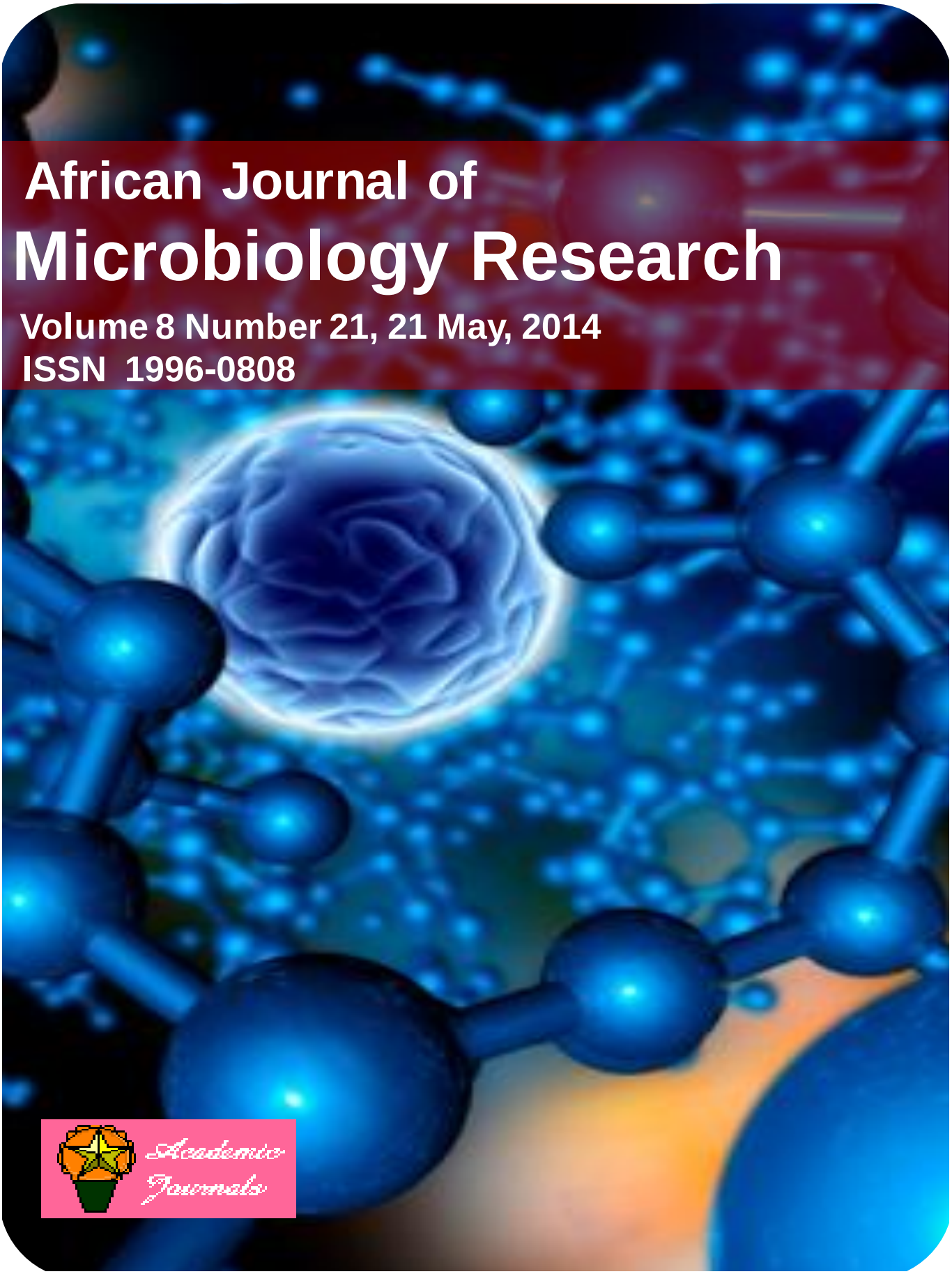

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Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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African Journal of Microbiology Research

Review

Antibacterial compounds in different species of *Datura*: A review

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The growing phenomenon of antibiotic resistance to pathogenic microorganisms has led to the concern of scientists on finding novel antimicrobial agents from natural sources. *Datura* species is a medicinal plant that has significant antibacterial properties and has been widely used to treat various diseases such as diabetes, leucoderma, skin disorders, ulcers, bronchitis, jaundice, hysteria, insanity, heart disease, fever, piles, etc. In this review, we focused on the antibacterial characteristics of plant with special reference to phytocompounds studied by various scientists in different species of *Datura*. Studies showed that maximum antibacterial work has been done on *Datura metal, Datura inoxia* and *Datura stramonium* against *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*. Leaves were found to be most promising part as a source for antibacterial activities. Steroidal (5[1], 7[1] dimethyl 6[1]– hydroxyl 3[1], phenyl 3 α-amine β–yne sitosterol) and β-carboline(1,7 dihydroxy-1-methyl 6,8-dimethoxy β-carboline) alkaloids are the two antibacterial compounds isolated from *D. metel.* Research showed that the *Datura* species are very promising plants for isolation of new antibacterial compounds.

Key words: *Datura*, antibiotic resistance, phytomedicine, antibacterial compound.

INTRODUCTION

Medicinal plants used as sources for therapeutic agents represent a rapidly expanding area of health science (Chopra et al., 1956). It is now believed that traditional use of the medicinal plants has fewer side effects over allopathic medicine. Such promising facts led to development of herbal derived medicines all over the world (Pal and Shukla, 2003). *Datura* as a medicinal plant, is a genus of nine species (*Datura ferox, Datura inoxia, Datura metel, Datura stramonium, Datura ceratocaula*, *Datura discolor*, *Datura leichhardtii*, *Datura quercifolia* and *Datura wrightii*) of vespertine flowering plants belonging to the family Solanaceae (Nightshade family). The name *Datura* comes from the early Sanskrit Dustura or Dahatura (Mann, 1996). They are commonly called thorn apple, stink weed, devil's apple, jimson weed, angel's trumpet, etc. (Heiser, 1969; Avery et al.,1959). All the species are woody, stalked leafy annuals and short lived perennials which can reach up to two meters in height. The leaves are alternate with lobed or toothed margin. The flowers are erect, trumpet shaped, 5-20 cm long and 4-12 cm broad at the mouth, colors vary from white to yellow, pink and pale purple. The fruit is spiny capsule. They normally have 12 pairs of chromosomes (Howard, 1989; Liogier, 1995; Burkill, 2000;

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Bonde, 2001; Stevens et al., 2001). It is very difficult to differentiate between species of *Datura,* because some species, such as *D. wrightii* and *D. inoxia*, are almost similar in appearance. They can change their size and shape of leaf, flower and fruit depending on location. When growing in a moist location, they grow half as tall as a person, but when growing in a very dry location, they only grow into a thin and short plant (Preissel and Preissel, 2002).

All *Datura* plants were commonly known to contain tropane alkaloids such as scopolamine, hyoscyamine and atropine (Oliver, 1986). Because of the presence of these substances, it has been used for a long time in some cultures as a poison and hallucinogen (Adams and Garcia, 2005). It is also widely used in phytomedicine to cure diseases and was regarded as antispasmodic, intoxicant, germicidal, anodyne antipyretic, antiseptic, antiphlogistic, antiproliferative, antidiarrhoeal, antihelminthic, alexiteric and useful in leucoderma, skin disorders, insanity, catarrh, ulcers, bronchitis, heart disease, jaundice and for fever and piles (Agharkar, 1991; Duke and Ayensu, 1985; Ali and Shuab, 1996; Dabur et al., 2004; Chopra et al., 1968, 1986).

In the present review, we studied antibacterial compounds from different species of *Datura*

Antibacterial compounds in *Datura Inoxia*

D. inoxia is also known as thorn-apple, moonflower, sacred *Datura*. The species was first described by English botanist Philip Miller in 1768. Many studies were done on their antibacterial activity and phytocompounds. Gachande and Khillare (2013), showed antimicrobial activity of ethanolic leaf extract of *D. inoxia* against *Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Proteus vulgaris and Salmonella typhi.* Jamdhade et al. (2010) studied the antibacterial activity of aqueous extracts of different parts of *D. inoxia* (root, stem, leaf, seed and fruit coat) against five human pathogenic bacteria viz. *Bacillus megaterium, Bacillus cereus, E. coli, S. typhi* and *S. aureus.* The results indicated that aqueous extract of leaf showed potential antibacterial activity against the tested pathogens. Kaushik and Goyal (2008), also investigated antibacterial activity of *D. inoxia* (leaf, stem and root) ethanol, methanol, hexane and ethyl acetate extracts against Gram-negative bacteria (*E. coli* and *S. typhi*) and Gram-positive bacteria (*B. cereus*, *B. subtilis* and *S. aureus*). Extracts prepared from leaves were shown to have better efficacy than stem and root extracts. Among all the extracts, methanolic extract was found to be most active against all the bacterial species tested.

Alwan et al. (2011), studied *in vitro* antibacterial activity of *D. innoxia* dried leaves against *E. coli* and *S. aureus.* The antibacterial activity of *D. innoxia* ethanolic extract showed that *E. coli* was more sensitive than *S. aureus*, also *E. coli* was more sensitive to ciprofloxacin. Phytochemical analysis of both *D. innoxia* powder leaves and alcoholic extract revealed the presence of alkaloids, phenols, glycosides, tannins, resins, saponins, flavonoids and steroids. Mathur et al. (2013), studied antibacterial activity of aqueous and ethanolic extracts of different parts of *D. inoxia* (leaves, fruits, roots and flowers) against pathogenic bacteria *E. coli, Pseudomonas aeruginosa* and *S. aureus.* Both aqueous and ethanolic extracts of the plant parts have shown mild to strong antimicrobial activity. Joshi and Kaur (2013), worked on antimicrobial activity of ethanol, methanol and aqueous extract of *D. innoxia* leaf against four pathogenic bacteria: *E. coli, Staphylococcus epidermidis, P. aeruginosa,* and *B. subtilis.* Results showed that the extracts of *D. innoxia* have potent antimicrobial activity against pathogens. Phytochemical analysis revealed the presence of alkaloids, flavonoids and tannins in the extract.

Antibacterial compounds in *Datura metel*

This species is also known as devil's trumpet, it was first described by Linnaeus in 1753 and studied by many scientists for its antibacterial properties and phytocompounds. Okwu and Igara (2009), isolated and identified a new antibacterial steroidal alkaloid (5^1, 7^1 dimethyl 6^1– hydroxyl 3^1, phenyl 3 α-amine β- yne sitosterol (Figure 1 and Table 1) from *D. metel* leaves ethanolic extract using ^{13}C, ^1H NMR, IR and MS spectroscopic data. Compound showed antibacterial activity against *S. aureus, P. aeruginosa, Proteus mirabis, S. typhi, B. subtilis* and *K. pneumonia* but could not inhibit *E. coli.*

Vadlapudi and Kaladhar (2012), evaluated antimicrobial activity of aerial parts of *D. metel* L. against the resistant pathogens such as *L. acidophilus, P. marginalis, Pseudomonas syringae, P. aeruginosa, S. mutans, Streptococcus salivarius* and *S. aureus*. The plant parts were extracted using methanol, hexane and chloroform solvents. Results of agar well diffusion method revealed that methanol and chloroform extracts exhibited promising antimicrobial activity than hexane extracts.

Ethanolic extract from leaf of *D. metel* showed potential antimicrobial activity against *B. subtilis, E. coli, S. aureus, P. vulgaris* and *S. typhi* (Gachande and Khillare, 2013). Sakthi et al. (2011), studied antibacterial potentiality of ethanol and ethyl acetate extracts of mature leaves of *D. metel* against *S. aureus, B. subtilis, B. cereus, E. coli, S. typhi, Shigella flexneri, Klebsiella pneumoniae, Vibrio cholera* and *P. aeruginosa*. The ethanol extract of *D. metel* showed maximum zone of inhibition against *P. aeruginosa, E. coli* and *B. subtilis*. *S. aureus* showed less zone of inhibition. The ethyl acetate extract of *D. metel* showed maximum zone of inhibition against *E. coli*. There was no zone of inhibition against *P. aeruginosa*. Phytochemical analysis revealed the presence of compounds like alkaloids, tripenoid, steroids, flavonoid,

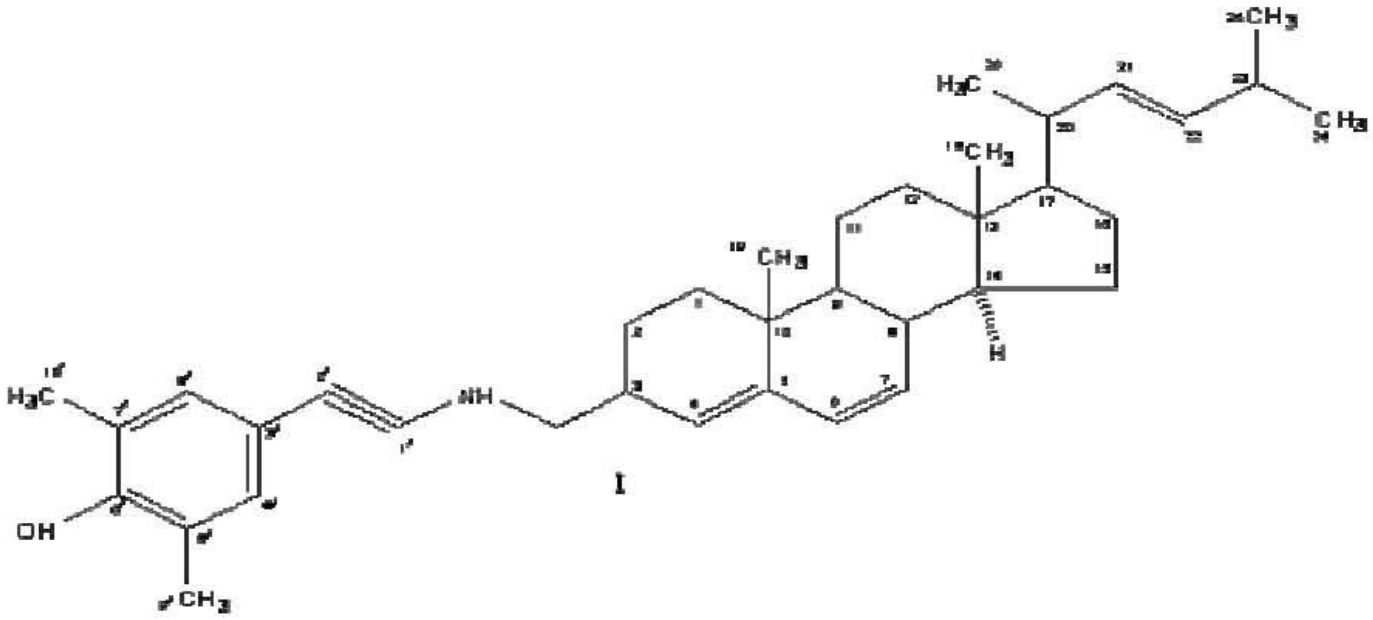

Figure 1. Steroidal alkaloid - 5¹, 7¹ dimethyl 6¹– hydroxyl 3¹, phenyl 3 α- amine β- yne sitosterol (Okwu and Igara, 2009).

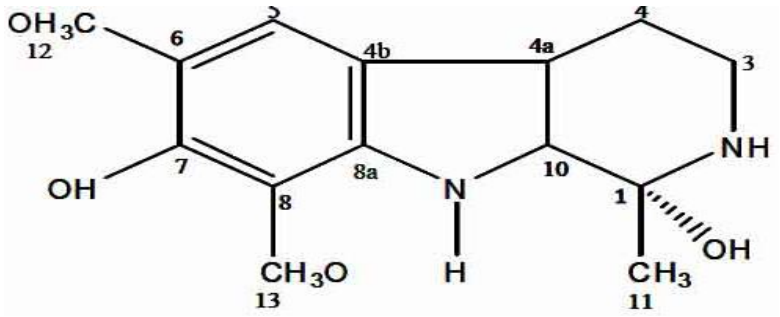

Figure 2. β-Carboline alkaloid-1,7-dihydroxy-1-methyl 6,8-dimethoxy β-carboline (Okwu and Igara, 2011).

triterpenes, phenolic compounds and tannins in the extracts showing antibacterial properties.

Jamdhade et al. *(*2010), investigated antibacterial activity of aqueous extracts of different parts of *D. metel* (root, stem, leaf, seed and fruit coat) against five human pathogenic bacteria viz. *Bacillus megaterium, B. cereus, E. coli, S. typhi* and *S. aureus.* The results indicated that aqueous extract of leaf were most effective against all the tested pathogens. Okwu and Igara (2011), isolated a new β-carboline alkaloid (1, 7 dihydroxy-1- methyl 6, 8 dimethoxy β -carboline) (Figure 2 and Table 1) from *D. metel* (leaves). The structure was analyzed using NMR spectroscopy in combination with IR and MS spectral data. Antibacterial studies showed that the isolated compound successfully inhibited the *P. aeruginosa, K. Pneumonia, S. aureus, P. mirabilis, E. coli, B. subtilis and S. typhi.*

Bharathi et al. (2010), studied antimicrobial activity of ethyl acetate and methanol extracts of *D. metel* leaf, stem and root against HIV associated opportunistic infections causing bacterial pathogens. All parts of plant extracts showed inhibitory activity against *P. aeruginosa, K. pneumoniae, E. coli, S. aureus* and *S. typhi,* but methanol leaf extract showed better antibacterial activity. Phytochemical screening of the plant revealed the presence of saponins, tannins, phenolic compounds, alkaloids, carbohydrates, anthraquinones, protein and aminoacids, fixed oil and fats, glycosides. Akharaiyi (2011) investigated antibacterial efficacy of crude aqueous and ethanol extracts of leaf, stem bark and roots of *D. metel* against *S. hemolytic, P. aeruginosa, E. coli, S. aureus, K. pneumoniae, B. cereus, S. typhi* and *S. dysenteriae.* All the test organisms were susceptible to the plant aqueous and ethanol extracts with various degree of sensitivity. The root extracts of the plants showed no antibacterial activity while the leaf extracts exhibited more therapeutic effect than the stem bark

Table 1. Summary of review showing comparative work of different scientist in different species of *Datura*.

S/N	Species	Plant part	Solvent	Pathogens effected	Nature of phytochemicals	Molecular formula/name of compound	Instrument used	Reference
1	*Datura metel*	Leaf	Ethanol	*Staphylococcus aureus, Pseudomonas aeruginosa, Proteus mirabis, Solmonella typhi* and *Bacillus subtilis*	Steroidal alkaloid	C36 H46 O2N (5^1, 7^1 dimethyl 6^1– hydroxy 3^1, phenyl 3 α - amine β – ynesitosterol)	^{13}C, ^1H NMR, IR and MS spectroscopic data.	Okwu and Igara (2009)
		Leaf	Ethanol	*Klebsiella pneumonia Pseudomonas aeruginosa, Klebsiella pneumonia, Staphylococcus aureus, Proteus mirabilis, Escherichia coli, Bacillus subtilis* and *Salmonella typhi*	β-carboline alkaloid	C14 H20 O4 N2 (1, 7 dihydroxy-1-methyl 6, 8 dimethoxy β-carboline)	NMR spectroscopy in combination with IR and MS spectral data	Okwu and Igara (2011)
		Leaf	Ethanol	*Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Proteus vulgaris* and *Salmonella typhi*	-	-	-	Gachande and Khillare (2013)
		Leaf	Ethanol and ethyl acetate	*Pseudomonas aeruginosa, Escherchia coli* and *Bacillus subtilis*	Alkaloids, tripenoids, steroids, flavonoids, triterpenes, phenolic compound and tannins	-	-	Sakthi et al. (2011)
		Leaf	Methanol	*Pseudomonas aeruginosa, Klebsiella pneumonia, Escherichia coli, Salmonella typhi*and *Staphylococcus aureus*	Saponins, tannins, phenolic compound, alkaloids, carbohydrates, anthraquinones, protein and amino acid, fixed oil and fats, glycosides	-	-	Bharathi et al. (2010)
		Leaf	Aqueous, ethanol	*Streptococcus* β *hemolytic, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, Bacillus cereus, Salmonella typhi*and *Streptococcus dysenteriae*	Saponins, flavonoids, tannins, phenols, alkaloids, glycosides, terpenoids and steroids	-	-	Akharaiyi (2011)

Table 1. Contd

		Aerial parts	Methanol and chloroform	*Lactobacillus acidophilus, Pseudomonas marginalis, Pseudomonas syringae, Pseudomonas aeruginosa, Streptococcus mutans, Steptococcus salivarious* and *Staphylococcus aureus*.	-	-	-	Vadlapudi and Kaladhar (2012)
		Leaf	Aqueous	*Bacillus megaterium, Bacillus cereus, Escherichia coli, Salmonella typhi* and *Staphylococcus aureus.*	-	-	-	Jamdhade et al. (2010)
		Leaf	Ethanol	*Bacillus subtilis, E.coli, Staphylococcus aureus, Proteus vulgaris* and *Salmonella typhi*	-	-	-	Gachande and Khillare (2013)
		Leaf	Ethanol	*Pseudomonas aeruginosa, Klebsiella pneumonia* and *Escherichia coli*	Saponins, tannins, alkaloids and glycosides	-	-	Banso and Adeyemo (2006)
		Leaf	Ethanol	*Pseudomonas aeruginosa*	-	-	-	Kumar et al. (2010)
		Branches and leaves	Benzene	*Enterobacter*, *Micrococcus luteus, Pseudomonas aeruginosa*, *E. coli*, *Staphylococcus aureus* and *Klebsiella pneumonia.*	-	-	-	Gul et al. (2012)
2	*Datura stramonium*							
		Leaf	Ethanol	*Staphylococcus aureus, Salmonella typhi* and *Pseudomonas aeruginosa*	Alkaloids, glycosides, reducing sugars, tannins, steroids, terpenoids, phenols, flavonoids, proteins, saponins, amino acids	-	-	Reddy et al. (2009)
		Leaf, fruit, stem, root and callus	Methanol	*Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa.*	-	-	-	Sharma et al. (2013)
		Leaf	Methanol	*Staphylococcus aureus, Escherichia coli*, *Pseudomonas aeruginosa*	Flavonoids, phytosterols and alkaloids	-	-	Sharma and Sharma (2013)

Table 1. Contd

		Leaf	Aqueous	*Bacillus megaterium, Bacillus cereus, Escherichia coli, Salmonella typhi*and *Staphylococcus aureus*	-	-	-	Jamdhade et al. (2010)
		Leaf	Ethanol	*Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Proteus vulgaris* and *Salmonella typhi*	-	-	-	Gachande and Khillare (2013)
		Leaf	Ethanol	*Escherichia coli and Staphylococcus aureus*	alkaloids, phenols, glycosides, tannins, resins, saponins, flavonoids and steroids	-	-	Alwan et al. (2011)
		Leavs,fruit, root and flower	Aqueous and ethanol	*Escherichia coli, Staphylococcus aureus and Pseudomonas aeruginosa*	-	-	-	Mathur et al. (2013)
3	*Datura innoxia*							
		Leaf	Ethanol, methanol and aqueous	*Escherichia coli, Staphylococcus epidermidis, Pseudomonas aeruginosa,* and *Bacillus subtilis.*	Alkaloids, flavonoids and tannins	-	-	Joshi and Kaur (2013)
		Leaf	Methanol	*Escherichia coli, Salmonella typhi,Bacillus cereus*, *Bacillus subtilis*and *Staphylococcus aureus*	-	-	-	Kaushik and Goyal (2008)
		Leaf	Aqueous	*Bacillus megaterium, Bacillus cereus, Escherichia coli, Salmonella typhi*and *Staphylococcus aureus*	-	-	-	Jamdhade et al. (2010)
		Leaf	Ethanol	*Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Proteus vulgaris, Salmonella typhi*	-	-	-	Gachande and khillare (2013)
4	*Datura ferox*							
		Leaf, stem and fruit coat	Aqueous	*Bacillus megaterium, Bacillus cereus, Escherichia coli, Salmonella typhi* and *Staphylococcus aureus*	-	-	-	Jamdhade et al. (2010)
5	*Datura ceratocaula*	-	-	-	-	-	-	- -

Table 1. Contd

6	*Datura discolor*	-	-	-	-	-	-	-	-
7	*Datura leichhardtii*	-	-	-	-	-	-	-	-
8	*Datura quercifolia*	-	-	-	-	-	-	-	-
9	*Datura wrightii*	-	-	-	-	-	-	-	-

extracts. Phytochemical analysis revealed the presence of saponin, flavonoids, tannins, phenols and alkakoids, glycocides, steroids and terpenoids of which steroids, terpenoids and tannins were absent in the ethanol extract.

Antibacterial compounds in *Datura stramonium*

D. stramonium is commonly known as jimsonweed, thorn-apple and moon flower. It was scientifically described and named by Swedish botanist Carl Linnaeus in 1753 and was studied by many scientists for their antibacterial properties and phytocompounds. Ethanolic leaf extract from *D. stramonium* showed potential antimicrobial activity against *B. subtilis, E. coli, S. aureus, P. vulgaris and S. typhi* (Gachande and Khillare, 2013). The antimicrobial activity of *D. stramonium* (leaf ethanolic extract) studied by Banso and Adeyemo (2006), were assessed against *P. aeruginosa, K. pneumonia* and *E. coli.* The plant showed significant antibacterial activity against the tested pathogens. Phytochemical analysis revealed the presence of saponins, tannins, alkaloids and glycosides. Kumar et al. (2010), investigated the antibacterial activity of *D. stramonium* (leaf ethanolic extract) against three standard microorganisms, *E. coli, Bacillus amyloliquefaciens* and *P. aeruginosa.* The plant has been found to show antibacterial activity against *P. aeruginosa* but not against *E. coli* and *B. amyloliquefaciens*.

The *in vivo* and *in vitro* antimicrobial activity of different parts of *D. stramonium* (leaf, fruit, stem, root and callus) were investigated by Sharma et al. (2013), against *E. coli, S. aureus* and *P. aeruginosa.* All the solvent extracts (ethanol, methanol, petroleum ether and aqueous) showed significant antimicrobial activity against all the tested micro-organisms. Methanolic extract was most active against all micro-organisms, whereas all the extracts showed significant activity against *P. aeruginosa.* Jamdhade et al. *(*2010), studied antibacterial activity of aqueous extracts of different parts of *D. stramonium* (root, stem, leaf, seed and fruit coat) against five human pathogenic bacteria viz. *B. megaterium, B. cereus, E. coli, S. typhi* and *S. aureus.* The results indicated that aqueous extract of leaf were most effective against all the tested pathogens.

The phytochemical analysis and antimicrobial activities of *in vitro* grown callus *and D. stramonium* methanolic extracts of root, stem, leaves, fruits, callus and crude metabolite rich fractions were studied by Sharma and Sharma (2013), against *E. coli*, *S. aureus* and *P. aeruginosa*. The crude extracts from *D. stramonium* were analyzed for moisture, starch, carbohydrate, ascorbic acid, lipid, proline, crudeprotein, phenols, DNA, RNA, chlorophyll and carotenoid in plant parts and callus. The results indicated that methanolic leaf extract exhibited better antimicrobial activity against *S. aureus, E. coli*, *P. aeruginosa.* In the metabolite rich fraction (flavonoids, phytosterols and alkaloids), greatest bactericidal activity was exhibited by flavonoids against *P. aeruginosa.* Gul et al. (2012), studied antibacterial activity of *D. stramonium* branches and leaves samples in three different solvents benzene, chloroform and ethanol against *Enterobacter*, *Micrococcus luteus, Pseudomonas aeruginosa*, *E. coli*, *S. aureus* and *K. pneumonia.* All the solvent extracts showed significant antibacterial activity against tested pathogens. Comparative minimum inhibitory concentration of benzene, chloroform and ethanol extract determined that benzene extract was very effective against all bacterial strains. The antimicrobial activity of ethanolic leaf extract of *D. stramonium* L. (Solanaceae) were evaluated by Reddy et al. (2009), against *K. pneumoniae, S. typhi, S. aureus, P. vulgaris, P. aeruginosa* and *E. coli.* The alcoholic extract of the plant has shown promising antimicrobial activity and effectively inhibited the growth rate of *S. aureus, S. typhi, P. aeruginosa* and *E. coli* but *K. pneumonia* and *P. vulgaris* were found resistance. Phytochemical studies revealed the presence of alkaloids, glycosides, reducing sugars, tannins, steroids, terpenoids, phenols, flavonoids, proteins, saponins and amino acids in the extract.

Antibacterial compounds in *Datura ferox*

This species of *Datura* is also called long-spined thorn-apple and fierce thorn apple. The species was first described in 1756 by Linnaeus and has been studied by various scientists for their antibacterial activity and phytocompounds. Gachande and Khillare (2013), investigated that *D. ferox* (leaf ethanolic extracts) showed potential antimicrobial activity against *B. subtilis, E. coli, S. aureus, P. vulgari and S. typhi*. The antibacterial activity of aqueous extracts of different parts of *D. ferox* (root, stem, leaf, seed and fruit coat) were investigated by Jamdhade et al. *(*2010), against five human pathogenic bacteria viz. *B. megaterium, B. cereus, E. coli, S. typhi* and *S. aureus.* The results indicated that aqueous extract of stem, leaf and fruit coat were most effective against all the tested pathogens.

Antibacterial compounds in other *Datura* species

No work on antibacterial activity or antibacterial phytocompounds was found reported from *Datura leichhardtii*, *Datura quercifolia*, *Datura discolor*, *Datura ceratocaula* and *Datura wrightii*.

CONCLUSION

Different species of *Datura* contains variety of antibacterial compounds that can be formulated into a drug (herbal antibiotic). From the above review and Table 1, it is clear that maximum work has been performed on antibacterial properties of *D. metal, D. inoxia, D. stramanium* and few on *D. ferox* but no such work has been found in *D. leichhardtii*, *D. quercifolia*, *D. discolor*, *D. ceratocaula* and *D. wrightii*. Also, there were very less studies which show characterization of phytocompounds. *Datura metel* is the only species in which characterization of antibacterial compounds has been observed. Many antibacterial work on *Datura* plant were found against *E. coli, S. aureus, P. aeruginosa* but very few against *B. subtilis, S. typhi, K. pneumonia*. It has been observed that leaves are the most promising part as source for antibacterial activities and also, ethanol and methanol were the most promising solvents to extract phytocompounds showing broad spectrum antibacterial properties. In the present review, alkaloids from *Datura* plant were found to be a major secondary metabolites which show antibacterial properties. Other phytocompounds such as terpenoid, phenols, glycosides, tannins, resins, saponins, flavonoids and steroids also act as promising agents. As *Datura* plant is known for its poisonous and hallucinogen properties since long time, there is need to test its compounds for safety purpose before developing a antibacterial drug (herbal antibiotic) from it.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Lithium chloride affects mycelial growth of white rot fungi: Fungal screening for Li-enrichment

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The enrichment of edible mushrooms with lithium (Li) may be a strategy to provide forms of lithium that are more soluble and bioavailable for humans. Therefore, it is important to determine which species of fungi are able to grow in the presence of Li, and which concentrations of Li allow fungal growth. Twelve white rot fungi strains were grown in potato dextrose agar media, supplemented with 0 to 1.65 g L^{-1} lithium chloride (LiCl). The fungal growth rate, morphological alterations of the colonies, changes in the length of the lag phase, fungal dry mass, changes in hyphae diameter and cell length were evaluated. Most fungi had decrease in their growth rates and dry mass, and had macroscopic/microscopic morphological alterations at increasing LiCl concentration. Generally, the fungi were sensitive to LiCl. However, *Pholiota nameko* was moderately tolerant to LiCl and *Pleurotus ostreatusroseus* tolerate the highest LiCl level tested, suggesting that it is the most appropriate fungus for Li-enrichment.

Key words: Fungal enrichment, mycelial morphology, screening.

INTRODUCTION

Lithium has an irregular distribution in the Earth's crust (Rybakowski, 1995; Aral and Vecchio-Sadus, 2008). As a result, some populations have a low dietary lithium intake (Rybakowski, 1995). Low levels of lithium in the blood have been related to the occurrence of some psychiatric disorders, (Schrauzer and Shrestha, 1990; Severus et al., 2009) and lithium compounds such as, lithium carbonate, are commonly used to treat bipolar disorder (Rapoport et al., 2009). It has been shown that the rates of rape and homicide are higher in counties with low levels of lithium in drinking water supplies (Dawson et al., 1970). The development of foods with high lithium availability could be a way to increase lithium intake.

Some Basidiomycetes are able to absorb and accumulate minerals, such as lithium, in mushrooms (Widmer, 1999; Bayramoglu et al., 2002; Figlas et al., 2010; de Assunção et al., 2012). The highest lithium content of wild mushroom was 12 mg kg^{-1} found in *Thelephora vialis* (Yin et al., 2012). However, the lithium content found in wild mushroom seems to vary among different fungi species and regions (Vetter, 2005; Yin et al., 2012; Falandysz and Borovička, 2013). The content

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of lithium in mushrooms found in China (Yin et al., 2012) were higher than values found in Hungary and Italy (Vetter, 2005; Giannaccini et al., 2012). This could be due to a higher lithium concentration in China soil than Hungary and Italy soils. Indeed, de Assunção et al. (2012) showed that the lithium levels found in the Li-enriched mushrooms were proportional to the lithium levels found in the substrate. These authors also showed that the enrichment of substrate with a soluble lithium salt (LiCl), do not affect the biological efficiency (productivity) of *Pleurotus ostreatus.*

Lithium is toxic, depending on the concentration, to animals, plants and microorganism (Aral and Vecchio-Sadus, 2008) which can be limitant for fungal Li-enrichment. Richter et al. (2008) tested the growth of 40 species of fungi in culture media with 1.5, 3 and 6 g L^{-1} of LiCl and observed that the growth rate of most of the fungi greatly decreased at 3 g L^{-1} of LiCl and above. The white-rot fungus, *P. ostreatus*, decreased its growth rate by 90% in 1.5 g L^{-1} of LiCl and its growth was inhibited at higher concentrations of LiCl (Richter et al., 2008). Wildman (1991) also observed that culture media with 6 g L^{-1} of LiCl inhibited *Trichoderma* spp. growth. As the sensitivity or tolerance of fungi to LiCl varies considerably, it is important to determine the concentration of this salt that allows mycelial growth and development. This will allow us to choose the fungi species more appropriate for fungal Li-enrichment.

MATERIALS AND METHODS

Microorganism

All the fungi species chose for the screening are cultivated at industrial scale. We also selected only fungi that produce mushrooms on non-composted substrate because this procedure is easier and cheaper than on composted substrates. The fungi used were *P. ostreatus* (PLO 06), *P. ostreatus* (P.98), *Pholiota nameko* (PH 01), *Pleurotus ostreatusroseus* (PLO 13), *Pleurotus citrinopiliatus* (PLO A), *Grifolla* sp. (GF), *Grifolla frondosa* (GF-JP), *Ganoderma subamboinense* var. *laevisporum* (GR 117), *Pleurotus eryngii* (PLE 04), *Hericium herinaceum* (HE 01), *Lyophillum shimeji* (LY 01) and *Lentinula edodes* (UFV 73). All these fungi belong to the collection of the Laboratório de Associações Micorrízicas, Departamento de Microbiologia, BIOAGRO, UFV. Two isolates of *P. ostreatus* were included by presenting different commercially important characteristics, such as white cap by PLO 06 and the light brown cap by P.98. Mushrooms of these species are sources of protein, fiber, vitamins and minerals (Barros et al., 2008; de Assunção et al., 2012). We incubated these fungi in potato dextrose agar (PDA, Fluka Analytical, St. Loius, Missouri, USA) at 22 ± 1°C for seven days.

Culture media and cultivation conditions

Fungi were grown on PDA containing 0, 0.30, 0.60, 0.90, 1.20 or 1.65 g L^{-1} of LiCl. This salt of lithium has previous been used for Li-enrichment of mushrooms (de Assunção et al., 2012). The culture medium was autoclaved at 121°C for 20 min. Plugs of inoculum 5 mm in diameter were cut from the perimeter of an actively growing fungus colony. Inoculum plugs were firmly placed with the mycelium side down in the center of the experimental plates. Six replicate plates were prepared for each LiCl concentration and fungus, and were then incubated at 22 ± 1°C.

Lag phase and growth rate

After incubation, the colonies were observed daily to determine the start of mycelial growth. The fungal growth rate was determined by measuring the colony's diameter in two directions that were perpendicular to each other. Measurements were made for 45 days or until maximum Petri dish colonization. The measurements were made every 48 h for fungi with high growth rates (PH01, PLO 06, P.98, PLO 13, PLO A, PLE 04, GR 117) and every 72 h for fungi with low growth rates (GF, GF-JP, UFV 73, HE and LY 01).

Colony morphology

The morphological characteristics of colonies were qualitatively evaluated using the following criteria: a) medium color alteration, b) colony color change, c) mycelial density decrease and d) colony growth appearance (uniform or uneven).

Hyphae diameter and septa distance

For diameter and septa distance measurements, we used epifluorescence microscopy to observe fungus samples stained with calcofluor. To standardize the measurements, the samples that were used were all young hyphae (hyphae in the border of coverslips). Photos were taken with a digital camera FUJIX HC-300Z and then they were processed with the software Image Pro Plus. For septa distance measurements, we used the photos 400x amplified and for hyphae diameter, we used the photos 1000x amplified. Measurements using photos more amplified are more precise and accurate. All the measurements were made using the software Image Pro Plus. These measurements were made because some fungi alter hyphae morphology under inappropriate environmental conditions and because the microscopic appearance can aid understanding how the fungi adapt to environments with LiCl.

Biomass

To determine the mycelial dry mass, the entire contents of Petri dish (mycelium + culture media) were put in a bottle with distilled water and heated in a water bath (1 to 5 min) to dissolve all culture medium. Then, the solution was filtered and the mycelium was dried in an oven at 80°C until a constant weight was reached.

Statistical analysis

The experiment used a randomized design. The data, except for the macroscopic morphological data, were subjected to analysis of variance, and the averages were compared by Tukey's test ($p < 0.05$) using Saeg software (version 9.1, Universidade Federal de Viçosa).

RESULTS

The fungi were affected differently by the LiCl. Among the

fungi assayed, five strains increased the lag phase (Figure 1). The *H. herinaceum* (HE 01) and *L. shimeji* (LY 01) strains showed a 4- to 5-fold increase in the lag phase at 0.3 g L^{-1} LICl ($P < 0.001$). Strains of both *Grifolla* (GF and GF-JP; $P < 0.001$) and *P. ostreatus* (PLO 06; $P < 0.001$) increased their lag phases at 0.60 and 0.90 g L^{-1} LICl, respectively; however, this increase was only up to 3-fold. The increase in the lag phase at low concentrations of LiCl indicated that *H. herinaceum*, *L. shimeji* and both strains of *Grifolla* are sensitive to LiCl.

The addition of LiCl to the culture media reduced the biomass and growth rates of most fungi (Figures 2 and 3). *P. ostreatus* (PLO 06; $P < 0.001$), *Lentinula edodes* (UFV 73; $P < 0.001$), *H. herinaceum* (HE 01; $P < 0.001$), *L. shimeji* (LY 01; $P < 0.001$), *P. eryngii* (PLE 04; $P < 0.001$) and *P. ostreatus* (P.98; $P < 0.001$) had their growth rates decreased at 0.30 g L^{-1} of LiCl and, for the majority of fungi, this reduction occurred at 0.60 g L^{-1} of LiCl. Ten strains had their growth inhibited by LiCl. Only *P. nameko* (PH 01) and *P. ostreatusroseus* (PLO 13) were able to grow in media enriched with 1.65 g L^{-1} of lithium (Figure 2). Biomass production (Figure 3) seemed to be a parameter more sensitive to LiCl than the growth rate (Figure 2), although sometimes they responded similarly. For *P. citrinopiliatus* (PLO A) and *P. eryngii* (PLE 04), the biomass decreased more than the growth rate. The biomass of *P. nameko* was completely different from the growth rate. While small changes in the growth rate were observed, the biomass began to decrease at 1.20 g L^{-1} LiCl and reduced by 90.4% at the highest level of LiCl tested. Overall, the majority of fungi tested were inhibited by LiCl. Considering the fungal biomass, growth rate and lag phase, *P. nameko* and *P. ostreatusroseus* strains were the fungi that best tolerated LiCl.

Interestingly, LiCl affected the hyphae diameter and the septa distance differently (Figures 4, 5, 1S and 2S). *P. nameko* (PH 01; $P < 0.001$), *Grifolla* sp. (GF; $P = 0.019$), *H. herinaceum* (HE 01; $P = 0.004$), *G. subamboinense* var. *laevisporum* (GR 117; $P = 0.002$) and *P. eryngii* (PLE 04; $P = 0.001$) showed changes in the septa distance and *P. nameko* (PH 01; $P = 0.004$), *G. frondosa* (GF-JP; $P < 0.001$), *P. ostreatusroseus* (PLO 13; $P < 0.001$), *P. citrinopiliatus* (PLO A; $P = 0.004$) and *P. eryngii* (PLE 04; $P < 0.001$) altered their hyphae diameter when LiCl was added to the media culture. Though *L. edodes* (UFV 73; Septa distance $P = 0.112$; hyphae diameter $P = 0.506$) and *L. shimeji* (LY 01; septa distance $P = 0.566$; hyphae diameter $P = 0.108$) did not have altered septa distance or hyphae diameter, *L. edodes* and *L. shimeji* were sensitive to LiCl (Figures 2 and 3).

P. nameko (PH 01), *H. herinaceum* (HE 01), *P. ostreatus*(PLO 06), *L. shimeji* (LY 01), *G. subamboinense* var. *laevisporum* (GR 117), *P. citrinopiliatus* (PLO A) and *P. eryngii* (PLE 04) altered their macroscopic morphology when LiCl was added to the culture media (Figure 3S and Table 1). Additionally, the macroscopic morphology alterations varied among the fungi. A decrease in mycelial density was observed in four fungi (Table 1). This data, plus the dry mass data, clearly show that many fungi tested are sensitive to LiCl. *P. nameko* (PH 01) showed the highest decrease in mycelium density, and it was very difficult to see the hyphae fungus in the Petri dish at 1.65 g L^{-1} of LiCl (Figure 3S). A change in colony color was observed in three of the fungi tested, namely, *P. nameko* (PH 01), *G. subamboinense* var. laevisporum (GR117) and *P. citrinopilatus* (PLO A) (Table 1 and Figure 3S). *P. ostreatus* (PLO 06) and *P. eryngii* (PLE 04) were the only fungi that changed the color of the medium. This may suggest that these species secrete some compound in the presence of LiCl, even at the lowest LiCl level tested.

DISCUSSION

The increase in the lag phase is common when microorganisms are exposed to adverse environmental conditions (Swinnen et al., 2004; Yates and Smotzer, 2007). In this study, the addition of LiCl to the culture media increased the lag phase and decreased the growth rate of most of the fungi tested (Figures 1 and 2), showing that LiCl, at certain concentrations, can be toxic for the fungi. This information is important for production of Li-enriched mushrooms, because colonization of the substrate by the fungus is one of the key steps of mushrooms production. A high growth rate allows fast substrate colonization, reducing the possibility of contaminant growth. As observed by Richter et al. (2008), some common contaminants such as, *Aspergillus niger* and *Trichoderma* sp. are resistant to higher LiCl levels than those used in our experiments. Additionally, quick colonization reduces the incubation time and, reduces the time needed to produce mushrooms.

Knowing which species of white rot fungi are more sensitive to LiCl, it is important when selecting fungi tolerant to LiCl. Here we sorted the fungi in four groups (Table 2). Most fungi tested were sensitive to LiCl. Indeed, when compared with other fungi groups (Richter et al., 2008), basidiomycetes seems to be a group, in general, more sensitive to LiCl. The genus *Pleurotus* seems to be a group slightly more tolerant to LiCl (Table 2). Richter et al. (2008) showed that the *P. ostreatus* strain (MAD 542) was able to grow in 1.5 g L^{-1} of LiCl. However, those authors used media containing 20 g malt, while in this study, we used PDA media, which can influence the LiCl toxicity. Also, the addition of 0.5 g kg^{-1} of LiCl to coffee husks, it does not alter mushroom formation of *P. ostreatus* (de Assunção et al., 2012).

The most tolerant fungi are *P. nameko* (PH 01) and *P. ostreatusroseus* (PLO 13). *P. nameko* did not change its growth rate at any LiCl level tested, although this fungus decreased its dry mass by 70.73% at 1.20 g L^{-1} LiCl

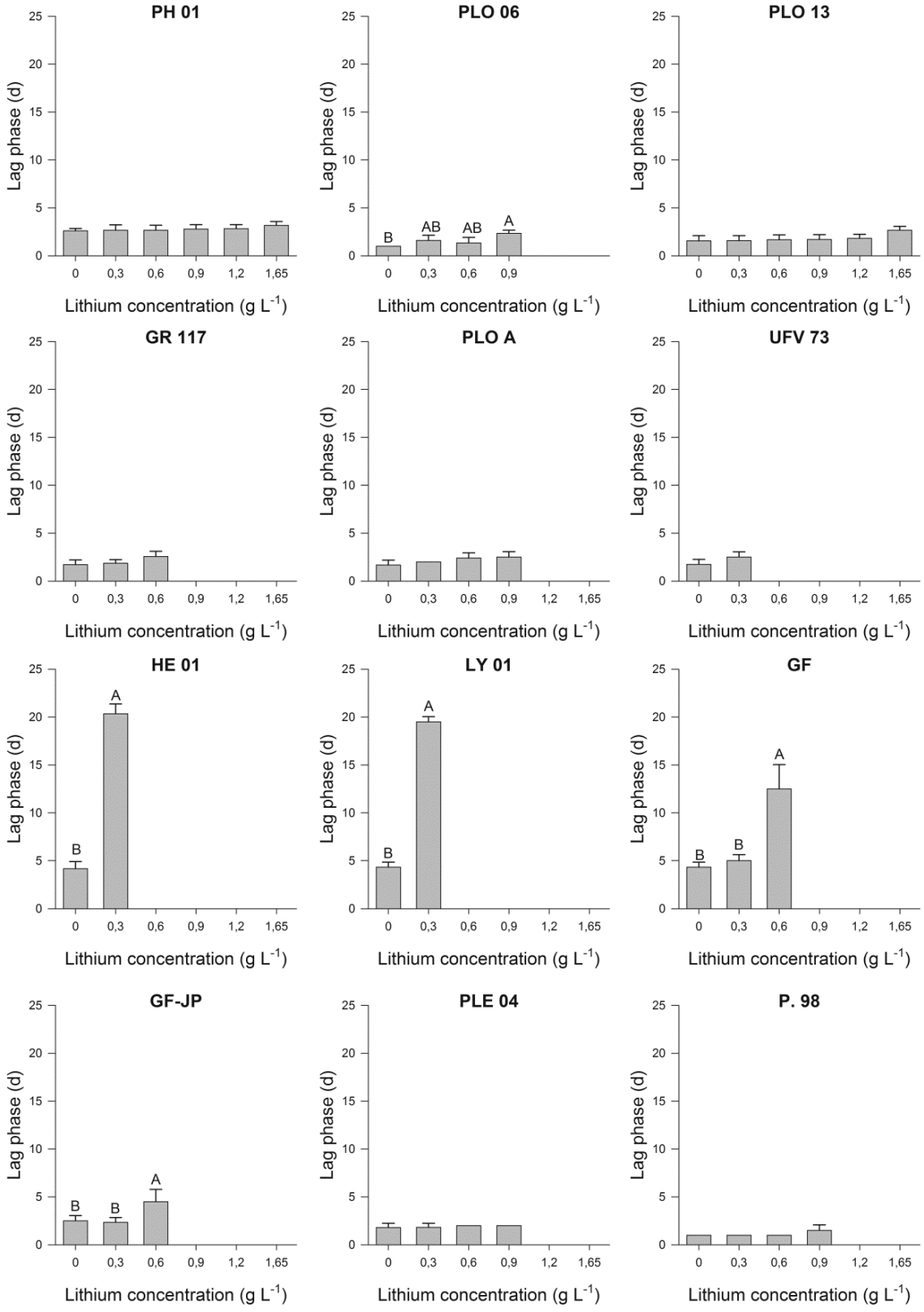

Figure 1. Lag phase of white rot fungi in culture media supplemented with LiCl. Mean growth of 6 plates. Missing bars indicate fungal growth inhibition. Letters above columns compare individual fungi by LiCl level (Tukey´s test; $p \leq 0.05$).

