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# A Study on the Prevalence of Vancomycin-resistant *Enterococci* and Their Antibiotic Resistance Pattern in Recreational Waters in Guilan Province, Iran

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## ABSTRACT

**Background and Aim:** *Enterococcus faecalis* is a major opportunistic pathogen that causes nosocomial infections in humans, especially in immunocompromised and elderly people. This bacterium can survive and grow in harsh conditions and low-nutrient environments, so it is usually found in water and can easily be transmitted via the fecal-oral route. Due to the high usage of antibiotics, many antibiotic-resistant strains of *E. faecalis* have been evolved, especially vancomycin-resistant ones (VRE). Water-borne VRE is an environmental and health problem. Since the monitoring of recreational waters is so important in human health, the aim of the present study was to investigate the prevalence of VRE isolates and their antibiotics patterns in the environmental samples from recreational waters in Guilan Province, Iran.

**Materials and Methods:** The environmental samples were obtained from recreational waters in six cities in Guilan Province, North of Iran, 4 stations in Anzali wetland, and 5 main rivers entering Anzali wetland from January to September 2019. *E. faecalis* samples were identified by microscopic analysis, biochemical tests, and molecular identification. Antibiotic resistance patterns of the isolates were determined by an antibiogram test. The molecular identification of the isolates was performed using polymerase chain reaction (PCR) with specific primers for the *ddlE* gene.

**Results:** Overall, in 268 samples, *Enterococci* were detected in 154 samples (57.46%), of which 35 isolates (29.68%) were VRE. From VRE isolates 32 isolates (91.42%) belonged to *E. faecalis*, 2 isolates (5.71%) belonged to *E. faecium,* and one isolate (2.86%) belonged to other *Enterococcus* species.

**Conclusion:** This study shows the high prevalence and antibiotic resistance rate of VRE strains of *E. faecalis* in water resources in Guilan province, which can be alarming and needs to be considered.

**Keywords:** Antibacterial drug resistance, *Enterococcus faecalis*; Vancomycin-resistant *Enterococci*; Water pollution

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## 1 Introduction

*Enterococci* are widely distributed in the environment. These bacteria are adequately tolerant to harsh conditions. They can survive and grow in a low-nutrient environment, so they are present almost everywhere, from water to soil, sewage, air, etc. **(1)**. Their primary source is humans' and warm-blooded animals' intestines **(2)**; however, they can resist outside the intestine for long periods **(3)**. Since they are resistant to different conditions such as low pH, freezing, moderate heat treatment, etc., they have been considered a contamination indicator for some types of food products as well as water **(4, 5)**. These bacteria are not highly pathogenic and cause nosocomial infections, such as urinary tract infections, intra-abdominal infections, endocarditis, and bacteremia **(6)**. *Enterococcus faecalis* (*E. faecalis*) and *En-*

*terococcus faecium* (*E. faecium*) are the most isolated *Enterococcus* species in clinical samples **(7, 8)**.

The opportunistic pathogen *Enterococcus faecalis* (*E. faecalis*) causes nosocomial infections in humans, especially in immunocompromised and elderly people **(9)**. Due to its ability to survive and grow in limited nutrients and various environmental conditions, the bacterium is usually found in water and can easily be transmitted via the fecal-oral route.

High consumption of antibiotics is an important driver for the increasing antibiotic resistance, which is one of the greatest threats to public health globally **(10-12)**. First reports on vancomycin-resistant *Enterococci* (VRE) emerged in the 1980s **(13)**. Since then, VRE strains have been isolated from patients as well as environmental samples.

Since vancomycin is the first-line drug for treating multi-drug resistant *Enterococci*, the epidemiology of VRE in the environment is of great importance. Indeed, the determination of the antibiotic resistance pattern of the VRE isolates is essential to tackle the spread of the strains. In this regard, in the present study, we investigated the prevalence of vancomycin-resistant isolates and their antibiotic-resistant patterns in the environmental samples from recreational waters in Guilan Province, Iran.

