Microbial quality of bottled water and their molecular characterization in Jeddah, Saudi Arabia

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Abstract: The microbiological quality of fifty one bottled water samples representing 17 domestic bottled water brands and three different water desalination plants from Jeddah Saudi Arabia, were analyzed. Results of microbial analysis revealed that none of the 51 samples were found to contain fungal growth, 9 (17.6%) of samples of bottled water (3 brands) and one from three of water desalination plants were contaminated with 6 different species of bacteria. Bacterial identification was conducted. Results showed that 5.88% (3/51 bottles) and 3.9 % (2/51 bottles) of the bottled water from retail stores and processing plants were contaminated by *Staphylococcus aureus* and *Pseudomonas aerugenosa* respectively and 1.96% (1/51 bottles) were contaminated by either *Pseudomonas flourescens*, *Anthracoid*, *Micrococcus*, Gram negative bacilli non-fermenter spp. or *Stenotrophomonas maltophilia*. In this study genetic fingerprinting of water-borne isolates ***S. aureus*****,** Gram negative bacilli non-fermenter spp., *P. aeruginosa* **and** *P. flourescenses* isolated from different bottled water samples, were studied using RAPD markers. Such information will be useful in their classification, ecology and diagnosis. The number of bands obtained by PCR reactions may serve in future studies for comparison purposes.
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Key wards: RAPD-PCR fingerprinting, *Staphylococcusaureus, Stenotrophomonasmaltophilia,* bottled water.

1. Introduction

The water source may be springs, municipal systems or other sources that are considered to be safe of sanitary quality and fit for human consumption. Bottled water is not necessarily safer than tap water. (**Warburton, 2000**) reported that bottled and municipal water may contain the same microorganisms since both can originate from the same sources. (**Warburton, 2000**) reported that under improper and/or prolonged storage of bottled water, bacteria can grow to levels that may be harmful to human health. Water from springs, wells, boreholes, municipal supplies, bottled water and other sources are known vehicles for enteric pathogens such as bacteria, parasites and viruses (**Manaia *et al.*, 1990**).The presence of these micro-organisms can have an impact on the health of travelers, immuno-compromised persons and infants if bottled water is used for formula preparations. Bottled water has been implicated as the source of outbreaks of cholera, typhoid fever as well as traveler's disease in countries such as Portugal and Spain (**Mavridou, 1992; Warburton *et al.*, 1992**). Norovirus (previously known as Norwalk-like viruses) sequences were detected in three European brands of mineral water (**Beuret *et al.*, 2002**).

The presence of *P. aeruginosa* is unacceptable because this species is an opportunistic pathogen, has been implicated in foodborne and waterborne diseases, and is now considered as a primary infectious agent (**Warburton, 1993, Elaichouni *et al.*, 1994, Römling *et al.*, 1994**). This organism is also capable of multiplying abundantly in low-nutrient water (**Gonza´*et al.*, 1987**, **Moreira *et al.*, 1994**) and can, therefore, colonize bottled waters. *P. aeruginosa* has been suggested as a surrogate indicator for the presence of other opportunistic pathogens (**Geldreich,** 1992). In nature, *S. maltophilia* exists in a wide variety of environments, being found in water, soil and plant material (**Denton & Kerr, 1998; Ryan** *et al.*, **2009**). *S. maltophilia* also occurs in man-made water systems, and most problematic is its presence in devices and reagents in hospital settings (**Denton & Kerr, 1998; Looney *et al.*, 2009**). Increasingly isolated by clinical microbiology laboratories, *S. maltophilia* is associated with a spectrum of diseases, including infections of the lung, blood, heart, urinary tract, eyes, CNS, skin and soft tissue (**Falagas *et al.*, 2009; Looney *et al.*, 2009**). *Stenotrophomonas* (formerly *Xanthomonas*), a gram negative bacillus is noted for its high degree of antibiotic resistance and pathogenic potential. It can be isolated from water sources including rivers, wells, a hypertrophic lake, bottled water, sewage, sink traps, factory residues and a variety of soil, plant and food materials. (**Connie and Manuselis, 1995; Denton and Kerr, 1998)**.