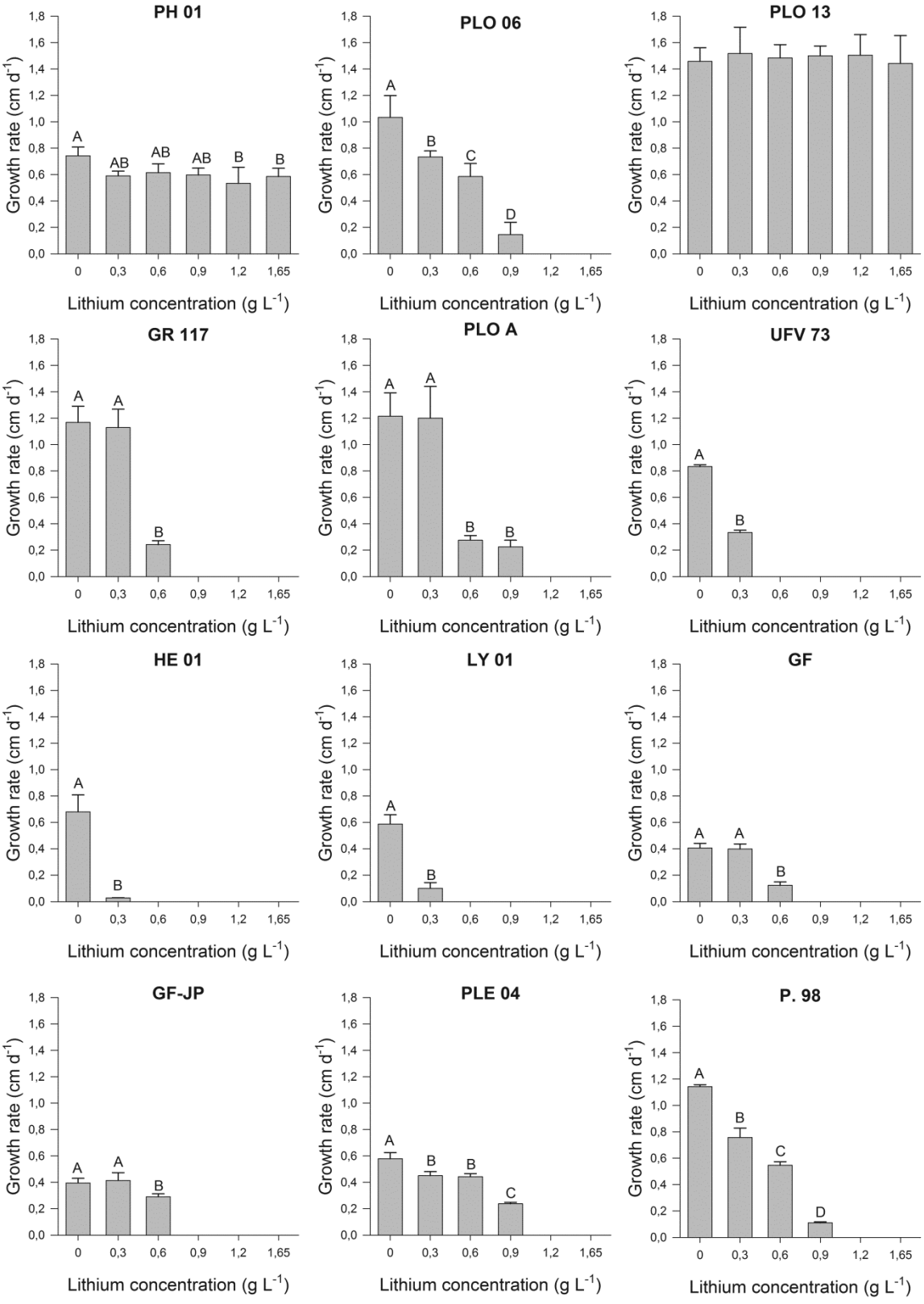

Figure 2. Mycelial growth rate of white rot fungi in culture media supplemented with LiCl. Mean growth of 6 plates. Missing bars indicates fungal growth inhibition. Letters above columns compare individual fungi by LiCl level (Tukey´s test; $p \leq 0.05$).

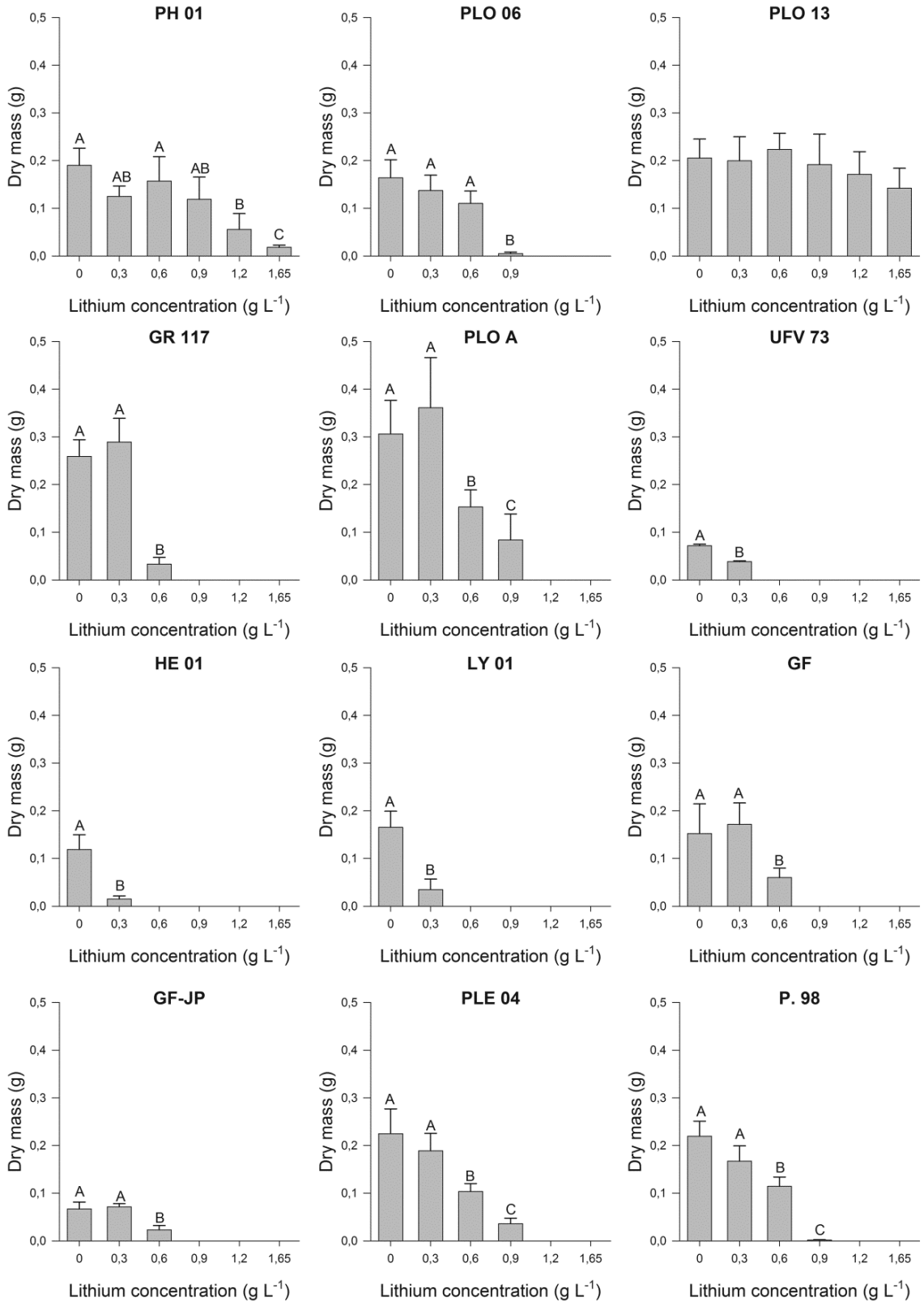

Figure 3. Mycelial dry mass of white rot fungi in culture media supplemented with LiCl. Mean growth of 6 plates. Missing bars indicates fungal growth inhibition. Letters above columns compare individual fungi by LiCl level (Tukey´s test; $p \leq 0.05$).

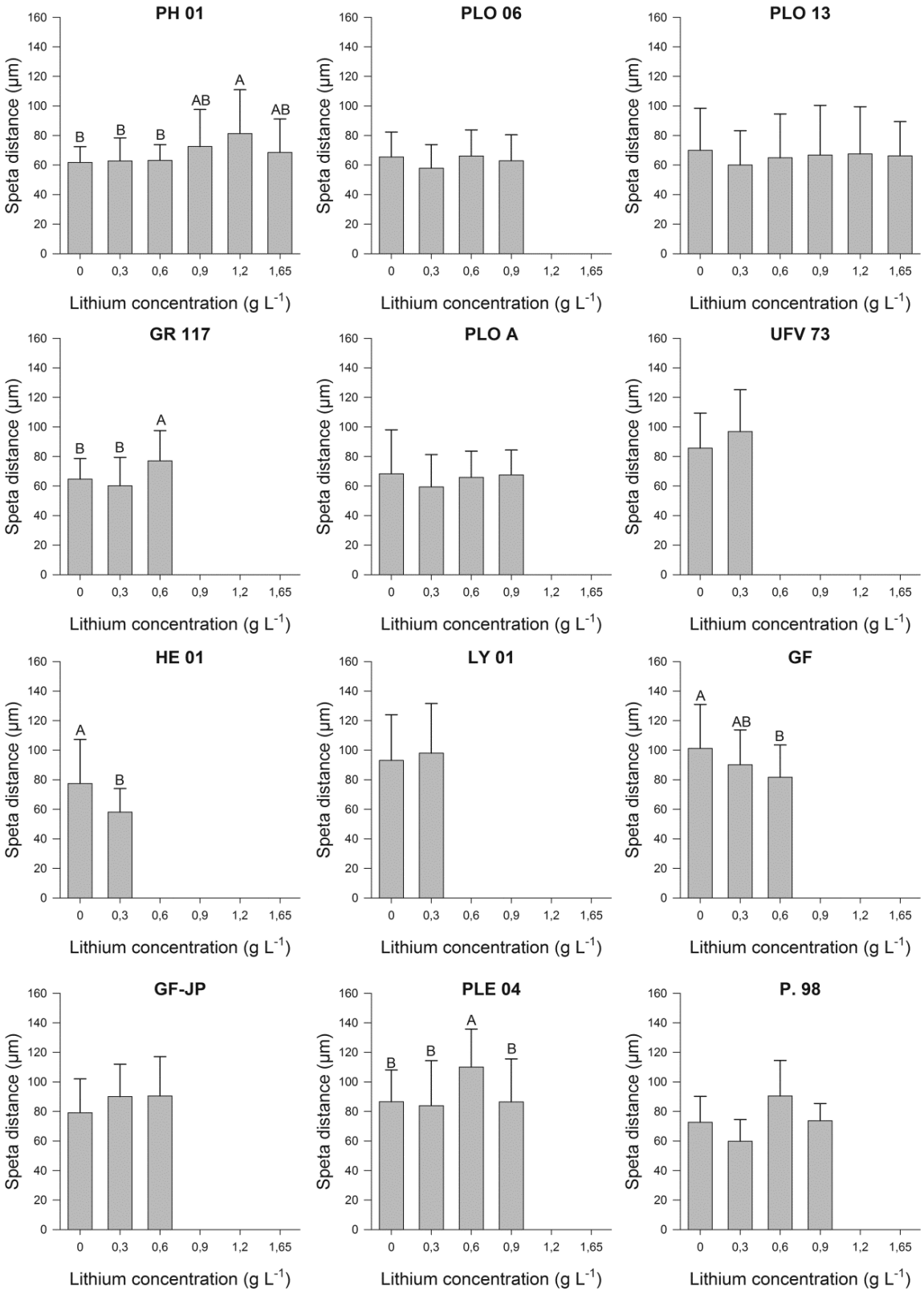

Figure 4. Septa distance of hyphae of white rot fungi in culture media supplemented with LiCl. Mean growth of 6 plates. Missing bars indicate fungal growth inhibition. Letters above columns compare individual fungi by LiCl level (Tukey´s test; $p \leq 0.05$).

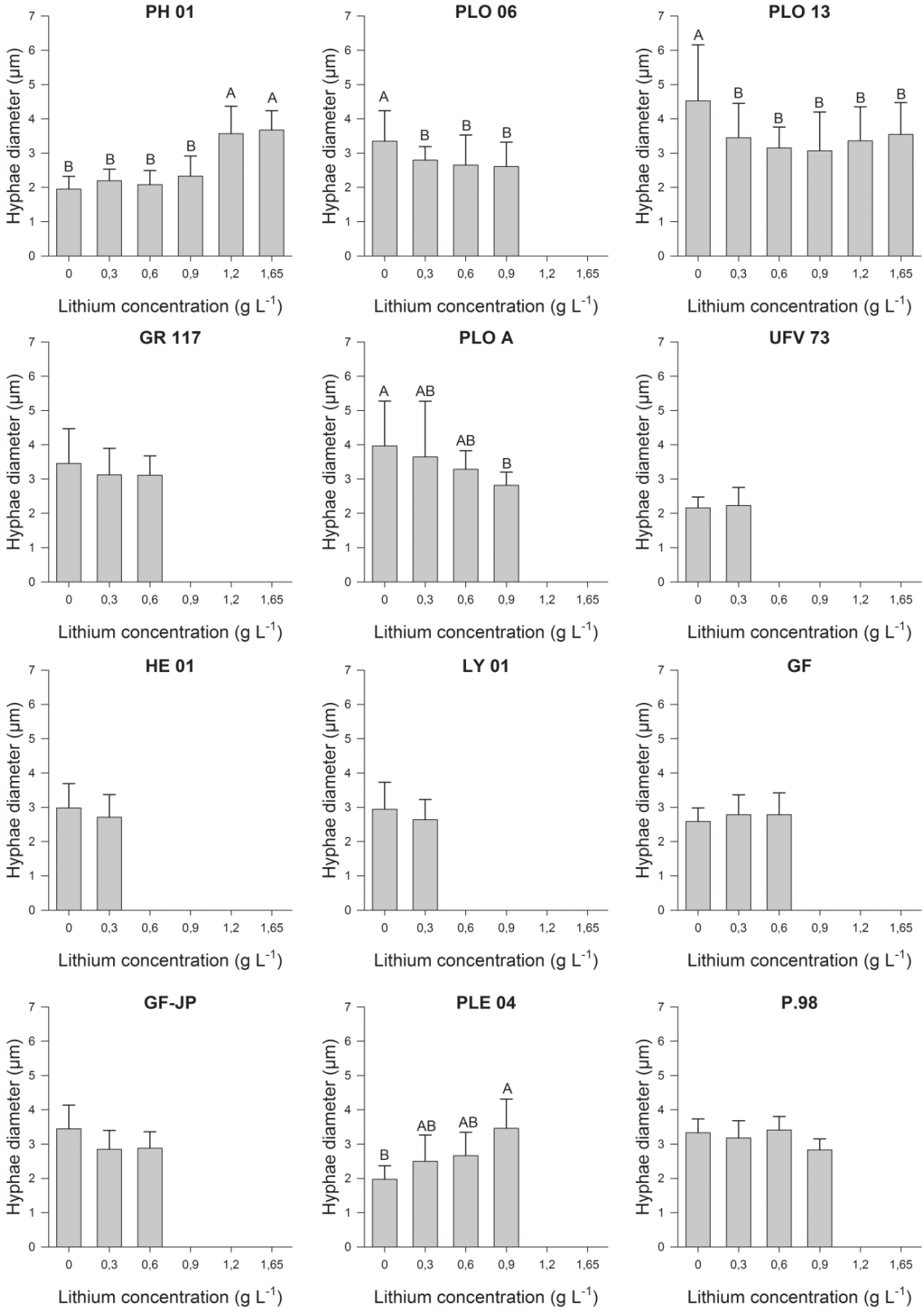

Figure 5. Hyphae diameter of white rot fungi in culture media supplemented with LiCl. Mean growth of 6 plates. Missing bars indicate fungal growth inhibition. Letters above columns compare individual fungi by LiCl level (Tukey´s test; $p \leq 0.05$).

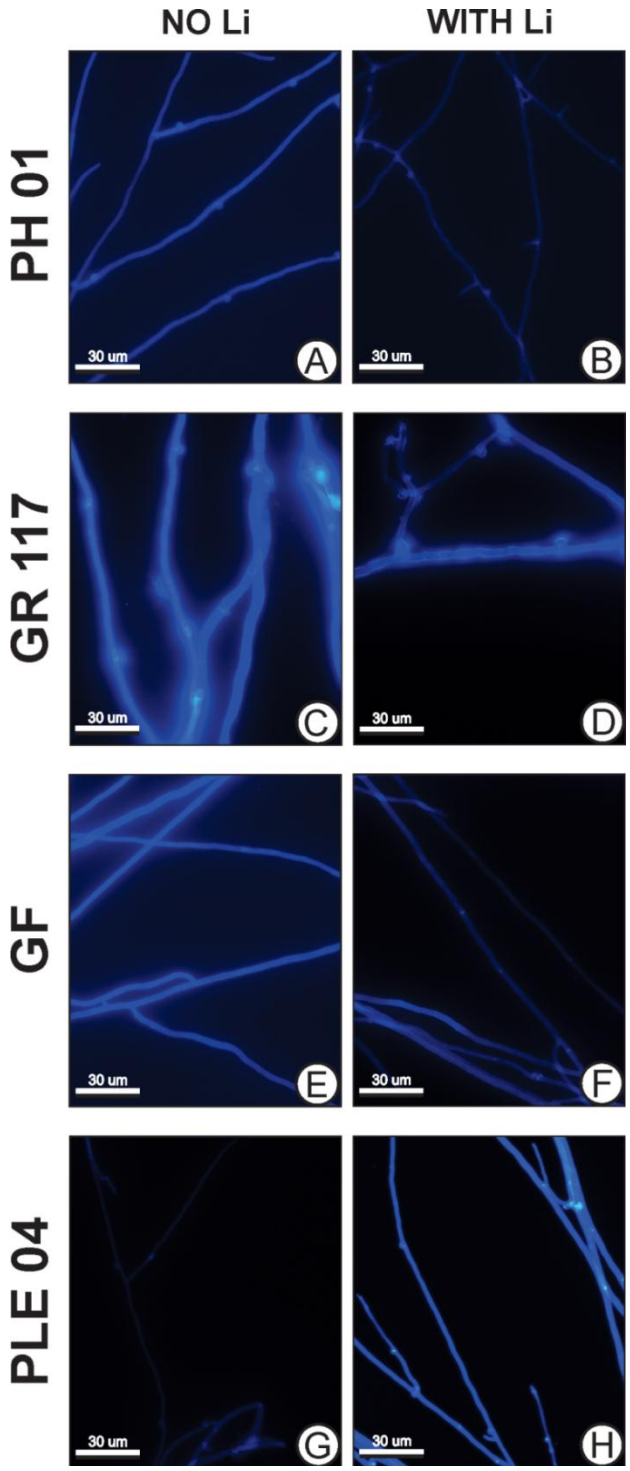

Figure 1S. Septa distance of white rot fungi in culture media supplemented with LiCl.

(Figure 3). This behavior suggests that *P. nameko* directs much of it energy to radial mycelial growth to look for environments with more suitable conditions. *P. ostreatusroseus* tolerated all the LiCl levels tested. Only the hyphae diameter decreased in the presence of LiCl (Figure 4), but the growth rate (Figure 2) and dry mass (Figure 3) did not change. As Li absorption by mushrooms can be linear and is directly related to the concentration of Li available in the substrate (de Assunção et al., 2012), further mycelial and mushroom production studies at higher LiCl level than we tested should be performed with this fungus, because production of mushrooms with high Li concentration can be interesting.

The morphological, macroscopic and microscopic changes were evaluated to begin to understand the response of fungi to LiCl. The modification of colony color suggests that some fungi are producing some compounds which are able to growth in the presence of LiCl. This hypothesis is further supported by the media color modification that occurred during the growth of *P. ostreatus* (PLO 06) and *P. eryngii* (PLE 04) strains (Table 1 and Figure S3). Further metabolomic studies should be performed to identify the intracellular or extracellular compounds responsible for the changes in color.

Some fungi increased their septa distance and hyphae diameter, while others decreased them (Figures 4, 5, 1S and 2S), showing that the fungi uses different adaptive strategies in environments with LiCl. Different taxonomic groups of fungi have previously been shown to decrease their septa distance under stressful conditions (Thrane et al., 1999; Turner and Harris, 1999; McIntyre et al., 2001; Denisov et al., 2011). The septum compartmentalizes the hypha without harming communication among them due to the presence of pores in the septum. (van Driel et al., 2008). When the cell is damage, the fungus may block the septa pores as a way to protect the colony (van Driel et al., 2008). Thus, colony with more septa will be more protected. We suggested that this strategy could be used by some fungi in environments with LiCl. Those fungi strains may increase the number of septa to reduce the time required to stock any damage that the colony may suffer due to the presence of the LiCl. Moreover, this strategy could also be used to reduce the portion of the colony that may suffer some damage.

Alteration in hyphae diameter is another morphological modification made by fungi (McIntyre et al., 2001; Tripathi et al., 2009; Dávila Costa et al., 2011; Denisov et al., 2011). Decreased hyphae diameter may decrease the energy required to produce a cell. The fungi could use this energy to look for a more suitable environment (e.g. redirect this energy for mycelial growth). On the other hand, increased hyphae diameter could be related to increased cell wall thickness. Chitin synthesis has been shown to increase when filamentous fungi are exposed to Calcofluor White (Ram et al., 2004). Additionally, yeast increases the chitin in its cell wall in response to stress (Lagorce et al., 2002). This increase in chitin content may be due to a higher chitin level in the lateral walls.

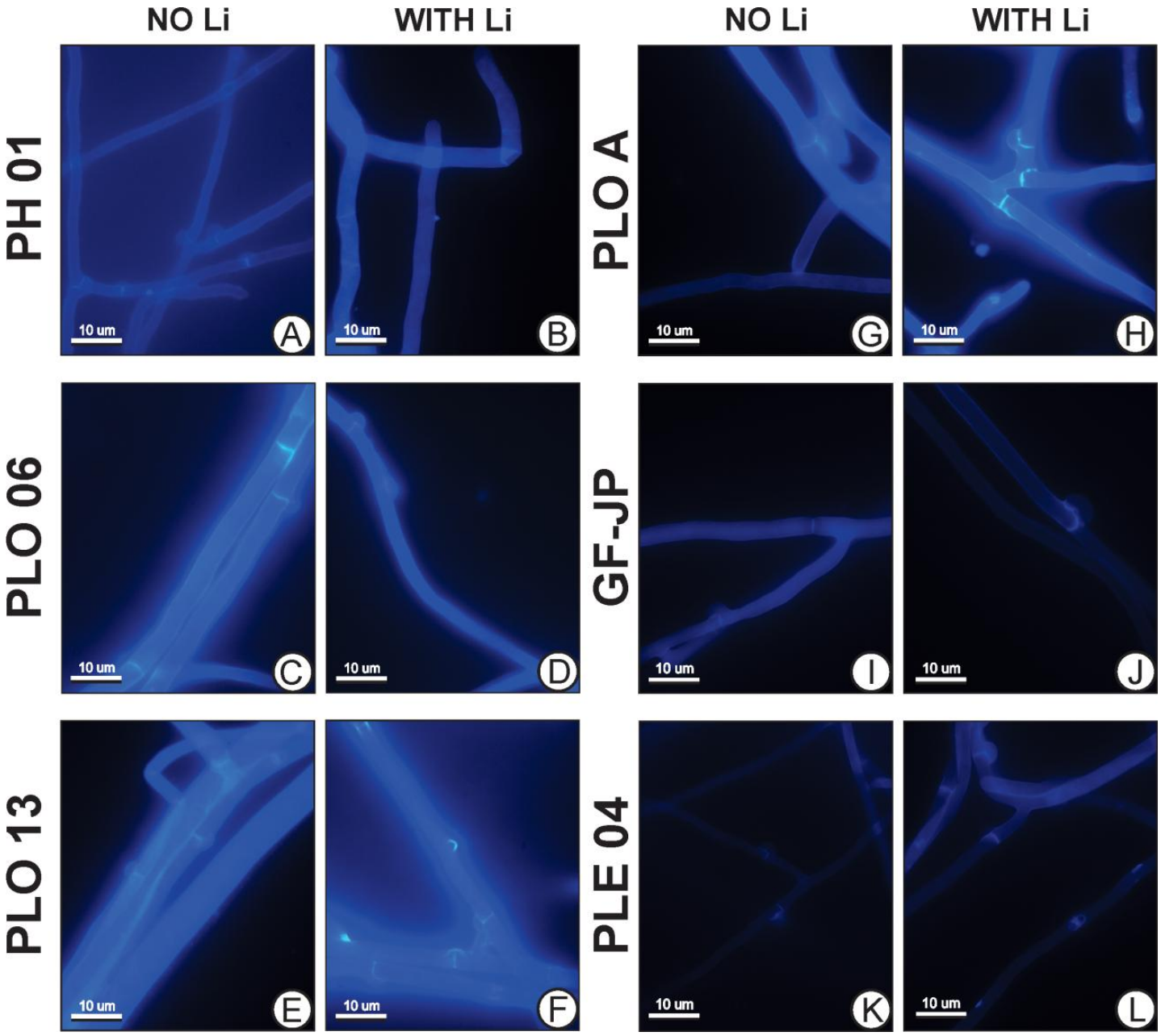

Figure 2S. Hyphae diameter of white rot fungi in culture media supplemented with LiCl.

Therefore, some white-rot fungi may protect the cells increasing the chitin content of those. This could be a strategy to avoid or reduce the entrance of Li into the cell. Increased hyphae diameter may also be related to cell swelling as observed by Lanfranco et al. (2002) where an ericoid ascomycete was treated with Zn. Indeed, the entrance of Li into the cell increases the osmolality of the cell, inducing the entrance of water. This consequently will result in cell swelling. The elucidation of these modifications could help to understand the process of fungal tolerance of LiCl and may help develop Li-enrichment methodology.

This is a preliminary and screening study. Other edible fungi may be more tolerant to LiCl, and thus more appropriate for Li-enrichment. Indeed, Richter et al. (2008) showed that *Trametes versiculor*, a fungus usually used in Chinese medicine, grows well in LiCl concentration up to 1.5 g L^{-1}. The aim of our research line is to produce Li-enriched mushroom. From this starting point, further studies should be performed. These include

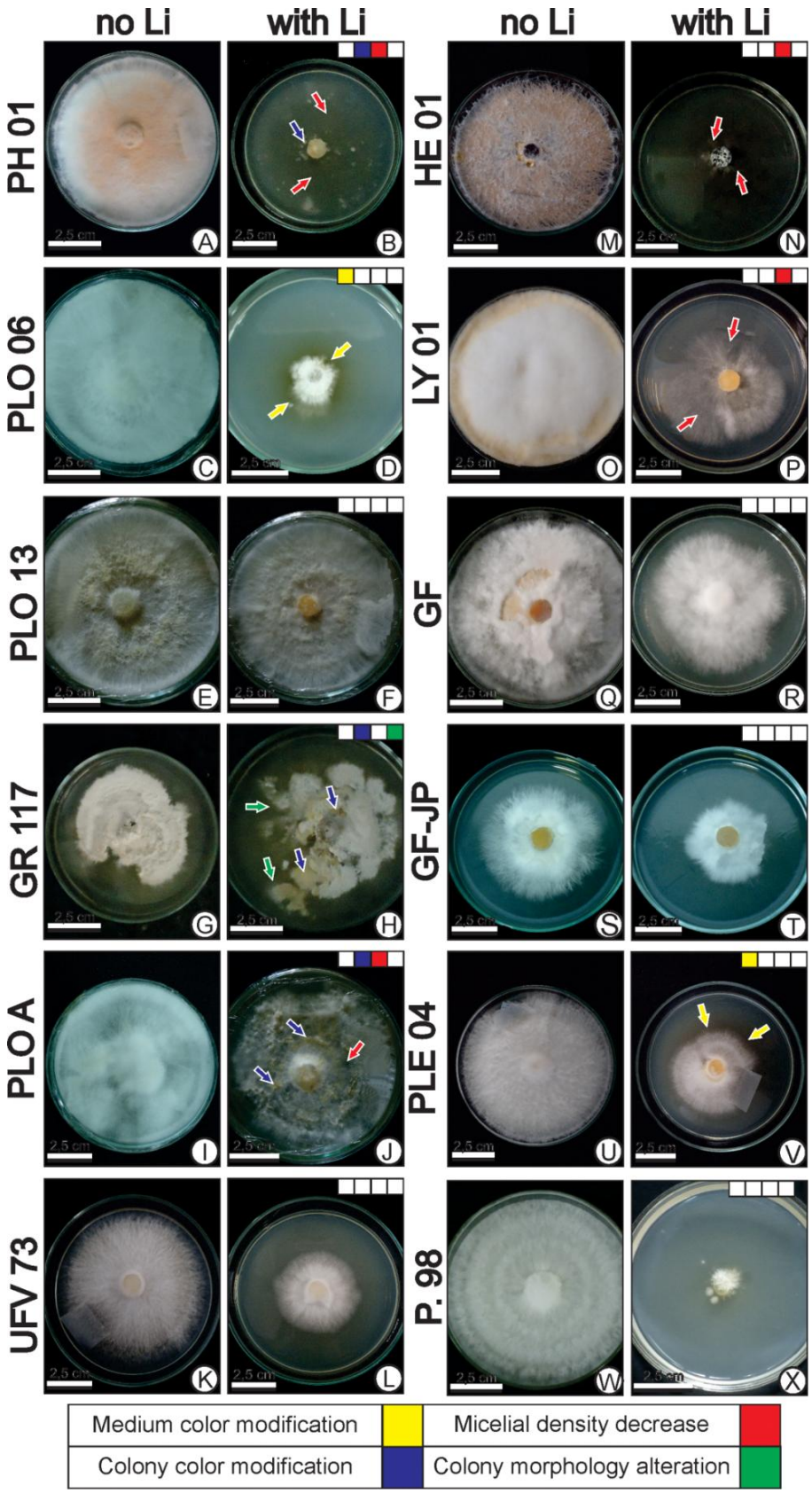

Figure 3S. Mycelial morphologyof white rot fungi in culture media supplemented with LiCl.

Table 1. Fungal macroscopic morphological alterations observed due to the addition of lithium chloride (LiCl) in the culture media.

Strain	Medium color alteration	Colony color alteration	Mycelial density decrease	Colony morphology alteration
PH 01		The mycelium light brown color that is intrinsic to this strain began to disappear with the increasing of LiCl	The mycelium thickness decrease with the increasing of LiCl and above 1.2 g L^{-1} the mycelium become so thin that was difficult to visualize the same in the Petri dish.	
PLO 06	Up to 0.3 g L^{-1} of LiCl we observed appearance of light orange color in the medium below the mycelium. The intensity of the orange do not changed with the increasing of Li concentration			
PLO 13				
GR 117		In higher concentrations of LiClwe observed the appearance of mycelium with light brown color.		In higher concentrations of LiCl we observed that the colony growth was not uniform.
PLO A		With the increase in LiCl concentration we observed an increase in the appearance of light brown mycelium.	Mycelium thickness decrease from 0.9 g L^{-1} of LiCl	
UFV 73				
HE 01			We observed thin mycelium at the lowest concentration of LiCl tested.	
LY 01			We observed thin mycelium at the lowest concentration of Li tested.	
GF				
GF-JP				
P. 98				
PLE 04	Up to 0.3 g L^{-1} of LiCl we observed appearance of light orange color in the medium below the mycelium. The intensity of the orange do not changed with the increasing of Li concentration.			

evaluation of mushroom production, measurement of the bioaccumulation of Li at different LiCl levels, the use of other sources of lithium and the effect of other substrate composition in the accumulation of Li.

Conclusions

Most of the Basidiomycetes strains used here showed sensitivity to LiCl. *P. ostreatusroseus* (PLO 13) was the only strain that was tolerant to all LiCl level tested, making it a promising fungus for future Li-enrichment research.

The fungi tested presented different morphological changes in the presence of LiCl at different concentrations. Elucidation of these various adaptive strategies may lead to new approaches for Li-enrichment.

Conflict of Interests

The author(s) have not declared any conflict of interests.

Table 2. Ranking of fungi sensitive to LiCl.

Group
Intolerant (growth up to 0.3 g L^{-1})
Hericium herinaceum
Lyophillum shimeji
Lentinula edodes
Sensitive (growth up to 0.6 g L^{-1})
Grifollafrondosa (GF-JP)
Grifollafrondosa (GF)
Ganoderma subamboinense var. laevisporum
Moderately sensitive (growth up to 0.9 g L^{-1})
Pleurotus ostreatus (PLO 06)
Pleurotus ostreatus (P. 98)
Pleurotus eryngii
Pleurotus citrinopiliatus
Resistant (growth up to 1.65 g L^{-1})
Pholiota nameko
Pleurotus ostreatusroseus

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Full Length Research Paper

Epidemiological trends in pathogens from the 2012 Thai flooding disaster

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Fungi and bacteria in water samples taken from various flooded areas in Narathiwat Province, Thailand, were investigated. They were isolated by filtration from water samples, and identified by examining macroscopic and microscopic features for fungi and using biochemical methods for bacteria. Nine species of filamentous fungi and two yeast species were isolated. Water contaminated with dermatophytes contained *Trichophyton mentagrophytes* (44%), *Trichophyton rubrum* (19%) and *Microsporum canis* (15%). The yeast *Candida albicans* was also found (75.5%). Water samples were contaminated with fungi, identified as non-cutaneous mycoses. The dominant fungi were *Aspergillus niger* (73%), *Cladosporium* spp. (58%) and *Aspergillus flavus* (41%). Thirteen bacterial strains were isolated from the samples; Gram-negative bacteria were most prevalent. The three dominant Gram-negative bacteria were *Escherichia coli* (62.5%), *Klebsiella pneumoniae* (61%) and *Enterobacter* spp. (59.5%). The two most abundant Gram-positive bacteria were *Corynebacterium* spp. (59.5%) and *Bacillus* spp. (not *Bacillus cereus*) (52.5%). These results suggest contaminated flood areas may be a transmission route for pathogens, and increase the risk of abnormal skin conditions among people exposed to the area.

Key words: Flood, Thailand, fungus, bacteria, yeast.

INTRODUCTION

Climate change represents a serious problem. There is an increase in the Earth's surface temperature, heat waves, droughts, storms and floods, as well as other frequent and costly natural disasters (Friel et al., 2011). Flooding causes many different health problems, including shortage of food and clean water, and a decreased sense of general well-being (McMichael et al., 2006; Bich et al., 2011; Friel et al., 2011). A study on the health impacts of the devastating flood that occurred in 2008 in Hanoi, Vietnam, revealed higher incidences of dermatitis, pink eye, dengue fever and psychological problems, in the communities that were severely affected, when compared with less-affected communities (Bich et al., 2011). A previous report on the health effects of flooding in Pakistan, in 2010, found that the most frequently reported conditions were skin diseases (18.3%), acute respiratory

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infections (15.1%) and acute diarrhea (13.3%) (CDC, 2012).

In Thailand, floods are frequent natural disasters. Regions to the south are faced with flash floods from heavy rains all year round. Southern Thailand is located on the peninsula bordered by the Andaman Sea to the West and the Gulf of Thailand to the East. The South has a tropical monsoon climate. Due to its geographical and climatic features, the South has become extremely vulnerable to flood disasters. Narathiwat Province is among those regularly affected. According to the Office of Natural Resources and Environmental Policy and Planning, Ministry of Natural Resources and Environment, Thailand (http://www.onep.go.th/index.php?option=com_content&task=view&id=4457&Itemid=266), in December 2012, devastating floods hit Narathiwat, affecting 25,158 people. Floods covered large areas of agricultural plantations (rubber, coconut, tropical fruit, rice), animal farms, water resources, roads and residential land. Heavy rain continued across its 13 districts, and in many parts of the province, flood water remained stagnant for weeks. People living in areas prone to flooding are at higher risk of contracting skin infections because of exposure to stagnant pools of flood water, which represent breeding sites for pathogens that cause waterborne skin infections. Poor hygiene standards among the displaced also contribute to this problem. Skin problems are among the most common diseases caused by flooding, especially superficial fungal infections. A previous study conducted in Thailand showed that the most commonly occurring dermatosis during the 2006 flood crisis was eczema. It accounted for 34.3% of the total skin problems and the great majority of these cases comprised chronic irritant foot dermatitis. Skin maceration of toe web spaces (Hong Kong foot), due to chronic irritant dermatitis with associated bacterial infections were prevalent. Only a few cases of fungal infection were reported. Topical anti-inflammatories, with antibacterial and antifungal agents, were used to treat skin conditions (Vachiramon et al., 2008). The highest level of total bacterial contamination was found in samples of flood water collected during the 2011 Thai flood. Moreover, one sample of flood water was found to be positive for *Leptospira* spp. (Chaturongkasumrit et al., 2013). A study carried out in Chiang Mai, Thailand, in 2011, found that water samples collected from seven flood crisis areas in Chiang Mai were contaminated with free-living amoebae (Wannasan et al., 2013). Flood water samples collected from flood zones in central Thailand, in 2011, were contaminated with human enteric viruses-norovirus (14%), rotavirus (9%), and hepatitis A (7%) (Ngaosuwankul et al., 2013).

Some data exist on the identification of microorganisms in skin specimens from patients during the floods in Thailand. Other studies have shown the presence of different types of microorganisms in water samples during flood crises. However, the microorganisms responsible for skin problems in flooded areas have not been examined. Therefore, the purpose of this study was to identify microorganisms from flooded areas of Narathiwat Province in December 2012.

MATERIALS AND METHODS

Sample collection

This study was conducted in Narathiwat Province, southern Thailand. Four main flood-affected districts of Narathiwat (Chanae, Waeng, Su-ngai Kolok, and Sukhirin) were selected for water sampling and testing. Sampling locations in each district included rubber plantations, water resources, roads and houses. A total of 200 water samples (200 mL each) were taken from flooded areas in December 2012, and stored in clean, sterile, 250 mL bottles with screw caps that were sealed and sent to the laboratory for the identification of any microorganisms. With the naked eye, moderately turbid water from rubber plantations, water resources, roads, and houses, were observed. Turbid water samples were allowed to settle for 30 min at room temperature. The water samples were fairly clear after the sedimentation process. Only clear water samples with virtually no sediment were collected and examined.

Isolation and identification of fungi

Water samples (100 mL each) were filtered through sterile 0.45 µm membrane cellulose nitrate filters (47 mm diameter). The filters were transferred to Petri dishes containing sterile Sabouraud Dextrose Agar (SDA) medium supplemented with chloramphenicol (50 mg/L) and gentamycin (25 mg/L) after autoclaving. Three replications were used per water sample. Petri dishes were incubated at 25°C for 7 days and examined daily for the presence of fungal colonies. The number of colonies was recorded and the fungi were subcultured for purity and identification (Gonçalves et al., 2006; Mbata et al., 2008). Fungi were identified by examining their macroscopic and microscopic structures.

Yeasts were identified by the following tests:

Germ tube test: For the identification of *Candida albicans*. The suspected yeast colonies were inoculated into 0.5 mL human serum in a small tube and incubated at 37°C for 2 h. After incubition, a drop of the yeast-serum mixture was placed on a glass slide, covered with a cover slip, and examined (Bhavan et al., 2010).

Carbohydrate assimilation test measures the ability of yeast to utilize different carbohydrates as the sole source of carbon aerobically. The sugars used were dextrose, maltose, sucrose, lactose, galactose, melibiose, cellobiose, inositol, xylose, raffinose, trehalose and dulcitol. Yeast cultures were suspended in saline, and basal medium (1.5 mL) containing 67.8% yeast nitrogen base was added. This was then added to 13.5 mL of molten, cooled agar containing 2% agar powder, mixed well, poured into a Petri dish and allowed to solidify. Then, paper disks soaked in 20% solutions of the various sugars were placed on the plates. Yeast growth was examined after incubating the plates at 25°C for 10-24 h (Bhavan et al., 2010).

Carbohydrate fermentation test detects the ability of microorganism to ferment a specific carbohydrate. Fermentation is noted by the production of acid and gas. The sugars used were dextrose, maltose, sucrose, lactose, galactose, and trehalose. 5 mL of carbohydrate (pH 7.4) containing 1% peptone, 1% sugar, 0.3% beef extract and 0.5% NaCl, 0.2% bromothymol blue in distilled water medium were prepared in a sterilized Durham tube. 0.2 mL of saline suspension was added to the tube of the suspected yeast, and incubated at 37°C for 10 days. After incubation, the tubes were exa-

examined for acid and gas production (Bhavan et al., 2010).

Chlamydospore formation: The suspected yeast cultures were inoculated on corn meal agar plates containing 1% Tween 80 and covered with cover slips. After incubation at 25°C for 3 days, the plates were examined for chlamydospores (Kim et al., 2002).

Isolation and identification of bacteria

Water samples (100 mL each) were filtered to isolate any bacteria. The filters were then placed on Trypticase Soy Agar (TSA) plates, and incubated to obtain a bacterial colony count. Distinct colonies were selected from the plates and subcultured on TSA to isolate a pure, single colony for identification. Unknown bacteria were identified to genus and species level busing biochemical methods as follows:

Gram staining is a common method used to differentiate two large groups of bacteria. It involves three steps: staining with crystal violet, decolorization and counterstaining with safranin. Gram-positive bacteria appear blue-black or purple, while gram-negative bacteria appear red or pink (Nester et al., 2007).

Catalase test is used to detect the existence of catalase enzyme in bacteria. Catalase activity is revealed by bubble formation after the addition of a drop of 3% hydrogen peroxide (Alexander and Strete, 2001).

Oxidase test is used to differentiate bacteria that produce cytochrome oxidase enzyme. Oxidase activity was determined by the oxidation of N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (TMPD). The filter paper was moistened with 1% TMPD. Then, the bacterial colony was picked up and smeared onto the filter paper. If oxidase was present in the colony, it would oxidize the reagent and the colony on the filter paper would turn dark blue (Alexander and Strete, 2001).

Methyl red test is used to identify bacteria that ferment glucose to stable acid. Methyl Red Voges Proskauer Broth (MRVP Broth) was used. Broth was inoculated and incubated at 30°C for 5 days to allow stable acid to be produced. After incubation, methyl red indicator was added. Red indicates a positive result, while yellow is negative (Leboffe and Pierce, 1995).

Gelatin hydrolysis test detects the capacity of bacteria to produce enzyme gelatinases. Gelatin is solid at room temperature. If bacteria make gelatinase, gelatin is hydrolyzed and turns to liquid. To carry out the test, gelatin was set in a test tube. The gelatin was stabbed with an inoculated needle to the base of the tube, and incubated at 25°C for 2 days. The tube was then placed in a refrigerator for 30 min or on ice for 15 min. The result is positive if the gelatin remains liquid (Haas and Defago, 2005).

Citrate test detects the ability of bacteria to use citrate as carbon and energy source. A single isolated colony was selected and lightly streaked onto the surface of an agar slant. The agar was then incubated at 35°C for 24 h. After incubation, bromothymol blue indicator was added. The result is positive if the agar turns blue and negative if no color change is observed (Forbes et al., 1998)

Nitrate test is used to determine the ability of bacteria to reduce nitrate to nitrite. Nitrate test tubes were arranged using premade nitrate broth, inoculated, and incubated at 37°C for 24 h. After incubation, several drops of N-N-dimethyl-1-napthylamine and an equal amount of sulfanilic acid were added and mixed. A red color, which should develop within a few minutes, indicates a positive result for nitrate reduction. If no color develops, zinc dust is added. Zinc catalyzes the reduction of nitrate to nitrite. A color change to red indicates a negative result and no color change indicates a positive result (Gusberti and Syed, 1984).

RESULTS

The identification of fungi and bacteria isolated from water taken from different flood zones in Narathiwat Province, in December 2012, is shown in Tables 1 and 2, respectively. In this study, we tested 200 samples from a range of flooded areas. Nine species of filamentous fungi and 2 yeast species (*C. albicans* and *Trichosporon* spp.) were isolated from the water samples by filter methods. The results showed the dominant isolated dermatophyte was *Trichophyton mentagrophytes* (44%). *Trichophyton rubrum* and *Microsporum canis* accounted for 19 and 15%, respectively. Other cutaneous mycoses found in the water samples were *C. albicans* (75.5%). The dominant isolated non-cutaneous mycoses were *Aspergillus niger* (73%), *Cladosporium* spp. (58%), and *Aspergillus flavus* (41%). Other fungi, including *Trichosporon* spp., *Penicillium* spp. (not *Penicillium marneffei*), *Aspergillus nidulans* and *Rhizopus* spp. were also isolated. The distribution of each of the 11 fungi across 4 different flood zones is shown in Table 1 and Figure 1. These fungi were mainly found in water samples taken from rubber plantations and houses.

