Prevalence and Removal Efficiency of Enterococcal Species and Vancomycin-resistant Enterococci of a Hospital Wastewater Treatment Plant

Fatemeh Karimi, Mohammad Reza Samarghandi, Reza Shokoohi, Kazem Godini, and Mohammad Reza Arabestani

Abstract

Simultaneous presence of various antibiotics and bacteria in hospital wastewaters creates a suitable environment, in which the bacteria, such as enterococci become resistant to the antibiotics. The aim of this study was to evaluate the performance of different units of the hospital wastewater treatment plant (HWTP) to remove Enterococcus spp and Vancomycin-resistant Enterococcus (VRE). The study was performed on the 27 samples collected from HWTP in Hamedan, Iran during December 2014 to August 2015. Enterococcus spp and VRE were identified by biochemical tests and then the isolates were confirmed by PCR. Finally, the antibiotic susceptibility test was performed using disk diffusion methods. Of the 27 samples examined, 315 a total of enterococcal isolates were obtained. Of the 315 isolates of enterococci investigated, 162 (51.42%) were identified as E. faecium, 11 (3.5%) as E. faecalis, 11 (3.5%) as E. gallinarum, 7 (2.22%) as E. casseliflavus, 4 (1.26%) E. avium, and 9 (2.85%) isolates VR E. faecium. The results of antibiotic susceptibility testing showed that of the total 315 isolates, 146 (46.34%) were resistance to tetracycline, 9 (2.85%) were resistance to vancomycin and Teicoplanin. Lower antibiotic resistance was seen with Nitrofurantoin 2 (1.26%). This study indicates a high prevalence of multidrug resistance among E. faecium isolated from HWTP, thus, it could be considered as a threat to the health and safety of wastewater workers and even public health.

Keywords: Vancomycin-Resistant Enterococci, Hospital Wastewater Treatment Plant, Enterococcus faecium, Enterococcus faecalis

1. Introduction

Enterococci are Gram-positive cocci and common commensal organisms in the intestines of humans and some animals. Despite the weak virulence of these bacteria, they are responsible for most community and hospital acquired infections. Living in their principal place, Enterococci are not harmful. The entrance of these bacteria into the blood, urinary, and each body part, especially in susceptible persons, can cause the infections of urinary tract, blood, intra-abdominal, and pelvic as well as Endocarditis and Meningitis (1, 2). Two species of enterococci, which are more involved in enterococcal infections than other ones are E. faecalis and E. faecium. The most important reason of the enterococci’s importance is their inherent resistance to common antibiotics and their ability to acquisition of resistance to almost all available antibiotics (3).

The environment of wastewater can grow microbes besides providing a suitable place for bacteria to resist to antibiotics because significant amounts of various antibiotics are discharged into wastewater immethodically and makes hospital wastewater treatment plants (HWTP) one of the main sources of bacteria to the environment (4, 5). Conventional wastewater treatment processes usually do not have enough efficiency in bacteria and antibiotics removal, so that concentrations of nanograms to micrograms per liter of antibiotics have been detected in surface and underground waters as well as other water sources. The entrance of antibiotics into biological wastewater treatment plants not only increases the bacterial resistance but also destroys bacterial population, which, in turn, disturbs the biological treatment processes and decreases efficiency (6, 7). The use of water recycled from treated hospital wastewaters for different goals has recently increased owing to continuing droughts and limitation of water storage. As a result, this phenomenon increases the exposure of workers and staphylococcus with VRE and other pathogens, which may exist in this recycled wastewater (8); so that the transfer of resistant genes among enterococci in HWTP has also been reported (9).
sistance frequency of Enterococci in clinical samples (10, 11). Also, in view of the possibility of the presence of VRE in wastewater, as a suitable environment for bacteria growth and creation of resistance in other bacteria, Ahmadi et al. and Talebi et al. (12, 13) studied the prevalence and frequency of Enterococci’s resistance in wastewater treatments. Considering the hospital wastewater is a proper environment of simultaneous presence of bacteria and antibiotics, the current research was first conducted on a wastewater treatment plant in Iran. Therefore, considering the importance and effects of antibiotic resistance on human health and the role of hospital wastewaters in spread of resistant genes, this research was carried out to survey the detection and isolation of VRE from a hospital wastewater treatment plant (HWTP) in Hamadan.

