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Examination of the incidence of *Staphylococcus aureus* and their carriage of vancomycin-resistance genes

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Abstract

The aim of this study was to investigate the carriage of vancomycin-resistance genes by *Staphylococcus aureus* found in drinking water supplies. A total of 100 samples (potable water and faucet swabs) were analyzed for the presence of *S. aureus* and their carriage of vancomycin-resistance genes. Mannitol salt agar was used for the isolation of staphylococci, and confirmation of *S. aureus* was carried out by means of Gram staining technique, growth on blood agar, production of catalase and coagulase. Antibiotic susceptibility testing was performed by disk diffusion method, and polymerase chain reaction (PCR) was used to investigate the presence of vancomycin-resistance genes (*vanA*, *vanB*, *vanC*, *vanS*, *vanY*). Staphylococci were recovered from 25% of water samples (n = 75), of which seven samples were positive for *S. aureus*, on the other hand 76% (n = 25) of the faucet swabs yielded typical staphylococcal colonies, with 53% being positive for *S. aureus*. The disk diffusion method showed that all *S. aureus* resistance to penicillin G, and about 66.6% (n = 51 isolates) were resistance to oxacillin. Of all 51 *S. aureus* isolates, only 14% were resistant to vancomycin by disk diffusion method, however, the DNA extracted from all confirmed *S. aureus* did not yield any PCR products with all the primers used for detecting vancomycin-resistance genes. Vancomycin-resistant *S. aureus* remains rare. The disk diffusion method may give false-resistance with vancomycin, therefore, caution is required with the investigation and interpretation of vancomycin susceptibility testing by agar diffusion method.

Keywords: *Staphylococcus aureus*, vancomycin-resistance genes, water, biofilm.

INTRODUCTION

Staphylococcus aureus remains an important pathogen that is associated with various hospital-and community-acquired infections, and has been generally regarded as a public health problem (Casey et al., 2007). *S. aureus* is recognized as an important agent of food poisoning. This form of infection results after the ingestion of one or more performed staphylococcal enterotoxins (SEs) on food that has been previously contaminated with the bacteria (Seo and Bohach, 2007).

Multidrug-resistant *S. aureus* is wide spread in the environment and has been recovered from foodstuffs, nasal mucosa and skin of healthy humans, clinical cases, food-producing animals, food-catering and aquatic environments (Acco et al., 2003; Normanno et al., 2007; Kluytmans, 2010; Abulreeh and Organji, 2011), this may highlight the potential public health problems associated with the ubiquity of antibiotic-resistant *S. aureus* in the environment.

Staphylococci in potable water may be regarded as a natural flora or one of the genera that are commonly found in water supplies known as heterotrophic plate count (HPC) bacteria (Allen et al., 2004). Despite the suggestion that it is not possible to establish health-based standards for the presence of HPC bacteria in

Table 1. Properties and sequences of the primers for vancomycin-resistance genes.

Primer pair	Sequence	Annealing temperature (°C)	Product size
vanA/*vanA*1	5'-ATGAATAGAATAAAAGTTGCAATAC 5'-CCCCTTTAACGCTAATACGAT	62	1029
vanB/*vanB*1	5'-CCCGAATTTCAAATGATTGAAAA 5'-CGCCATCCTCCTGCAAAA	59	457
vanC/*vanC*1	5'-GCTGAAATATGAAGTAATGACCA 5'-CGGCATGGTGTTGATTTCGTT	58	811
vanS/*vanS*1	5'-AACGACTATTCCAAACTAGAAC 5-GCTGGAAGCTCTACCCTAAA	60	1094
vanY/ *vanY*1	5'-ACTTAGGTTATGACTACGTTAAT 5'-CCTCCTTGAATTAGTATGTGTT	55	866

drinking water, the incidence of high concentration of *S. aureus* in water intended for human consumption may represent potential health hazards, especially if these strains possess determinants of antibiotic resistance and are able to produce enterotoxins (Percival et al., 2004). Pavlov et al. (2004) found that *S. aureus* isolated from drinking water were the most virulent and resistant to multi-antibiotics among all of the HPC bacteria recovered from water supplies in South Africa. Furthermore, water contaminated with *S. aureus* was reported to cause food poisoning when used to cool boiled eggs (Anon., 1972).