Bacteria were isolated by filtration from water samples. Isolated strains were identified by conventional biochemical testing. A list of the 13 isolated bacterial strains is shown in Table 2 and Figure 2. Five Gram-positive bacterial strains were isolated (*Bacillus* spp. (not *Bacillus cereus*), coagulase-negative staphylococci, coagulase-positive staphylococci, *Corynebacterium* spp., and *Micrococcus* spp.). The dominant isolated Gram-positive bacteria in the water were *Corynebacterium* spp., which accounted for 59.5%, and *Bacillus* spp. (not *B. cereus*), which accounted for 52.5%. Eight gram-negative bacterial strains were isolated: *Acinetobacter baumannii*, *Enterobacter* spp., *Escherichia coli*, *Klebsiella pneumoniae*, *Morganella morganii*, *Pseudomonas aeruginosa*, *Pseudomonas* spp. and *Vibrio cholerae*. The most abundant Gram-negative bacteria in the water were *E. coli*, accounting for 62.5%, *K. pneumoniae*, accounting for 61%, and *Enterobacter* spp., accounting for 59.5%. Both Gram-positive and negative bacteria were found mostly in water samples from rubber plantations and houses.

DISCUSSION

During flood events, several types of microorganisms are present in the flood water, such as human enteric viruses (Ngaosuwankul et al., 2013), free-living amoebae (Wannasan et al., 2013), and bacteria (Chaturongkasumrit et al., 2013). Contaminated flood water indicated a high risk of health problems. This is the first study to investigate the prevalence of both fungi and bacteria in affected areas during the flood crisis in Thailand. This research found that water samples collected from various flooded areas in Narathiwat Province, in December 2012, contained high levels of microorganisms (fungi and bacteria). Flooded areas are a source of potential pathogen transmission to humans.

Flood conditions contribute to the growth and spread of pathogens, and direct contact with contaminated water

Table 1. Fungi identified in water from different flood zones in Narathiwat Province, December 2012.

Fungi	Rubber plantation (n=50)	Water resource (n=50)	Road (n=50)	House (n=50)	Total	Percentage (%)
Aspergillus flavus	28	16	15	23	82	41
Aspergillus nidulans	9	6	4	1	20	10
Aspergillus niger	45	38	22	41	146	73
Candida albicans	47	35	30	39	151	75.5
Cladosporium spp.	40	37	15	24	116	58
Microsporum canis	11	3	6	10	30	15
Penicillium spp. (not *P. marneffei*)	17	8	2	14	41	20.5
Rhizopus spp.	8	6	0	2	16	8
Trichophyton mentagrophytes	26	13	16	33	88	44
Trichophyton rubrum	13	6	8	11	38	19
Trichosporon spp.	15	10	11	14	50	25

Table 2. Bacterial organisms identified in water from different flood zones in Narathiwat Province, December 2012.

Bacteria	Rubber plantation (n=50)	Water resource (n=50)	Road (n=50)	House (n=50)	Total	%
Acinetobacter baumannii	1	0	0	3	4	2
Bacillus spp. (not *B. cereus*)	36	22	19	28	105	52.5
Coagulase-negative staphylococci	25	19	16	21	81	40.5
Coagulase-positive staphylococci	28	17	11	22	78	39
Corynebacterium spp.	38	25	23	33	119	59.5
Enterobacter spp.	30	36	21	32	119	59.5
Escherichia coli	34	38	20	33	125	62.5
Klebsiella pneumoniae	33	31	22	36	122	61
Micrococcus spp.	19	10	11	17	57	28.5
Morganella morganii	10	6	8	12	36	18
Pseudomonas aeruginosa	24	9	11	16	60	30
Pseudomonas spp.	27	15	14	15	71	35.5
Vibrio cholerae	5	7	11	10	33	16.5

can cause skin conditions. People exposed to flood water contaminated with pathogens are at increased risk of developing skin problems.

Fungi are eukaryotic organisms that are ubiquitous in nature. Most fungi grow terrestrially, but they can be found in every habitat, including aquatic environments. The results of this study showed that water samples collected from different flooded areas were contaminated with 9 species of filamentous fungi and 2 yeast species. Cutaneous mycoses are superficial fungal infections of the skin, hair or nails. Dermatophytes are a group of filamentous fungi that are the most common cause of cutaneous mycoses. The present study showed that the examined water was contaminated with the dermatophytes *T. mentagrophytes* (44%), *T. rubrum* (19%), and *M. canis* (15%). *T. mentagrophytes* and *T. rubrum* are the two main causative agents of tinea pedis (Hong Kong foot) (Rippon, 1988). However, causative agents of tinea pedis in outpatients attending the Institute of Dermatology, Bangkok, Thailand, differed from previous reports. The dermatophytes were the secondary cause of tinea pedis (36.8%), comprising *T. mentagrophytes* (18.4%), *T. rubrum* (13.2%), and *E. floccosum* (5.2%) (Ungpakorn et al., 2004).

A report from Nigeria showed that the most common dermatophytes isolated from athletics kits stored in Nigeria University's Sports Center were *T. mentagrophytes*, *T. rubrum* and *E. floccosum*. These fungi are often associated with tinea pedis among athletes in Nigeria (Essien et al., 2009). Rafiei and Amirrajab (2010) found that dermatophytes isolated from indoor public swimming pools in Ahwaz, Iran, were *T. mentagrophytes*, *T. rubrum*,

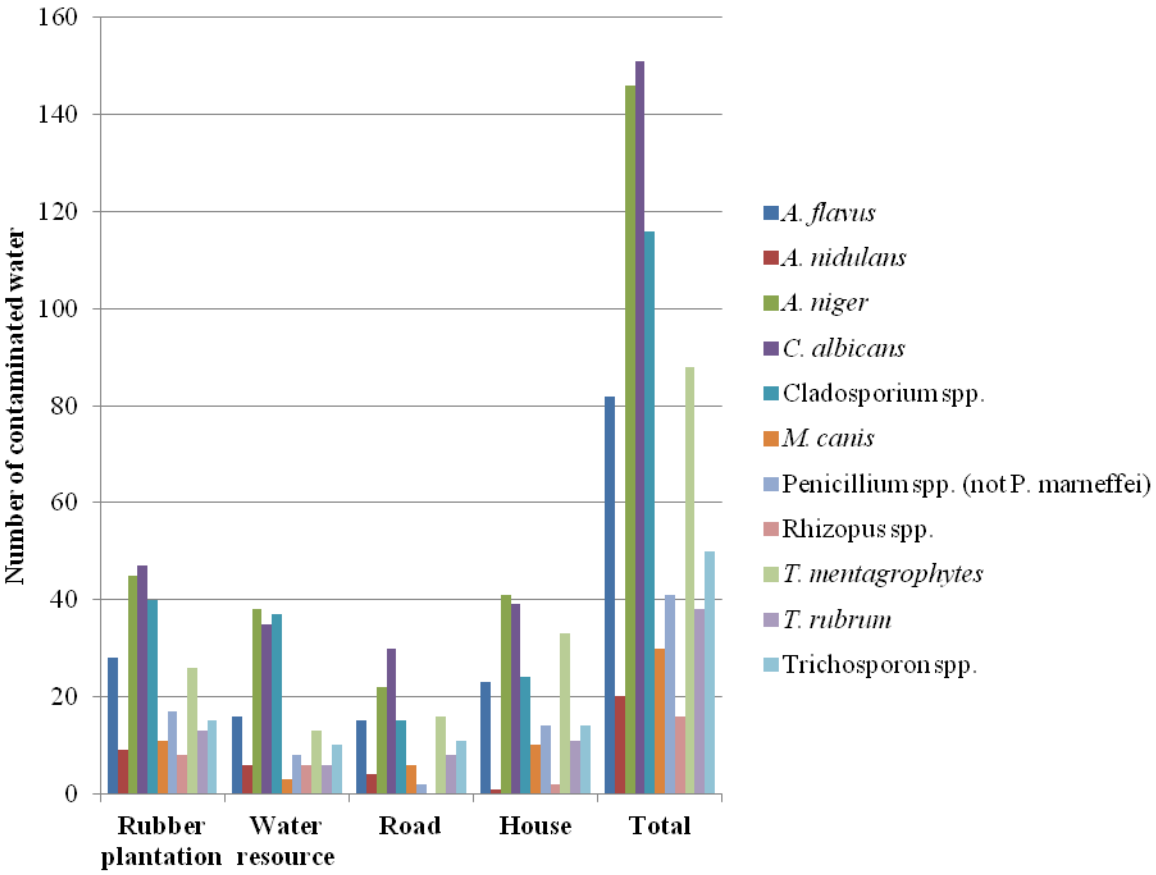

Figure 1. Prevalence of fungal species in water from different flood regions in Narathiwat Province, December 2012.

T. verrucosum and *E. floccosum*. The results from skin specimens from patients presenting with itching and skin maceration at the web spaces of the toes (16 cases) revealed 12.5% positive fungal growth (2 cases), comprising *Trichosporon mucoides* and non-spore forming hyaline fungi (Vachiramon et al., 2008).

Cutaneous mycoses can also be caused by the yeast *Candida* spp. From water tests, the highest frequency of fungi in our samples was *C. albicans*, accounting for 75.5%. The presence of *C. albicans* in this study was significant; the level is similar to that recorded in other studies. *Candida* species have been reported as common pathogens in community and hospital tap-water samples. Nine of the 14 isolated yeasts discovered in water were *Candida* species (Arvanitidou et al., 1999).

Mbata et al. (2008) demonstrated the presence of *Candida* species from the Yardenit Baptismal site on the Jordan River, Israel, as highly significant. There are also clinical-study data from workers at Ahvaz University of the Medical Sciences, Iran. The prevalence of cutaneous mycoses among the workers was 10.2%. The most common fungal disease was pityriasis versicolor (Tinea versicolor). Candidiasis due to *C. albicans* was the second most-common type of cutaneous mycosis. All patients with candidiasis were exposed to humidity during the day (Mahmoudabadi and Izadi, 2011).

The present study showed the water samples contaminated with fungi are non-cutaneous mycoses. The dominant fungi were *A. niger*, *Cladosporium* spp. and *A. flavus*, which accounted for 73, 58 and 41%, respectively. The most abundant filamentous fungi in water from the Jordan River in Israel were *Aspergillus* spp., which accounted for 32.5%, followed by *Penicillium* spp. (26.2%) (Mbata et al., 2008). Similarly, *Cladosporium*, *Penicillium* and *Aspergillus* dominated the fungi isolated from raw and treated water from the municipal water supply system in sub-tropical Australia (Sammon et al., 2010).

Flood water commonly contains high levels of bacteria that can cause skin infections. In this study, a total of 13 bacterial strains (8 strains of Gram-negative and 5 of Gram-positive bacteria) were isolated from water samples. The most prevalent bacteria found were gram-negative (56.44%). The three dominant isolated Gram-negative bacteria were *E. coli*, *K. pneumonia* and *Enterobacter* spp., accounting for 62.5, 61 and 59.5%, respectively. The two most frequent Gram-positive bacteria were *Corynebacterium* spp. (59.5%) and *Bacillus* spp.

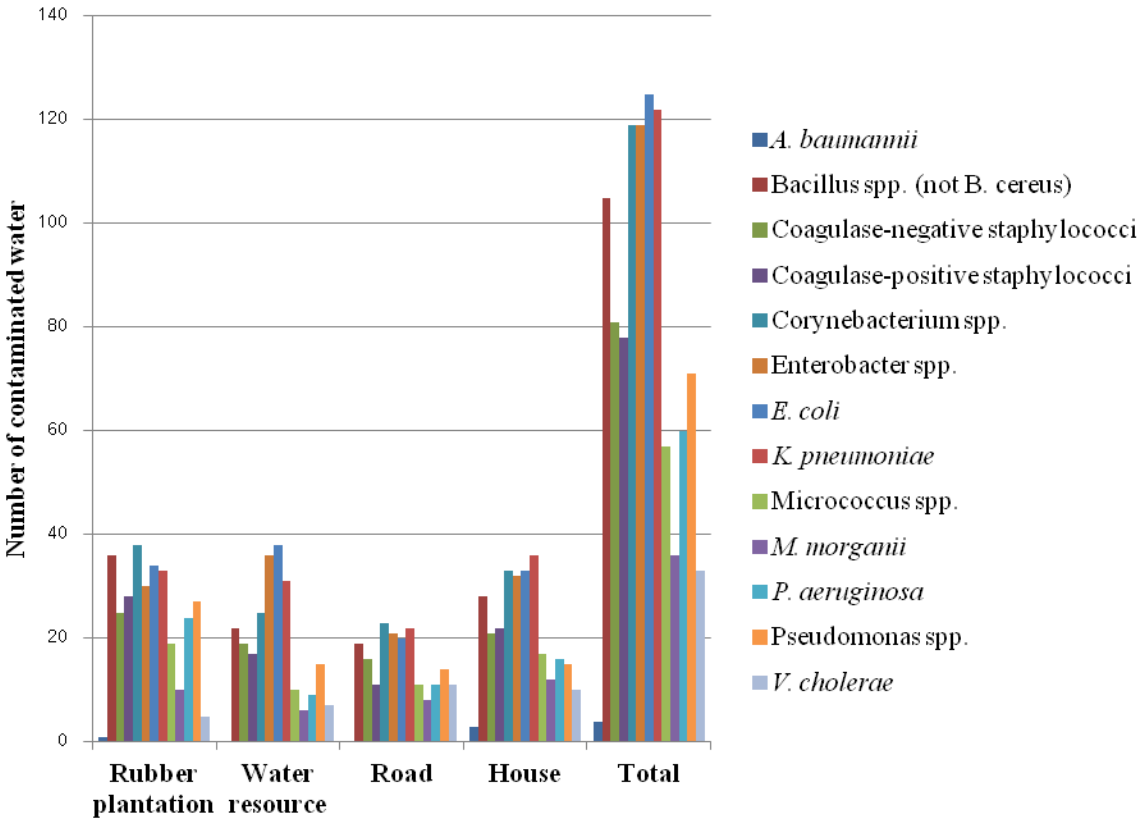

Figure 2. Prevalence of bacterial species in water from different flood regions in Narathiwat Province, December 2012.

(not *B. cereus*) (52.5%). Assessment of total bacterial contamination in various water sources during the 2011 Thai flooding disaster showed that the flood water and river water samples from the central part of Thailand contained the highest level of contamination (4.08-6.44 log cfu/mL) when compared with tap water (2.66-4.72 log cfu/mL) and filtered tap water (2.75-3.93 log cfu/mL) (Chaturongkasumrit et al., 2013).

Previous studies have shown that several species of water-borne Gram-negative bacteria are linked to skin and soft-tissue infections after tsunamis and floods. One clinical study found Gram-negative bacteria were the most common bacteria isolated from the traumatic wounds of tsunami victims in southern Thailand in 2004 (95.5%). The five dominant isolated Gram-negative bacteria were *Aeromonas* spp., *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *Proteus* spp. The most common Gram-positive bacteria were *Staphylococcus* spp. (*Staphylococcus aureus* and coagulase-negative staphylococci). Most skin and soft-tissue infections among the tsunami victims were polymicrobial infections (71.8%) (Hiransuthikul et al., 2005). During the 2006 flood crisis in Thailand, Gram-negative bacilli were the most prevalent microorganism found in the skin specimens of patients presenting with itching and skin maceration at the web spaces of the toes (14 of 16 skin specimens). Gram-positive bacilli, *Corynebacterium* spp. and *Staphylococcus* spp. were also recorded. The increased prevalence of irritant dermatitis might be a result of over-exposure to contaminated water, friction, high humidity and unhygienic surroundings (Vachiramon et al., 2008).

Water turbidity, a measure of the cloudiness of water, is used to indicate water quality and measure the risk of contamination (Schwartz et al., 2000). The relationship between water turbidity and microbial contamination has been studied. Higher turbidity levels are often associated with higher contamination levels (LeChevallier et al., 1991; Clark et al., 1992; Chaturongkasumrit et al., 2013). In this study, water samples from various flooded areas appeared moderately turbid to the naked eye. When these samples were examined, several different types of fungi and bacteria were isolated. Fungi and bacteria were mainly found in water samples taken from rubber plantations and houses. There may be a correlation between the number of microorganisms and level of water turbidity in these areas. However, the turbidity level and the number of fungi and bacteria were not measured in this study.

The recommended regimen for treating skin infections is the use of topical anti-inflammatories with antibacterial

and antifungal agents. The fungi and bacteria that cause skin infections should be investigated in patients pre-treatment. Regrettably, it remains impractical because many people have skin infections during a flood disaster and need prompt treatment. Further investigation of the prevalence of fungi and bacteria in flooded areas during disasters would be beneficial for treating skin infections, and may also help physicians prepare effective medications in the future.

Conflict of interest

The authors declare that they have no conflict of interest.

ACKNOWLEDGMENTS

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Full Length Research Paper

Antimicrobial studies of the crude extracts from the roots of Chenopodium ambrosioides Linn.

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Antibacterial and antifungal activities of *Chenopodium ambrosioides* Linn have been studied for their medicinal potential by agar well diffusion method. Five bacterial strains, *Escherichia coli, Klebsiella pneumoniae* (Gram negative bacteria), *Staphylococcus aureus, Bacillus subtilis* and *Staphylococcus epidermidis* (Gram positive bacteria) as well as five fungal strains, *Aspergillus niger, Aspergillus parasiticus, Trycophyton horzianum, Rhizopus tolenapur* and *Aspergillus flavus* , were used to study the antimicrobial potential of the crude methanolic extract along with n-hexane, ethyl acetate, dichloromethane, n-butanol and aqueous fractions from the roots of *Chenopodium ambrosioides* Linn. The tested bacterial strains were taken from Center for Phytomedicine and Medicinal Organic Chemistry (CPMMOC) University of Peshawar, Pakistan; they were previously collected from hospital patients while the antifungal strains were collected from Center for Biotechnology and Microbiology (CBM) University of Peshawar, Pakistan which were also in advance collected from hostel patients of Khyber Teaching Hospital, University road Peshawar. The selected strains were tested against crude extract and its fractions. Zone of inhibition were measured by using National Committee for Clinical Lab Standards (NCCLS) method in which for antibacterial activities, Streptomycine while for antifungal activities, Miconazole were used as standard drugs. Dimethyl sulphoxide (DMSO) was used as negative control in both cases. All fractions remained inactive against *K. pneumonia* while other fractions showed good to non-significant activities against other bacterial strains. The n-hexane fraction showed moderate activity against *A. niger* while all other fractions showed low activity against antifungal strains. The Statistical Package for the Social Sciences (SPSS) calculations by using test statistics "t" shows the 'p' value of lower than α=0.05, while the confidence interval (CI), 95% is also significant.

Key words: Antibacterial, antifungal, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Staphylococcus epidermidis*, *Klebsiella pneumonia*, *Aspergillus niger, Aspergillus parasiticus, Trycophyton horzianum, Rhizopus tolenapur* , *Aspergillus flavus,* modest, significant.

INTRODUCTION

Chenopodium Ambrosioides Linn, being a therapeutic plant, is widely used in the traditional medicinal system in Asia, Europe and America especially as an antihelmintic agent and as a remedy for parasitic disorders (Monzote et al., 2009; Gadano et al., 2002). In Pakistan, the plant is widely distributed in Peshawar, Baluchistan, Dir, Swat, Kohala, Kashmir and Rawalpindi (Nasir et al., 1972; Nisar et al., 2013). This plant is a member of an important plant

family, *Chenopodiaceae,* which have an elevated importance for phytochemical investigation and medicinal evaluation. The family *Chenopodiaceae* consists of 102 genera and 1400 species (Manske et al., 1965). At the beginning of the 19th century, the essential oil from this plant, known as 'Baltimore oil' was used for treating patients with worms (Monzote et al., 2009). The essential oil obtained from this plant has been reported to have antifungal (Kumar et al., 2007; Jardim et al., 2008) and insecticidal activities. Ascaridole was the first compound isolated from this plant in 1895 by a German Pharmacist, named Hüthig. Ascaridole is the main constituent of the essential oil of this plant together with carvacrole and cryophyllene oxide, and the toxic effects of these compounds on mitochondria have been reported (Monzote et al., 2009). Other components isolated from the essential oil of this plant are limonene, transpinocarveol, ascaridole-glycol, aritasone, β-pynene, myrcene, phelandrene, alcanphor and α-terpineol (de Pascual et al., 1981).

However, literature does not provide any information regarding the use of the whole plant or different parts of the plant for phytochemical studies or medicinal evaluation. Major studies were carried out on the essential oil of this plant together with the leaves extract. Due to this reason, we studied the antimicrobial potential of the extracts from the roots of this medicinal plant.

MATERIALS AND METHODS

Plant collection

The plant was collected from Peshawar, Pakistan in June and its different parts were separated. The plant was identified by Dr. Abdur Rashid, Department of Botany, University of Peshawar, having assigned voucher number BOT20056 (PUP). The plant roots were shade dried. After shade dryness, the plant roots were grinded and converted to powdered form. The powdered plant roots crude methanolic extract was concentrated at 40°C through vacuum distillation by using rotary evaporator. This methanolic extract was further concentrated till complete dryness in water bath were extracted with methanol for three times by maceration for four nights each and thus methanolic crude extract was obtained. The at same temperature. The dried methanolic crude extract was further dissolved in distilled water and was further fractionated using n-hexane, ethyl acetate, dichloromethane and n-butanol solvent systems at the end aqueous fractions. All the five fractions together with crude extract were tested for antibacterial and antifungal activities.

Antibacterial bioassay

The antibacterial activity was checked by the agar well diffusion method (Nisar et al., 2010; Shah, 2014). In this method, one loop full of 24 h old culture containing approximately 10^4-10^6 CFU (colony forming units/ml) was spread on the surface of Mueller-Hinton agar plates. Wells were dug in the medium with the help of sterile metallic cork borer. Stock solution of the test samples in the concentration of 22 mg/ml was prepared in the dimethyl sulphoxide (DMSO) and 150 µl dilutions were added in their respective wells. The antibacterial activity of samples ware compared with standard drug, streptomycin. The concentration of streptomycin was 2 mg/ml. The standard drug streptomycin and DMSO were used as positive and negative control. The amount of growth in each well was determined visually by comparing with the growth in the controlled wells. Antibacterial potential of sample was then determined as per criteria mentioned in Table 1. Percent growth inhibition was calculated with reference to positive control.

Table 1. Criteria for determination of antibacterial activity.

Entry	Diameter (mm)	Activity
1	Below 9	No activity
2	9-12	Non-significant
3	13-15	Low
4	16-18	Good
5	Above 18	Significant

Antifungal bioassay

The antifungal activity was determined by the Agar Well Diffusion Method (Nisar et al., 2011; Nisar et al., 2013). In this method, Miconazole was used as the standard drug. The samples were dissolved in DMSO (24 mg/ml). Sterile Sabouraud dextrose agar medium (7 ml) was placed in a test tube and inoculated in a sample solution (40 µg/ml) kept in slanting position at room temperature overnight. The fungal culture was then inoculated on the slant. The samples were incubated for 7 days at 30°C and growth inhibition was observed. The percent growth inhibition was calculated with reference to the negative control by applying the formula:

Inhibition (%) = (Linear growth of the negative control - Linear growth of sample)/100 × 100.

Growth in medium containing crude extract and fractions was determined. The results were evaluated by comparing with the Table 2.

RESULTS AND DISCUSSION

Antibacterial bioassay

Antibacterial activity was studied against various human pathogens including *Escherichia coli, Klebsiella pneumoniae* (Gram negative bacteria), *Staphylococcus aureus, Bacilus subtilis* and *Staphylococcus epidermidis* (Gram positive bacteria) as shown in Figure 1. The tested bacterial strains were taken from the culture of Microbiology Laboratory, PNRL (Pakistan Nuclear

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Table 2. Criteria for determination of antifungal activity.

Entry	Percent inhibition	Activity
1	30-40	Low
2	50-60	Moderate
3	61-70	Good
4	Above 70	Significant

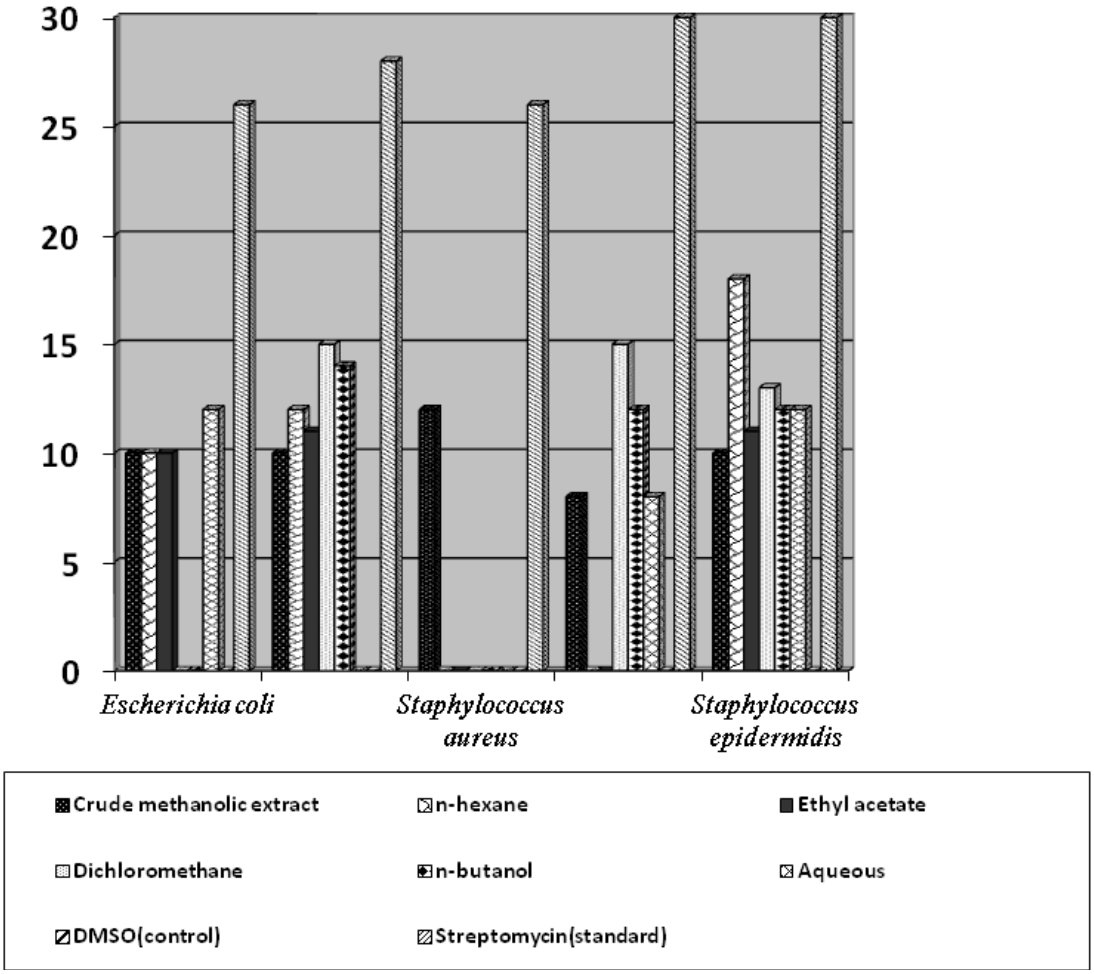

Figure 1. The graphical representation of antibacterial activities (mean only).

Research Laboratories, Peshawar, Pakistan); they were previously isolated from hospital patients. The diameter of zone of inhibition (mm) of samples against the bacteria is given in the Table 3.

The crude extract showed non significant activity against bacterial strains. The n-hexane fraction showed good to low activities against all bacterial strains. It showed good activity against *S. epidermidis* and low to non significant against four other bacterial strains. The ethyl acetate fraction showed low and non significant activities. This fraction showed non significant activity against *K. pneumoniae,* low against *E. coli* and *B. subtilis* while no activity against *S. aureus* and non significant against *S. epidermidis.* Dichloromethane fraction showed no activity against *E. coli* and *S. aureus,* while low activity against *B. subtilis, S. epidermidis* and *K. pneumonia.* The n-butanol fraction showed no activities against *S. aureus and E. coli*, non significant activities against *S. epidermidis and*

Table 3. Diameter of zone of inhibition (mm) against bacterial strains.

Sample/fraction	*Escherichia coli*	*Klebsiella pneumoniae*	*Staphylococcus aureus*	*Bacilus subtilis*	*Staphylococcus epidermidis*
Crude methanolic extract	10 ± 1.03	10 ± 0.23	12 ± 0.35	08 ± 0.32	10 ± 1.06
n-hexane	10 ± 1.54	12 ± 0.70	00 ± 0.11	00 ± 00	18 ± 0.85
Ethyl acetate	10 ± 0.28	11 ± 0.39	00 ± 00	00 ± 00	11 ± 0.32
Dichloromethane	00 ± 00	15 ± 1.23	00 ± 0.89	15 ± 0.81	13 ± 0.65
n-butanol	00 ± 00	14 ± 0.85	00 ± 00	12 ± 1.32	12 ± 0.62
Aqueous	12 ± 1.50	00 ± 00	00 ± 1.23	08 ± 0.54	12 ± 1.35
DMSO(control)	00	00	00	00	00
Streptomycin(standard)	26 ± 0.11	28 ± 0.13	26 ± 0.56	30 ± 0.61	30 ± 0.98

The data is represented as triplicate with ±SD.

Table 4. One-sample statistics of antibacterial activities.

	N	Mean	Std. deviation	Std. error mean
Sample mean	40	9.38	9.189	1.453

Table 5. One-sample test of antibacterial activities.

Sample mean	Test value = 0					
	t	df	Sig. (2-tailed)	Mean difference	95% Confidence interval of the difference	
					Lower	Upper
Sample mean	6.452	39	.000	9.375	6.44	12.31

B. subtilis while low activities against *K. pneumoniae* and *S. aureus* while the aqueous fraction remained non significant against *S. epidermidis* and *E. coli* and non activities against all other bacterial strains. Streptomycin was used as a standard drug, it showed zone of inhibition (mm) 26, 28, 26, 30 and 30 against *E. coli, K. pneumoniae, S. aureus, B. subtilis* and *S. epidermidis*, respectively.

The good activities of n-hexane fraction against *S. epidermidis* shows the presence of some good antibacterial novel drugs in this fraction which may lead to isolation of many good antibiotics.

SPSS antibacterial interpretation

The SPSS antibacterial calculations are listed below (Tables 4 and 5).

From SPSS output, the total size is 40, mean is 9.38, standard deviation is 9.189 (measure of dispersion), the 'P' value is 0.000 which is less than the level of significance, α=0.05. Therefore on the basis of sufficient evidence, it is concluded that antibacterial activities are significant, whereas the 95% confidence interval (CI) is 6.44, 12.31.

Antifungal bioassay

Antifungal activities were performed against the five fungal strains including *A. niger, Aspergillus parasiticus, Trycophyton horzianum, Rhizopus tolenapur* and *Aspergillus flavus* (Figure 2). The inhibition (%) of samples against fungal strains is shown in the Table 6.

The crude as well as all the fractions showed from none to low activities against all fungal strains. The best activities are of ethyl acetate fraction against *A. niger* and *R. tolenapur* with inhibition (%) of 40 and 35, respectively, which on further chemical investigations will lead to isolation of antifungal chemicals.

SPSS antibacterial interpretation

SPSS interpretation of antifungal activities is given in Tables 7 and 8.

The SPSS output of the antifungal activities of the plant

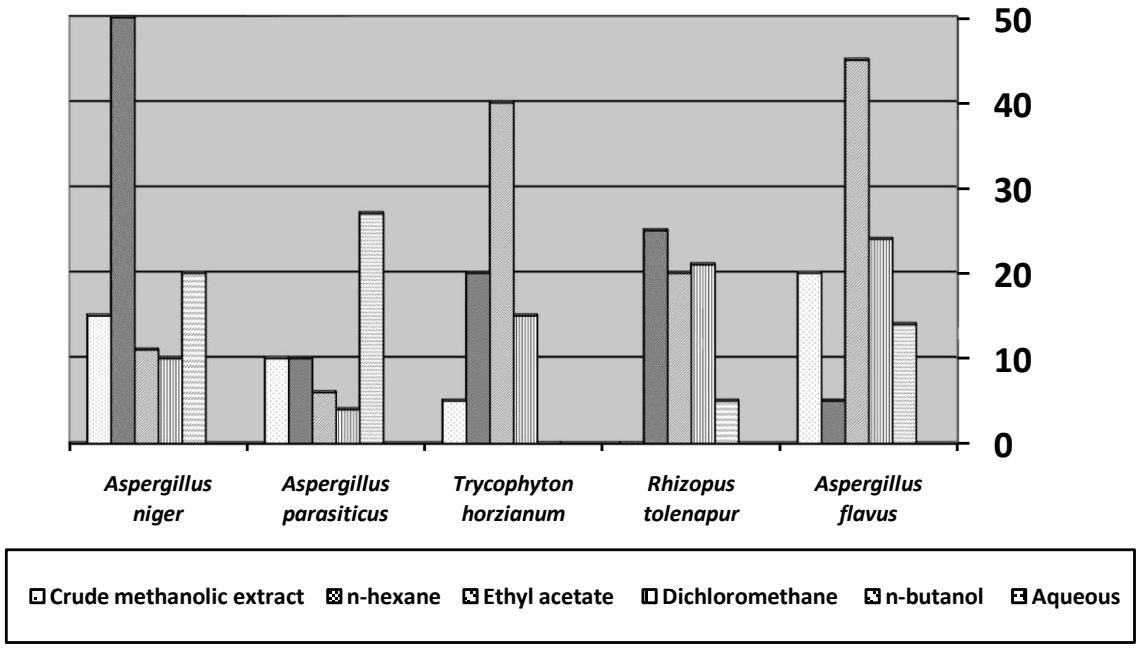

Figure 2. The graphical representation of antifungal activities (mean only).

Table 6. Percent inhibition (mm) of samples against fungal strains.

Sample/fraction	*Aspergillus niger*	*Aspergillus parasiticus*	*Trycophyton horzianum*	*Rhizopus tolenapur*	*Aspergillus flavus*
Crude methanolic extract	15 ± 0.23	10 ± 0.58	05 ± 0.55	00 ± 00	20 ± 0.21
n-hexane	50 ± 0.51	10 ± 0.29	20 ± 0.65	25 ± 0.65	05 ± 0.19
Ethyl acetate	11 ± 0.20	06 ± 0.12	40 ± 1.13	20 ± 0.63	45 ± 0.12
Dichloromethane	10 ± 0.32	04 ± 0.82	15 ± 0.24	21 ± 0.52	24 ± 0.51
n-butanol	20 ± 0.22	27 ± 0.54	00 ± 00	05 ± 0.12	14 ± 0.58
Aqueous	10 ± 0.17	15 ± 0.54	21 ± 0.21	18 ± 1.58	20 ± 0.59
DMSO	00	00	00	00	00
Miconazole	100	100	100	100	100

The data is represented as triplicate with SD±.

Table 7. One-sample statistics of antifungal activities.

N	Mean	Std. deviation	Std. error mean
30	16.87	12.099	2.209

Table 8. One-sample test of antifungal activities.

Data	Test value = 0					
	t	df	Sig. (2-tailed)	Mean difference	95% confidence interval of the difference	
					Lower	Upper
Data	7.635	29	.000	16.867	12.35	21.38

samples of total size is 30, mean is 16.87, standard deviation is 16.87 (measure of dispersion), the ‘P’ value by test statistics ‘t’ is 0.000 , in this case is lower than the level of significance, α = 0.05. Considering these values, the antifungal activities of the plant are significant. The CI 95% is 12.35 and 21.38.

Conclusion

The above results confirm the antimicrobial strength of the crude extracts of the roots of this plant, which is also supported by SPSS calculations, and supporting the traditional medicinal use of this plant extracts. The results also show the importance of screening plants as a potential source of bioactive compounds. However, further studies are required to investigate this important medicinal plant for isolation of novel compounds.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Screening for hepatitis B virus (HBV), hepatitis C virus (HCV) and syphilis infections among asymptomatic students of a private university in Western Delta, Nigeria

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This study was aimed at screening for non-treponemal antibodies, anti-hepatitis C and hepatitis B surface antigen markers among asymptomatic students of Western Delta University, Oghara, Nigeria. Venous blood was collected from 318 students of 26 years average age who did not show any visible signs or symptoms of any of the three infections. Blood samples were processed and screened for hepatitis C, hepatitis B and syphilis by rapid test methods. Blood samples were collected and processed in the Microbiology and Biotechnology Laboratory of Western Delta University, Nigeria between June 2013 and October, 2013. Anti-hepatitis C assay was done by a one step hepatitis C virus test strip, hepatitis B assay was done by a one step hepatitis B surface antigen test strip and non-treponemal antibodies was done by syphilis ultra rapid test strip. Ninety three (29.3%), 36 (11.3%), 6 (1.9%), 3 (0.9%) and 0.0% number of students belonged to 21-25 (24 years average), 15-20 (19 years average), 26-30 (27 years average), 36-40 (40 years average), 41-45 (44 years average) and 31-35 (30 years average) age groups respectively. Nine (5.1%) male students out of the 117 (55.7%) screened in the 21-25 age bracket tested positive to hepatitis B (produced antibodies against HBsAg) while 0.3 (1.7%) female students of the same age group produced antibodies to *Treponema palladium* antigen (tested positive to syphilis). Seropositivity to HBsAg was also recorded by 3 (8.3%) male students out of 36 (11.3%) screened in the 26-30 years age group. On the whole, 12 (3.8%) male and 3 (0.9%) female students tested positive to hepatitis B and syphilis, respectively. Hepatitis due to HBV and syphilis infection are prevalent within the university under study and indeed, Oghara town though in apparent low occurrence. The relevant health authorities should ensure a constant sero-epidemiological surveillance as well as institute counseling and vaccination programmes.

Key words: Syphilis, hepatitis B, hepatitis C, seroprevalence, asymptomatic students, university.

INTRODUCTION

Most cases of viral hepatitis in humans are caused by four viruses. These include hepatitis B virus, hepatitis C virus, hepatitis A virus and hepatitis D virus or delta hepatitis (CDC, 1991). There are, however, distinct differences in these viral agents in terms of their epidemiologic, immunologic and clinical characteristics (Robinson, 1990). For drug users, hepatitis viruses B and C are the agents of concern because the major risk of

infection is by blood-borne transmission especially through contaminated needles (CDC, 1991).

Hepatitis B virus (HBV) is a double stranded DNA virus which is transmittable by three main mechanisms which include: (1) percutaneous (2) sexual contact and (3) mother-to-child (CDC, 1991). Hepatitis B surface antigen (HBsAg) has been isolated in all body fluids such as blood products, saliva, semen and vaginal fluids (CDC, 1991). After the initial exposure, the incubation period averages 60-90 days and sometimes, 6 weeks to 6 months (CDC, 1991). Infected persons may be sources of infection to others before the onset of symptoms which include loss of appetite, vague abdominal discomfort, weight loss, fever, nausea, vomiting and diarrhoea. Others are dark tea-coloured urine, muscle aches and skin rashes prior to the development of clinical jaundice (CDC, 1990).

Hepatitis C virus (HCV) was first isolated in 1988 (CDC, 1990). Previous studies have confirmed that the hepatitis C virus is the viral agent that causes non-A, non-B hepatitis (Benenson, 1990) and it is associated with both acute and chronic liver disease. HCV, like HBV is a blood-borne pathogen which is transmitted by direct contact with infected blood products, contaminated needles or syringes, sexually as well as through vaginal secretions, semen or saliva (Benenson, 1990). Symptoms of HCV hepatitis include vague abdominal discomfort, anorexia, nausea, vomiting and jaundice with elevated liver enzymes (CDC, 1990).

Syphilis is a systemic infection that has been known since the 15th century. It is caused by the spirochaete-*Treponema pallidium* which is spread by contact with infectious lesions during sexual intercourse (Hook and Marra, 1992). About 12 million people worldwide are infected with syphilis each year and most of them live in developing countries (WHO, 2001).

HCV, HBV and syphilis infection are very common in HIV-infected persons (Soriano et al., 2008; Laurent et al., 2003). Patients with HCV and HBV co-infection with HIV have an increased risk of progression of HCV and HBV-related liver diseases (such as chronic hepatitis, cirrhosis and hepatocellular carcinoma) when compared with HCV mono-infected patients (Konopnicki et al., 2005; Chen et al., 2006; Omland et al., 2009; Ananthakrishnan et al., 2010).

There is paucity of documented or published literature (information) on the seroprevalence of HBsAg, anti-HCV and non-treponemal antibody (syphilis) in Nigeria and perhaps Africa. The purpose of this work was therefore to study the seroprevalence of HBsAg, anti-HCV and non-treponemal antibody (syphilis) markers among asymptomatic students of a private University in Western Delta, Nigeria with the under mentioned objectives: (a). Analyse the sex and age distribution of Western Delta University students recruited for the study, (b). Determine the presence of HBsAg, Anti-HCV and syphilis antibody markers among apparently healthy students recruited for the study.

MATERIALS AND METHODS

Informed consent

The researchers obtained a written informed consent from students who were mainly adults and able to make independent decision. Students were given an informed consent questionnaire which they filled and signed. Oral informed consent was also given by students engaged in the study.

Sampling

Four millilitres (4ml) of venous blood was collected by vein puncture with sterile 5ml needles and syringes from a total of 318 students aged 15-44(have age 26yrs) and made up of 108 (34.0%) males and 210 (66.0%) females of Western Delta University, Oghara, Nigeria. Subjects were grouped into nine departments which included Geology, Accounting, Mass communication, Computer Science, Microbiology and Biotechnology, Business Administration, Biochemistry, Economics and Political science. Subjects were also grouped into 15-20, 21-25, 26-30, 31-35, 36-40 and 41-45 age brackets with the average age for each age group recorded. Venous blood samples were dispensed into appropriately labelled ethylene-diamine tetra acetic anti-coagulated blood containers and properly mixed with the anticoagulant. Blood samples were allowed to stand on working bench for about 30minutes to allow for proper plasma separation by force of gravity.

Processing of samples

One step hepatitis B surface antigen test strip (rapid test procedure)

A rapid one step test for the qualitative detection of HBsAg in the plasma samples was done according to the method initially prescribed by Blumberg et al. (1971). The kit for this rapid test is commercially available.

Anti HCV Assay

One step hepatitis C virus test strip rapid test procedure

A rapid one step test for the qualitative detection of antibodies to hepatitis C virus in the plasma samples was carried out according to the method described by Choo et al. (1989). The kit for this rapid test is commercially available

Syphilis ultra rapid test strip procedure

The syphilis ultra-rapid test strip assay was carried out according to the procedure described by Johnson and Taylor–Robinson (1994). The kit for this rapid test is commercially available.

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Table 1. Sex and age distribution of Western Delta University students recruited for the study.

Departments sampled	Sex/No of students	15-20 yrs n=93 (29.3%) Av. age=19 years	21-25 yrs n=117 (55.7%) Av. age=24 years	26-30 yrs n=36 (11.3%) Av. age=27 years	31-35 yrs n=0 (0.0%) Av. age=30 years	36-40 yrs n=06 (1.9%) Av. age=40 years	41-45 yrs n=03 (0.9%) Av. age=44 years
Geology	M(33)	06	24	03	00	00	00
n=54(17.0%)	F(21)	06	12	03	00	00	00
Accounting	M(18)	12	00	06	00	00	00
n=57(17.9%)	F(39)	09	24	06	00	00	00
Mass.Com	M(06)	03	00	00	00	03	00
n=24(7.6%)	F(18)	09	06	03	00	00	00
Comp.Sci	M(06)	00	06	00	00	00	00
n=27(8.5%)	F(21)	06	15	00	00	00	00
Microbiology	M(15)	06	06	00	00	03	00
n=63(19.8%)	F(48)	12	30	06	00	00	00
Bus.Admin	M(06)	00	06	00	00	00	00
n=30(9.4%)	F(24)	03	12	09	00	00	00
Biochemistry	M(09)	00	06	00	00	00	00
n=27(8.5%)	F(18)	13	09	00	00	00	00
Economics	M(09)	00	09	00	00	00	00
n=21(6.6%)	F(12)	00	12	00	00	00	00
Pol.Sci	M(06)	03	00	00	00	00	03
n=15(4.7%)	F(09)	09	00	00	00	00	00
TOTAL	M108 (34.0%)	27(29.0%)	60(33.9%)	09(25.0%)	0(0.0%)	06(100.0%)	03(100.0%)
n=318	F210 (66.0%)	66(71.0%)	117(66.1%)	27(75.0%)	0(0.0%)	0(0.0%)	0(0.0%)

Av.age: Average age.

RESULTS

Table 1 shows the sex and age distribution of students recruited for the study. Out of the total of 318 students screened, 108 (34.0%) and 210 (66.0%) were males and females, respectively. In decreasing order, 177(55.7%), 93(29.3%), 36(11.3%), 6(1.9%), 3(0.9%) and 0(0.0%) number of students belonged to 21-25(24yrs average), 15-20 (19yrs average), 26-30 (27yrs average), 36-40 (40yrs average), 41-45(44yrs average) and 31-35 age groups respectively. Out of the total recruited in each age group, 60(33.9%), 27(29.0%), 09(25.0%), 06(100.0%), 03(100.0%) and 0(0.0%) represented male students in 21-25,15-20, 26-30, 36-40, 41-45 and 31-35 age brackets respectively while 117(66.1%), 66(71.0%), 27(75.0%), 0(0.0%), 0(0.0%) and 0(0.0%) represented female students in the same age groups respectively. Table 2 shows the seroprevalence of hepatitis C (HCV) antibody, hepatitis B surface antigen (HBsAg) and non-treponemal antibody (syphilis) among students recruited for the study. None of the 93(29.3%) male and female students of the 15-20 age group recordedsero-reactivity for HBsAg, HCV and syphilis. The highest number of volunteering students which was 177 (55.7%) belonged to the 21-25 age group of which 9 (5.1%) male students tested positive for hepatitis B surface antigen. In this same age group, the male students recorded 0 (0.0%) and 0 (0.0%) antibody *in vitro* response to HCV and syphilis assays, respectively. The female students in the

Table 2. Sero-prevalence of hepatitis C (HCV) antibody, hepatitis B surface antigen (HBsAg) and non treponemal antibody (syphilis) among students recruited for study.

Age brackets/sex of students	Sex	HBsAg	HCV	Syphilis
15-20yrs	M	0(0.0)	0(0.0)	0(0.0)
n=93 (29.3%)	F	0(0.0)	0(0.0)	0(0.0)
21-25yrs	M	09(5.1%)	0(0.0)	0(0.0)
n=117 (55.7%)	F	0(0.0)	0(0.0)	03(1.7%)
26-30yrs	M	03(8.3%)	0(0.0)	0(0.0)
n=36 (11.3%)	F	0(0.0)	0(0.0)	0(0.0)
31-35yrs	M	0(0.0)	0(0.0)	0(0.0)
n=0 (0.0%)	F	0(0.0)	0(0.0)	0(0.0)
36-40yrs	M	0(0.0)	0(0.0)	0(0.0)
n=06 (1.9%)	F	0(0.0)	0(0.0)	0(0.0)
41-45 yrs	M	0(0.0)	0(0.0)	0(0.0)
n=03 (0.9%)	F	0(0.0)	0(0.0)	0(0.0)
Total	M	12(3.8%)	0(0.0)	0(0.0)
n=318	F	0(0.0)	0(0.0)	03(0.9%)

21-25 age bracket recorded 0 (0.0%), 0 (0.0%) and 3 (1.7%) seropositivity to HBsAg, anti-HCV and non-treponemal antibody, respectively.