## 2.Materials and Methods

### Sample Collection

In this cross-sectional study, sampling was performed from January to December 2019 in Guilan province, Iran. Samples were obtained from 20 natural swimming places, 4 stations in 4 main areas of Anzali wetland, and 5 main rivers entering the wetland, whose details are presented in Table 1. In each season, 40 samples were collected from 20 swimming places (160 in total in one year), 12 samples were collected from 4 main areas of Anzali wetland (3 samples from each region and a total of 48 samples in one year), and 15 samples were obtained from 5 rivers entering Anzali wetland (3 samples were collected at specified intervals and a total of 60 samples in one year) (Table 2). Collectively, 268 samples were obtained and entered the study.

**Table 1.** Sampling sites' names and locations.

| Sampling sites | City/Town | Site's name | Location (UTM*) |
|---|---|---|---|
| Swimming Places | Astara | Sadaf beach resort | 39S E0314219N4246168 |
| | Astara | Safir e omid beach resort | 39S E0315016 N4254644 |
| | Chobar(Talesh) | Beach park | 39S E0316030 N4229067 |
| | Goruq (Talesh) | Goruq beach resort | 39S E0321687N4189920 |
| | Gisum (Talesh) | Gisum beach | 39S E0328401 N4171642 |
| | Anzali | Matin beach | 39S E0355253N4151996 |
| | Anzali | Pasdaran peach park | 39S E0358072N 4150927 |
| | Anzali | Sahel e ghoo beach resort | 39S E0367956 N4148400 |
| | Anzali | Taleb abad beach | 39S E03721116N4147603 |
| | Anzali | Jafrud beach | 39s E0383284N4146271 |
| | Rasht | Morvarid-e-khazar beach resort | 39S E0389521N4145553 |
| | Rasht | Haji bekandeh beach | 39S E0393849N4145223 |
| | Rasht | Amin abad beach | 39S E0395187N4145153 |
| | Kiashahr | Kiashahr beach | 39S E0409042N4143641 |
| | Astane-e-ashrafieh | Asgarabad beach | 39S E0417999N4140070 |
| | Lahijan | Saharkhiz beach resort | 39S E0431430N4131561 |
| | Langarud | Chamkhaleh | 39S E0434659N4120964 |
| | Rudsar | Taraneh e darya beach resort | 39S E0437608N4112988 |
| | Kelachay | Negin e shomal beach resort | 39S E0448873N4102317 |
| | Chaboksar | Gole e sorkh beach resort | 39S E0458530N4094627 |

| Sampling sites | City/Town | Site's name | Location (UTM*) |
|---|---|---|---|
| Stations in Anzali wetland | Anzali | Outlet chanel | 39S E0364283N4148079 |
| | Anzali | West (abkenar) | 39S E0358114N4146039 |
| | Anzali | Central ( Sorkhankol) | 39S E0363128N4143754 |
| | Rasht | East (pirbazar) | 39S E0367428N4140646 |
| | Rasht | Pirbazar | 39S E0369161N4136398 |
| Rivers entering Anzali wetland | Rasht | Psikhan | 39S E0367921N 4139878 |
| | Someh sara | Masouleh Rudkhan | 39S E0355186N4137506 |
| | Someh sara | Siah Darvishan | 39S E0359776N 4139371 |
| | Someh sara | Morghak and Khalakai | 39S E0347523 N4142832 |

* UTM: Universal Transverse Mercator

**Table 2.** The number of samples collected from different sites in different seasons.

| Season / Site | Spring | Summer | Fall | Winter | Total |
|---|---|---|---|---|---|
| **Swimming Places** | 40 | 40 | 40 | 40 | **160** |
| **Stations in Anzali wetland** | 12 | 12 | 12 | 12 | **48** |
| **Rivers entering Anzali wetland** | 15 | 15 | 15 | 15 | **60** |
| **Total** | 67 | 67 | 67 | 67 | **268** |