Vancomycin (glycopeptides) has become an approved and highly recommended antibiotic worldwide for the treatment of *S. aureus* infections, particularly methicillin-resistant *S. aureus* (MRSA) (Sotozono et al., 2013). Although vancomycin-resistant *S. aureus* is rare, the emergence of strains with decreased susceptibility and/or vancomycin intermediate resistance has been reported (Stienkraus et al., 2007; Gould, 2008; Shang-Yi et al., 2012).

The aim of the present study was to investigate the incidence of *S. aureus* and their carriage of vancomycin-resistance genes in potable water intended for human consumption in Makkah, Saudi Arabia.

METHODOLOGY

Sampling

A total of 100 samples were examined in this study for the presence of vancomycin-resistant *S. aureus*. These samples consisted of potable tap water from households (50 samples), potable water from households main reservoir or tank (25 samples) and households faucets swabs (25 samples). All water samples were collected into sterile polypropylene bottles. Faucets were internally swabbed using sterile cotton swabs. All samples were packed in ice and kept in darkness during transport; bacteriological assays were begun within six hours on the same day as sampling.

Isolation and identification of *Staphylococcus aureus*

S. aureus was recovered from potable water samples by means of membrane filtration technique. A volume of 100 ml of water from each sample was filtered onto a 0.45 µm membrane filter. The membrane filter was placed onto mannitol salt agar (Oxoid, Basingstoke, UK), and incubated at 34°C for 48 h. Each of the faucets swab was streaked onto mannitol salt agar (Oxoid) and incubation was at 34°C for 48 h (Abulreesh and Organji, 2011). Typical staphylococcal colonies on mannitol salt agar (colonies surrounded by yellow zones) were subcultured onto Blood agar plates (Saudi Prepared Media Laboratory, Riyadh, Saudi Arabia). The identification of the colonies was carried out using the following tests: Gram staining, observation of type of haemolysis on blood agar plates, production of catalase and coagulase using the BBL staphyslide latex test (Becton, Dickinson and Company, Maryland, USA). The *S. aureus* NCTC 12989 was used as a control strain.

Antibiotic susceptibility testing

Antibiotic susceptibilities were tested by the disk diffusion method according to the guidelines of the British Society for Antimicrobial Chemotherapy (BASC, 2010) using Mueller-Hinton agar (Oxoid) (Abulreesh and Organji, 2011). Six commercial antimicrobial disks (Mast Diagnostics, Bootle, UK) were used: Erythromycin (60 µg ml^{-1}), Rifampicin (15 µg ml^{-1}), Penicillin G (2 IU), Kanamycin (1000 µg ml^{-1}), Vancomycin (5 µg ml^{-1}) and Oxacillin (1 µg ml^{-1}) (Bio-Rad, Hercules, USA). *S. aureus* NCTC 12989 used a control to ensure the accuracy of testing.

Detection of vancomycin-resistance genes by PCR

PCR was performed on all confirmed *S. aureus* isolates to detect vancomycin-resistance genes. DNA was extracted by suspending a loop of *S. aureus* colony in a 100 µl sterile, pure water and boiling for 5 min. The suspension was then centrifuged for 5 min at 1260 *g*, and 10 µl of the supernatant were used as target DNA. Primers *vanA*, *vanB*, *vanC*, *vanS* and *vanY* (Bioneer, Alameda, USA) were used to detect vancomycin-resistance genes (Miele et al., 1995), the sequence of these primers and the expected product size is listed in Table 1. The PCR reaction mixture contained 10 mM Tris-

Table 2. Isolation of *Staphylococcus aureus* from potable water and faucets swabs.

Sample type: Source	No. of samples tested	No. with typical staphylococcal colonies (%)	No. with confirmed *S. aureus*	% of samples with *S. aureus*
Potable water:				
Household faucets	50	15 (30)	6	40
Reservoir/tank	25	4 (16)	1	25
Faucet swabs	25	19 (76)	10	53
Total	100	38	17	44.7

Table 3. Antibiotic susceptibility of *Staphylococcus aureus* isolated from potable water by disk diffusion method.

