African Journal of Microbiology Research

Volume 10 Number 10, 14 March 2016
ISSN 1996-0808

Academic Journals

ABOUT AJMR

The African Journal of Microbiology Research (AJMR) is published weekly (one volume per year) by Academic Journals.

The African Journal of Microbiology Research (AJMR) is provides rapid publication (weekly) of articles in all areas of Microbiology such as: Environmental Microbiology, Clinical Microbiology, Immunology, Virology, Bacteriology, Phycology, Mycology and Parasitology, Protozoology, Microbial Ecology, Probiotics and Prebiotics, Molecular Microbiology, Biotechnology, Food Microbiology, Industrial Microbiology, Cell Physiology, Environmental Biotechnology, Genetics, Enzymology, Molecular and Cellular Biology, Plant Pathology, Entomology, Biomedical Sciences, Botany and Plant Sciences, Soil and Environmental Sciences, Zoology, Endocrinology, Toxicology. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles are peer-reviewed.

Contact Us

Editorial Office: ajmr@academicjournals.org

Help Desk: helpdesk@academicjournals.org

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

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

Editors

Editorial Board Members

Editorial Board Members

Editorial Board Members

Editorial Board Members

African Journal of Microbiology Research

Table of Contents: Volume 10 Number 10 14 March, 2016

ARTICLES

academicJournals

Vol. 10(10), pp. 312-318, 14 March, 2016
DOI: 10.5897/AJMR2015.7616
Article Number: 400DC7457650
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Antimicrobial activity of seaweeds of Pernambuco, northeastern coast of Brazil

Renata Carla Corrêa Alves[1], Paula Fernanda Figueiredo das Mercês[1], Isabel Renata Arruda de Souza[2], Clébia Maria Alves de Almeida[1], Ana Paula Sant'Anna da Silva[1], Vera Lúcia de Menezes Lima[1], Maria Tereza dos Santos Correia[1], Márcia Vanusa da Silva[1] and Alexandre Gomes da Silva[3]*

[1]Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Pernambuco (UFPE), Av. Prof. Moraes Rego, 1235, Cidade Universitária, CEP 50670–901, Recife. PE, Brazil.
[2]Centro de Tecnologias Estratégicas do Nordeste/Ministério da Ciência, Tecnologia e Inovação (CETENE/MCTI), Av. Prof. Moraes Rego, 01, Cidade Universitária, CEP 50740–540, Recife, Brazil.
[3]Núcleo de Bioprospecção e Conservação da Caatinga, Instituto Nacional do Semiárido/Ministério da Ciência, Tecnologia e Inovação (INSA/MCTI), Av. Francisco Lopes de Almeida, s/n, Serrotão, CEP 58429-970 , Campina Grande, PB, Brazil.

Received 8 June, 2015; Accepted 10 August, 2015

The antibacterial efficacy of various solvent extracts of marine algae *Caulerpa racemosa*, *Ulva lactuca* (Chlorophyta), *Jania adhaerens* (Rhodophyta), *Padina gymnospora* and *Sargassum polyceratium* (Phaeophyta) against some selected gram-positive and gram-negative human pathogenic bacteria was screened. Crude extracts were prepared from the selected marine algae using different solvents namely, hexane, chloroform, ethyl acetate and methanol and were tested for their antibacterial activity against human pathogenic bacteria using disc diffusion method. Minimum inhibitory concentration (MIC) was also determined for selected solvent extracts for all the bacterial species. A suitable positive control was also maintained. Among the five marine algae screened *C. racemosa* and *U. lactuca* were found to be more active. It was observed that the ethyl acetate extracts of all the five marine algae showed higher inhibitory activity for the selected bacterial species than other solvent extracts. The results revealed that the crude ethyl acetate extracts seem to be a good source material in identifying the effective pure antibacterial compound(s) in all the five marine algae and particularly, *C. racemosa* and *U. lactuca*. The present study showed that the ethyl acetate extracts of marine algae such as *C. racemosa*, *J. adhaerens*, *P. gymnospora*, *S. polyceratium* and *Ulva lactuca* exhibited good antimicrobial activity. But the ethyl acetate extracts of *C. racemosa* and *U. lactuca* possessed highest antibacterial activity than others and so it could be useful in seeking active principles against human pathogenic bacteria.

Key words: Seaweeds, antimicrobial activity, marine macroalgae, human bacterial pathogens.

INTRODUCTION

Bacterial infection causes high rate of mortality in human population and aquaculture organisms. Preventing disease outbreaks or treating the disease with drugs or chemicals tackles these problems. Nowadays, the use of antibiotics increased significantly due to heavy infections and the pathogenic bacteria becoming resistant to drugs

is common due to indiscriminate use of antibiotics. It becomes a greater problem of giving treatment against resistant pathogenic bacteria (Mahida and Mohan, 2007). The search of new antimicrobial drugs from natural source became an obligation.

There are reports of macroalgae derived compounds that have a broad range of biological activities, such as antibiotic, antiviral, antineoplastic, antifouling, anti-inflammatory, cytotoxic and antimitotic (Jones et al., 2008; Maleki et al., 2008; Tambekar and Dahikar, 2011). The first to observe antimicrobial substances secreted by algae was Harder (Harder, 1917). However, it was not until the 1970s that large-scale screening of antimicrobial activity was carried out (Mahida and Mohan, 2007; Jones et al., 2008) and in the past few decades, macroalgae are attracting increasing attention as a new source for bioactive compounds (Arvinda Swamy, 2011).

Nowadays, infectious diseases are responsible for a high morbidity and mortality rate and are consider as a public health problem because of their frequency and their severity. For the treatment of these diseases, people often use synthetic drug. But, bacteria developed a resistance mechanism to fight against most of the synthetic family of antibiotics. The resistant of microbes is due to indiscriminate utilization of commercial antimicrobial medicines supported by many scientists investigation for modern antimicrobial substances from several medicinal plants and seaweeds (Alagesaboopathi and Kalaiselvi, 2012). There are several bioactive compounds which are produced by seaweeds and they also possess the ability to prevent the disease caused by some gram negative and gram positive pathogenic bacteria (Kolanjinathan et al., 2009).

In the present study, antibacterial efficacy of various organic solvent extracts of the seaweeds *Caulerpa racemosa*, *Ulva lactuca* (Chlorophyta), *Jania adhaerens* (Rhodophyta), *Padina gymnospora* and *Sargassum polyceratium* (Phaeophyta) against some clinically important gram-positive and gram-negative human pathogenic bacteria species is reported.

MATERIALS AND METHODS

Collection of algae

In this study, a total of five seaweed species (Table 1) were collected by hand picking from the submerged marine rocks at Paiva Beach (08º 15'10.50" S e 34º 56'51.80" W) and Pedra do Xareu Beach (08º 18'00.30" S e 34º 56'34.86" W), Cabo de Santo Agostinho municipality, Pernambuco State, Brazil (Figure 1) during low tide in December 2010 and January 2011. All samples were brought to laboratory in plastic bags containing sea water to prevent evaporation. Some of the collected seaweeds were preserved for identification. Seaweeds were identified by Dra. Paula Regina Fortunato do Nascimento, expert in macroalgae, Universidade Federal Rural de Pernambuco, Brazil. Voucher specimens of each species have been deposited at Instituto Agronômico de Pernambuco Herbarium (IPA) (Table 1).

Extract preparation

Algal samples were cleaned of epiphytes and extraneous matter, and necrotic parts were removed. Plants were washed with seawater and then in fresh water. The seaweeds were transported to the laboratory in sterile polythene bags at 0°C temperature. In the laboratory, samples were rinsed with sterile distilled water and were shade dried, cut into small pieces and powdered in a mixer grinder. The algal powdered samples were extracted using four different solvents hexane, chloroform, etyl acetate and methanol. 100 g of powdered algal material were extracted in Soxhlet extractor at 40°C containing 1000 mL of solvent separately using all the four solvents. The material was refluxed for about 36 to 48 h until saturation and the resulting extracts were evaporated in a rotary flash evaporator. The obtained extracts were collected in a clean Petri dish and weighed.

Test organisms

The antimicrobial activity of the seaweeds extracts were tested against the following microorganisms: three Gram-positive bacteria *Bacillus subtilis* (UFPEDA 82), *Micrococcus luteus* (UFPEDA 100) and *Staphylococcus aureus* (UFPEDA 02), the two Gram-negative bacteria *Escherichia coli* (UFPEDA 224) and *Klebsiella pneumoniae* (UFPEDA 396). All strains were provided by Departamento de Antibioticos, Universidade Federal de Pernambuco (UFPEDA) (Table 1) and maintained in Nutrient Agar (NA) and stored at 4□C.

Antibacterial assay

Antibacterial activity was evaluated by agar diffusion method (Bauer et al., 1966). Twenty milligrams of crude extract was dissolved in 1 mL of 10% of Di Methyl Sulphoxide (DMSO). From this stock solution, 10 µL of each extract was loaded on sterile antibiotic discs (6 mm diameter) (Hi-media company) and air-dried. After drying, discs were placed on the Tryptic soy agar. Chloramphenical antibiotics disc and disc loaded with 10 µL of respective solvent were used as positive and negative control respectively. Each sample was used in triplicate for the determination of antibacterial activity.

Determination of the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC)

The minimal inhibitory concentrations (MICs) of all extracts and reference antibiotic (Chloramphenicol) were determined by microdilution techniques in Mueller-Hinton broth (Merck) following the protocol established by the CLSI (NCCLS, 2009) for bacteria. Inoculates were prepared in the same medium at a density adjusted to a 0.5 McFarland turbidity standard [10^8 colony-forming units (CFU)/mL] and diluted 1:10 for the broth microdilution procedure. Microtiter plates were incubated at 37°C and the MICs were recorded after 24 h of incubation. Minimum inhibitory concentration

*Corresponding author. E-mail: alexandre.silva@insa.gov.br. Tel: +558333156450.

Table 1. Selected seaweeds from Brazilian coast.

Order	Species	Local	Date	Voucher
Rhodophyceae	*Jania adhaerens* J.V. Lamour.	Paiva Beach, Cabo de Santo Agostinho, Pernambuco State	January 2011	IPA 91010
Ulvophyceae	*Caulerpa racemosa* (Forssk.) J.Agardh	Pedra do Xaréu Beach, Cabo de Santo Agostinho, Pernambuco State	December 2010	IPA 91011
Phaeophyceae	*Padina gymnospora* (Kütz.) Sonder	Pedra do Xaréu Beach, Cabo de Santo Agostinho, Pernambuco State	December 2010	IPA 91012
Phaeophyceae	*Sargassum polyceratium* Mont.	Pedra do Xaréu Beach, Cabo de Santo Agostinho, Pernambuco State	December 2010	IPA 91013
Ulvophyceae	*Ulva lactuca* L.	Gaibu Beach, Cabo de Santo Agostinho, Pernambuco State	January 2011	IPA 91014

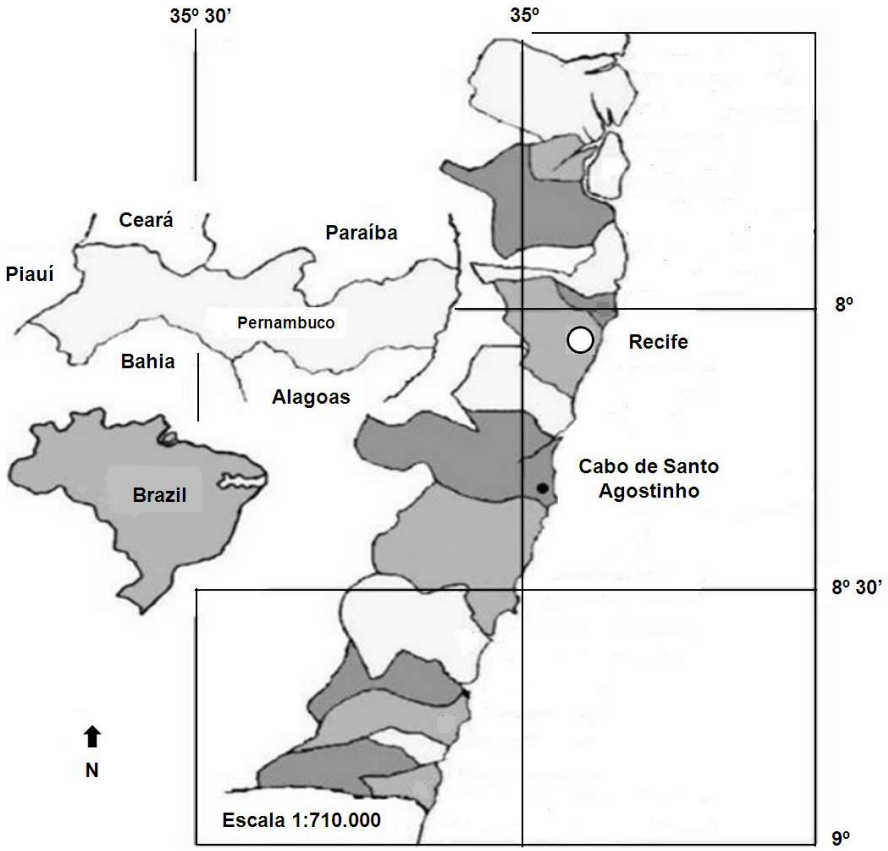

Figure 1. Geographical localization of Pedra do Xaréu Beach and Gaibu Beach, Cabo de Santo Agostinho municipality, Pernambuco state, Brazil.

Table 2. Results of antimicrobial activity of 18 extract crude of five seaweeds of Brazilian coast, determined by agar disc diffusion method (inhibition zone in mm).

Seaweeds	Solvents	Zone of Inhibition (mm)				
		Bacilus subtilis	***Micrococcus luteus***	***Staphylococcus aureus***	***Escherichia coli***	***Kleibsiela pneumoniae***
Jania adhaerens J.V. Lamour.	Hexane	14.33±0.58	10±0.00	-	-	-
	Chloroform	15.33±0.58	8.67±0.00	-	-	-
	Ethyl acetate	15±0.00	14.33±0.58	-	-	-
	Methanol	13±0.00	9.33±0.58	-	-	-
Caulerpa racemosa (Forssk.) J.Agardh	Hexane	16±0.00	16±0.00	16.33±0.58	15.33±0.58	10±0.00
	Chloroform	17±0.00	17±0.00	17±0.00	15±0.00	10.67±0.58
	Ethyl acetate	17±0.00	19±0.00	17±0.00	15.33±0.58	12.33±0.58
	Methanol	15.33±0.58	18±0.00	18±0.00	16.33±0.58	13±0.00
Padina gymnospora (Kütz.) Sonder	Hexane	16±0.00	16.33±0.58	-	-	-
	Chloroform	16±0.00	15.33±0.58	-	-	-
	Ethyl acetate	17±0.00	15±0.00	-	-	-
	Methanol	15±0.00	13±0.00	-	-	-
Sargassum polyceratium Mont.	Hexane	17±0.00	12±0.00	10±0.00	16.33±0.58	6.67±0.58
	Chloroform	15±0.00	15.33±0.58	8.67±0.00	11.33±0.58	8.67±0.58
	Ethyl acetate	17±0.00	16.33±0.58	15.33±0.58	12.33±0.58	10.67±0.58
	Methanol	17±0.00	11±0.00	12±0.00	15±0.00	8.67±0.58
Ulva lactuca L.	Hexane	18±0.00	15±0.00	14±0.00	12±0.00	10.67±0.58
	Chloroform	19±0.00	18±0.00	15±0.00	10±0.00	8.67±0.58
	Ethyl acetate	20±0.00	23±0.00	18±0.00	15±0.00	10.67±0.58
	Methanol	20±0.00	20±0.00	17±0.00	9.33±0.58	10±0.00
Chloramphenicol	-	20±0.00	19±0.00	21±0.00	17±0.00	17±0.00

corresponded to the minimum extract concentration that inhibited visible bacterial growth. Afterwards, cultures were seeded onto MHA and incubated for 24 h at 37°C to determine the minimum bactericidal concentration (MBC) which corresponded to the minimum concentration of extract that caused the bacteria elimination. The antibiosis (bacteriostatic or bactericidal) activity is determined by the ratio of MBC/MIC. When the ratio of MBC/MIC is ≤ 2, the active fractions were considered as bactericidal, and when the ratio was higher ≥ 2 was considered bacteriostatic. Finally if the ratio is ≥ 16 the fractions were considered as ineffective (Traczewski et al., 2009; Sader et al., 2009).

RESULTS

The antibacterial activity of various solvent extracts of *C. racemosa*, *U. lactuca* (Chlorophyta), *J. adhaerens* (Rhodophyta), *P. gymnospora* and *S. polyceratium* (Phaeophyta) on five different human bacterial pathogens are presented in Tables 2 and 3. Seaweeds extract of *C. racemosa*, *S. polyceratium* and *U. lactuca* demonstrated good antimicrobial activity against all gram-positive and gram-negative pathogenic bacteria. *J. adhaerens* and *P. gymnospora*

Table 3. Results of antimicrobial activity of 20 extract crude of five seaweeds of Brazilian coast, determined by the agar-dilution methods (Minimum Inhibitory Concentration, MIC, in mg/mL).

Seaweeds	Solvent	Microorganisms														
		MIC					MBC					MBC/MIC				
		B.s.	***M.l.***	***S.a.***	***E.c.***	***K.p.***	***B.s.***	***M.l.***	***S.a.***	***E.c.***	***K.p.***	***B.s.***	***M.l.***	***S.a.***	***E.c.***	***K.p.***
Jania adhaerens J.V. Lamour.	Hexane	12.5	12.5	25	12.5	25	12.5	25	12.5	12.5	12.5	1	2	2	1	1
	Chloroform	6.25	12.5	25	12.5	25	6.25	25	12.5	12.5	12.5	2	2	2	1	1
	Ethyl Acetate	6.25	6.25	12.5	12.5	12.5	6.25	12.5	12.5	12.5	12.5	1	2	1	1	1
	Methanol	6.25	6.25	12.5	12.5	12.5	6.25	12.5	12.5	12.5	12.5	1	2	1	1	1
Caulerpa racemosa (Forssk.) J.Agardh	Hexane	1.56	3.12	1.56	3.12	6.25	1.56	3.12	3.12	3.12	6.25	1	1	2	1	1
	Chloroform	3.12	3.12	1.56	3.12	6.25	3.12	3.12	3.12	3.12	6.25	1	1	2	1	1
	Ethyl Acetate	1.56	6.25	0.78	3.12	3.12	1.56	6.25	0.78	3.12	6.25	1	1	1	1	2
	Methanol	1.56	6.25	0.78	3.12	3.12	1.56	6.25	0.78	3.12	6.25	1	1	1	1	2
Padina gymnospora (Kütz.) Sonder	Hexane	6.25	12.5	12.5	25	50	6.25	12.5	25	50	50	1	1	2	2	1
	Chloroform	6.25	12.5	12.5	25	50	6.25	12.5	25	50	50	1	1	2	2	1
	Ethyl Acetate	3.12	6.25	12.5	50	50	3.12	6.25	12.5	50	50	1	1	1	1	1
	Methanol	6.25	6.25	12.5	50	50	6.25	6.25	12.5	50	50	1	1	1	1	1
Sargassum polyceratium Mont.	Hexane	12.5	6.25	12.5	12.5	25	12.5	6.25	12.5	25	25	1	1	1	2	1
	Chloroform	12.5	6.25	12.5	12.5	25	12.5	6.25	12.5	25	25	1	1	1	2	1
	Ethyl Acetate	6.25	3.12	6.25	12.5	12.5	6.25	6.25	6.25	50	12.5	1	2	1	4	1
	Methanol	6.25	6.25	6.25	12.5	12.5	6.25	6.25	6.25	50	12.5	1	1	1	4	1
Ulva lactuca L.	Hexane	0.39	0.78	1.56	3.12	6.25	0.39	3.12	3.12	6.25	12.5	1	4	2	2	2
	Chloroform	0.39	0.78	1.56	3.12	6.25	0.39	3.12	3.12	6.25	12.5	1	4	2	2	2
	Ethyl Acetate	0.39	0.39	0.78	1.56	3.12	0.39	1.56	1.56	3.12	3.12	1	4	2	2	1
	Methanol	0.39	0.39	0.78	1.56	6.25	0.39	1.56	1.56	6.25	6.25	1	4	2	4	1
Choramphenicol	-	0.09	0.04	0.09	0.19	0.19	0.09	0.04	0.09	0.39	0.39	1	1	1	2	2

B.s. = Bacilus subtilis; *M.l. = Micrococcus luteus*; *S.a. = Staphylococcus aureus*; *E.c. = Escherichia coli*; *K.p. = Klebsiella pneumoniae.*

demonstrated antimicrobial activity against only *Bacillus subtilis* and *Micrococcus luteus*. Among the four solvents tested, ethyl acetate and methanol extracts exhibited maximum inhibition on the growth of the tested bacterial species. As observed, the ethyl acetate extracts of all the five marine algae showed the highest inhibitory activity for the chosen bacterial strains followed by other solvent extracts.

