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RESEARCH ARTICLE

# High Frequency of Vancomycin-Resistant Enterococci in Sewage and Fecal Samples of Healthy Carriers

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**Abstract:**

***Objectives:***

Enterococci are part of the microbial flora of the gastrointestinal tract of animals and human and can be released into the environment through fecal materials. These microorganisms play an important role in the dissemination of antibiotic resistance genes. Vancomycin-Resistant Enterococci (VRE) have been obtained in municipal sewage, hospital and agricultural wastes and healthy carriers. The aim of this study was to investigate the frequency of VRE in sewage and fecal samples of healthy carriers.

***Methods:***

This study was performed on fecal specimens of 100 healthy carriers and 100 samples of sewage in Zanjan Province. Fecal and sewage samples were cultured on Trypticase Soy Agar and biochemical tests were performed for Enterococci identification. Antimicrobial susceptibility testing was performed as CLSI guidelines and vancomycin resistance was determined using the agar dilution method.

***Result:***

Of 200 cultured samples, 141 isolates of Enterococci were detected. 64 isolates were detected from fecal and 77 were isolated from the sewage samples. Antibiotic resistance profile of fecal isolates was as follows: tetracycline (57.8%), ciprofloxacin (54.7%), phosphomycin (54.7%), erythromycin (51.5%), chloramphenicol (12.5%), amoxicillin (21.8%) and gatifloxacin (23.5%). Also, for the sewage samples, the most resistance of antibiotic was detected against ciprofloxacin (76.6%) followed by tetracycline (74%), erythromycin (68.8%), and phos-phomycin (61%). According to Agar dilution method, among 141 isolates of Enterococci, 15 (10.6%) isolates were vancomycin resistant, 11 of sewage isolates (14.3%) and 4 of the carrier isolates (6.2%).

***Conclusion:***

Our study describes the high frequency of VRE in municipal sewage and healthy carriers. Regarding the importance of VRE strains in the clinical and environmental settings, it seems necessary to address the issue.

**Keywords:** Enterococci, Healthy carrier, Sewage, Vancomycin resistant, Agricultural wastes, Antimicrobial susceptibility, Environment.

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|---|---|---|---|

## 1. INTRODUCTION

Enterococci are opportunistic and nosocomial human pathogens that can cause urinary tract infections, burn wound and surgical site infections, bacteremia and sepsis, endocarditis, cholecystitis, peritonitis, neonatal meningitis, and others [1, 2]. *Enterococcus faecalis* is one of the most frequently isolated microbial flora in the human gastrointestinal tract, while *Enterococcus faecium* isolated with less frequency [3]. The emergence of Multidrug-Resistant (MDR) Enterococci has become a serious problem in healthcare settings worldwide [4]. The intrinsic resistance to various antimicrobials and dissemination of resistant genes by horizontal transfer are currently thought to play a major role in the development of MDR Enterococci. These MDR isolates are associated with increased mortality and costs due to prolonged hospitalization, need for surgery and prolonged treatment with antibiotics [5].

A common regimen for treatment of serious enterococcal infections is the synergistic combination of cell wall inhibitors as penicillin, ampicillin or vancomycin with aminoglycosides such as streptomycin or gentamycin. However, Vancomycin Resistant Enterococci (VRE) have caused significant problems

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for antibiotic therapy [6]. VRE were first isolated in the UK and France in 1986. Since that time, hospital outbreaks of VRE have been reported from the UK, Ireland, Italy and Finland [7]. VRE carriage has also been reported among out-patients in Germany and patients that newly admitted to hospital in France [8, 9]. According to Van der Auwera *et al*., fecal cultures of 11 (28%) out of 40 healthy individuals who were not health care staff and who had not taken antibiotics in the previous year showed a heterogeneous collection of vancomycin-resistant *E. faecium* [10]. In the Netherlands, 5 to 10% of healthy people were colonized with VRE and a study in a cattle-rearing region in France revealed that 11.8% of non-hospitalized people and 37% of hospitalized patients were carriers of VRE [11 - 13].

