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# ANTIMICROBIAL RESISTANCE PATTERNS AND VIRULENCE DETERMINANTS OF CLINICAL ENTEROCOCCUS ISOLATES IN PAKISTAN

**J. Ullah[a], A. Aziz[a], A. Ullah[a], I. Ullah[a], A. Jabbar[a], M. Umair[b], M. Ullah[b], H. Ullah[c], M. Ullah[b], I. Ali[b]**

[a] *The University of Haripur, KPK, Pakistan*
[b] *Khyber Medical University, Peshawar, KP, Pakistan*
[c] *Saidu Group of Teaching Hospitals, Saidu Sharif, Swat, KP, Pakistan*

**Abstract.** *Background*. The current study was designed to determine antibiotic resistance profile,detection of antimicrobial resistance and virulence-related genes among enterococcus species. *Materials and methods*. Altogether, one hundred fifty enterococcal isolates were collected from various clinical specimens and identified by Polymerase chain reaction (PCR). Antibiotic susceptibility testing and MICs of vancomycin were carried out as per CLSI guidelines. A series of PCR reactions were used to screen vancomycin-resistant genes *(vanA*, *vanB*, and *vanD)* and virulence-related genes *(esp, ace, asa1, gelE & cylA)* among VRE enterococcus species. *Results*. The isolated enterococcal strains comprised 62.6% *E. faecalis*, 33.4% *E. faecium*, and 4% of other species. Overall enterococcus showed a high level of resistance; 94% to erythromycin, followed by ciprofloxacin 82.6%, levofloxacin 70%, and vancomycin 16%. The 57.4% of the isolates were recovered from hospitalized patients and 96% of the enterococcus isolates were multi-drug resistant. The MICs of vancomycin-resistant strains remained in the range of 32 μg/ml to 256 μg/ml for the majority of the isolates. The vancomycin-resistant phenotypes *vanA*, *vanB*, and *vanD* were found in 29.2%, 37.5%, and 33.3% isolates respectively. Regarding virulence determinants the observed percentages were as follows; *esp*: 16.6%, *asa1*: 70.8%, *gelE*: 25%, *ace*: 33.3%, and *cylA*: 25%. *Conclusion*. The majority of the isolates were *E. faecalis* and multi-drug resistant. The VRE isolates carried antimicrobial resistance and virulence-related genes, and *vanA*, B, D phenotypes were the most common among VRE isolates.

***Key words:*** *antimicrobial resistance, Enterococcus, minimum inhibitory concentration, vancomycin resistant enterococci, virulence factors, antimicrobial resistance gene.*

## СТРУКТУРА УСТОЙЧИВОСТИ К АНТИМИКРОБНЫМ ПРЕПАРАТАМ И ФАКТОРЫ ВИРУЛЕНТНОСТИ КЛИНИЧЕСКИХ ИЗОЛЯТОВ ЭНТЕРОКОККА В ПАКИСТАНЕ

**Улла Д.[1], Азиз А.[1], Улла А.[1], Улла И.[1], Джаббар А.[1], Умейр М.[2], Улла М.[2], Улла Х.[3], Улла М.[2], Али И.[2]**

[1] *Университет Харипура, КПК, Пакистан*
[2] *Хайберский медицинский университет, Пешавар, КПК, Пакистан*
[3] *Группа Сайду клинической больницы, Сайду-Шариф, Сват, КПК, Пакистан*

**Резюме.** Настоящее исследование было разработано для определения у разных видов энтерококков профиля устойчивости к антибиотикам, выявления устойчивости к противомикробным препаратам и генов, связанных с вирулентностью. *Материалы и методы*. Всего было собрано 150 изолятов энтерококков из различных

**Адрес для переписки:**
Али Ихсан
Тел.: (091) 921-77-03.
E-mail: ihsan.ipms@kmu.edu.pk; ihsanmicro@gmail.com

**Contacts:**
Ihsan Ali
F1 Phase-6 Rd, Phase 5 Hayatabad, Peshawar,
Khyber Pakhtunkhwa 25100.
Phone: (091) 921-77-03.
E-mail: ihsan.ipms@kmu.edu.pk; ihsanmicro@gmail.com

