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Isolation and identification of *Enterococcus faecalis* with vancomycin resistant gene from Ghalerudkhan River, Iran, 2022

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Abstract

Vancomycin-resistant enterococci (VRE) are highly concerning bacteria listed on the Global Priority List of Antibiotic-Resistant Bacteria by the World Health Organization. Their resistance to antibiotics poses a significant threat to public health due to environmental pressures. This bacterium can survive and grow in harsh conditions and low-nutrient environments, and can easily be transmitted via the fecal-oral route. Water-borne VRE is an environmental and health problem. The aim of the present study was to investigate the prevalence of VRE isolates and their antibiotic patterns in environmental samples from the Ghale Rudkhan River in Guilan Province, Iran. The water samples were collected from the river and tested for the presence of Enterococci using bile esculin azide agar. After collection, the samples were identified using biochemical tests and molecular techniques with specific primers for the ddlE. Antibiotic resistance patterns of the isolates were determined using an antibiogram test. Furthermore, molecular identification of the vancomycin-resistant isolates was conducted using polymerase chain reaction (PCR) with specific primers for the *vanA* and *vanB* genes.Out of 263 samples, Enterococci were found in 154 samples (69%) after a confirmatory ddlE gene PCR analysis. Among these isolates, 130 (84%) were identified as VRE. The results indicated that nearly all of the isolates had vancomycin-resistant genes, with 89 isolates (68.46%) carrying *vanA* and 111 isolates (85.38%) carrying *vanB*. This study reveals that many of the Enterococcal isolates obtained were resistant to streptomycin, kanamycin, gentamicin, and vancomycin. It also found that VRE carrying *vanA* and *vanB* genes are present in the Ghale Rudkhan River and that the sources of VRE are spread throughout the river basin. The high prevalence and antibiotic resistance rate of VRE strains of *E. faecalis* in water resources in Guilan province are concerning and require attention.

Key words: *Enterococcus faecalis;* Antibiotic resistance; VRE; Water pollution

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Introduction

Antibiotics have been instrumental in saving countless lives over the last century (Kandeel et al. 2022). However, overuse and misuse of antibiotics have resulted in the development of bacteria resistant to antibiotics. This has become a significant threat to human health (Nemattalab et al.2022, Rohani et al. 2023). These resistant bacteria are responsible for causing around 700,000 fatalities globally each year (Zhou et al. 2023). Some strains of bacteria are capable of carrying resistance genes through plasmids, which can then be transmitted between various bacterial species. This transmission of genes can lead to a decreased susceptibility to commonly used antibiotics (Im et al. 2023). In the case of Enterococci, these plasmid-mediated genes have contributed to the emergence of Vancomycin-Resistant Enterococci (VRE), which are a major concern in clinical settings (Ferchichi et al. 2021). *Enterococcus* faecalis is a gram-positive anaerobic bacterium that can be a member of a healthy human gut and skin microbiome (Mechmechani et al.2022, Da Silva et al.2022) and a hospital-adapted multidrug-resistant opportunistic nosocomial pathogen (Tatta et al. 2023). *E. faecalis* leads to life-threatening systemic infections such as endocarditis, meningitis, surgical wounds, abdominal and pelvic infections, and urinary tract infections, and can be linked to periprosthetic joint infections (Voit et al.2022, Ali et al. 2022, Zhou et al. 2023). *E. faecalis* is known to develop resistance to various antibacterial agents, including vancomycin, daptomycin, and linezolid. This resistance may be attributed to its thick cell wall (Zhou et al. 2023). Enterococci, especially multidrug-resistant and vancomycin-resistant ones, are major causes of nosocomial infections in immunocompromised patients (Zheng et al. 2009). These bacteria can transfer antimicrobial resistance genes to other pathogenic organisms in the human gut, posing a threat to public health (Mirzaie et al. 2023).