Maximum activities were recorded in the green marine algae *U. lactuca* ethyl acetate (23 ± 0.00 mm) and methanol (20 ± 0.00 mm) extracts and *C. racemosa* ethyl acetate (19 ± 0.00 mm) and methanol (18 ± 0.00 mm) extracts when compared to other solvent extracts as well as

various solvent extracts of the marine algae *J. adhaerens*, *P. gymnospora* and *S. polyceratium* (Table 2). Less inhibitory effects for all the test organisms were recorded in the *J. adhaerens* and *P. gymnospora*. Among the five groups of marine algae tested, maximum activities were recorded in green marine algae *U. lactuca* and minimum activity was recorded in red marine algae. All the four solvent extracts of the marine algae, *J. adhaerens* and *P. gymnospora* were not revealed any zone of inhibition against *Staphylococcus aureus*, *Escherichia coli* and *K. pneumonia*e.

Of the five marine algae screened in the present study for their antibacterial activity, *U. lactuca* and *C*. *racemosa* were observed to be more active than *J. adhaerens*, *P. gymnospora* and *S. polyceratium* gainst human pathogens in the control of their growth.

There were also specific antibacterial activities with reference to either the known solvent extract effective to a number of bacterial strains or specific effect of marine algae to some bacterial pathogens. The ethyl acetate extract of *C. racemosa* and *U. lactuca* showed excellent antibacterial activity. Specifically hexane extracts of *C. racemosa* and *U. lactuca* indicated inhibition of bacteria such as *B. subtilis* and *S. aureus*. In *P. gymnospora* species hexane extract shows prominent activity against bacteria *B. subtilis*. It was observed that hexane extracts of *J. adhaerens* and *S. policeratium* produced broad spectrum antibacterial activity against *S. aureus* and *B. subtilis*. Ethyl acetate extract of all the five marine algae exhibited activity against *B. subtilis*, *M. luteus* and *S. aureus*.

Table 2 shows the MIC, MBC and MBC/MIC ratio value of the extracts from algae. These MIC and MBC values were demonstrated to range from 0.39-50 mg/mL. A minimum value of MIC as 0.39 mg/mL was observed for *B. subtilis* to all organic extracts of *U. lactuca*. Among various crude solvent extracts tested, ethyl acetate extracts of all the five marine algae performed better than the other solvent extracts.

Almost all organic extracts of the five seaweed showed bactericidal action.

DISCUSSION

There is high expectation that organisms from the marine environment will yield a vast array of new pharmaceutical compounds with novel activities that will provide new drugs in the fight against a number of microbial pathogens currently developing resistance conventional antibiotic therapies.

In this study, green algae had higher inhibition activity than the red and brown algae. But the brown algae exhibited the moderate inhibition growth when compared with green algae. Some previous investigations revealed higher antibacterial activity in the extracts of brown algae than the red algae extract (Reichelt and Borowitzka, 1984).

Reichelt and Borowitzka (1984) and Salvador et al. (2007) screened many species of algae for their antibacterial activity. They reported that the members of the red algae exhibited high antibacterial activity. In contrast the green algae (Clorophyceae) were the most active species. Present results are in accordance with those of Kandhasamy and Arunachalam (2008) who reported that green algae (Chlorophyceae) were the most active division than others.

Antimicrobial activity depends on both algal species and the solvents used for their extraction. In our study it was reported that the green algae (*U. lactuca* and *C. racemosa*) showed antibacterial activity against several Gram-negative and Gram-positive bacteria. Maximum activities were recorded in the green algae *U*. *lactuca* and *C. racemosa* against *S. aureus* in ethyl acetate and metanol extracts when compared to other solvent extracts of the marine algae *J. adhaerens*, *P. gymnospora*, *S. polyceratium*.

Perez et al. (1990) observed that the extract of *U. lactuca* had no antibacterial activity. In contrast, results of our study shows that *U. lactuca* inhibited mostly all the organisms in all the solvents tested. To *C. racemosa,* the literature reports the presence essentially of alkaloids, terpenoids and steroids. It is reported that these compounds possess therapeutical applications (Güven et al., 2010; Ornano et al., 2014).

A few workers tried using different solvents for screening the antimicrobial activity of seaweeds and made comparisons. Martinez–Nadal et al. (1966) mentioned that benzene and diethyl ether were suitable solvents for extracting the antibiotic principle. In another study, acetone was found best among several solvents used for extracting antibacterial substances (Patra et al., 2009).

Some other studies performed in the extraction of seaweeds using chloroform and ethyl acetate also exhibited good antibacterial activity (Vonthron-Sénécheaus et al., 2011). It was reported that methanol extracts of seven different seaweeds tested showed broad spectrum antibacterial activity against human pathogenic bacteria (Jebasingh et al., 2011; Kandhasamy and Arunachalam, 2008; Kannan et al., 2010; Rajasulochana et al., 2009). This kind of less or more activity could also be attributed to the sequential extraction of marine algae using solvents from low polar to high polar.

Differences between the results of the present investigation and results of other studies may be due to the production of bioactive compounds related to the seasons, method, organic solvents used for extraction of bioactive compounds and differences in assay methods. Among the seaweed species screening in this paper, *C. racemosa* is the species most studied. Investigation of phytochemicals of *C. racemosa* led to the isolation of several secondary metabolites related to different categories of natural products: indol derivatives, indan

derivatives, sesquiterpenoid derivatives, diphenyl pentadiene derivatives, terpenoids and fatty acids (Ornano et al., 2014). Liu et al. (2013a) discoveried two rare antifungal prenylated para-xylenes, caulerprenylols A and B. The same authors (Liu et al., 2013b) isolated two bisindole alkaloids, racemosins A and B, and one well-known pigment in the genus *Caulerpa*, caulerpin. The antimicrobial activity of organic extracts of C. racemosa is due to the action of these compounds.

Finally it can be concluded from the study that extracts of algal species used in the present investigation showed better antibacterial activity against pathogens used. In general, the ethyl acetate and methanol extracts of all the five marine algae showed antibacterial activity against both gram positive and gram negative bacteria with very well-known higher levels of antibacterial activity of *U. lactuca* and *C. racemosa*. It is thus concluded from this study that the ethyl acetate extract of marine alga, *U. lactuca* and *C. racemosa* could be used for further investigation to identify actual components against human bacterial pathogens.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Alagesaboopathi C, Kalaiselvi N (2012). Antimicrobial activities of the root, stem and leaf extracts of *Argemone mexicana* L. Int. J. Biosci. 2(5):61-68.

Arvinda Swamy ML (2011). Marine algal sources for treating bacterial diseases. Adv. Food Nutr. Res. 64:71-83.

Bauer AW, Kirby MDK, Sheries JC, Truck M (1966). Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45:493-496.

Güven KC, Percot A, Sezik E (2010). Alkaloids in marine algae. Mar. Drugs 8(2):269-284.

Harder R (1917). Ernährungsphysiologische Untersuchungen an Cyanophyceen, hauptsächlich am endophytischen Nostoc punctiforme. Z. Bot. 9:145.

Jebasingh SEJ, Rosmary S, Elaiyaaja S, Sivaraman K, Lakshmikandan M, Murugan A, Raja (2011). Potencial antibacterial activity of selected green and red seaweeds. J. Pharm. Biomed. Sci. 5:1-7.

Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL (2008). Global trends in emerging infectious diseases. Nature 452:990-993.

Kandhasamy M, Arunachalam KD (2008). Evaluation of in vitro antibacterial property of seaweeds of southeast coast of India. Afr. J. Biotechnol. 7:1958-1961.

Kannan RRR, Arumugam R, Anantharaman P (2010). Antibacterial potential of three seagrasses against human pathogens. Asian Pac. J. Trop. Biomed. 11:890-893.

Kolanjinathan K, Ganesh P, Govindarajan M (2009). Antibacterial activity of ethanol extracts of seaweeds against fish bacterial pathogens. Eur. Rev. Med. Pharmacol. Sci. 13:173-177.

Liu AH, Liu DQ, Liang TJ, Yu XQ, Feng MT, Yao LG (2013a). Caulerprenylols A and B, two rare antifungal prenylated para-xylenes from the green alga *Caulerpa racemosa*. Bioorg. Med. Chem. Lett. 23:2491-2495.

Liu AH, Liu DQ, Liang TJ, Yu XQ, Feng MT, Yao LG (2013b). Racemosins A and B, two novel bisindole alkaloids from the green alga *Caulerpa racemosa.* Fitoterapia 91:15-20.

Mahida Y, Mohan JSS (2007). Screening of plants for their potential antibacterial activity against *Staphylococcus* and *Salmonella* spp. Nat. Prod. Radiance 6:301-305.

Maleki S, Seyyednejad SM, Damabi NM, Motamedi H (2008). Antibacterial activity of the fruits of Iranian *Torilis leptophylla* against some clinical pathogens. Pak. J. Biol. Sci. 11:1286-1289.

Martinez-Nadal NGC, Casillas Rodriguez–Perrazza JR, Torreera L (1966). Antibiotic properties of marine algae *Cymoplia barbata*. Bot. Mar. 9:21-26.

NCCLS (2009). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, Wayne, National Clinical and Laboratory Standards Institute. 65 pp.

Ornano L, Donno Y, Sanna C, Ballero M, Serafine M, Bianco A (2014). Phytochemical study of *Caulerpa racemosa* (Forsk.) J. Agarth, an invading alga in the habitat of La Maddalena Archipelago. Nat. Prod. Res. 28(20):1795-1799.

Patra JK, Patra AP, Mahapatra NK, Thatoi HN, Das S, Sahu RK, Swain GC (2009). Antimicrobial activity of organic solvent extracts of three marine macroalgae from Chilika Lake, Orissa, India. Malays. J. Microbiol. 5: 128-131.

Perez RM, Avila JG, Perez S, Martinez A, Martinez G (1990). Antimicrobial activity of some American algae. J. Ethnopharmacol. 29:111-116.

Rajasulochana P, Dhamotharan R, Krishnamoorthy P, Murugesan S (2009). Antibacterial activity of the extracts of marine red and brown algae. J. Am. Sci. 5:20-25.

Reichelt JL, Borowitzka MA (1984). Antimicrobial activity from marine algae: Results of a large-scale screening programme. Hydrobiologia 116/117:158-168.

Sader HS, Rhomberg PR, Jones RN (2009). Nine-hospital study comparing broth microdilution and Etest method results for vancomycin and daptomycin against methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. 53: 3162-165.

Salvador N, Gómez Garreta A, Lavelli L, Ribera L (2007). Antimicrobial activity of Iberian macroalgae. Sci. Mar. 71:101-113.

Tambekar DH, Dahikar SB (2011). Antibacterial activity of some Indian ayurvedic preparations against enteric bacterial pathogens. Int. J. Phytomed. 2:24-29.

Traczewski MM, Katz BD, Steenbergen JN, Brown SD (2009). Inhibitory and bactericidal activities of daptomycin, vancomycin, and teicoplanin against methicillin-resistant *Staphylococcus aureus* isolates collected from 1985 to 2007. Antimicrob. Agents Chemother. 53:1735-1738.

Vonthron-Sénécheau C, Kaiser M, Devambez I, Vastel A, Mussio I, Rusig AM (2011). Antiprotozoal activities of organic extracts from french marine seaweeds. Mar. Drugs 9:922-933.

academicJournals

Vol. 10(10), pp. 319-323, 14 March, 2016
DOI: 10.5897/AJMR2015.7817
Article Number: 78CF16757652
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Comparative evaluation of antibacterial activity of induced and non-induced *Cajanus cajan* seed extract against selected gastrointestinal tract bacteria

Shandu Jabulani Siyabonga, Manqele Ayanda, Maliehe Tsolanku Sydney and Shode Francis Oluwole

Department of Biochemistry and Microbiology, Faculty of Science and Agriculture, University of Zululand, Private Bag X1001 KwaDlangezwa 3886, South Africa.

Received 19 October 2015; Accepted 25 February, 2016

Gastrointestinal tract (GIT) infections are major causes of mortality and morbidity world-wide, especially in developing countries. *Cajanus cajan* extracts possess therapeutic properties. In this study, the induced and non-induced antibacterial properties of *C. cajan* seeds were evaluated against bacterial strains implicated in GIT infections by Disc diffusion method and Micro-well dilution assay. *C. cajan* produced phytoalexins after the seeds were elicited with native flora and silver nitrate. At 100 mg/ml, the ethyl acetate extract produced zones of inhibition (14 to 16 mm) against *Staphylococcus aureus* (ATCC 25925), *Klebsiella pneumoniae* (ATCC 31488) and *Salmonella typhimurium* (ATCC 700030). The minimum inhibitory concentration (MIC) values obtained using micro well dilution method were 6.5, 12.5, and 12.5 mg/ml for *S. aureus* (ATCC 25925), *K. pneumoniae* (ATCC 4352) and *S. typhimurium* (ATCC 700030) and 25 mg/ml for all bacterial strains in the ethyl acetate extract ($AgNO_3$ induced seeds), respectively. The results thus indicated that *C. cajan* seed extract do possess antibacterial activity.

Key words: Seeds, antimicrobial, phytoalexins, phytoanticipins.

INTRODUCTION

Gastrointestinal tract (GIT) infections are major cause of mortality and morbidity world-wide, especially in developing countries where more than 1.5 billion episodes of infections result in more than 3 million deaths annually (The United Nations Children's Fund (UNICEF)/World Health Organization (WHO), 2009). GIT infections are transmitted mainly through contaminated food and water (Mahadeva, 2013). Plant parts, as extracts and in various forms have been utilized in previous years as medicine for treatments of GIT infections caused by pathogens and metabolic disorders (Brahmachari, 2012). Although seed extracts are excellent sources of therapeutic phytochemicals, they have rarely been used as medicine (van Wyk et al., 2009).

*Corresponding author. E-mail: Shanduj@unizulu.ac.za. Tel: +27768810728.

However, with more than 70% of microorganisms causing infections having resistance to some antibiotics, the prohibitive costs of treatments consequent upon this resistance and the side effects of allopathic medicine, the use of seed extracts with potency against microbial infections have gained momentum (WHO, 2004).

According to Satapathy et al. (2012) and Harbone (1999), seed extracts possess antimicrobial substances that include prohibitions, pre-inflectional metabolites and phytoanticipins. *Cajanus cajan*, also known as pigeon pea, is a leguminous plant of the Fabbaceae family (Krishna and Bhatia, 1985). *C. cajan* extracts have health benefits as therapeutic agents in diseases such as sickle cell anemia (Egunyomi et al., 2009), hepatic disorder (Kundu et al, 2008), hyperlipidermia (Dai et al., 2013) and GIT infections (Luo et al., 2010). Moreover, both the seeds and leaves of *C. cajan* have hypoglycemic potential as edible foods (Jaiswal et al., 2008). In this study, the induced and non-induced antibacterial properties of the *C. cajan* seed extract were evaluated against bacterial strains implicated in GIT infections.

MATERIALS AND METHODS

Pretreatment of seeds

The seeds of *C. cajan* purchased from Nigeria by Professor O. O. Shode were washed with distilled water, divided into 6 groups of 250 g and placed in six different 1000 ml Erlenmeyer flasks and labeled 1 up to 6.

Biotic elicitation

Biotic elicitation was performed as described by Dahiya et al. (1984), whereby the seeds of groups 1 and 2 were soaked in distilled water for 2 h in their respective flasks. After soaking, the seeds were dried using a paper towel and then incubated overnight in moist conditions. After overnight incubation, the seeds were ground using an electric grinder.

Abiotic elicitation

The seeds of groups 3 and 4 were abiotically elicited as described by Dahiya (1987), whereby the seeds were soaked in 0.1 M Silver Nitrate ($AgNO_3$) for 2 h in their respective flasks, were dried using paper towel and incubated in the dark at room temperature for 6 days. After 6 days of incubation, the seeds were ground to powder using an electric grinder.

Non-elicited group

The seeds of groups 5 and 6 were taken directly from the package. These seeds were not subjected to any form of treatment and were ground to powdery form using an electric grinder.

Extraction

Two hundred and fifty grams of powdered seeds were steeped in ethyl acetate (300 ml), incubated in a shaking incubator (200 rpm) at room temperature for 3 days. After 3 days the extracts were filtered using vacuum filtration system and concentrated using a rotary evaporator at 45°C. All the concentrated extracts were kept in the refrigerator (4°C) for antibacterial study.

Susceptibility testing

Reviving of microorganisms for susceptibility test

The bacterial cultures obtained from the culture bank were evaluated for purity by sub-culturing and were incubated overnight at 37°C. The pure cultures were then transferred to nutrient broth and 1 ml of each bacterial species was pipetted into 9 ml of nutrient broth in separate test tubes labeled with the corresponding bacteria and incubated overnight at 37°C. After incubation, the bacterial cultures were standardized according to McFarland's standards using a spectrophotometer at a wavelength of 620 nm (Andrews, 2001).

Preparation of paper discs for susceptibility test

Ten microliters of all the seed extracts were pipetted into separate sterile 6 mm paper discs and were left to absorb the extract for 10 min at room temperature.

Disc diffusion method for susceptibility test

Disc diffusion method described by Ezeifeka et al. (2004) was used for susceptibility testing. The bacterial cultures were spread on Muller Hinton agar using a spread plate technique, whereby a sterile cotton swab was deepened into bacterial cultures, spread evenly throughout the plates and left to dry for 30 min. After drying, each disc impregnated with the seed extract was placed at the center of each plate and the plates were incubated overnight at 37°C. The procedure was repeated thrice.

Minimum inhibitory concentration (MIC)

Preparation of different concentrations

Different concentrations of ethyl acetate seed extracts induced with native flora and silver nitrate were prepared using 10% DMSO. The stock extract of 900 mg/ml from seeds induced with normal flora was used to prepare different extract concentrations of 100, 50, 20, 10 and 5 mg/ml. The stock extract of 619 mg/ml from seeds induced with silver nitrate was used to prepare different extract concentrations of 100, 50, 20, 10 and 5 mg/ml.

Disc diffusion method for MIC

Blank paper discs were separately impregnated with extracts of different concentrations for 5 min. The Muller Hinton agar plates were spread with bacterial cultures. Disc diffusion method described by Ezeifeka et al. (2004) was used to determine the minimum concentration that inhibits the bacteria species. Paper discs impregnated with different concentration were placed on plates alongside antibiotic discs (vancomycin and neomycin) used as positive controls. The plates were then incubated overnight at 37°C. This disc diffusion assay was repeated three times.

96 micro well dilution assay for MIC

96 micro well dilution method adopted from Andrews (2001) was

Table 1. Zones of inhibition from susceptibility testing.

Extract	*S. aureus* (ATCC 25925) (mm)	*K. pneumoniae* (ATCC 31488) (mm)	*S. typhimurium* (ATCC 700030) (mm)
$AgNO_3$ induced extract	25	24	25
normal micro flora induced extract (mm)	32	30	32

Table 2. Antibacterial activities of different concentrations of ethyl acetate seed extract induced with normal micro flora.

Extract concentrations (mg/ml)	*S. aureus* (ATCC 25925) (mm)	*K. pneumoniae* (ATCC 31488) (mm)	*S. typhimurium* (ATCC 700030) (mm)
100	16	15	16
50	8	0	9
20	0	0	0
10	0	0	0
5	0	0	0

Table 3. Antibacterial activities of different concentrations of ethyl acetate seed extract induced with $AgNO_3$.

$AgNO_3$ induced extract concentrations (mg/ml)	*S. aureus* (ATCC 25925) (mm)	*K. pneumoniae* (ATCC 4352) (mm)	*S. typhimurium* (ATCC 700030) (mm)
100	15	14	15
50	8	0	7
20	0	0	0
10	0	0	0
5	0	0	0

used to determine the MIC value of the extract. 50 µl of nutrient broth was pipetted into 96 micro wells, 50 µl of the seed extract was added in all the wells in the first row, mixed thoroughly and a 3 fold serial dilution was performed throughout the columns. 20 µg/ml of vancomycin and neomycin were used as positive control and 10% dimethyl sulfoxide (DMSO) as a negative control. 50 µl of the bacterial culture was added to all the wells and the micro well plate was incubated overnight at 37°C. After overnight incubation, 20 µl 0.2 mg/ml of *P*-iodonitrotetrazodium violet (INT) was added on the wells and the wells were wrapped with a parafilm and incubated at 37°C for 30 min.

Minimum bactericidal concentration (MBC)

MBC of the extract was determined by Kirby-Bauer method adopted from Elaissi et al. (2012), whereby the micro wells that did not turn pink were used to find the minimum bactericidal concentration. A loopful of bacterial cultures in MIC, with no color change were streaked on the agar plate and incubated overnight at 37°C.

RESULTS

Susceptibility testing

All the ethyl acetate extracts of seeds induced with $AgNO_3$ and normal micro flora did show antibacterial activity on all bacterial species (*Staphylococcus aureus* (ATCC 25925), *Klebsiella pneumonia* (ATCC 31488) and *Salmonella typhimurium* (ATCC 700030)). The results from Disc diffusion method are shown in Tables 1, 2 and 3.

Micro-dilution assay

S. aureus had the lowest MIC value (6.5 mg/ml) on normal micro flora induced seeds while all species used had the MIC of 25 mg/ml on ethyl acetate extract ($AgNO_3$ induced seeds) (Table 4).