A total of 36 (11.3%) students were in the 26-30 age group of which 3 (8.3%), 0 (0.0%) and 0 (0.0%) male students showed reactivity to HBsAg, anti-HCV and syphilis antibody, respectively. The female students in this bracket recorded 0 (0.0%) each, reactions to HBsAg, anti-HCV and syphilis antibody, respectively. The 31-35 age group had no student recorded in it. In the 36-40 bracket, out of 6 (1.9%) students, both the male and female students recorded 0(0.0%) sero-reactions to HBsAg, anti-HCV and non-treponemal antibody respectively. Similarly and lastly, all the 3 (0.9%) students in the 41-45 age group tested negative to hepatitis B, hepatitis C and syphilis. In all, 12 (3.8%) male and 3 (0.9%) female students tested positive to hepatitis B and syphilis respectively. No student tested positive to hepatitis C.

DISCUSSION

Screening tests do not mean the prevalence of infection but the prevalence of the markers which are important from epidemiological perspective (Silvio and Johnson, 2011). Hence this work sought to unravel the seroprevalence markers of HBsAg, anti-HCV and non-treponemal antibodies (syphilis) among healthy (asymptomatic) students of Western Delta University, Oghara. Apart from presentation or observance of symptoms, the risk factors involved in the disease caused by the above agents justify screening for the diseases or their markers in populations (Shiell and La, 2001; Castelnuovo et al., 2006).

In this study, none of the 318 students screened tested positive to hepatitis C. This is almost consistent with 1.64, 1.6 and 1.3% as reported by Guimaraes et al. (2007), Cad-Saude et al., (2008) and Moukoko et al., (2014) respectively. It is near consistent because previous authors worked on much larger sample sizes to represent their various populations. The anti-HCV finding in this study is not in tandem with prevalence rates of 66.0, 49.1, 32.5, 22.4 and 16.0% as reported by previous authors (Lohiya et al., 1986; Kulik, 1999; Klinkenberg et al., 2003; De-Souza et al., 2004; Tabibian et al., 2008). The 0 (0.0%) anti-HCV response recorded in this study is also not in agreement with 27.3%, 19.7%, 18.1%, 11.1%, 9.3%, 8.3% and 3.0% prevalence rates as published by Vassilopoulos et al. (2008), Almeida and Predrose (2004), Chang et al. (1993), Palumbo et al. (2007), Majori et al. (2008) and Silvio et al. (2008) respectively, respectively.

The overall 0 (0.0%) anti-HCV prevalence rate recorded however in this study was almost consistent with the 0.4% antibody HCV prevalence rate recorded by Cad-Saude et al. (2008) and at variance with 41.2, 12.5, 6.8, 4.5, 2.7 and 2.63% prevalence rates recorded by some authors (Zhao et al., 2011; Vasilopoulou and Hadjichristodoulou, 2008; Chang et al., 1993; Silvio and Johnson, 2008; Majori et al., 2008; Guimaraes et al., 2007).

In this study, out of the 12 (3.8%) HBsAg seroprevalence rate recorded, 9 (5.1%) and 3 (8.3%) prevalence rates occurred among male students in the 21-25 and 26-30 age groups, respectively. The seroprevalence rate of hepatitis B in this study is low and this result is consistent with prevalence rates of 3.0 and 3.5% as reported in similar studies by some authors (Carey et al., 2007; Moukoko et al., 2014). Conversely, the result obtained is at variance with hepatitis B prevalence rates of 16.0, 18.0, 19.7, 22.4 and 49.1% as published by previous

authors in similar studies (Tabibian et al., 2008; Chang et al., 1993; Almeida and Pedroso, 2004; De-Souza et al., 2004; Kulik, 1999). No female students recorded positive reactivity to HBV antigen among all the age groups. Conversely 3 (0.9%) syphilis seroprevalence rate found in this research occurred in female students all of whom belonged to the 21-25 age bracket.

The occurrence of HBsAg and syphilis in both male and female students may be incidental and may have no direct connection with gender although this was not tested statistically. This opinion is supported by Zhao et al. (2011) who did not establish any gender association with the disease or microbial agents described in this study. Besides, it has been established that variations in prevalence rates may be due to different patterns of injection used by drug users and different geographical locations (Cad-Saude et al., 2008). Vellinga et al. (1999) however reported that risk factors for hepatitis B infection among people with intellectual disability include male gender, old age and geographical location.

Syphilis disease has been associated with migration and population. Seroprevalence rate of 3 (0.9%) for syphilis was recorded in this study. This low prevalence may be due to the fact that the students concerned (or most of the students) are not migrants from place to place. According to Macpherson and Gushulak (2008), syphilis has been associated with migration and the international movement of disease. The 0.9% syphilis seroprevalence is inconsistent with 1.1, 1.1, 1.5, 3.35, 3.3 and 19.6% prevalence rates published by some authors (Guimaraes et al., 2007; Cad-Saude et al., 2008; Silvio and Johnson, 2008; Takada et al., 2003; Carey et al., 2007; Zhao et al., 2011).

Findings in this study showed no case of co-infection between any of the three microbial agents. This is in contrast to some reports which established a significant association between HCV and syphilis infection (Hwang et al., 2000; Adjei et al., 2008; Wu et al., 2010). A similar situation was seen in the 3 (8.3%) HBsAg seropositive cases in the 26-30 age group. The only 3 (1.7%) seropositive reactivity for syphilis which was recorded by all females in the 21-25 age bracket had no record of co-infection with any of the other agents.

The non-association of HCV infection with syphilis infection as recorded in this study is however consistent with some reports which stated that a majority of studies suggest that HCV is not associated with syphilis in general but with genital ulcers inherent in syphilis infection (Marx et al., 2003). The students who were seropositive for HBV antigen and syphilis antibodies did not show physical signs of the disease. It is possible sooner than later, they will. This deduction is corroborated by Konopnicki et al. (2005), Chen et al. (2006), Omland et al. (2009) and Ananthakrishnan et al. (2010) who reported that patients with HCV and HBV co-infection have an increased risk of progression of HCV and HBV related liver disease (chronic hepatitis, cirrhosis and hepatocelular carcinoma) when compared with mono-infected patients. Although students used for this investigation were not screened for anti-retroviral (HIV) antibodies, the obtained results somewhat suggest that students and indeed subjects in and around Oghara town may be almost free from the diseases implicated. This is against the background of documented reports that state that HCV, HBV and syphilis infection are very common in HIV-infected persons (Soriano et al., 2008; Laurent et al., 2003).

Conclusion

Overall occurrence or prevalence rates of 3.8% for HBsAg and 0.9% for syphilis in this study indicate that hepatitis due to HBV and syphilis infection are being harboured by students of Western Delta University and perhaps, residents within and around Oghara town though in apparent low occurrence. Health authorities of Ethiope West LGA and indeed, Delta State should therefore start off or ensure constant seroepidemiological surveillance in order to monitor the prevalence of hepatitis, syphilis and other infections within the study area. Counseling and vaccination programs would also help to control their transmission.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Phenotypic characterization of phenol degrading microorganisms isolated from olive mill waste

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The growth of the olive oil production in Saudi Arabia particularly in Al Jouf region in recent years has been accompanied by an increase in the discharge of associated processing waste. Olive mill waste is produced through the extraction of oil from the olive fruit using the traditional mill and press process. Deterioration of the environment due to olive mill wastes disposal is a serious problem. When olive mill waste is disposed into the soil, it affects soil quality, soil micro flora and also toxic to plants. The aim of this work is to isolate microorganism (bacterial or fungal strains) from OMW capable of degrading phenols. Olive mill wastewater, olive mill waste and soil (beside oil production mill) contaminated with olive waste were used for isolation of phenol tolerant microorganisms. Four strains (two fungal and two bacterial) were isolated from olive mill waste. The isolated strains were *Candida tropicalis* and *Phanerochaete chrysosporium* (fungal strains) and *Bacillus* sp. and *Rhodococcus* sp. (bacterial strains). These strains were able to degrade phenols and could be used for bioremediation of olive mill waste.

Key words: Bioremediation, bacteria, fungi, Sakaka.

INTRODUCTION

In the last few years, human activities have changed a large number of ecosystems. Protection of the environment against the damage caused by contamination is of great importance. During the previous

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Abbreviations: OMW, Olive mill waste; **OMWW,** olive mill wastewater; **SOMW,** solid olive mill waste; **COD,** chemical oxygen demand; **BOD,** biochemical oxygen demand; **TSS,** total suspended solids; **TKN,** total Kjeldahl nitrogen; **TP,** total phosphorus.

years, different researches were carried out to study the risks of OMW pollution. More than 30 million cubic meters of olive oil has been produced by Mediterranean countries (Roberta and Giuseppe, 2012) which account for 95% of the total olive oil production worldwide (European Commission, Directorate-General for Agriculture and Rural Development, 2012). Olive mill waste (OMW) is the by-product generated during olive oil production (Mekki et al., 2009). Disposal of OMW is an environmental issue facing the olive oil producing countries due to the generation of huge quantities in a short period of time.

The growth of the olive oil production in Saudi Arabia particularly in Al Jouf region in recent years has been accompanied by an increase in the volumes of associated processing waste. Olive fruits contain only 2% of phenol (Rodis et al., 2002). OMW is phytotoxic (Saravanakumar et al., 2009) and has disastrous effect on human, so it causes a number of ecological and acute environmental problems (Maria et al., 2013). Earlier studies showed that, OMW has several advantages. It has antibacterial, antiviral and antifungal activities due to its phenolic content (Vagelas et al., 2009; Thabet et al., 2008; Cristina, 2006; Anna et al., 2011).

Previous studies revealed chemical characterization of OMW (Niaounakis and Halvadakis, 2006) and application of biological treatments for the reduction of its high organic carbon contents (Diamadopoulos and Paraskeva, 2006). Most of these studies described treatments based on the use of yeasts (Papanikolaou, 2008) or white rot fungi (Laconi, 2007). The screening degrading microbial communities that proliferate on OMW is so far not done in Saudi Arabia. In this study, the isolation and phenotypic characterization of OMW degrading microorganisms generated from olive oil mills in Al Jouf was done.

MATERIALS AND METHODS

Sample collection

Al Jouf is located in the north-western part of Saudi Arabia, 29° 58′ 11″ N, 40° 12′ 0″ E. Field trips were carried out to collect OMW during the period of September to October, 2013. Soil and solid olive mill waste samples were collected in clean and sterile plastic bags. The bags were appropriately sealed, labeled and dated. The samples were transported to the laboratory in ice and processed within 24 h of collection.

Physico-chemical analyses of OMWW

Samples were collected to study the physico-chemical characteristics of the OMW. The physico-chemical analyses included: pH, chemical oxygen demand (COD), biochemical oxygen demand (BOD), total suspended solids (TSS), total Kjeldahl nitrogen (TKN) and total phosphorus (TP). The analyses were carried out according to the American Public Health Association for Examination of Water and Wastewater (2006).

Sample culturing

Isolation of microorganisms was carried out by using three olive mill wastewater (OMWW), two soil mixed with olive mill waste and three solid olive mill waste (SOMW). 3 g of SOMW was inoculated into 45 ml on chemically defined medium (CDM). The contents of the CDM per liter were NH_4NO_3 (2.0 g), KH_2PO_4 (0.5 g), K_2HPO_4 (1.0 g), $MgSO_4.7H_2O$ (0.5 g), $CaCl_2.2H_2O$ (0.5 g), KCl (0.1 g), yeast extract (0.06 g), NaCl (5.0 g), Resazurin (0.0001 g), cysteine hydrochloride (0.5 g), trace element solution (10 ml), vitamin solution (10 ml) and phenol (3 mM), pH 7.2 ± 0.1 at 25°C. All components were added to distilled water and volume was brought up to 1.0 L except phenol. The medium was mixed thoroughly and gently heated until it dissolved and then autoclaved. After autoclaving, 3 mM phenol was added using Whitman's filter paper. After 5 days of incubation, the sample was subjected to serial dilutions (10^{-1}, 10^{-2}, $10^{-3,}$ 10^{-4}, 10^{-5} and 10^{-6}) of SOMW made in sterile saline (0.85% w/v). Soil samples were incubated for five days before isolation of microorganisms for the possible isolation of a wide variety of microorganisms from soil. As nutrients become depleted or are made less available by the drying out of the soil, vegetative cells of some of the microorganisms could produce reproductive spores which can withstand a wider variety of deleterious conditions such as radiation and lack of nutrients and water. Like reproductive spores, endospores will germinate when growth conditions return, and generations of vegetative cells will again thrive as long as appropriate nutrients are available. So, our motive was to provide the favorable environment for the microorganisms to get enough nutrition and sufficient growth of microorganisms that are present in the soil. A 5 ml aliquot of each diluted sample was spread on chemically defined medium (CDM) agar (15-20% w/v) plates. Dilution of each soil sample was analyzed in triplicate. The plates were inverted and incubated for 3 days at 35°C. Results were recorded as colony forming unit (CFU). Colonies growing on the plates were counted and the density of microorganisms in the original sample was estimated by multiplying the colony count times the dilution. The single colonies were streaked onto nutrient agar plates, incubated at 35°C overnight and then the pure isolates were stored on Luria broth (LB) agar. Slant supplemented with phenol was used as sole carbon source at 4°C for future use. The same procedures were carried out using 5 ml of OMWW and soil mixed with olive mill waste. Malt extract media (Sigma-Aldrich) was used for isolation of fungi.

Characterization of isolates

Selected isolated colony samples were characterized by Gram-stain and by observed cell morphology (shape and size) using a Leica DMD108 digital microimaging, Leica Microsystems, Germany.

Characterization of isolates

Selected isolated colonies were characterized by Gram-stain and by observing cell morphology (shape) using a light microscope. To obtain various groups of isolates, colonies that showed different color plate morphologies, different colony size, shape and textures were chosen. Isolates were tested for the presence of the enzyme catalase by aseptically transferring a small amount of cells onto a glass slide and adding 2-3 drops of 3% H_2O_2. The observed production of bubbles was considered as positive test for catalase.

Table 1. Physico-chemical characteristics of OMWW.

Parameter	Unit	Minimum	Maximum	Average
pH		4.2	4.9	
COD	mgO_2/L	112,980	187,600	167,500
BOD	mgO_2/L	35,490	69,530	57,576
BOD/COD		0.31	0.37	0.34
TSS	mg/L	18,930	25,640	21,768
TKN	mgN/L	465	786	564
TP	mgP/L	118	154	134
Oil and grease	mg/L	4,532	6,435	5,213
Phenol	mg/L	2,143	2,879	2,543

COD, chemical oxygen demand; **BOD,** biochemical oxygen demand; **TSS,** total suspended solids; **TKN,** total Kjeldahl nitrogen; **TP,** total phosphorus

The phenotypic characteristics of all isolates studied were determined and compared with phenotypic data of known organisms described in the Bergey's Manual of Systematic Bacteriology. Varieties of biochemical tests were conducted according to the standard determinative bacteriology procedure (Beishier, 1991; Smibert and Krieg, 1994). Biochemical profiles for isolates include tests for fermentation of some carbohydrates and H_2S acid production. The number and types of positive tests were tabulated for the isolates and used to construct biochemical phenotype profiles of the cultures which were compared amongst the isolates.

RESULTS AND DISCUSSION

OMWW characteristics

Table 1 shows the characteristics of OMWW discharged during the production of oil. The pH of OMWW was found to be slightly acidic ranging from 4.2 to 4.9. The average concentration of organic load represented by COD, BOD and TSS was 167,500, 57,576 and 21,768 mg/l, respectively. The nutrient concentration of TKN and TP ranged from 465 to 786 and 188 to 154 mg/l with average of 564 and 134 mg/l, respectively. The average concentration of oil and grease and phenol was 5,213 and 2,543 mg/l, respectively.

The BOD/COD ratio of OMWW ranged from 0.31 to 0.37 with an average of 0.34. This result indicates that the biodegradability of OMWW was very poor. This may be attributed to the presence of phenolic compounds which hinder the activity of microorganisms.

Isolation and identification of microorganisms

Four strains of microorganisms were isolated from OMW and were screened for phenol degradation in Ramsay modified medium with 3 mM phenol at 35°C. The result for the screening of phenol degrading microorganisms is presented in Table 2.

The 4 strains which were able to degrade phenol are LW1A, OMSW2B, SW1A and SW2B. Out of the four strains, two were bacteria and two were fungi. LWI, OMSW2B, SWIA and SW2B were identified as *Candida tropicalis*, *Phanerochaete chrysoporium*, *Bacillus* sp. and *Rhodococcus* sp., respectively (Figures 1-4). *Rhodococcus* sp. (Figure 1) is non-motile, non-spore forming, aerobic Gram-positive filamentous rod bacteria.

P. chrysosporium (Figure 2) shows a pattern of septate hyphae, has some branching. At the end of the hyphae rests chlamydospores, the conidiophore gave rise to round asexual blastoconidia.

Candida tropicalis (Figure 3) shows the absence of terminal chlamydospores.

Bacillus sp. belong to genus Bacillus, Gram positive, rod shaped member of Firmicutes, catalase positive and oxidase negative bacteria.

It has been shown that the concentration of phenol is very important in order to determine the efficiency of phenol biodegradation. High concentration of phenol may contribute to toxic effects thus reduced biodegradation rates. However, low concentration of phenol below the threshold cannot support growth and degradation will not occur (Cornelissen and Sijm, 1996). Therefore, we recommended that, the study of different initial phenol concentration is very important in the determination of phenol biodegradation efficiency. According to Maria et al. (2013), phenol may act as a substrate and may also act as an inhibitor. Self-inhibition was also reported by Saéz and Rittmann (1991) where high concentration of substrate inhibits its own degradation. The utilization of phenol as sole carbon sources by microorganisms found in this study may probably be due to the presence of enzymes which is able to degrade phenol. The enzymes which are responsible for phenol degradation can also be studied by isolation, identification and characterization for further information. Continuous and fed-batch culture system can be used to study the performance of phenol

Table 2. Putative main characteristics of phenol degrading microorganisms isolated from Sakaka Olive Mill.

Feature	*Fungi*		*Bacteria*	
	Candida tropicalis	***Phanerochaete chrysosporium***	***Bacillus* sp.**	***Rhodococcus* sp.**
Morphology	*Oval*	septate hyphae	Rods	filamentous rods
Pigmentation	*None*	*None*	*None*	*None*
Gram stain	ND	ND	+	+
Motility	-	-	+	-
Oxidase	ND	ND	-	-
Catalase	ND	ND	+	+
Temp range (°C)	30-45	30-45	30-45	30-45
pH range	5-9	5-9	5-9	5-9
Acid production				
Sucrose	-	ND	-	-
Glucose	+	ND	+	+
Lactose	ND	ND	-	-
Maltose	+	ND	+	-
Mannitol	+	ND	-	+
Casein	+	ND	-	+
Starch	+	ND	-	-
Esculin	-	ND	+	+
H_2S production	ND	ND	ND	+

+ Positive, - negative, ND not determined.

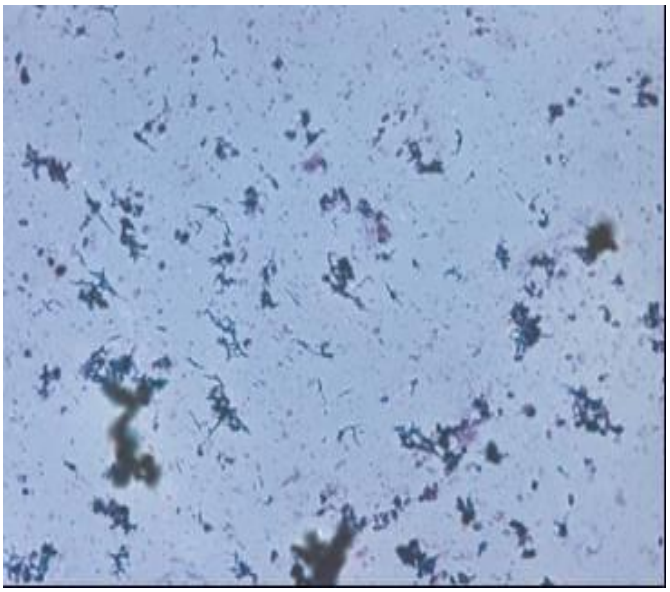

Figure 1. *Rhodococcus* sp. isolated from SW2B.

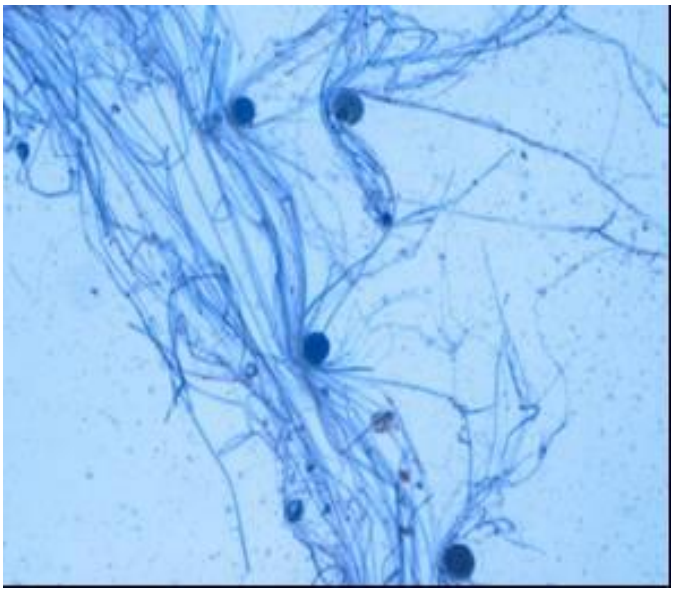

Figure 2. *Phanerochaete chrysosporium* isolated from OMSW2B

biodegradation in different fermentation modes. The incubation temperature of microorganisms is an important factor to determine the biodegradation efficiency of phenol. This is because at high temperature which is above optimum temperature, the microbial activity declined due to enzyme denaturation (Hiba et al., 2014). However, at low temperature below optimum, the microbial activity is relatively slow or no microbial activity because the movement of molecules is slower and there is not enough energy to start a chemical reaction.

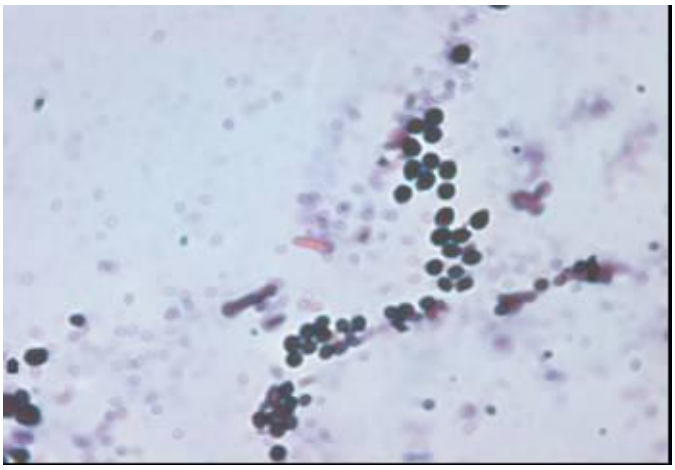

Figure 3. *Candida tropicalis* isolated from LW1A.

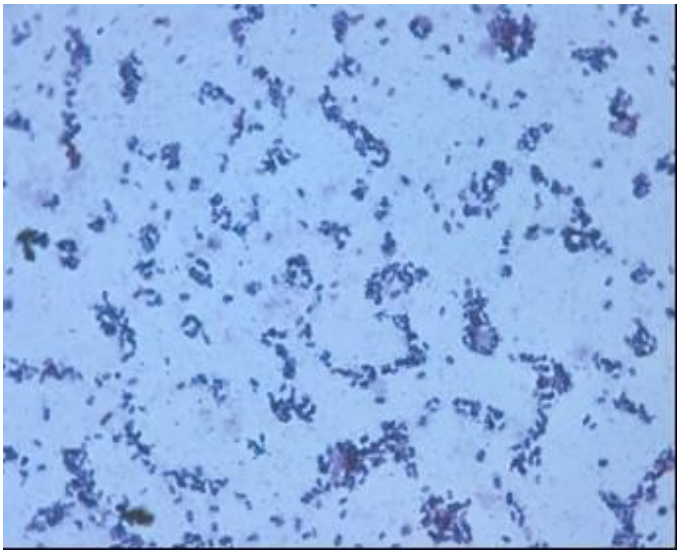

Figure 4. *Bacillus* sp. isolated from SW1A.

Conclusions

Bioremediation system of OMWW was conducted using microorganisms and phenol degrading bacteria grown in OMWW at the expense of its constituents and transformed into an organic liquid of high fertilizing value. Results from screening showed that 6 strains were able to grow in Ramsay medium and have capability to degrade phenol and its compounds. Phenol and its derivatives are organic pollutants which pollute the environment considerably. Therefore, the study of new approaches should be continued for this research in order to find out a suitable condition to degrade phenol. The reaction of the degradation mechanism can also be studied in order to increase the degradation activity. Besides, the enzymes which is responsible for phenol degradation can also be studied by isolation, identification and characterization for further information.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Assessment of chemical and bacteriological quality of pipe-borne water from various locations in Delta State University, Abraka, Nigeria

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Eighteen samples, consisting of six samples each, from three different locations that were 250, 500 and 750 away from the drinking water source in Delta State University, Abraka Campus, were collected and analyzed for their microbial and chemical quality using standard methods. Total viable counts were carried out using the pour plate method, while the most probable number was determined with the multiple tube fermentation technique. The total viable counts increased with distance away from the water source and were high for all the water samples, exceeding the 2.0 Log_{10}cfu/ml set limit for drinking water. The isolated organisms were *Micrococcus* sp., *Chromobacterium* sp., *Streptococcus* sp., *Pseudomonas aeruginosa* and *Staphylocosus aureus*. Chemical parameters analyzed were pH, chloride, alkalinity, carbon-dioxide, calcium, magnesium, zinc, iron, copper, potassium, total hardness, total dissolved solids, total suspended solids and total solids. The results obtained from each parameter were compared with the quality standard for drinking water laid down by the World Health Organisation and Federal Environmental Protection Agency (FEPA), Nigeria. The analyses revealed that there were increases in some of the parameters with distance away from the water source while some of the parameters studied were within the approved standard, others were above or below. It is thus imperative for our drinking water to be properly treated prior to consumption.

Key words: Water, microbial, chemical, parameters, standards.

INTRODUCTION

Water supply is the general process required for the provision of water from public water system to individual buildings and subsequent distribution of such water to various parts of such buildings. The water from public supply system to buildings is supplied through pipes. The strength of the pipes, water carrying capacity, life and

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durability of pipes, joining process, maintenance and repairs affect the quality of water being supplied. Piped water remains susceptible to biological and/or chemical contamination. Portable water supply system requires not only pipes, but many fittings and valves which add considerably to their functionality (Roberge, 1999).

Drinking water system thus provides habitat for microorganisms which are sustained by organic and inorganic nutrients present on the surface of the pipes or in the conveyed water. Maintaining the distribution system will require maintenance and survey procedures to prevent contamination and also remove and prevent the accumulation of internal deposits (Sobsey, 1989).

The safety of drinking water therefore depends on a number of factors which include quality and source of water, effectiveness of treatment and integrity of the distribution system that transfer the water to containers. The traditional approach to varying the bacteriological and chemical safety of piped water supply has relied on sampling strategies based on the end product, that is, tap water (WHO, 2003; Craun et al., 1997).

The objectives of this study, therefore, are to determine the bacteriological, and ascertain the chemical quality of piped water distribution system and suggest ways to reduce corrosion and increase portability of water for human consumption.

MATERIALS AND METHODS

Six samples from each of the three different locations (designated A - C, and were 250, 500 and 750 m away from the borehole), were collected with 500 ml sterile conical flasks, corked with cotton wool wrapped with aluminium foil and were transported immediately to the laboratory for analyses.

Identification of bacterial isolates

The identification of the sample microorganisms were based on cultural, morphological and biochemical characteristics according to the schemes of Cowan and Steel (1974), Buchanan and Gibbons (1974) and MacFaddin (1980). The result of each test was observed and recorded.

Bacteriological analysis

The total aerobic count (TAC) was carried out as described by Anon (1994). The sample water was serially diluted with distilled water after which 0.1 ml aliquots of 10^{-1} and 10^{-3} dilutions, respectively were dispensed into separate Petri dishes. Molten plate count agar cooled to 450°C was dispensed into each plate and incubated for 48 h at 37°C. The growths were observed and counted.

Estimation of coliforms

Coliforms were estimated using the five tube most probable number (MPN) technique. The lauryl sulphate broth used has high nutrient quality and the presence of phosphate buffer in this medium enhances rapid growth and increased gas production of slowly lactose fermenting coliform bacteria. It also inhibits the growth of undesired bacteria. The numbers of positive tubes were compared with MPN index table. Aliquots of the water samples were also incubated on centrinide agar which is selective for *Pseudomonas* sp. and also violet red bile which is a selective medium for *Escherichia coli.* Degradation of lactose to acid is indicated by the pH indicator, neutral red, which changes to red, and also by precipitation of bile acids. The appearance of the colonies on the plates is red, surrounded by reddish precipitation zones.

Chemical analyses

Determination of carbon-dioxide

Twenty millilitres (20 ml) of water sample was dispensed into a sample vial using a sterile syringe and two drops of phenolphthalein indicator was added. The content of the vial was mixed thoroughly after which it was titrated with carbon dioxide reagent B (0.02 N sodium hydroxide solution) until a pink colour was observed. The test result was read directly from the scale on the titrator barrel and recorded.

Determination of chloride

Ten millilitres (10 ml) of water sample was dispensed into the sample vial and three drops of chloride A reagent (5% potassium chromate) was added as indicator and mixed thoroughly. The mixture was titrated with chloride turned to a faint permanent brick-red colour. The result was read directly from the scale of the titration barrel and recorded.

Determination of alkalinity

Five millilitres (5 ml) of the water sample was pipette into the sample vail and a tablet of BCG-MR indicator (Bromocresol green-methyl red) was added and allowed to dissolve. The green colour was titrated with alkalinity reagent B (0.1%) sulphuric acid0 until solution turned purple. A post end colour was red. The result was read and recorded.

Determination of total hardness

Three millilitres (3 ml) of the water sample was dispensed into the vial using a syringe. The vial was inserted into the spectrophotometer chamber and scanned blank after which it was removed from the chamber and the sample transferred into Ca hardness UDV (unit dose vial). The vial was mixed vigorously for about 10 s and inserted into the chamber. The sample was scanned and results were recorded in mg/L.

Calcium hardness

Calcium hardness was recorded as one third of the total hardness in milligram per litre.

Magnesium hardness

Magnesium hardness was recorded as two third of the total hardness in milligram per litre.

Determination of electrical conductivity

Electrical conductivity was determined using a conductivity meter. The probe was dipped into a beaker containing the sample until a stable reading was obtained and recorded in µs/cm.

Determination of pH

The pH of the water samples were determined using the Hanner microprocessor pH meter standardized with a buffer solution of 4 to 9. The results were obtained using a stable reading.

Determination of zinc

Ten millilitres (10 ml) of the water sample was dispensed into a clean tube with the help of a syringe. The sample was scanned black after which 0.1 g of sodium ascorbate and 0.5 g of zinc buffer powder were added and mixed thoroughly for 1 min. Three drops of 10% sodium cynate, 1 ml of zinc indicator solution (5.0 ml zinc indicator solution and 17.8 ml methyl alcohol) and four drops of formaldehyde solution (37%) were added, capped and mixed thoroughly. The vial was inserted into the Smart spectrophotometer and readings were recorded in mg/L.

Determination of copper

The vial was rinsed with water sample after which 3 ml of the sample was dispensed into the vial with the help of a sterile syringe and was capped, its content mixed thoroughly and allowed to stand for 5 min. This was followed by further mixing to re-suspend the settled precipitate after which it was immediately inserted into the spectrophotometer chamber and scanned. The results were recorded in mg/L.

Determination of cadmium

The tube was rinsed with sample water after which 10 ml of sample was dispensed into it and scanned blank. The tube was removed from the chamber and 1.0 ml buffered ammonia reagent, two drops of 10% sodium citrate, 0.5 ml of PAN indicator, and 0.5 ml of stabilizing reagent were added, capped and mixed thoroughly. The tube was inserted into the chamber and its content scanned.

Determination of iron

The tube was rinsed with the sample water and filled to 10 ml line of the vial and scanned blank. With the help of a syringe, 0.5 ml of iron reagent 2 powder was added and mixed thoroughly for 30 s. The solution was allowed to stand for 30 s for maximum colour development after which the sample vial was inserted into the spectrophotometer chamber and scanned. Results were read and recorded in mg/L.

Determination of total dissolved solids

The electrode of the Hanna's instrument (Model TDS 1) was rinsed with distilled water after which it was dipped back into the water sample in a clean beaker. The total dissolved solids were read by slightly sliding the knob on top of the instrument. The result was read and recorded.

Determination of total solids

A clean, dry and flat silica disc was weighted (W1) and 50 ml of the water sample was dispensed into it. The content of the disc was evaporated in a water bath. With the help of a forceps, the disc was transferred into an oven set at 105°C for 3 h after which it was removed, left to cool and re-weighed. The process was repeated till a constant weight was obtained (W2). Total solid (mg/L) = (W2-W1) 20,000 mg/L.

Determination of total suspended solids

The difference between the total solids and total dissolved solids is equal to the total suspended solids.

SS = TS - DS mg/L

RESULTS AND DISCUSSION

The organisms isolated from the water samples presented in Table 1 were *Micrococcus* sp.*, Chromobacterium* sp.*, Streptococcus* sp.*, Pseudomonas aeruginosa* and *Staphylocosus aureus.* These are reported water resident organisms (Benka-Coker and Olimani, 1995; Edema et al., 2006; Ukpong, 2008). Some of the organisms are reported causal agents of some water-borne diseases. Thus, their presence in water could pose some effects on human health.

The results obtained in the analyses of the water samples as presented in Table 2 shows that the mean values of carbon-dioxide in the water samples, which ranged from 11 - 23 ppm, were not within the World Health Organization approved standard of 50 ppm. Hung and Hsu (2004) reported that carbon-dioxide quickly combines with water form carbonic acid, a weak acid. Thus the presence of carbon-dioxide in water may have negative effects depending on the water pH. If the water has a high pH value, the carbonic acid will act to neutralize it, but if the water is acidic, the carbonic acid will act o neutralize it, but if the water is acidic, the carbonic acid will make it even more acidic.

The mean values of chloride content, that ranged from 8.17 to 12.5 ppm, was below the 200 ppm maximum range for standard water, and so, has no adverse health impact when present in water for consumption and other domestic uses.

The mean values of alkalinity of the water samples, which ranged from 10.67 to 16 ppm, was below the set standard of 100 ppm which would have no adverse effect on human health while the mean values of pH, which ranged from 5.38 to 6.32, were below the acceptable limit of 6.5-8.5. This calls for the treatment of such water necessary prior to consumption in order to avoid the associated adverse health implications.

The mean values of electrical conductivity of the water samples, that ranged from 10.32 to 31.82 µs/cm, was

Table 1. Identification of bacterial isolates.

	A	B	C	D	E
Shape	Cocci in clusters	Rod	Cocci in chains	Rod	Cocci in clusters
Gram reaction	+	-	+	-	+
Aerobic growth	+	+	+	+	+
Anaerobic growth	-	+	+	-	+
Endospore production	-	-	-	-	-
Motility test	-	+	-	+	-
Catalase test	+	+	-	+	+
Oxidase test	+	+	-	+	-
Glucose fermentation	-	+	+	-	+
Organism identified	*Micrococcus* sp	*Chromobacterium* sp.	*Streptococcus* sp.	*Pseudomonas aeruginosa*	*Staphylococcus* sp.

Table 2. Average values of water parameters of samples.

Parameter	A (250 m)	B (500 m)	C (750 m)	Set standards
CO_2	8.83	11.00	23.00	50 ppm
Cl (ppm)	10.67	12.50	8.17	200
Alk (ppm)	12.00	16.00	10.67	100
Cond (µs/cm)	10.38	31.82	12.42	1000
pH	5.85	6.32	5.38	6.5 - 6.8
Hardness (ppm)	21.83	27.83	21.00	100
TDS (ppm)	0.57	1.92	0.80	-
TSS (ppm)	0.22	0.50	0.16	-
TS (ppm)	0.79	2.42	0.96	500
Mn (ppm)	14.55	17.55	14.00	30
Ca (ppm)	7.25	8.77	7.00	75
Zn (ppm)	0.09	0.10	0.18	5.0
Cu (ppm)	0.23	0.27	2.25	1.0*
K (ppm)	0.24	0.63	0.63	10.00
Cd (ppm)	0.09	0.07	0.23	0.003*
Fe (ppm)	3.63	1.38	1.66	0.30*
Total aerobic counts	2.35	2.67	3.56	-
Coliforms (cfu/ml)	0.33	2.33	1.33	0.00*

* = Above set limits.

within the acceptable limit of 10.00 µs/cm set by the World Health Organisation and Nigeria for drinking water.

range of 500 ppm. Storey and Ashbolt (2003b) reported that solids can either be suspended or dissolved solids and together are referred to as total solids. Solids in water samples can vary significantly with season and rainfall. Events and abnormal changes in the amount and type of solids, whether total or dissolved can provide information on the pollution level of the water. Solids can also affect the taste and appearance of the drinking water.

The showed that the mean values of total solids present in the water samples were within the acceptable

The mean values of zinc concentration, which ranged from 0.09 to 0.18 ppm, were within the set limit of 5 ppm. While the copper concentration of the samples from locations A (0.23 ppm) and B (0.27ppm) were within set limits, water samples from Location C (2.25 ppm) was above the acceptable limit of 1.0 ppm.

The mean values of value for potassium concentration, which ranged from 0.24 to 0.63 ppm, were within the acceptable standard of 1.0 ppm. However, the mean

values of values for cadmium (0.09 – 0.23 ppm) and iron (1.38 – 3.63 ppm) were above the set limits of 0.003 and 0.30 ppm, respectively. Florea and Busselberg (2006) and Hung and Hsu (2004) reported that some trace elements are potentially toxic. Zinc and copper are essential elements for the maintenance of the body's metabolic activities but copper contaminated water could pose health hazards such as abdominal pains, nausea, vomiting, diarrhoea, headache and dizziness as reported by Chinwe et al. (2010). Copper poisoning principally influences formation of liver cirrhosis known as non-India childhood cirrhosis (WHO, 2003). Jerup (2003) reported that some trace elements are potentially toxic because they act on the cell membrane or interfere with the cytoplasmic or nuclear functions when they enter into the cell, hence their entry into the human body could result in malfunctioning of the body systems. Therefore copper, zinc, cadmium and other trace metals have adverse effects in humans if present in water samples in very high concentration. Cadmium, for instance, derives its toxicological properties from its similarity with zinc, an essential micronutrient in humans. Cadmium is bio-persistent and once absorbed by humans, remains resident for many years, although it is eventually excreted.

The mean values of total aerobic counts (2.35 – 3.56Log_{10}cfu/ml) of the water samples were high. According to the World Health Organization (2003) report, a high aerobic count does not itself present a risk to human water supply system. A particular feature of the *Pseudomonas aeruginosa* is its ability to grow in low nutrient water. Warburton (1992) reported that the *Pseudomonas* strains present in water usually do not have the same genetic pattern as those in clinical cases during gastrointestinal infections. Though, Allen et al. (2000) reported that water for human consumption is required to be free from any bacteria that may pose a health risk, the presence of *Pseudomonas* in these water samples may not pose adverse health hazard due to their genetic constitution.

The presence of biofilms in the drinking water distribution system may play a role in the presence of potential pathogens in drinking water pipes. This contamination can occur due to defective joints, back siphonage, rusted pipelines crossing over the sewage pipes and low/high pressure in the pipelines. For water to be wholesome, it should not present a risk of infection or contain unacceptable contamination of chemicals hazardous to health and should be aesthetically be acceptable to consumers.

The mean values of coliform counts (0.33 - 2.33log_{10}cfu/ml) were higher than the set standard of 0.0. This could have been due to mixing-up of water and sewage where the water pipes are broken. Being indicator organisms of faecal contamination and the causal organisms of many water-borne diseases, it is therefore pertinent to treat the water with physical and/or chemical methods prior to use for domestic uses. The university community draws her drinking water from these locations and this could lead to outbreak of water-borne infections if treatment options are not employed.

The Pearson moment correlation coefficients presented in Table 3 revealed strong correlations between the tested parameters. CO_2 was strongly correlated to Zn, Co, K, Cd and TAC; Cl was strongly correlated to alkalinity, conductivity, pH, Mn, Co, hardness, TDS, TSS and TS; Alkalinity was strongly correlated to pH, Mn, Ca, hardness, TDS, TSS, TS, Cl and coliform counts; conductivity was strongly correlated to Cl, alkalinity, Mn, Ca, hardness, TDS, TSS, TS and K; pH was strongly correlated to Cl, alkalinity, conductivity, Mn, Co, hardness, TDS, TSS and TS; Mn was strongly correlated to Cl, alkalinity, conductivity, pH, hardness, TDS, TSS, TS and coliform counts; Ca was strongly correlated to Cl, alkalinity, conductivity, pH, Mn, TDS, TSS. TS and coliform counts; Hardness was strongly correlated to Cl, alkalinity, conductivity, pH, Mn, Ca, TDS, TSS, TS and coliform counts; TDS, TSS and TS were strongly correlated to Cl, alkalinity, conductivity, pH, Mn, Ca, hardness and coliform counts; TDS was strongly correlated to TSS and TS; Zn was strongly correlated to CO_2, K and total aerobic counts; Cu was strongly correlated to CO_2, K, Cd and total aerobic counts; K was strongly correlated to CO_2, conductivity, TDS, TS, Zn, Cu, total aerobic counts and coliform counts; Cd was strongly correlated to CO_2, Cu and total aerobic counts; Fe was not correlated to all parameters; TAC was strongly correlated to CO_2, Zn, Cu, K and Cd while CC was strongly correlated to alkalinity, Mn, Ca, hardness, TDS, TSS and TS and K.

Student t-test at 95% confidence level revealed that there was a statistically significant difference between the values in the locations. The parameters increased with distance away from the water source (borehole).

Conclusion

The need for suitable water for human consumption can never be overemphasized. The water parameters were found to vary with distance away from the water source. There is need to maintain water quality during transport either by chemical and/or physical treatments to avert water related diseases which are harmful to the health of man.

Conflict of Interests

The author(s) have not declared any conflict of interests.

Table 3. Pearson moment correlation coefficient for the tested parameters.

Parameter	CO_2	Cl (ppm)	Alk (ppm)	Cond (µs/cm)	pH	Mn (ppm)	Ca (ppm)	Hardness (ppm)	TDS (ppm)
CO_2	1								
Cl (ppm)	-0.83802	1							
Alk (ppm)	-0.58349	0.932098	1						
Cond (µs/cm)	-0.29041	0.765485	0.94657	1					
pH	-0.78615	0.996033	0.96063	0.819704	1				
Mn (ppm)	-0.50154	0.89235	0.995237	0.9735	0.928971	1			
Ca (ppm)	-0.48976	0.886146	0.993823	0.976512	0.923865	0.999908	1		
Hardness (ppm)	-0.47286	0.877048	0.9915	0.980484	0.916316	0.999461	0.999814	1	
TDS (ppm)	-0.21925	0.716095	0.920292	0.997292	0.775364	0.954047	0.958023	0.963372	1
TSS (ppm)	-0.52015	0.90191	0.997114	0.968322	0.936766	0.999766	0.99938	0.998516	0.947337
TS (ppm)	-0.28207	0.759858	0.943728	0.999962	0.81469	0.971473	0.9746	0.978736	0.997895
Zn (ppm)	0.999155	-0.85974	-0.61637	-0.32949	-0.81088	-0.53667	-0.52518	-0.50868	-0.25916
Cu (ppm)	0.992157	-0.89965	-0.68043	-0.40774	-0.85724	-0.60575	-0.5949	-0.5793	-0.33949
K (ppm)	0.618038	-0.08898	0.27783	0.572787	5.25E-16	0.370151	0.382718	0.400456	0.631515
Cd (ppm)	0.967003	-0.94938	-0.77114	-0.52461	-0.91766	-0.7054	-0.69572	-0.68174	-0.46058
Fe (ppm)	-0.52424	-0.02532	-0.38569	-0.66263	-0.11417	-0.47379	-0.48569	-0.50245	-0.71591
Total aerobic counts	0.993349	-0.76962	-0.48609	-0.17829	-0.70976	-0.39859	-0.38611	-0.36826	-0.10545
Coliforms (cfu/ml)	0.142162	0.420956	0.720923	0.9059	0.5	0.785046	0.793376	0.804963	0.93459

Table 3. contd.

Parameter	TSS (ppm)	TS (ppm)	Zn (ppm)	Cu (ppm)	K (ppm)	Cd (ppm)	Fe (ppm)	Total aerobic counts	Coliforms (cfu/ml)
TSS (ppm)	1	1							
TS (ppm)	0.966112	0.966112	1						
Zn (ppm)	-0.55481	-0.55481	-0.32126	1					
Cu (ppm)	-0.62283	-0.62283	-0.39978	0.996456	1				
K (ppm)	0.349957	0.349957	0.579897	0.585206	0.514923	1			
Cd (ppm)	-0.72058	-0.72058	-0.51718	0.976656	0.991264	0.39736	1		
Fe (ppm)	-0.45462	-0.45462	-0.66912	-0.4888	-0.41368	-0.99346	-0.28999	1	
Total aerobic counts	-0.41835	-0.41835	-0.16972	0.987777	0.971165	0.704449	0.931235	-0.61881	1
Coliforms (cfu/ml)	0.771454	0.771454	0.909551	0.101361	0.017318	0.866025	-0.11471	-0.91745	0.255193

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Full Length Research Paper

Analysis of the distribution of phytoplankton and enteric bacteria in Efteni Lake, Turkey

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Efteni Lake is a small and shallow lake located at the Western Black Sea Region of Turkey that is under severe environmental threat in terms of receiving land-based sources of pollutants especially of diffuse character. No field investigations related to phytoplankton distribution or enteric bacteria in the lake have been conducted prior to this study. To fill this gap in order to better understand the present status of microbiological water quality, the progress of the eutrophication process and to support the investigations relating to the rehabilitation of the lake as well as water quality management, a field study for determining the instantaneous spatial distribution of enteric bacteria and phytoplankton groups has been realized. The results of these investigations indicate that the lake is currently in transition from mesotrophic to eutrophic state. Enteric bacteria with multiple antibiotic resistances that may threat the public health as well as livestock were identified. The antibiotics to which these bacteria were found to be resistant were more commonly used for livestock rather than humans. This finding indicates that livestock is the more important source of faecal contamination than human-based faecal contamination. This study is the first step of a more comprehensive rehabilitation planning study for the lake.

