Identification of Two Honey- Sensitive Pathogens Isolated from Musk Lake, Jeddah

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Abstract: Two bacterial pathogens, *Exiguobacterium* ET and *Staphylococcus aureus* ET, have been isolated from wastewater, Musk lake, Jeddah, KSA, biochemically characterization using the API system, and molecularly identified using the partial sequencing of the 16S rRNA gene (approx. 1000 bp). The 16S rDNA nucleotides of the orange colonies showed 98% similarity to *Exiguobacterium sp*., while those of the yellow colonies exhibited 99% similarity to *S. aureus*. API system differentiated between them in 3 biochemical tests, arginine hydrolysis, urease production and utilization of raffinose. Both of them are sensitive to Amikacin (30 µg/disc) and Amoxycillin (30 µg/disc). On the other hand, their resistance and sensitivity to Nalidixic acid (30 µg/disc) and Sulfamethoxazole (50 µg/disc) are distinguishable. Diluted honey (60- 80%) was highly potential to inhibit the growth of the isolates. Neat honey (100%) reduced the optical density (OD_{550} is not less than 0.28 for both strains) of the pathogens but not as much as the diluted one (OD_{550} is not more than 0.03 upon using 70% of honey for both strains).
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1. Introduction

Wastewater offers a source of water that could drastically reduce the utilization of precious natural water resources. Sources of wastewater can include collected storm water, runoff, industrial wastewaters, domestic grey water and sewages (Bahlaoui et al., 1997). Polluted and/or untreated waters have a large health risk by causing water born disease (Anderson, 1996). Despite large advances in water and wastewater treatment, world born diseases still pose a major world-wide threat to public health. It has been reported that water-born pathogens infect around 250 million people each year resulting in 10-20 million deaths (Anderson, 1996). Many of these infections occur in developing nations which have lower levels of sanitation, problems associated with low socioeconomic conditions and less public health awareness than in more developed nations. However, the health risk for the public comes from microbial pathogens, toxic chemicals and heavy metals (Fliermans, 1996).

The contamination of foods by water containing known toxin producing *Staphylococcus aureus* and other *Staphylococcus* spp. can cause outbreaks of food poisoning, as this genus is one of the major bacterial agents causing food born disease in human worldwide (Le-Loir et al., 2003; EFSA, 2010). The genus *Exiguobacterium* is a newly described bacterium which was explained by Collins *et al*., 1983. This genus has 17 described species and more than 294 strains and new members are still adding to it. Members of this genus had firmly been identified as the genera such as *Corynebacterium, Brevibacterium* and *Staphylococcus* (Rodrigues et al., 2006) because of some similarities in peptidoglycan structure or fatty acid profile (Karami et al., 2011). *Exiguobacterium* can also be found in many sources of water bodies (Kim et al., 2005; Karami et al., 2011).

One of the most effective antimicrobial agents is honey. It has been shown to exert antimicrobial action against a broad spectrum of fungi and bacteria (Cooper et al., 2002). The reasons for this antimicrobial activity include a relatively low water activity (0.56-0.59), low pH (3.2-4.5), the production of hydrogen peroxide on dilution (as a result of the presence of the enzyme glucose oxidase) and the presence of phytochemical components including flavonoids and phenolic acids (Cooper et al., 2002). Honey has been used from ancient times as a method of accelerating wound healing (Zumla and Lulat, 1989) and has been mentioned in the Torah, Bible and Quran (Namias, 2003).

Traditionally, bacterial identification is performed by isolating the organism and studying it phenotypically to elucidate its Gram staining, culture and biochemical characteristics (Woo et al., 2001). Nowadays, PCR based techniques are commonly used for typing, as they are easy, fast and cost effective (Vázquez-Sánchez et al., 2012). Among such techniques, amplification and sequencing of the 16S rRNA gene, which had proved to be a sufficient successful tool for identification of many bacterial species including members belonging to the genus *Exiguobacterium* (Chaturvedi and Shivaji, 2006) and

Staphylococcus (Woo et al., 2001; Kajikazawa et al., 2007).

In the current study, two different bacterial isolates, *Exiguobacterium* ET and *Staphylococcus aureus* ET, have been isolated from Musk lake (Jeddah, KSA), characterized and molecularly identified using the 16S rRNA gene. Besides, the antibacterial effect of some antibiotics as well as honey against these pathogens has been tested.

2. Materials and Methods

Sampling, bacterial isolation and enumeration

Water samples were taken from Musk lake, Jeddah, KSA, directly to sterilized bottles in December, 2011. Water samples were diluted up to 10^{-6} in sterile distilled water before cultivation on nutrient agar (NA), Difco, Detroit, USA, plates. Petri-dishes then incubated for 24h at 35 °C and the number of general viable bacteria was counted.