In the Kingdom of Saudi Arabia, the quality of drinking water had received some attention (**Saudi Arabian Standards Organization (SASO), 1984; Hashim, 1990; Garawi and Al-hendi, 1993; Gulf Cooperation Council Standard (G.C.C.S.), 1993; Abdel Magid, 1997; Moghazi and Al-Shoshan, 1999**). SASO (1984) developed drinking water standards for both bottled and unbottled (municipal) water to define a quality of water that is safe and acceptable to the consumer. These standards set limits for the permissible and the maximum contaminant level of chemical elements and indicator organisms that endanger the health of consumers. A substantial number of these standards are based on the World Health Organization (**WHO, 1971**) international guidelines for drinking water. Random amplified polymorphic DNA (RAPD) markers, which are based on the amplification of discrete DNA fragments in the genome by the use of oligonucleotide primers with random sequences, have been largely used to identify physiological races of fungi **(Guthrie *et al*., 1992)**. RAPD-PCR method, when compared with biochemical methods is cheap, simple, more sensitive and faster. Little is known concerning the genetic diversity that exists in bacterial populations in bottled water in Saudi Arabia. In this study genetic fingerprinting of the water-borne isolates *S. aureus*, Gram negative bacilli non-fermenter spp., *P. aeruginosa* and *P. flourescenses* isolated from different bottled water samples in Jeddah was evaluated using RAPD markers. Such information will be useful in their classification, ecology and diagnosis. The aim of this study is screening and RAPD fingerprinting of microbial pathogens isolated from bottled water in Jeddah, Saudi Arabia.

2.Materials and methods

Water samples, Media and Culture Conditions:

Twenty domestic bottled water brands in Jeddah; Saudi Arabia, were analyzed for microbial contamination. Seventeen different commercial bottled water (non-carbonated) products were randomly selected from various retail outlets. Bottled water of the same batch was bought (3 x 1.5 l or 3 x 19 l) of each type. Nine samples obtained from three different water desalination plants were also analyzed. Samples from different water desalination plants were stored at room temperature (25-30°C) for one day and analysis was conducted. All brand names has been replaced with code names and randomly chosen number to avoid any commercial consequences. Domestic bottled water brands were coded as follow 13 A, B, C. For bacterial identification, one ml sample was inoculated in Blood Agar Plate (BAP), another one ml was inoculated in chocolate agar. All samples were incubated at 37°C. Bacterial identification was conducted using the API system of bacterial identification. Coliforms were identified with conventional biochemical tests (**Forbes *et al*., 1998**). The membrane filter technique was employed for fungi (**Clesceri *et al.*, 1998**). A volume of 100 ml of the samples was filtered through membrane filters with 0.45 µm pores (Millipore, Massachusettes, USA). The membranes were placed on Sabouraud Dextrose Agar (SDA, Difco, Maryland, USA) supplemented with streptomycin (50 µg/ml). The plates were incubated at room conditions of temperature 20 to 24 ºC and examined daily during one week.

Isolation of total DNA from isolated strains:

Total DNA was isolated according to **Sambrook *et al.*, (1989)**. The quantity and purity of the obtained DNA were determined using UV-absorbance at 260 and 280 nm using spectrophotometer (Shimadzu UV-VIS model UV-240).