Enterococci are part of the microbial flora of the gastro-intestinal tract of animals and human and can be released into the environment through fecal materials [14]. The sewage contains various materials, such as antimicrobial agents, patho-genic and saprophytic microorganisms, organic materials, nutrients *etc*., that facilitates the Enterococci survival and results in Enterococcal release into the environment [15, 16]. Anti-biotics are extensively used for human therapy, animal farming and agricultural purposes. These applications made antibiotics to be released in large amounts in natural ecosystems and this is maybe the main reason for the high incidence of antibiotic resistance detected in environmental bacteria [13, 17]. Resi-dues of antibiotics and antibiotic resistance genes were detec-ted from human environments and farms and they must be considered as important pollutants [13]. Dissemination of resis-tant genes among microbial flora and pathogenic bacteria in sewage can play a major role in the development of MDR isolates [16]. Methods to reduce the amount of antimicrobial agents and a load of resistant bacteria in wastewaters originated from hospitals and farms include the optimization of dis-infection procedures and management of wastewater and manure [17]. Vancomycin-resistant Enter-ococci were reported in sewage and wastewater treatment plants in previous studies [14, 15]. These resistant isolates may be transmitted to human through contaminated water, food or vegetables [14, 17].

Regarding the vancomycin resistance in Enterococci iso-lates may result in emerging and persistence of nosocomial infections, the present study aimed to investigate the frequency of VRE in sewage and fecal samples of healthy carriers.

## 2. MATERIALS AND METHODS

### 2.1. Bacterial Isolation and Identification

From June to October 2017, a total of 200 individual samples of sewage and fecal including 100 fecal specimens of healthy carriers and 100 samples of urban sewage water were collected randomly in Zanjan Province. Stool specimens were collected from healthy people (carriers) with no history of diarrhea and antibiotic therapy for at least 1 month. The age of healthy people was between 15 to 50 years. Sewage samples were collected in sterile bottles from six different urban sewage waters and kept at 4°C until processing. All samples were cultured onto Trypticase Soy Agar (Merck, Germany) supp-lemented with 5% of sheep blood and incubated at 37°C for 24h. All isolates were phenotypically identified to the genus level using conventional biochemical tests as gram staining, catalase, growth on bile esculin agar, growth on 6.5% of NaCl, growth on 45°C and PYR. Confirmation of presumptive Enterococci isolates was performed using PCR, targeting the 16S rRNA gene with the following primers and amplicon size 733 bp: E1 (5'-TCAACC GGGGAGGGT-3') and E2 (5'-ATTACTAGCGATTCCGG-3') [18]. Verified isolates of Enter-ococci were preserved at −70°C in Trypticase Soy broth (Merck, Germany) containing 20% (v/v) glycerol for further analysis.

### 2.2. Antimicrobial Susceptibility Testing

Susceptibility of isolates to the following antibiotics was examined using the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines: Chloramphenicol (30 μg), Fosfomycin (200 μg), Erythromycin (15 μg), Gatifloxacin (5 μg), Ciprofloxacin (5 μg), Amoxicillin (10 μg) and Tetracyclin (10 μg). Multidrug resistance (MDR) was defined as resistance to three or more different classes of antibiotics.

### 2.3. Minimum Inhibitory Concentration Determination of Vancomycin

The MIC of vancomycin was determined using the Agar dilution method according to CLSI guidelines. Blood agar media with two-fold serial dilutions of vancomycin (256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5 and 0.25 μg/ml) were prepared. Then, the media were inoculated with Enterococci isolates overnight culture containing $5 \times 10^6$ CFU/mL and were incubated at 37°C for 24 h. The MIC was calculated as the lowest concentration that inhibited visible growth of the organism.

### 2.4. Statistical Analysis

The data were analyzed with SPSS version 17.0 software (SPSS, Inc., Chicago, IL). A chi-square test was used to determine the statistical significance of the data. A *P*-value of $< 0.05$ was considered significant.

## 3. RESULTS

### 3.1. Identification of Enterococci

From 200 cultured samples of sewage and fecal, 141 Enterococci were isolated. 64 (64%) isolates were detected from fecal samples of carriers and 77 (77%) were isolated from the sewage water samples. All Enterococci isolates were identified using biochemical tests and 16S rRNA. Of 100 healthy people participated in our study, 51 (51%) were younger than 30 years, 36 (36%) were 30-40 years and 13 (13%) were 40-50 years. The sex distribution was 56 (56%) female and 44 (44%) male.