Morphological characterization

Pure cultures were examined for colony morphology. Cell morphology was tested using Gram and Malachite green stains.

Biochemical methods

Biochemical characterization was performed using API 20E kit (Biomereux, France). 0.1 ml of overnight cultures has been used to inoculate each well, containing freeze-dried test substrate. The inoculated strips were incubated at 35°C for 24 h. For the sensitivity test, the following antibiotics were purchased from Sigma co. and their concentrations are in µg/disc: Norfloxacin (10), Amikacin (30), Amoxycillin (30), Nalidixic acid (30) and Sulfamethoxazole (50).

Source and dilution of honey

The honey (Gannah Bee Honey, Al-Farouk apiaries co., Cairo, Egypt) used in this study was diluted in sterile nutrient broth (Difco, Detroit, USA) to different concentrations, 10-80% (v/v) in order to determine the minimum inhibitory and/or the most effective concentration of the used honey. 50 µl of each bacterial culture (approximate $O.D_{550}$= 0.9) was added to each dilution. Tubes were then incubated at 35 °C for 48h (Agbaje et al., 2006). Optical densities were detected at 550 nm using Genesys 10 UV spectrophotometer, Thermo Scientific, USA. Two sets of controls were employed; one was a row of positive tubes containing only the growth medium and each of the microorganism, while the other was a negative control which consists of a row of tubes containing different concentration of honey with no organism (Agbaje et al., 2006).

DNA extraction, purification and amplification

DNA was extracted from the bacterial cultures and purified using EZNA bacterial DNA extraction kit (Omega Biotek, USA). The 16S rDNA region was partially amplified (approx 1000 bp) by polymerase chain reaction (PCR) using the forward primer (FP) 5'-AGAGTTTGATCMTGGCTCAG-3' and the reverse one (RP) 5'-TACGGYTACCTTGTTACGACTT -3'. The PCR mixture consists of 30 pmol of each primer, 100 ng of DNA, 200 µM dNTPs, 1.5 mM magnesium (Mg) Cl2, 20 mM potassium chloride (KCl), 10 mM trishydrochloric acid (HCl), pH 8.3, and 2.5 U of Taq polymerase (Pharmacia Biotech, USA). The 50 µl PCR mixture containing tube was placed in the DNA thermocycler, Gene cycler TM BIO- RAD, USA. The PCR conditions were as follows: initial denaturation of DNA at 95°C for 3 min and then 30 cycles of three-step PCR amplifications consisting of denaturation at 94°C for 1 min, primer reannealing at 55°C for 1 min and extension at 72°C for 2 min. Samples were subjected to an additional extension at 72°C for 10 min at the end of the amplification cycles (Ausubel et al., 1999). The amplicons were finally purified using QIA quick PCR purification kit (QIAgen, USA).

Gel electrophoresis

Ten µl of PCR products, mixed with loading buffer, were loaded on a 2% w/v agarose gel and electrophoresed with 1X TEA (Tris EDTA Acetate) buffer. DNA was visualized by UV transillumination after staining with ethidium bromide (0.5 µg/ml). The molecular sizes of the amplified DNA fragments were estimated using DNA molecular weight marker X (Roche Applied Science, Germany).

DNA sequencing and data analysis

The purified PCR products have been sequenced using the BigDye Terminater cycle sequencing kit (Applied Biosystems, USA) in ABI Prism 3730 sequencer (Perkin Elmer, Applied Biosystem, USA). Sequences were deposited in the GenBank and the accession numbers were obtained. After obtaining the sequences, homology search was performed against DDBJ (DNA Data Base Japan), using Blast program to find the sequences producing significant alignment with the obtained ones. Similarity percentages among the sequences were obtained using Biology WorkBench software version 3.2.

3. Results

Water samples were taken from Musk lake, Jeddah, KSA, in December, 2011. The total bacterial viable count was $8x10^6$. Orange and yellow colonies represented almost 60% of the total count. Therefore, two of these colonies were picked up as representatives for the environmental sample.

Table 1 represents the results of the morphological examination, biochemical tests performed using API system and sensitivity test using some antibiotics. Although both cultures have a lot of common characteristics, they are differing from each other with respect to colony and cell morphology.

Besides, *Staphylococcus aureus* ET (yellow colonies) is unique to hydrolyze arginine and urea. On the other hand, only *Exiguobacterium* ET (orange colonies) can utilize raffinose. The sensitivity test was also distinguishable for some extent, especially for nalidixic acid (30) and sulfamethoxazole (50). *S. aureus* ET was sensitive to nalidixic acid (30) and resistant to sulfamethoxazole (50), while *Exiguobacterium* ET was resistant to the first antibiotic and sensitive to the second one, respectively.