Key words: Antibiotic resistance, enteric bacteria, biological water quality, Efteni Lake, eutrophication, phytoplankton distribution.

INTRODUCTION

Efteni Lake located at the North-Western part of Turkey within the Western Black Sea Watershed is a small and quite shallow and natural lake with a surface area of 180 ha and an average depth of less than 2 m (Figure 1). The lake is an important wetland for birds nesting and breeding. The lake had been larger and deeper years ago and was connected to a stream system. However, the lake lost most of its surface area after it was drained to gain agricultural land (Figure 2). Currently, the lake faces severe environmental threat because of the

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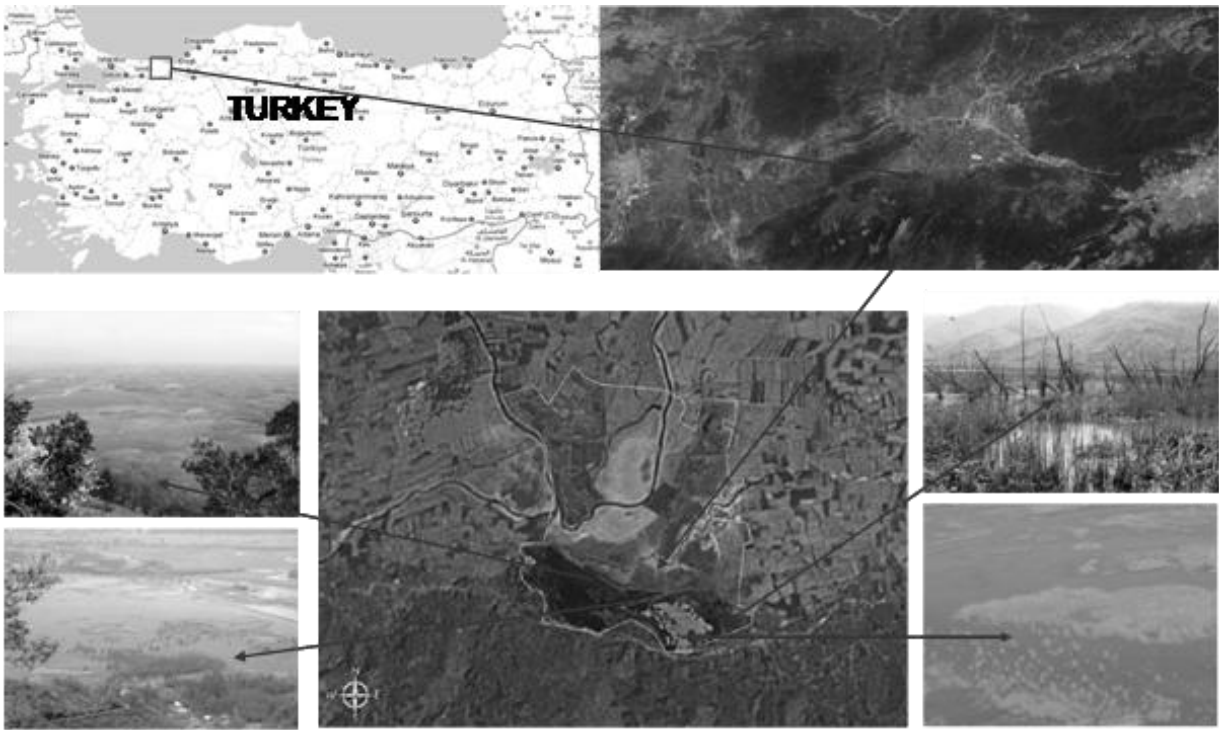


Figure 1. The study area.

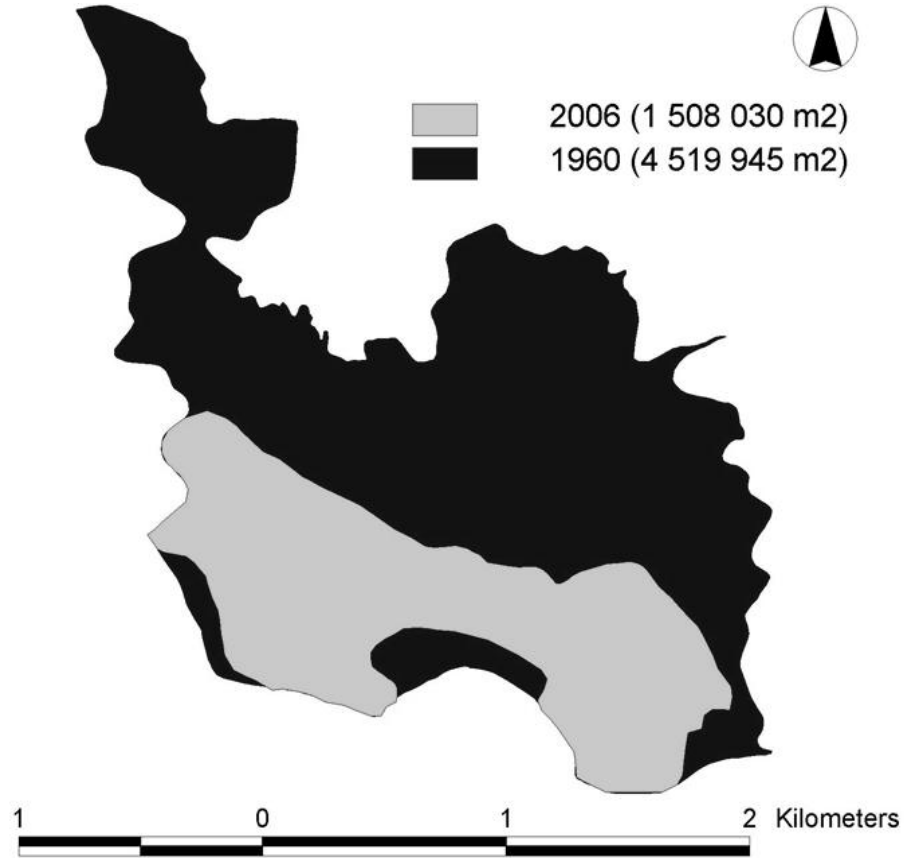


Figure 2 Efteni Lake before (1960) and after (2005) being drained.

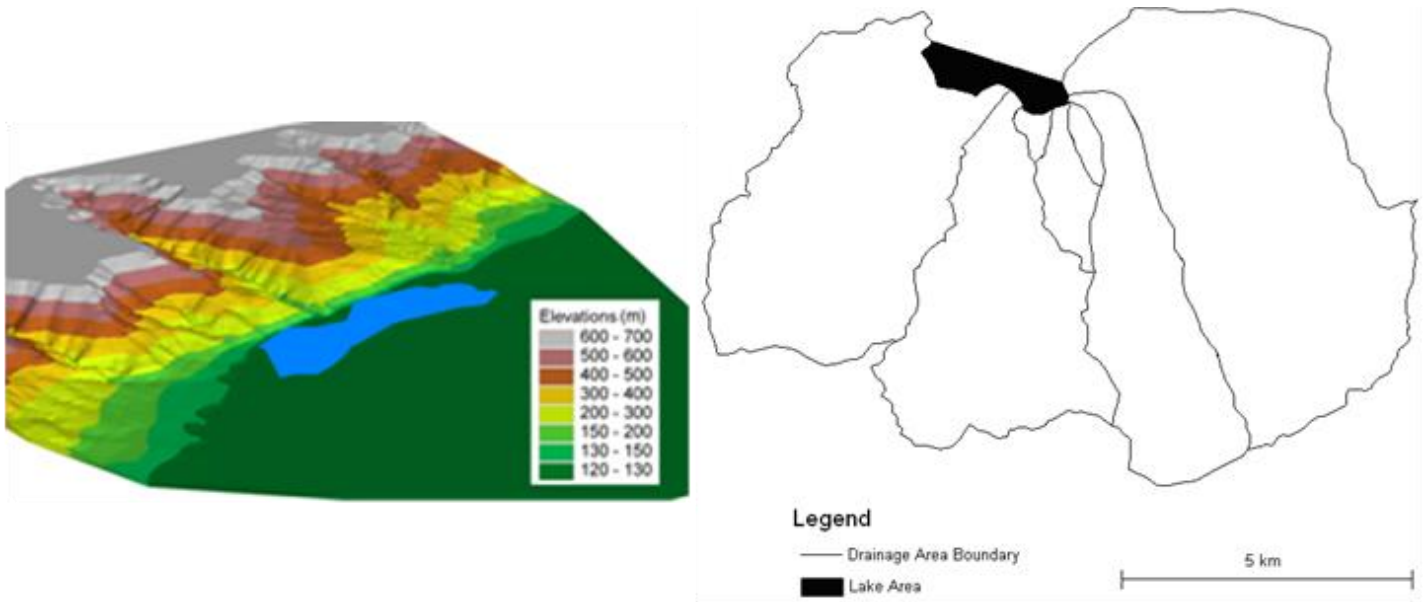


Figure 3. Drainage area of Efteni Lake.

decrease of its circulation and assimilation capacity as well as increased input of nutrient loads resulting from intensive agricultural activities and livestock breeding.

Although the lake was once used as a drinking water resource, water quality of it has been deteriorated especially from the biological point of view. Unfortunately, no historical data on the nutrient and biological parameters such as phytoplankton species distribution, microbiological pollution and macro invertebrates exist as reference conditions. The aim of this study is to conduct a biological monitoring prior to further deterioration of the system and to identify the existence of primary producers that play an important role in the eutrophication of the lake and the prevalence of enteric bacteria used as the indicators of faecal pollution.

Species distribution of phytoplankton may play an important role and shifts in phytoplankton to bloom forming species are observed in eutrophic aquatic ecosystems (Smith, 2003). Sources of different nutrient inputs to aquatic ecosystems are also important regarding their common effects on eutrophication; however, identification of significant sources leads to developing strategies for reducing them which is the first step of combating with eutrophication (World Health Organisation and European Commission, 2002; Environment Agency, 2002).

Enteric bacteria can be used as an indicator to identify the faecal inputs from humans and livestock to the aquatic systems as well as to monitor the biological water quality (Sigee, 2005). Many countries consider the occurrence of *Enterococcus faecalis* as a serious threat on human health and discussions are still ongoing to use it as an indicator (Kinney et al., 1977; Pinto et al., 1999; Dionisio et al., 2000; Arvanitidou et al., 2001; Blanch et al., 2003; Kim et al., 2010).

The aim of this study is to better understand the present status of microbiological water quality, the progress of the eutrophication process for supporting the investigations related to the rehabilitation and water quality management in the Efteni Lake.

MATERIALS AND METHODS

Delineation of the catchment

The first step of the analysis was on the delineation of the catchment area of the Efteni Lake that has significantly been reduced through drainage operations by means of diversion of large rivers feeding the system. Initially, a digital elevation model of the drainage area was created using digitized 1:25000 scaled topographical maps. ESRI's ArcGIS software with 3D Analyst extension was utilized to create the Digital Elevation Model (DEM) that was used to delineate the drainage area of the Lake (Figure 3) using the methods as described by Erturk et al. (2004) and Erturk et al. (2006). There are seven small streams flowing into the lake.

Based on hydrological calculations estimated flushing time of the lake is 15 days in the spring, less than a week in the winter, more than two months in the summer. Considering the amount of water in the lake, estimated flushing time, corresponding nutrient inputs, accessibility to the lake and other environmental factors such as water temperature, bathymetry and migration of birds, spring season was selected to conduct the field study in order to obtain a feasible abundance of as many organism groups as possible. Since there was no bathymetry data available, two survey teams were established; a land team that surveyed the coastline and the nearest and shallow part of the littoral zone, and a water team that surveyed the relatively deeper parts of the lake by a boat. The field data together with several maps and remotely sensed data that presented the coastline in different seasons (dry and wet) were

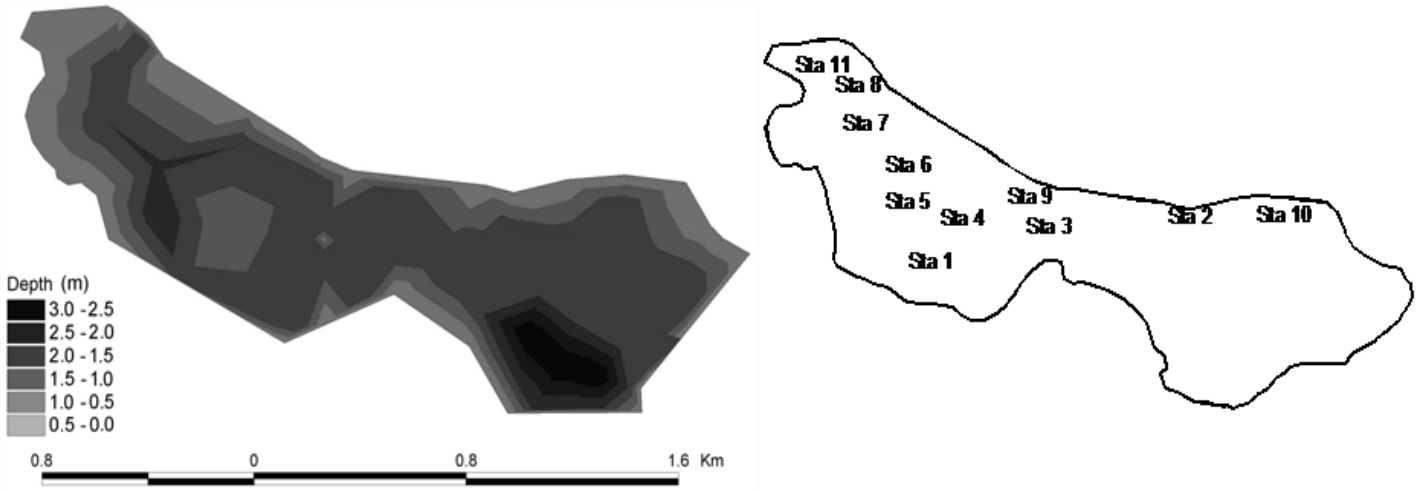


Figure 4. Bathymetry and Monitoring stations.

used to compile a bathymetrical data set for the lake. Then, isobaths were created using ArcGIS software (Figure 4). Considering the stream inflows, bathymetry and the location of reed assemblages, 11 sampling stations were selected to represent the lake (Figure 4).

Physicochemical water quality parameters

Fluoride, chloride, nitrite + nitrate nitrogen, PO_4-P, and sulphate were analyzed by a Dionex, ICS 1500 model ion chromatograph (Standard Methods 4110, 2005), TKN, NH4-N and TP were analyzed respectively based on standard methods APHA/AWWA/WEF (2005), (4500-org-N, Nitrogen & B, Macro Kjeldahl Method; 4500-NH3, Nitrogen & C titrimetric method and 4500-P, B & 4500-P, D).

Images were processed to obtain 60 clusters at 0.98 convergence threshold using 50 iterations. Afterwords, clusters were analysed in order to define their membership of Land Use Land Change (LULC), clusters belonging to the same LULC class were combined using recode operation. After recoding, four needed LULC classes were obtained and area information was extracted.

Biological water quality parameters

One litre of samples collected for chlorophyl-a measurements were filtered *in-situ* with 0.45 µm pore-sized GF/C Whatman filters and after addition of 90% acetone, the samples were stored for 24 h at +4°C under dark conditions. Chl-a (µg/L) and carotene concentrations were calculated using absorbance values obtained through spectrophotometric measurements (APHA/AWWA/WEF, 2005-Method 10200 H, Spectrophotometric). Phytoplankton counts were conducted by drop method using a NIKON YS-2 research microscope with the same method as applied by Ozman Say (2010).

Quantitative data related to enteric bacteria were obtained by multiple tube fermentation method. From each station water samples (100 ml) were collected using sterile screw capped glass bottles, and stored in cold bags at 4ºC until analysis in the laboratory. Numbers of total coliform bacteria were enumerated by multiple-tube fermentation methods within Lauryl Sulphate Broth (Merck). If necessary, water samples were diluted ten times (10x). Faecal growth was achieved by monitoring the acidification and gas production during growth in Brilliant Green Lactose Broth (Merck) at 44±0.5°C for 24±3 h and sub-cultured on Eosine Methylene Blue agar (Merck) plates from the fermentation tubes in order to isolate the coliforms. For control, the same samples were incubated in Chromocult Coliform® agar for 24 h. The detailed analyses for identification, the standard water and wastewater methods were applied as described in APHA/AWWA/WEF (2005) and Geissler et al. (2000).

Since many infectious bacterial groups that can develop resistance to antibiotics were identified in the lake, their resistance to several antibiotics was also tested. The minimum inhibition concentration was determined by the disk diffusion method in Mueller-Hinton medium in accordance with the Clinical and Laboratory Standards Institute CLSI guidelines (CLSI, 2003). Nine antimicrobial agents were selected as representatives of important classes of antimicrobials: Chloramphenicol (C^{30}), Tetracycline (T^{30}), Nalidixic acid (NA^{30}), Ampicillin (A^{10}), Imipenem (I^{10}), Ceftazidime (CA^{30}), Amikacin (AK^{30}), Streptomycin (S^{10}), Amoxicillin (AC^{30}) and Trimethoprim/sulfamethoxazole (CO^{25}). The results were separately interpreted by using the breakpoints from the CLSI guidelines for the family Enterobacteriaceae and non-fermenters.

RESULTS AND DISCUSSION

In Efteni Lake, spring is the season with relatively strong water circulation and high nutrient inputs providing a relatively homogenous distribution of phytoplankton. There exists as a high correlation between nutrient concentrations and Chl-a. In this study, highest Chl-a (2.95 mg/m^3) was measured at Station 5 (Figure 5). The spatial distribution of TN:TP and Chl-a is illustrated in Figure 6. When the nutrient ratios at different stations are examined, it is seen that TN/TP ratios are quite convenient for the uptake of N and P that will be utilized by organisms at Station 5.

TN:TP ratios have been used as a basis for estimating nutrient limitation and low TN:TP ratios (less than about 7:1) are indicative of nitrogen limitation, whereas ratios

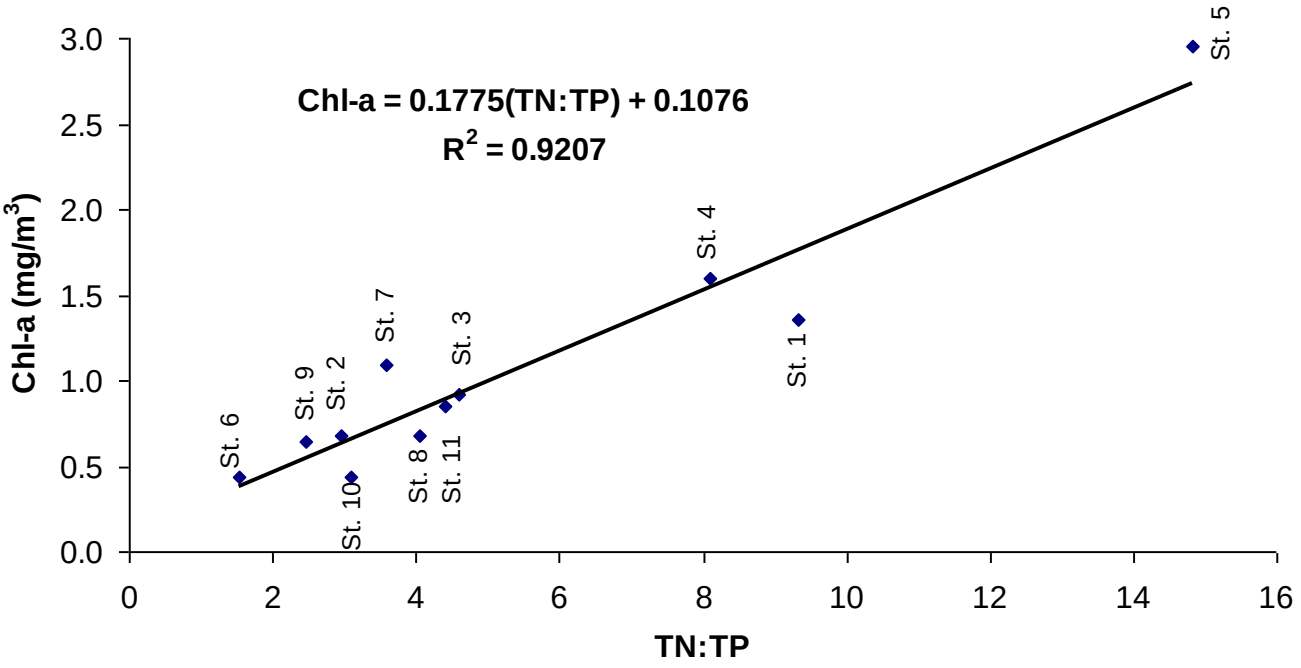


Figure 5. Chl-a and TN:TP ratios at the sampling stations.

greater than 10:1 are increasingly indicative of phosphorus limitation (USEPA, 2000; Wang et al., 2005; Camargo and Alonso, 2006). According to N:P ratios that are less than 10 with the exception of Station 5 and the high correlation between N:P ratio and the Chl-a (R^2 = 0.92), it can be stated that nitrogen is the limiting nutrient for Efteni Lake. Not only the N:P ratio, but also Si has an important role in the nutrient composition. Growth of diatoms depends on silicon. For this reason, Si, which exists in the water column and accumulates at the sediment, is one of the parameters that affect N and P concentrations especially at the transition periods extending from winter to spring.

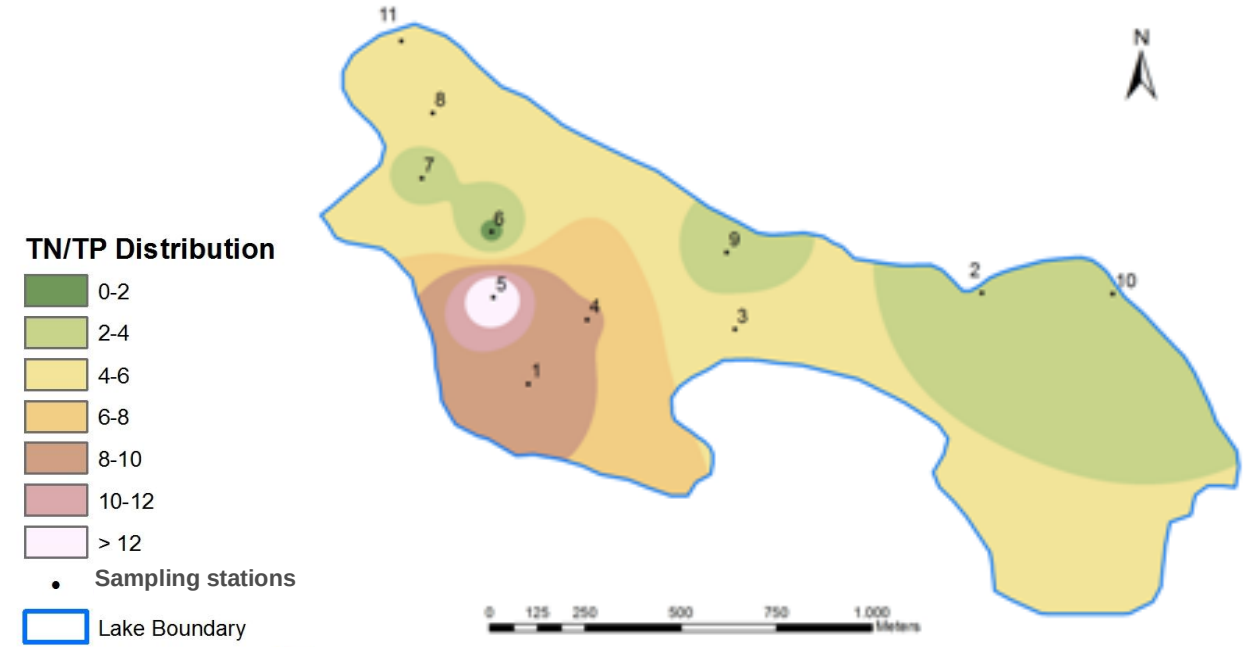
Although silicate measurements are not available, existing dominant diatom species with its numbers greater than a million observed at Stations 1, 5 and 12 indicate intensive nutrient input and probable increase in plankton. The quantity of phytoplanktonic organisms during sampling is given in Table 1. Frequencies in Table 1 are calculated by dividing the number of stations, where each species were found by the total number of stations and converting the results to percentage. Because the survey was conducted in spring, *Bacillariophyceae* (Diatoms) was the dominant phytoplankton group as expected; however, *Dinophyceae,* and *Chlorophyceae* were also observed.

Gymnodinium aeruginosum, which is a mixotrophic organism, was observed at each station. This species, which is quite high in number (2 393 600 organism/L at Station 5), may cause a negative effect since it may consume dissolved oxygen and may result in anaerobic conditions especially in the dark period of diurnal cycle. It is one of the permanent species in the regions where light intensity is low. The prevalence and abundance area of this specie can change depending on the high aquatic vegetation. It is considerable that this species exists at Station 5, which has the highest intensity among the other stations. The regions where *Chrysophyta* increases are observed are the mixing zone of stream inputs. This indicates that the lake is affected by the external inputs. Although it is rarely seen observation of *Dinophyta* is an indicator of external input to the lake.

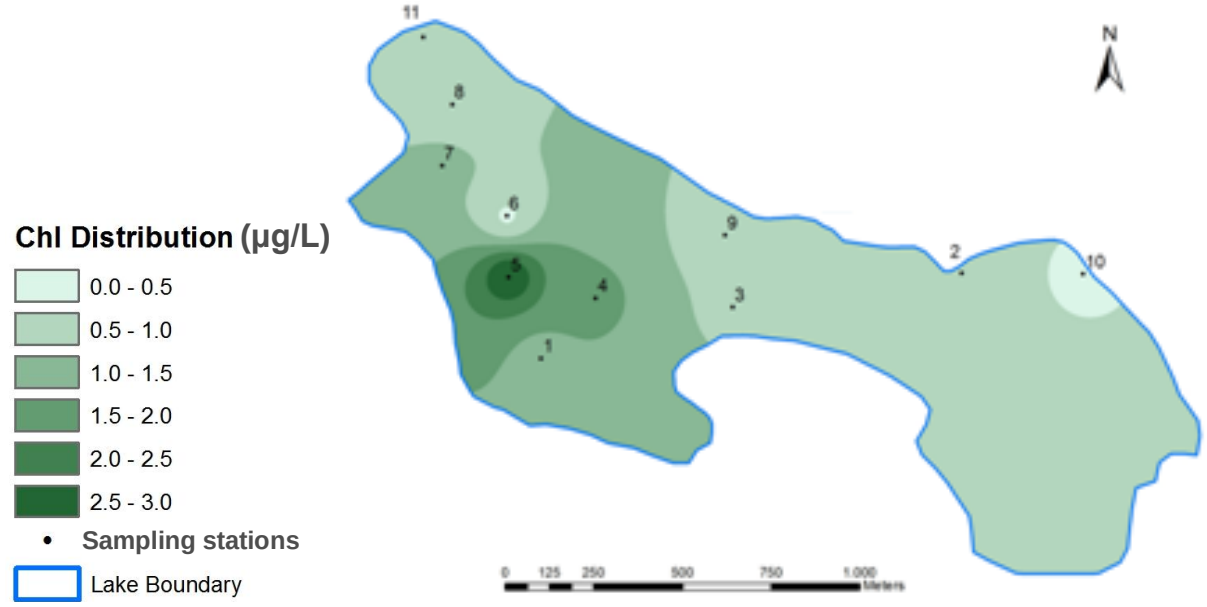
As seen from Table 2, special care should be paid to Stations 1, 5 and 10. Important increase in the number of plankton is reported in these stations, even though the temperatures were quite low (around 11ºC) during the sampling period. The microphotographs of different phytoplankton are illustrated in Figure 7.

Bacterial counts for each sampling station are given in Table 3. The spatial distribution of their dominance is illustrated in Figure 8. In all stations *Enterococcus fecalis* were dominant; *Klebsiella pneumoniae* and *Pseudomonas aeroginosa* were detected as well.

It is important to note that *E. faecalis* was identified in each station, whereas *Escherichia coli* were identified in only 4 stations. *Enterococci* are Gram-positive cocci that can survive under harsh conditions in nature. *E. faecalis* is a very diverse species of *Enterococci*. *E. Coli* is a well known indicator of microbiological contamination in natural waters; however, as stated previously by Kinney et al. (1977), Pinto et al. (1999), Dionisio et al. (2000), Arvanitidou et al. (2001), Blanch et al. (2003) and Kim et al. (2010); many countries consider the occurrence of *E.*



(a)



(b)

Figure 6. Map of TN:TP (a) and Chl-a (b) distribution.

faecalis as a serious threat on human health and discussions are still ongoing to use it as an indicator.

Bacteria from genera *Pesudomanas* and *Klebsiella* can develop resistance to antibiotics as well. Both of them were observed in some of the stations of the Efteni Lake. The resistance of bacteria to several antibiotics was tested as well (Table 4). In this study, the resistances of *E. faecalis, E. coli, Pseudomonas aeroginosa, Proteus mirabilis* and *Krebsiella pneumoniae* to Chloramphenicol (C^{30}), Tetracycline (T^{30}), Nalidixic acid (NA^{30}), Ampicillin (A^{10}), Imipenem (I^{10}), Ceftazidime (CA^{30}), Amikacin (AK^{30}), Streptomycin (S^{10}), Amoxicillin (AC^{30}) and

Table 1. The presence of phytoplanktonic organisms in the sampling stations.

Species name	Phytoplankton Group	Station No											Frequency (%)
		1	2	3	4	5	6	7	8	9	10	11	
Aulacoseira sp.	Bacillariophyceae	+		+		+			+				36
Cocconeis fasciolata	Bacillariophyceae	+											9
Cymbella lanceolata	Bacillariophyceae	+	+	+	+	+					+		54
Epithemia argus	Bacillariophyceae	+											9
Fragilaria crotonensis	Bacillariophyceae	+	+	+	+	+			+	+	+	+	81
Fragilaria ulna	Bacillariophyceae				+				+		+	+	36
Gomphonema acuminate	Bacillariophyceae				+								9
Melosira varians	Bacillariophyceae	+	+	+	+	+	+	+	+	+	+		90
Navicula capitata	Bacillariophyceae			+		+	+	+	+		+		54
Navicula lanceolata	Bacillariophyceae	+		+					+			+	36
Navicula trivialis	Bacillariophyceae		+					+		+	+	+	45
Navicula tuscula	Bacillariophyceae		+		+				+		+		36
Nitzschia linearis	Bacillariophyceae					+				+			18
Nitzschia acicularis	Bacillariophyceae		+			+	+			+	+	+	54
Synedra capitata	Bacillariophyceae	+											9
Cosmarium sp.	Chlorophyceae			+									9
Pediastrum tetras	Chlorophyceae	+			+	+							27
Scenedesmus acuminatus	Chlorophyceae					+							9
Gymnodinium aeruginosum	Dinophyceae	+	+	+	+	+	+	+	+	+	+	+	100
Gymnodinium aeruginosum	Dinophyceae	+	+	+	+	+	+	+	+	+	+	+	100
Synura sp.	Synurophyceae	+	+		+	+		+				+	54

Table 2. Number of phytoplankton in sampling stations (x1000).

Phytoplankton	Station No										
	1	2	3	4	5	6	7	8	9	10	11
Diatoms	1017.6	185.6	563.2	167	147.2	217.6	508.8	76.8	83.2	1587.2	166.4
G. aeruginosum	-	259.2	83.2	414.72	2393.6	563.2	393.6	819.2	230.4	-	57.6

Trimethoprim/sulfamethoxazole (CO^{25}) were tested. Tetracycline, Ampicillin and Amoxicillin are commonly used antibiotics for animals. Their use is an important opportunity for the development of enteric bacteria such as *E. faecalis* with multiple antibiotic resistances. In this study, *E. faecalis* that is resistant to environmental conditions is found to be the dominant species among the identified ones. As seen in Table 4, all the bacterial samples were resistant to Ampicillin and most of the samples were resistant to Tetracycline and Amoxicillin. On the other hand, all bacterial samples were sensitive to Imipenem that is commonly used for humans rather than animals. Considering these facts, it is possible to conclude that the effect of livestock on faecal contamination and faecal nutrient loads in the study area is more important than the anthropogenic sources. This complies with field study logs where cattle were observed near the lake.

Conclusions

Field investigations related to biological water quality of the Efteni Lake were conducted for the first time. Prior to this study, there were no investigations of microalgal flora of the lake. The abundance and distribution of phytoplankton as well as the chlorophyll-a and nutrient concentration suggested that the lake is in a transition condition leading to a eutrophic level from mesotrophic status. Bacteria identified in the lake include infectious pathogens that endanger public health and indicate faecal contamination. From these findings, it is clear that the lake should be rehabilitated before its conditions get worse. TN/TP ratios and their correlation with Chl-a indicate that the primary production in the lake is more likely based on nitrogen limitation rather than phosphorus. This finding must be considered during the rehabilitation studies of the lake. However, field

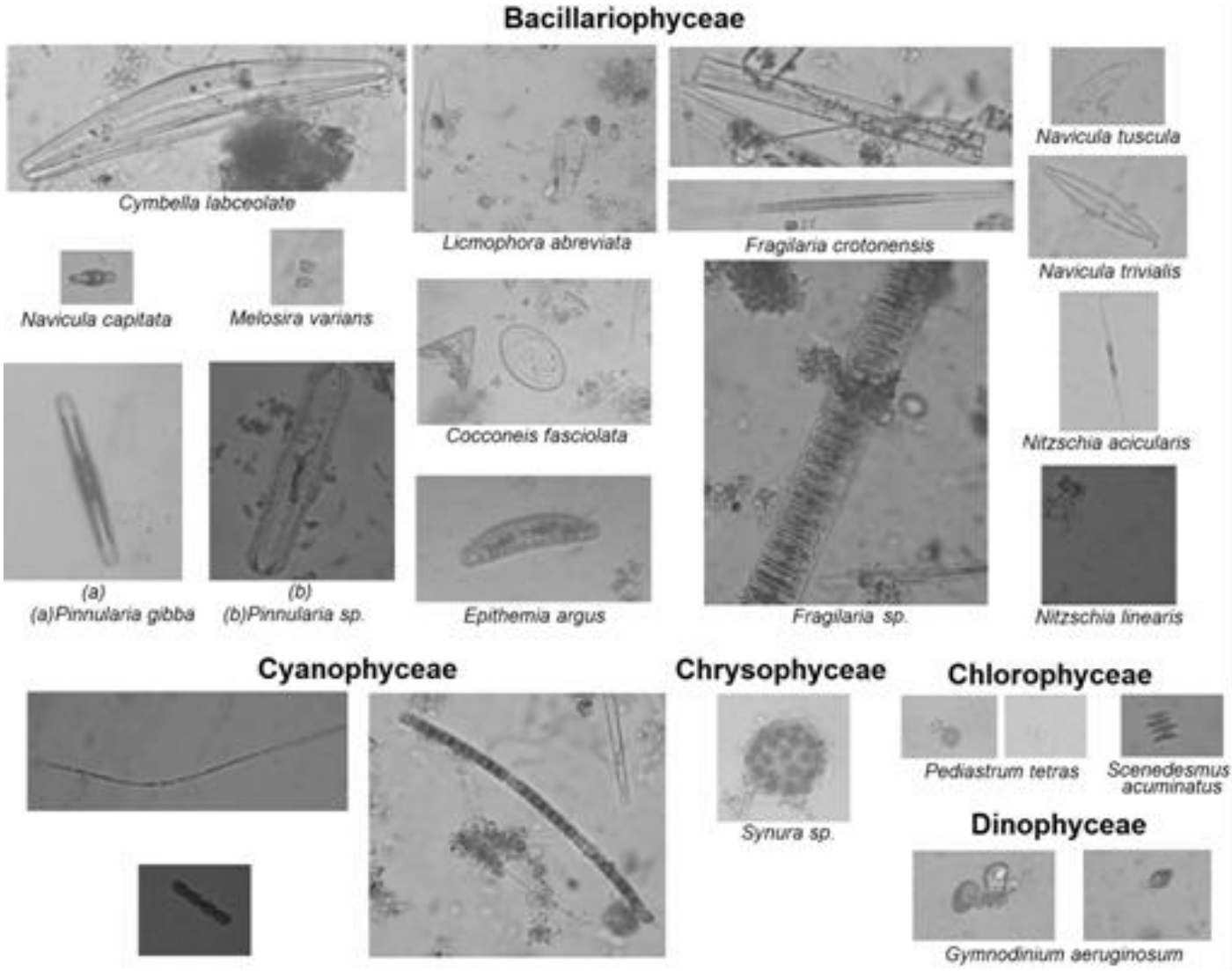


Figure 7. Microphotographs of Phytoplankton.

Table 3. Enteric bacteria counts.

Station	Percentage in total count					Total count (CFU)
	Enterococcus faecalis	*Escherichia coli*	*Pseudomonas aeroginosa*	*Proteus mirabilis*	*Krebsiella pneumoniae*	
1	40	40	20	None	None	4300
2	40	40	20	None	None	2400
3	80	None	None	20	None	1100
4	60	None	25	10	5	2900
5	80	None	None	20	None	1200
6	80	None	None	20	None	1100
7	60	None	20	20	None	4600
8	80	None	None	20	None	1100
9	30	30	20	10	10	11000
10	30	30	20	10	10	12000
11	50	None	30	30	10	11000

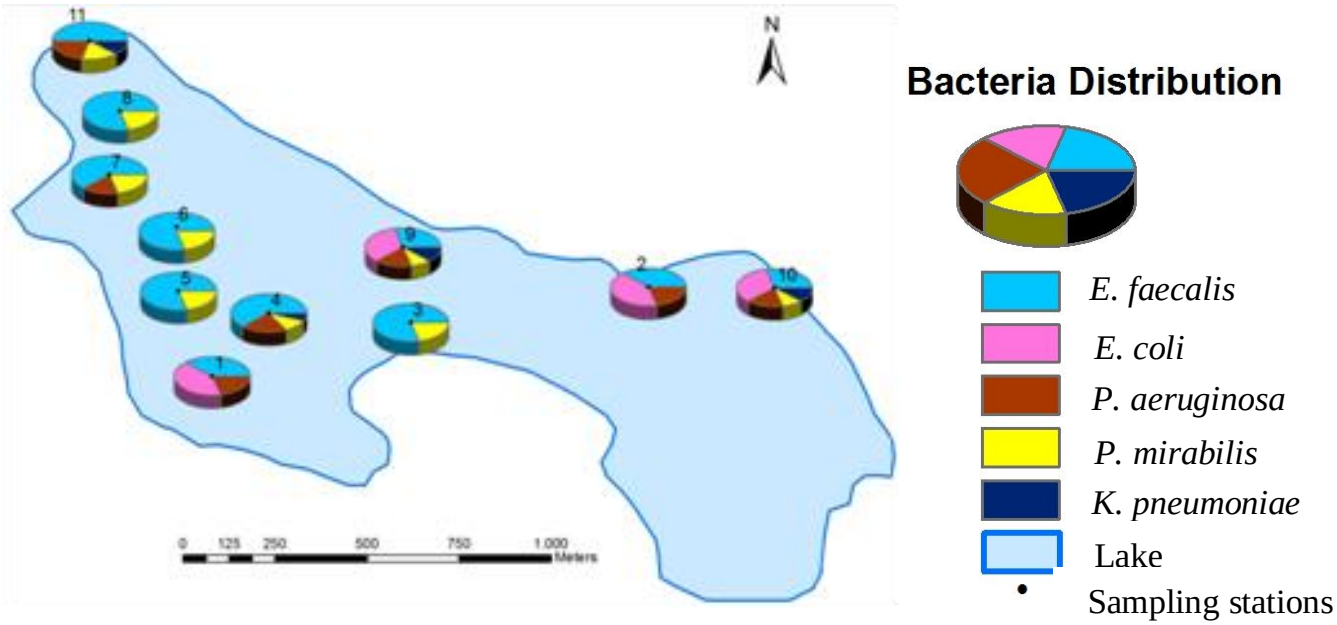


Figure 8. The spatial distribution of bacterial dominance.

Table 4. Test results for antibiotic resistance.

Station	Bacteria	Antibiotic									
		C^{30}	AK^{30}	S^{10}	CO^{25}	NA^{30}	AC^{30}	T^{30}	A^{10}	CA^{30}	I^{10}
1	*E. fecalis*	R	R	R	S	S	R	R	R	R	S
	E. coli	R	R	R	S	R	R	R	R	S	S
	P. aeroginosa	S	S	R	S	R	R	R	R	R	S
2	*E. fecalis*	R	R	R	S	S	R	R	R	R	S
	E. coli	R	R	R	S	R	R	R	R	S	S
	P. aeroginosa	S	S	R	S	R	R	R	R	S	S
3	*E. fecalis*	R	R	R	S	S	R	R	R	R	S
	P. mirabilis	R	R	S	R	R	S	S	R	S	S
4	*E. fecalis*	R	R	R	S	R	R	R	R	R	S
	P. aeroginosa	S	S	R	S	S	R	R	R	S	S
	P. mirabilis	R	R	S	S	R	R	S	R	S	S
	K. pneumoniae	S	S	S	S	S	S	R	R	S	S
5	*E. fecalis*	R	R	R	S	S	R	S	R	S	S
	P. mirabilis	S	R	S	S	S	R	S	R	S	S
6	*E. fecalis*	R	R	R	S	R	R	S	R	S	S
	P. mirabilis	S	R	S	S	R	S	R	R	S	S
7	*E. fecalis*	R	R	R	S	R	R	R	R	R	S
	P. aeroginosa	R	S	R	S	S	R	R	R	S	S
	P. mirabilis	R	R	S	S	S	R	R	R	R	S

Table 4. Contd.

8	*E. fecalis*	S	R	R	S	R	R	S	R	R	S
	P. mirabilis	R	R	R	R	R	R	R	R	S	S
9	*E. fecalis*	R	R	R	R	R	R	R	R	S	S
	E. coli	S	R	R	R	R	R	S	R	S	S
	P. aeroginosa	R	S	S	S	S	R	S	R	R	S
	P. mirabilis	S	R	R	S	R	R	R	R	S	S
	K. pneumoniae	S	R	S	R	R	R	S	R	S	S
10	*E. fecalis*	S	R	R	S	S	R	R	R	R	S
	E. coli	R	S	R	R	R	R	R	R	S	S
	P. aeroginosa	S	R	S	S	R	R	R	R	R	S
	P. mirabilis	R	R	R	S	R	R	S	R	S	S
	K. pneumoniae	R	R	S	S	R	R	S	R	R	S
11	*E. fecalis*	R	R	R	R	S	R	R	R	S	S
	P. aeroginosa	R	R	S	S	S	R	R	R	R	S
	P. mirabilis	S	R	R	S	R	R	S	R	S	S
	K. pneumoniae	S	R	S	R	R	R	S	R	S	S

R: Resistant, S: Sensitive, C^{30}: Chloramphenicol, T^{30}: Tetracycline, NA^{30}: Nalidixic acid, A^{10}: Ampicillin, I^{10}: Imipenem, CA^{30}: Ceftazidime, AK^{30}: Amikacin, S^{10}: Streptomycin, AC^{30}: Amoxicillin, CO^{25}: Trimethoprim/sulfamethoxazole

monitoring of physicochemical and biological parameters together with laboratory based bioassays should be done for detailed further planning of rehabilitation.

The antibiotic resistance tests have shown that faecal pollution depends more on livestock rather than humans. Therefore, livestock can be considered as the main source of faeces based nutrient loads. These findings should be considered for development of diffuse pollution reduction strategies and rehabilitation planning studies as well as for ecological model development for such planning studies. Thus, animals should be prevented to reach close to the lake in order to reduce faecal contamination as well as nutrient loads to the lake.

In this study, sources of diffuse pollution were identified; however diffuse loads were not quantified. It is beneficial to quantify the nutrient and bacterial loads and their effects on the Efteni Lake using advanced data analysis including detailed calculations and models if management and rehabilitation plans for the lake are to be developed.

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Full Length Research Paper

Production and characterization of exopolysaccharides (EPS) from mangrove filamentous fungus, *Syncephalastrum* sp.

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In this study, the production and characterization of exopolysaccharide from *Syncephalastrum* sp. was carried out. This is the first report on the production of exopolysaccharides from *Syncephalastrum* sp. Totally, four different fungi (*Aspergillus niger, Aspergillus flavus, Penicillium expansum* and *Syncephalastrum* sp.) were screened for exopolysaccharide production. Among these, maximum polysaccharide producing species was selected for further large scale production. Different concentrations (6, 8 and 10%) of sucrose were also used to increase polysaccharide production. Overall results depicted that 8% of sucrose concentration exhibited higher amount of polysaccharide production. Further, structure of polysaccachride was confirmed by FT-IR and NMR spectroscopy. Based on this spectroscopy results, the polysaccharide produced by *Syncephalastrum* sp. was 3 linked β-D-galactopyronosyl units. The polysaccharide produced by this species composed of galactose as predominant monosccharides in the cell wall of *Syncephalastrum* sp. and their idealized structures were established.

Key words: Exopolysaccharide, mangroves, filamentous fungi, *Syncephalstrum*, β-D-galactopyronosyl.

INTRODUCTION

Polysaccharides are relatively complex carbohydrates consisting of multiple monosaccharides joined together and often branched. Polysaccharides have a characteristic which allows them to produce a material that makes them to stick each on other surfaces. Polysaccharides derived from plants and seaweeds have been in use for thousands of years. However, over the past 20 years, a new class of microbial products: the microbial polysaccharides have grown in industrial importance (Laroche and Michaud, 2007). They can function in foods as viscosifying agents, stabilizers, emulsifiers and gelling agents. These products can be used as alternatives to other synthetic or natural water-soluble polymers. Many bacteria and fungi often secrete

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polysaccharides as an evolutionary adaptation to help them adhere to surfaces and to prevent them from drying out (Mathur and Mathur, 2006).

