fusarium oxyspoum* f. sp. *cubense* (Foc), is one of the most important and destructive diseases of banana, and is known to be a major biotic limiting factor for the development of the present banana industry. Biocontrol on the destructive disease, the use of antagonist as biocontrol agents (BCAs) against Foc, constitutes an effective option for the management of the disease. The effectiveness of biocontrol agents depends on a range of biological and physico-chemical factors, including the type and properties of the biocontrol agents, the obstacles to the initial colonization of antagonists, as well as the variation factors after initial colonization. Various strategies can be implemented to optimize the biocontrol efficacy, such as the use of endophytes from banana plants as BCAs (favorably *Bacillus* spp.), the development of water and nutrition retaining agent, the application of proper carrier for BCAs, the restoration of soil biodiversity, and combined management of nematodes disease and *Fusarium* wilt. In this review, elements affecting the biocontrol efficacy of Fusarium wilt are analyzed in detail, and strategies to promote the biocontrol effects are proposed. Besides, the concept of "post-indigenousness" and "post-indigenous microbes" were firstly suggested.

Key words: *Fusarium* wilt of banana, biocontrol, *Fusarium oxysporum* f. sp. *cubense* (E.F. Smith) Snyder & Hansen, *Bacillus* sp., endophtic bacteria.

INTRODUCTION

Fusarium wilt of banana, also called Panama disease, is caused by *Fusarium oxysporum* f. sp. *cubense* (E.F. Smith) Snyder & Hansen, shortly as Foc (Stover, 1962). *Fusarium* wilt is known to be one of the most important and destructive diseases of banana, which is especially serious in the Central and South Americas, parts of Africa (Viljoen, 2002), Sri Lanka, Burma, Thailand, Indonesia and the Philippines, resulting in heavy enormous economic losses each year (Ploetz, 1994; Stover et al., 1987). In the recent ten years, banana plantation areas in China, influenced by Fusarium wilt, have decreased dramatically, especially in Guandong, Hainan, Fujian provinces.

*Correspondence authors. E-mail: jhming_326@163.com, zhc081@126.com. Tel: 86-898-66754695, 86-898-66707697.

#Authors contributed equally to this work.

The fungus infects banana plants through the roots and invades the vascular tissue (xylem), causing external symptoms like gradual wilting, progressive yellowing of banana leaves (spreads from leaf margins, and from older leaves to younger leaves), eventual collapse at the petiole, and longitudinal splitting of the outer leaf sheaths in the pseudostem (Yin et al., 2011). The distinguishing internal symptoms of the disease is the typical discoloration of vascular tissues (in roots, corm, pseudostem, fruit stalk) varying from light yellow to dark brown, which appears first in the outer or oldest leaf sheath, then extends up to the pseudostem (Ploetz, 2006). Eventually, the disease leads to the death of banana plants.

The pathogenic isolates of Foc have been traditionally grouped into four physiological races based on patho-genicity to host cultivars under field condition (Fourie et al., 2009). *F.* oxysporum f. sp. cubense race 1 (Foc R1) attacks 'Gros Michel', 'Lady Finger' (AAB) and 'Silk' (AAB) varieties. Foc R2 infects cultivar 'Bluggoe' (ABB), while Foc R3 infects Heliconia spp. (a close relative of banana). And Foc R4 is able to attack cultivar Cavendish and all cultivars that are susceptible to Foc1 and Foc2 (Persley, 1987; Ploetz, 1990).

Banana production was once severely threatened by *Fusarium* wilt caused by Foc R1. The disease almost devastated the 'Gros Michel'-based banana industry in the Central American and Caribbean during the mid-1900s (Ploetz, 1994). This adversity persisted until the introduction of Cavendish cultivars, which are unsus-ceptible to Foc R1 and Foc R2 in tropical banana-growing regions (Fernández-Falcón et al., 2003). However, the prosperity did not last long. The Cavendish cultivars had then been found highly susceptible to a new race of Foc, Foc R4 (Ploetz and Pegg, 2000). Economic losses world-wide are enormous. It brings to the spiteful reality that the banana industry is once again threatened by *Fusarium* wilt.

Fusarium wilt is destructive because it seriously ham-pers banana production and is difficult to manage. *F. oxysporum* are able to infect more than 100 plants, and are divided into more than 120 host-specific forms, known as formae speciales (Minerdi et al., 2008). *Foc*, generally considered to be one of the most destructive formae speciales of *F. oxysporum* (Ploetz, 1990), is soil-borne and has strong saprophytic ability. The pathogen can survive in the soil for several decades by producing spores (specifically, chlamydospores), which will re-infect the susceptible banana plants (Stover, 1962). This adds to the difficulty of disease management.

Till date, results from disease management studies have been disappointing. No effective and efficient control strategies available can satisfy the needs for the manage-ment of disease worldwide. Although, Huang et al. (2012) reported that Chinese leek-banana rotation is an efficient way for controlling banana *Fusarium* wilt, the method is not economical because Chinese leek is in low need in the market. Beside, crop rotation tends to have limited effec-

tiveness once a disease outbreak occurs because of its soil-born nature and strong vitality (Fravel et al., 2003). Soil disinfection using chemicals or pesticides are not recommended due to environmental and human health concerns. And fumigated soils with methyl bromide can be re-infected in two or three years in fields with susceptible cultivars (Stover, 1958). Developing resistant banana cultivars may solve this problem but such efforts are progressing slowly (Stover and Buddenhagen, 1986; Hwang and Ko, 2004). Considering the urgency of the disease, a complementary approach for managing *Fusarium* wilt involves the biological control (or biocontrol), an important component of integrated disease management programs, specifically, in this case, is to use naturally occurring antagonists and active substances (viruses, bacteria, fungi, active substances of natural origin) as biocontrol agents (BCAs) for disease management.

When compared with other approaches, the use of BCAs is proved to be an ecologically safe strategy for disease management. However, the technology is still in its infancy. Enormous efforts should be still contributed to the development of effective and safe BCAs for comer-cial application. This review would focus on the elements affecting the biocontrol efficacy of *Fusarium* wilt, the points needed to be considered in the development of BCAs, and the corresponding strategies to improve the biocontrol efficacy, aiming to provide guidance for the biocontrol of *Fusarium* wilt and point the way forward for enhanced utilization of BCAs.

ELEMENTS AFFECTING THE BIOCONTROL OF FUSARIUM WILT AND CORRESPONDING STRATEGIES TO IMPROVE THE BIOCONTROL EFFICACY

It is a systematic work to use biological agents to control banana *Fusarium* wilt. In order to achieve ideal results, we should at least take into account the following elements: the type and properties of the biocontrol agents, the obstacles to the initial colonization of antagonists, as well as the variation factors after initial colonization.

The type and properties of the biocontrol agents

The first step to achieve effective biocontrol is to find the suitable potential biocontrol agents (BCAs). A primary consideration in the selection of antagonists as BCAs for field application is their type and properties, which directly or indirectly affect the biocontrol efficacy, the production process, the post-processing, as well as the storage and transportation. Production methods for biocontrol agents must be at low cost and yield viable, highly effective propagules of high concentrations. Also, these propagules, such as spores, must be amenable to long-term storage as dry preparations (Jackson, 1997).

Previous studies and experiences implied that *Bacillus*

species serves as ideal candidates for viable BCAs (Yilmaz et al., 2005; Bertagnolli et al., 1996; Szczech and Shoda, 2004; Farhana et al., 2011; Govindasamy et al., 2011; Tan et al., 2013). *Bacillus* spp. strains are advantageous as BCAs because they are tolerant to adverse environmental stresses by producing endospores, such as heat and desiccation (Schallmey et al., 2004). At present, a variety of strains of *Bacillus* spp. have been extensively applied as BCAs against soil-borne plant diseases (Gurr et al., 2005), including Rhizoctonia (Yu et al., 2002) and *Fusarium* (Schisler er al., 2002; Sun et al., 2011), which have been proven to bring about high biocontrol efficacy. In addition, using *Bacillus* spp. strains as BCAs, the post-processing costs are easy to control, and the storage and transportation conditions needed could be easily fulfilled (Schallmey et al., 2004). On the other hand, the application of other microorganisms as BCAs, such as nonpathogenic *F. oxysporum* (Nel and Steinberg, 2006), *Trichoderma* spp. (Thangavelu et al., 2004), has been demonstrated to result in high cost of production, storage and transportation.

Obstacles to the initial colonization of antagonists

The second key point needed to be considered is the colonization of antagonistic microbes. The biocontrol efficacy of the BCAs largely depends on the ability of the antagonistic microbes to colonize the plant root and the rhizosphere, and to produce substances which inhibit pathogens. The colonization of antagonistic microbes is hampered by several natural barriers. Obstacles to the initial colonization refers to the first set of natural barriers encountered by the antagonistic microbes after the application of BCAs, including the predation and phagocytosis from soil protozoa (Ekelund et al., 2001; Ronn et al., 2002), inhibition from the exudates of indigenous microbes (Bolwerk et al., 2003) or plant roots (Chao et al., 1986), the competition with indigenous microbes for ecological sites, nutrients and energy.

Normally, influenced by these barriers, the population of most antagonistic microbes reduced drastically in the first 2 to 3 days after the application of the BCAs (Christoffersen et al., 1995). However, BCAs usually must maintain a certain population of the antagonistic microbes to obtain acceptable levels of disease suppression (Wang et al., 2011). Thus, in order to achieve satisfactory biocontrol efficacy, appropriate measures should be taken to help the antagonistic microbes get through this hard time. One of the alternatives is to increase the original population of the antagonistic microbes in the BCAs, ensuring that a considerable part of the population survive this adverse phase under the regulation of environmental factors. Another recommendation is to apply BCAs repeatedly to maintain certain levels of the antagonistic microbes. Repeated applications of BCAs can be achieved by distributing the BCAs through the irrigation water.

Predation and phagocytosis from soil protozoa

Predatory protozoa in the soil to some extent act as the regulator of the population of soil bacteria and fungi (Bird et al., 2003; Ingham et al., 1985). They prey selectively on certain bacteria and fungi, thus exert an effect on the population and diversity of their prey (Burke et al., 2003). The antagonistic microbes in the BCAs, of course, are affected inevitably by this regulation. One proposed solution to counteract this regulation is to inoculate the BCAs directly into the leaves or stems of banana plant through drip injection, but not to apply them into the soil. Specifically, the approach means to inject the BCAs intermittently into the plant vessel from up to down under the law of gravity, and let them spread into the cell eventually. However, one leading problem of this method is the increased costs. Advanced techniques are needed to be developed in order to reduce the costs to an acceptable level for large-scale field applications.

Besides, studies have shown that the spatial hetero-geneity of soil may have something to do with the stability of the predator-prey relationship, since certain soil structures are beneficial for the survival of bacteria, for example, in the case that the spaces in the soil are suitable for bacteria to live in but are inaccessible to protozoa (Wardle, 2006). Soil aggregates are clumps of soil particles that are held together by moist clay, organic matter (like roots and fibrous roots), gums (from bacteria and fungi) and by fungal hyphae. Study revealed that well-aggregated soil is thought to be a very desirable habitat for bacteria (Wardle, 2006). Bacteria tend to accumulate inside soil aggregates because this environ-ment provides them with micro-ecological sites that are not accessible to certain protozoa, that is to say, bacteria in this environment are less likely to be eaten by soil animals like protozoa and mites. Such knowledge gives us some clues on the application of BCAs- to make the carrier of BCAs into the structure of soil aggregates. This approach can to some extent protect the antagonistic microbes from the prey of indigenous predatory protozoa, thus conserving a substantial number of the microbes as a result. One recommendation currently, is to apply resin-coated controlled-release materials as the carrier of BCAs (Ko et al., 1996; Lunt, 1968).

Inhibition from the exudates of indigenous microbes or plant roots

A substantial amount of studies have reported that certain exudates of indigenous microbes and plant roots exert an inhibitory effect on heterologous microorganisms (Quintana et al., 2009; Bais et al., 2006; Hirsch et al., 2003). In addition, plants tend to develop an induced systemic resistance to heterologous microorganisms (Pieterse et al., 1998). These effects exert strong negative influences on the colonization of the heterologous antagonistic microbes in banana plants. To overcome these difficulties,

a proposal is to screen active antagonistic microbes from the endophytic strains of healthy banana plants (Lian et al., 2008). The use of endophytes as antagonist is of great interest because of their ability to colonize plant tissue and produce antibiotics *in situ* (Sessitsch et al., 2004).

Of course, the endophytes isolated from a certain plant tend to be plant tissue and variety specific. That is to say, the endophytes from one cultivar might not be endophytic to another cultivar due to genetic diversity, which may stand in the way of the colonization process. In fact, the variety of banana cultivars in China is relatively uniform geographically, mostly Brazil and Williams, both belonging to *Musa* AAA group. Normally, it would not be difficult for the endophytes to colonize the varieties from the same group. Our recent studies have preliminarily confirmed the feasibility of endophytes (Wang et al., 2011).

When the BCAs have to be applied into soil, to overcome the inhibition from indigenous microbes and plant roots, or the "induced systemic resistance" mentioned above, a recommended option is to introduce a cover for the antagonistic microbes, which shelters the microbes from antimicrobial agents and environmental stress by acting as a physical barrier. For example, to entrap the biocontrol agents in capsule-like materials (Candela and Fouet, 2006) or coated and controlled-release materials mentioned above. The advantages of entrapped BCAs include: a controlled release of agent (controlled by the environment and the properties of the entrapment materials); easy handing and prolonged shelf life; protection against extreme environmental conditions.

The competition with indigenous microbes for ecological sites, nutrients and energy

The colonization of the antagonists is also impacted tremendously by indigenous microbes through competition for ecological sites, nutrients and energy (Hyakumachi, 2000). The competition, creating a nutrient-limiting environment, determines how different microbial populations coexist in the same ecosystem. Competition for essential resources is a factor that determines the survival of all organisms (Hyakumachi, 2000). The biocontrol fails if the antagonistic organisms are out-competed by other indigenous microbes in the soil for a limited essential resource. Thus, the antagonists must have the ability to use energy substrates and nutrients at low concentrations. Studies revealed that strains of *Pseudomonas fluorescens* and *Trichoderma harzianum* showed antagnostic effect on Foc. (Rujappan et al., 2002; Saravanan et al., 2003).

Obstacles to the long-term colonization of antagonists: The variation factors after initial colonization

The variation factors after initial colonization means the factors that affect the long-term colonization of antagonists. *Fusarium* wilt is a typical soil-borne disease. To achieve ideal biocontrol efficacy, the antagonists must colonize the rhizosphere soil and sustainedly occupy favorable ecological sites in the environment. Previous studies have illustrated that the long-term colonization of antagonists is correlated to soil moisture (or the water content of soil), temperature, soil aeration, pH, soil salinity, as well as the continuous supply of nutrients (Sessitsch et al., 2004; Bashan et al., 1991; Bevivino et al., 2005; Cavaglieri et al., 2005; de-Bashan and Bashan, 2008).

According to latest studies, rhizosphere bacteria exist not in the traditional recognized status (planktonic cells), but in biofilms (Webb et al., 2003). A biofilm is "a structured community of microorganisms encapsulated within a self-developed polymeric matrix and adherent to a living or inert surface" (http://en.wikipedia.org, 20090205), formed by an aggregate of microorganisms in which cells adhere to each other and/or to a surface. In nature, biofilms constitute a protected growth modality allowing bacteria to survive in hostile environments (Rinaudi and Giordano, 2010). In this case, we believe biofilm is the best mode for antagonists to occupy heterogeneous habitats, acting as a physical barrier sheltering the antagonists from antimicrobial agents and environmental stress. Thus, favorable conditions should be provided to facilitate the formation of biofilm in practice. As indicated by Chang and Halverson (2003), the formation of rhizosphere bacterial biofilm is mainly affected by water moisture, nutritional status and environmental parameters.

Soil moisture

One of the most significant requirements for biofilm formation is sufficient moisture. In banana plantations, soil moisture affects the structure of soil aggregates, and is significantly related to soil aeration, temperature, entropy and pH. Banana plants need plenty of water and fertilizer to grow (Goenaga and Irizarry, 1998), due to their large stems and leaves, shallow roots, rapid growth and high yield. They do best in areas of high humidity (50% or more) and require regular applications of fertilizer. The growing periods and yield of banana plants depend largely on the rational management of fertilizer and water. In China, areas under banana cultivation are mostly dry slopes, platforms, plateaus, or plains in short of water. Irrigation in these areas is normally not convenient (Xia, 2011). The large-scale banana plantations are mostly watered through well irrigation, ditches-guided irrigation, showering irrigation via perforated pipe, or microjet irrigation (Chen, 2001). These irrigation means, although solve the water shortage problem to some extent, are far from enough to satisfy the sustained demand of banana plants for continuous water supply, especially in the vegetative stage, flower bud differentiation stage, flowering and fruit ripening stages. Besides, the irrigation means mentioned above are all based on high costs, which tend to benefit the large-scale plantations owners more and are relatively uneconomical to the smallholders.

Banana plant is also vulnerable to water logging, and the

soil aeration condition directly determines the growth status of its root system (Goenaga and Irizarry, 1998). Waterlogging reduces yield and plant size, restricts root growth, causes shallow root systems, and stops the active uptake of nutrients (Aguilar et al., 2008). In a nutshell, banana plant is vulnerable to both blood (waterlogging) and drought. In this dilemma, most plantations have difficulty in maintaining a balanced water supply through-out the whole growing season, which inevitably results in the intermittent shortage of water. This problem will, undoubtedly, lead to changes in soil microbial commu-nities, mostly, dramatic decrease in the population of soil microbes (which are irreversible for at least one growing season). This is the fundamental reason why most BCAs do not have a sustained effect in practice, according to our continued investigation in the recent 4 years (Guo et al., unpublished data).

Sustained water supply in rhizosphere soil leads to favorable rhizosphere environments for the biofilms formation of antagonists. Through biofilms, the population of antagonists in the rhizosphere can maintain a balance for quite a long time, which in turn prolongs the persis-tence of the colonization of the of antagonists. In conclu-sion, from our point of view, the population of antagonists is mostly dependent on the stability of sustained water supply in soil. In standard large-scale plantations, this demand can be satisfied through microjet irrigation and drip irrigation facilities. However, in small plantations, where it is not profitable and feasible to set up these facilities, a recommended option is to apply water retain-ing agents in rhizosphere soil.

There are two types of water retaining agents nowadays, one is chemical and the other is bioactive. Poly-γ-glutamic acid (Zeng et al., 2013), in short γ-PGA, act as a new kind of bioactive water retaining agent in agricultural and environmental applications. It is a biodegradable, water- soluble amino acid polymer with a molecular weight ranging from about 10,000 up to 2 millions, generated by the microbial fermentation in nature (Zeng et al., 2013). It is normally composed of about 5,000 glutamic acids molecules or mono units, with a free carboxyl group on the α-carbon atom of each repeating unit (Do et al., 2011).

Because of the repulsion of the negatively charged carboxyl, the chain space is extended to be particularly large. The interaction between molecules is strong even when γ-PGA is in low concentrations, which makes it an ideal material for super absorbent (Candela and Fouet, 2006). Studies have revealed that γ-PGA can effectively absorb soil water, and can reach a maximum water absorption coefficient of 1108.4 (Tsujimoto et al., 2010). Due to its nature of economical costs, biodegradability and water absorption, γ-PGA, as a water retaining agent, can be used to change soil aggregates structure, maintain soil moisture, and conserve fertilizer in soil. In our study, we have found several γ-PGA-producing strains that are antagonistic against Foc (Guo et al., unpublished data)

under the application for a patent. Further studies are still under way to screen more efficient strains. Undoubtedly, the application of these bacteria as BCAs would bring more efficiency.

Nutritional status and environmental parameters

Nutritional status and several environmental parameters are also key factors that influence the formation of biofilm (Pan et al., 2010), which further affect the persistence of the colonization of the antagonists, including carbon source, amount of nitrate, phosphate, calcium and mag-nesium as well as the effects of osmolarity and pH (Rinaudi et al., 2006).

Availability of nutrition in the form of glucose results in increased biofilm formation of bacteria (Shera et al., 2006; Revdiwala et al., 2012). Increased levels of phosphate, Ca^{2+}, Mg^{2+} enhance biofilm formation, whereas osmotic agents, such as NaCl and sorbitol negatively affect biofilm formation through an osmotic effect as their concen-trations increase (Rinaudi et al., 2006). Besides, tempera-tures and pH are also factors that affect biofilm formation (Pettit et al., 2010), and the effects differ from one bacte-rial species to another. Taken together, the nutritional and environmental requirements for biofilm formation appear to be rather species specific. Thus a specific antagonist should be tested for the optimal nutritional conditions for the biofilm formation, according to which various sugars, osmotic agents or salts should be supplemented to the medium for cultivation to provide feasible conditions for biofilm formation.

Currently, most banana plantations are in the condition of imbalanced soil nutrition (such as redundant nutrition in rhizosphere) and improved salinity (due to years of continuous cultivation and preference in employ of N, P fertilizer) (Zhong et al., 2011), which stand in the way of biofilm formation. Inadaptability to these conditions would definitely result in the rapid decline in the population of the antagonists, thus weakening the biocontrol efficacy.

The development of time release technology, also known as sustained-release (SR), provides an alternative for this problem. The BCAs are recommended to be introduced into a controlled-release system with sustained release of nutrition, for example, the water retaining agent or the slow-release fertilizers. In this system, the nutrients are provided slowly and steadily for an extended duration, which not only protect the antagonists and facilitate the biofilm formation, but also put banana plants in the way they prefer to be fed and helps them grow well. Conside-ring the water shortage problem, it is desirable to develop a water and nutrition retaining agent to maintain both water and nutrition at the same time. In addition, as men-tioned above, high salinity exerts negative influence on the formation of biofilms through an osmotic effect. A recommended approach is to use halo-tolerant microbes from mangrove and marine source as antagonists against Foc pathogens (Xu and Dai, 2007).

OTHER FACTORS THAT HELP IMPROVE THE BIOCONTROL EFFICACY AGAINST *FUSARIUM* WILT OF BANANA

Pay attention to the evolutionary and geographic diversity of Foc

By comparing DNA sequences of nuclear and mitochondrial genes, O'Donnell et al. (1998) concluded that *Fusarium* wilt of banana is caused by pathogenic strains with independent evolutionary origins, and the Foc races isolated are geographically distinct. A recent field experiment conducted by us supports this conclusion as well, which found that a biocontrol agent proved to have excellent effect in a field in Ledong, Hainan (having a control effect of 99%) exhibited poor performance in the field just 17 km around (having a control effect of 23%). Preliminary analysis revealed that the colony morphology and virulence of Foc R4 in the two fields showed significant difference (Mo et al., 2013). Therefore, the evolutional and geographic diversity of the pathogen should be taken into consideration in the screening of antagonists against Foc. In the first screening round, it is recommended to use pathogens from various evolutionary origins and geographic areas as targets, in order to improve the biocontrol efficacy.

Take into account the integrated management of banana nematodes

Banana nematodes constitute another major threat to banana production all over the world, which cause yield losses of up to 30 to 60% in many countries (Roderick et al., 2012). The fundamental reason that leads to nematode disease and *Fusarium* wilt is basically in agreement with previous studies (Zhong et al., 2011; Palomares-Rius et al., 2011), both due to the loss of biodiversity in soil. Banana plants that are infected with nematodes tend to have enhanced susceptibility to *Fusarium* wilt, even in those lines showing *Fusarium* tolerance or resistance (Ammar, 2007). Thus, the two diseases could be controlled jointly, and combined management of the two diseases would save us lots of efforts and costs (Pararu et al., 2009). To improve biocontrol efficacy, BCAs against both disease are encouraged to be developed.

Consider addition of non-antagonistic microbes into the biocontrol agents

Microbes that do not have antagonistic effect to pathogens may act as potential biocontrol agents by occupying the ecological sites of the pathogen or competing with the pathogen for nutritious and infection sites (Nel et al., 2006). Therefore, it is recommended to add certain non-antagonistic microbes into the biocontrol agents. Non pathogenic *F. oxysporum* strains can be developed as biocontrol   agents. They are able to compete for nutrients in the soil, which affect the rate of chlamydospore germination of the pathogen (Kidane and Laing, 2010). They can also compete with the pathogens for infection sites on the root, and can trigger plant defense reactions, inducing systemic resistance. To give an example, Fo47, a non-pathogenic *F. oxysporum* strains, have been successfully applied as biocontrol agent in the field. Studies revealed that the general pattern of colonization in soil was similar for the pathogenic strain and Fo47. However, Fo47 grew faster than the pathogenic strain and, as a consequence, colonized the rhizosphere earlier (Michielse and Rep, 2009).

Considering using secondary metabolites for biocontrol agents

Some antagonistic microbes produce antagonistic substances only under the nutrient-poor conditions (Opelt and Berg, 2004). Based on our experience, this kind of microorganisms cannot act as viable biocontrol agents directly (Mo et al., 2013). A better choice is to take advantage of its secondary metabolites. If they are used as potential viable biocontrol agents, relatively poor culture conditions should be applied in the dual-culture assay during the second screening round, for example, cultured in the water agar. Only the microbes that exhibit antagonistic characteristics in the water agar should be chosen for the third screening round.

Avoid using actinomycetes as viable biocontrol agents

Actinomycetes is not recommended to be applied as viable biocontrol agents for managing *Fusarium* wilt of banana in Chinese banana plantations, because the favorable conditions needed for actinomycetes to survive is not desirable for the growth of banana roots (Jayasinghe and Parkinson, 2008). However, it is feasible to use the secondary metabolites of Actinomycetes as biocontrol agents.

Introducing limitation factors as many as possible in the screening of antagonists

In the second round of screening for potential BCAs, it is recommended to introduce limitation factors as many as possible, such as extreme pH and temperature. The antagonistic stains that are tolerant to a wide range of environmental conditions have greater market potential as biocontrol agents against Foc. In other words, the advantageous and competitive microbes have greater chance to colonize heterogeneous habitats and become "indigenous inhabitants" in this environment. The process for the microbes to become "indigenous inhabitants" in heterogeneous habitats is defined by us as "post-indigenousness",   and these microbes are designated as "post-indi-

genous microbes".

As we all know, the most typical feature of strains belonging to *Bacillus* family is that they can form spores to get through adverse environmental conditions (Vos et al., 2009), which enable them to survive in a board range of environmental conditions. The utility of strains of *Bacillus sp.* as biocontrol agents cannot only obtain sustained colonization in banana plants, but also facilitate the post-processing, storage and transportation. Therefore, antagonistic strains from *Bacillus* spp. are a recommended choice as biocontrol agents in field application.

Restoring the soil biodiversity in banana plantations to control *Fusarium* wilt indirectly

The most serious problem for banana plantations with years of continuous cultivation is the loss of soil bio diversity (Zhong et al., 2011). Especially, the loss of keystone species in the microbial communities would severely destroy the ecological balance of the environ ment (Walker, 1992), which offers favorable conditions for the accumulation of Foc pathogens. The toxins secreted by these pathogens first attack the roots of banana plants, and then spread to stem and leaves, eventually resulting in outbreak of *Fusarium* wilt (Kuo and Scheffer, 1964). The growth of banana plants is closely related to the bio-diversity in the rhizosphere. Under constant supply of normal nutrition, the roots of the banana plants are well developed. The exudates from the root hairs or root tip promote the growth of rhizosphere organisms (Bais et al., 2006), which in turn enhance the biodiversity of soil.

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REFERENCES

Aguilar EA, Santos PJA, Tamisin LL (2008). Root characteristics of five local banana cultivars (*Musa sp.*) under waterlogged condition. Phil. J. Crop Sci. 33(3):14-23.

Ammar MI (2007). Fusarium species associated with corm rots and wilt of banana (*Musa sp.*) under Egyptian conditions. Egypt. J. Phytopathol. 35(2):81-98.

Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. Ann. Rev. Plant Biol. 57:233-266.

Bashan Y, Levanony H, Whitmoyer RE (1991). Root surface colonization of non-cereal crop plants by pleomorphic *Azospirillum brasilense* Cd. J. Gen. Microbiol. 137:187-196.

Bertagnolli BL, Soglio FKD, Sinclair JB (1996). Extracellular enzyme profiles of fungal pathogen *Rhizoctonia solani* isolate 2B-12 and of two antagonists, *Bacillus megaterium* strain B153-2-2 and *Trichoderma harzianum* isolate Th008. I. Possible correlation with inhibition of growth and biocontrol. Physiol. Mol. Plant Pathol. 48:145-160.

Bevivino A, Peggion V, Chiarini L, Tabacchioni S, Cantale C, Dalmastri C (2005). Effect of *Fusarium* verticillioides on maize-root-associated *Burkholderia cenocepacia* populations. Res. Microbiol. 156: 974-983.

Bird DM, Oppermana CH, Davies KG (2003). Interactions between bacteria and plant-parasitic nematodes: now and then. Int J Parasitol. 33:1269-1276.

Bolwerk A, Lagopodi AL, Wijfjes AH, Lamers GE, Chin-A-Woeng TF, Lugtenberg BJ, Bloemberg GV (2003). Interactions in the tomato rhizosphere of two Pseudomonas biocontrol strains with the phyto pathogenic fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Mol. Plant Microbe In. 16(11): 983-893.

Burke RA, Molina M, Cox JE., Osher LJ, Piccolo MC (2003). Stable carbon isotope ratio and composition of microbial fatty acids in tropical soils. J. Environ. Qual. 32:198-206.

Candela T, Fouet A (2006). Poly-gamma-glutamate in bacteria. Mol. Microbiol. 60:1091-1098.

Cavaglieri L, Orlando J, Etcheverry M (2005). In vitro influence of bacterial mixtures on *Fusarium verticillioides* growth and fumonisin B1 production: effect of seeds treatment on maize root colonization. Lett. Appl. Microbiol. 41:390-396.

Chang WS, Halverson LJ (2003). Reduced water availability influences the dynamics, development, and ultrastructural properties of *Pseudomonas putida* biofilms. J. Bacteriol. 185:6199-6204.

Chao WL, Nelson EB, Harman GE, Hoch HC (1986). Colonization of the rhizosphere by biological control agents applied to seeds. Phytopathol. 76:60-65.

Chen HB (2001). Banana production and demand in China. In: Molina AB, Roa VN, Maghuyop MAG (eds.), Advancing banana and plantain R & D in Asia and the Pacific-Vol. 10, Proceedings of the 10th INIBAP-ASPNET Regional Advisory Committee meeting held at Bangkok, 2000/11/10-11, INIBAP-ASPNET, Los Baños, pp.92-93.

Christoffersen K, Ahl T, Nybroe O (1995). Grazing of nonindigenous bacteria by nano-sized protozoa in a natural coastal system. Microb. Ecol. 30:67-78.

de-Bashan LE, Bashan Y (2008). Joint immobilization of plant growth-promoting bacteria and green microalgae in alginate beads as an experimental model for studying plant-bacterium interactions. Appl. Environ. Microbiol. 74:6797-6802.

Do TH, Suzuki Y, Abe N, Kaneko J, Itoh Y, Kimura K (2011). Mutations suppressing the loss of DegQ function in Bacillus subtilis (natto) poly-γ-glutamate synthesis. Appl. Environ. Microbiol. 77(23): 8249-58.

Ekelund F, Rønn R, Christensen S (2001). Distribution with depth of protozoa, bacteria and fungi in soil profiles from three Danish forest sites. Soil Biol.Biochem. 33(4-5): 475-481.

Farhana MSN, Bivi MR, Khairulmazmi A (2011). Effect of carbon sources on bacterial production of metabolites against *Fusarium oxysporum* and *Colletotrichum gloeosporioides*. Int. J. Agric. Biol. 13: 1-8.

Fernández-Falcón M, Borges AA, Borges-Pérez A (2003). Induced resistance to *Fusarium* wilt of banana by exogenous applications of indoleacetic acid. Phytoprotection. 84:149-153.

Fourie G, Steenkamp ET, Gordon TR, Viljoen A (2009). Evolutionary relationships among the *Fusarium oxysporum* f. sp. *cubense* vegetative compatibility groups. Appl. Environ. Microbiol. 75: 4770-4781.

Fravel D, Olivain C, Alabouvette C (2003). *Fusarium oxysporum* and its biocontrol. New Phytol. 157: 493-502.

Goenaga R, Irizarry H (1998). Yield of banana grown with supplemental drip-irrigation on an Ultisol. Expl. Agric. 34:439-444.

Govindasamy V, Senthilkumar M, Magheshwaran V, Kumar U, Bose P, Sharma V, Annapurna K (2011). *Bacillus* and *Paenibacillus spp.*: Potential PGPR for Sustainable Agriculture. Microbiol. Monographs. 18: 333-364.

Gurr GM, Wratten SD, Kehrli P, Scarratt S (2005). Cultural manipulations to enhance biological control in Australia and New Zealand: progress and prospects. In: Mark, S.H., ed. "Proceedings of the second International Symposium on Biological Control of Arthropods", Davos, Switzerland, pp.154-166.

Hirsch AM, Bauer WD, Bird DM, Cullimore J, Tyler B, Yoder JI (2003). Molecular signals and receptors: controlling rhizosphere interactions between plants and other organisms. Ecology. 84: 858-868.

Huang YH, Wang RC, Li CH, Zuo CW, Wei YR, Zhang L, Yi GJ (2012). Control of Fusarium wilt in banana with Chinese leek. Eur. J. Plant Pathol. 134(1):87-95.

Hwang SC, Ko WH (2004). Cavendish banana cultivars resistant to *Fusarium* Wilt acquired through somaclonal variation in Taiwan. Plant Dis. 88: 580-588.

Hyakumachi M (2000). Studies on biological control of soilborne plant pathogens. J. Gen. Plant. Pathol., 66: 272-274.

Ingham RE, Trofymow JA, Ingham ER, Coleman DC (1985). Interactions of bacteria, fungi, and their nematode grazers: effects on nutrient cycling and plant growth. Ecol. Monogr. 55:119-140.

Jackson MA (1997). Optimizing nutritional conditions for the liquid culture production of effective fungal biological control agents. Journal of Industrial Microbiology & Biotechnology, 19:180-187.

Jayasinghe BATD, Parkinson D (2008). Actinomycetes as antagonists of litter decomposer fungi. Appl. Soil Ecol. 38:109-118.

Kidane E.G., Laing MD (2010). Integrated Control of Fusarium Wilt of Banana (Musa spp.). In: Dubois T (Eds), Proc. IC on Banana & Plantain in Africa, Acta Hort. 879, ISHS, pp. 315-321.

Ko BS, Cho YS, Rhee HK (1996). Controlled release urea from rosin-coated fertilizer particles. Ind. Eng. Chem. Res . 35(1):250-257.

Kuo MS, Scheffer RP (1964). Evaluation of fusaric acid as a factor in development of *Fusarium* wilt. Phytopathology. 54:1041-1044.

Lian J, Wang ZF, Zhou SN (2008). Response of endophytic bacterial communities in banana tissue culture plantlets to *Fusarium* wilt pathogen infection. J. Gen. Appl. Microbiol. 54:83-92.

Lunt OR (1968). Modified sulphur coated granular urea for controlled nutrient release. Tans. Ninth Int Congr. Soil Sci. 3:337-383.

Michielse CB, Rep M (2009). Pathogen profile update: *Fusarium oxysporum*. Mol. Plant Pathol. 10: 311-324.

Mo KL, Zeng T, Chen HQ, Guo G, Zeng HC (2013). Optimization of solid-state fermentation for two Streptomyces stains against banana Fusarium wilt and selection of its additive. Chinese Agricultural Science Bulletin 29:97-102 (in Chinese)

Minerdi D, Moretti M, Gilardi G, Barberio C, Gullino ML, Garibaldi A (2008). Bacterial ectosymbionts and virulence silencing in a *Fusarium oxysporum* strain. Environ. Microbiol. 10:1725-1741.

Nel B, Steinberg C, Labuschagne N, Viljoen A (2006). Isolation and characterization of nonpathogenic *Fusarium oxysporum* isolates from the rhizosphere of healthy banana plants. Plant Pathol. 55:207-216.

Nel B, Steinberg C, Labuschagne N, Viljoen A (2006). The potential of nonpathogenic *Fusarium oxysporum* and other biological control organisms for suppressing *Fusarium* wilt of banana. Plant Pathol. 55:217-223.

O'Donnell K, Kistler HC, Cigelnik E, Ploetz RC (1998). Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. Proc. Natl. Acad. Sci., USA. 95:2044-2049.

Opelt K, Berg G (2004). Diversity and antagonistic potential of bacteria associated with bryophytes from nutrient-poor habitats of the Baltic Sea Coast. Appl. Environ. Microbiol. 70(11):6569-6579.

Palomares-Rius JE, Castillo P, Navas-Cortés JA, Jiménez-Díaz RM, Tena M (2011). A proteomic study of in-root interactions between chickpea pathogens: the root-knot nematode Meloidogyne artiellia and the soil-borne fungus Fusarium oxysporum f. sp. ciceris race 5. J. Proteomics. 74(10): 2034-51.

Pan Y, Breidt F Jr, Gorski L (2010). Synergistic effects of sodium chloride, glucose, and temperature on biofilm formation by Listeria monocitogenes serotype 1/2a and 4b strains. Appl. Environ. Microbiol. 76(5): 1433-1441.

Pararu P, Dubois T, Coyne D, Viljoen A (2009). Dual inoculation of *Fusarium oxysporum* endophytes in banana: effect on plant colonization, growth and control of the root burrowing nematode and the banana weevil. Biocontrol Sci. Techn. 19:639-655.

Persley GJ (1986). Banana and Plantain Breeding Strategies. In: Persley, G.J., De Langhe, E.A., eds. Proceedings of an international workshop held at cairns, Australia. State Mutual Book and Periodical Service, Ltd. pp.13-17.

Pettit RK, Repp KK, Hazen KC (2010). Temperature affects the susceptibility of Cryptococcus neoformans biofilms to antifungal agents. Med. Mycol. 48(2) 421-426

Pieterse CM, van Wees SC, van Pelt JA, Knoester M, Laan R, Gerrits H,

Weisbeek PJ, van Loon LC, (1998). A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. Plant Cell 10:1571-1580.

Ploetz RC (1990). Population biology of *Fusarium oxysporum* f.sp. *cubense*. In: Ploetz, R.C., ed. *Fusarium* wilt of banana. St Paul: APS Press, pp. 63-67.

Ploetz RC (1994). Panama disease: return of the first banana menace. Int. J. Pest Manage. 40(4): 326-336.

Ploetz RC (2006). *Fusarium* Wilt of banana is caused by several pathogens referred to as *Fusarium oxysporum* f. sp. *Cubense*. Phytopathology 96(6):653-656.

Ploetz RC, Pegg KG (2000). Fungal disease of the root, corm, and pseudostem: *Fusarium* wilt. In: Jones DR, ed. Diseases of banana, abaca and enset. London: CAB International, pp. 143-157.

Quintana N, El Kassis EG, Stermitz FR, Vivanco JM (2009). Phytotoxic compounds from roots of *Centaurea diffusa* Lam. Plant Signal Behav. 4:9-14.

Revdiwala S, Rajdev BM, and Mulla S (2012). Characterization of bacterial etiologic agents of biofilm formation in medical devices in critical care setup. Crit. Care Res. Pract. doi:10.1155/2012/945805

Rinaudi L, Fujishige NA, Hirsch AM, Banchio E, Zorreguieta A, Giordano W (2006). Effects of nutritional and environmental conditions on *Sinorhizobium meliloti* biofilm formation. Res Microbiol. 157: 867-875.

Rinaudi LV, Giordano W (2010). An integrated view of biofilm formation in rhizobia. FEMS. Microbiol. Lett. 304(1): 1-11.

Roderick H, Mbiru E, Coyne D, Tripathi L, Atkinson HJ (2012). Quantitative digital imaging of banana growth suppression by plant parasitic nematodes. PLoS One. 7(12):e53355.

Ronn R, McCaig AE, Griffiths BS, Prosser JI (2002). Impact of protozoan grazing on bacterial community structure in soil microcosms. Appl. Environ. Microbiol. 68(12):6094-6105.

Rujappan K, Vidhyasekaran P, Sethuraman K, Baskaran TL (2002). Development of powder and capsule formulations of *Pseudomonas fluorescens* strain Pf-1 for control of banana wilt. Z. Pflanzenk. Pflanzen. (Journal of Plant Diseases and Protection) 109:80-87.

Saravanan T, Muthusamy M, Marimuthu T (2003). Development of integrated approach to manage the fusarial wilt of banana. Crop Prot. 22:1117-1123.

Schallmey M, Singh A, Ward OP (2004). Developments in the use of Bacillus species for industrial production. Can. J. Microbiol. 50:1-17.

Schisler DA, Khan NI, Boehm MJ, Slininger PJ (2002). Greenhouse and field evaluation of biological control of Fusarium Head Blight on Durum Wheat. Plant Dis. 86:1350-1356.

Sessitsch A, Reiter B, Berg G (2004). Endophytic bacterial communities of field-grown potato plants and their plant-growth-promoting and antagonistic abilities. Can. J. Microbiol. 50:239-249.

Shera J, Sriprakash KS, McMillan DJ (2006). The nutritional requirements for biofilm formation by Group A streptococcus. International Congress Series. 1289:139-142.

Stover RH (1958). Studies on Fusarium wilt of bananas: III. Influence of soil fungitoxins on behavior of *F. oxysporum* f. cubense in soil extracts and diffusates. Can. J. Bot. 36(4):439-453.

Stover RH (1962). Fusarial Wilt (Panama Disease) of Bananas and Other Musa Species. Kew, UK: Commonwealth Mycological Institute.

Stover RH, Buddenhagen IW (1986). Banana breeding: Polyploidy, disease resistance and productivity. Fruits 40:175-191.

Stover RH, Simmonds NW (1987). Bananas, 3rd ed. Longmans, London, United Kingdom, pp.29-30

Sun BJ, Peng M, Wang YG, Zhao J, Xia QY (2011). Isolation and characterization of antagonistic bacteria against *fusaium* wilt and induction of defense related enzymes in banana. Afr. J. Microbiol. Res. 5(5):509-515.

Szczech M, Shoda M (2004). Biocontrol of Rhizoctonia damping-off tomato by *Bacillus subtilis* combined with *Burkholderia cepacia*. J. Phytopathol. 152:549-556.

Tan S, Dong Y, Liao H, Huang J, Song S, Xu Y, Shen Q (2013). Antagonistic bacterium Bacillus amyloliquefaciens induces resistance and controls the bacterial wilt of tomato. Pest Manag. Sci. Jan. 21. doi: 10.1002/ps.3491

Thangavelu R, Palaniswami A, Velazhahan R (2004). Mass production of *Trichoderma harzianum* for managing *Fusarium* Wilt of banana. Agr. Ecosyst. Environ. 103:259-263.

Tsujimoto T, Kimura J, Takeuchi Y, Uyama H, Park C, Sung MH (2010). Chelation of calcium Ions by Poly (gamma-Glutamic Acid) from *Bacillus subtilis* (Chungkookjang). J. Microbiol. Biotechnol. 20: 1436-1439.

Viljoen A (2002). The status of Fusarium wilt (Panama disease) of banana in South Africa. S. Afr J. Sci. 98:1-4.

Vos P, Garrity G, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer KH, Whitman WB, Eds., (2009). Bergey's Manual of Systematic Bacteriology, The Firmicutes. New York, NY: Springer-Verlag, 3: 1-17.

Waite BH (1977). Inoculation studies and natural infection of banana varieties with races 1 and 2 of *Fusarium oxysporum* f. *cubense*. Plant Dis. Rep. 61:15-19.

Walker C (1992). Systematics and taxonomy of the arbuscular endomycorrhizal fungi (Glomales): a possible way forward. Agronomie. 12:887-897.

Wang T, Duan YJ, Liu BT, Guo G, Zhou DB, Tan X, Zhang XY, Huang MJ (2011). The Colonization of Two Strains of Antagonistic Bacteria of Fusarium oxysporum in Banana. Gen. Appl. Biol. 30(3):342-350 (in Chinese).

Wardle DA (2006). The influence of biotic interactions on soil biodiversity. Ecol. Lett. 9:870-886.

Webb JS, Givskov M, Kjelleberg S (2003). Bacterial biofilms prokaryotic adventures in muhicellularity. Curt. Opin. Microbioll. 6:578-585.

Xia YK (2011). The economic research of banana production technology in China. Dissertaion for Ph.D., Hainan University, Supervisor: Guo JC, Jin ZQ, pp. 35-36 (in Chinese).

Xu XL, Dai P (2007). Isolation and identification of two entophytic bacteria strains against *Fusarium oxysporum*. J. Fruit Sci. 24: 483-486. (in Chinese)

Yilmaz M, Soran H, Beyatli Y (2005). Antimicrobial activities of some *Bacillus spp.* strains isolated from the soil. Microbiol. Res. 161: 127-131.

Yin XM, Jin ZQ, Xu BY, Ma WH, Fu YG, Wang JB (2011). Characterization of early events in banana root infected with the GFP-tagged *Fusarium oxysporum* f. sp. *cubense*. Acta horticulturae, (897): 371-376.

Yu GY, Sinclair JB, Hartman GL, *et al* (2002). Production of iturinA by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani*. Soil Biol. Biochem. 34:955-963.

Zeng W, Lin Y, Qi Z, He Y, Wang D, Chen G, Liang Z (2013). An integrated high-throughput strategy for rapid screening of poly (γ-glutamic acid)-producing bacteria. Appl. Microbiol. Biotechnol. 97(5): 2163-2172.

Zhong S, He YD, Zeng HC, Mo YW, Jin ZQ (2011). Effects of banana wilt disease on soil nematode community structure and diversity. Afr. J. Biotechnol. 59(10):12759-12767.

Full Length Research Paper

Incidence of *Candida albicans* in diabetic patients with a dental prosthesis in Northeast Mexico

Garza-Garza Luis Carlos, Gallegos-Canales Anakaren, Elizondo-Garza Nemesio, Martínez-González Gustavo Israel and García-Palencia Patricia*

Department of Oral Microbiology/Molecular Biology, Facultad de Odontología, Universidad Autónoma de Nuevo León, Monterrey, México.

Diabetes is an important health problem with a high morbidity and mortality. There are infections that are common in patients with diabetes and others that have greater severity and complications. A study was carried out to determine if there is a higher incidence of *Candida albicans* in patients with diabetes who use total and partial dental prostheses in comparison with patients without diabetes. Samples were obtained from 43 patients seven days after receiving a total or partial dental prosthesis. Afterwards, samples from the dentures were cultured in CHROMagar to determine the presence of *Candida*. The frequency of different types of *Candida* was analyzed and it was found that no significant difference exists between patients with diabetes and those without diabetes with regard to the presence of *C. albicans*.

Key words: *Candida albicans*, dental prosthesis, diabetes mellitus, CHROMagar.