DISCUSSION

Phytochemicals are naturally occurring and biologically active substances that are chemically derived from plants (Alasalvar and Shahidi, 2013). According to Satapathy et al. (2012) and Harbone (1999), seed extracts contain different groups of antibacterial phytochemicals which include prohibitions, pre-inflectional metabolites and phytoanticipins. Phytoalexin is a term originally coined by Muller and Borger (1940), describing low molecular

Table 4. Minimum inhibitory concentration (MIC) (mg/ml) of *C. cajan* seed extract on selected bacterial species.

Bacterial species	MIC (ethyl acetate extract) (mg/ml)	MIC (ethyl acetate extract ($AgNO_3$ induced)) (mg/ml)
S. aureus	6.5	25
K. pneumoniae	12.5	25
S. typhimurium	12.5	25

weight antibacterial compounds that are synthesized *de novo* and accumulate in plant after being exposed to bacterial infections (Dakora and Phillips, 1996). Phytoanticipins are low molecular weight antimicrobial compounds that are present in plants before challenged by microorganisms or produced after infection solely from pre-existing constituents (van Etten et al., 1994).

There was no antibacterial activity in the non-induced seeds and that implied that there was no phytoanticipins presence in the seeds of *C. cajan*. This is supported by van Etten et al. (1994) who state that phytoanticipins are present in the plant before the plant is challenged by elicitors and some phytoanticipins play a role in defense mechanism and some do not.

Muller and Borger (1940) explained that phytoalexins are antimicrobial compounds that could not be performed in plant tissues or be released from preexisting plant constituents but are produced through microbial elicitation and their production requires microbial elicitation. Phytoalexins are not only produced through biotic elicitation but also through abiotic elicitation such as irradiation of using short ultraviolet light, treatment with heavy metal ions and non-biological elicitors (Grayer and Kokubun, 2001). This gave the reason why production of phytoalexins with both abiotic and biotic elicitors was successful.

The antibacterial activity of in the induced seeds of *C. cajan* indicates that phytoalexins were produced because of abiotic and biotic elicitation. Even though the extract can inhibit bacterial growth, when using different concentrations, the highest concentration (100 mg/ml) was required. The higher the concentration, the more the bacterial inhibition. This applied to both extract from $AgNO_3$ induced and native flora induced seeds. *K. pneumoniae* (ATCC 31488) was resistance to lower concentrations (x<100 mg/ml). Gram-negative bacteria, in addition to a thin peptidoglycan layer (2 to 7 nm), possesses about 7 to 8 nm of the outer membrane. This outer membrane composes of additional protective lipopolyssachride layer that exhibits toxicity and antigenicity against antibacterial or chemotherapeutic agents (Martinko and Madigan, 2006). It was concluded that the high resistance shown by *K. pneumoniae* (ATCC 31488) was due to this layer.

The MIC values of extracts of the seeds elicited with native flora were lower than the extracts of the seeds elicited with $AgNO_3$. This could be linked to the idea proposed by Harborne (1999) that phytochemicals that are induced biotically and abiotically are different in activity. Phytoalexins produced during abiotic elicitation are stress related not infection related. Even though these phytoalexins can inhibit bacterial growth, they are not as effective as phytoalexins produced through elicitation by bacteria.

The mechanism by which abiotic elicitation affects phytoalexin production is not clear, but the following two mechanisms are possible; abiotic elicitors may act by simply injuring plant cells which then stimulates the phytoalexin biosynthetic pathway or abiotic elicitors may cause the host plant to release a constitutive elicitor which triggers phytoalexins formation Dakora and Phillips (1996). One or both of these mechanisms could be the possible reasons why the zones of inhibition from the abiotic elicited extract were smaller than the zones produced by biotic elicited extract since abiotic elicitation is not as direct as abiotic elicitation when it comes to phytoalexin production.

Antibacterial compounds impose bactericidal, bacteriostatic and bacteriolytic effects on exponentially growing microbial species (Martinko and Madigan, 2006). *C. cajan* seed extracts did show the bacteriostatic effect on all bacterial strains and not bactericidal effect since all the bacterial species from the MIC assay did not give MBC. Bacterial strains did grow when the MBC was evaluated. Some phytoalexins are considered not to be stable, since some bacteria can detoxify phytoalexins into less toxic compounds or furthermore into compounds that can suppress establishment of defense response in plants (González-Lamothe et al., 2009).

Conclusion

It can be concluded that *C. cajan* elicited seeds, produce phytoalexins through biotic and abiotic elicitation and do demonstrate antibacterial activity. Although the seed extract showed the bacteriostatic and not bactericidal effect, they can be used as potential therapeutic sources for treatment of GIT infections.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors would like to thank the staff from University of Zululand, Department of Biochemistry and Microbiology in collaboration with Department of Chemistry for all the assistance given.

Abbreviations

GIT, Gastrointestinal tract; **DMSO,** dimethyl sulfoxide; **MIC,** minimum inhibitory concentration; **MBC,** minimum bactericidal concentration.

REFERENCES

Alasalvar C, Shahidi F (2013). Dried fruits-phytochemicals and health effects. Oxford. Willey-Blackwell. pp. 1-15, 325-347, 444-453.

Andrews JM (2001). Determination of inhibitory concentrations. J. Antimicrob. Chemother. 48:5-16.

Brahmachari G (2012). Bioactive Natural Products, Opportunities and Challenges in Medicinal Chemistry, World Scientific Publishing Co. (Pte). Ltd (UK): pp. 400-599.

Dahiya J, Strange R, Bilyard K, Cooksey C, Garratt P (1984). Two isoprenylated isoflavone phytoalexins from *Cajanus cajan*. Phytochemistry *23*(4):871-873.

Dahiya JS (1987). Reversed-phase high performance liquid chromatography of *Cajanus cajan* phytoalexins. J. Chromatogr. 409. 355-359.

Dai FJ, Hsu WH, Huang JJ, Wu SC (2013). Effect of pigeon pea (*Cajanus cajan* L.) on high-fat diet-induced hypercholesterolemia in hamsters. Food Chem. Toxicol. 53:384-391.

Dakora F, Phillips D (1996). Diverse functions of isoflavonoids in legumes transcend anti-microbial definitions of phytoalexins. Physiol. Mol. Plant Pathol. 49(1):1-20.

Egunyomi A, Moody JO, Eletu OM (2009). Antisickling activities of two ethnomedicinal plant recipes used for the management of sickle cell anaemia in Ibadan, Nigeria. Afr. J. Biotechnol. 8(1):20-25.

Elaissi A, Chemli R, Rouis Z, Salem N, Salah K, Aouni M, Farhat F, Chemli R, Harzallah-Skhiri F, Khouja, M. (2012). Chemical composition of 8 eucalyptus species' essential oils and the evaluation of their antibacterial, antifungal and antiviral activities. BMC Complement. Altern. Med. 12:81.

Ezeifeka GO, Orji MU, Mbata TI, Patrick AO (2004). Antimicrobial activities of *Cajanus cajan, Garcinia kola and Xylopia aethopica* on pathogenic microorganism. Biotechnology 3(1):41-43.

González-Lamothe R, Mitchell G, Gattuso M, Diarra MS, Malouin F, Bouarab K (2009). Plant antimicrobial agents and their effects on plant and human pathogens. Int. J. Mol. Sci. 10(8):3400-3419.

Grayer RJ, Kokubun T (2001). Plant-fungal interactions: The search for phytoalexins and other antifungal compounds from higher plants. Phytochemistry 56:253-263.

Harborne JB (1999). The comparative biochemistry of phytoalexin induction in plants. Biochem. Syst. Ecol. 27(4):335-367.

Jaiswal D, Rai PK, Kumar A, Watal G (2008). Study of glycemic profile of *Cajanus cajan* leaves in experimental rats. Indian J. Clin. Biochem. 23(2):167-170.

Krishna TG, Bhatia CR (1985). Vicillin from *Cajanus cajan* seeds. Phytochemistry 24(10):2201-2203.

Kundu R, Dasgupta S, Biswas A, Bhattacharya A, Pal BC, Bandyopadhyay D, Bhattacharya S, Bhattacharya S (2008). *Cajanus cajan* Linn. (Leguminosae) prevents alcohol-induced rat liver damage and augments cytoprotective function. J. Ethnopharmacol. 118(3):440-447.

Luo M, Liu X, Zu Y, Fu Y, Zhang S, Yao L, Efferth T (2010). Cajanol, a novel anticancer agent from Pigeonpea [*Cajanus cajan* (L.) Millsp.] roots, induces apoptosis in human breast cancer cells through a ROS-mediated mitochondrial pathway. Chem. Biol. Interact. 188(1):151-160.

Mahadeva UU (2013). Diagnostic histopathology of infections of the luminal gastro-intestinal tract: “small blue dots”, worms and sexually transmitted infections of the distal gastro-intestinal tract. Diagn. Histopathol. 19(3):72-79.

Martinko JM, Madigan JT (2006). Brock-Biology of Microorganisms.11thed. Pearson Prentise Hall. USA. pp. 702-708.

Muller KO, Borger H. (1940). Experimentalle untersuchungen uber diephyophora-resistenz der kartoffel. Arb. Bio Reichsanst. Land-forst-wirtsch, Berlin-Dahlem 23:189-231.

Satapathy PP, Achary V, Panda BB (2012). Aluminum-induced abiotic stress counteracts *Fusarium* infection in *Cajanus cajan* (L.) Millsp. J. Plant Interact. 7(2):121-128.

van Etten HD, Masnfield JW, Bailey JA, Farmer EE (1994). Two classes of plant antibiotics: Phytoalexins versus phytoanticipins. The Plant Cell pp. 1191-1192.

van Wyk BE, van Oudtshoorn B, Gerickle N (2009). Medicinal Plants of South Africa. Tien Wash Press (Pte). Ltd, Singapore. pp. 284-287.

The United Nations Children’s Fund (UNICEF)/World Health Organization (WHO) (2009). Diarrhoea; Why Children Are Still Dying and What Can Be Done. http://whqlibdoc.who.int/publications/2009/9789241598415_eng.pdf

World Health Organisation (WHO) (2004). The World Health Report. The Problem of Antibiotic Resistance. Geneva. http://www.who.int/en/.

academicJournals

Vol. 10(10), pp. 324-329, 14 March, 2016
DOI: 10.5897/AJMR2015.7551
Article Number: A62647C57654
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

In vitro antitumoral activity of soluble protein extracts of *Bacillus thuringiensis*

Franco-Molina M. A.*, Mendoza-Gamboa E., Roman-Calderon M. E., Zapata-Benavides P., Rivera-Morales L. G., Zapata-Monsivais L., Coronado–Cerda E. E., Sierra-Rivera C. A., Tamez-Guerra R.[1] and Rodríguez-Padilla C.

Laboratorio de Inmunología y Virología. Departamento de Microbiología e Inmunología. Facultad de Ciencias Biológicas de la Universidad Autónoma de Nuevo León, San Nicolás de los Garza, N. L. México.

Received 22 April, 2015; Accepted 15 November, 2015

There are many studies about the antitumour effects of *Bacillus* toxins from different strains or subspecies in different parts of the world. Proteins that selectively kill tumor cells *in vitro* have potential as anticancer agents. The aim of this study was to evaluate the cytotoxic activity of soluble proteins extracts (SPE) from Mexican strains of *B. thuringiensis* on murine lymphoma L5178YR cell line. *In vitro*, L5178YR cells were treated with different concentrations of specific primers (SPE) from *B. thuringiensis* (0 to 39.85 μg/mL) and cellular viability was evaluated by MTT method and orange acridine/ethidium bromide staining. The mechanism of cell death was evaluated through caspase-3 activation by flow cytometry and TUNEL assays. The study results shows that SPE from *B. thuringiensis* (GM1-3 h, GM1-24 h, GM1-48 h, GM18-3 h, GM18-24 h, GM18-48 h, HD512-3 h, HD512-24 h, and HD512-48 h) affected the cell viability of L5178YR in a dose-dependent manner which presented higher cytotoxic effect of SPE collected at 3 h independent of the strain used; and the 7% SDS-PAGE presented an electrophoretic profile of proteins in a range of 10 to 100 kDa of the SPE *B. thuringiensis*. The cytotoxicity is through a mechanism of apoptosis because the caspase-3 activation and TUNEL assays corroborated this result. In conclusion, SPE derived from early culture (3 h) of *B. thuringiensis (Bt)* GM1, GM18 and HD-512 have *in vitro* cytotoxic potential on murine lymphoma L5178YR cell line through a mechanism of cell death by apoptosis.

Key words: *Bacillus thuringiensis,* soluble protein extracts, cancer.

INTRODUCTION

The entomopathogenic *Bacillus thuringiensis*, a gram-positive bacterium, is naturally found in the soil. It is characterized by crystal production during sporulation, containing Cry proteins, encoded by the *Cry* genes, with a wide division into classes and subclasses according to their insecticide activity, and presently classified according to the percent amino acid identity between Cry protein sequences (Mezzomo et al., 2015; Mohamed et

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

al., 2010; Palma et al., 2014a). Besides the cry proteins, known as δ-endotoxins, *B. thuringiensis* isolates can synthesize other toxins, such as β-exotoxin, phospholipases, proteases, chitinases and enterotoxins (Berlitz et al., 2012).

B. thuringiensis also produces proteins during its vegetative state. Some of these are insecticidal, and are called vegetative insecticidal proteins (VIP). VIP were named as such because, unlike the Cry and Cyt proteins, they are mainly produced during the vegetative growth phase of δ-endotoxins, *B. thuringiensis* cultures, although their secretion can also be extended into the sporulation stage. vip genes have shown no DNA sequences homology to cry and cyt genes, suggesting they bind to different receptors (Estruch et al., 1996; Lee et al., 2003). Four basic types of VIP toxins (Vip I, II, III, and IV) have been described, although they can be present in a variety of forms within each class (Yu et al., 1997).

The Cry and Cyt proteins toxins of *B. thuringiensis* have different activities including; antimicrobial (Cahan et al., 2008), insecticidal (Höfte and Whiteley, 1989), toxicity against nematode (Wei et al., 2003), antitumoral, (Chan et al., 2012; Jung et al., 2007) and adjuvant of the immune system (Román Calderón et al., 2007). *B. thuringiensis* spore crystals have shown toxicity for lymphocytes and promoting cytotoxic and genotoxic effects for the erythroid lineage of bone marrow at high concentrations which is not commonly found in the environment, indicated that these *B. thuringiensis* spore crystals (Mezzomo et al., 2015) were not harmless to mammals (Mezzomo et al., 2015; Okumura et al., 2014).

Since 1970s, Prasad and Shethna had carried out research on the antitumour effects of *B. thuringiensis* toxins from different strains or subspecies (Wong, 2010). Recently, the named cell-free supernatant of *B. thuringiensis* demonstrated as a suitable biostimulation agent for enhancing chlorpyrifos biodegradation in chlorpyrifos-contaminated soils (Aceves-Diez et al., 2015). But scarce studies about the anticancer activity of soluble protein extracts (SPE) from *B. thuringiensis* have been carried out; the aim of this study was to determine the cytotoxic effect of SPE from Mexican strains of *B. thuringiensis* on lymphoma murine L5178YR.

MATERIALS AND METHODS

B. thuringiensis strains

The *B. thuringiensis* strain GM1 (serovar 7 *aizawai,* isolated of garden soil from México), GM18 (serovar 24A24B *neolenensis,* isolated of agriculture soil from México*)* and HD-512 (serovar 15 *Dakota*) were from the culture collection of Howard Dulmage. All strains were kept under fry-dry in the Bank of Laboratory of Immunology and Virology from Faculty of Biological Science of University Autonomous of Nuevo León, México. They were grown at 30°C on nutrient broth (pH 7.6) consisting of meat extract (10 g), polypeptone (10 g), NaCl (2 g), and distilled water (1000 mL). The cultures were kept in a rotatory shaker at 14 x g by 3, 24, and 48 h at 30°C. Then the supernatants of strains were collected by centrifugation at 7519 x g by 10 min. The SPE were precipitated by acetone in a proportion of 1:1 of supernatant and incubated 30 min at 70°C. Thereafter, centrifuged at 30,074 x g/30 min, the supernatant was eliminated and the pellet was resuspended in sterile distilled water and washed three times with sterile distilled water and stored at 20°C until use. The treatments were named according to the strain and time recollection; GM1-3h, GM1-24h, GM1-48h, GM18-3h, GM18-24h, GM18-48h, HD512-3h, HD512-24h and HD512-48h.

MTT method

L5178YR cells obtained from ATCC (American Type Culture Collection) were cultured in RPMI medium with 10% FBS, and 5 × 10^3 cells/well were plated on 96 flat-bottom well plates, and the SPE of *B. thuringiensis were* diluted in the same medium and added at concentrations ranging from 0 to 39.85 μg/mL. The plates were then incubated for 24 h at 37°C, and 5% CO_2 atmosphere. Thereafter, the supernatants were removed, and cells were washed twice with RPMI-1640 medium. Cell viability was determined by the MTT method. Quantification was obtained by the absorbance reading at a wavelength of 570 nm and cellular viability was expressed as percentage.

Acridine orange/ethidium bromide

L5178YR cancer cells were briefly seeded at 1x10^6 cells/well into 6-well plates in RPMI-1640 with 10% FBS and treated with SPE of *B. thuringiensis* at doses mentioned previously. After one washing with phosphate-buffered saline (PBS), the cells were stained with 2 □L of a mixture (1:1) of acridine orange-ethidium bromide (100 μg/mL) in PBS. The cells were incubated for five minutes in the dark at room temperature and washed with phosphate-buffered saline; then were viewed under a Nikon inverted fluorescent microscope (TE-Eclipse 300) with an attached camera and photographs were taken under fluorescent conditions. Detection of apoptosis was based on morphological and fluorescent characteristics of the stained cells. Viable cells were indicated by bright green color, apoptotic cells by orange/brown color, and necrotic cells by red color**.**

Protein determination

Protein concentration was measured by the method of Bradford using bovine serum albumin as standard (Bradford, 1976).

SDS-PAGE

The protein profile of the soluble supernatant from each strain evaluated was performance according to the Laemmli method gel electrophoresis (Lane, 1978).

Flow cytometry analysis

L5178YR cells (1x10^6) were treated with SPE of *B. thuringiensis* at doses mentioned previously, and after 24 h of incubation, the active caspase-3 was detected using the PE-conjugated active caspase-3 apoptosis kit (BD Pharmingen™), by flow cytometry analysis (Accuri Flow Cytometer, Beckton Dickinson).

TUNEL assay

Nuclear DNA fragmentation of apoptotic cells was measured by the

Table 1. Relative cell viability of L5178YR treated with SPE of *B. thuringiensis* by MTT. L5178YR ($5x10^3$ cells) were treated with different concentrations of SPE from *B. thuringiensis*, incubated for 24 h at 37°C, and 5% CO_2 atmosphere. Cell viability was determined by the MTT method. Quantification was obtained by the absorbance reading at a wavelength of 570 nm and cellular viability was expressed as percentage. Results were given as the mean ± SD of three independent experiments (*p<0.05).

Treatments	6.65 μg/mL RCV (%)	26.55 μg/mL RCV (%)	39.85 μg/mL RCV (%)
GM1-3 h	36.76*	2.75*	8.69*
GM18-3 h	78.15	13.04*	8.80*
HD512-3 h	60.65*	14.10*	16.12*
GM1-24 h	61.19*	64.80*	34.89*
GM18-24 h	71.58	55.67*	57.05*
HD512-24 h	94.80	105.30	38.81*
GM18-48 h	89.18	57.68*	55.04*
HD512-48	67.89*	63.73*	44.64*
Control	100	100	100

TUNEL assay (DeadEnd Fluorometric TUNEL System; Promega, Madison, WI). Briefly, culture medium was discarded, cells were fixed in 4% formaldehyde, permeabilized in 0.2% Triton X-100 and incubated with TdT incubation buffer for 60 min in a 37°C humidified incubator for 3'-OH labeling. Immediately**,** analyze samples under a fluorescence microscope using a standard fluorescein filter was set to view the green fluorescence of fluorescein at 520 ± 20 nm; view red fluorescence of propidium iodide at >620nm.

Statistical analysis

Data represent the mean ±SD of triplicates from three independent experiments. Statistical differences were obtained using the analysis of variance, and the Dunnett's tests (SPSS v. 17.0 program). The results were considered statistically significant if the *p value was <0.05.

RESULTS

Cell viability assays

The SPE of *B. thuringiensis* treatments (GM1-3h, GM1-24h, GM1-48h, GM18-3h, GM18-24h, GM18-48h, HD512-3h, HD512-24h, and HD512-48h) affected the cell viability of L5178YR in a dose-dependent manner, except *Bt* HD512-24h at doses of 6.65 and 26.55 μg/mL (Table 1). We selected the SPE of *B. thuringiensis* treatments from GM1 3h, GM-18 3h, and HD-512 3h, because were the best treatments that affected the cell viability to carry out the experiments with acridine orange/ethidium bromide staining, shown that these treatments induced cell death by apoptosis characteristic by orange color in cells (Figure 1).

