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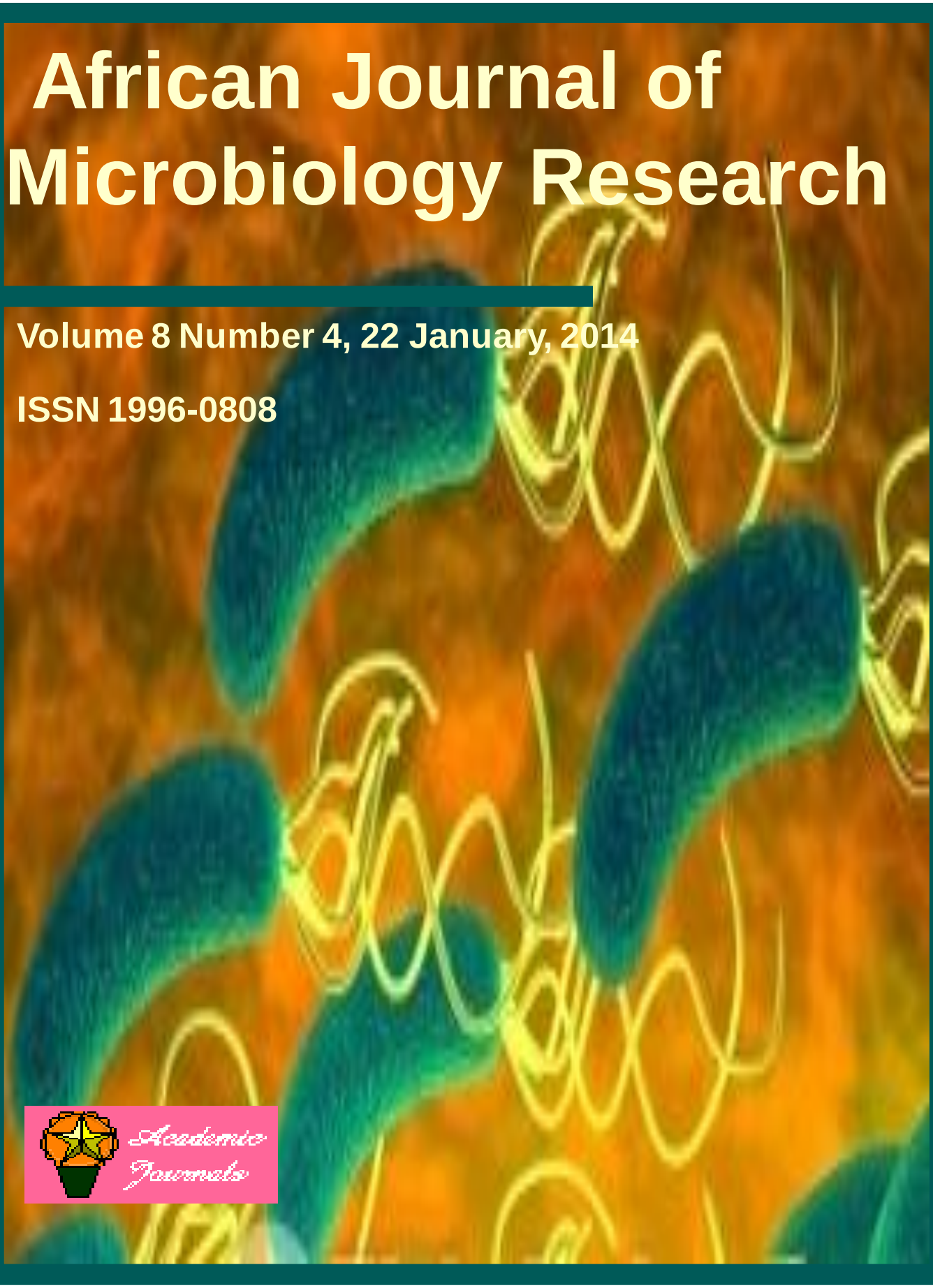

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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: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

Full Length Research Paper

Multi-drug resistant *Klebsiella pneumoniae* causing urinary tract infections in children in Pakistan

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Multi-drug resistant (MDR) *Klebsiella pneumoniae* has been associated with different types of infections and the most important aspect is the emergence of MDR strains particularly in hospitalized children. They have the proficiency to cause bacteremia, septicemia and urinary tract infections (UTI). The objective of this study was to determine the susceptibility of MDR *K. pneumoniae* causing UTI in children. One thousand and fifteen (1015) urine samples were collected aseptically. Specimens were cultured on blood agar, MacConkey agar and cysteine lactose electrolyte deficient (CLED) agar. Antimicrobial susceptibility was determined using Kirby-Bauer disc diffusion method as per CLSI 2011 guidelines. Of the 1015 urine specimens, 230 (22.6%) were positive for bacterial growth. Out of these positive cultures predominantly Gram-negative rods (90%) were isolated and major pathogens were *K. pneumoniae* (40%) and *Escherichia coli* (33%). Antimicrobial susceptibility pattern of *K. pneumoniae* showed that more than 70% of these pathogens were resistant to cephalosporins, 69% to ciprofloxacin and amoxicillin-clavulanic acid and 63% to norfloxacin and nalidixic acid while most effective drugs were pipracillin-tazobactam and meropenem. This study concludes that MDR-*K. pneumoniae* is a great threat particularly in children in our locality.

Key words: Multi-drug resistant (MDR), *Klebsiella pneumoniae*, children, antimicrobial resistance (AMR).

INTRODUCTION

Urinary tract infections (UTIs) have become one of the most common disease encounters in clinical practice. There is an estimation that over 150 million UTIs cases occur worldwide annually (Gobernado et al., 2007). This is associated with a high risk of morbidity, mortality, extra financial budget and even, fatal consequences particularly in children (Hasan et al., 2007). UTIs are mainly caused by Gram negative rods (GNR) which account for 80 to 85% and the leading causative organisms are *Escherichia coli* (75 to 95%) and *Klebsiella pneumoniae* (Tanvir et al., 2012).

K. pneumoniae are very often isolated in hospital set up and a significant proportion being multidrug resistant (MDR) that become a formidable challenge nowadays (Rampure et al., 2013). The ability of MDR *K. pneumoniae* to easily spread makes this bacterium an important nosocomial pathogen (Ramirez et al., 2012). These pathogens are also responsible for an important cause of children infections such as bacteremia, septicemia and urinary tract infections (UTIs) (Manisha and Pratibha, 2012). Treatment of UTI patients infected with *K. pneumoniae* has become more difficult due to increasing antimicrobial resistance which severely limits the therapeutic options. These strains have typically been resistant to two or more different classes of antibiotics such as β-lactams, β-lactamase inhibitors, aminoglycosides

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Table 1. Frequency of urinary isolates (n = 230) from UTI patients.

Organism (n = 230)	Frequency	(%)
Gram-negative rods (n = 209)		
Klebsiella pneumoniae	92	40
Escherichia coli	75	32.6
Pseudomonas aeruginosa	18	7.8
Acinetobacter spp.	15	6.5
Citrobacter spp.	5	2.1
Proteus mirabilis	4	1.7
Gram-positive cocci (n = 21)		
Enterococcus faecalis	13	5.6
Coagulase negative staphylococci (CoNS)	5	2.2
Staphylococcus aureus	3	1.3

and quinolones. This is believed that the best empirical therapy to treat mild to moderate infections due to MDR *K. pneumoniae* would be the ertapenem (Brink, 2008). This is expected that MDR *K. pneumoniae* will become more and more resistant to antimicrobials with the passage of time because of the generation of their new mutant strains (Khameneh and Afshar, 2009). Therefore, we design this study to determine the frequency and antimicrobial resistant pattern in clinical isolates of *K. pneumoniae* in a tertiary care hospital, Lahore.

MATERIALS AND METHODS

Prior to start of the study, permission was taken from ethical review committee, School of Allied Health Sciences, tertiary care hospital Lahore, Pakistan.

Study population

A total of 1015 urine specimens suspected for UTIs were collected from children (age: 0 - 12 years) in a tertiary care hospital Lahore, Pakistan. Mid stream urine samples were collected aseptically.

Identification of isolates

Urine specimens were cultured on blood agar, MacConkey agar and cysteine lactose electrolyte deficient (CLED) agar and incubated at 35°C for 24 h. The isolates were preliminary identified on the basis of morphology and cultural characteristics. Gram-positive isolates were biochemically identified by catalase, slide and tube coagulase and DNAse test whereas, Gram-negative isolates were biochemically identified by cytochrome oxidase and confirmed by API 20E and 20NE (BioMerieux France).

Antimicrobial susceptibility testing

Antimicrobial susceptibility of *K. pneumoniae* isolates was performed by Kirby-Bauer disk diffusion method using Mueller-Hinton agar (Oxoid UK), according to Clinical Laboratory Standards Institute (CLSI) 2011 guidelines. The plates were prepared and incubated at 35°C for 24 h. Implanted antibiotics were amikacin (30 µg), amoxicillin-clavulanic acid (20 µg/10 µg), cefotaxime (30 µg), nalidixic acid (30 µg), cefuroxime (30 µg), piperacillin-tazobactam (100/10 µg), nitrofurantoin (300 µg), norfloxacin (10 µg), cefixime (5 µg), ceftazidime (30 µg), ciprofloxacin (5 µg) and meropenem (10 µg).The interpretation of susceptibility results were done as per CLSI 2011 guidelines (Wikler et al., 2009). Statistical analysis was done using SPSS 16.0.

RESULTS

Of 1015 urine specimens, 230 (22.6%) were positive for bacterial growth. Out of these positive cultures (n = 230), 209 GNR and 21 GPC were isolated. Among GNR major pathogens were *K. pneumoniae* (n = 92) followed by *E. coli* (n = 75), *P. aeruginosa* (n = 18) and *Acinetobacter spp* (n=15) whereas among Gram positive Cocci (GPC), *Enterococcus faecalis* (n = 13) was the main pathogen (Table 1).

Antimicrobial susceptibility pattern showed that more than 70% of *K. pneumoniae* were resistant to cephalosporins, 69% to ciprofloxacin and amoxicillin-clavulanic acid and 66% to nalidixic acid and norfloxacin. Most effective drugs were piperacillin-tazobactam followed by meropenem that showed resistance of 5.4 and 14.1%, respectively (Figure 1).

DISCUSSION

Organisms causing UTIs particularly in children are becoming a public health problem. In present study, 22.6% (n=230) urine specimens were positive for significant bacterial growth which is in accordance with previous studies (Ahmad, 2013; Chaudhary et al., 2013). However few data suggested variable results ranging from 9 to 34% of urine positive cultures (Oh et al., 2013; Jahanzeb et al., 2008). This difference could be due to the difference in samples size, handling and processing of sample techniques.

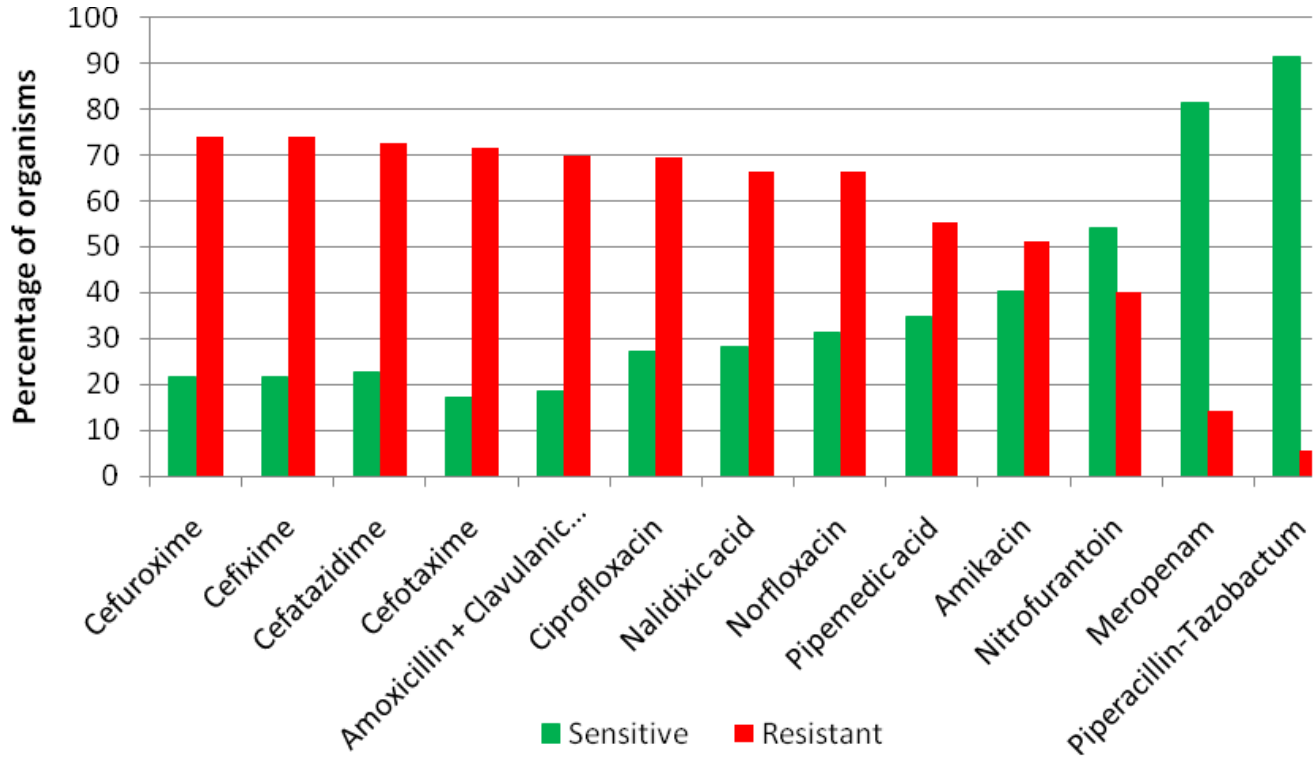

Figure 1. Overall percent susceptibility pattern of *K. pneumoniae* in which high drug resistance was observed against cephalosporins, Amoxicillin+clavulanic acid and ciprofloxacin whereas most effective drug was piperacillin-tazobactam.

Overall, GNR are the commonest cause of UTIs as compare to GPC. In this study, 91% of GNR were isolated which are comparable with previous studies conducted in Iran (90.3%), Turkey (89%) and Australia (96%) (Ayazi et al., 2010; Mehr et al., 2004; Arslan et al., 2002). This has been identified in earlier studies that *Enterobacteriaceae* are the predominant pathogens in UTI patients. These organisms are part of gut normal flora and individuals can easily get infected with poor personal hygiene (Afsharpaiman et al., 2012). Overall, *E. coli* still remains the most frequent isolated pathogens in UTIs but in the present study, the predominant pathogen was *K. pneumoniae* (40%). These results are in agreement with studies conducted in the Children hospital Lahore, Pakistan and Turkey (Ejaz et al., 2006; Biyikli et al., 2004). Contrary to this study, various other data also documented that *E. coli* was the predominant pathogen in UTIs patients (Al-Momani, 2006; Qureshi, 2005). In the current study, *K. pneumoniae* showed high resistance against commonly used antibiotics (cephalosporins, ciprofloxacin, amoxicillin- clavulanic acid and amikacin). These findings are almost in accordance with previous studies conducted in Iran, Pakistan, Mexico and India that reported MDR *K. pneumoniae* (Rampure et al., 2013; Langarizadeh et al., 2011; Ullah et al., 2009). Contrary few studies from Turkey, Korea and Iran showed low to moderate resistance (Yoon et al., 2011; Jalalpoor, 2011; Senel et al., 2010). Spread of MDR- *K. pneumoniae* is associated with inappropriate infections control practices. Various other factors includes contaminated intravenous catheters, environment surfaces and colonized hands of health care staff. Substandard and disinfection practices are also common. Another major factor to acquire resistance in our setup is the irrational use of empirical therapy which is not according to WHO criteria (Hannan et al., 2013).

It is concluded that MDR *K. pneumoniae* play a crucial role in spreading UTIs and these pathogens are also extended from the hospital to community. Now, it is the need of the hour to improve infections control practices, avoid irrational use of antibiotics and empirical regime should be revisited to prevent further resistance.

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African Journal of Microbiology Research

Full Length Research Paper

Activity of conessine at various temperatures and pH on inhibition of germination of *Bacillus cereus* and *Bacillus stearothermophilus* spores

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This work reports the activity (at various conditions) of conessine isolated from methanolic extract of *Holarrhena floribunda,* on the inhibition of the germination of two *Bacillus* spores species. This activity was studied by treating spores of *Bacillus cereus* T and *Bacillus stearothermophilus* CNCH 5781 with effective concentrations of conessine at various temperature, pH and treatment times. The inhibition of germination was evaluated by the culture of treated spores on agar medium and the number of colony obtained was compared with that of control culture (not treated with conessine). We found that conessine used at 50 and 100 µg/ml for 20 min each decreased considerably the germination of spore of *B. cereus* T and *B. stearothermophilus* CNCH 5781. The maximum temperature of conessine activity for *B. cereus* T was at 30 and 60°C for *B. stearothermophilus* CNCH 5781 spores. Furthermore, the activity of conessine was sensitive to pH change and was more effective at pH 6 on both bacterial spore strains. The treatment of spores with conessine at various lengths of time demonstrated that, the activity of the compound on both bacterial spores was strongly related to the bacterial species. This study suggested that the activity of conessine on the inhibition of germination of *Bacillus* spore depends on physico-chemical factors and the bacterial species.

Key words: Conessine, germination, spores, *Bacillus*.

INTRODUCTION

In many food industries, bacterial spores are forms of microbial contaminants that are most harmful. First, they are difficult to be removed because of their high resistance to physical and chemical agents used in food sterilization. Furthermore, the inactivation of bacterial spores requires high temperatures often combined with pressure (Kramer and Gilbert, 1984; Gerhardt and Marquis, 1989). This causes significant losses of protein and vitamin in foods. At last, several foodstuffs have a short shelf life because, in industry, they are pasteurized to avoid a change in their organoleptic characteristics, leaving within them, a high content of spores.

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The existence of bacterial spores is not a problem exclusive to only industries. The microbiological quality of many local foods, to craft production in Cameroon comes into play with the high concentrations of bacterial spores.

Such is the case with many sold soups for consumption by children which many studies have revealed spore concentrations exceeding the standard (Bougnom, 2005; Feudjio, 2005). Alarming results were found in several honey samples across the country, as well as the suspected offending food of infant botulism (Etoa and Adegoke, 1996)

However, it should be noted that, although is a major contaminant, bacterial spore itself is of no danger because it can not cause any harm due to its very low metabolism as compared to a vegetative cell. In addition, bacterial spores cannot divide to give new ones. But, spores can respond to specific compound called germinant to give a vegetative cell by the process called germination (Moir, 1990). For instance, spores of *Bacillus cereus* can produce after germination, Gram-positive bacteria widely spread in several foods and drugs, are able to grow in aerobic and anaerobic conditions as well. Furthermore, during germination, some bacterial spore's species can produce food-spoiling toxins. This is the case of *B. cereus* vegetative cell that causes two different types of food poisoning: the emetic syndrome cause by production of non-protein heat stable toxin and the diarrheal syndrome due by an entero-toxin (Granum, 1994). Spores of *Bacillus stearothermophilus*, on its own, can give germination heat resistant vegetative cells that are non pathogenic, but cause outbreaks of several foods.

Control of pathogenic and toxigenic spore strains could result from the ability, either to stop completely spore germination, so that subsequent growth and multiplication could not occur. This kind of process is already applied in some canned food and drug using nysin or tylosin, two antibiotics produce by microorganisms (Meyer et al., 1988). However, few studies concerning the effect of non-germination of bacterial spores by plant compounds have been reported.

In order to promote the use of plant extract to decrease germination of bacterial spore, the investigation done by Bogne (2008) showed that methanolic extract of *Holarrhena floribunda* can decrease germination. At some concentrations of the extract, number of colonies obtained from treated spores was statistically lower than those of non spore control treated with the extract. This activity was later ascribed as conessine (Bogne, 2008), an alkaloid often present in various Apocynaceae that many studies has revealed its important anti-amoebic, antibacterial and antifungal activities (Burn, 1915).

The aim of this work was to evaluate the effect of temperature, pH and exposure time on conessine activity against the germination of spores of *B. cereus* and *B. stearothermophilus*.

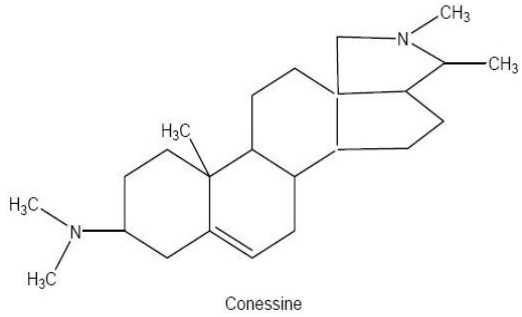

Figure 1. Structure of conessine revealed by spectroscopy (Bogne, 2008).

EXPERIMENTAL

Conessine

Conessine was obtained from the Microbiology Laboratory of the University of Yaoundé I. The methanolic crude extract of *H. floribunda* was used to obtain the molecule using a 72-h maceration of stem bark in methanol. The crude extract was acidified with 5% HCl, the aqueous solution obtained was brought to alkaline pH with ammonia and extracted with ethyl acetate (EA). The methanolic, EA and the remaining fraction resulting from the precedent operations were screened for antigermination activity. The bioactive fraction (EA) was flash-chromatographied on aluminia column under eight solvent systems composed of hexane (Hex) and ethyl acetate (EA) as solvents. The Hex-EA fraction (25-75%) from our active extract was purified by flash chromatography on aluminia column gradiently eluted with Hex-EA to obtain ten fractions (FA1 to FA10). The active fraction FA1 was crystallized from acetone to yield shiny pink crystals identified as conessine (Figure 1) on the basis of spectroscopic data and comparison with reported data (Bogne, 2008).

Bacterial strains

Activity of conessine against germination was evaluated on two *Bacillus* spores: *B. cereus* T spores, obtained from the culture collection of the Microbiology Laboratory of the Institute of food research of Reading, UK and *B. stearothermophilus* CNCH 5781 spores, obtained from the Institut Appert of Paris. These materials were maintained at 4°C before use.

Spore production and purification

The spores used in this work were preliminarily produced from spore stocks in two steps. Firstly spore stocks were heat-activated at 80°C for 10 min (Neyman and Buchanan, 1985) and spread on plate nutrient agar. The plates were incubated for 24 h at 35°C for *B. cereus* and 63°C for *B. stearothermophilus*, and vegetative cells were obtained. Secondly, the spores were obtained from vegetative cells. Spores of *B. cereus* were obtained according to the protocol described by Johnson et al. (1982) and those of *B. stearothermophilus* were obtained as described by Kim and Naylor (1966). Spores of both species were purified according to the standard method of Long and Williams (1958). Cleaned spores were suspended in distilled sterile water and stored at 4°C for three months.

Determination of effective concentration of conessine for inhibition of germination

The determination of effective concentration of conessine inhibiting germination was done according to the method described by Bogne (2008). Fifteen microliters of heat-activated spores (at 1.8×10^7 spores/ml) of both species were treated at various concentrations of conessine of 100, 50, 25 and 0 µg/ml at 30°C for 20 min. After treatment, in order to evaluate germination, 100 µl of appropriated decimal dilution of treated culture were spread on GPB (*gélose glucosé au pourpre de bromocrésol*). The plates were incubated for 24 h at 35°C for *B. cereus* and 63°C for *B. stearothermophilus*. The number of colonies was enumerated and expressed in percentage germination as compared to that of non treated spore control, subjected to same conditions. The lesser the percentage of germination, the higher the activity of conessine (Hanlin and Slepecky, 1985).

Percent germination (%) = (Number of colony of experimental culture / number of colony of control culture) x 100

Determination of conessine activity at various temperatures

Fifteen microliters of heat-activated spores (at $1.8x10^7$spores/ml) of each bacterial was treated at effective concentration of conessine (50 µg/ml for *B. cereus* and 100 µg/ml for *B. stearothermophilus*) at temperatures of 30, 40, 50 and 60°C for 20 min. Control heat-activated spores none exposed to conessine were treated in the same conditions. Appropriate decimal dilution was spread on agar medium and culture was done as described above. Colonies were enumerated and percentage of germination calculated.

Determination of conessine activity at varying pHs

Fifteen microliters of heat-activated spores (at $1.8x10^7$ spores/ml) of each species were treated at effective concentration and optimum temperature activity of conessine (50 µg/ml at 30°C for *B. cereus* and 100 µg/ml at 60°C for *B. stearothermophilus*) at various pH of 5; 6; 7 and 8 for 20 min. pH were obtained by adding little amounts of HCl 0.2% and NaOH 0.4%. Control heat-activated spores none exposed to the conessine were treated at the same condition. After treatment, the pH of the medium was neutralized and final volume completed at 500 µl. One hundred microliters of appropriated decimals dilution was spread on agar medium and culture was done as described above. Colonies were enumerated and percentage of germination calculated.

Influence of treatment time on activity of conessine

Fifteen microliters of heat-activated spores (at $1.8x10^7$ spores/ml) of each species were treated at effectiveness concentration, optimum temperature and pH of activity of conessine as described above, but at different times of <1; 10; 20; 30; 40 min. Control heat-activated spore not exposed to the conessine was treated at the same condition. After each treatment time, neutralization of the pH of the medium and culture of 100 µl of appropriated decimal dilution were done as described above. Colonies were enumerated and percentage of germination calculated as already described.

Statistical analysis

The experiments were conducted in triplicate and the results expressed in terms of means. The difference between the control and treatments was made using a one-factor ANOVA and the Student Newman-Keuls test with IBM SPSS 20.0 for window at a 95% confidence level.

RESULTS

Figure 2 presents the results of effect of concentration of conessine, on decrease of percent germination of spores of *B. cereus* and *B. stearothermophilus*. These results show that, some amount of percent germination obtained from cultured treated spores were statistically lower than those of control spore (not treated) at some concentration of conessine, this depend on the bacterial species (at all concentrations of conessine for *B. cereus* spores and only at 100 µg/mL for spores of *B. stearothermophilus*). This suggests that conessine can decrease germination with a strong differential sensitivity depending both on bacterial species and conessine concentration. *B. cereus* appears more sensitive, with maximum decrease of germination observed at 50 versus 100 µg/ml. of conessine for spores of *B. stearothermophilus*. Over 50 µg/ml the effect of conessine on spores of *B. cereus* remained constant.

The comparisons of percent germination of control spore, with those of experimental spores treated at 20 min with effective concentration of conessine (50 µg/ml for *B. cereus* and 100 µg/ml for *B. stearothermophilus*) at various temperatures are shown in Figure 3. The results obtained showed that, inhibition of germination of spores of *B. cereus* is effective only at 30°C. Temperatures equal and more than 40°C did not allow an inhibitory activity of conessine on germination of this strain.

For spores of *B. stearothermophilus,* temperatures of 30, 50 and 60°C showed the percent germination obtained from culture of spores treat with conessine statistically lower than those of control spore (non treated with conessine). These temperatures allowed effective activity of conessine. The maximum activity of compound against germination of spore of *B. stearothermophilus* CNCH 5781 is observed at 60°C.

The percent germination obtained after culture of control spores of *B. stearothermophilus* (non treat with conessine) was greater with increased temperature more than 50°C. For the study of the effect of pH on conessine activity, bacterial spores were treated with 50 µg/ml of conessine at 30°C for *B. cereus* and 100 µg/ml at 60°C for *B. stearothermophilus* at various pHs. The results illustrated in Figure 4 show that, although all the pH used allow an antigerminative effective activity of conessine on all spore species, conessine maximum pH of activity was 6 on both species.

The results of Figure 5 show that, conessine activity depends on treatment time of spores. So, the activity of conessine was effective after 20 min of treatment on spores of *B. cereus* and 10 min on spores of *B. stearothermophilus*.

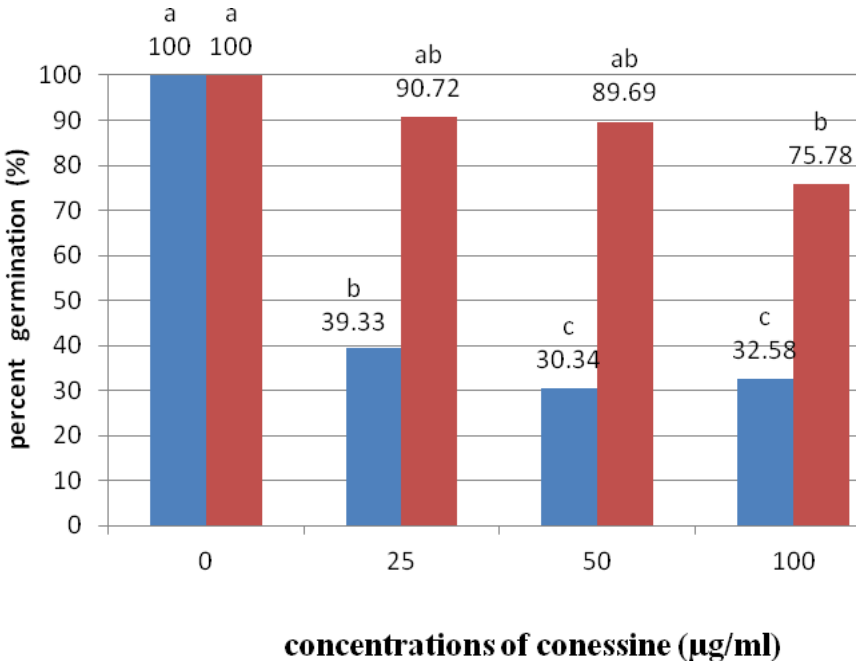

Figure 2. Percent germination of spore of *B. cereus* ( ) and *B. stearothermophilus* ( ) at various concentrations of conessine on germination. Different letters square of recovery percentage indicate significant difference using Student Newman-Keuls test ($p<0.05$).

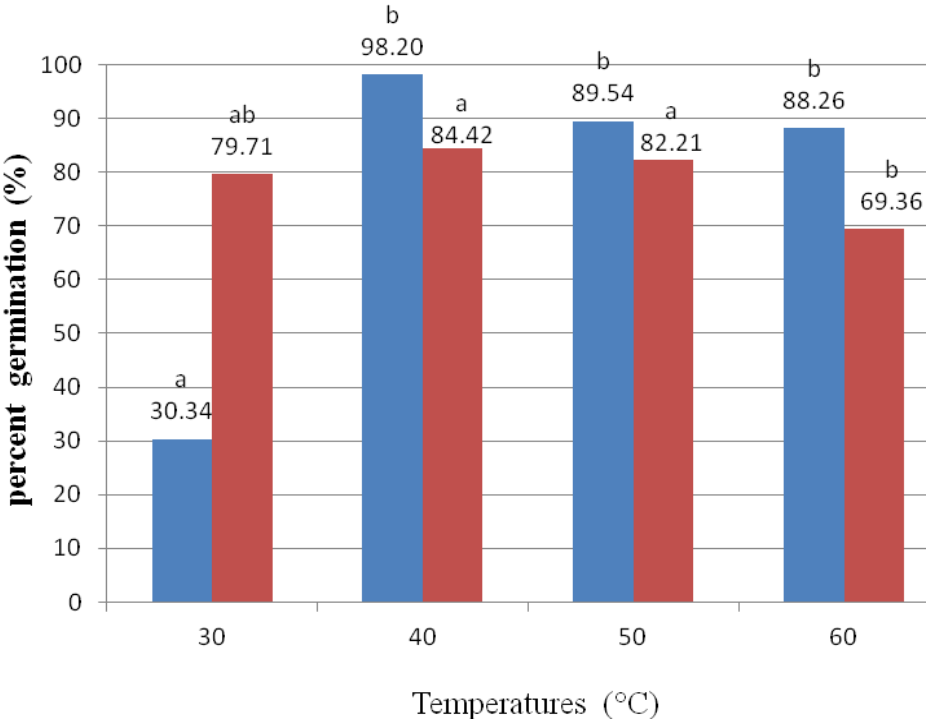

Figure 3. Percent germination of spore of *B. cereus* ( ) and *B. stearothermophilus* ( ) at various temperatures of treatment with conessine. Different letter squares of recovery percentage indicate significant difference using Student Newman-Keuls test ($p<0.05$).

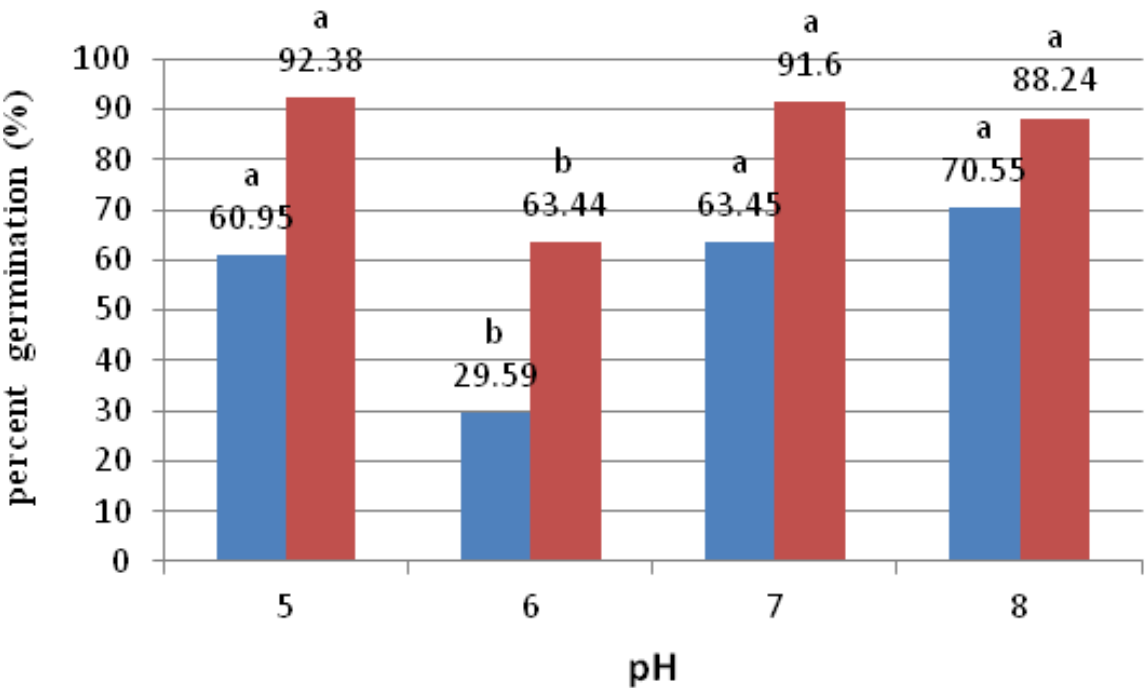

Figure 4. Percent germination of spore of *B. cereus* ( ) and *B. stearothermophilus* ( ) at various pH treatment of conessine. Different letter squares of recovery percentage indicate significant difference using Student Newman-Keuls test ($p<0.05$).

At 40 min of treatment, the activities began to slow down.

It was also seen that antigerminative activity of conessine was not immediate because no activity was observed after incubation time which is less than 1 min. Activity of conessine therefore needed treatment time which depended on bacterial species.

DISCUSSION

The results obtained in this study confirm the previous work of Bogne (2008), where it was shown that conessine inhibits germination of spores of *B. cereus* and *B. stearothermophilus*. We think that, conessine could bind to spore surface layer (coat and exosporium), thus contributing to reinforce the dormancy of spore and their resistance to respond to germination. This activity was already observed by Edima et al. (2010) who treated spores with some Cameroonian beers. Another hypothesis could be that, conessine specifically reacts with germination sites of spore and therefore acting as specific inhibitor of germination agents.

Although conessine inhibits germination of two bacterial spores, spores of *B. cereus* were more sensitive than those of *B. stearothermophilus*. Difference of sensitivity of conessine could be explained by possible difference in number and accessibility on sites of fixation of antigerminative substances on spores of different species (Wolgamott and Durham, 1971). Constance activity of conessine on spores of *B. cereus* treated at the concentrations equal or more than 50 µg/ml may be explained by saturation of those active sites on spores.

This work also shows that treatment of spores of both species with conessine is more effective at temperature ranges of 28-35°C for *B. cereus* and 55-65°C for *B. stearothermophilus*. This may be due to the fact that, germinant receptors are generally proteins (Gould, 1970). So, at temperature of optimal growth, those receptors would have specific conformation to react with germinant or inhibitor of germination like conessine.

In addition, lack of conessine activity observed at temperatures equal or more than 40°C on spores of *B. cereus* and at 40°C on spores of *B. stearothermophilus* may be due to the fact that, conessine did not reach or did not attach to its fixation sites at those temperatures. On the other hand, the increase of numbers of colonies of control spores of *B. stearothermophilus* exposed at temperature more than 50°C may be explained by the continuation of activation step already observed on those spores by Etoa (1985).

Activity of conessine depends on pH medium. This factor can influence both compound state (solubility and ionization state) and site of fixation on spore. In this report, we can say that, effect of conessine depending on

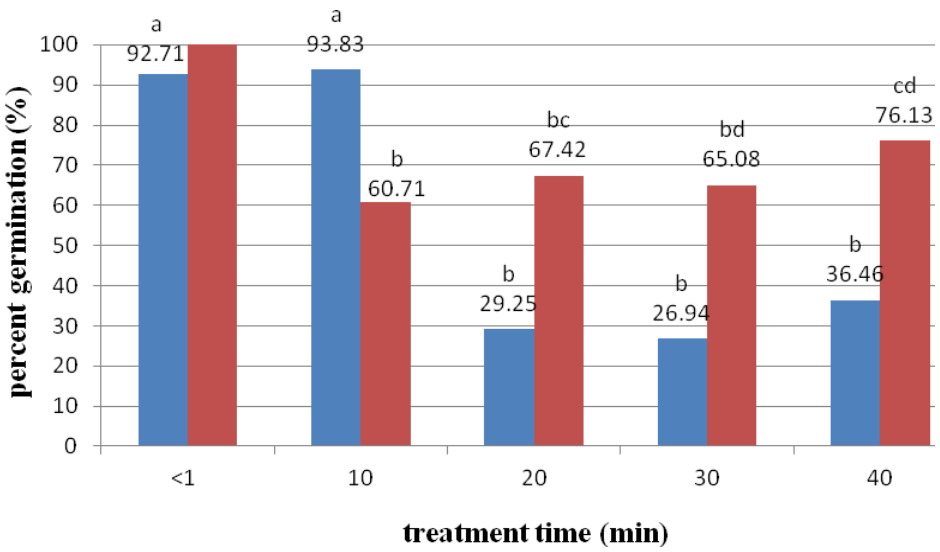

Figure 5. Percent germination of spore of *B. cereus* ( ) and *B. stearothermophilus* ( ) at various treatment time of conessine. Different letter squares of recovery percentage indicate significant difference using Student Newman-Keuls test ($p<0.05$).

pH is due to difference of solubility in solvent used at different pH. Indeed, it was shown that, alkaloid are more soluble in polar solvents (Bruneton, 1999). So, conessine would be more soluble in polar solvents used at acid pH (5 and 6) as compared to the neutral and basic pH used (7 and 8). However, at pH 5, medium would be more acidic to alter spore coats because acid activates spores germination by damage in an irreversible manner spores coats. Then a maximum activity was observed at pH 6 as compared to pH 5.

