



## Isolation and identification of *Enterococcus* species and determination of their susceptibility patterns against antibiotics and heavy metals in coastal waters of Iran

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### ABSTRACT

Recreational waters should be considered as risks for Enterococcal infections in regions with high utilization and long exposure periods. The aim of this study was to investigate the resistance of Enterococci to selected antibiotics and some heavy metals [ $\text{Pb}^{+2}$ ,  $\text{Fe}^{+2}$ ,  $\text{Zn}^{+2}$  and  $\text{Cr}^{+2}$ ]. A total of 75 Enterococcal isolates was obtained from 54 bathing and fishing water samples from 9 bathing and fishing areas in Bandar Abbas (Iran). *Enterococcus raffinosus* and *Enterococcus faecium* were the most prevalent species. Disk diffusion method was performed to determine the high level resistance to selected antibiotics, and Micro dilution method was used to detect the sensitivities of the strains against different concentrations (0.005-20 mM) of heavy metals. Antibiotic resistance patterns were observed in isolates. All of the isolates were resistant to Penicillin. Many of them exhibited high resistance to Streptomycin and Kanamycin in 68.08% and 65.96% respectively. The results suggest that recreational waters may contribute to the dissemination of Enterococcal species that exhibit resistance to several antibiotics which are used to treat community-acquired infections.

**Keywords:** Minimum inhibitory concentration, Minimal bactericidal concentration, *Enterococcus*, Recreational waters, Heavy metals.

### INTRODUCTION

Enterococci are Gram positive, Catalase negative, Oxidase negative and facultative anaerobic cocci that do not form spores and are found in individuals, groups and strain forms (Fisher and Philips 2009; Obri-danso and Jones 1999) and can be separated from animal and environmental sources (Fisher and Philips 2009). Enterococci can survive in extreme environmental conditions such as high temperature (5-65 °C),

pH (4.5-10.0) and NaCl rate which enable them to clone in vast ranges. Enterococci are important human pathogens in both community and hospital infections. They also exhibit intrinsic resistance to various antibiotics. In recent years, Enterococcal infections have become a major therapeutic challenges because of their increased incidence and the spread of strains that have acquired resistance to several antimicrobial agents (Arvanitidou and Tsakris 2001). In addition to antibiotics, heavy metals are among other antimicrobial agents that Enterococci have acquired resistance to them. Some sources of resistant Enterococci may be bathing waters. The presence of Enterococcal strains that exhibit antibiotic resistance in these environments is of particular interest because of the possible link between community-acquired Enterococcal infections and recreational activities. There have been few reports of the presence and antibiotic resistance of Enterococci in water of bathing beaches (Arvanitidou and Tsakris 2001). This study evaluated antibiotic and heavy metal resistance patterns of Enterococcal species cultured from waters of 9 recreational areas in Iran.

## MATERIALS AND METHODS

Sampling was performed during a six-month period from August to January 2012. A total number of 54 sea samples were collected from 9 points of 3 regions in coastal areas of Bandar Abbas. Sampling regions were selected according to their popularity for bathing and fishing activities. All samples were transported to laboratory in a container filled with ice. Samples were analyzed for the presence of Enterococci. Temperature, salinity and pH of sea samples were measured just after sampling and the amount of dissolved Oxygen were estimated by Iodometry technique.

### Microbial examination

To enumerate the bacteria, 3-tube MPN method was applied. All the tubes with turbidity in probable test were transmitted to Pfizer Selective Enterococcus Agar (PSA) for confirmative test. After 24 hours incubation at 35°C, formation of brown-black colonies with brown corona suggested positive plates. Catalase test was performed on all Gram-positive cocci suspicious of being Enterococci. Growth in presence of 40% Bile Esculin, growth in the presence of 6.5% NaCl and growth in 45°C were performed for the final identification of Enterococci. For identification of species bacteria were tested for their ability to Hypurate hydrolysis, fermenting lactose, raffinose, sucrose, mannitol and arabinose and their ability to move and producing H<sub>2</sub>S.