### Bacterial Isolation and Detection

Following the sampling, the most probable number (MPN) method was used to estimate the concentration of viable *Enterococci* in water samples utilizing replicate growth in Azide Dextrose Broth (Merck, Germany) (14). The grown bacteria were transferred to Pfizer Selective *Enterococcus* (PSE) Agar for confirmatory testing. After 24 hours of incubation at 35°C, the formation of blackish-brown colonies was considered *E. faecalis*. Then, biochemical tests, including esculin hydrolysis test, growth in Trypticase Soy Broth (TSB) medium containing 6.5% salt (NaCl), bile tolerance test, heat resistance test, growth at temperatures of 10°C and 45°C, growth at pH=9.6, tellurite reduction, motility test, H2S production, and sugar fermentation tests.

### Antimicrobial Susceptibility Testing

For antibiotic susceptibility determination of the isolates, the Kirby-Bauer disk diffusion method on Mueller-Hinton agar was performed based on the guidelines of the Clinical and Laboratory Standards Institute (CLSI) **(15, 16)**. Standard antimicrobial drugs (Mast, UK), including vancomycin (30 µg), kanamycin (30 µg), streptomycin (30 µg), erythromycin (15 µg), amikacin (30 µg), ampicillin (10 µg), gentamicin (120 µg), ciprofloxacin (5 µg), and chloramphenicol (30 µg) were used for this aim. *E. faecalis* (ATCC 10541) was used as a control to standardize antibiotic susceptibility testing.

### Molecular Confirmation of VREs

Using ExiPrep Plus Bacteria Genomic DNA Kit, genomic DNA was extracted according to the manufacturers' instructions. Then, the quality of the extracted DNA was determined using agarose gel electrophoresis.

For molecular identification of the *E. faecalis* isolates, a 475-bp fragment corresponding to the *ddlE* gene was amplified using specific primers. The forward and reverse primers sequence was: 5'-CACCTGAAGAAACAGGC-3', and 5'-ATGGCTACTTCAATTTCACG-3', respectively **(17)**. *E. faecalis* JH2-2 reference strain was used as the control. The PCR reactions were set up according to the manufacturer's recommendations. Each reaction contained 1X PCR buffer, 1.5 mM of MgCl2, 1 µL of the isolated bacterial DNA, 0.2 mM of each dNTPs, and 0.25 µM of each primer. The cycling conditions were set to 95°C for 2 min, followed by 45 cycles at 95°C for 5 s, 50°C for 30 s, and 72°C for 1 min.

## 3.Results

### Sample Collection

Based on morphological, microscopic, and biochemical traits, from 268 samples, 154 were identified as *Enterococcus* spp. Isolated *Enterococci* formed blackish-brown colonies on PSE Agar and Bile Esculin Agar media (Figure 1A). Gram staining and micro-

scopic observation of the isolates showed single or short chains of Gram-positive cocci (Figure 1B). The total number of *Enterococci* counted in this study is illustrated in Table 3.

**Table 3.** The number of *Enterococci* bacteria isolated from samples collected from each site in each season.

| Season / Site | Spring | Summer | Fall | Winter | Total |
|---|---|---|---|---|---|
| **Swimming Places** | 39 | 42 | 107 | 95 | 283 |
| **Stations in Anzali wetland** | 457 | 299 | 389 | 538 | 1683 |
| **Rivers entering Anzali wetland** | 290 | 222 | 249 | 222 | 983 |
| **Total** | 786 | 563 | 745 | 855 | 2949 |

### Antibiotics Susceptibility Tests

The antibiotic susceptibility pattern of isolated *Enterococcus* species showed that 100% of isolates from swimming pools were resistant to streptomycin. The prevalence of the resistant isolates to the evaluated antibiotics was as follows: ciprofloxacin 22.2%, gentamicin 56.5%, erythromycin 21.2%, kanamycin 77.7%, chloramphenicol 18.1%, ampicillin 14.1%, and vancomycin 35.3%.

Isolated *Enterococcus* species from rivers leading to Anzali wetland had the antibiotic-resistance pattern as follows: streptomycin 96.1%, ciprofloxacin 19.2%, gentamicin 57.6%, erythromycin 23%, kanamycin 73%, chloramphenicol 15.3%, ampicillin 19.2%, and vancomycin 46.1%.