INTRODUCTION

Diabetes is an important health problem with a high morbidity and mortality. It currently affects more than 285 million people worldwide according to the Mexican Federation of Diabetes. Diabetes mellitus is a group of metabolic disorders that are characterized by an increase in blood glucose levels (hyperglycemia). There are infections that are common in patients with diabetes and others which have greater severity and complications (Federación Mexicana de Diabetes, 2012).

A dental prosthesis is an artificial element that is used to restore the anatomy of one or several teeth, also restoring the relationship between the maxillaries, at the same time correcting the vertical dimension and improving both natural dentition and periodontal structures

(Rendon, 2007). Prostheses can be classified according to their characteristics, such as the type of support, the material used, and the type of restoration that will be performed; however, they can also be classified as unremovable (fixed prothesis) and removable (removable partial tooth supported, total mucosal supported and over denture prosthesis). The objective of placing a partial or total prosthesis is to provide function, phonation, swallowing, esthetics, and safety for the patient who uses it.

Candidiasis is a frequent disorder, especially in patients with diabetes. It is caused by excessive growth of *Candida* in the mouth, digestive tract, vagina and other tissues. The risk factors for candidiasis are type 1 diabetes mellitus, characterized by autoimmune destruction

*Correcponding author. E-mail: cd_patygpalencia@yahoo.com.mx. Tel: +52 (81) 83 29 4000. Ext: 3168. Fax: +52 (81) 83 29 4000. Ext: 1718.

of Langerhans islet B cells by T cells that cause a lack of production of insulin; type 2 diabetes, in which a family history of the disease as well as other factors such as age >40 years, obesity, the use of drugs (corticosteroids) are present, and gestational diabetes with poor glycemic control in which vaginal candidiasis is more frequent (Nowakowska et al., 2004). Immuno-suppressive states and drugs can also cause candidiasis. A prevalence of denture stomatitis of 15% to over 70% has been reported in denture wearers and can be caused by poor denture hygiene, continual and nighttime wearing of removable dentures, accumulation of denture plaque, and bacterial and yeast contamination of the denture surface. Another cause is mucosal trauma produced by poor-fitting dentures. These factors can increase colonization of the denture and oral mucosal surfaces by *Candida albicans*, which can produce an opportunistic infection (Gendreau and Loewy, 2011). *Candida albicans* has been identified as the most frequent etiological agent in oral candidiasis although other studies have isolated *Candida dubliniensis*, *Candida parapsilosis*, *Candida krusei*, *Candida tropicalis* and *Candida glabrata* in the pathogenesis of candida-associated denture stomatitis (Liebana Ureña, 2002; Salerno et al., 2011).

The aim of this study was to determine if there is a greater incidence of *C. albicans* in patients with diabetes in comparison with patients without diabetes when they use a dental prosthesis.

MATERIALS AND METHODS

Study population

We carried out a comparative, open, observational, prospective, cross sectional study in the Total Prosthesis Clinic of the Universidad Autónoma de Nuevo León Dental School. The population consisted of 11250 patients that came for consultation in the prosthesis clinic. To calculate sample size we used the following formula:

$$n = \frac{Nz^2 pq}{e^2(N-1) + z^2 pq} \quad n = \frac{11250\,(1.96)^2\,(0.55)(0.45)}{(0.15)^2\,(11250-1) + (1.96)^2\,(0.55)(0.45)} =$$

42.10 ≈ 43 patients

with a margin of error of 0.15 and a 95% confidence interval. The population sample consisted of 43 patients (13 men and 30 women) with a mean age of 67 years (range 45 to 85).

The study was approved by the Bioethics Committee of the School of Dentistry of the UANL with registration number SSPI-010613, Page 00008. Patients provided informed consent and were assured of the confidentiality of their personal data and medical history. Men and women with (n =12) and without diabetes (n = 31) with partial or total dental loss and a dental prosthesis evaluated from August to December 2011 were included. Out of town patients and those less than 44 or greater than 85 years of age were excluded. Patients who did not follow or complete the study protocol or who died during the study were removed.

The presence of diabetes was determined by patient interview. Since *C. albicans* is part of the normal flora of the oral cavity, we determined its quantitative presence and that of other species of *Candida*. The independent variables of the study were the presence of diabetes as a nominal qualitative variable. The dependent variables were the quantitative presence of *Candida*, determined by counting the number of colony forming units (CFU), and the identification of the species of *Candida* present.

Protheses were studied at seven days because patients were programmed for revision at that time for examination and necessary adjustments. The opportunity was used to take samples at this time.

Procedures

In the first phase, the prostheses were washed with soap and water, and disinfected with Microdacyn MR for 1 min. They were then rinsed with sterile distilled water to ensure that the prosthesis was clean before giving it to the patient. The device was then placed in the patient´s mouth.

After seven days, the patient returned to the clinic and a sample was obtained using a sterile swab dipped in distilled water. The swab was placed in an Eppendorf tube containing phosphate buffer as a transport medium. Samples were taken to the molecular biology laboratory where 100 µL of the sample were seeded in a Petri dish with previously prepared CHROMagar. Seeding was carried out with a sterile glass pipette.

Samples were incubated at 37°C for 24 to 48 h in CHROMagar™ media and up to 72 h to improve colony pigmentation. Four types of *Candida* were identified by pigmentation. *Candida albicans* colonies appear green in CHROMagar, *C. tropicalis* metallic blue, *C. krusei* fuzzy pink and other species, white to mauve. A macroscopic count of CFU was performed according to the color of the colony.

Statistical analysis

Descriptive statistics were applied to obtain means, medians, standard deviations and 95% confidence intervals of the variable UFC for *Candida*; frequencies and percentages for the presence of diabetes in patients were also determined. Cross tabulation was carried out between the presence of diabetes and the *Candida* values obtained; the mean difference (95% confidence interval) for the presence of *Candida* in each of the groups was subsequently tested. All statistical procedures were performed using Microsoft Excel 2007 and SPSS, achieving a 95% confidence interval for all cases.

RESULTS

We evaluated 13 (30.2%) men and 30 (69.8%) women with (n = 12; 28%) and without diabetes (n = 31; 72%). Forty two patients had upper dentures and 36 lower dentures. The mean pH among patients with and without diabetes was 7.25 and 7.26, respectively ($P = 0.4828$). Of the total patients 30 (71.4%) had *Candida* in the upper denture. In patients with diabetes mellitus, 9 (75%) had some form of *Candida* in the upper denture. In patients without diabetes mellitus, 21 (70%) had *Candida* in the upper denture. Of all the patients studied, *C. albicans* was present in 11 patients (26.2%). In patients with diabetes, *C. albicans* was present in 3 (25%). Of the
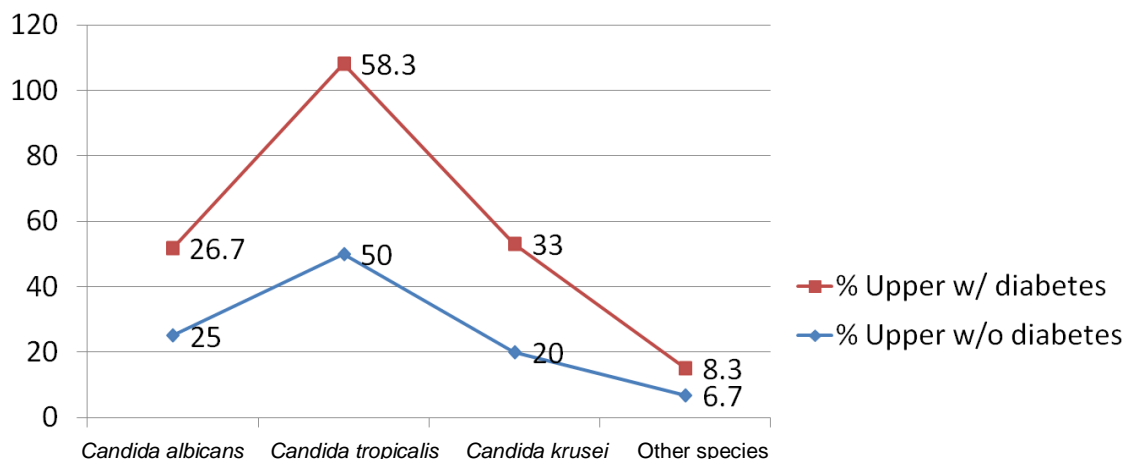
Figure 1. Relationship of percentages of patients with and without diabetes with an upper dental prosthesis from the Total Prosthesis Clinic with different types of *Candida* analyzed after 7 days of use between August and December, 2011.
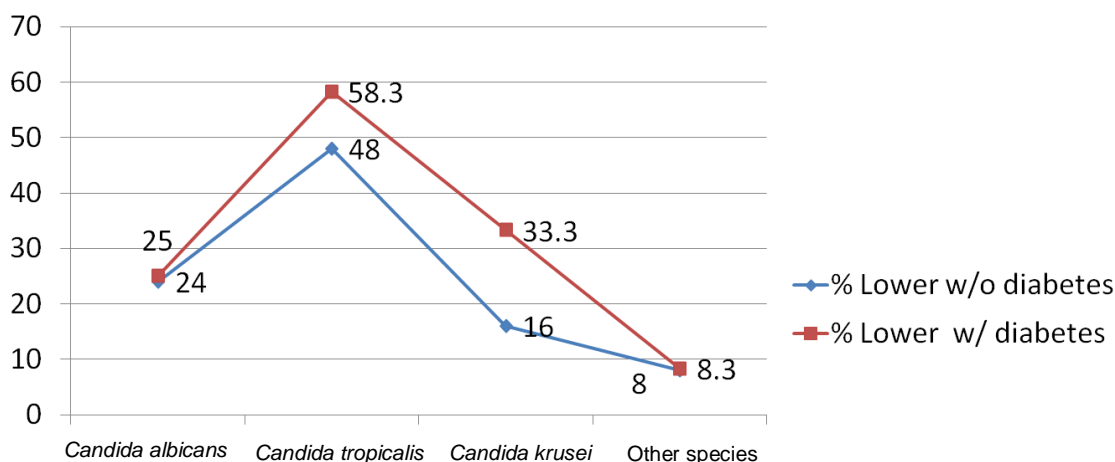
Figure 2. Relationship of percentages of patients with and without diabetes with a lower dental prosthesis from the Total Prosthesis Clinic with different types of *Candida* analyzed after 7 days of use between August and December, 2011.

patients without diabetes mellitus only 8 (26.7%) had *C. albicans*.

In patients with diabetes mellitus, 7 (58.3%) had *C. tropicalis*. *C. krusei* was found in 10 (23.8%) patients. This strain of *Candida* was found in 6 (20%) patients that did not have diabetes mellitus and in 4 (33.3%) patients with diabetes (Figure 1). It was also observed that 3 (7.1%) patients in the study had other species of *Candida*. In patients with diabetes mellitus, only 1 (8.3%) had other species of *Candida*. Of the patients who did not have diabetes mellitus, 2 (6.7%) presented other species of *Candida*.

Regarding those with lower dentures, it was observed that 25 (67.6%) had *Candida*. In patients with diabetes mellitus, 9 (75%) had some form of *Candida*. Of the

patients who did not have diabetes mellitus, 16 (64%) had some forms of *Candida*. In patients with lower dentures, 9 (24.3%) had *C. albicans*. In patients with diabetes mellitus, 3 (25%) had *C. albicans*. In patients without diabetes mellitus, 6 (24%) had *C. albicans* (Figure 2).

It was found that 19 (51.4%) patients had *C. tropicalis*. Of the patients who had diabetes mellitus, 7 (58.3%) had *C. tropicalis*. Of the patients who did not have diabetes mellitus, 12 (48%) had *C. tropicalis*.

C. krusei was present in 8 (21.6%) patients. In patients with diabetes mellitus, 4 (33.3%) had *C. krusei*. Of the patients who did not have diabetes mellitus, 4 (16%) had *C. krusei*. As for other species of *Candida*, growth was observed in 3 (8.1%) patients with lower dentures. In

patients with diabetes mellitus, only one (8.3%) had other species of *Candida*. Of the patients who did not have diabetes mellitus, 2 (8%) had other *Candida* species.

DISCUSSION

We found an incidence of *Candida* in 71.4% of samples taken from 43 patients. This is in contrast with Zaremba (2006), who isolated *Candida* in 59.4% of 32 patients with prosthesis and Daniluk et al. (2006), who found an incidence of 66.7% in samples from patients without diabetes, and 43.8% in patients with diabetes. In the present study, 26.2% of the sampled patients had *C. albicans* and in those with diabetes only 25% had the fungus. Pfaller et al. (1996) evaluated the use of CHROMagar as a differential culture medium that allows the isolation of yeasts and simultaneously identifies colonies of *C. albicans, C tropicalis* and *C krusei.* They found that more than 95% of the values and clinical isolates of *Candida* species were correctly identified based on colony morphology and CHROMagar pigmentation. The study by Odds et al. (1994) found that the specificity and sensitivity of the medium in the identification of *C. albicans*, *C. tropicalis* and *C. krusei* exceeded 99% for all three species.

The study by Mata de Henning et al. (2001) showed that 65% of patients had *Candida* species in 20 patients studied by swabbing the prosthesis in the area in contact with the palatal mucosa. However, their study differs from ours because they observed *C. albicans* in 50% of cases and found one case with a species that was not *albicans*.

In a study by Belazi (2005), of the 128 diabetic and 84 nondiabetic patients, *Candida* was observed in the oral cavity of 64% of the diabetic patients. However, in contrast with the present research, they found a higher frequency of *C. tropicalis*. There was also a difference with Daniluk et al. (2006), where it was determined that 43.8% of diabetic patients had *C. albicans*. In the present study, only 25% had a positive result.

We had a small number of patients with diabetes in our clinic and we believed that there would be a greater incidence of *C. albicans*, but this was not the case. It would be convenient to perform this study in a greater number of patients.

Our results indicate that colonization by *C. albicans* is independent of the presence of diabetes mellitus in patients with total and/or partial dentures. Thus, no significant relationship between diabetic and nondiabetic patients with total and/or partial dentures was shown. *C. albicans* was not as frequent as other types of *Candida*, since *C. tropicalis* was found in 52.4% of samples, which was the highest and most significant finding in this study.

REFERENCES

Belazi M, Velegraki A, Fleva A, Gidarakou I, Papanaum L, Baka D, Daniilidou N, Karamitsos D (2005). Candidal overgrowth in diabetic patients: potential predisposing factors. Mycoses. 48 :192-196.

Daniluk T, Tokajuk G, Stokowska W, Fiedoruk K, Sciepuk M, Zaremba ML, Rozkiewicz D, Cylwik-Rokicka D, Kedra BA, Anielska I, Górska M, Kedra BR (2006). Occurrence rate of oral Candida albicans in denture wearer patients. Adv. Med. Sci. 51 Suppl 1 : 77–80.

Diabetes y la salud oral (2007). [On line] Available at: http://www.ada.org/3356.aspx [Accessed February 3, 2007]

Federación Mexicana de Diabetes (2012). Diabetes Atlas de la FID 5ª Edición. Available at: http://www.fmdiabetes.org/fmd/des/IDFATLAS2012.pdf. Accesed August 2012.

Gendreau L, Loewy ZG (2011). Epidemiology and etiology of denture stomatitis. J. Prosthodont. 20: 251-260.

Liebana Ureña J (2002). Microbiología Oral, 2da ed. Madrid: McGraw-Hill/Interamericana de España, pp. 487-493.

Mata de Henning M, Perrone, M (2001). Factores determinantes de patogenicidad en relación a la ecología de Cándida albicans en cavidad bucal. Acta. Odontol. Venez 39 [On line] Available at: http://www.scielo.org.ve/scielo.php?pid=S0001-63652001000200010&script=sci_arttext. [Accessed October 10, 2000]

Nowakowska D, Kurnatowska A, Stray-Pedersen B, Wilczyński J (2004). Species distribution and influence of glycemic control on fungal infections in pregnant women with diabetes. J Infect. 48: 339-346.

Odds FC, Bernaerts R (1994). CHROMagar Candida, a new differential isolation medium for presumptive identification of clinically important Candida species. J. Clin. Microbiol. 32: 1923-1929.

Pfaller MA, Houston A, Coffmann S (1996). Application of CHROMagar Candida for rapid screening of clinical specimens for Candida albicans, Candida tropicalis, Candida krusei, and Candida (Torulopsis) glabrata. J. Clin. Microbiol. 34:58-61.

Rendon YR (2007). Prótesis parcial removible, Conceptos actuales, Atlas de diseño. Buenos Aires: Editorial Médica Panamericana.

Rozman C, Cardellach F (2008). Farreras-Rozman Medicina Interna Vol II, 16ªEd. Barcelona: Editorial Elsevier, pp 1955-1964.

Salerno C, Pascale M, Contaldo M, Esposito V, Busciolano M, Milillo L, Guida A, Petruzzi M, Serpico R (2011). Candida-associated denture stomatitis. Med. Oral. Patol. Oral. Cir. Bucal. 16: e139-143.

Zaremba ML, Daniluk T, Rozkiewicz D, Cylwik-Rokicka D, Kierklo A, Tokajuk G, Dabrowska E, Pawińska M, Klimiuk A, Stokowska W, Abdelrazek S (2006). Incidence rate of Candida species in the oral cavity of middle-aged and elderly subjects. Adv. Med. Sci. 51 Suppl 1: 233–236.

Full Length Research Paper

Antimicrobial activity of *Syzygium aromaticum* extracts against food spoilage bacteria

Muhammad Saeed[1], Muhammad Nadeem[2*], Moazzam Rafiq Khan[1], Muhammad Asim Shabbir[1], Aamir Shehzad[1] and Rai Muhammad Amir[1]

[1]National Institute of Food Science and Technology, University of Agriculture, Faisalabad-Pakistan.
[2]Department of Food Science, University College of Agriculture and Environmental Sciences, The Islamia Uuniverity Bahawalpur, Bahawalpur-Pakistan.

In this study, the emphasis was on extraction of aqueous and methanolic extract from whole clove (*Syzygium aromaticum*) that can be efficiently used as an antimicrobial agent with an ultimate objective of developing replacements for the synthetic chemical additives in food products. Antimicrobial activity of extract revealed that the solvent extract of clove has a great potential for the inhibition of microbial load. The value of antimicrobial activites of solvent extract ranged from 12 to 17 mm in the disc diffusion method as compared to aqueous extract i.e ranged from 12 to 16 mm. Minimum Inhibitory Concentrations were found from 17 to 23 mm for solvent extract and 13 to 17 mm for aqueous exract. The sensory characteristics of bread showed that the treatments had a highly significant effect on volume, color of crust, symmetry of form, evenness of bake, character of crust, grain, color of crumb, taste and texture and overall acceptability of bread. Significantly lower total scores for the bread were exhibited by the bread supplemented with 2 and 2.5% clove extract. The results revealed that bread containing up to 1% clove extract is acceptable.

Key words: Antimicrobial activity, Clove, *Syzygium aromaticum,* food spoilage bacteria, bread, total plate count.

INTRODUCTION

The distinguished inhibitors of microorganisms are plant essential oils and their extracts. The Spices are reputed to possess several medicinal and pharmacological properties and hence find position in the preparation of a number of medicines. Spices impart aroma, colour and taste to food preparations and sometime mask undesirable odours. Volatile oils give the aroma, and oleoresins impart the taste (Proestos et al., 2008).

Due of their antimicrobial nature, spices are used to improve taste and enhance shelf life. Some of spices are also known to contribute to the self-defence of plants against infectious organisms (Kim et al., 2003). There is considerable potential for utilization of natural antimicro-

bials in foods, especially in fresh fruits and vegetables. Extract derived from spices and plants have antimicrobial activity against *Listeria monocytogenes, Salmonella typhimurium, Escherichia coli* O157:H7, *Shigella dysenteriae, Bacillus subtilis* and *Staphylococcus aureus* at levels between 0.2 and 10µ ml^{-1} (Burt, 2004).

Presently, the major problem is that we can not use chemical preservatives safely now a day due to carcinogenic nature of these chemicals. Residual toxicity is increased due to these chemicals. Due to these reasons, consumers lean to be doubtful of chemical additives and thus the requirement has been increased for natural and adequate preservatives (Skandamis et al., 2001). As a

*Corresponding author. E-mail: mnadeem11@gmail.com. Tel: +923457855994.

consequence, natural antimicrobials are receiving a good deal of attention for a number of micro-organism-control issues. Reducing the need for antibiotics, controlling microbial contamination, improving shelf-life extension technologies to eliminate undesirable pathogens, decreasing the development of antibiotic resistance by pathogenic micro-organisms or strengthening immune cells in humans are some of the benefits (Fisher and Phillips, 2008).

Clove belongs to a tree Eugenia caryophyllata (*Syzygium aromaticum*), is used as a spice in almost all the world's fare. Bud Oil of Clove has natural behavior and the main properties include antioxidant, insecticidal, antifungal and antibacterial properties. By tradition, it has been used in food preservation as flavoring and antimicrobial substance (Velluti et al., 2003). It has a very major role in spice trade and is highly appreciated for their therapeutic properties. Cloves are an excellent source of manganese. They are also a very good source of dietary fiber, vitamin C, vitamin K, and Ω-3 fatty acids and a good source of magnesium and calcium. Cloves consist of a significant amount of proteins, iron, carbohydrates, calcium, phosphorus, potassium, sodium and hydrochloric acid. They are also rich in vitamins A and C, manganese, and dietary fiber (Kim et al., 1998).

The most important constituent of clove is the phenyl-propene eugenol due to which it has strong characteristic aroma. Major parts of clove consist of eugenol comprises 70 to 90 % and remaining 15% consist of dry weight (Shobana and Naidu, 2000). Molds, yeast and bacterial growth could be inhibited by the application of clove essential oil (Burt, 2004). Micro-organisms like *Alternaria* sp., *Aspergillus* sp., *Canninghamella* sp., *Lactobacillus* sp. *Fusarium* sp., *Clostridium* sp; *Mucor* sp., *Salmonella* sp. *Penicillium* sp. *Bacillus* sp. could be repressed by using clove essential oil (Soliman and Badeaa, 2002). The cloves are antimutagenic, anti-inflammatory, antioxidant, antiulcerogenic, antithrombotic and antiparasitic. The essential oil extracted from the dried flower buds of clove are used for acne, warts, scars and parasites (Miyazawa and Hisama, 2003; Srivastava and Malhotra, 1991; Chaieb et al., 2007b).

B. subtilis is not a human pathogen but is responsible for causing Ropiness, a sticky and stringy consistency caused by bacterial production of long-chain polysaccharides in spoiled bread dough (Priest et al., 1988). *B. subtilis* has been associated with outbreaks of food poisoning but the exact nature of its involvement has not been established. *B. subtilis*, like other closely related species in the genus, *B. licheniformis, B. pumulis,* and *B. megaterium*, have been shown to be capable of producing lecithinase, an enzyme which disrupts membranes of mammalian cells. However, there has not been any correlation between lecithinase production and human disease in *B. subtilis* (Collins et al., 1991). Considereing the importance clove, the present project has been designed to to analyze the physico-chemical properties of clove extract in order to study the inhibitory effect of clove extract against food spoilage bacteria.

MATERIALS AND METHODS

Sample preparation and extraction

Fresh whloe clove samples were purchased from the local grain market of Faisalabad, Punjab, Pakistan. The samples were obtained from retail spice-sellers in the amount of 1 kg. Grinding was done with grinder MJ-176P in the laboratory grinder. The samples were kept in closed containers after being chopped into small pieces (1 mm). Two types of clove extract were prepared for aqueous and methanolic extracts.

For the preparation of aqueous extract, 150 ml of distilled water was added into 25 g of chopped clove and the mixture was left for agitation in shaker incubator for 8 h at 300 rpm at 39°C. Afterwards, it was filtered. The dark colored extract obtained at the end of this process was used for furthure analysis. The sample extract was kept in the refrigerator (4°C) for accomplishment of further analysis (Wilson, 1995).

For the preparation of solvent extract, 150 ml of methanol was added into 25 g of chopped clove and the mixture was left for agitation in shaker incubator for 8 h at 300 rpm and 39°C. Afterwards, it was filtered and the methanol was vaporized in rotary evaporator (60°C). The dark colored oily extract obtained at the end of this process was used form for the analysis. The sample extract was kept in the refrigerator (4°C) until the analysis was accomplished (Wilson, 1995).

Physicochemical analysis of extract

Electronic digital type pH meter of Wellium model- Inolab pH 720, WTW 82362 was used for pH determination. Total Acidity, Referactive index, Specific gravity and Brix value was determined by following the methods described in AOAC (2007).

Isolation of bacteria

Glassware should be autoclave before the start of experiment. Spoiled bread samples were collected from different bakeries located in vicinity of Faisalabad city. The samples were drawn in sterilized screw capped bottles and preserved for further studies. Six sterilized test tubes were taken and labeled as 10^{-1}, 10^{-2}, 10^{-3}… 10^{-6}. Serial dilutions for each sample were made by the method as recommended by Cappuccino and Sherman (1996). Nutrient agar, Mueller Hinton agar (MH agar) and Potato dextrose agar (PDA) were prepared according to the methods of NCCLS (2000). In each Petri plate 10ml of medium and 1ml of respective dilution were added carefully. Six sterilized test tubes were taken and labeled as 10^{-1}, 10^{-2}, 10^{-3}… 10^{-6}. Medium and inoculum were mixed immediately by given to and for shaking and circular movement lasting 5 to 7 s. Medium was allowed to solidify. After solidifying inverted Petri plates were placed in incubator at 37°C for 24 to 48 h for bacterial isolation by following the method given by Cappucino and Sherman (1996).

Morphological examination of bacteria

The representative colonies, showing catalase negative and Gram positive, were randomly picked from higher dilution (10^{-5}) of nutrient agar plates. The culture isolates were morphologically observed under microscope by following the method described by Cappuccino and Sherman (1996). Gram's Staining was done according to the method described by Becker et al. (2003).

Purification of isolates

The colonies which appeared after 48 h incubation on nutrient agar

Table 1. Mean values for physiochemical analysis of aqueous and methanolic extracts

Physicochemical	Methanolic	Aqueous
Total acidity (%)	0.45 ± 0.02	0.25 ± 0.25
pH	5.20 ± 0.21	6.70 ± 0.24
Specific gravity	1.025 ± 0.23	1.015 ± 0.26
Refractive index	1.53 ± 0.22	1.52 ± 0.22
Brix	9.80 ± 0.24	9.80 ± 0.23

Values are given as mean ± standard deviation

plates were subjected to morphological examination. Pure growth of *B. subtilis* were then transferred to potato dextrose agar and incubated at 37°C and preserved in refrigerator at 4°C for further use. The results of colony characteristics were recorded by following the method as described by Harrigan and McCance (1990).

Determination of antibacterial activity

Microbiological methods used for the determination of antimicrobial activity of clove extract were disc diffusion method and agar well diffusion method.Treatments T1, T2, T3, T4 and T5 for aqueous extract and methanolic extract were used at concentration 0.5, 1, 1.5, 2 and 2.5% respectively. Antimicrobial activity was determined by standard disc diffusion method as described by saeed et al. (2007). The inoculum suspension of each bacterial strain was swabbed on the entire surface of Mueller-Hinton agar (MHA, pH 7.3 ± 0.1, Difco). Sterile 6 mm filter paper discs (Schleicher & Schuell) immersed with clove extract was aseptically placed on MHA surfaces. The plates were left at ambient temperature for 15 min to allow excess prediffusion of extracts prior to incubation at 37°C for 24 h. Diameter of inhibition zones was measured. Each experiment was done in triplicate.

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration was determined by agar well diffusion method as described by Ogata et al. (2000). The plates containing Mueller Hinton agar medium were spread with either 0.1 ml of the bacterial noculum. Wells (8 mm in diameter) were cut from agar plates using a sterilized stainless steel borer and were filled with 0.1 ml of clove extract. The plates were incubated at either 37°C for 24 h and the diameter of resultant zone of inhibition was measured. Each combination antimicrobial agent was repeated three times. Microorganisms showing a clear zone of more than 6mm were considered to be inhibited.

Preparation of bread

The breads were prepared from straight grade flour containing clove methanolic extract from 0.5 to 2.5% by method according to the procedure as described in AACC (2000).

Physical analysis of bread

The color of bread was determined with the help of Color meter as described by Rocha and Morais (2003). The textural study of bread was conducted by using Texture analyzer (Model TA-XT2, Stable Microsystems, Surrey, UK) as described by Piga et al. (2005). The water activity of bread was determined by using Hygropalm water activity meter (Rotronic Hygropalm water activity meter, series num-

ber 601089738) as described by Piga et al. (2005). The loaf volume of the bread was determined using a rapeseed displace-ment method by the method of Hussain (2009).

Total plate count of bread

Six sterilized test tubes were taken and labeled as 10^{-1}, 10^{-2}, 10^{-3}… 10^{-6}. Nine milimliters of normal saline was poured into each test tube. One grams of crushed spoiled bread sample was shifted into the first test tube and contents were mixed well by gentle shaking. 1m shifted contents from each dilution on to the surface of separate plate count agar plates. Serial dilutions for each sample were made by the method as recommended by Cappuccino and Sherman (1996). One mililiters shifted contents from each dilution on to the surface of separate plate count agar plates and spread well and incubated at 37°C for 24 h. Average number of colonies was counted from those dilutions that showed the colonies size ranging from 30 to 300 with the help of colony counter. The total plate count was calculated using the following formula

Total Plate Count = Average number of colonies × dilution factor/volume factor

Sensory evaluation

The prepared bread loaves were evaluated by a panel of judges for external characteristics such as volume, crust color, symmetry of form, evenness of bake and internal characteristics like grain, crumb color, aroma, taste and texture by following the method of Land and Shepherd (1988).

Statistical analysis

The data obtained from each parameter was subjected to a statistical analysis using analysis of variance techniques to determine the level of significance in different parameters according to the method described by Steel et al. (1997).

RESULTS AND DISCUSSION

Physicochemical analysis of extracts

The results indicated that acidity of clove extracts differed significantly. The results given in the Table 1 indicated that total acidity was found to be 0.45 and 0.25 for methanolic extract and aqueous extract respectively. With increase in solvent concentration, acidity was increased. The results of present sudy are in agreement with the findings of Burt (2004) who reported that acidity ranged from 0.20 to 0.55%. The results were also in agreement with the findings of Hammer et al. (1999). They studied 13 different spices solvent extract and reported that titrateable acidity ranged from 0.35 to 0.55.

The results for pH of clove extract (Table 1) indicated significant variations for pH of clove extract. The results indicated that pH was ranged from 5.20 to 6.70 for methanolic extract and aqueous extract respectively. The results of present study are in concordance with the findings of Koiche and Bouras (2010) who reported that pH of the clove extract was ranged from 5.0 to 5.50. Chaieb

Table 2. Effect of clove aqueous and methanolic extracts on antibacterial activity.

Treatment	Aqueous extract	Methanolic extract
T_0 = control without methanolic extract	-	-
T_1= 0.5% methanolic extract	12.33 ± 0.22	14.00 ± 0.23
T_2= 1.0% methanolic extract	14.66 ± 0.24	14.66 ± 0.25
T_3= 1.5% methanolic extract	14.85 ± 0.25	15.33 ± 0.28
T_4= 2.0% methanolic extract	15.00 ± 0.25	16.00 ± 0.26
T_5= 2.5% methanolic extract	16.66 ± 0.26	17.66 ± 0.29

Values are given as Mean ± Standard Deviation, T_0= Control (without methanolic extract).

et al. (2007) described their results for pH of clove aqueous extract was ranged from 6.50 to 6.90. The results for specific gravity of clove extracts (Table 1) indicated the significant differences for specific gravity.

The results showed that specific gravity was ranged from 1.025 to 1.015 for methanolic extract and aqueous extract respectively. The results of present study are in agreement with the findings of Kumar et al. (2007a) who described that the specific gravity of methanolic extract was ranged from 1.02 to 1.028. Kim et al. (1995) described that the specific gravity of clove extract was ranged from 1.01 to 1.029.

The results pertaining to referective index of clove extracts (Table 1) indicated significant variations of refracttive index. The results showed that refractive index ranged from 1.533 to 1.523 for methanolic extract and aqueous extract respectively. The results of presented study are in agreement to the findings of Dorman et al., 2000 who reported that refractive index of clove extract was ranged from 1.520 to 1.535. These results were also in agreement with the findigs of Gutierrez et al. (2009) who reported the refrective index of clove extract was ranged from 1.518 to 1.536.

The results for brix of clove extract showed that brix of clove extracts had non significant differences. The results (Table 1) indicated that brix of methanolic and aqueous extracts was 9.80 and 9.80 respectively. The results of present study are in contradiction to the findings of Cowan, 1999 who reported that brix of spices extract are in the range of 8.50 to 9.0. The difference in the results might be due to the chemical reaction of solvents with sugars during the mixing operations.

Microbiological analysis of extract

Determination of antibacterial activity

The results for antibacterial activity of aqueous clove extract have been presented in Table 2. The results indicated that aqueous extract of clove extract showed highly significant variations as compared to methnolic extract for antibacterial activity. The highest zone of inhibition of antibacterial activity for clove aqueous extract was showed by T_5 (16.66 mm) followed by T_4 (15.00 mm).

Minimum antibacterial activity was showed by T_1 (12.33 mm).

The results for antibacterial activity of methanolic clove extract (Table 2) indicated that clove methanolic extract showed significant results as compared to acueous extract for antibacterial activity. Methanolic extract have highest antibacterial activity at T_5 (17.66 mm) and minimum zone of inhibition was showed by T_1 (14.00 mm). The present results showed that methanolic extract exhibited the strongest inhibitory activity (14 to 17.66 mm) as compared to results of aqueous extract (12 to 16 mm). It is evident from the results that the diameter of inhibition increased significantly as concentration of extract increased. Diameter is showed in millimeter (mm)

The results of present study are in agreement with the findings of Meena and Sethi (1994) who reported that different extracts of clove showed strong antibacterial acti-vity against *P. vulgaris* and *B. subtilis.* They reported the inhibition diameter in the range of 12 to 25 mm. Their results also in concordance with the findings of (Kumar, 2007a) who stated that *S. cumini* showed good activity against S. *aureus* and *B. subtilis.* Both aqueous and methanol extracts of *S. lineare* and *T. asiatica* showed a strong antimicrobial activity against food spoilsge bacteria.

Similar results were found in the previous study of Mytle and others (2006) that determined the inhibitory effect of clove extract against *B. subtilis* on chicken frankfurters. *B. Subtilis* was inoculated at low (102 to 103 CFU/g) or high cell numbers (104 to 106 CFU/g), and stored at 5°C for 2 wk or at 15°C for 1 wk .All strains of *B. subtilis* and grew on control frankfurters at 5 and15°C, but growth was inhibited under both storage conditions in the presence of either 1 or 2% clove oil.

S. aromaticum, *P. granatum, S. cumini* and *T. asiatica* produced the largest zones of inhibition against *B. subtilis, S. aureus* and *S. epidermidis.* (Bevilacqua et al., 2010) reported good antibacterial activity in clove metha-nolic extract against *E. coli* using aqueous and methanol extracts. The ethanolic extracts of clove, cumin, and kaffir lime peels showed the broadest antibacterial activity by inhibiting growth of all bacterial strains tested (the dia-meter of inhibition zone, 8 to 22 mm), while the extracts of cardamom, cinnamon, and kaffir lime leaves inhibited the growth of almost all strains (7 to 12 mm), except for *S.*

Table 3. Effect of clove aqueous and methanolic extracts on MIC.

Treatment	Aqueous extract	Methanolic extract
T_0	-	-
T_1	14.00 ± 0.22	14.66 ± 0.28
T_2	16.00 ± 0.25	15.66 ± 0.27
T_3	17.00 ±0.23	17.00 ± 0.25
T_4	19.00 ± 0.24	20.00 ± 0.26
T_5	21.00 ± 0.27	23.00 ± 0.27

Values are given as Mean ± Standard Deviation, T_0= Control (without methanolic extract), T_1= 0.5% methanolic extract, T_2= 1.0% methanolic extract, T_3= 1.5% methanolic extract, T_4= 2% methanolic extract, T_5= 2.5% methanolic extract.

Typhimurium, S. London, and *Serratia marcescens*. Plant-derived antimicrobial compounds have been recognized as a means of inhibiting undesirable bacteria and numerous research articles have described the antimicrobial properties of plant extracts. Clove extract has shown inhibiting activity against bacteria (Kildeaa, 2004).

Minimum inhibitory concentration (MIC)

The results pertaining to MIC of aqueous extract have been given in the Table 3 showed highly significant differences beween aqueous extract and methanolic extract for minimum inhibitory concentration. The results indicated that highest value of MIC with aqueous extract was shown by T_5 (21 mm) and minimum value was shown by T_1 (17 mm).

The results for MIC of methanolic extract (Table 3) showed highly significant variations among aqueous extract and methanolic of MIC. Highest value of MIC methanolic extract was shown by T_5 (23 mm) and minimum value 17 mm was shown by T_1. Results showed that MIC value of methanolic extract is greater as compared to aqueous extract because methanol also acts as a natural antimicrobial agent.

The results of present study are in concordance with the findings of Kim et al. (1994) who showed that the methanolic extract of clove showed inhibitory activity against all the six food associated bacteria in which the diameter of zone of growth inhibition varied between 15 to 25 mm (in clove) and 15 to 20 mm (in garlic). The clove ethnaolic extract showed highest diameter of zone of inhibition of 32 mm against *E. coli* followed by *S. aureus* (21 mm) and B. *subtilis* (23 mm) (Burt, 2004).

The clove ethanolic extract showed similar zone of inhibition of 20 mm diameter against *B. megaterium* and *B.* sp*haericus*. The minimum inhibitory activity was recorded against *B. polymyxa*. Our results substantiate the findings of Soliman and Badeaa (2002) that demonstrated the antibacterial activity of clove ethanolic extract against *E. coli, S. aureus* and *B. subtilis* and found that the highest antibacterial activity was against *B. subtilis*.

The MIC values of the clove methanolic extract tested against *L. monocytogenes* ranged in between 20 to 25 mm for selected ethanol extracts ranged from 0.25 to 11.75 mg/mL. It is apparent from the results that the MIC values are high for lavender and verbena, explaining the extent of resistance offered by *L. monocytogenes*, against these ethanol extracts. This study revealed that clove extract showed maximum activity against *L. monocytogenes* with MIC value 0.25 mg/mL followed by mint timija extract with MIC value of 0.315 mg/mL*,* indicating that clove and mint timija showed excellent antimicrobial activity against *L. monocytogenes*. *L. monocytogenes* is fairly sensitive to all ethanol extracts except lavender and verbena and was showing moderate MIC values against rosemary, geranium and camomile. The MIC values were used as guide for the treatment and battle against undesirable microorganisms. The results obtained showed that the MIC values varied according to the extracts and indicated that clove exhibited the strongest antibacterial activity, followed by mint timija. Similar result has been reported by Meena and Sethi (1994).

Analysis of bread

Based on the results obtained during study, it is stated that methanolic extracts had great antibacterial activity and zone of inhibition than aqueous extract. So bread was prepared from methanolic extracts**.**

Physical analysis of bread

The results pertaining to color of bread have been given in the Table 4. The results indicated that the color value of bread differed significantly due to increasing concentrations of extract. The results illustrated that color of breads prepared from T_0 (control) had the minimum color value that is, 173.0 and its value increased gradually as concentrations of extract increased. The darker color was due to dark oily color of extract. The bread prepared with T_1 was found as best value of color near to control. The breads prepared from T_5 concentration got the maximum color value that is, 186.67. The results of present study are in concordance to the finding of Holley and Patel (2005) who reported that color value of cereal products increase with increase in the concentration of spices extract.

The results regarding the water activity results indicated highly significant variations for water acticity of bread. The results indicated that water activity of breads ranged from 0.77 to 0.83. The maximum water activity was found in the bread prepared with T_5 while the minimum water activity was found in the bread prepared with T_0. The water activity was increased as the percentage of extract increased (Dragland, 2003). The results pertaining to the texture of bread (Table 4) showed highly significant results for texture. The results indicated that the texture (firmness) of bread ranged from 1721 to 2043. Maximum

Table 4. Effect of clove methanolic extract on physical characteristics of bread.

Treatment	Color	Water activity	Texture	Loaf volume
T_0	173.00 ± 0.25	0.77 ± 0.25	1721.3 ± 0.28	620.0 ± 0.25
T_1	175.67 ± 0.26	0.79 ± 0.24	1814.7 ± 0.21	580.3 ± 0.22
T_2	176.67+1.23	0.80 ± 0.25	1920.7 ± 0.23	550.30 ± 0.24
T_3	180.00+2.09	0.80 ± 0.26	2016.0 ± 0.25	520.30 ± 0.21
T_4	183.00+0.76	0.81 ± 0.27	2029.7 ± 0.26	490.30 ± 0.26
T_5	186.67+0.91	0.83 ± 0.26	2043.0 ± 0.27	440.00 ± 0.28

Values are given as Mean $\pm$ Standard Deviation, T_0= Control (without methanolic extract), T_1= 0.5% methanolic extract, T_2= 1.0% methanolic extract, T_3= 1.5% methanolic extract, T_4= 2% methanolic extract, T_5= 2.5% methanolic extract.

Table 5. Effect of clove methanolic extract on external characteristics of breads.

Treatment	Volume	Crust color	Symmetry of form	Evenness of bake	Crust character
T_0	8.80 ± 0.21	8.20 ± 0.26	4.80 ±0.21	2.60 ± 0.25	2.90 ± 0.28
T_1	7.95 ± 0.25	7.60 ± 0.23	4.05 ±0.22	2.60 ± 0.23	2.40 ± 0.21
T_2	7.60 ± 0.22	7.10 ± 0.28	3.80 ±0.23	2.30 ± 0.26	2.30 ±0.27
T_3	6.30 ±0.23	5.70 ± 0.25	3.60 ±0.24	2.20 ± 0.24	2.20 ± 0.22
T_4	5.30 ±0.24	4.30 ±0.24	3.30 ±0.25	2.20 ± 0.22	1.90 ± 0.23
T_5	4.30 ±0 .26	3.40 ± 0.21	3.30 ±0.26	2.20 ± 0.27	1.80 ± 0.25

Values are given as Mean $\pm$ Standard Deviation, T_0= Control (without methanolic extract), T_1= 0.5% methanolic extract, T_2= 1.0% methanolic extract, T_3= 1.5% methanolic extract, T_4= 2% methanolic extract, T_5= 2.5% methanolic extract.

firmness value of bread was 2043 prepared with T_5 (2.5%) methanolic extract. Minimum firmness value of bread prepared with T_0 (control) extract concentration was 1721. The results of present study are in agreement with the findings of Piga et al. (2005) who reported that firmness value is increased as the percentage of spices essential oil increasd.

The results for loaf volume of breads containing methanolic extract (Table 4) indicated highly significant results for loaf volume of bread. The results revealed that the maximum loaf volume (620 ml) was found by the bread produced from T_0 (control) followed by the T_1 extract (580 ml) while the minimum loaf volume (440 ml) was found in the T_5 extract bread. The results indicated that loaf volume of bread was affected significantly by different concentrations level of extract. It is obvious from results that loaf volume of bread containing methanolic extract was higher than prepared from the bread having no extract.

The decrease in loaf volume of the bread may be attributed due to the reduction in wheat structure forming proteins and low ability of dough to entrap air. The protein quantity, alpha amylase activity, damaged starch and genetic factors might have significant effect on bread volume and baking quality for different composite flours (Burt et al., 2007).

Sensory evaluation of bread

The sensory evaluation of bread for various attributes such as volume, colour, symmetry of form, evenness of bake, character of crust, grain, and colour of crumb, aroma, taste and texture was carried out. The product was evaluated by a panel of judges and the results are described below.

External characteristics of breads

The results pertaining regarding volume of breads have been given in Table 5 revealed that methanolic extract showed highly significant effect on the scores assigned to loaf volume of the breads. The results revealed that the scores assigned to volume of breads were affected significantly by the level of extracts concentrations. The results that the scores assigned to loaf volume of breads ranged from 4.30 to 8.80. The judges assigned maximum scores to the volume of control breads (8.80) followed by the breads prepared from the T_1 (0.5% extract). However, the breads prepared from T_5 (*2.5%*) got minimum scores with respect to volume. Decrease in volume was observed after baking of breads. In the present study the breads prepared from methanolic extract showed variable trends as breads from T_1 and control got statistically closest scores for volume where as breads from other conc. got significantly lower scores for volume of breads.

Crust color is an important sensory parameter concerning the consumer's acceptability of bread. The results (Table 5) indicated that the clove methanolic extract showed significant effect on the scores given by judges to crust color of the breads. The results indicated that there was a significant decrease in scores assigned to crust

Table 6. Effect of Clove methanolic extract on internal characteristics of bread.

Treatment	Grain	Crumb color	Aroma	Taste	Texture
T_0	14.00 ± 0.23	8.40 ±0.25	8.70 ± 0.27	15.30 ± 0.21	13.80 ± 0.24
T_1	12.20 ± 0.22	7.50 ± 0.28	7.10 ± 0.26	14.80 ± 0.22	12.80 ± 0.25
T_2	11.30 ± 0.21	6.50 ± 0.27	6.20 ± 0.23	13.30 ±0.24	11.10 ± 0.26
T_3	9.80 ± 0.25	5.20 ± 0.26	5.20 ± 0.24	12.84 ± 0.25	9.10 ± 0.28
T_4	8.30 ± 0.26	4.20 ± 0.29	4.40 ± 0.25	11.30 ± 0.26	7.80 ± 0.26
T_5	7.20 ± 0.27	3.30 ± 0.22	3.40 ± 0.21	10.20 ± 0.27	6.50 ± 0.23

Values are given as Mean ± Standard Deviation, T_0= Control (without methanolic extract), T_1= 0.5% methanolic extract, T_2= 1.0% methanolic xtract, T_3= 1.5% methanolic extract, T_4= 2% methanolic extract, T_5= 2.5% methanolic extract.

color of breads prepared from different extract concentrations as compared to control bread. The breads prepared from T_0 got significantly the highest scores for crust color followed by the breads produced from T_1. The results also indicated that bread prepared with T_5 has minimum crust color value. Crust colour of the bread was light brown which darkened progressively with the increasing level of extract concentration. The darkened colour of crust may be due to the Maillard reaction taking place during baking of loaves, due to high lysine contents. The results of the present study are comparable with those of Lee and Shibamoto (2000) who studied the behavior of different spices extract during bread making process and found that the addition of extract has a positive effect for the crust color of sourdough breads.