The fungal kingdom estimated with 1.5 million species contains a diverse range of species presenting polysaccharides that possess various biological activities and chemical properties exploitable for commercial applications. Glucans, chitin and mannans are particularly abundant in the fungal cell wall. Well characterized immunoactive polysaccharides produced by fungus include alpha and beta glucans. For the production of microbial polysaccharides, the main interest is extracellular polysaccharides (EPS). EPS are widely secreted by various marine organisms, including plants, animals, diatoms, microalgae and bacteria (Decho, 1990; Gutierrez et al., 1996; De Philippis et al., 1998; Philippis and Vincenzini, 1998). The EPS produced by these organisms have been explored for various biotechnological applications, such as anti-tumor agents, anticoagulants (heparin analogues) and wound dressings for eye and joint surgery. Apart from that, EPS are also important as emulsion stabilizers (in food and thixotrophic paints), flocculants (in water clarification and ore extraction), foam stabilizers (in beer and fire-fighting fluids), gelling agents (in cell and enzyme technology and foods), hydrating agents (in cosmetics and pharma-ceuticals), and as inhibitors of crystal formation in frozen foods and sugar syrups (Colwell et al., 1986; Sogawa, 1998; Sutherland, 1985). It is likely that hitherto unexplored groups of marine microorganisms produce novel and useful EPS.

Secondary metabolites of marine microbes have been well investigated to discover a lot of natural products of fascinating biological and chemical interest and few reports dealing with the isolation and chemical characterization of polysaccharides of marine microbial origin could be found to date. Hence an attempt has been made to explore the possible potential of polysaccharide production and the structural characteri-zation of an extracellular polysaccharide isolated from *Syncephalastrum* sp.

MATERIALS AND METHODS

Isolation

The fungi were isolated from the mangrove rhizosphere soil of *Rhizopora annamalayana* Kathir, the only endemic species to India. They were sub-cultured in malt extract yeast extract agar medium containing (g/l): malt extract 20, yeast extract 4, agar 20, pH 5.3.

Microorganisms

Isolated colonies like *Aspergillus niger, Aspergillus flavus, Penicillium expansum* and *Syncephalastrum* sp. were grown on potato dextrose agar (PDA) plates at 28°C and incubated for 5 days. After that time, the plates were maintained at 4°C until used. They were transferred once every two weeks to maintain availability and stability for extra cellular polysaccharides production.

Screening for polysaccharide producing microorganism

The fungus was inoculated in a large test tube containing 15 ml of the screening medium (g/100 ml): sucrose, 6; $NaNO_3$, 0.3; KCl, 0.05; $MgSO_4.7H_2O$, 0.005; KH_2PO_4, 0.1; $FeSO_4.5H_2O$, 0.005 an initial pH 6 and incubated at 30°C as static liquid medium. Five days old cultures were heated at 80°C for 15 min. Then two volumes of ethanol were added to the supernatant and the resulting precipitate was collected by centrifugation (10,000 xg, 5 min). The precipitate was dissolved in water and the phenol H_2SO_4 method of Dubois et al. (1956) was used for quantitative estimation of the sugar contents.

Inoculum

Actively growing cells from a newly prepared PDA plate were inoculated in 250 ml flask containing 50 ml liquid medium. Liquid cultures were incubated for 72 h at 28°C in an incubator-shaker. After the incubation phase, 50 ml liquid cultures were used for the production medium.

Culture media and cultivation of *Syncephalastrum* sp.

Maximum polysaccharide producing fungus was grown in the 250 ml Erylinmeyer flask containing 100 ml of the medium. Various concentrations of sucrose: 6, 8 and 10% were added individually with the following composition of $NaNO_3$, 0.3; KCl, 0.05; $MgSO_4.7H_2O$, 0.005; KH_2PO_4, 0.1 and $FeSO_4.5H_2O$, 0.005 (g/100 ml). The media were adjusted to pH 6 and fermentation periods were maintained at 15 days. After that, flasks were autoclaved at 120°C for 15 min and then allowed to cool.

Estimation of mycelial growth

Mycelial growth of *Syncephalastrum* sp. was expressed as dry cell weight (DCM). The mycelia were harvested at the end of incubation periods and then filtered through pre weighed Whatman No.1 filter paper followed by drying at 80°C for approximately 48 h in an oven, after which DCW were measured.

Isolation and purification of polysaccharides

The culture broth was heated for 15 min at 60°C and then cell free solution was obtained by centrifugation (8900 xg, 15 min). The crude polysaccharide was separated from the supernatant by the addition of two volumes of ethanol and the precipitate around the stirrer. The precipitation procedure was repeated thrice and the final product was dried at 60°C and ground to a fine powder. In an old culture, the precipitate did not wound around the stirrer and had to be collected and washed by centrifugation (Leal and Ruperez, 1978).

Analytical techniques

Total sugars were determined by the phenol-sulfuric acid method of Dubois et al. (1956) and reducing sugars by the di-nitro salicylic

Table 1. Effect of different concentrations of sucrose on the production of exopolysaccharides.

Substrate concentration	6% of sucrose	8% of sucrose	10% of sucrose
Volume of filtrate (ml)	91	75	83
Weight of mycelium	1.61	2.12	1.86
Weight of polysaccharides (g/100ml)	0.69	1.2	0.97
Carbohydrate (g/100ml)	10.25	14.43	12.65
Reducing sugar (g/100ml)	1.76	2.82	1.94
Protein (U/ml)	0.83	2.30	1.16
Nitrogen (g/ml)	0.13	0.36	0.18

acid method of Miller (1972) with D-glucose as the standard. Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard. The nitrogen content was calculated from the protein values by dividing the factor 6.25 because 1 mg of nitrogen is equal to 6.25 mg of protein. So this is protein nitrogen (Ghai et al., 1981).

FT-IR spectroscopy

FT-IR was used to investigate the vibrations of molecules and polar bonds between the different atoms. Structures of polysaccharides, such as monosaccharides types, glucosidic bonds and functional groups can be analyzed using FT-IR spectroscopy. Samples were mixed with KBr and pressed into pellets of 13 mm size and infrared spectrum was recorded using Perkin-Elmer IR spectrophotometer (Model IR 577).

NMR analysis

The proton spectra at 400 MHZ and proton decoupled ^{13}C NMR spectra at 100 MHZ were recorded at room temperature on DR x 400 NMR spectrometer using 10 mm sample tube. Sample were prepared by dissolving about 10 mg of the sample in 0.5 ml of ethanol-d containing 1% TMS for ^{1}H and 0.5 ml of sample in 2.5 ml of ethanol-d and a few drop as TMS for ^{13}C. The solvent ethanol-d also provided the integral field frequency lock signal.

RESULTS

Screening for the polysaccharides producing microorganisms

In this study, 4 different fungal strains such as *A. flavus*, *A. niger*, *P. expansum* and *Syncephalastrum* sp. were used for the production of extracellular polysaccharides. Among these, *Syncephalastrum* sp. produced maximum amount of polysaccharides. Hence this strain was selected for further studies.

Effect of sucrose on the production of extracellular polysaccharides

Three different concentrations of sucrose *viz*., 6, 8 and 10% were used for the production of extracellular polysaccharides from *Syncephalastrum* sp. Among these concentrations of sucrose tested, 8% gave maximum production of polysaccharides (1.2 g/100 ml) and biomass (2.12 g/100 ml) which was achieved within the 15 days of incubation periods (Table 1). Ten percent of sucrose also gave satisfactory amount of polysaccharides (0.97 g/100 ml) and biomass production (1.86 g/ 100 ml).

Chemical composition of culture filtrate

Total carbohydrates, reducing sugar, total protein and nitrogen of the culture filtrates were estimated and the results are presented in Table 1. Higher level of carbohydrates (14.43 g/ 100 ml), reducing sugar (2.82 g/100 ml), protein (2.30 U/ml) and nitrogen (0.36 g/ml) were recorded in 8% sucrose concentration at 15 day of incubation periods. At 10% sucrose concentration, the levels of carbohydrates, reducing sugar, protein and nitrogen declined.

FT-IR spectroscopy

Configuration of the polysaccharide fraction was ascertained using FT-IR spectroscopy. The sharp peak at 1067 cm^{-1} was assigned to C-O-C mode. Cyclic C-H stretching vibration appeared at 2934 cm^{-1} and assigned to the stretching vibration of the OCH_3 (methoxy) observed at 1383 cm^{-1}. The obvious absorption peaks at 825 cm^{-1} revealed the existence of β-galatopyranosyl linked IR spectrum of polysaccharide units as shown in Figure 1.

^{1}H NMR spectroscopy

In the ^{1}H-NMR spectrum of polysaccharide unit, methoxy carbon was observed in the region, 3.45 to 3.60 ppm integrals. The signals around 3.38 to 3.42 ppm were assigned to methylene proton of β-D-galactopyronosyl units. Anomeric porton H-1 was observed at 5.11 ppm, and it was attached with carbon atom C-1. The remaining methane protons were observed at 4.53, 3.14 and 3.12 ppm, respectively. ^{1}H- NMR spectrum of polysaccharide is shown in Figure 2.

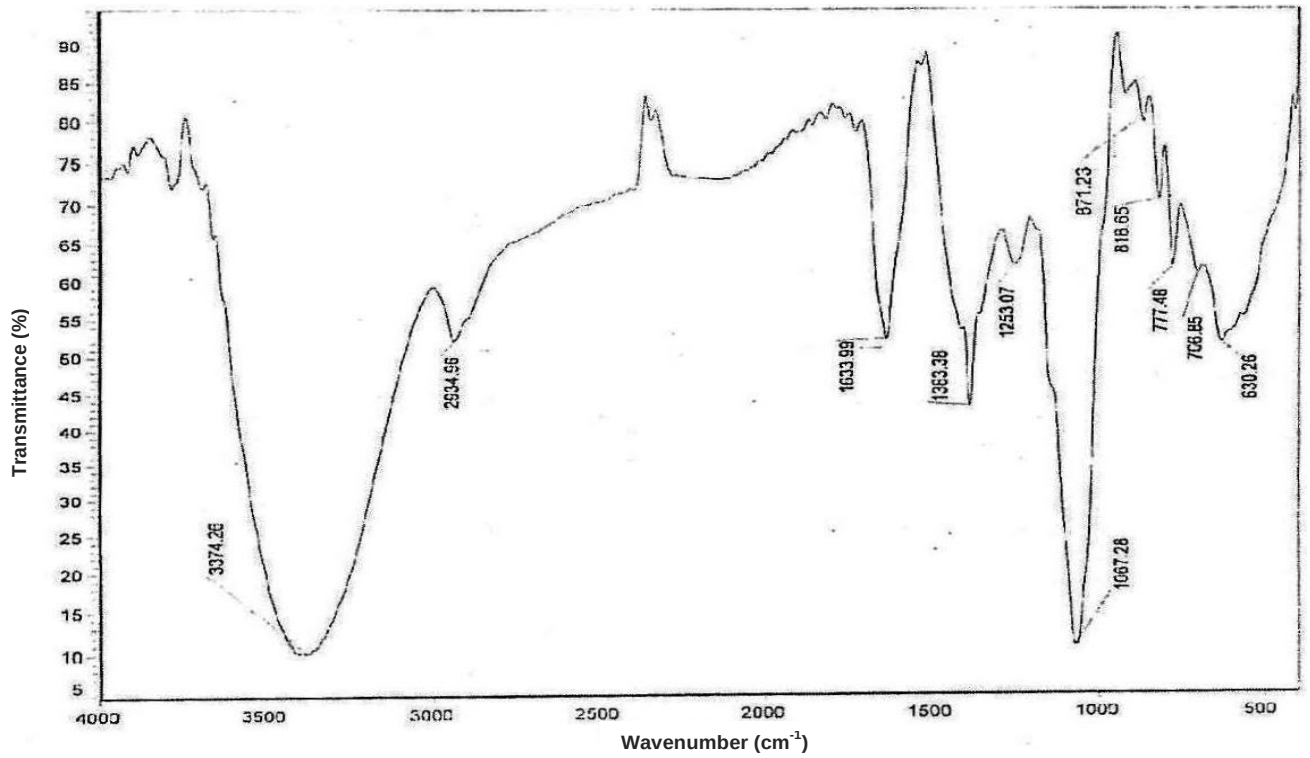

Figure 1. FT-IR spectrum of exopolysaccharides from *Syncephalastrum* sp.

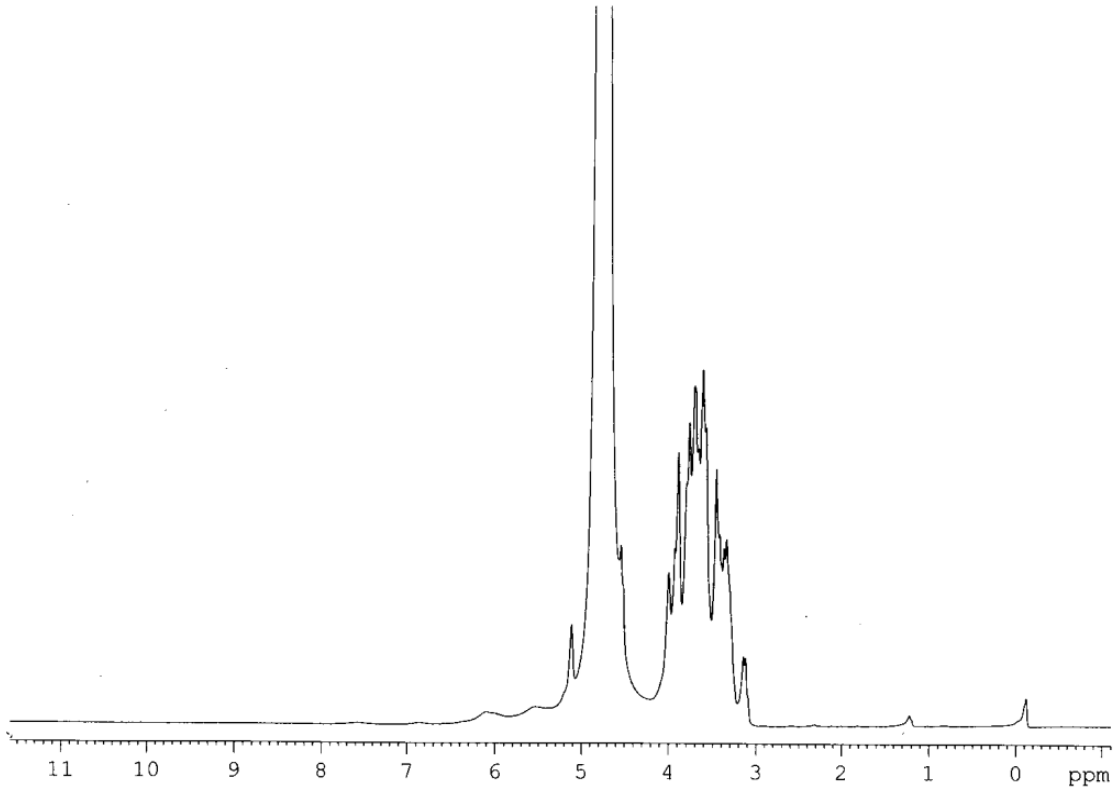

Figure 2. ^{1}H spectrum of exopolysaccharides from *Syncephalastrum* sp.

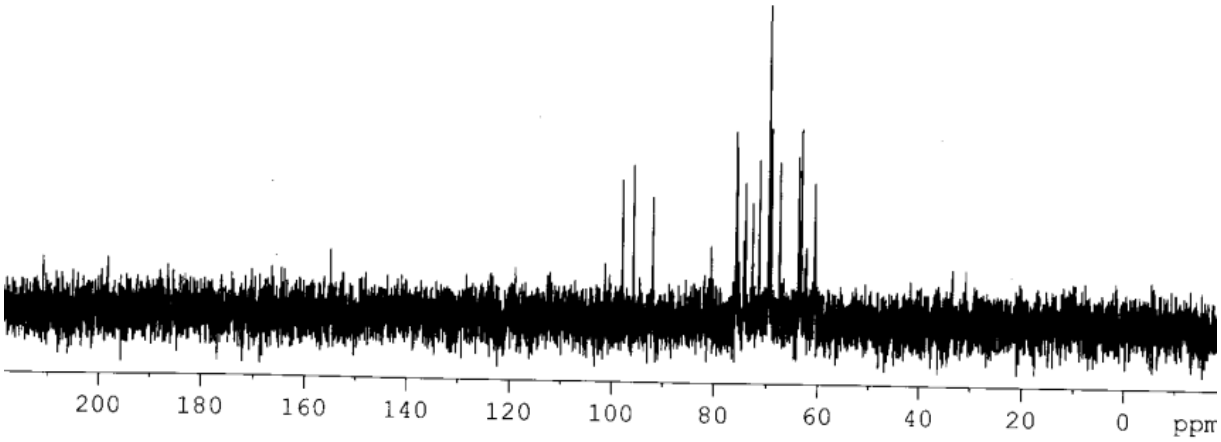

Figure 3. ^{13}C spectrum of exopolysaccharides from *Syncephalastrum* sp.

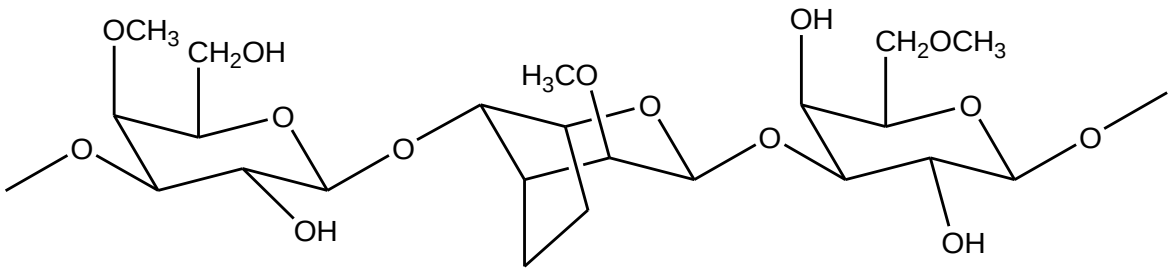

Figure 4. 3 linked β-D-galactopyronosyl units.

The ^{13}C NMR spectrum of *Syncephalastrum* polysaccharides samples showed that, 18 signals were assigned to carbon of polysaccharides unit (Figure 3). The signals at 102.00, 69.16, 80.63, 67.53, 74.11 and 60.69 ppm corresponded to the 3 linked β-D-galactopyronosyl units, while the signals at 98.03, 69.61, 75.90, 75.72 and 71.40 ppm are attributed to the 4-linked 3,6-anhydrno-α-L-galactopyranosyl units. Moreover, the additional signals (95.87, 80.63, 63.32 and 62.35 ppm) in the spectra revealed the presence of 4-Omethyl-α-L-galactopyrynosyl unit. The anomeric carbons were observed at 102.00 to 95.90 ppm which was attached with bridging oxygen atom in polysaccharide units. Based on the TLC, FT-IR and NMR spectroscopy revealed that cell wall of *Sycephalastrum* sp containing 3 linked β-D-galactopyronosyl units (Figure 4) and galactose as predominant monosccharides in polysaccharide units.

DISCUSSION

Most of the EPS produced by fungi are highly hygroscopic β-glucans, suggesting that its production could be related with tolerance to desiccation; similarly to that observed and described in bacteria. Recently, there has been a growing interest in studying the EPS production of a wider array of fungi, not only from the standpoint of comparative biology but also with the expectation of finding better EPS producing systems for use in various biological applications. Besides the health benefits, EPS represent a valid alternative to plant and algal products considering that their properties are almost identical to those employed in food, pharmaceutical and cosmetic industries (Sutherland, 2002).

In the present study, different levels of sucrose *viz*., 6, 8 and 10% were used to induce extracellular polysaccharide production in *Syncephalastrum* sp. Among the three different levels tested, 8% of sucrose gave maximum amount of polysaccharides (1.2 g/100 ml) and mycelia growth (2.12 g/100 ml) production on the 15th day of incubation. Similar results also observed by Leal-Ruperez (1978) who reported maximum production of 86.9 mg of polysaccharides/100 ml observed at 10% of sucrose concentration, at 15 days of incubation by *A. niger* in Egypt, 513 mg/ 100 ml. Because, this polysaccharide could be cell wall component, the microorganisms continue to produce when there is a little demand for cell wall synthesis. It could also be cytoplasmic

reserve that is produced in larger amounts than the fungus which is capable of storage (Leal et al., 1979).

Whereas, Leal-Serrano et al. (1980) recorded the maximum amount of polysaccharides isolated from *Aurobasidum pullulans* after the 15 days of incubation. Similar results were also observed by Senthilkumar and Murugesan (2010). The disparity in yield may be due to the difference in the strain.

Previous studies have studied the utilization of several carbon source viz., glucose and sucrose in 13 species of *Aspergillus* (Leal and Ruberze, 1978) as well as nitrogen sources namely L-alanine, L-aspargine, L-aspartic acid, L-glutamine, L-glutamic acid, L-leucine, L-phenlalanine, L-valine, potassium nitrate and di-ammonium tartarate in *Aspergillus nidulans* (Ruberez and Leal, 1979). Leal-Serrano et al. (1980) proved that among the carbon sources investigated for poly-saccharides production maltose (68.5 mg/100 ml) and mannose (66.5 mg/ 100 ml) proved to be the best, whereas mannitol (12.4 mg/ 100 ml) gave the lowest polysaccharide yield. Interestingly, in the present study, 6 to 10% sucrose as carbon source yielded fairly high level of extracellular polysaccharide (0.69 to 1.2 g/ 100 ml) in 15 days of incubation in *Syncephalastrum* sp. (Table 1). There was a clear trend that EPS have larger carbo-hydrates contents; in the present study, total carbo-hydrate contents was recorded (10.25 to 14.43 g/ 100 ml) in culture filtrate, but the reducing sugar (1.76 to 2.82 g/ 100 ml), protein (0.68 to 2.30 U/ml) and nitrogen (1.13 to 0.36 g/ 100 ml) content in culture filtrate was very low as compared to total carbohydrate content recorded in all the three different levels of sucrose. Similar results were also observed by Leal and Ruberez (1978) and Senthilkumar and Murugesan (2010) had less similar carbohydrate and protein values in *A. nidulans* and *A. niger*. Griffin (1996) investigated the proximate composition of fungus containing 16 to 85% carbohydrate, 0.2 to 87% lipids, 14 to 44% protein, 1 to 10% DNA, 0.15 to 0.3% RNA and 1 to 29% ash content.

Polysaccharide fraction could be ascertained with the help of IR-spectroscopy. Based on the TLC, FT-IR and NMR spectroscopy revealed that cell wall of *Syncephalastrum* sp. contain 3 linked β-D-galacto-pyronosyl units, and galactose as predominant mono-sccharides in polysaccharide units which was conformity with the observations of Archer et al. (1977) on *Monilinia frutigena*. The polysaccharide obtained from the three isolates of *Nectria cinnabarina* was similar to the polysaccharides found in species of *Gibberella* and *Fusarium* (Jikibara et al., 1992; Ahrazem et al., 2000) and *Penicillium vermoesenii* (*Gliocladium vermoesenii*) (Ahrazem et al., 1999) since they have similar residues, mainly 2,6-di-O-substituted galactofuranose (!2,6)- Gal*f*-(1!), and terminal glucopyranose (Glc*p*-(1!), and almost identical "H-NMR spectra. The differences found in the polysaccharide from *N. cinnabarina* and species of *Sesquicillium* and *Nectria* with *Sesquicillium* anamorphs were in agreement with the separation of these species according to their morphological characters (Samuels and Rossman, 1979) and support the creation of the genus *Bionectria* (Rossman et al., 1999).

Conclusion

The developments of microbial polysaccharides are best viewed in the context of their maximum utility in food and medical industry. The most promising developments seem possible in therapeutic and cosmetic applications as these compounds have been described as immunomodulators, antitumorogenic and antiviral (AIDS) agents for the treatment of hypercholesterolemia and agents for stabilization of glycemia. Moreover, specific action as food additives has been ascribed for glucan oligosaccharides. But, to the best of our knowledge, it is the first time that they have been described 3 linked β-D-galactopyrynosyl chain in *Syncephalastrum* sp. This species was able to produce a high level of EPS in production medium containing 8% sucrose concentration. This study has also confirmed that the concentration of EPS produced by *Syncephalastrum* sp. was dependent on the carbon sources. Further, immunomodulatory effects in animals are still under study.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Efflux pump genes and chlorhexidine resistance: Clue for *Klebsiella pneumoniae* infections in intensive care units, Egypt

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One of the serious and growing challenges for infection control programs worldwide is hospital acquired infections. *Klebsiella pneumoniae* is considered the second most common cause of hospital-acquired Gram-negative blood stream infections. Chlorhexidine is a topical antiseptic agent, widely used in different applications in hospitals. Healthcare associated infection rates were lower after chlorhexidine-containing detergent hand washing when compared with using plain soap or an alcohol-based hand rinse. Intensive exposure of hospital pathogens to biocides may result in the emergence of resistance not just to the biocides but also to antibiotics. We aimed to investigate the susceptibility of multi-drug resistant *K. pneumoniae* isolates to chlorhexidine and to correlate chlorhexidine susceptibility and its association with both the efflux pump genes (cepA, qacDE, qacE), and resistance to later-generation anti-Gram negative antibiotics. Fifty-six strains of *K. pneumoniae* were isolated from blood specimens in intensive care units, Suez Canal University Hospital, Ismailia, Egypt. Antibiotic sensitivity profiles were determined by disc diffusion method. Minimal inhibitory concentration (MICs) of 1% chlorhexidine was assessed by the agar dilution method. The effect of efflux pumps was determined by repeating the susceptibility in the presence of the efflux pump inhibitor carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (10 mg/L). Polymerase chain reaction (PCR) was used for identifying efflux pump genes. The MICs of chlorhexidine ranged from 4 - 256 mg/L. Most isolates carried the cepA gene. The MICs of chlorhexidine was significantly reduced on addition of CCCP. Carriage of efflux pump gene cepA affect chlorhexidine susceptibility in ICU related *K. pneumoniae* infections.

Key words: *Klebsiella pneumoniae*, chlorhexidine, efflux pump, ICU.

INTRODUCTION

Health care associated infections (HAI) have direct or indirect extensive health effects and large economic burden (Valbona et al., 2004). Despite the lower number of intensive care units (ICU) patients as compared to those in other clinics, the ICUs have the highest rate of HAI among all the units. This can be attributed to frequent use of antibiotics and invasive techniques such as central venous catheterization, mechanical ventilation and urinary catheterization for long periods (Akalın, 2001).

Klebsiella pneumoniae is considered the second most common cause, after *Escherichia coli*, of hospital-acquired

Gram-negative bloodstream infection (BSI) (Meatherall et al., 2009). It causes about 8% of hospital-acquired infections including pneumonia, wound infections, diarrhoea and urinary tract infections (Gupta, 2002). Furthermore, it can lead to a poor prognosis in critically ill patients with bacteremic community-acquired pneumonia (Lin et al., 2010).

The severity of BSI has been associated with multi drug resistant (MDR) strains producing extended-spectrum beta-lactamase enzymes (ESBLs). This is associated with increased morbidity and mortality especially in intensive care units (ICUs), neonatal units and surgical wards (Khanfar et al., 2009). BSI is considered a major problem in critically care units as it is associated with increased rates of hospitalization, and increased length of stay and hospital costs (Pien et al., 2010).

Hand contamination of Health Care Workers (HCWs) is one of the most common modes of transmission of *K. pneumoniae* in ICUs. Others include direct patient contact and respiratory-tract care; all of which contaminate the fingers of caregivers. Whereas, *Staphylococcus aureus* account for 11% of isolates, Gram-negative bacilli accounted for about 15%. Duration of patient-care activity was strongly associated with the intensity of bacterial contamination of HCWs' hands (Samore et al., 1996).

In order to combat these infections, the staff frequently wash their hands with cationic biocides (such as chlorhexidine), which produces a 98-100% reduction in the number of patients infected with *K. pneumoniae* (Casewell and Phillips, 1977). Chlorhexidine is a topical antiseptic agent with a broad spectrum of actions, widely used in different applications in hospitals such as surface cleaning, hand disinfection and skin preparation before invasive procedures (Milstone et al., 2008). HAIs rates were lower after antiseptic hand washing using a chlorhexidine-containing detergent when compared with hand washing with plain soap or use of an alcohol-based hand rinse (Doebbeling et al., 1992).

Even with the considerable reductions in *K. pneumoniae* infections with the wide use of biocides, infections caused by multi-resistant *K. pneumoniae* are increasing, and this raises concerns that intensive exposure of hospital pathogens to biocides may result in the emergence of resistance not just to the biocides but also to antibiotics (Buehlmann et al., 2011; Chong et al., 2011). The concentrations of the biocides used clinically are often higher than those required to inhibit the organism. However, many factors, such as the local dilution policy, presence of organic matter, formation of biofilms and length of exposure, could comprise the effective concentration of the biocide so that resistance may become a problem (Smith and Hunter, 2008; Tumah, 2009).

As there is little information on reduced activity of chlorhexidine toward *K. pneumoniae* and its relationship with resistance to antibiotics, this study aimed to investi-gate the activity of chlorhexidine on clinical isolates of MDR *K. pneumoniae*. And also study the correlation bet-ween chlorhexidine activity and its association with both the efflux pump genes (cepA, qacDE and qacE), which have been linked to antiseptic 'resistance', and resistance to later-generation anti-Gram negative antibiotics (Kucken et al., 2000).

MATERIALS AND METHODS

Bacterial strains

Fifty-six strains of *K. pneumoniae* were isolated from blood specimens in patients with BSIs at intensive care units in Suez Canal University from November 2012 to June 2013. All blood cultures were examined by the hospital's microbiology laboratory using the BacT/ALERT® system (bioMe´rieux, France) and the isolated strains were confirmed by the API 20E strips (bioMe´rieux, Basingstoke, UK).

Antibiotic susceptibility

Antibiotic susceptibility testing was performed using the Kirby–Bauer Disk Diffusion test, according to the criteria of the National the Clinical and Laboratory Standards Institute (CLSI) (National Committee for Clinical Laboratory Standards, 2002). The antibiotics used were cefotaxime, cefoxitin, colistin, chloramphenicol, gentamicin, polymyxin B, ciprofloxacin, ceftriaxone, chloramphenicol, amikacin, trimethoprim, piperacillin-tazobactam, ceftazidime, imipenem and meropenem (Sigma, Poole, UK). These are the commonly used generation of antibiotics for the Gram negative strains in the infirmary.

Minimum inhibitory concentration (MIC) of chlorhexidine

The common hospital biocide used was 1% chlorhexidine gluconate, a member of the biguanide family (Sigma, St. Louis, MO, USA). The MIC of chlorhexidine was determined using agar dilution in accordance with the 2002 guidelines of the CLSI. Serial two fold dilution of chlorhexidine digluconate on Muller-Hinton (MH) agar containing 0- 256 mg/mL was used. *K. pneumoniae* ATCC13883 (MIC of chlorhexidine 16 mg/mL) and *Escherichia coli* ATCC25922 (MIC of chlorhexidine 2 mg/mL) were included in each experiment as control strains.

Efflux pumps inhibitor and reduced biocide susceptibility

In order to determine the activity of efflux pump, 10 mg carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (Sigma aldrich, USA) was dissolved in 10 ml of dimethyl sulphoxide (DMSO) to make 10 µmol concentration. This was then added to Muller Hinton agar at 10 mg/L in plates containing increasing concentrations of chlorhexidine for minimum inhibitory concentration (MIC) determination. Decrease in chlohexidine MIC indicated the presence of an efflux

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Table 1. PCR primer sets, specificities and amplicon sizes.

Antiseptic resistance genes	Primers set	Amplicon size (bp)
cepA	FW: 5'CAACTCCTTCGCCTATCCCG3', RV: 5'TCAGGTCAGACCAAACGGCG 3' [18]	1051
qacDE	FW:5' GCCCTACACAAATTGGGAGA 3', RV: 5'CTGCGGTACCACTGCCACAA 3' [19]	370
qacE	FW: 5'GCCCTACACAAATTGGGAGA 3', RV: 5'TTAGTGGGCACTTGCTTTGG 3' [19]	350

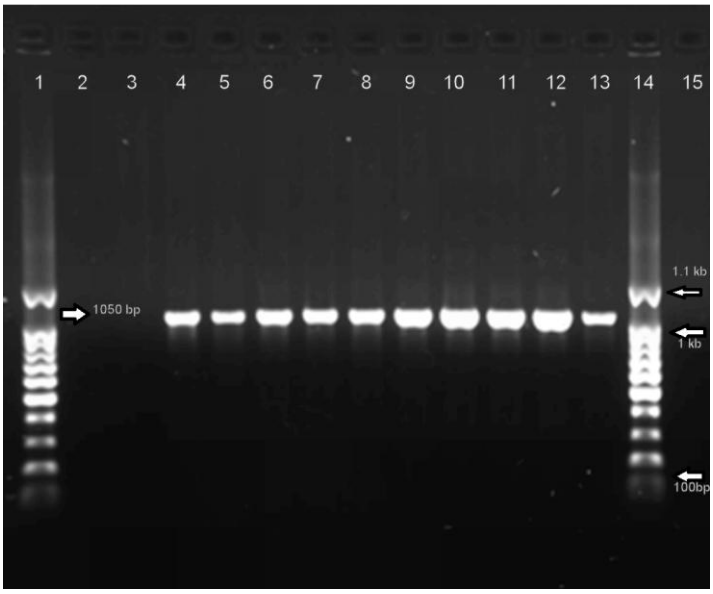

Figure 1. Agaroe gel 1.5% electrophoresis showing PCR amplicons of cepA gene. Lane 1, 14: 100 bp DNA ladder, 2: Negative contol, 3: Negative sample for cepA gene, 4: positive control, 5-13: samples positive cepA gene, 15: NTC.

pump.

Polymerase chain reaction (PCR)

The antiseptic resistance genes cepA, qacDE and qacE were screened by PCR. The primer sequences, specificities and predicted amplicon sizes are listed in Table 1. Each PCR reaction was done in a final volume of 25 µl reaction volume. The reaction mixture contained 5 µl of template DNA, 12.5 µl of OneTaq® Quick-Load® 2X Master Mix [20 mM Tris-HCl, 22 mM KCl, 22 mM NH_4Cl, 1.8 mM $MgCl_2$, 5% Glycerol, 0.06% IGEPAL® CA-630, 0.05% Tween® 20, 0.2 mM dNTPs, 1X Xylene Cyanol, 1X Tartrazine, 25 unit/ml OneTaq® DNA Polymerase, pH 8.9 at 25°C] (New England Biolabs), 2.5 µl each primer (25 pmoles), 2.5 µl water (PCR-grade). Positive and Non-template controls were included in each experiment (positive control was a known resistant *K. pneumoniae* isolate from a surgical wound previously tested as described before by Fang et al. (2002) and Khazama et al. (1998). The optimal cycling conditions were as follows: 5 min at 95°C, 95°C for 45s, (66 °C for 30s for cepA, 49˚C for 40s for both qacE and qacDE), 72°C for 1 min for 35 cycles and final extension step 72°C for 10 min (Fang et al., 2002; Khazama et al., 1998) using Eppendorf Mastercycler® nexus PCR thermal cycler. PCR products were analyzed on agarose gel 1.5% (Promega, Madison, USA). The gel was stained with ethidium bromide and photos were taken using the documentation system syngene G.Box (Syngene, UK).

RESULTS

Antiseptic resistance genes

Forty two isolates contained the cepA gene and, in every case, the size of gene fragment was 1051 bp. All samples were negative for both qacE and qacDE genes by PCR (Figures 1 and 2). Table 2 shows the individual levels of chlorhexidine, susceptibility with the presence of antiseptic resistance genes cepA, qacE and qacDE. High MICs (64-128 mg/L) of chlorhexidine were found in 15 and 12% isolates, respectively. On the other hand, qacDE and qacE antiseptic resistance genes were not

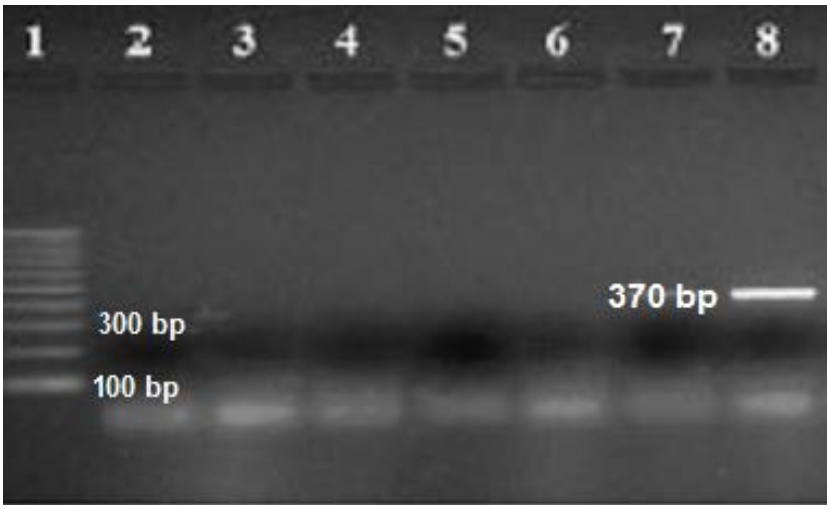

Figure 2. Agaroe gel 1.5% showing amplicons of PCR of qacDE gene. Lane 1: 100 bp DNA Ladder, 2: Negative control, 3-6: Negative samples for cepA gene, 8: positive control, 7: NTC.

Table 2. Effect of carbonyl cyanide m-chlorophenyl hydrazone (CCCP in 10 mg/L) on the minimum inhibitory concentration (MIC) of chlorhexidine in association with CepA gene.

Number of strains	MIC chlorhexidine only (mg/L)	MIC of chlorhexidine with 10 mg/L CCCP	CepA gene	qacDE	qacE
2	16	4	+	-	-
1	16	8	+	-	-
3	32	8	+	-	-
5	32	16	+	-	-
13	64	8	+	-	-
3	128	64	+	-	-
5	128	32	+	-	-
2	128	8	+	-	-
1	256	64	+	-	-
2	64	16	-	-	-
1	32	16	-	-	-
1	16	4	-	-	-

Table 3. Minimal inhibitory concentrations (MICs) for chlorhexidine in different *K. pneumoniae* strains in the presence of different efflux pumps genes.

Number of strains	MICs (mg/L) chlorhexidine	cepA+qacE - qacDE -	cepA -qacE + qacDE+	cepA -qacE - qacDE +	cepA - qacDE - qacE -
0	0	-	-	-	-
0	2	-	-	-	-
3	4	2	0	0	0
3	8	2	0	0	0
4	16	3	0	0	0
12	32	10	0	0	0
19	64	16	0	0	0
13	128	8	0	0	0
1	256	1	0	0	0

Table 4. The minimum inhibitory concentration (MIC) of chlorhexidine correlated with antibiotics sensitivity pattern among *K. pneumoniae* isolates.

Antibiotic	MIC (mg/L) range
Piperacillin-tazobactam	1- 4
Cefotaxime	0.01-64
Ceftriaxone	0.01- 64
Ceftazidime	0.1- 0.2
Imepenem	0.2- 1.1
Meropenem	0.021- 0.071
Amikacin	0.2 - 1
gentamycin	4 - 64
Trimethoprim	2.4 - 128
cefoxitin	16 - 128
ciprofloxacine	0.4- 2
rifampcin	16- 128
Polymixin B	2 - 16
Chloramphenicol	1- 32
colistin	8- 64

found in any isolate.

Efflux pumps inhibitors and reduced biocid susceptibility

On addition of CCCP (10 mg/L) to the MICs of the chlorhexidine, we found that the MICs of chlorhexidine reduced in 39 isolates (Tables 2). This was associated with the presence of the cepA gene in 35 isolates. No reduction of MICs was observed at concentration 4-8 mg/L. In association with cepA gene, reduction on MICs occurred in 3 strains at concentration 16 mg/L, 8 strains at concentration 32 mg/L, 13 strains at concentration 64 mg/L, 10 strains at concentration 128 mg/L and one strain at concentration 256 mg/L. In the absence of cepA gene, the reduction on MICs on addition of CCCP occurred in only 4 strains (2 of them from concentration 64 to 16 mg/L, one from concentration 32 to 16 mg/L and one from concentration 16 to 4 mg/L).

Antibiotic sensitivity pattern

The susceptibility of 56 isolates of *K. pneumoniae* was determined by disc diffusion methods. All isolates were sensitive to imipenem and meropenem. The MICs of chlorhexidine ranged from 4 - 256 mg/L as shown in Table 3 which were higher than the control strains *K. pneumoniae* ATCC13883 (MIC of chlorhexidine 16 µg/ml) and *E. coli* ATCC25922 (MIC of chlorhexidine 2 µg/ml).

The MIC for all used antibiotics in the ICU was determined for the isolates. Increased MIC range for most of the antibiotics was noted (Table 4). The resistance of some isolates to ceftazidime, cefotaxime and ciprofloxacin was noted in traditional disc diffusion method. Most isolates were sensitive to third generation cephalosporines. A similar consistent antibiotic sensitivity pattern for the 4 isolates (with decreased MIC for chlorehexidine and absent resistant genes) was shown.

DISCUSSION

Chlorhexidine is a widely used topical antiseptic that has been used worldwide since the fifties of the last century. Its safety has been proved with high efficacy in many clinical applications. These include preoperative skin preparation, vaginal antisepsis and body washes to prevent neonatal sepsis. Its major role in infection control has been followed over years with high satisfactory results (Hugo and Longworth, 1964; McDonnell and Russell, 1999).

Reduced susceptibility to disinfectants among bacteria often involves the action of active or over expressed efflux pumps (Russell et al., 1998). Many products contain chlorhexidine in different concentrations and forms. Chlorhexidine affects membrane integrity at low concentrations, whereas at high concentrations it precipitate cytoplasmic contents, resulting in cell death (Hugo and Longworth, 1964; McDonnell and Russell, 1999).

Unlike Abuzaid and his co-workers (2012), we did not examine other biocides as they are not commonly used in our hospital. In our hospital, chlorhexidine is considered to be an essential component of infection control strategies and the most widely used disinfectant in hospitals, so high MICs of chlorhexidine may offer an advantage to nosocomial pathogens. So this study evaluates the relationship between reduced susceptibility to chlorhexidine and the carriage of antiseptic resistance genes, cepA, qacDE and qacE, as well as identify the role of efflux pumps in conferring reduced susceptibility. Moreover, this may be of great help in optimizing the best concentration for this antiseptic.

Increasing frequency of hospital infection leads to overuse and pressure of biocides, similarly to antibiotics. The linkage between bacterial resistance and the use of biocides has been suggested (Block and Furman, 2002). They have observed a significant inverse relationship between the intensity of chlorhexidine use and overall susceptibility of organisms (*S. aureus*, coagulase-negative staphylococci,*K.pneumoniae,P.aeruginosa,Acinetobacter baumannii* and *Candida albicans*) to this antiseptic (Smith et al., 2008; Abuzaid et al., 2012). We examined the most common pathogenic species in the ICU (*K. pneumoniae* followed by *E. coli* and *S. aureus*) according to the infection control unit records. In our study, MICs of chlorhexidine ranged from 4 - 256 mg/L.

The MIC assays performed in the presence of CCCP showed a considerable decrease in the MICs of chlorhexidine for almost all the strains (39 isolates) and this was usually associated with the presence of cepA except in four cases (35 isolates). These results were in agreement with that of Abuzaid and his co-workers (2012) who

examined the effect of CCCP on the MIC of different hospital biocides. They found that it reduced the MICs of chlorhexidine considerably with presence of cepA gene in most of the isolates (except five strains).

On the other hand, no strains carried the other 2 efflux pump genes (qacE and qacDE) unlike Abuzaid et al. (2012) who detected qacDE gene in 34 isolates; and qacE w in only one isolate out of 64 isolates.

The chlorehexidine susceptibility was notably reduced in most isolates similar to the decrease reported by Abuzaid el al. (2012). However, a different pattern of antiseptic resistance genes carriage was noted. This may be explained by the exposure to a significant amount of chlorehexidine in the ICU. Moreover, *K. pneumoniae* isolates were obviously resistant to the commonly used antibiotics in contrast with those of Abuzaid et al. (2012) and in accordance with Koljalg et al. (2002).

Thus, measures were applied in line with the decisions of the infection control committee. Intensive care unit staff was trained regularly and frequently. The staff was trained on hand hygiene and personal protective equipment to limit the transmission of infections. Also, we recommend evaluating and applying the possibility of predicting chlorhexidine susceptibility as a routine work with multidrug resistant organisms. However, the clinical importance of this is considered when other factors are taken into account, such as biofilm formation, which already compromises the activity of the biocide.

The presence of decreased MIC for chlorehexidine in 4 isolates without any resistant gene was an interesting finding. This was incoherent with previous results reported by Abuzaid et al. (2012). However, a similar consistent antibiotic sensitivity pattern with the lowest MIC for most of the antibiotics used was shared by these isolates.

Conclusion

The carriage of efflux pump gene cepA affects chlorhexidine activity on *K. pneumoniae* infections in the ICU related infections. However, the study should be extended to include other species in the same ward. Also, other antibiotics should be tested (other than the commonly used antibiotics in the hospital). Further evaluation against biofilm formation should be planned with the hospital team.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Production and characterization of alkaliphilic alpha-amylase from *Bacillus subtilis* A10 isolated from soils of Kahramanmaras, Turkey

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Bacillus subtilis **A10 isolated from orchard soil, Kahramanmaras, Turkey. The enzyme synthesis was observed between pH 7.0-11.0, with an optimum 37°C. The amylase was purified by fractional ammonium sulfate precipitation and sephadex G-100 column. Analysis of the enzyme with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed a single band which was estimated as 46.9 kDa. The enzyme presented an optimum activity at pH 8.5 and 45°C. Its thermal stability between 20-50°C was about 89.5% for 30 min. The pH stability was observed between pH 7.0 and 10.0 with an average of 84.9% of retaining activity for 15min. The activity of the enzyme was inhibited by SDS and EDTA by 48.4 and 75.9%, respectively. On the other hand, Na_2SO_3 and β-Mercaptoethanol did not effect the enzyme activity. This alkaliphilic amylase is suitable for waste-paper, starch and bioethanol industries.**

Key words: *Bacillus subtilis,* alpha amylase, alkaliphilic, enzyme.

INTRODUCTION

Amylase (EC 3.2.1.1) is one of the hydrolytic enzyme that catalyzes the hydrolysis of α-glucosidal bonds in starch, glycogen and related polysaccharides (Reddy et al., 2003). They are widely used in various industries such as food, textile, paper, detergent, and bewerage etc. Amylases constitute approximately 25% of the todays enzyme market (Elayaraja et al., 2011; Haq et al., 2012). Alpha amylases degrade α-1,4-glucosidic linkages by endo acting and produce oligosaccharides such as maltose, glucose and alpha limit dextrin. Common sources of the enzyme for industrial need is microorganisms because of their short fermentation period and bulk production of enzyme (Gupta et al., 2003). Microbial amylases are also applicative in pharmaceutical and biotechnological industries (De Souza et al., 2010; Naidu and Saranraj, 2013). Among the microorganisms, the genus *Bacillus* is one of the highly potential industrial agents since they are extracellular enzyme producers and generally regarded as safe etc. (Schallmey et al., 2004).