Antibiotic susceptibility pattern of isolated *Enterococcus* species from Anzali wetland showed that 96.6% of isolated *Enterococci* were resistant to streptomycin, 34.4% were resistant to ciprofloxacin, 55.1% were resistant to gentamicin, 31% were resistant to erythromycin, 58.6% were resistant to kanamycin, 24.1% were resistant to chloramphenicol, 20.6% were resistant to ampicillin, and 41.3% were resistant to vancomycin (Table 4).

**Table 4.** Antibiotic susceptibility among *E. faecalis* isolates.

| Antibiotics / Site | S (No; %) | CIP (No; %) | GEN (No; %) | ERY (No; %) | KAN (No; %) | C (No; %) | AMP (No; %) | VAN (No; %) |
|---|---|---|---|---|---|---|---|---|
| **Swimming Places** | 100 | 22.2 | 56.5 | 21.2 | 77.7 | 18.1 | 14.1 | 35.3 |
| **Stations in Anzali wetland** | 96.6 | 34.4 | 55.1 | 31 | 58.6 | 24.1 | 20.6 | 41.3 |
| **Rivers entering Anzali wetland** | 96.1 | 19.2 | 57.6 | 23 | 73 | 15.3 | 19.2 | 46.1 |

Abbreviations: S: Streptomycin; C: Chloramphenicol; CIP: Ciprofloxacin; GEN: Gentamicin; ERY: Erythromycin; KAN: kanamycin; AMP: ampicillin; VAN: vancomycin

### Molecular Identification of the Isolates:

As mentioned previously, the *ddlE* gene was amplified to identify vancomycin-resistant *E. faecalis* isolates molecularly. Figure 1A represents the DNA extraction from the isolates, and Figure 1B shows the results of the *ddlE* gene amplification. As shown in Figure 1, the *ddlE* gene has been amplified in VRE isolates but not in the control strain. As the positive control for PCR reaction, we used primers that could amplify a 500 bp segment of the *fliC* gene of *Salmonella typhi* (*S. typhi*).

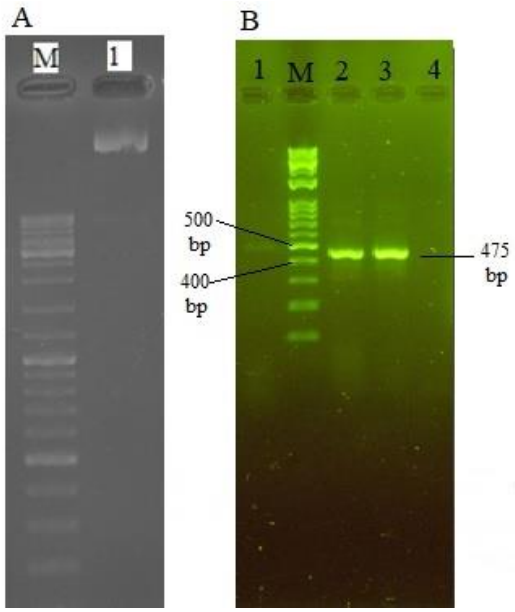


**Figure 1.** A (Left image): Genomic DNA Extraction, M: 1 kb DNA Marker, 1: bacterial DNA; B (Right image): Amplification of *ddlE* gene by PCR, 1: positive control (a segment of *fliC* gene of *S. typhi*) sample, M: 1 kb DNA Ladder, 2. *ddlE* gene from one of the test samples, 3. *ddlE* gene from an *Enterococci* strain (*E. faecalis* (ATCC 10541), 4: negative control

## 4.Discussion

*Enterococci* are common pathogens that cause severe nosocomial infections **(18-20)**. In the present study, *Enterococci* isolates were obtained from environmental samples from recreational waters, mainly swimming places in Guilan Province, Iran. We chose to investigate these places because many people, both travelers, and natives, go there for swimming, and therefore, monitoring the microbial load of these places is very important.