Symmetry of form is an important bread parameter in deciding the characteristics like uneven top, low ends and shrunken sides of the bread. The results pertaining to the effect of different clove methanolic extract concentration indicated that scores assigned to evenness of bake differ significantly due to variation in extract concentration (Table 5). It is evident from the results that breads prepared from T_0 clove extract got significantly the highest scores. The scores for symmetry of form were assigned to be the lowest (3.3) to the breads prepared form T_5. It was also evident from the data that the score for symmetry of form decreased proportionally with increase in the concentration of clove extract. The results of present study are in in agreement with the findings of Chavan et al. (1991) who reported that the score assigning to symmetry of form of bread decreased as the concentration level of methanolic extract increased. Burt (2007) also found that spices extracts significantly reduce the score for symmetry of form of bread.

The evenness of bake reflects that all sides including top and the bottom are uniformly baked and it also reflects the intensity of baking whether the sides having lighter or darker shade. The results pertaining regarding the effect of clove methanolic extract indicated that scores assigned to evenness of bake showed non significant variations (Table 5). The results for the evenness of bake of breads indicated that evenness of bake among breads varied from 2.0 to 2.6 prepared from different cocentration of methanolic extract. The scores assigned

to evenness of bake decrease significantly. The loaf should be evenly baked on all sides, including the bottom. Pan breads should be evenly colored with no light or burned spots. The shade of the sides and bottom should conform to that of the crust.

The results regarding scores given by judges to crust character of sourdough breads made from clove methanolic extract at different concentration have been presented in Table 5. The results showed that methanolic extract exhibited highly significant effect on scores given to crust character of the breads. The mean scores assigned by the panelists to the crust character of breads prepared from methanolic extract with the difference in concentration showed that the breads prepared from T_0 and T_1 got the highest scores 2.90 and 1.80 respectively for crust character.

Internal characteristics of bread

The results (Table 6) showed that methanolic exhibited highly significant effect on scores given to grain of the breads. The scores assigned by the panelists to the grain of breads prepared from clove extract with the difference in concentration ranged from 7.20 to 14.00 among the breads. The breads from T_5 (7.20) got significantly lower scores while maximum scores were given to the control breads (14.0). The breads prepared from T_1 and T_2 got almost similar scores for grain.

A soft creamy white crumb color is preferred in white breads. However, in some geographic areas, a bright white color is also preferred for the bread crumb. The results for the effect of clove methanolic extract on crumb color of breads have been given in Table 6 indicated that clove extract showed significant effect on crumb color of the breads. The crumb color got significantly the highest scores for breads prepared from the T_0 (8.40) followed by the T_1 bread (7.50). The scores given to the breads decreased with the increase in extract concentrations. The results for crumb color of bread prepared from different concentration levels of clove extract indicate that clove extract significantly affected the crumb color of breads. The scores assigned by the panelists to the crumb color of breads prepared from different concentration of extract. Results indicated that breads prepared with T_0 got

Table 7. Effect of clove methanolic extract on Total Plate Count of bread.

Treatment	Mean
T_0	6.5×10^2
T_1	5.6×10^2
T_2	5.0×10^2
T_3	4.4×10^2
T_4	3.4×10^2
T_5	3.1×10^2

T_0= Control (without methanolic extract), T_1= 0.5% methanolic extract, T_2= 1.0% methanolic extract, T_3= 1.5% methanolic extract, T_4= 2% methanolic extract, T_5= 2.5% methanolic extract.

significantly the highest scores (8.40) for crumb color while the breads prepared with T_5 were ranked at the bottom (3.30) by the judges. It is also evident from the results that breads prepared from T_1 is close to control value followed by T_2 extract concentration.

The results of aroma (Table 6) showed that aroma of breads prepared from different clove extract concentrations significantly affected the scores given to aroma of breads. The scores assigned to the aroma of different breads indicated that breads prepared from T_0 extract got statistically the highest scores (8.7) for aroma followed by breads from T_1 (7.10) whereas the minimum aroma scores were assigned to the breads prepared from T_5. The results are in concordance to the findings of Meena (1994) who reported that aroma of the food products decreased as the concentration of extract increased.

The results of scores allocated to taste of the bread samples prepared from different clove extract concentrations (Table 6) indicated significant effect of clove extract. The scores assigned to taste of breads prepared from clove extract showed that the breads prepared from control i.e. T_0 extract got the highest scores (15.30) for taste by the panelists. It is obvious from the results that breads prepared from control were graded at the bottom with respect to taste scores. The results in Table 6 further exposed that breads prepared from T_1 (14.80) and T_2 (13.30). There was a decline in assigning the scores to breads by increasing the level of extract concentration.

Texture and appearance are two major sensory characteristics of the cereal products. The textural properties of a food has been described as that group of physical characteristics that are sensed by the feeling of touch, disintegration and flow of the food under the application of a force, time and distance. The resuts for texture of breads prepared from different extract concentrations (Table 6) indicated that scores given to texture of breads differed significantly due to differences in extract concentration level. The scores given to the texture of breads prepared with extract indicated that the breads from T_0 extract got the maximum scores for texture (13.80) and the scores decreased progressively as the level of extract

concentration increased. The breads prepared from T_5 got the minimum scores by the judges for texture.

Microbial analysis

Total plate count

The results pertaining to total plate count of bread have been presented in the Table 7. The results indicated the highly significant values for Total plate count of bread prepared with clove methanolic extract.

The results indicated that the total plate count of all the bread samples decreased significantly. Decrease in the total plate count occurred from 6.5×10^2 to 3.1×10^2 for all breads. Bread with clove methanolic extract T_1 (0.5%) had TPC value 5.6×10^2. TPC for bread with 1% extract decreased from 6.5×10^2 to 5.6×10^2 and 5.6×10^2 to 3.1×10^2 from the rest. The best results of TPC were obtained from the bread made with 0.25% methanolic extract. Additions of clove extract which is used as anti-microbial substance, showed decrease in the growth rate of microorganisms.

It is evident from the results that there was decrease in the total plate count occurred as the extract level increased. All the results were in the agreement with the Lewis and ausubel (2006) and Lane et al. (1991) who showed there was significant effects on spices extract addition on the TPC of bread.

Conclusion

The preliminary study of this project revealed that spices can be used as antural antimicrobial in food products due to their less lethal effects as compared to synthetic chemical additives. Consumers are are very conscious about food safety nowadays. The Present study reveals that there is a great potential of using spices extract as natural antimicrobials for controlling food spoilage and pathogenic bacteria. Our results signify the fact that natural products like spices can be seen as alternatives to chemical preservatives used in various food industries so as to minimize their side effects and simultaneously improving the shelf life of the food products. The inhibitory factor responsible for the antimicrobial activity can further be identified and used as an alternative to currently used drugs against the pathogenic microbes. Nowadays microbes are increasingly developing resistance against the drugs in use. To combat against these drug resistant microbes, a large library of novel compounds is required. Natural products from plants may give us a solution to this alarming problem.

REFERENCES

AACC (2000). Approved Methods of the American Association of Cereal Chemists. American Association of Cereal Chemists, Inc; St Paul, Minnesota, USA. antimicrobial systems (pp. 265–295). Boca Raton, Florida: CRC Pre

AOAC (2007). Official Methods of Analysis of Association of Official Analytical Chemists International. In: Horwitz, W. (Ed.), 17th ed. AOAC Press, Arlington, VA, USA.

Becker N, Petrie D, Zgomba M, Boase C, Dahl C, Lane J, Kaiser A (2003). Mosquitoes and their control. Kluwer Academic/Plenum Publishers, New York.

Bevilacqua A,Corbo MR, Sinigglia M (2010). Combining eugenol and cinnamaldehyde to control the growth of *Alicyclobacillus acidoterrestris*. Food Control 21(2): 172–177.

Burt S (2004). Essential oils: their antibacterial properties and potential applications in foods – a review. Int J Food Microbiol 94: 223–253.

Burt SA, Der Zee RV, Koets AP, De Graaff AM, Van F, Knapen WG (2007). Carvacrol induces heat shock protein 60 and inhibitssynthesis of flagellin in Escherichia coli O1 57:H7. Appl. Environ. Microbiol. 73: 4484–4490.

Cappuccino JG, Sherman N (1996). Microbiology: A Laboratory Manual. The Benjamin Cummings Pub. Co. Inc. New York.

Chaieb K, Zmantar T, Ksouri R, Hajlaoui H, Mahdouani K, Abdelly C, Bakhrouf A (2007b). Antioxidant properties of the essential oil of Eugenia caryophyllata and its antifungal activity against a large number of clinical Candida species. Mycoses 50: 403–406.

Chavan JK, Shinde VS, Kadam SS (1991). Utilization of expeller pressed partially defatted peanut cake meal in the preparation of bakery products. Plant Foods Hum. Nutr., 41(3):253-9.

Clarke CI, Chober TJ,Angst E, Arendt EK (2003). Use of response surface methodology to investigate the effects of processing conditions on sourdough wheat bread quality. Eur. Food Res. Technol. 217:23-33.

Collins NF, Kirshner LAM, von Holy A (1991). A characterization of *Bacillus* isolates from ropy bread, bakery equipment and raw materials. S. Afr. J. Sci. 87:62–66.

Cowan MM (1999). Plant products as antimicrobial agents. Clin. Mic.Rev. 12: 564-586.

Dorman HJD, Surai D, Deans SG (2000). *In vitro* antioxidant activity of a number of plant essential oils and phyto constituents. J. Essential Oil Res. 12: 241–248.

Dragland S, Senoo H, Wake K (2003). Several culinary and medicinal herbs are important sources of dietary antioxidants. *J Nutr* 2003; 133: 1286-1290.

Gutierrez J, Barry-Ryan C, Bourke P (2009). Antimicrobial activity of plant essential oils using food model media: Efficacy, synergistic potential and interactions with food components. Food Microbiol. 26: 142–150.

Hammer KA, Carson CF, Riley TV (2000). Antifungal effects of Melaleuca alternifolia (tea tree) oil and its components on Candida albicans, Candida glabrata and Saccharomyces cerevisiae. J Antimicrob Chemother 53, 1081–1085.

Harrigan WF, McCance ME (1976). Laboratory Methods in Food and Dairy Microbiology. Academic Press Inc. London.

Holley RA, Patel D (2005). Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. Food Microbiol. 22(4).

Hussain, S. 2009. Utilization of flaxseed as a functional food. Ph.D. Thesis, Deptt. National Institute of Food Sci. Technol., Uni. Agric. Faisalabad.

Kildeaa MA, Allanb GL, Kearney RE (2004). Accumulation and clearance of the anaesthetics clove oil and AQUI-S_ from the edible tissue of silver perch (Bidyanus bidyanus). Aquaculture 232: 265–277.

Kim HM, Lee EH, Hong SH, Song HJ, Shin MK, Kim SH Shin TY (1998). Effect of *Syzygium aromaticum* extract on immediate hyper-sensitivity in rat. J. Ethnopharmacol. 60: 125-131.

Kim HK, Kim JR, Ahn YJ (2003). Acaricidal activity of clove bud oil compounds against Dermatophagoides farinae and Dermato-phagoides pteronyssinus (Acari: Pyroglyphidae). J. Agric. Food Chem. 51, 885–889.

Kim SY, Kim JH, Kim SK, Oh MJ, Jung MY (1994). Antioxidant activities of selected oriental herb extracts. J. Am. Oil Chem. Society, 71: 633–640.

Kumar R, Dubey NK, OP, Tiwari YB. Tripathi, Sinha KK (2007a). Evaluation of some essential oils as botanical fungitoxicants for the protection of stored food commodities from infestation. J. Sci. Food Agric. 87:1737–1742.

Land DG, Shepherd R (1988). Scaling and ranking methods. In: Sensory Analysis of Foods. Piggott JR, ed. Elsevier Applied Science, New York. p. 155-185.

Lane BW, Ellenhorn MJ, Hulbert TV (1991). Clove oil ingestion in an infant. Hum. Exp. Toxicol. 1991: 10:291–294.

Lee KG, Shibamoto T (2000). Antioxidant property of aroma extract isolated from clove buds [*Syzygium aromaticum* (L.) Merr. Et Perry]. Food Chem. 74: 443-448.

Lewis K, Ausubel F (2006). Focus on antibacterials. Nature Biotech. 24(12):1453 1602

Meena, M.R. and V. Sethi. 1994. Antimicrobial activity of essential oils from spices. J. Food Sci. Technol. Mysore 31(1), 68–70.

Microscopic Examination and Staining of Microorganisms and Appendix 3: Preparations of stains and reagents. In: Baker FJ, Breach MR, editors. Medical Microbiological Techniques. Butterworth Heinemann; 1980. pp. 14, 509.

Miyazawa M, Hisama M (2003). Antimutagenic activity of phenyl-propanoides from clove (Syzygium aromaticum). J. Agric. Food Chem. 51(22): 6413-6422.

Mytle N, Anderson GL, Doyle MP, Smith MA (2006). Antimicrobial activity of clove (Syzygium aromaticum) oil in inhibiting *Listeria monocytogenes* on chicken frankfurters. Food Control. 17, 102–107.

NCCLS (2000). Performance standards for antimicrobial disk susceptibility tests. Approved standard, 7th ed. NCCLS document M2-A7. NCCLS, Wayne, Pa. www.nccls.org.

Ogata M, Hoshi M, Urano S, Endo T (2000). Antioxidant activity of eugenol and related monomeric and dimeric compounds. Chem. Pharm. Bull. 48: 1467–1469.

Piga A, Catzeddu P, Farris S, Roggio AT, Sanguinetti ES (2005). Texture evaluation of Amaretti cookies during storage. Eur. Food Res. Technol. 221: 387-391.

Priest FG, Goodfellow M, Todd C (1988). A numerical classification of the genus *Bacillus*. J. Gen. Microbiol. 134: 1847-1882.

Proestos C, Boziaris I, Kapsokefalou SM, Komaitis M (2008). Natural antioxidant constituents from selected aromatic plants and their antimicrobial activity against selected pathogenic microorganisms. Food Technol. Biotechnol., 46: 151–156.

Rocha ANCN, Morais AMMB (2003). Shelf life of minimally processed food apple determined by colour changes. Food Controls. 14:13-20.

Saeed S, Tariq P (2007). Antimicrobial activities of Emblica officinalis and Coriandrum sativum against Gram-positive bacteria and *Candida albicans*. Pak. J. Bot., 39(3): 913-917.

Shobana S, Naidu KA (2000). Antioxidant activity of selected Indian spices Prostaglandins, Leukotrienes and Essential Fatty Acids, 62(2): 107-110.

Skandamis P, Koutsoumanis K, Fasseas K, Nychas GJE (2001). Inhibition of oregano essential oil and EDTA on E.coli O157:H7. Italian J. Food Sci. 13 55–65.

Soliman KM, Badeaa RI (2002). Effect of oil extracted from some medicinal plants on different mycotoxigenic fungi. Food and Chemical Toxicol. 40: 1669– 1675.

Srivastava AK, Srivastava SK Syamsundar KV (2005). Bud and leaf essential oil composition of *Syzygium aromaticum* from India and Madagascar. Flav. Fragr. J. 20: 51-53.

Steel RGD,Torrie JH, Dicky DA (1997). Principles and Procedures of Statistics. A Biometrical Approach. 3rd Edi. McGraw Hill Book Co. Inc., New York.

Velluti A, Sanchis V, Ramos AJ, Marı'n S (2003). Inhibitory effect of cinnamon, clove, lemongrass, oregano and palmarose essential oils on growth and fumonisin B1 production by *Fusarium proliferatum* in maize grain. Int. J. Food Microbiol. 89: 145-154.

Wilson R (1995). Aromatherapy for vibrant health and beauty. In: A guide to understanding and using aromatherapy for vibrant health and beauty. Garden City Park, New York: Avery Publishing group. ISBN: 08952-627-6. (http://www.oiganic.com).

Full Length Research Paper

Influence of multi drug resistance Gram negative bacteria in liver transplant recipient

Mohamed Abdel Aziz[1], Iman El-Kholy[2], Amin Abdo[3] and Samy Selim[1,4]*

[1]Microbiology and Botany Department, Faculty of Science, Suez Canal University, Ismailia, P.O. 41522, Egypt.
[2]Ain Shams University Specialized Hospital, Cairo, Egypt.
[3]Gomohory Hospital, Ministry of Health, Yemen.
[4]Department of Medical Laboratory Sciences, College of Applied Medical Science, Al Jouf University, Sakaka, P.O 2014, Saudi Arabia.

Infection is a common cause of morbidity and mortality after liver transplantation, which are often caused by Gram negative bacteria and the most frequently occurring infectious complications after liver transplantation (LT). The aim of this study was to investigate, incidence, pathogenic spectrum, and risk factors for bacteria due to multidrug resistant (MDR) Gram-negative bacteria, and its impact on mortality after LT. In total, 150 consecutive patients who underwent liver transplantation between January 2012 and March 2013, 115 isolates of bacteria and 10 isolates fungal from 80 patients. MicroScan® microbiology and API20 were used to identify the all isolates. Bacterial infections represented the most frequent event (92.0%) and fungal infections (8.0%). The presumed sources of infection were the Urine (41 events, 51%), followed by the bail drain (14 events, 17.5%). Infections of the sputum, ascetic fluid, and blood site accounted for 13.8, 5.0 and 6.3%, respectively. Antibiotics the most susceptible antibiotic against *Pseudomonas aeruginosa* was colistin (94.1%). Patients with Gram-negative bacilli after liver transplantation show a significantly worse prognosis. Gram-negative bacteria after liver transplantation have been a major problem in our center.

Key words: Gram-negative bacteria, multi drug resistance, liver transplantation, mortality.

INTRODUCTION

Infection is a common cause of morbidity and mortality after liver transplantation (LT). The effect of infections in LT is higher compared with recipients of other organs. Bacterial infections are among the most relevant causes of morbidity and mortality after organ transplantation. The high prevalence of multidrug-resistance among bacterial pathogens causing infections in solid organ transplant recipients is an additional concern. In a recent report, infections with multidrug-resistant Gram negative bacteria were associated with higher mortality among liver transplant recipients (Shi et al., 2009). Emergence of nosocomial infections, especially *Acinetobacter baumannii,*

Pseudomonas aeruginosa with multidrug resistance have become a major problem among transplant recipients in hospital settings (Diab et al., 2002, 2004; Patel et al., 2010). These infections can result in a wide range of complications, including bacteremia, pneumonia, urinary tract infection and peritonitis. The aim of this study was to investigate the clinical characteristics, mortality, and outcomes among liver transplant recipients with Gram-negative infection. Over the past 30 years, colistin use has been limited due to concerns regarding its toxicity along with the development of newer antibiotics with better safety profiles (Jain et al., 2004). However, the increasing

*Corresponding author. E-mail: sadomm2003@yahoo.com.

Table 1. Microorganisms distribution of 125 isolates in 80 patients after liver transplant (L T) recipients.

Microorganisms isolated	Total number of Microorganisms isolated (%)
Bacteria	**115 (92%)**
Pseudomonas aeruginosa	34 (29.6%)
Klebsiella pneumonia	25 (21.7%)
Escherichia coli	24 (20.9%)
Acinetobacter spp	13 (11.3%)
Staphylococci	12 (10.4%)
Coagulase-negative staphylococcus	5
Staphylococcus aureus	7
Enterobacter spp	5 (4.3%)
Aeromonas hydrophila	2 (1.7%)
Fungi	**10 (8%)**
Candida species	7 (70%)

incidence of multidrug-resistant *A. baumannii* in addition to a lack of new antimicrobial agents has reawakened interest in the utilization of colistin due to its good activity against this organism.

MATERIALS AND METHODS

In total, 150 consecutive patients (50 female and 100 male) who underwent liver transplantation between January 2012 and March 2013 at the Ain Shams University Specialized Hospital, Cairo, Egypt, were enrolled in this study. Patients range age from 18 to 70 years. Specimens included blood, pus, tissues and body fluids, urine, sputum and peritoneal dialysate.

The strains had been isolated on blood agar, desoxycholate citrate agar, thiosulphate citrate bile salts sucrose agar tests for growth at 30, 37°C were performed in brain heart infusion broth in a water bath. Sheep blood (5%) agar plates were used to detected hemolysis, gelatin stab method was used to test for gelatin lique-faction. The susceptibility tests were read after overnight incubi-tion, and the tests for hemolysis and growth at 30, 37°C were read on the first and second day. For gelatin liquefaction the incubation temperature was 22°C. Antimicrobial susceptibility was determined by the Kirby- Bauer disk diffusion method (Selim, 2011; Selim et al., 2012, 2013). The interpretive criteria followed the latest National Committee for Clinical Laboratory Standards (NCCLS) recommend-dations (NCCLS, 2002). Identified at least to genus level by MicroScan® microbiology, biochemical tests and API 20E system (bioMe´rieux, France), in a previous study and cryopreserved in 20% glycerol at -80°C. Working cultures were maintained in Luria Bertani (LB) agar and broth. We collected data from hospital and operative records of each liver transplantation recipient, including perioperative demographic and clinical characteristics (that is, age, gender, Child-Pugh), operative variables (that is, operation time, blood loss) and clinical events with 6 months post transplant (that is, duration of initial intubation, intensive care unit (ICU) stay, reoperation, dialysis, and rejection).

Statistical analysis

The data was analyzed using Chi-square test, Z-test and two way classification of ANOVA.

RESULTS AND DISCUSSION

In total, 150 patients (median age 40 years) were inclu-ded in this study and contributed a total of 64.4, 80 patients positive infection, events 70 cases are negative. 125 isolated, bacterial infections represented the most frequent (115 events, 92%). followed by fungal infections (10 events, 8.0 %).

Bacterial infections

Among the bacterial infections, the most frequent patho-gen was *Pseudomonas aeruginosa* with 34 isolates (29.6%).The second most common isolated organism was *Klebsiella pneumonia* (25 events, 21.7.0%), followed by *E. coli* (24 events, 20.9%), *Acinetobacter baumannii* (13 events, 11.3%), *Staphylococcus* spp (12 events, 10.4%), *Enterobacter* spp (5 events, 4.3%), *Aeromonas* spp (2 events, 1.7%) The majority of the bacterial infec-tions occurred during the first 6 months, with the highest incidence of infection during the first 30 days (30 events/ month), Table 1.

Fungal infections

In total, 10 events of fungal infections were documented. In 7 cases (70.0%), Candida species were isolated (5 events of *Candida albicans* and 2 cases with *Candida kruseii*).. Infections with *Aspergillus* accounted for 30 %of fungal infections (3 cases of *Aspergillus fumigatus*). One case with pulmonary involvement was documented during the first 30 days.

Manifestation of infection

The most common site of pathogen isolation overall was the urine (41 events, 51%), followed by the bail draine

Table 2. Postoperative Gram negative bacteria isolates from various sites in 80 patients after LT.

Site	*Pseudomonas aeruginosa* 34 in (25)	*Klebsiella pneumonia* 25 in (20)	*E.coli* 24 in (20)	*Acinetobacter* spp. 13 in (9)	*Aeromonas hydrophila* 2 in (2)	*Enterobacte* Spp. 5 in (4)	Total
Urine	16 (15)	13(11)	12(11)	2(2)	1(1)	2(1)	46(41)
Bile drain	9 (5)	3(2)	4(3)	3(2)	1(1)	1(1)	21(14)
Sputum	4(2)	5(4)	3(3)	2(1)	None	1(1)	15(11)
Fluid	None	2(1)	2(1)	3(2)	None	None	7(4)
Blood	3(2)	1(1)	None	1(1)	None	1(1)	6(5)
Abdominal	2(1)	1(1)	3(2)	2(1)	None	None	8(5)
Total	34(25)	25(20)	24(20)	13(9)	2(2)	5(4)	103(80)

Total = isolates of bacteria/number of patients.

Table 3. Antibiotic resistance profiles of predominant Gram-negative bacilli in 80 recipients after LT.

Antimicrobial agents	*Pseudomonas aeruginosa* (n=34)	*Klebsiella pneumonia* (n=25)	*E. coli* (n=24)	*Acintobacter* spp (n=13)	*Enterobacter* spp (n=5)	*Aeromonas hydrophila* (n=2)
Amoxicillin-clavulanate	17.6%(6/34)	20%(5/25)	16.7%(4/24)	92.3%(12/13)	40%(2/5)	0%(0/2)
Ampicillin-sulbactam	23.5%(8/34)	20%(5/25)	20.8%(5/24)	100%(13/13)	20%(1/5)	0%(0/
Piperacillin-tazobactam	11.8%(4/34)	16%(4/25)	20.8%(5/24)	100%(13/13)	60%(3/5)	100%(2/2)
Ceftriaxone	17.6%(6/34)	28%(7/25)	25%(6/24)	92.3%(12/13)	20%(1/5)	50%(1/2)
Cefepime	20.6%(7/34)	16%(4/25)	16.7%(4/24)	84.6%(11/13)	20%(1/5)	50%(1/2)
Ceftazidime	17.6%(6/34)	16%(4/25)	16.7%(4/24)	100%(13/13)	20%(1/5)	50%(1/2)
Imipenem	94.1%(32/34)	80%(20/25)	83.3%(20/24)	100%(13/13)	100%(5/5)	100%(2/2)
Meropenem	88.2%(30/34)	88%(22/25)	83.3%(20/24)	100%(13/13)	100%(5/5)	100%(2/2)
Gentamicin	44.1%(15/34)	52(13/25)	62.5%(15/24)	100%(13/13)	40%(2/5)	100%(2/2)
Amikacin	11.8%(4/34)	8%(2/25)	8.3%(2/24)	100%(13/13)	20%(1/5)	0%(0/2)
Netilmicin	14.7%(5/34)	20%(5/25)	8.3%(2/24)	92.3%(12/13)	40%(2/5)	50%(1/2)
Colistin	5.9%(2/34)	72%(18/25)	29.2%(7/24)	7.7%(1/13)	20%(1/5)	0%(0/2)

Values in the parentheses are number (n) of antibiotic resistance isolates/number of total isolates tested, respectively.

(14 events, 17.5%). Infections of the sputum, blood and ascetic fluid site accounted for 13.8, 6.3 and 5.0%, respectively. Other common sites of infection included the abdominal (6.3%) (Table 2).

Antimicrobial resistance of main Gram-negative bacilli

The antibiotic resistance patterns of predominant Gram negative bacilli were listed in Table 3. The most susceptible antibiotic against *p. aeruginosa* was colistin (94.1%). Imipenem was the most resistance antibiotic against all isolates (80 to 100%).

All antimicrobial agents showed relative low susceptibility against *Acinetobacter* spp except colistin. Piperacillin/tazobactam (84%) high antibiotic susceptibility to *K. pneumonia*.

Phenotypic identification

All isolates were subjected to simplified phenotypic tests as described in Table 4. Briefly, tests for growth at 30, 37°C were performed in brain heart infusion broth in a water bath. Sheep blood (5%) agar plates were used to detected hemolysis, gelatin stab method was used to test for gelatin liquefaction. The susceptibility tests were read after overnight incubation, and the tests for haemolysis and growth at 30, 37°C were read on the first and second day. For gelatin liquefaction the incubation temperature was 22°C.

DISCUSSION

Studies of infectious complications after liver transplantation exist in the literature (Piselli et al., 2007).

Table 4. Phenotypic characteristics of Gram negative bacteria isolates from patients after LT.

Parameter	*Pseudomonas aeruginosa* N=34	*Klebsiella pneumonia* N=25	*E.coli* N=24	*Acinetobacter* spp. N=13	*Aeromonas hydrophila* N=2	*Enterobacter* spp. *N=5*
Hemolysis	+	-	-	2	**1**	-
Lipase	+	-	-	-	-	-
Proteolytic	+	-	-	-	-	-
Gelatinase	+	-	-	-	-	-
Catalase	+	+	+	+	+	+
Congo red	30	23	20	11	2	5

N= number of isolates.

However, most of these data are older than 10 years and all of the studies were performed in the United States and Europe. Therefore, we analyzed the incidence, presentation, and risk factors of infections in liver transplant recipients in Egypt population treated between 2012 and 2013, and compared our findings with data worldwide.

The 1-year survival in our population was 89%, similar to that of other transplant groups. The incidence of infection during the period of 12 months was comparable to data by an Italian study (Garbino et al., 2005), who reported infections in 56% of patients during the first year, and somewhat lower than data surveyed by Kusne et al. (1998) from the University of Pittsburgh Medical Center between 1984 and 1985, who found infections in83% of the patients, or results from a Swiss single-center study, who reported that 80% of patients developed infections after liver transplantation (Garbino et al., 2005).

Bacterial infections were the most frequent event in our population, as found by other groups (9, 4). In our study, the proportion of bacterial events was higher (92.0%) in comparison with 47% observed in the Swiss study (Garbino et al., 2005), 48% reported by Torbenson et al. (1998), about 54% at the Pittsburgh Medical Center, or 57% in the Italian study (Piselli et al., 2007). Infection by Gram-negative enteric organisms was the main cause of bacterial infection in our population, which shows that the epidemiology of infections at our hospital is similar to those of Western Europe and the United States. In accordance with other studies (Piselli et al., 2007, Garbino et al., 2005), most bacterial infections occurred within the first month, and the bloodstream was the leading infection site. Other significant locations were urinary tract infections, and abdominal and surgical site infections.

Fungal infections accounted for 8.0% in our patients which compared with other studies that showed infection rates between 12 and 29% (Piselli et al., 2007). Pappas et al. (2006) reported a rate of 4% of invasive fungal infections during the first 100 days post transplantation. Most of the infections in our study were attributed to Candida species and peaked during the first month. In summary, liver transplant recipients within the first month are most susceptible to nosocomial infections similar to those seen in non-immunosuppressed surgical patients.

Positive cultures by routine investigations, obtained according to the protocols of harvesting systematic samples after the transplant, or the identification of a microorganism of the normal human flora were not taken into account, unlike some studies that included such events (Dawwas et al., 2007). Multiple screening cultures likely result in an over diagnosis of infection; however, screening can be the only early guidance for early treatment of infection.

The literature lacks randomized controlled studies for bacterial prophylaxis comparing the efficacy of different antibiotic regimens in transplant recipients. Most centers used amoxicillin-clavulanate or a second- or third-generation cephalosporin against bacterial infections. We used amoxicillin-clavulanate in combination with gentamicin, a standard prophylaxis, and reported an infection rate that was comparable with data worldwide. As described previously, most infections were caused by Gram-negative aerobic bacteria such as *E. coli*, which are inhabitants of the digestive tract.

However, the incidence of multiple-antibiotic-resistant bacteria is increasing, so the choice of prophylaxis also has to take into consideration the bacterial isolates that are commonly present in the center performing the transplantation. Therefore, it is difficult to compare our results with other studies reports regarding prophylaxis and infection rate, because many other variables affect infection rates. In one of the largest studies with more than 1200 patients, more than 8 types of antibiotics or antibiotic combinations were used for surgical prophylaxis (Asensio et al., 2008); however, the most important risk factor for surgical site infection was the choledochojejunal reconstruction (Asensio et al., 2008).

The recent study could confirm the data by other groups and supports the impact of post-transplant infections on morbidity and mortality of liver recipients. Infections were mainly of bacterial origin and occurred more frequently during the early post-transplant period. The bloodstream, abdomen, urinary tract, and drain were the most common sites of infection. Parenteral nutrition and a prolonged stay in the ICU were associated significantly with an increased infection rate. Early detection and treatment of infections is essential to obtaining a better outcome in

liver transplant patients. The choice of prophylactic regimen has to take into account the bacterial isolates that are commonly present in the center performing the transplantation. Our results provide additional insight into the risk of infection after liver transplantation and could motivate performance of new studies concerning the understanding and improved prophylaxis of post-transplant infections.

Conclusion

Infection due to MDR Gram-negative bacilli are common after LT, and associated with allograft acute rejection, post-transplant reoperation, and abdominal infection. Patients with MDR Gram-negative bacillus have significantly worse prognosis, and are associated with pre LT bacterial infections, renal dysfunction, post- LT abdominal infection, and prolonged endotracheal intubation. The increasing isolates of MDR Gram-negative bacilli pose a great challenge for clinical treatment.

REFERENCES

Asensio A, Ramos A, Cuervas-Mons V (2008). Effect of antibiotic prophylaxis on the risk of surgical site infection in orthotopic liver transplant. Liver Transpl. 14: 799-805.

Dawwas MF, Gimson AE, Lewsey JD, Copley LP, Meulen JH (2007). Survival after liver transplantation in the United Kingdom and Ireland compared with the United States Gut. 56: 1606-1613.

Diab AM, Abdel Aziz MA, Selim SA (2002). Plasmid encoded transferable antibiotic resistance in Gram-negative bacteria isolated from drinking water in Ismailia city. Pak J. Biol Sci, 5(7):774-779.

Diab AM, Abdel Aziz MH, Selim SA, El-Alfay S., Mousa MA (2004). Distribution, Involvement and Plasmid Characterization of Aeromonas spp. Isolated from Food Staffs and Human Infections. Egyptian J. Biol. 6:12-20.

Garbino J, Romand JA, Pittet D, Giostra E, Mentha G, Suter P (2005). Infection and rejection in liver transplant patients: a 10-year Swiss single- centre experience. Swiss Med Wkly.135: 587-593.

Jain R, Danziger LH (2004). Multidrug-resistant *Acinetobacter* infections: an emerging challenge to clinicians. Ann Pharmacother. 38: 1449–1459.

National Committee for Clinical Laboratory Standards (NCCLS) (2002). Performance Standards for Antimicrobial Susceptibility Testing. 12th Informational Supplement. M100 -S12.Wayne, PA: NCCLS.

Pappas PG, Andes D, Schuster M (2006). Invasive fungal infections in low-risk liver transplant recipients: a multi-center prospective observational study. Am J Transplant. 6: 386-391.

Patel G, Perez F, Bonomo RA (2010). Carbapenem-resistant Enterobacteriaceae an *Acinetobacter baumannii* assessing their impact on organ transplantation. Curr. Opin. Organ. Transplant. 15: 676.

Piselli P, Zanfi C, Corazza V (2007). Incidence and timing of infections after liver transplant in Italy. Transplant Proc. 39: 1950-1952.

Selim SA (2011). Chemical composition, antioxidant and antimicrobial activity of the essential oil and methanol extract of the Egyptian lemongrass Cymbopogon proximus STAPF. Int. J. Fats Oils (Grasas y Aceites), 62(1):55-61.

Selim SA, El Alfy S, Al-Ruwaili M, Abdo A, Al Jaouni S (2012). Susceptibility of imipenem-resistant Pseudomonas aeruginosa to flavonoid glycosides of date palm (*Phoenix dactylifera* L.) tamar Growing in Al Madinah, Saudi Arabia. Afr. J. Biotechnol. 11(2):416-422.

Selim SA, Abdel Aziz M.H, Mashait MS, Warrad MF (2013). Antibacterial activities, chemical constitutes and acute toxicity of Egyptian *Origanum majorana* L., *Peganum harmala* L. and *Salvia officinalis* L. essential oils. J. Pharm. Pharmacol. 7(13):725-735.

Shi SH, Kong HS, Xu J, Zhang WJ, Jia CK, Wang WL, Shen Y, Zhang M, Zheng SS (2009). Multidrug resistant Gram-negative bacilli as predominant bacteremic pathogens in liver transplant recipients. Transpl Infect. Dis. 11: 405–412.

Torbenson M, Wang J, Nichols L, Jain A, Fung J, Nalesnik MA (1998). Causes of death in autopsied liver transplantation patients. Mod. Pathol. 11: 37-46.

Full Length Research Paper

Phosphate solubilization by a few fungal strains belonging to the genera *Aspergillus* and *Penicillium*

Jyoti Saxena[1], Paramita Basu[2]*, Vanaja Jaligam[3] and Shalini Chandra[4]

[1]Biochemical Engineering Department, BT Kumaon Institute of Technology, Dwarahat, Uttarakhand 263653, India.
[2]Department of Pharmaceutical and Biomedical Sciences, Touro College of Pharmacy, New York, NY 10027, USA.
[3]Charles River Laboratories, 20401 Seneca Meadows Pkwy Germantown, MD 20876, USA.
[4]Department of Statistics, Banasthali University, Distt. Tonk, Rajasthan 304022, India.

Many phosphate solubilizing fungi (PSF) are found in soil and their introduction in the rhizosphere of crops not only increases the availability of phosphorus from insoluble sources of phosphate but also increases the efficiency of phosphate fertilizers such as superphosphate and rock phosphate. Studies on 2 strains belonging to *Aspergillus niger*, and 1 strain each of *Aspergillus flavus*, *Penicillium aurantiogriseum* and *Penicillium claviformis* with special reference to phosphate solubilization were performed in this communication. All the fungal strains showed halo zone around them on Pikovskaya plates. Quantitative estimation of 3 indigenous strains along with the other 2 non-indigenous strains taken as reference strains was done in two nutrient broths namely: Czapek's Dox and Pikovskaya containing dicalcium phosphate (DCP) as insoluble source of phosphorus. Out of the two media selected for the study, Pikovskaya broth supported better solubilization. The insoluble phosphates DCP, TCP (tricalcium phosphate) and hypt (hydroxyapatite) were tested, DCP was least solubilized by all strains but there was no significant difference in solubilization of TCP and hydroxyapatite. There was significant negative correlation between pH and phosphate solubilized in all forms of insoluble phosphates. Types of nitrogen sources and metal ions were also screened during the study. $(Na_4)_2SO_4$ supported more solubilization than $NaNO_3$, whereas phosphate solubilizing activity decreased (except in *A. niger* ATCC 282) in presence of Mn^{+2} and Fe^{+3} but presence of Al^{+3} did not have statistically significant effect on solubilization. Though various species of *Penicillium* have been reported for phosphate solubilization in literature but to the best of our knowledge this is first report of phosphate solubilization by *P. claviformis.*

Key words: *Aspergillus, Penicillium,* phosphate solubilisation, dicalcium phosphate, tricalcium phosphate, hydroxyapatite.

INTRODUCTION

Phosphorus (P) is the second most important macro-nutrient required for plant growth next to nitrogen. In particular, Indian soils are poor to medium in available phosphorus (Hasan, 1994). Besides, there is also the problem of its fixation in nature. As soon as it is applied to soil in the form of fertilizer, a large amount of it quickly combines with other chemicals forming compounds which do not release the phosphorus for plants. In acidic soil it is fixed by free oxides and hydroxides of aluminium (Al) and iron (Fe), while in alkaline soil by calcium (Ca). In order to reduce P deficiencies and ensure plant producti-vity, large quantities of expensive chemical phosphate

*Corresponding author. E-mail: paramita.basu@touro.edu.

fertilizers are applied worldwide every year.

Microorganisms play critical role in natural P cycle, and the use of phosphate-solubilizing microorganisms (PSMs) has been proposed as a low-cost input to increase the agronomic effectiveness of insoluble phosphates. Several scientific reports showed that microorganisms such as bacteria, fungi, and actinomycetes were indeed able to promote the P solubilization and increase crop yields (Whitelaw, 2000; Oberson et al., 2001; Hamdali et al., 2008; Minaxi et al., 2010). These PSMs render insoluble phosphate into soluble form through the process of acidification, chelation, and exchange reactions (Assailed et al., 2003).

Out of all microbes, fungi are superior to their bacterial counterpart for P solubilzation both on precipitated agar and in liquid (Kucey, 1983a; Banik and Dey, 1982; Singhal et al., 1994; Whitelaw et al., 1997; Sheshadri and Ignacimuthu, 2004). Fungal hyphae are able to reach greater distances in soil more easily than bacteria. Furthermore, it has been observed that PSB upon repeated sub-culturing lose the phosphate solubilizing activity (Halder et al., 1990a; Illmer and Schinner, 1992) but such losses have not been observed in PSF (Kucey, 1983b).

Among the fungal genera with the phosphate solubilization ability are *Aspergillus, Penicillium, Trichoderma, Mucor, Candida, Yeast, Discosia, Eupenicillium* and *Gliocladium* (Xiao et al., 2008; Rahi et al., 2009). The strains from the genera *Aspergillus* and *Penicillum* are among the most powerful phosphate solubilizers. Solubilization of PSF depends on the insoluble inorganic phosphate source, type of carbon, nitrogen and metal ions in soil, as well as on culture conditions (Kucey, 1983b; Nahas, 2007; Jain et al., 2012).

Thus, in view of above facts, there is an increasing realization to explore the possibilities of utilizing a viable alternative for chemical fertilizers for sustainable agriculture. The present study is an effort to see the solubilization pattern of a few fungi belonging to Aspergilli and Penicillia on insoluble forms of phosphate salts of calcium and the effect of different nitrogen sources and metal ions on P solubilization.

MATERIALS AND METHODS

Organisms

Two known organic acid producing fungal strains namely: *Aspergillus niger* ATCC 282 and *Penicillium aurantiogriseum* MTCC 2285 were obtained from IMTECH, Chandigarh, India in lyophilized state and maintained in laboratory on Czapek's Dox Agar slants at 4°C in a refrigerator. The other 3 strains namely: *A. niger, A. flavus* and *P. claviformis* were isolated from the rhizosphere region of wheat fields of Banasthali, India.

The soil was collected from rhizosphere region of 5 wheat plants of 3 agricultural fields in sterilized polythene bags and composite mixtures of soil were prepared from each field. Serial dilution and enrichment methods were used for isolation of fungi. The phosphate solubilizing species were identified based on morphological characteristics by manuals and expertise available in the department. These were designated as *A. niger* bv, *A. flavus* bv

and *P. claviformis* bv. Due to lowest solubilization capacity of different phosphate sources, *A. flavus* was not selected for further studies.

Media used

The following media have been used:

i) Pikovskaya agar medium obtained from Hi Media Labs was used to determine the halo formation.
ii) To see the effect of 3 different forms of insoluble phosphatesnamely: DCP, TCP and hypt, Pikovskaya broth having following composition Glucose 10.0, Tricalcium phosphate 2.0, $(NH_4)_2SO_4$ 0.5, KCl 0.2, $MgSO_4$. $7H_2O.1$, $MnSO_4$ Trace, $FeSO_4$ Traces, Yeast extract 0.5, Distilled wáter 1000 ml (pH 7.0) was used. All these salts being insoluble in water, 0.5 g of each form of phosphate was added in 100 ml of broth in separate flasks.
iii) For experiments on effect of different nitrogen sources and metal ions modified Czapek's Dox broth was used (composition: Sucrose 30.000, Sodium nitrate 2.000, Dipotassium phosphate 1.000, Magnesium sulphate 0.500, Potassium chloride 0.500, Ferrous sulphate 0.010, Final pH (at 25°C) 7.3 ± 0.2 in which soluble KH_2PO_4 was replaced by insoluble $CaHPO_4.2H_2O$ as it is dominant in alkaline soils of study area). Czapek's being a synthetic medium, it is easy to replace or add different ions. To see the effect of nitrogen source, $NaNO_3$ was replaced by equal amount of $(NH_4)_2SO_4$. For metal ions viz. Fe^{+3}, Al^{+3} and Mn^{+2}, 0.1 g of $FeCl_3$, $Al_2(SO_4)_3$ and $MnSO_4$, respectively were added separately to modified Czapek's Dox broth.

Solubilization of phosphate on solid medium

The strains were subjected to screening test for their phosphate solublization potential. Pikovskaya's medium containing TCP was prepared and poured into sterilized Petri plates. The isolates were spotted on these plates and incubated at 28°C for 4 to 7 days. Those showing halo zones around the colonies were supposed to be phosphate solubilizing ones. Solubilizing efficiency (S.E) was calculated according to Nguyen *et al.* (1992) by following formula:

$$\% \text{ Solubilization efficiency (SE)} = \frac{\text{Diameter of solubilization zone (S)}}{\text{Diameter of the colony}} \times 100$$

Microdetermination of phosphorus:

Two ml of 10^5 spores/ml of sterilized water was used for inoculation of flasks. Control flasks were also prepared with 2 ml of sterilized water instead of spore suspension. The flasks were incubated at 28 ± 2°C and 120 rpm in an incubator shaker for 2, 4, 6, 8, 10 and 12 days. After completion of respective incubation periods, cultures in triplicate were harvested and filtered with Whatman filter paper no. 42 into separate 250 ml beakers to separate the broth containing phosphate from fungal mat. Chen's method (Chen et al., 1956) was used for quantitative estimation of solubilized phosphate. Absorbance at 820 nm was read by spectrophotometer. Standard curve was plotted to determine the values. The pH of fungal filtrate was also taken using a glass electrode pH meter.

All the glassware used for biochemical studies were soaked in chromic acid overnight. Thereafter they were washed with distilled water, rinsed with dilute HCl, washed again with distilled water and then dried in glassware drying oven to remove organic matter from the glasswares, especially phosphorus, if present.

Statistical analysis

A statistical analysis for the comparison of phosphate solubilization
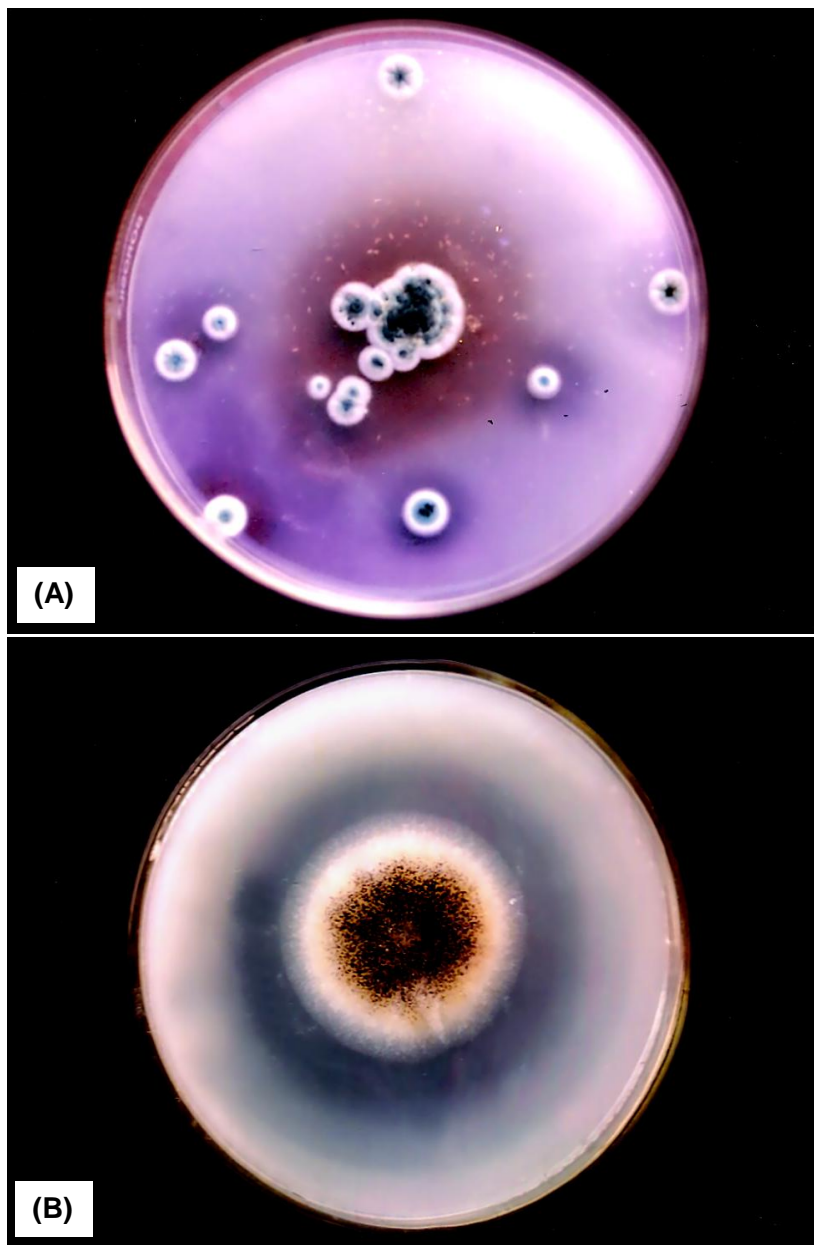
Figure 1. (A) *Aspergillus niger* bv showing halo zone. (B) *Penicillium aurantiogriseum* MTCC 2285 showing halo zone.

process by different strains has been done using two-way and three-way incomplete ANOVA. For the multiple pair wise comparisons Tukey test and l.s.d. were used.