### 3.2. Susceptibility to Antimicrobial Agents

Antibiotic resistance of 77 Enterococci isolated from urban sewage water samples and 64 Enterococci isolated from fecal samples of healthy carriers were determined by the disk diffu-sion method. Antimicrobial resistance patterns of isolates are presented in Tables **1** and **2**. In all, 135 (135/141, 95.7%) isolates were resistant to one or more of tested antimicrobial

**Table 1. Antimicrobial resistance of 77 Enterococci isolated from sewage samples.**

| Antimicrobial Agents | No. (%) of Resistant | No. (%) of Intermediate | No. (%) of Susceptible |
|---|---|---|---|
| Erythromycin | 53 (68.8) | 23 (29.9) | 1 (1.3) |
| Amoxicillin | 31 (40.2) | 5 (6.5) | 41 (53.2) |
| Chloramphenicol | 24 (31.2) | 3 (3.9) | 50 (64.9) |
| Ciprofloxacin | 59 (76.6) | 9 (11.7) | 9 (11.7) |
| Gatifloxacin | 25 (32.5) | 22 (28.6) | 30 (38.9) |
| Tetracycline | 57 (74) | 0 | 20 (26) |
| Fosfomycin | 47 (61) | 3 (3.9) | 27 (35.1) |

agents. Among the sewage isolates, the most antibiotic resistance was detected against ciprofloxacin (76.6%) followed by tetracycline (74%), erythromycin (68.8%), and phosphomycin (61%). Chloramphenicol and amoxicillin showed the highest activity against isolates and 64.9% and 53.2% of isolates were susceptible to these antibiotics, respectively. The most antibiotic resistance in carrier samples was detected against tetracycline (57.8%), ciprofloxacin and phosphomycin (54.7%). Similar to sewage isolates, chloramphenicol and amoxicillin showed the highest activity against carrier isolates of Enterococci.

Prevalence of antibiotic resistance varied among carrier and sewage isolates, and the frequency of resistance to amoxicillin, chloramphenicol and ciprofloxacin in sewage isolates was significantly higher than carriers ($P<0.05$).

Among sewage and carrier isolates, a total of 89 (63.1%) isolates were resistant to at least three different classes of antimicrobial agents and considered as Multidrug Resistance (MDR): 57 of sewage isolates (74%) and 38 of the carrier isolates (59.4%). The most prevalent MDR pattern was resistance to tetracycline, ciprofloxacin and phosphomycin.

### 3.3. Determination of Minimum Inhibitory Concentration of Vancomycin

Vancomycin breakpoint was based on the CLSI cutoff for Enterococci (Resistant, MIC of ≥ 32µg/ml; Susceptible, MIC of ≤ 4; Intermediate, MIC of 8-16). Based on the Agar dilution method, the range of vancomycin MIC for Enterococci isolates varied from 0.25 to 256 µg/ml (Table **3**). Among 141 isolates of Enterococci, 15 (10.6%) isolates were vancomycin resistant: 11 of sewage isolates (14.3%) and 4 of the carrier isolates (6.2%).

## 4. DISCUSSION

The emergence of vancomycin and multiresistant Enterococci, as an important nosocomial pathogen, has become a serious problem in healthcare settings worldwide [4, 16]. Enterococci are usually isolated from the environment, sewage, human and animals faeces. Treatment of VRE infections is complicated in USA, European and Asian countries such as Turkey, India and Iran [16, 19, 20]. Sewage contains bacterial pathogens causing risks to human health unless measures are taken to control the hazard. Enterococci as fecal bacteria have an excellent ability for adaption to adverse environmental conditions [21]. In our study, 95.7% of sewage and fecal Enterococci were resistant to one or more antimicrobial agents and 63.1% were multidrug resistant. The most frequent resistance in sewage Enterococci was found against ciprofloxacin (76.6%) and tetracycline (74%). High-level resistance to tetracycline and ciprofloxacin have been reported in previous studies [14, 22]. The high incidence of tetracycline resistance find in this survey is most probably due to the widespread use of tetracycline in animal feed in our country which eventually could end-up in sewage [22]. In a study conducted by Cupáková and Lukášová in Sweden, more than 95% of sewage isolated Enterococci were resistant to more than one antibiotic [23]. Borhani and colleagues also reported that all or most of sewage Enterococci were resistant to 5 or 6 antibiotics. They have isolated VRE from sewage on different occasions in Tehran [21]. Recovery of sewage VRE has been shown in other studies. High prevalence of vancomycin-resistant Enterococci (60%) in untreated sewage samples was reported in Swedish [14]. The use of avoparcin glycopeptide as a feed additive is considered to have created a pool of VRE in Europe [14]. In the USA and Portugal, the rate of VRE isolation from farm wastewater and hospital sewage samples