RAPD analysis:

PCR-GOLD Master-Mix Beads (BIORON, Germany, Cat. No. 10020-96) were used for PCR technique. Each bead contains all of the necessary reagents, except primer and DNA template, for performing 25 µl PCR amplification reactions. Two different primers were used in the present study. The first primer (B-08) sequence was 5'-GTC CAC ACG G-3' and the second primer (B-11) sequence was 5'-GTA GAC CCG T-3'. All primers were supplied by Operon Technologies Company, Netherlands. To each Ready To Go PCR bead, 12 ng of the used random primer and 40 ng of the purified DNA sample were added. The total volume of the amplification reaction was completed to 25 µl using sterile distilled water. The amplification protocol was carried out as follows: Denaturation at 95^{o}C for five min; thirty-five cycles each consists of the following segments: Denaturation at 95^{o}C for one min; primer annealing at 32^{o}C for two min. according to GC ratio of each primer, and incubation at 72^{o}C for two min. for DNA polymerization. At the end, hold the PCR at 4^{o}C till analysis. The amplified DNA products from RAPD analysis were electrophoreses on 1% agarose gel and 1 X TBE buffer at consistent 100 volt for about 2 hrs. The different band sizes were determined against 100 bp ladder and the separated bands were stained with 0.5 µg/ml ethidium bromide and photographed using both Polaroid Instant Camera and UV transeleminator.

3. Results And Discussion

Microbiological analysis

Twenty domestic bottled water brands in Jeddah; Saudi Arabia were analyzed for microbial contamination. Seventeen different commercial bottled water (non-carbonated) products were randomly selected from various retail outlets. Bottled water of the same batch was bought (3 x 1.5 l or 3 x 19 l) from each type. The microbiological quality of fifty one bottled water samples representing 17 domestic bottled water brands, and three of water desalination plants from Jeddah Saudi Arabia, were determined. Results revealed that one water desalination plants (2 samples) from three different water desalination plants were contaminated with 2 different species of bacteria (2/9 bottles) (22.22 %) and none were contaminated with fungal growth (Table 1 and Table 1) .Results of microbial analysis are (showed in Table 1) revealed that none of the 51 samples was found to contain fungal growth and (9/51 bottles) 17.6 % of them were contaminated with 6 different species of bacteria (Table 2).

Table 1: Microbiological analysis of domestic bottled water brands and water desalination plants in Jeddah, Saudi Arabia.

Microorganism type	Number of positive samples (percentage)	
	Bottled mineral water (n=51)	Water desalination plants (n=9)
Fungi	0 (0.0%)	0 (0.0%)
Bacterial contaminant	9 (17.6%)	2 (22.22%)

Table 2: Isolated bacterial strainsand their distribution in bottled mineral water

Identified bacterial strains	Contaminated samples	
	Number	%
Staphylococcus aureus	3(i.e.,13A, 14A and water desalination |plant 46 B)	5.88
Pseudomonas aeruginosa	2 (i.e., 13 C and 15 A)	3.9
Pseudomonas flourescens	1 (i.e.,18 A)	1.96
Antharcoid	1 (i.e.,14B)	1.96
Gram negative bacilli non-fermenter spp.	1 (i.e., 13B)	1.96
Stenotrophomonasmaltophilia	1(i.e., water desalination plant 46 A)	1.96

Isolation and identification of bacterial strains

The isolated strains was phenotypically and biochemically characterized using standard techniques (Gram staining, colony shape, size and color on nutrient agar plate, catalase and oxidase tests, etc.), according to Bergey's Manual of Determinative Bacteriology (**Holt *et al.*, 1994**). Additional biochemical and physiological characteristics were determined using the API 20NE and API 20E system (BioMerieux, Lyon, France). Results in showed that 5.88% and 3.9 % of the bottled water from retail stores and processing plants were contaminated by *Staphylococcus aureus* and *Pseudomonas aeruginosa* respectively and 1.96% were contaminated by either of *Pseudomonas flourescens*, *Anthracoid*, *Micrococcus*, Gram negative bacilli non-fermenter spp. or *Stenotrophomonas maltophilia* (Table 2).