RESULTS

Phosphate solubilization by different strains

The screening of phosphate solubilizing fungi was done on Pikovskaya agar plates. All the fungi selected for the study showed a clear halo zone around them (Figure 1 A and B). *A. flavus* solubilized 46.5 µg/ml DCP and nearly half the amount of TCP and hydroxyapatite, hence

discarded for further studies. The percent phosphate solubilization efficiency of this strain was lowest i.e. 90% in comparison to other strains which was in between 300 to 380%, which shows that percent efficiency was directly related to P solubilized in medium.

Three forms of most commonly found insoluble phosphates namely: DCP, TCP and hydroxyapatite were chosen to see the effect of solubilization by different fungal strains in Pikovskaya broth. The results are depicted in Table 1. In general, the amount of soluble phosphate increased with the increase of incubation period in all types of insoluble phosphates solubilized by all the strains,

Table 1. Comparison of solubilization of different forms of insoluble phosphates.

Strain	Days	Dicalcium phosphate		Hydroxyapatite		Tricalcium phosphate	
		Amount of phosphate solubilized (µg/ml)	pH	Amount of phosphate solubilized (µg/ml)	pH	Amount of phosphate solubilized (µg/ml)	pH
A. niger ATCC 282	0	8.00	7.00	8.50	7.00	9.03	7.00
	2	26.50	5.11	19.00	5.03	16.51	4.78
	4	45.91	3.63	29.12	3.50	27.51	3.39
	6	54.25	3.10	52.14	3.25	32.48	3.14
	8	56.50	2.87	72.23	3.06	63.00	2.98
	10	57.12	2.75	77.40	2.71	67.50	2.62
	12	58.40	2.73	78.11	2.48	**80.22**	2.54
A. niger bv	0	8.00	7.00	8.52	7.00	9.04	7.00
	2	24.22	5.80	17.30	5.75	20.00	4.47
	4	40.12	3.72	25.51	3.43	31.40	2.94
	6	49.71	3.31	47.00	2.97	36.10	2.70
	8	55.73	3.25	62.33	2.75	64.00	2.59
	10	56.81	3.04	68.89	2.25	67.55	2.51
	12	57.41	2.96	72.00	2.20	**74.01**	2.50
P. aurantiogriseum MTCC 2285	0	8.00	7.00	8.51	7.00	9.02	7.00
	2	26.05	5.80	19.50	5.27	21.00	5.19
	4	31.63	4.61	30.50	3.85	32.51	3.70
	6	36.14	4.25	25.82	3.40	32.23	3.06
	8	39.74	3.80	56.90	3.05	60.90	3.22
	10	36.72	3.62	66.48	2.86	75.66	2.82
	12	40.21	4.32	72.33	3.16	**77.70**	3.35
P. claviformis bv	0	8.00	7.00	8.42	7.00	9.00	7.00
	2	24.07	5.78	21.42	5.68	20.03	5.25
	4	38.50	4.58	32.20	4.28	35.52	4.55
	6	35.11	3.99	30.05	3.80	28.51	3.47
	8	32.72	3.91	63.20	3.52	76.60	3.64
	10	46.01	4.25	83.51	3.32	69.70	3.03
	12	30.52	3.95	69.49	3.56	**75.01**	3.13
Control	0	8.00	7.00	8.52	7.00	9.01	7.00
	2	8.32	6.99	9.00	6.68	9.25	6.99
	4	8.50	6.98	9.25	6.72	9.75	6.98
	6	10.00	6.86	10.81	6.86	10.81	6.83
	8	11.41	6.74	9.75	6.74	11.75	6.72
	10	10.60	6.72	11.50	6.68	10.30	6.65
	12	10.50	6.63	11.65	6.61	12.11	6.62

although the amount varied with the phosphate type and the fungal strain. The pattern of solubilization of different forms of insoluble phosphates was significantly different in all four strains with F= 2.579 and p-value < 0.1. The solubilization of TCP was highest, closely followed by hydroxyapatite, while DCP was most difficult to solubilize. However, there was no significant difference in solubilization of TCP and hydroxyapatite at 5% level of significance. *A. niger* strains solubilized phosphates efficiently for first 6 days, then the rate of solubilization finally attained an almost constant values. However, the values increased in DCP whereas; there were more fluctuations in case of TCP and hydroxyapatite. But the *Penicillium* spp. did not give constantly increasing values at all, as shown by more evident fluctuations in solubilized phosphate concentrations. Also, after 10 to 12 days of incubation

Figure 2. Solubilization pattern of Dicalcium Phosphate by (A) *A. niger* ATCC 282 , (B) *A. niger bv*, (C) *P. aurantiogriseum* MTCC 2285, (D) *P. claviformis bv* in two nutrient media.

there was a slight increase in pH.

Table 1 also showed that the rise in the available phosphate concentration was accompanied by concomitant decrease in pH of the medium. It decreased from 7 at day zero to 2.20 at 12th day in case of *A. niger* bv solubilizing hydroxyapatite. In other strains also the decline in pH from 7 to 2 to 4 was observed. In all, statistical analysis revealed a significant negative correlation (-0.879 with p value<0.01) between amount of phosphate solubilized and pH values.

Microdetermination of phosphorus in liquid medium

Phosphate solubilizing activity of four strains was observed on Czapek's Dox and Pikovskaya broth containing DCP as the insoluble phosphate source (Figure 2 A-D). There was a significant difference between amounts of phosphate solubilized in vitro by all the four strains in Czapek's Dox and Pikovskaya broth with t = 3.370 and p-value<0.01. It was found that Pikovskaya broth overall supported better solubilization than Czapek's Dox broth.

The results of effect of $NaNO_3$ and $(NH_4)_2SO_4$ as nitrogen sources and Mn^{+2}, Al^{+3} and Fe^{+3} as metal ions on phosphate solubilization by all four strains is depicted in Figures 3 to 4. There was a significant difference bet-

ween amounts of phosphate solubilized by all the four strains in case of nitrate and ammonium ions as nitrogen source with t=-2.471 and and p value<0.05. $NaNO_3$ proved inferior nitrogen source to $(NH_4)_2SO_4$ in supporting phosphate solubilization activity.

Mostly, in all the experimental fungal strains, phosphate solubilizing activity decreased (except in *A. niger* ATCC 282) in presence of Mn^{+2} and Fe^{+3} as compared to control but presence of Al^{+3} did not have statistically significant effect at 1% level of significance on these strains. The pattern of solubilization of different forms of insoluble phosphates in presence of metal ions was significantly different in all four strains. Al^{+3} did not make any remarkable difference but Fe^{+3} and Ca^{+2} had an inhibitory effect on phosphate solubilization.

DISCUSSION

In general, TCP supported highest phosphate solubilizing activity, closely followed by TCP and hydroxyapatite while DCP was least solubilized. Calcium phosphates are the dominant insoluble inorganic phosphates present in neutral to alkaline soils. The soil of study area is also alkaline (Anamika et al., 2007), therefore, locally isolated strains are expected to solubilize these forms of phosphates well. Out of three insoluble inorganic phosphate sources,
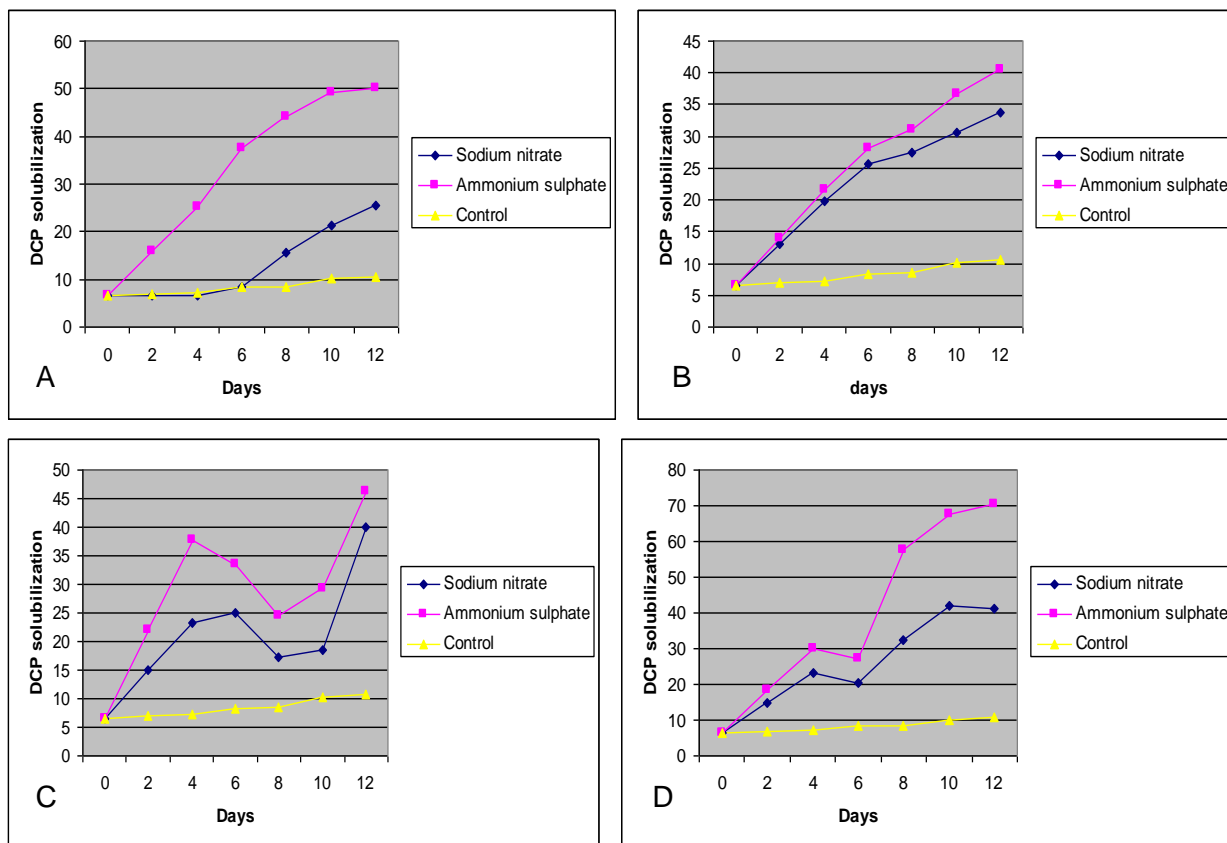
Figure 3. Solubilization pattern of Dicalcium Phosphate by (A) *A. niger* ATCC 282 , (B) *A.niger bv*, (C) *P.aurantiogriseum* MTCC 2285, (D) *P.claviformis bv* in the presence of NO_3^- and NH_4^+ as nitrogen sources.

TCP was solubilized the most which is in agreement with Narsian et al. (1995). This could be due to the difference in chemical structure and charge of three forms of phosphates. $CaHPO_4^-$ gives HPO_4^- after release of phosphate while $Ca_3(PO_4)_2$ gives PO_4^{-3} which bears more negative charge. This would react more readily with or have higher affinity for H^+ present in water in dissociated form, thus go into solution more easily. This was further supported from the data obtained from control samples which did not have inocula. For all three forms of insoluble phosphates, the control showed a very small and gradual increase in available phosphorus concentration with increase in number of days of incubation. This may be explained by the phenomenon of leaching, an abiotic process, caused due to formation of traces of organic acids due to chemical interaction between the components of media (Johnston, 1954).

The rise in the available P concentration was accompanied by decrease in pH of the medium. This drop in pH indicates the production of organic acid (Pradhan and Sukla, 2005; Jain et al., 2011). *A. niger* is known to produce citric, oxalic and gluconic acids (Fomina et al., 2004), thus lowering the pH of medium and facilitating the solubilization. The rate of fall in pH was sharp and uniform during the first 8 days and then it gradually lowered

to attain a more or less stable value. These results coincide with Halder and his team mates (Halder et al., 1990b). The lower values of pH in case of *Aspergillus* spp. in comparison to *Penicillium* spp. may be due to less organic acid produced by Penicillia. After 10 to 12 days of incubation there was a slight increase in pH in all the cases which can be explained by the start of autolysis phase (Halder et al., 1990a; b).

In the present study, NH_4^+ was found to be a good nitrogen source in comparison to NO_3^-. Our results corroborate with Jain et al. (2011) who also reported NO_3^- as inferior nitrogen source for phosphate solubilization. The possible reason could be the acid production in form of H^+ release in response to the assimilation of cations such as NH_4^+, which has been reported as a well known phenolmenon in fungi (Kucey, 1983a; b; Roos and Luckener, 1984). Asea et al. (1998) attributed higher phosphate concentration in plant amended with NH_4^+ to better development of roots and acidification of rhizosphere. Roos and Luckner (1984) have reported that the presence of NH_4^+ in growth medium of *P. cyclopium* resulted in the development of inorganic acid following an operation of NH_4^+/H^+ exchange mechanisms.

The phosphate solubilizing activity decreased (except in *A. niger* ATCC 282) in presence of Mn^{+2} and Fe^{+3} as
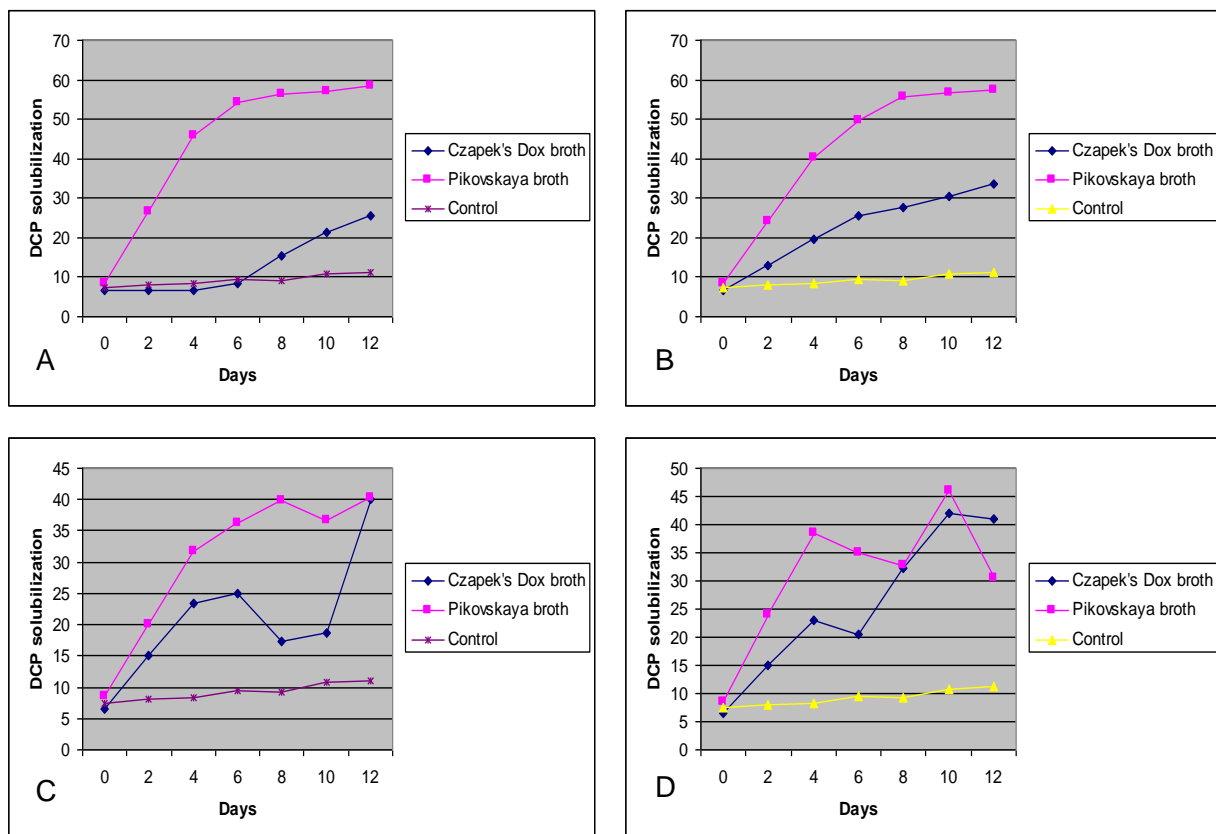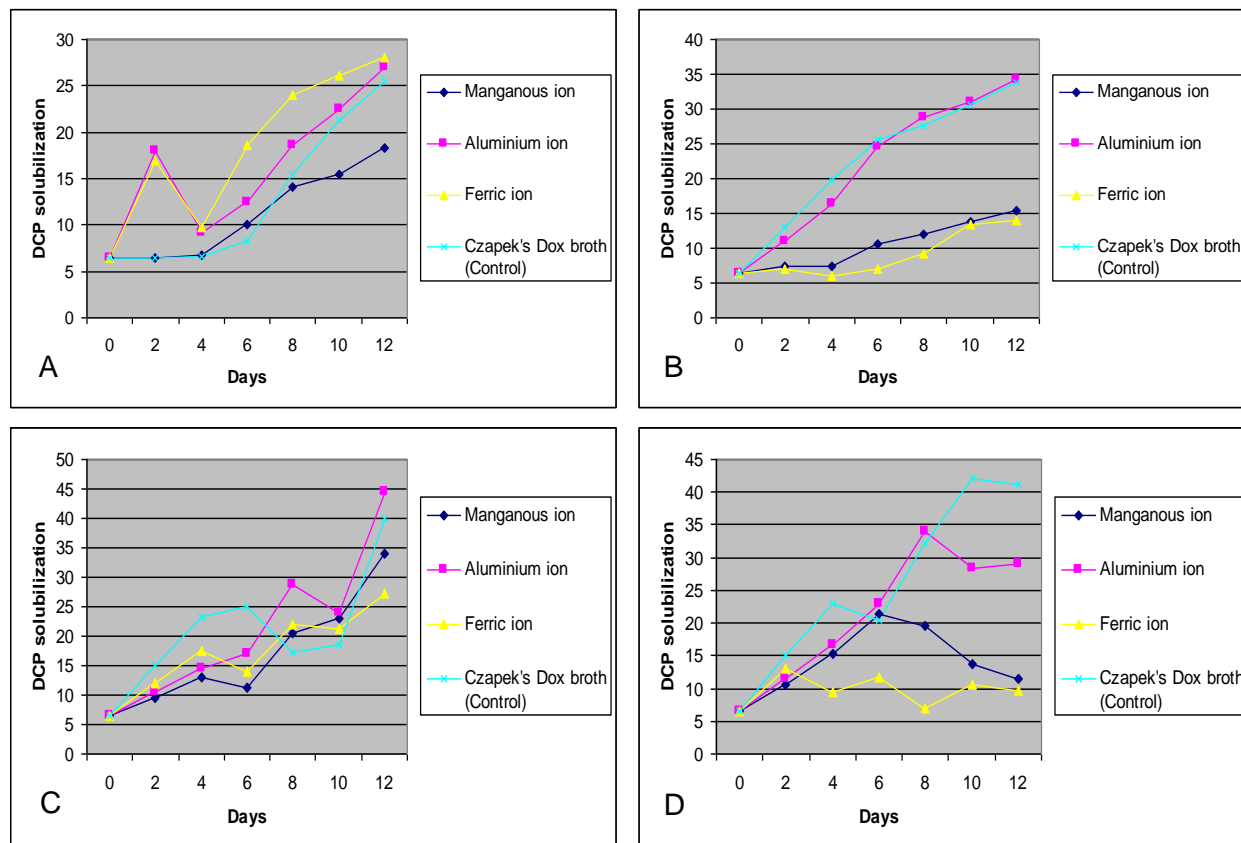
Figure 4. Solubilization pattern of Dicalcium Phosphate by (A) *A. niger* ATCC 282 , (B) *A.niger bv*, (C) *P.aurantiogriseum* MTCC 2285, (D) *P.claviformis bv* in the presence of Mn^{+2}, Al^{+3} and Fe^{+3} as metal ions.

compared to control but presence of Al^{+3} did not have statistically significant effect on these strains. Gaur and Sachar (1980) found that Al^{+3}, Fe^{+3} and Ca^{+2} were inhibitory to growth and activity of fungi or caused a change in pH of the medium, which in turn affected phosphate solubilization. In the present study Al^{+3} did not make any remarkable difference but the other two ions had inhibitory effect. Sayer et al. (1995) observed the activity of *P. simplicissimum* and *A. niger* in presence of 8 insoluble metal compounds. Cobalt phosphate was the most toxic whereas, zinc phosphate was the least toxic and most resistant to solubilisation, while zinc oxide was the most readily solubilized compound.

The type of insoluble phosphate present in the soil of a particular locality seems to affect the efficiency of phosphate solubilization by microbes present locally or applied as biofertilizer. Also, NH$_4^+$ which was proved to be a better source of nitrogen, could be considered while choosing nitrogen fertilizer if these microbes were simultaneously applied in the same soil as a part of integrated management of nutrients. More studies are in offing with regard to metal ions which could play important role in selecting the biofertilizer, if any of the salts containing these ions were present in the soil.

The advantage of using fungi as bioinoculant includes their tolerance to high concentrations of potentially toxic metals (Sayer et al., 1995), and better acid and alkali tolerance than bacteria (Chuang et al., 2007), although fungi might be inferior to bacteria in their ability to colonize plant root. Overall, fungi may have a much better potential to serve as an agent to convert insoluble inorganic P into a soluble form (for example, HPO$_4^{2-}$, H$_2$PO$_4^-$) usable by plants in low or high soil pH.

The present study has generated useful information about the phosphate-solubilizing fungi under control and stressful conditions *in vitro*. It is expected that these fungal strains could serve as suitable candidates for solubilizing P in rigorous environments. However, since the conditions in soil are much more complex than those in vitro, further study of environmental factors affecting phosphate solubilization by these strains in soil should be of practical importance for crops.

REFERENCES

Achal V., Savant VV, Reddy MS (2007). Phosphate solubilization by a wild type strain and UV induced mutants of Aspergillus tubingensis. Soil Biol. Biochem. 39: 695–699.

Anamika SJ, Sharma V (2007). Isolation of tri-calcium phosphate solubilizing bacterial strains from semi arid agriculture soils of Rajasthan, India. J. Pure and Appl. Microbiol. 1 (2): 269-280.

Assailed N,Vassileva M (2003). Biotechnological solubilization of rock phosphate on media containing agro-industrial wastes. Appl. Microbiol. Biotechnol. 61: 435–440.

Asea PEA, Kucey RMN, Stewart JWB (1988). Inorganic phosphate solubilization by two Penicilium species in solution culture and soil. Soil Biol. Biochem. 20: 459-464.

Banik S, Dey RK (1982). Available phosphate content of an alluvial soil as influenced by inoculation of some phosphate solubilizing microorganisms. Plant Soil. 69: 53-364.

Chen PS, Toribara TY, Warner H (1956). Microdetermination of phosphorus. Anal. Chem. 28: 1756-1758.

Chuang C, Kuo YL, Chao CC, Chao WL (2007). Solubilization of inorganic phosphates and plant growth promotion by Aspergillus niger. Biol. Fertil. Soils. 43: 575–584.

Fomina M, Alexander IJ, Hillier S and Gadd GM (2004). Zinc phosphate and pyromorphite solubilization by soil plant-symbiotic fungi. Geomicrobial. J. 21: 351-366.

Gaur AC, Sachar S (1980). Effect on rock phosphate and glucose concentration on phosphate solubilization by Aspergillus awamori. Curr. Sci. 49: 553-554.

Halder AK, Mishra AK, Bhattacharyya P, Chakrabarty PK (1990). Solubilization of rock phosphate by Rhizobium and Bradyrhizobium. J. Gen. Appl. Microbiol. 36: 81-92.

Halder AK, Mishra AK and Chakrabarty PK (1990). Solubilization of phosphate by Aspergillus niger. Sci. Cult. 56: 455-457

Hamdali H, Hafidi M, Virolle MJ, Ouhdouch Y (2008). Rock phosphate solubilizing Actinomycetes: screening for plant growth promoting activities. World J. Microbiol. Biotechnol. 24: 2565–2575.

Hasan R (1994). Phosphorus research in India. In: Dev A (ed). Proceedings of Group Discussion, IARI Publication. New Delhi. pp. 15-21.

Illmer P, Schinner F (1992). Solubilization of inorganic phosphate by microorganism isolated from forest soil. Soil Biol. Biochem. 24: 389-395.

Jain R, Saxena J, Sharma V (2011). Determination of the organic acids in fermentation media by HPLC. Int. J. Biol. Sci. Eng. 2 (4): 261-265.

Jain R, Saxena J, Sharma V (2012) Solubilization of inorganic phosphates by Aspergillus awamori S19 isolated from agricultural soil of semi-arid region. Annals Microbiol. 62: 725-735.

Johnston NW (1954). The solubilization of insoluble phosphates II. A quantity and comparative study of the action of selected aliphatic acids on TCP. J. Sci. Tech. 36: 49-65.

Kucey RMN (1983a). Phosphate solubilizing bacteria and fungi in various cultivated and virgin Alberta soils. Can. J. Soil Sci. 63: 671-678.

Kucey RMN (1983b). Effect of Penicillium bilaji on the solubility and uptake of phosphorus and micronutrients from soil by wheat. Can. J. Soil Sci. 68: 261-270.

Minaxi SJ (2010). Disease suppression and crop improvement in moong beans (Vigna radiata) through Pseudomonas and Burkholderia strains isolated from semi arid region of Rajasthan. BioControl. 55 (6): 799-810.

Nahas E (2007). Phosphate solubilizing microorganisms: Effect of carbon, nitrogen, and phosphorus sources. In First International Meeting on Microbial Phosphate Solubilization, E. Velázquez (ed) 16-19 July 2002, Salamanca, Spain. pp. 111-115.

Narsian V, Thakkar J, Patel HH (1995). Mineral phosphate solubilization by Aspergillus aculeatus. Ind. J. Exp Biol. 33: 91-93.

Oberson A, Friesen DK, Rao IM, Buhler S, Frossard E (2001). Phosphorus transformations in an oxisol under contrasting land-use system: The role of the microbial biomass. Plant Soil. 237: 197-210.

Pradhan N, Sukla LB (2005). Solubilization of inorganic phosphate by fungi isolated from agricultural soil. Afr. J. Biotechnol 5: 850-854.

Rahi P, Vyas P, Sharma S, Gulati A (2009). Plant growth promoting potential of the fungus Discosia sp. FIHB571 from tea rhizosphere tested on chickpea, maize and pea. Indian J. Microbiol. 49: 128-133.

Roos W, Luckner M (1984). Relationship between proton extrusion and fluxes of ammonium ions and organic acids in Penicillium cyclopium. J. Gen. Microbiol. 130: 1007-1014.

Sayer JA, Raggett SC, Gad GM (1995). Solubilization of insoluble metal compounds by soil fungi: Development of a screening method for solubilizing activity and metal tolerance. Mycol. Res. 99: 987-993.

Sheshadri S, Ignacimuthu S (2004). Effect of nitrogen and carbon sources on the inorganic phosphate solubilization by different Aspergillus niger strains. Chem. Eng. Comm. 191: 1043-1052.

Singhal R, Gupta R, Saxena RK (1994). Rock phosphate solubilization under alkaline conditions by Aspergillus foetidus and A. japonicus. Folia Microbiol. 39: 33-36.

Whitelaw MA, Hardenand TA and Bender GL (1997). Plant growth promotion of wheat inoculated with Penicillium radicum sp nov. Aus. J. Soil Res. 35: 291-300.

Whitelaw MA (2000). Growth promotion of plants inoculated with phosphate solubilizing fungi. Adv. Agron. 69: 99-151.

Xiao C, Chi R, Huang X, Zhang W, Qiu G, Wang D (2008). Optimization for rock phosphate solubilizing fungi isolated from phosphate mines. Ecol. Eng. 33: 187-193.

Full Length Research Paper

Identification of antagonistic bacteria for *Amorphorallus konjac* soft rot disease and optimization of its fermentation condition

Jinping Wu, Zili Ding, Zhenbiao Jiao, Rong Zhou, Xinsun Yang* and Zhengming Qiu

Hubei Academy of Agricultural Sciences, Wuhan 430064, China.

Soft rot disease of *Amorphorallus konjac*, an important and potentially destructive corms disease, is caused by *Pectobacterium* species. Now, the conventional methods of controlling the disease include the breeding variety and the chemical control, but the effects are uncomfortable. The aim of this study was to screen antagonistic bacteria for soft rot disease and optimize its fermentation conditions. The antagonistic bacterium (strain C12) confirmed *Bacillus subtilis* by the identification of Biolog system and analysis of 16S rDNA gene sequence. Antimicrobial spectrum of the strain C12 was determined by growth rate method, which could restrain the growth of 12 pathogens. The fermentation conditions of the strain C12 were studied by using the single-factor method. The optimal fermentation conditions for antagonistic bacteria were as follow: Medium initial pH 7.0, the fermentation temperature 31°C, the quantity of medium 50 mL in a 250 mL flask, the inoculation volume 2.5%, the incubation time 22 h and the rotation speed 180 rpm, the glucose as carbon source and yeast as nitrogen source. The fermentation liquor of the strain C12 was twice than the streptomycin in control effect of pot experiment. The research provides reference for controlling soft rot disease of *A. konjac*. The findings suggested that the strain C12 could be exploited as a biocontrol agent for soft rot pathogens.

Key words: Soft rot disease, antagonistic bacteria, characterization, fermentation condition, optimization.

INTRODUCTION

Soft rot disease is one of the destructive diseases of vegetables. It causes a greater total loss of produce than any other bacterial disease. The disease can be found on crops in the field, in transit, in storage and during marketing, and results in great economic losses (Bhat et al., 2010). The disease is conventionally controlled by cultivation measures (such as crop rotation, intercropping) and chemical control (Ronald et al., 2004). Chemical control is usually inappropriate because pathogens can develop resistance and also pesticides can pollute the environment. Therefore, people pay much attention to the biological control. It was reported that *Pseudomonas fluorescens* (Hendawy et al., 1998), *Lysobacter enzymogenes* (Folman et al., 2003), *Erwinia carotovora* subsp. *Betavasculorum* Ecb168 (Costa and Joyce, 1994), *E. carotovora* subsp. *carotovora* Ecc 32 (Seo et al., 2004) and *Streptomyces* (Zamanian et al., 2005) and so on could restrain the *Pectobacterium carotovorasub* sp. *carotovora*. *Amorphophallus konjac* K. Koch ex N.E.Br. (Araceae) originates in South East Asia (Hetterscheid

andIttenbach,1996)andisnowmainlydistributedthroughout Southern and South Eastern China and Vietnam (Brown and Aroids, 2000). China is the main producer of konjac with an area under cultivation of ~200 million acres (Xu et al., 2001) and has approximately 400 factories devoted to the production of konjac flour and related goods (En, 2008). Due to the increasing demand for konjac flour, konjac is now regarded by the Chinese government as an agronomically important crop which has great potential in both domestic and international markets (WFS, 2003). Projects involving planting konjac in mountainous regions of Southern China have been implemented by provincial governments to help combat rural poverty (WFS, 2003). But the soft rot disease of konjac could cause losses between 30 and 50% in the total production, some as high as 80% and even the complete destruction (Xiu et al., 2006). This disease is becoming the bottleneck of konjak industry. No konjac cultivars resistant to the soft rot disease have been reported so far. Recent researches have focused on biological control. Zhou et al. (2004) found that the extracts from *Orostachys fimbriatus* had the antibacterial activities against *Pectobacterium* spp.. Sheng et al. (2007) used the antibiotic extracted from microbial product to prevent and treat soft rot in konjac. *Bacillus subtilis* BSn5 from calli tissue of konjac and *Serratia marcescens* strain 21-2 from rotten corms of konjac showed antibacterial activity against *Pectobacterium carotovorasub* sp. *Carotovora* (*P.c.c*) (Zhou et al., 2007; Wu et al., 2012). But until now effecttive ways to control this pathogen in the field have not been available. The primary objective of this study is to screen and indentify the antagonistic bacteria against from *P.c.c* the soil of the rhizosphere of konjac, and grope the optimal fermentation conditions, so that the antagonistic bacteria could be quickly developed as a biological control agent of soft rot disease of konjac.

MATERIALS AND METHODS

Strains and culture medium

The strain of *P.c.c* (Registry number: FJ463871) and other pathogens were provided by the laboratory. PSA medium (200 g·L^{-1} potato, 18 g·L^{-1} sucrose) and KMB medium (20 g·L^{-1} peptone, 15 mL glycerol, 2.5 g·L^{-1} K$_2$HPO$_4$, 0.73 g·L^{-1} MgSO$_4$) were reference of the microbiology experiment (Fang, 1998).

Isolation of bacterial antagonist

One gram of soil sample, collected from rhizosphere soil of konjac, was suspended in 10 ml of sterile water and vortexed for 45 s. The sample was serially diluted and 100 µL of each dilution was added to molten PSA agar maintained at 55°C along with 10^8 cfu·mL^{-1} of *P.c.c* and poured in sterile petri dishes. After incubation at 28°C for 2 days, bacterial colonies showing zones of inhibition were selected. Further, the antagonistic bacteria were inoculated onto PSA at 180 rpm, 28°C for 24 h. The cell-free supernatants were obtained by centrifugation at 5000×g for 15 min and filtered on a 0.45 µm Mill`ipore filter (Millipore Co., USA). Culture supernatants were added in the wells, made in PSA using sterile metal cylinders, and

the plates were incubated at 28°C for 24 h. Bacterial colonies showing zones of inhibition were reselected (Fang, 1998). The streptomycin (0.24 mg·mL^{-1}) (ZHONGNONG, Co., China) was controlled to compare the effect of the antagonistic bacteria. The experiment was repeated three times.

Inhibition of the different fungi

Inhibition of the different fungi for antagonistic bacteria was determined by growth rate method. The bacteriostasis rate was calculated by the antibacterial circle diameter.

The bacteriostasis rate (%) = (Control bacteria colony diameter-Treatment bacteria colony diameter)/(Control bacteria colony diameter-The colony diameter)

Identification of the strain based on 16S rRNA and Biolog plates

The sequence of the 16S rDNA was obtained from the total DNA of the antagonistic bacteria by polymerase chain reaction (PCR) amplification with sense primer 5'-AGAGTTTGATC CTGGCTCA G-3' and antisense primer 5'-CGGCTACCTTGTTACGACTTC-3' (Weisburg et al., 1991). The PCR amplification conditions were as follows: one denaturation step (3 min at 95°C), 35 cycles of amplification (30 s at 95°C, 30 s at 50°C, 1.5 min at 72°C), and a final elongation step of 10 min at 72°C. PCR products were inserted into the pGEM T-easy vector for sequencing. The Biolog system was used to support the 16S rDNA genus identification. All protocols for preparation and identification of microorganisms are outlined in the Biolog™.

Fermentation conditions of the antagonistic bacteria

The antagonistic bacteria grew in KMB medium for these experiments. The fermentation conditions of the antagonistic bacteria were studied by using the single-factor method. Different factors were regulated by the requirement of experiment. The antagonistic bacteria was cultured at 31°C, pH 7.0, broth content 50 mL/250 mL, inoculum concentration 2.5%, the fermentation cycle 22 h and the shaker revolution 180 rpm. Different factors: pH (2-11), fermentation temperature (22, 25, 28, 31, 34, 37 and 40°C), liquid volume (30, 50, 70, 100, 130 and 150 mL medium in 250 mL triangular flask), inoculation amount (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%) (volume fraction), culture time (12, 18, 24, 30, 36, 42 and 48 h), rotation speed (150, 180, 210, 240 and 270 rpm), carbon source (glucose, fructose, lactose, mannitol, maltose, sucrose and glycerin) and nitrogen source (NaNO$_3$, C$_2$H$_5$NO$_2$, Yeast, (NH$_4$)$_2$SO$_4$, KNO$_3$, Tryptone, (NH$_4$)H$_2$PO4, NH$_4$NO$_3$, L-Glu). The optical density (OD) was surveyed by spectrophotometer (INESA, Co., China). Each treatment was repeated for three times.

Pot experiments

A. konjac plants were grown in a greenhouse until the 3-month-old, where the temperature ranged between 25 and 30°C, and the humidity was more than 80%. The healthy plants were drenched with the 24-h-old-grown culture (approximately 1×10^7 cfu·mL^{-1}, 50 ml/plant) of the antagonistic bacterial. At the same time, the sterile distilled water and streptomycin (0.24 mg·mL^{-1}) were as the negative control. After one week, the plants were challenge-inoculated with 50 ml of *P.c.c* strain (approximately 1×10^7 cfu·mL^{-1}) by pouring the bacterial suspension around the root zone. The incidence of soft rot was recorded periodically over a period of time up to 30 days.
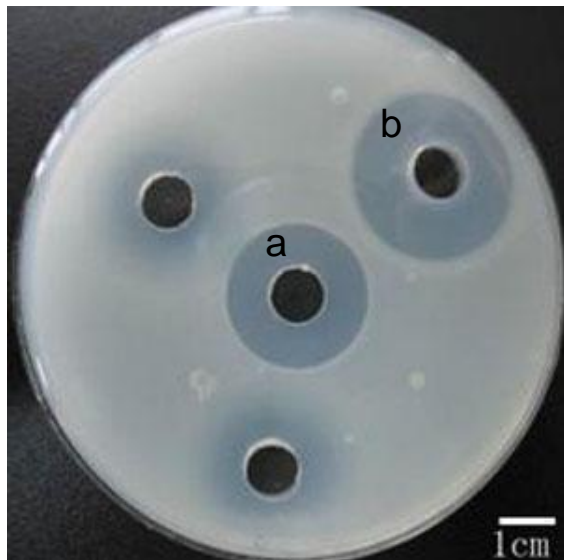
Figure 1. The antimicrobial activity in fermentation supernatants of strain C12 to *P.cc* on PDA medium in 9-cm-diameter Petri dish. The antimicrobial activity was determined by measuring the translucent inhibition zones after incubating the dish 24 h at 30°C. A. streptomycin; B, fermentation liquor of strain C12.

Each treatment was repeated three times. The data were analysed statistically.

RESULTS

Screening of antagonistic bacteria

Twenty-four antagonistic bacteria from the soil of the rhizosphere of konjac against *P.c.c* were obtained by zones of inhibition. The antagonistic effect was further studied by using sterile metal cylinders. The strain C12 had the largest inhibition zone diameter (about 2.6 cm) among the twenty-four antagonistic bacteria. The inhibition zone diameter of the streptomycin was about 2.1cm. The antibacterial circle diameter of the strain C12 was about 0.5 cm wider than the streptomycin (Figure 1).

Determination of the antifungal spectrum

The antifungal spectrum of the antagonistic bacteria strains C12 was determined by growth rate method (Table 1). The strains C12 could inhibit the 12 kinds of pathogens, and the inhibition rate of the 11 pathogens was above 50%. The inhibition rate was greater than 90% of the strawberry root rot (*Rhizoctonia solani*) and Fusarium Wilt of watermelon (*Fusarium oxysporum* f.sp. *niveum*). The results showed that strain C12 had a wide antifungal spectrum.

Identification the antagonistic bacteria

Similar degree of strain C12 with *B. subtilis* was 0.562 by

the BIOLOG system (Gin III 5.2 Microstation). The whole 1,420 bp nucleotides 16S rDNA of strain C12 (Registry number: JX960647) was aligned with all related sequences in the NCBI database by the Basic Alignment Search Tool (BLAST) program. The Max ident was 99% with *B. subtilis* in Genbank. So the strain C12 was proved to be *B. subtilis.*

Fermentation conditions of the antagonistic bacteria

The effect of pH value on cell density indicated that the strain C12 could grow under 5 < pH < 10 (Figure 2a). The strain C12 could grow in a wide pH range. The fermentation temperature has little influence on the growth of strain C12. The strain C12 grew best at 31°C (Figure 2b). Soft rot disease of konjac is easily occurred at 25-30°C. Therefore, the optimum growth temperature of the strain C12 was consistent with the outbreak temperature of soft rot disease.

Liquid volume had great influence in the growth of strain C12. The strains C12 grew best when liquid volume was 50 mL, and then liquid volume was 30 and 70 mL. So the ventilation volume was too large or small, which are not beneficial to the growth of strain C12 (Figure 2c). The strain C12 grew best under the inoculation amount 2.5%, then the 2.0and 3.0% (Figure 2d). Antagonistic bacteria strain C12 reached a maximum growth value when culture time in 22 h, then the strain C12 started aging period (Figure 2e). The antagonistic bacteria strains grew best at 180 rpm. When the shaking speed was more than 180 rpm, the growth of the strain C12 was gradually reduced (Figure 2f).

We could see that the carbon source utilization rate was glucose > sucrose > fructose > maltose > mannitol > lactose > starch > glycerin for antagonistic bacteria (Figure 2g). The highest utilization rate of antagonistic bacteria was yeast, then tryptone, and the other nitrogen source utilization rate was very low (Figure 2h).

Effects of biological bacteria against soft rot disease in pot experiments

30 trees konjac were experimented in each treatment. The plants were all deaths after 30 d by the water treatment, the survival rate of the plant was 16.7% by streptomycin, and the survival rate of the plant was 33.3% by the fermentation liquor of strain C12 (Table 2). The results of pot experiments illuminated that the strain C12 could effectively control soft rot disease of konjac.

DISCUSSION

The strain C12 was identified to be *B. subtilis* by the BIOLOG system and 16S rDNA. Because the bacterium *B. subtilis* produces a variety of antibacterial and antifungal antibiotics such as Zwittermicin-A, kanosamine and lipopeptides from iturin, surfactin and fenzycin fami-

Table 1. The antibacterial spectrum of strain C12.