We have also seen that, conessine activity depends on treatment time which also depends on *Bacillus* species. Antigerminative activity of conessine is not immediate, because no activity was observed after incubation time less than 1 min. The activity needed treatment time which depends on bacterial species. Spores of *B. cereus* which appeared above to be more sensitive needed more time as compared to those of *B. stearothermophilus*. This could be explained by different accessibility of conessine at the site of fixation of spores of both species. Further studies must been done on another *Bacillus* species and *Clostridium.*

Conclusion

In this work, results obtained suggest that, conessine considerably decreased germination of spores of both *B. cereus* T and *B. stearothermophilus* CNCH 5781. This activity depended on physico-chemical factors and the bacterial species. This compound could be used as food additive to extend food shelf-life by inhibiting bacterial spores growth, however, further studies must been done on another *Bacillus* species and *Clostridium*.

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Full Length Research Paper

Study on bovine mastitis and associated risk factors in Adigrat, Northern Ethiopia

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A cross sectional study was conducted from November, 2011 to April, 2012 in Adigrat, Ethiopia, with the objective of assessing the prevalence of bovine mastitis, the risk factors associated with the disease and identifying the bacteria responsible for the disease. A total of 322 cows were selected from 10 small holder dairy farms using simple random sampling method. California Mastitis Test (CMT), clinical examination of udder and teats and bacteriological examination were employed. The overall prevalence of mastitis at a cow level was 64.3% (207/322), from which 15 (31/322) and 85% (176/322) were clinical and subclinical, respectively. The quarter level prevalence of the disease was also 54% (696/1288) from which 20.5 (264/1288) and 33.5% (432/1288) were clinical and subclinical form, respectively. As compared to the others, the right hind quarters were affected with the highest infection rate (63.9%). The left hind quarters were the second with an infection rate of 59.3% followed by right front quarters (52.5%) and left front quarters (40.4%). Among the bacterial causes of bovine mastitis in the study area, *Staphylococcus aureus*, *Echerichia coli* and *Streptococcus agalactiae* were the major isolates with percentages of 51.7, 20.9 and 20.3, respectively. All the potential risk factors considered in this study namely, parity, age, stage of lactation and breed showed significant effects on prevalence of mastitis in the present study. The present study concludes that mastitis was a major health problem of dairy cows in the area. Hence, strategic control measures against the disease and regular surveillance measures are recommended.

Key words: Prevalence, bovine mastitis, risk factors, bacteria, Adigrat, Ethiopia.

INTRODUCTION

Ethiopia has the largest livestock population in Africa. Cows represent the largest population of cattle production of the country (CSA, 2007). Hence, development of the dairy sector in Ethiopia can contribute significantly to poverty alleviation and nutrition in the country (Mohamed et al., 2004). Nevertheless, the quality and quantity of milk in the country deteriorates due to various causes. Mastitis is an inflammation of the mammary gland and commonly associated with intra-mammary bacterial infection. It is considered as the most important disease among diseases of the dairy having zoonotic and economic impact (Omore et al., 1996; Al-Majali et al., 2008).

Bovine mastitis can be clinical with local (in some cases general) clinical signs and milk abnormalities or sub clinical with production losses and lowered milk quality. Clinical signs vary with the severity of the disease and generally include pain, heat and swelling of the affected quarter or half of the gland and abnormality of the milk either as clots or flakes and wateriness of the liquid phase (Radostits et al., 2000).

Bovine mastitis as a disease, has received little attention in Ethiopia, especially the sub clinical form. When modern dairy farming in the tropics was first adopted,

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mastitis was predicted to be important disease in dairy cattle and one of the most tasks of risk factors for animal health and production problems to allow effective control strategies to be adopted (Hunderra et al., 2005).

The disease has been reported by several authors in different parts of Ethiopia (Lakew et al., 2009; Gebreyohannes et al., 2010; Megersa et al., 2010). However, in some parts of Ethiopia, the disease is insufficiently investigated and information relating to its magnitude, distribution and risk factors is scant. Such information is important to envisage when designing appropriate strategies that would help to reduce its prevalence and effects (Megersa et al., 2010; Mekbib et al., 2010).

Even though the disease is known in Adigrat, documented information regarding the disease is unavailable. Therefore, the study was undertaken with the objective of assessing the prevalence of bovine mastitis, the risk factors associated with the disease and identifying the bacteria responsible for the disease.

MATERIALS AND METHODS

Study area

The study was conducted in dairy farms in Adigrat town located 898 km away from Addis Ababa in the north east direction, 14° 16' N and 39° 29' E, at an altitude with a range of 246-297 m above sea level. The minimum and maximum temperatures are 9.28°C and 21.91°C, respectively. The area receives a bimodal rainfall of 400 mm minimum and 600 mm maximum (CSA, 2010).

Study population

Cows from 10 small holder dairy farms of Adigrat were selected using simple random sampling method. The sample size was determined by the formula given by Thrusfield (2005) by assuming the expected prevalence to be 30% (Ministry of Agriculture, Personal information) while the statistical confidence level was 95%. Accordingly, the sample size of lactating cows was determined to be 322 (193 cross bred and 129 local breeds).

California mastitis test (CMT)

The California mastitis test (CMT) was carried out as a screening test for sub clinical mastitis. It was carried out as per the procedure of Quinn et al. (2004). A squirt of milk, about 2 ml from each half was placed in each of 2 shallow cups in the CMT paddle. An equal amount of the commercial CMT reagent was added to each cup. A gentle circular motion was applied to the mixtures in a horizontal plane for 15 s. Then depending on CMT results, cases were categorized as either positive based on degree of jell formation or negative which did not show jell formation. Positive CMT-cows were defined as having at least one CMT-positive quarter.

Sample collection and handling

Samples were collected aseptically as described by Quinn et al. (2004). They were collected before milking. Udders and especially teats were cleaned and dried before sample collection. Each teat end was swabbed with cotton soaked in 70% ethyl alcohol. Samples were taken in sterile glass vials and closed with screw caps. The vials were marked with a permanent marker, so that the markings were easy to read when the vials were placed in rack. The first streams of milk were discarded and 10 ml of milk was collected into horizontally held vial. After collection, the sample was placed in an icebox and transported to the laboratory for analysis.

Bacterial culture

Milk samples were examined following standard procedure where about one standard loop full (0.01 ml) of each milk samples was streaked on 10% sheep blood agar. Plates were incubated aerobically at 37°C for up to 72 h and checked for any bacterial growth.

Suspected colonies were identified morphologically, microscopically and biochemically according to Quinn et al. (2004) and NMC (2004).

Data analysis

The data was entered to Microsoft excel spread sheet and analyzed using statistical software (SPSS version 17) program and total prevalence was calculated by dividing the number of positive cows/quarters by the total number of cows/quarters tested (Thrusfield, 1995). The Chi-square (χ^2) test was applied to test the existence of association between mastitis and risk factors such as breed, age, lactation stage and parity. In all chi-square test applications, a probability level of $p<0.05$ was considered statistically significant.

RESULTS

Prevalence of mastitis

A total of 322 lactating cows were examined for presence of mastitis out of which 207 (64.3%) cows were found to be affected with clinical and subclinical mastitis based on clinical and CMT diagnosis. Out of the total 207 cows affected, 176 (85%) were positive for subclinical mastitis and 31 (15%) for clinical mastitis.

The result shows that 31(15%) of the cows and 264 (20.5%) of the quarters were found to have clinical mastitis whereas 176 (85%) of the cows and 432 (33.5%) of the quarters were affected with subclinical mastitis. Out of the 1288 quarters examined, 696 (54%) were positive for mastitis (Table 1).

As compared to the others, the right hind quarters were affected with the highest infection rate (63.9%). The left hind quarters were the second with an infection rate of 59.3% followed by right front quarters (52.5%) and left front quarters (40.4%) (Table 2).

Bacteriological examination

Out of the total samples collected from affected quarters for bacterial isolation, 681 samples showed growth on 10% sheep blood agar. The relative prevalence of various bacterial species isolated from cases is shown in

Table 1. Prevalence of clinical and subclinical mastitis at a cow and quarter level.

Form of mastitis	Total examined cows	Total affected cows (%)	Total examined quarters	Total affected quarters (%)
Clinical	322	31 (15%)	1288	264 (20.5%)
Subclinical	322	176 (85%)	1288	432 (33.5%)
Total	322	207 (64.3%)	1288	696 (54%)

Table 2. Quarter level prevalence of mastitis.

Quarter	Quarters examined	Affected quarters	Prevalence (%)
Front right	322	169	52.5
Front Left	322	130	40.4
Hind Right	322	206	63.9
Hind Left	322	191	59.3
Total	1288	696	54

Table 3. Bacteria isolated from mastitis affected quarters.

Organisms isolated	Frequency	Percentage
Staphylococcus aureus	361	51.7
E. coli	146	20.9
Streptococcus agalactiae	142	20.3
Other gram positive *cocci* and rods	32	4.6
Other gram negative rods	17	2.5
Total	698	100

Table 3. S*. aureus* was the most predominant isolates constituting 51.7% of all the isolates followed by *E. coli* (20.9%), *S. agalactiae* (20.3%), other gram positive cocci and bacilli (4.6%) and other gram negative rods (2.5%).

Risk factors for mastitis

Prevalence of mastitis with respect to specific risk factors was determined as the proportion of affected cows out of the total examined. Four variables were considered as potential risk factors for the occurrence of mastitis. The association of mastitis with parity, lactation stage, age and breed was statistically significant ($p<0.05$). Prevalence of mastitis in cows delivered 1 to 3 calves was 39.7%, four to seven calves was 69.8% and greater than 7 calves was 81.8%.

Mastitis prevalence was highest in early lactation (74.3%) and also higher in late stage of lactation (65%) but lower in mid lactation (46.7%). Cows older than 10 years were affected the highest (79.6%), followed by 6 to 9 years (57.5%) and 1 to 5 years (41.7%). Cross bred cows were affected at higher rate (71%) than local breeds (54.2%) (Table 4).

DISCUSSION

The present study shows an overall mastitis prevalence of 64.3%, as determined by CMT and clinical examination of udders. The finding is comparable with previous reports of 63% by Geresu (1989) in Addis Ababa and 71% by Mekbib et al. (2010) in Holetta town.

However, the overall prevalence is higher than the previous findings of other authors in different regions of Ethiopia like 34.9% by Biffa et al. (2005) in Southern Ethiopia, 52.8% by Hunderra et al. (2005) around Sebeta and 46.7% by Abera et al. (2010) in Adama. The difference in prevalence reports of mastitis in the present study and other reports could probably be due to difference in breeds, management practices, geographic areas, level of production and differences in study methods.

The prevalence of subclinical mastitis was higher (176, 85%) than clinical mastitis 31(15%). A similar observation of the dominance of subclinical mastitis was observed by several studies: 10.3 and 33.8% by Delelesse (2010); 4.9 and 30.6% by Moges et al. (2012); 19.6 and 55.1% by Zeryehun et al. (2013) for clinical and subclinical mastitis, respectively. The finding strengthened the claim

Table 4. Prevalence of mastitis with respect to different risk factors.

Risk factor		Animals examined	Number of animals affected	Prevalence (%)	χ^2 (P-value)
Parity number	1-3 calves	68	27	39.7	23.94(0.000)
	4-7 calves	232	162	69.8	
	> 7 calves	22	18	81.8	
Lactation Stage	Early(1-4 month)	121	88	74.3	13.77(0.001)
	Mid(5-7 month)	75	35	46.7	
	Late (> 7 month)	126	82	65.0	
Age	Young (1-5 years)	24	10	41.7	22.76 (0.000)
	Young adult (6-9 years)	146	84	57.5	
	Old (≥10 years)	142	113	79.6	
Breed	Cross	193	137	71.0	8.7 (0.0032)
	Local	129	70	54.2	

made by Ojo et al. (2009) that subclinical mastitis remains the most economically damaging and zoonotic potential disease for dairy industry.

The overall quarter prevalence of mastitis (54%) found in this study was higher than the finding of Mekbib et al. (2010) who reported quarter prevalence rate of 44.9% but lower than the report made by Kifle and Tolossa (2008) in Selale, Ethiopia, Zeryehun et al. (2013) in and around Addis Ababa, Ethiopia, who reported 63.1 and 62.3%, respectively.

As compared to the others, the right hind quarters were affected with the highest infection rate (63.9%). The left hind quarters were the second with an infection rate of 59.3% followed by right front quarters (52.5%) and left front quarters (40.4%). The result shows that the hind quarters are affected more than the front quarters. This could be attributed to the high production capacity of the hind quarters (Radostits et al., 1994) and the high chance of getting fecal and environmental conta-mination (Sori et al., 2005).

In the study, the predominant organisms isolated from mastitis were *S. aureus* (51.3%) followed by *E. coli* (20.9%) and *S. agalactiae* (20.3%). This is in agreement with the report of Ayano et al. (2013). The predominance and primary role of *S. aureus* isolate in bovine mastitis has also been reported in other studies (Mekbib et al., 2010; Atyabi et al., 2006). The high prevalence of this organism may be associated with its frequent coloni-zation of teats, its ability to exist intra-cellular and localize within micro abscesses in the udder and hence resistant to antibiotic treatment (MacDonald, 1997). The organism is well adapted to survive in the udder and usually establishes a mild sub clinical infection of long duration from which it shed in milk facilitating trans-mission to healthy animals mainly during milking (Radostits et al., 2007).

The study reveals the prevalence of mastitis to be affected significantly ($p<0.05$) with age. The prevalence of mastitis was highest in old cows (79.6%) followed by young adults (57.5%) and young cows (41.7%). This is in agreement to the findings of previous works by Moges et al. (2011) and Zeryehun et al. (2013). The highest prevalence in older cows is because of their largest teats and more relaxed sphincter muscles, which increase the accessibility of infectious agent in the cows' udder (Radostits et al., 2007). Cows with more than 7 calves (81.8%) were at greater risk than those of cows having 4-7 calves (69.8%) and cows having 1-3 calves (39.7%). Similar finding was reported by Radostits et al. (2007) and Mekbib et al. (2010). Early lactation stage had higher relative prevalence (74.3%) than late (65%) and mid (46.7%) lactation stage; the difference is statistically significant ($p<0.05$). This result aligns with reports made elsewhere (Delelesse, 2010; Moges et al., 2011; Zeryehun et al., 2013). Absence of dry cow therapy regime could possibly be the major factor contributing to high prevalence at early lactation (Schalm et al., 1971). Cross bred cows were affected (71%) at higher rate than local breeds (54.2%). The difference is statistically significant ($P< 0.05$). This agrees to the findings of Moges et al. (2011).

The present study shows that mastitis was a major health problem of dairy cows in the area hence warrants serious attention. The high prevalence clearly indicated lack of strategic control measures against the disease, as well as poor surveillance measures. It is therefore important that farmers should ensure strict personal hygiene and that of animals, and general sanitary condition of the farms should be improved and maintained. Since the bacteria isolated from cows' milk samples in the present study are types that cause both contagious and environmental mastitis, correct and good milking tech-

niques are essential in the prevention strategies. Furthermore, regular screening for the detection of subclinical mastitis and proper treatment of the clinical cases as well as appropriate treatment of cows during dry and lactation period should be practiced.

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Full Length Research Paper

Antimicrobial susceptibility patterns of community-acquired Gram-negative uropathogens

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Drug resistance is now a public health crisis and global problem. This study was performed to identify the antibiotic susceptibility of urinary tract infections (UTI) in Sanqar City of Kermanshah, Iran. Urine specimens of 891 ambulatory patients over 18 years of age with clinically suspected UTI were referred from Physicians' Office (MD) to the Sanqar Hospital (Kermanshah, Iran) for urine culture from September 1, 2011 to December 31, 2012. These samples were cultivated in agar-blood and McConkey agar. For culture positive samples, antibiogram test was done by disk diffusion method in Muller-Hinton agar plate. The relevant results were reported based on Clinical and Laboratory Standards Institute (CLSI) criteria. Of the 891 urine specimens, 379 cases were urinary culture positive (42.28%). The most common uropathogens were *Escherichia coli* (78.1%) and *Klebsiella pneumoniae* (15%). High susceptibility patterns to: ciprofloxacin (95.3%), amikacin (93.9%), nalidixic acid (92.2%), gentamicin (89.2%) and nitrofurantoin (83.8%) among the *E. coli* isolates identified were observed. Ciprofloxacin and nalidixic acid are the most suitable antibiotics for the empirical treatment used for ambulatory patients over 18 years of age with urinary tract infections in the geographical area of this study.

Key word: Antibiotic resistance, uropathogens, antibiogram, antimicrobial susceptibility.

INTRODUCTION

Uncomplicated urinary tract infections are the most common infections encountered after respiratory infections (Schito et al., 2009). These infections if not treated lead into the increase of mortality rate, disability, hospitalization and economical costs (van et al., 2010). Unsuitable treatment can be due to the resistance of uropathogens to antibiotics. Today, drug resistance is a public health crisis and a global problem (Xiao et al., 2011).

Drug resistance model of uropathogens is different in various regions (Schito et al., 2009; Xiao et al., 2011; Gupta et al, 2001; Alós et al., 2005; Khameneh et al., 2009; Foxman, 2010; Kashef et al., 2010; Baue et al., 1996). Thus, epidemiological study of bacterial resistance is an important instrument to control the development of bacteria resistance. There was a logical association between experimental prescription (without urine culture) of the antibiotics and increase in the bacterial resistance (Gupta et al., 2001).

For proper use of antibiotics in treating urinary tract infections in each region, it is required to have a detailed knowledge of uropathogens and their drug sensitivity. This study was performed to find the antibiotic susceptibility pattern in urinary tract infections in Sanqar City located in the north east of Kermanshah, Iran.

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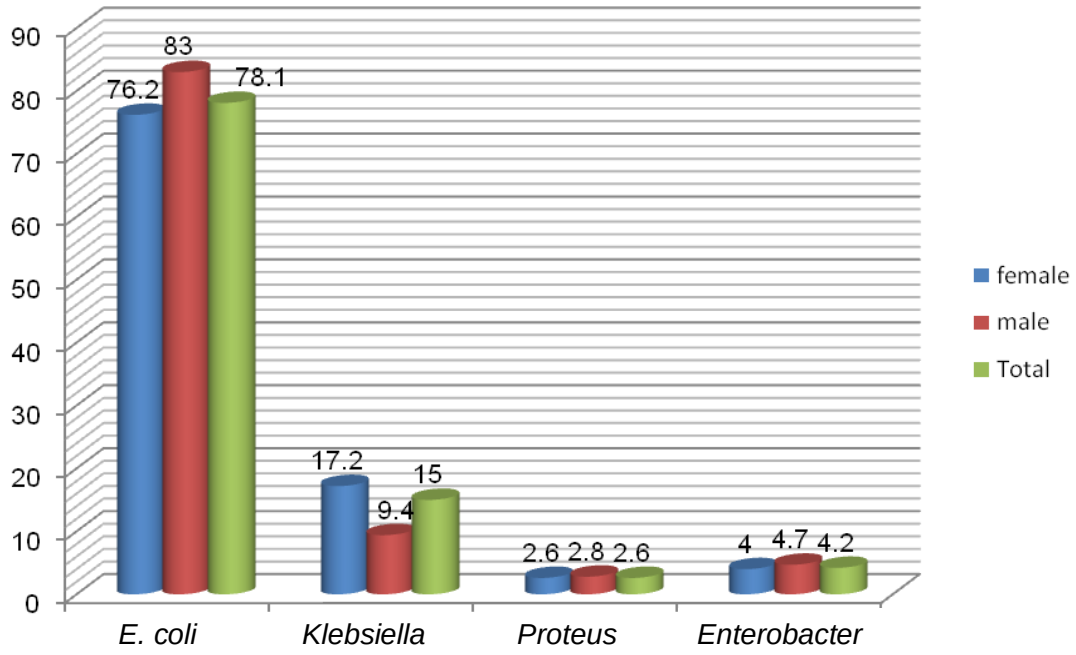

Figure 1. Frequency of isolated bacteria from positive urine samples according to patient gender.

MATERIALS AND METHODS

The Sanqar City with about 998,985 people is located in the northeastern of Kermanshah province, Iran. Imam Khomeini hospital is the only hospital in this city and is one of the subsets of Kermanshah University Medical Sciences. This observational study was carried out on 891 subjects which included 534 females and 285 males. The subjects were ambulatory patients, over 18 years of age with clinically suspected urinary tract infection, referred from Physicians' Office (MD) to Imam Khomeini hospital's laboratory of Sanqar City for urine culture from September 1, 2011 to December 31, 2012. Patients who received antimicrobials within the previous two weeks, who were suffering from renal disorders, diabetes mellitus, persons infected with HIV, on corticosteroid therapy and pregnant women were excluded; In these cases, the patient were asked to take a urine sample.

Urine specimens were collected by the mid-stream urine method in sterile dishes in the hospital laboratory. These samples were cultured in blood agar and McConkey agar for 24 h at 37°C. Then, the colonies in the medium were counted and the colonies with more than 10^5 CFU/ml of a single uropathogen was considered as culture positive. A confirmation test (TSI, triple sugar iron agar) was performed for identifying the organism.

In culture positive samples, to determine the sensitivity and resistant antibiotic of isolated specimens, sensitivity test was performed by Kirby-Bauer method in Muller-Hinton agar (Bauer et al., 1996). After incubation and determination of zone of inhibition, we determined the growth rate of the sensitive and resistant microorganisms. The applied antibiotic discs were made by Iran medicine Antibody Company.

The applied discs for bacteria isolated from culture media included antibiotics commonly used for treatment of uncomplicated UTI: cephalexin (CF, 30 µg), Ciprofloxacin (CP, 5 µg), Ampicillin (AM, 5 µg), Nitrofurantoin (NI, 50 µg), Gentamicin (GM, 10 µg), Trimethoprim-Sulfamethoxazole (SXT, 30 µg), nalidi-xic acid (NA, 30 µg), Amikacin (AK, 30 µg). After incubation for 24 h, Muller-Hinton agar was investigated and its results were reported based on the National Committee on Clinical Laboratory Standards (NCCLS) criteria (CLSI, 2012). The collected data was statistically analyzed using the SPSS 18 software. The findings were reported in number of isolates tested against each antimicrobial agent and percentage of isolates susceptible to antimicrobial agent.

RESULTS

Over a 12-month period (September 1, 2011 to December 31, 2012), 819 urine samples from Ambulatory patients over 18 years of age with clinically suspected UTI were analyzed, of which 65.2% (534/819) were females and 34.8% (285/819) males. The overall prevalence of positive urine culture was 46.28% (379/819). It was 51.12% (273/534) for females and 37.19% (106/253) for male's subjects.

Most urinary pathogens isolated were *Escherichia coli* 78.1% (296/379), *Klebsiella pneumoniae* 15.04% (57/379) for men and women. Distribution of pathogens isolated from culture samples showed no statistically significant difference in both sexes (Fisher's Exact Test = 3.861, P = N.S) (Figure 1).

Analysis according to patient age showed that the highest prevalence of positive urine culture was in the age group 25 to 34 years (158 cases, 41.69%) followed by the age group 18 to 24 years (78 cases, 20.58%). *E. coli* infections were more prevalent in the age group 25 to 34 years (129 cases, 81.65%), *K. pneumoniae* in the age group 34 to 44 years (11case, 16.92%), *Proteus mirabilis* in the age group 19 to 24 years (3 cases, 3.85%) and *Enterobacter aerogenes* in the age group 45 to 60 years

Table 1. Frequency of isolated bacteria from positive urine samples according to patient age.

Age (years)	Organism				
	E. coli	*Klebsiella pneumoniae*	*Proteus mirabilis*	*Enterobacter aerogenes*	Total (%)
19-24 n (%)	59 (75.64)	12 (15.38)	3 (3.85)	4 (5.13)	78 (20.58)
25-34 n (%)	129 (81.65)	19 (12.03)	5 (3.16)	5 (3.16)	158 (41.69)
35-44 n (%)	50 (76.92)	11 (16.92)	1 (1.54)	3 (4.62)	65 (17.15)
45-60 n (%)	40 (75.47)	9 (16.98)	1 (1.89)	3 (5.67)	53 (13.98)
>60 n (%)	18 (72)	6 (24)	0 (0)	1 (4)	25 (6.6)
Total n (%)	296 (78.10)	57 (15.04)	10 (2.64)	16 (4.22)	379 (100)

Table 2. Resistance frequency of Gram-negative bacteria isolated from positive urine samples to commonly used antibiotics.

Bacteria	*E. coli* (n = 296)		*Klebsiella pneumoniae* (n = 57)		*Proteus mirabilis* (n = 10)		*Enterobacter aerogenes* (n = 16)	
	Isolated[1]	R[2] (%)	Isolated	R (%)	Isolated	%	Isolated	R (%)
Cephalexin	138	46.62	29	50.88	4	40	5	-
Ciprofloxacin	14	4.73	2	3.51	0	0	0	0
Ampicillin	251	84.8	55	96.49	9	90	15	-
Nitrofurantoin	48	16.22	16	28.07	3	30	7	43.75
Gentamicin	32	10.81	12	21.05	0	0	5	31.25
Co- Trimoxazol	115	38.85	21	36.84	3	30	5	31.25
Nalidixic acid	23	7.77	6	10.53	0	0	0	0
Amikacin	18	6.08	3	5.26	1	10	0	0

[1]Number of isolates tested against each antimicrobial agent. [2]Percentage of isolates resistance to antimicrobial agent.

(3 cases, 5.67%) (Table 1).

The highest antibiotic resistance of *E. coli* strains was reported to ampicillin (84.8%), cephalexin (46.62%) and trimethoprim-sulfamethoxazole (38.85%). Among *K. pneumoniae* strains isolated, the highest drug resistance was found for ampicillin (96.49%), cephalexin (50.88%), trimethoprim- sulfamethoxazole (36.84%) and nitrofurantoin (28.07%).

P. mirabilis strains showed the highest resistance to Ampicillin (90%), Cephalexin (40%), trimethoprim-sulfamethoxazole and nitrofurantoin (30%), *E. aerogenes* showed the highest resistance to nitrofurantoin (43.75%) and gentamicin (31.25%) (Table 2).

DISCUSSION

In this study, of the 819 urine samples from ambulatory patients over 18 years of age with clinically suspected UTI, 379 (46.28%) had positive urine culture. The results of our study show that the overall prevalence of positive urine culture UTI was 46.28%. It was higher in female subjects (51.12%) than in males (37.19%).

The reason for males being less prone to UTIs may be attributed to their longer urethra. These results are in agreement with other studies carried out around the world (Akram et al., 2007; Shaifali et al., 2012; Jeremy et al., 2011).

Our study shows that highest prevalence of positive urine culture was in the age group 25 to 34 years (41.69%) followed by the age group of 18 to 24 years (20.58%). In other words, more cases of UTIs were recorded among young and middle age patients (20-49 years, 51.04%) which are similar to trends reported in other studies (Akram et al., 2007; Alós et al., 2005; Jeremy et al., 2011). This may be because sexual activity is more common among these age groups.

In our investigation, the most common pathogens isolated from positive urine cultures were *E. coli* strains (78.1%) and *K. pneumoniae* (15.04%). Other studies carried out in various regions have also shown that the most common uropathogens were *E. coli* and *K. pneumoniae*. It can be due to the fact that Gram negative bacteria in are abundantly present in urinary tract system. The prevalence percentages of pathogens isolated in different regions are in agreement with our study (Kurtaran et al., 2010; Farajnia et al., 2009; Mashouf et al., 2009; Moinzadeh et al., 2013). Several studies have reported lesser or a higher prevalence (Bours et al, 2010; Ranjbar et al, 2009), because of the different quality and

quantity of treatment of urinary infections and society health level in various regions.

In this study, *E. coli* strains showed the highest antibiotic resistance to Ampicillin (84.8%), Cephalexin (46.62%) and Trimethoprim-Sulfamethoxazole (38.85%). In most of the studies performed in Iran and other countries (despite the percentage different), the highest antibiotic resistance of *E. coli* were found for ampicillin, trimethoprim- sulfamethoxazole and nalidixic acid. In other studies after Ampicillin, the highest resistance was for cephalexin and co-trimoxazol (McLoughlin and Joseph, 2003; Yüksel et al., 2006; Grude et al., 2005; Farrell et al., 2003; Moinzadeh et al., 2013; Kashef et al., 2010). Although, these results differ from some published studies from Iran, they are consistent with those of the highest antibiotic resistance of *E. coli* which were found to be Trimethoprim-Sulfamethoxazole 90% (Pourakbari et al., 2012), 66% (Valavi et al., 2013) and Nalidixic acid 59.7% (Ayatollahi et al., 2013).

In our study, *E. coli*, *K. pneumoniae* and *P. mirabilis* strains had the highest percentages of resistance to ampicillin (84.8-96.49%) and cephalexin (40-50.88%), followed by trimethoprim- sulfamethoxazole (30-38.85%); this is similar to the result reported by Bours et al. (2010). While, other similar studies conducted in iran have shown a high resistance to ampicillin and trimethoprim-sulfamethoxazole, respectively (Mashouf et al., 2009; Farrell et al., 2003; Bauer et al., 1996; Khameneh et al., 2009; Moinzadeh et al., 2013). This difference could be due to improper use of antibiotics by patients or physicians (according to the drug culture in Iran), and also because of the increased use of antibiotics in livestock breeding.

E. aerogenes strains had the highest resistance to ampicillin (93.75%) and Nitrofurantoin (43.75%), followed by gentamicin and trimethoprim- sulfamethoxazole (31.25%). These results are similar to that of Moinzadeh et al. (2013) and Kashef et al. (2010) but differ from Moinzadeh et al. (2013) and Valavi et al. (2013) that show nalidixic acid (100%) and ceftriaxon (71.4%). Resistance of *K. pneumoniae* (96.49%) to ampicillin is nearly similar to the results of the study performed in Arabia during 1999 to 2002 (Kader et al., 2004). Limitation of our study is, not considering ESBL.

Conclusion

In this study, Ciprofloxacin and Nalidixic acid were the most suitable antibiotics for the empirical treatment for ambulatory patients with urinary tract infections in the study's geography region (Sanqar City, Kermanshah, Iran).

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Full Length Research Paper

Affectivity of *Spodoptera littoralis* nucleopolyhedrovirus (*Spli*NPV) against first and second instar larvae of the cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae)

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Preliminary bioassays were conducted to determine the virus activity against the 1st and 2nd instar larvae of the cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae). First instar larvae were more susceptible to the virus than the second instar larvae. Mortality response of the larvae was greater at the highest concentration of the virus than at lowest concentrations. The lethal concentration that killed 95% of the tested larvae (LC95%) was 2.32 x 10^6. The obtained mortality rates of *S. littoralis* first instar (1st) larvae were 0, 15.15, 16.77, 43.57, 82.44 and 96.94%, for the tested concentrations of control (2 x 10^2, 2 x 10^3, 2 x 10^4, 2 x 10^5 and 2 x 10^6 PIB/ml). However, second instar (2nd) larvae were less susceptible to the virus than the first instar larvae with the mortality rate of 0, 4, 9.39, 25.33, 43.24, and 71.63%, for the same tested concentrations, respectively. In control treatment, no mortality was recorded. The high concentration levels caused a high rate of mortality. The same trend of mortality is similar to those found by other studies.

Key words: Biological control, virus, *Spli*MNPV, Noctuidae, cotton leafworm, *Spodoptera littoralis.*

INTRODUCTION

The increased awareness of environmental pollution and the demand for safe food production have led to growing interests in use of biological control agents such as baculoviruses in plants' protection. *Spodoptera littoralis* (Boisd.) is an important key pest on clover and many vegetable crops in Saudi Arabia (Albarrak, 2009). In fact, the cotton leafworm is a major limiting factor affecting crop and vegetable production in many other countries. *S. littoralis* is one of the most destructive agricultural lepidopterous pests within its subtropical and tropical regimes (Hosny et al., 1986). It can attack numerous economically important crops all year round.

Chemical is used for controlling the species of *S. littoralis*. This control has been used especially on cotton. Up to 1968, methyl-parathion was used for *S. littoralis*, but then resistance to this compound developed. The chemicals that have been used such as organophosphorus, pyrethroid and other insecticides have appearance of resistance and cross resistance in many cases (Issa et al., 1984). The control of this insect is usually done by excessive use of chemical pesticides which may lead to the development of pesticide resistance. Also, the excessive use of chemicals will lead to negative impacts on the environment and non-target species.

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The microbial control agents in pest management such as viruses are environmental friendly, as well as other microbial control agents such as bacteria, fungi, and nematode; but for their successful use, a specialist's knowledge is needed (Lacey and Kaya, 2000).

Baculoviruses are promising alternatives for reducing dependency on harmful chemical insecticides in plant protection. They are safe, specific, environmental friendly bio-agents, and effective against certain insect pests (Burges et al., 1980). These viruses are specially designed to survive outside their host and can persist in crevices and soil for years (Millar, 1997). They have the greatest potential for use as microbial insect pest control agents. Therefore, Szewczyk et al. (2009) described that these viruses are excellent for their species specificity as biopesticides.

*Spli*MNPV is a means of controlling cotton leafworm, *S. littoralis* (Boisd.). The objective of this study is to provide a clearer understanding of the product, which makes instars larvae of *S. littoralis* more susceptible to the virus (*Spli*MNPV) under laboratory condition.

MATERIALS AND METHODS

Source of test insect

S. littoralis, female moths, were collected by light trap from Al-waseel, Riyadh, Saudi Arabia. Collected moths were transferred to plastic cages with piece of cotton wetted with sugar solution (10%) for egg laying in the laboratory. The obtained eggs from the moths were kept in plastic containers until hatching.

Mass rearing of the cotton leafworm

The colony of the cotton leafworm was established and maintained at the rearing laboratory at the Economic Entomology Research Unit (EERU), Plant Protection Department, King Saudi University. The laid egg masses were transferred into plastic cups, and covered with tissue paper until egg hatching. Neonate larvae were reared in group in plastic cups with a thin layer of semi-artificial diet described by Shorey and Hale (1965). The semi-artificial diet described by Shorey and Hale (1965) contained a mixture of 250 g white bean, 20 g agar, 15 g ascorbic acid, 1.2 g sorbic acid, 80 g yeast, 5 g natrium benzoate and 1200 ml distilled water. All ingredients were cooked (except of ascorbic acid) and mixed, then poured into plastic cups (D: 6.2 cm); ascorbic acid was added when the mixture cooled down at 60°C and then poured in plastic cups and stored at 5°C.

Third instar larvae were reared individually to pupation using the same diet in plastic cups, to avoid cannibalism. Pupae were transferred to a plastic box (L: 18 cm, W: 12 cm, and H: 6.5 cm) until emergence. Five pairs of moths were transferred to each jar and supplied with 10% sugar solution for mating and producing fertilized eggs. The colony was maintained under controlled conditions of 25 ^{o}C and 60-70% RH.

Virus inocula

The virus source is the commercial product Littovir®. It was obtained from Biocontrol, Switzerland, Distributer, AlRashed est. for Trading and Agriculture, Riyadh, Saudi Arabia. Polyhedral Inclusion Body (PIB) of *Spli*MNPV was $2x10^{12}$ PIB/L (Ralf-Udo et al., 2008). Dilutions of virus suspensions were prepared, as required by dilution in a glass tubes (Lab. Glass, India).

Bioassay for determination of lethal concentrations (LC) values of *Spli*MNPV

In order to determine the lethal concentration of *Spli*MNPV at 95-99%, diet surface treatment bioassays were conducted, in which six viral concentrations of 2×10^2, 2×10^3, 2×10^4, 2×10^5 and 2×10^6 PIB/ml were tested against first and second instar larvae of *S. littoralis*. In this bioassay, the artificial diet was spread in bioassay plates (5 x 10 x 1 cm) (LICEFA, Bad Salzuflen (DE), Germany), divided into 50 cells using special cover plate. One milliter of each virus treatment was spread on the surface of artificial diet in each bioassay plate. After dryness of the viral film, fifty larvae of each instar were transferred into plate cells individually. The bioassay plate was covered by two layers of tissue paper and glass cover fixed with rubber band to prevent larvae from escaping. In untreated control treatment was used distilled water only. The experiment was incubated at 25°C and 70% R.H. Larval mortality was recorded daily up to 15 days, and LC value was calculated according to probit analysis (Finney, 1971).

Larval mortality was observed daily according to the following initial signs: gradual changes in colour (cuticula showing a pale, whitening colour) and milkiness (Tanada and Kaya, 1993).

RESULTS AND DISCUSSION

Mortality response of larvae *S. littoralis to Spli*MNPV concentrations

In order to determine the lethal concentration (95-99%) of *Spli*MNPV that kill the tested first and second instar of *S. littoralis* larvae, five successive concentrations were tested. Data presented in Table 1 shows that first instar larvae were more susceptible to the S*pli*MNPV.

The mortality rates of *S. littorali*s first instar (1^{st}) larvae (Table 1) were 0, 15.15, 16.77, 43.57, 82.44, and 96.94% respectively, at concentration levels of 2×10^2, 2×10^3, 2×10^4, 2×10^5 and 2×10^6 PIB/ml, respectively. However, second instar (2^{nd}) larvae showed to be less susceptible to the virus than the first instar larvae with the mortality rate of 0, 4, 9.39, 25.33, 43.24, and 71.63%, for the same viral concentration, respectively. No mortality was recorded in case of the untreated control. The obtained result of high rate of mortality depends on the concentration levels, is similar to that found by Ignoffo (1966).

Related to this result, Wilson et al (2000) also mentioned that second instar more susceptible to *Ac*MNPV than four instar larvae of cabbage looper, *Trichoplusiani*. Also, Cherry et al., (2000) reported that the susceptibility of *Helicoverpha armigera* depends on the larvae age when *Hear*NPV applied. Also mentioned that earlier instar has speed of kill by *As*NPV than older instar larvae of *Agrotis segetum* (Bourner et al., 1992), and *Cf*MNPV on spruce budworm larvae (Duan and Otvos, 2001). These bioassays determined *Spli*MNPV concentration, which caused percentage mortality of 90

Table 1. Mortality responds of *S.littoralis* larvae to different concentration of SpliMNPV (Means ± SE)[a,b,c].