In the present study, *S. aureus* was detected in 5.88% (3/51 bottles) only of the bottled water from retail stores and processing plants. Higher detection rate of *S. aureus* was reported by **EL-Batouti (2002)** (10.7%). This suggests that the bottled water may be subjected to contamination, not only from the containers, but also from the physical surroundings and the people who come in contact with any part of the bottling operation (United States Environmental Protection Agency, 2008). However, **Selka (1988)** found that only 3.3% of the bottled water from retail stores and processing plants contained coagulase-positive *S. aureus*. **Mavridou (1992)** in Scotland and **Abdel Karem and Hassan (2000)** in Egypt revealed similar findings. *P. aeruginosa* has been advocated as a mean of monitoring the hygienic quality of drinking water. It is used to assess the quality of bottled water as its presence suggests non-compliance with GMP (**Warburton *et al.*, 1998**). Contamination may be the result of colonization of the bottling plant equipment; as rubber seals, lining or coating, washers, and even disinfecting soap, that may all provide nutrients for this organism. It may grow in low-nutrient water such as deionized and distilled water and reach 10^4 CFU / ml in mineral water thus increasing the public health risks. The presence of *P. aeruginosa* may suppress standard coliform enumeration procedures and can degrade water color, turbidity, and taste **(Murray *et al.*, 1999)**. The present study revealed that (2/51bottles) 3.9 % of the examined bottled water samples were contaminated by *P. aeruginosa*. **Warburton *et al.* (1998)** reported that 1.2 % of the examined samples were contaminated by *P. aeruginosa*. **Richards *et al.* (1992)** isolated *P. aeruginosa* from 4% of bottled water samples. On the other hand, **Hernandez-Duquino and Rosenbeg, 1987, Manaia *et al.*, 1990, Hunter, 1993, Papapetropoulou *et al.*, 1994 and EL-Batouti, 2002,** isolated *P. aeruginosa* from 5.7%, 10.2%, 11%, 18.8%, and 29% of the examined bottled water

samples, respectively. It was also found to be the predominant isolated strain by **Tamagnini and Gonzalez (1997)**. Two out of four brands had a mean bacterial count for *P. aeruginosa* **(Karem and Hassan, 2000)**. It has been shown that the adherence, survival and colonization of *P. aeruginosa* on plastic bottle surfaces especially Polyvinyl Chloride (PVC) enhances the capacity of bacteria to resist disinfection and starvation **(Tamagnini and Gonzalez, 1997).** *P. aeruginosa, P. stutzeri*, *P. fluorescens* and *P. putida* have been commonly isolated from drinking bottled water **(Hernandez-Duquino and Rosenberg, 1987)** and bottled mineral water (**Rosenberg and Hernandez-Duquino, 1988 and Venieri *et al*., 2006**). In their study on five brands of French mineral water, **Mary *et al.* (2000)** have identified the following Pseudomonal species: *P. maltophilia*, *P. fluorescens* and *P. alcaligenes*. The species *P. aeruginosa, P. testoalcaligenes*, *P. maltophilia*, *P.diminuto*, *P. fluorescens*, and *P. vesicular* were isolated from domestic bottled water in Greece where *P. aeruginosthe* highest incidence **(Venieri *et al.*, 2006). Wilkinson and Kerr (1998)** determined the prevalence of *Stenotrophomonas maltophilia* in both carbonated and non-carbonated commercially available bottled water. Three samples yielded *S. maltophilia* and a further eight revealed *Pseudomonas* species. Non-fermentative Gram-negative bacilli can cause serious healthcare-associated infections and are often resistant to multiple antibiotics. **Memish *et al.* (2012)** examined resistance rates among these bacteria from different regions of Saudi Arabia.