Pathogenic bacteria	The inhibition rate (%)	Pathogenic bacteria	The inhibition rate (%)
Alternaria alternata	86.7	*Fusarium graminearum*	72.9
Colletotrichum acutatum	55.0	*Rhizoctonia bataticola*	80.0
Colletotrichum gloeosporioides	78.0	*Rhizoctonia solani*	98.7
Myrothecium inundatum	45.8	*Sclerotinia sclerotiorum*	86.7
Fusarium oxysporum f.sp. batatas	68.3	*Sphaeronaemella fragariae*	51.2
Fusarium oxysporum f.sp. *niveum*	94.9	*Sclerotium rolfsii Sacc*	71.8
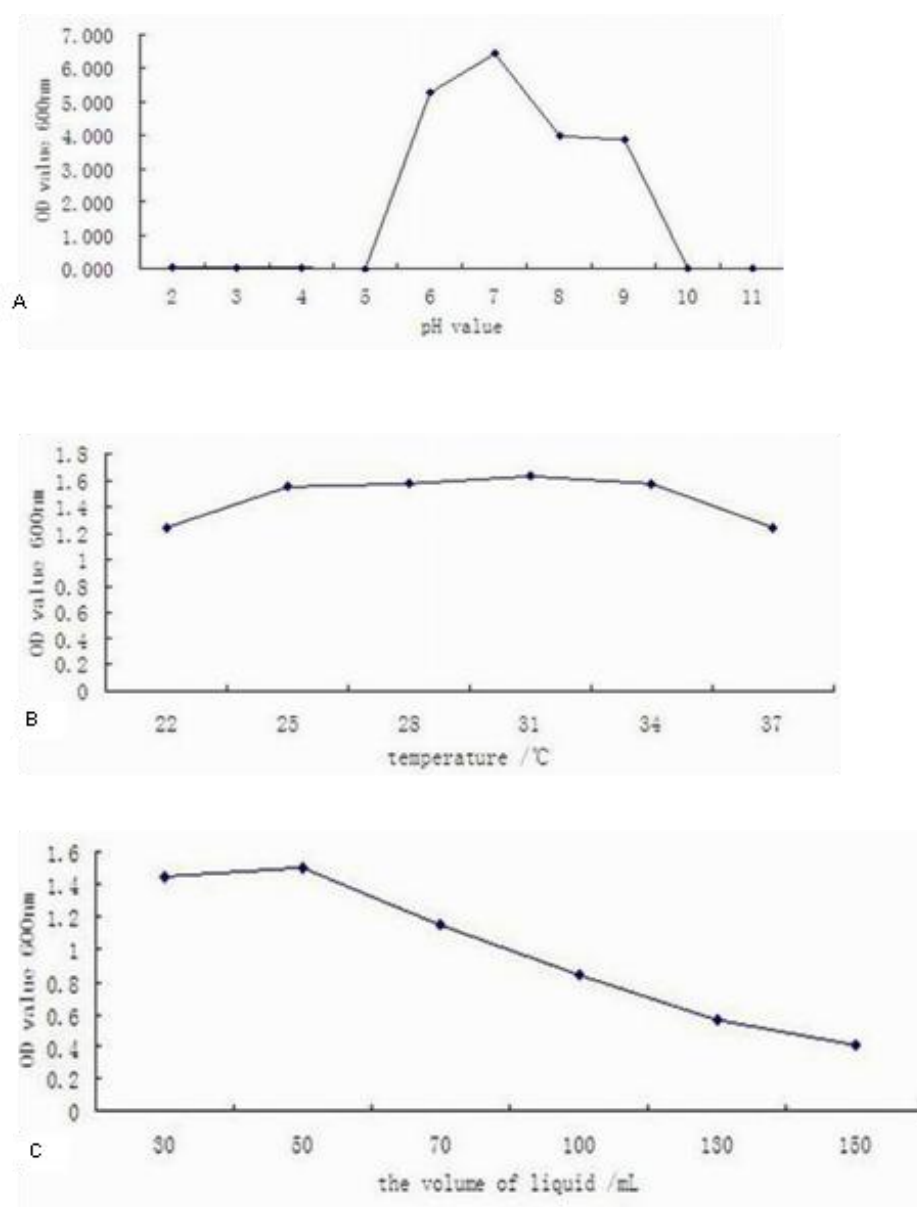
Figure 2. Effect of different factor on cell density for the strain C12. a, Effect of pH value on cell density; B, effect of temperature on cell density; C, effect of the volume of liquid on cell density; D, effect of inoculation volume on cell density; E, effect of incubation time on cell density; F, effect of rotation speed on cell density; G, effect of different C-source on cell density; H, effect of different N-source on cell density.
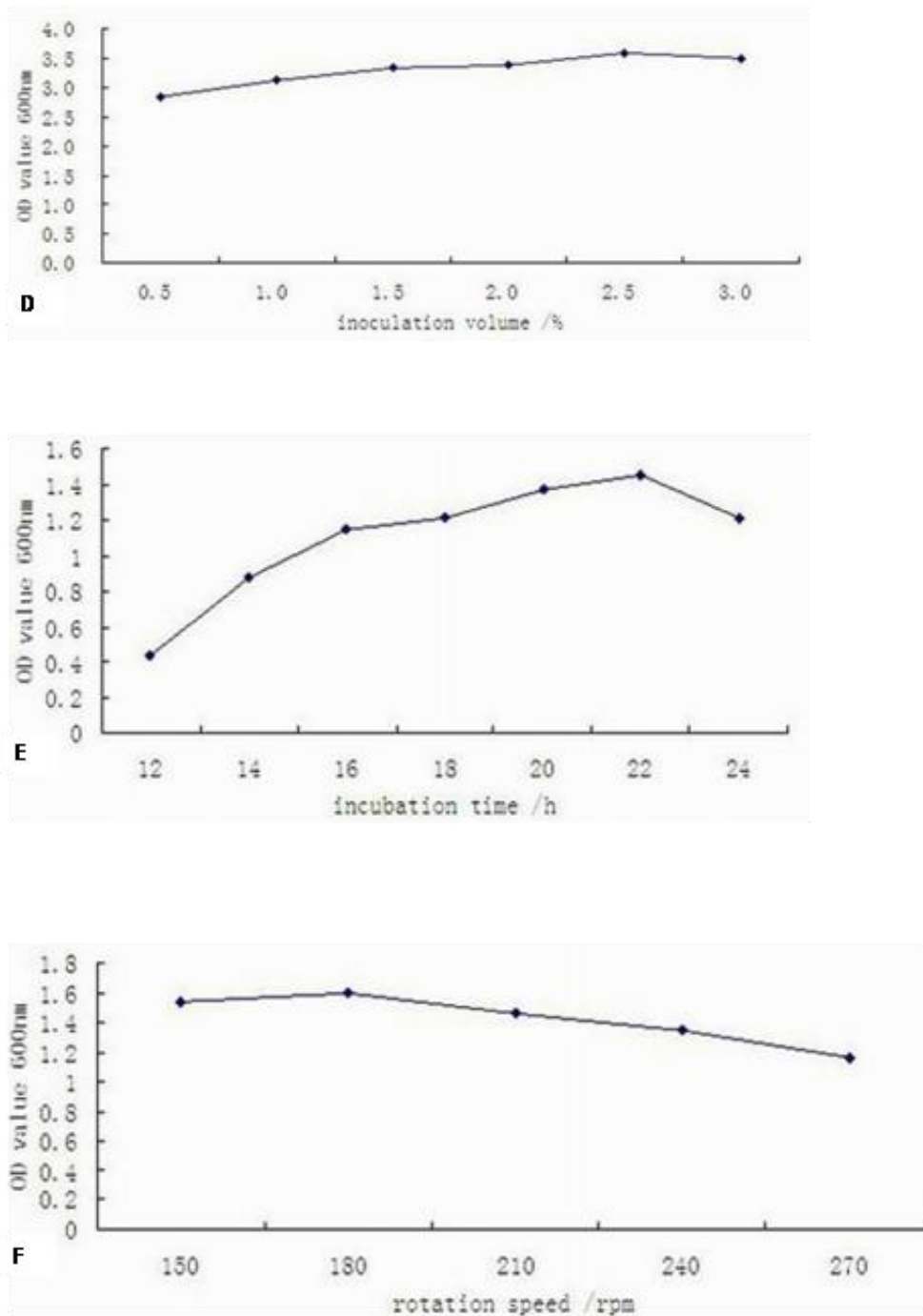
Figure 2. Contd.

families, so the strain C12 not only inhibited the soft rot pathogen of konjac but also exhibit strong inhibitory activity against various plant pathogenic fungi, such as *R. solani*, *Alternaria alternate*, *Fusarium graminearum* among others.

Many bacteria could be used as biological control agents. Among them, *B. subtilis* is now widely recognized as a powerful tool in bio-control as a prevalent soil

inhabitant. As a soil-dwelling rhizobacterium, naturally present in the immediate vicinity of plant roots, *B. subtilis* is able to maintain stable contact with higher plants and promote their growth. In addition, *B. subtilis* had broad host range, formed endospores and produced different antibiotics with a broad spectrum activity. Soft rot disease of konjac was a soil-borne disease, and almost no che-mical measure was effective in the control of this disease
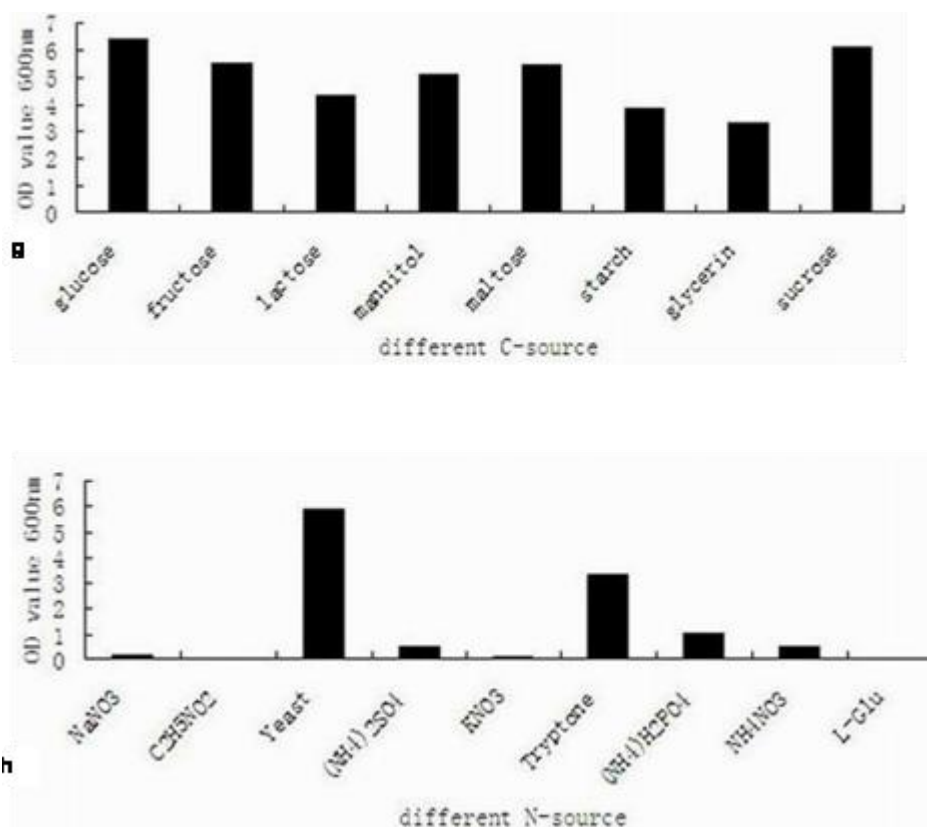
Figure 2. Contd.

Table 2. Control efficiency of antagonistic bacteria against soft rot of konjac in the greenhouse.

Treatment	Concentration	Inoculate time (d)	The number of inoculated plants	Survival rate (%)
C12	1×10^7 cfu·mL^{-1}	30	30	33.3
Streptomycin	0.24 mg·mL^{-1}	30	30	16.7
CK	—	30	30	0

(Krzysztofa et al., 2007). Therefore, the strain C12 was expected to be a bio-control bacterium for soft rot disease of konjac.

The optimal medium volume in the flask and the rotary speed were around 50 mL and 180 rpm, respectively. Our study revealed that rotary speeds, medium volume in the flask are related to the dissolved oxygen (DO) in shaken flasks (Yan et al 2012). A proper DO level was beneficial to the growth of antagonistic bacteria. Lai et al. (2005) found that the shear effect on cell morphology such as mycelium growth or pellet formation was closely influenced by the DO level.

Carbon sources and nitrogen sources played an important role in the growth of the strain C12, not only because of limiting the supply of an essential nutrient is an effective means of restricting growth but also because of the choice of limiting nutrients can have specific metabolic and regulatory effects (Doull and Vining, 1990;

Elibol, 2004). The initial pH, the fermentation temperature, the inoculation volume, the incubation time and so on were disadvantage to the growth of the strain C12 when they were too large or small. The information obtained is considered fundamental and useful for developing a cultivation process for efficient production of antibiotics on a large scale for the strain C12.

ACKNOWLEDGMENTS

REFERENCES

Bhat KA, Masood SD, Bhat NA, Bhat MA, Razvi SM, Mir MR, Wani N, Habib M (2010). Current Status of Post Harvest Soft Rot in Vegetables: A Review. Asian J. Plant Sci. 9: 200-208.

Brown D, Aroids (2000). Plants of the Arum Family Timber Press, Portland, Oregon. 97.

Costa JM, Joyce EL (1994). Derivation of Mutants of *Erwinia carotovora* subsp. *betavasculorum* deficient in export of pectolytic enzymes with potential for biological control of potato soft rot. AEM. 60: 2278-2285.

Doull JL, Vining LC (1990). Nutritional control of actinorhodin production by *Streptomyces coelicolor* A3(2): suppressive effects of nitrogen and phosphate. Appl Microbiol. Biotechnol. 32: 449-454.

Elibol M (2004). Optimization of medium composition for actinorhodin production by *Streptomyces coelicolor* A3(2) with response surface methodology. Process Biochem. 39: 1057-1062.

En SC (2008). Current production of konjac: problems and strategies. J. Anhui Agric. Sci. 36: 14792-14793.

Fang ZD (1998). The Research Methods of Plant Disease. Beijing, China Agric. Press. 179.

Folman LB, De KM, Postma J (2003). Characterization of *Lysobacter enzymogenes* (Christensen and Cook, 1978) strain 3.1T8, a powerful antagonist of fungal disease of cucumber. Microbiology Res. 158: 107-115.

Hendawy HE, Zeid IM, Mohamed M (1998). The biological control of soft rot disease in melon caused by *Erwinia carotovora* subsp. *carotovora* using *Pseudomonas fluorescens.* Microbiol. Res. 153: 55-60.

Hetterscheid WL, Ittenbach S (1996). Everything you always wanted to know about *Amorphophallus* but were afraid to stick your nose into. Aroideana 19: 7-129.

Krzysztofa N, Mariusz B, Michał O (2007). Multicellular behaviour and production of a wide variety of toxic substances support usage of *Bacillus subtilis* as a powerful biocontrol agent. Acta Biochimica Polonica. 54: 495-508.

Lai LS, Tsai TH, Wang TC, Cheng TY (2005). The influence of culturing environments on lovastatin production by *Aspergillus terreus* in submerged cultures. EMT. 36: 737-748.

Ronald CS, Hae RC, Margriet MH, Pim L, Jaap MT (2004). Genetic variation in *Zantedeschia* spp. (*Araceae*) for resistance to soft rot caused by *Erwinia carotovra* subsp. *carotovora*). Euphytica 135: 119-128.

Sheng DX, Jia Y, Ran LP, Teng JX, Chen RC (2007). The control effect for different antibiotics to soft rot of *Amorphophallus konjac*. Anhui Agric. Sci. Bull.13: 153-155.

Seo ST, Furuya S, Iiyama K (2004). Characterization of an antibacterial substance produced by *Erwinia carotovora* subsp. *carotovora* Ecc 32. J. Gen. Plant Pathol. 70: 273-277.

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991). 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173: 697-703.

World of Food Science(WFS) (2003). Part II: Konjac-A Program to Help Reduce Poverty in Northern Guangdong, China. Accessed 31 Aug 2009. Available at: http://www.worldfoodscience.org /cms/?pid=1003561.

Wu JP, Liu XY, Diao Y, Ding ZL, Hu ZL (2012). Authentication and characterization of a candidate antagonistic bacterium against soft rot of *Amorphophallus konjac*. Crop protection. 34: 83-87.

Xiu JH, Ji GH, Wang M, Yang YL, Li CY (2006). Molecular identification and genetic diversity in Konnyaku's soft rot bacteria. Acta Microbiologica Sinica 46(4): 522-525.

Xu L, Xu HH, Huang TS, Du TF, He XR, Shen LH (2001). New Techniques of Cultivation for Good Agricultural Practice (GAP) and Industrializing Develop-mentonthe Chinese Rare-medicinal Herbs. Xie He Medical University, Beijin (in Chinese).

Yan X, Huang L, Wang X, Yao M, Kang Z (2012). *Saccharothrix yanglingensis* sp. Nov., an antagonistic endophytic actinomycete isolated from cucumber plant. Antonie van Leeuwenhoek. 101: 141-146.

Zamanian S, Shahidi GH, Saadoun I (2005). First report of antibacterial properties of a new strain of *Streptomyces plicatus* (strain 101) against *Erwinia carotovora* subsp. *carotovora* from Iran. Biotechnology 4: 114-120.

Zhou Y, Liu SY, Zou LF (2004). The inhibiting effect of extracts from some wild plants on plant pathogens. Chin. Wild Plant Resour. 23: 30-32.

Zhou Y, Chen L, Chai XL, Yu ZL, Sun M (2007). Isolation of endophytic antagonistic bacterium from *Amorphophallus konjac* and research on its antibacterial metabolite. Acta Microbiol. Sin. 47: 1076-1079.

Full Length Research Paper

Evaluation of the potency of some entomopathogenic bacteria isolated from insect cadavers on *Anopheles arabiensis* Giles (Order: *Dipthera*; Family: *Culicidae*) mosquito larvae in Nigeria

Omoya, F.O.* and Akinyosoye, F.A.

Department of Microbiology, Federal University of Technology, Akure, Ondo State, Nigeria.

The laboratory evaluation of larvicidal activities of some bacteria namely; *Pseudomonas aeruginosa, Bacillus polymyxa* and and *Bacillus subtilis* was assessed against the second and fourth instar of *Anopheles arabiensis* collected from South West Nigeria. Concentrations ranging from 1.3×10^7 cfu/mL to 6.5×10^7 cfu/mL were tested on the larvae for a period of 48 h. The disparity in the activities was monitored with *B. subtilis* displaying the highest activity in both the second and fourth instar with LC_{50} of 0.865 and 2.361 mg/mL respectively. *P. aeruginosa* showed LC_{50} of 1.931 and 4.205 mg/mL while the least activity was recorded in *B. polymyxa* with LC_{50} of 5.776 and 7.403 mg/mL. There were significant differences in the LC_{50} value of the bacteria on the tested instars. Values obtained from the fourth instar group were significantly different from those obtained from the second instar group in all the treatments. Finding from this study show that *B. subtilis* may be a potential biocontrol agent of *A. arabiensis,* the main malaria vector in Nigeria.

Key words: Biocontrol, larvicidal activities, *Anopheles* mosquito larvae, malaria.

INTRODUCTION

Mosquito (Diptera) is one of the social insects that has posed serious problems to the health of man by serving as a vector to the etiologic agents of diseases such as malaria, dengue fever, filariasis and Japanese encephalitis which remain endemic in the sub-sahara region of developing countries (Omoya et al., 2009). Malaria is most challenging among the afore mention diseases considering the morbidity-mortality ratio. According to Breman et al. (2004), globally, 300 to 500 million cases of infection and over one million deaths are reported annually; 90% of these occur in tropical Africa. Nigeria is known for its high prevalence of malaria and available records show that about 50% of the population suffer from at least one episode of malaria each year. Malaria accounts for over 45% of all out-patient cases in Nigeria

(Federal Ministry of Health, 2001). The global increase of malaria toward the end of the 1970s coupled with the inadequate results of malaria eradication strategies in many countries especially the tropical countries have led to concerted search for new methods of control. This was due largely to the emergence of strains of *Plasmodium falciparum* resistant to chloroquine and sulphadoxine-pyrimethamine (SP), the mainstays of treatment (Trape et al., 1998). The use of chemical insecticides as prophylaxis measure in applications such as coils, sprays, insecticide-treated nets (ITNs) have posed threat to human health and the ecosystem (Philip, 2001).

An important alternative measure to chemical insecticides is biological control measure which involves the regulation of pest population using natural control agents

such as predators, nematodes and microbial insecticides (Merritt et al., 2005). It is the use of one biological organism to control another; releasing beneficial bacteria, fungi or arthropods to limit pest infestation. Weinzierl et al. (2005) noted that the organisms used in microbial insecticides are essentially non-toxic and non pathogenic to non-target organisms. The safety offered by microbial insecticides is their greatest strength. Bacteria and fungi have been shown to kill mosquitoes to varying degrees (Orduz and Axtel, 1991; Su et al., 2002). *Bacillus thuringiensis* var *isrealiensis* (BTI) and *B. sphaericus* are being used in worldwide field test designed to control mosquitoes' population (Philip, 2001). These microorganisms have their own limitations which include low persistence of the bacterial larvicidalcystal protein in warm environment as a result of sunlight inactivation. The direct use of BTI cells also has its drawbacks as its cells do not exhibit stable habitation in their environment. In addition, the strain of mosquito existing in a particular region appears to differ from that in another place. Thus, BTI may not be effective for use in all regions where mosquitoes are problematic. Therefore, the isolation and development of bacterial strains with larvicidal activity having a broad host range specifically, stable habitation and non-hazardous properties is desired. In order to achieve these, we investigate the larvicidal activities of *Pseudomonas aeruginosa*, *Bacillus polymyxa* and *Bacillus subtilis* on different instars of *Anopheles* mosquito larvae at varying concentrations under laboratory condition.

MATERIALS AND METHODS

Insect rearing

The mosquito larvae were collected from stagnant waters. They were selected and differentiated using both physical and molecular characterisations. The A*. arabiensis* larvae were reared in a meshed cage at 25°C and 70% relative humidity under 14L:10D photoperiod with slight modifications according to Zhong et al. (2006). They were fed daily with Tetramin® fish food. This allowed them to reach maturity stage and where offered blood meal. Eggs laid on wet filter papers were transferred to water trays. Larvae were fed and sorted for bioassays.

Entomopathogenic bacteria

This study was conducted at the Federal University of Technology Akure, Nigeria. Cockroaches and housefly were collected into sterile containers from their natural breeding habitats (cupboards for cockroach and housefly around the refuse dumps) in Akure, Nigeria. In the laboratory, adult cockroaches were placed inside a sterile Petri dish containing 10 mL of sterile water each (in triplicate). The Petri dish was properly shaken to ensure good washing away of particles that were on the cockroaches. 1 ml was taken from the wash water, serially diluted to 10^{-4} and 0.1 ml of the 10^{-4} serial dilution was pour plated using molten nutrient agar and potato dextrose agar. Incubation was done at 37°C for 24 h and the plates were observed for growth. Identification of the bacterial isolates was done using cultural, morphological and biochemical

characteristics according to the methods of Holt et al. (1994). The identification of fungi was by comparison of the observed morphological characteristics with those described by Onions et al. (1995), after examination under the microscope. The same proce-dure was repeated using housefly. *B. subtilis, P. aeruginos*and *B. polymyxa* were selected for bioassay due to high larvicidal activity recorded on preliminary assay conducted in our laboratory.

Cultivation of bacteria

A basal medium containing K_2HPO_4 (17.4 g), NH_4SO_4 (1.98 g), $MgSO_4$ (0.48 g), $FeSO_4.7H_2O$ (0.0025 g) and glucose (2.0 g) in 100 mL of sterile distilled water was used. Each isolate was inoculated into10 mL of sterile basal medium, incubated at 37°C for 24 h. The cells were centrifuged at 12.168×10^3 g for 15 min (Centrifuge MSE Minor 35) and re-suspended into 2 mL sterile water. The cells were counted and diluted. At inoculation onto mosquito, the diluted cells were pour plated into nutrient agar, incubated and counted using colony counter.

Susceptibility of *A. arabiensis* larvae to *P. aeruginosa, B. polymyxa* and *B. subtilis*

One hundred (100) *A. arabiensis* larvae were used for each concentration in this experiment. The mosquito larvae were surface sterilised in separate Petri dishes using 75% alcohol and rinsing with sterile water. There were four replicates and control per treatments with 25 mosquito larvae in each container. The mosquito larvae were starved for 24 h prior to inoculation. Each mosquito larva was inoculated with the cells of bacterial isolates at varying cell loads. Incubation was carried out for 48 h. The cadavers were removed daily and counted.

Statistical analysis

All data were analysed using analysis of variance (ANOVA), Duncan Multiple Range Test was used to separate mean while Probit analysis (Finney, 1971) was used to determine the LC_{50} that will kill 50% of test population and resistance ratio.

RESULTS AND DISCUSSION

Based on our previous findings (Omoya et al., 2010) and by the new outcomes, we selected the tested microorganisms for evaluation in the successive bioassay. Cell population and incubation time were seen to affect the degradation and subsequently lead to increase in the percentage mortality (Figures 1 to 3). This implies that there is need to increase the contact time to effectively eradicate mosquito larvae particularly when low cell number is used. The degradation rate of mosquito larvae was rapid when sufficient high cell number was used. *B. subtilis* exhibited higher larvicidal activity than both *P. aeruginosa* and *B. polymyxa*. This observation was noticed after 24 h of infesting the larvae with the organisms. In all the treatments, the percentage mortality recorded was seen to increase with increase in concentration with *B. Subtilis* treatment showing the highest percentage mortality. Although, there was no significant
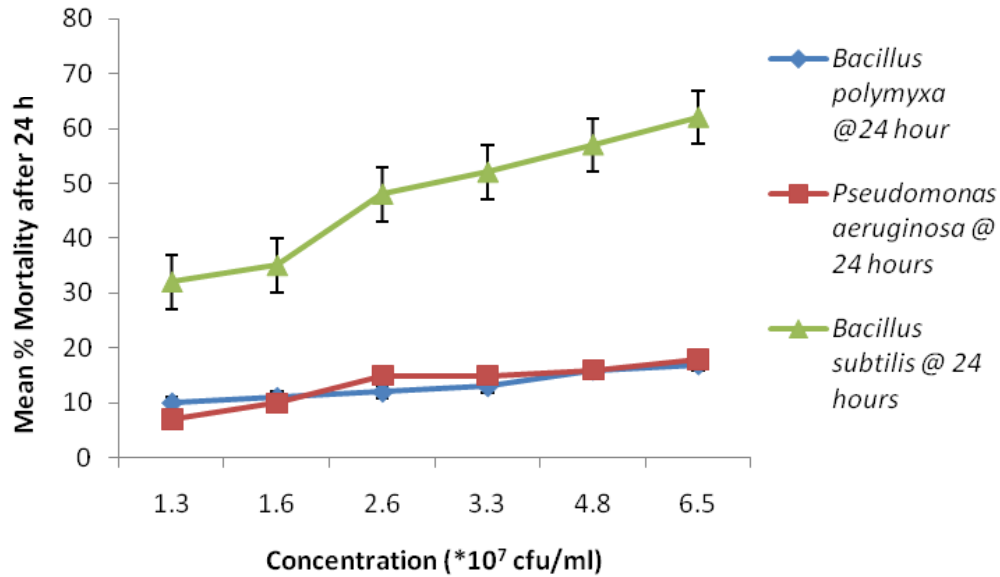
Figure 1. Effect of different concentration of bacterial isolateson the mortality of 2nd instar *Anopheles* mosquito larvae at 24 h.
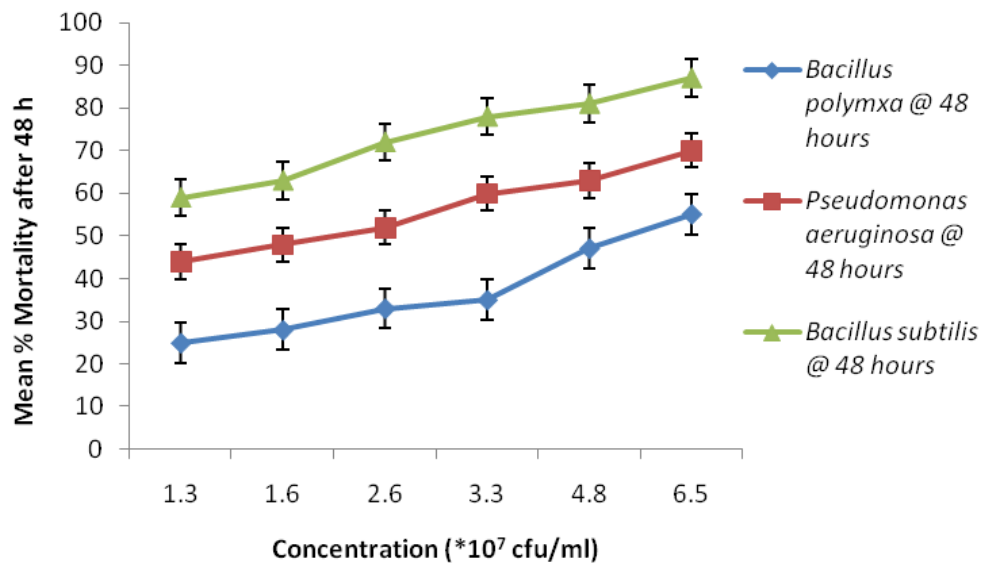
Figure 2. Effect of different concentration of bacterial isolateson the mortality of 2nd instar *Anopheles* mosquito larvae at 48 hours

difference in percentage mortality of *P. aeruginosa* and *B. polymyxa* at 24 h of exposure (Figure 1), at 48 h of exposure to all the treatments, gradual increase in percentage mortality was recorded and results from each treatment were significantly different from each other (Figure 2). This might be as a result of increase in the feeding rate of the larvae which is in agreement with the results of Gunasekaran et al. (2004). In Figure 3,

decrease in percentage mortality of the inoculated mosquito larvae was observed. This could be as a result of decrease in the ingestion rate due to the age of the larvae. During this present study, the mosquito larvae showed greatest susceptibility to *B. Subtilis* when compared to the other tested bacterial isolates. Toxin concentration of 4 to 5 folds of *B. polymyxa* was necessary to induce the same effect of 50% mortality
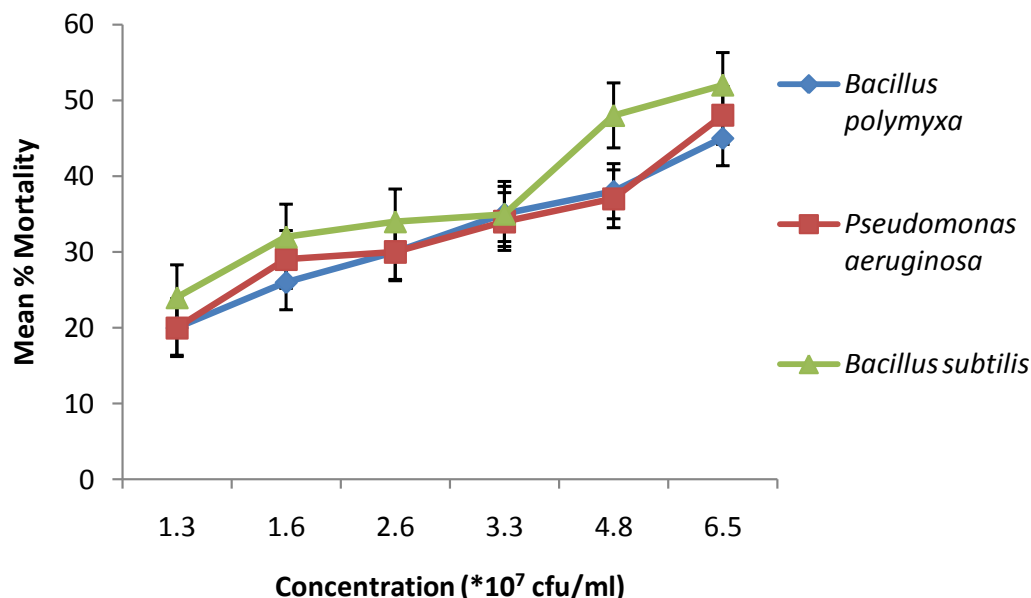
Figure 3. Effect of different concentration of bacterial isolateson the mortality of 4th instar *Anopheles* mosquito larvae.

Table 1. Relative potency of *Bacillus subtilis, Pseudomonas aeruginosa* and *Bacillus polymyxa* on second instar.

Line name	LC_{50}	Lower limit	Upper limit	1	2	3	Index	RR	Slope	LC_{50}
Bacillus subtilis	0.862	0.433	1.219	*			100	1	1.249	0.862
Pseudomonas aeruginosa	1.931	1.23	2.501		*		44.64	2.24	0.926	1.931
Bacillus polymyxa	5.776	4.442	9.415			*	14.92	6.701	1.116	5.776

LC_{50}, lethal concentration at which the extract kill 50% of the organisms at a given time.*1, 2 and 3 showed a significant difference in the test organisms; RR, resistance ratio: resistance ratio (RR) is calculated by dividing the LC_{50} of the most potent microorganisms with other susceptible strains.

Table 2. Relative potency of *Bacillus subtilis, Pseudomonas aeruginosa* and *Bacillus polymyxa*on fourth instar.

Line name	LC_{50}	Lower limit	Upper limit	1	2	3	Index	RR	Slope	LC_{50}
Bacillus subtilis	2.36	0.433	1.219	*			100	1	1.021	2.36
Pseudomonas aeruginosa	4.205	3.805	5.586		*		33.25	1.78	1.623	4.205
Bacillus polymyxa	7.403	4.442	9.415			*	16.78	3.34	1.862	7.403

LC_{50}, lethal concentration at which the extract kill 50% of the organisms at a given time.*1, 2 and 3 showed a significant difference in the test organisms; RR, resistance ratio: resistance ratio (RR) is calculated by dividing the LC_{50} of the most potent microorganisms with other susceptible strains.

(LC_{50}) on the larvae when compared to *B. Subtilis* while 2 folds of *P. aeruginosa* concentration will cause the same effect and resistance ratio (RR) showed that the three tested organisms in second instar varied. *P. aeruginosa* showed that the resistance ratio (RR) values was 2.24 folds above that of *B. substilis* while *B. Polymyxa* was 6.70 folds above *B. substilis* (Table 1). Variation in the LC_{50} and RR values was recorded in Table 2. Toxin concentration of above 3 folds of *B.*

polymyxa was necessary to induce the same effect of 50% mortality (LC_{50}) on the larvae when compared with *B. subtilis* while 2 folds of *P. aeruginosa* concentration will cause the same effect and RR values of *P. aeruginosa* was 1.78 folds above that of *B. substilis* while *B. polymyxa* was 3.34 folds above *Bacillus substilis* in fourth instar. The observed difference in the susceptibility might be due to their ingestion rate (Sun et al., 1980). Hence more mortality of the mosquito larva was recorded

in the second instar treatments. Therefore, from the data obtained in this study, we conclude that the application of *B. Subtilis* to kill malaria vector larvae could significantly reduce parasite transmission, be economical and therefore lead to reduction of malaria risk. Hence this method of biological control has potential as a new strategy for malaria control in Nigeria.

REFERENCES

Breman JG, Alilio MS, Mills A (2004). Conquering the intolerable burden of malaria: what's new, what's needed: A summary. Am J Tropical Med. Hygiene, 71: 1–15.

Finney DJ (1971). Probit analysis.1st edition.Cambridge University Press. Cambridge. London. Pp 100 - 121

Federal Ministry of Health (2001). National Strategic Plan for Roll Back Malaria in Nigeria 2001 – Abuja: Federal Ministry of Health, Nigeria. Pp 67.

Gunasekaran K, Boopathi PS, Vaidyanathan K (2004). Laboratory and field evaluation of Teknar HP-D, a biolavicidal formulation of *Bacillus thuringiensis*ssp.*isrealensis* against mosquito vectors. *Actatropica* 92: 109-118

Holt JG, Kreig NR, Sneath PHA, Staley JT, Willliams ST (1994). Bergey'sManual of Determinative Bacteriology. 9thEdn., Lippincott Williams and Wilkins, Baltimore, ISBN-10: 0683006037.

Merritt RW, Lessard JL, Wessell KJ., Hernandez O, Berg MB, Wallace JR, Novak JA, Ryan J, Merritt BW (2005). Lack of effects of *Bacillus sphaericus* (Vectolex) on non-target organisms in a mosquito-control program in southeastern Wisconsin: A 3-year study. J. Am. Mosquito Control Assoc. 21: 201-212.

Omoya FO, BE Boboye, Akinyosoye FA (2009). Mosquito-Degradative-Potential of Cockroach and Mosquito Borne Bacteria. J. Med. Sci. 9 (4): 202-207.

Omoya FO, Boboye BE Akharaiyi FC, Akinyosoye FA (2010). Biocontrol of Anopheles mosquito larvae with Bacteria isolated from Housefly (Muscadomestica). J. Pure Appl. Microbiol. 4: 23-30

Onions AH, Allsop D, Eggins HOW (1995). Smith's Introduction to Industrial Mycology. 2nd edition. The pitman press, Bathsebath. pp. 65-92.

Orduz S, Axtel RC (1991). Compatibility of *Bacillus thuringiensis*var. israeliensis and *B. sphaericus*for managing *Anopheles* mosquito larvae. Proceedings of the 3th International Conference on Biopesticides, Apr. 2002, Kuala Lumpur, Malaysia, pp: 102-111.

Philip RS (2001). Current status of malaria and potential for control.Clin. Microbiol. Rev., 14: 208-226.

Su X, Zuo F, Guo Q, Huang J, Chen TX (2001). A report on a mosquito killing fungus, *Pythiumcarolimanium.* Fungal Diversity, 7: 129-113.

Sun C, Georghiou GP, Weiss K (1980). Toxicity of *Bacillus thuringiensis*var.*isrealensis* to mosquito larvae variously resistant to conventional insecticides. Mosquito news, 40 : 614-618

Trape JF, Pison G, Preziosi MP, Enel C, Desgrées du Lou A, Delaunay V, Sam B, Lagarde E, Molez JF, Simondon F (1998). Impact of chloroquine resistance on malaria mortality.CompetesRendus de l'Academie des Sciences – Serie III. Life Sci. 321: 689-97.

Weinzierl R, Henn T, Koehler PG, Tucker CL (2005). Microbial Insecticides Entomology and Nematology Series, ENY-275 (IN081), Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida.http://edis.ifas.ufl.edu.

Zhong D, Temu EM, Guda T, Gouagna L, Menge D, Pai A, Githure J, Beier JC, Yan G (2006). Dynamics of Gene Introgression in the African Malaria Vector *Anopheles gambiae.* Genetics, 172: 2359-2365.

Full Length Research Paper

Typing of *Candida* species isolated from blood cultures and analysis of their *in vitro* antifungal susceptibilities

Nurten Baran[1], Ismail Serkan Salman[1], Sureyya Gul Yurtsever[1]*, Rahim Ozdemir[1], Serdar Gungor[1], Senel Yurtsever[2] and Mustafa Demirci[1]

[1]Department of Medical Microbiology, Ataturk Training and Research Hospital, Izmir Katip Celebi University, Izmir, Turkey.
[2]Department of Dermatology, Tepecik Training and Research Hospital, Izmir, Turkey.

The aim of the present study was to investigate antifungal susceptibility of Candida strains isolated from blood cultures in our tertiary hospital. Patients hospitalized between December 2008 and April 2011 whose more than one blood cultures revealed growth of *Candida* species and Candida strains isolated in these cultures were included in the study. In order to identify isolated yeast species, appearance and configuration of the colonies, germ tube test results and their morphological appearance in corn flour Tween 80 agar were evaluated. Within the study period, among 65 Candida strains isolated, 36 (55.4%) *Candida parapsilosis*, 17 (26.2%) *Candida albicans*, 6 (9.2%) *Candida glabrata*, 4 (6.2%) *Candida tropicalis*, and 2 (3.1%) *Candida lusitaniae* isolates were identified. According to Fungifast susceptibility panel, antifungal susceptibility rates were as follows: amphotericin B and flucytosine (100%) fluconazole (93.8%), itraconazole (87.6%) and voriconazole (96.9%). Antifungal susceptibility rates of Candida isolates based on E-test method were as follows: amphotericin B (100%), voriconazole (92.3 %) itraconazole (53.8%) and fluconazole (89.2%). In consideration of higher morbidity, mortality and economic burden of the cases with fungemia, measures against emergence of these infections convey crucial importance. Typing of fungi isolated from intensive care units in particular, and their antifungal susceptibility tests should be done regularly to reveal resistance patterns of pathogens, and any increase in resistance (if any) over time, must be determined with scientific methods. We think that similar studies will guide the clinician in planning treatment of Candida infections especially in patients at risk.

Key words: Candidemia, candida, *in vitro*, microbial sensitivity tests.

INTRODUCTION

Hematologic infections due to *Candida* species in the world are becoming increasingly important. In the United States, *Candida* spp. yeasts ranked fourth among the most frequently isolated microorganisms from blood cultures (Martin et al., 2005). In recent years, the incidence of nosocomial Candida infections increased due to increasing number of patients receiving chemotherapy and other immunosuppressive therapies, innovations in transplantation surgery, use of broad-spectrum antibio-

tics, higher number of patients hospitalized in the intensive care units and invasive procedures performed on patients (Lunel et al.,1999; Cheng et al.,2004). Although fungemias are not frequently encountered clinical entities, these infections present therapeutic challenges with their inherent higher risks of mortality, and requirement for longer hospital stays (Ostrosky-Zeichner et al., 2003).

Due to differences in antifungal use and infection control strategies, distribution and antifungal susceptibility of

Candida spp. differ between countries and hospitals (Pfaller et al., 2003; Dimopoulos et al., 2008). In species other than Candida albicans, especially in Candida krusei and Candida glabrata resistance against azole group antifungal agents develops which further complicates choice of treatment in candidemias. Antifungal susceptibility tests guide the antifungal therapy during episodes of fungemia.

In this study, our aim was to investigate antifungal susceptibility of Candida strains isolated from blood cultures in our tertiary hospital.

MATERIALS AND METHODS

Our study included patients hospitalized in various services of our tertiary hospital between December 2008 and April 2011 whose more than one blood cultures sent to Medical Microbiology Laboratory revealed growth of Candida spp. and also Candida strains isolated in these cultures. Among the same results obtained from the cultures of the same patient, only one blood culture results were included in the analysis.

In order to identify isolated yeast spp., appearance and configuration of the colonies, germ tube test results and their morphological appearance in corn flour Tween 80 agar were evaluated. In addition to these conventional methods, commercial Fungifast (ELİTech France SAS) kit which provided quick identification and antifungal susceptibility results were used in accordance with the manufacturer's instruction.

Antifungal susceptibility tests were performed using E test method. For this purpose, CLSI M27-A M44-A guidelines published by CLSI were followed. As a culture medium, Mueller-Hinton agar (Oxoid CM0337) (GM-MH) containing 2% glucose and 5 µg/ml methylene blue (RM 956-Himedra) was used. In the study, for E-test application, yeasts was suspended in 0.85% NaCl so as to achieve 0.5 McFarland turbidity standard. Surface of GM-MH Agar were scratched in zigzags with sterile cotton swab to provide homogenous growth of Candida. E-test strips (bioMerieux AB, Sweden) containing fluconazole, itraconazole, amphotericin B and voriconazole were applied on agar surface using sterile forceps. E-test strips containing culture medium were incubated at 35°C. Incubation was maintained up to 24 or 48 h till distinct appearance of inhibition ellipse which indicated growth of Candida. The first intersection point between the inhibition ellipse and the scale on the strip where a significant inhibition was observed was evaluated as minimal inhibitor concentration (MIC).

RESULTS

Within the study period, among 65 Candida strains isolated, 36 (55.4%) Candida parapsilosis, 17 (26.2%) Candida albicans, 6 (9.2%) Candida glabrata, 4 (6.2%) Candida tropicalis, and 2 (3.1%) Candida lusitaniae isolates were identified. In addition, two standard Candida strains (C. krusei ATCC 6258 and C. albicans ATCC 90 029) were included in the study. Distribution of the patients among services is shown in Table 1, and of isolates of Candida spp. in Table 2.

In the study, according to Fungifast susceptibility panel, antifungal susceptibility rates were as follows: amphotericin B and flucytosine (100%) fluconazole (93.8%), itraconazole (87.6%), and voriconazole (96.9%).

Table 1. Distribution of the patients among hospital departments.

Department	Number of patients (%)
Anaesthesia Intensive Care Unit	37 (56.9)
Internal Medicine	8 (12.3)
Cardiovascular Surgery	7 (10.8)
Neurosurgery	1 (6.2)
General Surgery	4 (6.2)
Haematology	2 (3.1)
Urology	2 (3.1)
Emergency Medicine	1 (1.5)
Infectious diseases	1 (1.5)
Nephrology	1 (1.5)
Neurology Intensive Care Unit	1 (1.5)

Table 2. Candida spp. isolated from blood cultures and their distribution.

Candida spp.	Number of patients (%)
C. parapsilosis	36 (55.4)
C. albicans	17 (26.2)
C. glabrata	6 (9.2)
C. tropicalis	4 (6.2)
C. lusitaniae	2 (3.1)

Antifungal susceptibility rates of Candida isolates based on E-test method were as follows: amphotericin B (100%), voriconazole (92.3%) itraconazole (53.8%) and fluconazole (89.2%).

Using E-test in the study, MIC range of amphotericin B for C. albicans was 0004-0125 µg/ml, and for non-albicans Candida spp. it was 0.003-0.38 µg/ml, respectively. In our study, amphotericin B resistance was not observed in none of the 65 isolates. MIC_{50}, and MIC $_{90}$ values of amphotericin B against C. albicans were 0.047 and 0.125 µg/ml, while for non-albicans Candida spp. MIC50, MIC 90 values were 0.064 and 0.19 µg/ml, respectively.

MIC range of fluconazole for C. albicans and non-albicans Candida spp. were 0.38-64 and 0.19-256 µg/ml, respectively. Seven of the sixty five isolates in our study (10.7%) were resistant to fluconazole. Four C. albicans, one C. tropicalis and two C. glabrata strains were resistant strains to fluconazole. In our study, a dose-dependent susceptible strain for fluconazole was not detected. Fluconazole MIC 50 and MIC 90 values for Candida albicans, and non-albicans Candida spp. were detected as 0.75 vs. 64 µg/ml, and 0.50 vs. 4 µg/ml, respectively.

MIC range of itraconazole was 0023-32 µg/ml for C. albicans, and 016-32 µg/ml for non-albicans Candida spp. In our study, 17 of 65 isolates (26.1%) were resistant to itraconazole. However, 13 (20%) of these strains were susceptible to itraconazole in a dose-dependent manner. Five strains of C. albicans (29.4%) twelve (25%) of non-

albicans *Candida* spp. were resistant. Among all non-albicans *Candida* spp., all *C. glabrata* (100%), three *C. tropicalis* (75%), and three *C. parapsilosis* (8.3%) strains were resistant to itraconazole. One *C. albicans*, two *C. lusitaniae*, and ten *C. parapsilosis* strains were suscepti-ble to itraconazole in a dose-dependent manner. MIC50 and MIC 90 values of itraconazole against *C. albicans* were 0125 and 32 µg/ml, and for non-albicans *Candida* spp. MIC 50 and MIC 90 values of the drug were 0125 and 32 µg/ml, respectively.

MIC range, MIC50 and MIC 90 of voriconazole against *C. albicans*, and non-albicans *Candida* spp. were 0012-32, 0 094 and 32 vs. 0012-32, 0 032 and 12:25 µg/ml, respectively. A total of five (7.6%) strains were found to be resistant among 65 strains investigated for their sus-ceptibilities to voriconazole. Four of these resistant strains belonged to the *C. albicans*, and one to the *C. tropicalis* spp. None of the strains of *C. parapsilosis*, *C. glabrata* and *C. lusitaniae* spp. were resistant to voriconazole. According to Fungifast susceptibility panel, all (100%) strains of *C. albicans*, and non-albicans *Candida* spp. were susceptible to amphotericin B, while the corresponding susceptibility rates for fluconazole (88.2 vs. 95.8%), itraconazole (82.3 vs. 89.6%) and voriconazole (94.1 vs. 97.9%) were also estimated as indicated in parentheses.

We used E-test in our study, and detected rates of susceptibility of *C. albicans* and non-albicans Candida spp. to amphotericin B (100 vs. 100%), fluconazole (76.5 vs. 93.7%), itraconazole (64.7 vs. 50%) and voriconazole (76.5 vs. 97.9%) as indicated in parentheses.

DISCUSSION

Since fungemias are often severe, rapidly progressive, and treatment-resistant diseases which are difficult to diagnose, they cause serious morbidity and mortality. Literature studies have been emphasizing potential differences among regions, and patient groups served by hospitals (Martin et al, 2005; Cheng et al., 2004; Ostrosky-Zeichner et al., 2003).

In many studies performed, various causative agents of candidemia varying from country to country, annually in the same country, and also among the hospitals were reported. According to some population based studies in the United States and Europe four strains of Candida, namely *C. albicans, C. glabrata, C. parasilosis* and *C. tropicalis* are responsible for about 95% of the cases of candidemia (Tan et al., 2008). In a study performed by the International Fungal Surveillance Group which inclu-ded 32 nations, and related data recorded between years 1992 and 2001, observed that *C. parapsilosis* candidemia took the first place among cases of candidemia (Pfaller et al., 2004). In our study in 36 (55.4%) of 65 strains of Candida, *C. parapsilosis* was identified and ranked first with more than twice the number of *C. albicans* isolates. *C. albicans* was identified in 17 (26.2%) isolates and took the second place. Subsequently 6 (9.2%) *C. glabrata*, 4

(6.2%) *C. tropicalis*, 2 (3.1%) *C. lusitaniae* strains were identified in order of decreasing frequency.

The International Fungal Surveillance Group isolated 6082 strains of *Candida* spp. during a study lasting for 12 years, and detected susceptibility of *Candida* spp. to fluconazole as 90%, while rates of dose-dependent sus-ceptibility, and resistance to this antifungal medication were 7 and 3%, respectively. In the same study, *C. glabrata* was determined as the least fluconazole susceptible strain (Pfaller et al., 2004). Matta et al. (2007) used mic-rodilution antifungal susceptibility test in their investi-gation in Brazil conducted between 1995 and 2003 with 1000 Candida isolates obtained from blood cultures, and reported susceptibility rates for amphotericin B, fluconazole, itraconazole, and voriconazole as 100, 97, 93, and 99.7%, respectively. In our country, resistance to amphotericin B seems to have changed among geogra-phic regions. Yucesoy et al. (2000) investigated *in vitro* susceptibilities of blood culture isolates of *Candida* spp. to antifungal agents, and all strains of *Candida* spp. were susceptible to amphotericin B. However, according to another study performed by Dograman et al.(2000) resis-tance against amphotericin B was reported for ten *C. tropicalis* spp, two *C. albicans* spp, and one *C. parapsilosis* spp. Strains. In studies performed in our country, regions with higher resistance against azole antifungals were detected. In an investigation, significant *in vitro* resistance against azole antifungals was not detected in isolates identified (Arikan et al., 2001). Koc et al. (1999) retrospectively evaluated yeast growth in blood cultures for one year, and found MIC 50 vs. MIC 90 values of fluconazole for *C. glabrata* and *C. krusei*, which are mostly encountered *Candida* spp. after *C. albicans* as 64 vs.64 and 64 vs. 128 µg/ml, respectively.