Parameter	R	IR	S	NCTC 12989†
	N (%)	N (%)	N (%)	
OX	16 (76.2)	0.0 (0.0)	5 (23.8)	S
VA	2 (9.5)	0.0 (0.0)	19 (90.5)	S
KA	0.0 (0.0)	0.0 (0.0)	21 (100)	S
PG	21 (100)	0.0 (0.0)	0.0 (0.0)	R
RP	0.0 (0.0)	0.0 (0.0)	21 (100)	S
E	0.0 (0.0)	0.0 (0.0)	21 (100)	S

R: Resistant, IR: Intermediate resistance, S: sensitive, OX: Oxacillin, VA: Vancomycin, KA: Kanamycin, PG: Penicillin G, RP: Rifampicin, E: Erythromycin, N: number of isolates tested, †: Control strain (*Staphylococcus aureus* NCTC 12989)

HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.15 mM (each of) dNTP, 1.2 U of Taq polymerase (ABgene, Surry, UK). The final reaction volume 0.05 ml contained 50 pmol of each primer and 500 ng of *S. aureus* DNA. The PCR program consisted of initial denaturation step at 94°C for 3 min; this was followed by denaturation at 94°C for 30 s, primers annealing at appropriate temperature (Table 1) for 2 min, and DNA extension at 72°C for 2 min. After the last cycle, the reaction was terminated by incubation at 72°C for 6 min and the products were stored at 4°C. PCR products (5.0 µl) were analysed by 1% agarose gel (Bioline, London, UK) electrophoresis and made visible by ethidium bromide (0.5 mg ml^{-1}) staining and UV transillumination (Miele et al., 1995).

RESULTS

Isolation of *Staphylococcus aureus* from potable water and faucet swabs

In potable water, staphylococci were detected in 30% of the samples from household faucets, and in 16% of the samples from household reservoirs/tanks. High recovery rate of staphylococci was observed in household faucet swabs (76%) (Table 2). *S. aureus* was present in six water samples out of 15 samples taken from household faucets (40%) and one water sample out of 4 samples taken from household reservoirs/tanks (25%). In faucet swabs, *S. aureus* was detected in 53% of the samples (10 out of 19) that yielded typical staphylococci colonies (Table 2).

Antibiotic susceptibility testing by disk diffusion

Antibiotic susceptibility patterns of *S. aureus* isolates from potable water and faucet swabs are shown in Tables 3 and 4, respectively. All *S. aureus* isolates from water and from faucet swabs were resistant to penicillin G, but sensitive to kanamycin and rifampicin (Tables 3 and 4). Resistance to oxacillin was exhibited by 76.2% and 60% of the *S. aureus* recovered from potable water (Table 3) and faucet swabs, respectively (Table 4). Small number of *S. aureus* recovered from water (9.5%, n = 2.0) exhibited resistance to vancomycin (Table 3), while 16.6% (n = 5.0) of the isolates derived from faucet swabs were resistant to vancomycin (Table 4). No isolates from water were resistant to erythromycin, however, two isolates from faucet swabs showed resistance and intermediate resistance, respectively to erythromycin (Table 4).

Detection of vancomycin-resistance genes by PCR

No vancomycin-resistance genes were detected in all 51 *S. aureus* isolates recovered from potable water and faucet swabs.

DISCUSSION

Staphylococci are ubiquitous with widespread distribution in the environment, and their presence in aquatic

Table 4. Antibiotic susceptibilities of *Staphylococcus aureus* isolated from faucet swabs by disk diffusion method.

Parameter	R	IR	S	NCTC 12989†
	N (%)	N (%)	N (%)	
OX	18 (60)	0.0 (0.0)	12 (40)	S
VA	5 (16.6)	0.0 (0.0)	25 (83.3)	S
KA	0.0 (0.0)	0.0 (0.0)	30 (100)	S
PG	30 (100)	0.0 (0.0)	0.0 (0.0)	R
RP	0.0 (0.0)	0.0 (0.0)	30 (100)	S
E	1.0 (3.3)	1.0 (3.3)	28 (93.33)	S

R: Resistant, IR: Intermediate resistance, S: sensitive, OX: Oxacillin, VA: Vancomycin, KA: Kanamycin, PG: Penicillin G, RP: Rifampicin, E: Erythromycin, N: number of isolates tested, †: Control strain (*Staphylococcus aureus* NCTC 12989).

environments is well established (Percival et al., 2004). In the present study, 46% of water samples collectively yielded staphylococci, with 65% of these isolates confirmed as *S. aureus* (Table 2). Similar and higher percentages of *S. aureus* occurrence in water were reported worldwide (Harakeh et al., 2006; Faria et al., 2009), including Makkah, Saudi Arabia (Mihdhdir, 2009; Abulreesh and organji, 2011). Although *S. aureus* can be found among the genera that normally exist in potable water as HPC bacteria (Allen et al., 2004), there are many reasons for potential concern when *S. aureus* are present in drinking water supplies. Common food preparation practices such as washing boiled potatoes, pasta, shelfish, and cooling of boiled eggs could possibly leave these food items contaminated with *S. aureus*. If these food items used for preparation of salads are left at room temperature, or improperly refrigerated, the possibility of staphylococcal food intoxication certainly exists if these *S. aureus* contaminants were toxigenic.

