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Full Length Research Paper

Streptococcus pseudopneumoniae as an emerging pathogen from patients with respiratory diseases

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Streptococcus pseudopneumoniae is a mischaracterized species of Streptococci that is usually overlooked during examination of sputum samples of patients with chest infections. The association of this organism with lower respiratory tract diseases is still unclear and its isolation and description is underestimated in our locality. To our knowledge, there are no published studies on the isolation of *S. pseudopneumoniae* from pathological specimens in Egypt. The aims of this study were to isolate *S. pseudopneumoniae* from sputum specimens of patients admitted to Chest Department of Mansoura University Hospitals (MUHs) and to differentiate it from *Streptococcus pneumoniae* and other viridans group Streptococci, also to determine its prevalence and associated risk factors. Sixteen isolates of *S. pseudopneumoniae* were diagnosed phenotypically by optochin susceptibility and bile solubility tests followed by genotypic characterization by multiplex polymerase chain reaction (PCR). All of the isolates were subjected to antibiotic susceptibility testing using the disk diffusion method. The prevalence of *S. pseudopneumoniae* among studied patients was 4.9% (16/329). All of the examined isolates were found to be positive for *aliB*-like ORF2 and negative for *cpsA* and *lytA* genes by multiplex PCR. Elevated resistance rate of the isolates was recorded for erythromycin, penicillin and co-trimoxazole. Infection by *S. pseudopneumoniae* was found to be significantly associated with Chronic Obstructive Pulmonary Diseases (COPD). Although, *S. pseudopneumoniae* recorded a low prevalence in this study, their elevated antibiotic resistance together with the association with COPD and their pure isolation from sputum samples underline the necessity of spending more effort for their detection and characterization in the microbiology laboratories.

Key words: Chronic obstructive pulmonary diseases (COPD), optochin, respiratory diseases, *Streptococcus pseudopneumoniae*.

INTRODUCTION

Streptococcus pseudopneumoniae as suggested by DNA-DNA homology studies is recorded as a species of the *Streptococcus mitis/oralis* members of viridans group Streptococci; they have some similar features to *Streptococcus pneumoniae* (Arbique et al., 2004). The principle characteristics of *S. pseudopneumoniae* are the lacking of pneumococcal capsule, bile insolubility, indeterminate sensitivity or resistance to optochin after

incubation in CO_2 (5%), while sensitivity to it after incubation in ambient air, they give positive results with antigen detection and DNA probe hybridization tests (Keith et al., 2006).

S. pseudopneumoniae was firstly described in sputum samples of patients with lower respiratory tract infection, particularly those with Chronic Obstructive Pulmonary Diseases (COPD) (Laurens et al., 2012). The clinical relevance and pathogenic potential of this organism has not been clearly determined except in few studies that were conducted on mouse models with sepsis and peritonitis, so the correct identification and rapid detection of this emerging pathogen in clinical setting is very important for determination of its disease potential (Harf-Monteil et al., 2006).

S. pseudopneumoniae is usually misidentified in our laboratories as we usually rely on optochin sensitivity testing with 5% CO_2 for detection of *S. pneumoniae* and miss the incubation in ambient air which is the key point for identification of *S. pseudopneumoniae.* Biochemical reactions are usually used to differentiate typical *S. pneumoniae* from *S. pseudopneumoniae* and other related Streptococci; several molecular techniques have also been used (Rolo et al., 2013).

There are several polymerase chain reaction (PCR)-based assays that target specific pneumococcal virulence factors for the typical *S. pneumoniae*, such as autolysin A (*lytA*) which is the major pneumococcal autolysin (Simões et al., 2010) and the capsular polysaccharide biosynthesis gene A (*cpsA*) which is a conserved pneumococcal capsular polysaccharide gene (Bentley et al., 2006), also *aliB*-like ORF2 (a gene that usually found in the capsular region of non-capsulated *pneumococci*) (Hathaway et al., 2004); these genetic markers have been used to differentiate *S. pneumoniae* from other related species (Rolo et al., 2013).

Unknown putative genes, specific intergenic DNA sequences, or specific regions of the 16S rRNA, have also been proposed to be pneumococcal species-specific (El Aila et al., 2010).

To the best of our knowledge, there are no published studies on the isolation of *S. pseudopneumoniae* from pathological specimens in Egypt. So the aims of the present study were to isolate *S. pseudopneumoniae* from sputum samples of patients admitted to Chest Department of Mansoura University Hospitals (MUHs) and to differentiate it from *S. pneumoniae* and other viridans group Streptococci by phenotypic and genotypic tests as well as to determine the prevalence of this overlooked pathogen and the risk factors of its acquisition.

METHODOLOGY

Design of the study

Cross sectional descriptive study was conducted on 381 patients showing signs of respiratory tract infection (Horan et al., 2008) during period extending from the first of September, 2015 to the end of August, 2016. All patients, enrolled in this study, were admitted to the Chest Department of MUHs. The protocol of this research was approved by the ethical committee in the Faculty of Medicine, Mansoura University, with code number R/16.10.08.

Sample collection

Sputum samples were collected from all studied patients (one sample from each patient) under complete aseptic condition. The samples were processed in the Unit of Microbiology Diagnostic and Infection Control in the Department of Medical Microbiology and Immunology, Faculty of Medicine, Mansoura University.

Microscopic examination

Prepared films stained with Gram from sputum specimens were microscopically examined. Specimens with >25 leukocytes and <10 squamous epithelial cells/×100 microscopic field were incorporated in this study and subjected to advance processing, whereas specimens with >10 epithelial cells/×100 microscopic field were not included in the research (Goyal et al., 2007).

After microscopic examination of the collected 381 sputum samples, 329 samples fulfilling aforementioned criteria were subjected to further processing and 52 samples were excluded.

Culture and identification

The 329 sputum specimens were cultivated on 5% sheep blood agar, chocolate agar (Oxoid, UK), and MacConkey's agar (HiMedia Laboratories, India) plates by taking a loopful from the purulent portion of each sputum specimen using calibrated bacteriological loop that carry 0.01 ml. Cultivation on blood agar and chocolate agar plates were done in duplicate; one was incubated in CO_2 enriched atmosphere using a candle jar for 24 h at 37°C and the other was incubated aerobically at 37°C for 24 h. MacConkey's agar plates were incubated aerobically at 37°C for 24 h (Zhou et al., 2011). Significant count of bacteria has been determined as being ≥10^5 CFU/ml, whereas bacteria < 10^5 CFU/ml was considered to be non-significant count of normal flora (Amissah and Pappoe, 2014). Isolated organisms were identified according to standard microbiological methods. Alpha hemolytic colonies were selected for Gram staining, colonies that showed Gram positive cocci arranged in pairs or chains were subjected to further identification by pneumococcal capsule detection test (capsules were detected by observing a halo around Pneumococci with India ink at ×400 magnification) (Harf-Monteil et al., 2006), optochin susceptibility and bile solubility tests.

Optochin susceptibility test

The test was done in duplicate; one plate was incubated in 5% CO_2 and the other in ambient air (O_2 atmosphere) at 35°C for 18 to 24 h. Inhibition zones around the disks were measured and interpreted according to the recommended standards of CLSI (2014). Positive test showed a zone of inhibition of 14 mm or more around the disk (Koneman et al., 2006).

Bile solubility test

The test was conducted with bile solubility reagent (10%) (Remel, USA). Clearing of the test suspension within 3 h was considered to

Table 1. Primers used in the multiplex PCR of the studied isolates

Gene	Primer name	Primer sequence	Size, bp	References
cpsA	*cpsA* F2 *cpsA* R2	AGCAGTTTGTTGGACTGACC GTGTGAATGGACGAATCAAC	613 bp	Simões et al. (2011)
lytA	*lytA* 1145 *lytA* 750	AATCAAGCCATCTGGCTCTA GGCTACTGGTACGTACATTC	395 bp	Messmer et al. (1997)
aliB-like ORF2	104_Fl3.6 104_b832.10	AGATGCCAAATGGTTCACGG GAAACTCTTCGTTTACTGGG	290 bp	Hathaway et al. (2004)
16S rRNA	16S rRNA F2 16S rRNA R2	ACATGCTCCACCGCTTGTG GCTCTGTTGTAAGAGAAGAACG	522 bp	Simões et al. (2011)

bp, Base pair.

be positive test (Wessels et al., 2012).

All *S. pseudopneumoniae* isolates included in this study were phonotypically suspected as being capsule free, sensitive to optochin in ambient air but intermediate or resistant by incubation in 5% CO_2 and bile insoluble.