SDS-PAGE

The SDS-PAGE of SPE of *B. thuringiensis* from GM1 3h, GM-18 3h and HD-512 3h shows an electrophoretic profile of proteins in a range of 10 to 100 kDa. Being of special interest, the presence of protein major the amount of molecular weight of 16.69 kDa (Figure 2).

Apoptosis assays

In order to confirm the type of cellular death induced by SPE of *B. thuringiensis*, shown by TUNEL assay the presence of apoptosis (Figure 3). This data was corroborated by caspase-3 activation in treatments with GM1-3h at 26.55 μg/mL (20.4%) GM1-3h at 39.85 μg/mL (28.9%), GM18-3h at 26.55 μg/mL (23.4%), GM18-3h at 39.85 μg/mL (19 %), HD512-3h at 26.55 μg/mL (27.3%) and HD512-3h at 39.85 μg/mL (36.9%) characteristic of apoptosis (Figure 4).

DISCUSSION

Parasporins are parasporal proteins produced by *B. thuringiensis* that are capable of killing cancer cells (Okassov et al., 2015; Okumura et al., 2014), similar to this study results obtained with SPE from *B. thuringiensis* that shows cytotoxic activity on L5178YR murine lymphoma.

In the literature reviewed, no data was referred to the *in vitro* action of SPE of *B. thuringiensis* strains GM1, GM18 and HD-512. Studying vegetative proteins from Malaysian, strains of *B. thuringiensis israelensis* (*Bt* 11, *Bt* 12, *Bt* 15, *Bt* 16, *Bt* 17, *Bt* 21 and *Bt* 22) and *Bacillus sphaericus* H-25 strains (Bs 1 and Bs 2) were found, and screened for cytotoxic activity being indiscriminately cytotoxic to both CEM-SS (human T lymphoblastoid) and HeLa (human uterus cervical cancer) cell lines (Ramasamy et al., 2008).

The SPE collected at 3 h shows several proteins

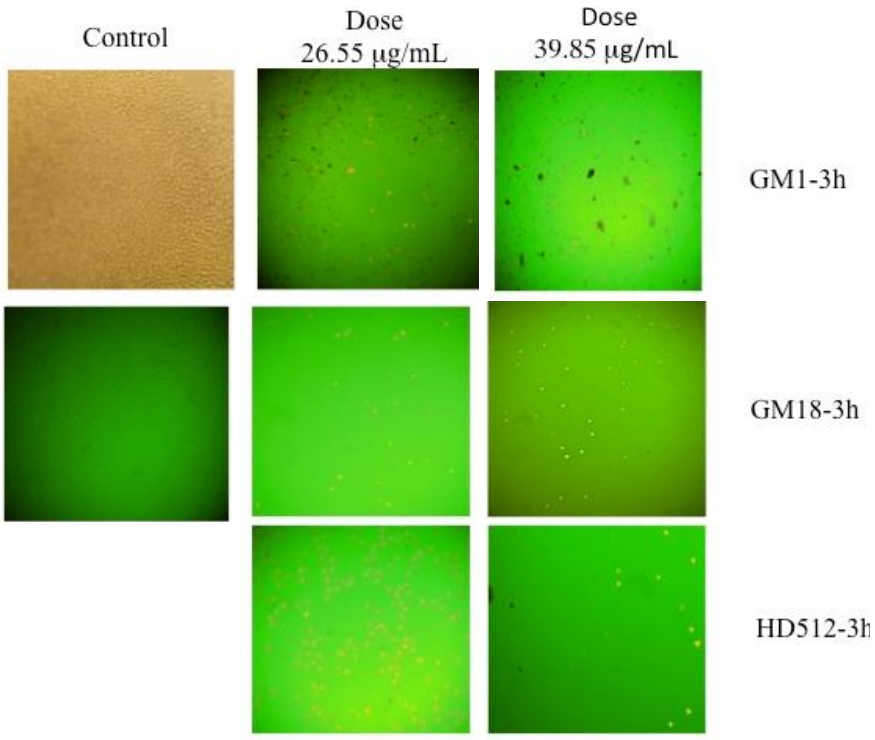

Figure 1. Relative cell viability of L5178YR treated with SPE of *B. thuringiensis* by acridine orange/ethidium bromide staining. L5178YR ($1x10^6$ cells) were treated with different concentrations of SPE from *B. thuringiensis*, incubated for 24 h at 37°C, and 5% CO_2 atmosphere. Cell viability was determined by acridine orange/ethidium bromide method. The cells were viewed under fluorescent microscope. Viable cells were indicated by bright green color, apoptotic cells by orange/brown color, and necrotic cells by red color.

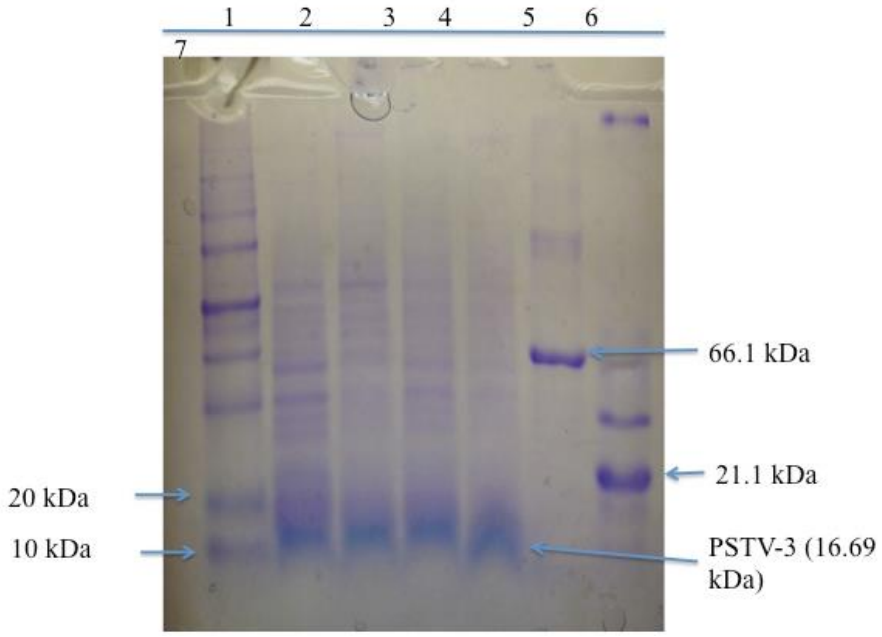

Figure 2. 7% SDS-PAGE of SPE of *B. thuringiensis*. Lane 1: Molecular weight marker Promega V849A, Lane 2 and 3: GM1, Lane 4: GM-18, Lane 5: HD-512, Lane 6: BSA; Lane7: Trypsin inhibitor.

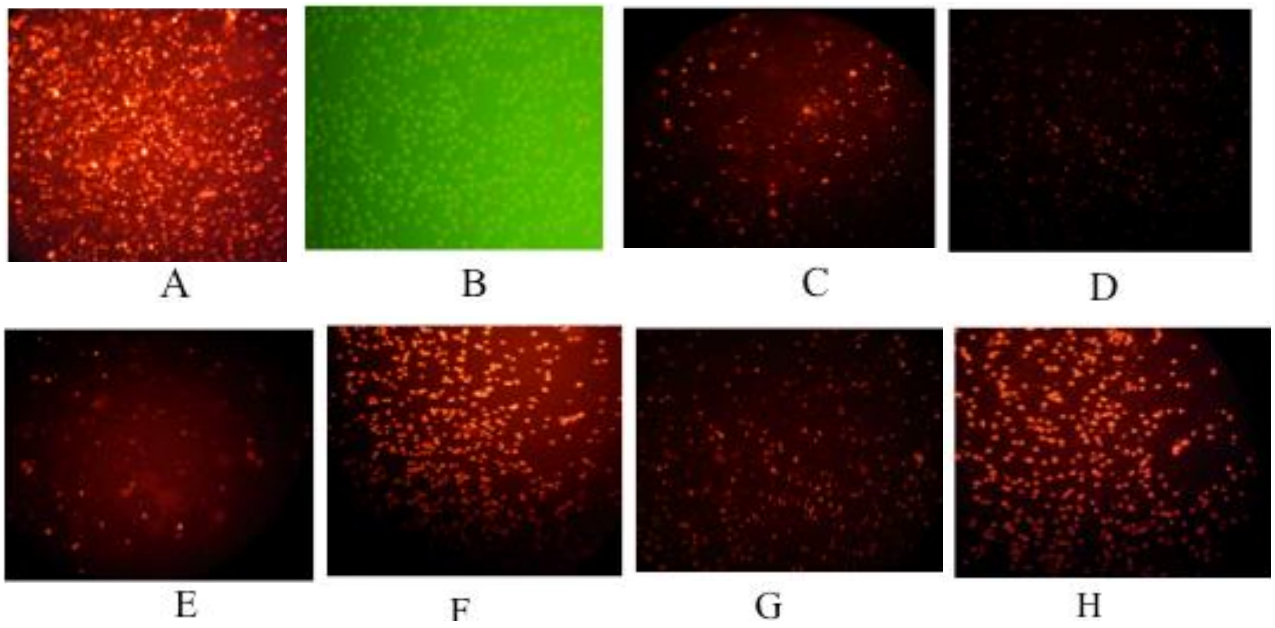

Figure 3. L5178YR stained by the TUNEL labeling. A) L5178YR TUNEL positive control cells showing abundant red color characteristic of celular death, B) untreated control L5178YR cells showing abundant green color characteristic of viability, C) GM1-3h 26.55 µg/mL, D) GM1-3h 39.85 µg/mL, E) GM18-3h 26.55 µg/mL, F) GM18-3h 39.85 µg/mL, G) HD512-3h 26.55 µg/mL, H) HD512-3h 39.85 µg/mL.

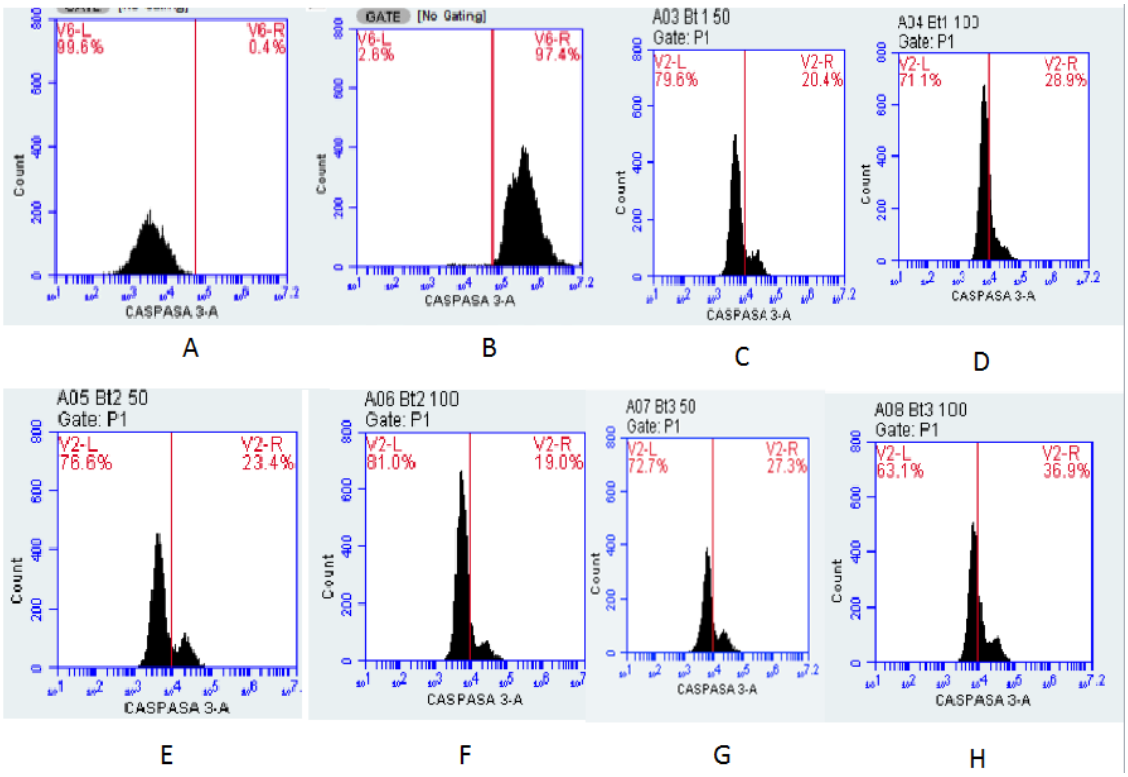

Figure 4. Representative flow cytometry histograms of caspase-3 activation in apoptotic L5178YR cells. L5178YR cells treated with different doses of SPE from *B. thuringiensis* to determine the activation of capsase-3 by flow cytometry analysis, Accuri Flow Cytometer (Beckton Dickinson). The histograms shown the distribution of: A) caspase-3 negative control cells, B) doxorrubicin positive control, C) GM1-3h 26.55 µg/mL, D) GM1-3h 39.85 µg/mL, E) GM18-3h 26.55 µg/mL, F) GM18-3h 39.85 µg/mL, G) HD512-3h 26.55 µg/mL and H) HD512-3h 39.85 µg/mL.

detected by SDS-PAGE in a range of 10 to 100 kDa with cytotoxic activities open the gates to study the sequencing and synthesis of amino acids from each protein being of special interest the proteins with a molecular weight of 16.69 kDa by its abundant expression. Similar to this as reported by Palma et al. (2014b), that describes the insecticidal activity of a novel *B. thuringiensis* Cry-related protein with a deduced 799 amino acid sequence (~89 kDa) and ~19% pairwise identity 95-kDa-aphidicidal protein (sequence number 204) and ~40% pairwise identity to the cancer cell killing Cry proteins (parasporins Cry41Ab1 and Cry41Aa1), respectively. This determined that SPE of *B. thuringiensis* induced cellular death through apoptosis. It has been reported that PS1Aa1 induces apoptosis through caspase-3 in HeLa cells (Okassov et al., 2015).

Conclusion

The SPE derived from early culture (3 h) of *B. thuringiensis* strains GM1, GM18, and HD-512 have *in vitro* cytotoxic potential on L5178YR lymphoma murine cell line through mechanism of apoptosis. This could offer another modality of cancer therapy, as a novel tumoricidal agent derived from prokaryotic cells but definitive assessment must wait until these observations are clarified through experiments in several tumor cancer cell lines *in vitro* and *in vivo*.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

This study was supported by the Laboratory of Immunology and Virology, Faculty of Biological Sciences, University Autonomous of Nuevo León (UANL), México, in collaboration with "Red Temática de Inmunología en Cancer y Enfermedades Infecciosas with register number 253053. CONACYT.

REFERENCES

Aceves-Diez A, Estrada-Castañeda K, Castañeda-Sandoval LM (2015). Use of *Bacillus thuringiensis* supernatant from a fermentation process to improve bioremediation of chlorpyrifos in contaminated soils. J. Environ. Manage. 157:213-219.

Berlitz DL, Giovenardi M, Charles JF, Fiúza LM (2012). Toxicity intraperitoneal and intragastric route of *Bacillus thuringiensis* and *Melia azedarach* in mice. Arq. Inst. Biol. (Sao Paulo) 79(4):511-517.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Cahan R, Friman H, Nitzan Y (2008). Antibacterial activity of Cyt1Aa from *Bacillus thuringiensis* subsp. *israelensis*. Microbiology 154(Pt 11):3529-3536.

Chan KK, Wong RS, Mohamed SM, Ibrahim TAT, Abdullah M, Nadarajah VD (2012). *Bacillus thuringiensis* parasporal proteins induce cell-cycle arrest and caspase-dependant apoptotic cell death in leukemic cells. J. Environ. Pathol. Toxicol .Oncol. 31(1):75-86.

Estruch JJ, Warren GW, Mullins MA, Nye GJ, Craig JA, Koziel MG (1996). Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. Proc. Natl. Acad. Sci. U.S.A. 93(11):5389-5394.

Höfte H, Whiteley HR (1989). Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. 53(2):242-255.

Jung YC, Mizuki E, Akao T, Côté JC (2007). Isolation and characterization of a novel *Bacillus thuringiensis* strain expressing a novel crystal protein with cytocidal activity against human cancer cells. J. Appl. Microbiol. 103(1):65-79.

Lane LC (1978). A simple method for stabilizing protein-sulfhydryl groups during SDS-gel electrophoresis. Anal. Biochem. 86(2):655-664.

Lee MK, Walters FS, Hart H, Palekar N, Chen, JS (2003). The Mode of Action of the *Bacillus thuringiensis* Vegetative Insecticidal Protein Vip3A Differs from That of Cry1Ab Delta -Endotoxin. Appl. Environ. Microbiol. 69(8):4648-4657.

Mezzomo BP, Miranda-Vilela, AL, Barbosa LC, Albernaz, VL, Grisolia CK (2015). Hematotoxicity and genotoxicity evaluations in Swiss mice intraperitoneally exposed to *Bacillus thuringiensis* (var *kurstaki*) spore crystals genetically modified to express individually Cry1Aa, Cry1Ab, Cry1Ac, or Cry2Aa. Environ. Toxicol.

Mohamed A, Grlko N, Junker M, Bulla L (2010). *Bacillus thuringiensis*: A genomics and proteomics perspective. Bioeng. Bugs 1(1):31-50.

Okassov A, Nersesyan A, Kitada S, Ilin A (2015). Parasporins as new natural anticancer agents: a review. J. BUON 20(1):5-16.

Okumura S, Koga H, Inouye K, Mizuki E (2014). Toxicity of parasporin 4 and health effects of pro-parasporin-4 diet in mice. Toxins 6(7):21152126.

Palma L, Muñoz D, Berry C, Murillo J, Caballero P (2014a). *Bacillus thuringiensis* Toxins: An Overview of Their Biocidal Activity. Toxins 6(12):3296-3325.

Palma L, Muñoz D, Berry C, Murillo J, de Escudero IR, Caballero P (2014b). Molecular and insecticidal characterization of a novel Cry-related protein from *Bacillus thuringiensis* toxic against *Myzus persicae*. Toxins 6(11):3144-3156.

Ramasamy B, Nadarajah VD, Soong ZK, Lee HL, Mohammad SM (2008). A preliminary study of the bioactivity of vegetative proteins extracted from Malaysian *Bacillus thuringiensis* isolates. Trop. Biomed. 25(1):64-74.

Román Calderón ME, Alcocer González JM, Franco Molina MA, Tamez Guerra RS, Rodríguez Padilla C (2007). Adjuvant effects of cristal proteins from a Mexican strain of *Bacillus thuringiensis* on the mouse humoral response. Biologicals 35(4):271-276.

Wei JZ, Hale K, Carta L, Platzer E, Wong C, Fang SC, Aroian RV (2003). *Bacillus thuringiensis* crystal proteins that target nematodes. Proc. Natl. Acad. Sci. U.S.A. 100(5):2760-2765.

Wong SYR (2010). *Bacillus Thuringiensis* Parasporal Proteins And Their Effect On Human Cancer Cells : An Overview. IeJSME 4(2):3-9.

Yu CG, Mullins MA, Warren GW, Koziel MG, Estruch, JJ (1997). The *Bacillus thuringiensis* vegetative insecticidal protein Vip3A lyses midgut epithelium cells of susceptible insects. Appl. Environ. Microbiol. 63(2):532-536.

academicJournals

Vol. 10(10), pp. 330-338, 14 March, 2016
DOI: 10.5897/AJMR2015.7879
Article Number: 128717757661
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Diversity of fungi in sediments and water sampled from the hot springs of Lake Magadi and Little Magadi in Kenya

Anne Kelly Kambura[1]*, Romano Kachiuru Mwirichia[2], Remmy Wekesa Kasili[1], Edward Nderitu Karanja[3], Huxley Mae Makonde[4] and Hamadi Iddi Boga[5]

[1]Institute for Biotechnology Research, Jomo Kenyatta University of Agriculture and Technology, P. O. Box 62000 - 00200, Nairobi, Kenya.
[2]Embu University College, P. O. Box 6 - 60100, Embu, Kenya.
[3]International Centre of Insect Physiology and Ecology (ICIPE), P. O. Box 30772 - 00100, Nairobi, Kenya.
[4]Pure and Applied Sciences, Technical University of Mombasa, P. O. Box 90420 - 80100, GPO, Mombasa, Kenya.
[5]Taita Taveta University College, School of Agriculture, Earth and Environmental Sciences, P. O. Box 635-80300 Voi, Kenya.

Received 9 December, 2015; Accepted 26 February, 2016

Lake Magadi and Little Magadi are saline, alkaline lakes lying in the southern part of Kenyan Rift Valley. Their solutes are supplied by a series of alkaline hot springs with temperatures as high as 86°C. Previous culture-dependent and independent studies have revealed diverse prokaryotic groups adapted to these conditions. However, very few studies have examined the diversity of fungi in these soda lakes. In this study, amplicons of Internal Transcribed Spacer (ITS) region on Total Community DNA using Illumina sequencing were used to explore the fungal community composition within the hot springs. Operational taxonomic units (OTUs) were analyzed using QIIME 1.8.0, taxonomy assigned via BLASTn against SILVA 119 Database and hierarchical clustering was done using R programming software. A total of 334, 394 sequence reads were obtained from which, 151 OTUs were realized at 3% genetic distance. Taxonomic analysis revealed that 80.33% of the OTUs belonged to the Phylum *Ascomycota,* 11.48% *Basidiomycota* while the remaining consisted of *Chytridiomycota, Glomeromycota* and early diverging fungal lineages. The most abundant *Ascomycota* groups consisted of *Aspergillus* (18.75%), *Stagonospora* and *Ramularia* (6.25% each) in wet sediment at 83.6°C, while *Penicillium* and *Trichocomaceae (*14.29% each) were dominant in wet sediment at 45.1°C. The results revealed representatives of thermophilic and alkaliphilic fungi within the hot springs of Lake Magadi and Little Magadi. This suggests their ability to adapt to high alkalinity, temperature and salinity.