We observed a relatively high resistance level to different antibiotics, including vancomycin, kanamycin, streptomycin, erythromycin, amikacin, ampicillin, gentamicin, ciprofloxacin, and chloramphenicol, among these isolates. Of the isolates, 46.1% were resistant to vancomycin, which is relatively high. Almost all strains ( 98%) were resistant to streptomycin. Among the studied antibiotics, ampicillin and chloramphenicol had the highest antibacterial effect on the isolates, 18%, and 19%, respectively.

The prevalence of the VREs and their antibiotic-resistant patterns among clinical as well as environmental samples is variable in different regions **(21-24)**. Same as the present study, Alipour *et al.* examined the presence of *Enterococcus* spp. as well as their antibiotic resistance patterns in samples from a river and coastal waters in Mazandaran Province, Iran. Of 70 isolated *Enterococci*, 68.6% and 20% belonged to *E. faecalis* and *E. faecium*, respectively. They reported a high resistance rate to chloramphenicol, ciprofloxacin, and tetracycline **(25)**. In 2019, Mazaheri *et al.*, investigated the prevalence of VREs among dried vegetable samples in Tehran, Iran, which they found that 48% of the isolates were VREs **(26)**. In a study by Roberts *et al.* on the prevalence of VREs in crows and their environment, 24.5% and 55% of the crows and environmental samples, respectively, were VRE positive **(27)**.

Rezvani *et al.* studied the prevalence of *Enterococcus* spp. and their antibiotic-resistant patterns in gastroenteritis patients. They found *Enterococcus* spp. in 37% of samples; most of them belonged to *E. faecalis* (91 %), and 9 % belonged to *E. faecium*. The prevalence of VREs among *Enterococci* isolates was relatively low (6 %), all of which belonged to *E. faecalis* **(28)**. Zavaryani *et al.* assessed the susceptibility of the clinical *Enterococcus* isolates to five antibiotics, including vancomycin, gentamicin, teicoplanin, fosfomycin trometamol, and quinupristin/dalfopristin among 400 *Enterococcus* species. In their study, teicoplanin and vancomycin were the most effective antibiotics, while Quinupristin/dalfopristin was the least effective against the clinical samples **(29)**.

Khanmohammadi *et al.* investigated the prevalence of VREs among two different sets of samples, fecal and clinical samples. The rate of VREs among fecal samples (52%) was higher than in clinical isolates (32%) **(30)**. As can be seen, different results are being reported from different samples. This variation can be related to the types of samples (clinical or environmental), geographical location of sample collection, the treatment strategies exploited in each region (high rate of antibiotics consumption), sewage disposal, etc. However, due to the high spread rate of antibiotic-resistant bacteria and the horizontal transfer of antibiotic-resistant genes, it is a real public health concern.

In the present study, the *E. faecalis*-specific ddl gene (ddlE. faecalis) was used as the specific gene to identify *E. faecalis* strains **(31)**. *ddl* encodes D-alanine:D-alanine ligases and related glycopeptide resistance proteins **(32)**. D-alanine--D-alanine ligase is an essential enzyme for the peptidoglycan biosynthesis. This enzyme dimerizes D-Ala before its incorporation in peptidoglycan precursors **(33)**. Mutation in this gene results in the deficiency of bacterial growth. This gene is widely used for the identification of different bacterial species, including various species of *Enterococci* as well as other species **(34-40)**.

## 5. Conclusion

A relatively high prevalence of VREs in the studied places poses a serious epidemiological threat and a

risk to public health. The possibility of horizontal gene transfer among the bacteria may transmit resistant genes from VREs to other bacteria; therefore, it is necessary to think about the necessary measures to determine the source of the pollution and at the same time prevent swimming in places with a high level of bacterial pollution until the problem is addressed.

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## Author Contribution

H.K. and M.F.G. carried out the experiment. H.K. wrote the manuscript with support from A.H. K.I and A.H. supervised the project.

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None.

## Conflict of Interest

The authors declared that there is no conflict of interest regarding this article.

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