**Table 2. Antimicrobial resistance of 64 Enterococci isolated from fecal samples.**

| Antimicrobial Agents | No. (%) of Resistant | No. (%) of Intermediate | No. (%) of Susceptible |
|---|---|---|---|
| Erythromycin | 33 (51.5) | 10 (15.6) | 21 (32.8) |
| Amoxicillin | 14 (21.8) | 0 | 50 (78.2) |
| Chloramphenicol | 8 (12.5) | 3 (4.7) | 53 (82.8) |
| Ciprofloxacin | 35 (54.7) | 18 (28.1) | 11 (17.2) |
| Gatifloxacin | 15 (23.5) | 16 (25) | 33 (51.5) |
| Tetracycline | 37 (57.8) | 0 | 27 (42.2) |
| Fosfomycin | 35 (54.7) | 5 (7.8) | 24 (37.5) |

**Table 3. The range of vancomycin MIC for sewage and carrier isolates.**

| Vancomycin MIC | | |
|---|---|---|
| **MIC (µg/ml)** | **No. (%) of Sewage Isolates** | **No. (%) of Carrier Isolates** |
| **0.25-0.5** | 19 (24.7) | 21 (32.8) |
| **1-2** | 35 (45.4) | 24 (37.5) |
| **4** | 12 (15.6) | 15 (23.4) |
| **64** | 8 (10.4) | 3 (4.7) |
| **128** | 2 (2.6) | 1(1.5) |
| **256** | 1 (1.3) | 0 |

has been reported 6% and 20%, respectively [24, 25]. In our study, vancomycin-resistant Enterococci in sewage samples (14.3%) were detected with higher frequency than some previous studies in Iran [3, 22]. Talebi and colleagues were investigated the occurrence, stability and antibiotic resistance of 593 Enterococci in six samples collected from three urban Sewage Treatment Plants (STPs) in Tehran, Iran. According to their results, the most prevalent species in all three STPs were *E. faecium* followed by *E. hirae* and *E. faecalis* and 45% of isolates were resistant to 1 or more tested antibiotics [22]. These resistant Enterococci could pass through STPs to surface waters. As reported in previous studies, antibiotic resistant enterococci in the environment could serve as a resistance genes reservoir and can be transferred to other bacterial pathogens [22]. Furthermore, the presence of vancomycin-resistant Enterococci in sewage, farm animals, municipal and hospital STPs, suggesting that VRE can be transmitted to humans through the food chain and water [14, 22, 24].

VRE carriage has been reported among out-patients in Germany and patients that newly admitted to hospital in France [8, 9]. We detected VRE in 6.2% of fecal samples of healthy carriers. In a study conducted by Tabatabaei and colleagues in Iran, fecal VRE colonization in hospitalized children was 16.9% [26]. The prevalence of fecal VRE colonization among kidney transplant patients was reported 13.6% in Brazil [5]. Some previous studies have also reported a high frequency of VRE in carriers: 11% in French [12], 12% in Germany [8], and 28% in a Belgium [10]. According to Gambarotto and colleagues study, a total of 37% of the hospitalized patients and 11.8% of the subjects from the community were VRE carriers [12]. National survey data have indicated the prevalence of VRE in 0-59% of isolates in 126 adult ICUs from 60 US hospitals [27]. As confirmed by several previous studies, the presence of VRE in the fecal samples of humans in the community suggests that VRE are part of the gut microbial flora and can be acquired in the community [8, 10 - 12]. A possible source of VRE in fecal carriers could be the consumption of contaminated food, as VRE has been reported in farm animals and animal product-based foodstuffs. Furthermore, previous use of vancomycin or broad-spectrum cephalosporins, prolonged hospitalization and chronic dialysis were reported as important risk factors for VRE colonization [5, 11, 12].

Chloramphenicol showed the highest activity against isolates and 64.9% of sewage and 82.8% of fecal isolates were susceptible to chloramphenicol. Similar results have been reported by Talebi and colleagues from Iran [22]. The low incidence of chloramphenicol resistance most probably due to chloramphenicol is not prescribed as commonly as other antibiotics in Iran [22]. Chloramphenicol resistance was also reported with low frequency in Switzerland and New Zealand [28, 29].

## CONCLUSION

According to our results, 14.3% and 6.2% of sewage and fecal samples were carried vancomycin-resistant Enterococci, respectively. Due to increasing resistance rate of Enterococci to vancomycin and most common antibiotics, appropriate surveillance and control measures are essential to reduce the amount of antimicrobial agents and a load of resistant bacteria in wastewaters.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

## HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

## CONSENT FOR PUBLICATION

Not applicable.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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