Instars of larvae	Concentration level of virus (*Spli*MNPV)					Control
	10^2	10^3	10^4	10^5	10^6	
1st instar	15.15±5.89[a]	16.77±3.64[a]	43.57±9.75[b]	82.44±7.92[c]	96.94±1.87[d]	0±0[a]
2nd instar	4±1.33[a]	9.39±6.34[a]	25.33±13.38[b]	43.24±2.43[c]	71.63±3.31[d]	0±0[a]

[a]Concentration level of *Spli*MNPV of diet surface. 3 replicates; 50 larvae per treatment per replicates; 50 larvae untreated control per replicate. [b]Instar of *S. littoralis* are 1st and 2nd instar larvae. [c]Means within columns with the same letter are not significantly different (P>0.05) by LSD.

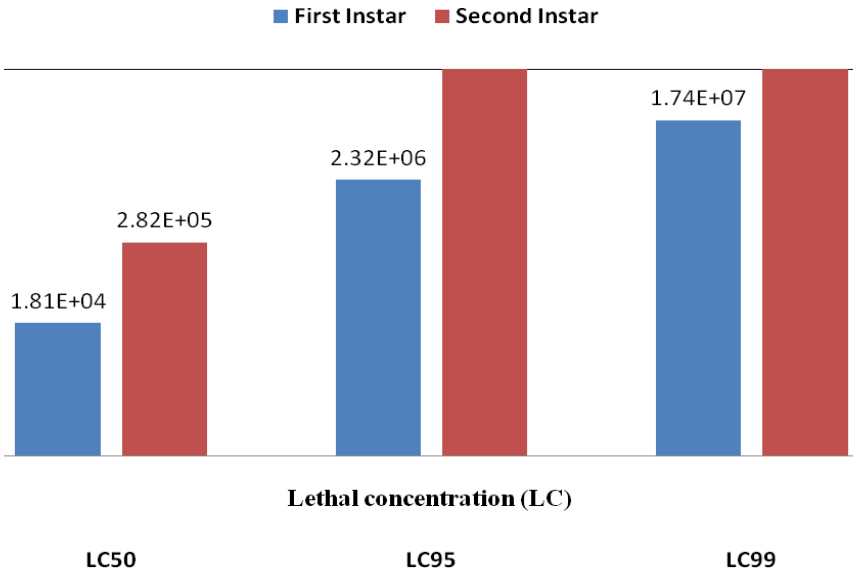

Figure 1. The susceptibility of first and second instars larvae to *Spli*MNPV measured by lethal concentration (LC) value.

and 95% based on Shapiro (1992) study. From this study, the calculated lethal concentration at 95% (LC_{95}) was $2.32x10^6$ PIB/ml, which was used for the further UV exposure tests in the presence or absence of the additives.

The obtained viral symptoms shown in Figure 1, is typical to that described by Tanada and Kaya (1993). The sign of infected larvae by virus was pale color, milky and at the end, the body of larva was melted. The death of *S. littoralis* larvae due to *Spli*MNPV appears in approximately 8-8.5 days after treatment for third instars and in approximately 3-3.5 days for neonates (Toprak et al., 2005).

The virus infection occurred when larvae ingested the Polyhedral Inclusion Body (PIB) and it reached the insect's midgut (Moore and Manousis, 1986). According to Petrik et al., (2003), releasing of *Spli*MNPV virions (OBv) in the midgut were infectious to the larvae, they entered and spread in the midgut in order to cause the infection.

The tested larvae were highly sensitive and susceptible. The highest concentration of the virus caused great mortality to the larvae rather than lower concentrations within a short time of 4 days after application. The young larvae can be killed by virus within 2-4 days (Ignoffo, 1966) cit. (Toprak et al., 2005). The potential of *S. littoralis* MNPV as a biological control has been described; also this virus has potential to control beet armyworm *S. exigua,* and fall armyworm *S. fregiperda* (Simón et al., 2004). Another virus such as *Se*MNPV is only able to cause infection in beet armyworm (*S. exiqua*) (Yanase et al., 1998b?). In case of this experiment, the susceptibility of cotton leafworm, *S. littoralis* to *Spli*MNPV occurred especially in young instar larvae. This is similar to the result of *Af*MNPV and *Ac*MNPV when this virus was applied to the neonate larvae of codling moth (Lacey et al., 2002). Virus infection in insect larvae occurs within 5-15 days. Liquefied symptom is the character of virus infection, because virus spread in tissues and organs of the larvae (El Salamouny, 2007).

In the control group, distillated water was used; it was shown that there was no mortality. It was concluded that

control (distilled water) and treatments (virus concentrations) were significantly different and LC95% of virus concentration was 2.32 x 10^6 PIBs/ml (Figure 1).

Conclusion and recommendation

First instar larvae of *S. littoralis* were highly susceptible to the *Spli*MNPV than the second instar larvae; the highest concentrations caused high rate of mortality and 2 x 10^6 is the most effective concentration of larvae mortality.

*Spli*MNPV is the greatest microbial insect pest control agent but the activity of this virus is still limited due to the degradation of its activity under field conditions (UV radiation). Therefore, there is need to have some virus protection, such as natural UV protectant (based on some plant derived materials), to increase the persistence of this virus in adverse harsh sunny conditions in the Kingdom of Saudi Arabia, for it to be effective.

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Full Length Research Paper

In-vitro efficacy of polymyxin B with rifampin, colistin and doxycycline against extensively drug resistant *Acinetobacter baumannii*

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***Acinetobacter baumannii* is an opportunistic Gram negative coccobacillus that can grow easily in moist as well as dry conditions. During the last decade, *A. baumannii* emerged as one of the most resistant opportunistic pathogens responsible for nosocomial infections including ventilator associated pneumonia. The bug remains an important and difficult to treat pathogen whose pan-drug resistant nature has created a serious challenge. This has restricted the choice of treatment modalities. Currently, it appears as if all the available antibiotics are failing against this pathogen while single antibiotic therapy is certainly not working anymore. Thus, there is a strong need, thus, to explore new regimens to combat this resistant organism. A wide range of various combinations of drugs should therefore be tested for their synergistic activity against this pathogen. This study was aimed to find some effective combinations against extensively drug resistant (XDR) *A. baumannii* by combining various antibacterials. The microdilution checkerboard titration method was used for this purpose and fractional inhibitory concentrations (FICs) were calculated. *In-vitro* synergy was found in polymyxin B-colistin (n = 3; 15%) and polymyxin B-rifampin (n = 3; 15%) combinations. Only additive effect was noted with polymyxin B-doxycycline (n = 12; 60%), polymyxin B-rifampin (n = 11; 55%), and polymyxin B-colistin (n = 13; 65%). However, antagonism was detected in the polymyxin B-rifampin combination in one of the 20 strains evaluated for the purpose. Polymyxin B in combination with rifampin and colistin may be exploited against XDR *A. baumannii*. Synergy between polymyxin B and colistin have been demonstrated in only 15% of strains, this fully warrants the testing of more combinations.**

Key words: *Acinetobacter baumannii*, extensively drug resistant, fractional inhibitory concentration.

INTRODUCTION

The genus *Acinetobacter* is a ubiquitous group of micro-organisms (Giamarellou et al., 2008) and is found in the environment. *Acinetobacter baumannii* was considered to be a pathogen of low grade pathogenecity and was ignored whenever isolated from clinical specimens until the 1970s (Zarrilli et al., 2009). It has recently emerged as one of the most troublesome nosocomial pathogens globally and has become a major cause of health care-associated and community-acquired infections (Talbot et al., 2006; Davis et al., 2005). Management of MDR/XDR

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Abbreviations: *A. baumannii*, *Acinetobacter baumannii*; **API 20NE,** analytical profile index 20 Non-Enterobactericeae; **ATCC,** American type culture collection; **FIC,** fractional inhibitory concentration; **FICI,** fractional inhibitory concentration index; **MBC,** minimal bactericidal concentration; **MDR,** multi-drug resistant; **MIC,** minimal inhibitory concentration; **PDR,** pan-drug resistant; **XDR,** extensively drug resistant.

A. baumannii infections is a big challenge for physicians and clinical microbiologist. The organism's capability to survive in hospital settings and to persist for long periods of time on various surfaces makes it a frequent cause of healthcare associated infections. Another problem regarding *A. baumannii* is its ability to cause a wide spectrum of infections which include wound infections, bacteremia, pneumonia, urinary tract infections, etc (Manchanda et al., 2010). *A. baumannii* is proven to have the capability to form biofilms that is believed to play central part in the process of colonization (Pour et al., 2011).

During the early 1970s, the clinical isolates of *A. baumannii* were usually susceptible to various antibiotic classes (Bergogne-Berezin and Towner, 1996). However, since 1975, increasing resistance to almost all groups of antibacterials started appearing (Manchanda et al., 2010; Montefour et al., 2008). From the year 2000 to date, various combinations of antibiotics have been evaluated for their synergistic activity or otherwise to combat this resistant pathogen (Rodriguez-Hernandez et al., 2000; Montero et al., 2004; Saballs et al., 2006; Tan et al., 2011).

The focus of this study was to determine the synergistic effect of polymyxin B with either of the antibiotics: rifampin, colistin and doxycycline by the checkerboard microtitration technique.

MATERIALS AND METHODS

Bacterial strains

The extensively drug resistant *A. baumannii* (XDR-AB) phenotype was identified as an *A. baumannii* strain, resistant to all classes of the traditional antibiotics except tigecycline and polymyxin B using the disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI, 2012) recommendations. These strains were isolated from various clinical samples, and collected from various patients hospitalized in Lahore, Pakistan in 2012. The strains were identified by their morphological and biochemical characteristics and, later by using API 20-NE (BioMerieux, France). The selected strains were stored in microbanks at -80°C.

Antimicrobial agents and minimal inhibitory concentration determination

The base materials of antimicrobial agents used in combinations were: polymyxin B (Glaxosmith Kline pharmaceuticals), rifampin (Pacific pharmaceuticals), colistin (Forest pharmaceuticals) and doxycycline (Pfizer Global pharmaceuticals). Stock solutions of antibiotics were prepared in their respective solvents (water for polymyxin B, colistin and doxycycline, methanol for rifampin) according to the CLSI 2012 guidelines and stored at -20°C for one week. Minimal inhibitory concentrations (MICs) of all strains for each antibiotic were determined by a standard agar dilution method. Bacterial inoculum equivalent to 0.5 McFarland ($5x10^8$) was prepared and diluted 1:10 to achieve the final concentration of $5x10^7$ CFU/ml. The concentration range of various antibiotics was prepared; 0.125 to 8.0 µg/ml (polymyxin B and colistin), 0.5 to 64 µg/ml (doxycycline), 0.06 to 64 µg/ml (rifampin) in Mueller Hinton agar. The plates were inoculated with the bacterial suspensions using multipoint inoculators (MAST Diagnostics UK). The same was incubated for 24 h at 37°C. Lowest concentration at which bacterial growth was inhibited was noted after incubation (CLSI 2012). *Escherichia coli* ATCC 25922 was used as a control strain. MIC results were read and interpreted according to the CLSI breakpoint criteria for *A. baumannii*. Since there are no CLSI interpretation criteria of rifampin, available, relevant to *A. baumannii*, the breakpoints for this antibiotic were based on the MIC standards of CLSI for Gram positive bacteria (CLSI, 2012).

Synergy testing

The synergistic activity of the antibiotic combinations was determined using the microdilution checkerboard titration method. The range of concentrations was chosen according to the previously determined MIC of each antibiotic for each isolate. Concentrations used ranged from 0.06xMIC to 8xMIC for each antibiotic. The interpretation of the checkerboard synergy testing results was determined by the method of Orhan et al. (2005). FICs and FICI were calculated for each antimicrobial combination using the formulas below:

$$\Sigma FIC \text{ or } FICI = FIC\ A + FIC\ B$$

Where,

$$FIC\ A = \frac{\text{MIC of drug A in the combination}}{\text{MIC of drug A alone}}$$

and

$$FIC\ B = \frac{\text{MIC of drug B in the combination}}{\text{MIC of drug B alone}}$$

The combination was considered synergistic when the ΣFIC was ≤0.5, additive when the ΣFIC was >0.5 to ≤1.0 indifferent when the ΣFIC was >1.0 to <2, and antagonistic when the ΣFIC was ≥2 (Orhan et al., 2005).

RESULTS

The detail of the various clinical materials from which *A. baumannii* was originally isolated is shown in Figure 1. The highest number of *A. baumannii* strains were isolated from central venous catheter tips (n=9; 45%) followed by pus (n=8; 40%), urine (n=1; 5%), high vaginal swab (n=1; 5%) and body fluids (n=1; 5%). Major isolation from CVC tips is mainly due to the capability of *A. baumannii* to survive on dry as well as moist conditions and also grow well on tubings of catheters and ventilators. The second highest source was pus which indicates its ability to colonize open wounds and from where it can invade into the blood stream to cause life threatening bacteremia. All isolates were resistant to all the classes of antimicrobials except polymyxin B (100% susceptible) and doxycycline (85% susceptible). However, all were resistant to colistin irrespective of their susceptibility or otherwise to polymyxin B and doxycycline. Their susceptibility patterns are shown in the Figure 2.

MIC_{90} for polymyxin B, colistin, doxycycline and rifampin was 1, 8, 64 and 2 µg/ml respectively. Their MIC ranges and susceptibility rates are shown in Table 1. MIC for ATCC *E. coli* 25922 for polymyxin B, colistin, doxycy-

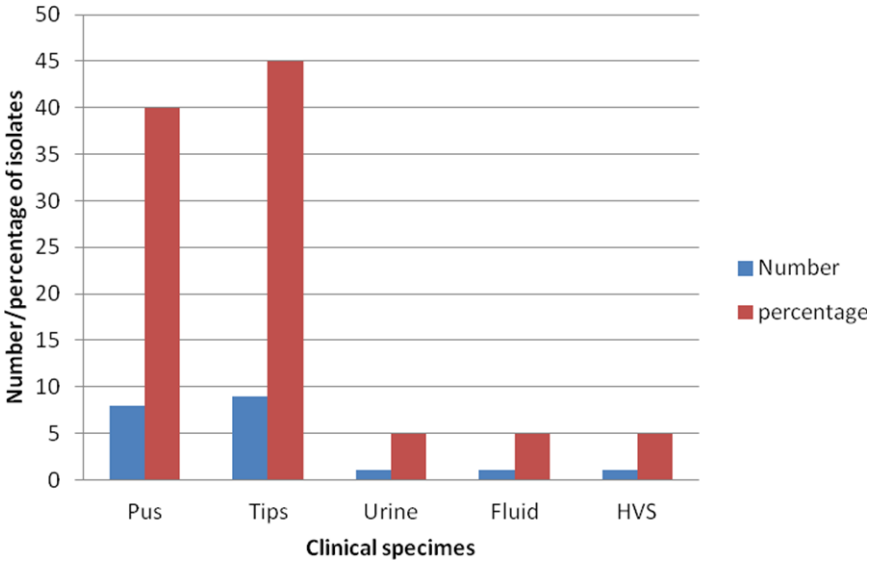

Figure 1. Sources of XDR *A. baumannii* isolates.

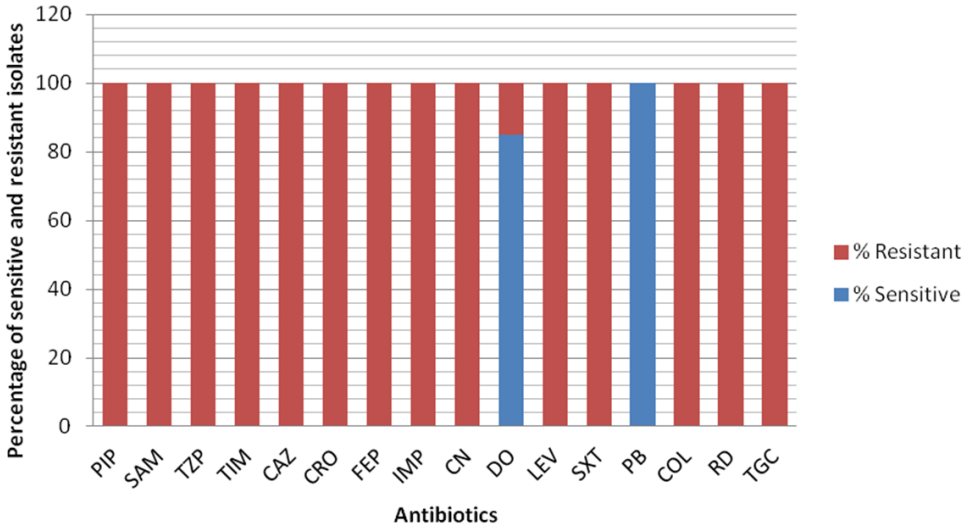

Figure 2. Susceptibility of *A. baumannii* isolates to various antibiotics. PIP: piperacillin, SAM: ampicillin-sulbactam, TZP: piperacillin-tazobactam, TIM: ticarcillin-clavulanic acid, CAZ: ceftazidime, CRO: ceftriaxone, FEP: cefepime, IMP: imipenem, CN: gentamicin, DO: doxycycline, LEV: levofloxacin, SXT: cotrimoxazole, PB: polymyxin B, COL: colistin, RD: rifampin, TGC: tigecycline.

cline, and rifampin are also shown in Table 1. MICs of XDR *A. baumannii* for four tested antibiotics by broth microdilution method are shown in Table 2.

The results of the microtitration checkerboard method are shown in Table 3. Three isolates (15%): AB-02, AB-14, and AB-20 strains showed synergistic effect in polymyxin B-rifampin combination while 55% additive effect was seen in this combination. In the case of AB-02 and AB-20, the MIC of polymyxin B in combination was reduced to one fourth as compared to the individual MICs against these isolates. The MIC of rifampin was reduced to one eighth and one fourth for AB-02 and AB-20 respectively. For the isolate AB-14, the MIC of polymyxin B and rifampin was reduced to one eighth and one fourth respectively.

Indifference was detected in 25% of the isolates while AB-08 showed antagonism (5%) in this combination of antimicrobials. The MIC of polymyxin B was increased

Table 1. Minimal inhibitory concentrations (MICs), susceptibility rates and quality control (QC) ranges of XDR *A. baumannii* (n=20) and *E. coli* ATCC 25922 versus 4 different antibiotics.

Antibiotic	XDR *A. baumannii* (n=20)						*E. coli* ATCC [a] 25922 (n=1)	
	MIC (µg/ml)			Susceptibility rate (%) [b]			MIC (µg/ml)	MIC QC Ranges[d] (µg/ml)
	Range[c]	MIC_{50}	MIC_{90}	Susceptible	Intermediate	Resistant		
Polymyxin B	0.5 to 1.0	0.5	1.0	100	-	-	0.5	0.25-2.0
Rifampin	1.0 to 8.0	2.0	2.0	-	-	-	8.0	4.0-16
Colistin	4.0 to 8.0	4.0	8.0	-	-	100	1.0	0.25-2.0
Doxycycline	1.0 to 64	1.0	64	85	-	15	1.0	0.5-2.0

a: American Type Culture Collection; b: susceptibility was interpreted according to Clinical Laboratory Standard (CLSI) 2012 guidelines. c: Susceptibility range given by CLSI 2012 guidelines. d: Quality control ranges provided by CLSI 2012 guidelines against ATCC reference strain.

Table 2. Minimal inhibitory concentrations (MICs) of XDR *A. baumannii* (n=20) versus 4 different antibiotics by broth microdilution method.

Isolate no.	MIC (µg/ml)			
	Polymyxin B	Colistin	Doxycycline	Rifampin
AB-01	0.5	1.0	0.5	1.0
AB-02	0.5	2.0	32	2.0
AB-03	1.0	2.0	0.5	1.0
AB-04	0.5	1.0	0.25	1.0
AB-05	0.5	1.0	0.25	1.0
AB-06	0.5	2.0	0.5	1.0
AB-07	1.0	1.0	0.5	1.0
AB-08	0.5	2.0	0.25	1.0
AB-09	0.5	1.0	0.5	2.0
AB-10	0.5	1.0	0.5	1.0
AB-11	0.5	2.0	0.25	2.0
AB-12	0.5	4.0	0.5	1.0
AB-13	0.5	2.0	0.5	1.0
AB-14	0.5	2.0	32	8.0
AB-15	0.5	2.0	0.5	2.0
AB-16	1.0	2.0	0.25	2.0
AB-17	0.5	2.0	0.25	1.0
AB-18	0.5	2.0	0.25	1.0
AB-19	0.25	2.0	0.5	1.0
AB- 20	1.0	2.0	64	8.0

four times for AB-08 in combination with rifampin and showed antagonism. In polymyxin B-colistin combination, three strains: (15%) AB-06, AB-19, and AB-20 showed synergistic effect. In all the three isolates showing synergism, MIC of colistin was reduced by one fourth in combination with polymyxin B. For AB-19, MIC was dropped to one eighth, while for the rest of the two isolates it was reduced to one fourth in combination.

Additive effect in this combination was found to be 65%, and indifference was 20% while there was no antagonism detected in this combination. In the case of polymyxin B-doxycycline combination, no synergism or antagonism was shown. Additive effect was 60% while the remaining 40% was indifference shown in this combination.

DISCUSSION

A. baumannii has become a major challenge due to its multiple drug resistance. In recent years, more and more cases of mortality and morbidity due to MDR/XDR *A. baumannii* have come to light (Queenan et al., 2012; Karaiskos et al., 2013).

Several studies have been done, both *in-vitro* and *in-vivo*, to demonstrate the synergism of two or more antibiotics in combination against resistant pathogens (Gunderson et al., 2003; Lim et al., 2011; Fiori and Van

Table 3. Synergy test results of antibiotic combination by checkerboard microtitration method.

Strain no.	PB - RD				PB - COL				PB - DO			
	Conc. of PB	Conc. of RD	∑ FIC	Activity	Conc. of PB	Conc. of COL	∑ FIC	Activity	Conc. of PB	Conc. of DO	∑ FIC	Activity
AB-01	MIC	1/2 MIC	1.5	ID	1/2 MIC	1/2 MIC	1.0	ADD	1/2 MIC	MIC	1.5	ID
AB-02	1/4 MIC	1/8 MIC	0.375	S	1/4 MIC	1/2 MIC	0.75	ADD	1/2 MIC	1/4 MIC	0.75	ADD
AB-03	1/2 MIC	1/4 MIC	0.75	ADD	1/2 MIC	MIC	1.5	ID	1/2 MIC	1/4 MIC	0.75	ADD
AB-04	MIC	1/2 MIC	1.5	ID	1/2 MIC	1/4 MIC	0.75	ADD	1/2 MIC	MIC	1.5	ID
AB-05	1/2 MIC	1/8 MIC	0.625	ADD	1/2 MIC	1/2 MIC	1.0	ADD	1/2 MIC	MIC	1.5	ID
AB-06	1/2 MIC	1/4 MIC	0.75	ADD	1/4 MIC	1/4 MIC	0.49	S	1/2 MIC	1/2 MIC	1.0	ADD
AB-07	1/2 MIC	1/4 MIC	0.75	ADD	1/2 MIC	MIC	1.5	ID	MIC	1/2 MIC	1.25	ID
AB-08	4 MIC	1/2 MIC	4.5	AG	1/2 MIC	MIC	1.5	ID	1/2 MIC	MIC	1.5	ID
AB-09	1/8 MIC	1/2 MIC	0.62	ADD	1/2 MIC	MIC	1.5	ID	1/2 MIC	1/2 MIC	1.0	ADD
AB-10	1/2 MIC	1/4 MIC	0.75	ADD	1/2 MIC	1/4 MIC	0.75	ADD	1/2 MIC	1/4 MIC	0.75	ADD
AB-11	MIC	1/8 MIC	1.125	ID	1/2 MIC	1/2 MIC	1.0	ADD	1/4 MIC	1/2 MIC	0.75	ADD
AB-12	MIC	1/4 MIC	0.75	ADD	1/8 MIC	1/2 MIC	0.62	ADD	1/8 MIC	1/2 MIC	0.62	ADD
AB-13	1/2 MIC	1/4 MIC	0.75	ADD	1/8 MIC	1/2 MIC	0.62	ADD	1/2 MIC	1/2 MIC	1.0	ADD
AB-14	1/8 MIC	1/4 MIC	0.37	S	1/2 MIC	1/2 MIC	1.0	ADD	1/4 MIC	1/2 MIC	0.62	ADD
AB-15	1/8 MIC	1/2 MIC	0.62	ADD	1/2 MIC	1/4 MIC	0.75	ADD	1/4 MIC	MIC	1.25	ID
AB-16	1/16 MIC	1/2 MIC	0.56	ADD	1/2 MIC	1/2 MIC	1.0	ADD	1/4 MIC	MIC	1.25	ID
AB-17	MIC	1/4 MIC	1.25	ID	1/2 MIC	1/4 MIC	0.75	ADD	1/8 MIC	MIC	1.125	ID
AB-18	MIC	1/4 MIC	1.25	ID	1/2 MIC	1/4 MIC	0.75	ADD	1/2 MIC	1/2 MIC	1.0	ADD
AB-19	1/2 MIC	1/2 MIC	1.0	ADD	1/8 MIC	1/4 MIC	0.37	S	1/2 MIC	1/4 MIC	0.75	ADD
AB- 20	1/4 MIC	1/4 MIC	0.5	S	1/4 MIC	1/4 MIC	0.5	S	1/2 MIC	1/8 MIC	0.62	ADD

AB: *A. baumannii*, S: synergism, ID: indifference, ADD: additive effect, AG: antagonism, PB: polymyxin B, RD: rifampin, COL: colistin, DO: doxycycline, FIC: fractional inhibitory concentration, Conc.: concentration.

Dijck, 2012). Antimicrobial resistance in *A. baumannii* has considerably increased in the recent past (Lockhart et al., 2007). In our study, 15% of XDR *A. baumannii* strains showed resistance to doxycycline. In a study done in 2006, doxycycline resistance was reported to be 22% for *A. baumannii* (Elmanama, 2006).

In yet another study, 8% strains showed resistance to doxycycline (Timurkaynak et al., 2006). All strains were susceptible to polymyxin B while all of them were resistant to colistin. The susceptibility of XDR *A. baumannii* to polymyxin B is found to be 100% in other studies as well (Kuo et al., 2012; Lim et al., 2011). Colistin resistance has been reported from various regions of the world. Colistin resistance was found to be 40.6% in Spain (Arroyo et al., 2009). In Kuwait, colistin resistance was found to be 12% (Al-Sweih et al., 2011). In a study done by Chang et al. (2012) 10.4% colistin resistance was found. In another study colistin resistance was found to be 7.1% (Rodriguez et al., 2010). Although the incidence of colistin resistance is low worldwide in contrast to our findings, it has been proved through *in-vitro* experiment that the rate of resistance development to colisitin is rapid among *Acinetobacter* (Tan et al., 2007). Colistin is being used against MDR and XDR Gram negative organisms especially *Pseudomonas* and *A. baumannii* due to its relatively low neurotoxicity

and nephrotoxicity as compared to polymyxin B and aminoglycosides. So far no heteroresistance has been reported in polymyxin B, this could be the main reason behind the 100% susceptibility to it (Mammina et al., 2012; Adams et al., 2009; Li et al., 2006) . In this study we intentionally took XDR strains of *A. baumannii* because these organisms have become a major problem and are untreatable in our setup. These isolates were resistant to colistin, however this might not represent the actual percentage of colistin resistance in our setup, as we did not take random isolates. In addition to their resistance to various antimicrobials, all XDR *A. baumannii* were resistant to tigecycline as well. Decreased susceptibility to tigecycline is linked with efflux pumps, over expressed by MDR/XDR *A. baumannii* (Ruzin et al., 2007). Tigecycline resistance among MDR *A. baumannii* was found to be 78% by Li et al. (2007) and 66% by Navon-Venezia et al. (2007). Al-Sweih et al. (2011) in contrast, reported 13.6% tigecycline resistance among 250 *Acinetobacter* isolates. The most probable reason for these contrasting results to our study could be the XDR strains in comparison with the susceptible and MRD strains which were used in the above mentioned studies. It is reported that *A. baumannii* showing resistance to multiple antimicrobial agents are notorious for reduced susceptibility to tigecycline (Ruzin et al., 2007).

Rifampin has been reported in various studies to have synergistic activity with different antibiotics against *A. baumannii* (Pachon-Ibanez et al., 2010). Thus synergistic activity of polymyxin B-rifampin in combination was found to be 15% in our study, although these strains were resistant to rifampin alone. Tan et al. (2011) reported 19% synergism in polymyxin B-rifampin combination against *A. baumannii* by the checkerboard microtitration method and 56% by the time kill assay. These results are in accordance with our study when compared with the checkerboard microtitration method. Lim et al. (2011) found the highest synergistic activity in a polymyxin B-rifampin combination out of all the tested combinations (41.9%) by time kill assay. Carl et al. (2010) reported 60% bactericidal activity in polymyxin B-doripenem-rifampin triple combination against MDR *A. baumannii* in a time kill assay. All MDR *A. baumannii* strains were resistant to carbapenems and rifampin when tested alone (Urban et al., 2010). Manikal et al. (2000) found a 50% synergistic effect of polymyxin B-rifampin combination against *A. baumannii* and a 50% additive effect by the checkerboard microtitration method. Antagonism was also noted in the polymyxin B-rifampin combination and was found to be 5%, however the additive effect was found to be 55%. In contrast to our study, none of the above mentioned studies reported antagonism of polymyxin B with rifampin (Tan et al., 2011; Lim et al., 2011; Urban et al., 2010).

Polymyxin B-colistin combination also showed 15% synergism. To our knowledge, a polymyxin B-colistin combination has not yet been tested against XDR *A. baumannii*. The highest additive effect was noted in this combination (65%). Although both the antibiotic agents have the same site of action, more extensive research is needed to find an effective combination showing higher rates of synergism against XDR *A. baumannii*. In the case of the polymyxin B-doxycycline combination, only additive effect/indifference (60/40%) was found. Our results are in accordance with another study in which doxycycline in combination with other antibiotics against *A. baumannii* showed either additive effect or indifference (Timurkaynak et al., 2006). There are several reports about the synergistic activity of polymyxin B when used in combination therapies with imipenem, rifampin and azithromycin (Yoon et al., 2004; Wareham and Bean, 2006). It is noted that the results of synergy tests are highly strain and method dependent and *in vitro* synergy may or may not translate into *in-vivo* benefit (Pankey and Ashcraft, 2009).

It is concluded that polymyxin B-rifampin and polymyxin B-colistin combinations have demonstrated synergism against XDR *A. baumannii* by the method used, that is, checkerboard microtitration. However, the gold standard method for synergy testing is time-kill assay, that is, our study limitation. More antibiotic combinations should be tested e.g., tigecycline in order to find more effective combinations against XDR *A. baumannii*.

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Full Length Research Paper

Production, purification and characterization of cellulase from *Streptomyces* sp.

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High cellulase producing *Streptomyces* strain C188 was isolated from a Saudi Arabia soil sample and identified as *Streptomyces longispororuber* by 16S rDNA sequencing. Enzyme productivity by this strain in carboxymethyl cellulase liquid medium reached 8540 U/L after 96 h of incubation at 30°C. Cellulase productivity in tested strain was improved (25084 U/L) by supplementation of the carboxymethyl cellulase liquid medium with 1% corn steep liquor and pH 6.5 (maintained throughout the incubation period using 0.05 M phosphate buffer). Purification of cellulase enzyme was carried out by ammonium sulfate precipitation, diethylaminoethyl cellulose and Sephadex G-75 gel filtration chromatography. The final preparation had 13.5% activity recovery and approximately 38.5-fold purification. The purified enzyme migrated in a single band with molecular weight of 42 kDa on SDS-PAGE. Maximum enzymatic activity was observed at pH 6-6.5 and 50°C, while maximum stability was obtained at pH 6.5 and up to 60°C.

Key words: Cellulase, *Streptomyces longispororuber*, purification.

INTRODUCTION

Cellulase is one of the most important industrial enzymes. Cellulases have attracted interest because of their diversity of applications. In 2001, the world market for enzymes was over $1.5 billion; this was doubled by the year 2008. The United States and Europe each consume 30% of the world output of enzymes. Approximately 75% of industrial enzymes are used for hydrolysis and depolymerization of complex natural substances (Kirk et al., 2002).

Major industrial applications of cellulases are in the textile industry for "biopolishing" of fabrics such as production of the stonewashed look of denims, and in household laundry detergents to improve fabric softness and brightness. Moreover, they are used in animal feeds to improve nutritional quality and digestibility, in processing of fruit juices, and in baking; de-inking of paper is yet another emerging application (Ponnambalam et al., 2011).

In addition, cellulase enzymes are involved in enzymatic hydrolysis of cellulose, one of the most abundant organic materials that can be converted to products with significant commercial interest. Bioconversion of cellulose to monomeric sugars has been intensively studied as researchers seek to produce bioethanol and bio-based products, food and animal feeds, and many valuable chemicals (Barros et al., 2010).

Cellulase enzymes are produced from plant, animal and microbial sources. For commercial production, microbial enzymes have the enormous advantage of being scalable to high-capacity production by established fermentation techniques (Tahtamouni et al., 2006). A wide variety of microorganisms are known for their ability to

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Abbreviations: CMC, Carboxymethyl cellulase; **CSL,** corn steep liquor.

produce cellulase enzymes. *Streptomyces* sp. is one of the best known enzyme producers (El-Sersy et al., 2010)

This study focused on isolation of a high cellulase-producing *Streptomyces* strain from soil samples collected in Saudi Arabia, and improvement of enzyme productivity by supplementation with organic nitrogen sources and pH optimization. The enzyme was purified and characterized.

MATERIALS AND METHODS

Isolation of *Streptomyces* isolate

A high cellulase-producing *Streptomyces* strain C188 was isolated from a soil sample in Saudi Arabia as described by Jaradat et al. (2008). The strain was purified by streak-plating on starch-nitrate agar (20 g soluble starch, 2.0 g KNO_3, 1.0 g K_2HPO_4, 0.5 g $MgSO_4$, 0.5 g NaCl, 3.0 g $CaCO_3$, 0.01 g $FeSO_4$, 0.01 g $MnCl_2$, 0.01 g $ZnSo_4$, 20 g agar per liter).

Growth condition and enzyme production

Enzyme productivity by the tested strain was determined as described by El-Sersy et al. (2010). In brief, a spore suspension from 4 to 5 days old cultures was prepared in normal saline (0.85% NaCl solution) and used as an inoculum for a final count of 10^3 CFU/mL in 50 mL carboxymethyl cellulose (CMC) liquid medium (1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g NaCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot 7H_2O$, 0.3 g NH_4NO_3, 10.0 g CMC per liter, pH 7) in 250 mL Erlenmeyer flasks and incubated in a rotary shaking-incubator (S19R-2, Sheldon Mfg, USA) at 250 rpm and 30°C for 96 h. After incubation, a known volume of culture broth was centrifuged at 12,000 × *g* for 20 min and the cell pellet was washed twice with distilled water and dried in hot air oven at 100°C to a constant weight. The dry cell weight per liter of culture broth was used to determine microbial growth. The activity of cellulase enzyme in the culture filtrate was determined as described in the procedure below.

Enzyme assay

Cellulase activity was quantified according to Miller (1959). A reaction mixture composed of 0.2 mL crude enzyme solution and 1.8 mL 0.5% CMC in 50 mM sodium phosphate buffer (pH 7.0) was incubated at 50°C in a shaking water bath (GFL 1083, Germany) for 30 min. The reaction was terminated by adding 3 mL 3,5-dinitrosalicylic acid reagent (Sigma Aldrich, USA). The color was developed by boiling the mixture for 5 min. Optical densities were measured at 575 nm by using spectrophotometer (Labomed UVD-3200, UK) against a blank containing all the reagents minus the crude enzyme. Results were interpreted in terms of enzyme activity in which one unit (U) of enzyme activity was defined as the amount of enzyme that liberates 1 µmol glucose per minute under the above assay conditions.

Genetic identification of the selected *Streptomyces* isolate by 16S rDNA analysis

Isolation of the whole genomic of the selected strain was carried out as described by Pospiech and Neumann (1995). PCR amplification of the 16S rDNA gene was conducted using 2 primers, StrepF: 5-ACGTGTGCAGCCCAAGACA-3 and StrepR: 5-ACAAGCCCTGGAAACGGGGT-3 (Edwards et al., 1989). The PCR mixture contained 30 pmol each primer, 100 ng chromosomal DNA, 200 µM dNTPs and 2.5 units Taq polymerase in 50 µL polymerase buffer. Amplification was conducted in an automated thermocycler (Techne, TC-5000, UK) for 30 cycles of 1 min at 94°C, 1 min annealing at 53°C and 2 min extension at 72°C. Amplified products were analyzed by agarose gel electrophoresis and the remaining mixture was purified using QIA quick PCR purification reagents (Qiagen, USA). The 16S rDNA gene was sequenced on both strands via the dideoxy chain termination method (Sanger et al., 1977) with a Terminator Cycle Sequencing kit (Applied Biosystems 3500 Genetic Analyzer, Applied Biosystems, USA). The BLAST search program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to assess the degree of DNA similarity and sequence alignment. Sequence alignment was performed and the neighbor joining phylogenetic tree was constructed using the TREE VIEW program.

Factors affecting cellulase production

The effect of organic nitrogen source on growth and cellulase production was investigated by supplementing the CMC fermentation medium with different organic nitrogen sources and inoculating with 0.5 mL of the spore suspension (10^5 CFU/mL). After incubation, the supernatant was assayed for cellulase activity. Microbial growth was quantified by dry cell weight determination.

To study the effect of initial pH, a set of flasks containing CMC broth with initial pH values from 5.0 to 9.0 was prepared and inoculated with the test strain. To determine the effect of pH maintenance over the incubation period, the ingredients of the CMC medium were dissolved in 0.05 M phosphate buffer at varying pH values. At the end of the incubation period, microbial growth and enzyme activity were evaluated as described above.

Purification of the cellulase enzyme

The strain was cultivated in modified CMC broth at 30°C for 4 days. The microbial culture was centrifuged (12000 × *g* for 30 min at 4°C); the supernatant was collected and the enzyme was precipitated with ammonium sulfate salt (pH 5). The precipitated fraction of the enzyme between 30 and 70% ammonium sulfate saturation was collected by centrifugation (10000 × *g*, 20 min, 4°C), then dissolved in 0.02 M sodium acetate buffer (pH 4.8) and dialyzed against the same buffer. The prepared solution, a crude enzyme preparation, was applied to a column (2.5 × 15 cm) of DEAE-cellulose (Sigma Aldrich) pre-equilibrated with the same buffer at 4°C. Elution from the column was performed with buffer containing increasing concentrations of NaCl from 0.05 to 1 M. Approximately 3 mL fractions were collected in test tubes and the enzymatic activity of each fraction was estimated. The fraction containing enzymatic activity was purified by gel filtration after dialysis against distilled water, followed by 0.02 M sodium acetate buffer, pH 5.2 for 24 h at 4°C. The clear sample, obtained after centrifugation, was loaded onto a column (2.5 × 30 cm) of Sephadex G-75 (Sigma Aldrich) and allowed to diffuse. The eluate was collected in 2-mL fractions and monitored for enzyme activity. The active fractions were collected and dialyzed overnight against the same buffer. The homogeneity of the purified enzyme and its molecular mass were measured by SDS-PAGE.