2. Method

2.1. Study Site

The HWTP is located in Hamadan, Iran. The HWTP utilizes an extended aeration activated sludge system including the following units: screens, aeration tank, secondary sedimentation basin, and chlorination. The plant produces 150 m$^3$ of wastewater and the treated effluent is piped to landscaping sites for the reuse in spray irrigation. Additionally, the treated effluent had average values of 76.6 mg L$^{-1}$ COD, 35 mg L$^{-1}$ BOD, 1 mg L$^{-1}$ Chlorine Residual, and 256 mg L$^{-1}$ suspended solids.

2.2. Sample Collection

A total of 27 grab samples were collected, 3 samples were collected per month, lasted 9 months between December 2014 and August 2015, and was done from the influent, secondary sedimentation basin, and effluent. The samples were collected in 1 liter sterile polyethylene bottles and transported to the laboratory at 4°C within 4 hours for processing (14).

2.3. Bacteria Isolation

The influent and secondary sedimentation samples (volume 200 mL) with dilution, respectively, 100 and 10, and the effluent sample (volume 200 mL) were filtered through a 0.45-µm Millipore membrane. Next, the filters were transferred gradually to a Brain-heart infusion agar plate (Difco Laboratories, Detroit, Michigan, USA) and kept in an incubator at 37°C for 2 hours. Then, the filters were transferred to the special plate Membrane Enterococcus agar (ME agar) (DIFCO, USA) and kept at 37°C for 36 - 48 hours in order to isolate total enterococci. The filters, then, were held on Bile-esculin agar plates (DIFCO, USA) at 45°C for 2 hours. Finally, the black colonies, taken from on the filters, were purified on blood agar (15).

2.4. Biochemical Characterization

After purification of the colonies in order to identify the genus Enterococcus, Gram stain test, catalase test, growth in the presence of NaCl 6.5%, Bile-esculin hydrolysis test, and Pyrrolidonyl-beta-naphthylamide (PYR) test was performed. Then, verified colonies were identified at the species level by means of fermentation of carbohydrates: L-arabinose, lactose, D-sorbitol, D-mannitol, L-sorbose, Lactose, Sucrose, hydrolysis of arginine and hippurate, hemolysis, motility, presence and absence of pigment, tetracylum 0.01%, and tellurite 0.04% reduction-assay (16, 17).

2.5. Antimicrobial Susceptibility Testing

According to the clinical and laboratory standards institute (CLSI 2014) guidelines, disk diffusion method was used to determination of antimicrobial susceptibilities of Enterococcus isolated strains (18), for the antibiotics including Linezolid (30 µg), Tetracycline (30 µg), Vancomycin (30 µg), Norfloxacin (10 µg), Ciprofloxacin (5 µg), quinupristin/dalfopristin (synercid) (15 µg), Erythromycin (15 µg), Teicoplanin (30 µg), Ampicillin (10 µg), Chloramphenicol (30 µg), and Nitrofurantoin (300 µg) (Mast Group Ltd, Merseyside, U.K, ENG). Minimum Inhibitory Concentration (MIC) of the vancomycin-resistant enterococci (VRE) isolates was determined by using an E-test. Quality control strains for performing antibioretical tests were used by the E. faecalis ATCC 29212.

2.6. DNA Extraction

First, 3 to 5 colonies from the bacterial fresh culture were added to 500 µL of sterile distilled water in a 1.5 mL micro tube; then, a homogenous suspension was prepared from the bacterium by using a shaker. Next, the bacterial suspension was boiled at 100°C for 10 - 15 minutes in a water bath. Finally, the strains were centrifuged (14000 rpm) for 5 minutes and the supernatant was transferred to a microtube as the extracted DNA (19).