Maintenance of the selected alpha hemolytic Streptococci

For isolates preservation, skim-milk tryptone glucose glycerol (STGG) medium was used (O'Brien et al., 2001). Pure growth of each isolate was collected and dispensed into 1.5 ml vials containing 1.0 ml of STGG medium. After that, the suspension was stored at -70°C for further testing. Before starting any further experiment, subculture of the isolates on blood agar was done twice to allow isolates to restore their viability (Charalambous et al., 2003).

Multiplex PCR for genotypic characterization of *S. pseudopneumoniae* isolates

Design of specific gene primers for multiplex PCR assay

A multiplex PCR was performed for all of studied alpha hemolytic Streptococci in order to detect internal fragments of the following four genes: *cpsA, lytA*, *aliB*-like ORF2 and 16S rRNA gene as a positive internal control (Rolo et al., 2013). The primer sequences and expected band sizes have been used in accordance with previous studies (Table 1).

DNA extraction

DNA was extracted using QIAamp® DNA Mini kits, QIAGEN (Germany) according to the manufacturer's instructions.

Multiplex PCR technique

The multiplex PCR was done in a 10 µl volume with 1×PCR buffer (10 mM Tris-HCl, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$), 0.12 mM of dNTPs Mix (2 mM each), 1 U/µl of Taq DNA polymerase (Fermentas) and 0.5 *p*mol/µl of each primer (Simões et al., 2011). 1 µl of extracted DNA was added as DNA template. PCR was done with the following reaction conditions: a pre-denaturation step of 4 min at 94°C; 35 cycles at 94°C for 30 s, 52°C for 45 s, and 72°C

for 1 min; a final extension step of 5 min at 72°C; and soaking at 16°C. The PCR products were analyzed after running on 2% agarose gel containing ethidium bromide at 80 volts for about 2 h on comparison to DNA standard marker: #SMO373, 50 bp DNA Ladder, Thermo Scientific Inc., USA (Richter et al., 2013; Hajia et al., 2014). Distilled water was incorporated in every run of the multiplex PCR as a negative control.

Testing for antimicrobial sensitivity

S. pseudopneumoniae isolates were subjected to antibiotic sensitivity testing as recorded by the recommendations of CLSI using disk diffusion method on Muller-Hinton agar (Oxoid, UK) supplemented with 5% sheep blood using the following antibiotics: penicillin, amoxicilline/clavulinic acid, erythromycin, clindamycin, cefotaxime, co-trimoxazole, ciprofloxacin, linezolid, cefepime, vancomycin, meropenem and teicoplanin (Forbes et al., 2007). The inhibitory zone limits of the tested antimicrobials were referred to CLSI (2014) guidelines.

Analysis of data

The data were entered and analyzed statistically with Statistical Package of Social Science (SPSS) using software version 21 (SPSS Inc., Chicago, IL, USA). Descriptive statistics were described as mean, standard deviation (SD), minimum, maximum and percentage. Inter-group comparison of categorical data was done using Chi square test ($\chi2$-value). Odds ratio (OR) and confidence interval (CI) were used to calculate the risk factors. For all of the previous statistical tests, the threshold of significance is fixed at 5% (p-value <0.05 was considered to be statistically significant).

RESULTS

Out of the studied 329 sputum samples, 286 yielded single pathogen whereas 13 yielded more than one pathogen (2 pathogens) and mixed insignificant growth of normal respiratory tract flora was detected in the remaining 30 samples. The total number of the isolated respiratory pathogens was 312. Sixty two isolates (62/312, 19.9%) were alpha hemolytic Streptococci whereas the remaining 250 isolates were *Klebsiella*

Table 2. Multiplex PCR results of the examined alpha hemolytic Streptococcal isolates.

| Gene | Alpha hemolytic Streptococci (number=62) | | |
	S. pseudopneumoniae (number=16) % (number positive/screened)	S. pneumoniae (number=21) % (number positive/screened)	Viridans group Streptococci (number=25) % (number positive/screened)
cpsA	0.0 (0/14)	90.5 (19/21)	0.0 (0/20)
lytA	0.0 (0/14)	100 (21/21)	0.0 (0/20)
aliB-like ORF2	100 (14/14)	14.3 (3/21)	20 (4/20)
16S rRNA	87.5 (14/16)	100 (21/21)	80.0 (20/25)

species (59/312, 18.9%), *Candida* species (50/312, 16.0%), *Staphylococcus aureus* (41/312, 13.1%), *Escherichia coli* (23/312, 7.4%), *Pseudomonas aeruginosa* (21/312, 6.7%), *Streptococcus pyogens* (18/312, 5.8%), *Enterococci* (12/312, 3.8%), MRSA (10/312, 3.2%), *Citrobacter* species (6/312, 1.9%), *Haemophilus influenzae* (6/312, 1.9%) and *Moraxella catarrhalis* (4/312, 1.3%).

Among the isolated 62 alpha hemolytic Streptococci, 16 isolates were presumptively recognized as being *S. pseudopneumoniae* by phenotypic tests, as all these isolates lacked the pneumococcal capsules, showed resistance to optochin (zone diameter <14 mm) in 5% CO_2 incubation, but they were sensitive to optochin (zone diameter between 18 to 25 mm) when incubated in ambient air; also they were found to be bile insoluble. Regarding the colony morphology of the *S. pseudopneumoniae* on 5% sheep blood agar, they were observed to be small (about 1 mm in diameter), shiny, smooth, and domed, with entire edges. Few colonies having depressed centers, seem to be a smaller version of the *S. pneumoniae* draftsmen colonies. The remaining 46 alpha hemolytic Streptococci were identified as *S. pneumoniae* (21 isolates) and other viridans group Streptococci (25 isolates) accounted for 6.7 (21/312) and 8.0% (25/312) of the isolated respiratory pathogens, respectively. The percentage of the isolated *S. pseudopneumoniae* in relation to other detected respiratory pathogens was 5.1% (16/312) and its prevalence among studied patients was 4.9% (16/329).

The multiplex PCR assay which was conducted on the studied alpha hemolytic Streptococci showed that DNA from 2/16 of the putative *S. pseudopneumoniae* isolates and 5/25 of the viridans group Streptococci were negative for 16S rRNA gene, so these extracts must have been inhibitory. *S. pseudopneumoniae* PCR results for the other targets therefore were determined out of the 14 (non-inhibitory) samples. All of the phenotypically identified *S. pseudopneumoniae* isolates were negative for *cpsA* and *lytA* genes, but they were positive for *aliB*-like ORF2 (Table 2 and Figure 1).

Antibiotic sensitivity testing of the studied 16 *S. pseudopneumoniae* isolates showed that all of them were sensitive to linezolid, vancomycin and teicoplanin, on the other hand, co-trimoxazole, penicillin and erythromycin recorded the lowest sensitivity for those isolates (Table 3).

The distinctive data of patients with culture positive sputum for *S. pseudopneumoniae* (16 patients) showed that their mean age was 58.06 years (range 17-76 years); 56.2% were males, 68.8% were presented with COPD with infected exacerbation. On the other hand, only 37.5% of them showed positive X-ray for infiltration and 31.2% recorded elevated leukocyte counts (>11 × 10^9 cells/L). *S. pseudopneumoniae* was the only detected pathogen in 68.8% of sputum cultures of those patients, whereas *H. influenza* was the most common co-pathogen detected in sputum culture of patients that showed other bacterial growth along with *S. pseudopneumoniae* (Table 4).

On comparing patients with culture positive sputum for *S. pseudopneumoniae* with patients who showed the carriage of other alpha hemolytic Streptococci (*S. pneumoniae* and viridans group Streptococci) in their sputum samples, COPD with infected exacerbation and previous hospitalization with COPD were found to be more significantly associated with the acquisition of *S. pseudopneumoniae* infections (P= 0.006 and 0.01, respectively). On the other hand, there was no statistically significant difference regarding presence of asthma, pneumonia, bronchiectasis, bronchogenic carcinoma or smoking history as risk factors (Table 5).

DISCUSSION

S. pseudopneumoniae may often be overlooked during examination of sputum samples of patients with respiratory disorders particularly in our locality. Few studies have been conducted on the isolation and description of this organism; the prevalence of *S. pseudopneumoniae* is determined in few researches that have been considered to be unique for certain areas of the world and this research was one of the first attempts to isolate this organism from patients with respiratory diseases in our locality.