In our study, we evaluated antifungal susceptibilities using E-test method, and commercial Fungifast suscepti-bility panel. Based on standardization techniques, and experiences gathered, correlation between E-test, and referenced methods as for *Candida* spp and azole antifungals is at an acceptable level. Besides, E-test is a relatively valuable method in the determination of MIC value for amphotericin B, and it is one of the reliable methods for the identification of resistant isolates. In a multicenter study, Pfaller et al. (2000) detected a 86-100% concordance between results of E-test, and macro-dilution test methods with respect to amphotericine B, fluconazole, flucytosine, and itraconazole. In our study, we performed E-test method using GM-MH agar instead of RPMI 1640 culture medium. In a study, the authors reported that reference macrodilution method, and E-test method used with RPMI 1640 medium are far from being practical methods that can be used routinely by every laboratory (Lee et al., 2009). In this study, for 182 iso-lates, GM-MH of fluconazole was used, and in compa-risons with E-test and reference macrodilution methods, MIC values of these tests were concordant at a level of 82.9%. In our study, antifungal susceptibility was evaluated

by E-test method and 100, 92.3 53.8, and 89.2% of Candida isolates were found to be susceptible to amphotericin B, voriconazole, itraconazole, and fluconazole, respectively. This 100% susceptibility to amphotericin was in agreement with other studies performed in this country and abroad. Although higher rates of resistance against fluconazole were detected in our study when compared with rates in developed countries, these rates are closer to those reported in research centers localized in different regions of our country.

Susceptibility rate of all *Candida* spp. to itraconazole was 53.8%. Concordance of this susceptibility rate detected for itraconazole in literature results is controversial. In our study, itraconazole-resistance detected in all strains of *C. glabrata* spp. when compared with other drugs, resistance of all *C. glabrata* strains to itraconazole and higher rates of itraconazole-resistance among strains of non-albicans *Candida* spp. to itraconazole is a striking phenomenon. Besides, detection of 10 (27.7%) dose-dependent susceptible and three (8.3%) resistant strains of *C. parapsilosis* spp., higher incidence of nasocomial infection in our hospital caused by this agent, and also the fact that it was the most commonly isolated strain in this study increase the importance of this etiologic factor. Susceptibility rates for voriconazole appear to be in concordance with the literature findings. In our study, all strains were susceptible to both itraconazole and fluconazole at the same time. All five strains resistant to voriconazole were also resistant to fluconazole, and itraconazole.

Fungifast susceptibility panel, and E-test results were compared, and a p value of 0.001 was determined for susceptibility estimations of non-*C. albicans* to itraconazole, and p <0.05 was accepted as the level of significance. Therefore, when compared with the E-test method, Fungifast susceptibility rating method was found to be insufficient for the determination of susceptibility to itraconazole. A statistical difference was not found between two tests with respect to amphotericin B, fluconazole and voriconazole.

When susceptibilities of all isolates identified in our study were evaluated, susceptibility of 89.2% of Candida isolates to fluconazole demonstrates drug's suitability for initial antifungal therapy for patients with candidemia in our hospital in consideration of its relative lack of toxicity, ease of use, affordable cost and availability. All strains of *Candida* spp. responsible for candidemia were found to be susceptible to amphotericin B. In this regard, it is a suitable drug for patients with higher risk of mortality.

In conclusion, in consideration of higher morbidity, mortality, and economic burden of the cases with fungemia, measures against emergence of these infections convey crucial importance. Typing of fungi isolated from intensive care units in particular, and their antifungal susceptibility tests should be done regularly to reveal resistance patterns of pathogens, and any increase in resistance (if any) over time, must be determined with scientific methods.

We think that similar studies will guide the clinician in planning treatment of Candida infections especially in patients at risk.

REFERENCES

Martin D, Persat F, Piens MA, Picot S (2005). Candida species distribution in bloodstream cultures in Lyon, France, 1998-2001. Eur. J. Clin. Microbiol. Infect. Dis. 24:329-333.
Lunel FMV, Meis JFGM, Voss A (1999). Nosocomial Fungal Infections: Candidemia. Diagn. Microbiol. Infect. Dis. 34:213-220.
Cheng MF, Yu KW, Tang RB, Fan YH, Yang YL, Hsieh KS, Ho M, Lo HJ (2004). Distribution and antifungal susceptibility of Candida species causing candidemia from 1996 to 1999 Diagn. Microbiol. Infect. Dis. 48:33-37.
Ostrosky-Zeichner L, Rex JH, Pappas PG, Hamill RJ, Larsen RA, Horowitz HW, et al (2003). Antifungal susceptibility survey of 2,000 bloodstream Candida isolates in the United States. Antimicrob. Agents Chemother. 47:3149-3154.
Pfaller MA, Diekema DJ (2004). Twelve years of fluconazole in clinical practice: global trends in species distribution and fluconazole susceptibility of bloodstream isolates of Candida. Clin. Microbiol. Infect. 10 Suppl 1:11-23.
Dimopoulos G, Ntziora F, Rachiotis G, Armaganidis A, Falagas ME (2008). C. albicans Versus Non-Albicans Intensive Care Unit-Acquired Bloodstream Infections: Differences in Risk Factors and Outcome. International Anesthesia Research Society 106:523-529.
Tan TY, Tan AL, Tee NWS, Ng LSY (2008). A Retrospective Analysis of Antifungal Susceptibilities of Candida Bloodstream Isolates From Singapore Hospitals. Ann. Acad. Med. 37:835-840.
Matta DA, Almeida LP, Machado AM, Azevedo AC, Kusano EJU, Travassos NF, Saloma R, Colombo AL (2007). Antifungal susceptibility of 1000 Candida bloodstream isolates to 5 antifungal drugs: results of a multicenter study conducted in Sao Paulo, Brazil, 1995–2003. Diagn. Microbiol. Infect. Dis. 57:399-404.
Yucesoy M, Karaman M, Yulug N (2000). In vitro antifungal susceptibility of Candida species isolated from blood cultures. Ankem Derg. 14:71-78.
Dograman F, Aktaş AE, Tuncel E, Ayyıldız A, Yiğit N (2000). Detection of antifungal susceptibility of Candida species isolated from newborn blood cultures by standart macrodilution and E test method. XXIX. Turkish Microbiology Congress, Congress book, Antalya, p.365.
Arıkan S, Arslan Ş, Hasçelik G, Günalp A (2001). In vitro antifungal susceptibility of yeasts isolated from blood cultures in Hacettepe University Hospital. Microbiol. Bult. 35:433-441.
Koc AN, Erdem F, Çetin N (1999). Retrospectif evaluation and antifungal susceptibility of yeasts isolated from blood cultures. 4. Antimicrobial Chemotherapy Days Summary book, Istanbul p.212.
Pfaller MA, Messer SA, Houston A, Mills K, Bolmström A, Jones RN (2000). Evaluation of the Etest method for determining voriconazole susceptibilities of 312 clinical isolates of Candida species by using three different agar media. J. Clin. Microbiol. 39:3715-3717.
Lee SC, Lo HJ, Fung CP, Lee N, See LC (2009). Disc diffusion test and E-test with enriched Mueller-Hinton agar for determining susceptibility of Candida species to voriconazole and fluconazole. J. Microbiol. Immunol. Infect. 42:148-153.

Full Length Research Paper

Isolation and identification of microorganism from polyhouse agriculture soil of Rajasthan

Nakuleshwar Dut Jasuja[1]*, Richa Saxena[1], Subhash Chandra[1] and Suresh C. Joshi[2]

[1]Department of Biotechnology and Allied Sciences, Jayoti Vidyapeeth Women's University, Jaipur- 303007, Rajasthan, India.
[2]Department of Zoology, University of Rajasthan, Jaipur-302004, India.

The present work deals with the isolation and characterization of microorganisms from polyhouse agriculture soil of Jharna village (Rajasthan). Several bacteria and fungi were isolated from polyhouse soil, using serial dilution method. These bacterial isolates were *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacter aerogenes, Shigella* sp., *Proteus mirabilis, Bacillus anthracis, Bacillus subtilis, Staphylococcus aureus* and *Staphylococcus epidermidis* species which were further identified on the basis of colony morphology, Gram staining, biochemical tests and using selective and differential media. Identification of fungi such as *Aspergillus niger, Aspergillus flavus, Fusarium oxysporum, Trichoderma* sp*.* and *Rhizopus* sp*.* was carried out by culturing on potato dextrose and sabouraud's dextrose agar media and microscopic method. Microorganisms play an important function in biodegradation of solid agriculture waste and also help in the crop production.

Key words: Bacteria, fungi, agriculture, polyhouse.

INTRODUCTION

Microorganism are frequently present in soil, manure and decaying plant tissues which are able to degrade wastes that are correlated with the substrate organic matter (Alexander, 1977). Agriculture soil is a dynamic medium in which a large number of pathogenic and non-pathogenic bacterial and fungal flora live in close association. Microbes in the soil are the key to carbon and nitrogen recycling. Microorganisms produce some useful compounds that are beneficial to soil health, plant growth and play an important role in nutritional chains that are important part of the biological balance in the life in our planet (Paul and Clerk, 1966; Kummerer, 2004). Polyhouses are basically naturally ventilated climate controlled. Poly-

house cultivation has been evolved to create favorable micro-climates, which favours the crop production and could be possible all through the year or part of the year as required. Wherein, off season crops are also grown under a favorable controlled environment and other conditions viz. temperature, humidity, light intensity, ventilation, soil media, irrigation, fertigation and other agronomical practices throughout the season irrespective of the natural conditions outside. Therefore, the soil of polyhouse may have different conditions for growth of microorganism than natural environment. The present study aimed to isolate effective microorganisms that are present in polyhouse soil and find out the optimize culture

*Corresponding author. E-mail: nakuljasuja@gmail.com. Tel: +91-9414658277.

Figure 1. (a) and (b) Collection of soil sample from polyhouse of Jayoti Vidyapeeth Women's University, Jaipur.

conditions.

MATERIALS AND METHODS

Collection of soil samples

Agricultural soil sample were collected from polyhouse of Jayoti Vidyapeeth Women's University campus, located at East Longitude-75° 27' 38", North Latitude-26° 49' 34", 450-500 m above sea level, weather: Temperature - 28- 34° C in summer, 12°- 18° C in winter, Jaipur, Rajasthan (Figure 1). Soil samples were taken with the help of sterile spatula, in sterile plastic bags. The samples were brought to the microbiology laboratory.

Determination of physiochemical properties of soil

Freshly collected soil samples were taken for determination of physiochemical properties. The moisture content of the sample was measured in a hot air incubator at 105°C to constant weight. The pH, temperature, humidity, and air pressure was determined using digital pH meter, thermometer, hygrometer and barometer, respectively (Pramer and Schmidt, 1964; Iyengar and Bhave, 2005).

Isolation of microorganism

The microorganisms were isolated by serial dilution technique on Potato Dextrose Agar (PDA) and Nutrient Agar Media (NAM). In this technique, a sample suspension was prepared by adding 1.0 g sample to 10 ml distilled water and mixed well for 15 min and vortexed. Each suspension was serially diluted 10^{-1} to 10^{-6}. 0.1 ml was pipetted onto plates with PDA and NAM media, spread with a glass spreader and incubated at 28°C for fungal and 37°C for bacterial observation. Each colony that appeared on the plate was considered as one colony forming unit (cfu) (Waksman, 1927; Nazir, 2007).

Identification of fungi

The fungal isolates were identified by morphological examination and its characteristics. Morphological characteristics were examined under microscope (Onion et al., 1981).

Optimization of culture condition for fungi

For the determination of optimum condition of isolated fungi, three media were used (Potato dextrose agar media, Sabouraud's dextrose agar, Czapack Dox agar). The media were adjusted to pH 4 to 7. For optimization of the incubation period, the culture plates were incubated at 28°C for 4 to 7 days (Bhattacharyya and Jha, 2011; Azzaz et al., 2012).

Identification and characterization of Bacteria

Gram staining was performed to check the morphology of the cells and spore chain morphology was identified by spore staining technique. The pure culture was grown on nutrient agar medium and transferred to Mac-conkey agar medium, EMB agar medium, Endo-Agar medium and mannitol salt agar medium for differentiate and identified bacteria The plates were incubated at 37°C in the incubator and readings were taken 24 h after inoculation. The bacterial isolates were biochemically characterized by catalase test, oxidase test, urease test, motility test, TSI test, nitrate reduction test and IMVIC test (Collins and Lyne, 1989; Harold, 2002; Zaved et al., 2008).

RESULTS AND DISCUSSION

This study revealed that polyhouse soil samples were analyzed with respect to different types of bacteria and fungi. The common bacteria, *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacter aerogenes, Shigella* sp., *Proteus mirabilis, Pseudomonas aeruginosa, Bacillus anthracis, Bacillus subtilis,* and *S. epidermidis* are dominating species in the soil samples. Similarly, when the soil samples were tested for different types of fungi, *Aspergilllus niger, Aspergillus flavus, Trichoderma* sp., *Fusarium oxysporum, Rhizopus* sp. were dominating species in soil samples. The physiochemical properties of soil play an important role in the growth of microorganism. The polyhouse soil was slightly acidic. The humidity, moisture content, and temperature were 35, 45.5 and 32°C, respectively (Table 1).

In the present study, the isolated fungi were identified on the basis of cultural, microscopic and morphological characteristics (Figure 2). Earlier work reported that for maximum growth of fungi, potato dextrose agar was most favorable (Maheshwari, 2000). The isolated fungi were

Table 1. Physiochemical properties of soil samples.

Physiochemical properties of soil	Observation
pH	6.5 ±1.0
Temperature	32^0C
Humidity	35% Fair
Moisture content	45.5 %
Air pressure	743 mb
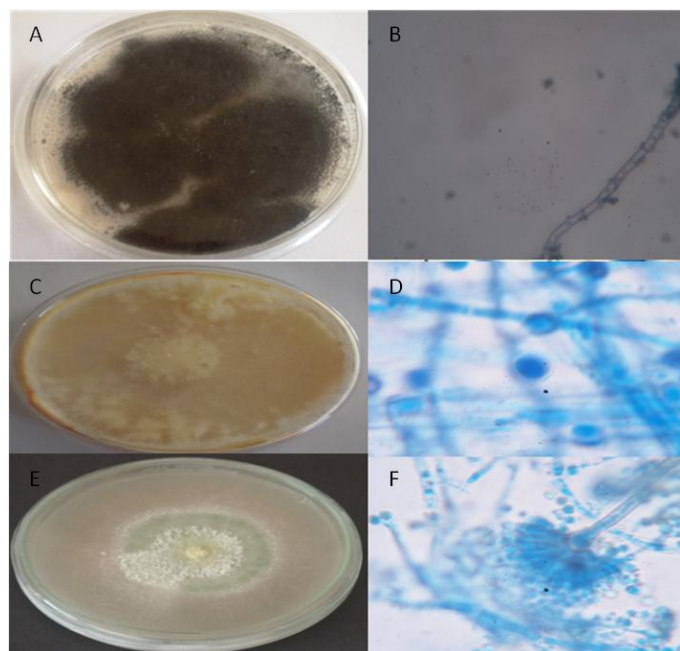
Figure 2. Identified fungi and their microscopic image. A, B; *Aspergillus niger*, C, D; *Fusarium* sp., E, F; *Aspergillus flavus*.

cultured in PDA, SDA and Czapeck Dox agar media. It was observed that the PDA and SDA media were most suitable for good growth of *A. niger*, *A. flavus* and *Trichoderma* sp., *Rhizopus* sp. and *Fusarium oxysporum* at optimum range of pH 6 to 8 and a suitable incubation period was 4 to 7 days, respectively. However, czapeck dox agar medium was also suitable for *A. niger, A. flavus*, *Trichoderma* sp., *Rhizopus* sp. and *F. oxysporum*. Most of the fungi show favorable growth in alkaline side of neutral pH but they can also tolerate the pH range from 6 to 8 (acidic to basic) (Table 2). The enzymatic activities and use of bacteria, actinomycetes and fungi in the decomposition of organic matter provides beneficial metabolic products to the soil (Tiquia et al., 2002; Singh et al., 2003). Similarly, Karthik et al. (2011) also reported the isolation, identification of microorganisms such as *Bacillus* species from agricultural waste dump soil for screening of pectinase producing activity. The isolation of various fungal, bacterial species showed that the agricul-

tural soil is quite rich in microbial flora. In agriculture process, soil microorganisms such as bacteria and fungi may play important roles in soil fertility and in the form of loss and gain in the production of grains, fruits, vegetables. Moreover, it also helps to maintain or enhance the environment quality and conserve natural resources. Most of the bacteria such as *Bacillus anthracis*, *B. subtilis*, and *S. aureus* and *S. epidermidis* isolated in this study have been reported by other researchers (Amir and Pineau, 1998; Okoh et al., 1999). Several bacteria were isolated from agriculture soil samples. Identification and characterization of isolated bacteria were performed by morphological, microscopically (Figure 3), biochemical tests such as shape, arrangement, colonies, temperature, growth, indole production test, ethyl red and Voges-proskauer test, citrate utilization test, urease test, catalase test, Mac-conkey's test, TSI test, growth at 37°C (Table 3). Further, selective and differentiate media were prepared and the isolated bacteria were inoculated under sterilized conditions incubated at 37°C for 18 to 24 h and the results were recorded (Table 4). In different fermentation media, isolates showed different level of growth. The growth of *S. aureus* was observed on mannitol salt agar medium. In the presence of high salt concentration, *S. aureus* fermented mannitol producing acid which changes the pH turning phenol red to yellow (Figure 4). The present study, concluded on isolation and identification of bacterial and fungal floras with optimum pH, temperature and other cultural condition. These isolated polyhouse soil bacteria and fungi are also used in the seasonal and off season crop production with different environment condition. These microorganisms are to supply nutrients to crop, to encourage plant growth; for example, through the production of plant hormones and to control or inhibit the activity of plant pathogen. This study provides knowledge on microorganisms of Rajasthan semi arid soil which is used in polyhouse and produce seasonal and off seasonfoods and vegetables which are not able to grow in normal environment condition.

Conclusion

The environment where we live is the habitat for various microorganisms; mostly bacteria and fungi which are used for various industrial applications like enzymes production, fabric manufacturing, bioremediation, pharmaceutical production, etc. Micro-organisms play an important role in composting of organic waste and can be an important contributor to optimal agricultural waste. This study revealed the isolation and identification of diversity of microorganisms which are present in agricultural soil habitat.

ACKNOWLEDGEMENT

Table 2. Effect of pH and incubation period on the growth of isolated fungi on PDA, SDA, Czapek - Dox agar media at 28°C temperature.

pH of the Medium	Isolated fungi	PDA Media Incubation period (days)				SDA Media Incubation period (days)				Czapek Dox agar Media Incubation period (days)			
		4	5	6	7	4	5	6	7	4	5	6	7
6	*Aspergillus niger*	+1	+2	+3	+4	+2	+2	+3	+4	+2	+3	+4	+4
	Aspergillus flavus	+1	+2	+3	+4	+1	+2	+3	+4	+2	+2	+3	+4
	Trichoderma sp.	+2	+3	+4	+4	+2	+3	+4	+4	+2	+2	+3	+4
	Fusarium oxysporum	+1	+1	+2	+3	+1	+2	+3	+4	+2	+3	+3	+4
	Rhizopus sp.	+1	+2	+3	+4	+1	+2	+3	+4	+1	+2	+3	+4
7	*Aspergillus niger*	+2	+3	+4	+4	+2	+3	+4	+4	+2	+3	+4	+4
	Aspergillus flavus	+2	+2	+3	+4	+2	+3	+3	+4	+1	+2	+3	+4
	Trichoderma sp.	+3	+3	+4	+4	+2	+3	+4	+4	+1	+2	+3	+4
	Fusarium oxysporum	+1	+2	+3	+4	+1	+2	+3	+4	-	-	-	-
	Rhizopus sp.	+1	+2	+3	+4	+1	+2	+3	+4	+1	+1	+2	+3
8	*Aspergillus niger*	+1	+2	+3	+4	+2	+2	+3	+4	-	+2	+3	+4
	Aspergillus flavus	+2	+2	+3	+4	+1	+2	+3	+4	-	+1	+2	+3
	Trichoderma sp.	+3	+3	+4	+4	+2	+3	+3	+4	-	+2	+3	+4
	Fusarium oxysporum	-	-	-	-	-	-	-	-	-	-	-	-
	Rhizopus sp.	+1	+1	+2	+2	+1	+1	+1	+2	-	-	+2	+3

- No growth, +1 poor growth, +2 moderate growth, +3 good growth, +4 massive growth.
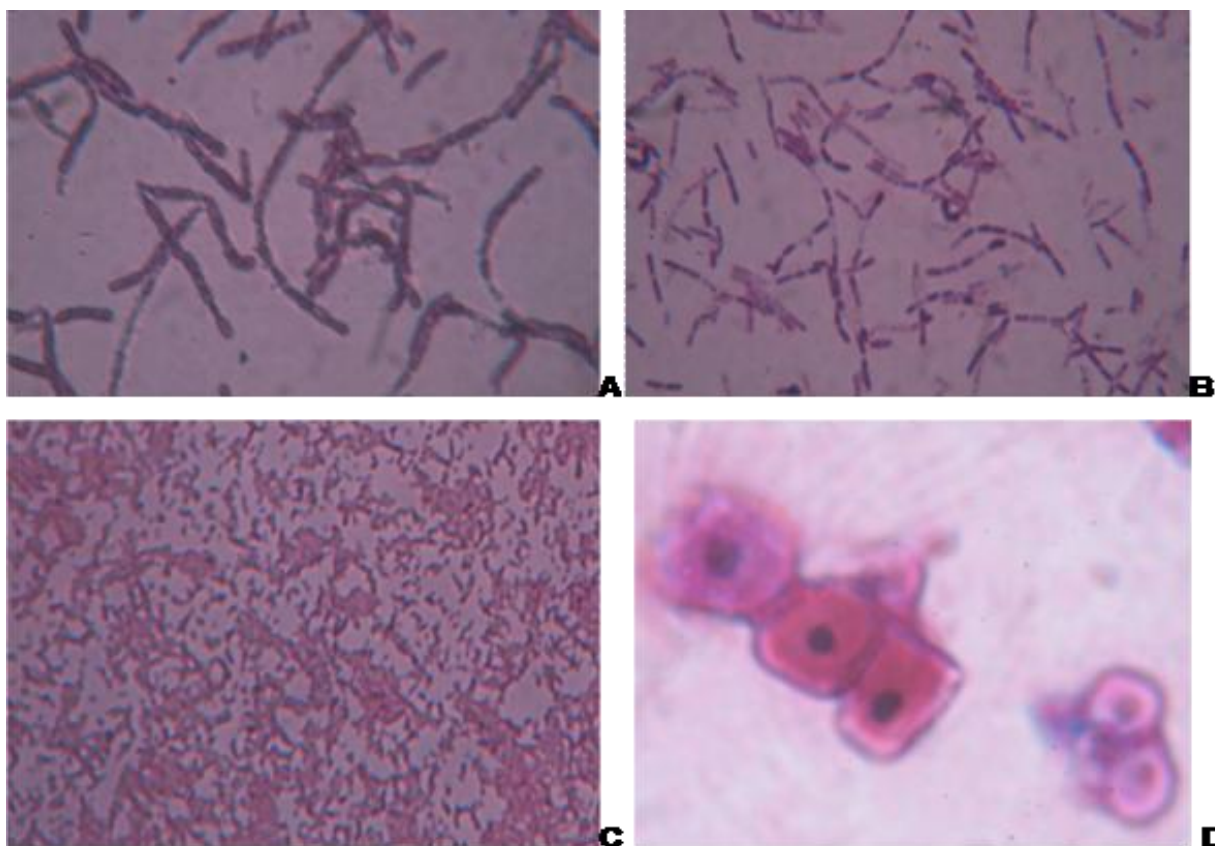
Figure 3. Microscopic images showing A. *Bacillus anthracis,* B. *Bacillus subtilis* C. *Pseudomonas aeruginosa*, D. *Klebsiella pneumoniae.*

Table 3. Biochemical characterization of bacterial isolates.

Identified bacteria	Gram stain	Oxidase	Catalase	Nitrate reductase	VP	MR	Ind	Citrate	Motility	Urease
E. coli	-ve bacilli	-ve	+ve	+ve	-ve	+ve	+ve	-ve	Motile	-ve
Klebsiella pneumoniae	-ve bacilli	-ve	+ve	+ve	+ve	-ve	-ve	+ve	Non motile	+ve
Enterobacter aerogenes	-ve, bacilli	-ve	+ve	+ve	+ve	-ve	-ve	+ve	Motile	+ve
Shigella sp.	-ve rods	-ve	+ve	+ve	-ve	+ve	-ve	-ve	Non motile	-ve
Proteus mirabilis	-ve, bacilli	-ve	+ve	+ve	-ve	+ve	-ve	-ve	Motile	+ve
Pseudomonas aeruginosa	-ve, bacilli	+ ve	+ve	+ve	-ve	-ve	– ve	+ve	Motile	-ve
B. anthracis	+ve, bacilli	-ve	+ve	-ve	+ve	-ve	-ve	-ve	Non motile	+ve
B. subtilis	+ve, bacilli	-ve	+ve	-ve	+ve	-ve	-ve	+ve	Motile	+ve
Staphylococcus aureus	+ve , cocci in clusters	-ve	+ve	-ve	-ve	-ve	-ve	-ve	Non motile	+ve
Staphylococcus. epidermidis	+ve , cocci	-ve	+ve	-ve	-ve	-ve	-ve	-ve	Non motile	+ve

+ve Positive, -ve negative, VP: Voges- Proskauer test, MR: Methyl Red test, In: indole test.

Table 4. Growth of bacterial isolates on different media.

Identified bacteria	Mac- Conkey agar media	EMB agar media	TSI agar media
E. coli	LF	Metallic sheen	A/A, Gas (+), H_2S (-)
Klebsiella pneumoniae	LF	Dark	A/A, Gas (+), H_2S (-)
Enterobacter aerogenes	LF	Dark	A/A, Gas (-ve), H_2S (-)
Shigella sp.	NLF	Colorless	A/ALK Gas (-), H_2S (-)
Proteus mirabilis	NLF	Colorless	A/Alk, Gas (+), H_2S (+)
Pseudomonas aeruginosa	NLF	Light	Alk /no change, Gas (-), H_2S (-)

LF: Lactose fermentation, NLF: lactose non fermentation, A: yellow slant or butt, Alk: slant or butt red.

Figure 4. (A). *Staphylococcus aureus* and (B). *Staphylococcus epidermidis* on mannitol salt Agar media.

REFERENCES

Alexander M (1977). Introduction to Soil Microbiology. 2nd Edition. John Wiley & Sons, New York. pp. 423-437.

Amir H, Pineau R (1998). Influence of plants and cropping on micro-biological characteristics of some new Caledonian Ultramafic soils. Aust. J. Soil Res. 36:457–470.

Azzaz HH, Murad HA, Kholif AM, Hanfy MA, Abdel Gawad MH (2012). Optimization of culture conditions affecting fungal cellulase production Res. J. Microbiol. 7:23-31.

Bhattacharyya PN, Jha DK (2011). Optimization of cultural conditions affecting growth and improved bioactive metabolite production by a subsurface *Aspergillus* strain TSF 146". Int. J. App. Biol. Pharma. Technol. 2:133 – 143.

Collins CH, Lyne PM, Grange GM (1989). Collins and Lyne Microbiological methods, 6th Edition. Butterworth, London.

Harold JB (2002). Microbiological Applications. Laboratory Manuals in General Microbiology, 8th Edition. McGraw - Hill Higher Education.

Iyengar S, Bhave PP (2005). In-vessel composting of household wastes. Waste Management, pp.1-11.

Karthik JL, Kumar G, Rao Bhaskara KV (2011). Screening of pectinase producing microorganisms from agricultural waste dump soil. Asian J. Biochem. Pharma. Res. 2:329- 337.

Kummerer K (2004). Resistance in the Environment. J. Antimicrob. chemother. 45:311- 320.

Maheshwari R, Bharadwaj G, Bhat MK (2000). Thermophilic fungi: Their physiology and enzymes. Microbiol. Mol. Biol. Rev. 64:461-488.

Nazir N, Mirza JH, Akhtar N, Bajwa R, Nasin G (2007). Some studies of thermophilic and thermotolerant fungi from Lahore, Pakistan. Mycopath. 5:95-100.

Okoh LA, Badejo MA, Nathaniesl IT, Tian G (1999). Studies on the bacteria, fungi and springtails (collembola) of an agroforestry arboretum in Nigeria. Pedobio. 43:18-27.

Onion AHS, Allsop D, Eggins HOW (1981). Smiths introduction to industrial mycology.   7th Edition. Edward-Arnold Publishers Ltd. London p.398.

Paul EA, Clark FE (1996). Soil Microbiology and Biochemistry. 2nd Edition. New York. Academic press. pp. 225-229.

Pramer DE, Schmidt EL (1964). Experimental soil microbiology burges publishing company Minneapolis, 15. Minnesota, USA.Sons Ltd. Beecles and London. pp. 31-32.

Singh A, Sharma S (2003). Effect of microbial inocula on mixed solid wasted composting, vermicomposting and plant response. Compost Sci. Uti. 11:190-199.

Tiquia S, Wan J, Tam N (2002). Microbial population dynamics and enzyme activities during composting. Compost Sci. Util. 10:150-161.

Waksman SA (1927). Principles of soil microbiology, Batimore; William & Wilkins co.

Zaved HK, Rahman Mizanur M, Rahman Mashir M, Rahman A, Arafat SMY, Rahman MS (2008). Isolation and characterization of effective bacteria for solid waste degradation for organic manure KMITL Sci. Tech. J. 8:44-55.

Full Length Research Paper

A monoclonal antibody-based antigen-capture enzyme-linked immunosorbent assay (ELISA) for the detection of *bluetongue virus*

Hui-qiong Yin[#], Shu Yang[#], Wen-chao Li, Jun Liu, Rui Wang and Jin-gang Zhang*

Viral Detection Laboratory of National Center of Biomedical Analysis, Institute of Transfusion Medicine, the Academy of Military Medical Sciences, Beijing 100850, China.

A monoclonal antibody-based antigen-capture enzyme-linked immunosorbent assay (ELISA) was developed for the detection of *bluetongue virus (BTV)* in cell culture lysates and blood samples from sheep. The monoclonal antibody 3E2 and 1C11 specific to BTV VP7 were used as capture antibody and detection antibody, respectively. The assay has detected BTV 1-22 specifically, and had no cross-reactivity with the closely related epizootic hemorrhagic disease virus (EHDV) serotypes 5. The limit of sensitivity of the assay was 9 ng/ml for purified recombinant BTV VP7 and $10^{0.5}$ $TCID_{50}$/ml for BTV-5. The coefficient of variation (CV) of intra-assay and inter-assay range from 3.45 to 6.10%. The developed antigen-capture ELISA showed good coincident rate (100%) with INGEZIM BTV DAS in 5 serotypes BTV and 8 blood samples from sheep. Therefore, the antigen-capture ELISA may be useful for testing large number of samples in a convenient and short time.

Key words: *Bluetongue virus*, antigen-capture enzyme-linked immunosorbent assay (ELISA), serotypes, monoclonal antibody.

INTRODUCTION

Bluetongue (BT) is an insect-borne viral disease of ruminants. Among domestic animals, clinical disease occurs most often in sheep, and can result in significant morbidity. The economic consequences of the outbreak were dramatic. For instance, in the Netherlands, the estimated total net costs of the 2006 and 2007 outbreak were 200 million Euros (Velthuis et al., 2010). It has been included in the World Organization for Animal Health (OIE) list of notifiable diseases (formerly List A) (OIE, 2011). The distribution of BT is determined by the geographic distribution of the arthropod vector and extends globally between latitudes 35°S and 53°N (Martin et al., 2008; Orru et al., 2004). Outbreaks have occurred in many countries in northern and western Europe since 2006 (Saegerman et al., 2008; Carpentera et al., 2009; Kampen and Ortega et

al, 2010).

Bluetongue virus (BTV) is the prototype member of the genus *Orbivirus* within the family *Reoviridae*. Thus far 26 serotypes are recognized. BTV is non-enveloped with a double shelled structure and a double-stranded (ds) 10 segment RNA genome. The virus contains 7 structural proteins and its genome encode also for 5 non structural proteins: NS1, NS2, NS3, NS3a and NS4. The outer capsid proteins, VP2 and VP5, are the serotype determinants and are responsible for generation of serotype-specific neutralizing antibody. The antibodies against VP7, the major core protein, will specifically detect the whole BTV serogroup. And the genomic segment 5, encoding NS1, is the most highly conserved of the 10 segments (Roy, 1989).

*Corresponding author. E-mail: zhangjg@nic.bmi.ac.cn.

[#]Authors contributed equally and are co-first authors for this work.

Traditionally, laboratory confirmation of BTV is done by intravenous egg inoculation followed by passages in mammalian cells (World Organization for Animal Health, 2008). Virus isolation is tedious and may take up to 5 weeks for completion. Consequently, alternative methods for virus detection have been sought, which include enzyme-linked immunosorbent assay (ELISA), immuno-electron microscopy, reverse transcription polymerase chain reaction (RT-PCR) (Yin et al., 2008), real-time RT-PCR (Yin et al., 2010), bio-barcode assay (Yin et al., 2011) and so on. ELISA techniques have a number of advantages including being economical, specific and rapid. Additionally, large number of clinical or laboratory samples could be screened by the assay in a very short time during sero-epidemiological studies. In this study, a monoclonal antibody-based antigen-capture ELISA was developed for detecting of VP7 protein.

MATERIALS AND METHODS

Monolonal antibody to VP7 of *bluetongue virus*

The gene of serogroup-specific antigen VP7 was amplified from BTV-5 (China Animal Health and Epidemiology Center, China) and cloned into pET-DsbA (Promega, Madison, U.S.A). Then, the recombinant VP7 was expressed in *Esherichia coli* BL21 (DE3) pLysS and purified using a His-tag affinity chromatography column on Ni^{2+}-nitrilotriacetate (NTA) resin (Qiagen, Hilden, Germany) (Li et al., 2007).

BTV-5 propagated in confluent monolayers of baby hamster kidney (BHK)-21 cells was purified through gradient centrifugation. Balb/c mice were immunized with the purified BTV-5 antigens. Splenocytes from the immunized mice were fused with SP2/0 myeloma cells, and positive hybridoma clones were screened through the expressed recombinant VP7. Monoclonal antibodies (MAb) 3E2 and 1C11 specific to BTV VP7 were prepared and determined to IgG2b (κ) (Yang et al., 2008).

The antigen-capture ELISA

In the antigen-capture ELISA, flat bottom, 96-well plates were incubated with 3E2 MAb and incubated overnight at 4°C. After three washes with PBST, 1% bovine serum albumin (BSA) blocking solution was added to the wells and incubated at 37°C for 60 min and washed three times with PBST. VP7 or BTV samples were added to the wells and incubated at 37°C for 60 min. After three washings, horseradish peroxidase-conjugated 1C11 MAb was added to the wells and incubated at 37°C for 30 min. The solutions were developed by adding 3,3',5,5'-tetramethylbenzidine (TMB) at room temperature. Reactions were stopped after 15 min by the addition of 2 M H$_2$SO$_4$. The absorbance in each well was read at 450 nm wavelength on an ELISA reader. A value twice (or more) the mean OD value of the negative antigen control was considered as the positive/negative cut-off value (positive to negative (P/N) ratio≥2).

The working dilutions of the capture and detection antibodies were selected by chequer board titration, and the best and most satisfactory result was obtained at a 1:2000 dilution for the capture antibody and a 1:1000 dilution for the detection antibody, while the dilution of the antigen (recombinant VP7) and negative control was a 1:10 dilution. At this dilution, the protein content of the controls was between 10 and 15 μg per well, and the P/N ratio was 4.0. These dilutions of reagents were followed throughout the study.

Evaluation of the antigen-capture ELISA

The specificity of the antigen-capture ELISA was confirmed by performing it to detect BTV serotypes 1-22 and the closely related orbivirus epizootic hemorrhagic disease virus (EHDV), using reference strains of serotype 5 (China Animal Health and Epidemiology Center, China).

The sensitivity of the assay was evaluated by conducting the antigen-capture ELISA to detect the recombinant VP7 protein at different concentrations and viral 10-fold serial dilutions of a BTV-5 strain cultured in BHK-21 cell, respectively.

The antigen-capture ELISA was carried out to detect strong-positive sample (0.25 μg/mL VP7), weak-positive sample (0.010 μg/ml VP7) and negative sample (distilled water) to determine the reproducibility of the assay. The samples were tested on 5 separate occasions, with 3 identical samples each time. The coefficients of variation (CV) were analyzed according to sample to negative (S/N) ratio (OD$_{450}$ value).

Five serotypes of BTV cultured in BHK-21 cell were 10-fold diluted respectively, and these BTV samples and eight sheep blood samples (a gift from China Animal Health and Epidemiology Center) were processed using the antigen-capture ELISA and INGEZIM BTV DAS (INGENASA, Madrid, Spain) to investigate the coincidence rate.

RESULTS

The specificity

Different serotypes BTV and EHDV-5 were detected using the antigen-capture ELISA, and the results showed that BTV serotypes 1-22 in the infected cell culture supernatant were all detected as positive, whereas EHDV 5 tested as negative (Table 1).

The sensitivity

VP7 of BTV at different concentrations was detected using the antigen-capture ELISA, and the results indicated that the assay could detect VP7 at concentrations as low as 9 ng/ml protein. Then, 10-fold serial diluted BTV-5 was tested using the assay. Results showed that titers as low as 100.5 TCID50/ml BTV-5 were detected positively (Table 2).

The reproducibility

The reproducibility of the antigen-capture ELISA was evaluated by detecting the VP7 protein. And the results showed that the CVs (%) of the strong-positive sample, weak-positive sample and negative sample were 3.79, 4.55 and 3.45 intra-test respectively, and 5.38, 6.10 and 4.88 inter-test, respectively (Table 3).

Coincidence analysis

Five serotypes of 10-fold diluted BTV and eight sheep blood samples were detected using the antigen- capture ELISA and INGEZIM BTV DAS. And the results indicated

Table 1. Detection of BTV and EHDV strains cultured in BHK-21 cell with the antigen-capture ELISA.

| Viral sample | BTV serotype | | | | | | | | | | | | | | | | | | | | | | EHDV serotype |
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	5
Results	P[a]	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	N[b]

[a] P: positive; [b] N: negative.

Table 2. Detection of BTV VP7 and BTV at different concentrations with the antigen-capture ELISA.

| Sample | The recombinant VP7 of BTV (µg/ml) | | | | | | BTV-5 (TCID$_{50}$/ml) | | | | |
	5	0.5	0.05	0.01	0.009	0.008	10^3	10^2	10^1	$10^{0.5}$	10^0
Results	P[a]	P	P	P	P	N[b]	P	P	P	P	N

[a] P: positive; [b] N: negative.

Table 3. Reproducibility of the antigen-capture ELISA for positive and negative samples.

| Sample | CV[a] (%) of S/N[b] of OD$_{450}$ value | |
	Intra-test	Inter-test
Strong-positive sample (0.25µg/ml VP7)	3.79	5.38
Weak-positive sample (0.01µg/ml VP7)	4.55	6.10
Negative sample (distilled water)	3.45	4.88

[a]CV: Coefficient of variation; [b]S/N: sample to negative ratio.

that the coincidence rate of the antigen-capture ELISA and INGEZIM BTV DAS was 100% in detecting BTV-5, 14, 16, 21, 22 ($10^{1.57}$, $10^{1.81}$, $10^{2.67}$, $10^{2.93}$, $10^{3.50}$ TCID$_{50}$/ml) and these blood samples (Table 4).

DISCUSSION

Monitoring and controlling of BTV infection in cattle, sheep and goats remains a top priority in BTV-endemic and epidemic countries interested in exporting livestock free of this disease or in restricting the introduction of new serotypes into existing endemic populations. The recent outbreaks of BT in Europe, 2006 to 2010, emphasize the need for surveillance and monitoring of BT, and the clinical and laboratory diagnosis of BT.

Serological assays provide evidence of earlier animal exposure to BTV. VP7 has a highly conserved sequence, displays antigenicity across all serotypes, and is the major group-specific antigen (Mertens et al., 2005). Not surprisingly, VP7 is frequently used in immunoassays designed to detect BTV (Nagesha et al., 2001; Reddington et al., 1991). Many countries use ELISAs that use Abs raised against BTV to detect the virus, even though ELISAs that utilize MAbs that specifically recognize VP7 have greater specificity (Afshar et al., 1992; Reddington et al., 1991).

A recent study demonstrated that the sensitivity of the polyclonal antibody-based sandwich ELISA was estimated to be between $10^{2.4}$ and $10^{2.6}$ TCID$_{50}$/ml with different serotypes of BTV (Chank et al., 2009). In the present study, the analytical detection limit of the antigen-capture ELISA for VP7 protein and BTV-5 was 9 ng/ml VP7 and $10^{0.5}$ TCID$_{50}$/ml BTV-5, respectively. The sensitivity of the assay was compared with the real-time RT-PCR

Table 4. Detection of BTV strains cultured in BHK-21 cell and sheep blood samples with the antigen-capture ELISA and INGEZIM BTV DAS[a].

Sample	BTV serotype					Sheep blood sample							
	5	14	16	21	22	1	2	3	4	5	6	7	8
The antigen-capture ELISA	P[b]	P	P	P	P	P	N[c]	N	N	N	N	P	N
INGEZIM BTV DAS	P	P	P	P	P	P	N	N	N	N	N	P	N

[a] INGEZIM BTV DAS: double antibody sandwich ELISA for detection of BTV VP7 protein purchased from INGENASA (Spain); [b] P: positive; [c] N: negative.

(Yin et al., 2010). As expected, real-time RT-PCR (10^{-1} TCID$_{50}$/ml BTV-1) was found to be at least 10 times more sensitive than the antigen-capture ELISA for detection of BTV in cell culture.

The analytical specificity of the assay has been studied with EHDV, since the virus is closely related to BTV. The results indicate that the 22 serotypes of BTV reference strains in our laboratory tested were detected specifically, with no cross-reactivity against the closely related orbivirus EHDV.

The antigen-capture ELISA developed in this study is based on MAbs specific to BTV VP7, which further improves the specificity of the assay of antibody-based ELISA. The assay is a simple and rapid test for high-throughput detecting BTV in cell culture and blood samples, and will be helpful at least for national purpose at least in China.

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REFERENCES

Carpentera S, Wilsona A, Mellora PS (2009). Culicoides and the emergence of *bluetongue virus* in northern Europe. Trends. Microbiol. 17(4):172-178.

Kampen H, Werner D (2010). Three years of bluetongue disease in central Europe with special reference to Germany: what lessons can be learned? Wien. Klin. Wochenschr. 122(3):31-39.

Li WC, Lv MM, Yang S, Yin HQ, Shi LJ, Ma YY, Zhang JG (2007). Molecular cloning and sequence analysis of the S7-ORF of *bluetongue virus 5*. Letters in Biotechnology. 18(3):384-387.

Mertens PPC, Maan S, Samuel A (2005). Oribivirus, Reoviridae. In: Eighth Report of the International Committee on the Taxonomy of Virus. Virus Taxonomy, Classification and Nomenclature of Viruses Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), London. pp.466-483.

Nagesha HS, Wang LF, Shiell B, Beddome G, White JR, Irving RA (2001). A single chain Fv antibody displayed on phage surface recognises conformational group-specific epitope of bluetongue virus. J. Virol. Methods. 91(2):203-207.

Orrù G, Santis PD, Solinas F, Savini G, Piras V, Caporale V (2004). Differentiation of Italian field and South African vaccine strains of *bluetongue virus* serotype 2 using real-time PCR. J. Virol. Methods. 122(1):37-43.

Ortega J, Crossley B, Dechant JE, Drew CP, Maclachlan NJ (2010). Fatal *bluetongue virus* infection in an alpaca (Vicugna pacos) in California. J. Vet. Diagn. Invest. 22(1):134-136.

Reddington JJ, Reddington GM, MacLachlan NJ (1991). A competitive ELISA for detection of antibodies to the group antigen of *bluetongue virus*. J. Vet. Diagn. Invest. 3(4):144-147.

Roy P (1989). *Bluetongue virus* genetics and genome structure. Virus. Res. 13(3):179-206.

Saegerman C, Berkvens D, Mellor PS (2008). Bluetongue epidemiology in the European union. Emerg. Infect. Dis. 14(4):539-544.

Tabachnick WJ, Robertson MA, Murphy KE (1996). Culicoides variipennis and bluetongue disease. Research on arthropod-borne animal diseases for control and prevention in the year 2000. Ann. N. Y. Acad. Sci. 791:219-226.

Velthuis AGJ, Saatkamp HW, Mourits MCM, de Koeijer AA, Elbers ARW (2010). Financial consequences of the Dutch bluetongue serotype 8 epidemics of 2006 and 2007. Prev. Vet. Med. 93(4):294-304.

World Organization for Animal Health (2008). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. pp.158-174.

World Organization for Animal Health (2011). OIE Listed diseases 2011. OIE, Paris, France.

Yang S, Li WC, Lv MM, Yin HQ, Ma YY, Liu M, Zhang JG (2008). Preparation and characterization of group specific monoclonal antibody against *bluetongue virus*. Lett. Biotechnol. 19(6):866-869.

Yin HQ, Zhang H, Shi LJ, Yang S, Zhang GP, Wang SQ, Zhang JG (2010). Detection and quantitation of *bluetongue virus* serotypes by a TaqMan probe-based real-time RT-PCR and differentiation from epizootic hemorrhagic disease virus. J. Virol. Methods. 168(1-2):237-241.

Yin HQ, Jia MX, Shi LJ, Yang S, Zhang LY, Zhang QM, Wang SQ, Li G, Zhang JG (2011). Nanoparticle-based bio-barcode assay for the detection of *bluetongue virus*. J. Virol. Methods. 178(1-2):225-228.

Yin HQ, Zhang GP, Zhang H, Zhang JG (2008). A pair of novel primers for universal detection of the NS1 gene from various *bluetongue virus* serotypes. Virol. Sin. 23(1):68-72.