Staphylococci are well-known for their ability to produce biofilm formation on different surfaces such as water line pipes in dental clinics (Lancellotti et al., 2007) and on stainless steel pipes milk processing plants (Michu et al., 2011), this feature is considered as one of the main virulence factors of nosocomial staphylococcal-related infections (Piette and Verschraegen, 2009). In this study, 76% of the faucet swabs samples (n = 25) yielded typical staphylococci, of which 53% were confirmed as *S. aureus* (Table 2). Abulreesh and Organji (2011) reported higher recovery rate of staphylococci from faucet swabs and faucet filter swabs. The presence of *S. aureus* in biofilm formation within drinking water distribution system may, in part, cause aesthetic and hygienic problems as staphylococci within the biofilm consortia can inherit resistance to disinfectants, and their long term persistence, together with other HPC bacteria, can deteriorate the overall microbiological quality of potable water. Further potential waterborne pathogens may take refuge within the biofilm formation and survive for longer periods, with the possibility of acquiring resistance to antibiotics due to transferrable resistance genes (Lee and Kim, 2003; Parsek and Singh, 2003; Zhu et al., 2008).

Resistance to antimicrobial agents is a major public health concern as resistant bacteria can disseminate in the environment with possible transmission to human through contaminated food and water. In the current study, all *S. aureus* that were recovered from water (Table 3) and faucet swabs (Table 4) exhibited resistance to penicillin G, while resistance to oxacillin was observed in 76.2% and 60% of the isolates from water and faucet swabs, respectively. *S. aureus* is well-known for its remarkable resistance to β-lactam agents. Thus, methicillin-resistant *S. aureus* (MRSA) is currently the most commonly identified antibiotic-resistant pathogen worldwide (Ippolito et al., 2010), and their presence in drinking water and biofilm is widely reported (Harakeh et al., 2006; Lancellotti et al., 2007; Abulreesh and Organji, 2011).

Vancomycin has been successfully used for the last 50 years for the treatment of staphylococcal infections, particularly the cases involving MRSA. However its reliability has been questioned by the emergence of *S. aureus* strains that exhibit complete or intermediate resistance (Holmes et al., 2012). Various published accounts, using disk diffusion method reported the incidence of vancomycin-resistant strains in clinical (Mehdinejad et al., 2008), food (Ateba et al., 2010) and water and biofilm (Lancellotti et al., 2007; Abulreesh and Organji, 2011) specimens.

In the current study, few isolates from water (9.5%, n = 2.0) and from faucet swabs (16.6%, n = 5.0) exhibited resistance to vancomycin by disk diffusion method (Tables 3 and 4). In the United States, the Clinical and Laboratory Standard Institute (CLSI) only approve methods that determine minimum inhibitory concentrations (MIC) values for the detection of vancomycin-resistance in staphylococci due to its accuracy; this is also standardized in other countries such as Japan and European Union (Srinivasan et al., 2002). Therefore, the use of disk diffusion method for investigating resistance to vancomycin in staphylococci may not be appropriate

and possibly yield false-resistance patterns. This was the case in the current study, when all *S. aureus* isolates yielded no PCR products with all primers used, suggesting the absence of vancomycin-resistance genes despite five isolates exhibited resistance when tested by disk diffusion technique. Thus, caution is required when investigating and interpreting vancomycin-resistance in *S. aureus*.

The first isolation of *S. aureus* with *vanA* was reported in Michigan Hospital in 2002 (Anon., 2002), subsequently, several other strains were reported (Chang et al., 2003; Anon., 2004; Zhu et al., 2008), however all these isolates were of clinical origins. Despite the sporadic occurrence of *S. aureus* that carry vancomycin-resistance genes, it is generally accepted that these strains are rare (Srinivasan et al., 2002; Holmes et al., 2012; Askari et al., 2013; Culos et al., 2013). The DNA isolated from all 16 isolates reported in this study did not give any PCR products with the primers *vanA*, *vanB*, *vanC*, *vanS* and *vanY*. This result further confirms the rarity of vancomycin-resistant *S. aureus* in general and in the environment in particular.

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