S. pseudopneumoniae could be easily isolated and characterized by few phenotypic and genotypic tests
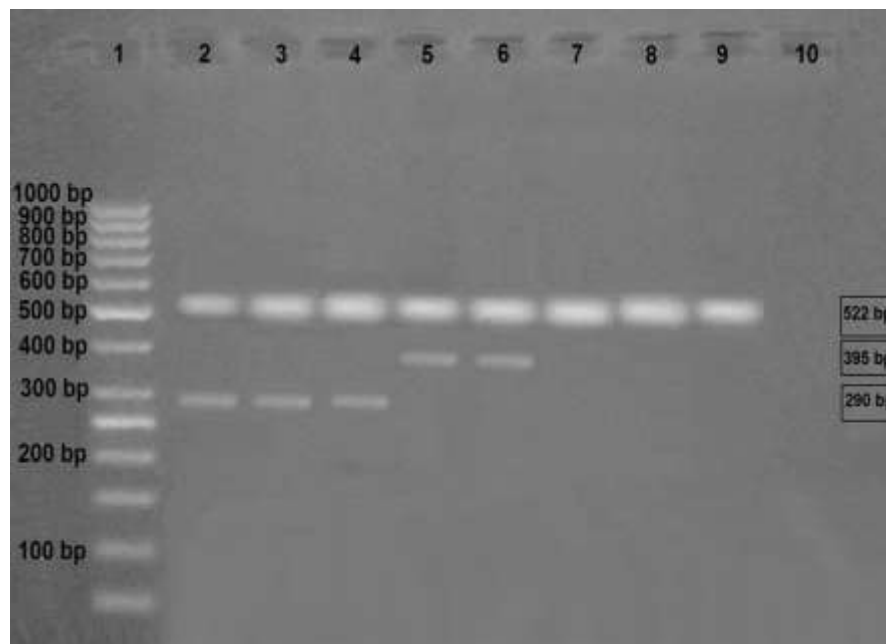
Figure 1. Multiplex PCR results of the studied alpha hemolytic Streptococci including *S. pseudopneumoniae* isolates. Lane 1 shows the DNA standard marker. All lanes (2 to 9) show 522 bp fragments of positive internal control. Lane 10 is the negative control (distilled water). Lanes 2, 3 and 4 show the 290 bp bands from positive *S. pseudopneumoniae* isolates to *aliB*-like ORF2 gene. Lanes 5 and 6 show the 395 bp bands from positive *S. pneumoniae* isolates to *lytA* gene. Lanes 7, 8 and 9 show only the bands of positive internal control with absence of bands of other target genes from negative isolates of viridans group Streptococci.

Table 3. Antibiotic sensitivity pattern of the studied 16 *S. pseudopneumoniae* isolates.

Antibiotic tested	*S. pseudopneumoniae* (number=16)	
	% (number sensitive/screened)	% (number resistant/screened)
Penicillin	37.5 (6/16)	62.5 (10/16)
Amoxicilline/clavulinic acid	56.2 (9/16)	43.8 (7/16)
Erythromycin	43.8 (7/16)	56.2 (9/16)
Clindamycin	56.2 (9/16)	43.8 (7/16)
Cefotaxime	87.5 (14/16)	12.5 (2/16)
Co-trimoxazole	25.0 (4/16)	75.0 (12/16)
Ciprofloxacin	62.5 (10/16)	37.5 (6/16)
Linezolid	100 (16/16)	0.0 (0/16)
Cefepime	93.8 (15/16)	6.2 (1/16)
Vancomycin	100 (16/16)	0.0 (0/16)
Meropenem	93.8 (15/16)	6.2 (1/16)
Teicoplanin	100 (16/16)	0.0 (0/16)

particularly the optochin sensitivity test and specific genes detection by multiplex PCR assay.

The prevalence of *S. pseudopneumoniae* among studied patients with respiratory tract disorders was 4.9%; this percentage approximates what has been recorded in previous studies at which this pathogen was isolated from nearly 4% of the studied cases (Keith et al., 2006; Mohammadi and Dhanashree, 2012). On the other hand, lower percentage of *S. pseudopneumoniae* (nearly 1%) has been observed by other researchers (Harf-Monteil et al., 2006; Swathi et al., 2015). The variability of results among various studies may be due to the

Table 4. distinctive features of patients with culture positive sputum for *S. pseudopneumoniae*.

Item	Study group (n=16)	
	(Number positive/screened)	%
Age (years)		
Mean ± SD	58.06±15.03	-
Min-Max	17-76	-
Sex		
Male	(9/16)	56.2
Female	(7/16)	43.8
Clinical diagnosis		
COPD with infected exacerbation.	(11/16)	68.8
Lung abscess	(1/16)	6.2
Bronchogenic carcinoma, COPD	(1/16)	6.2
Pneumonia	(1/16)	6.2
Asthma	(1/16)	6.2
Bronchiectasis	(1/16)	6.2
Chest X ray for infiltration		
Positive	(6/16)	37.5
Negative	(10/16)	62.5
Elevated leukocyte		
Positive	(5/16)	31.2
Negative	(11/16)	68.8
Acompanied bacteria in sputum culture		
No other bacteria	(11/16)	68.8
H. influenzae	(4/16)	25
E. coli	(1/16)	6.2

Table 5. Patients risk factors for acquisition of *S. pseudopneumoniae* in relation to patients infected with other alpha hemolytic Streptococci.

Risk factor	Patients with culture positive sputum for *S. pseudopneumoniae* (number=16) % (no. positive/screened)	Patients with culture positive sputum for other alpha hemolytic Streptococci (number=46) % (no. positive/screened)	P value	Odds ratio and confidence interval
Previous hospitalization with COPD	62.5 (10/16)	23.9 (11/46)	0.01*	5.30 (1.36-21.66)
COPD with infected exacerbation	68.8 (11/16)	26.1 (12/46)	0.006*	6.23 (1.55-26.42)
Asthma	6.2 (1/16)	21.7 (10/46)	0.2	0.24 (0.01-2.15)
Pneumonia	6.2 (1/16)	26.1 (12/46)	0.1	0.19 (0.01-1.65)
Bronchiectasis	6.2 (1/16)	17.4 (8/46)	0.4	0.32 (0.01-2.94)
Smoking	56.2 (9/16)	58.7 (27/46)	0.9	0.90 (0.25-3.31)
Bronchogenic carcinoma	6.2 (1/16)	15.2 (7/46)	0.6	0.37 (0.02-3.53)

difference in patient health care and the associated manner of infectious agent distribution in different localities.

Checking for specific genetic markers by multiplex PCR revealed the absence of *cpsA* and *lytA* genes in all studied isolates of *S. pseudopneumoniae,* whereas they were found to be, respectively positive in 90.5 and 100% of the isolated *S. pneumoniae*. These findings support a previous published study at which *cpsA* was confirmed to be a specific capsular polysaccharide gene exclusively harbored by *S. pneumoniae* as it is commonly linked with the capsular operon and absent in *S. pseudopneumoniae*

isolates as being non capsulated (Park et al., 2010). Similarly, *lytA* gene which has been considered to be one of the diagnostic markers that differentiate *S. pneumoniae* from other closely related species including *S. pseudopneumoniae* was also found to be absent in all isolates of *S. pseudopneumoniae* that have been examined in other researches (Rolo et al., 2013).

aliB-like ORF2 gene was detected in all studied *S. pseudopneumoniae* isolates supporting the observations recorded in previous researches that have mentioned this gene as being specific for non-capsulated Pneumococci and hence *S. pseudopneumoniae* as being non capsulated (Hathaway et al., 2004). There were three isolates of the examined *S. pneumoniae* found to be positive for *aliB*-like ORF2 gene; our results are in agreement with previous findings that have recorded the same observation (Simões et al., 2011) which could be explained by the lack of ability of those isolates to produce the capsule *in vitro* (Scott et al., 2012) as a result of capsular genes mutation (Melchiorre et al., 2012).

A great resistance rate of the studied *S. pseudopneumoniae* isolates was recorded for co-trimoxazole (75.0%), penicillin (62.5%) and erythromycin (56.2%) which was consistent with previous studies at which the same resistance pattern has been observed among studied isolates (Keith and Murdoch, 2008; Mohammadi and Dhanashree, 2012). Other studies have also documented a high resistance rate among *S. pseudopneumoniae* isolates to erythromycin (60%) without any recorded resistance to penicillin (Keith et al., 2006) that was in contrast with our finding and may be due to different antibiotic policy in other countries at which penicillin is used within limits. Unfortunately, there is no recorded information about isolation and antibiotic sensitivity profile of *S. pseudopneumoniae* in our locality to be compared with our findings. The observed high resistance of this organism to penicillin and other classes of antibiotics highlights its role as one of the respiratory tract pathogen that should not be neglected particularly during selection of the appropriate antibiotic that could be used in dealing with respiratory tract infection.