Key words: Fungi, hot springs, temperature, DNA, diversity.

INTRODUCTION

Fungi have colonized diverse habitats such as tropical regions (Hawksworth, 1991), extreme environments such as deserts, areas with high salt concentrations (Vaupotic et al., 2008), ionizing radiation (Dadachova et al., 2007), deep sea sediments (Raghukumar and Raghukumar, 1998) and ocean hydrothermal areas (Le Calvez et al.,

2009). Most fungi grow in terrestrial environments, though several species live partly or solely in aquatic habitats, such as the chytrid fungus *Batrachochytrium dendrobatidis*, a parasite that has been responsible for a worldwide decline in amphibian populations (Brem and Lips, 2008). In most ecosystems, fungi are the major decomposers, playing an essential role in nutrient cycling as saprotrophs and symbionts that degrade organic matter into inorganic molecules (Barea et al., 2005; Lindahl et al., 2007; Gadd, 2007). While there are well-known examples of bacteria that can grow in a variety of natural environments including hot springs and geysers where temperatures can reach 100°C, eukaryotes are much more sensitive because, above 65°C, their membranes become irreparably damaged (Magan and Aldred, 2007). However, mesophilic thermo-tolerant fungi exist. For example, some *Deuteromycetes* isolated from thermal springs have maximum growth temperature of 61.5°C (Magan, 2006).

The presence of fungi in extreme alkaline saline environments has been recognized by culture-dependent methods, with the majority showing similarity to terrestrial species (Mueller and Schmit, 2006; Salano, 2011; Ndwigah et al., 2015). Culture-independent methods have revealed highly novel fungal phylotypes such as *Chytridiomycota* and unknown ancient fungal groups (Yuriko and Takahiko, 2012).

pH tolerance in fungi has been attributed to efficient control of proton movement into and out of the cells, and is able to meet necessary energy requirements (Magan, 2006). The exact diversity and function of fungi in extreme environments is still poorly understood. The aim of this study was to explore the fungal diversity within the hot springs of Lake Magadi and Little Magadi in Kenya using metagenomic analysis.

MATERIALS AND METHODS

Study site

Lake Magadi is a hyper saline lake that lies in a naturally formed closed lake basin within the Southern part of the Kenyan Rift Valley. It is approximately 2 °S and 36 °E of the Equator at an elevation of about 600 m above sea level (Behr and Röhricht, 2000). The solutes are supplied mainly by a series of alkaline springs with temperatures as high as 86°C, located around the perimeter of the lake. Samples analyzed in this study were collected from 3 hot springs: one hot spring within the main Lake Magadi (02° 00′ 3.7″S 36° 14′ 32″ E at an elevation of 603 m, a temperature of 45.1°C and pH 9.8), and two hot springs within Little Magadi "*Nasikie eng'ida"*: Hot spring 1 - 01° 43′ 28″ S 36° 16′ 21″ E, at an elevation of 611 m, a temperature of 83.6°C and pH 9.4); and Hot spring 2 - 01° 43′ 56″ S 36° 17′ 11″ E, at an elevation of 616 m, temperature of 81°C and pH of 9.2 (Table 1).

Measurements of physicochemical parameters

Geographical position of each site in terms of latitude, longitude and elevation was taken using Global Positioning System (GARMIN eTrex 20). The pH for each sampling point was measured with a portable pH-meter (Oakton pH 110, Eutech Instruments Pty. Ltd) and confirmed with indicator strips (Merck, range 5-10), Temperature, Electrical Conductivity (EC), Total Dissolved Solids (TDS) and dissolved oxygen (DO) were measured on site using Electrical Chemical Analyzer (Jenway - 3405) during sampling. *In situ* temperature was recorded once for each study site and assigned to all the sample types for that site.

Sample collection

All samples were collected randomly in triplicates from each hot spring. Water samples were collected using sterile 500 ml plastic containers that had been cleaned with 20% sodium hypochlorite and UV-sterilized for one hour. Wet sediments were collected by scooping with sterilized hand shovel into sterile 50 ml falcon tubes. All samples were transported in dry ice to the laboratory at Jomo Kenyatta University of Agriculture and Technology. Water for DNA extraction (500 ml) was trapped on 0.22 µM filter papers (Whatman) and stored at -80°C. Pellets for DNA extraction were obtained from water samples by re-suspending the filter papers in phosphate buffer solution (pH 7.5), and centrifuging 5 ml of the suspension at 13000 rpm for 10 min.

DNA extraction

Total community DNA was extracted in triplicates; pellets from water samples and 0.2 g of sediment samples as described by (Sambrook et al., 1989). The DNA extracted from triplicate samples was pooled during the precipitation stage, washed, air dried and stored at -20°C.

Amplicon library preparation and sequencing

PCR amplification of ITS region was done using ITS1 (TCCGTAGGTGAACCTGCGG) and TS4 (TCCTCCGCTTATTGATATGC) primers with barcode according to (White et al., 1990). Amplification proceeded in a 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) with initial heating at 94°C for 3 min, followed by 28 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 40 s and extension at 72°C for 1 min, after which a final elongation step at 72°C for 5 min was performed. Polymerase chain reaction (PCR) products were visualized on 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple samples were pooled together in equal proportions based on their DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads (Agencourt Bioscience Corporation, MA, USA). The pooled and purified PCR product was used to prepare DNA library by following Illumina sequencing protocol (Yu and Zhang, 2012). Sequencing was performed at Molecular Research DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq platform following the manufacturer's guidelines.

*Corresponding author. E-mail: annnderitu@gmail.com. Tel: +254721235147.

Table 1. Physico-chemical parameters of sampling stations in Lake Magadi and Little Magadi measured before sampling.

Parameter	Latitude °S	Longitude °E	Elevation (m)	Temperature (°C)	pH	EC (mS/cm)	TDS (mg/L)	Dissolved oxygen (mg/L)
Hot spring 1	02° 00′ 3.7″	36° 14′ 32″	603	45.1	9.8	0.03	1	12.4
Hot spring 2	01° 43′ 28″	36° 16′ 21″	611	83.6	9.4	1	1	0.04
Hot spring 3	01° 43′ 56″	36° 17′ 11″	616	81	9.2	1	1	0.71

Sequence analysis, taxonomic classification and data Submission

Sequences obtained from the Illumina sequencing platform were depleted of barcodes and primers using a proprietary pipeline (www.mrdnalab.com, MR DNA, Shallowater, TX) developed at the service provider's laboratory. Low quality sequences were identified by denoising and filtered out of the dataset (Reeder and Knight, 2010). Sequences which were < 200 base pairs after phred20-based quality trimming, sequences with ambiguous base calls, and those with homopolymer runs exceeding 6bp were removed. Sequences were analyzed by a script optimized for high-throughput data to identify potential chimeras in the sequence files, and all definite chimeras were depleted as described previously (Gontcharova et al., 2010). De novo OTU clustering was done with standard UCLUST method using the default settings as implemented in QIIME pipeline Version 1.8.0 at 97% similarity level (Caporaso et al., 2010a). Taxonomy was assigned to each OTU using BLASTn against SILVA SSU Reference 119 database at default e-value threshold of 0.001 in QIIME (Quast et al., 2013).

Statistical analysis

Diversity indices (Shannon, Simpson and Evenness) for each sample were calculated using vegan package version 1.16-32 in R software version 3.1.3 (R Development Core Team, 2012). Community and Environmental distances were compared using Analysis of similarity (ANOSIM) test, based upon Bray-Curtis distance measurements with 999 permutations. Significance was determined at 95% confidence interval (p=0.05). Calculation of Bray-Curtis dissimilarities between datasets and hierarchical clustering were carried out using the R programming language (R Development Core Team, 2012) and the Vegan package (Oksanen et al., 2012). To support OTU-based analysis, taxonomic groups were derived from the number of reads assigned to each taxon at all ranks from domain to genus using the taxa_summary.txt output from QIIME pipeline Version 1.8.0. Obtained sequences were submitted to NCBI Sequence Read Archive with SRP# Study accessions: SRP061806.

RESULTS

Wet sediment and water samples were randomly collected at three different locations in hot springs of Lake Magadi and Little Magadi. The hot springs temperatures ranged from 45.1 to 83.6°C while pH ranged from 9.2 to 9.8. The TDS was beyond measurement using the Electrical Chemical Analyzer; hence the readings appeared as one on the sampling equipment. The metadata collected before sampling is summarized in Table 1. Temperature measurement showed a gradient from hot spring in the main Lake Magadi, with the springs at Little Magadi measuring between 81 and 83.6°C. Cation analysis of the water samples showed that the levels of calcium range between 0.33-0.62 ppm, iron (<0.01 - 0.014 ppm) and magnesium (<0.02 - 0.026 ppm). Sodium levels were very high (11,300, 17,300 and 17,700 ppm) and potassium levels were 225, 458 and 287 ppm. Anion analysis showed that phosphorus range between 2.72 to 6.31 ppm. Chloride levels were high in all samples ranging from 4000 to 4640 ppm (Table 2).

Sequence data

The raw data from the sediments and water samples (three sediment and one water sample) consisted of 548,639 sequences, of which 334, 394 sequences were retained after removing sequences with different tags at each end for quality filtering and denoising. After removing singletons, chimeric sequences and OTUs of non-fungal organisms (<200 base pairs after phred20-based quality trimming, sequences with ambiguous base calls, and those with homopolymer runs exceeding 6 bp), a total of 151 fungal OTUs recovered at 3% genetic distance, were included in the final analysis.

Composition and diversity of fungal communities

Based on BLASTn searches in SILVA SSU Reference 119 database, 151 OTUs were identified, most of which had their best matches against accessions in SILVA database. These 151 OTUs spanned 5 phyla namely; *Ascomycota*, *Basidiomycota, Fungi unspecified phylum*, *Chytridiomycota* and *Glomeromycota*. Sediment samples collected from 81°C had the highest number of OTUs (135 OTUs) while 88 OTUs were shared among all sample types (Figure 1).

The shared OTUs were distributed among the phyla; *Ascomycota* (up to 42.9% relative abundance in sediment sample at 45.1°C), *Fungi unspecified phylum* (up to 6.2% relative abundance in sediment sample at 83.6°C), *Basidiomycota* (up to 3.3% relative abundance in sediment sample at 81°C), *Chytridiomycota* and *Glomeromycota* (up to 1.5% relative abundance each in water sample at 81°C. OTUs belonging to the Phylum *Ascomycota* were the most abundant and were represented

Table 2. Chemical analyses of samples from the Hot Springs Lake Magadi and Little Magadi.

Parameter	Sampling stations		
Chemical properties	**Hot springs 45.1°C**	**Hot springs 81°C**	**Hot springs 83.6°C**
pH	9.61	9.2	9.41
EC (mS cm^{-1})	30.3	30.5	29.9
Ammonium (ppm)	0.94	2.66	2.57
Calcium (ppm)	0.62	0.53	0.33
Magnesium (ppm)	<0.02	0.026	<0.02
Potassium (ppm)	287	458	225
Phosphorus (ppm)	6.31	4.17	2.72
Nitrate N (ppm)	0.53	0.67	0.67
Nitrates (ppm)	2.35	2.98	2.97
Sulphur (ppm)	129	107	58.9
Sulphates (ppm)	387	322	176
Iron (ppm)	<0.01	0.012	0.014
Manganese (ppm)	0.016	0.012	<0.01
Zinc (ppm)	<0.01	<0.01	<0.01
Boron (ppm)	9.3	15.5	8.06
Copper (ppm)	0.043	<0.01	<0.01
Molybdenum (ppm)	0.14	0.12	0.071
Sodium (ppm)	17700	17300	11300
Chlorides (ppm)	4000	4640	4220
Bicarbonates (ppm)	14200	17500	17100

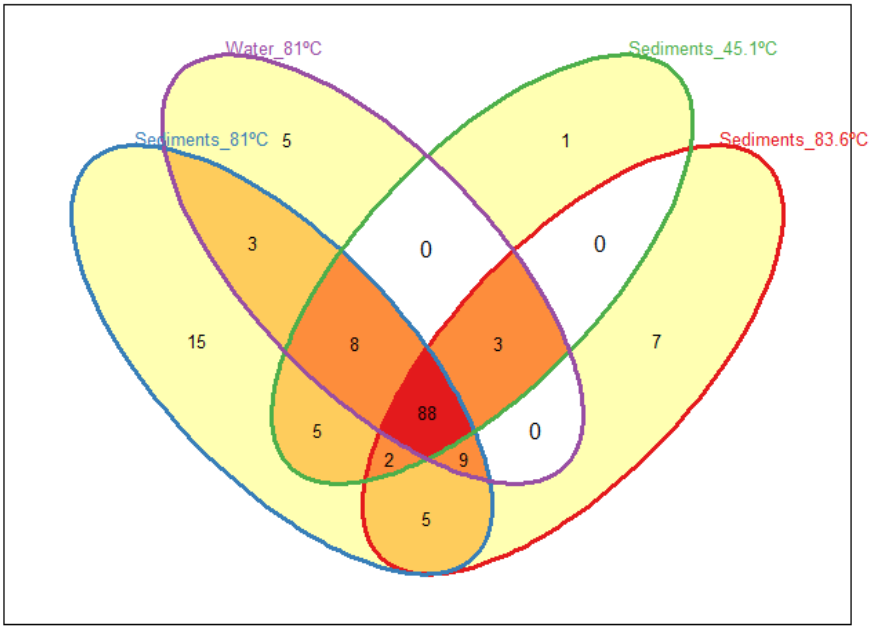

Figure 1. Venn diagram showing the distribution of unique and shared OTUs within various sample types in the three sampling sites. The number of OTUs in each hot spring is indicated in the respective circle.

by the most genera as shown in Figure 2. In sediments at 45.1°C the OTUs were affiliated to the genus *Aspergillus, Penicillium* and *Trichocomaceae*. *Aspergillus oryzae* was the most abundant species with a relative abundance of

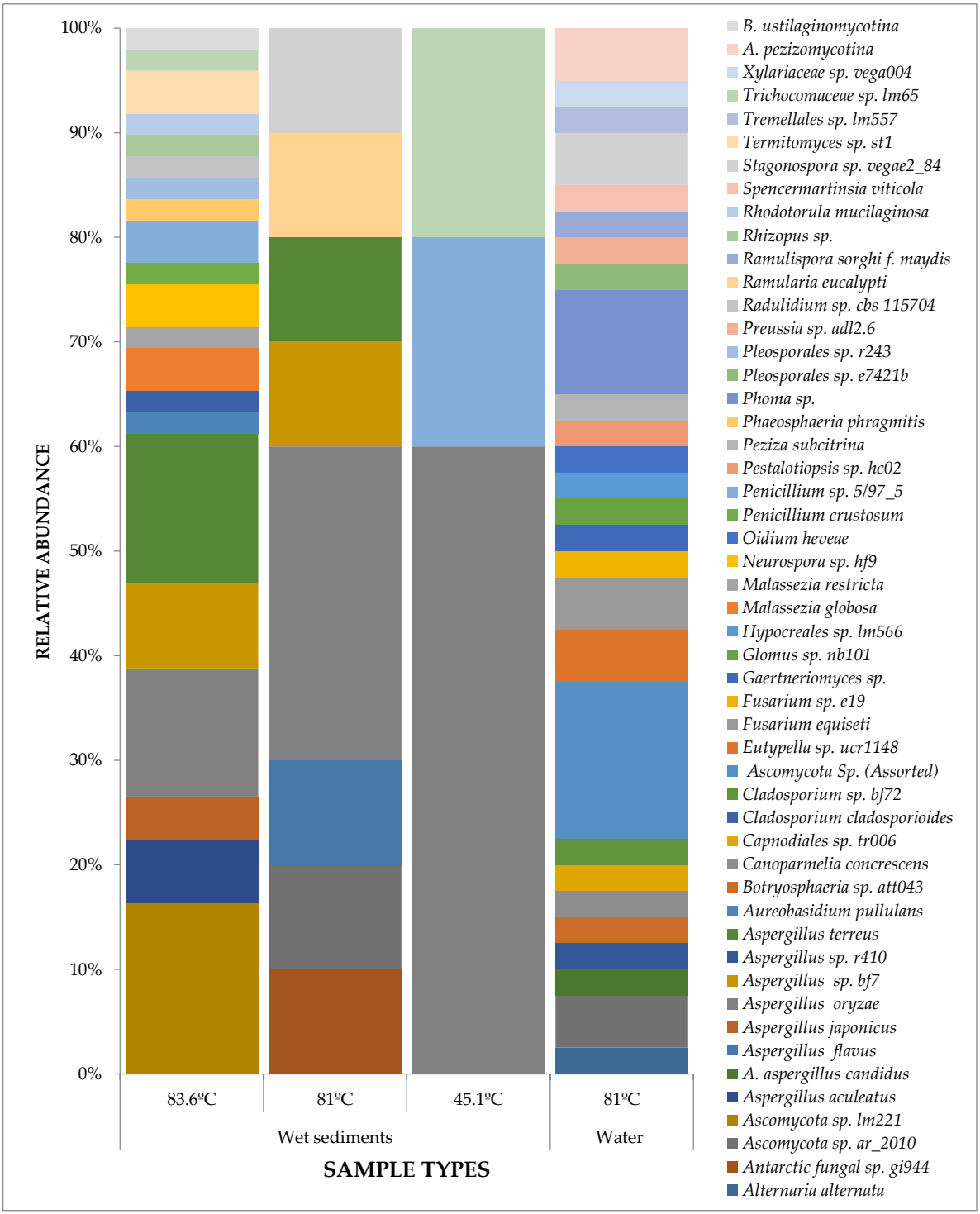

Figure 2. Relative abundance of the most predominant fungal species in various samples collected from the hot springs.

42.86%. Other species present were *Penicillium* sp. 5/975 and *Trichocomaceae* sp. lm65 with 14.29% relative abundance (Figure 2). However, in the sediment sample at 83.6°C genera represented were *Aspergillus*, *Aureobasidium*, *Cladosporium*, *Malassezia*, *Neurospora*, *Penicillium*, *Phaeosphaeria*, *Pleosporales*, *Radulidium* and *Trichocomaceae*. *Basidiomycota* phylum comprised *Rhodotorula* and *Termitomyces* species (Figure 2). *Malassezia* sp., *Neurospora* sp., *Ascomycota* sp. lm221, *Aspergillus aculeatus, Aureobasidium pullulans* and

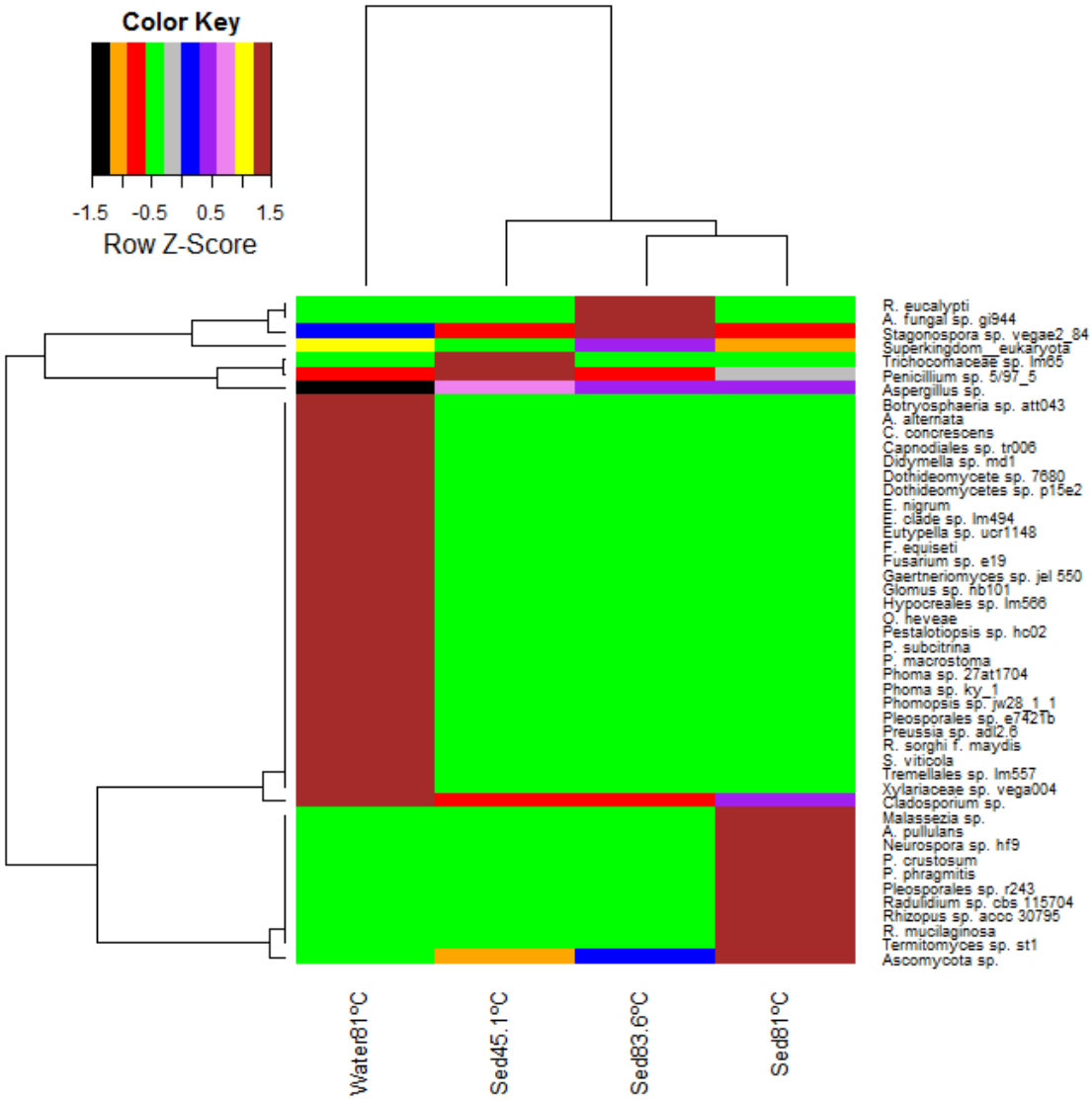

Figure 3. Hierarchical clustering of DNA samples collected from the three hot springs of Lake Magadi and Little Magadi. Species level was chosen to be used in hierarchical clustering to assess the relationships between samples and taxa.