Full Length Research Paper

The extraction on polysaccharide of sporocarp and static culture optimization conditions of *Morchella esculenta* from Qinghai-Tibetan Plateau

Ke-long Chen[1], Xu Su[1, 2*], Yu-ping Liu[3], Heng-sheng Wang[1], Zhao-meng Li[1], Jian-min LI[1] and Ben La[1]

[1]Key Laboratory of Education Ministry of Environments and Resources in Qinghai-Tibetan Plateau, School of Geography and Life Science, Qinghai Normal University, Xining 810008, China.
[2]State Key Laboratory Breeding Base-Key Laboratory of Qinghai Province for Plateau Crop Germplasm Innovation and Utilization, Qinghai University, Xining 810016, China.
[3]Department of Ideological and Political Theory Teaching and Research, Qinghai Normal University, Xining 810008, China.

In this paper, adopting orthogonal design method, the *Morchella esculenta* mycelia from Qinghai-Tibetan plateau was preliminary studied in different growing conditions such as pH, temperature, carbon sources, nitrogen sources and growth factors. The results showed that the *M. esculenta* mycelium liquid fermentation pH was between 6.0 to 7.0, cultivating optimum temperature 24°C, training required for 1.2% of potassium nitrate nitrogen (KNO$_3$), carbon source was 4.5% soluble starch, growth factor biotin was 0.1%. At the same time, we also took the plateau *M. esculenta* polysaccharide fermentation liquid extraction, the extraction time was optimal combination 2 h, precipitated with ethanol concentration of 90% extraction ratio 1:20, the highest yield of polysaccharides.

Key words: Qinghai-Tibetan Plateau, *Morchella esculenta*, sporocarp, mycelium, static culture.

INTRODUCTION

Polysaccharide is the material basis of life, it has a comparable information function of nucleic acid or protein, involved in cell-cell recognition, regulation of immune function and intercellular substance during transport, cell transformation, apoptosis, and most non-toxic. The research found that polysaccharides had anti-tumor and immunomodulatory (Zhang et al., 2007), anti-bacterial, anti-viral (Li et al., 2003), antioxidant and anti-aging, and many other pharmacological activities, it had become the ideal drug and natural sources of antioxidants. Polysaccharides exist in the mycelium, sporocarp and fermentation broth, which consists of more than 10 monosaccharide glycoside linkage to a polymer made of polymers

with complex biological activity and function. Among them, the most important one is immunomodulatory activity. Recently, fungal polysaccharides have been widely used into immunodeficiency diseases, autoimmune diseases, cancer and other diseases and clinical treatment and medical field for other purposes such as preparation of pharmaceutical materials, drug delivery agents, blood plasma substitutes. In recent years, the study of fungal polysaccharide and its compound has attracted more and more attention. So it has become one of the hot research areas such as molecular biology, medicine, food science, etc.

Morchella esculenta is famous for edible fungus with rich

*Corresponding author. E-mail: xusu8527972@126.com.

Table 1. The level table of L_9 (3^4) on nutritional conditions

Level	Factor			
	A (%)	B (%)	C (%)	D (%)
1	0.3 KNO$_3$	0.25 Biotin	10	2.5 C$_6$H$_{12}$O$_6$
2	0.5 Peptone	0.5 VB$_2$	20	3 Corn flour
3	0.3 Soy flour	0.75 IAA	30	3.5 Soluble starch

Note: The content of other components is respectively 0.5% KH2PO4, 0.1% MgSO4 and 100mL H2O.

nutritious and delicious taste, which is welcomed by the international market. The content of amino acid lies on the first of all kinds of edible fungi. *M. esculenta* is internationally recognized as valuable medicinal fungus, its medicinal value of Chinese "broad spectrum of bacteria" and "compendium of materia medica" and other classics have been reported. Traditional medicine thought *M. esculenta* was natural, sweet and benefited the stomach and phlegm and lung airflow at ease. Modern medical research showed that *M. esculenta* polysaccharide is effective medicinal ingredients; it has enhanced immunity, anti-fatigue, anti-viral, tumor suppression and many other effects (Ren and Zhang, 1999).

In this article, on the basis of single factor experiment, static culture conditions were optimized and extraction technology of polysaccharide was selected adopting the orthogonal experiment. This research will provide the theoretical foundation for the antioxidant of natural polysaccharide development.

MATERIALS AND METHODS

Static culture conditions optimized of *M. esculenta* mycelium.

Test materials

Tested strains: the optimal use of screening strain Y2 (North mountain forestry farm, Huzhu, Qinghai, gathered on May 3, 2005, by isolating stalk of *Morchella vulgaris* to obtain the pure strain).

Culture medium

Mother Culture Media: PDA integrated medium (potato 200 g, glucose 20 g, KH$_2$PO$_4$ 1 g, MgSO$_4$·7H$_2$O 1 g, VB$_1$ 50 μg, agar 15 g, chloramphenicol 0.5 g), add distilled water to 1000 ml, pH 6.5, 121°C, sterilize 20 min.

Liquid seed medium: PDA medium without agar.

Test method

Let the above medium become into plate medium, then take a different number of punch block *M. esculenta* mycelium, and take them to plant in the fresh medium (five repeats of each group). Finally place them in incubator.

Determination on dry weight of colonies

Training 120 h, place the colonies and medium from the plates in a beaker with distilled water, each plate processing corresponds to a beaker, them the beaker is heated in the dry box. When the medium melted, mycelium will singled out with a sterile glass rod and the excess water was absorbed by the filter paper. Then place them in a weighing bottle and dry at 60°C until constant weight. At the end, they will be weighed (Wang et al., 2010). Based on the preliminary experiments of single factor, the orthogonal experiment was made by selecting the more important factor, including nitrogen (A), the type and concentration of growth factors (B), inoculum (C), carbon (D), pH (E), incubation time (F), incubation temperature (G), ventilation (H), impact on dry weight of *M. esculenta* mycelium. As there were more experimental factors and there are in order, the orthogonal experiment carried out twice (Wang, 2006). In the vicinity of the optimum value of each factor, three levels were taken and four factors and three levels orthogonal experiments were done with indicators for dry weight of mycelium. According to the L_9(3^4) cross-table, the technology of polysaccharide extraction was optimized (Tables 1 and 2).

Polysaccharide extraction of *M. esculenta* fermentation liquor

Tested strains

The optimal screening strain Y2.

Media and culture conditions

Make media by applying the above, the best media and culture conditions obtained by the experiments.

Determination of polysaccharide content

M. esculenta fermentation broth was filtered after 21 days of culture. Then the filtrate was concentrated in a water bath at 90°C to the original volume of 1/3, slowly add 3 volumes of 80% ethanol solution, stand and overnight. The next day, the precipitation is extracted by centrifugation, dissolve in a small amount of hot water and wash three times with ethanol, centrifuge for 15 min with 3000 rpm. The precipitate was dried naturally in order to obtain polysaccharide fermentation broth of *M. esculenta*.

Extraction technology of polysaccharide of *M. esculenta* sporocarp

Tested materials

M. esculenta sporocarp: collected from North mountain forestry farm of Huzhu in Qinghai Province.

Table 2. The level table of L_9 (3^4) on culture conditions.

Level	Factor			
	E	F (h)	G (°C)	H
1	6.0	72	20	（1）
2	6.5	96	24	（2）
3	7.0	120	28	（3）

Note: (1) Three levels of ventilation volume were respectively 150, 250 and 350 ml can bottles with 100 ml culture medium. (2) The content of other components is respectively 0.5% KH_2PO_4, 0.1% $MgSO_4$ and 100 ml H_2O.

Table 3. The orthogonal table of polysaccharide extraction of *Morchella esculenta* sporocarp.

Factor	Level		
	1	2	3
Extraction ratio (ml)	1:20	1:30	1:40
Extraction time (h)	2	4	6
Concentration of ethanol precipitation (%)	75	90	100

Test Method

Best extraction selection: select three main factors impact on polysaccharide extraction of *M. esculenta* sporocarp including extraction ratio, extraction time and ethanol concentration. The orthogonal experiment was made by using $L_9(3^4)$ and regarding the ratio of *M. esculenta* polysaccharide as investigated indicators (Table 3).

Extraction method: dry *M. esculenta* sporocarp in 60°C and grind them for used. Take 9 parts of *M. esculenta* sporocarp with the weight of 10 g, then place them into 1000 ml beaker, which was added 20 times with distilled water (200 ml), 30 times (300 ml), 40 times (400 ml), and finally take them into thermostatic water bath with the water temperature controlled at about 95°C. They were extracted with water volume and extraction time under orthogonal test. Centrifuge for 10 min with 4000 r/min speed, take the supernatant, extract precipitate again by the above method, concentrate them, the above extract was concentrated according to orthogonal 3-fold volume, added various concentrations of ethanol precipitation, overnight, discarded the supernatant liquid centrifugal, derived from sediments into the Petri dish, dried at 60°C in the roasting oven, and get morel fruiting polysaccharides (Meng et al., 2013).

RESULTS AND ANALYSIS

Static culture conditions optimized of *M. esculenta* mycelium

Through the intuitive analysis of data (Tables 4 and 6), the static culture conditions of *M. esculenta* mycelium was selected out by orthogonal test. Namely, when use water as solvent and take dry weight as evaluation indicators, the best mix programme was $A_1B_2C_2D_3$ and $E_2F_3G_2H_2$. What is more, 0.3% KNO_3, 0.5% VB_2, 10% vaccination volume, 3.5% soluble starch as carbon source, pH 6.5, 120 min training time, the most suitable temperature for 24°C and ventilation volume for 250 ml. Analysis of variance

table (Tables 5 and 7) showed that the impact extent order of various factors on dry weight of mycelium is: carbon source > growth factor > nitrogen > inoculation, incubition temperature > pH > incubation time > ventilation. The carbon source, growth factors, nitrogen, temperature, pH and incubation time have significantly effect on the dry weight of *M. esculenta*.

Study on extraction technology of polysaccharide from *M. esculenta* sporocarp

The results were shown in Table 8. When the ratio of extraction was 1:30, extraction time was 2 h and concentration of ethanol was 90%, the production of polysaccharides yield was the highest and reached for 13.1%, which was basically consistent with the result reported by Wu and An (Wu and An, 2005), but it was lower than Li and Qiu (Li and Qiu, 2005) who reported. Thus, we thought that it was a certain relationship with the extraction method of polysaccharide fermentation liquid. Li and Qiu (Li and Qiu, 2005) used a method of enzyme extraction, so extraction yield of polysaccharide could be increased significantly through this method. They determined the optimum conditions of pectinase action were 15% enzyme dosage, 50°C and 3 h and the optimal conditions of cellulase enzyme were 15% enzyme dosage, 45°C and 3 h. But it could be seen from the tables, the polysaccharides yield is little difference when extraction ratio were 1:20, 1:30 and 1:40. If considering the practicality of technology, the choice of 1:20 was the best extraction ratio. Extraction time had the greater impact on the extraction of polysaccharide. When extraction time was 2 h, the average yield of polysaccharide was the highest, so 2 h should be used for the best extraction time. At the same

Table 4. The design scheme and results of orthogonal test L_9 (3^4) about medium formula of *Morchella esculenta*.

Number	Factor				Dry weight of mycelium (mg/100ml)			Sum	Average value (mg/100ml)
	A	B	C (%)	D (%)	I	II	III		
1	1 (0.3% KNO$_3$)	1(0.25% Biotin)	1(10)	1(2.5% C$_6$H$_{12}$O$_6$)	217.3	210.4	213.2	640.9	213.6
2	1	2 (0.5%VB$_2$)	2(20)	2(3% Corn flour)	205.6	214.3	203.2	623.1	207.7
3	1	3(0.75% IAA）	3(30)	3(3.5% Soluble starch)	215.4	207.3	209.6	632.3	210.8
4	2 (0.5% Peptone)	1	2	3	220.0	218.4	210.6	649	216.3
5	2	2	3	1	204.6	212.3	217.5	634.4	211.5
6	2	3	1	2	178.3	186.2	179.4	543.9	181.3
7	3 (0.3% Soy flour)	1	3	2	185.4	190.2	196.3	571.9	190.6
8	3	2	1	3	207.1	203.1	210.8	621	207.0
9	3	3	2	1	196.3	189.7	192.4	578.4	192.8
T_1	1896.3	1861.8	1805.8	1853.7					
T_2	1827.3	1878.5	1850.5	1738.9					
T_3	1771.3	1754.6	1838.6	1902.3					
X_1	210.7	206.8	200.6	206.0					
X_2	203.0	208.7	205.6	193.2					
X_3	196.8	195.0	204.3	211.4					
R	13.9	13.8	5.0	18.2					
Optimization parameters	A_1	B_2	C_2	D_3					

Table 5. The variance table of orthogonal experiment on *Morchella esculenta* medium formulation.

Source of variation	SS	df	MS	F	P
Block	3559.26	8	444.91	19.53	<0.001
A	871.19	2	435.59	19.12	<0.001
B	1004.52	2	502.26	22.05	<0.001
C	119.09	2	59.55	2.61	0.1
D	1564.47	2	782.23	34.34	<0.001
The experimental error	409.97	18	22.78		
The total variation	3969.23	26	152.66		

time, ethanol concentration was 90%, the average yield of polysaccharide was the highest, so it was the best to select 90% ethanol precipitation. As could be seen from the above results, the optimum combination of extraction of *M. esculenta* polysaccharide was that, extraction time was 2 h, 90% ethanol precipitated and extraction ratio was 1:20. Meanwhile, by orthogonal analysis of variance table (Table 9) showed that it had reached significantly that ethanol concentration had effected on the yield of polysaccharide.

Conclusion

M. esculenta sporocarp growing on the ecological environment requirements was extremely strict and has obvious season. Because mycelium and sporocarp of *M. esculenta* were different, they were not the same with demand for nutrients, temperature, pH, moisture, light, oxygen and so on. In different nutritional conditions, the shape, color and other cultural characteristics of myce-

lium, which were growing in the same strain of *M. esculenta*, are not identical. These differences reflected the diversity of physiological characteristics about *M. esculenta*. This diversity caused the volatility of domesticated conditions and increased the difficulty of domestication. At present, the artificial cultivation could still not achieve commercialized cultivation and wild resources of *M. esculenta* was very limited, but the demand for *M. esculenta* became more and more, so we must find another way in order to do the sustainable development and utilization of *M. esculenta* resources. Through the experiment, we found that the nutrients contained in mycelium produced by liquid fermentation were basically consistent with those from sporocarp, so mycelium and mycelium polysaccharide should be obtained by the massively artificial liquid fermentation and those products were developed and utilized. Because Qinghai province had the unique geographical environment and location, and *M. esculenta* resources were rich and various, so the wild resources of *M. esculenta* were realized sustainable development. On

Table 6. The orthogonal test (L_9 (3^4)) design scheme and results of *Morchella esculenta* culture conditions.

Number	Factor				Dry weight of mycelium (mg/100ml)			Sum	Average value mg/100ml
	E	F (h)	G (°C)	H(ml)	I	II	III		
1	1(6.0)	1(72)	1（20）	1(150)	164.3	172.1	168.4	504.8	168.3
2	1	2(96)	2（24）	2(250)	177.2	179.5	182.3	539	179.7
3	1	3(120)	3（28）	3(350)	160.4	173.6	168.2	502.2	167.4
4	2(6.5)	1	2	3	185.4	190.7	179.6	555.7	185.2
5	2	2	3	1	167.2	173.3	178.1	518.6	172.9
6	2	3	1	2	193.5	187.2	191.6	572.3	190.8
7	3 (7.0)	1	3	2	158.4	167.2	170.2	495.8	165.3
8	3	2	1	3	180.1	186.2	178.3	544.6	181.5
9	3	3	2	1	190.7	186.4	193.2	570.3	190.1
T_1	1546	1556.3	1621.7	1593.7					
T_2	1646.6	1602.2	1725	1607.1					
T_3	1610.7	1644.8	1516.6	1602.5					
X_3	179.0	182.8	168.5	178.0					
R	11.2	9.8	16.5	1.5					
Optimization parameters	E_2	F_3	G_2	H_2					

Table 7. Analysis of variance table of orthogonal experiment on *Morchella esculenta* culture conditions.

Sources of variation	SS	df	MS	F	P
Block	2317.43	8	289.68	12.81	<0.001
E	577.60	2	288.80	12.77	<0.0001
F	435.33	2	217.66	9.63	<0.0001
G	1294.20	2	647.10	28.62	<0.001
H	10.30	2	5.15	0.23	0.7985
Experimental error	406.97	18	22.61		
Total variation	2724.40	26	104.78		

Table 8. The experimental results and intuitive analysis on polysaccharide extraction of *Morchella esculenta* sporocarp.

Number	Extraction ratio A	Extraction time (h) B	Concentration of ethanol precipitation (%) C	Polysaccharide production (g)	Polysaccharide yield (%)
1	1	1	1	1.21	12.1
2	1	2	2	1.20	12.0
3	1	3	3	1.24	12.5
4	2	1	2	1.32	13.1
5	2	2	3	0.70	7.0
6	2	3	1	1.10	11.1
7	3	1	3	1.20	12.0
8	3	2	1	1.05	10.5
9	3	3	2	1.09	12.9
K_1	1.217	1.243	1.120		
K_2	1.040	0.983	1.203		
K_3	1.113	1.143	1.047		
R	0.177	0.260	0.156		
Optimization parameters	A_1	B_1	C_2		

Table 9. Analysis of variance table of orthogonal experiment on polysaccharide extraction.

Source of variation	SS	df	MS	F	P
Block	0.19	6	0.03	0.89	0.62
A	0.05	2	0.02	0.67	0.60
B	0.10	2	0.05	1.46	0.41
C	0.04	2	0.02	0.52	0.66
The experimental error	0.07	2	0.04		
Total variance	0.26	8	0.03		

On the one hand, liquid fermentation of *M. esculenta* were studied and related products were developed in order to research and develop mycelium polysaccharides and related products; On the other hand, the relevant departments should formulate corresponding policies to manage and control the collection, purchase, sales, and actively establish production and experimental base for the implementation of artificial and artificial cultivation so as to achieve the purpose of effective protection and rational utilization of *M. esculenta* resources and realize sustainable development.

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REFERENCES

Feng Z, Qun G, Ling-lei K, Yu-yun Z (2007). The experimental research on anti-tumor activity of polygonati polysaccharide. China Pract. Med. 2(21): 95-96.

Li J, Qiu D (2005). Research bulletin on *Morchella esculenta* polysaccharide by enzymatic extraction. Hebei For. Sci. Technol. (1): 1-2.

Li X, Jian-hua R, Mou-cheng W (2003). Recent pharmacological studies on polysaccharide from *Grifola fronadosa*. Natural Product Research and Development. 15(4): 364-367.

Meng C, Shi H, Zhao J, Li C, Guo M (2013). Studies on the extraction technology of *Morchella esculenta* polysaccharide with rich Selenium. Sci. Technol. West China 12(2): 83-84.

Ren G, Zhang S (1999). The progress of research on *Morchella*. J. Yanan Univ. 18(1):57-64.

Wang G, Dong L, Zhang H (2010). The study of optimization conditions about fermentation medium shallow of *Morchella*. Northern Hortic. (7): 173-175.

Wang S (2006). Studies on the biological characteristics and domestiction of *Morchella* from Huzhu northern mountain forest farm in Qinghai province. Xining: Qinghai Normal University. pp. 46-65.

Wu Q, An J (2005). Study on extraction and separation of polysaccharide of *Morchella esculenta* cultured by fermentation. Food Sci. 26(1): 116-120.

Full Length Research Paper

Molecular characterization of *Trichoderma longibrachiatum 21PP* isolated from rhizospheric soil based on universal ITS primers

Mohammad Shahid[1]*, Mukesh Srivastava[1], Antima Sharma[1], Anuradha Singh[1], Sonika Pandey[1], Vipul Kumar[1], Neelam Pathak[2] and Smita Rastogi[2]

[1]Biocontrol Laboratory, Department of Plant Pathology, Chandra Shekhar Azad University of Agriculture and Technology, Kanpur-208002, Uttar Pradesh, India.
[2]Department of Biosciences, Integral University, Lucknow 226026, Uttar Pradesh, India.

Trichoderma species are morphologically very similar and were considered for many years as a single species. A new strain *Trichoderma longibrachiatum 21PP* was isolated from a rhizospheric soil sample collected from Kaushambi district of an Indian State (Uttar Pradesh). The universal primers (ITS-1& ITS-4) were used for the amplification of 28S rRNA gene fragment that produced a sharp band of about 700 bp on the gel. The amplified gene fragment was then sequenced (664 bp) and then deposited in GenBank with the Accession No. JX978542. Thus, the molecular identification of the specified strain enabled us for further characterization as this strain of *Trichoderma* species can be used as a biocontrol agent against pathogenic fungi such as *Fusarium, Alternaria, Sclerotinia*, etc. It is proposed that the identified strain *T. longibrachiatum 21PP* be assigned as the type strain of a species of the genus together with the 28S rRNA gene sequence search in Ribosomal Database Project, small subunit rRNA and large subunit rRNA databases.

Key words: 28S ribosomal RNA gene, *Trichoderma,* polymerase chain reaction, phylogenetic analysis, DNA isolation, DNA sequencing.

INTRODUCTION

Accurate and definitive fungal identification is essential for correct disease diagnosis and treatment of associated fungal infections. Characterization of fungal species using classical methods is not as specific as the genotyping methods. Genotypic techniques involve the amplification of a phylogenetically informative target such as the small-subunit (28S) rRNA gene (Woese and Fox, 1977). rRNA is essential for the survival of all cells, and the genes encoding the rRNA are highly conserved in the fungal and other kingdoms. The sequences of the rRNA and proteins comprising the ribosome are highly conserved throughout evolution because they require complex inter-

and intramolecular interactions to maintain the protein-synthesizing machinery (Sacchi et al., 2002; Hillis et al., 1991; Woese, 1987). *Trichoderma* spp. is common soil inhabitants and is effective in providing biocontrol of soil borne pathogens due to its antagonistic behaviour. The major aspect of successful biological control strategies includes the production, formulation and delivery system of bioagents. The Internal Transcribed Spacer (ITS) regions of the rDNA are perhaps the most widely sequenced DNA regions in fungi. It has typically been most useful for molecular systematic study at species level, and even within species (Ospina-Giraldo et al., 1998; Kubicek et al.,

*Corresponding author. Email: shahid.biotech@rediffmail.com.

Table 1. Gene sequence with universal primers.

Primer Name	Sequence (5'-3')	Length	T_m (°C)	GC content (%)
ITS-1	TCTGTAGGTGAACCTGCGG	19	53	58
ITS-4	TCCTCCGCTTATTGATATGC	20	50	45

2000; Kulling et al., 2002; Lee and Hseu, 2002). Kindermann et al. (1998) attempted a first phylogenetic analysis of the whole genus of *Trichoderma* using sequence analysis of the ITS-1 region of rDNA.

The study aims at identifying a potential strain of Trichoderma species as it can help us in the commercial-lization and eventually developing a better solution to the eradication of soil borne pathogens. Here, the method of isolation and identification of an unknown fungus from the Indian Kaushambi (25° 31′ 50.68″ N, 81° 22′ 38.25″ E) district using 28S rRNA gene sequence is described (Srivastava et al., 2008; Yadav et al., 2009) to charac-terize the strain *Trichoderma longibrachiatum 21PP* as a member of the *Trichoderma* spp. We initiated a systema-tic screening program to catalogue the microbial compo-sition of the Indian district Kaushambi.

MATERIALS AND METHODS

Isolation of *Trichoderma* from soil sample

Rhizospheric soil samples were collected from various experimental fields of an Indian district Kaushambi (25° 31′ 50.68″ N, 81° 22′ 38.25″ E). The strain was isolated and identified in Potato Dextrose Agar (PDA) with low sugar medium by serial dilution method (Johnson and Crul, 1972; Nirenberg, 1976).

DNA isolation of *Trichoderma*

Pure culture of the target fungal was grown overnight in liquid PDB for the isolation of genomic DNA using a method described by Hiney et al. (1992).

Identification of *Trichoderma*

The isolated strain was then identified at molecular level using PCR amplification of the specific gene sequence with universal ITS-1 (forward) and ITS-4 (reverse) primers. It is then deposited to the Indian Type Culture Collection (ITCC), IARI (New Delhi, India).

Molecular characterization

The total genomic DNA was extracted from isolate of *Trichoderma* based on Cetrimide Tetradecyl Trimethyl Ammonium Bromide (CTAB) mini extraction method as was described by Crowhurst et al. (1995) in their study.

Agarose gel electrophoresis

Ten microlitre of the reaction mixture was then analyzed by submarine gel electrophoresis using 1.0% agarose with ethidium bromide at 8 V/cm and the reaction product was visualized under a UV transilluminator.

Amplification of isolated DNA using ITS

The internal transcribed spacer (ITS) regions of the rDNA repeat from the 3′ end of the 28S and the 5' end of the 28S gene were amplified using the two primers, ITS-1 and ITS-4 which were syn-thesized on the basis of conserved regions of the eukaryotic rRNA gene (White et al., 1990). The PCR-amplification reactions were performed in a 50 µl mixture containing 50 mM KCl, 20 mM Tris HCl (pH 8.4), 2.0 mM MgCl₂, 200 µM of each of the four deoxynucleo-tide triphosphates (dNTPs), 0.2 µmM of each primer, 40 ng/µl of template and 2.5 U of Taq polymerase (Fermentas). The cycle parameters included an initial denaturation for 5 min at 94°C, followed by 40 cycles of denaturation for 1 min at 94°C, primer annealing for 2 min at 55°C, primer extension for 3 min at 72°C, and a final extension for 10 min at 72°C. Amplified products were separated on 1.2% agarose gel in TAE buffer, pre-stained with ethidium bromide (1 µg/ml) and electrophoresis was carried out at 60 V for 3 h in TAE buffer. One Kb ladder (MBI, Fementas) was used as a marker. The gel was observed in a transilluminator over ultraviolet light. The desired bands were cut from the gel with minimum quantity of gel portion using QIAGEN gel extraction kit.

Purification of PCR product

The PCR product was purified by QIAGEN gel extraction kit using the protocol described in the manufacturer's manual.

DNA sequencing of the 28S rDNA fragment

A pair of universal ITS primers ITS-1 (forward) and ITS-4 (reverse) was used for sequencing of the amplified product and this step was carried out at the Merck Laboratory (Bangalore, India). The details of the primers are mentioned in Table 1.

Sequence analysis and phylogeny

A comparison of 28S rRNA gene sequence of the test strain against nucleotide collection (nr/nt) database was done using BLAST program (Zhang et al., 2000). Sequences that shared about 90% similarity with the test sequence were selected for a multiple se-quence alignment that was carried out using ClustalW (Thompson et al., 1994). Subsequently, an evolutionary distance matrix was generated from these nucleotide sequences in the dataset. A phylogenetic tree was then constructed using the Neighbor Joining method (Saitou and Nei, 1987). Phylogenetic and molecular evolu-tionary analyses were conducted using MEGA (Molecular Evolu-tionary Genetics Analysis) version 4.0 (Tamura et al., 2007). The 16S rRNA gene sequence of test strain was compared with a different set of sequence database such as small subunit ribosomal RNA (SSU rRNA) and large subunit ribosomal RNA (LSU rRNA)
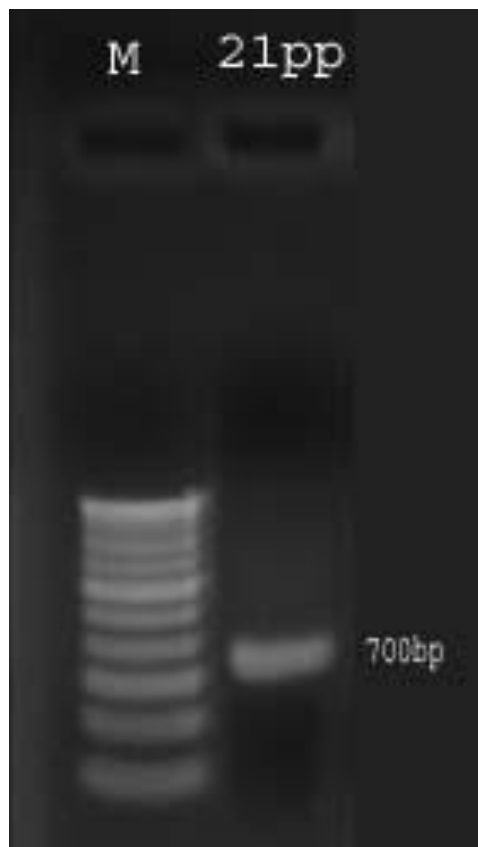
Figure 1. Shows the amplified PCR product of the *Trichoderma longibrachiatum 21PP*.

using Ribosomal RNA BLAST (Altschul et al., 1997). 28S rRNA gene sequence of the test strain was also compared against those sequences, in Ribosomal Database Project (Cole et al., 2009) by using the RDP Classifier check program (Wang et al., 2007). The annotated information for the sequence in the database to which 28S rRNA aligns is used for fungal identification.

RESULTS

Rapid identification of microorganisms is necessary in the pathological laboratory in an attempt to proceed for the preparation of a bioformulation that can be useful for the farmers. The rRNA based analysis is a central method in pathology used not only to explore microbial diversity but also to identify new strains. The genomic DNA was extracted from isolated fungal strain *T. longibrachiatum* 21PP and universal ITS-1 (19F) and ITS-4 (20R) primers were used for the amplification and sequencing of the 28S rRNA gene fragment. A total of 664 bp of the 28S rRNA gene was sequenced (Figure 1).

The sequenced gene fragment of the specific strain was then searched for other closely related and similar templates using BLAST program. Sequences that shared 90% similarity to the test sequence were selected for a multiple sequence alignment. Figure 2 shows the phylogenetic relationship of the test strain (*T. longibrachiatum*

21PP) with others strains of *Trichoderma*. Comparison of test strain against known sequences of SSU and LSU rRNA databases showed that the gene sequence of the isolate *21PP* has 90% sequence similarity (Score=664 bits, Expect=0.0) with 28S rRNA gene sequence of *Trichoderma* (GenBank Acc. No.: JX978542). Thus, it can be concluded from the above data that the isolate *21PP* is a member of the genus *T. longibrachiatum*.

Conclusion

The study is focused on the identification of bioagent *T. longibrachiatum 21PP* at species level. Biocontrol activity of fungal strains in laboratory is generally determined by biological assays. To check the presence or absence of these biocontrol agents is a need to develop some efficient method. In past, serial dilution method had some disadvantages as it cannot distinguish between indigenous and artificially introduced strains that is why the first step of utilizing the full potential of fungal strains is identified at molecular level.
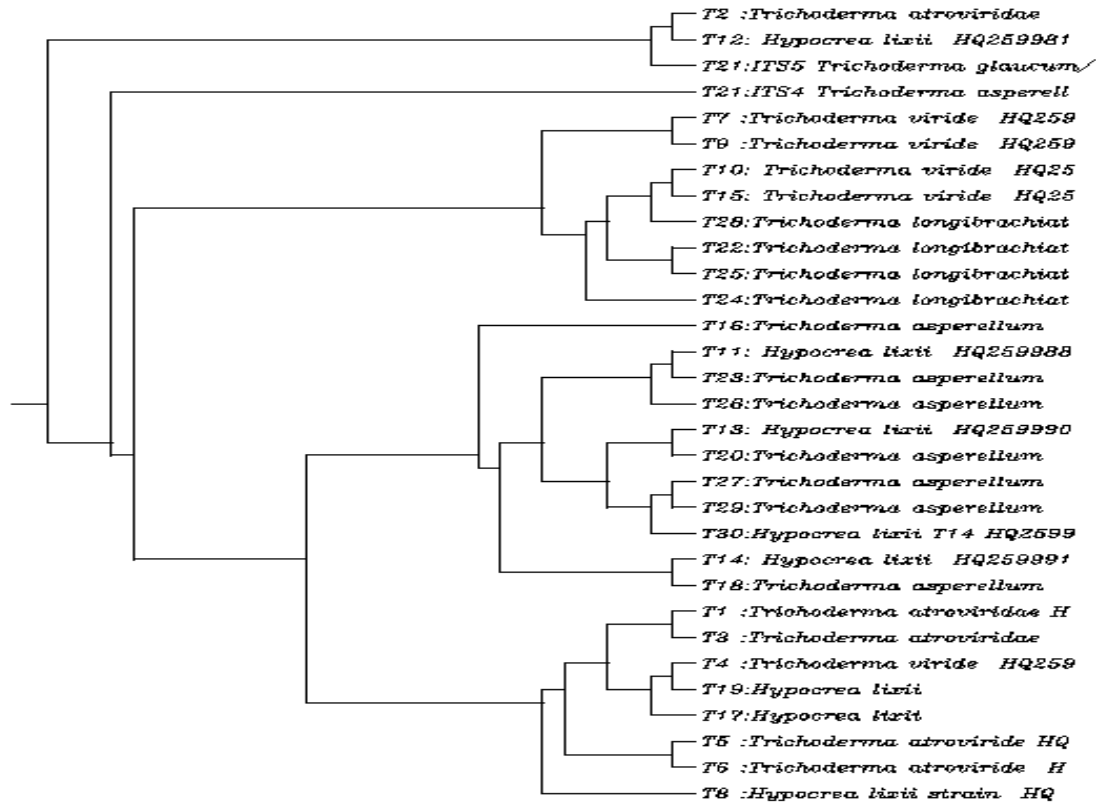
ACKNOWLEDGEMENT

Figure 2. Shows the phylogenetic relationship of the test strain with other strains of *Trichoderma*.

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REFERENCES

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25(17): 3389-3402.

Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed--Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM (2009). The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res. 37: D141-D145.

Crowhurst RN, King FY, Hawthorne BT, Sanderson FR, Choi-Pheng Y (1995). RAPD characterization of *Fusarium oxysporum* associated with wilt of angsana (*Pterocarpus indicus*) in Singapore. Mycol. Res. 99: 14-18.

Hillis DM, Moritz C, Porter CA, Baker RJ (1991). Evidence for biased gene conversion in concerted evolution of ribosomal DNA. Science 251: 308-310.

Hiney M, Dawson MT, Heery DM, Smith PR, Gannon F, Powell R (1992). DNA probe for *Aeromonas salmonicida*. Appl. Environ. Microbiol. 58(3): 1039-1042.

Johnson LF, Crul EA (1972). Methods of research on ecology of soil borne pathogens, Minneapolis U.S. Burgess Pub. 247.

Kindermann J, Ayouti YE, Samuels GJ, Kubicek CP (1998). Phylogeny of the genus *Trichoderma* based on sequence analysis of the Internal Transcribed Spacer region 1 of the rDNA cluster. Fungal Gen. Biol. 24: 298-309.

Kubicek CP, Mach RL, Peterbauer CK, Lorito M (2000). *Trichoderma*. From genes to biocontrol. J. Plant Pathol. 83: 11-23.

Kulling CM, Szakacs G, Kubicek CP (2002). Phylogeny and evolution of the genus *Trichoderma*: a multigene approach. Mycol. Res. 106: 757-767.

Lee C, Hseu T (2002). Genetic relatedness of *Trichoderma* sect. *Pachybasium* species based on molecular approaches. Can. J. Microb. 48: 831-840.

Nirenberg HI (1976). Studies on the morphological and biological differentiation in Fusarium section Liseola. Mitt Biol Bundesanstalt fur Land-Forstw Berlin-Dallem, 169: 1-117.

Ospina-Giraldo MD, Royse DJ, Thon MR, Chen X, Romaine CP (1998). Phylogenetic Relationships of *Trichoderma harzianum* Causing Mushroom Green Mold in Europe and North America to Other Species of *Trichoderma* from World-Wide Sources. Mycologia 90(1): 76-81.

Sacchi CT, Whitney AM, Reeves MW, Mayer LW, Popovic T (2002). Sequence Diversity of *Neisseria meningdidis* 16S rRNA Genes and Use of 16S rRNA Gene Sequencing as a Molecular Subtyping Tool. J. Clin. Microbiol. 40(12): 4520-4527.

Saitou N, Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4(4): 406-425.

Srivastava S, Singh V, Kumar V, Verma PC, Srivastava R, Basu V, Gupta V, Rawat AK (2008). Identification of regulatory elements in 16S rRNA gene of Acinetobacter species isolated from water sample. Bioinformation 3(4): 173-176.

Tamura K, Dudley J, Nei M, Kumar S (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24(8): 1596-1599.

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.

Wang Q, Garrity GM, Tiedje JM, Cole JR (2007). Naïve Bayesian

Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. Appl. Environ. Microbiol. 73(16): 5261-5267.

White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal DNA for phylogenetics. In: Innes MA, Gelfand DH, Sninsky JJ, White TJ (Eds.), PCR protocols: a guide to methods and applications. Academic Press, Inc., San Diego, Calif. 315-322.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acid Res.18: 6531-6535.

Woese CR (1987). Bacterial evolution. Microbiol. Rev. 51: 221-271.

Woese CR, Fox GE (1977). Phylogenetic structure of the prokaryotic domain: the primary kingdoms. Proc. Natl. Acad. Sci. USA, 74: 5088-5090.

Yadav V, Prakash S, Srivastava S, Verrna PC, Gupta V, Basu V, Rawat, AK (2009). Identification of *Comamonas* species using 16S rRNA gene sequence. Bioinformation 3(9): 381-383.

Zhang Z, Schwartz S, Wagner L, Miller W (2000). A greedy algorithm for aligning DNA sequences. J. Comput. Biol. 7(1-2): 203-214.

Full Length Research Paper

Correlation between architect hepatitis C virus (HCV) core antigen and HCV Ribonucleic acid levels in Anti-HCV reactive patients in Turkey

BURUK Kurtulus[1], BAYRAMOGLU Gulcin[1], KAKLIKKAYA Nese[1]*, AKYUZ Zeynep[1], KAYA Selcuk[2], KOKSAL Iftihar[2], CAN Gamze[3] and AYDIN Faruk[1]

[1]Karadeniz Technical University Medical Faculty, Department of Medical Microbiology, Trabzon, Turkey.
[2]Karadeniz Technical University Medical Faculty, Department of Infectious Diseases, Trabzon, Turkey.
[3]Karadeniz Technical University Medical Faculty, Department of Public Health, Trabzon, Turkey.

Quantitative tests for the detection of hepatitis C virus ribonucleic acid (HCV-RNA) levels and HCV core antigen have been mainly used for the management of patients with HCV infection. The aim of this study was to evaluate the correlation between HCV core antigen and HCV RNA levels in patients reactive for anti-HCV antibodies. Three hundred and twenty-five anti-HCV reactive sera samples were included in the study. HCV core antigen and HCV RNA levels were determined using the Architect HCV Ag test and Abbott RealTime[TM] HCV RNA test (RT-PCR), respectively. The correlation coefficient between the levels of HCV core antigen and HCV RNA test results was calculated using Spearman's rank test, and linear regression analysis was applied. One hundred and sixteen of the 325 samples were detected positive by both methods. Three additional samples by RT-PCR, and 4 samples by Architect HCV Ag, the negative samples were found positive by the other method. All of these contradictory results were obtained from the low level HCV RNA or HCV core antigen including samples. A correlation coefficient (r) was determined as 0.899 between the levels of HCV core antigen and HCV RNA (p<0.0001). The sensitivity, specificity, positive predictive value and negative predictive value of the HCV core antigen test were 97.48, 98.06, 96.67 and 98.54%, respectively, using the HCV RNA test as a reference. The Architect HCV core antigen test exhibits a good correlation with the HCV RNA test. It can be used as an alternative method, especially when the HCV RNA test is unavailable.

Key words: Anti- hepatitis C virus, Architect hepatitis C virus Ag test, hepatitis C virus RNA.

INTRODUCTION

The most frequently used screening method in the diagnosis of hepatitis C virus (HCV) infections is the detection of anti-HCV antibodies in serum or plasma. Nevertheless, anti-HCV antibodies reached detectable levels in the serum after a long window period of HCV infection. Additionally, even if the virus is eliminated from the blood its presence may persist for many years, for which reason this method cannot be used for the differentiation of active or past infection or for monitoring antiviral therapy. At the same time, tests used for determining anti-HCV antibodies can give false positive results for various reasons (Chevaliez, 2011; Richter 2002; Alter et al., 2003).

*Corresponding author. E-mail: nkkaya@yahoo.com. Tel: +90 532784 8648. Fax: +90 462 377 5340.

Table 1. Comparison of HCV RNA and HCV core antigen tests' results.

HCV Core Ag	HCV RNA		
	Positive (%)	Negative (%)	Total (%)
Positive	116 (35.69)	4 (1.23)	120 (36.92)
Negative	3 (0.92)	202 (62.16)	205 (63.08)
Total	119 (36.61)	206 (63.39)	325 (100)

The recombinant immunoblot test is used in order to determine true positive results. It has disadvantages, such as giving a large number of indeterminate results and being a time-consuming procedure (Makuria et al., 2012). For these reasons, HCV RNA tests are the most commonly used technique in the confirmation of HCV infections and in the monitoring of antiviral therapy. Tests used for the detection of HCV RNA have high sensitivity and specificity, and low detection limits such as 15 to 30 IU/ml and can give quantitative results. However, these tests also require special technical and laboratory facilities, carry a risk of contamination and also impose high costs on users (Chevaliez, 2011; Richter 2002; Alter et al., 2003).

A large number of immunologically-based tests have been developed over the last 20 years for the detection of HCV core antigens in plasma or serum as potential alternatives to HCV RNA tests. In first-generation HCV core antigen tests, there is no preliminary procedure intended to differentiate between antigen and antibody. Therefore, HCV core antigens can only be detected in the period up to the emergence of anti-HCV antibodies using first-generation tests (Takahashi et al., 1992; Aoyagi et al., 1999; Tanaka et al., 1995; Kashiwakuma et al., 1996; Icardi et al. 2003). With second-generation tests, preliminary procedures aimed at distinguishing HCV core antibodies and anti-HCV antibody complexes are performed beforehand. HCV core antigens can thus be detected during seroconversion. Preliminary procedures are performed manually in these tests carried out on microplates, and the lowest detectable limit is about 1.5 pg/ml (Tobler et al., 2005; Tanaka et al., 2006; Fabrizi et al., 2005).

A fully automated system has recently been developed by ABBOTT Diagnostics (Abbott Park, IL, USA) for the detection of HCV core antigen in serum or plasma including preliminary procedures for differentiation of antigen-antibody complexes. This test also provides quantitative results. The lowest detectable limit in the test is 3 fmol/l (0.06 pg/ml). This value is approximately 25 times lower than that for previous tests (Mederake et al., 2009).

The purpose of this study was to compare the results of the architect HCV core antigen test, which has newly been introduced in Turkey with the HCV RNA results obtained from the Abbott RealTime™ HCV RNA test (Abbott Molecular Inc., Des Plaines, IL, USA) and to determine the correlation between the two.

MATERIALS AND METHODS

In this study, HCV RNA test and HCV core antigen test were consecutively and simultaneously administered on three hundred and twenty-five sera samples between June 2010 and July 2012. All the samples were anti-HCV reactive.

Anti-HCV levels in the serum samples reaching our laboratory were determined using the chemiluminescent microparticle immunoassay (CMIA) technique with Architect Anti-HCV kits (Abbott Diagnostics, Wiesbaden, Germany) on an Architect I2000SR (Abbott Diagnostics, Abbott Park, IL, USA) device. Sample/cutoff values (S/CO) ≥1 were regarded as reactive.

HCV core antigen levels in serum were investigated using the Architect HCV Ag test (Abbott Diagnostics, Wiesbaden, Germany) with CMIA technology following the manufacturer's recommendations. Since the HCV core antigen test and Anti-HCV antibody test were investigated on the same device, it was subjected to daily maintenance using a 0.5% sodium chloride solution in order to prevent cross-contamination. The lowest level detectable by the test, 3 fmol/l (0.06 pg/ml), was adopted as the cut-off value.

HCV RNA isolation was performed using the Sample Preparation System (Promega Corporation Madison, WI, USA) on an Abbott m2000sp platform (Abbott Molecular Inc., Des Plaines, IL, USA). HCV RNA levels were determined using the Abbott m2000rt Instrument System (Abbott Molecular Inc., Des Plaines, IL, USA) and Abbott RealTime™ HCV test (Abbott Molecular Inc., Des Plaines, IL, USA). This test, based on the quantitative determination of the HCV viral load in plasma using the real time polymerase chain reaction method (RT-PCR) after reverse transcription, was performed with a 0.2 ml plasma sample, in line with the manufacturer's instructions. The test's lowest detection limit, 30 IU/ml, and values above that were regarded as positive.

Statistical analysis was performed using SPSS 13.0 (Series no: 9069728). Descriptive data were expressed as number and percentage. The Spearman rank test and linear regression analysis were used to determine the correlation between HCV RNA and HCV core antigen levels.

RESULTS

One hundred and sixteen of the 325 samples were detected positive by the two tests. HCV RNA levels were greater than 30 IU'ml in 119 of the 325 samples (35.69%). HCV core antigen levels were greater than 3 fmol/l in 120 samples (36.92%) (Table 1). When the HCV RNA test was regarded as a reference test, HCV core antigen test sensitivity, specificity, PPV and NPV rates were calculated as described by Akobeng AK (2007), and found as 97.48, 98.06, 96.67 and 98.54%, respectively. The Spearman's correlation coefficient (r) was determined as 0.899 (p<0.0001) at comparison of HCV RNA and HCV core antibodies (Figure 1). HCV core antigen was deter-
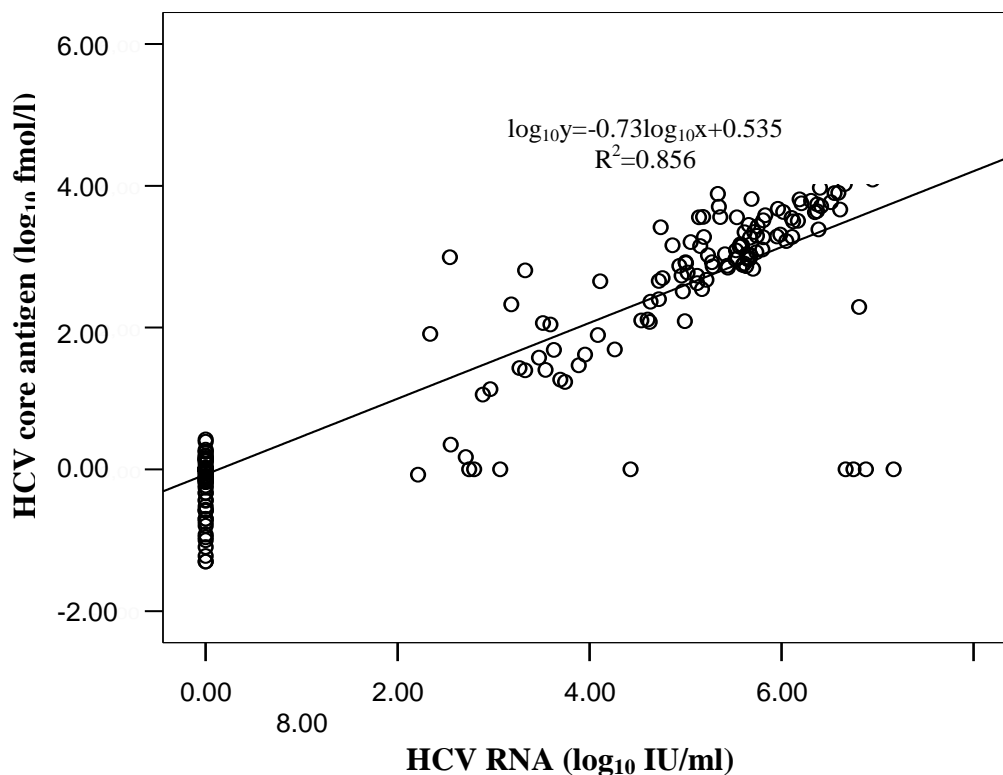
Figure 1. Comparison of HCV RNA and HCV core antigen levels (n=325). (The graph was prepared on the basis of the logarithm of the results obtained in both tests).