In this study, *S. pseudopneumoniae* was the only pathogen recovered from 11/299 (3.7%) of specimens that were positive both by smear-microscopy and culture for bacterial pathogens and it was the predominant one in samples that showed growth of other co pathogen. This observation should not be neglected as it may be an indirect evidence of the important clinical role of this pathogen in the acquisition of respiratory tract infection. This observation was also supported by the presence of pulmonary infiltrate in 37.5% and elevated leukocyte count in 31.2% of cases with culture positive *S. pseudopneumoniae*. Although a high resistance rate of *S. pseudopneumoniae* to various classes of antibiotics has been documented in previous studies, most of them suspected this organism as a colonizing bacterium of the respiratory tract or a weak opportunistic pathogen that is usually associated with potentially pathogenic organisms in patients with chronic conditions (Laurens et al., 2012). Subsequent studies have supported our finding that this organism may have a real clinical importance depending on the observed high resistance rate for this organism together with its involvement in various types of invasive diseases (Rolo et al., 2013).

In this study, the actual risk factors associated with *S. pseudopneumoniae* infection were assessed by comparing patients with culture positive sputum for *S. pseudopneumoniae* with patients who revealed other alpha hemolytic Streptococci (*S. pneumoniae* and viridans group Streptococci) in their sputum cultures. COPD with infected exacerbation and previous hospitalization with COPD were significantly associated with increase in the acquisition of *S. pseudopneumoniae* in spite of large number of studied cases suffering from COPD in this research. The association of COPD with *S. pseudopneumoniae* infection has been documented by previous studies (Keith et al., 2006; Harf-Monteil et al., 2006), however other studies have recorded that *S. pseudopneumoniae* were more frequently associated with bronchitis and pneumonia rather than COPD (Laurens et al., 2012). To determine the actual role of *S. pseudopneumoniae* in COPD exacerbation, cohort studies for patients during periods of exacerbation and stability of COPD is recommended to observe the appearance of new *S. pseudopneumoniae* isolates in the exacerbation periods that could confirm the association of infection with new bacterial strains and COPD exacerbation (Sethi et al., 2002).

Future studies seems to be necessary to determine the actual habitat, epidemiology, colonization of *S. pseudopneumoniae* and if it is associated with respiratory diseases other than COPD particularly pneumonia.

Conclusion

The pure growth of *S. pseudopneumoniae* from sputum samples together with the great percentage of antibiotic resistance exhibited by those isolates observed in the present study should raise our attention to the clinical importance of this organism. The initial data recorded in this research concerning the prevalence, the genetic characterization, the antibiotic sensitivity pattern and the associated risk factors of *S. pseudopneumoniae* were the first in our locality as regarding this organism. Future studies should be encouraged to allow further description of *S. pseudopneumoniae* especially in relation to lower respiratory tract diseases; this may change the notion that they are just atypical pneumococci as it has been previously believed in our area.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Halo-thermophilic bacteria and heterocyst cyanobacteria found adjacent to halophytes at Sabkhas, Qatar: Preliminary study and possible roles

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This study was conducted to investigate the halo-thermophilic bacteria and cyanobacteria adjacent to the halophytic plants, *Suaeda virmiculata*, *Limonium axillare* and *Tetraena qatarense*, and the microbial functionalities in Sabkhas of Qatar. These soils are alkaline and highly saline, and their moisture contents varied throughout the year. A significant presence of thermo-halophilic bacteria was found when selective media was used; however, bacterial populations were highest in soil samples taken adjacent to *L. axillare* as compared to those taken adjacent to other halophytes. They were lowest in samples taken close to *S. virmiculata*. Microscopic examinations revealed that the bacterial cells of isolated strains were Gram-positive rods with pointed ends that occurred singly, in pairs or in short chains. Most were bacilli, either *Bacillus thuringenses* or *Bacillus cereus*. These can form endospores to survive until more favorable environmental conditions allow them to resume growth and activity. Moreover, when Sabkhan soils were transferred to the laboratory under natural conditions, only cyanobacteria grew, and some produced biofilms. The most recognizable cyanobacteria were *Anabaena* and *Nostoc*. Some produced heterocysts and akinetes, which play important roles in soil biology and nitrogen fixation. The possible roles of these microorganisms in saline environment in Sabkhan soil appear to be support of halophyte growth by alleviating salt stress and other extreme environmental conditions.

Key words: Halo-thermophilic bacteria, halophytes, Sabkhas, cyanobacteria, ecophysiology.

INTRODUCTION

Wildlife, crops and even the biodiversity of the Arabian Gulf region in general and in the State of Qatar in particular, face many harsh abiotic factors which include drought, high salinity and high temperatures. Meteorological reports confirm that annual rainfall could be well below 80 mm, electrical conductivity of soil saturated extract (ECe) could be above 200 dSm^{-1}, and summer temperatures are known to exceed 50°C (Abdel-Bari et al., 2007; Yasseen and Al-Thani, 2013). These environmental factors can cause a host of changes in the

Figure 1. Part of the study site, northeast of Qatar.

microbial community and the biodiversity in desert ecosystems and habitats (Berg et al., 2014; Classen et al., 2015; Tang et al., 2015; Zeglin, 2015). The abundance and activities of soil microorganisms are determined by these environmental factors and the presence of organic matter, pollution, etc. (Sarig et al., 1999; Katterer and Andren, 2001; Vidali, 2001; Whitford, 2002; Mohammadipanah and Wink, 2015). In fact, the microbial community is considered as a primary part of the soil biota, and it plays very important roles in nutrient availability, thereby affecting the nutrient cycle. These microorganisms rely considerably on the soil for various life activities such as nutrition, growth, pharmaceutical needs, and possibly metabolic activities (Richer et al., 2012; Gougoulias et al., 2014).

Many microbial communities and activities were recently reported to be well adapted to desert and saline environments (Mohammadipanah and Wink, 2015), and they might play various roles in alleviating the impact of harsh abiotic stresses, thereby contributing considerably to stress tolerance in plants. Such mechanisms could be associated with the activities of many living organisms (bacteria, fungi and cyanobacteria) in various parts of plant and soil systems through biological activities such as the biosyntheses of polymers, compatible solutes and plant growth-promoting hormones as well as the activation of phytohormone-degrading enzymes (Spaepen et al., 2007; D'Ippolito et al., 2011; Qurashi and Sabri, 2011; 2012; Ruppel et al., 2013; Ahemad and Kibret, 2014; Glick, 2014; Oteino et al., 2015; Shrivastava

and Kumar, 2015).

Five decades ago, a pioneer study on soil bacteria and actinomycetes in the desert of Kuwait gave bases for further investigations in the field of soil microbiology in the Arabian Gulf States (Hashem and Al-Gounaim, 1973). In other studies in the area, *Streptomyces* was discovered in the Qatari desert soil (Al-Thani, 2007). Biodiversity and the roles of microorganisms in desert ecosystems is continuously investigated, and new species have been isolated from the desert and salt marshes (Al- Zarban et al., 2002a, b).

This study aimed to investigate the microbial communities close to halophytic plants such as *Suaeda*, *Limonium* and *Tetraena* and assess the possible functions of these microorganisms in the ecosystem of the Sabkhas of Qatar. Thus, the ultimate goal of successfully restoring habitats and attaining their sustainability can be achieved if extremophiles such as thermo-halophilic bacteria and cyanobacteria close to or associated with native plants and their roles in the Sabkhas ecosystem is determined.

MATERIALS AND METHODS

Description of the study sites and climatic data

This study was conducted in the Sabkhas area (Figure 1) in northeast Qatar (Figure 2) at 25°38'13.59" N latitude and 51°30'02.31" E longitude. The Qatari climate is arid or semi-arid,

Figure 2. Map of Qatar.

the annual mean air temperature is 27°C, and the average annual free water evaporation ranges from 1750 to 2150 mm. The average minimum temperature of 22°C is attained in January, and the average maximum of 47°C is reached in July. Thus, the area has a desert climate with cool winters and hot dry summers. The average annual precipitation is 81 mm, and the rain falls normally during the winter and spring months (November to April).

Sampling

The site is dominated by desert shrubs, but only three native halophytes were chosen for this study: *Suaeda virmiculata*, *Limonium axillare* and *Tetraena qatarense* (previously known as *Zygophyllum qatarense*). All grow in the same ecosystem but differ in their ecophysiological adaptations. Soil samples were collected using a soil auger 5 cm in diameter at random depths between 0 and 10 cm under selected individual plants on 30th April 2016 (representing the end of the wet season and the beginning of the dry season). Ten individual plants from each species were chosen, and soil samples were collected 5 m apart adjacent to these plant species. Controls were obtained from the open spaces between the plants. Each soil sample was placed in a well-sealed polyethylene bag and then transported to the laboratory. Soil type, microbiological and physicochemical analyses were conducted after removing stones, roots and organic debris, and sieving soil samples through a 2-mm mesh.