### Antibiotic sensitivity testing

Antimicrobial resistance patterns of Enterococci to 9 antimicrobial agents were performed with Ampicillin, Amikacin, Streptomycin, Gentamicin, Choloramphenicol, Kanamycin, Penicillin G, Vancomycin and Sulfamethoxazol using Standard Disk Diffusion Method (Kirby-Bauer sensitivity test). All tests were performed on Mueller-Hinton agar and results were read after 24 hours incubation at 37°C. 10<sup>4</sup> CFU of bacteria was used to spot the medium. The zone diameter measured around each disk was considered as resistant, intermediate or sensitive according to the zone size diameter with the ranges provided by the disk manufacturer. The antimicrobial agents were obtained from PadtanTeb Co (Tehran, Iran).

### Heavy metals susceptibility testing

To determine the resistance of isolated species to the heavy metals such as  $Pb^{+2}$ ,  $Fe^{+2}$ ,  $Zn^{+2}$  and  $Cr^{+2}$ , Micro dilution method was applied. To supply different concentrations of the metals, 1000 ppm standard solution of each metal was used. To assess the Minimum Inhibitory Concentration (MIC), variable dilutions of each metal were supplied. To determine the MIC, 96-well format microplates were used. Microplates incubated at 37°C and turbidity surveyed optically after 24 hrs. For each metal, the first well without turbidity assumed as MIC. The Minimal Bactericidal Concentration (MBC) of each metal that completely causes the death of bacteria, determined by culturing of 10µg of wells without turbidity in Bile Esculin Agar and plates incubated at 37°C for 24 hours.

## RESULTS

In this research, sampling performed from 9 points of 3 regions in western coastal areas of Bandar Abbas over a six-month period from August to January. Of 54 surveyed samples, 47 samples identified as *Enterococcus* according to their morphologic, biochemical and microscopic characteristics. Among 47 *Enterococcal* samples, 7 different species were identified, of which *Enterococcus raffinisus* was the most prevalent, accounting for 19 (40.4%) of isolates and *Enterococcus faecium* was found in 18 instances (38.2%). The following were identified at lower frequencies: *Enterococcus minutia*(5), *Enterococcus gallinarum*(2), *Enterococcus hire*(1), *Enterococcus pseudoavium*(1) and *Enterococcus saccharolyticus*(1). Antibiotic susceptibility patterns of isolated *Enterococci* suggested that 100% of these species were resistant to Penicillin. Isolated *Enterococci* resistance to Streptomycin, Kanamycin, Amikacin, Gentamicin, Ampicillin and Chloramphenicol were 68.08%, 65.96%, 29.79%, 10.64%, 6.38% and 2.12% respectively. The least resistance among selected antibiotics referred to Sulfametoaxol and Vancomycin. Among four selected metals [ $Pb^{+2}$ ,  $Fe^{+2}$ ,  $Zn^{+2}$  and  $Cr^{+2}$ ], the most MIC and MBC referred to  $Pb^{+2}$  and  $Fe^{+2}$ , and the least MIC and MBC referred to  $Zn^{+2}$  and  $Cr^{+2}$ . MICs suggested that the most average referred to species. *Faecium*, *E. gallinarum*, *E. hirae* and *E. saccharolyticus*, and the least average referred to species *E. minutia* (Table 1). MBC of isolated *Enterococcal* species suggested that species *E. faecium*, *E. gallinarum*, *E. hirae* and *E. saccharolyticus* were the most resistant, and the least resistance referred to species *.mundtii* (Table 2).