The first-line treatment for systemic Enterococcal infections is glycopeptide antibiotics, primarily vancomycin (Van) and teicoplanin (Tei) (Mwikuma et al.2023). The use of glycopeptide antibiotics, especially vancomycin, has increased significantly in medical institutions. Unfortunately, this has led to the emergence of vancomycin-resistant enterococci (VRE) (Nishiyama et al.2017). These enterococci can coexist in the same area, and it's possible that they can share antibiotic-resistance genes with other members of microbial communities in natural environments such as soil and water (Taylor et al. 2012). Vancomycin-resistant enterococci strains (VRE) have five resistance genes that produce products responsible for resistance to glycopeptide antibiotics. Among these five genes, *vanA* and *vanB* are more common, especially in *E. faecalis* and E. faecium. Strains with *vanA* gene are resistant to both vancomycin and tycoplanin, whereas strains with *vanB* gene are resistant only to vancomycin but sensitive to Teicoplanin(Honarm et al.2012, Moosavian et al.2018).Since vancomycin is the preferred medication for treating multi-drug resistant Enterococci, therefore, understanding the epidemiology of VRE in the environment is crucial and is essential to determine the antibiotic resistance pattern of VRE isolates to combat the spread of these strains (Kalantari et al. 2022). When enterococci resistant to clinically important antimicrobials are present in aquatic environments close to human communities, the risk of opportunistic infection by these bacteria increases both in medical institutions and in the general community. 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 the Ghale Rudkhan River in Guilan Province, Iran.

Material and Methods

Study Design and Samples

In this study, river water samples were collected from the Ghalerudkhan River which flows through Fouman City. Sampling has been performed five times from ten sites of the river and a total of 50 samples in 5 months. Water samples were collected in sterile polyethylene bottles stored on ice in a coolbox and transported to the microbiology laboratory immediately for water and microbial quality analysis. The experiments

were conducted within 5 hours after sampling. Temperature and pH were also determined using a thermometer (PDQ 400; Comark Thermometers, London, England), and a pH meter (Gen wey, London, England) respectively.

Enumeration and isolation of bacteria

The study involved analyzing water samples from the river to detect the presence of *E. faecalis* bacteria. The samples were subjected to a Most Probable Number (MPN) assay, which determined the concentration of *E. faecalis* in the sample. Dilutions of the sample were incubated in tubes containing Azide Dextrose broth and were then placed on Bile Esculin agar. After incubation, colonies with brown halos were identified as *E. faecalis*. The tubes were incubated for 24 to 48 hours at 35°C ± 0.5°C (Nishiyama et al.2015).

Antimicrobial Susceptibility Testing

For the determination of antibiotic susceptibility, the Kirby-Bauer disk diffusion method was used on Mueller-Hinton agar according to CLSI guidelines. Standard antimicrobial drugs (Mast, UK), including erythromycin (15 µg), vancomycin (30 µg), gentamicin (120 µg), ampicillin (10 µg), kanamycin (30 µg), streptomycin (30 µg), amikacin (30 µg), ciprofloxacin (5 µg), and chloramphenicol (30 µg) were used for this aim. To standardize antibiotic susceptibility testing, a control sample of *E. faecalis* (ATCC 10541) was used (Hsueh et al. 2012, Humphries et al. 2021).

Chromosomal and Plasmid DNA Extraction

All chromosomal DNA of *E. faecalis* strains were extracted using a high pure DNA template preparation kit (Roche, Germany) according to the manufacturer's instructions to determine the frequency of *vanA* and *vanB* genes.

Amplification of *vanA* and *vanB* Genes by PCR

PCR analysis was performed to detect vancomycin-resistant genes (*vanA* and *vanB*) using specific primers (Table 1). The PCR reactions were conducted in a final volume of 25 µL, which contained 200 µM of nucleoside triphosphate, 10 pmol of each primer, 1 µg of extracted DNA, and one unit of Taq DNA polymerase. The amplification process involved pre-incubation at 94ºC for five minutes, followed by 30 cycles of denaturation at 94ºC for 60 seconds, annealing at 63ºC for 60 seconds, extension at 72ºC for 60 seconds, and a final extension at 72 ºC for 10 minutes. The PCR products of *vanA* and *vanB* genes were confirmed by electrophoresis analysis on a 1.5% agarose gel containing DNA-safe stain. The DNAs were visualized under UV light, and a 100-bp DNA ladder (Takara, Otsu, Japan) was used as the molecular size marker (Erbas et al. 2016).