Soil analysis

Each sample was subjected to three analyses.

Soil moisture (%SM)

Soil moisture content was determined by drying 10 g of the sample at 105°C for 48 h and re-weighing, then calculating gravimetric soil moisture.

Soil salinity (EC)

Salinity was measured in a suspension of soil in distilled water (1:10) using an autoranging EC/temperature meter. Expressed as dSm^{-1}, soil salinity was evaluated according to the method described by Landon (1991).

Soil pH

Soil pH was measured using a glass electrode inserted into a 1:2 (w/w) soil: water mixture.

Table 1. Soil properties around the roots of the studied plants (mean ± standard deviation and range across 10 samples).

Parameter	*Suaeda virmiculata*	*Limonium axillare*	*Tetraena qatarense*	Control
Soil water content (%)	18.17 ± 2.22	21.50 ± 1.06	17.27 ± 4.63	20.67 ± 2.80
	(15.11 - 22.89)	(19.76 - 22.89)	(8.88 - 22.09)	(16.47 - 25.30)
pH of soil suspension	8.44 ± 0.11	8.37 ± 0.21	8.56 ± 0.10	8.53 ± 0.08
	(8.21 - 8.58)	(8.07 - 8.72)	(8.41 - 8.70)	(8.42 - 8.66)
EC of soil suspension (dSm^{-1})	14.02 ± 2.45	17.37 ± 1.51	16.51 ± 3.45	16.67 ± 1.62
	(10.62 - 18.50)	(15.33 - 19.62)	(8.83 - 19.83)	(14.74 - 18.50

Microbiological analysis

The procedure used was described by Bassiri (2014). A plate count method was used to estimate the population of heterotrophic bacteria, in colony-forming units (CFUs). Nutrient agar (NA) and nutrient agar plus 2 M NaCl (2MNA) were prepared and separately inoculated with 200 µL of a 10-fold soil dilution (using sterilized distilled water). After incubation at 35 and 60°C for 7 and 3 days, respectively, bacterial populations were determined for each plate. All cultures were performed in duplicate.

Cyanobacteria were examined using a light microscope; after transferring soils from Sabkhas, sterilized distilled water was added and the mixture was exposed to 3-4 weeks of natural light and temperature conditions in the laboratory.

RESULTS AND DISCUSSION

Ecophysiology of halophytes at Sabkhas

The soils of the Sabkhas in Qatar are mostly sandy loam with rock and gypsum, and they are dry during the summer and highly saline throughout the year. An evaluation of soil moisture, pH and salinity (Table 1), revealed the soils to be alkaline (pH 8.5), dry (absolute water content of less than 22%), and highly saline (up to 17.5 dSm^{-1}) under natural environmental conditions, which promote halophytes like *S. virmiculata*, *L. axillare, T. qatarense* and possibly others to grow and thrive in such habitats. However, EC measurements can differ depending on the methods used. For example, the EC of a saturated soil extract (ECe) exceeds 200 dSm^{-1} among Sabkha soils if the method described by Richards (1954) is used. In fact, adopting one particular method relies on the estimated moisture content in the soil, but the moisture content of soils from the Sabkhas in Qatar vary between summer and winter (Al-Busaidi et al., 2006).

The taxonomic descriptions and habitats of the studied plants have been well-documented (Abdel-Bari, 2012). *Suaeda* spp. are represented in the local flora of Qatar by two species: *Suaeda aegyptiaca* and *S. vermiculata*. The latter is common among coastline vegetation by the edges of mangrove forests between the tidal zone and higher grounds. Specifically, it is widespread along the eastern coastline of the Qatar peninsula. *Limonium* spp. are represented by a single species, *L. axillare*, in the

Qatari flora. This species is widespread on the coastline of the Sabkhas and is usually associated with *Halopeplis*. It is common in central Qatar and Doha and appears after the seasonal rains. *T. qatarense* (syn. *Zygophyllum qatarense*) is widespread; it is the most common plant in Qatar and occurs in all types of habitats, both the rocky and sandy soils at the coastline and inland, forming large communities. It can grow in saline soils and in the Sabkhas. The primary features of the studied plants are shown in Table 2. All are succulents found in moist, saline and dry habitats; however, only *L. axillare* has salt glands, and it has lower water content than the other two, which have no salt glands. It seems that *S. vermiculata* and *T. qatarense* have more succulent habits of diluting the extra salts absorbed from the environment, while *L. axillare* extrudes extra salt using salt glands (Yasseen and Abu-Al-Basal, 2008).

In fact, the physiological and biochemical characteristics of the halophytes examined in this study were not measured; however, the data reported by Abdel-Bari et al. (2007) and many others (Yasseen and Al-Thani, 2007; Yasseen and Abu-Al-Basal, 2008; Yasseen and Al-Thani, 2013; Yasseen, 2016) showed that these plants accumulate substantial amounts of Na^{+} and Cl^{-} to achieve a water balance between the plants and their environment by lowering the solute and water potentials of their tissues (Flowers and Yeo, 1986; Yasseen and Abu-Al-Basal, 2008).

In fact, these plants absorb ions that are abundant in their growth media, and the range of these concentrations exceeds the range set by Chapman and Pratt (1961) for normal plant growth. These ions at such high concentrations could inhibit or impair protein transporters, leading to nutrient imbalances (low K^{+} and Mg^{2+}); however, the acceptable Ca^{2+} concentrations found in the shoot systems in these plants could support resistance mechanisms by providing various functions (Nilsen and Orcutt, 1996; Orcutt and Nilsen, 2000; Mengel et al., 2001) such as maintaining internal membrane structures, regulating ion homeostasis for stomatal control, activating enzymes such as ATPase and other proteins (Yasseen and Abu-Al-Basal, 2008). Other physiological and biochemical variables were negatively affected under these soil and environmental

Table 2. Primary features of the studied plants and thermo-halophilic bacterial (THB) counts of bacteria found adjacent to three halophytic plants.

Variable	Plant species		
	S. vermiculata	*L. axillare*	*T. qatarense*
Family	Chenopodiaceae	Plumbaginaceae	Zygophyllaceae
Habitat	Moist saline soils, Sabkhas	Coastline and Sabkhas	All types of habitats, dry-saline soils
Life form	Undershrub	Undershrub	Undershrub
Halophyte/Xerophyte	Halophyte / Xerophyte	Halophyte	Xerophyte
Succulence	Shoots and leaves	Large fleshy leaves	Leaves and petioles
Plant water content* (%)	77 - 88	64 - 72	80 - 88
Salt glands	No salt glands	Salt glands on both leaf surfaces	No salt glands
THB (cfu-g^{-1} x 10^2)**	0.4	7.2	0.65

*The range among ten individuals of each species is given. **THB (cfu-g^{-1} x 10^2) for the control was 3.3.

conditions, which included photosynthetic pigments, total soluble sugars and nitrogen as compared to glycophytes and crop plants grown under normal soil conditions (Alhadi et al., 1999; Abdel-Bari et al., 2007). Moreover, the studied plants showed various abilities to accumulate compatible organic solutes, such as proline, to provide various physiological and biochemical functions (Yasseen, 2016).

Microbiology and possible roles

The arid salty soils of the study location contain a considerable number and variety of bacterial communities, but little is known about microbial functionalities in Qatari soils. Investigations of various groups of bacterial extremophiles were performed using NA at 35°C, 2 M NaCl (2MNA) at 35°C, NA at 60°C and 2 M NaCl (2MNA) at 60° to isolate heterotrophic, halophilic, thermophilic and thermo-halophilic bacteria, respectively. Incubation clearly showed that the bacterial populations of heterotrophic (2.5×10^4 cfu-g^{-1} dry soil), halophilic (1.5×10^4 cfu-g^{-1} dry soil) and thermophilic (3×10^2 cfu-g^{-1} dry soil) bacteria did not differ between the samples taken adjacent to the halophytes. Normally, such outcomes can be attributed to the use of a NA medium that is not selective; therefore, the types and numbers of colonies were nearly the same. However, when selective media were used, such as 2MNA at 60°C, selective for extremophiles, populations of thermo-halophilic bacteria were highest in soils adjacent to *L. axillare* and lowest in soils adjacent to *S. virmiculata* (Table 2).

The bacterial cultures shown in Figures 3 and 4 indicate that colonies of most strains were 1 to 2 mm in diameter, circular, smooth or mucoid, slightly raised and non-pigmented. However, some pigmented colonies were found in halophilic cultures incubated at 35°C. Selected isolated colonies were characterized by Gram stain, shape and morphology, and were observed using a light microscope. To obtain a diverse assortment of isolates,

Figure 3. Culture of halophilic bacteria grown in nutrient agar in 2 M NaCl at 35°C.