Amylases working at pH values 8.0 or higher have potentials for harsh conditions as in textile and detergent industries (Saxena et al., 2007). Most of the amylases from bacterial and fungal (Comlekcioglu et al., 2010) strains have an optimum pH between 5.0-7.5, therefore they are not appropriate to use in many industrial applications (Das et al., 2004). So, alkaline amylases to meet

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industrial demand have temptated the search for microbial strains propagating the enzymes with desired properties. In this study, we report purification and some properties of an alkaliphilic alpha amylase produced by a newly isolated *Bacillus* sp.

MATERIALS AND METHODS

Organisms and cultivation conditions

Bacillus sp. A10 isolated from the soil samples collected from orchard located at hillside of the Ahir mountain, Kahramanmaraş, Turkey. The soil samples were pasteurised in water bath at 80ºC for 10 min for selection of Gram-positive spore forming bacteria, *Bacillus* sp. (Lennete et al., 1985; Hamilton et al., 1999). The isolated strains were monitored for amylase production on agar plate composed of Na_2HPO_4 6 g, KH_2PO_4 3 g, NaCl 0.5 g, $MgSO_4$ 0.24 g, $CaCl_2$ 0.01 g, peptone 3 g, 1% (w/v) soluble starch (Merck), and Agar 15 g (Shibuya et al., 1986). The starting pH of the medium was 9.5 arranged with 10% Na_2CO_3 after sterilization. Amylase producing strains were selected after allowing the plates to iodine solution's vapour (Hols et al., 1994). Amylase positive strains were stored at 4°C on agar slope until enzyme production processes.

Identification of the microorganisms

Microorganisms were identified on the basis of 16S rDNA sequence analysis as well as its morphological and biochemical properties such as colony morphology, gram staining, spore bearing, motility, catalase production and acid production from glucose, xylose and manitol (Ratanakhanokchai et al., 1999). The polymerase chain reaction (PCR) product was sequenced by a commercial company (Refgen, Ankara, Turkey) using automatic sequencer. The analyses of the nucleotide sequence were performed by Clone Manager 5 and homology search was carried out by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence of the *B. subtilis* A10 was registered on GenBank, NCBI (Accession Number: KJ433550).

Enzyme production

The selected strain, *Bacillus* sp. A10, was cultivated in minimal medium (M9) containing 1% soluble starch. The initial pH of the medium was adjusted to 9.0 with 10% Na_2CO_3 after sterilization. The medium was inoculated with a 10% of fresh overnight cell culture. The medium was then left incubation for 3 days at 37°C with shaking at 250 rpm. After removal of bacterial cells by centrifugation (Hettich Mikro 22R) (4020 *g*) for 30 min at 4°C. The supernatant was used for further investigations.

Purification of enzyme

The cell free supernatant was applied to fractionated ammonium sulfate precipitation for enzyme purification. The ammonium sulfate crystals were added to the supernatant to make the desired saturation (40-90%) at 4°C. Four hours later, the precipitate was removed by centrifugation at 4020 *g* and 4°C, for 30 min. The precipitates produced in different ammonium sulfate saturation were resuspended with 100 mM phosphate buffer at pH 7.6 (McTigue et al., 1995). They were then dialysed against the same buffer for 48 hours with several changing. The fractions were checked for amylase activity on agar-starch (1%) petri dish by dropping 10 μL. Then the fractions showing amylase activity were pooled and concentrated in Amicon Ultra-15 Filter Units tubes by centrifugation at 4020 *g*. The concentrated suspension was applied to Sephadex G-100 gel Filtration Column (1cm Diameter × 40 cm Height). First of all, the column was equilibrated with 100 mM phosphate buffer at pH 7.6. The enzyme suspension (1.5 mL) was eluted at a flow rate 14 mL/h using the same buffer at room temperature. The fractions (0.5 mL each) were checked again on a agar-starch petri dish to determine the fractions including enzyme. The fractions giving the largest hydrolytic zones were then gathered together.

Homogeneity, molecular weight determination and zymography

The homogeneity and molecular weight of the enzyme was tested by SDS-PAGE (10%) (Laemmli, 1970). As a molecular weight marker, protein mixture SDS6H2 (SIGMA) containing porcine myosin (200 kDa), *E. coli* β-Galactosidase (116 kDa), rabbit muscle Phosphorylase b (97 kDa), bovine albumin (66 kDa), ovalbumin (45 kDa), bovine erythrocytes carbonic anhydrase (29 kDa) was used and the bands were detected by staining with Coomassie brilliant blue R-250.

Zymography analysis of the enzyme was carried out by SDS-PAGE containing soluble starch (1%). After electrophoresis process, the gel was subjected to the renaturation solutions (Saul et al., 1990) prior to incubation. The incubation of gel was carried out in a plastic storage box after lining the gel on a glass plate at 45°C for 2 h. Activity bands were obtained by soaking the gel in iodine solution (KI: 5 g/L, I_2:0.5 g/L) (Hashim et al., 2004).

Enzyme assay

α-Amylase activity of the supernatant and the purified enzyme were assayed by the detection of reducing sugars (Miller, 1951). The reaction mixture contained 400 μL of 1% soluble starch and 100 μL of enzyme solution. The enzymatic reaction was stopped with 500 μL of 3,5-dinitrosalicylic acid after 30 min incubation at 45°C, and absorbance was measured at 550 nm in a Perkin Elmer Lambda EZ 150 Spectrophotometer. One unit of amylase activity was defined as the amount of enzyme liberating 1μmol reduced sugars per min under assay conditions.

Effect of pH and temperature on activity and stability

The optimum pH and optimum temperature for amylase activity were assayed at different pH values ranging from 3 to 11.5 and temperatures from 4-90°C for 30 min. The buffers used were as follows: Citrate-phosphate (pH :3.0-5.5), Na-phosphate buffer (pH 6.0-7.5), Tris-HCl buffer (pH: 8.0-9.0) and Borax-NaOH (pH: 9.5-11.5). Temperature stability was performed by pre-incubating the enzyme at temperatures between 4 and 90°C for 30 min. in optimum pH. For the pH stability, the enzyme was also pre-incubated at different pH ranged 4.0 to 11.0 at optimum temperature for 15 and 30 min. Then the remaining acitivity was determined under the standart assay conditions. The effect of NaCl on the activity was tested by adding the enzyme into the substrate containing different NaCl, while the stability of enzyme was tested by pre-incubating the enzyme in different NaCl concentration (0.1-4.0 M) at optimum pH and temperature for 30 min, All the experiments were conducted three times and mean values were taken.

Effect of some chemicals and inhibitors on enzyme activity

The effect of chemicals suchs as metal ions, chelaters, detergents and inhibitors on enzyme activity were tested by pre-incubating the

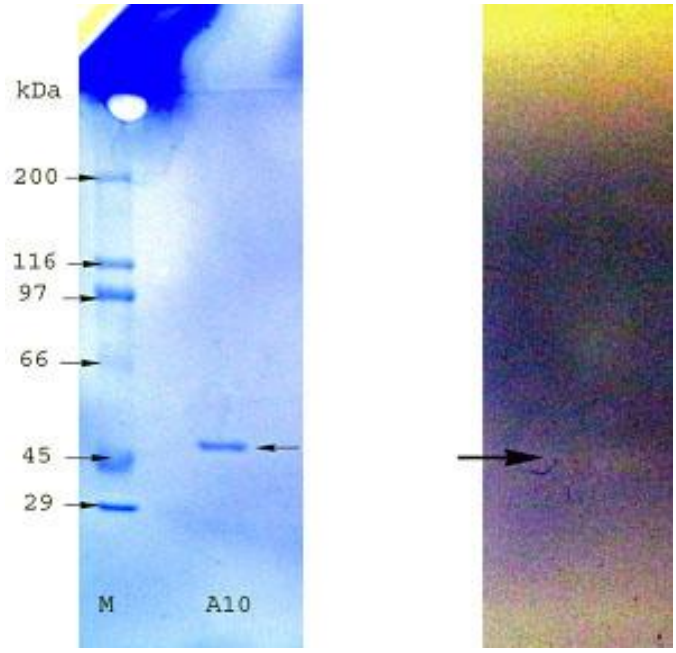

Figure 1. SDS-Page and Zymogram Analysis of α-amlyase A10. M: Protein mixture SDS6H2 (SIGMA) Porcine myosin (200 kDa), *E. coli* β-Galactosidase (116 kDa), rabbit muscle phosphorylase b (97 kDa), Bovine albumin (66 kDa), Ovalbumin (45 kDa), Bovine erythrocytes Carbonic Anhydrase (29 kDa).

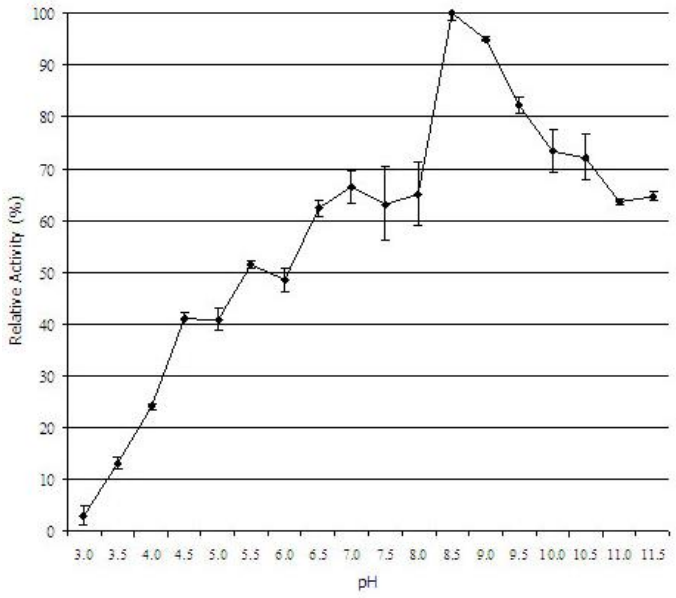

Figure 2. Effect of pH on the activity of *Bacillus subtilis* A10 α-amylase.

amylase in different concentration of the material at optimum pH and temperature for 30 min. The remaining activity was determined according to the control tubes which were not containing any additives.

Analysis of the end products

For the determination of amylase action on soluble starch thin layer chromatography analysis was performed on 'Aluminium oxide 60 F_{254} neutral' TLC plates (Merck). Firstly, soluble starch was digested with amylase at 45°C for 60 min of incubation. Cold ethanol was added to the mixture to stop the reaction after incubation. Development was carried out with a solvent system of butanol-acetic acid-water (3:1:1,by volume). Then the spots were visualized by spraying 20% sulphuric acid in ethanol and keeping the plates in an oven at 120°C for 30 min.

RESULTS

Total of 247 isolates were tested for α-amylase production on agar plates containing soluble starch (Shibuya et al., 1986). Among them, total 231 amylase positive isolates were selected after application of iodine vapour. The highest amylolitic potential showing strain A10 was chosen for enzyme production (Bernhardsdotter et al., 2005). The strain was aerobic, Gram positive, rod shaped, motility and catalase positive and spore forming. Although the enzyme synthesis by *Bacillus subtilis* A10 was observed between pH 7.0-11.0, with an optimum 37°C, the maximum amylolitic potential was at pH 9.0 on agar plate.

The nucleotide sequence of 16S rDNA gene of the selected strain A10 was determined and the sequence analysis showed that the strain A10 shared more than 98% of its identidy with diferent *Bacillus subtilis*. Then the organism was named as *Bacillus subtilis* A10.

Determination of molecular weight and zymography

SDS-PAGE analysis revealed a single band for the purified α-amylase, indicating this enzyme has been purified to near homogeneity by the fractional ammonium sulfate precipitation and Sephadex G-100 chromatography (Figure 1). Its molecular weight was estimated as 46.9 kDa. Zymogram analysis was accomplished by SDS-PAGE including soluble starch. The gel was subjected to the renaturation solution as described by Saul et al. (1990). Activity band was observed after soaking the gel in iodine solution (Hashim et al., 2004).

Properties of the α-Amylase

The optimum pH was determined with four different buffer systems. Although the organism showed maximum enzyme production at pH 9.0, the α-amylase showed the maximal relative activity at pH 8.5 (Figure 2). The α-amylase also presented a mean activity around 73.4% in between pH 6.5 and 11.5. Optimum temperature of the α-amylase was observed at 45°C with an average 71% activity between 25 and 55°C (Figure 3).

The pH stability of the α-amylase was determined by pre-incubating the enzyme at 45°C for 15 and 30 min. The remaining activity was surveyed by standart assay method. The highest stability was in between pH 7.0 and

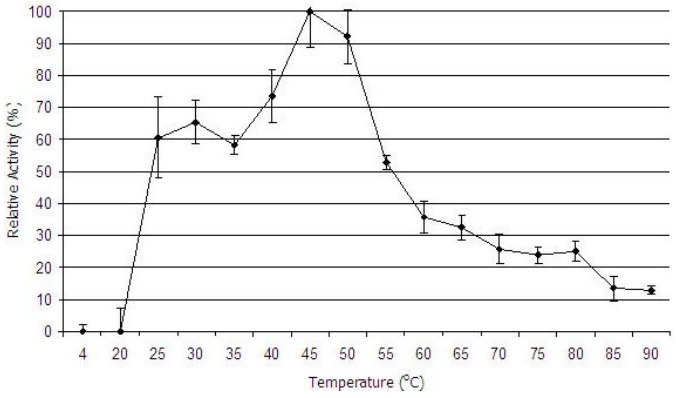

Figure 3. Effect of temperature on the activity of *Bacillus subtilis* A10 α-amylase.

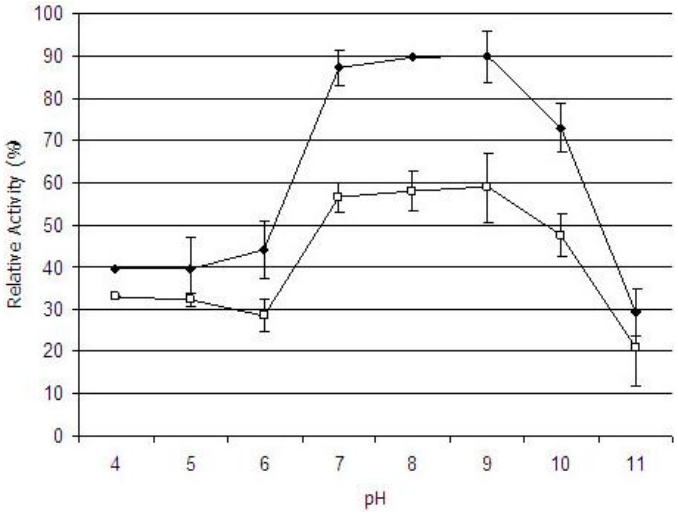

Figure 4. Effect of pH on the stability of *B. subtilis* A10 α-amylase. The enzyme was preincubated in different buffers for 15 (♦) and 30 (□) min. The buffers used were 100 mM Citrate-phosphate (pH :4.0-5.0), Na-phosphate buffer (pH 6.0-7.0), Tris-HCl buffer (pH: 8.0-9.0) and Borax-NaOH (pH: 10.0-11.0).

10.0 with an activity average 84.9% for 15 min. The enzyme was also stable over 50% remaining activity for 30 min (Figure 4). In both preincubation period, the maximal remaining activity was at pH 8.0 and 9.0. For thermal stability estimation, the enzyme was preincubated at diferent temperatures (4-90°C) for 30 min at pH 8.5. The enzyme was highly stable in between 4 and 50°C with an average 91.5% remaining activity (Figure 5).

To detect the effect of NaCl on α-amylase activity and stability, different NaCl concentration ranging from 0.1 to 4.0M were used. The maximal activity and stability were obtained in the presence of 1M (5.85%) NaCl (Figure 6) .

Effect of some chemicals on enzyme activity

The α-amylase was preincubated at 45°C for 30 min in

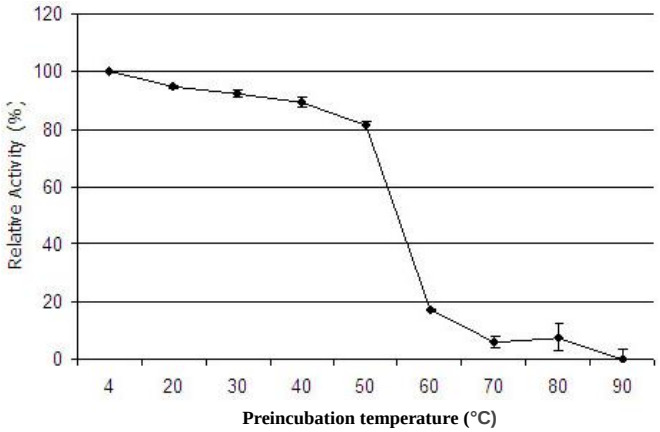

Figure 5. Effect of temperature on the stability of *B. subtilis* A10 α-amylase. The enzyme was pre-incubated at temperatures from 4 to 90ºC for 30 min at optimum pH. Remaining activity (%) was determined under standard assay condition.

the presence of chemicals prior to standart assay reactions. The enzyme activity was inhibited in the presence of $ZnCl_2$, urea, KCl, EDTA, $CaCl_2$, and SDS to 35.2, 24.3, 35, 51.6, 46.35 and 24.1%, respectively. Among the substances tested, Na_2SO_3 and β-Mercaptoethanol did not effect the enzyme activity indeed (Figure 7).

Analysis of the end product of enzyme action

Enzymatic hydrolysis product from soluble starch were analysed by TLC using aluminium oxide plate. After 60 min incubation of reaction mixture at 45°C, glucose, maltose and other longer oligosaccharides were the main products produced (Figure 8).

DISCUSSION

Among the microorganisms, the genus *Bacillus* are one of the extracellular enzyme producing bacteria and they have taken an important place in various industrial application. Amylases are one their significant hydrolytic enzymes for industries. This study reports that isolation of microorganism producing amylase, production, purification, and characterization of α-amylase.

The isolated strain *Bacillus subtilis* A10 for α-amylase production showed a growth mainly in the alkaline range between pH 6.5 to 11.0 giving the largest colony at pH 10.0 as in the findings of Johnvesly and Naik (2001). Alkaliphilics grow best above pH 8.0 and cannot grow or grows poorly around neutral pH (Horikoshi, 1999), therefore, we called the organism A10 is an alkaliphilic.

Bacillus subtilis A10 α-amylase enzyme was calculated as 46.9 kDa with SDS-PAGE analysis. Similar results for alkaliphilic α-amylase between 42 to 70 kDa have been

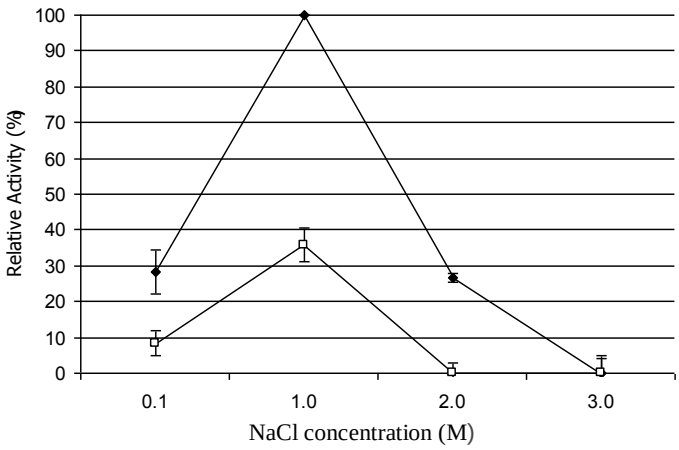

Figure 6. Effect of salt concentration on enzyme activity (♦)and stability (□) of *Bacillus subtilis* A10 α amylase.

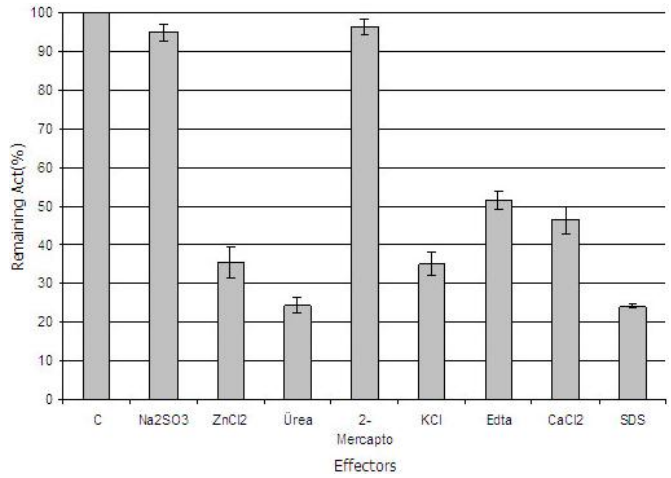

Figure 7. Effect of Some Chemicals on the activity of of *B. subtilis* A10 α-amylase.

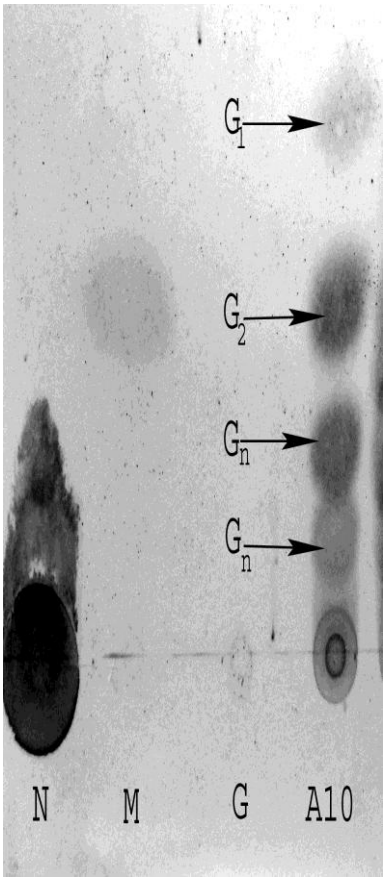

Figure 8. TLC of enzyme products from *Bacillus subtilis* A10. N: Untreated soluble starch; G: Glucose M: Maltose, A10: Enzyme substrate mixture (G_1: Glucose, G_2: Maltose, G_n: oligosaccharides).

reported by Horikoshi (1971); Igarashi et al. (1998); Ben et al. (2001); Das et al. (2004) and Annamalai et al. (2011). Many alkaline α-amylases from different *Bacillus* sp. were reported that optimal temperatures were around 40 to 70°C. The amylase from *Bacillus subtilis* A10 presented also an optimum temperature at 45°C as in the previously reported alkaline amylases (Igarashi et al., 1998; Lin et al., 1998; Cordeiro et al., 2002; Bernhardsdotter et al., 2005).

Amylase A10 is alkaline, but not thermostable as most of amylases reported earlier. Its thermostability at pH 8.5 up to 50°C for 30 min is also sufficient with a remaining activity over 80% for most of the industrial applications using amylases. Das et al. (2004) and Saxena et al. (2007), reported that urea highly denatured the amylase and their findings support our results. Although the denaturation action of urea (8M), uneffected amylase from *Thermus* sp. was also reported by Shaw et al. (1995).

The enzyme A10 was also presented a similar results to *Thermus* sp. amylase (Shaw et al., 1995) in presence of Ca^{2+} and EDTA. There was no Ca^{2+} activation was observed, on the other hand, sensitivity to EDTA was about 50%. Although the inhibiton with EDTA indicates that the enzyme requires metal ions, but enzyme may not need Ca^{2+} for activity or stability due to the adaptation to environment deficient in Ca^{2+} ions since carbonates production. The effect of Zn^{2+} on amylase activity is variable. It could be an effective inhibitor or ineffective at al (Kim et al., 1995; Mamo and Gessesse, 1999; Demirkan et al., 2005). The inhibition with Zn^{2+} was explicated as the competition in between exogenous and protein associated cations (Lin et al., 1998). Sodium sulfite is a chemical agent used for deinking processes in wastepaper industries. Uninhibitory effect of sodyum sulfite makes the enzyme rewarding for paper industries. This findings were in agreement with Krishnan and Chandra's (1983) findings too. Additionally, β-mekaptoethanol did not affect the enzyme substantially (Ozcan et al., 2010). According to the TLC plate, that the glucose band observed pale in

comparison with maltose and longer oligosaccharides. This is probably due to liberation of longer fragments at the beginning of hydrolysis of starch (Das et al., 2004).

Conclusion

Amylases are one of the most important enzymes for industrial applications. Microorganisms are the most efficient sources for enzyme production. The enzyme A10 α-amylase was produced alkaliphilic *Bacillus subtilis* A10 those are accepted as GRAS status. The enzyme is alkaline with an optimum pH 8.5. Its stability up to 50°C and activity in the presence of sodyum sulfite makes the enzyme merit in waste-paper industries for brightening processes. Its amylolytic action makes it worthy in starch industries as well as bioethanol production.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Semen and microbial characteristics of two breeds of turkeys in an arid tropical environment of Bauchi State, Nigeria

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A study was conducted at the poultry unit of Abubakar Tafawa Balewa University, Bauchi, Teaching and Research Farm to investigate semen characteristics and the sensitivity of semen microbes to some antibiotics in exotic (large white – LW) and local (indigenous – I) breed of turkeys for a period of six months. Data were analysed using one way analysis of variance (ANOVA). Results show significant breed differences (P≤0.01) in live weight (15.24±0.88 kg and 6.53±0.53 kg) for LW and I breeds, respectively. Significant breed differences were also recorded for semen volume (0.35±0.05 and 0.18±0.02 ml), total sperm per ejaculation (7.43±1.22 and 2.77±0.29×10^8/ml), daily sperm output (1.02±0.17 and 0.39±0.05×10^8/ml), total live spermatozoa (5.68±1.12 × 10^8 and 1.99±0.27×10^8/ml), total live normal spermatozoa (4.57±0.99 ×10^8 and 1.76±0.24×10^8/ml) for LW and I breeds, respectively. In the LW breed also, sperm concentration correlated positively (r = 0.79) with semen volume, total live spermatozoa (r= 0.77) with total spermatozoa per ejaculation, daily sperm output (r=0.68) with total live spermatozoa, daily sperm output (r=0.52) with semen motility and live weight (r=0.54) with semen volume. In the I breed, there was significant and positive correlation between total live normal spermatozoa (r=0.76) with total spermatozoa per ejaculation, daily sperm output (r=0.91) and total spermatozoa per ejaculation, daily sperm output (r=0.84) and total live normal spermatozoa. *Enterobacter* spp. was the only microbe isolated from the semen of both LW and I breeds of turkey and was susceptible to Ciproxine (Cip) and Gentamycin (GN). There were turkey breed differences in semen characteristics in our environment, and they compare favourably with those obtained elsewhere in the tropics. The LW breed appears to have a higher reproductive potential than the I breed. Ciproxine and Gentamycin could be used in the control of bacteria in turkey semen in our environment.

Key words: Turkey semen, microbial characteristics, breeds, arid tropical environment.

INTRODUCTION

Artificial insemination (AI) is a very important practice by modern turkey breeding systems. It is one of the animal production technologies that augment production and returns from livestock at a faster rate and enhance cross Breeding programmes. The evaluation of the semen of any animal species gives an excellent indicator of its

quality and is sine-quanon to an effective artificial insemination programme, but there is paucity of information on this aspect for the Nigerian indigenous species of poultry and turkey in particular.

Rapid human population growth and low protein intake are some of the major problems facing developing countries like Nigeria. With a population of about 174 million (PRB, 2013) and with over 70% of the population living on less than a dollar a day (Watts, 2006), Nigeria is the most populous country in Africa. Nigerians own a variety of farm animals with poultry being the highest in number and 80 - 90% of these flocks owned by small scale farmers (Ebangi and Ibe, 1994). Poultry offers an avenue for rapid transformation in animal protein consumption. The average Nigerian consumes 9 grams of animal protein per capita per day as compared to over 50 g per capita per day in North America and Europe (Boland et al., 2013). The poultry population in Nigeria is estimated at 140 million (Adeleke et al., 2010), producing about 268,000 metric tons of poultry products annually (FAO, 2013). Indigenous poultry constitute more than 90% of total poultry in Nigeria (Gueye, 1998). Sonaiya (1999) confirmed that of the 82.4 million chickens in Nigeria, commercial holdings accounted for only 10 million chickens or 11%. Philips (1996) indicated that nearly every household keeps poultry, thus protein from poultry sources is still available to most families.

Research on the indigenous breeds of poultry is increasing in Nigeria. There are some reports on studies carried out on chickens (Oke and Ihemeson, 2010; Peters et al., 2010; Adeleke et al., 2011); guinea fowl (Butswat et al., 2001) and turkeys (Peters et al., 1997; Zahraddeen et al., 2005; Ironkwe and Akinola, 2010; Ilori et al., 2011; Yakubu et al., 2013). Turkey production is both an important and a profitable agricultural industry, with a rising global demand for its products (Anandhl et al., 2012). Indigenous turkey is the least produced among domesticated poultry species in Nigeria numbering about 1.05 million (Yakubu et al., 2013), despite its greater potential as a meat bird than the chicken (Shingari and Sapra, 1993; Ajayi et al., 2012).

At present, the production of exotic poultry species is unaffordable by many Nigerians. The indigenous breeds are numerous, better adapted and cheaper to raise (Fisinin and Zlochevskaya, 1989). These birds are natural foragers and scavengers and always range farther. Indeed, they thrive best where they can move about freely feeding on seeds, fresh grass, locusts, crickets, grasshoppers, worms, slugs and snails (Singh and Sharma, 2012). It is therefore, necessary that more research be conducted on them. There is scarcity of information on semen and microbial characteristics and the reproductive performance of indigenous turkeys in Nigeria. To improve local stock, knowledge of their reproductive potential is relevant, and to derive fullest benefits from breeding turkeys, proper knowledge of their sperm output is essential (Butswat, 1994). Semen collected is normally preserved in specially compounded diluents pending insemination (Niba et al., 2002). Antibiotics and sometimes antimycotics are often added for the diluents before conservation. This is done to protect the semen content from destruction before insemination. Quite often, antibacterial application is done without prior knowledge of the type of microorganisms prevalent in semen. Microorganisms have a deleterious effect on sperm function, both directly by altering the structure of the sperm, by affecting its motility (Depuydt et al., 1998) or by provoking a premature acrosome reaction (Kohn et al., 1998), the putrefaction of diluents components of biological origin, and the utilization of metabolic substrates (Lamming, 1984), and indirectly stimulating the production of antibodies that can be directed against the sperm glycocalyx complex (Kurpisz and Alexander, 1995). Most ejaculates collected from healthy animals are contaminated with bacteria to some extent. Some reports indicate that metabolic products such as endotoxins from some bacteria appear to have detrimental effects on the survival of sperm (Almond and Poolperm, 1990). Hence, semen quality and the quantity of viable sperm cells may be reduced with bacterial contamination.

The aim of this study is to improve breeding practices in turkeys, by establishing a baseline for assessing and comparing the semen characteristics of the two genotypes. The objective was therefore designed to evaluate the semen characteristics and microbial flora, and the sensitivity of semen microbes to antibiotics in the two genotypes.

MATERIALS AND METHODS

Climate and vegetation of the study site

Bauchi State is in north eastern Nigeria. It occupies a total land area of 49,259.01 km^2 (5.3% of Nigeria) and is situated between latitude 9° and 12°30' North, and between longitudes 8°50' and 11° East. According to the 1991 census, Bauchi State has a population of 2,826,444 inhabitants. The society is primarily agrarian and agriculture contributes about 75% to the State's economy. The climate is characterized by two well defined seasons: The rainy season (usually May to October) and the dry season (usually November to April). The vegetation is Sahel/Sudan in the north, guinea savannah in the central and western zones of the state (IAR/BSADP, 1996). The mean annual rainfall is 905.33 mm with an annual temperature range of 11-14°C. The annual rainfall is between 700 and 1250 mm in the north and south – south west zones, respectively. Mean monthly hours of sunshine are about 300

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h (highest) in December and about 150.1 h (lowest) in August. August records the highest relative humidity of 65.5% and February records the lowest, 16.5% (Kowal and Knabe, 1972).

Breed description and management of experimental birds

Two breeds of turkeys were used in the study. The word 'breed' is used loosely to distinguish the birds.

The indigenous breeds of turkeys were not well defined. Various color phases exist with black and white occurring as pure colors. Brown color also exists and has numerous pale barring and mottling of the feathers especially of the tail, primaries, secondaries and wing coverts; a metallic sheen of the plumage usually accompany the black and brown color phases. Yearling males weigh between 4.6 - 4.8 kg and females 2.4 - 3.2 kg (Malia, 1998).

The large white (LW) exotic breed are heavy, broad-breast, white in color and with a well developed snood, dewlap and caruncles, which are bright red in color. There is little amount of blue skin around the eyes. All birds are flightless, markedly tame and of a calm disposition thereby being easily approached. Beaks, shanks and feet are pink tan. Mature body weights for the toms range from 25 - 35 kg and for the hens 8 -12 kg.

The exotic (LW) breed was sourced from ZARTECH Ltd. Farms, Ibadan, Oyo State while the indigenous (I) breed was purchased from some local farmers in Bauchi. Four animals of each breed, age between 9 and 11 months old and weighing averagely 11 and 4.2 kg respectively were used in this study. All the toms were housed singly in a netted pen in well ventilated poultry houses and fed a standard breeder diet (17% crude protein, 2900 kcal of metabolizeble energy/kg). The experimental diets in mash form and fresh clean drinking water were provided *ad libitum*. The ambient relative humidity was 50-60%. All groups of toms were trained for semen ejaculation on breed basis over a period of three weeks.

Tetracolivit (an anti-stress containing Oxytetracycline, vitamin and minerals) was administered in water for one week following stocking. On the seventh day, the birds were dewormed using Piperazine Citrate. Deworming was repeated after one week. Birds were treated against external parasites (fowl lice) using *dichlovos* as active ingredient in a devlap spray and drops were sprayed in saw dust and pen walls. During the training period, *Trisulmycine Forte* (containing Trimethoprim and Sulphadiazine as active ingredients) was used to control attacks against respiratory diseases.

Semen collection and evaluation

Semen collection was carried out by two persons using the double hand lumbar massage method of Burrows and Quinn (1937) and Watson (1990).

Semen volume was obtained using a graduated conical test tube calibrated to the nearest 0.01 ml. Semen motility was evaluated using a haemocytometer and a light microscope with warm stage in accordance with the methods outlined by Sorensen (1976) and Sexton (1981).

Sperm count was carried out using a haemocytometer 450x magnification. The number of sperm counted in five large diagonal squares multiplied by 10^6 (N x 10^6) equalled sperm concentration/ml. Total spermatozoa per ejaculation were obtained by multiplying ejaculate volume and concentration.

Percent live/dead spermatozoa and percent normal/abnormal sperm cells were determined using the technique of Ernst and Ogasawara (1970). A mixture of Eosine/Nigrosine was used as dye/stain mixed with semen freshly collected. Thereafter, on microscopic examination, normal and abnormal morphology were observable. Cells that were dead or damaged were seen to be stained while live cells absorbed no dye colour.

Total live spermatozoa were obtained by multiplying total spermatozoa per ejaculation and percent live spermatozoa. Total live and normal spermatozoa were obtained by multiplying total live spermatozoa and percent normal sperm (Sexton, 1986).

Daily sperm output was measured by dividing total spermatozoa per ejaculation by the interval of semen collection (7 days) (Brillard and de Reviers, 1985). Weights of birds were determined using a weighing scale.

Semen microbial analysis

Isolation and identification of micro-organisms

A loopful of the test semen was aseptically streaked on nutrient agar (NA) plates (Oxoid Ltd.) in triplicates and incubated aerobically at 37°C for 24 h for bacteria and on already prepared potatoes dextrose agar (PDA) plates (Oxoid Ltd.) in triplicates and incubated at 25°C for 5 days for fungi. This was repeated for 10 collections. Bacterial colonies of discrete cultural characteristics were carefully picked and purified by repeated sub-cultures on nutrient agar plates and their morphology was studied. Pure cultures were then preserved on nutrient agar plates and used for Gram staining. Gram stained slides were identified after microscopy using Bergey's Manual of determinative bacteriology (Buchannan and Gibbons, 1974).

Sensitivity of semen microbes to antibiotics

Using a sterile wire loop, a loopful of the test organisms was picked up and streaked according to standard medical laboratory techniques, (SMLT) (Harrigan and McCance, 1976). A sterile bent glass rod spreader was used to make a fine lawn and the antibiotic discs (Poly-Tes-Multo-disks PS 003 gram negative) were used according to agar diffusion method and the results reported according to SMLT. When more than one level of a particular antibacterial was available, the maximum dosage was tested. The antibiotics used for the study were Ciproxine (Cip) – 5 mcg, Gentamycine (GN) -10 mcg, Tetracycline (TE) -50 mcg, Nalidixic acid (NA) -30 mcg, Ampicillin (AM) -25 mcg, Cefuroxine (CF) -20 mcg, Norbactin (NB) – 10 mcg and Cotrimoxazole (CO) -50 mcg. The effectiveness of each antibacterial tested was determined by measuring the minimum inhibitory zone (mm) surrounding each disc (Sexton et al., 1989b). The minimum inhibitory zone is the diameter surrounding the sensitivity disc in which no growth occurred and is measured from one side of the circular zone to the other side as seen from the underside of the agar plate.

Interpretation of susceptibility or resistance to antibacterial treatment was aided by data collected with test cultures provided by the manufacturers. A susceptible score indicated that the micro-organism in semen was most likely affected by antibacterial while a resistant score indicated that micro-organisms were not affected.

Data analysis

Breed effects on the various semen characteristics were determined using analysis of variance (ANOVA) (Ryan et al., 1985).

RESULTS

Live weight

The overall live weight for the two breed of turkeys was 10.89 ± 0.5 kg. Analysis of variance showed a significant difference ($P \leq 0.01$) in weights among the two breeds of turkey used for the study, being 15.24 ± 0.88 and 6.5 3 ± 0.53 kg for LW and I, breed respectively (Table 1).

Table 1. Mean semen characteristics by breed.
±

Semen parameter	Overall	Exotic (LW)	Indigenous (I)	SEm±	LOS
Live weight (kg)	10.89±0.51	15.24±0.88	6.53±0.53	0.73	**
Volume (ml)	0.26±0.03	0.35±0.05	0.18±0.02	0.04	**
Motility (%)	82.95±1.14	84.63±0.96	81.27±2.07	1.61	NS
Conc. x (10^8 sperm/ml)	1.99±0.16	2.27±0.26	1.73±0.18	0.22	**
Tot. Sperm/ejac x 10^8	5.19±0.16	7.43±1.22	2.77±0.29	0.95	*
Tot. live Spz x 10^8	3.84±0.64	5.68±1.12	1.99±0.27	0.90	*
Tot. live Nor Spz. x 10^8	3.16±0.56	4.57±0.99	1.76±0.24	0.79	**
Daily sperm output x 10^8	0.70±0.10	1.02±0.17	0.39±0.05	0.14	NS
Live Spz (%)	83.53±0.96±	84.91±1.38	82.43±1.34	1.36	NS
Dead Spz (%)	16.48±0.96	15.39±1.38	17.57±1.34	1.36	NS
Normal Spz (%)	86.71±0.81	88.29±1.08	85.13±1.18	1.14	NS
Abnormal Spz (%)	13.16±0.81	11.96±1.11	14.36±1.17	1.15	NS

Tot. = Total, ejac. = ejaculate, Spz = spermatozoa, Nor.= normal; *- P≤0.05; **-P≤0.01; NS- not significant; LOS- Level of significance.

Semen characteristics

The overall mean of the various semen characteristics studied in breed of turkeys are shown in Table 1. The effects of breed on semen volume, total sperm per ejaculation and daily sperm output were significantly different (P≤0.01). Total live spermatozoa and total live normal spermatozoa were also significantly different (P≤0.05), the values for the LW are 5.68 ± 1.12 and 4.57 ± 0.99 as compared to 1.99 ± 0.27 and 1.76 ± 0.24 (x10^8 sperm/ml) for I breed. The highest yield of 1.1 ml good quality semen was obtained among the LW breed while 0.1 ml was obtained in the I breed. The effects of breed on the other semen characteristics namely percent motility, sperm concentration per ml, percent live/dead and percent normal/abnormal sperm cells were however not significant. Semen volume for the LW was 0.35 ± 0.05 ml as against 0.18 ± 0.02 ml for the I breed. The overall motility percentage was 82.95 ± 1.14 (%). Similarly, the concentration of sperm cells was 2.27 ± 0.26 and 1.73 ± 0.18 (x10^8 sperm/ml) for LW and I breeds respectively. Total live spermatozoa, total live normal spermatozoa and daily sperm output were higher for LW than for I breeds (5.68 ± 1.12 vs 1.99 ± 0.27, 4.57 ± 0.99 vs 1.76 ± 0.24 and 1.02 ± 0.17 vs 0.39 ± 0.05 x 10^8/ml respectively. In the case of total sperm per ejaculation, LW had the highest value while I breed had the lowest (7.43 ± 1.22 and 2.77 ± 0.29 x 10^8/ml sperm cells, respectively.

The overall percent live and dead cells were 83.52 ± 0.96 and 16.48 ± 0.96%, respectively. On the other hand, the overall percentage normal as compared to abnormal sperm cells was 86.71 ± 0.81 and 13.16 ± 0.81%, respectively.

Relationship between semen characteristics

The correlation coefficients among the studied parameters are presented in Table 2 and showed that correlations between some parameters studied were weak and non significant (p≤0.05) while some parameters showed negative relationships. Highly significant (p≤0.01) and strong associations were observed between most parameters with r values ranging from 0.91 between daily sperm output (DSO) and total spermatozoa (TS), DSO and total live normal sperm (TLN), concentration (CON) and semen volume (SV), total live spermatozoa (TLS) and total spermatozoa (TS), total live normal spermatozoa (TLN) and total spermatozoa (TS) to 0.68 between DSO and TLS. However, correlations between daily sperm output and semen motility percentage (r = 0.05) and between body weight and semen volume (r = 0.54) were low but still significant (P≤0.05).

Semen microbiology

Data on sensitivity test of semen microbes to some antibiotics (Gram negative) are shown in Table 3. *Enterobacter* spp. was the only microbe isolated from the semen of both LW and I breeds of turkey. Among the antibiotics tested on bacterial isolates, only Ciproxine (Cip) and Gentamycin (GN) were found to be effective. The mean millimeter zone of inhibition for 3 replicates was 27 and 15.03 mm for Ciproxine and Gentamycin, respectively.

DISCUSSION

Live weight, semen characteristics and relationship between semen traits

Livestock breeders everywhere are interested in old and new factors that can affect the breeding capacity of the male of any species. Live weight obtained for the exotic breeds used in this study was lower than the 25.8 kg reported by Noirault and Brillard (1999). The ranking in live weights of the breeds studied agrees with those

Table 2. Correlation matrix between semen characteristics measured on genotypes.

Genotype		SV	SM	NS	AS	CON	TS	TLS	TLN	DSO	BDW	Li %
	SM	-0.210										
	NS	-0.328	0.181									
	AS	0.302	-0.192	-0.992**								
	CON	0.793**	-0.104	-0.229	0.219							
	TS	-0.183	0.426	0.042	-0.056	-0.227						
Exotic (LH)	TLS	-0.196	0.274	0.016	-0.034	-0.209	0.772**					
	TLN	-0.109	0.113	0.287	-0.280	-0.176	0.070	0.339				
	DSO	-0.177	0.052*	0.221	0.284	-0.274	0.444	0.679**	0.012			
	BDW	0.541*	0.007	-0.226	0.253	-0.109	0.180	0.098	-0.237	-0.121		
	Li (%)	-0.224	-0.241	-0.020	-0.004	-0.349	0.323	0.245	-0.012	0.149	0.233	
	DD (%)	0.224	-0.241	-0.020	0.004	-0.349	0.323	0.245	-0.012	0.149	0.233	-1.000**
	SM	-0.238										
	NS	-0.063	0.115									
	AS	0.062	-0.115	-1.000								
	CON	0.170	-0.489	0.163	-0.164							
	TS	0.347	0.005	0.275	0.275	0.245						
Local (I)	TLS	-0.263	0.260	0.174	0.174	0.388	0.165					
	TLN	-0.521*	0.065	0.350	-0.349	0.241	0.755**	0.192				
	DSO	-0.492	0.065	0.334	-0.333	0.160	0.905**	0.220	0.835**			
	BDW	-0.064	0.288	-0.310	0.309	0.003	-0.330	-0.235	-0.138	-0.213		
	Li (%)	-0.430	0.164	-0.065	-0.066	0.096	-0.045	0.033	0.092	0.193	0.264	
	DD (%)	0.430	0.164	0.065	0.066	0.096	0.045	-0.033	0.092	-0.193	-0.264	-1.000**

SV = Semen volume, SM = Semen motility, NS = Normal spermatozoa, AS = Abnormal spermatozoa, CON = Sperm concentration, TS = Total spermatozoa, TLS= Total live spermatozoa, TLN = Total live normal spermatozoa, DSO = Daily sperm output, BDW = Body weight, Li (%) = Live percentage, DD (%) = Dead percentage. * = $P \leq 0.05$, ** = $P \leq 0.01$.

of Oluyemi and Roberts (1988). The higher live weight obtained in the LW also suggests that the breed has a higher potential for use in breeding programmes in this environment since live weight has been linked to testes weight (Yakubu et al., 2012). Furthermore, earlier studies with crosses between indigenous hens and exotic cooks resulted in better progeny live weights (Ebangi and Ibe, 1994).

Quantitative evaluation of semen is an aspect of reproductive status assessment in males. Cecil and Bakst (1988), Egbunike and Nkanga (1999) and Etchu et al. (2013) have all reported that spermatozoa output, measured by volume and concentration; tend to vary with breed, nutrition and season. The overall results of the seminal traits for the two breeds showed that the LW breed was superior to the I breed. There was a significant difference between breeds in their yields of semen. The exotic toms (LW) yielded the highest volume of semen (0.35 ± 0.05 ml). This value was however lower than those reported (0.56 ml) by Sexton (1981) and obtained (0.43±0.12 ml) by Noirault and Brillard (1999) and Bonato et al., 2011 (1.16 ± 0.05 ml) in ostriches but higher than the value (0.31 ml) reported by Taras et al. (1997). The overall mean yield of semen in this study is lower than the 0.5ml reported by Hafez (1995) but within the range of 0.25 - 2ml given by Cerolini et al. (2003) for chickens.

Sperm concentration is influenced by breed, nutrition, season and even method of ejaculation (Butswat et al., 2001). The overall mean concentration of semen for all breeds in this study (1.99 ±

Table 3. Results of antibacterial disc survey on micro-organisms of Turkey semen.