Many biochemical and molecular methods have been used in epidemiological investigations of human and bovine staphylococcal infections (**Hartstein *et al*., 1989; Prevost *et al*., 1991; Kapur *et al.*, 1995**). Because of their sensitivity and speed of performance, PCR based typing systems represent useful techniques for the study of *S. aureus* of animal and hospital origin and for monitoring their spread. Random amplified polymorphic DNA (RAPD) markers, which are based on the amplification of discrete DNA fragments in the genome by oligonucleotide primers with random sequences, have been largely used to identify physiological races of fungi **(Guthrie *et al*., 1992)**. With this technique a DNA fingerprint may define individual in a very fast and reliable way. RAPD-PCR method, when compare with biochemical methods is cheap, simple, more sensitive and faster. Apart from the study of antibiotic resistance **(Ikeh, 2003)**, little is known concerning the genetic diversity that exists in bacterial populations in bottled water in Saudi Arabia.

In this study genetic fingerprinting of the water-borne isolates *S. aureus***,** Gram negative bacilli non-fermenter spp., *P. aeruginosa* and *P. flourescenses* isolated from different bottled water samples in Jeddah was evaluated using RAPD markers. Such information will be useful in their classification, ecology and diagnosis. Two primers were selected for this study. To score fingerprints, one band has assumed to be corresponded to one locus. Results indicated that the amplification reactions with primer 1 generated 0, 15, 7, and 14 bands of *S. aureus***,** Gram negative bacilli non-fermenter spp., *P. aeruginosa* and *P. flourescens* strains respectively (Fig 1). Primer 2 generated 2, 13, 2, and 10 bands of *S. aureus***,** Gram negative bacilli non-fermenter spp., *P. aeruginosa* and *P. flourescens* trains respectively (Fig 2) (Table 3). A total of 61 allelic bands ranging between 200 and ~3000 bp were amplified as shown in Table 3, Figure 1 and 2 for primers B-8 and B-11. Results indicated that the use of two primers generated a total of 2 amplified fragments from 350 to 4000 bp, 28 amplified fragments from 150 to 4000 bp, 9 amplified fragments between 350 to 3000 bp and 24 amplified fragments between 250 to 4000 bp. From *Staphylococcus aureus,* Gram negative bacilli non-fermenter spp*., P. aeruginosa* and *P. flourescenses* respectively.

Kim *et al*. (2011) reported that the DNA fingerprinting patterns did not reveal genetic similarity between the water-borne and clinical *P. aeruginosa* isolates. In other study, where sewage samples from Hospital São Vicente de Paulo and water samples from the Passo Fundo river were compared with regard to the susceptibility profile of *P. aeruginosa* isolates, **Fuentefria *et al.* (2008)** had demonstrated significant differences between the water and hospital effluent samples using antimicrobial susceptibility tests and RAPD analysis. And the use of various primers for *P. aeruginosa* generated a total of 131 amplified fragments between 200 to 300 bp, this was more than the number of fragments generated from *Staphylococcus aureus* and *Escherichia coli* while using the same number of primers **(Onasanya *et al.*, 2003; Salehi *et al*., 2008 and Shehata, 2008)**.

Studies have suggested that some *P. aeruginosa* clinical isolates are phenotypically, genotypically, chemotaxonomically and functionally indistinguishable from environmental isolates, **Römling *et al*. (1994)** reported that a clone frequently isolated from cystic fibrosis patients was also detected at a high frequency in aquatic environments. **Alonso *et al*. (1999)** reported that both oil-contaminated soil isolates and clinical isolates of *P. aeruginosa* showed pathogenic and biodegradative

properties. **Wolfgang *et al.* (2003)** reported that the genomes of *P. aeruginosa* strains, representing distinct clinical or environmental sources, are highly conserved.

Table 3: Number of DNA polymorphic bands in bacterial isolates amplified with two oligonucleotide primers.