Protein determination

Quantitative determination of protein was carried out by adding 5 mL Bradford dye reagent (0.1 g/L Sigma Coomassie Brilliant Blue G-250, 1.6 mol/L phosphoric acid, 0.8 mol/L ethanol) to 100-µL aliquots of the protein samples, incubated at room temperature for 20 min**,** and absorbance was measured at 280 nm (Bradford, 1976).

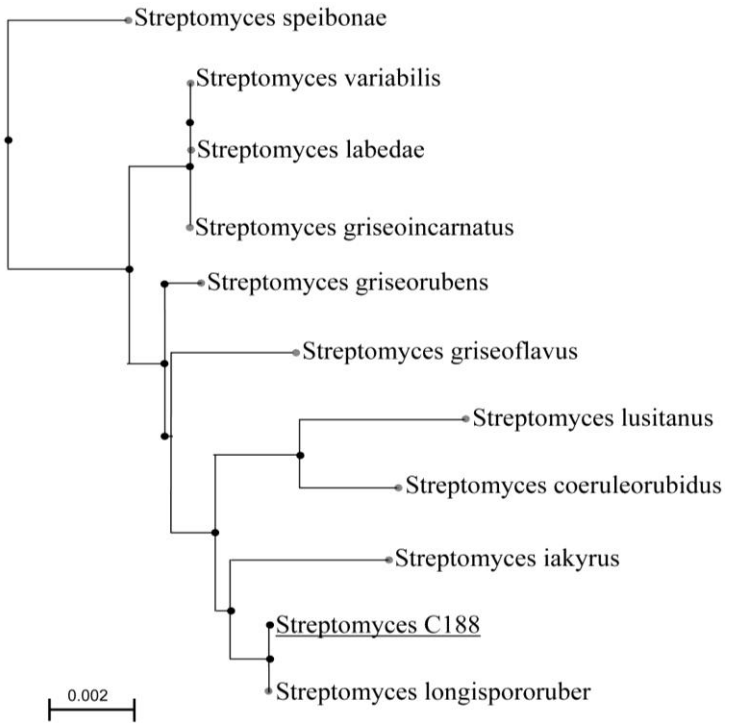

Figure 1. Neighbor-joining tree showing the phylogenetic position of *Streptomyces* C188 and related species based on partial 16S rRNA gene sequences. The scale bar indicates a 0.002 substitution per nucleotide position.

Polyacrylamide gel electrophoresis

SDS-PAGE of partially purified and pure enzyme samples was carried out as described by Sambrook et al. (1989). The proteins were stained with 0.25% Coomassie brilliant blue G-250 in aqueous solution containing 25% methanol and 5% glacial acetic acid and destained with the same solution without dye. The molecular mass of the purified enzyme was compared to standard proteins of molecular weights (MWs) between 36 and 118 kDa.

Factors affecting cellulase activity and stability

To study the effect of temperature on enzyme activity, 900 µL of 1% CMC in 20 mM phosphate buffer (pH 7) was mixed with 100 µL pure enzyme and the optimum temperature for cellulase activity was determined between 30 and 80°C. Enzyme thermostability was studied by incubating the CMC solution in 50 mM phosphate buffer (pH 7) at temperatures from 30 to 90°C for 30 min, and then residual cellulase activity was determined.

The effect of pH on enzyme activity was studied by preparing 1% CMC solution in 50 mM phosphate buffer adjusted at pH from 5 to 9, in which 900 µL samples were mixed with 100 uL of pure enzyme samples, and enzyme activity was evaluated as described. Enzyme stability at various pH levels was evaluated by preparing the pure enzyme in 50 mM phosphate buffer at pH from 5 to 9, incubated at 25°C for 30 min, and then the residual activity was determined.

Statistical analysis

Statistically significant differences between means were tested by analysis of variance and student's *t*-test by using InStat-ANOVA software. The differences between means were considered statistically significant when the test yielded a value $P < 0.05$.

RESULTS

Genetic identification of the tested *Streptomyces* isolate (C188)

The 1,426 bp sequence obtained from the test strain was aligned with all presently available 16S rRNA gene sequences in the GeneBank databases. The results show high similarity (98 to 100%) to the *Streptomyces* 16S rRNA genes. In addition, the tested nucleotide sequence of *Streptomyces* C188 shows 100% similarity to *Streptomyces longispororuber* (Accession no. NR_041147.1). The phylogenetic tree (Figure 1) revealed that *S. longispororuber is* the closest isolates in similarity to the tested strain.

Factors affecting cellulase productivity by test strain

Enzyme production by the test strain in CMC liquid medium reached 8540 U/L after 96 h incubation at 30°C. Improvement of cellulase productivity by *Streptomyces* C188 was carried out by studying the effect of organic nitrogen sources and pH on enzyme production.

Table 1. Effect of different organic nitrogen sources on growth and cellulase productivity by *S. longispororuber* in CMC broth medium at 30°C for 96 h.

Nitrogen source (concentration, %)	Cellulase productivity (U/L ± SD)	Dry cell weight (g/L)	Specific enzyme productivity (U/g)
Control	8540 ± 165	6.8	1255.9
CSL* (1%)	15490 ± 374	8.4	1844.1
CSL* (2%)	10510 ± 212	9.2	1142.4
CSL* (3%)	11480 ± 325	10.6	1083.0
Peptone (1%)	240 ± 66	4.8	50.0
Peptone (2%)	290 ± 74	5.6	51.8
Tryptone (1%)	380 ± 76	5.8	65.5
Tryptone (2%)	530 ± 87	6.7	79.1
Yeast extract (1%)	920 ± 94	7.1	129.6
Yeast extract (2%)	640 ± 62	7.3	87.7
CSL* (1%) + Peptone (1%)	6570 ± 184	8.8	746.6
CSL* (1%) + Tryptone (1%)	10320 ± 158	9.6	1075.0
CSL* (1%) + Yeast extract (1%)	8680 ± 246	9.2	943.5

CSL* = Corn steep liquor

Table 2. Effect of initial pH on growth and cellulase productivity by *S. longispororuber* in modified CMC broth at 30°C and 96 h.

pH	Cellulase productivity (U/L ± SD)	Dry cell weight (g/L)	Specific productivity (U/g)
5	1026 ± 105	4.8	213.8
5.5	11482 ± 392	6.4	1794.1
6	11482 ± 328	6.6	1739.7
6.5	15668 ± 404	7.1	2206.8
7	8551 ± 275	7.2	1187.6
7.5	6310 ± 245	7.4	852.7
8	4656 ± 212	6.8	684.7
8.5	3443 ± 196	6.2	555.3
9	2541 ± 146	5.6	453.8
9.5	1387 ± 124	5.2	266.7

Effect of organic nitrogen source

The highest level of cellulase productivity (15490 U/L) by *Streptomyces* C188 was obtained in the presence of 1% corn steep liquor (Table 1). Therefore, the modified CMC broth containing 1% corn steep liquor was used for further studies.

Effect of pH

The modified CMC broth medium was prepared with a wide range of initial pH (5-9.5). A good cellulase productivity was obtained at pH 5.5-7.5, with the highest level (15668 U/L) at initial pH 6.5 (Table 2).

Further studies were performed with 0.05 M phosphate buffer (pH 5.5-7.5) to control the pH of the fermentation medium throughout the incubation period. The greatest enzyme productivity (25084 U/L) was obtained when the fermentation pH was maintained over the incubation period at pH 6.5 (Table 3).

Purification of cellulase enzyme

The purification of cellulase enzyme is summarized in Table 4. The final preparation had 13.5% activity recovery and approximately 38.5-fold purification. The results of SDS-PAGE showed that the partially purified protein samples migrate 4 bands with relative molecular masses between 40 and 45 kDa. The final preparation of the purified

Table 3. Maintaining the pH throughout the fermentation process using 0.05 M phosphate buffer, and its effect on growth and cellulase productivity by *S. longispororuber* in modified CMC broth at 30°C for 96 h.

pH	Cellulase productivity (U/L ± SD)	Dry cell weight (g/L)	Specific productivity (U/g)
6	15628 ± 422	6.8	2298.2
6.5	25084 ± 586	7.3	3436.2
7	18197 ± 378	7.8	2332.9
7.5	11480 ± 312	7.6	1510.5

Table 4. Purification of cellulase enzyme from *S. longisporobur* culture supernatant.

Purification step	Total protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Yield (%)	Purification fold
Crude extract	32135	38420	1.2	100	1.00
Ammonium sulfate (30 - 70%)	2645	25043	9.5	65.2	7.9
DEAE-Cellulose	318	8768	27.6	22.8	23
Sephadex G-75	112	5175	46.2	13.5	38.5

cellulase enzyme had a single protein band with approximate relative molecular mass of 42 kDa (Figure 2).

Effect of temperature and pH on cellulase activity and stability

Effect of temperature

Maximum enzymatic activity was obtained at incubation temperature of 50°C. While, the thermal stability of the cellulase enzyme was up to 55°C and reduction in the activity was reached to 15% when the temperature raises up to 60°C (Figure 3).

Effect of pH

Regarding pH, maximum enzymatic activity (less than 10% reduction) was obtained at pH range 5.5 - 7, with optimum pH range at 6 - 6.5. In case of enzyme stability, enzyme preparation retained 85% or more of its activity between pH 5.5 and 7.5, while the optimum pH is 6.5 (Figure 4).

DISCUSSION

The present study focused on isolation of a high cellulase-producing *Streptomyces* isolate from soil samples of Saudi Arabia. The results of the screening by Congo red test and dinitrosalicylic acid assay revealed that the isolate coded C188 produces promising level of cellulase (8540 U/L). Genetic identification of the selected isolate was carried out by analysis of 16S rDNA gene. Based on the nucleotide sequence of the16S rDNA gene and the phylogenetic analysis, the organism is most similar to *S. longispororuber.*

Improvement of cellulase productivity by *Streptomyces* C188 was carried out by studying the effect of different nutritional factors and pH of the fermentation medium. Modification of the fermentation medium is an essential stage in the design of successful laboratory experiments, pilot scale development and manufacturing processes (Stanbury et al., 2000). Cellulase enzymes are inducible, so the presence of carboxymethyl cellulose induces enzyme production in addition to its role as carbon source. Trials were carried out to improve the cellulase productivity by adding different carbon additives (glucose, lactose, sucrose, maltose, arabinose) to the fermentation medium, but none of the tested carbon sources improved enzyme production (data not shown). This observation is well in agreement with that reported by Gautam and his collegues (2010). Accordingly, we used the same basic CMC liquid, containing carboxymethyl cellulose as carbon source, for further studies on the effect of other factors.

For studying the effect of nitrogen source, supplementation of the fermentation medium with different organic nitrogen sources was carried out. A significant increase in the enzyme productivity (15490 U/L) by the tested strain was observed in the presence of 1% corn steep liquor as compared to that of the control ($P < 0.05$). The benefit of corn steep liquor over other nitrogen sources is not only improving enzyme production but also reducing the cost of enzyme production, especially at large scales (Da Vinha et al., 2011).

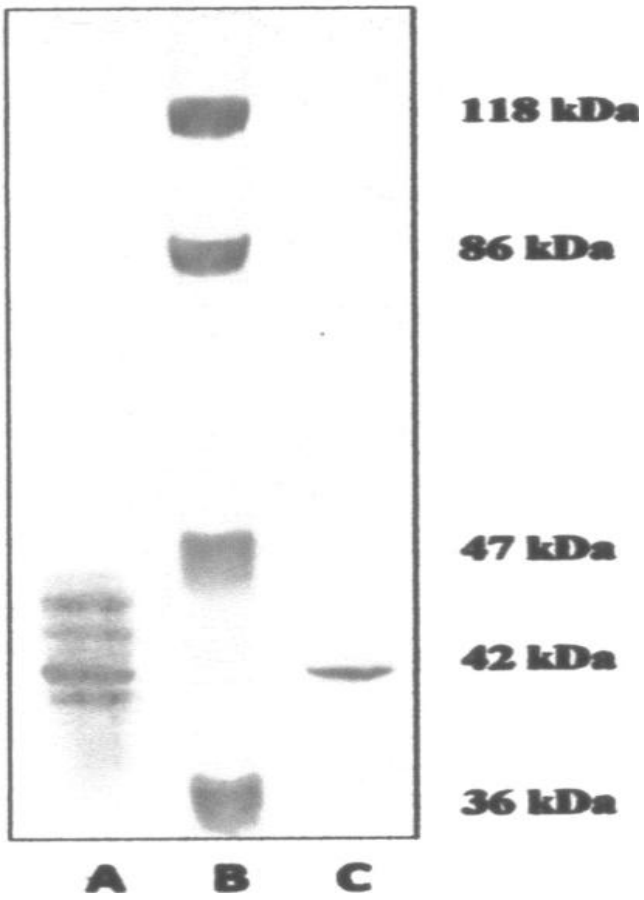

Figure 2. SDS-PAGE of (A) Partially purified protein sample, (B) molecular mass standards and (C) final preparation of the purified cellulase enzyme (β-galactosidase, 118 kDa; Bovine serum albumin, 86 kDa; Ovalbumine, 47 kDa; Carbonic anhydrase 36 kDa).

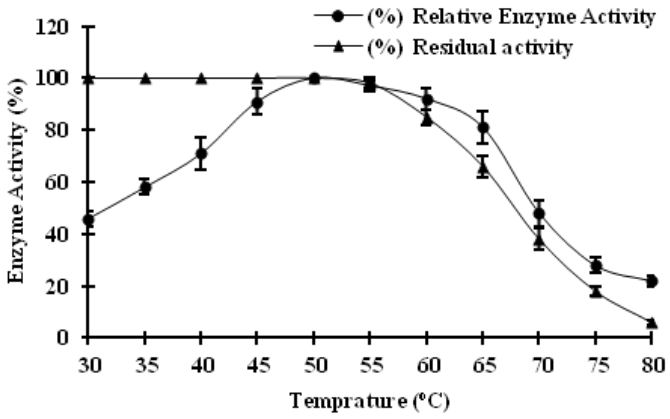

Figure 3. Effect of temperature on the enzyme activity and stability.

The pH of fermentation medium modulates microbial growth and enzyme production (Odeniyi et al., 2009). The

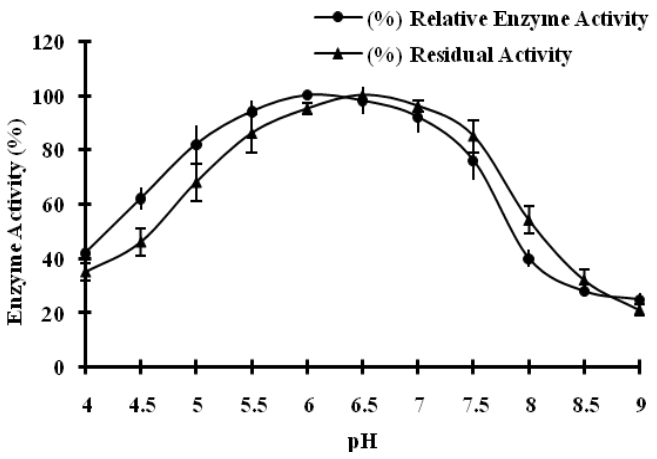

Figure 4. Effect of pH on the enzyme activity and stability.

suitable initial pH of the fermentation medium for production of cellulase enzyme by *Streptomyces* sp. was mainly located within acidic range between 5.5 and 6.5 (El-Sersy et al., 2010; Jaradat et al., 2008). However, some *Streptomyces* sp. as that isolated from East African Soda Lakes have an optimal pH of 8 (Solingen et al., 2001). In the case of *Streptomyces* C188, highest level of enzyme production was at initial pH 6.5. Regardless of the initial pH employed, the final pH of the spent media was in the alkaline range; a characteristic result in cultures of *Streptomyces* (Chen et al., 1979). In the present study, controlling the pH of the fermentation medium by 0.05 M phosphate buffer at pH 6.5 increased the enzyme productivity of *Streptomyces* C188 by 1.8 times as compared to that of the control.

Along the enzyme purification steps, a gradual increase in the specific activity to finally reach 46.2 U/mg was observed. Although the yield was only about 13.5% but over 97% of the extracted protein was removed during the purification steps and the enzyme was purified with an increase in purification fold more than 38. This decrease in yield might be due to denaturation of enzyme during the purification steps or other reasons (Begum et al., 2009).

Homogenous purified enzyme preparation was obtained as analyzed by SDS–PAGE with estimated MW to be about 42 kDa, similar to cellulase enzyme produced by *Streptomyces reticuli* 21 (Wachinger et al., 1989) and close to that produced by *Streptomyces lividans*, MW- 36 kDa (Wittmann et al., 1994). However, it was larger than 24-27 kDa of the cellulases from *Streptomyces ruber* (El-Sersy et al., 2010) and smaller than the second cellulase enzyme (MW-119 kDa) produced by *Streptomyces viridobrunneus* (Da Vinha et al., 2011).

The cellulase activity was affected by the pH and temperature of the reaction mixture. It was observed that the optimum condition of enzyme activity by *Streptomyces* C188 was pH 6-6.5 and 50°C which are in agreement with that reported by other investigators (Lima et al., 2005). However, a slightly higher optimum temperature

(55-60°C) was observed for the cellulase activity by *S. malaysiensis* (Nascimento et al., 2009) and *Streptomyces* sp. *J12* (Jaradat et al., 2008) and lower optimum temperature by *S. ruber* (El-Sersy et al., 2010).

In the case of the optimum pH, a more acidic pH (pH 4 and 4.9) was observed for the cellulase activity by *S. malaysiensis* (Nascimento et al., 2009) and *S. viridobrunneus* (Da Vinha et al., 2011). In addition, wider range of optimum pH (between 5.5 and 7.5) was reported for the cellulase activity by *S. reticuli* (Wachinger et al., 1989). The variation in the optimum pH and temperature for the cellulase activity may be due to the difference in the types of the producer strains.

In the present study, cellulase enzyme is moderately thermostable (up to 55°C). Comparatively, its optimum thermal stability was slightly higher than that produced by *S. viridobrunneus* (Da Vinha et al., 2011) and lower than that produced by *Mucor circinelloides* (Saha et al., 2004). Regarding the effect of pH on the enzyme stability, the optimum pH in the present study (6.5) was similar to that produced by *Bacillus sublitis* YJ1 (Yin et al., 2010).

Thus, a high-cellulase producing *Streptomyces* isolate, identified as *S. longispororuber*, was isolated from Saudi Arabia, and its enzyme productivity was improved by modifying the nutritional conditions and pH of the fermentation medium. The produced cellulase enzyme has MW of 42 kDa and its maximum enzymatic activity was obtained at 50°C and pH 6.5, while the maximum stability was at 55°C and pH 6-6.5.

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Full Length Research Paper

Prokaryotic biodiversity of halophilic microorganisms isolated from Sehline Sebkha Salt Lake (Tunisia)

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North of Tunisia consists of numerous ecosystems including extreme hypersaline environments in which the microbial diversity has been poorly studied. The Sehline Sebkha is an important source of salt for food. Due to its economical importance with regards to its salt value, a microbial survey has been conducted. The purpose of this research was to examine the phenotypic features as well as the physiological and biochemical characteristics of the microbial diversity of this extreme ecosystem, with the aim of screening for metabolites of industrial interest. Four samples were obtained from 4 saline sites for physico-chemical and microbiological analyses. All samples studied were hypersaline (NaCl concentration ranging from 150 to 260 g/L). A specific halophilic microbial community was recovered from each site and initial characterization of isolated microorganisms was performed by using both phenotypic and phylogenetic approaches. The 16S rRNA genes from 77 bacterial strains and two archaeal strains were isolated and phylogenetically analyzed and belonged to two phyla Firmicutes and gamma-proteobacteria of the domain *Bacteria.* The results show that the Sehline Lake harbored novel prokaryotic diversity, never reported before for solar salterns. In addition, diversity measurement indicated an increase of bacterial diversity with rising salinity gradient, which is probably due to competition between bacteria and others species.

Key words: Sebkha, bacteria, extremophiles, biodiversity, screening.

INTRODUCTION

Previous studies on the microbiology of hypersaline environments showed that halophilic members of the domain Archaea were dominant, whereas those of the domain Bacteria represented limited components (Litchfield and Gillevet, 2002; Ochsenreiter et al., 2002; Baati et al., 2008; Hedi et al., 2009). Nevertheless, other studies have demonstrated that members of the bacteria domain play an important role in hypersaline environments (Antoin et al., 2000). Such extremophiles were described by their ability to produce compounds of industrial interest and biotechnological products (biopolymers, exopolysaccharides, hydrolases, amylases, cellulases, proteases and lipases). In addition, halophilic organisms play important roles in fermenting fish sauces and in transforming and degrading waste and organic pollutants (Grant et al., 1998; Boone and Garrity, 2001). Although molecular diversity studies have been carried out in other hypersaline environments, this work represents the first one where such a

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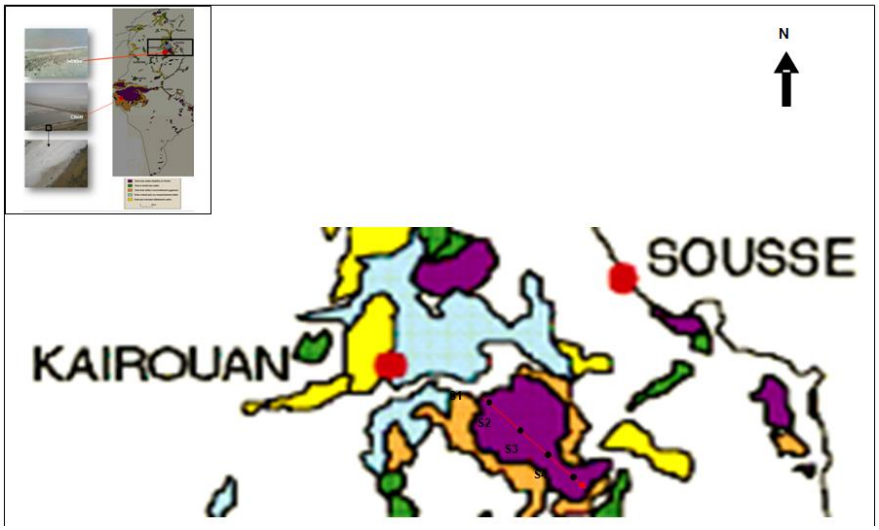

Figure 1. Location map of the Sebkha Sehline Lake (Tunisia) and sampling points.

study was performed on the anoxic sediment underlying microbial mats in Mediterranean salterns. North of Tunisia consists of numerous ecosystems including extreme (e.g. hypersaline) environments (Monastir, Tunisia) in which the microbial diversity has been poorly studied. The largest saline lake named Sehline Sebkha, located in North of Tunisia covers nearly 16 km^2.

Sehline Sebkha salt lake is a hypersaline environment in the north east part of Tunisia which is considered as a thalassohaline habitat, putting it in the same category as the Great Salt Lake or solar salterns (Rodriguez-Valera et al., 1988). Physico-chemical conditions of the Sebkha revealed that this extreme environment showed high salinity, high radiations (U-V) and changes in temperatures and dryness which make it relevant to be studied by microbiologists. The saltern lake is also an important source of salt for food, providing a wide set of ecological niches for halophilic microorganisms.

However, no study with regards to its microbial diversity has been undertaken so far. The purpose of this research was to chemically analyse salt and brine samples collected from the lake, to isolate novel extremely halophilic aerobic or facultative anaerobic microorganisms that develop in it, and to examine their phenotypic features and their physiological and biochemical characteristics with the aim to screen for metabolites of industrial interest produced by the novel halophilic isolates.

MATERIALS AND METHODS

Sample collection

The strains were isolated aseptically from mixed water and sediments of the Sehline Sebkha (Figure 1). Taking into account the *in situ* physico-chemical conditions and level of wastewater pollutants, the Sebkha was divided into four experimental sites. Water and sediment samples were collected at the surface and at various depths (0.1, 0.2 and 0.3 m), within each site.

Physico-chemical analysis of the samples

The physico-chemical analysis of water and soil samples from the hypersaline Sehline Sebkha (Tunisia), performed by standard methods (Trussel et al., 1989); are reported in Table 1. Cl^- was quantified by titration with $AgNO_3$, Mg^{2+} was quantified by atomic absorption spectrophotometry, Na^+ was quantified by flame spectrophotometry, and Ca^{2+} was quantified by complexometry using EDTA. Temperature and pH were measured *in situ*.

Enrichment and isolation

Considering the importance of salinity within the Sebkha, we focused our isolation procedures particularly on extreme halophilic archaea and bacterial microbiota.

Enrichment cultures and isolation procedures to recover aerobic or facultative anaerobic extremely halophilic microorganisms were performed in medium containing (per liter): NaCl, 250 g; $MgCl_2.6H_2O$, 13 g; $MgSO_4.7H_2O$, 20 g; KCl, 4 g; $CaCl_2.2H_2O$, 1 g; NaBr, 0.5 g; $NaHCO_3$, 0.2 g; yeast extract, 5 g; tryptone, 8 g; and glucose, 1 g. This medium was selected because it contains the majority of components which can be utilized by halophilic microorganisms. The pH was adjusted to 7.2 and 8.2 with 10 M NaOH before autoclaving. Enrichment cultures were subcultured several times under the same conditions.

Strains were grown in 100 mL of medium in 250 mL Erlenmeyer flasks in a rotatory shaker under agitation at 150 rpm. The adequate temperature chosen for growth was 37°C (average temperature at the sampling sites for isolation at 12 h in the morning). Aliquots (100 µL) of 10^{-1} - 10^{-4} dilutions were plated onto agar medium.

After two weeks of incubation (at least) at 37°C (plates incubated in a humid steam room adding distilled water to avoid dryness), red, orange-red, pale-pink, yellowish, cream, white, and also transparent colonies were observed. Different colonies were picked and restreaked several times (three times at least) to obtain pure cultures.

Characterization and identification of isolates

The isolates that showed different phenotypic characteristics and phylogenetic signatures (Amplified rDNA Restriction Analysis; ARDRA, 16S rRNA gene sequences), were chosen for further characterization. Isolated strains were examined for colony and cell morphologies and cell motility. Colonial morphologies were described by using standard microbiological criteria such as pigmentation, colonial elevation, consistency and opacity. Gram-staining was carried out with the method described by Dussault (1955). The temperature for cultures growth was 37°C and NaCl concentration growth was limited to 25%. The pH tolerance of each isolate was tested in medium with pH values of 7.2 and 8.2.

Biochemical tests of bacteria

Chitinase, cellulase, xylanase, protease and curdlanase activities were analysed for each representative species of the bacteria using colonies of the strains. For each test, a mixture of 50% agar (4% agar dissolved in sodium acetate 0.1 M, pH 5) and 50% enzymatic substrate such CM-Curdlan-RBB, CM-Cellulose-RBB, CM-Xylan-RBB or CM-Chitine-RBV (LOEWE Biochemica GmBH Laboratory), was prepared in plate. Test protease of bacteria was examined in medium containing 50% nutrient agar and 50% half-skim milk. All the tests were supplemented with 10% NaCl. Microorganisms showing clearing zones after 48 h of incubation at 37°C were considered as enzymatic producers.

PCR amplification of 16S rDNA

The PCR amplification and restriction endonuclease digestions were performed as previously described (Hedi et al., 2009). The DNA from bacterial cultures was extracted using a Wizard Genomic DNA Purification Kit (Promega). The 16S rRNA gene of the isolated strain was amplified by adding 1 µL of extracted DNA to a thermocycler microtube containing 5 µL of 10 × taq buffer, 0.5 µL of each 50 mM Fd1 and Rd1 primers, 5 µL of 25 mM $MgCl_2.6H_2O$, 0.5 µL of 25 mM dNTPs, 0.5 µL of Taq polymerase (5 U μL^{-1}), and 38 µL of sterilized distilled water. Universal primers Fd1 and Rd1 (Fd1, 5-AGAGTTTGATCCTGGCTCAG-3 and Rd1, 5-AAGGAGGTGATCCAGCC-3) were used to obtain a PCR product of 1.5 kb corresponding to base positions 8 - 1542 based on *Escherichia coli* numbering of the 16S rRNA gene (Winker and Woese, 1991).

The reactions were put in a thermal reactor thermocycler (BIOMetra, Leusden, The Netherlands), denatured for 1 min at 95°C and subjected to 30 cycles for 20 s at 95°C, 30 s at 55°C, and 1 min and 30 s at 72°C. This was followed by a final elongation step for 5 min at 72°C. The PCR products were analyzed on 1% (w/v) agarose gels and sent to GATC (Germany) for sequencing using universal primers Fd1 and Rd1 described previously. Sequence data were imported into the BioEdit version 5.0.9 sequence editor (Hall, 1999); base-calling was examined, and a contiguous sequence was obtained. The full sequence was aligned using the RDP Sequence Aligner program (Maidak et al., 2001). The consensus sequence was manually adjusted to conform to the 16S rRNA gene secondary structure model (Winker and Woese, 1991). A nonredundant BLAST search (Altschul et al., 1997) identified its closest relatives. Sequences used in the phylogenetic analysis were obtained from the RDP (Maidak et al., 2001) and GenBank databases (Benson et al., 1999). Sequence positions and alignment ambiguities were eliminated and pairwise evolutionary distances were calculated using the method of Jukes and Cantor (1969). A dendrogram was constructed using the neighbour-joining method (Saitou and Nei, 1987). Confidence in tree topology was determined using 100-bootstrapped trees (Felsenstein, 1985).

Restriction endonuclease digestions

The PCR amplification and restriction endonuclease digestions were performed as previously described (Hedi et al., 2009). Enzymatic digestions were performed by incubating 5 µL of the PCR products with 10 U of each endonuclease and the corresponding enzyme buffer. Digestions were incubated for one hour at 37°C for *Alu*I, *Hae*III, and *Rsa*I and products were analyzed on 2% (w/v) agarose gels.

RESULTS

Physico-chemical analysis of the samples

The temperature at the sampling sites was 21°C at 8 h in the morning. The pH of sediment samples was between 8.6 and 9.1 and may be considered as weakly alkaline. The highest values of moisture and salt saturation content were found in the S2 sample. Sodium and chloride were the most abundant ions. Sulfate and magnesium content were found higher also in the four samples when compared with others ions (Table 1). The total salt composition of the S4 sampling site was higher than the other sampling sites (Table 1). Total ionic composition of the lake differed depending on the area sampled. Taking into account the mineral composition of the lake, with regard to its concentration in Na^+, K^+, Ca^{2+}, Mg^{2+}, SO_4^{2-} and Cl^-, it is clear that halophilic microorganisms should inhabit this lake, thus justifying studies on the microbial survey of it.

Microbiological analyses

After several dilutions and subculturing in the same liquid medium under aerobic conditions, colonies were isolated in the agar medium containing 25% NaCl. The total number of extremely halophilic bacteria in the salt samples of sites 1 and 3 (3.49×10^4 - 7.3×10^5 CFU/g, respectively) was higher than in the salt samples of sites 2 and 4 (4.1×10^3 - 3.2×10^4 CFU/g, respectively). The high concentration of salt limited the number of strains. The 126 strains isolated belong to 11 different genera within Bacteria and Archaea domains (Table 3). The total strains give an idea about the distribution of major microbial groups that inhabit the Sebkha, taking into account the salinity of the culture medium used for isolation. The number of genera found decreased specially in sites 2 and 4, because of increase of ecosystem salinity (Rodriguez-Valera, 1993).

On the basis of phenotypic characteristics (macro and microscopic analysis), physiological analysis (NaCl, pH), biochemical tests (chitinase, cellulase, curdlanase, xylanase and protease) and molecular approaches [16S analysis, ARDRA], only 79 isolates were selected for characterization and examined in greater details. These strains have been identified by analyzing sequences of genes encoding for the 16S rRNA (Figure 2). The others

Table 1. Physico-chemical characteristics of the sediment samples.

Characteristic	S1	S2	S3	S4	Average
Colour of sampling site	Cream	Dark-cream	Cream	Dark-cream	
pH	8.66	9.11	8.73	8.60	8.77
Moisture (%)	17.58	20.91	15.24	17.44	17.79
Saturation (g/l)	282. 5	300	250	230	265.6
Electric conductivity (ms/cm)	189.30	181.00	187.60	185.20	185.77
Anions (mg/g)					
Chloride	98.00	102.00	101.20	101.80	100.51
Carbonate	0 .52	0.08	0.33	0.09	0.25
Bicarbonate	0.06	0.24	0.19	0.20	0.17
Sulfate	34.42	46.03	40.51	39.90	40.21
Cations (mg/g)					
Sodium	98.00	104.00	10 1.00	101.00	101.00
Potassium	5.80	8.60	6.00	6.00	6.6
Calcium	0.93	1.14	1.04	1.04	1.04
Magnesium	32.27	30.61	30.19	33.95	31.75
Total	270	292.7	280.46	283.98	281.53

strains were a repeated isolates showing the same ARDRA profiles analyses.

Colonial and cell morphology

The dominant bacterial population in hypersaline environment comprised motile or non motile, Gram-positive microorganisms and most of them were spore-forming bacteria. Most colonies on agar media were 0.5 - 2 mm in diameter after three weeks of incubation. These colonies were smooth, circular, low-convex, transparent or translucent and entire. Cells of all strains isolated were short, long and swollen rods and occurred in singles, pairs or short chains. Colonial pigmentation from these samples ranged from blood-red to pale-pink. Optimum growth occured at, 25% (w/v) NaCl, 37°C, and two pH (7.2 and 8.2) (Table 2). No growth was observed at NaCl concentrations of less than 15% (w/v) for the majority of isolates, thus suggesting that these isolates should be considered as extremely halophilic according to the definition of Ventosa et al. (1998).

Biochemical tests

To identify and characterize the enzymatic capabilities of the isolated strains, some biochemical tests were conducted. Large zones of clearing around the growing bacteria were observed. Others strains were also unable to form clearing zones. These results suggested that enzymes may be secreted by the strains into the culture medium. Results reveal also that many isolates were able to produce chitinase and curdlanase (Table 4). Moreover, most of them exhibited cellulase activity. A few number of the strains chosen were able to produce protease and xylanase. In addition, all isolates showed variability in degradation of enzymes that reflect inter- and intra-specific polymorphism.

Phylogenetic analysis

Based on the enzymatic digestion profiles obtained, 79 representatives bacteria of the 126 isolates were chosen for taxonomic and phylogenetic studies. To determine their phylogenetic position, the 16S rRNA gene sequence of each strain was analyzed and phylogenetic trees were constructed (Figure 2). Phylogenetic trees are presented as two clades of bacteria, Archaea are presented by two genus (*Haloferax* and *Natrinema*). The 16S rRNA gene sequences of strains have been deposited in the GenBank database (as the accession numbers ranged from KC142043 to KC142106). The phylogenetic analysis indicated that the first clade is made of up a wider variety of genera including *Halomonas* (*Halomonas koreensis*, *Halomonas salina*, *Halomonas halmophila*, *Halomonas eurihalina*, *Halomonas elongata* and *Halomonas sinaiensis*), *Pseudomonas*, *Halovibrio* and is dominated by isolates related to genera *Salicola* (Figure 2). The second clade is a group of isolates related to two genera (*Marinococcus* and *Bacillus*), including *Halobacillus karajensis*, *Halobacillus trueperi*, *Halobacillus yeomnijeoni*, *H. salinus*, *Marinococcus halophilus*,

Table 2. Phenotypic features of the 24 representatives strains studied.

Sampling sites (S)/ depth (cm)/ characteristics	**Strain (7SPE)**							
	2421 S2/40	**411 S4/10**	**121 S1/20**	**1132 S1/10**	**2412 S2/40**	**105 S1/0**	**4212 S4/20**	**323 S3/20**
Taxonomical status	*Halobacillus* sp.	*Halobacillus* sp.	*Marinococcus* sp.	*Bacillus* sp.	*Pontibacillus* sp.	*Marinococcus* sp.	*Bacillus* sp.	*Halovibrio* sp.
Colonial morphology	Circular	Circular	irregular and spreading	Circular	Circular	Circular	Circular	Circular
Colony size	0.5 mm	1 mm	1-2 mm	2 mm	1 mm	1mm	0.5 mm	0.3 mm
Colony	convex	flat	flat	convex	flat	convex	flat	convex
Colony density	Opaque matt	Opaque matt	Transparent glistening	Opaque matt	Opaque matt	Opaque matt	Opaque matt	Translucent matt
Pigmentation	Cream	Cream	Cream	White	Cream	Reddish pleomorphic	Cream	White
Cell shape	Pleomorphic	Pleomorphic	Pleomorphic	Pleomorphic	Pleomorphic		Pleomorphic	Pleomorphic
Cell arrangement	Rods long chains	Cells single and paired cells	Rods single and paired cells	Cells single and paired cells	Rods long chains	Rods single and paired cells	Rods single and paired cells	Cells single and paired cells
Chains	+	-	-	-	+	-	-	-
Motile	-	-	+	-	+	-	+	-
Cell size; length and width (µm)	2-5 X 1.5	3-7.5 X 1.5	1-4 X 1	1-3 X 0.5	1-4 X 1	1-5 X 2	1-4 X 1	1-4 X 0.5
Growth at 37°C, pH 7.2								
0% NaCl	-	-	-	-	-	-	-	-
2% NaCl	-	-	-	-	-	-	-	-
5% NaCl	+	+	+	-	+	+	+	+
8% NaCl	+	+	-	+	+	+	+	+
10% NaCl	+	+	+	+	+	+	+	+
15% NaCl	+	+	+	+	+	+	+	+
25% NaCl	+	+	+	+	+	+	+	+
30% NaCl	-	-	-	-	-	-	-	-
Growth at 37°C								
pH 4.5	-	-	-	-	-	-	-	-
pH 6	+	+	+	+	+	+	+	+
pH 7	+	+	+	+	+	+	+	+
pH 7.5	+	+	+	+	+	+	+	+
pH 8	-	-	-	-	-	-	-	-

Table 2. Contd.