2.7. Detection of Enterococcus Species by PCR

After isolation and DNA extraction, resistant and susceptible isolates were verified by the use of PCR (Polymerase chain reaction). The composition of PCR reaction mixture included: 10 µL of 2x Taq Master mix (Parstous Biotech CO. Iran), 5 µL sterile double distilled water, 1 µL of reverse primer (Table 1), 1 µL of forward primer, and 3 µL of DNA sample. The optimum conditions of PCR were as follows: an initial denaturation step for 5 minutes at 95°C followed by 30 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute and a final cycle of 72°C for 10 minutes in a Bio-Rad Thermal Cycler (USA) (10). In the case of each primer, X PCR assay

was performed individually \( E. \text{faecalis} \): \( F \{\text{ATCAAGTACGGTAGTCTTCTTATTG}, \ R \{\text{TCCGATCAGACGACCTGACGGTAC}\} \), \( E. \text{avium} \) \( F \{\text{TGGGCAAGACCGGATCC}\}, \ R \{\text{TCGCATCACAAGCACCAATC}\} \)

p of VRE isolates resistance to Tetracycline, 22.22% (\( n = 2 \)) isolates were resistant to Teicoplanin at influent, 11.11% (\( n = 1 \)) isolates were resistant to Chloramphenicol and Enterococcus \( \text{E. faecium} \) was performed individually (21). In previous studies VRE has been detected in influent and biological treatment (activated sludge reactor, aeration tank and secondary sedimentation), however, it has not been reported in the effluent of tertiary treatment, of course, in the case of a suitable chlorination (8). Besides, most European studies in which the presence of VRE in wastewater has been evaluated have reported a prevalence rate of 52% of non-VRE enterococci were evaluated from tertiary treatment with chlorination (23-25).

### 3. Results and Discussion

In the current study, based on different standard biochemical tests and PCR methods relating to detection of species and genus of Enterococcus from the obtained 315 isolates over 9 times of sampling from the HWTP, 6 species of Enterococcus (\( E. \text{faecalis} \), \( E. \text{hiraee} \), \( E. \text{faecalis} \), \( E. \text{gallinarum} \), \( E. \text{casseliflavus} \) and \( E. \text{avium} \)) were identified in total from Hamadan HWTP, from which 1 species (VRE \( E. \text{faecium} \)) was resistant to vancomycin. 208 (66.03%) isolates were from influent, 86 isolates (27.3%) were from the secondary sedimentation basin and 21 (6.66%) isolate were from the effluent (Table 1 and Figure 1). Vancomycin is a strong treatment antibiotic against gram-positive cocci such as Enterococcus, particularly resistant Enterococci. This antibiotic is prescribed for treatment of Enterococci resistant to penicillin or a patient who is susceptible penicillin. Resistance against glycopeptides significantly reduces the treatment properties in Enterococcal infections. Thus, this category of acquired antibiotic resistance is very important in terms of medicine (21).

In this study, prevalence of enterococcal population in the HWTP in Hamadan was evaluated. According to Table 1E. \text{faecium} \) was the only vancomycin resistant enterococci species, and other species did not show any resistance to this antibiotic. These isolates were highly resistant to vancomycin with MIC \( \geq 256 \) mg/L. Among VRE \( E. \text{faecium} \) isolates \( n = 9 \), 6 (66/67%) isolates were found in influent samples and 3 (33/33) isolates were found in Secondary Sedimentation Basin. No VRE isolates were found in effluent (Table 1). All 9 VRE \( E. \text{faecium} \) isolates were resistant to antibiotics such as Ampicillin, Erythromycin, Ciprofloxacin, Penicillin, and Streptomycin, which are used for treatment of Enterococcal infections in all stages of wastewater treatment, however, streptomycin in secondary sedimentation basin (Figure 2). Among VRE \( E. \text{faecium} \) isolates \( n = 9 \) only 11.11% (\( n = 1 \)) isolates were resistant to Chloramphenicol and 22.22% (\( n = 2 \)) isolates were resistant to Teicoplanin at influent. When the wastewater passed the different processes of plant, percentage of VRE isolates resistance to Tetracycline, Synercid, Streptomycin, Norfloxacin, and Gentamycin decreased so that there were no resistant VRE isolate in effluent. All of the isolates were totally sensible to Linezolid further (Figure 2).