TABLE 1. The morphological and biochemical tests of the two bacterial isolates.

Tests	*Staphylococcus aureus* ET	*Exiguobacterium* ET
Conventional methods:		
Colony color	Yellow	Orange
Gram reaction	Positive	Positive
Cell shape	Cocci	Rods
Spore formation	None	None
Catalase production	Positive	Positive
API System:		
Utilization of:		
D-Glucose	Positive	Positive
D- Fructose	Positive	Positive
D-Mannose	Positive	Positive
Maltose	Positive	Positive
Lactose	Positive	Positive
Mannitol	Positive	Positive
Raffinose	Negative	Positive
Trehalose	Positive	Positive
Sucrose	Positive	Positive
Pyruvate	Positive	Positive
Melibiose	Negative	Negative
Xylose	Negative	Negative
Xylitol	Negative	Negative
α-Methyl-D-glucoside	Negative	Negative
N-acetyl-glucosamine	Negative	Negative
Nitrate reduction	Positive	Positive
Production of:		
Arginine hydrolase	Positive	Negative
Urease	Positive	Negative
Alkaline phosphatase	Positive	Positive
Antibiotic tolerance (µg/disc):		
Norfloxacin (10)	Resistant	Resistant
Amikacin (30)	Sensitive	Sensitive
Amoxycillin (30)	Sensitive	Sensitive
Nalidixic acid (30)	Sensitive	Resistant
Sulfamethoxazole (50)	Resistant	Sensitive

As can be shown in Table 2, honey has been proved to be an effective antibacterial agent, especially after dilution to a certain limit (60-80%). The ODs for both strains were dramatically decreased upon using diluted honey (not less than 60%). It can be said that the minimum inhibitory concentration for honey against both species is 60%, while the most effective one is 70%. Interestingly, neat honey (100%) was not so effective (ODs were decreased to 0.28 and o.32 for *S. aureus* and *Exiguobacterium*, respectively) as much as the diluted ones, 60 and 70%, where the ODs decreased to 0.05-0.03 and 0.055-0.023 for *S. aureus* and *Exiguobacterium*, respectively.

TABLE 2. Sensitivity of the two isolates against the antibacterial effect of different concentrations of honey.

Honey concentration % (v/v)	Optical Density of bacterial cultures at 550 nm	
	***S. aureus* ET**	***Exiguobacterium* ET**
0	1.2	1.13
40	0.53	0.49
50	0.35	0.24
60	0.05	0.055
70	0.03	0.023
80	0.033	0.029
100	0.28	0.32

The 16S rDNA universal primers, FP and RP, have been used to amplify and sequence around 1000 bp of the 16S rRNA genes from the newly isolated bacteria, *Staphylococcus aureus* ET and *Exiguobacterium* ET (Figure 1). The homology search of the obtained DNA sequences revealed that the yellow colonies are belonging to *Staphylococcus aureus* with a similarity percentage of 99% and the orange ones to *Exiguobacterium sp* with 98% similarity. The sequences were deposited in the GenBank and accession numbers are given in Table 3.

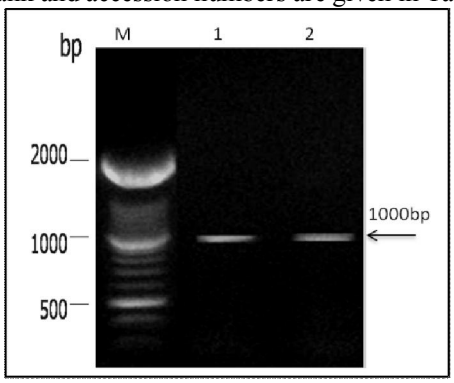

Figure 1. Gel electrophoresis of approx. 1000 bp of the 16S rRNA gene. M, DNA molecular weight marker X; 1, *Staphylococcus aureus* ET; and 2, *Exiguobacterium* ET.

TABLE 3. Accession numbers of the newly isolated bacteria.