Figure 4. Thermophilic bacteria cultured using nutrient agar and incubated at 60°C. The white colonies are thermophilic *Streptomyces,* which predominate in desert soils.

Figure 5. Gram positive bacilli, non-spore-forming long bacteria isolated from nutrient agar in 2 M NaCl after incubation at 60°C for 2 days. Magnification 1000x (scale, 10 μm).
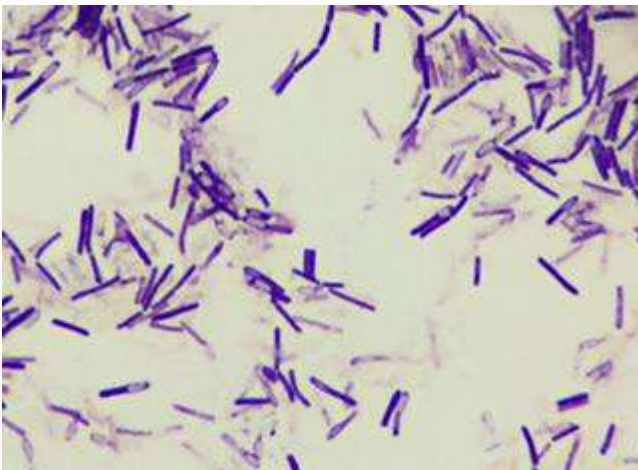
Figure 6. Gram-positive spore-forming single bacilli: thermophilic bacteria incubated in nutrient agar at 60°C for 2 days. Magnification 1000x (scale, 10 μm).

Figure 7. Cyanobacteria grew after soils were transferred to the laboratory, the mixture of soil and sterilized distilled water was left under natural light and temperature for 3 to 4 weeks. Some produced a biofilm on the inner surface of the glass bottle, producing bubbles of oxygen.

colonies that displayed different morphologies such as off-white, orange-red or cream colors, non-circular colony shapes, and mucoid textures were chosen. Microscopic examinations revealed that cells of isolated strains were Gram-positive rods with pointed ends that occurred singly, in pairs, or in short chains. In fact, most isolates belonged to the genus *Bacillus;* they were rod-shaped, Gram-positive, non-spore-forming long bacteria (Figure 5) isolated from 2MNA incubated at 60°C for 2 days. Some were spore-forming (Figure 6), and almost all strains formed endospores after growth on NA at 60°C. Spores were ellipsoidal or sometimes spherical (0.7 to 1.5 pm in diameter) and were located at a central to subterminal position. The frequency of spore-forming

cells was higher among thermophilic than halophilic cells. Thermo-halophilic bacteria (as extremophiles) are those living organisms that are boosted and can survive in the extreme environmental conditions normally found in the Arabian Gulf region.

The severe environmental conditions of the desert in the Arabian Gulf regions have not only negatively impacted and imposed a great deal of constraints on the microbial community, they differentiate the types of microbial species. Some Gram-positive bacilli can form endospores to endure the severe periods until more favorable conditions allow them to resume growth. Cyanobacteria grew in these Sabkhas when soils were transferred to the laboratory and settled under natural environmental conditions, and some produced a biofilm on the inner surface of the glass bottle, producing bubbles of oxygen (Figure 7). These cyanobacteria were examined and confirmed to be *Anabaena* and *Nostoc* (Figure 8).

Interestingly, thermo-halophilic bacteria counts were high adjacent to the halophyte *L. axillare* as compared to the others, bearing in mind that this plant is well adapted to saline environments and has salt glands to extrude extra salts (Yasseen and Abu-Al-Basal, 2008). This plant is predominant at the coastal line and Sabkhas, while *S. vermiculata* and *T. qatarense* can be found in other habitats of inland areas in addition to the Sabkhas (Abdel Bari et al., 2007). Such outcomes might reflect the roles that these bacteria play in the life of halophytes at harsh environments of the Arabian Gulf region. More
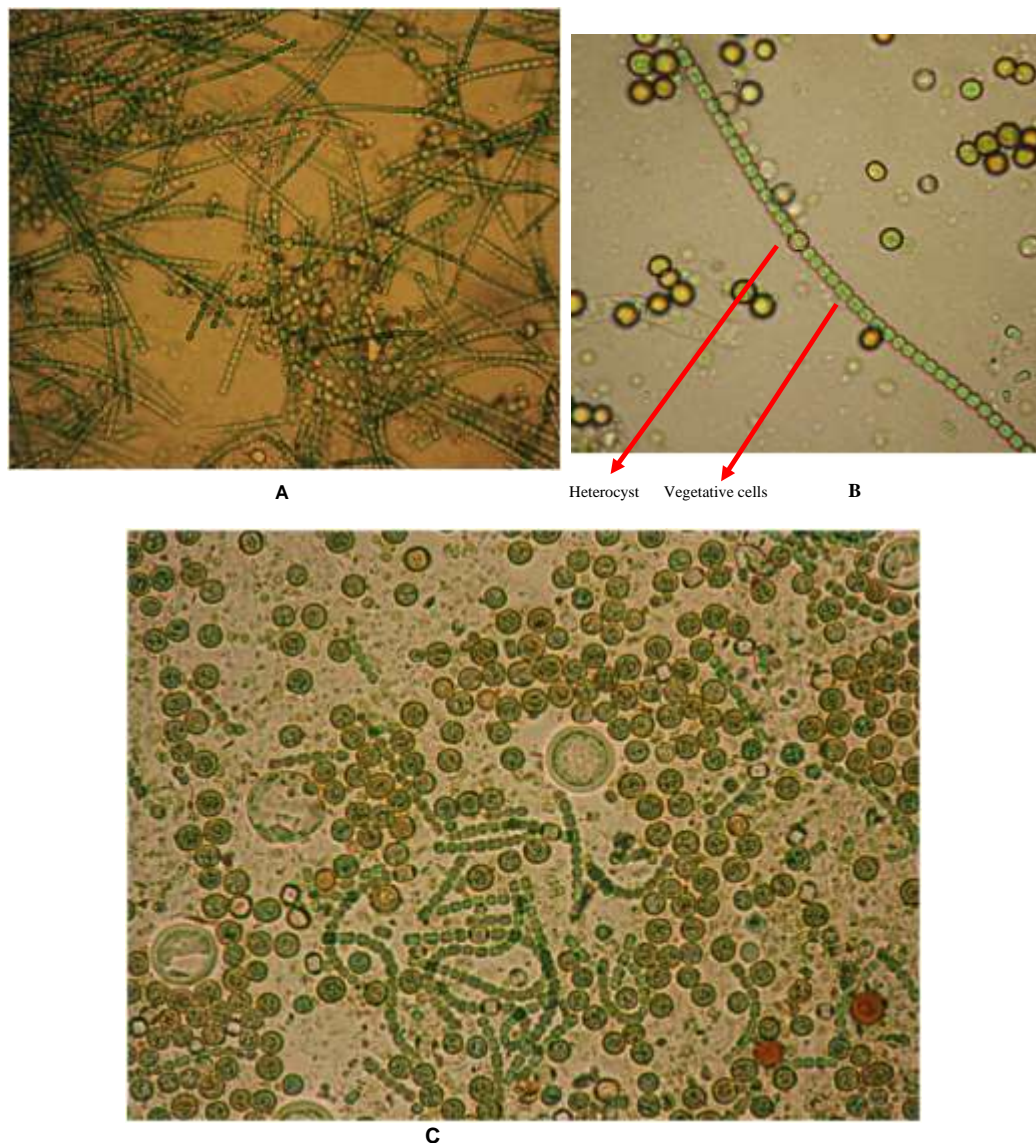
Figure 8. *Anabaena* and *Nostoc* genera are filamentous with heterocysts and akinetes. These were the most common cyanobacteria found after Sabkha soil incubation under natural conditions. Magnification 10x (A); 400x (B and C).

investigation is needed to look at the ecological aspects of these bacteria and the type of relationships with these halophytes. Microorganisms isolated from the selective NA media used with these soil samples belonged to the *Bacillus* genus. *Bacillus thuringenses* is reportedly the most common bacilli isolated from Qatari soils, followed by *Bacillus cereus* (Umlai et al., 2016). Microorganisms in soil adjacent to or associated with halophytes play many possible important roles supporting and promoting their growth through various mechanisms and methods (Yuan et al., 2016; Al-Thani and Yasseen, 2017). These roles have been discussed widely across the world as a result of the extreme desert environment, but microbial flora's influential roles in governing key life bioprocesses of

surface and subsurface soils is very interesting, which cover various aspects of economic, agriculture and health sectors. In fact, during the last two decades, a huge number of articles and reports of experimental work have suggested many possible mechanisms and methods adopted by microorganisms in alleviating the harsh abiotic stresses facing plants in general and crops in particular (Al-Thani and Yasseen, 2017). These mechanisms include: (a) establishing a biofilm (Qurashi and Sabri, 2012), (b) producing polymers (exopolysacharides) (Qurashi and Sabri, 2011), (c) chemotaxis (D'Ippolito et al., 2011), (d) accumulating endogenous osmolytes such as compatible organic solutes (Fernandez-Aunión et al., 2010; Shrivastava and

Kumar, 2015; Vílchez et al., 2016), (e) producing phytohormones and the enzymes degrading them (Spaepen et al., 2007; Kazan, 2013; Glick, 2014), (f) fixing nitrogen (Ahemad and Kibret, 2014), (g) solubilizing phosphate (Oteino et al., 2015), (h) secreting various regulatory chemicals around the rhizosphere (Ahemad and Kibret, 2014), (i) removing organic (petroleum hydrocarbons) and inorganic (heavy metals) contaminants from soil and water (Yasseen, 2014), (j) producing antibiotics (Ahmed et al., 2013; Bizuye et al., 2013) and (k) increasing carbon utilization efficiency (Gougoulias et al., 2014).