It was shown that \( E. \text{faecium} \) was the most common enterococcal isolate followed by \( E. \text{hiraee} \). The presence of \( E. \text{faecium} \) in every sampling and wastewater treatment plant indicated the capability of this organism to survive and resist in the wastewater. Furthermore, it was shown that all of the VRE strains were \( E. \text{faecium} \) that have important role to disseminate and establish antibiotic resistance within the enterococcal populations. VRE \( E. \text{faecium} \) removal efficiency was investigated for the first time for a HWTP in Iran; it should be noted that the effluent of this plant is used for irrigation of the green space of the hospital. Similar studies have reported the presence of VRE in wastewater (22, 23).

Among the non-resistance enterococci to vancomycin (\( n = 306 \)), \( E. \text{faecalis} \) had the highest frequency of resistance to Tetracycline and Synercid and \( E. \text{gallinarum} \) was completely resistant to Synercid and Ampicillin. \( E. \text{hiraee} \) was the only species that was sensible to all antibiotics. Furthermore, \( E. \text{casseliflavus} \) shows 100% resistance to Tetracycline. \( E. \text{avium} \) was just quite sensible to the Ampicillin, and Teicoplanin (Table 2). The frequency distribution of the enterococcal strains illustrated that the influent and effluent had the highest and lowest prevalence rates, respectively (Table 1).

The results showed that \( E. \text{avium} \) had the lowest prevalence rates. \( E. \text{faecium} \) was the most common species isolated from the influent and the secondary sedimentation basin, comprising 107 (33.96%) influent, 36 (11.42%) secondary sedimentation basin and 19 (6.03%) effluent, followed by \( E. \text{hiraee} \) and \( E. \text{faecalis} \). The frequency of VRE strains isolated from the 2 parts of wastewater treatment plants was similar to an average of 3% VRE from the total enterococcal isolations. It has claimed that different parts of plants are effective in VRE removal (24); similarly, in our study, it was observed that the different parts of the plant are entirely efficacious in total enterococci and VRE removal and the system had a suitable performance (Table 1).

In previous studies VRE has been detected in influent and biological treatment (activated sludge reactor, aeration tank and secondary sedimentation), however, it has not been reported in the effluent of tertiary treatment, of course, in the case of a suitable chlorination (8).
Table 1. The Prevalence of Different Species of Enterococci Isolated from Hospital Wastewater Treatment Plant (HSTP)*

<table>
<thead>
<tr>
<th></th>
<th>Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. faecium</td>
</tr>
<tr>
<td>Influent</td>
<td>107 (33.96)</td>
</tr>
<tr>
<td>Secondary Sedimentation Basin</td>
<td>36 (11.42)</td>
</tr>
<tr>
<td>Effluent</td>
<td>19 (6.03)</td>
</tr>
</tbody>
</table>

*Values are expressed as No. (%).

Figure 1. Electrophoresis Products PCR

The prevalence of the VRE from the wastewater treatment plants varies in different countries. In the USA, VRE has still reported in influent and secondary sedimentation parts, however, it was completely removed by the UV-disinfected process (26).

In our study, VRE has the prevalence of approximately 3%. VRE was observed in influent and secondary sedimentation and it was treated completely through chlorination.
Table 2. Prevalence of Antibiotic-Resistant Enterococci Among 293 Isolates from the Wastewater Treatment Plant

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>E. faecalis (N = 35)</th>
<th>E. faecium (N = 162)</th>
<th>E. gallinarum (N = 11)</th>
<th>E. avium (N = 4)</th>
<th>E. hirae (N = 87)</th>
<th>E. casseliflavus (N = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>12 (34.28)</td>
<td>78 (48.14)</td>
<td>5 (45.45)</td>
<td>2 (50)</td>
<td>0</td>
<td>3 (42.85)</td>
</tr>
<tr>
<td>Teicoplanin</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>27 (77.14)</td>
<td>104 (64.19)</td>
<td>5 (45.45)</td>
<td>3 (75)</td>
<td>0</td>
<td>7 (100)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>6 (17.14)</td>
<td>17 (10.49)</td>
<td>0</td>
<td>1 (25)</td>
<td>0</td>
<td>2 (28.57)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>25 (71.42)</td>
<td>73 (45.06)</td>
<td>5 (45.45)</td>
<td>3 (75)</td>
<td>0</td>
<td>5 (71.42)</td>
</tr>
<tr>
<td>Linezolid</td>
<td>6 (17.14)</td>
<td>3 (1.85)</td>
<td>0</td>
<td>1 (25)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Synercid</td>
<td>27 (77.14)</td>
<td>83 (51.23)</td>
<td>11 (100)</td>
<td>3 (75)</td>
<td>0</td>
<td>5 (71.42)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>15 (42.85)</td>
<td>61 (37.65)</td>
<td>11 (100)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>0</td>
<td>3 (1.85)</td>
<td>0</td>
<td>1 (25)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>13 (37.14)</td>
<td>61 (37.65)</td>
<td>3 (27.27)</td>
<td>2 (50)</td>
<td>0</td>
<td>2 (28.57)</td>
</tr>
</tbody>
</table>