Sampling sites (S)/ depth (cm)/ characteristics	Strain (7SPE)							
	3212 S3/20	**116 S1/10**	**1211 S1/20**	**2224 S2/20**	**1131 S1/10**	**2215 S2/20**	**3128 S3/10**	**3030 S3/0**
Taxonomical status	*Salicola* sp.	*Halomonas* sp.	*Halomonas* sp.	*Halomonas sp*.	*Halomonas* sp.	*Halomonas* sp.	*Halomonas* sp.	*Halomonas* sp.
Colonial morphology	Irregular and spreading	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Colony size	0.5 mm	1 mm	1-2 mm	2 mm	1 mm	2mm	1 mm	1-2 mm
Colony	Convex	Flat	Flat	Convex	Flat	Convex	Flat	Slightly raised
Colony density	Opaque matt	Opaque matt	Opaque matt	Opaque matt	Opaque matt	Opaque matt	Opaque matt	Translucent
Pigmentation	Reddish	Cream	Cream	White	Cream	Cream	Cream	Glistening white
Cell shape	Pleomorphic Cells	Pleomorphic cells	Pleomorphic cells	Pleomorphic cells	Pleomorphic cells	Pleomorphic cells	Pleomorphic rods	Pleomorphic Cells
Cell arrangement	Single and paired cells	Long chains	Single and paired cells	Single and paired cells	Long chains	Long chains	Paired cells and long chains	Single and paired cells
Chains	-	+	-	+	+	+	+	-
Motile	-	-	-	-	-	-	+	-
Cell size; length and width (µm)	2-5 X 0.3	1-5 X 1	1-4 X 0.5	1-3 X 1.5	1-4 X 1.5	1-5 X 1	2-5 X 1	1-4 X 1
Growth at 37°C, pH 7.2								
0% NaCl	-	-	-	-	-	-	-	-
2% NaCl	-	-	-	-	-	-	+	-
5% NaCl	+	+	-	-	+	+	+	-
8% NaCl	+	+	+	+	+	+	+	-
10% NaCl	+	+	+	+	+	+	+	+
15% NaCl	+	+	+	+	+	+	+	+
25% NaCl	+	+	+	+	+	+	+	+
30% NaCl	-	-	-	-	-	-	-	-
Growth at 37°C								
pH 4.5	-	-	-	-	-	-	-	-
pH 6	+	+	+	+	+	+	+	+
pH 7	+	+	+	+	+	+	+	+
pH 7.5	+	+	+	+	+	+	+	+
pH 8	-	-	-	-	-	-	-	-

Table 2. Contd.

Sampling sites (S)/ depth (cm)/ characteristics	Strain (8SPE)					
	1262 S1/20	**236 S2/30**	**1359 S1/30**	**422 S4/20**	**607 S6/0**	**338 S3/30**
Taxonomical status	*Halobacillus* sp.	*Halobacillus* sp.	*Halobacillus* sp.	*Gracilibacillus* sp.	*Halovibrio* sp.	*Salicola sp*.
Colonial morphology	Irregular and spreading	Circular	Irregular and spreading	Circular	Circular	Circular
Colony size	0.5 mm	1 mm	1-2 mm	2 mm	1 mm	2 mm
Colony	convex	flat	flat	convex	flat	convex
Colony density	opaque matt	opaque matt	opaque matt	opaque matt	opaque matt	opaque matt
Pigmentation	reddish	cream	reddish	white	cream	cream
Cell shape	pleomorphic	pleomorphic	pleomorphic	pleomorphic	pleomorphic	pleomorphic
Cell arrangement	Cells single and paired cells	Cells single and paired cells	Cells paired and long chains	Cells single and paired cells	Cells single and paired cells	Cells paired cells
Chains	-	-	+	+	-	-
Motile	-	+	+	-	-	-
Cell size; length and width (µm)	2-5 X 0.3	1-5 X 1	1-4 X 1	1-3 X 0.5	1-4 X 1	1-5 X 0.5
Growth at 37°C, pH 7.2						
0% NaCl	-	-	-	-	-	-
2% NaCl	-	-	-	-	-	-
5% NaCl	+	+	+	+	+	+
8% NaCl	+	+	+	+	+	+
10% NaCl	+	+	+	+	+	+
15% NaCl	+	+	+	+	+	+
25% NaCl	+	+	+	+	+	+
30% NaCl	-	-	-	-	-	-
Growth at 37°C						
pH 4.5	-	-	-	-	-	-
pH 6	+	+	+	+	+	+
pH 7	+	+	+	+	+	+
pH 7.5	+	+	+	+	+	+
pH 8	-	-	-	-	-	-

Table 3. Distribution and taxonomic characteristics of micro-organisms isolated from the 4 sites sampled in Sehline Sebkha Lake.

Organisms	Number of strains / site				Number of strains
	S1	S2	S3	S4	
Halobacillus	4	2	0	4	10
Marinococcus	2	0	0	0	2
Pontibacillus	0	5	0	1	6
Bacillus	1	0	2	1	4
Salicola	8	3	16	7	34
Yeomjeonicoccus	4	0	0	0	4
Halomonas	4	8	2	0	14
Gracibacillus	0	0	0	1	1
Halovibrio	0	0	1	0	1
Pseudomonas	0	0	0	1	1
Others (*Archaea*)[a]	0	1	1	0	2
Total of strains	23	19	22	15	79

[a]Data not shown.

Marinococcus halotolerans, *Gracilibacillus halophilus*, *Pontibacillus chungwhensis*, *Pontibacillus marinus*, *Bacillus halophilus* and *Bacillus quingdaonensis* (Figure 2). The largest clusters are dominated by bacteria related to *Salicola* species, particularly *Salicola marasensis* and *Salicola salis*. The first group of bacteria are members of the Class Gammaproteobacteria, the second one are related to the Class Bacilli.

All strains shared more than 97% identity with their closest phylogenetic relative (Table 3) thus suggesting that they may be considered at the same species level until the results of DNA/DNA hybridization studies will be performed to validate or not their affiliation (work under progress).

Only two strain representatives of domain *Archaea* were identified as *Haloferax* sp. and *Natrinema sp.*, but these microorganisms have not been further characterized

DISCUSSION

In recent years, a number of extremophilic micro-organisms ranging from aerobes to anaerobes have been isolated. Research on microorganisms from extreme environments also intensified with the recognition of a third domain of life (*Archaea*) by Woese and Fox (1977). Investigations on the microbial ecology of various hypersaline environments have been largely extended during the last decades. In contrast to halotolerant micro-organism which do not require NaCl for growth but can grow under saline conditions, halophiles must have NaCl for growth (Ventosa et al., 1998). Both molecular and microbiological studies revealed the presence of moderately to extremely halophilic microorganisms in a wide range of these saline environments (Cayol et al., 1994; Oren, 2002a, b; Demergasso et al., 2004; Ventosa, 2006).

In the present study, we described microbial diversity among Bacteria and Archaea domains within four sites of two pH media (7.2 and 8.2) and high salinity (25%). The microbial communities in the four studied sites were different in terms of diversity and phylogenetic distribution of the 16S rRNA sequences. The differences between the samples indicated that microbial diversity may be strongly influenced by physical and chemical parameters in the four sites, particularly differences in salt concentration and in ions specification. Analysis of soil samples from the four sites studied is reported in Table 1. They differ from those of the other hypersaline environments studied so far (Hedi et al., 2009). Sodium and chloride concentrations in the four sites are higher than those of the Dead Sea in Israel, in particular (Oren, 1993). Waters of the Dead Sea and the Great Salt Lake in the USA, are slightly acidic (pH between 6 and 7), but the pH of the four sites studied are up to 8, and should be therefore considered as weakly alkaline (Oren, 1993). On the other side, the pH of Lakes Wadi Natrun and Magadi (in Kenya) are considered as highly alkaline environments (pH 11) (Jeon et al., 2005).

A total of 126 extremely halophilic strains have been isolated. Among them, 79 strains (77 *Bacteria*, 2 *Archaea*) with different pigmentations (cream, white, yellowish, and reddish-orange) as observed with colonies on agar plates have been further characterized. All bacterial strains were found as Gram-positive rods. The phylogenetic analysis indicate that all isolates were members of ten genera of the domain *Bacteria* including *Salicola*, *Pontibacillus*, *Halomonas*, *Marinococcus*, *Bacillus*, *Gracibacillus*, *Halobacillus*, *Yeomjeonicoccus*,

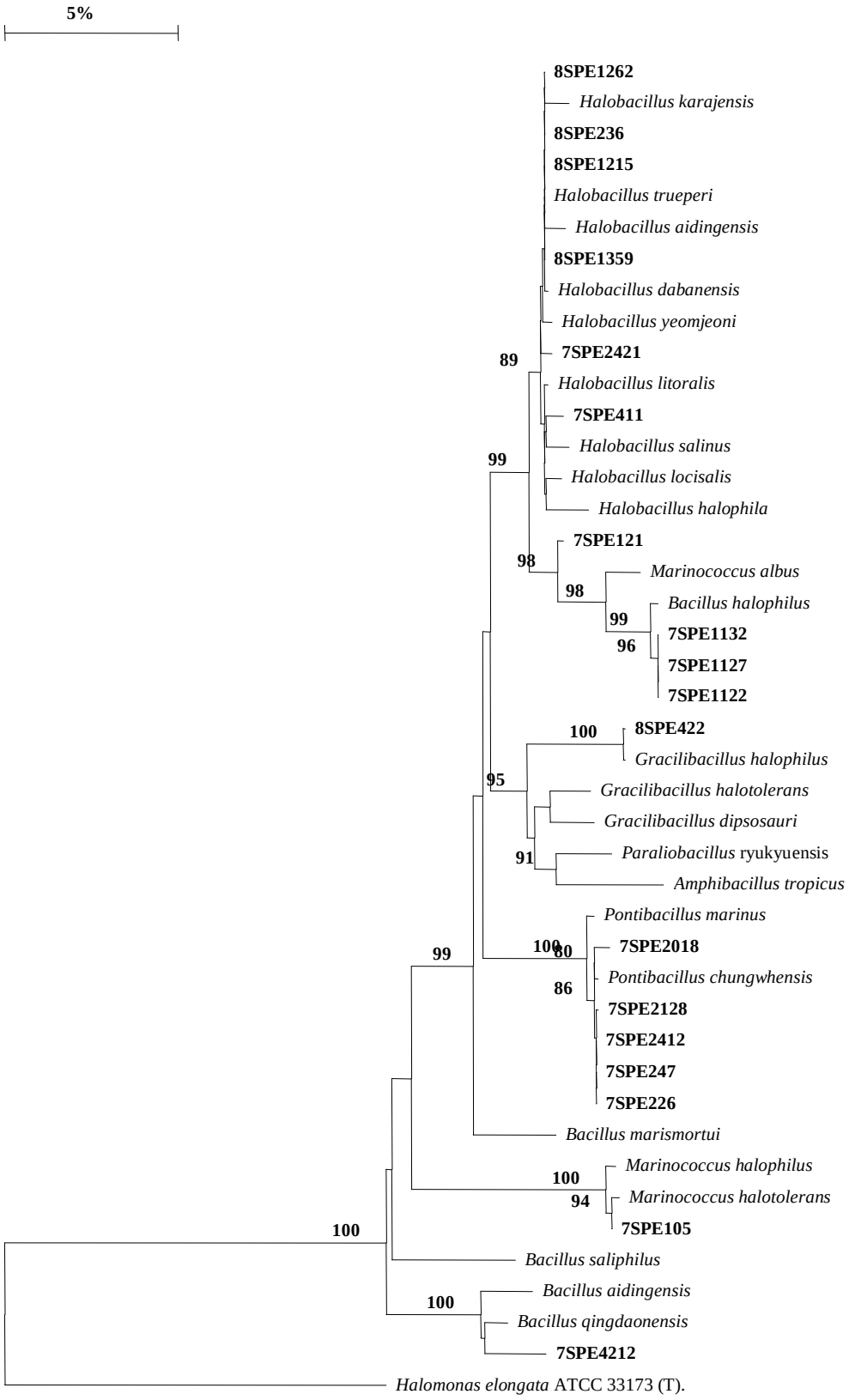

Figure 2. 16S rRNA gene-based phylogenetic trees of the bacterial domain, including the 16S rDNA sequences from sediments sample of Sehline Sebkha. The topologies of phylogenetic trees build using the maximum-likehood and maximum-parsimony algorithms were similar to those of the tree constructed by neighbour-joining analyses.

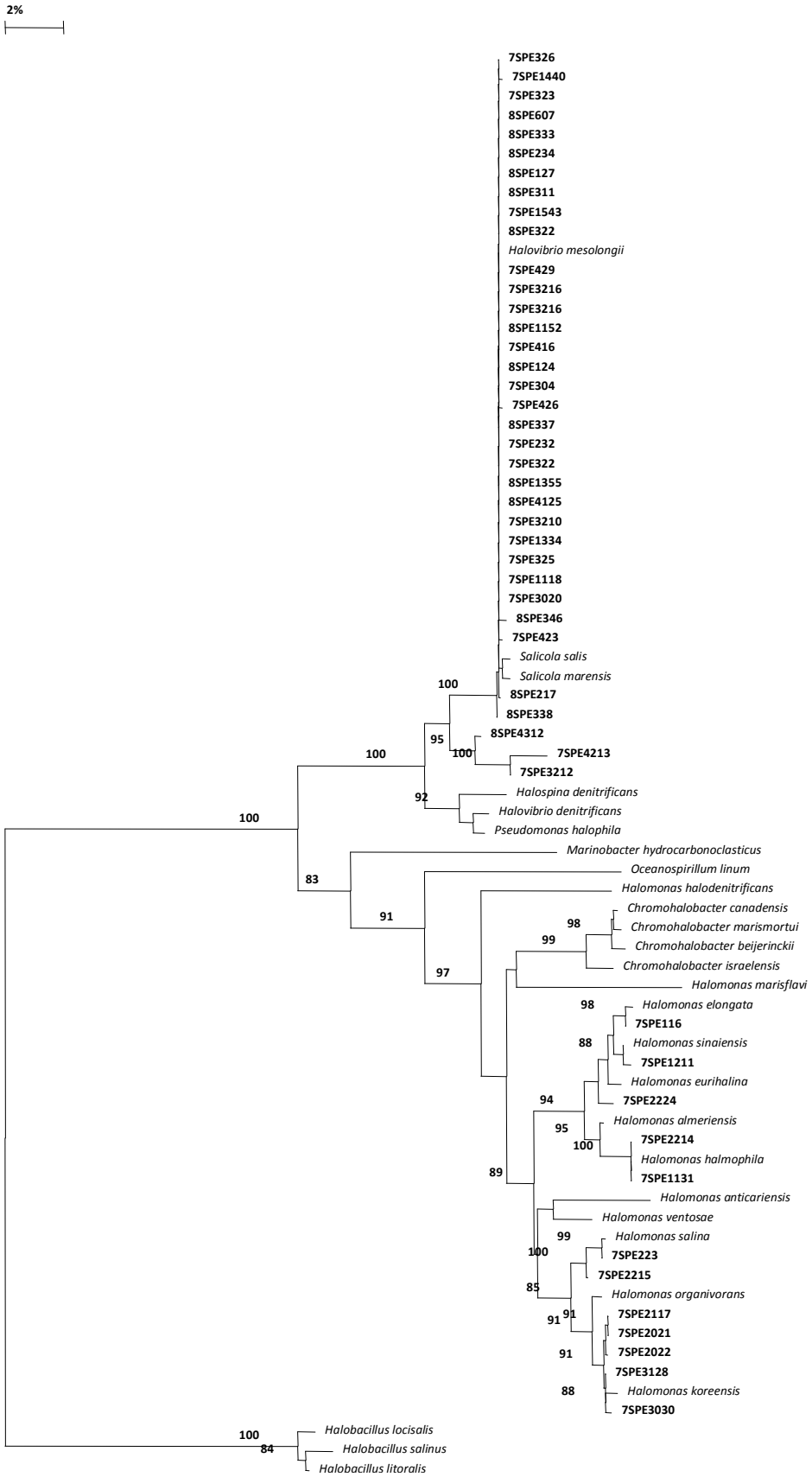

Figure 2. Contd**.**

Table 4. Enzymatic tests.

Isolates	Chitinase	Cellulase	Curdlanase	Protease	Xylanase
7SPE 1132	-	+	+	-	-
7SPE 3216	-	-	+	-	-
7SPE 2128	+	-	-	-	-
7SPE 429	+	+	+	+	-
7SPE 323	+	+	+	-	+
7SPE 423	-	-	+	+	-
7SPE 1440	+	+	+	-	-
7SPE 2421	-	+	+	-	-
7SPE 2214	-	+	+	-	-
7SPE 416	-	-	+	-	-
7SPE 2224	-	-	+	-	-
7SPE 326	+	-	-	-	-
7SPE 411	-	-	-	+	-
7SPE 325	-	-	+	-	-
8SPE 322	+	+	+	-	-
8SPE 337	-	+	-	-	-
8SPE 4214	-	+	-	-	-
8SPE 217	-	+	-	-	-
8SPE 338	-	+	-	-	-
8SPE 333	-	+	-	+	-
8SPE 1215	+	-	-	-	-
8SPE 346	-	-	-	+	-
8SPE 311	-	-	+	-	-
8SPE 1131	+	+	+	-	+

(+) Positive activity; (-) negative activity.

Halovibrio and *Chromohalobacter*. Members of the genera *Salicola*, *Pontibacillus, Marinococcus* and *Halobacillus* are considered as aerobic microorganisms, whereas those of genus *Halomonas* are considered as facultative anaerobes having the possibility to use nitrate as terminal electron acceptor under anaerobic conditions (Martínez-Cánovas et al., 2004). All these micro-organisms may use various organic compounds including sugars as substrates and should be considered as chemoorganotrophs. Almost all these isolates were detected on the surface of sediments (0.1 - 0.2 m) of each biotope.

The distribution of the microflora that inhabits the lake is reported in Table 3. *Salicola* species were distributed in all the 4 sites studied and represented the major strains isolated, especially in site 3. The large number of the genus *Halomonas* and *Salicola* may be due to the culture media used, which may have favoured the species growth and thus do not reflect their real distribution within the lake. Members of this genus together with those of genera *Halomonas*, *Gracibacillus*, *Bacillus*, *Halovibrio*, *Chromohalobacter*, *Yeomjeonicoccus*, *Pontibacillus*, *Marinococcus*, and *Halobacillus*, have also been isolated from other saline environments including athalassohaline and thalassohaline lakes and marine waters (Javor, 1989; Ventosa et al., 1998; Grant et al., 2001; Arahal and Ventosa, 2005).

With regards to the Archaea population, it could be noticed that all the 16S rRNA gene sequences obtained were affiliated with the Halobacteriales order of the Euryarchaeota. Among the halophilic micro-organisms isolated, only two originated from sites 2 and 3 pertaining to the domain *Archaea* (data not shown). Due to the industrial and economic importance of halophilic enzymes, the chitinase, cellulase, curdlanase, protease and xylanase activities of 24 extremely halophilic bacteria were screened. Ten of them produced two or more enzymes. They belong to genera *Halomonas*, *Salicola*, *Halobacillus* and *Bacillus*. Among the strains tested, only six strains were unable to produce any of the above enzymes. They are members of genera *Salicola* and *Marinococcus*. The others strains produced only one enzyme and they are members of genera *Salicola*, *Halomonas*, *Halobacillus* and *Pontibacillus*. Industrial enzymes obtained from halophiles might be used for improving garments during textile processing. Proteolytic enzymes from *Halobacterium* also play an important role in the brine fermentation of one type of traditional fish sauce (Quesada et al., 1990, 1993).

During an extensive search on different hypersaline

habitats in Spain and Marocco focused on the screening of new exopolysaccharide (EPS)-producing bacteria, several strains were isolated from saline soils and described as new species belonging to the genus *Halomonas* (Thongthai et al., 1992; Bouchotroch et al., 2001; Jones, 2001; Martínez-Cánovas et al., 2004). Similar to what we observed in our experiments, a minority of these micro-organisms isolated were identified as members of genera *Gracibacillus*, *Halovibrio*, *Marinococcus* and *Pseudomonas*. Several other aerobic or facultative anaerobic, moderately halophilic bacteria have been classified within genera related to the order *Bacillales* (Spring et al., 1996). The use of these microorganisms has been underlined (production of compatible solutes, halophilic enzymes, biopolymers and bioremediation processes) and reviewed in detail (Ventosa et al., 1998; Margesin and Schinner, 2001; Mellado and Ventosa, 2003). The potential industrial use of this collection of halophiles that we have got will be screened for molecules of industrial interest than enzymes (e.g. EPS and PHA, etc) (work in progress).

Finally, we believe that studies on these bacteria should be emphasized as they constitute a source of halostable enzymes (Table 4) which may be used in different pharmaco-chemical industries (Jones, 2004; Quesada et al., 2004).

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Full Length Research Paper

Relationship between fungal community and physico-chemical characteristics in the Hokersar Wetland, Kashmir Himalayas

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Relative effect of some physico-chemical parameters of water on the occurrence of water borne fungi was studied in a high altitudinal wetland. The number of conidial fungal species was enumerated in water samples collected monthly during the period of March 2008-February 2009. There was marked seasonal fluctuation in the occurrence number of individuals in each species. The maximum number of individuals was found during summer to early autumn, while there was a decline in the number of individuals during late autumn and early winter seasons. Principal component analysis showed a high inverse relationship between number of fungal species and pH and dissolved oxygen, while abundance was positively related to temperature, nitrate nitrogen and total phosphorus clustered together. Finally, it seems from the results that fungal communities are more influenced by the seasonal variation. More studies should be carried out to elucidate the effects of water variables on the community structure of fungi in other water systems in Kashmir Himalayas.

Key words: water borne fungi, water systems, fungal species, seasonal variation.

INTRODUCTION

The aquatic ecosystem comprises of variety of biota. Fungal community is one of them. Several physico-chemical factors of aquatic ecosystem influence the composition and activity of the fungal community. Of these, fluctuation in temperature, hydrogen-ion concentration, oxygen content, dissolved organic and inorganic matter, phosphate and sulphate concentration have been found to be important factors for the occurrence and distribution of individual species of water borne conidial fungi in the fresh water stream (Ingold, 1975; Nilsson, 1964). Within a stream, site differences are closely correlated with altitude or other factors associated with it, which may include differences in water chemistry (Fabre, 1998; Raviraja et al., 1998).

The occurrence and degradative ability of water borne conidial fungi colonizing on submerged leaf litter is influenced by the hydrogen ion concentration (pH) of water (McKinley and Vestal, 1982). While working in an arctic lake they found a progressive decline of fungi with increasing acidity and their almost complete absence at pH 4.0-3.0. These fungi require a fresh oxygenated environment for their occurrence (Webster and Towfic, 1972). Increase in fungal species number is related with increasing dissolve oxygen and dissolve organic matter of the aquatic systems (Kaushik and Hynes, 1971). Several studies indicate that in lotic ecosystems, leaf litter decomposition and fungal activity can be affected by the concentration of nutrients (e.g. nitrogen and phosphorus) in the water (Suberkropp and Chauvet, 1995; Sridhar and Barlocher, 2000; Grattan and Suberkropp, 2001;

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Rosemond et al., 2002). Aquatic hyphomycetes might obtain inorganic nutrients (nitrogen and phosphorus) not only from their organic substrate (leaf litter, wood debris, etc.) but also directly from water passing by riverine areas (Suberkropp, 1995; Suberkropp and Chauvet, 1995).

Temporal variations have been studied in recent past to see the variation in different aquatic ecosystems. Lefevre et al. (2012), for example studied temporal variation of small eukaryotic communities with main emphasis on zoosporic fungi and found that the overall structure of the eukaryotic community was similar between the 2 lakes, at lower taxonomic levels, community composition differed. Recent studies have also monitored a high diversity of zoosporic fungal sequences in fresh water ecosystems (Lefevre et al., 2007, 2012; Lepere et al., 2008; Chen et al., 2008; Luo et al., 2011; Monchy et al., 2011).

Previously no substantial work has been carried out on the association between water quality and fungal community. In order to fill the gap we carried out this study to elucidate the relationship between physico-chemical features and distribution pattern of water borne conidial fungi in a fresh water high altitudinal wetland of Kashmir Himalaya. We predicted that seasonality in physico-chemical characteristics of water will have drastic impact on the community ecology of conidial fungi. We also assumed that water temperature is an important factor influencing the fungal communities.

MATERIALS AND METHODOLOGY

Study area

Hokersar wetland (34° 06' N lat., 74° 05' E long.), located in Kashmir Himalaya is a protected wildlife reserve, situated about 10 km south of Srinagar city at an altitude of 1584 m (a.m.s.l.). The wetland harbours about two million migratory waterfowl during winter that migrate from Siberia and the Central Asian region. The wetland is fed by two inlet streams-Doodhganga (from east) and Sukhnag Nalla (from west). The water drains out through an outlet channel having a needle gate to regulate the water level during winter. The lake attains a maximum depth of 2.5 m in spring due to increase in discharge from the snow-melt water in the upper reaches of its catchment. The water depth at autumn is minimum of 0.7 m. The average rainfall, as observed from the nearest meteo-rological station at Srinagar is 650 mm and average temperature ranges from 7.5°C in winter to 19.8°C in summer.

Analysis of water quality

The physico-chemical parameters of water viz., water temperature (°C), water pH, dissolved oxygen (mg/L), dissolve organic matter (mg/L), dissolve inorganic matter (mg/L) and phosphate (mg/L) were analyzed following the methods of Trivedi and Goel (1986) and A.P.H.A. (1998).

Temperature was measured by using a centigrade thermometer by dipping it at a depth of 5-8 cm in water for 5 min, at the time of sample collection. pH was recorded on spot with the help of a digital portable pH meter (*Hanna*) periodically at the time of collection of samples.

Dissolved oxygen content was determined on the spot by making a composite sampling of water at each month following the Winkler method (APHA, 1998). Dissolved organic and inorganic matter (mg/L), phosphate (mg/L) were analyzed by following A.P.H.A. (1998).

Isolation of fungi

Water samples obtained from different sites were serially diluted five folds and then spread plate technique was followed for isolation of fungi in the study, spreading 0.1 ml inoculum from the serial dilution tubes on the Petri dishes containing Rose-Bengal Streptomycin Agar medium (Rice and Baird, 2005). Growing colonies were transferred to Petri dishes containing Potato Dextrose Agar (PDA), (MERCK, Germany), Malt Extract Agar (MEA) (Acumedia, USA), Czapek's dox Agar (CZ) and Czapek's Yeast Agar (CYA), 25% glycerol nitrate agar for identification, and then transferred everything to PDA for stock cultures. Plates were incubated at 25 to 37°C for one week in dark.

RESULTS AND DISCUSSION

Water temperature of wetland recorded during the study period indicates a marked seasonal variation (Table 1). The temperature of the wetland water ranges between 6.23-29.82°C. Fungal species were found temperature dependent, as fluctuation in the temperature also change the species density of water borne conidial fungi. Statistical analysis indicated a positive correlation (r = 0.929) of the fungal species with temperature (Table 2). It was noted that species number declines with the decreasing water temperature. It has been reported that raised temperature can lower diversity of hyporheic aquatic hyphomycetes (Bärlocher et al., 2008). Water transparency showed marked seasonal variation and ranges between 13-47 (Table 1), with minimum in spring and maximum in autumn. Statistical analysis showed a negative correlation (r = - 0.897) of fungal species with transparency. Dissolved oxygen content of water ranged between 3.2 - 11.6 mg/l (Table 1), with maximum in autumn and minimum in summer. A negative correlation (r = - 0.913) was found between the dissolved oxygen content of water and occurrence of species. There is no major study in the past which shows correlation between dissolved oxygen and occurrence of species in lentic water bodies. However, Medeiros et al. (2009) have shown that a decrease in oxygen concentration in streams affects the diversity and activity of aquatic hyphomycetes and consequently leaf litter decomposition. pH of water ranged between 7.4-8.9 (Table 2), with minimum in spring and maximum in autumn. Water pH had a close relationship with the occurrence of water borne conidial fungi. The number of fungal species had a negative correlation with pH, having values of r = - 0.923.

Conductivity showed reverse trend with other physico-chemical parameters and ranges between 210-381(Table 1), with maximum in summer and minimum in winter. A positive correlation coefficient (r = 0.884) was found

Table 1. Physico-chemical features of Hokersar wetland.

Parameter	Summer		Autumn		Winter	
	Site-I	Site-II	Site-I	Site-II	Site-I	Site-II
Temperature (°C)	28.08	14.47	7.31	27.94	14.73	7.51
Transparency (cm)	16	23	43	15.33	26.666	46
DO (mg/l)	3.283	6.53	10.43	3.653	6.91	10.747
pH	7.7033	8.1567	8.5867	7.73333	8.2133	8.65
Conductivity (µS/cm)	0.362	0.339	0.244	0.35	0.32	0.234
Ca (mg/l)	41.033	46.337	41.54	38.82667	43.7033	39.1567
Mg (mg/l)	13.0633	13.73	11.54333	12.8667	13.09	11.8
Alkalinity (mg/l)	141	308.67	270.3	133.33	302.33	260.33
Chloride (mg/l)	29	12.667	18	25.33	12.667	15
NO_3-N (µg/l)	0.383	0.304	0.238	0.413	0.268	0.223
Total phosphorus (µg/l)	0.357	0.208	0.15	0.361	0.182	0.126

Table 2. Species composition of different genera in different seasons and at different sites.

Species	Summer		Autumn		Winter	
	Site-I	Site-II	Site-I	Site-II	Site-I	Site-II
Penicillium spp.	7	3	2	5	2	1
Aspergillus spp.	6	2	1	4	1	1
Cladosporium spp.	5	3	2	4	2	0
Fusarium spp.	6	3	2	4	2	1
Verticillium spp.	4	2	1	3	0	0
Rhizopus spp.	2	2	1	2	1	0

between conductivity and number of individuals. Calcium content was minimum in winter and maximum in autumn. Statistical analysis indicated a positive correlation (r = 0.87) of the fungal individuals with magnesium.

Alkalinity shows marked seasonal variation and ranges between 107-330 (Table 1), with minimum in spring and maximum in summer. Chloride content ranges between 10-32 (Table 1), with minimum in spring and maximum in summer. Statistical analysis indicated a negative correlation (r = - 0.87) of the fungal species with temperature. Nitrate-nitrogen ranges between 206-465 (Table 1), with minimum in spring and maximum in autumn. Statistical analysis showed a positive correlation (r = 0.909) with the occurrence of species. It was noted that the maximum number of individuals occurred in summer and early autumn seasons when nitrate content of water was higher. The phosphorus content of the water varied between 134-390 µg/l (Table 1). The minimum value was recorded in autumn and maximum in summer. Statistical analysis showed a positive correlation (r = 0.934) with the occurrence of species. It was noted that the maximum number of individuals occurred in summer and early autumn seasons when phosphate content of water was higher (237 - 390 µg/l).

The result obtained during the present investigation revealed that the species composition of the water borne fungi varied considerably from season to season (Table 2), which would be attributed to the variation in physico-chemical characteristics of the habitat which have profound influence on the occurrence and distribution of water borne conidial fungi.

A perusal of seasonal occurrence of different species in the habitat indicates that the water borne fungi show a marked seasonal fluctuation in their occurrence. A maximum number of the fungal species was found during summer to early autumn, while number of species decline in late autumn to winter seasons. Occurrence of maximum number of individuals during summer and early autumn seasons in the present study may possibly be due to the increase in organic matter and more feasible temperature in such seasons (Khulbe and Durgapal, 1992). It can be attributed to the entry of sewage from the drains into the lake, as these genera have been reported frequently from the drain waters with maximum densities during higher pollution (Khulbe and Durgapal, 1994) and can therefore be inferred that these species are good indicators of pollution. In temperate regions, the aquatic fungal communities have been found to be effected by variations in temperature (Shearer, 1972; Iqbal and Webster, 1973; Suberkropp, 1992). Many investigators

Table 3. Correlation of fungal species with physico-chemical parameters of water.

Species	Temperature	Transparency	DO	pH	Conductivity	Ca	Mg	Alkalinity	Chloride	NO_3-N	TP
Penicillium	0.929**	-0.828*	-0.897*	-0.918**	0.801	-0.165	0.872*	0.465	-0.829*	0.909*	0.951**
Aspergillus	0.914*	-0.771	-0.858*	-0.882*	0.741	-0.268	0.901*	0.403	-0.870*	0.886*	0.934**
Cladosporium	0.890*	-0.893*	-0.913*	-0.923**	0.873*	0.069	0.728	0.589	-0.676	0.900*	0.915*
Fusarium	0.898*	-0.829*	-0.887*	-0.902*	0.815*	-0.064	0.825*	0.510	-0.762	0.877*	0.919**
Verticillium	0.860*	-0.779	-0.832*	-0.857*	0.746	-0.122	0.834*	0.443	-0.790	0.895*	0.914*
Rhizopus	0.776	-0.893*	-0.854*	-0.852*	0.884*	0.301	0.487	0.729	-0.464	0.841*	0.804

**Correlation is significant at the 0.01 level (2 tailed); *correlation is significant at the 0.05 level (2 tailed).

have observed similar maxima during summer periods (Sridhar and Kaveriappa, 1984) and suggested that after rainfall the large amounts of various leaf detritus get transferred into the stream through rain wash from distant places and stream gets greater abundance of these fungi. Table 3 summarizes the relationship between number of fungal species and different physico-chemical parameters (temperature, pH, transparency, conductivity, dissolved oxygen, calcium, magnesium, chloride, alkalinity, nitrate-nitrogen and total phosphorus) of Hokersar wetland.

There was a negative correlation with species number and pH within certain range (r = - .923). This indicates that high pH might not be suitable for these fungi. Barlocher and Rosset (1981) suggested that pH close to 7.0 favour higher numbers of fungal species. The occurrences of water borne conidial fungi also show negative correlation with temperature (r = -0.06454). This finding was found to be in support of Mer and Sati (1989) and Raviraja et al. (1998) studies. Water borne conidial fungi obtain phosphate and sulphate not only from the leaf litter, wood debris but also directly from water passing by riverine areas (Suberkropp, 1995; Suberkropp and Chauvet, 1995).

The result of the investigation shows a positive correlation between phosphate concentration and species occurrence (r = 0.9). It justifies the findings of Krauss et al. (2001), who also reported the stimulation of fungus activity at high P concentrations. In the present study, the impact of temperature, pH, transparency, conductivity, dissolved oxygen, calcium, magnesium, chloride, alkalinity, nitrate-N and total phosphorus content showed a marked influence on the occurrence and distribution of the water borne conidial fungi. In the last decade, different fungal groups have been used for assessing the impacts of pollution. The correlation analysis of fungal species with water quality can be used for assessing the potentialities of fungal communities as indicators of pollution. The roles of fungal communities have been a debatable aspect before researchers in recent time. Duarte et al. (2008), for example, showed that high diversity of fungi may mitigate the effects of pollution on plant litter decomposition. They play potentially crucial roles in nutrient and carbon cycling and interact with other organisms, thereby influencing food web dynamics (Wurzbacher et al., 2010).

Principal component analysis of physicochemical parameters

PCA was carried out to extract the most important physical parameters affecting the diversity of the microbial community. These physical parameters include the water temperature, transparency, dissolved oxygen, pH, conductivity, calcium, magnesium, alkalinity, chloride, nitrate-nitrogen and total phosphorus.

SPSS 16.0 and Pastprogramme were used to carry out principal component analysis to determine the main principal components from the original variables (Muller et al., 2001; Ogino et al., 2001; Van Der Gucht et al., 2001; Yang et al., 2001). Based on the Eigen values scree plot (Figure 1), the original 11 physico-chemical parameters were reduced to 2 main factors (factor 1 and factor 2) from the leveling-off point(s) in the scree plot as suggested by Cattell (1966). The factor corresponding to the largest Eigen value (13.7) accounts for approximately 80.6% of the total variance. The second factor corresponding to the second Eigen value (3.2) accounts for approximately 19.36% of the total variance. The remaining 3 factors have Eigen values of less than unity. The scree plot agrees well with the Kaiser criterion (Kaiser, 1960) where factors with an Eigen value greater than unity would be retained for further analysis (in this case, 2 principal components were retained). Further analysis of factor loadings showed that water temperature, dissolved oxygen, pH, nitrate- nitrogen

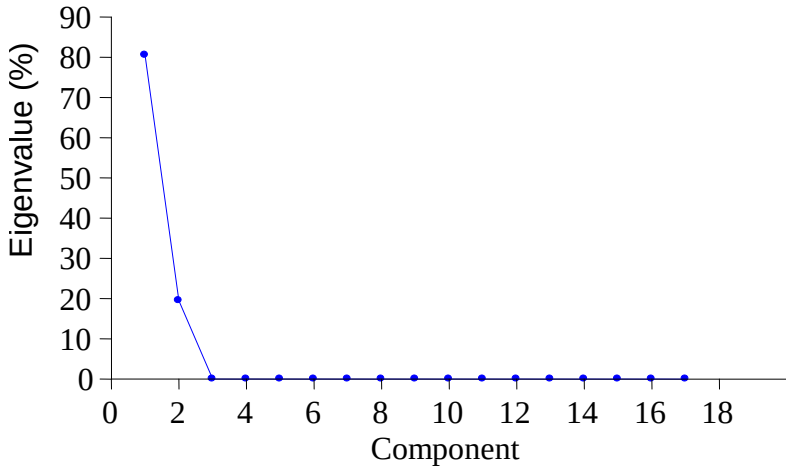

Figure 1. Eigen values scree plot for determining principal components for further analysis.

Table 4. Results of factor loading analysis to determine correlation between factors and variables.

Variable	Factor 1	Factor 2
Temp.	0.9985	-0.05513
Tran.	-0.9236	-0.3834
DO	-0.9849	-0.1729
pH	-0.9943	-0.1069
Cond.	0.8956	0.4449
Ca	-0.2075	0.9782
Mg	0.5834	0.8122
Alka.	-0.8132	0.582
Cl	0.747	-0.6648
NO_3-N	0.9975	0.07054
T Phos.	0.992	-0.1259
Peni.	0.9778	-0.2096
Aspe.	0.9778	-0.2096
Clad.	0.9977	-0.06776
Fusa.	0.9874	-0.1581
Verti.	0.9977	-0.06776
Rhiz	0.7985	0.6019

nitrogen, calcium and total phosphorus were the 5 major factors affecting the diversity of the microbial community (Table 4). For factor 1, water temperature, pH has the highest factor loading value (0.99), which shows that these are the most influential variables for the first factor or principal component. For factor 2, calcium has the highest factor loading value (0.97), and magnesium is a second influential variable with factor loading value of 0.81. Factor loadings can be interpreted as the correlation between the factors and the variables (physic-chemical parameters).