Antibiotics	Level or quantity (mcg)	Kill zone (mm)[a]	Sensitivity[b]
Ciproxine (cip)	5	27	+++
Gentamycin (GN)	10	15.03	++
Tetracycline (TE)	50	0	+
Nalidixic acid (NA)	30	0	+
Ampicilln (AM)	25	0	+
Norbactin (NB)	10	0	+
Cotrimoxazol (Co)	50	0	+

[a] = Values are the mean mm for 3 replicates; [b] = Organisms were in the highly sensitive range, if kill zone > 16 mm (+++), intermediate or slightly sensitive if kill zone is between 12 - 16 mm (++) and resistant or non sensitive if kill zone < 12mm (+).

0.16 x 10^8/ml) is lower than the value for low concentration semen (3.8 x 10^9/ml) reported by Moss et al. (1979) and far lower than that obtained (3.74 ± 70.83 x 10^9/ml) by Zahraddeen et al. (2005) in turkeys. There was no significant difference in the sperm concentration in the two breeds used in this study. These results are at variance with those obtained by Zahraddeen et al. (2005) in indigenous and exotic turkeys. Idi (2000) also obtained significant deference in sperm concentration using indigenous and exotic breeds of cocks in Bauchi. However, an explanation for the absence of significant differences in sperm concentration between breeds in this study may be due to the expertise of the semen collector as expertise is linked to increase in the flow of accessory fluids and hence reduction in concentration. Because total spermatozoa per ejaculation is the product of concentration and ejaculate volume, LW breed which has the highest volume of semen also had the highest total sperm yield (7.43±1.22x 10^8/ml). This value is however lower than those obtained (4.32±0.74 x 10^9/ml) by Noirault and Brillard (1999). Total sperm value for I breed was 2.77 ± 0.29 x 10^8/ml, much lower than the values (1.23 ± 0.15 x 10^9ml) reported by Idi (2000) for indigenous cocks.

Motility is the semen evaluation parameter that is normally first used to indicate the presence of live spermatozoa in a semen sample. Sexton (1981) reported excellent motility scores of 80% and above. The overall mean motility scores of 82.95 ± 1.14, and individual breed scores of 84.63 ± 0.96 and 81.27 ± 2.07 (%) for LW and I were higher than the values of 67 and 45% obtained by Onuora (1982) for guinea fowls and those reported by Nwagu et al. (1996) in chickens. The motility percentage scores were also higher than those obtained by Butswat et al. (2001) for local and exotic guinea fowls (38.5±2.8 Vs 51.6 ± 1.7%) and Idi (2000) for local and exotic cocks (67.97 ± 1.40 vs. 67.58 ± 1.2%) but were similar to the values of 84.6% obtained by Taras et al. (1997). The values were however not significant confirming the findings of Zahraddeen et al. (2005) in turkeys but at variance with the findings of Butswat et al. (2001) using guinea fowls and Egbunike and Nganga (1999) and Etchu et al. (2013) using chickens.

The overall mean number of live sperm cells (3.84 ± 0.64 x 10^8/ml) which is equivalent to 83.52 ± 0.96 in ordinary percentages and number of dead cells which is 16.48 ± 0.96% is rather high, although percentage motility is comparable to the minimum score of 70-80% viable spermatozoa normally required in a semen sample for insemination. Both LW and I breeds had dead cells below the maximum 20% that results in poor fertility.

The overall mean normal sperm cells (86.71 ± 0.81%) and individual breed values of 88.29 ± 1.08 and 85.13 ± 1.18% for the LW and I breeds respectively, are lower than those reported by Idi (2000) and Etchu et al. (2013) but higher than those (78.52 – 79.58%) reported by Nwagu et al. (1996). Butswat et al. (2001) reported means of 33.4 ± 0.76 and 19.5 ± 0.001 for total abnormal sperms of local black variety and exotic pearl variety of guinea fowls, respectively. Gbadamosi and Egbunike (1999) also obtained means of 10.66 ± 0.24 and 12.80 ± 0.30% for total abnormal sperms of exotic and local cocks, which were higher than the values (3.05 ± 0.47 and 2.38 ± 0.55%) by Idi (2000) for Barred Plymouth Rock and indigenous cocks, respectively. Mean abnormal sperm number in this study for the LW and I breeds (11.96 ± 1.11 Vs 14.36 ± 1.17%) respectively, compares favourably with those of Zahraddeen et al. (2005) who obtained 11.19 ± 0.73 and 13.61 ± 0.73% for abnormal cells among exotic and local turkey semen in Bauchi. Herbert and Acha (1995) attributed spermatozoa abnormalities to deformities or accidents and that radiations could cause both deformities and dead cells. These workers further elucidated that abnormalities may also occur as artefacts caused by the staining procedure.

The levels of daily sperm output (DSO) obtained from males of the two breeds (LW and I) differed significantly. The overall mean DSO (0.70 ± 10.0 x 10^8) and individual breed values of 1.02 ± 0.17 and 0.39 ± 0.05 (x 10^8/ml) for LW and I respectively were lower than the values obtained (0.62 ± 0.11 x 10^9 sperm) by Noirault and Brillard (1999) for large white turkeys and 0.73 ± 0.21 x

10^9 obtained by Etchu et al. (2013) for broiler breeder cocks. Noirault and Brillard (1999) also observed that, following a rest period of 2 days or more, 3 days of daily semen collection are necessary for turkey males to reach their DSO base level of approximately 1.6 -1.9 x 10^9 spermatozoa per ejaculation. Their values were however well above those observed by Cecil et al. (1988) in large turkeys (0.52 x 10^9 sperm per male).

The genotypes showed a positive correlation between DSO and semen motility, total spermatozoa, total live spermatozoa and total live normal spermatozoa. This implies that as one trait increases, the other traits also increase. This corresponds with the findings of McDaniel et al. (1995) who noted that the evaluation of the male chicken for breeding soundness must be based on semen motility and concentration. Daily sperm output could therefore serve as a useful indicator of the quality and quantity of viable semen in turkeys. Positive and significant coefficients were also obtained between semen volume and concentration and between body weight and semen volume. However, there was a negative correlation between semen volume and total live normal spermatozoa. This is an indication that volume may not be a good indicator of semen quality. This finding is in agreement with the results of Oke and Ihemeson (2010), who obtained a negative correlation between semen concentration and semen volume in different chicken genotypes and concluded that volume may not represent an excellent indicator of semen viability and fertility.

Generally, the semen of LW showed better quality over that of I and it is therefore more capable of giving good fertility. Selection of genotype with large testicular size and better semen quality will be good for breeding programme. Foote (1980) and Herbert and Adejumo (1993) attributed these differences to genetic make-up of the breeds as well as pre and post natal growth and development.

Semen microbiology

Virtually all semen samples are contaminated at the time of collection (Almond and Poolperm, 1990). Poultry semen becomes heavily contaminated with bacteria as it issues from the papillae on the wall of the cloaca during collection. Sexton et al. (1980) reported that turkey semen collected by artificial ejaculation contains on the average 1300 x 10^6 bacteria/ml. The effects of bacterial contaminants in semen have been reported (Revell and Glyssop, 1989, Sone, 1982). Some reports indicated that metabolic products, such as endotoxins from some bacteria and fungi appear to have detrimental effects on the survival of sperm. Watson (1990) observed that not only pathogen but, other microflora can have adverse effects on the fertility of semen by the production of toxins and by utilisation of metabolic substrates. For example, *Aspergillus* spp. are known to be associated with the secretion of a toxic fungal metabolite, aflatoxin. Clarke et al. (1987) observed that ingestion of aflatoxin contaminated feed can lead to widespread reproductive abnormalities in male chicken including a reduction in circulating hormonal levels of testosterone. In another experiment, Clarke and Ottinger (1989) reported that the percentage free testosterone in the peripheral circulating blood was significantly higher in aflatoxin-treated male chickens than the control. They however, stressed that this was not due to direct inhibition of the ability of the testicular cells to produce testosterone but rather a change in the rate of clearance of testosterone from the blood. There is also a direct influence of bacteria on fertilisation (conception) especially if the number of bacteria reaching the site of fertilisation in the oviduct results in the step-wise decrease in sperm counts during transit to the oviduct. Regardless of whether or not bacterial contamination reduces semen quality, interferes with fertilisation or causes uterine infection, it is clear that “infected” semen reduces the overall success of an artificial insemination (AI) programme. Several micro-organisms identified in poultry semen include *Staphylococcus albus, Staphylococcus aureus*, *Escherichia coli*, *Proteus* spp., *Hemolytic streptococci* spp., *Diphtheroid bacilli* and *Bacillus* spp. (Sexton et al., 1980). *Enterobacter* spp. were the only bacteria isolated from the two breeds of turkeys used in this study. The use of antibiotics to prolong the survival of spermatozoa and the reduction of bacterial load has been extensively studied (Laing, 1970). *Pseudomonas aeruginosa*, *E. coli* and *Candida stellatoidea* were the isolates reported and of all the Gram positive and negative antibiotics tested on these microbes only Ciproxine, Nobactin, Nalidixic acid, Chloramphenicol, Ampicillin and Gentamycin were found to be effective on one or both microbes. Sexton et al. (1980) also reported that Gentamycin, Kanamyan, Neomycin and Tobramyan were the only antibacterial tested which controlled microbial growth in turkey semen without affecting sperm viability for up to 24 h of storage at 5°C. Sexton (1988a) found higher fertility in semen stored in Gentamycin + Minnesota Turkey Grower Association (MTGA) extender than in MTGA alone. He opined that the beneficial effect of Gentamycin is from a mechanism other than an antibacterial one. He further confirmed that antibiotics in semen extenders act by either chelating metallic cations to slow spermatozoa metabolism, removing toxic cations from solutions or maintaining the shelf life of the extender (Sexton, 1980).

The superior effect of Gentamycin was also confirmed in this study, where only Ciproxine and Gentamycin were susceptible among the Gram negative antibiotics tested on *Enterobacter* spp. It is therefore suggested that the tested antibiotics and many others could be explored further for their use and inclusion in diluents preparation for handling turkey semen in our environment.

Conclusion

From the result of this investigation on the reproductive potentials of the breed of turkeys studied, there is a clear

indication of breed differences in semen characteristics and thus reproductive potentials. The LW breed had better potentials than the indigenous breeds of turkeys in Bauchi, north east Nigeria. Irrespectively of the breed, all the turkey toms used were potentially fertile and as such their semen could be used for any AI programs in inseminating the hens. They will be effective with a guarantee of having fertile eggs which will subsequently hatch to healthy poults, all conditions being equal. The tested antibiotics could be used in the control of bacteria in semen samples of turkey within the study area.

Conflict of interest

The authors declare that they have no conflict of interest.

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Molecular identification and antibiotics resistance genes profile of *Pseudomonas aeruginosa* isolated from Iraqi patients

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Pseudomonas aeruginosa **is one of the leading Gram-negative organisms associated with nosocomial infections. The increasing frequency of multidrug-resistant *P. aeruginosa* (MDRPA) strains affects the efficacious antimicrobial options which are severely limited. In this prospective study, forty two different types of samples collected from patients with multiple types of infections attainted to AL-Kadhyimia Teaching Hospital-Baghdad-Iraq, from February, 2012 to August, 2012. Morphological characteristics, biochemical testing by Vitek 2 Compact for Gram-negative Identification, card 2GN and amplification of species-specific 16SrDNA gene were used to identify *P. aeruginosa*. The antibiotic sensitivity profiles of the strains were determined against ten antibiotics belonging to different classes using the BioMérieux Vitek2 compact system AST card. In order to identify the genes implicated in antimicrobial resistance mechanisms, DNA was extracted from collected samples and resistance genes *PstS, blaOXA-50, blaOXA-2* and *bla IMP-13* were amplified using polymerase chain reaction (PCR). The results showed that there were difference in number of *P. aeruginosa* isolates that were identified using Vitek 2 Compact for Gram-negative Identification, card 2GN (25/42) and 16SrDNA gene(29/42), but it was not statistically significant (*p* value= 1.000). Also, it showed that the highest resistance percentages of strains to tested antibiotics was for Ceftriaxone (96.55%) and the lowest resistant percentages was (17.24%) for Meropenem. Seven strains out of twenty nine (24%) were resistant to all tested antibiotics and were identified as a multidrug resistance strain (MDR). The study detects the presence of *PstS* gene and *blaOXA-50* in (65.5% and 93% of strains, respectively), but it did not identify the presence of *blaOXA-2* or *blaIMP 13* at any of the tested strains of *P. aeruginosa*. There was no significant relationship between the presence of *bla*OXA-50 and resistance to Meropenem, Imipenem, Ceftazidime and Cefepim (*p* value 1.000, 1.000, 0.474, and 0308, respectively). We concluded that Meropenem is the most effective antibiotic and can be considered as the drug of choice against *P. aeruginosa.* This study is the first report presence of *PstS* and *blaOXA50* in *P. aeruginosa* in Iraq. The presence of *bla*OXA-50 is important in order to identify and track the spread of multidrug-resistant *P. aeruginosa* clones since *bla*OXA-50 may be potential clonality marker.**

Key words: *Pseudomonas aeruginosa*, molecular identification, antibiotic resistance genes.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen. Its infections in hospitals mainly affect the patients in intensive care units and those having catheterization, burn, and/or chronic illnesses (Yetkin et al., 2006). The ability of *P. aeruginosa* to survive on minimal nutritional requirements and to tolerate a variety of physical conditions has allowed this organism to persist in both community and hospital settings (Lister et al., 2009). Thou, It infects healthy tissues rarely but when defenses are compromised, it can infect different tissues. This explains why most infections are nosocomial (Mesaros et al., 2007). *P. aeruginosa* characterized by inherent resistances to a wide variety of antimicrobials. Its intrinsic resistance to many antimicrobial agents and its ability to develop multidrug resistance and mutational acquired resistance to antibiotics through chromosomal mutations imposes a serious therapeutic problem (Gales et al., 2001; Gorgani, 2009; Al-Grawi, 2011). A number of antimicrobial agents, including several Beta–lactams are active against *P. aeruginosa*. Extended-spectrum Penicillins, often used to treat infections caused by this bacterium.

Although most Cephalosporins are not active against *P. aeruginosa,* Ceftazidime, a third generation agent, and Cefepime, a fourth-generation agent, have excellent and about equivalent activity. Of Carbapenems, a class of broad-spectrum β-lactam antibiotics, Meropenem has slightly greater activity against *P. aeruginosa* (Ayalew et al., 2003; Shah and Narang, 2005; Baldwin et al., 2008). Of the fluoroquinolones agents, Ciprofloxacin is the most active against *P. aeruginosa*. Finally, the Aminoglycosides have been mainstays in the treatment of these infections (Hauser and Sriram, 2005; Katzung et al., 2009). However, *P. aeruginosa* adaptive ability causes difficulties for the sensitivity of microbial identification methods and it has become necessary to develop genotype-based characterization systems capable of accurately identifying these bacteria despite any phenoltypic modifications. So, molecular identification eliminates the problem of variable phenotype and allows for more accurate identification of bacteria (Drancourt et al., 2000).16S rDNA genes are highly conserved among all organisms and they possess various unique species-specific regions that allow for bacterial identification. Polymerase chain reaction (PCR) is highly sensitive, specific and rapid method which vastly improved the detection of *P. aeruginosa* especially when using species-specific primer for 16SrDNA (Spilker et al., 2004).

In the present study, we used two methods to identify *P. aeruginosa,* Vitek2 system and molecular technique using PCR to amplified species-specific 16SrDNA. Also, we study the relationships between the presence of resistance genes *PstS, blaOXA-50, blaOXA-2 and blaIMP-13* and the sensitivity to ten antibiotics.

Table 1. Distribution of isolates according to their sources.

Bacteria	No. of isolates	Source
Pseudomonas aeruginosa	13	Wound
	2	Eye
	3	Urine
	6	Ear
	1	CSF
	3	Burn
	1	Sputum
Burkholderiacepacia	6	Urine
Escherichia coli	2	Stool
Enterobacter	2	Urine
Klebsiella pneumonia	2	Wound
Proteus mirabilis	1	Urine

MATERIALS AND METHODS

Collection of samples

Forty two different types of samples were collected from patients with multiple types of infections attainted to AL-Kadhyimia Teaching Hospital from February 2012 to August 2012, Table 1.

Identification of *P. aeruginosa*

Collected samples were cultured on MacConkey agar and incubated overnight at 37°C. The selected colonies were cultured on 0.03% cetrimide agar and King B agar. Gram stain was done to examine cell shape and Gram reaction (Atlas et al., 1995). Also, Oxidase test was done (Mcfadden, 2000). The isolates were identified with Vitek 2 Compact system for Gram-Negative Identification, card 2GN (bioMérieux-France). Antibiotics susceptibility was examined using the BioMérieux VITEK2 compact system AST card (bioMérieux-France) according to manufacturer instructions, Table 2.

Molecular study

Genomic DNA was extracted from *P. aeruginosa* strains using DNA extraction kit (Promega, USA) following manufacture instructions. The sequences of primer sets used in PCR to amplify species-specific 16S rDNA gene for *P. aeruginosa* and resistance genes were shown in Table 3 (Spilker et al., 2004; Carciunas et al., 2010).

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Table 2. Vitek 2 Gram negative susceptibility card 30 contents.

Antibiotic	Concentration	Calling range	
		≤	≥
Amikacin	8, 16, 64	2	64
Gentamicin	4, 16, 32	1	16
Tobramycin	8, 16, 64	1	16
Cefepime	2, 8, 16, 32	1	64
Ceftazidime	1, 2, 8, 32	1	64
Ceftriaxone	1, 2, 8, 32	1	64
Ciprofloxacin	0.5, 2, 4	0.25	4
Levofloxacin	0.25, 0.5, 2, 8	0.12	8
Meropenem	0.5, 4, 16	0.25	16
Imipenem	2, 4, 16	1	16

Table 3. The sequences and molecular size of primers used in PCR reaction.

Gene	Sequence of forward Primer(5´- 3´)	Sequence of reverse primer (5´- 3´)	Product size(bp)
PstS	GGCTTTCGAGCAGAAGTACG	ATGTAGCCGTCCTTGACCAC	606
blaOXA-50	GAAAGGCACCTTCGTCCTCTAC	CAGAAAGTGGGTCTGTTCCATC	400
blaOXA-2	ATACACTTTTTGCACTTGATGCAG	TGAAAAGATCATCCATTCTGTTTG	510
IMP-13	AGACGCCTATCTAATTGACACTCC	CCACTAGGTTATCTTGAGTGTGACC	311
16S rDNA *of P. aeruginosa*	GGGGGATCTTCGGACCTCA	TCCTTAGAGTGCCCACCCG	956

Briefly, five master mixes (each of 25 µl per reaction) were prepared, one for each gene as in the following: 1X of 5XPCR buffer (Promega, USA), 200 µm of dNTPs (Promega,USA), 25 pmol of each primer (Alpha, USA), 1 U/reaction of Green Go Taq DNA polymerase (Promega,USA). The above components were mixed well by pipetting several times after each addition. Two microliter (equivalent to 100ng) of DNA was added for each reaction tube, except the no template control tube. DNA extracted from *Escherichia coli, Burkholderia cepacia, Enterobacter* and *Klebsiella pneumonia* were used as *P. aeruginosa* negative controls in testing the specify of primer set for amplifying 16S rDNA gene specific for *P. aeruginosa*. PCR reaction tubes were transferred into thermal cycler (eppendroff, Germany) that was programmed as following: 94◦C for 2 mints (X1), (92°C for 1 mint, 56°C (or 55°C) for 1 mint, 72°C for 1 mint) (X30) and final extension at 72°C for 10 mints. Amplified products were electrophoresed on 1.5% agarose for 90 mints at 4 V/cm.

Statistical analysis

Data were analyzed using SPSS version 16 and Microsoft Office Excel 2007. Nominal data were expressed as number and percent. Fischer Exact test was used for comparison of frequency. *P*-value less than 0.05 were considered significant.

RESULTS

Isolation and identification of *P. aeruginosa*

This study included 29 *P. aeruginosa* strains isolated from different clinical samples.The strains were identified using a variety of techniques, which are morphological characteristics; biochemical testing using automated Vitek2 GN, pigment production, and molecular technique**.** The identifications probabilities that was performed using the commercially available identification Vitek 2 GN card were ranged from 86 to 99%.

In this study, 16S rDNA species-specific primers for *P. aeruginosa* were used. Twenty nine strains identified as P*. aeruginosa* after amplification of 16S rDNA genes using PCR technique (Figure 1).

Antimicrobial sensitivity

The results of antibiotic sensitivity tests among *P. aeruginosa* strains using BioMérieux VITEK2 compact system AST card were shown in Table 4. The percentage of resistant strains to each antibiotic shown in Figure 2. Twenty eight strains (96.5%) were found to be resistant to ceftriaxone, 8 (27.59%) strains resistant to ceftazidime and gentamicin, 7 (24.14%) strains resistant to cefepim, imipenem, amikacin, tobramycin, ciprofloxacin and levofloxacin and 5 (17.24%) strains were resistant to meropenem. According to that, seven (24%) strains were considered as MDR.

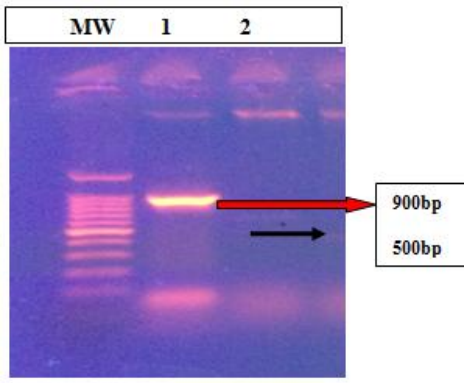

Figure 1. Agarose gel electrophoresis of amplified products of 16SrDNA gene of *P. aeruginosa*. Lane MW: molecular weight ladder of 100bp. Lane 1: amplified products of 16SrDNA gene (956bp), Lane 2: no template control.

Occurrences of *PstS*, *blaoxa50*, *blaoxa2* and *imp13*, and their relation with sensitivity to meropenem, imipenem, ceftazidime and cefepime

The results of amplification of common sequence of *psts, blaoxa50, blaoxa2* and *imp13* in relation to sensitivity patterns of meropenem, imipenem, ceftazidime and cefepime were shown in Figures 3, 4, 5 and 6.

Figure 7 show the results of agarose gel electrophoresis of amplification of *psts* and *blaoxa5*0. Amplification of common sequence of *blaoxa2* gene and *imp13 gene* revealed that all isolates were negative. In this study there were no significant relationship between *psts and bla*OXA-50 presence and resistance to meropenem, imipenem, ceftazidime and cefepim (*P* value 0.281, 1.000, 0.375, 0.375 and 1.000, 1.000, 0.474, 0308, respectively).

DISCUSSION

Isolation and identification of *P. aeruginosa*

In disease process in which delay in the initiation of appropriate therapy has significant consequences for patient outcomes, diagnostic tests such as PCR offer more rapid and sensitive results compared with traditional phenotypic laboratory methods. It was shown that when this rapid test is used, there is significant reduction in the time to initiating effective antimicrobial therapy, decreesed mortality and decreased hospital costs (Goff et al., 2012).

In this study, from Forty Two isolates, 25 isolates were identified as *P. aeruginosa* using Vitek 2 system. The correct identification rates of *P. aeruginosa* using this automated technique were 90.7%. Results from other investigators indicate that the Vitek ID-GNB cards correctly identified 85.3 to 100% of *P. aeruginosa* strains (Funke et al., 1998; Jossart and Courcol, 1999). Joyanes et al., tested 146 routinely isolated strains with the Vitek 2 system and ID-GNB cards and found correct identification rates of 91.6% (Joyanes et al., 2001). Using the same vitek identification card, Ines et al. (2009) found that correct identification rates of *P. aeruginosa* were 90.1%.

Molecular identification was done using primers targeted the variable regions in the 16S rDNA gene, which is a sequence offered a useful method for the identification of bacterial genus- or species (Spilker et al., 2004; Drancourt et al., 2000). From Forty Two isolates, 29 isolates were identified as *P. aeruginosa* using PCR. Spilker et al. (2004) tested 42 culture collection strains (including 14 *P. aeruginosa* strains and 28 strains representing 16 other closely related *Pseudomonas* species) and 43 strains that had been previously identified as belonging to 28 non-pseudomonal species also recovered from cystic fibrosis patient sputum. Based on these 85 strains, the specificity and sensitivity of used 16S ribosomal DNA (rDNA) sequence data to design PCR assays were 100%.

This variation that occurred may be due to the fact that the phenotypic test systems have potential inherent problems, example, (i) not all strains within a given species may exhibit a particular characteristic, (ii) the same strain may give different results upon repeated testing, and (iii) the corresponding databases are limited (Bosshard et al., 2006). The difference in the results of Vitek2 and those found by 16S rDNA for identification of *P. aeruginosa* isolates was not statistically significant (*P* value = 1.000). However, the rate of incorrect identifications showed that there was a need for a more precise method such as molecular methods of identification (Burd, 2010).

Distribution of *P. aeruginosa* isolates according to its sources

The incident of infection with *P. aeruginosa* varies according to the site of infection**.** It affect when introduced into areas devoid of normal defenses example, when membranes and skin are disrupted by direct tissue damage, when intravenous or urinary catheter are used (Cao et al., 2004).

In this regard, the isolates distributions in the current study (Table 1) were in agreement with that reported by local and global studies. In a study conducted by R'auf (2003), the highest percentage of *P. aeruginosa* were recorded among burn infections followed by wound (41.7%) and ear infection (28%) while Miteb (2006) in Najaf found highest percentages of *P. aeruginosa* were obtained from wound (44%) followed by burn (30%), urine (20.5%), ear (4.2%). Al-Derzi (2012) in the North of Iraq (Mosul and Duhok) revealed that the most common *P. aeruginosa* isolates come from purulent specimens

Table 4. Results of Vitek 2 antibiotic sensitivity test.

Strain number	Sample type	Antibiotic									
		MEP	IMP	CAZ	CEF	CRO	GEN	AK	TOB	CIP	LEV
PS 1	Ear swab	R	R	R	I	R	R	R	R	R	R
PS 2	Ear swab	R	R	R	R	R	R	R	R	R	R
PS 3	Ear swab	R	R	R	R	R	R	R	R	R	R
PS 4	Ear swab	R	R	R	R	R	R	R	R	R	R
PS 5	Wound	R	R	R	R	R	R	R	R	R	R
PS 6	Burn	I	R	R	R	R	R	R	R	R	R
PS 7	Urine	S	R	S	S	R	S	S	S	S	S
PS 8	Wound	S	I	I	I	R	S	S	S	S	S
PS 9	Urine	S	S	S	S	R	S	S	S	S	S
PS 10	sputum	S	S	S	S	R	S	S	S	S	S
PS 11	Ear swab	S	S	R	R	R	S	S	S	S	I
PS 12	Burn	S	S	S	S	R	S	S	S	S	S
PS 13	Urine	S	S	S	S	R	S	S	S	S	S
PS 14	Burn	S	S	R	R	R	R	R	R	R	R
PS 15	CSF	S	S	S	S	R	S	S	S	S	S
PS 16	Ear swab	S	S	S	I	R	R	I	S	S	I
PS 17	Lens	S	S	S	S	R	S	S	S	S	S
PS 18	wound	S	S	S	S	R	S	S	S	S	S
PS 19	wound	S	S	S	S	R	S	S	S	S	S
PS 20	Wound	S	S	S	S	R	I	S	S	S	S
PS 21	Wound	S	S	I	S	R	S	S	S	S	S
PS 22	Wound	S	S	S	S	R	S	S	S	S	S
PS 23	Lens	S	S	S	S	R	S	S	S	S	S
PS 24	Wound	S	S	S	I	R	S	S	S	S	S
PS 25	Wound	S	S	s	S	R	S	S	S	S	S
Ps26	Wound	s	s	s	s	R	S	s	S	S	s
Ps27	Wound	S	s	s	s	R	s	s	S	S	s
Ps28	Wound	S	s	s	s	I	I	s	S	S	s
Ps29	wound	s	s	s	S	R	S	S	S	S	s

MEP: Meropenem, IMP: Imipenem; CAZ: Ceftazidime; CEF: Cefepime; CRO: Ceftriaxone; AK: Amikacin; TOB: Tobramycin; GEN: Gentamicin; CIP: Ciprofloxacin; LEV: Levofloxacin.

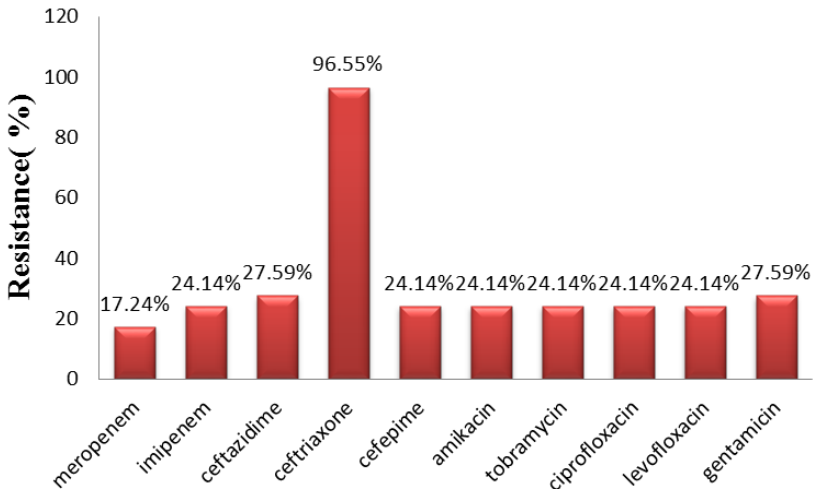

Figure 2. Percentage of *P. aeruginosa* resistant isolates to ten antibiotics.

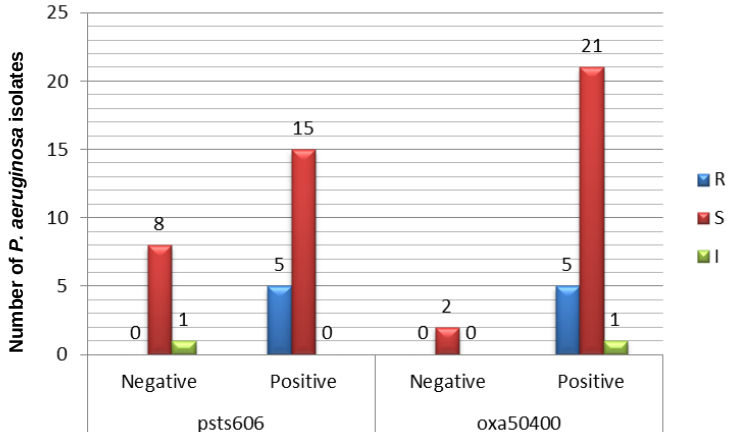

Figure 3. Presence of *psts* and *blaoxa 50* and its relation with sensitivity to meropenem.

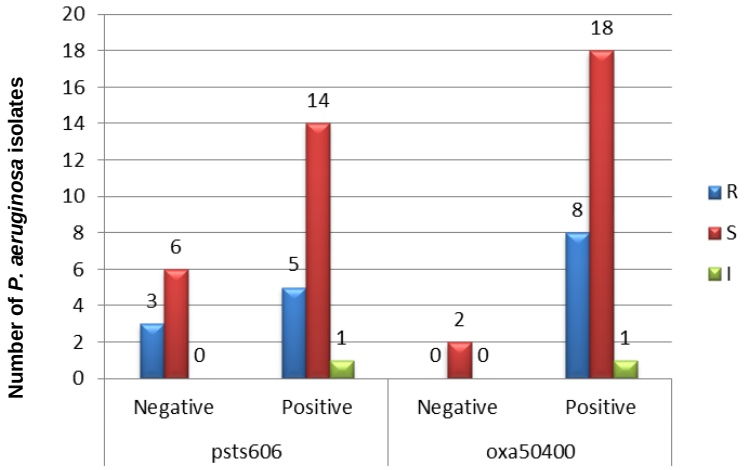

Figure 4. Presence of *psts* and blaoxa *50* and its relation with sensitivity to imipenem

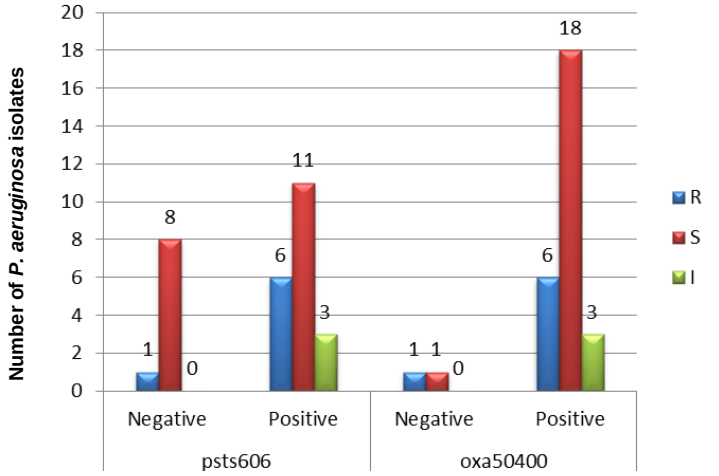

Figure 5. Presence of *psts* and *blaoxa 50* and its relation with sensitivity to ceftazidime.

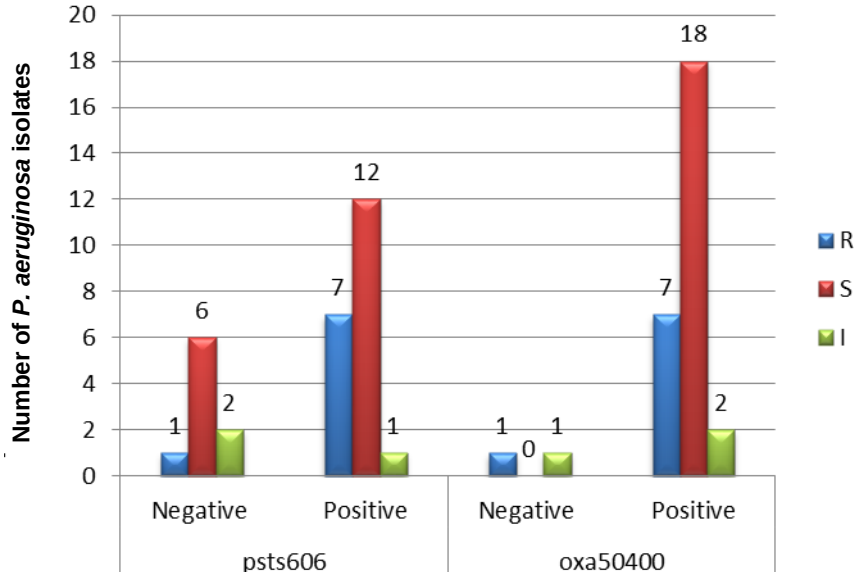

Figure 6. Presence of *psts* and *blaoxa 50* and its relation with sensitivity to cefepime.

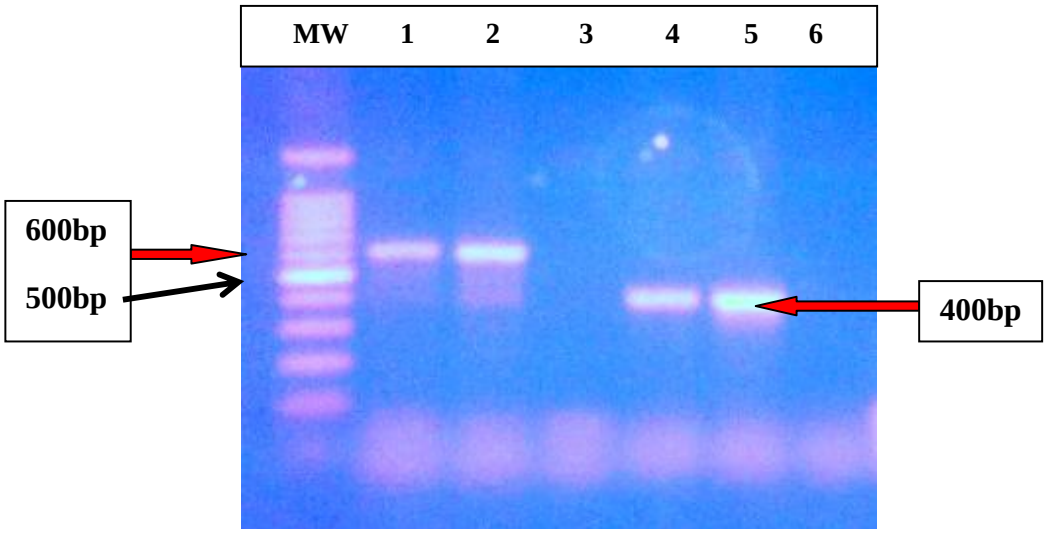

Figure 7. Agarose gel electrophoresis of amplified products of *psts* and *blaoxa50* of *P. aeruginosa*. Lane MW: molecular weight ladder of 100bp. Lane 1, 2: amplified products of *psts* (606 bp). Lane 3, 6: no template control. Lane 4, 5: amplified products of *blaoxa50* (400 bp).

collected from skin wounds and burns (44.4%) followed by isolates from urine (31.8%) and ear discharge specimens (12.4%). Also, the study of Hasan et al. (2012) in Kurdistan region of Iraq found highest percentage of *P. aeruginosa* was obtained from burn samples (%10.9) whereas the lowest percentage were obtained from Otitis samples (%1.81). Manhal indicated that *P. aeruginosa* constituted 7.3 % of hospital contamination in Iraq (Hassan et al., 2012; Manhal, 2006; Rauf, 2003; Miteb, 2006; Al-Derzi, 2012).

The discrepancy in the percentages of *P. aeruginosa* isolated from urine samples, septic surgical wound exudates and burn samples could be attributed to the difference in numbers of clinical specimens investigated in different studies. It is important to say that there was no burns ward in the hospital during sample collection period.

Antibiotic resistance of *P. aeruginosa*

Molecular methods can be used to identify antimicrobial-resistant organisms directly in a variety of clinical sam-

ples to optimize therapy early in the course of infectious illnesses (Tenover, 2010). Moreover, it enables the detection of resistance determinants in viable but not cultivable microorganisms (Volkmann et al., 2004).

In this study (Table 3), Meropenm MIC ranged from ≤0.25 to ≥16 µg/ml and it appeared to have the lowest resistance percent (17.24%) among tested antibiotics. This may be contributed to meropenem being a newly introduce drug in Iraq and there is certain prescription criteria in hospitals that it should not be use until it's the only antibiotic available and because it is expensive, this limits its use in private sector but the appearance of meropenem resistant strain despite previously mentioned restrictions is a great concern because it's considered as one of last-line agents and its resistance puts to threat its clinical effectiveness in treating MDR infections.

The activity of meropenem against *P. aeruginosa* is comparable to that of imipenem in this study. Imipenem resistance was 24.4% with MIC ≤1 to ≥16µg/ml. Lautenbach et al. (2010) referred to it as the greater intrinsic activity of meropenem over imipenem which may be explained, at least in part, by improved stability against common serine β-lactamases. In cases when porin synthesis is suppressed and Imipenem resistance emerges, Meropenem retains its activity on *P. aeruginosa,* suggesting the existence of another undefined route of Meropenem transport through the outer membrane.

We found that there were about 27.59% of *P. aeruginosa* isolates resistant to Ceftazidime MIC ≤1- ≥64 µg/ml and about 24.41% of them were resistant to cefepime MIC ≤1 to ≥64µg/ml. The highest resistance percent was for ceftriaxone (96.55%) MIC 16 to ≥64 µg/ml. Cefepime is less susceptible to destruction by β-lactamases than earlier generation Cephalosporins and thus remains active against bacterial mutants that produce such enzymes. In addition, cefepime may require Gram-negative bacteria to have more than one mutation to become resistant and it has a lower affinity than other Cephalosporins for binding many of the common bacterial β-lactamases (Jazani et al., 2010). The low susceptibility percentage of Ceftriaxone may be due to its extensive use in clinical practice in Iraq. Excessive use of broad-spectrum antibiotics in hospitals has led to the emergence of highly resistant strains of *P. aeruginosa* (Al-Grawi, 2011).

Imipenem and Ceftriaxone antibiotic sensitivity pattern were close to the antibiotic sensitivity pattern of Rabea , which found that resistance of burn isolated pseudomonas isolates in Najaf were 20%, 100% respectively but far from other antibiotics sensitivity pattern were (100%) found to be resistant to Ceftazidime, Cefepim and 85% of isolates were found to be resistant to Gentamicin and 75 of isolates were found to be resistant to Tobramycin and Ciprofloxacin; 60 of isolates were found to be resistant to Levofloxacin and Amikacin (Rabea, 2010).

Al-Derzi pattern of resistance revealed that Amikacin had the highest sensitivity (89.7%) followed by Imipenem, Tobramycin, Ceftazidim, Ciprofloxacin and Gentamicin (85.6, 84.1, 70.1, 76.1 and 79.1%, respectively) (Rauf, 2003), while Hassan et al. (2012) showed that all isolates were completely susceptible to Ciprofloxacin, Imipenin and all isolates were completely resistance to Gentamicin and 13.95% of the isolates were resistant to Amikacin (Hassan et al., 2012; Miteb, 2006).

Binnet and Uraz (2008) in Turkey found that the susceptibility of *P. aeruginosa* strains were as follows: 55% Amikacin, 68% Cefepim, 73% Ceftazidim, 76% Ciproflaxacin, 37% Gentamicin, 84% Meropenem, 47% Tobramycin and 84% Imipenem. Jazani et al. (2010) in Iran determined the rates of resistance to antibiotics as follows: Gentamicin 73%, Ceftazidime 83%, Tobramycin 84%, Amikacin 40%, Imipenem 27%, Ciprofloxacin 65% and 75.4% of isolates were resistant to Cefepime. In a retrospective study was carried out of Gram-negative isolate in Saudia Arabia, they found that *P. aeruginosa* susceptibility significantly declined after 2007, especially for carbapenem (66% in 2004 to 26% in 2009), ceftazidime (69% in 2004 to 44% in 2009) and ciprofloxacin (67% to 49%) (Al-Johani et al., 2010). Resistance rates (%) to fluoroquinolones, third generation cephalosporins and carbapenems in invasive isolates of *P. aeruginosa* from various European countries, according to the data reported by the EARSS surveillance system for year 2005 (EARSS, 2006) were as follows: France (27, 9, 140), Germany (22, 11, 24), Spain (14, 6, 17), Sweden (6, 5, 18) and UK (8, 3, 9) (EARSS, 2006).

In this study, MDR represent 24% of tested isolates. The definition of MDR organisms can vary from study to study; this study used the USA definition MDR *P. aeruginosa* as those strains that are resistant to all of the agents in two or more classes of antibiotic defined as (1) beta-lactam (including carbapenem agents), (2) aminoglycoside and (3) quinolone agents.

Occurrence of *PstS*

In this study, *PstS* was identified in 65.5% of all tested isolates. It is presents in 85.7% MDR and 59% of non-MDR isolates. Craciunas et al. (2010) in Romania found that *PstS* gene is present in almost all tested isolates.

PstS proteins are the cell-bound phosphate-binding elements of the ubiquitous bacterial ABC phosphate uptake mechanisms. PstS proteins are induced by phosphate deprivation in *Pseudomonas* non-fermenters species (Morales et al., 2007). The presence of this periplasmic phosphate binding protein (PstS) confers a highly virulent phenotype of MDR isolates of *P. aeruginosa*. Also the development of multi-drug resistance in *P. aeruginosa* clinical isolates might be related to the overproduction of *PstS* proteins (Zaborina et al., 2008).

Occurrences of *blaOXA-50* and *blaOXA-2*

The *bla*OXA-50 gene is naturally occurring in that *P. aeruginosa* species since that *bla*OXA-50 may be another potential clonality marker for *P. aeruginosa*. This aspect is important in order to identify and track the spread of multidrug-resistant *P.aeruginosa* (Aktas et al., 2005). The prevalence of *bla*OXA-50 in clinical strains of *P. aeruginosa* raises an alarm, as the high frequency of horizontal gene transfer among bacteria may likely introduce Class D β-lactamases to other co-inhabiting bacteria species (Girlich et al., 2004; Kong et al., 2005).

*bla*OXA-2 was the first class D β–lactamases to be discovered (Dale et al., 1985). The first characterized class D β-lactamases was referred to as oxacillinases because they commonly hydrolyse the isoxazolyl penicillins, oxacillin and cloxacillin (Sun et al., 2003). *OXA-2* has evolved variants, which confer resistance to extended-spectrum cephalosporins including ceftazidime and cefotaxime. These variants arise from single amino acid substitutions, which alter the substrate specificity of the enzyme (Paetzel et al., 2000).

A *bla*OXA-50 gene was identified in 93% of the *P. aeruginosa* strains tested and there were lack in presence of *blaoxa* -2. *bla*oxa-50 and *bla*oxa-2genes were found mainly in *P. aeruginosa* isolates from Turkey (Aktas et al., 2005; Kolayli et al., 2005), France (Bert et al., 2002) and Romania (Carciunas et al., 2010). In this study there were no significant relationship between *bla*OXA-50 presence and resistance to meropenem, imipenem, ceftazidime and cefepim (*P* value 1.000, 1.000, 0.474, and 0308, respectively). This may indicate the presence of other resistance mechanisms such isolates may express other β-lactam resistance mechanisms like other types of β-lactamases such as ESBLs or express MexAB-OprM efflux pump, or may exist simultaneously or in various combinations.

Occurrence of *IMP13*

In this study, the results of PCR amplification showed the lack of *IMP13* in tested strains. That was similar to what was recommended by Craciunas which also did not find *IMP13* among its isolates (Carciunas et al., 2010). IMP-type metallo-B-lactamases (MBLs) were the first acquired MBLs detected in Gram-negative pathogens, in the early 1990s, and is among the most relevant due to their worldwide distribution (Queenan and Bush, 2007). Since MBL production may confer resistance to virtually all clinically available β-lactams, the continued spread of MBL is a major clinical concern (Walsh, 2003). Several IMP-type variants have been described (Bebrone, 2007). *IMP-13* was first detected in clinical isolates of *P. aeruginosa* from Italy (Toleman et al., 2003) where *IMP-13* has become a widespread carbapenem resistance determinant, even involved in relatively large outbreaks (Santella et al., 2010). *IMP-13* was also occasionally detected in *P. aeruginosa* isolates from other European countries, including Romania (Mereuta et al., 2007), France (Cuzon et al., 2008) and Austria (Duljasz et al., 2009).

Conclusion

The molecular method (16SrDNA gene amplification) offered a useful method for the identification of bacteria used for genus - species - level identification. The isolates included in this study showed the presence of *Psts* and *blaoxa50* genes and lack the presence of *blaoxa2* and *IMP13*. More efforts are needed to control the spread of carbapenem resistant *P. aeruginosa* strains.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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