Cladosporium cladosporioides are unique to sediment samples at 83.6°C. In the sediment sample collected at 81°C, *Aspergillus* sp. (*terreus, oryzae, flavus* and *Aspergillus* sp. bf7) within the phylum *Ascomycota* were the most abundant with *Aspergillus oryzae* scoring 18.75%. Other taxa represented in the sample included *Ascomycota* sp. ar_2010, *Ramularia eucalypti,* and *Stagonospora* sp. vegae284 each scoring a relative abundance of 6.25%. *Antarctic fungal* sp. gi944 of *Fungi unspecified phylum* also scored a relative abundance of 6.25% (Figure 2). The water sample collected at 81°C was found to harbor a higher diversity of fungi with low species richness as shown in Figure 2.

Hierarchical clustering between samples collected from Lake Magadi revealed sediment samples from the two hot springs in Little Magadi "*Nasikie eng'ida"* to be closer than the sample from the hot spring in the main lake. Majority of the groups at species level included *Aspergillus*, *Ascomycota*, *Penicillium*, *Neurospora*, *Termitomyces*, *Malassezia*, *Trichocomaceae*, *Stagonospora*, *Ramularia* and *Hypocreales* (Figure 3). The dendogram shows relationship between the four samples.

Fungal richness and diversity indices

Richness (S) estimated the water sample (81°C) to be the richest site, constituting 35 taxa. Sediment samples from the three sites had Evenness (J') scores close to 1

Table 3. Diversity indices computed on all OTU-based fungal taxonomic units obtained from samples collected from the hot springs of Lake Magadi and Little Magadi.

Sample	No. of sequences after filtering	No. of OTUs	Richness (S)	Shannon (H')	Simpson (1/D)	Evenness (J')
Wet sediment (81°C)	112, 262	61	9	1.90	4.92	0.739
Wet sediment (45.1°C)	59,138	7	21	2.63	9.98	0.663
Wet sediment (83.6°C)	80,702	16	4	1.28	3.27	0.897
Water (81°C)	82,292	67	35	2.66	5.06	0.410
Total	**334, 394**	**151**	**69**			

(0.663 – 0.897), hence showing evenness in their number of taxa members than the water sample (81°C). Simpson (1/D) also indicated the sediment 45.1°C to harbor the most diverse taxa (9.98). The Shannon's index (H' = 1.28–2.63) indicated low variation in the level of diversity among the sediment and water samples (Table 3).

Analysis of similarity and distance based redundancy analysis at genus level showed connectivity of distance matrix with threshold dissimilarity of 1 indicating that data of the four samples are connected ([1] 1 1 1 1), hence there were no significant differences in community structure in the samples at 95% level of confidence (P value=0.05).

DISCUSSION

The significance of fungal communities in the hot springs of hypersaline lakes is unclear, mainly because data on the fungal species in these habitats is limited. Using traditional culture-based methods, researchers reported relatively low levels of diversity for fungal communities in extreme environments (Salano, 2011). In this study we used high-throughput sequencing technology in order to comprehensively analyze fungal communities within the hot springs. The high sensitivity of Illumina sequencing enables the detection of rare species, thus provides more detailed information on fungal diversity in these habitats. Members of *Ascomycota* were more frequently identified in the hot springs than those of *Basidiomycota*, whereas members of *Chytridiomycota* and *Glomeromycota* represented only a small proportion of the hot spring fungal communities. The abundance of *Ascomycota* is similar to the abundance of fungi determined in the previous study on soda soils at the edge of several lake basins, where filamentous fungi that could grow at high ambient pH values were isolated (Alexey et al., 2015). The results in that study revealed 100 strains of fungi with various degrees of alkali tolerance and taxonomic affinity within *Ascomycota* (Alexey et al., 2015). Additionally, 6.2% of the fungi detected in wet sediment 83.6°C were unspecified Phylum. These may be undiscovered and possibly indigenous species in the hot springs. Some of the groups in this study are similar to those recovered from a previous culture dependent study conducted on the Hot spring in main Lake Magadi (Salano, 2011). The isolates recovered in that study belonged to *Aspergillus, Penicillium*, *Neurospora, Polyozellus multiplex, Pycnoporus* sp., *Teratosphaeria, Acremonium, Talaromyces*, *Sagenomella, Paecilomyces* and *Aphanoascus* genera (Salano, 2011). Filamentous fungi like *Aspergillus* and *Penicillium* are attractive organisms for production of useful protein and biological active secondary metabolites. They have high secretion capacity and are effective hosts for the production of foreign proteins (Tsukagoshi et al., 2001).

Penicillium genera were found in wet sediments 45.1 and 81°C with relative abundance of 14.29 and 3.28% respectively. This is similar to previous studies in hypersaline water of salterns that revealed different species of *Aspergillus, Penicillium* and diverse non-melanised yeasts (Gunde-Cimerman et al., 2005). Another study that used morphological and molecular techniques to identify a series of halotolerant fungi from hypersaline environments of solar salterns revealed 86 isolates of 26 species from salt ponds, which were identified as *Cladosporium cladosporioides*, nine *Aspergillus* sp., five *Penicillium* sp. and the black yeast *Hortaea werneckii* (Cantrell et al., 2006). *Rhodotorula mucilaginosa,* a yeast species and *Rhizopus* sp. 30795, a *Zygomycota* were found in wet sediment at 81°C while unclassified *Antarctic fungal* sp. gi944 dominated wet sediment at 83.6°C. Other plant pathogenic fungi recovered included *Fusarium* sp., *Cladosporium cladosporioides, Aspergillus flavus, Aspergillus japonicas* and *Aspergillus oryzae.* Most of these organisms may have found their way to the hot springs through various dispersal mechanisms or may be adapted in these extreme environments.

According to Frontier (1985), harsh environments experiencing one or more extreme conditions tend to harbor fewer species. In contrast, wet sediments at hot spring 45.1°C were found to have the least OTUs (107 OTUs) as compared to higher temperature samples, distributed within *Aspergillus oryzae* (42.86%), *Penicillium* sp. 5/97_5 (42.28%) and *Trichocomaceae* sp. lm65 (42.28%). Although water samples at 81°C were found to harbor a higher diversity of fungi with lower species richness, wet sediments showed a lower diversity with

high abundance of present groups. This could be due to high abundance of organic matter and lower oxygen levels which favored decomposition processes; hence the groups present have sufficient carbon sources (Neira et al., 2001; Buee et al., 2009). The widespread fungal groups within the wet sediments may therefore be degraders of organic matter (Edgcomb et al., 2011a; Nagahama et al., 2011; Burgaud et al., 2013; Coolen et al., 2013).

This study reveals the presence of moderate and weak alkalitolerant fungi such as *Alternaria alternata, Penicillium* sp., *Cladosporium* sp. and *Fusarium* sp. previously reported to grow optimally at neutral or below neutral pH values. These species have previously appeared in existing reports on the alkalitolerant and halotolerant fungi (Kladwang et al., 2003; Gunde-Cimerman et al., 2009). They have therefore been considered as transition species in the alkaline environments, since they are also known to inhabit neutral soils worldwide. *Hypocreales* and *Pleosporaceae* have been reported as strong alkalitolerants and effective alkaliphiles inhabiting soda soils at the edge of lake basins (Alexey et al., 2015). In this study, *Hypocreales* sp. lm 566 was identified in water samples at 81°C while *Pleosporales* sp. was found in wet sediment and water samples at 83.6 and 81°C respectively. Other interesting groups recovered from this study include *Pestalotiopsis* sp., *Neurospora* sp., and *Xylariaceae* sp. These have been reported to have various applications in Biotechnology industries (Russell et al., 2011; Roche et al., 2014; Healy et al., 2004; Posada et al., 2007).

Conclusion

This study presented fungal diversity analysis of samples collected from the hot springs of Lake Magadi and Little Magadi, using Illumina Sequencing Technology. The results revealed representatives of thermophilic and alkaliphilic fungi within the hot springs, suggesting their ability to adapt to a multi-extreme sampling environment due to high pH, temperature, and salinity. Culture dependent studies in future will help us unravel the survival mechanisms used by these polyextremophilic fungi.

Conflicts of Interests

The authors have not declared any conflict of interests. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Abbreviations

ITS, Internal Transcribed Spacer; **OTUs,** Operational Taxonomic Units; **DNA,** Deoxyribonucleic Acid; **QIIME,** Quantitative Insights into Microbial Ecology.

REFERENCES

Alexey AG, Marina LG, Sofiya AB, Alfons JMD, Elena NB (2015). On the diversity of fungi from soda soils. Fung. Divers. 76(1):27-74.

Barea JM, Pozo MJ, Azcón R, Azcón-Aguilar C (2005). Microbial co-operation in the rhizosphere. J. Exp. Bot. 56(417):1761-778.

Behr HJ, Röhricht C (2000). Record of seismotectonic events in siliceous cyanobacterial sediments (Magadi cherts), Lake Magadi, Kenya. Int. J. Earth Sci. 89:268-283.

Brem FM, Lips KR (2008). *Batrachochytrium dendrobatidis* infection patterns among Panamanian amphibian species, habitats and elevations during epizootic and enzootic stages. Dis. Aquat. Org. 81(3):189-202.

Buee M, Reich M, Murat C, Morin E, Nilsson RH, Uroz S, Martin F (2009). Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. New Phytol. 184:449-456.

Burgaud G, Woehlke S, Rédou V, Orsi W, Beaudoin D, Barbier G, Biddle J, Edgcomb VP (2013). Deciphering the presence and activity of fungal communities in marine sediments using a model estuarine system. Aquat. Microb. Ecol. 70:45-62.

Cantrell SA, Martí´nez CL, Molina M (2006). Characterization of *fungi* from hypersaline environments of solar salterns using morphological and molecular techniques. Mycol. Res. 110:962-970.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushma FD, Costello E K, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky, J R, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R (2010a). QIIME allows analysis of high-throughput community sequencing data. Nat. Meth. 7:335-336.

Coolen MJL, Orsi WD, Balkema C, Quince C, Harris K, Sylva SP, Filipova-Marinova M, Giosan L (2013). Evolution of the plankton paleome in the Black Sea from the Deglacial to Anthropocene. Proc. Natl. Acad. Sci. USA 110:8609-8614.

Dadachova E, Bryan RA, Huang X, Moadel T, Schweitzer AD, Aisen P, Nosanchuk JD, Casadevall A (2007). Ionizing radiation changes the electronic properties of melanin and enhances the growth of melanized fungi. PLoS One 2(5):e457.

Edgcomb VP, Beaudoin D, Gast R, Biddle JF, Teske A (2011a). Marine subsurface eukaryotes: the fungal majority. Environ. Microbiol. 13:172-183.

Frontier S (1985). Diversity and structure in aquatic ecosystems. Oceanogr. Mar. Biol. 23:253-312.

Gadd GM (2007). Geomycology: Biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bio-weathering and bioremediation. Mycol. Res. 111(Pt 1):3-49.

Gontcharova V, Youn E, Sun Y, Wolcott RD, Dowd SEA (2010). Comparison of bacterial composition in diabetic ulcers and contralateral intact skin. Open Microbiol. J. 4:8-19.

Gunde-Cimerman N, Butinar L, Sonjak S, Turk M, Uršic V, Zalar P, Plemenitaš A (2005). Halotolerant and halophilic fungi from coastal environments in the Arctics. In: Gunde-Cimerman N, Oren A, Plemenitaš A (eds) Adaptation to life at high salt concentrations in Archaea, Bacteria and Eukarya. Springer, Netherlands. pp. 397-423.

Gunde-Cimerman N, Ramos J, Plemenitaš A (2009). Halotolerant and halophilic fungi. Mycol. Res. 113:1231-1241.

Hawksworth DL (1991). The fungal dimension of biodiversity: magnitude, significance and conservation. Mycol. Res. 95:641-655.

Healy PC, Hocking A, Tran-Dinh N, Pitt JI, Shivas RG, Mitchell JK, Kotiw M, Davis RA (2004). Xanthones from a microfungus of the genus *Xylaria*. Phytochemistry 65(16):2373-2378.

Kladwang W, Bhumirattana A, Hywel-Jones N (2003). Alkaline-tolerant fungi from Thailand. Fung. Divers. 13:69-83.

Le Calvez T, Burgaud G, Mahé S, Barbier G, Vandenkoornhuyse P (2009). Fungal diversity in deep sea hydrothermal ecosystems. Appl. Environ. Microbiol. 75(20):6415-6421.

Lindahl, BD, Ihrmark K, Boberg J, Trumbore SE, Högberg P Stenlid J,

Finlay RD (2007). Spatial separation of litter decomposition and mycorrhizal nitrogen uptake in a boreal forest. New Phytol. 173(3):611-620.
Magan N (2006). Fungi in extreme environments: Environmental and microbial relationships. In: Christian PK, Irina SD (eds) The Mycota IV. Springer, Netherlands. pp. 85-104.
Magan N, Aldred D (2007). Environmental fluxes and fungal interactions: maintaining a competitive edge. In: van West P, Avery S, Stratford M (eds) Stress in Yeasts and Filamentous Fungi. Elsevier Ltd, Amsterdam, Netherlands. pp. 19-35.
Mueller GM, Schmit JP (2006). Fungal biodiversity: what do we know? What can we predict? Biodivers. Conserv. 16:1-5.
Nagahama T, Takahashi E, Nagano Y, Abdel-Wahab MA, Miyazaki M (2011). Molecular evidence that deep-branching fungi are major fungal components in deep-sea methane cold-seep sediments. Environ. Microbiol. 13:2359-2370.
Ndwigah FI, Boga IH, Wanyoike W, Kachiuru R (2015). Characterization, Enzymatic Activity, and Secondary Metabolites of Fungal Isolates from Lake Sonachi in Kenya. IOSR J. Pharm. Biol. Sci. 10:65-76.
Neira C, Sellanes J, Levin LA, Arntz WE (2001). Meiofaunal distributions on the Peru margin: Relationship to oxygen and organic matter availability. Deep Sea Res. Part I. 48:2453-2472.
Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Henry MHS, Wagner H (2012) vegan: Community Ecology Package.
Posada F, Aime MC, Peterson SW, Rehner AS, Vega EF (2007). Inoculation of coffee plants with the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales). Mycol. Res. 111:748-757.
Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucl. Acids Res. 41(DI):D590-D596.
R Development Core Team (2012). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing: Vienna, Austria.
Raghukumar C, Raghukumar S (1998). Barotolerance of fungi isolated from deep-sea sediments of the Indian Ocean. Aquat. Microb. Ecol. 15(2):153-163.
Reeder J, Knight R (2010). Rapidly denoising pyrosequencing amplicon reads exploiting rank-abundance distributions. Nat. Meth. 7:668-669.
Roche CM, Loros JJ, McCluskey K, Glass NL (2014). *Neurospora crassa*: Looking back and looking forward at a model microbe. Am. J. Bot. 101(12):2022-2035.
Russell JR, Huang J, Anand P, Kucera K, Sandova AG, Dantzler KW, Hickman D, Jee J, Kimovec FM, Koppstein D, Marks DH, Mittermiller PA, Núñez SJ, Santiago M, Townes MA, Vishnevetsky M, Williams NE, Vargas MP, Boulanger LA, Bascom-Slack C, Strobel SA (2011). Biodegradation of polyester polyurethane by endophytic fungi. Appl. Environ. Microbiol. 77:6076-6084.
Salano O (2011). Isolation and characterization of fungal communities from L. Magadi. MSc Thesis, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya.
Sambrook KJ, Fritsch EF, Maniatis T (1989). Molecular Cloning: A Laboratory Manual. 2nd Ed. Cold Spring Harbor Laboratory, New York, USA.
Tsukagoshi N, Kobayashi T, Kato M (2001). Regulation of the amylolytic and hemi-cellulolytic genes in *Aspergilli.* J. Gen. Appl. Microbiol. 47:1-19.
Vaupotic T, Veranic P, Jenoe P, Plemenitas A (2008). Mitochondrial mediation of environmental osmolytes discrimination during osmoadaptation in the extremely halotolerant black yeast *Hortaea werneckii*. Fung. Genet Biol. 45(6):994-1007.
White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR Protocols: a guide to methods and applications. Academic Press, New York, USA. pp. 315-322.
Yu K, Zhang T (2012) Metagenomic and Metatranscriptomic Analysis of Microbial Community Structure and Gene Expression of Activated Sludge. PLoS One 7(5):e38183.
Yuriko N, Takahiko N (2012). Fungal diversity in deep-sea extreme environments. Fung. Ecol. 5:463-471.

academicJournals

Vol. 10(10), pp. 339-347, 14 March, 2016
DOI: 10.5897/AJMR2015.7871
Article Number: D567CE457672
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Phenotypic and genotypic detection of β-lactams resistance in *Klebsiella* species from Egyptian hospitals revealed carbapenem resistance by *OXA* and *NDM* genes

Amira Mohamed EL-Ganiny[1]*, Areej Mostafa EL-Mahdy[2,3], Hemat Kamal Abd EL-Latif[1], Ramadan Hassan Ibrahem[2] and Heba Ibrahem Abdelsabour[1]

[1]Microbiology and Immunology Department, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt.
[2]Microbiology Department, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt.
[3]Princess Norah Bint Abdelrahman, Department of Pharmaceutical Sciences, Riyadh, KSA.

Received 2 December, 2015; Accepted 9 February, 2016

The β-lactams are frequently used antibiotics and are essential in treatment of serious bacterial infections. The emergence of β-lactamases has been an ongoing serious therapeutic problem. To overcome this problem, scientists utilized several β-lactamases inhibitors such as clavulanic acid and tazobactam that are used in combination with antibiotics. However, pathogenic bacteria have acquired additional mechanisms of resistance such as the acquisition of extended spectrum beta-lactamases (ESBL) and carbapenemases that cannot be inhibited by current inhibitors. Carbapenem-resistant Enterobacteriaceae (CRE) has become an international health threat. In the present work, 100 clinical *Klebsiella* strains were isolated, identified, and their antibiotic profiles were determined by the disc diffusion method. β-lactam resistance was evaluated using phenotypic and genotypic methods. More than 50% of the *Klebsiella* isolates exhibited resistance to tetracyclin, ceftriaxone, (pipracillin/tazobactam), azetreonam, ofloxacin, cefepime and cefoperazone/sulbactam. *Klebsiella* isolates were less resistant to gentamicin (32.4%) and amikacin (14.7%). Few isolates were resistant to meropenem and imipenem (6.86%). Among the 100 clinical isolates, 50% were ESBL producers and 32% were AmpC producers. The 7 imipenem resistant isolates were carbapenemase producers. PCR showed that carbapenem resistance may be due to NDM gene that was present in 43% of the isolates and OXA gene that was found in 28% of the isolates. KPC genes were not detected in any of the isolates. Antibiotic resistance is a worldwide problem that poses a major threat to therapeutic efficacy of available antibiotics, including carbapenem. Determination of causes of resistance is essential for better treatment options.