To determine which sampling points were closely related, a plot of factor coordinates for all observations (cases) was constructed using the factors obtained from factor loading analysis. Figure 2 shows the cluster of sampling points (as affected by all 11 physical parameters). The cases (sampling points) that are clustered near each other have similar characteristics with respect to the factors. As can be seen from Figure 2 there are 5 distinct fungal species when projecting all the cases onto the factor plane. All the fungal species are located on the positive side of this dimension- toward nitrate-nitrogen and total phosphorus content. Thus, it appears that nitrate-nitrogen and total phosphorus plays a major role in shaping the number of the microbial community toward the middle of the summer. pH and dissolved oxygen on the other side shows inverse relationship with number of fungal species. Fungal species are close to summer season, which indicates that they thrive well in higher temperatures.

Conclusions

With the data obtained, it can be concluded that occurrence and distribution of the water borne conidial fungi is governed by interaction of temperature, pH, transparency, conductivity, dissolved oxygen, calcium, magnesium, chloride, alkalinity, nitrate-N and total phosphorus of wetland water. The major environmental factors affecting fungal diversity in this particular wetland appear to be pH, temperature, nitrate-nitrogen and total phosphorus based on PCA analyses. Based on the PCA and correlation analysis the environmental conditions (pH, temperature, nitrate-nitrogen and total phosphorus) dictate the proliferation of fungal communities within different seasons of the year. Environmental conditions vary at each location within wetland. These changes in

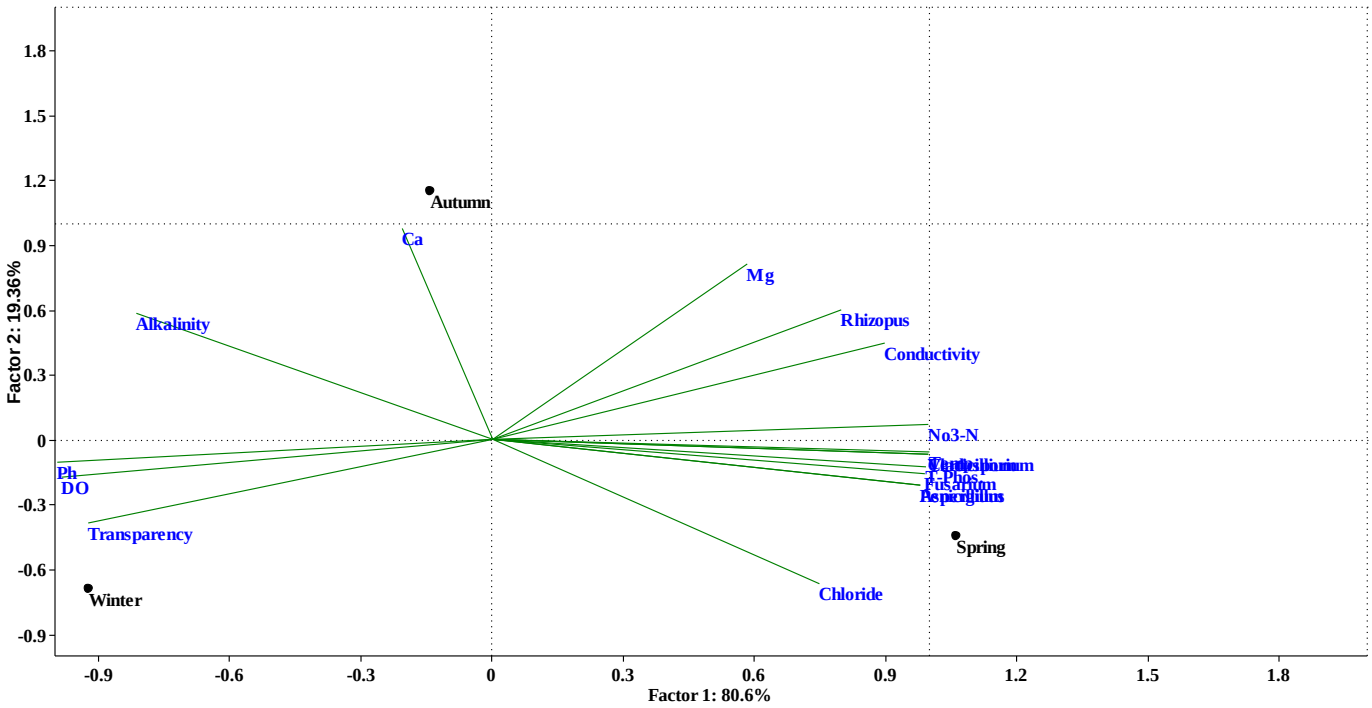

Figure 2. Two-dimensional plot of the PCA performed for the whole data set, including physicochemical, seasonal and biological data. Temp, temperature; Do, dissolved oxygen; pH; Cond, conductivity; Ca, calcium; Mg, magnecium; Alka, alkalinity; Cl, chloride; NO_3, nitrate- nitrogen; T Phos, total phosphorus, Peni, penicillium; Aspe, aspergillus; Clad, Cladosporium; Fusa, fusarium; Verti, Verticillium;Rhiz, rhizopus; Summer; Autumn; Winter.

environmental conditions do have a major effect on number of fungal individuals, even at the microclimate level.

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Full Length Research Paper

Staphylococcus aureus nasal carriage in centers of Casablanca (Morocco)

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The aim of this study was to determine the prevalence of nasal carriage of *Staphylococcus aureus* among patients and personnel of private centers at Casablanca, and to determine the resistance pattern of isolates. The carriage of virulence toxin genes by the methicillin resistant strains was also investigated. This study was conducted from November 2008 to February 2009. Nasal swabs were taken from 145 and 42 patients and personnel respectively. The susceptibility testing to 16 antibiotics was performed using the agar disc diffusion method. Minimum inhibitory concentrations (MICs) of oxacillin were determined by the agar dilution method for all strains demonstrating resistance to cefoxitin. In addition, resistant isolates were examined for the existence of the *mecA* gene by polymerase chain reaction (PCR). Furthermore, the carriage of 22 virulence toxin genes among strains showing resistance to cefoxitin was investigated by PCR Multiplex. The prevalence of nasal carriage of *S. aureus* was 32.4% (n=47) and 38.1% (n=16) in patients and personnel, respectively. Patients' strains showed 16 resistance patterns against only 4 in personnel strains. No *S. aureus* isolates were found to be resistant to lincomycin, pristinamycin, gentamicin, chloramphenicol, trimethoprim-sulfamethoxazole, rifampicin and vancomycin, while over 90% (n = 59) were resistant to penicillin G. For the other antibiotics, the percentage of resistance varied between 2.63 and 18.75%. One *S. aureus* (1.6%) was methicillin resistant by possession of *mecA* gene. This isolate harboured the staphylococcal enterotoxin genes *sec*, *sed*, *sell*, *selm*, *selo*, *ser* and toxic shock syndrome toxin gene (*tst*). Investigation of *S. aureus* nasal carriage and characterization of isolates among patients undergoing hemodialysis is important to develop infection prevention and to limit the spread of methicillin resistant *S. aureus* (MRSA) strains.

Key words: Hemodialysis centers, patients, personnel, *Staphylococcus aureus*, nasal carriage, antibiotic susceptibility, methicillin resistance, toxin genes.

INTRODUCTION

The anterior are the primary reservoir of *Staphylococcus aureus* in humans and its nasal carriage is recognised as a major risk factor for the development of both community-acquired and nosocomial infections (Boelaert et al., 1995; Koziol-Montewka et al., 2001), particularly in patients who are undergoing long-term hemodialysis

(Watanakunakorn et al., 1992). Infections complications are the main cause of morbidity and the second cause of mortality after cardiovascular diseases in chronic hemodialyzed patients. *S. aureus* is by far the most frequent bacterium implicated in these infections, especially in septicemia (Forestier et al., 2007; Koziol-Montewka et al., 2001; Koziol-Montewka et al., 2006).

Several factors are likely to depress the immune system of these patients, and thus, make them more susceptible to infection, such as old age, concurrent debilitating illnesses, long-term stay in hospital, repeated antibiotic treatment and specific immune defects associated with renal dysfunction (Koziol-Montewka et al., 2001). Therefore, recognition of persons colonized or infected with *S. aureus* is recommended for preventing the spread of the organism within hospitals or in communities. The emergence and dissemination of methicillin-resistant *S. aureus* (MRSA), which is also often multi-drug-resistant renders the treatment of staphylococcal infections more challenging.

Methicillin resistance in *S. aureus* is conferred by carriage of the *mecA* gene that codes for the penicillin binding protein PBP2a, with very low affinity to beta-lactam antibiotics (Beck et al., 1986; Katayama et al., 2000). Many *S. aureus* strains, especially MRSA produces a variety of extracellular toxins and virulence factors (Hu et al., 2008) that contribute to its pathogenic potential, including staphylococcal enterotoxins (SEs), SEs-like, Panton-Valentine leukocidin (PVL), toxic shock syndrome toxin-1 (TSST-1), exfoliative toxin (ET), hemolysins, and coagulase (Hu et al., 2008).

Increasing resistance to the drugs has recently raised the concerns of both microbiologists and clinicians, especially in the case of MRSA strains. Data on the epidemiology of antibiotic resistance are relevant, as they should provide a basis for the selection of empiric use of antimicrobial agents, either for therapy or for prophylaxis, especially patients receiving hemodialysis are at particular risk for the development of invasive infections caused by staphylococci (Watanakunakorn et al., 1992).
The purpose of this study was, on one hand, to define the frequency of *S. aureus* nasal carriage from patients and personnel of three private hemodialysis centers at Casablanca (Morocco) and to investigate antibiotic resistance rates with the choice of the method suited for the detection of the MRSA; on the other hand, to estimate the frequency and to access the virulence potential of methicillin resistant *S. aureus* isolates.

MATERIALS AND METHODS

Sampled persons

Nasal swabs were collected from patients and personnel (medical staff was not excluded) of three private hemodialysis centres in Casablanca during four months (November 2008 to February 2009). Each person was asked to give informed consent prior to specimen collection. Characteristics of the two study groups are noted (age, sex, treatment period with hemodialysis, history of hospitalisation, antibiotic therapy, and history of underlying diseases such as diabetes).

Nasal swabs

Nasal specimens for culture were taken with sterile, cotton tipped swabs and were obtained by 5 rotations in each anterior nary. All nasal swabs were quickly sent to the bacteriological laboratory in closed boxes and were processed on the day of sampling.

Isolation of *S. aureus* from nasal swabs

The study was conducted at the Laboratory of Molecular Bacteriology of the Pasteur institute, Casablanca, Morocco. Nasal swabs were screened for the presence of *S. aureus*. The swabs were inoculated and streaked on Chapman agar (local production according to its composition), and incubated aerobically at 36°C for 18 to 24 h. Only one isolate from each individual was included. After incubation, media were investigated and presumptive *S. aureus* colonies were identified, based on colony morphology, mannitol fermentation, catalase test and Gram staining, coagulase activity on rabbit plasma (Bio-Mérieux, Marcy l'Etoile, France), and production of clumping factor (Pastorex Plus-Staph, Bio-Rad, Marnes-la-Coquette, France). The homogeneous strains were stored at 4°C in nutrient agar until use, and at -20°C in glycerol stocks using commercial Cryobilles (AES Laboratoire; France).

Methicillin sensitivity

Methicillin susceptible *S. aureus* strains (MSSA) were differentiated from MRSA using agar screen plates on Muller-Hinton (M-H) agar (Bio-Rad, France) containing 30 µg/ml of cefoxitin disc as recommended by the French Microbiological Society Antibiogram Committee (CA-SFM 2009). Minimum inhibitory concentrations (MICs) to oxacillin were determined by the agar dilution procedure according to the CA-SFM guidelines (2009), for all strains showing a growth inhibition zone diameter ≤ 27 mm after incubation at 36°C for 18-24 h. The range of dilution used was 0.004 to 128 mg/L and the breakpoint for the definition of oxacillin resistance was MICs ≥ 2 µg/mL. MRSA reference strain U2A1593 and one methicillin-susceptible *S. aureus* reference strain U2A1594 provided by Pasteur Institute (Paris, France) were used as controls.

Antibiotic susceptibility testing

Antibiotic sensitivity of all *S. aureus* strains was performed on the isolates using the agar disc diffusion method on M-H medium (Bio-Rad, France) according to the recommendations given by the CA-SFM (2009). Disks loaded with the following antimicrobial agents (Bio-Rad, France) were used for susceptibility testing: cefoxitin (30 µg), penicillin G (6 µg), tetracycline (30 µg), erythromycin (15 µg), lincomycin (15 µg), pristinamycin (15 µg), kanamycin (30 µg), tobramycin (30 µg), gentamicin (10 µg), chloramphenicol (30 µg), rifampicin (5 µg), fosfomycin (50 µg), pefloxacin (5 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), fusidic acid (10 µg) and vancomycin (30 µg). In addition, *mecA* negative strains

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Table 1. Details of personnel and hemodialyzed patients *Staphylococcus* carriers and non-carriers

Parameter	*S. aureus* carriers	*S. aureus* non-carriers
Number of patient and percentage (n= 145)	47 (32.4%)	98 (67.6%)
Mean age		
Sex	22 men (46.8%) 25 women (53.2%)	
Number of personnel and percentage (n= 42)	16 (38.1%)	26 (61.9%)
Mean age		
Sex	8 men (50%) 8 women (50%)	

with reduced susceptibility to cefoxitin were submitted to the action of the following betalactamins antibiotics: cefalotin, amoxicillin and amoxicillin+clavulanic acid. Any penicillin G susceptible strain (inhibition diameter greater or equal to 29 mm) was submitted to a chromogenic test (cefinase test) for confirmation. The performance of the susceptibility testing was monitored by the quality control strains *S. aureus* ATCC 25923.

All isolates of *S. aureus* with reduced susceptibility to cefoxitin were grown in brain heart infusion media at 36°C overnight. Their genomic DNA used for PCR was extracted by using a standard phenol-chloroform procedure as described by Sambrook et al. (1989). In addition, the *nuc* gene responsible for the production of thermostable nuclease was detected by PCR assay (see *nuc* and *mecA* genes detection) in order to confirm that the isolates were indeed *S. aureus* and not other staphylococcal species.

nuc and *mecA* genes detection

The presence of the *mecA* gene was determined by Polymerase Chain Reaction methods (PCR) in isolates that showed cefoxitin zone sizes smaller or equal to 27 mm in order to confirm the assumption that the methicillin (cefoxitin) resistant strain was a MRSA. Duplex PCR was performed for the simultaneous detection of the *nuc* (encoding for the *S. aureus* specific thermonuclease) and the *mecA* (encoding for the PBP2a) genes using protocols and primers as described by Chesneau et al. (1993) and Vannuffel et al. (1995), respectively. The control organisms included *S. aureus* U2A 1594 (MSSA negative *mecA*) and *S. aureus* U2A 1593 (MRSA, positive *mecA*).

Virulence profile of *S. aureus* strains

To assess the virulence potential of strains demonstrating resistance to cefoxitin (CMI value of oxacillin ≥ 2 µg/mL), several Multipex PCRs for the parallel detection of the presence of genes coding for: the classical staphylococcal enterotoxins A, B, C and D (*sea, seb, sec* and *sed*) SEs, and SE*ls* H, K, L, M, O, P, Q and R (*seh, selk, sell, selm, selo, selp, selq* and *selr*), the toxic shock syndrome toxin-1 (*tst*), the exfoliative toxins A, B and D (*eta, etb* and *etd*), the Panton Valentine leukocidin (PVL) (*luk*S-PV; *luk*F-PV), the Luk-M leukocidin (*luk*M), the epidermal cell differentiation inhibitor A, B and C (*edin* A, B and C) and β- hemolysin (*hlb*) as described previously (Holtfreter et al., 2007; Jarraud et al., 2002; Tristan et al., 2003), were performed. Positivity was verified using reference strains *S. aureus* ATCC19095 (*sec, seh, sell, seg, sei, selm, seln, selo* and *seu*); FRI913 (*sea, sec, see, selk, sell, selq* and *tst*); ATCC14458 (*seb*). However, control chromosomal DNA samples for *sed*, *selr, selp, luk*-PV, *luk*M, *eta*, *etb*, *etd*, *edin* A/B/C and *hlb* genes were obtained from our standard laboratory controls.

RESULTS

Nasal carriage

In this study, we investigated a total of 42 personnel members (20 men (47.6%) and 22 women (52.4%)) and 145 patients (73 men (50.3%) and 72 women (49.7%)), who were between 15 and 94 years of age. The mean time on dialysis treatment was 72 months (range 3-264 months). Among hemodialyzed patients, 30 have stayed in a hospital or clinic at least 3 days during the current year (or year of study). Nasal screening identified 47 (32.4%) and 16 (38.1%) *S. aureus* carriers among patients and personnel respectively with a male to female ratio of 0.8 and 1, respectively (Table 1).

Detection of MRSA

All 63 *S. aureus* isolates were tested for cefoxitin resistance using a disk diffusion method and most (82%) were found to be susceptible to cefoxitin. For the remaining 11 cefoxitin nonsusceptible patient's isolates, MICs to oxacillin were determined (Table 2) and detection of the *mecA* gene was done. Four of the 11 strains had MIC to oxacillin higher than 2 µg/mL but only one strain (D11) was *mecA* carrier (Figure 1). The remaining strains were *mecA* negative and expressed resistance to cefalotin and amoxicillin and susceptibility to amoxicillin-clavulanic acid.

Antibiotic susceptibility

On the whole, 18 various resistance phenotypes are found for the 63 *S. aureus* strains (Table 3). The patient's isolates showed the greatest number (16 against only 4 in personnel isolates). Wild-type phenotype (*S. aureus* susceptible to all antimicrobial agents tested) represented a small fraction, that is, two personnel strains (3.4%) of the all 63 isolates tested. Multiresistance to more than three antibiotic classes was not observed in personnel isolates while 2 (4.3%) and 3 (6.4%) *S. aureus* patient's isolates showed multiresistance to 4 and 5 antibiotics respectively (Table 3).

Table 2. Range of methicillin (oxacillin) MICs for 11 isolates of MRSA (Diameter zone to cefoxitin ≤ 27 mm) from hemodialyzed patients by agar dilution method.

Strain code	Inhibition diameter of cefoxitin (in mm)	oxacillin MICs values (µg/mL)
D4	24	1.0
D6	26	1.0
D11	**15**	**16**
D20	**24**	**8.0**
D21	25	0.5
D46	26	0.5
D52	25	0.5
D59	**24**	**8.0**
D80	24	0.5
D88	**24**	**2.0**
D93	**24**	**0.5**

MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*.

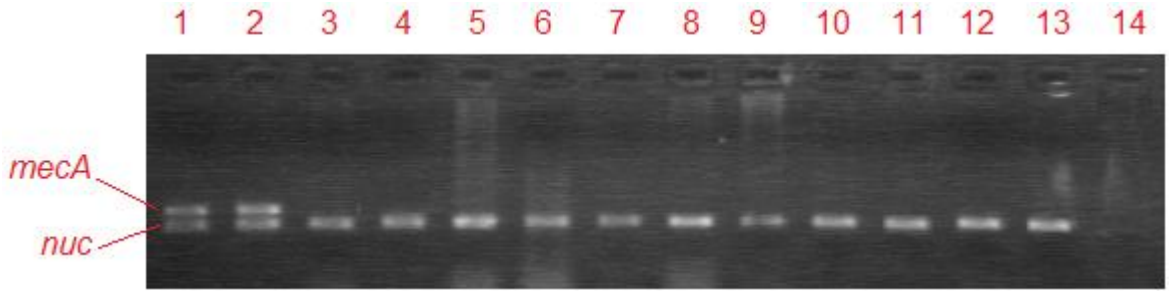

Figure 1. Detection of *nuc* and *mecA* genes by PCR duplex. Lanes 1 and 13, positive control *mecA* (MRSA strain U2A1593) and negative control *mecA* (MSSA strain 1594), respectively; lane 2: *mecA* positive strain (D11); lines 3 to 5: *S. aureus* BORSA (D20, D59, D88); lines 6 to 12: the others *S. aureus* isolates (D4, D6, D21, D46, D52, D80 and D93); line 14: control negative.

Table 3. Resistance profile of *S. aureus* strains isolated from personnel (n=16) and hemodialyzed patients (n=47) to 16 tested antibiotics.

Resistance phenotype profile	Frequency in personnel isolates (n = 16)	Frequency in hemodialyzed patients isolates (n=47)
Wild-type	0	2 (4.2%)
PG	9 (56.2%)	22 (46.8%)
RF	-	1 (2.1%)
Total	**9 (56.2%)**	**23 (49%)**
PG-Fox	3 (18.7%)	6 (12.8%)
PG-TE	-	3 (6.4%)
PG-E	3 (18.7%)	-
PG-Pef	-	1 (2.1%)
PG-FA	-	1 (2.1%)
E-RF	-	1 (2.1%)
Total	**6 (37.5%)**	**12 (25.5%)**

Table 3. Contd.

PG-TE-RF	1 (6.25%)	2 (4.2%)
PG-RF-FA	-	1 (2.1%)
PG-E-K	-	1 (2.1%)
PG-K-FA	-	1 (2.1%)
Total	**1 (6.25%)**	**5 (10.6%)**
PG-E-K-Fox	-	1 (2.1%)
PG-K-TE-FA	-	1 (2.1%)
Total	**0**	**2 (4.2%)**
PG-K-TM-Fox-FA*	-	1 (2.1%)
PG-K-TE-Fox-RF	-	1 (2.1%)
PG-TE-RF-Pef-FA	-	1 (2.1%)
Total	**0**	**3 (6.4%)**

PG: Penicillin G, GM: Gentamicin, K: Kanamycin, TM: Tobramycin, TE: Tetracycline, L: Lincomycin, E: Erythromycin, PT: Pristinamycin, C: Chloramphenicol, Pef: Pefloxacin, Fos: Fosfomycin, Fox: Cefoxitin, FA: Fusidic acid, RF: Rifampicin, VA: Vancomycine and SXT: Trimethoprim-sulfamethoxazole. * *mecA*(+).

Table 4. *S. aureus* strains antibiotic susceptibility pattern in hemodialyzed patients and personnel

Antimicrobial	Antimicrobial resistance rate (%)				
	Patients strains				Personnel strain
	MSSA (n=43)	BORSA (n=3)	MRSA (n=1)	Total (n = 47)	MSSA (n = 16)
Penicillin G	43 (100%)	3 (100%)	1	47 (100%)	14 (87.5%)
Lincomycin	-	-	-	0	0
Erythromycin	1 (2.3%)	1 (33.3%)	1	3 (6.4%)	3 (18.75%)
Pristinamycin	-	-	-	0	0
Kanamycin	3 (6.9%)	2 (66.6%)	1	6 (12.8%)	0
Tobramycin	0	-	1	0	0
Gentamicine	-	-	-	0	0
Chloramphenicol	-	-	-	0	0
Tetracycline	7 (16.2%)	1 (33.3%)	-	8 (17%)	1 (6.25%)
Cotrimoxazole	0	0		0	0
Cefoxitin	-	3[a] (100%)	1[b]	4 (8.5%)	0
Rifampicin	6 (14%)	1 (33.3%)	-	7 (14.9%)	1 (6.25%)
Fosfomycin	-	-	-	0	0
Pefloxacin	2 (4.6%)	-	-	2 (4.25%)	0
Vancomycin	-	-	-	0	0
Fusidic Acid	5 (11.6%)	-	1	6 (12.8%)	0

a: Borderline *S. aureus* isolate (BORSA). b: *S. aureus* isolate harbouring the *mec*A gene.

In vitro, antibiotic susceptibilities of *S. aureus* isolates (MSSA, Borderline *S. aureus* (BORSA) and MRSA) are shown in Table 4. For all 63 isolates, no *S. aureus* isolates were found to be resistant to lincomycin, pristinamycin, gentamicin, chloramphenicol, trimethoprim-sulfamethoxazole, fosfomycin and vancomycin, while over 90% were resistant to penicillin G. For the other antibiotics, in all, the percentage of resistance is relatively low and varied between 4.25 and 18.75%. The greatest percentage in resistance was observed against erythro-

mycin (18.75%) and tetracycline (16.2%) for the personnel and patients isolates respectively.

As expected, the resistance rates among isolates from patients were higher compared with the rates among *S. aureus* isolates from personnel. All personnel isolates were found to be susceptible to tobramycin, kanamycin, pefloxacin and fusidic acid while resistance to these antibiotics was noted among patients' isolates. Resistance profiles also differ between MSSA, BORSA and MRSA isolates with regard to some antibiotics and to resistance frequency (Table 4).

Virulence profile

The MRSA strain D11 harboured the enterotoxin genes *sec*, *sed*, *sell*, *selm*, *selo*, *ser* in combination with *tst*. D59 BORSA strain had none of the toxin genes investigated while enterotoxin gene *selo* was detected in D20 and D88 BORSA isolates in combination with *selm* and *tst* respectively.

DISCUSSION

A major part of dialysis-related infection is endogenous and related to high frequency nasal and skin carriage of *S. aureus*. Dialysis patients generally tend to have a higher carrier rate of *S. aureus* (32 to 82%) than other hospital patients and the personnel working in dialysis (Herwaldt, 2003; Herwaldt et al., 2003).

We revealed that 32.4 and 38.1% of hemodialyzed patients and personnel respectively, were *S. aureus* carriers. This percentage is close to that reported by Saxena and Panhotra (2003) among hemodialyzed patients in Saudi Arabia (38%), but was lower than that found in the studies of Edoh et al. (2003) in Ivory Coast where it reaches 85.7 and 87.5% in hemodialyzed patients and personnel respectively. In a more recent study, Oumokhtar and colleagues (2012) have found, in Fez city (Morocco), the rate prevalence of 42.9%.

As with ordinary strains of *S. aureus*, some patients harbour MRSA on their skin or nose and are at increased risk of developing infection. The emergence and dissemination of MRSA that often demonstrate multi-drug resistance is of great global concern, particularly in healthcare settings. Increased surveillance, including screening of high-risk patients, has been recognized as an important component of effective infection control programs to limit the spread of MRSA in hospitals and in the community. So, the choice of the suitable method for the detection of MRSA is crucial. The study conducted by Gueudet and Lemble (2004) on the comparison of five usual techniques for detection of MRSA, has demonstrated that the disk diffusion method using cefoxitin 30 µg, on M-H agar plate has a specificity of 100%. In our study, among the 11 *S. aureus* isolates that demonstrating resistance to methicillin by the disc diffusion test with cefoxitin, only one isolate harboured *mecA* gene and three were BORSA isolates, whereas seven strains were susceptible to oxacillin. We confirm that the gold standard for determining if a strain of *S. aureus* is MRSA, MSSA or BORSA is to test the isolate for the minimum inhibitory concentration to oxacilin (or cefoxitin) and to define the presence of the *mecA* gene using PCR methods. So, any prevalence of MRSA, determined only by disk diffusion tests, should be undertaken with extreme caution since they can be erroneous.

The surveys of the frequency of methicillin resistance in *S. aureus* were particularly determined among hospital strains. The epidemiology of MRSA varies considerably on a global basis and even shows remarkable differences at regional level (Grundmann et al., 2006). In Europe, a north to south gradient has been reported, with the highest proportions of resistant isolates found in the Mediterranean countries (Stefani and Varaldo, 2003). Greece, Spain, Italy, Israel and Croatia have all reported prevalence of 25% or more for methicillin resistance within *S. aureus* blood culture isolates (Tiemersma et al., 2004).

The lowest overall MRSA proportion was found in Tunisia (18%) and Morocco (at Ibn Rochd hospital, Casablanca) (19%) (Borg et al., 2007). In recent study, conducted by Elhamzaoui et al. (2009), the rate of MRSA was 13.5% (by using agar diffusion method with cefoxitin 30) among *S. aureus* strains isolated from various samples collected in several care units in two Moroccan teaching hospitals. Lowest values are found among community *S. aureus* strains isolated from different specimens collected in several private laboratories, 1.9 and 1.4% rates are reported by studies of Belabbes et al. (2001) and Elazhari et al. (2011), respectively.

The rate of MRSA (by possession of *mecA* gene) in hemodialyzed patients revealed by our study was 2.1% (1/47). If we take into account the whole strains including personnel isolates, this percentage is only 1.6%. The strain was cultured from a 61 year-old female not suffering from diabetes. The patient had not a history of previous hospitalisation. This MRSA strain was susceptible to 9/16 tested antibiotics including vancomycin. Decreasing susceptibility of MRSA isolates to vancomycin, which is currently the drug of choice for MRSA infections are, reported (Hiramatsu, 1998), what is very worrying. MRSA strain D11 (Table 4) has the following phenotype of resistance: PG-K-TM-Fox-FA (resistance to penicillin G, kanamycin, tobramycin, cefoxitin and fusidic acid).

The presence of multi-drug resistance, which has been considered a characteristic feature of nosocomial MRSA (Diep et al., 2004; Fey et al., 2003; Okuma et al., 2002), suggested that the strain might be a member of Hospital-Acquired MRSA (HA-MRSA) since this group of staphylococci is commonly resistant to the majority of other non beta-lactam antibiotics (Fey et al., 2003). The

hypothesis was subsequently corroborated by the fact that the MRSA strain was negative for the PVL leukocidin gene by PCR. Indeed, the expression of PVL has been strongly associated with Community-Acquired MRSA (CA-MRSA) (Boyle-Vavra and Daum, 2007; Holmes et al., 2005). Nevertheless, the elucidation of the origin of the MRSA was not possible with certainty. The prevalence of MRSA (*mec*A +) strains (2.1 or 1.6%) in our study was inferior to those reported by other authors: Ternois et al. (1993), Kresken et al. (2004) and Edoh et al. (2003) found 13, 20.7 and 22.7%, respectively. Although our rate was slightly inferior to result (5.3%) published by Askarian et al. (2009). In more recent work, Omokhtar and co-workers (2012) found 3.3% MRSA among hemodialyzed nasal carriage *S. aureus* in Morocco.

All the others *S. aureus* isolates were found to be susceptible to lincomycin, pristinamycin, gentamicin, chloramphenicol, trimethoprim-sulfamethoxazole, rifampicin and vancomycin (Table 4). The rate of nasal colonization of BORSA in hemodialyzed patients sampled revealed to be 6.4% (n=3). The mechanism of resistance exhibited by BORSA includes excessive penicillinase production or plasmid mediated inducible methicillinase (Chambers, 1997; Santhosh et al., 2008). The low level of oxacillin resistance in these strains is thought to be due to their hyperproduction of extracellular β-lactamase since clavulanic acid restore their susceptibility to amoxicillin.

The clinical impact of carriage of these strains by patients undergoing hemodialysis should be considered. Possible risk factors for patients harbouring BORSA in a case control study by (Balslev et al., 2005) showed that, in comparison to the controls, BORSA infected patients were more prone to severe skin infections, were more often hospitalized, and had more bed-days.

The MRSA D11 has accumulated enterotoxins genes (*sec*, *sed*, *sell*, *selm*, *selo* and *ser*) and *tst* gene that would increase its pathogenic potential. D20 and D88 isolates harboured the enterotoxin genes *selo* in combination either with *ser* or *tst* respectively.

Enterotoxins and toxic shock syndrome toxin (TSST-1) are important virulence factors, and as pyrogenic toxin superantigens, they have profound effects on their host. Thus, the circulation of TSST-1 producing *S. aureus* among patients prone to staphylococcal infections is a worrying issue. Furthermore, these bacterial populations with variable virulence represent a new challenge in terms of pathogenicity, treatment, and prevention of transmission.

Although colonized patients have no signs or symptoms of infection, they can still serve as a source from which transmission may occur. Colonized personnel can also serve as reservoirs. Hence, adequate establishment of staphylococcal nasal carrier status accompanied by characterization of cultured isolates along with anti-biotic susceptibility testing is crucial in patients particularly prone to infections caused by these bacteria, such as those receiving hemodialysis in order to develop infection prevention measures and treatment strategies.

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Full Length Research Paper

Comparison of the microbial quality of pork and poultry meat with or without grill marinade available in Polish retail markets

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During April and September 2012, a study on pork and poultry meat was undertaken to assess the differences in microbial quality of pork and poultry between the marinated and unmarinated products available in local trade network. Examination of the meats revealed that the vast majority of samples (98.5%) were of satisfactory microbiological quality according to Commission Regulation (EC) No 2073/2005, as amended. Unsatisfactory quality was due to the presence of *Salmonella* spp. in 5 pork necks with grill marinade. *Campylobacter* spp. was detected in 81% of poultry meat samples, and *Listeria monocytogenes* in 43% of all types of analysed meat. The total bacterial count was from 1.3 x 10^5 CFU/g for neck without marinade to 7.9 x 10^7 CFU/g for neck with marinade 1. The number of *Enterobacteriaceae* ranged between < 10 CFU/g (for neck without marinade) and 1.4 x 10^7 CFU/g (for chicken shashlik). The one-way ANOVA showed the significant influence of marinade on the number of *Enterobacteriaceae* and total bacterial count in all tested meat samples. No significant differences were observed taking into account the mean number of lactic acid bacteria and yeast (p>0.05). The relatively high contamination of marinated and unmarinated pork and poultry meat with food spoilage microorganisms and pathogens decreases the quality and constitutes a public health hazard but it is comparable with meat quality of developed countries.

Key words: Microbial quality, meat, pathogens, grill marinade.

INTRODUCTION

Grilling is a form of cooking dishes without fat, on grill placed over heat source. Nowadays, it is a very fashionable way of preparing food. In Poland, grilling tradition started in the first half of the 1990s. Initially, consumers usually bought grilling meat products, such as thin sausage, black pudding and bacon. Currently, the most popular are: neck-ends, knuckle ribs and small pieces of chicken: wings, legs, breast fillets. The demand for this kind of products is growing by up to 30% in the peak season between April and September. Simultaneously, a decrease in sale (by 20-25%) of traditional meat for polish dinner, the loin and ham can be observed. Consumers are more willing to get new products, especially products previously seasoned or marinated. Industrial marinades include: oil, water, spices, organic acids and glucose or sucrose.

With the marinade, time of meal preparation is reduced significantly, and the dishes have a wonderful aroma, a deep and attractive colour, enhanced flavour and juiciness fragility (Björkroth, 2005). During the heat

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treatment marinated meat evenly grills and does not burn up. In the available commercial offer, ready seasoned and marinated products constitute over a quarter of all of meat on the grill.

Despite the large variety of grill equipment facilities, the most popular used during the spring and summer in Poland are charcoal grills. These are the oldest types of grills, with temperatures of 260°C, suggesting a safe food preparation. However, the minimum time required for a trained fire up the grill is about 20-25 min.

As people are leading a very busy life style and not finding sufficient time for grilling, there is a high risk of food poisoning and when the meat is uncooked. Therefore, one of the most important issues, in addition to sensory value, is to ensure the microbiological safety of meat, spices and marinades. Marinating has been considered to increase safety of meat products. However, it may not necessarily have an effect on the survival of some enteric pathogens, like *Campylobacter* spp. (Björkroth, 2005). Moreover, the critical components, such as spices can decrease the microbial quality of marinades and have influence on their safety. In a study conducted by Dababneh (2013), 87.5% of the spices and herbs from local market were unsatisfactory because of the large population of fungi (87.5%) and coliforms (37.5%). Although plant materials used for producing commercially available herbs and spices contain natural antimicrobial substances, their microbiological quality is very diverse (Nokwanda and Ijabadeniyi, 2013). Micro-organisms isolated most often from spices are: non sporing bacteria, spore-forming bacteria, pathogenic bacteria, yeast and moulds. Particularly high microbial load was detected in spices like: black pepper, mustard, fenugreek, chilli,marjoram, cumin, red pepper, basil (Dababneh, 2013; Keller et al., 2013; Nokwanda and Ijabadeniyi, 2013; Van Doren et al., 2013b), commonly used in grill marinades.

Taking into account this fact, the Laboratory of Microbiology, Department of Refrigeration and Food Quality, Institute of Agricultural and Food Biotechnology has undertaken research aimed at determining the differences in microbial quality of pork and poultry between the marinated and unmarinated products available in local trade network.

MATERIALS AND METHODS

Sampling

A total of 340 samples of 10 types of commercially available meat were analyzed for their microbiological quality. The samples of meat were delivered to the Laboratory of Microbiology in isothermal containers, maintaining the temperature at 0 - 2°C, in the period from April to September 2012 and processed within 24 h. The material included 160 samples of pork and 180 samples of poultry meat. The pork consisted of 8 samples of neck without marinade and 24 samples of neck in three different types of marinades (herbs and garlic (1), paprika (2) and Bavarian (3)). Poultry meat consisted of 6 samples of chicken schnitzels, 6 samples of chicken schnitzels in paprika marinade, 6 samples of chicken wings, 6 samples of chicken wings in herbal paprika marinade, 6 samples of chicken stew and 6 samples of chicken shashlik. Microbiological analyses were carried out in 5 replicates.

Microbiological and statistical analyses

The meat was evaluated in terms of food safety and hygiene of the process. All microbiological parameters were sampled and analysed following Polish Standards (PN-EN ISO 4833: 2003; PN-EN ISO 6579: 2003 + A1:2007; PN-EN ISO 6888-1: 2001; PN-ISO 7954: 1999; PN-EN ISO 11290-2:2000+ A1:2005+ Ap1:2006; PN-ISO 10272-1:2007; PN-ISO 21528-2: 2005) and Commission Regulation (EC) No 2073/2005 of 15.11.05 on the microbial criteria for foodstuffs, as amended. The pathogens investigated included the presence of: *Salmonella* spp., *Listeria monocytogenes*, and poultry *Campylobacter* spp. Additionally, total bacterial count (TBC), the number of *Enterobacteriaceae* (E), lactic acid bacteria (LAB), coagulase-positive *Staphylococcus* (CPS), yeast and moulds were enumerated. API-tests (Biomerieux), Microgen Listeria ID (MicrogenBioproducts), and Singelpath (Merck) were used for biochemical and serological determination. All microbiological tests were carried out in 5 replicates, and the results expressed as log10 CFU/g. Data analysis was performed using STATISTICA software package (StatSoft, 2012). A one-way ANOVA analysis of variance was used for determination of the effect of marinades and spices on microbial quality of pork and poultry available in local trade network. The significance differences were indicated by ANOVA when $p= 0.05$ or less and $F>1$.

RESULTS

The frequency of detection of the pathogens in the tested meat samples is shown in Table 1. Examinations revealed that the vast majority of samples (98.5%) were of satisfactory microbiological quality according to Commission Regulation (EC) No 2073/2005 as amended. Unsatisfactory quality was caused by the presence of *Salmonella* spp. in 5 samples of necks in marinade 2. The spiced chicken shashlik had the highest prevalence of *L. monocytogenes* and *Campylobacter* spp.