**Table 1.** The MIC and MBC results.

Metal concentration Bacteria	Fe (mg/l)		Pb (mg/l)		Zn (mg/l)		Cr (mg/l)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>E. raffinosus</i>	500	700	700	800	300	400	400	500
<i>E. faecium</i>	800	900	800	900	500	600	500	600
<i>E. mundtii</i>	500	700	700	800	300	400	200	300
<i>E. gallinarum</i>	700	800	800	900	400	500	400	500
<i>E. hirae</i>	700	800	800	900	400	500	300	400
<i>E. pseudoavium</i>	500	700	700	800	300	400	300	400
<i>E. saccharolyticus</i>	700	800	800	900	500	600	500	600

## DISCUSSION

Identification of Enterococcal species in recreational waters may help to clarify their ecological characteristics (Arvanitidou and Tsakris 2001). As a measure of infection control, it is essential to differentiate Enterococci from other Gram positive bacteria. In previous studies which were performed in other regions such as Greece, Turkey and western coasts of Caspian Sea (Guilan, Iran), the most predominant species consisted of *E. faecalis* and *E. faecium*, but in this study the most isolated species consisted of *E. faecium* and *E. raffinosus*. The species with low frequency in this study identified as *E. mundtii*, *E. gallinarum*, *E. hirae*, *E. pseudoavium* and *E. saccharolyticus*, but in the study performed by Oliveira and Pinhata (South of Brazil) (2007), *E. hirae*, *E. durans*, *E. casseliflavus* and *E. avium* identified as species with low frequency. In another study which was performed by Arvanitidou, *E. casseliflavus*, *E. faecalis*, *E. pseudoavium*, *E. durans*, *E. hirae* and *E. gallinarum* were found with low redundancy (Arvanitidou and Tsakris 2001). In a report from Obri-Dansoin Greek coastal regions, *E. avium* and *E. raffinosus* were found to predominate in recreational waters and the most resistance to antimicrobial agents was found in *E. faecalis* and *E. faecium* species (Obri-danso and Jones 1999). According to Rice *et al.* (1995), as it is shown in this study, high resistance to Gentamicin occasionally occurs in Enterococci isolated from waters (Rice 2001). According to the study performed by Oliveira (recreational waters in southern regions of Brazil), the most resistance to antibiotics was seen in *E. faecalis* and *E. faecium* species, as the highest frequency of resistance observed against Streptomycin and Erythromycin in water samples and against Erythromycin and Tetracycline in sand samples (Oliver and Pinhata 2007). In this study, all samples were found to be resistant to Penicillin. These results suggested that recreational waters with high frequency of human fecal contamination could potentially be the sources of pathogens and then related with bacterial resistance spread. Environmental contamination may be one of the most difficulties in 21st century. In the last decade, different kinds of organisms such as bacteria, fungi and alga were seen and studied to identify efficient biological absorbents (Dutka-Malen and Courval 1990). Bacteria and fungi were isolated from industrial plants' soil samples which were resistant to heavy metals. Cr (VI) biotic community of these isolates was evaluated and they were found to be able to eliminate heavy metals during metallurgy and tannery. In a report from Erden *et al.*, 93% of isolates were resistant to Fe and Zn and 85% of them were resistant to Cr; whereas in this study, 92% of isolates were resistant to Zn, 91% to Pb, 85% to Fe and 83% to Cr. In a report from Kermanshahi *et al.* (2007), the most resistance referred to Zn which the maximum MIC in 2 of 3 industrial slops evaluated as 24 mMol which belonged to *Coryneform* and *E. faecalis*, and the least amounts evaluated as 8 and 12 mMol which belonged to *Citrobacter* and some *Bacillus* species respectively. The least observed resistance was to Copper. Most of the resistant bacteria, except those which were resistant to Copper, were Gram-positive. In this study, the most resistance waste  $Pb^{+2}$  and  $Fe^{+2}$  which their maximum MIC and MBC were 800 and 900 pap and the minimum resistance was to  $Zn^{+2}$  and  $Cr^{+2}$  which their maximum MIC and MBC were 500 and 600 ppm respectively.

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