Bacterial strains	No. of bands (primer B-8)	No. of bands (primer B-11)
Staphylococcusaureus	0	2
Non-fermentative Gram-negative bacilli	15	13
Pseudomonas aerugenosa	7	2
Pseudomonas flourescens	14	10

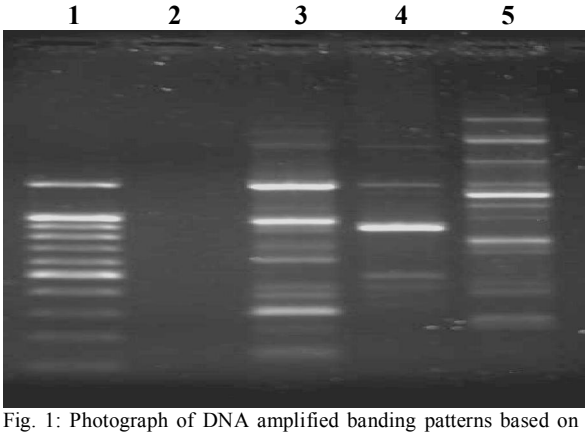

Fig. 1: Photograph of DNA amplified banding patterns based on RAPD for 4 different strains: *S.aureus*, Gram negative bacilli non-fermenter spp.,*P. aeruginosa* and *P.flourescens*(lanes: 2, 3, 4, 5 respectively) against 100 bp ladder DNA marker which has three distinct bands: 500, 1000, 2000 bp (lane 1) using primer B-08.

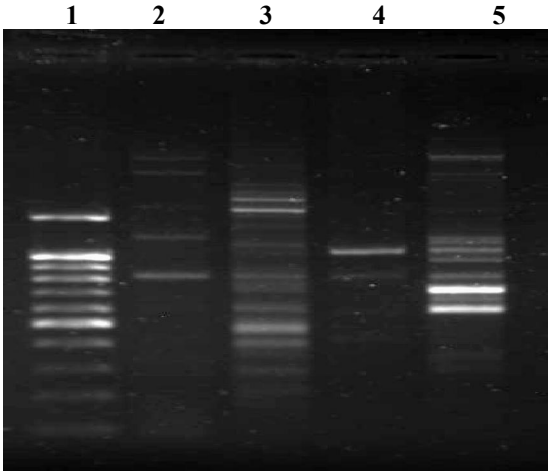

Fig. 2: Photograph of DNA amplified banding patterns based on RAPD for 4 different strains: *S.aureus*, Gram negative bacilli non-fermenter spp.,*P. aeruginosa* and *P.flourescens* (lanes: 2, 3, 4, 5 respectively) against 100 bp ladder DNA marker which has three distinct bands: 500, 1000, 2000 bp (lane 1) using primer B-11.

Salha *et al.* (2012) collected *P. aeruginosa* (24 isolates) from contact lens storage cases, contact lenses and contact lenses wearer in Saudi Arabia. A total of 48 amplified DNA fragments (from 3500 to 90 bp) were observed using three RAPD primers; B-01, B-11 (**primer 2**) and B-14.Whereas, 42 fragments were polymorphic and other 6 amplified fragments were commonly detected among all *Pa* isolates. The three primers showed a mean polymorphism of 87%, whereas, the polymorphic % B-01 primer was higher (89%) followed by primer B-11 and B-14 with 88 and 83%, respectively.

It is necessary to determine the effectiveness of this tool and primers studied here and other in clinical isolates. Primer 2 generated 2 bands (800 and 1000 bp) in *P. aeruginosa* while **Salha *et al.***, (2012) reported that using the same primer B-11 revealed 17 fragments, of which 15 were polymorphic with sizes ranging from 3500 to 90 bp. The total fragment numbers of the 24 isolates varied significantly in their amplified fragments: whereas isolate 11 revealed the highest with 13 fragments, followed by isolates 9 and 10 with 12, while six isolates revealed two for. *P. aeruginosa* (24 isolates) from contact lens storage cases, contact lenses and contact lenses wearer in Saudi Arabia.

4. Conclusion

In future study, water will be collected from different batches of the same manufacturer over a larger period of time. Hence, I believe this study point to the fact that bottled water can contain microorganisms but it does not prove a long term existing problem. The number of bands obtained by PCR reactions may serve future studies for comparison purposes.

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