Figure 2. Antimicrobial Resistance Patterns Among VRE E. faecium in Different Units of HWTP

So that chlorination plays an undeniable role in VRE removal. In a study done in USA also methicillin resistant *staphylococcus aureus* (MRSA) and VRE were identified in a wastewater treatment plant, in which chlorination has not been done (8). In addition, the presence of enterococci, which are sensitive to vancomycin is very important as it has bad consequences for public health and the environment. According to past studies, enterococci can stay alive for a long time in stool and tolerate warm weather and, in turn, these situations can result in the creation of resistance in bacteria (13).

In the current study, *E. faecium* was the most isolated species, which constituted 54.3% of all isolated species. In most studies conducted in Europe and Iran, *E. faecium* has had the highest prevalence (8, 27, 28). Increasing the presence of *E. faecium* in environmental samples has been reported in all around the world. It has claimed that the strains of *E. faecium* have a higher ability to gain resistance to different drugs; thus it is considered as a strong hospital pathogen around the world (27).

Based on international guidelines (CLSI), a combination of the antibiotic of Ampicillin and an aminoglycoside antibiotic (including Gentamicin, Streptomycin, and Kanamycin) is used for treatment of Enterococcal infections (29). In the present study, it was found that the amount of resistance to this antibiotic has an upward trend. Therefore, it is entirely required to pay more attention to antibiotic prescription. The findings showed that the least percentages of resistant *Enterococcus faecium* resistant to Ampicillin, Streptomycin, and Gentamicin, identified in different parts of the plants are approximately: 100%, 75%, and 35% respectively. Enterococcal strains, which are multidrug resistant, particularly *E. faecalis* and *E. faecium*, are considered as a major problems in treatment of Enterococcus infections owing to unsuitable prescription of antibiotics. All 9 VREs isolated from wastewater had multidrug resistance. Since multidrug resistance leads to a limitation in treatment of infections stemming from VRE, it is entirely vital to pay more attention to persons, particularly plant workers, that are exposure to wastewater.

Although wastewater is collected by piping systems, a small defect in the system can result in the contamination of natural waters through microorganisms and other resistant pathogens (27). Naturally, the health of the staff of wastewater treatment plants and the people, who use the treated wastewater, is very important. The results obtained by different studies have illustrated that the workers of wastewater plants have shown higher disease than ordinary people. The presence of Enterococcus and VRE has been proved in wastewater treatment plants; thus, the health and safety of workers is threatened by Enterococcus,
VRE, MRSA and 2 pathogens: Legionella and Aeromonas. It is very important to warn workers and staphylococcus about their health \(^{30, 31}\).

Enterococcus species can contaminate soil and water and stay alive for a long time; thus, its spread into the environment should be taken into account \(^{32}\). Furthermore, the resistant enterococci entered the environment through effluents can enter the food chain \(^{33}\). Therefore, closer control and monitoring over wastewater collection, treatment systems, and hospital wastewater plants are very important and, more importantly, irregular prescription of antibiotics should be prevented.

4. Conclusions

The present study dealt with the detection and removal of VRE in hospital wastewater in Hamadan City, Iran. The results indicated the presence of isolated VRE. \textit{faecium} at different units of the plant, which had multidrug resistant. Specifically, they were resistant to some antibiotics such as: Gentamicin, Aminoglycoside, and Streptomycin used for treatment of VRE infections. All workers and staphylococcus in wastewater treatment plants are in danger of exposure to VRE. We conclude that the process of chlorination plays a basic role in VRE removal; thus, the proper functioning of this unit should be thoroughly controlled.

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Footnote

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References