Table 2. Results from inconsistent HCV RNA and HCV core antigen tests.

Specimen number	HCV RNA level (IU/ml)	HCV core antigen level (fmol/l)
1	< 30	3.04
2	< 30	6.40
3	< 30	8.86
4	ND*	3.78
5	357	2.23
6	163	0.84
7	513	1.49

*ND: Not detected

mined as negative in four HCV RNA positive samples, and positive in three HCV RNA negative tests. Detailed results from the seven samples in which the HCV RNA and HCV core antigen tests were inconsistent are shown in Table 2.

DISCUSSION

This study compared HCV RNA test and Architect HCV core antigen test results in specimens with anti-HCV antibodies identified as reactive. The ability of Architect HCV core antigen test's to provide quantitative results is being fully automated, and having a low detection limit of 3 fmol/l represent superior features compared to previous HCV core antigen tests. The device's total working time, that is, maintenance once daily has been performed and is approximately 40 min; some five times shorter than that of previously manufactured tests (around 3.5 h) (Mederake et al., 2009; Ergunay K et al., 2011; Morota et al., 2009; Ross et al., 2010; Kesli et al., 2011; Park et al., 2010; Medici MC et al., 2011).

Positivity rates of HCV RNA, and HCV core antigen tests were found to be quite similar, 36.61 and 36.92%, respectively. When the HCV RNA test was taken as refe-

rence, the HCV core antigen test exhibited rather high sensitivity, specificity, PPV and NPV (97.48, 98.06, 96.67 and 98.54%, respectively). These values are slightly higher than those obtained in previously manufactured tests. Fabrizi et al. (2005) used the second-generation Ortho Trak-C test (Ortho-Clinical Diagnostics, Raritan, N.J., USA) and determined sensitivity, specificity, PPV and NPV of 92.7, 97.4, 94.7 and 96.5%, respectively, in hemodialysis patients.

HCV RNA and HCV core antigen test results were inconsistent in seven specimens in our study. The HCV RNA test was lower than 30 IU/ml or negative in four of these specimens in those HCV core antigen levels were positive with amount lower than 10 fmol/l. The manufacturers recommend that if HCV core antigens are measured at 3 to 10 fmol/l, the test should be repeated two times, and that if positivity above 3 fmol/l is seen in at least one of these tests, the test should be interpreted as positive. Samples with a level of 3-10 fmol/l were not retested in our study for economic reasons. This may be regarded as a limitation of the study. The possibility of there being insufficient RNA for quantification in specimens identified as HCV core antigen positive and with HCV RNA ≤30 IU/ml must also be borne in mind.

HCV RNA was positive in three samples although the HCV core antigen test results of them were below 3 fmol/l. HCV RNA levels in these three samples were 10^3 IU/ml or less. We therefore think that care is needed when interpreting HCV core antigen results if HCV RNA levels are ≤10^3 IU/ml.

When the correlation between HCV RNA and HCV core antigen levels was analyzed, the correlation coefficient between the two tests was quite high (r=0.899). Kesli et al. (2011) determined HCV RNA levels using the Qiagen HCV RNA test (Qiagen, Hilden, Germany) and identified a correlation with the Architect HCV core antigen test as 0.864. Ergünay et al. (2011) determined HCV RNA levels with COBAS Campliprep/COBAS Taqman HCV Real-time PCR (Roche Diagnostics, Germany) and calculated a correlation coefficient as 0.915. Mederacke et al. (2009) determined HCV RNA levels using Cobas Taqman or Amplicor HCV Monitor (Roche Diagnostics, Germany) and reported a correlation coefficient as 0.75. Medici et al. (2011) determined a correlation coefficient ranging from 0.713 to 0.870 for the HCV core antigen test and different HCV RNA kits. The values determined in our study and the results from other studies show that HCV RNA tests and the HCV core antigen test give quite compatible results.

In conclusion, the Architect HCV core antigen test and HCV RNA test produced highly compatible findings in our study. The HCV RNA test is still regarded as standard in the detection of active infection in individuals in whom anti-HCV antibodies are detected, in the confirmation of anti-HCV antibody tests and in the commencement and monitoring of treatment (Alter et al., 2003). However, we conclude that the HCV core antigen test can be used as an alternative to HCV RNA tests, particularly when the HCV RNA test is unavailable.

REFERENCES

Akobeng AK (2007). Understanding diagnostic tests 1: sensitivity, specificity and predictive values. Acta Paediatr. 96(3): 338-341.

Alter MJ, Kuhnert WL, Finelli L (2003). Centers for Disease Control and Prevention. Guidelines for laboratory testing and result reporting of antibody to hepatitis C virus. Centers for Disease Control and Prevention. MMWR Recomm. Rep. 52: 1-13.

Aoyagi K, Ohue C, Iida K, Kimura T, Tanaka E, Kiyosawa K, Yagi S (1999). Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. J. Clin. Microbiol. 37(6): 1802-1808.

Chevaliez S (2011). Virological tools to diagnose and monitor hepatitis C virus infection. Clin. Microbiol. Infect. 17(2): 116-121.

Ergunay K, Sener B, Alp A, Karakaya J, Hascelik G (2011). Utility of a commercial quantitative hepatitis C virus core antigen assay in a diagnostic laboratory setting. Diagn. Microbiol. Infect. Dis. 70:486-491.

Fabrizi F, Lunghi G, Aucella F, Mangano S, Barbisoni F, Bisegna S, Vigilante D, Limido A, Martin P (2005). Novel assay using total hepatitis C virus (HCV) core antigen quantification for diagnosis of HCV infection in dialysis patients. J. Clin. Microbiol. 43(1): 414-420.

Icardi G, Bruzzone B, Gota F, Torre F, Giannini E, Massone L, Li Bassi A, Lai PL, Picciotto A, Ansaldi F (2003). A new assay for hepatitis C virus (HCV) core antigen detection: an alternative to nucleic acid technologies in positive or indeterminate anti-HCV subjects? Ann. Ig. 15(6): 863-870.

Kashiwakuma T, Hasegawa A, Kajita T, Takata A, Mori H, Ohta Y, Tanaka E, Kiyosawa K, Tanaka T, Tanaka S, Hattori N, Kohara M (1996). Detection of hepatitis C virus specific core protein in serum of patients by a sensitive fluorescence enzyme immunoassay (FEIA). J. Immunol. Methods 190(1): 79-89.

Kesli R, Polat H, Terzi Y, Kurtoglu MG, Uyar Y (2011). Comparison of a newly developed automated and quantitative hepatitis C virus (HCV) core antigen test with the HCV RNA assay for clinical usefulness in confirming anti-HCV results. J. Clin. Microbiol. 49(12): 4089-4089.

Makuria AT, Raghuraman S, Burbelo PD, Cantilena CC, Allison RD, Gibble J, Rehermann B, Alter HJ (2012). The clinical relevance of persistent recombinant immunoblot assay-indeterminate reactions: insights into the natural history of hepatitis C virus infection and implications for donor counseling. Transfusion; doi: 10.1111/j.1537-2995.2011.03524.x.

Mederacke I, Wedemeyer H, Ciesek S, Steinmann E, Raupach R, Wursthorn K, Manns MP, Tillmann HL (2009). Performance and clinical utility of a novel fully automated quantitative HCV-coreantigen assay. J. Clin. Virol. 46(3): 210-215.

Medici MC, Furlini G, Rodella A, Fuertes A, Monachetti A, Calderaro A, Galli S, Terlenghi L, Olivares M, Bagnarelli P, Costantini A, De Conto F, Sainz M, Galli C, Manca N, Landini MP, Dettori G, Chezzi C (2011). Hepatitis C virus core antigen: analytical performances, correlation with viremia and potential applications of a quantitative, automated immunoassay. J. Clin. Virol. 51(4): 264-269.

Morota K, Fujinami R, Kinukawa H, Machida T, Ohno K, Saegusa H, Takeda K (2009). A new sensitive and automated chemiluminescent microparticle immunoassay for quantitative determination of hepatitis C virus core antigen. J. Virol. Methods. 157(1): 8-14.

Park Y, Lee JH, Kim BS, Kim do Y, Han KH, Kim HS (2010). New automated hepatitis C virus (HCV) core antigen assay as an alternative to real-time PCR for HCV RNA quantification. J. Clin. Microbiol. 48(6): 2253-2256.

Richter SS (2002). Laboratory assays for diagnosis and management of hepatitis C virus infection. J. Clin. Microbiol. 40(12): 4407-4412.

Ross RS, Viazov S, Salloum S, Hilgard G, Gerken G, Roggendorf M (2010). Analytical performance characteristics and clinical utility of a novel assay for total hepatitis C virus core antigen quantification. J. Clin. Microbiol. 48(4): 1161-1168.

Takahashi K, Okamoto H, Kishimoto S, Munekata E, Tachibana K,

Akahane Y, Yoshizawa H, Mishiro S (1992). Demonstration of a hepatitis C virus-specific antigen predicted from the putative core gene in the circulation of infected hosts. J. Gen. Virol. 73: 667-672.

Tanaka T, Lau JY, Mizokami M, Orito E, Tanaka E, Kiyosawa K, Yasui K, Ohta Y, Hasegawa A, Tanaka S (1995). Simple fluorescent enzyme immunoassay for detection and quantification of hepatitis C viremia. J. Hepatol. 23(6): 742-745.

Tanaka Y, Takagi K, Hiramatsu K, Naganuma H, Iida T, Takasaka Y, Mizokami M (2006). Fundamental and clinical evaluation of hepatitis B virus core-related antigen assay by LUMIPULSE f. Rinsho. Byori. 54(7): 692-698.

Tobler LH, Stramer SL, Lee SR, Baggett D, Wright D, Hirschkorn D, Walsh I, Busch MP (2005). Performance of ORTHO HCV core antigen and trak-C assays for detection of viraemia in pre-seroconversion plasma and whole blood donors. Vox. Sang. 89(4): 201-207.

Full Length Research Paper

Influence of general anaesthesias on the changes of bacterial flora in bronchial tree

Izabela Duda, Aleksandra Rudnik-Lipińska, Maria Damps, Ewa Musioł and Edyta Gogółka

Department of Anesthesiology and Intensive Care, Medical University of Silesia Katowice, Poland.

The aim of the study was to assess the influence of general anesthesia on bacterial growth in bronchial tree, depending upon the applied method of anesthesia. The artificial airway is the additional gate for respiratory tract infections and mechanical ventilation which can have an influence on postoperative complications such as pneumonia and atelectasis. Patients undergoing 4h surgeries were sampled. Due to selection of anesthetics, volatile or intravenous, patients were divided into groups VGA (volatile general anesthesia) and TIVA (total intravenous anesthesia). Material collected with mini-bronchoalveolar lavage method directly after intubation and just before extubation. In 40% of all patients no bacteria growth was noted in both time points. In VGA group, from the bacteria cultured in 61.9% of patients in first sample, 62.5% of colonies diminished or eradicated, only 6.25% multiplied. In TIVA group 42.9% patients presented bacteria in first sample. All bacteria got reduced. Length of hospitalization preceding surgery (p=0.036) and number of smoked cigarettes (p=0.028) significantly correlated with colonization of bacteria. General anesthesia has no influence on the respiratory tract microorganism contamination and can even favour the eradication of the colonizing bacterial flora.

Key words: Respiratory tract, general anesthesia, contamination.

INTRODUCTION

Natural defensive mechanisms, such as mucociliary transport, lysozyme activity, lactoferrins, macrophages, natural killer (NK) cells, as well as, specific humoral and cell response play significant roles in prevention and control of respiratory system infections (Ficker, 2008). After general anaesthesia of long duration, due to incorrect ventilation and retention of secretion in bronchial tree, the risk of pulmonary infections and atelectasis increases (Sachdev and Napolitano, 2012). Endotracheal intubation can cause mucosal injury, loss of cilia and promotes metaplasia of respiratory epithelium. That artificial respiratory tract is an additional entry of infection. Bacteria reach the lower respiratory tract mainly via aspiration from the upper part of pharynx, as well as leakage of secretion containing bacteria around the sealing collar, which slows down the tracheal flow of mucus. Inhalation of colonized bacteria from oropharynx, sinus cavities, nares, dental plaque can be a reason of postoperative lungs infections (Brusselaers et al., 2013).

Mechanical ventilation disturbs the correct cough reflex, sneezing, and efficient mucociliary transport. The mucociliary clearance rate in bronchial tree is optimal in the temperature of 34 to 40°C. Greater sensitivity of lower respiratory tract to decreased temperature brings about the threat that substitutive ventilation with dry and cold gases may lead to impairment of ciliated epithelium functions. That may promote the development of microorganisms in respiratory tract environment. Anaesthetics, inhalants and intravenous drugs, also affect the movement and function of cilia. The mechanism of impaired cleaning

*Corresponding author. E-mail: izaduda@neostrada.pl. Tel: 32 7894201.

of respiratory tract during anaesthesia has been suggested to exist, in the form of inhibition of Cl^- ions secretion and increased stickiness of mucus, caused by inhaled anaesthetics. Propofol, on the other hand, would enhance the activity of cilia by increasing the intracellular concentration of Ca^{2+} ions in cells of respiratory epithelium, stimulation of nitrogen oxide, and production of cGMP (Shirakami et al., 2000).

Some authors report inhibition of inflammatory reaction by anaesthetics being inhalants, or even their bactericidal properties noted in experiments *in vitro* (Molliex et al., 1998; Karabiyik et al., 2007). Among the risk factors for infections of lower respiratory tract in hospital conditions, apart from intubation, mechanical ventilation, surgery performed, one can also list prolonged hospitalization (Niedermann et al., 2005). Dependence has been proven to exist between the aetiology of hospital acquired pneumonia (HAP) and length of hospitalization. In early hospital infections, usually the dominating micro-organisms are those which are sensitive to antibiotics, in late ones the strains that dominate are resistant to treatment (Trouillet, 2012). The responsibility for infections of lower respiratory tract rests with pathogenic microorganisms, such as: Haemophilus influenzae, Staphylococcus aureus, Streptococcus pneumoniae, Moraxella catarrhalis, Pseudomonas aeruginosa, and in patients with hypoimmunity, also bacteria which colonize upper respiratory tract, such as: Streptococcus viridans, Neisseria species, Corynebacterium species (Ionas et al., 2002). The material for microbiological examinations concerning patients with suspected pulmonary pathology may be obtained by means of BAL (bronchoalveolar lavage) bronchoscopy or other, less invasive, techniques, such BPSB (blinded protected specimen brush) or mini-BAL (mini bronchoalveolar lavage).

The aim of the study was to assess the influence of mechanical ventilation used during general anaesthesia upon bacterial growth in bronchial tree, depending upon the applied method of anaesthesia: inhalation (VGA, volatile general anaesthesia), or purely intravenous administration (TIVA, total intravenous anaesthesia) and their influence on post-operative pneumonia development.

MATERIAL AND METHODS

Having obtained the consent of the Bioethics Committee of Medical University of Silesia in Katowice, Poland, the study comprised a group of 38 patients, operated in the Central University Hospital in Katowice, Poland, between May and November, 2011. Patients who have been qualified for the study met the physical status criteria for classes I and II according to ASA scale, also those patients had the predicted time of general anaesthesia exceeding four hours. Not included in the study have been those patients, whose general physical status was assessed according to ASA as ≥ class III, patients with history of chronic diseases of the respiratory system, namely asthma, COPD (Chronic Obstructive Pulmonary Disease), restrictive lesions, confirmed in spirometry, inflammatory and neoplastic tumours of the lungs. Patients who were currently treated with antibiotics as well as those who received recently anti-

biotic therapies have been excluded from the study. On the eve of the surgery, patients were consulted by an anaesthesiologist. Patients were informed about the study, potential threats, and gave informed consent for taking part in research study. Due to the selection of anaesthetics, patients have been divided into two groups.

Randomization was performed according to the number of patient's case history. In the VGA group, where patients were anaesthetized before the procedure by means of inhalants, 23 patients were studied, age range 41 to 78 years. In the TIVA group, 15 patients were studied, age range 29 to 66 years. Surgical procedures in that group were performed under general, total intravenous anaesthesia. All patients, 30 minutes before the scheduled procedure, were premedicated with midazolam in doses of 0.1 to 0.15 mg. kg^{-1} orally. After two-minute pre-oxygenation with 100% oxygen, anaesthesia was induced by means of two methods. In the VGA group, the following were used for inducing anaesthesia: midazolam in doses of 0.02 to 0 05 mg.kg^{-1} intravenously (i.v.), fentanyl 1 to 2 µg. kg^{-1} i.v., thiopental in doses of 4 to 5 mg. kg^{-1} i.v. rocuronium 0.8 mg. kg^{-1} i.v. Anaesthesia was sustained by a mixture of 50% oxygen with air and inhaled anaesthetic - sevoflurane (0.8 to 1.0 MAC), and repeated doses of fentanyl 0.1 to 0.2 mg i.v. and rocuronium 0.3 mg. kg^{-1} i.v. In the TIVA group anaesthesia was induced by propofol by means of infusion pump intravenous drop 1.5 to 2 mg. $kg^{-1}h^{-1}$, fentanyl in the dose of 0.1 mg i.v. After the cilial reflex subsided, rocuronium was administered in dose 0.8 mg. kg^{-1} i.v. After intubation anaesthesia was maintained by means of administration of propofol via pump in intravenous drop, at the rate of 6 mg. $kg^{-1}h^{-1}$, and of remifentanil at the rate of 0.05 to 0.25 µg. kg^{-1} $min.^{-1}$ the mixture of oxygen and air was used in proportion of 50/50%. Rocuronium was used for relaxation, in maintenance doses of 0.3 mg. kg^{-1} i.v.

Patients, in the supine position, after intubation were ventilated mechanically in the IPPV (intermittent positive pressure ventilation) mode, applying the volume of 8 ml kg^{-1} and frequency of 8-12 breaths per minute, depending upon end tidal CO_2, which was maintained at the range of 33-38 mmHg.

In order to provide qualitative and quantitative assessment of bacteria, the material from respiratory tracts has been collected twice: directly after induction of anaesthesia and again before the end of anaesthesia that is before extubation. All the procedures concerning tracheal intubation were performed by anaesthesiologist using surgical facemasks and sterile gloves after proper hands hygiene with sterile equipment (face mask, laryngoscope, single-use tracheal tube, single-use filter placed between the patient and the breathing circuit (a new filter for each patient) and standardised staff technique in order to minimise the contamination of tracheal tree. The mini-BAL technique has been used for collecting the material and was performed by the same single operator every time, carrying out a sterile procedure under full aseptic conditions.

The mini-BAL technique applied consisted of "blind" collection of washings from lower respiratory tract. After injecting, via endotracheal tube, of 20 ml of sterile solution of physiological saline, heated to body temperature, the patient was ventilated manually five times, by means of self-reinflating bag. After re-installation of the intrabronchial catheter, through endotracheal tube, the bronchoalveolar washings in the amount of 2 to 5 ml have been collected by means of aspirator directly to sterile test tubes. The material for the first test tube was obtained after intubation and stabilization of ventilation and circulation parameters, whereas the washings for the second test tube were collected after minimum four hours of anaesthesia, directly before extubation. Each test tube was immediately sent to microbiological laboratory for qualitative and quantitative assessment of bacterial flora. The time elapsing between collection of material and beginning of culture did not exceed 15 min. In laboratory of Central University Hospital, Katowice, Poland, the samples were processed in line with the generally accepted procedures mandatory in a microbiological laboratory. The material

Table 1. Data characterizing studied patients. Values are number (proportion) or mean (SD); n, number of patients.

Characteristic		Total number of patients	VGA group	TIVA group	p value
Patients		n=35	n=21	n=14	
Sex	Female	n=21 (60%)	n=12 (57.1%)	n=9 (64.3%)	
	Male	n=14 (40%)	n=9 (42.9%)	n=5 (35.7%)	
Average age		57.0 (11.31) (range 29-78)	59.81 (10.38) (range 41-78)	52.71 (11.67) (range 29-66)	p=0.21
Average BMI		24.99 (3.18)	24.83 (3.15)	25.22 (3.33)	
BMI 20-25		n=21	n=12	n=9	p=0.69
BMI 25-30		n=11	n=7	n=4	
BMI >30		n=3	n=2	n=1	
Smokers		n=12 (34.2%)	n=7 (33.3%)	n=5 (35.7%)	p=0.99
Number of smoked cigarettes		4.43 (7.25)	4.52 (7.40)	4.29 (7.30)	
Average number of days of hospitalization before the operation		9.31 (7.56) (range 1-32)	6.90 (6.46) (range 1-31)	12.93 (7.77) (range 2-32)	p=0.001
Type of operation					
Craniotomy		n=20	n=10	n=10	
Laparotomy		n=15	n=11	n=4	

obtained was cultured with use inoculating loop onto blood agar, chocolate agar, Chapman and MacConkey agar plates. Plates were incubated in the temperature of 35 to 37°C, in aerobic conditions. Then read after 48 h. Plates were visualized for growth by two different observers. CFU –colony forming unit was based on the laboratory formula: CFU= n $\times$ v where n stands for number of colony on space of plate, v means inverse of inoculating loop volume in 1 ml. Results more than 10^4 CFU implied infection of lower respiratory tract, results less than 10^4 CFU - bacterial colonization.

The statistical analysis has been prepared using the Statistica v8.0 package, by StatSoft. The graphic illustration of results has been prepared mainly by means of Statistica software, partly also by means of the graphics editor contained in the MS Office 2007 package. Verification of hypotheses concerning normal distribution of variables has been carried out using Kolmogorov-Smirnov test, and Shapiro-Wilk test. For variables having distribution consistent with normal distribution, the assessment of significance of differences between groups has been made by means of t-Student test. The use of that test required that the condition of variance homogeneity was met, which has been attained using Snedecor test. The significance of differences for variables, the distribution of which did not meet the condition of normality has been assessed by means of rank sum test by U Mann-Whitney. For analysis of significance of differences between groups resulting from division of the investigated sample, in accordance with a determined criterion for one variable in relation to another variable, the independence chi-squared test has been used. The condition required for the application of that test is that the number in each class established as a result of sample division must be greater than 8. For those subgroups, where the size of the group was smaller, the Yates's chi-squared independence test was used. In selected cases, for 2×2 tables, the Fisher's exact test has been used, while in case of tables of larger size that is, the test of highest likelihood. P value < 0.05 was considered statistically significant. Power analysis

calculation was conduct using G Power software. The sample size calculation was based on a 95% confidence level with a power of 80%.

RESULTS

From the total of 38 patients, three patients have been excluded from the study. Two of them due to too short operation time, not exceeding two hours, one because of difficult intubation and early death caused by circulatory insufficiency, within 24 h after the procedure. 35 patients who have been subject to final analysis are characterized in Table 1.

Of the 35 patients studied, 14 (40%) no micro-organisms have been isolated in both points in time assumed for material collection. In the VGA group that amounted 38.1% of patients (n=8), in the TIVA group to 57.1% of cases (n=8).

In the VGA group, the average time of general anaesthesia amounted to 239.90 min. The most frequently isolated flora was alpha-hemolytic Streptococcus. The list of micro-organisms isolated in washings obtained is provided in Table 2.

In 13 patients (61.9%), on material collected before the operation micro-organisms have been cultured. The respiratory tracts of nine patients were colonized by one species of bacteria, in four patients by two or more species of micro-organisms. Of the bacteria cultured on pre-operative material 62.5% of colonies diminished or

Table 2. Bacteria isolated from lower respiratory tract in the VGA group.

Case	Bacteria isolated	Number of colonies ml^{-1} in pre-surgery material, directly after intubation	Number of colonies ml^{-1} in post-surgery material directly before extubation
1.	Streptococcus alpha-hemolytic	10^4	10^4
2.	Streptococcus alpha-hemolyti	10^4	none
3.	Streptococcus alpha-hemolytic	10^3	10^3
	Streptococcus alpha-hemolytic	10^3	10^3
4.	Neisseria species	10^3	none
	Staphylococcus coagulase-negative	10^3	10^3
5.	Streptococcus alpha-hemolytic	10^3	none
	Moraxella species	10^3	none
6.	Streptococcus alpha-hemolytic	10^3	none
7.	Streptococcus alpha-hemolytic	10^3	none
8.	Streptococcus alpha-hemolytic	10^3	none
	Streptococcus pneumonia	10^3	10^6
9.	Klebsiella oxytoca	none	10^3
	Haemophilus influenzae	none	10^3
10.	Streptococcus pneumoniae	10^3	10^3
11.	Streptococcus pneumoniae	10^3	none
12.	Staphylococcus epidermidis	10^4	10^3
	Acinetobacter calcoaceticus baumani complex	none	10^3
13.	Corynebacterium species	10^3	none

Table 3. Bacteria isolated from lower respiratory tract in the TIVA group.

Case	Bacteria isolated	Number of colonies ml^{-1} in pre-surgery material, directly after intubation	Number of colonies ml^{-1} in post-surgery material, directly before extubation
1.	Streptococcus pneumoniae	10^6	10^3
	Acinetobacter calcoaceticus baumanii complex	none	10^4
2.	Streptococcus pneumoniae	10^5	10^3
3.	Streptococcus alpha-hemolytic	10^4	10^3
4.	Streptococcus alpha-hemolytic	10^4	none
	Moraxella species	10^3	none
5.	Serratia marcescens	10^4	none
6.	Haemophilus influenzae	10^3	none

eradicated, 31.25% of colonies remained unchanged, and 6.25% multiplied. In one patient bacteria culture increased to bacterial index level which may signify infection. In two patients, sample two revealed additional bacterial strains, which were not present in the first sample, having a bacterial index of 10^3 CFU.

In the TIVA group the average time of general anaesthesia amounted to 247.14 min. The bacteria most often isolated in the material analysed are presented in Table 3. In six cases (42.9%), bacteria were cultured from bronchoalveolar washings obtained just after intubation. In four patients the respiratory tracts were colonised by one species of bacteria, in two cases by two strains of bacteria. In two patients the titre of sample one indicated infection, however, bacterial colonies were reduced after the anaesthesia ended. The bacterial index of bacteria isolated in samples collected after intubation got reduced in all cases. 57.1% of bacterial colonies underwent total eradication. In one patient in sample two additional bacterial colonies were detected, in the amount of 10^4 CFU, which were not present in sample one.

In the analysis, to determine the predictors of bronchial bacterial colonization we have assessed variables: age, sex, BMI, smoking habits, hospitalization time preceding surgery. Three of these variables proved to be statistically significant in analysed VGA group: BMI (p=0,048)
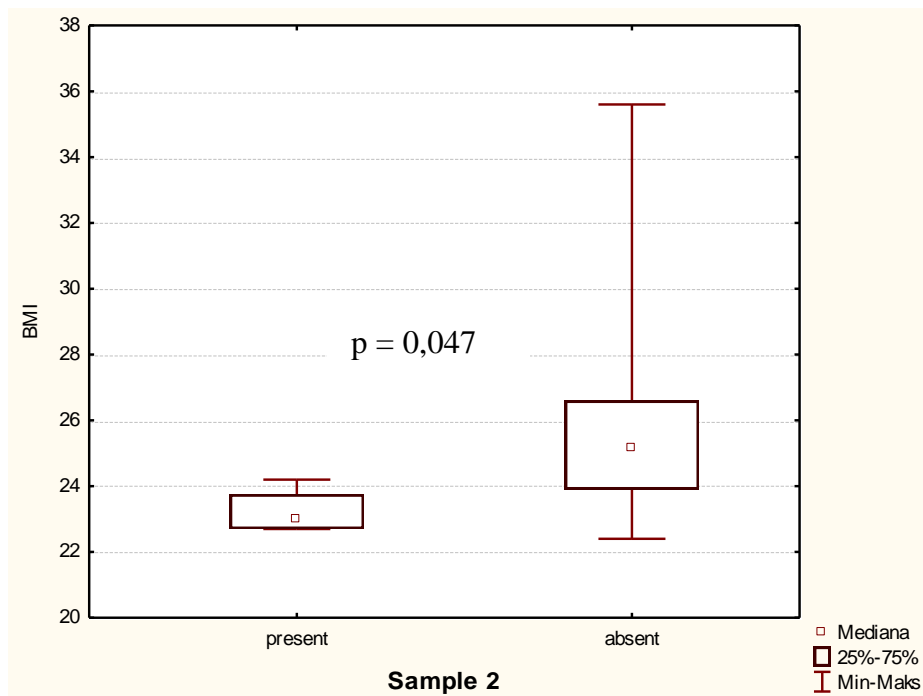
Figure 1. Comparison of bacteria presence in sample 2 according to BMI.

(Figure 1), hospitalization time preceding surgery (p = 0,036) and number of smoking cigarettes (p=0,028). Bacteria were cultured in smokers as well as non-smokers in both samples. Of the patients who had no bacteria cultured in samples more often were non-smoking patients (Figure 2 and 3). There was significant difference between patients smoking more cigarettes in comparison to patients smoking less in VGA group (Figure 4).

The complication in the form of bronchial spasm, which appeared immediately after administration of physiological saline and insertion of catheter to bronchial tree, occurred in nine patients (25.7%). Higher peak and plateau pressures were noted in respiratory tract, as well as changes on auscultation, in the form of wheezing, dry rales and absence of vesicular murmur. Bronchial spasm usually subsided after five to ten minutes after administration of 100 to 300 mg hydrocortison i.v. In two cases there was a significant drop of blood saturation to 84 and 90%, in case of ventilation with 100% oxygen. The patients required intravenous administration of aminophillin, repeated removal by sucking from intubation tube, and temporary change of ventilation mode to PCV (pressure controlled ventilation) mode.

DISCUSSION

Bacterial colonization of lower respiratory tract has been noted in 54.3% of patients in our study. Micro-organisms that have been most often isolated were Streptococcus alpha-hemolytic (37% of colonies cultured) and Streptococcus pneumoniae (18.5% of colonies cultured). As opposed to strains of bacteria, which dominate in VAP (ventilator associated-pneumonia) - the Gram negative bacteria, Gram positive bacteria dominated in our material. Probably time factor might play an important role there, especially the length of hospitalization preceding the operation. It is well known that prior hospitalization is a risk factor of bacterial colonization. Complications, in the form of post-operative pneumonia, depend also on the underlying disease and type of operation performed (Ficker, 2008). In our study, patients had the procedures of laparotomy and craniotomy. In such cases, suppression of the cough reflex, for example to prevent the increase of intracranial pressure or to restrain pain level might have promoted the retention of secretion in bronchial tree, and pulmonary infection. Decreased BMI<25 was also an independent risk factor for bronchial colonization. That conclusion differs from those noted in literature (Ionas et al., 2002). Despite the existing pneumonia risk factors in VGA and TIVA groups, no infections of lower respiratory tract have been recorded.

The amount of bacteria isolated in both groups, in the majority of cases decreased in relation to the initial bacteriological index. In literature the influence of inhalational anaesthetics: halothane, enflurane, isoflurane, as well as intravenous anesthetics upon the movement of cilia, and transport of mucus were presented. Inhalational anesthetics would inhibit ciliary movement and increase the volume of retained mucus. Propofol, due to the properties stimulating ciliary movement and influencing the mechanisms of secretion liquefaction would appear a safer drug in case of patients with history of pulmonary pathology
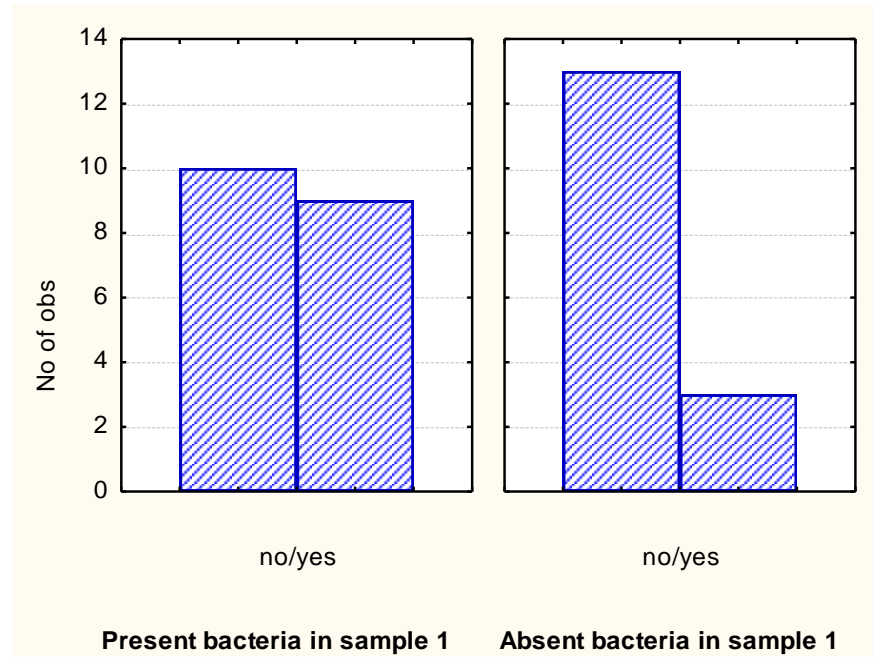
Figure 2. Comparison of bacteria presence in sample 1 according to smoking. No, non-smokers; Yes, smokers.
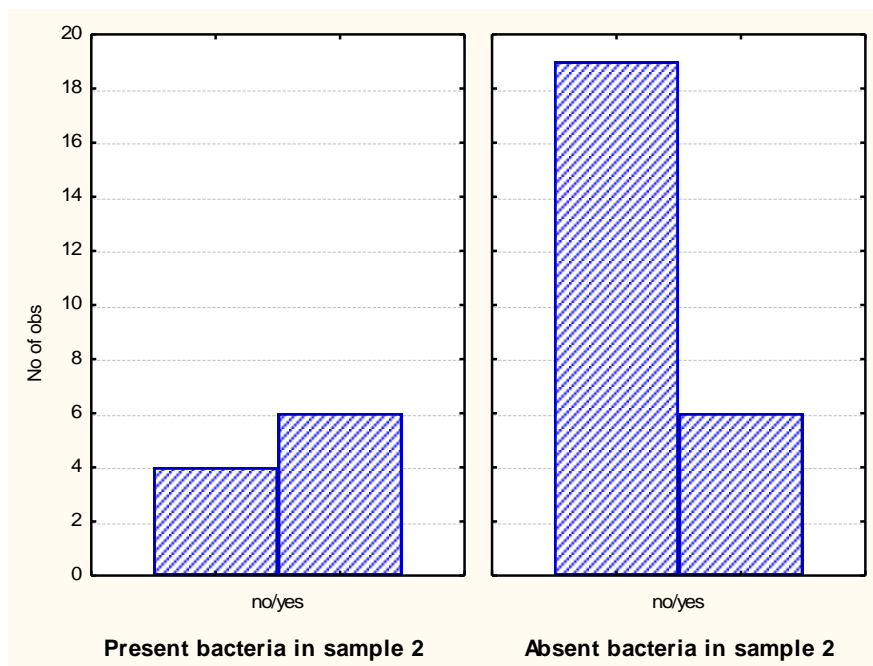

Figure 3. Comparison of bacteria presence in sample 2 according to smoking. No, non-smokers; Yes, smokers.

(Ledowski et al., 2006). In his study, Molliex in *in vitro* conditions demonstrated a direct influence of inhalational general anesthesia drugs upon bacterial cells of the Pseudomonas aeruginosa strain, a pathogen participa-

ting in hospital-acquired pneumonia. The author has proven a maximum inhibition of bacterial cultures after four hours of exposure to isoflurane, enflurane, halo-thane, greater in the concentration of anesthetic equal to
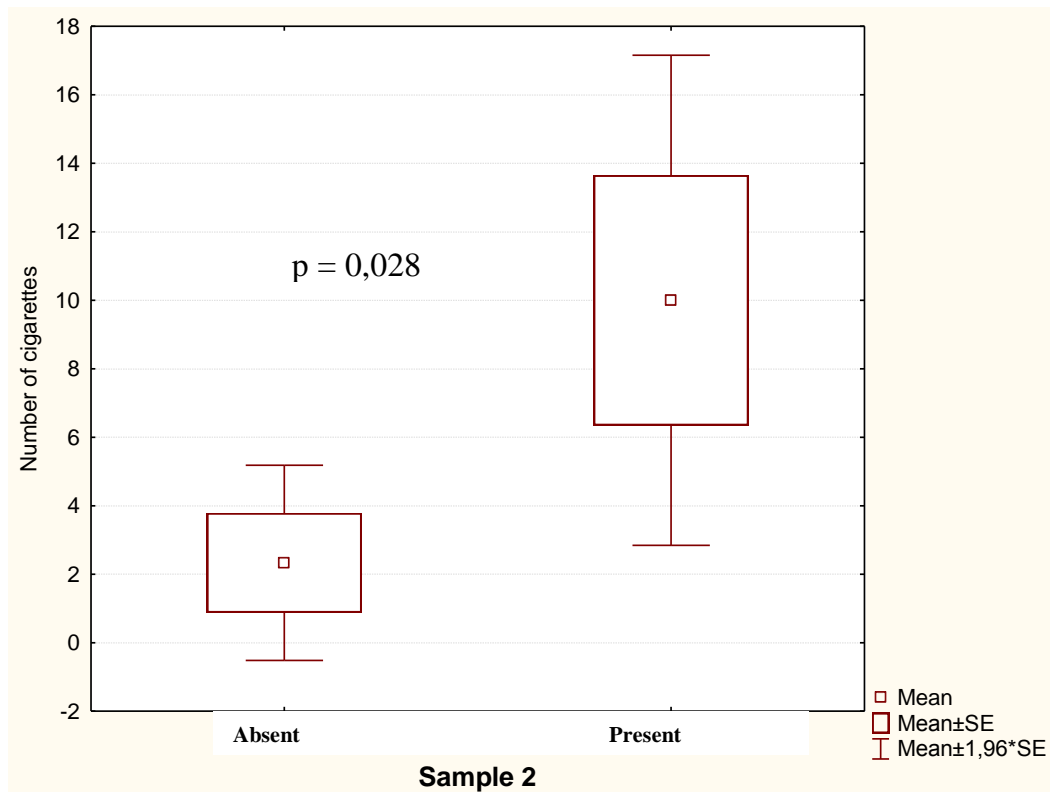
Figure 4. Comparison of bacteria presence in sample 2 according to number of cigarettes smoked per day.

2,0 MAC, in comparison with 1,0 MAC (Molliex et al., 1998).

Only in one patient, in the material examined was there an increase of bacteria to the value of 10^6 CFU and cultures of additional species from the family Klebsiella and Haemophilus influenzae. Suspicion of pneumonia, in accordance with the latest recommendations of American Thoracic Society, may be had on the basis of the following typical lesions detected in lung radiography, and minimum two of the following three criteria: body temperature, bronchial secretion, and leucocytosis. (American Thoracic Society, 2005). On the fifth day after operation, each patient participating in the project has been examined for parameters of inflammation - physical examination, leukocyte count, and taking body temperature. The woman patient whose bacteriological index could have indicated lung infection has been referred to her original ward and put under detailed observation. In the end, without signs of infection (no changes detected above lung fields on auscultation, leucocytosis, or elevated temperature). The woman has been discharged home.

Most studies concerning bacterial colonization of respiratory tract are devoted to patients with chronic obstructive pulmonary disease (COPD) (Domenech et al., 2012; King et al., 2013; Marin et al., 2012). A positive result of culture in one of our woman-patients, a compulsive

smoker (20 cigarettes a day) may be considered colonization of respiratory tract.

In order to avoid the drawbacks of bronchoscopy, such as steep cost, side effects, experience required because of the performance technique, a cheaper and quicker mini-BAL method has been used. The method labelled as mini-BAL is an alternative for traditional bronchoscopy. Examination sensitivity is assessed at about 82% (63 to 100%), whereas the specificity compared with that of a bronchoscopy is assessed at 66 to 96% (Campbell, 2000).

Conclusion

To sum up, none of the patients participating in the study manifested marks of pneumonia in the postoperative period. It appears that general anesthesia does not have influence upon the increase of bacterial contamination of bronchial tree, and may even promote eradication of the colonizing bacterial flora. In the existing literature, there is no explicit standpoint concerning the influence of general anesthesia with the use of inhalants or intravenous anesthetics upon changes of bacterial flora in bronchial tree, especially in clinical aspect. However, it is too early to draw ultimate conclusions. The study reported here should be treated as preliminary communication.
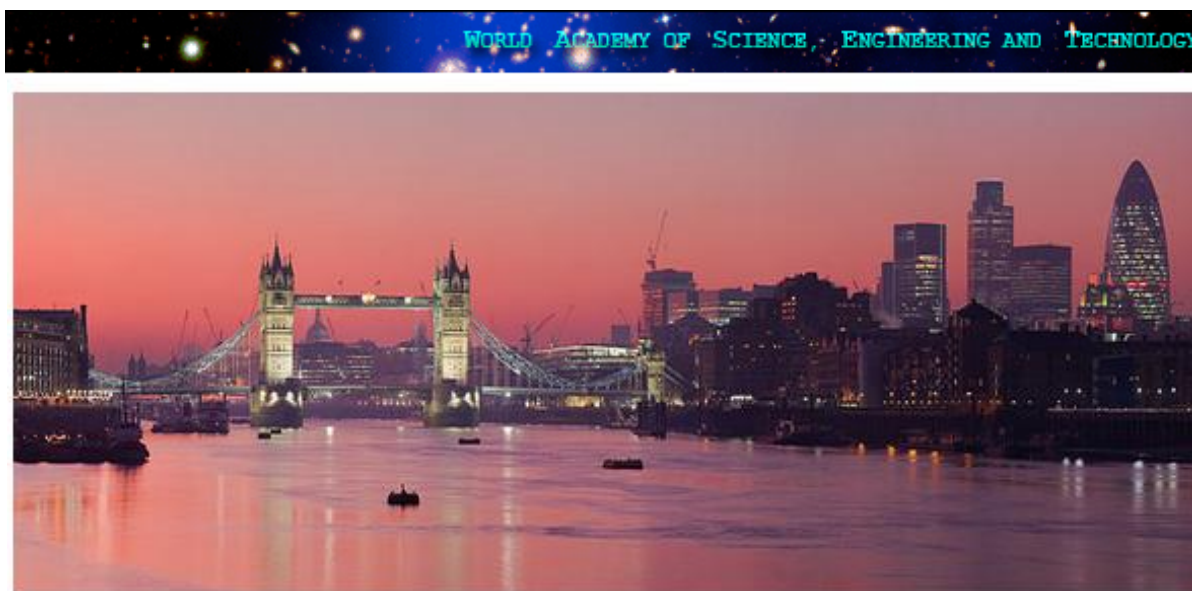
REFERENCES

American Thoracic Society; Infectious Diseases Society of America (2005). Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia. Am. J. Respir. Crit. Care Med. 171:388–416

Brusselaers N, Labeau S, Vogelaers D, Blot S (2013). Value of lower respiratory tract surveillance cultures to predict bacterial pathogens in ventilator-associated pneumonia: systematic review and diagnostic test accuracy meta-analysis. Intensive Care Med. 39(3): 365-375.

Campbell D (2000). Blinded invasive diagnostic procedures in ventilator-associated pneumonia. Chest. 117: 207S-211S.

Domenech A, Ardanuy C, Balsalobre L, Marti S, Calatayud L, De la Campa A G, Linares J (2012). Pneumococci can persistently colonize adult patients with chronic respiratory disease. J. Clin. Microbiol. 50(12): 4047-4053.

Ficker J (2008). Physiology and pathophysiology of bronchial secretion. Pneumonologie 62: S11-13.

Ioanas M, Angrill J, Baldo X, Arancibia F, Gonzala J, Baurer T, Canalis T, Torres A (2002). Bronchial bacterial colonization in patients with resectable lung carcinoma. Eur. Respir. J. 19(2): 326-332.

Karabiyik L, Turkan H, Ozisk T, Saracli MA, Haznedaroglu T (2007). Effects of sevofluran and/or nitrous oxide on bacterial growth in in vitro culture conditions. J. Anesth. 21:436-438.

King P, MacDonald M, Bardin PG (2013). Bacteria in COPD; their potential role and treatment. Trans. Resp. Med. 1:13.

Ledowski T, Paech MJ, Patel B, Schug SA (2006). Bronchial mucus transport velocity in patients receiving propofol and remifentanil versus sevoflurane and remifentanil anesthesia. Anesth. Analg. 102(5): 1427-30.

Marin A, Garcia-Aymerich J, Sauleda J, Belda J, Millares L, García-Nunez M (2012). Effect of bronchial colonisation on airway and systemic inflammation in stable COPD. COPD: Int. J. Chron. Obstruct. Pulmon. Dis. 9(2): 121-130.

Molliex S, Montravers P, Dureuil B, Desmonts J (1998). Halogenated anesthetics inhibit Pseudomonas aeruginosa growth in culture conditions reproducing the alveolar environment. Anesth. Analg. 86: 1075-1078.

Niedermann MS, Craven DE, Bonten MJ, Chastre J, Craig WA, Jakoby GA (2005). Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia. Am. J. Resp. Crit. Care Med. 171(4): 388-416.

Sachdev G, Napolitano LM (2012). Postoperative Pulmonary Complications: Pneumonia and Acute Respiratory Failure. Surg. Clin. North Am. 92(2): 321-344.

Shirakami G, Li D, Zhan X, Johns RA (2000). Propofol stimulates ciliary motility via the nitric oxide–cyclic GMP pathway in cultured rat tracheal epithelial cells. Anesthesiology 93(2): 482-488.

Trouillet JL (2012). Ventilator-associated pneumonia: a comprehensive review. Hosp. Pract. 40(2): 165-175.

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