Recent evidence suggested that all the above mechanisms could be utilized by these microorganisms to support and help native plants in their adaptation to severe environments, allowing them to resist abiotic stresses and promote the growth and productivity of adjacent plants through many methods (Hanin et al., 2016). For example, some bacterial strains can form a biofilm as a strategy to improve growth in crops such as wheat and chickpeas under severe environmental conditions (Afrasayab et al., 2010; Qurashi and Sabri, 2012). Moreover, Kasim et al. (2016) concluded that *Bacillus* bacteria (such as *Bacillus amyloliquifaciens*) could provide a biofilm to alleviate salt stress around the root systems of crops such as barley. Other methods might be at work in such a system such as the production of polymers (exopolysacharides) adopted by some bacteria to protect native plants against various types of stresses such as salinity, drought and extreme temperatures (Nicolson et al., 2000; Qurashi and Sabri, 2011; 2012; Aanniz et al., 2015; Sardaria et al., 2017).

Additionally, cyanobacteria found in Sabkhan soils produce heterocysts, which play significant roles in nitrogen fixation during nitrogen shortages in such environments (Redfield et al., 2002), especially those in Qatar (Ashore, 1991), to support halophytes coping with saline environments (Yasseen and Abu-Al-Basal, 2008). Akinetes, on the other hand, are produced by certain filamentous cyanobacteria to survive the harsh environments caused by high salinity, high temperatures and hard desiccation (Hori et al., 2003).

Conclusion

Having recognized the presence of bacilli and cyanobacteria adjacent to or associated with native plants in the Qatari peninsula, the roles of these microorganisms in the performance of these plants were concentrated on, and their aspects, including nutrition, pollution, production of antibiotics, etc were evaluated. One possible approach is to examine the origins of metabolic pathways in microorganisms and native plants and determine whether these pathways originate from native plants or microorganisms. Alternatively, some plant genes might have originated from prokaryotes. This

would require a thorough investigation at the molecular level, and horizontal gene transfer must be considered a possibility. Further identification of the molecular approach is needed for the bacterial strains found in the Sabkhas of Qatar.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Susceptibility to fungal infection: A comparison between *Capsicum annuum* and *Capsicum frutescens*

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Experiment was carried out to compare the susceptibility of *Capsicum annuum* and *Capsicum frutescens* on sale to fungal infection at Sharada and Rimi markets in Kano metropolitan area. A total of four fungal species were isolated from samples of *C. annuum* and *C. frutescens* collected from the two markets. Out of the colonies isolated, the most abundant colony was *A. niger* (84, 39.25%). This was followed by *R. stolonifer* (71, 33.17%) and *A. fumigatus* (37, 17.28). The least occurring colony was *A. flavus* (22, 10.28). The difference between the four species was statistically significant at P < 0.001. On the basis of location, more colonies were isolated from samples collected from Sharada market (126, 58.87%) than Rimi market (88, 41.12%). The results indicate that hot or sweet features have no influence on the fungal infection of the sample from the two locations. More isolates were counted on Mondays (133, 62.1%) while 81 (37.9%) were counted on Thursdays. The differences between Mondays and Thursdays exposure was statistically significant at P<0.05.

Key words: *Capsicum annuum, Capsicum frutescens,* fungi, Rimi, Sharada.

INTRODUCTION

Members of the family *Solanacea, Capsicum annuum* L. and *Capsicum frutescens* L., are species of genus *Capsicum* native to Southern North America and Northern South America (Norman, 1992; Williams et al., 1991; Alao, 2000; Jay et al., 2005). They are important as source of food as well as cash in Nigeria, particularly at the northern part of the country as in many tropical countries of the world (Alao, 2000; Yahaya, 2010; Ewekeye et al., 2013). *C. annuum* and *C. frutescens* are

vegetables of great importance in human nutrition and grows over a wide variety of soil types with rain fall between 100 to 160 mm per annuum. They are oxidizing specificity with rich source of vitamins (particularly vitamin C), poly phenols, chlorophylls, caratenoides, as well as various essential oils. David et al. (1985) and Jay et al. (2005) stated the nutritional values in ripe pepper as; protein (0.20 g/dry wt), fat (0.2 g/dry wt), carbohydrate (5.8 g/dry wt), calcium (0.7 g/dry wt), iron (5 mg/dry wt),

thiamin (0.5 mg/dry wt), riboflavin (0.15 mg/dry wt), nicotinamide (2.3 mg/dry wt), ascorbic acid negligible and vitamin A variable.

Large quantities of *C. annuum* and *C. frutescens* are produced in Nigeria however, in 1987, Opadokun observed that practically all fresh vegetables grown in Nigeria are consumed in this country and production is seasonal resulting in a glut during the season and scarcity at off seasons. However, because of their soft texture they are easily bruised or wounded as a result of harvesting, and other post-harvest handling operation such as packaging, transportation and storage (Kuku et al., 1980; FAO, 1989; Williams et al., 1991).

Traditionally, unlike other fruits and vegetables fresh *C. annuum* and *C. frutescens* have not been considered as high risk food in terms of causing food-borne diseases or illness (Williams, 1987; Mare, 1999; Bukar et al., 2009). However, report by Hayatu (2000) and Yahaya (2005) shows that *C. annuum* and *C. frutescens* are susceptible to a number of fungal disease most of which require mechanical damage or weakening of the body tissue before they can penetrate. They reported that pathogens can enter into the fruits through severed tissue and natural opening. Damaged *C. annuum* and *C. frutescens* are usually mixed with undamaged ones in the market and are sometimes washed together which predisposes them to be attack by mould thus reducing their shelf life and resulting in wastage of production (Tindal, 1992; Hayatu, 2000; Yahaya, 2005; Yahaya, 2010).

Like in other parts of the world, large quantity of perishables such as *C. annuum* and *C. frutescens* are grown in Kano state which serves as a source of food and cash to many families (Alao, 2000; Yahaya, 2005; Sani and Alao, 2006). However, the quantity and quality of *C. annuum* and *C. frutescens* is greatly reduced due to fungal infection. However, there is no accurate data to clarify between *C. annuum* and *C. frutescens* which one is more susceptible to fungal infection. Therefore, the main aim of this study was to identify the fungal species and compare their susceptibility in *C. annuum* and *C. frutescens* so as to provide baseline information which will be valuable in control.

MATERIALS AND METHODS

Study site

Rimi market

It is located at municipal local government area of Kano state, Nigeria. It is one of the busiest vegetable markets within the ancient city of Kano state. There are no vegetables grown in Kano state that are not found at Rimi market. Despite being one of the largest markets in Kano state, there are no good storage facilities in the market. Some marketers store their vegetables on the floor of the stores, while others kept their vegetables packed in baskets. Marketers hardly used chemicals on their vegetables. They however washed them either with hot water or detergents, while others sort and grade their vegetables. About 9% of vegetables used in Kano metropolis are from Rimi market.

Sharada market

It is located at Gwale local government area of Kano state, Nigeria and is one of the largest vegetable markets in Kano state however, lacks good storage facilities. Some marketers stored their vegetables on the bare floor of the stores, while others kept their vegetables packed in baskets or sacks. Marketers hardly used chemicals on their vegetables. They however washed them either with hot water or detergents, while others sort and grade their vegetables. About 20% of vegetables used in Kano metropolis are from Sharada market. The two markets share similar feature of being the busiest and popular vegetable markets within the six metropolitan local government of Kano State, with produce at affordable rate for the consumers.