The changes in counts of bacteria, yeast and moulds (CFU/g) for different types of meats are shown in Table 2. TBC displayed loads from 1.3×10^5 CFU/g (neck without marinade) to 7.9×10^7 CFU/g (neck with marinade 1). CPS were below the detection limit, except for three cases: neck with marinade 2, chicken wings in marinade and chicken shashlik, whose counts increased to 5.0×10^1 CFU/g, 8.0×10^1 CFU/g,1.0×10^2 CFU/g, respectively. The number of *Enterobacteriaceae* ranged between < 10 CFU/g (for neck without marinade) and 1.4×10^7 CFU/g (for spiced chicken shashlik), while LAB grew up to 1.9×10^7 CFU/g (neck with marinade 3). Moulds generally reached values between 10^1- 10^2 CFU/g with the highest values of 4.2×10^3 CFU/g for spiced chicken shashlik. In contrast, the viable counts of yeast were higher and grew over 10^6 CFU/g (neck with marinade 1).

The mean count of analysed microorganisms of marinated and unmarinated meat samples is depicted in Figure 1. In the present study, the average number of total bacterial count was within the range of 5.87 (chicken

Table 1. Frequency of isolated pathogens from tested meats.

Meat type	No. of meat samples	No (%) positive for			
		Listeria monocytogenes	*Salmonella* spp.	*Salmonella* Typhimurium/ Enteritidis	*Campylobacter* spp.
Neck without marinade	40	15 (37.5)	Not detected	-	not tested
Neck in marinade 1	40	10 (25.0)	Not detected	-	not tested
Neck in marinade 2	40	20 (50.0)	5 (12.5)	Not tested	not tested
Neck in marinade 3	40	10 (25.0)	Not detected	-	not tested
Chicken schnitzels	30	15 (50.0)	Not detected	-	15 (50.0)
Chicken schnitzels in marinade	30	10 (33.3)	Not detected	-	30 (100.0)
Chicken wings	30	10 (33.3)	Not detected	-	25 (83.3)
Chicken wings in marinade	30	15 (50.0)	Not detected	-	20 (66.7)
Chicken stew	30	10 (33.3)	Not detected	-	25 (83.3)
Spiced chicken shashlik	30	30 (100.0)	10 (33.3)	Not detected	30 (100.0)
Total	340	145 (42.6)	15 (4.4)		145 (80.6)

Table 2. Microbiological quality of tested meat.

Product	Counts of microorganisms [CFU/g]					
	TBC	LAB	E	CPS	Moulds	Yeast
Neck without marinade	1.3×10^5-7.8×10^6	1.7×10^3-6.5×10^6	< 10-2.3×10^3	< 10	< 10-5.0×10^1	< 10-3.8×10^3
Neck in marinade 1	8.5×10^6-7.9×10^7	1.3×10^4-8.1×10^6	< 10-8.0×10^5	< 10	< 10-1.0×10^3	1.4×10^3-1.3×10^6
Neck in marinade 2	6.8×10^6-2.8×10^7	9.6×10^4-9.3×10^6	5.0×10^2-4.0×10^5	< 10-5.0×10^1	< 10-2.1×10^2	5.6×10^2-1.1×10^6
Neck in marinade 3	3.9×10^6-5.6×10^7	7.1×10^5-1.9×10^7	3.4×10^2-1.0×10^4	< 10	< 10-3.1×10^2	2.8×10^2-1.5×10^5
Chicken schnitzels	2.9×10^5-1.6×10^7	< 10-3.5×10^5	2.5×10^2-3.6×10^5	< 10	< 10-1.9×10^2	8.2×10^2-2,0 $\times 10^5$
Chicken schnitzels in marinade	6.4×10^5-3.9×10^7	< 10-1.8×10^6	1.2×10^4-5.0×10^5	< 10	< 10-7.3×10^2	6.3×10^3-1.5×10^5
Chicken wings	1.4×10^5-4.4×10^6	< 10-2.0×10^4	2.3×10^3-2.6×10^4	< 10	8.0×10^1-5.5×10^2	4.9×10^2-2,3 $\times 10^5$
Chicken wings in marinade	1.5×10^6-3.7×10^7	1.2×10^2-6.2×10^5	4.8×10^4-5.1×10^5	< 10-8.0×10^1	7.0×10^1-2.7×10^3	3.5×10^3-2,7 $\times 10^5$
Chicken stew	4.6×10^2-6.1×10^7	< 10-3.4×10^5	5.0×10^3-2.1×10^5	< 10	< 10-2.0×10^2	6.2×10^3-1.2×10^5
Chicken shashlik	1.8×10^6-2.8×10^8	6.0×10^2-1.8×10^7	9.5×10^4-1.4×10^7	< 10-1.0×10^2	8.2×10^1-4.2×10^3	4.2×10^4-4.9×10^5

stew) -7.47 log10 CFU/g (chicken shashlik). The average count of *Enterobacteriaceae* ranged from 2.52 log10 CFU/g (neck unmarinated) to 5.87 log10 CFU/g (chicken shashlik). The population of LAB was 3.22 log10 CFU/g for chicken wings and 5.48 log10 CFU/g for chicken shashlik.

Irrespective of the analysed group of micro-organisms, the mean microbial load of meat (neck and chicken) with marinade and spices was

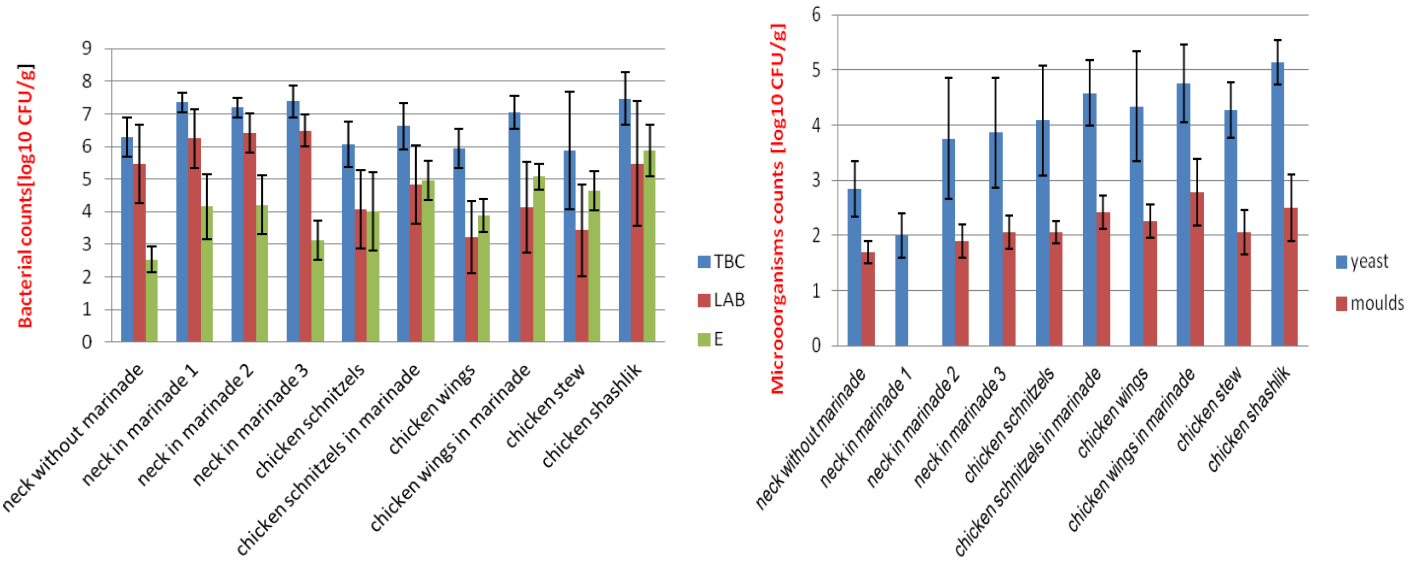

Figure 1. Mean count of analysed microorganisms (log10 CFU/g) of marinated and unmarinated meat samples (log10 CFU/g). a) Total bacterial counts (TBC), lactic acid bacteria (LAB), *Enterobacteriaceae* (E); b) Yeast and moulds.

higher compared to that without marinade and spices. The mean TBC for samples of neck with marinade 1, 2, 3 was 7.3, 7.19, 7.38, respectively, and for neck unmarinated was 6.28 log10 CFU/g. The mean TBC of marinade chicken schnitzels was 6.66; in contrast the mean TBC of chicken schnitzels without marinade was 6.07 log10 CFU/g. The mean number of *Enterobacteriaceae* in chicken shashlik was 5.87 as compared to 4.64 log10 CFU/g in chicken stew.

The one-way ANOVA showed the significance influence of marinade on the number of *Enterobacteriaceae* and total bacterial counts in all tested meat samples. No significant differences were observed taking into account mean number of lactic acid bacteria and yeast for meat with or without marinade ($p>0.05$).

DISCUSSION

According to the Regulative EU (Commission Regulation 2073/2005, as amended), the only parameters that can disqualify the tested meat is: the presence of *Salmonella* spp. (in pork) and the presence of *Salmonella* Typhimurium and Enteritidis (in poultry). These two serovars remain the most important in foodborne salmonellosis worldwide. International research indicates wide variation in the rates of meat samples contaminated by *Salmonella* spp. The presence of this pathogen in meat is dependent on a number of factors such as: the quality of meat, spices used or hygienic condition during production. The occurrence of *Salmonella* spp. in spices has been reported in many countries (Keller et al., 2013; Moreira et al., 2009; Van Doren et al., 2013a; Zweifel and Roger, 2012). Results obtained by the present study provided evidence that 4.4% of analysed samples were contaminated with *Salmonella* spp. None of the bacteria isolates belonged to *Salmonella* Enteritidis or Typhimurium. The survey performed by Greeson et al. (2013), Madden et al. (2011) and Zdragas et al. (2012) found 5.1, 13.7 and 39.5% samples of poultry meat to be positive for *Salmonella*, respectively. On the other hand, the results obtained by Chagas et al. (2013) indicated that 94.0% of the chicken carcass samples contained *Salmonella;* among these isolates, 32% were genotyped by multiplex polymerase chain reactions (mPCR) as *Salmonella* Enteritidis while none were identified as *Salmonella* Typhimurium. Furthermore, the prevalence of *Salmonella* in ground beef and pork was 0.4% (Schwaiger et al., 2012; Vipham et al., 2012).

L. monocytogenes in the current study was detected in 42.6% of analysed samples of meat that is comparable with the results obtained by other researchers. In the work conducted by Aarnisalo et al. (2008) the prevalence of *L. monocytogenes* in marinated broiler legs varied between 36 and 57% for the samples stored at 6 and 10°C, respectively. Survey performed by Goh et al. (2012) showed that the occurrence of *L. monocytogenes* in raw chicken samples was 20%.

There are no safety criteria for pork and poultry meat, in terms of total bacterial count, *Enterobacteriaceae*, lactic acid bacteria, molds, yeast and the presence of *Campylobacter* spp. in the Polish standard regulations.

The average number of total bacterial count in the current study was within the range of 5.87-7.47 log10 CFU/g. This level is in accordance with Cohen et al. (2007) and Andritsos et al. (2012), who found the mean value of 5.9 log10 CFU/g (poultry) and 6.8 log10 CFU/g (minced pork), respectively. As expected, in all marinated

pork and poultry meat tested, the higher total bacterial counts were observed. The reason for this fact is probably due to the use of spices, which could be a cause of increased microbial contamination. From the report of Steinhauserová et al. (2012), it is known that the quality and age of the raw meat play an important role. Some producers often try to “salvage” raw meat before its expiratory date. The salt content and low pH of the marinade slow down the multiplication of microorganisms and thus extend their shelf life.

The average count of *Enterobacteriaceae* in the present research ranged from 2.52 log10 CFU/g (neck unmarinated) to 5.87 log10 CFU/g (chicken shashlik). These values are close to the counts reported by some other researchers. Andritsos et al., (2012) indicated that samples of minced pork marketed in Greece contained *Enterobacteriaceae* at the level of 3.6 ± 1.2 log10 CFU/g. In another work (Kilonzo-Nthenge et al., 2013), *Enterobacteriaceae* numbers were between 3.26 (chicken wings) and 4.75 log10 CFU/g (chicken breast). Our results have shown different effect of marinades on the proportion of *Enterobacteriaceae* in meat microflora, from that observed by Finnish scientists. Finnish research showed that marination may extend sensory shelf-life of broiler meat by delaying the growth of *Enterobacteriaceae* that is associated with spoilage of poultry (Björkroth, 2005; Nieminen et al., 2012a). To our knowledge, this is the first report comparing the effect of grill marinade and spices on the microbial quality of pork and poultry meat available in Polish retail markets. More research in this field should be performed in order to determine the differences between the proportion of *Enterobacteriaceae* in marinated and unmarinated meat. Despite this fact, high level of total bacterial count and *Enterobacteriaceae* found in current research, independent of marinades and spices used, is an indication of fecal contamination and poor hygiene during processing. On the other hand, in the present study, we observed the low level of contamination with *Staphylococcus aureus* (2.0 log10 CFU/g). This level suggests strict personal hygiene of the workers (Cohen et al., 2007).

Lactic acid bacteria are considered to be the Specific Spoilage Organisms that contribute to the meat spoilage stored under packaging conditions in which the concentration of carbon dioxide is increased (Doulgeraki et al., 2010). *Lactobacillus*, *Leuconostoc*, and *Carnobacterium* are the most frequently encountered genera on marinated and unmarinated meat and play a significant role in the spoilage of refrigerated raw meat (Doulgeraki et al., 2010, Nieminen et al., 2012b). In a study conducted by Nieminen et al. (2012b), the LAB colony counts measured from marinated broiler fillets strips ranged from 8.2 to 9.2 log10 CFU/g. In the present research, the average count of LAB for poultry products with grill marinade ranged from 4.14 (chicken wings in marinade) to 5.48 log10 CFU/g (chicken shashlik).

Molds and yeasts constituted a minor portion of the microflora on meat. However, it is practically impossible to eliminate them completely from meat. Especially, spices added to meat can considerably contribute to total molds and yeast contamination. In the present study, the marinades and spices used have little influence on the numbers of molds and yeasts detected. Analysed samples were contaminated with molds and yeasts in values varying up to 2.78 and 5.13 log10 CFU/g, respectively. Similar results have been noted by Ismail et al. (2000). According to them, mycoflora has not been considered as playing a major role in spoilage, as it is seldom present in high numbers. The mean populations of yeast and molds observed by these researchers ranged from 1.65 to 5.12 log10 CFU/g. The growth of the mycoflora was not inhibited by marination.

Our analysis revealed that *Campylobacter* spp. was detected in poultry product with the prevalence of 38.9% and 61.1% for non marinated and marinated products, respectively. The data presenting contamination of chicken with *Campylobacter* spp. in industrialized nations are very diverse. In Alabama (USA), during the seven years of the study, the prevalence of *Campylobacter* spp. in retail broiler meat did not change and was at the level of 41.0% (Williams and Oyarzabal, 2012). Madden et al. (2011) reported, that in the Republic of Ireland, 84.3% of chicken samples were positive for *Campylobacter* spp. This result is in agreement with that presented by Moran et al. (2009). A year-long survey of fresh poultry products in Northern Ireland showed that 91.0% of chicken samples were contaminated by this pathogen. Luber and Bartelt (2007) obtained similar results. They analysed the prevalence of *Campylobacter* spp. in 100 German fresh broilers chicken breast, and found that 87% of the filets were *Campylobacter*-positive on the surface and 20.0% in the deep tissue. The research carried out in Finland by Perko-Mäkelä et al. (2000) and Björkroth (2005) showed that marinating may not have inhibiting effect on the survival of *Campylobacter* spp. This may be due to the buffering capability of meat quickly neutralizing the pH of the acidic marinade and resulting in dissociation of the lipophilic acids, making their antimicrobial effect invisible. The high occurrence of *Campylobacter* spp. in retail poultry meat suggests the high prevalence of this microorganism in broilers in farms. However, more research is needed to clarify the cause of the high numbers of this pathogen located in different parts of chicken meat. This kind of data may be useful for future study of risk assessment.

Conclusion

The relatively high contamination of marinated and unmarinated pork and poultry meat with food spoilage microorganisms and pathogens decreases their quality and constitutes a public health hazard, but it is comparable with the meat quality of developed countries.

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Full Length Research Paper

Investigation of *Anaplasma phagocytophilum* among agrarian residents and domestic animals in Anhui Province, China

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A total of 596 blood samples from agrarian residents, and 132 from goats, 12 from dogs and six from cattle were collected from 3 rural Counties including Guangde, Mingguang and Huaiyuan in Anhui Province. The survey questions presumed exposure risk of *Anaplasma phagocytophilum* were recorded for each participant and statistically analyzed. The antibodies against *A. phagocytophilum* were determined using an immunofluorescence assay (IFA) and the *A. phagocytophilum* 16S rRNA gene was amplified and analyzed for the DNAs of the blood samples of febrile participants and domestic animals using nested polymerase chain reaction (PCR). The average percentage of *A. phagocytophilum* was 44.6% for human, 33.3% for dogs, 0.8% for goats and 0% for cattle, respectively. The positive rate of the 16S rRNA gene (389 bp) of *A. phagocytophilum* in dogs, goats and cattle was 25.0, 33.3 and 0%, respectively. Two genotypes of *A. phagocytophilum* were identified and group A was dominantly endemic in Huaiyuan County while group B was mainly located in Guangde County. Living Mountain regions, outdoor activities, contacting animals, fever history, more than 3 h of working per day and more than 2 years of servers might be increased at risk of exposure *A. phagocytophilum* in these areas of Anhui Province.

Key words: *A. phagocytophilum*, seroepedemiology, prevalence, Anhui Province of China.

INTRODUCTION

Human granulocytic anaplasmosis (HGA) is an emerging tick-borne zoonoses that is caused by the obligate intracellular bacteria *Anaplasma phagocytophilum* (Chen et al., 1994; Walker et al., 2008; Chapman et al., 2006; Dumler et al., 2001; Petrovec et al., 1997). In 2006, an unusual nosocomial human-to-human transmission of HGA occurred in a hospital in Anhui Province (Zhang et al., 2008a). In this event, five relatives of the index patient and four medical workers who participated in rescuing the index patients were secondary infected for direct contacting with blood or respiratory secretions. Despite clear laboratory evidence of an outbreak of anaplasmosis, most cases of HGA is likely misdiagnosed or underrecognized because of limited epidemiological,

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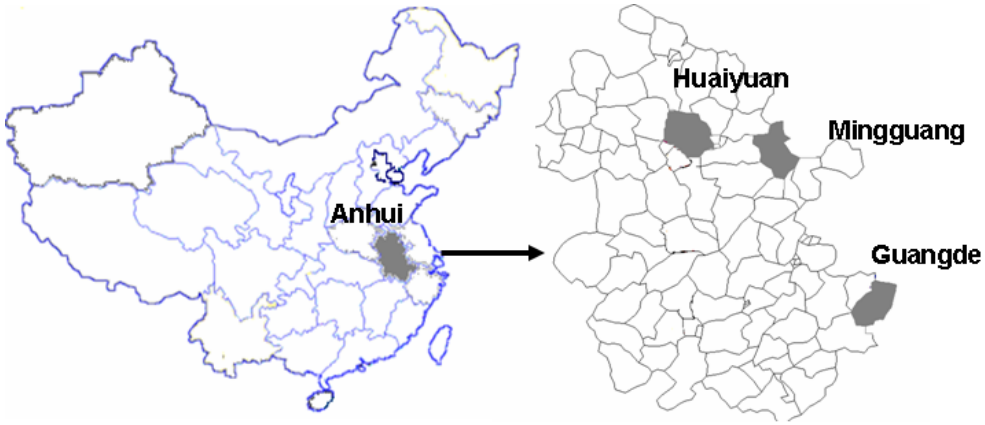

Figure 1. Map of Anhui Province and the 3 investigated sites.

clinical and microbiological data in these areas of Anhui Province (Wu et al., 2010; Cao et al., 2010). Therefore, an investigation to assess the epidemiologic status of emerging infectious diseases caused by *A. phagocytophilum* among farm residents and domestic animals in Guangde County (Figure 1), where the index patient from the nosocomial transmission of HGA lived, and in Huaiyuan County and Mingguang City in Anhui Province was undertaken by a collaboration project between the Department of Anaplasma and Rickettsiology, National Institute for Communicable Disease Control and Prevention, China CDC and the Department of Epidemiology, Centers for Disease Control and Prevention of Anhui Province from April to May in 2009.

MATERIALS AND METHODS

Ethics statement

The study and the protocol of field investigation were approved by the China CDC Institutional Review Board (No.201103). A written consent form was obtained from each participant before sampling blood. Parents informed consent on behalf of their child. All procedures of sampling animal blood were conducted to conform to institutional guidelines for the care and use of laboratory animals as described by the China CDC, Beijing, China, and confirmed to the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985). Sampling animal blood was conducted after the owners consented.

The survey sites and sampling

According to the geographic location and previous epidemiological information on rickettsia infection (Zhang et al., 2008a), Guangde County, which lies in the south areas of Anhui, was selected because the first outbreak of HGA in China occurred there (Figure 1). The other two sites studied were Huaiyuan County and Mingguang City, which are located in the north and east areas of Anhui Provence, respectively (Figure 1). Guangde County and Mingguang City are typical hill and mountainous areas and most local people make a living by farming, raising domestic animals and growing fruit trees. Huaiyuan County is a typical plain area, and crop farming is the primary occupation there. As in many other mountainous areas, goats, dogs and cattle are the most common domestic animals raised in Guangde County and Mingguang City, and all of these animals are bred outside during the day in spring and summer. A high density of ticks was observed on the body of animals although pesticides were being sprayed twice (morning and evening) a day to kill them.

In each County, three to five villages were chosen based on their geographic locations (for example, the eastern, southern, western, northern and central areas of each). Participants were randomly selected based on the odd or even number of each registration permanent resident. The information collected included demographics; gender; age; living areas; past medical history; occupation (outdoor activities or not, if unemployment such as retried people, housewives, students and preschool children); contact with domestic animals including dog, cat, cattle, sheep or goat; and exposure to ticks or bitten by ticks; length of working time per day and length of service time; All participants were asked if they had a fever on the day of the survey and whether they had high fever (temperature of 38.0≥°C) during the preceding 12 month and if so, we asked their body temperature and the main clinical manifestation such as myalgias or headache, and whether they had received any antibiotic, and if so, what types. Two ml sample of non-anticoagulated blood was collected for each participant. At the same time, three or five of goats or sheep or cattle, and 1 or 2 dogs were selected for sampling blood. Five-ml of non-anticoagulated blood was collected from each animal if the participant owned domestic animals and consented the animal blood sampling. In the local County CDC, Sera were separated to test antibody and the remaining blood clot of the febrile person and the animals were used to extract DNA using a DNeasy® Blood and Tissue Kit (QIAGEN, Cat No. 69506) according to the manufacturers' instructions. All the samples were stored at -20 or -40°C at local CDC and then transferred to the Department of Anaplasma and Rickettsiology, National Institute for Communicable Disease Control and Prevention, China CDC by air within 48 or 72 h for laboratory test.

Antibody assay

Immunofluorescence assays (IFA) were performed as previously described (Bakken et al., 2002; Raoult et al., 1985). *A. phagocytophilum* (Webster strain) crude antigen and its positive human serum were provided by the J. S. Dumler at the Johns Hopkins University School of Medicine, USA. In order to evaluate the

the reactivity with other rickettsiae, another commen 6 rickettsiae crude antigen were simultaneously tested by IFA. These rickettsiae antigen were as follows: *E. chaffeensis* (Arkansas strain) antigen was provided by Dr. Robert Massung at the United Stated CDC. *Rickettsia typhi*, *Orientia tsutsugamushi* types Karp, *Rickettsia heilongjiangensis*, *Bartonella henselae* and *Coxiella burnetii* were kindly provided by Dr. Didier Raoult from WHO Rickettsial Diseases Collaborating Center (Marseille, France).

The IFAs were simply performed as follows. The serum samples were diluted 1:40 (IgM) or 1:80 (IgG) in PBS containing 3% nonfat powdered milk, and 25 µl of the diluted serum was placed in a slide well and incubated for 60 min in a moist chamber at 37°C. After washing in PBS to remove unbound antibody, the slides were labeled with FITC-conjugated rabbit anti-human immunoglobulin (IgM or IgG; Sigma Co., NY, New York State, United States) as a secondary antibody, which was diluted 1:400 with Evans blue, for another 60 min in a moist chamber at 37°C. The slides were then washed in PBS to remove unbound secondary antibody. The slides were air dried at 37°C and examined using a fluorescent microscope (Nikon, Tokyo, Japan). Samples were interpreted as reactive if there was strong green fluorescence corresponding to bacterial morulae within the cells on the slide. PBS-milk and the mixed sera from healthy human (workers in our institute but not members of our laboratory) were used as negative control respectively. Samples that were reactive at the 1:80 screening dilution were deemed IgG positive based on the reference criteria (Bakken et al., 2002; Raoult et al., 1985) and IgM titer of 1:40 were considered positive. In order to reduce the perform errors, every 4 antigens mentioned above were spotted in different rows in the same slide. If a serum sample had reactivity with the other 6 reckettsial antigens mentioned above, further dilution and titration were conducted, and a two-fold or higher titer increase was read as positive.

Amplification and sequencing of the 16S rRNA gene

A previously developed nested PCR assay, based on the 16S rRNA gene (389bp) of *A. phagocytophilum,* was performed (Wen et al., 2002) with the DNAs extracted from febrile patients' blood and animals' blood as templates. Sterile deionised water and DNAs extracted from healthy person, goat, sheep, dog and cattle were used as a negative control, and the DNA of *A. phagocytophilum* strain Webster, which kindly provided by the J. S. Dumler at the Johns Hopkins University School of Medicine, USA, was used as a positive control. Positive results were confirmed by commercial sequencing (Shanghai Shengong Biotechnology Co.) using an ABI 3730 sequencing apparatus (Life Technologies, USA) and a Bigdye Terminator V3.1 sequencing kit. The sequences were compared with sequences in GenBank by Blast (http://www.ncbi.nlm.nih.gov/). All of the sequences obtained from the domestic animals or febrile patients were deposited in GenBank.

Statistical analysis

Statistical analysis was conducted using SAS software (version 9.1, SAS Institute, Inc., Cary, NC). A comparison of the prevalence in human and animals from different areas was performed using the $\chi 2$ and Fisher's exact tests. Age was converted into a categorical variable (2 -19, 20 - 29, 30 - 39, 40 - 49, 50 - 59 and >60 years of age). $\chi 2$ and Fisher tests were used to compare distributions of seropositivity or to examine association between pairs of categorical measures. Logistic regression analysis was used to calculate odds ratios for seropositivity among variables. The survey questions regarding variables "living plain areas", "living hilly regions", outdoor activity or works", "livestock breeding or contact domestic animals", "length of working hour per day" and "length of service time" were created to the associated with presumed risk among permanent residents of agrarian areas. The significance for these analyses was defined as a P value of 0.05. Phylogenetic analysis was conducted using MEGA 4.0 software and phylogenetic tree was constructed by using neighbor-Joining (NJ) methods.

RESULTS

Survey people and animals

From April to May in 2009(tick season), a total of 596 farm residents from the 3 sites investigated were enrolled in the study. Of those who participated, 244 were male (average age 50 years, range, 5 - 76 years), and 352 were female (average age 49 years, range, 4 - 66 years). Ninety five percent of people investigated were engaged in outdoor activity (92% of people for farming and 3% of person for feeding domestic animals and planting fruit trees) and their working time were all more than 3 h per day at least 4 days per week. Five percent of investigated individuals were preschool children, students, housewives and elderly people who were retired and could not work anymore. Ninety five percent of residents owned or contacted with domestic animals. Person reported that they had been bitten by ticks in the past two years accounted up 10.6% and all of the people recognized ticks but nobody could tell the species of the ticks. Nobody was bitten by lice or flea but all of the people recalled that they had been by mosquitoes during the last summer or autumn. About 7.7% residents recalled fever in the last 12 months and most people (85%) had headache and generalized myalgias but nobody could describe what drug had been used. Five residents had fever (≥38°C) on the day of their survey and all people had fatigue weakness and 3 of them had headache.

In this survey, a total of 132 blood samples were collected from 114 goats, 12 from dogs and 6 from cattle owned by the families of the participants during the tick season in 2009. A high density of ticks was noticed on the bodies of the animals although the pesticides were daily used. The detail geographic distribution of the animals was summarized in Table1.

Serological detection

The seroprevalence of *A. phagocytophilum* among people was shown in Table 1. There were 6 samples existing weak reactivity with *E. chaffeensis* at 1:80 cut off, and these samples were confirmed to be *A. phagocytophilum* by further titration using Fuller diagnose kit of *A. phagocytophilum* and *E. chaffeensis*, in which the 2 antigens of *A. phagocytophilum* and *E. chaffeensis* were spotted in the same well of the slice in order to avoid of laboratory errors. Overall, the average seropositive rate of *A. phagocytophilum* among farm residents was 44.6%. Of the 3 sites investigated, Guangde County

Table 1. Seroprevalence of *A. phagocytophilum* in humans and animals.

Area	Human %（No. of positive/No. of tested sera）				χ^2 test			Total			Animal% (No. of positive/No. of tested sera)		
	Male		Female										
	Seropositive rates%	95%CI	Seropositive rates%	95%CI	P value	OR	95%CI	Seropositive rates%	P value	OR(95%CI)	Goat	Cattle	Dog
Huaiyuan	9.3(10/108)	3.8-14.8	11.3(16/141)	6.1-16.5	1	0.8	0.35-1.83	10.4(26/249)	<0.0001[a]	0.08（0.05-0.14）	0(0/43)	0(0/1)	0(0/5)
Mingguang	40.3 (21/52)	27-53.6	69.5 (66/95)	60.2-78.8	0.37	0.3	0.15-0.6	59.2(87/147)	0.0006[b]	0.45（0.28-0.71）	0(0/38)	0(0/5)	57.1(4/7)
Guangde	83.3 (70/84)	75.3-91.3	71.5 (83/116)	63.3-79.7	0.59	2	0.99-4.0	76.5%(153/200)	<0.0001[c]	27.9（16.6-47.0）	2.0(1/51)	-	-
Total	41.4 (101/244)	35.2-47.6	46.9 (165/352)	23.7-33.1	0.82	1.8	1.3-2.5	44.6(266/596)	-	-	1.5(2/132)	0(0/6)	33.3(4/12)

a: Huaiyuan vs. Mingguang, b: Mingguang vs Guangde, c: Guangde vs Huaiyuan.

had the highest seroprevalence (76.5%), and Huaiyuan County had the lowest (10.4%). The seroprevalence of *A. phagocytophilum* in Guangde were significantly higher than in Huaiyuan (p<0.0001, OR 27.9, 95%CI 16.6, 47.0) and in Mingguang (p=0.0006, OR 0.45, 95%CI 0.28, 0.71), respectively. Moreover, the seroprevalence of *A. phagocytophilum* in Mingguang was higher than in Huaiyuan (p<0.0001, OR 0.08, 95%CI 0.05, 0.14). However, the differences between the male and the female residents in each County investigated was not statistically significant (Table 1). Statistical analysis of age distribution revealed that the seroprevalence differed across strata of age and the seroprevalence increased with age growth (Table 2). For the 5 people who were febrile on the day investigated in the study, 2 sera reacted with *A. phagocytophilum* in IFA testing. The IgG antibody titers of these 2 patients were 1:160 and 1:80 respectively while the IgM antibody titer were 1:80 and 1:80, respectively. However, no confirmed diagnoses were made because sera from the patients' covalent stage of illness were unavailable.

Considering the questionnaires in the study, Living Mountain or hill regions, outdoor activities, contacting animals, fever history in the last 12 month, more than 3 h of working time per day and more than 2 years of servers length were associated with the high seroprevalence of the participants (Table 3).

The total seropositive rate of the 3 species of animals in the study was calculated because of the limited number of samples (Table 1). The seroprevalence of *A. phagocyophilum* in dogs, goats and cattle was 33.3, 0.8 and 0%, respectively.

The seroprevalence between dogs and goats was statistically significant (p<0.001) as was the difference between dogs and cattle (P<0.001). However, the difference in seroprevalence between cattle and goats was not statistically significant (P=1.00). There was no reactivity with the six other common rickettsiae mentioned in the part of material and methods at 1:80 cut off, and only 1 dog sample showing weak reactivity with *E. chaffeensis* at 1: 80 dilution sera, but failed to be reactivity with *E. chaffeensis* at 1:160.

Molecular analysis

The 16S rRNA gene of *A. phagocytophilum* was PCR amplified from 33.3, 25.0 and 0% of the goat, dog and cattle blood samples, respectively. The 44 goat sequences deposited in GenBank included 24 sequences from Huaiyuan (GQ499896,GQ499897,GQ499898,GQ499899,GQ499900,GQ499901,GQ499902,GQ499903,Q499904,GQ499905,GQ499906,GQ499907,GQ499914,Q499909,GQ499895,GQ499910,GQ499911,GQ499912,GQ499913,GQ499915,GQ499916,GQ499917,GQ499918,GQ499930), 9 from Mingguang(GQ499922,GQ499923, GQ499921,GQ499924, GQ499927, GQ499925,GQ499926,GQ499932, GQ499928) and 11 from Guangde(GQ499885,GQ499886,GQ499887,GQ499888 GQ499889, GQ499890,GQ499891, GQ499892, GQ499893,GQ499894, GQ49993). The 3 dog sequences included 2 from Mingguang (GQ499919, GQ499920) and 1 from Huaiyuan (GQ499929).

Based on the analysis of Blast and alignments, 15 represented sequences were selected from the sequences obtained in the study for constructing the phylogenetic tree. In addition, some sequences of *A. phagocytophilum* identified in different ticks, animals, patients from different areas of China and counties around of China as

Table 2. Seroprevalence of *A. phagocytophilum* in humans by age.

Age group	Seropositivity rate% (No. of positive/No. of tested)	Age group					
		2 - 19	20 - 29	30 - 39	40 - 49	50 - 59	>60
2-19	28.8(15/52)	-	0.4[a] 0.7[b] (0.4, 1.5)[c]	0.06 0.5 (0.3, 1.0)	0.01 0.4 (0.2, 0.8)	0.009 0.4 (0.2, 0.8)	0.006 0.3 (0.1, 0.7)
20-29	35.7(40/112)		-	0.2 0.7 (0.4, 1.2)	0.03 0.6 (0.3, 0.9)	0.03 0.5 (0.3, 0.9)	0.02 0.4 (0.2, 0.9)
30-39	43.8(64/146)			-	0.3 0.8 (0.5, 1.3)	0.3 0.8 (0.5, 1.2)	0.2 0.6 (0.3, 1.2)
40-49	50.0(66/132)				-	0.9 1 (0.6, 1.6)	0.5 0.8 (0.4, 1.5)
50-59	51.0(53/104)					-	0.6 0.8 (0.4, 1.6)
>60	56.0(28/50)						

a: P value, b: odds ratio, c: 95%CI.

Table 3. Analysis of survey questions which presumed risks for *A. phagocytophilum* among agrarian residents.

Variable	No. (%) of resident					
	Total cohort (n=596)	IFA positive (n=266)	IFA negative (n=330)	OR	95%CI	P Value
Living plain areas	249(41.8)	26(9.8)	223(67.6)	0.05	(0.03,0.08)	<0.0001
Living hill or mountain areas	347(58.2)	240(90.2)	107(32.4)			
Outdoor activity	566(95.0)	261(98.1)	305(92.4)	4.3	(1.6,11.3)	0.002
Contact farm animals	566(95.0)	259(97.4)	307(93.0)	2.7	(1.2,6.6)	0.02
Tick bite in last 24 month	60(10.6)	25(9.4)	35(10.6)	0.9	(0.5,1.5)	0.6
Fever in last 12 month	46(7.7)	5(1.9)	41(12.4)	0.1	(0.05,0.4)	<0.0001
Working time >3 h per day	566(95.0)	261(98.1)	305(92.4)	4.3	(1.6,11.3)	0.002
Service time>2 years	536(90.0)	256(96.2)	280(84.8)	4.6	(2.3,9.2)	<0.0001

well as other areas of the world were included (Figure 2). There were two dominant genetic groups of *A. phagocytophilum* in the surveyed areas based on the Phylogenetic analysis and they were distributed in two distinct geographic areas. Group A was mainly found in Huaiyuan, which is in the north plain areas of Anhui Province, and Group B was found in Guangde, which is in the southeast. Both groups of *A. phagocytophilum* were found in Mingguang City, which is in east Anhui Province. Notably, a total of 10 sequences obtained from the animals in the study were 100% homologues with the sequences (EF211110) from patients involved in the nosocomial outbreak of anaplasmosis in Guangde County, Anhui Province, in 2006 (Zhang et al., 2008a) and the other sequences from patients in Yiyuan County (EU982709), Shandong Province (Zhang et al., 2011), and these sequences were grouped in the same group (group B), which was predominantly distributed in the Guangde area. None of the positive PCR results was found on the 5 DNA samples from the febrile person

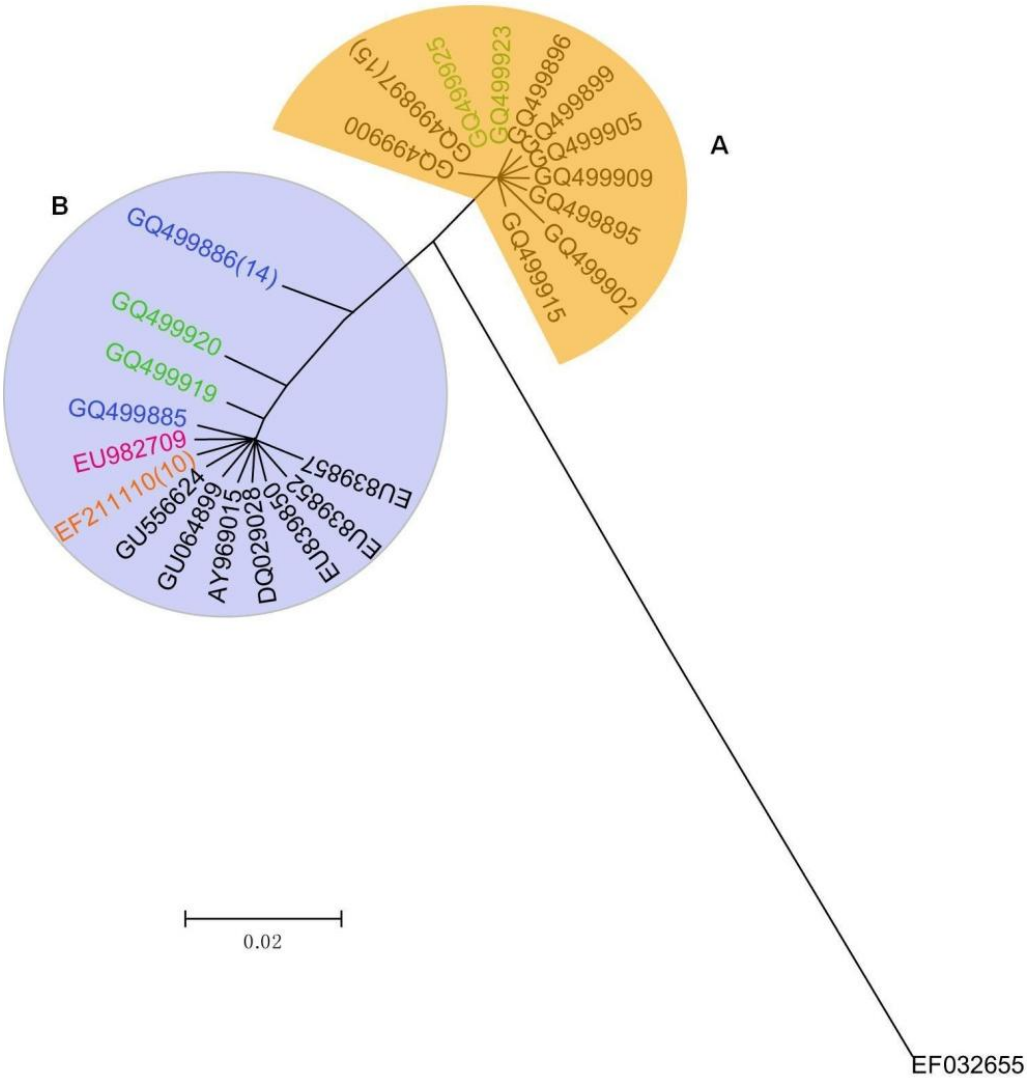

Figure 2. Phylogenetic analysis of *A. phagocytophilum* from goat, cattle and dog blood samples collected in Guangde, Mingguang and Huaiyuan Counties in Anhui Province. The tree was based on the partial segment sequences (389 bp) of *A. phagocytophilum* 16SrRNA by using neighbor-joining (NJ). In group A: green sequences were from goats in Mingguang County in this study, and the black sequences were from goats in Huaiyuan County in this study. In group B: orange sequences were from patients in unusual nosocomial outbreak of HGA in Anhui Province in 2006; purple sequences were from patients in Yiyuan County, Shandong Province; green sequences were from dogs in Mingguang County in this study; blue sequences were from goats in Guangde County in this study, and the black sequences were from different hosts, vectors and patients identified in other countries. The numbers in parentheses indicate the number of isolates in each genotype. EF032655: reference sequence (alpha proteobacterium SepB-6 16SrRNA).

investigated in the study.