Key words: *Klebsiella*, beta-lactams, ESBLs, carbapenem-resistant, NDM, OXA, antibiotic resistance

INTRODUCTION

Klebsiella species are important opportunistic pathogens causing community-associated and nosocomial infections (Brisse et al., 2006). β-lactam antibiotics are widely used in treatment of many bacterial infections. The persistent

exposure of bacterial strains to β-lactams induces production of β-lactamases which are encoded by several genes and selections for mutations (Chaudhary and Aggarwal, 2004). Some of β-lactamases have activity even against 3rd and 4th generation cephalosporins and monobactams, these are called the extended spectrum β-lactamases (ESBLs). ESBLs are a heterogeneous group of plasmid-mediated enzymes (Bush and Jacoby, 2010), which are prevalent in *Klebsiella pneumoniae*. They are frequently isolated from patients with septicemia, pneumonia, or urinary tract infection (Nordmann et al., 2009). It is clearly known that prevalence of ESBL producers in any hospital depends on various factors; antibiotic policies and types of disinfectants used, especially in the Intensive Care Unit (ICU) (Wollheim et al., 2011). More than 300 different ESBL gene variants have been described (Paterson and Bonomo, 2005).

AmpC β-lactamases are a group of enzymes widely found in *K. pneumoniae.* They inactivate cephalosporins such as ceftazidime and cefotaxime. Plasmid-mediated AmpC enzymes have been reported since 1980s (Bradford et al., 1997). Most ESBLs arise as a result of mutations in the genes of TEM, SHV and CTX-M (Bradford, 2001). Carbapenems (for example, imipenem and meropenem) are considered one of the few last resort therapies for serious infections caused by multidrug-resistant (MDR) Gram-negative bacteria. The emergence of novel β-lactamases with direct carbapenem-hydrolyzing activity has contributed to the increased prevalence of carbapenem resistant Enterobacteriaceae (Gupta et al., 2011). Carbapenemases are enzymes that vary in their ability to hydrolyze carbapenems, and they are poorly inhibited by clavulanate (Robledo et al., 2011).

Carbapenemases can be divided into two types based on their mechanism of action. The first (for example, KPC and OXA types) uses a serine residue active site that covalently attacks β-lactam ring. The second (for example, VIM and NDM types) are the Metallo-β-lactamases (MBLs) that use Zn atoms to break the β-lactam ring (Walsh et al., 2005). OXA family encoded carbapenemases are most commonly produced by *Acinetobacter* spp. but have also been reported in *K. pneumonia* and *E. coli* (Mendes et al., 2009). The most recent MBL, the New Delhi MBL (NDM) showed hydrolytic activity against all β-lactams and has originated in India in 2009 and rapidly spread worldwide (Yong et al., 2009; Li et al., 2014).

In the present study, the antimicrobial susceptibility of *Klebsiella* clinical isolates was evaluated using disc diffusion method, and the causes of increased resistance to β-lactam antibiotics were determined both phenol-typically and genotypically using polymerase chain reaction (PCR).

MATERIALS AND METHODS

Bacterial isolation

A total of 100 *Klebsiella* isolates were isolated from clinical specimens obtained from wounds, surgical wounds, urinary catheters, diabetic foot, burns, blood and sputum of patients admitted to Mansoura and Zagazig University Hospitals during the period from April, 2011 to November, 2011. All the isolates were collected under approved ethical standards. The isolates were identified and verified using standard biochemical reactions including: growth on MacConkey agar, citrate utilization, Voges Proskauer, methyl Red, and motility tests (Koneman et al., 1997).

Antibiotic susceptibility tests

The antibiotic resistance pattern of isolates was determined using the disk diffusion method according to the Clinical and Laboratory Standard Institute guidelines (CLSI, 2013). The antibiotic discs were obtained from Oxoid, UK. The tested antibiotics were ofloxacin (OFX, 5 µg), gentamicin (CN, 10 µg), aztreonam (ATM, 30 µg), imipenem (IPM, 10 µg), sulphmethoxazole/trimethoprim (SXT, 25 µg), amikacin (AK, 30 µg), cefepime (FEP, 30 µg), cefoperazone/sulbactam (SCF, 105 µg), cefrtiaxone (CRO, 30 µg), meropenem (MEM,10 µg), pipracillin/tazobactam (TPZ, 100/10 µg) and tetracycline (TE, 30 µg).

Detection of ESBLs producing isolates

Phenotypic detection of ESBL was done by modified double disc synergy test (DDST) according to Jarlier et al. (1998). Briefly, a disc of amoxicillin-clavulanate (20/10 µg) was placed on the surface of Muller Hinton agar (MHA) plates then discs of cefotaxime (30 µg) and ceftazidime (30 µg) were kept 20 mm apart from amoxicillin-clavulanate disc. The plates were incubated aerobically at 37°C overnight. The enhancement of the zone of inhibition of the cephalosporin discs towards amoxicillin-clavulanate disc was taken as evidence of ESBL production.

Determination of AmpC producing isolates

Phenotypic detection of AmpC was done according to Singhal et al. (2005). Briefly, *E. coli* ATCC 25922 was streaked on MHA plate. One colony of *Klebsiella* isolates were inoculated on a sterile moistened 6 mm disc. A cefoxitin disc was placed next to this disc (almost touching). The plates were incubated overnight at 37°C. A flattening of growth or the indentation of the cefoxitin inhibition zone was considered a positive test for the presence of AmpC mediated resistance.

Determination of MBLs producing isolates

Phenotypic detection of Metallo β-lactamases (MBLs) was done by combined disk test according to Yong et al. (2002). Briefly, MHA plates were surface inoculated with overnight broth culture of *Klebsiella* isolates. Two imipenem discs (10 µg) and two ceftazidime (30 µg) discs were placed on the plate surface. Five (5) µl of 0.5 M EDTA solution was added one imipenem and one ceftazidime disc. The inhibition zones of imipenem and ceftazidime and their EDTA-impregnated discs were compared after overnight

*Corresponding author. E-mail: amiraganiny@yahoo.com. Tel: 002-01145051974.

Table 1. Primers used in this study: Beta-lactamases specific primers.

Primer	Sequence (5'-3')	Primer Tm	Amplicon size (bp)	Annealing temp
SHV F	ACTATCGCCAGCAGGATC	58	355	53
SHV R	ATCGTCCCACCATCCACTC	58		
TEM F	GATCTCAACAGCGGTAAG	54	750	50
TEM R	CAGTGAGGCACCTATCTC	56		
CTXM-15 F	GTGATACCACTTCACCTC	54	255	49
CTXM-15 R	AGTAAGTGACCAGAATCAG	54		
NDM-1 F	GCACACTTCCTATCTCGACATGC	57	209	51.5
NDM-1 R	CCATACCGCCCATCTTGTCC	56		
OXA-9 F	CGTCGCTCACCATATCTCCC	56	315	51
OXA-9 R	CCTCTCGTGCTTTAGACCCG	56		
KPC F	GTATCGCCGTCTAGTTCTG	51	209	48
KPC R	CCTTGAATGAGCTGCACAGTG	54		

Table 2. Prevalence of *Klebsiella* species isolated from different sources.

Specimen type	NO of specimens	NO of isolates	Percentage %
Urine	83	41	49.4
Sputum	46	21	45.7
Catheters	35	16	45.7
Swabs	25	11	44
Blood	16	9	56.3
Diabetic foot	10	2	20
Total	215	100	46.5

incubation at 37°C. A zone size difference of ≥ 7 mm was taken as indicative of MBLs production.

Determination of carbapenemases producing isolates

Phenotypic detection of carbapenemases was done using modified hodge test [MHT] according to Lee et al. (2001). Briefly, a culture of *E. coli* ATCC 25922 was inoculated onto the surface of MHA plate. A 10 µg imipenem disc was placed at the center of the plate, the *Klebsiella* strains were streaked from edge of the disk to the periphery of the plate. The plate was incubated overnight at 37°C, presence of distorted inhibition zone 'cloverleaf shaped' of *E. coli* was considered as positive result for carbapenemase production by the test strain.

Genotypic detection of resistance genes

Colony PCR was done for detection of resistance genes in *Klebsiella* isolates, including the ESBLs genes (TEM, SHV, CTX-M), the Metallo- β- lactamase gene (NDM-1) and the carbapenamases genes (OXA-9 and KPC-1). The primers were designed and supplied from IDT (Intrgrated DNA Technologies, Coralville, Iowa, USA). The primers for NDM-1 and OXA-9, KPC were designed according to Geyerand Hanson (2013). All primers are listed in Table 1. The gDNA was prepared according to Nair and Venkitanarayanan (2006). The PCR mixture was prepared in a final volume of 50 µl and contained: 25 µl of 2× DreamTaq TM Green Master Mix (Thermo Fisher Scientific, http://www.thermoscientific.com/), One µl of each primer, 5µl gDNA and nuclease-free water to 50 µl. The cycling conditions were: initial denaturation at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 5 s, annealing for 30 s at temperature (5° below primer Tm) as indicated in Table 1 and extension at 72°C for 1 min and a final extension cycle at 72°C for 5 min.

RESULTS

Prevalence of *Klebsiella* among clinical specimens

In the present study, a total of 100 isolates were identified as *Klebsiella* species using conventional biochemical methods. Those strains were isolated from 215 clinical samples. The highest prevalence of *Klebsiella* isolates (56.3%) was observed in blood specimens, while the lowest prevalence (20%) was observed in diabetic foot specimens (Table 2).

Table 3. Antibiotic susceptibility patterns of *Klebsiella* isolates*

Antibiotic Disk	Klebsiella species (Total Number of isolates =100)					
	Resistant		Intermediate		Sensitive	
	n	%	N	%	N	%
IMP	1	1	6	6	93	93
MEM	1	1	6	6	93	93
AK	15	15	7	7	78	78
CN	33	33	11	11	56	56
FEP	55	55	0	0	45	45
SCF	57	57	5	5	38	38
OFX	57	57	8	8	35	35
TPZ	69	69	8	8	25	25
ATM	75	75	0	0	25	25
CRO	87	87	0	0	13	13
SXT	89	89	1	1	10	10
TE	99	99	0	0	1	1

IMP: imipenem, MEM: meropenem, AK: amikacin, CN: gentamicin, FEP: cefepime, SCF: (cefoperazone/ sulbactam), OFX: ofloxacin, ATM: aztreonam, TPZ: (pipracillin, tazobactam), CRO: ceftriaxone, SXT: (sulphamethoxazole/ trimethoprim), TE: tetracycline. Breakpoints wear according to CLSI (2013).

Determination of antimicrobial susceptibility (AMS) of *Klebsiella* isolates

The AMS pattern of *Klebsiella* isolates were determined by disc diffusion method, the diameters of inhibition zones were recorded and interpreted as resistant, intermediate or sensitive according to CLSI (2013). The results of AMS revealed that *Klebsiella* isolates exhibited high resistance to tetracycline, SXT, ceftriaxone (pipracillin/ tazobactam), azetreonam, ofloxacin, cefoperazone/ sulbactam and cefepime with percentages 97, 87.3, 85.3, 73.5, 67.6, 55.9, 55.9 and 53.9%, respectively. *Klebsiella* isolates exhibited less resistance to gentamicin (32.4%) and amikacin (14.7%). On the other hand the resistance was low to meropenem and imipenem (6.86%) for each (Table 3).

Phenotypic detection of β-lactamase enzymes

Detection of extended spectrum β-lactamase (ESBL) producers

Among 100 *Klebsiella* clinical isolates, 50 isolates were ESBL producers (50%). Enhancement of zone of inhibition between one of ceftazidime, cefotaxime and amoxicillin/ clavulanic disc indicate positive ESBL production (Figure 1A).

Detection of AmpC β-lactamase among Klebsiella isolates

AmpC β-lactamase test was carried out on *Klebsiella*. Thirty three isolates (33%) were AmpC producers. The positive AmpC activity appeared as either flattening or indentation of cefoxitin zone of inhibition (Figure 1B).

Detection of metallo β-lactamase (MBL)

MBLs test were carried out on isolates showing resistance to imipenem and meropenem using combined disk test. Seven *Klebsiella* imipenem resistant isolates (both intermediate (6) and resistant (1) isolates in AMS test), the seven isolates were MBL producers showing a zone size difference of more than 7 mm between imipenem and ceftazidime and their EDTA-impregnated disk (Figure 1C).

Detection of carbapenemases by modified Hodge test

The imipenem and meropenem resistant *Klebsiella* isolates were subjected to modified Hodge test. The seven tested isolates showed a distorted zone of inhibition (clover-leaf shaped zone) due to carbapenemase production (Figure 1D).

Genotypic detection of resistance genes using PCR

Detection of ESBLs genes (TEM, SHV and CTXM-15)

The resistance genes TEM, SHV and CTXM-15 were amplified from gDNA of the 50 ESBL producing *Klebsiella* isolates. For TEM gene, all tested isolates produced a 750 bp band (Figure 2A), for *SHV* gene, all the ESBL producing isolates gave a 355 bp band (Figure 2B). For CTXM-15 gene, 255 bp band was also detected in all tested ESBL-producing isolates (Figure 2C).

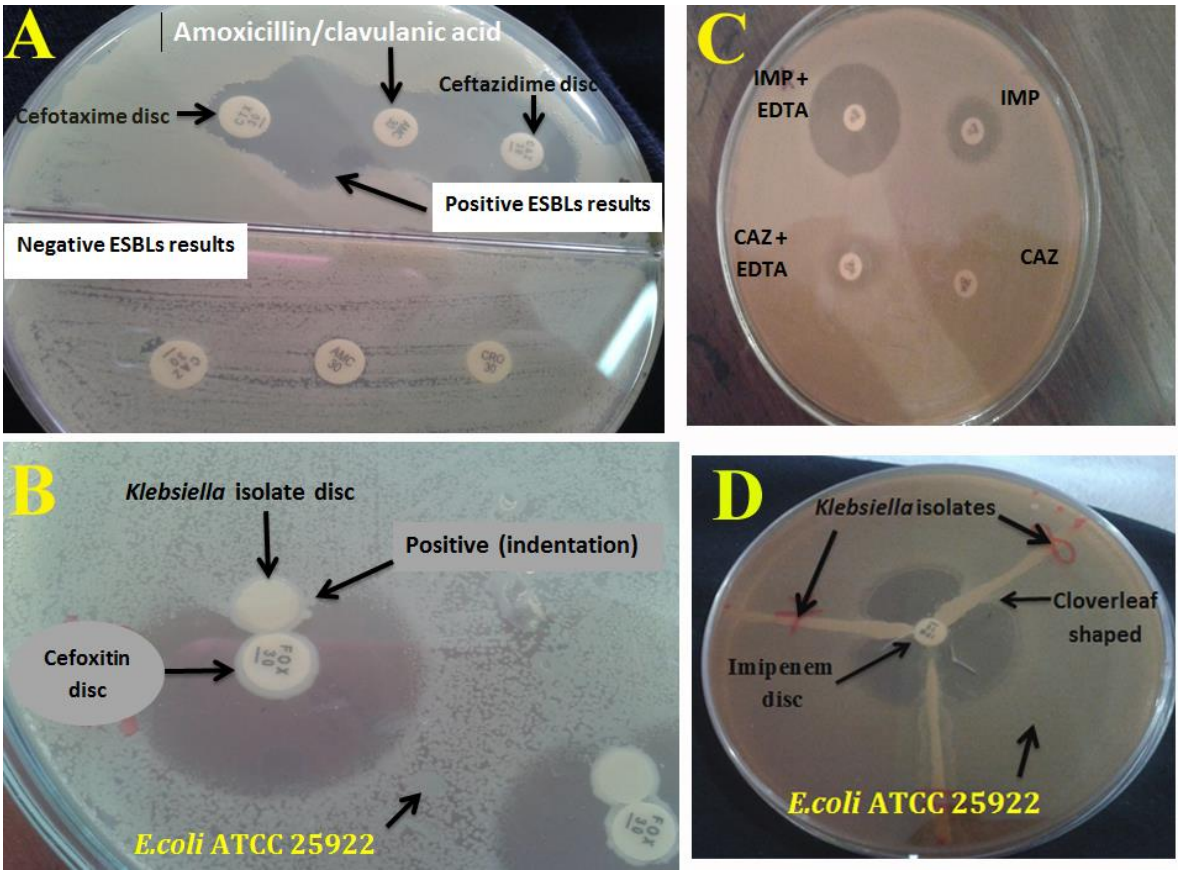

Figure 1. Phenotypic detection of ESBLs, AmpC, MBLs and Carbapenemases. A) Detection of ESBLs by modified double disc synergy test, enhancement of cephalosporin zone of inhibition towards Augmentin disc indicates positive test. B) Detection of AmpC: flattening or indentation of cefoxitin zone of inhibition was considered a positive test. C) Detection of MBLs by combined disk test, 7mm increase in zone of inhibition of EDTA impregnated disc indicates positive result. D) Detection of carbapenemases using Modified Hodge test, distorted inhibition zone 'cloverleaf shaped' was considered as positive result.

Detection of the Metallo-β-lactamase gene (NDM-1) and the carbapenemases genes (OXA-9 and KPC)

The resistance gene New Delhi Metallo β-lactamase (*NDM-1*) was amplified from gDNA of the seven imipenem and meropenem resistant *Klebsiella* isolates. It was observed that 3 isolates only harbored *NDM-1* gene of ampilcon size 209 bp (Figure 3a). The resistance genes of carbapenemase (*OXA-9* and *KPC*) were amplified from gDNA of the 7 imipenem and meropenem resistant *Klebsiella* isolates. OXA gene was observed in two isolates only (amplicon size 300 bp) as shown in Figure 3b, while *KPC* gene was not detected in any of the seven imipenem resistant isolates.

DISCUSSION

The *Klebsiella* species have been shown to be important opportunistic pathogens causing serious infections (Traub et al., 2000). Epidemics caused by MDR Klebsiella species have led to closures of hospital units or even whole hospitals (Casewell and Phillips, 1981).

In the present study *Klebsiella* species were isolated from various clinical specimens. The prevalence of *Klebsiella* in blood, urine, sputum, catheter, swabs and diabetic foot specimens were 56, 49.4, 45.7, 45.7, 44 and 20, respectively. Thosar and Kamble (2014) reported prevalence rate of 45 and 42.9% in sputum and urine, respectively. Our results were higher than those reported by Podschun and Ullmann (1998) and Acheampong et al. (2011). The relative high prevalence of *Klebsiella* may have been due to the increased resistance of these isolates which decreased the possibility of elimination of these isolates, leading to nosocomial outbreaks by these resistant isolates (Brisse et al., 2006).

In the present study, the result of AMS revealed that *Klebsiella* isolates exhibited high resistance to tetracycline (97 %), SXT (88.2%), ceftriaxone (85.3%), TPZ (73.5%), and aztreonam (73.5%). This high resistance rate is more than that reported previously (Acheampong et al., 2011; Midan et al., 2012; El- sharkawy et al., 2013). We

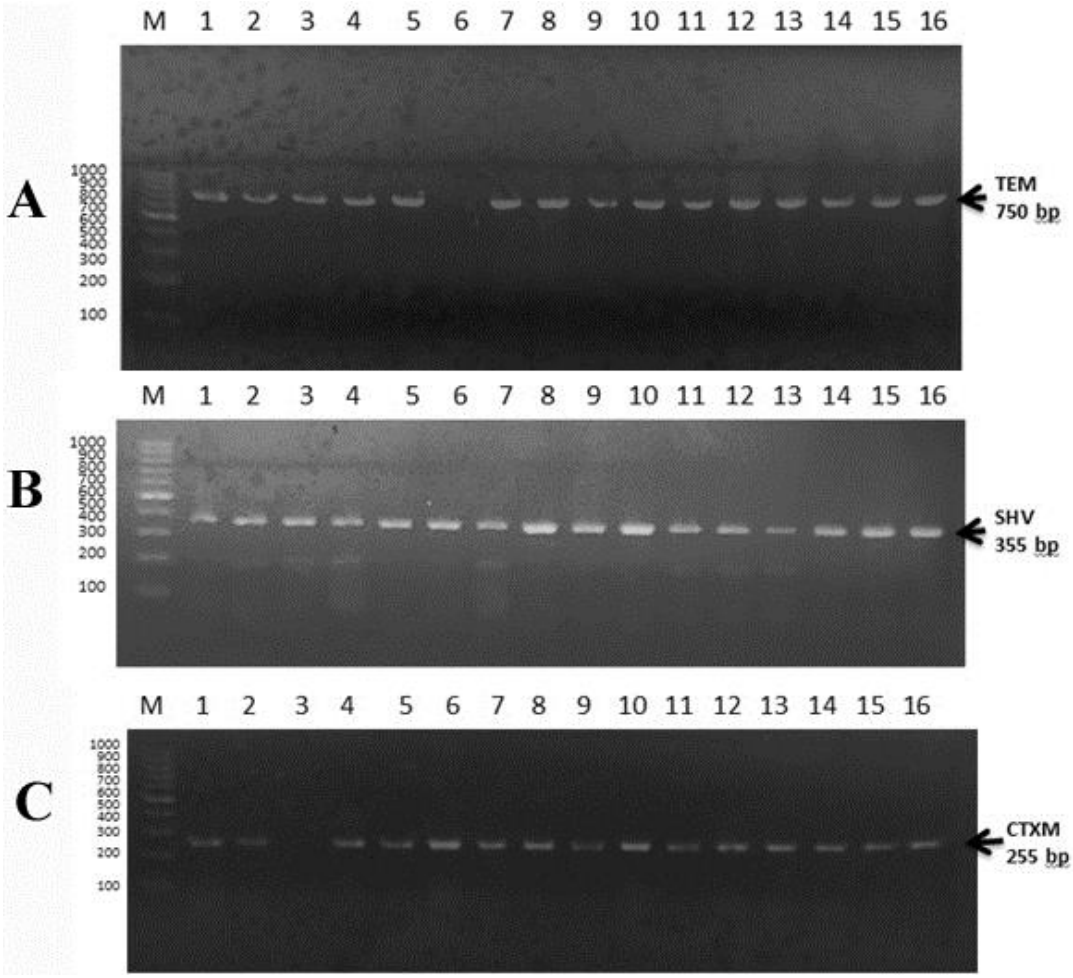

Figure 2. Genotypic detection of ESBLs genes using PCR . A) TEM gene, 750 bp band was detected in all isolates (lane 6 is a negative control), B) SHV, 355 bp band was detected in all isolates and C) CTX-M, 255 bp band was detected in all isolates (lane 3 is a negative control).

concluded that these agents have a limited capacity in treating *Klebsiella* infections. The tested strains exhibited resistance to ofloxacin by 63.7%, while resistance to gentamicin was 43.1%. The data reveals that resistance to quinolones and gentamicin was less than that reported for 3rd generation cephalosporins in this study (ceftriaxone, 87%). The excessive and over use of cephalosporins antibiotics lead to emergence of resistance and therefore shifting to alternative quinolones and aminoglycosides therapy (Sekowska et al., *2002*). Less use of quinolone and structural dissimilarities is one of the reasons of comparatively low resistances than cephalosporins. The emergence of resistance to 4th generation cephalosporins (cefepime) was detected in our study by (53%). This susceptibility rate could be attributed to the empirical use of this antibiotic in the treatment of nosocomial infections in Egyptian hospitals.