Experimental procedure

The methodology used in this study was similar to the one used by Yahaya (2005) and Yahuza and Yahaya (2016). The investigations lasted for a period of four months from September, 2014 to January, 2015. The procedure is described below.

Sample collection and handling

Five samples of *C. annuum* and *C. frutescens* each were obtained twice a week directly from Sharada and Rimi markets. The samples obtained were surface sterilized by immersion in 3% (v/v) sodium hypochlorite solution for 3 min. They were rinsed in three changes of running tap water and allowed to dry. Portions (2 mm) were cut with a sterilized scalpel. Cut pieces were placed on PDA and incubated at $25.7 \pm 2°C$ for four days.

Colony count and subculture

Each week, growth of fungal organisms was monitored and the number of isolates that appeared was recorded. Each distinct species was sub cultured into fresh PDA.

Pathogenicity test

All fresh samples were separately washed in 10% (v/v) sodium hypochlorite solution and rinsed in three changes of running tap water and allowed to dry. A ruler was used to mark a 2 mm diameter circle on each sample; a sterilized needle was used to streaked fungal hyphae on marked portions. Controls were inoculated with sterile distil water. Materials were placed on the laboratory bench. Sterilized forceps were used to remove portions from the diseased areas on the fourth day and placed on freshly prepared PDA plates and incubated at $25.7 \pm 2°C$ for four days. Fungal growth that appeared was recorded. Microscopic examination was carried out, for each examination; a streak of fungal mycelium was placed on a clean glass slide. One drop of cotton blue lactophenol was added and the cover slip placed. The slide was mounted on the microscope and observed at magnification of ×10, ×40 and ×100. Morphological characteristics of fungi isolated were determined and identified using the method described by Dorothea et al. (1976) using colonial and morphological characteristics.

Statistical analysis

Data collected on fungal species was analysed using analysis of variance (ANOVA). This was achieved using statistical software

Table 1. Total number of colonies counted from *C. annuum* *C. frutescens* collected from Sharada and Rimi market.

Location	*C. annuum*	*C. frutescens*	Total	Mean	% Abundance
Sharada	55	71	126	63	58.87
Rimi	39	49	88	44	41.12
TOTAL	94	120	214	107	100

Table 2. Total number of fungal colonies isolated from *C. annum C. frutescens* collected from Sharada and Rimi market.

Colonies	*C. annuum*	*C. frutescens*	Total	Mean	% Abundance
A. niger	38	46	84	42	39.25
A. flavus	09	13	22	11	10.28
A. fumigatus	14	23	37	18.5	17.28
R. stolonifer	33	38	71	35.5	33.17
Total	94	120	214	107	100

Table 3. Total number of fungal species counted on Mondays and Thursdays from *C. annum C. frutescens* collected from Sharada and Rimi markets.

Days	Sharada	Rimi	Total	Mean	% abundance
Monday	80	53	133	66.5	62.1
Thursday	46	35	81	40.5	37.9
Total	126	88	214	107	100

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RESULTS

A total of 214 were counted during the study at Rimi and Sharada markets. More colonies were counted from Sharada market (126, 58.87%), while 88 (41.12%) were counted from Rimi market (Table 1). The higher occurring species was *A. niger* with 84 colonies (39.25%). This was followed by *R. stolonifer,* 71 colonies (33.17%), and *A. fumigatus,* 37 colonies (17.28). The least occurring colony was *A. flavus* with 22 counts (10.28). Statistically, the differences between the four species were significant (P < 0.001) (Table 2).

Variation in the species isolated from *C. annuum* and *C. frutescens* collected from Sharada and Rimi markets

More species were isolated from *C. frutescense* (120, 56.07%) than *C. annuum* (94, 43.92%). *A. niger* was the highest occurring colony in both *C. frutescens* and C. *annuum*; 46 (21.49%) and 38 counts (17.75%),

respectively. The least occurring isolates in both *C. frutescens* and *C. annuum* was *A. flavus* with species count of 13 (6.07%) and nine (4.20%), respectively (Table 2).

Variation in isolate counted on Monday and Thursday in the two locations

Higher number of isolates were counted on Mondays, with 133 counts (62.1%) while 81 (37.90%) were counted on Thursdays. The differences between Mondays and Thursdays exposure was statistically significant at P<0.05 (Table 3).

Variation in the species isolates on Monday and Thursday at Sharada and Rimi markets

The higher occurring species isolated on Monday and Thursday was *A. niger* with number of occurrence of 51 (23.03%) and 33 (15.42%) respectively while the least occurring colony isolated on both Monday and Thursday was *A. flavus* with 13 (6.07%) and nine (4.20%), respectively (Table 4).

Table 4. Fungal species isolated on Monday and Thursday from *C. annum* and *C. frutescens* collected at Sharada and Rimi markets.

Days	*A. niger*	*R. stolonifer*	*A. fumigatus*	*A. flavus*	Total	Mean	%
Monday	51	40	22	13	127	63.5	59.34
Thursday	33	31	15	09	87	43.5	40.65
Total	84	71	37	22	214	107	100

Table 5. Total number of fungal species identifies from samples collected from Sharada and Rimi market.

Colonies	Sharada	%	Rimi	%	Total	Mean	% of abundance
A. niger	54	25.23	30	14.01	84	42	39.25
A. fumigatus	20	9.34	17	7.94	37	18.5	17.28
A. flavus	10	4.67	12	5.60	22	11	10.28
R. stolonifer	42	19.62	29	13.55	71	35.5	33.17
Total	126		88		214	107	100

Variation in the isolates counted from *C. annuum* and *C. frutescens* sampled from Sharada and Rimi markets

More counts of *A. niger* (54, 25.23%) were registered from Sharada market. The least occurring isolate at Sharada was *A. flavus* with 10 counts (4.67). At Rimi market *A. niger* was the highest occurring isolate with 30 counts (14.01 %), while *A. flavus* (14.01%) was the least occurring isolate (Table 5).

DISCUSSION

Almost all the *C. annuum* and *C. frutescens* samples collected from the two markets gave positive results for the presence of fungal species and each sample was contaminated with at least one of the known species of pathogenic fungi. The isolated fungi may have contaminated the *C. annuum* and *C. frutescens* either in the field or during postharvest handling operations. During the study, a total of 214 isolates were counted and recorded at Sharada and Rimi markets; out of this more isolates were counted at Sharada market (58.87%), while 41.12% were isolated at Rimi market. From the isolate counted during the study, four fungal species were identified that is, *A. niger*, *A. fumigatus*, *A. flavus*, *R. stolonifer,* while *A. niger* (84) was the highest occurring species.

The finding of this study could be related to the result of Hayatu (2000) and Yahaya (2005) who assessed fungal deterioration of some selected vegetables in some selected irrigation site of Kano State, Nigeria and found that 70% of losses in pepper were attributed to the activities of *A. niger*, *A. fumigatus* and *R. stolonifer*. The result of this study also support the finding of Yahaya and

Fatima (2009) who reported four fungal species associated with losses of sweet oranges in the two areas of study as *A. niger* (36.94%), *A. flavus* (17.83%), *Penicillium digitatum* (20.38%), and *Mucor* (24.84%).

The high number of isolates recorded from samples collected from Sharada market may be attributed to the nutrients effluent discharge in the surrounding household and industries around the market area, which infected the environment. Such contaminants may contain some effluents which might favour fungal growth as against the lower number of isolated counted from samples collected at Rimi market where the area is free from household and industrial effluents (Kuku et al*.,* 1980; IAR, 1985; O'Neil et al*.,* 1997).

In this study it is shown that more fungal species were counted on *C. frutescens* than in *C. annuum*. The high number of isolate counted from *C. frutescens* may be an indication that it is more susceptible to fungal infection. The higher isolates counted on Monday could be due to heavy activities with high influx of customers from different locations on the day for buying and selling. Statistical difference of P >0.05 was obtained between the two markets.

It can be concluded that the four fungal species namely *A. niger, A. fumigatus*, and *A. flavus,* and *R. stolonifer* are the common post-harvest fungi associated with losses of *C. annum* and *C. frutescens* on sale at the studied markets. The results obtained in this study indicate that more fungal isolates were counted on *C. frutescens* which is an indication that *C. frutescens* is more susceptible to fungal infection than *C. annuum*. At Rimi market area there is total absence of household and industrial effluents in the area surrounding the market and this might have accounted for the least colony count. Sharada site is the least suited for marketing of *C. annuum* and *C. frutescens* and other vegetables because

effluents from household and industries in the surrounding area were the source of infection. The effluents might contain toxic chemicals that on long time exposure could pose serious health hazards to the consumers.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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