Table 1. Primer Sequences Used for the Amplification of vanA and *vanB* Genes

Primer Designation	Primer Sequences	Size of Product, bp
ddlE	5'-CACCTGAAGAAACAGGC-3' 5'-ATGGCTACTTCAATTTCACG-3'	475-bp
vanA	5 '-TCTGCAATAGAGATAGCCGC-3' 5 '-GGAGTAGCTATCCCAGCATT-3'	450-bp
vanB	5'-GGGGGGGAGGATGGTGGGATAGAG-3' 5 '-GGAAGATACCGTGGCTCAAAC -3'	420-bp

Table 2. Antibiotic susceptibility among *E. faecalis* isolates

	S	CIP	GEN	ERY	KAN	C	AMP	VAN
Resistant	96.7	25.43	56.35	18.23	70.16	24.3	14.36	57.45
Intermediate	2.2	4.42	-------	4.97	0.55	3.31	------	14.36
Susceptible	1.1	70.15	43.64	76.8	29.29	72.39	85.64	28.19

Abbreviations: S: Streptomycin; C: Chloramphenicol; CIP: Ciprofloxacin; GEN: Gentamicin; ERY: Erythromycin; KAN: Kanamycin; AMP: Ampicillin; VAN: Vancomycin

2.6. Statistical Analysis

The analysis was conducted using SPSS version 21.0. The significance of differences was determined using Fisher's exact tests. The statistically significant difference was considered at $P < 0.05$.

3. Results

3.1. Bacterial Counts and Water Quality of the Ghale Rudkhan River

A total of 150 water samples, including ground and surface water, were collected from the village communities. The samples were taken from Ghale Rudkhan River and the pH and temperature of the water were measured. Based on differences in their colonial morphologies, 263 potential *Enterococcus* isolates were obtained. All isolates were identified as Gram-positive *Enterococcus* and also met the preliminary identification criteria for *Enterococcus* species. However, only confirmatory ddl gene PCR analysis revealed 69% as E. faecalis isolates.

3.2. Antimicrobial Susceptibility of E. faecalis isolates

In total, 181 E. faecalis isolates were obtained from 263 *Enterococcus* isolates. Among these isolates, the highest resistance rate was observed against Streptomycin (96.68 %), while the lowest rate was against Ampicillin (14.36 %). The results of antibiotic susceptibility testing are presented in Table 2.

3.3. Determination of Vancomycin-Resistant Genes

The study examined 130 Enterococcal isolates obtained from mEI agar or Van-mEI agar to determine the presence of vancomycin-resistant genes (*vanA*, *vanB*) using PCR analysis. The results showed that almost all of the isolates carried the vancomycin-resistant genes; with 89 isolates (68.46%) carrying *vanA* and 111 isolates (85.38%) carrying *vanB*.

In five isolates of vancomycin-resistant *E. faecalis*, neither of the two vancomycin-resistant genes was detected, that it may be the presence of other vancomycin-resistant genes such as *vanD* and *vanE* or their variants.

Discussion

New and improved methods for monitoring microbial pollution in freshwater recreational areas are being developed to provide faster and more reliable results (Saleem et al. 2023). Rivers that flow through areas with varying degrees of human activity may contain harmful microorganisms, including drug-resistant (MDR) ones, which can pose a risk to public health. Therefore, it is important to monitor these waters for safety purposes. The presence of fecal Enterococci and *E. coli*, also known as FIB (fecal indicator bacteria), are reliable indicators of water quality (Gotkowska-Płachta et al. 2023).

E. faecalis is a bacteria found in the intestines of healthy individuals that can survive in different environments such as soil, water, and food products (Cattoir et al. 2022). It can cause serious illness in people with weakened immune systems and has become resistant to many antibiotics (Elashiry et al. 2023). MDR Enterococci pose a significant threat in today's world as they are resistant to most of the latest antibiotics available to doctors in critical situations (Rana et al. 2023, Vase et al. 2023).

In this study, we explored the distribution of VRE and the resistance of *E. faecalis* in the Ghale Rodkhan River basin. Our findings indicated that enterococci were present throughout the basin. This highlights the importance of understanding the distribution of VRE and enterococci resistance to clinical antimicrobials in aquatic environments. These insights can help identify possible transmission routes for antibiotic-resistant bacteria and reveal bacterial dynamics in water environments. The reason we chose to investigate these areas is that they are popular among both native people and tourists for swimming. Therefore, monitoring microbial load in these places is crucial.The results presented in this report were from the analysis of data obtained the isolates are showing a relatively high resistance level to various antibiotics, including vancomycin, kanamycin, streptomycin, erythromycin, amikacin, ampicillin, gentamicin, ciprofloxacin, and chloramphenicol. The resistance to vancomycin is particularly high, with 71.81% of the isolates being resistant to it. Almost all strains (98.9%) showed resistance to streptomycin. Out of all the antibiotics studied, ampicillin