Resistance to amikacin was 14.7%, which correlated with results obtained by Yasmin (2012). Resistance to imipenem and meropenem were 6.86% but higher than those reported by El-sharkawy et al. (2013). Yasmin (2012) reported that all *Klebsiella* isolates were sensitive to imipenem and meropenem. According to Rahal et al. (2008), carbapenems are the most effective therapy for ESBL bacterial infections. However, their routine use can select resistant strains leading to the emergence of imipenem-resistant *K. pneumonia* strains.

In the present study, 50% of *Klebsiella* isolates were ESBLs producing. This result was quite similar to that reported by Haque and Salam (2010) and Aladag et al. (2013) who reported 57.9 and 55%, respectively, but was higher than the 40% detected in France (Branger et al., 1998) and 13% in Hong Kong (Ho et al., 2000). High occurrence of ESBLs in *Klebsiella* spp is of great concern since they spread easily, and strains become more efficient at acquiring more resistance plasmids (Chaudhary and Aggarwal, 2004).

In the present study, 32.4% of *Klebsiella* isolates were AmpC producers. This result was higher than that reported by Shivanna and Rao (2014), but lower than that

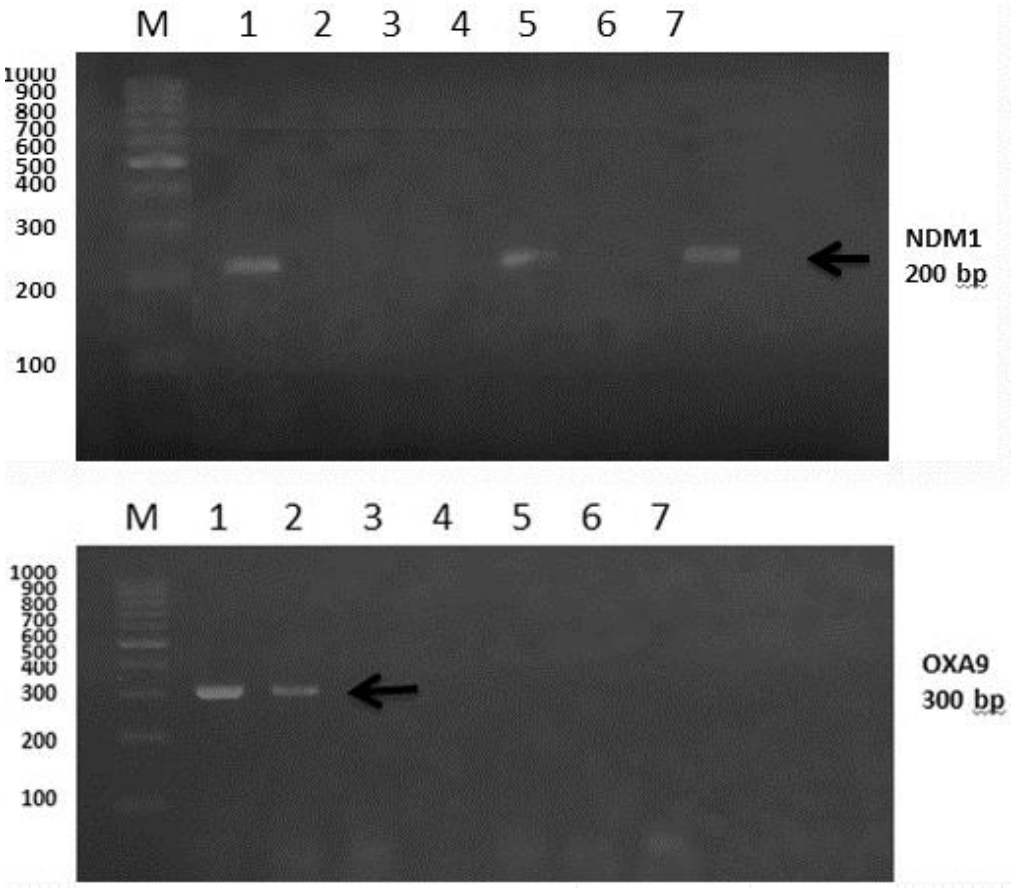

Figure 3. Genotypic detection of the NDM and OXA genes. For NDM, ~200 bp band was detected in 3 isolates only (lanes 1, 5 and 7). OXA gene, a 300 bp band was detected in 2 isolates only.

found by Akujobi et al. (2012) and Fam et al. (2013). The increased presence of plasmid mediated AmpC β-lactamases worldwide is becoming of great concern (Jacoby, 2009). Infections caused by AmpC-positive bacteria cause higher patient morbidity and mortality (Livermore, 2012), demanding new measures of *Klebsiella* infections management. 14.7% of our isolates were both ESBL and AmpC producers, a result that underlines the need for new measures of management of *Klebsiella* infections.

In the current study all *Klebsiella* isolates that exhibit resistance to imipenem and meropenem were MBL and carbapenemase producers by phenotypic detection tests. These results come in complete accordance with the results of Bora et al. (2014). It is reported that carbapenemase-producing *K. pneumoniae* exhibit resistance to important antibiotics, such as amino-glycosides and fluoroquinolones (Livermore et al., 2011). Uncontrolled clinical utility of carbapenems is a serious worldwide threat.

PCR was used in this study to detect the presence of TEM, SHV and CTX-M, these were detected in all the ESBL isolates (100%). Our results come in accordance with Kiratisin et al. (2008) who detected in ESBL-producing *K. pneumoniae*, CTX-M (99%), SHV (87.4%) and TEM (71.7%); but, were higher than that reported in India, where of the 64 ESBL-*Klebsiella* isolates, 48% had TEM and 20% had SHV (Jain et al., 2008). Another study in USA showed 93% of SHV production and 20% of TEM production among ESBL *Klebsiella* isolates (Bradford et al., 2004).

The incidence of Carbapenem resistant entero-bacteriaceae has been increasing worldwide (Bhaskar et al., 2013). The main mechanism of resistance to carbapenem is by enzyme production. The most commonly identified enzymes are the carbapenemases OXA, KPC and the MBL enzyme NDM. They have been identified in different countries (Pfeifer et al., 2012). In the seven imipenem resistance isolates, OXA-9 was detected in 2 isolates (28.5%). Kiratisin et al. (2008) reported OXA-10 in 11.8% of Klebsiella isolates in Thailand. However OXA genes were not detected in any of 15 *Klebsiella* isolates from Turkey (Bali et al., 2010).

None of our isolates have the carbapenem resistance gene KPC. Our results differed from those reported in USA by Bradford et al. (2004), who found KPC in 100%

of isolates. Also, Robledo et al. (2011) found KPC in 73% of 457 MDR Klebsiella isolates. In this study, the MBL NDM-1 was detected in 42.8% of imipenem resistant isolates. Chaudhary and Payasi (2013) detected NDM in 6.25% (in 24/150) of *Klebsiella* isolates.

We recommend that strict antibiotic policies and measures to limit unnecessary use of cephalosporins and carbapenems should be enforced to minimize the emergence of resistance to these antibiotics. Also rapid routine molecular detection of resistance determinants is essential to optimize therapy, improve outcomes, and limit the spread of such resistance.

Conflict of interest

The authors have not declared any conflict of interest

REFERENCES

Acheampong DO, Boamponsem LK, Feglo PK (2011). Occurrence and species distribution of *Klebsiella* Isolates: A case study at KomfoAnokye teaching hospital (Kath) in Ghana. Pelagia Res. Lib. 4:187-193.

Akujobi CO, Odu NN, Okorondu SI (2012). Detection of AmpC beta lactamases in clinical isolates of *Escherichia coli* and *Klebsiella*. Afr. J. Clin. Exp. Microbiol. 13:51-55.

Aladag MO, Dundar AUN, Durak Y, Gunes E (2013). Characterization of *Klebsiella pneumoniae* strains isolated from urinary tract infections: Detection of ESBL characteristics, antibiotic susceptibility and RAPD genotyping. Pol. J. Microbiol. 62(4):401-409.

Bali BE, Açık L, Sultan N (2010). Phenotypic and molecular characterization of SHV, TEM, and CTX-M and extended-spectrum β-lactamase produced by *Escherichia coli*, *Acinobacter baumanni* and *Klebsiella* isolates in a Turkish hospital. Afr. J. Microbiol. Res. 4:650-654.

Bhaskar M, Anand R, Harish, BN (2013). Prevalence of bla NDM-1 producing blood isolates of *Escherichia coli*, *Klebsiella* species and *Enterobacter* species in a tertiary care centre in South India. J. Microbiol. Res. Rev. 6:61-68.

Bora A, Sanjana R, Jha BK, Mahaseth SN, Pokharel K (2014). Incidence of metallo-beta-lactamase producing clinical isolates of *Escherichia coli* and *Klebsiella pneumonia* in central Nepal. BMC Res. Notes 7:557-564.

Bradford P, Urban C, Mariano N, Projan S, Rahal J, Bush K (1997). Imipenem resistance in *Klebsiella pneumonia* is associated with the combination of ACT-1, a plasmid-mediated AmpC β-lactamase and the loss of an outer membrane protein. Antimicrob. Agents Chemother. 41:563-569.

Bradford PA (2001). Extended-spectrum *β*-lactamases in the 21st century: Characterization, epidemiology and detection of this important resistance threat. Clin. Microbiol. Rev. 14:933-951.

Bradford PA, Bratu S, Urban C, Visali M, Marriano N, Landman D (2004). Emergence of Carbapenem-resistant *Klebsiella species* possessing the class A carbapenem hydrolyzing KPC-2 and inhibitor resistant TEM-30 β-lactamases in New York City. Clin. Infect. Dis 39:55-60.

Brisse S, Grimont F, Grimont PAD (2006). The genus *Klebsiella.* Prokaryotes. 6:159-196.

Branger C, Lesimple AL, Bruneu B, Berry P, Zechovsky NL (1998). Long term investigation of clonal dissemination of *K. pneumonia* isolates producing extended spectrum β-lactamases in a university hospital. J. Med. Microbiol 47:201-209.

Bush K, Jacoby GA (2010). Updated functional classification of β-lactamases. Antimicrob Agents Chemother. 54:969-976.

Casewell MW, Phillips I (1981). Aspects of the plasmid-mediated antibiotic resistance and epidemiology of *Klebsiella* species. Am. J. Med. 70:459-462.

Chaudhary M, Payasi A (2013). Antimicrobial susceptibility patterns and molecular characterization of *Klebsiella pneumonia* clinical isolates from North Indian patients. Int. J. Med. Med. Sci. 46:1218-1223.

Chaudhary U, Aggarwal R (2004). Extended spectrum beta lactamases (ESBL): An emerging threat to clinical therapeutics. Indian J. Med. Microbiol. 22:75-80.

Clinical and Laboratory Standard Institute (CLSI, 2013). Performance standards for antimicrobial susceptibility testing. Twenty-Third Informational supplements. CLSI document 2013; M100-S23. Wayne, Pennsylvania, USA.

El-sharkawy AA, Elkady LM, Mansour SA, Esmaeel NE (2013). Detection of extended-spectrum and plasmid-mediated AmpC β-lactamases in nosocomial *Klebsiella* isolates. J. Microbiol. Infect. Dis. 3:24-30.

Fam N, Gamal D, El Said M, El Defrawy I, El Dadei E, El Attar S, Sorur S, Ahmed S, Klena J (2013). Prevalence of plasmid-mediated *AmpC* genes in clinical isolates of *Enterobacteriaceae* from Cairo, Egypt. Br. Microbiol Res. J. 3:525-537.

Geyer CN, Hanson ND (2013). Rapid PCR amplification protocols decrease the turn-around time for detection of antibiotic resistance genes in Gram-negative pathogens. Diagn. Microbiol. Infect. Dis 77:113-117.

Gupta N, Limbago BM, Patel JB, Kallen AJ (2011). Carbapenem-resistant Enterobacteriaceae: Epidemiology and prevention. Clin. Infect. Dis 53:60-67.

Haque R, Salam MA **(**2010). Detection of ESBL producing nosocomial negative bacteria from tertiary care hospital in Bangladesh. PJMS 26:887-891.

HO PL, Tsang DNC, Que TL, Ho M, Yuen KY (2000). Comparison of screening methods for detection of extended spectrum β-lactamases and their prevalence among *E. coli* and *Klebsiella* spp. in Hong Kong. ActaPathol. Microbiol. Immunol. Scandinavica 108:237-240

Jacoby GA (2009). AmpC B-lactamases, Clin. Microbiol. Rev. 22:161-182

Jain A, Mondal R (2008). TEM and SHV genes in extended spectrum β-lactamase producing *Klebsiella* species and their antimicrobial resistance pattern. Indian J. Med. Res. 128:759-764.

Jarlier V, Nicolas MH, Fournier G, Philippon Al (1998). Extended spectrum β-lactamases conferring transferable resistance to newer β-lactamase agents in *Enterobacteriaceae* hospital prevalence and susceptibility patterns. Rev. Infect. Dis. 10: 867-878.

Kiratisin P, Apisarnthanarak A, Laesripa C, Saifon P (2008). Molecular characterization and epidemiology of extended spectrum β-lactamase producing *Escherichia coli* and *Klebsiella pneumonia* isolates causing health care associated infection in Thailand where CTX-M is endemic. Antimicrob. Agents Chemother. 52:2818-2824.

Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WC (1997). The *Enterobacteriaceae*. In color atlas and textbook of Diagnostic Microbiology, (5th Ed.). Lippincott, Philadelphia. pp. 171-252.

Li B, Xu X, Zhao Z, Wang M, Cao Y (2014). High prevalence of metallo-β-lactamase among carbapenem-resistant *Klebsiella pneumoniae* in a teaching hospital in China. Can. J. Microbiol. 60(10):691-695.

Lee K, Chong Y, Shin HB, Kim YA, Yong D, Yum JH (2001). Modified Hodge and EDTA- disk synergy tests to screen metallo-β-lactamase-producing strains of *Pseudomonas* and *Acinetobacter* species*.* Clin. Microbiol. Infect. 7:88-91.

Livermore DM (2012). Current epidemiology and growing resistance of Gram-negative pathogens. Korean J. Int. Med. 27:128-142.

Livermore DM, Warner M, Mushtaq S, Doumith M, Zhang J, Woodford N (2011). What remains against carbapenem-resistant Enterobacteriaceae? Evaluation of chloramphenicol, ciprofloxacin, colistin, fosfomycin, minocycline, nitrofurantoin, temocillin and tigecycline. Int. J. Antimicrob. Agents 37:415-419.

Mendes RE, Bell JM, Turnidge JD, Castanheira M, Jones RN (2009). Emergence and widespread dissemination of OXA-23, -24/40 and -58 carbapenemases among *Acinetobacter* spp. in Asia-Pacific nations: Report from the sentry surveillance program. J. Antimicrob. Chemother. 63:55-59.

Midan D, Swarnamoni D, Kalpana G (2012). A study of occurrence of antibiotic resistance reported against *Klebsiella* spp. In a tertiary care hospital in Assam, India. Int. Res. J. Pharm. 3:58-59.

Nair MK, Venkitanarayanan KS (2006). Cloning and sequencing of *ompA* gene of *Enterobacter sakazakkii* and development of an *ompA*-targeted PCR for rapid detection of *Enterobacter sakazakii*in infant formula.Appl Environ Microbiol 72:2539-2546.

Nordmann P, Cuzon G, Naas T **(**2009). The real threat of *Klebsiella pneumoniae* carbapenemases producing bacteria. Lancet Infect. Dis. 9(4): 228-236.

Paterson DL, Bonomo, RA (2005). Extended-spectrum β-lactamases: A clinical update. Clin. Microbiol. 18:657-686.

Pfeifer Y, Schlatterer K, Engelmann E, Schiller RA, Frangenberg HR, Stiewe D, Holfelder M, Witte W, Nordmann P, Poirel L (2012). Emergence of OXA-48-type carbapenemase-producing *Enterobacteriaceae* in German hospitals. Antimicrob. Agents Chemother. 4: 2125-2127.

Podschun R, Ullmann U (1998). *Klebsiella* spp. as nosocomial pathogens: Epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin. Microbiol. Rev 11:589-603.

Rahal JJ (2008). The role of carbapenems in initial therapy for serious Gram-negative infections. Crit Care 12 (Suppl 4):S5.

Robledo IE, Aquino EE, Va´zquez GJ (2011). Detection of the *KPC* gene in *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* during a PCR- based nosocomial surveillance study in Puerto Rico. Antimicrob. Agents Chemother. 55:2968-2970.

Sekowska A, Janika G, Klyszejko C, Wojda M, Wroblewskiand M, Szymankiewicz M (2002). Resistance of *K. pneumonia* strains producing and not producing extended spectrum β- lactamase (ESBL) type enzyme to selected non β-lactam antibiotics. Med. Sci. Mon. 8:100-104.

Shivanna V, Rao A (2014). Detection of AmpC β-lactamases among Gram negative clinical isolates. International J. Recent Trends Sci. Technol. 9:361-364.

Singhal S, Mathur T, Khan S, Upadhyay DJ, Chugh S, Gaind R, Rattan A (2005). Evaluation of methods for AmpC beta-lactamase in Gram negative clinical isolates from tertiary care hospitals. Indian J. Med. Microbiol. 23:120-124.

Thosar MG, Kamble VA (2014). Prevalence of *Klebsiella* species isolated from clinical samples in Vidarbha region of Maharashtra state India. Int. J. Pharm. BioSci. 3:40-45.

Traub WH, Schwarze I, Bauer D (2000). Nosocomial outbreak of cross-infection due to multiple-antibiotic-resistant *Klebsiella pneumonia*: Characterization of the strain and antibiotic susceptibility studies. Chemotherapy 46:1-14.

Walsh TR, Toleman MA, Poirel L, Nordmann P (2005). Metallo β-lactamase: The quite before the storm. Clin. Microbiol. Rev. 18:306-325.

Wollheim C, Guerra MI, Conte VD, Hoffman SP, Schreiner FJ, Delamare APL, Barth AL, Echeverrigaray S, Costa SO (2011). Nosocomial and community infections due to class A extended-spectrum beta-lactamase -producing *Escherichia coli* and *Klebsiella* spp. in Southern Brazil. Braz. J. Infect. Dis. 15:138-143.

Yasmin T (2012). Prevalence of ESBL among *E. coli* and *Klebsiella* spp. in a tertiary care hospital and molecular detection of important ESBL producing genes by multiplex PCR. MSc Thesis. Mymensingh Medical College, Bangladesh.

Yong D, Lee K, Yum JH, Shin HB, Rossolini GM, Chong Y (2002). Imipenem-EDTA disk method for differentiation of metallo-β-lactamase producing clinical isolates of *Pseudomonas spp*. and *Acinetobacter spp*. J. Clin. Microbiol. 40:3798-3801.

Yong D, Toleman MA, Giske CG, Cho HS, Sundman K,Lee K, Walsh TR (2009). Characterization of a new metallo-beta-lactamase gene, bla (NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. Antimicrob. Agents Chemother 53:5046-54.

African Journal of Microbiology Research

Related Journals Published by Academic Journals

- *African Journal of Biotechnology*
- *African Journal of Biochemistry Research*
- *Journal of Bacteriology Research*
- *Journal of Evolutionary Biology Research*
- *Journal of Yeast and Fungal Research*
- *Journal of Brewing and Distilling*

academicJournals