DISCUSSION

In 2006, we first reported the identification of HGA in China and first demonstrated nosocomial human-to-human transmission of HGA (Zhang et al., 2008a). In 2009, Zhou et al further reported the epidemic characterizes of HGA in this event and indicated that the infection rate of people contacting with the index patient after onset of illness was 14.3% while the incidence of people contacting the index patient with critical ill was 23.1% (Zhou et al., 2009). However, the seroepidemiological, clinical and microbiological information about HGA is very limited in these areas and the disease is likely underreported (Wu et al., 2010; Cao et al., 2010). Unlike HGA cases reported from US or European coun-

tries, Chinese HGA patients represented severe clinical manifestations and 52.1% of patients had systemic inflammation response syndrome (SIRS) and 34.2% of patients rapidly developed multiple organ dysfunction syndrome (MODS), and the fatality rate of Chinese HGA patients has been reported to be as high as 26.5% (Li et al.,2011). A more recent nationwide etiological investigation of HGA was conducted and a total of 46 confirmed and 16 probable HGA cases were obtained from 2009-2010. Among these cases, 41.2% of patients were diagnosed with multiple organ dysfunction syndrome (MODS), and the fatality rate was as high as 8.1% (Zhang et al., 2013). Four human HGA isolates and one tick isolate was obtained in this project mentioned above and the 16S rRNA gene (750bp) of the 5 Chinese HGA isolates were 100% identical to the sequences (EF211110) from patients involved in the nosocomial outbreak of anaplasmosis in Guangde County, Anhui province, in 2006 (Zhang et al., 2008a) and the other sequences from patients in Yiyuan County (EU982709), Shandong Province. This dominant sequence of HGA 16S rRNA gene accounted for up to 60% of the sequences identified in *H. longicornis* from Laizhou, Shandong Province (Zhang et al., 2013).

It is reported that HGA is a nonspecific febrile tick-borne illness and the infected patients generated specific antibody at 2 week and peaked at 4 week after the onset of clinical sympotoms (Bakken et al., 1996). Nearly 50% of patients maintain serum IFA antibody titers at 1:80 or higher for 18 months or longer and some people could remain seropositive for as long as 42 month (Bakken et al., 2002).

In this study, we conducted a broad seroepidemiological investigation of *A. phagocytophilum* among agrarian residents and domestic animals in Anhui Province; that was focused on Guangde County, where the index case of the nosocomial human-to-human transmission of HGA lived (Zhang et al., 2008a). The average seroprevalence (44.6%) of *A. phagocytophilum* among people in 3 surveyed sites is significantly higher than the 16.3% (Zhang et al., 2011) and the 2.8% (Cao et al., 2010) that was observed in a previously studies in Anhui. The reasons caused this big differences might be related with the different strains of *A. phagocytophilum* as antigens for IFA. In another words, the endemic strain of *A. phagocytophilum* in these areas is more genetically related with the *A. phagocytophilum* Webster strain used in this study.

Similarly, the seroprevalence (44.6%) obtained in the study is higher than the 8.8% of Tianjin areas (Zhang et al., 2008b) and it is also higher than the average seroprevalence 7.1% (from 2.4% in Henan Province to 17.6% in Shanxi Province) reported from a recent serological investigation in China (Hao et al., 2013) and higher than the average seroprevalance 14.1% of Beijing rural areas (Zhang et al. 2012). However, it is similar to the rates that have been reported in endemic areas in US (Dumler et al., 2005; IJdo et al., 2000). Notably, this study found that the seropositive rate of *A. phagocytophilum* among people in Guangde County is as high as 76.5% (with a cut off value of 1:80). We also found that the seroprevalence in humans from the mountainous areas (Guangde and Mingguang) was significantly higher than that from humans in the plain areas (Huaiyuan). In addition, contacting animals, outdoor working, fever history in the last 12 month, more than 3 h working per day and more than 2 year service were all independent risks of exposure to *A. phagocytophilum*. However, the seropositive rates between the males and the females in the investigated sites were not statistically significant.

As a zoonotic pathogen, a wide variety of *A. phagocytophilum* strains circulate in different animal systems and some may cause zoonoses and some may not infect human. In this study, 3 species of animals were included and the seropositive rate of *A. phagocytophilum* were 33.3% for dogs, 0.8% for goats and 0% for cattle and the PCR positive rates of 16SrRNA gene were 33.3% for dogs, 25.0% for goats and 0% for cattle. There were not any positive evidences of *A. phagocytophilum* identified in cattle, which we proposed that one of the reasons is the limited samples (6 blood samples). In addition, the *A. marginale* that was considered as one of the most common pathogen for cattle anaplasomosis was unavailable in our laboratory. A big difference was noted between the serological positive rates and PCR positive rates for goats and this might be related with the different immunogenicity between the domestic strains of Anaplasma in China and the abroad strain of *A. phagocytophilum* Webster used in the study. However, the seropositive rate and the PCR positive rate for dogs were as high as 33.3 and 33.3%, respectively. Furthermore, phylogenetic analysis demonstrated that these sequences from dogs were grouped as the same clad (Group B) with the sequences from patients involved in the nosocomial transmission of HGA in Guangde County in 2006 (Figure 2).

There are two varieties of *A. phagocytophilum* from animals based on the phylogenetic tree constructed in this study and a striking geographic distribution was noticed between these two genetic groups. Group B is represented by the sequences from patients in the nosocomial outbreak of HGA in 2006; a patient (EU 9827709) in Yiyuan County; and 60% of *Haemaphysalis longicornis* collected from Laizhou Bay (Zhang et al., 2013), Shandong province; and water deer (GU556624) from Korea and *H. longicornis* (GU064899) collected from Jeju Island in Korea. This clad was also detected in *Ixodes ovatus* (AY969015) in Japan (Ohashi et al., 2005). In addition, a sequence that is 100% homologous to group B was identified in an Italian patient (DQ029028) with HGA from Sicily (de la Fuente et al., 2005), in wild ruminant animals (EU839850) and in 2 horses (EU839857 and EU839852) that were infected with *A. phagocytophilum* in the Czech Republic (Zeman and Jahn, 2009).

Group A was mainly endemic in Huaiyuan County, but sequences that were 100% homologous were also identified in goats (HM439432) from Zhejiang Province and in goats (FJ389576) from southeast China (Zhou et al., 2010), in *H. longicornis* (GU064899) collected from Jeju Island in Korea, and in deer (AB454075) from the Nara park in Japan. However, no sequences from patients grouped into this clad.

As an emerging tick-borne infectious disease, this study is the first and largest serological survey of *A. phagocytophilum* in Anhui Province based on the special and unusual nosocomial outbreak of HGA in 2006. The local documents from Anhui Provincial CDC (Zhou et al., 2009; Wu et al., 2010; Cao et al., 2010) and the sero-logical and molecular evidences in the study indicated that the rural residents especially residents in mountain or hill are at increased risk of *A. phagocytophilum* exposure.

Further systematic surveillance, including the role of the vector and host and monitoring of the trans-mission of HGA in these areas should be performed in the future. Etiological investigation based on the isolation of agents is urgently needed and differential diagnosis of HGA in clinical practice should be emphasized.

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Full Length Research Paper

Evaluation of the probiotic properties of *Bacillus* spp. strains isolated from Tunisian hypersaline environments

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Bacillus **species are ubiquitous and diverse both in the terrestrial and marine ecosystems. In this study,** ***Bacillus*** **species were isolated from Tunisian hypersaline environments and their probiotic properties were studied. In total, 13 strains were identified as a** ***Bacillus*** **spp. Antagonism assay revealed an inhibitory effect of these strains against pathogenic bacteria. Cell surface hydrophobicity values ranged between 0.33 and 30%. The tested strains were able to produce extracellular enzymes such as amylase, lipase, caseinase, and lecithinase. Qualitative analysis of biofilm results show that seven strains were able to produce slime on Congo red agar. Furthermore, the investigated strains were fairly adhesive to glass and polystyrene with values ranging from 0.07 to 0.70 at 570 nm.**

Key words: *Bacillus* spp., hypersaline, antagonism, extracellular enzymes, adhesion.

INTRODUCTION

The domain of bacteria contains many types of halophilic and halotolerant microorganisms, spread over a large number of phylogenetic groups (Ventosa et al., 1998). Within the lineages of gram positive bacteria (Firmicutes), halophiles are found both within the aerobic branches (*Bacillus* and related organisms) and also within the anaerobic branches (Oren, 2002).

Due to their ability to form spores and withstand a range of variable environ-mental conditions, *Bacillus* spp. adapt easily to diverse habitats. *Bacillus* species can be found in marine environ-ment and are part of the microflora of several marine species (Hovda et al., 2007). Perfectly adapted to harsh conditions, halophilic bacteria have the ability to grow in environments with hight salt concentration. Their particu-larities are manifested by stable proteins and enzymes. Besides, marine bacteria are known to produce wide range of compounds, which have potential applications as bioactive compounds, probiotics and nutritional supple-ments (Watanabe et al., 1996). The term probiotic, means “for life”, originating from the Greek words “pro” and “bios”. Today probiotics are quite commonplace in health promoting “functional foods” for humans, as well as therapeutic, prophylactic and growth supplements in animal production and human health (Sullivan and Nord, 2002; Senok et al., 2005; Abdelkarim et al., 2012).

Bacillus spp. have been shown to possess adhesion abilities, produce bacteriocins (antimicrobial peptides)

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Abbreviations: NBSW, Nutrient broth sea water; **TBE,** Tris-borate-ethylenediaminetetraacetic acid; **TSB,** trypticase soy broth; **PBS,** phosphate buffered saline; **TSA,** trypticase soy agar; **CSH,** cell surface hydrophobicity; **DLVO,** Derjaguin, Landau, Verwey, and Overbeek; **CRA,** Congo red agar.

Table 1. Geographic location of bacteria isolation sites.

Site	Sebkhat El Meleh	Sebkhat Moknine	Sahline saltworks	Sebkhat Sidi El Heni	Bkalta saltworks	Sfax saltworks	Sebkhat El Adhibet
Geographic coordinates	33°19‘N 10°55’E	35°35‘N 10°56’E	35°45‘N 10°42’E	35°35‘N 10°30’E	35°35‘N 11°10’E	34°43‘N 10°44’E	33°06‘N 11°23’E

N, North; E, East.

and provide immunostimulation (Cherif et al., 2001; Barbosa et al., 2005). Several research articles demonstrate the benefits of using *Bacillus* to improve shrimp growth performance, survival, immunity, and disease resistance in aquaculture (Farzanfar, 2006). Systematic studies of *Bacillus* have always focused on the terrestrial *Bacillus,* although marine Bacilli are noted for their ability to produce different antibiotics, glucanases and cyclic acylpeptides (Oguntoyinbo, 2007).

Besides their enzyme production, marine Bacilli are also well known for the reduction of toxic heavy metals. Extremely halotolerant *Bacillus* strains were isolated from hypersaline environments (Oren, 2002). Their discovery could be of great biotechnological potential because many *Bacillus* isolates produce industrially important compounds.

In this study, halophilic *Bacillus* strains were isolated from Tunisian hypersaline environments and their probiotic properties were evaluated, in the aim to find more effective and environmentally friendly treatments for aquatic sector.

MATERIALS AND METHODS

Samples collection and bacterial characterization

Water samples were recovered from seven Tunisians hypersalines environments (Table 1). The samples were collected in 1 L sterile plastic bottles. The samples were immediately stored in a refrigerator for further microbial analysis. Bacterial strains were isolated according to the following procedure: 1 ml from samples were enriched 24 h at 30°C in nutrient broth sea water (NBSW) (salinity 34 g l^{-1} and pH 7.99) then speared on nutrient agar plate and incubated at 30°C. Only Gram and catalase positive rods were selected for further characterization.

Molecular identification of *Bacillus* strains

Total DNA was extracted from all the isolated strains according to the scheme described by Sambrook et al. (1989) and stored at -20°C. The two primers were (B-KF 5’-TCACCAAGGCRACGATGCG-3’ and B-KR: 5’CGTATTCACCGCGGCATG 3’) (Xi-Yang et al., 2006). Amplification was carried out using a 50-µl reaction volume: 2 µl DNA, 0.2 mM of each dATP, dGTP, dCTP and dDTP, 1x buffer solution, 1.5 mM $MgCl_2$, 1 mM of each primer (B-K1/F and B-K1/R1) and 1 unit of Taq DNA polymerase (Promega, USA). Each PCR program was conducted using a denaturation step of 3 min at 94°C, followed by 25 cycles of 94°C for 30 s, 63°C for 30 s and 72°C for 2 min, with an extension step of 72°C for 10 min. PCR products were separated by 1.5% agarose gel electrophoresis in Tris-borate-EDTA buffer (TBE) at 100 V. The sizes of DNA fragments were estimated using a 100-bp DNA ladder (Promega).

Antimicrobial activity using well diffusion agar assay (WDAA)

Potential probiotic strains were tested for their antagonistic activity using the well diffusion agar assay (WDAA) (Vaseeharan and Ramasamy, 2003) against four pathogenic strains: *Vibrio. alginolyticus* ATCC17749, *Vibrio. parahaemolyticus* ATCC17802, *Aeromonas hydrophila* ATCC7966 and *Salmonella typhymurium* ATCC 17802. The pathogenic bacteria were grown overnight in 10 ml of nutrient broth and then cultured for 24 h on nutrient agar at 30°C.

The common colonies from pure culture were suspended in 10 ml of physiological medium and well mixed during 5 min. 1 ml was spread over the agar plates. Potential probiotic strains were cultured in 10 ml nutrient broth for 24 h, 100 µl of the supernatant were introduced into the wells of the Muller Hinton MH agar medium and incubated for a period of 24 h at 30°C. Antibacterial activity was defined as the diameter (mm) of the clear inhibitory zone formed around the well.

Cell surface hydrophobicity

Hydrophobicity was measured by the hexadecane partitioning method of Van Loosdrecht et al. (1987). Bacterial cells grown overnight in trypticase soy broth (TSB) were washed with phosphate-buffered saline (7 mM Na_2HPO_4, 3 mM NaH_2PO_4 and 130 mM NaCl at pH 7.4) and resuspended in 4 ml of PBS, and the absorbance (OD_{600}) was determined (Abs1). One milliliter of hexadecane was added to each cell suspension and equilibrated for 10 min. Each suspension was re-incubated at 37°C for 30 min. The aqueous layer was removed and aerated to remove all traces of hexadecane, and absorbance (OD_{600}) was measured against a hexadecane-extracted PBS blank (Abs2). The hydrophobicity index (% adhesion to the solvent) was expressed as the ratio of absorbance of the hexadecane-extracted sample to absorbance of the sample before extraction. Percentage of adhesion was expressed as:

% adhesion = (1 - Abs2/Abs1)×100.

Phenotypic characterization of slime-producing bacteria

Qualitative detection of biofilm formation by tested strains was studied by culturing the strains on Congo red agar (CRA) plates as described previously (Chaieb et al., 2007). *Bacillus* strains were inoculated onto the surface of CRA plates, made by mixing 0.8 g Congo red with 36 g saccharose (Sigma) in 1 L of brain heart infusion agar, and were incubated for 24 h at 30°C under aerobic conditions and followed overnight at room temperature. Slime-producing bacteria appeared as black colonies, whereas non-slime

Table 2. Antagonistic activity of *Bacillus* spp. isolated strains in mm.

Strain	Pathogen			
	V. alginolyticus **ATCC 17749**	***V. parahemolyticus*** **ATCC 17802**	***S. typhimurium*** **ATCC 1408**	***A. hydrophila*** **ATCC 7966**
H1	12.1±0.36	10.1±0.12	*	*
H3	16.0±0.20	10.1±0.42	*	*
H4	*	12.2±0.25	*	*
M2	*	*	*	*
M5	14.1±0.10	12.1±0.12	22.0±0.06	16.4±0.21
Sa2	11.7±0.58	10.1±0.06	*	*
Sa3	*	10.0±0.00	*	*
SH1	*	12.1±0.31	10.1±0.42	17.0±0.00
SH3	*	10.2±0.20	18.0±0.06	*
BK2	12.2±0.25	*	18.0±0.00	*
SF1	12.1±0.12	10.1±0.06	*	*
CA	*	*	10.1±0.12	12.1±0.12
BC45	16.1±0.42	12.1±0.12	20.1±0.23	15.1±0.31

±, Standard deviation (n= 3); *, no activity.

producers remained non pigmented (Chaieb et al., 2007).

Biofilm formation in glass test tubes

For the biofilm formation assay, each *Bacillus* strain, was cultured in Subwoofer wireless transmitter (SWT) broth containing (per liter): 5 g of Bacto-Tryptone (Difco), 3 g of yeast extract (Difco), 3 ml of glycerol, 700 ml of filtered seawater, and 300 ml of distilled water, at 37°C with shaking and then transferred to glass test tubes. The cells were incubated without shaking for 10 h at 37°C, then stained with 1% crystal violet solution to visualize cells attached to the test tube (Wolfe et al., 2004). Thereafter, the tubes were rinsed with sterile distilled water. Biofilms formed at the air-liquid interface were stained purple. All the strains were tested in triplicate.

Semi quantitative adherence assay

Biofilm production by *Bacillus* spp. strains was determined using a semi-quantitative adherence assay on 96-well tissue culture plates, as described previously (Chaieb et al., 2007). Strains were grown in TSB supplemented with 1% (w/v) NaCl. Following overnight incubation at 30°C, the optical density at 600 nm (OD_{600}) of the bacteria was measured. An overnight culture, grown in TSB 1% at 37°C, was diluted to 1:100 in TSB supplement with 2% (w/v) glucose. A total of 200 µl of cell suspensions was transferred in a U-bottomed 96-well microtiter plate (Nunc, Roskilde, Denmark). Each strain was tested in triplicate. Wells with sterile TSB alone were served as controls. The plates were incubated aerobically at 37°C for 24 h. The cultures were removed and the microtiter wells were washed twice with PBS to remove non-adherent cells and dried in an inverted position. Adherent bacteria were fixed with 95% ethanol and stained with 100 µl of 1% crystal violet (Merck, France) for 5 min. The excess stain was rinsed and poured off and the wells were washed three times with 300 µl of sterile distilled water. The water was then cleared and the microplates were air-dried. The optical density of each well was measured at 570 nm (OD_{570}) using an automated Multiskan reader (Gio. De Vita EC, Rome, Italy). Biofilm formation was interpreted as highly positive ($OD_{570} \geq 1$), low grade positive ($0.1 \leq OD_{570} < 1$), or negative ($OD_{570} < 0.1$) (Knobloch et al., 2001).

Characterization of extracellular enzymes

The presence of exoenzymes was determined using the API Zym System (Bio-Mérieux) composed of 19 enzymatic substrates. The activities of various other enzymes were determined following inoculation of cultures onto trypticase soy agar (TSA) to which the following substrates had been added: 0.2% [wt/vol] strach for amylase, 1% [wt/vol] skim milk for caseinase, 1% Tween 80 for lipase, 5% [vol/vol] egg yolk for phospholipase (lecithinase) (Ben Kahla-Nakbi et al., 2006).

RESULTS

Bacterial characterization

Based on the results of the amplification of *Bacillus* 16s RNA gene, the thirteen tested strains yielded a PCR product with an amplicon sized around 1114 bp and were identified as *Bacillus* spp. (data not shown).

Antagonism assay

Potential probiotic strains have an inhibitory effect against pathogenic *Vibrio* strains *V. parahaemolyticus* ATCC17802, *V. alginolyticus* ATCC17749, *A. hydrophila* ATCC7966 and *S. typhymurium* ATCC 17802 (Table 2). The inhibitory zones were about 10-22 mm in diameter.

Cell surface Hydrophobicity (%)

The results of microbial adhesion to solvent are

Table 3. Qualitative and semi quantitative estimation of *Bacillus* spp. biofilm formation.

Strain	Hydrophobicity (%)	Phenotypes on CRA	Slime production	Glass surface	Polystyrene surface	
					Mean OD_{570} ± SD	Adhesion rate
H1	0.33	Orange	NP	A	0.14±0.10	++
H3	17.33	Orange	NP	A	0.13±0.06	++
H4	5.67	Black	P	A	0.07±0.04	-
M2	1.33	Orange	NP	A	0.07±0.02	-
M5	30.00	Pinkish red	NP	A	0.11±0.02	++
Sa2	4.00	Black	P	A	0.08±0.01	-
Sa3	26.00	Pinkish red	NP	NA	0.09±0.01	-
SH1	3.00	Black	P	A	0.17±0.01	++
SH3	3.67	Black	P	NA	0.09±0.02	-
BK2	19.67	Black	P	A	0.07±0.01	-
SF1	8.00	Pinkish red	NP	A	0.11±0.01	++
CA	1.33	Pinkish red	NP	NA	0.08±0.02	-
BC45	2.67	Black	P	A	0.70±0.01	++

NP, Slime non-producer; P, slime producer; NA, none adhesive; A, adhesive; -, non biofilm forming OD570≤0.1; ++, medium biofilm forming (0.1 ≤ OD570 < 1).

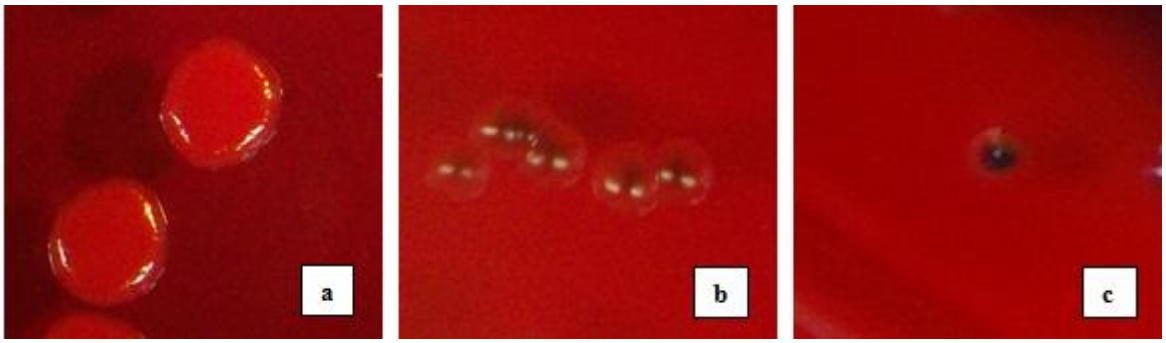

Figure 1. Morphotypes of *Bacillus* spp. strains based on the colorimetric scale obtained on Congo red agar: (a), orange colonies; (b), pinkish-red colonies with a darkening at the centre; (c), black colonies.

summarized in Table 3. Cells with hydrophobicity percentage greater than 70% as highly hydrophobic; from 70 to 30% as weakly hydrophobic and those with hydrophobicity lower than 30% as highly hydrophilic (Borges et al., 2008). We have found that the affinity to hexadecane was low suggesting a hydrophilic character for the majority of the studied strains. Only M5 strain was weakly hydrophobic.

Slime production

The ability of the tested strains to produce biofilm was assessed by culture on CRA plates. *Bacillus* strains (H1, H3 and M2) formed colonies with orange color. Other strains (M5, SA3, SF1 and CA) developed colonies with black center and red contour. H4, SA2, SH1, SH3, BK2 and BC strains were slime producing characterized by a black colonies (Figure 1).

Biofilm formation in glass surfaces

The majority of *Bacillus* spp. strains (10/13) were able to adhere to the glass giving a purple pellicule on the air-surface of the glass tube. We noted that both the intensity and the width of the pellicule differ from strain to strain. In fact, H3, M2 and SA2 strains are classified as faintly adherent with a very small purple pellicule. H1, H4 and SF1 strains showed a purple pellicule with medium width and were classified as moderately adherent. M5, SH1, BK2, and BC strains give a large purple pellicule and were classified as highly adherent. Other strains (SA3, SH3 and CA) were unable to adhere to the glass surface (Figure 2).

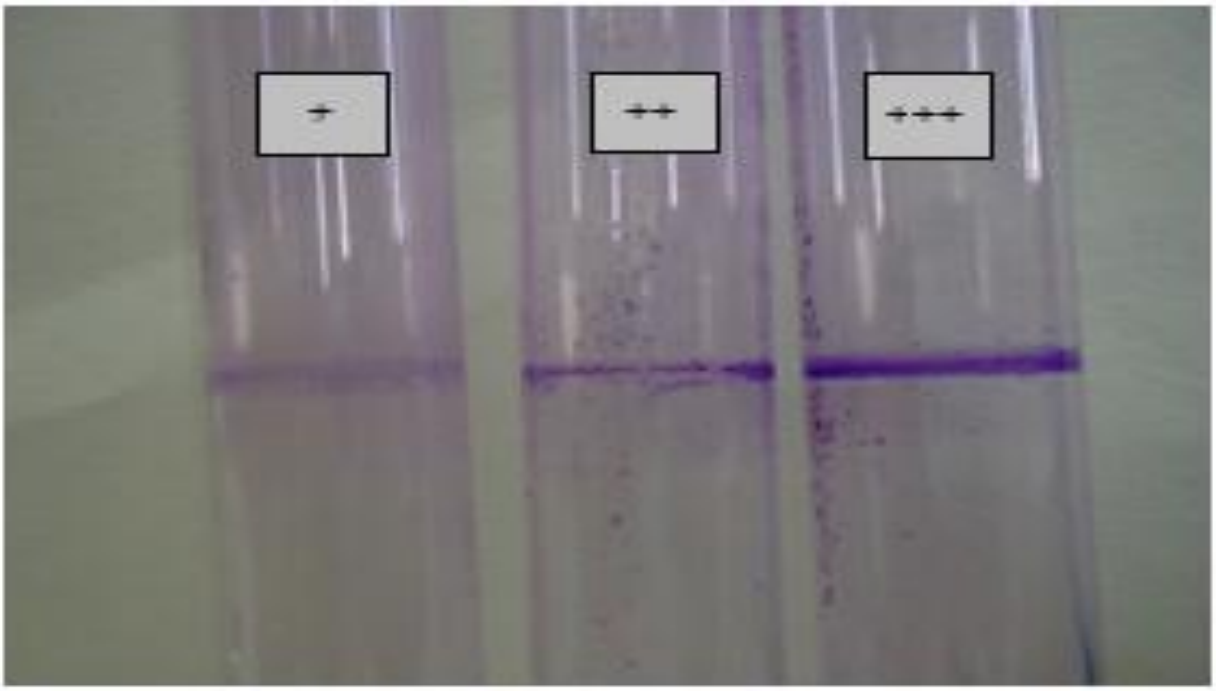

Figure 2. Adherence of *Bacillus* spp. strains on the surface of the glass tube. **+**, Slightly adherent; **++,** moderately adherent; **+++,** highly adherent.

Table 3. Qualitative and semi quantitative estimation of *Bacillus* spp. biofilm formation.

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M2	1.33	Orange	NP	A	0.07±0.02	-
M5	30.00	Pinkish red	NP	A	0.11±0.02	++
Sa2	4.00	Black	P	A	0.08±0.01	-
Sa3	26.00	Pinkish red	NP	NA	0.09±0.01	-
SH1	3.00	Black	P	A	0.17±0.01	++
SH3	3.67	Black	P	NA	0.09±0.02	-
BK2	19.67	Black	P	A	0.07±0.01	-
SF1	8.00	Pinkish red	NP	A	0.11±0.01	++
CA	1.33	Pinkish red	NP	NA	0.08±0.02	-
BC45	2.67	Black	P	A	0.70±0.01	++

NP, Slime non-producer; P, slime producer; NA, none adhesive; A, adhesive; -, non biofilm forming OD570≤0.1; ++, medium biofilm forming (0.1 ≤ OD570 < 1).

Biofilm formation in polystyrene surfaces

The results of the OD_{570} presented in the Table 3 showed that 46.1% of *Bacillus* spp. strains (6/13) were adhesive to polystyrene with values ranging from 0.11 to 0.70. The other strains were non- biofilm forming with an OD_{570} ≤1.

Enzymatic characterization

The tested *Bacillus* strains were able to produce exoenzymes such as amylase (92.3%) and lipase (84.6%). The caséinase was only produced by H1, M5, SA2, SA3 and SH1 strains. Excepting the H4 strains, all

Table 4. Extracellular enzymes products of *Bacillus* spp. strains.

Enzyme	H1	H3	H4	M2	M5	SA2	SA3	SH1	SH3	BK2	SF1	CA	BC45
Lipase	+	+	+	+	+	-	+	+	+	+	-	+	+
Caseinase	+	-	-	-	+	+	+	+	-	-	-	-	-
Lecitinase	-	-	+	-	-	-	-	-	-	-	-	-	-
Amylase	+	+	+	+	+	+	+	+	+	+	+	-	+
Phosphatase alcaline	*	*	*	*	+	*	*	+	*	*	*	*	+
Estérase(C4)	*	*	*	*	+	*	*	-	*	*	*	*	-
Estérase Lipase(C8)	*	*	*	*	+	*	*	+	*	*	*	*	-
Lipase(C14)	*	*	*	*	-	*	*	-	*	*	*	*	-
Leucine arylamidase	*	*	*	*	+	*	*	-	*	*	*	*	+
Valine arylamidase	*	*	*	*	-	*	*	+	*	*	*	*	-
Cystine arylamidase	*	*	*	*	+	*	*	-	*	*	*	*	+
Trypsine	*	*	*	*	+	*	*	-	*	*	*	*	-
α-chymotrypsine	*	*	*	*	-	*	*	-	*	*	*	*	-
Phosphatase acide	*	*	*	*	-	*	*	+	*	*	*	*	+
Naphtol-AS-BI-phosphohydrolase	*	*	*	*	+	*	*	+	*	*	*	*	+
α-galactosidase	*	*	*	*	-	*	*	-	*	*	*	*	-
β-galactosidase	*	*	*	*	-	*	*	+	*	*	*	*	-
β-glucuronidase	*	*	*	*	-	*	*	-	*	*	*	*	-
α-glucosidase	*	*	*	*	+	*	*	+	*	*	*	*	+
β-glucosidase	*	*	*	*	-	*	*	-	*	*	*	*	-
N-acétyl-β-glucosaminidase	*	*	*	*	-	*	*	-	*	*	*	*	-
α-mannosidase	*	*	*	*	-	*	*	-	*	*	*	*	-
α-fucisidase	*	*	*	*	-	*	*	-	*	*	*	*	-

+, Production; -, non production; *, non tested; N-AS-BI-P, naphtol-AS-BI-phosphohydrolase.

other tested bacteria were lecithinase negative (Table 4). Taking consideration of the previous results, only M5, SH1 and BC strains were selected for enzymatic characters studied on Api-ZYM system. Results showed that all investigated cells are able to assimilate the alkaline phosphatase, Naphtol-AS-BI-phosphohydrolase and α-glucosidase (Table 4).

DISCUSSION

In the present study, 13 bacteria strains have been isolated from different Tunisian hypersaline environments. The isolated strains were identified as *Bacillus* spp. according to Yang et al. (2006). In previous studies, eighty nine isolates were obtained from the sediments of four deep-sea, hypersaline anoxic brine lakes in the Eastern Mediterranean Sea and was dominated by representatives of the genus *Bacillus* (Terry et al., 2008). *Bacillus* spp. generally occur more frequently in sediments than in the water column, and *Bacillus* spores may account for up to 80% of the total heterotrophic flora (Hovda et al., 2007).

On diffusion agar assay only M5, SH1 and BC45 strains exhibited a zone of clearance against the tested pathogens. The inhibitory mechanism of the interaction was not characterized in this study. However, this result can be related to the ability of *Bacillus* to produce antibacterial compounds such as bacitracin, gramicidin S, polymyxin, and tyrothricin, which are active against a wide range of gram-positive and gram-negative bacteria (Ravi et al., 2007). On the other hand, other tested bacteria showed an antagonist effect but against all pathogens. It has been found that *Bacillus* species isolated from the soil are effective against Gram-positive and Gram-negative bacteria whereas their extensive inhibition effect is particularly against Gram-positive bacteria (Yilmaz et al., 2006).

Cell surface hydrophobicity (CSH) of the bacteria is one of the most important factors which govern the mechanism of bacterial adhesion to inanimate and biological surfaces. Furthermore, the surface charges and hydrophobicity of bacteria were influenced by the environmental condition (Vesterlund et al., 2005), explaining, in fact, the variation of bacteria capacity to adhere to substrates. All strains tested in this study grow on Congo red agar plates and gave after 18 to 24 h of incubation at 37°C three different morphotypes on the basis of the colour of the colonies obtained. The morphotype I is characterized by an orange colonies

(3/13 strains) morphotype II with pinkish-red colonies with a darkening at the centre (4/13 strains) and morphotype III which were considered as slime producers were characterized by black colonies (6/13 strains). Previous studies used this medium to study the phenotypic formation of biofilm for several bacteria including *Aeromonas* spp. and *Staphylococus* spp. (Saidi et al., 2011). On abiotic surfaces, the tested *Bacillus* strain were able to adhere to glass surface characterized by a purple pellicule on the air-surface of the glass tube, and most of them exhibit a high potential to adhere to polystyrene microplates with biofilms formation. This mechanism begins with the attachment of bacteria to abiotic surface, by means of pili, flagella or other materials, followed by the production of exopolysaccharides to form a glycocalyx (Wong et al., 2002). The DLVO (Derjaguin, Landau, Verwey, and Overbeek) theory of colloid stability has been used by several groups to try to explain attachment of micro-organisms to surfaces (Hermansson, 1999). Dan (2003) suggested that the DLVO approach to bacterial adhesion tended to treat bacterial cells as traditional colloidal particles, characterized by having an even surface and an evenly distributed surface charge. The problem remains that cells contain many complicated surface structures such as flagella, pili, fimbriae, glycoproteins, carbohydrates, teichoic acids and other biological materials composed of proteins in *Bacillus* species up to 9% of total cell proteins are associated with the cell wall. These complicated surface structures may exert their own localized cell surface charge at a microscopic cell surface level that could possibility mediate attachment through local electrostatic attraction despite the cell's having an overall electrostatic repulsion (Palmer et al., 2007). We noted that adhesion ability differ from strain to strain and from surface to another. In fact, there is no reliance between the slime producing ability on CRA plates and the adhesion power developed on polystyrene material. In fact, 4 strains characterized by orange colonies on Congo red agar (slime non producers), were adhesive to polystyrene microtiter plates.

Extracellular enzyme activity of the isolates revealed that the majority of the tested strains are able to produce amylase and lipase. Bal et al. (2009) found that all isolated marine *Bacillus* spp. possess extracellular enzymes amylase and protease. Some of other isolated *Bacilllus* spp. strains could able to produce caseinase. Previously studies reported that *Bacillus* bacteria secrete many exoenzymes, such as proteases, carbohydrolases and lipases, which are very efficient in breaking down a large variety of proteins, carbohydrates and lipids into smaller units (Ninawe and Selvin, 2009). The *Bacillus* strains (M5, H1 and BC45) analyzed in this study were heterogeneous on the basis of their exoenzymes profile tested on Api-ZYM system. However, α-glucosidase was produced by all tested stains. In fact, it is shown that members of the *Bacillus* genus are known to express a number of α-glucosidase exhibiting a diversity of substrate specificities (Sadler et al., 1984). As well, multiple research investigations were aimed on screening and purification of thermostable enzymes from *Bacillus* genus α-glucosidases from *Bacillus caldovelax* DSM 411, *Bacillus flavocaldarius* KP1228 (FERM-P9542), *Bacillus thermoamyloliquefaciens* KP1071 (FERM-P84776) and *Bacillus* sp. DG0303 have been well characterized (Ferrari et al., 1993; Kashiwabara et al., 2005). Similarly, alkaline phosphatase were produced by all tested stains. Thirty one strains of *Bacillus* spp. were isolated from soil sample, including 7 isolates of *Bacillus stearothermophilus* with one showing high production of alkaline phosphatase (Hamza and Hassan, 2005).

Conclusions

Halophilic bacterial communities isolated from Tunisian hypersaline environments have not been thoroughly studied. In this study, a great number of halophilic bacilli were isolated from distinct hypersaline environments of Tunisia in order to screen their antagonism effect against pathogens, adhesive ability and exoenzymes production capacities. The studied strains were identified as *Bacillus* spp. and were shown to have interesting properties, thus making them good candidates for biotechnological applications.

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