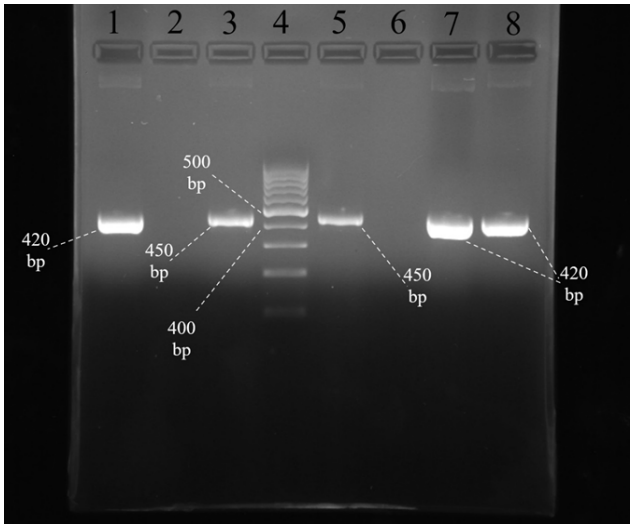

Figure 1. *vanB* gene, 2: negative control, 3: *vanA* gene, 4: 100 bp DNA Marker, 5: *vanA* gene, 6: negative control, 7, 8: *vanB* gene

showed the highest antibacterial effect on the isolates, with a resistance rate of 14.36%. The PCR analysis result showed almost all of the isolates carried the vancomycin-resistant genes, with 89 isolates (68.46%) carrying *vanA* and 111 isolates (85.38%) carrying vanB. The prevalence and antibiotic resistance patterns of VREs in clinical and environmental samples vary across regions (Markwart et al. 2019, Friedman et al. 2019 - Wada et al. 2020). In a study similar to the present one, Alipour et al. investigated the presence of *Enterococcus* spp, and their antibiotic resistance patterns in samples taken from a river and coastal waters in Mazandaran Province, Iran. Out of 70 isolated Enterococci, 68.6% and 20% were identified as *E. faecalis* and *E. faecium*, respectively. The study found a high resistance rate to chloramphenicol, ciprofloxacin, and tetracycline (Alipour et al. 2014). Zavaryani et al. conducted a study to evaluate the susceptibility of 400 *Enterococcus* species to five antibiotics, which were vancomycin, gentamicin, teicoplanin, fosfomycin trometamol, and quinupristin/dalfopristin. The results showed that vancomycin and teicoplanin were the most effective antibiotics against the clinical *Enterococcus* isolates, whereas quinupristin/dalfopristin was the least effective (Zavaryani et al. 2020). In another study, Mazaheri et al. investigated the prevalence of VREs among dried vegetable samples in Tehran, Iran, and found that 48% of the isolates were VREs (Mazaheri et al. 2019). In a study by Roberts et al., they collected and screened 245 samples for acquired *vanA*, *vanB*, and/or intrinsic *vanC1* genes, including 156 from crows and 89 from the environment, to determine the prevalence of VREs. Out of all the samples that were tested, 24.5% of the crow samples and 55% of the environmental/cow samples were found to be VRE-positive. Also, four strains of *vanA E. faecium* and multiple strains of vanC1 E. gallinarum were identified from crows at three locations. In addition, *E. faecium* carrying *vanA*, vanB, and vanC1, and other *Enterococcus* species carrying *vanA* and vanC1 were found in three different environments. All of these *Enterococcus* strains were found to be MDR (Roberts et al. 2019). Rezvani et al. investigated the incidence and antibiotic resistance patterns of *Enterococcus* spp. in patients with gastroenteritis. In the samples tested, *Enterococcus* spp. was found in 37% of them. The majority of these samples were identified as E. faecalis (91%), while the remaining 9% were identified as *E. faecium*. Among the *Enterococcus* isolates, the prevalence of VREs was relatively low (6%). All of the VREs identified belonged to E. faecalis (Rezvani et al. 2016). Khanmohammadi et al. investigated the prevalence of VRE among fecal and clinical samples. The prevalence of VRE in fecal samples (52%) was higher than in clinical isolates (32%) (Khanmohammadi et al. 2018).

Conclusion

The prevalence of VREs in the areas under study is relatively high, which poses a significant epidemiological threat and a risk to public health. There is a possibility of horizontal gene transfer among the bacteria, which may transmit resistant genes from VREs to other bacteria. Therefore, it is essential to take necessary measures to identify the source of the pollution and prevent swimming in areas with high levels of bacterial pollution until the issue is resolved.

Conflicted of Interest

No conflicts of interest

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