Bacteria	Accession numbers
Staphylococcus aureus ET	JX163859
Exiguobacterium ET	JX163860

Discussion

As expected from a highly polluted wastewater lake, high bacterial viable count ($8x10^6$ CFU/ml) was found in Musk lake, Jeddah, KSA, in December, 2011. However, two unique colonies, orange and yellow, were picked up as representatives for the taken environmental sample. The orange one is for the genus *Exiguobacterium* which was firstly found by Collins et al., 1983 to accommodate Gram positive and non-spore forming rods. This genus was found before by some researchers in polluted water bodies (Kim et al., 2005; Karami et al., 2011). Besides, genus *Staphylococcus* (yellow colonies) forms Gram –positive cocci that posses catalase- positive activity (Woo et al., 2001). Pathogens belonging to this genus have been recently isolated from fishery products (Vazquez-Sanchez et al., 2012), polluted water (Motsepe and Warwick, 2000) and sea water (Goodwin and Pobuda, 2009). Moreover, some studies have shown that *Staphylococcus aureus* count can be greater than that of coliforms and in some cases *S. aureus* is present in the absence of coliforms (Yoshe-Purer and Golderman, 1984). This finding is in parallel to the current results where the orange and yellow colonies were predominant in the total bacterial count. This suggests that staphylococci may be a suitable indicator in evaluating water quality (Yoshe-Purer and Golderman, 1984).

Although API system was successful to characterize the two species under test (*Exiguobacterium sp.* and *S. aureus*) giving results similar to great extent to those of Woo et al., 2001 and Kim et al., 2005, it was not so effective in differentiation between them. Only 3 tests were discriminative, arginine hydrolysis, urease production and raffinose utilization. This reflects the need to use more than one tool for discrimination among the environmental isolates. Sensitivity test using 5 different antibiotics showed that the infections by the pathogens under test can be healed using Amikacin (30) and Amoxycillin (30). However, differentiation between the two species is obvious using Nalidixic acid (30) and Sulfamethoxazole (50).

Honey has been shown convincingly to have a potent antibacterial activity. The holy Quran intimates honey as the healing for diseases. It occupied a prominent place in traditional medicine and its antimicrobial effect has been reported by a number of workers (Armon, 1981; Haffeejee and Mosa, 1985; Reynold, 1989; Hamid and Saeed, 1991). Interestingly, dilutions from 60 to 80% of honey inhibited the growth of the 2 pathogens to a very great extent (ODs were decreased to 0.03 and 0.029 for *Staphylococcus aureus* ET and *Exiguobacterium* ET, respectively). On the

other hand, undiluted honey showed less activity against both pathogens (ODs were not less than 0.28) despite its high osmotic pressure. This is may be due to hydrogen peroxide produced by the enzymatic activity of glucose oxidase in honey when diluted (Cooper et al., 1999). The current results of the diluted honey are in parallel with cooper et al., 1999 studies in Manuka and Pasteur honeys. They have found that the antibacterial activity of honey doesn't depend wholly on its high osmolarity (due to supersaturation with carbohydrates). It also depends on other factors such as the release of hydrogen peroxide, the presence of inhibine (Obaseiki-Ebror et al., 1983), an antibacterial substance in honey which can be reduced by sunlight, and acidity (Hamid and Saeed, 1991), pH 3.2-4.5. Therefore, in this research the minimum inhibitory concentration of honey is 60%, but the most effective ones are 70 and 80%. These results confirm the effectiveness of the diluted honey against Gram positive bacteria especially the toxic pathogen, *Staphylococcus aureus*, as indicated before by Cooper et al., 1999 and Agbaje et al., 2006.

Small rRNA gene sequencing, particularly the 16S rRNA sequencing in bacteria has led to advances on multiple fronts in microbiology. First, construction of phylogenetic tree for classification (Olsen et al., 1992; Olsen and Woese, 1993; Thompson et al., 1994). Second, it revolutionizes the classification of microorganisms and makes the classification of non cultivable microorganisms possible (Relman et al., 1990; Relman et al., 1992). Third, it helps to elucidate the relation of unknown bacterial species to known ones (Woo et al., 2001). In the current study, partial sequencing of the 16S rRNA gene (approx. 1000 bp) has successfully identified the two different Gram positive isolates, *Exiguobacterium* ET (98% similsrity to *Exiguobacterium sp*. in the database) and *Staphylococcus aureus* ET (99% similarity to *S. aureus* in the database). The use of that gene makes the turnaround time short. Besides, the 16S rRNA gene hypervariant region of the 5′ end, which has been sequenced in the current study, has been proved to be an efficient molecular chronometer which can be used effectively to distinguish between closely related species (Goto et al., 2002; Mohamed and ElSersy, 2009; ElSersy and Mohamed, 2011). However, many species belonging to S. *aureus* and other staphylococci have been successfully identified in previous studies using that gene (Golledge and Gordon, 1989; Olsen et al., 1992; Woo et al., 2001). Besides, Karami et al., 2011 have stated that sequencing of the 16S rRNA gene is a powerful tool for identification of many *Exiguobacterium* spp.

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