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клинических образцов. Эти изоляты были идентифицированных с помощью полимеразной цепной реакции (ПЦР). Тестирование чувствительности к антибиотикам и определение минимальной ингибирующей концентрации (МИК) ванкомицина проводились в соответствии с рекомендациями Института клинических и лабораторных стандартов (CLSI). Серия реакций ПЦР использовалась для скрининга генов устойчивости к ванкомицину *(vanA*, *vanB* и *vanD)* и генов вирулентности *(esp*, *ace*, *asa1*, *gelE* и *cylA)* среди видов ванкомицинрезистентных энтерококков (ВРЭ). *Результаты.* Выделенные штаммы энтерококков включали 62,6% *E. faecalis*, 33,4% *E. faecium* и 4% других видов. В целом энтерококки показали высокий уровень устойчивости: 94% — к эритромицину, за которым следуют ципрофлоксацин (82,6%), левофлоксацин (70%) и ванкомицин (16%). 57,4% изолятов были получены от госпитализированных пациентов, 96% изолятов энтерококков имели множественную лекарственную устойчивость. МИК для ВРЭ штаммов большинства изолятов варьировали в диапазоне от 32 мкг/мл до 256 мкг/мл. ВРЭ фенотипы в виде *vanA*, *vanB* и *vanD* были обнаружены у 29,2%, 37,5% и 33,3% изолятов соответственно. В отношении факторов вирулентности, получены следующие распределения: *esp* — 16,6%, *asa1* — 70,8%, *gelE* — 25%, *ace* — 33,3% и *cylA* — 25%. *Заключение.* Большинство изолятов были *E. faecalis* и имели множественную лекарственную устойчивость. ВРЭ изоляты несли гены устойчивости к противомикробным препаратам и гены, связанные с вирулентностью, а фенотипы *vanA, vanB, vanD* были наиболее распространены среди ВРЭ изолятов.

***Ключевые слова:*** *устойчивость к противомикробным препаратам, энтерококки, минимальная ингибирующая концентрация, ванкомицинрезистентные энтерококки, факторы вирулентности, ген устойчивости к противомикробным препаратам.*

## Introduction

Enterococci persistently emerged as important nosocomial pathogens globally and cause a wide range of infections such as bacteremia, meningitis, urinary tract infections, intra-abdominal and soft tissue infections, etc. [1]. The majority of the clinical enterococcal infections are caused by two species; *Enterococcus faecalis* and *Enterococcus faecium* [2]. Due to the frequent use of antibiotics in clinical practices, the emergence and spread of multi-drug-resistant enterococci such as vancomycin-resistant enterococci (VRE) has been observed [1]. Globally, this rapid emergence of VRE strains is considered a major public health concern. Besides increased morbidity and mortality of VRE infections, increased length of hospitalization and financial burden have also been reported [3].

The resistance to vancomycin in enterococci is mainly mediated by van gene phenotypes such as *vanA*, *vanB*, van*C*, *vanD*, and *vanE* genes, etc. The *vanA* and *vanB* have the highest clinical importance in enterococci among vancomycin-resistant phenotypes [4]. The spread of multi-drug-resistant enterococci strains and resistance-related genes has serious health implications. Furthermore, treatment options for VRE infections are quite limited including linezolid, teicoplanin, and fosfomycin [3]. Moreover, various virulence determinants associated with pathogenesis such as aggregation substance (Asa1), enterococcal surface protein (Esp), cytolysin (CylA), collagen binding protein *(ace)*, and gelatinase *(gelE)* are important for the progress of infection among these strains [5].

In Pakistan, the VRE is posing a challenge for clinicians as well as for hospital infection control practitioners. Despite its increased prevalence, data are scarce regarding its detailed characterization from Pakistan. The aim of the current study was to evaluate the frequency of enterococcal infections, antibiotic resistance profile, detection of antimicrobial resistance and virulence-related genes in clinical strains of enterococcus isolated from tertiary care hospital in the northwest of Pakistan.

## Materials and methods

*Bacterial isolates.* A total of one hundred and fifty (n = 150) non-repetitive enterococcal isolates were collected from various clinical specimens of patients admitted at a tertiary care teaching Hospital in Peshawar, Pakistan from January 2020 to February 2021. The isolates were re-identified at the Department of Medical Lab Technology, The University of Haripur by routine microbiological techniques [6]. The Polymerase chain reaction (PCR) was performed using specific primers ddl *E. faecium* and ddl *E. faecalis* to confirm the identity of *E. faecium* and *E. faecalis* as described elsewhere [7]. Ethical approval was obtained from the departmental ethical committee at the University of Haripur.

*Antimicrobial susceptibility testing.* Antimicrobial susceptibility was carried out using the Kirby Bauer disc diffusion method according to the guidelines of the Clinical Laboratory Standard Institute (CLSI, 2020) [8]. The antibiotic discs were obtained from (Oxide, England). The antibiotic discs and concentrations used were as follows; Vancomycin (30 µg), Linezolid (30 µg), Teicoplanin (30 µg), Gentamicin (10 µg), Penicillin (10 µg), Amoxicillin (10 µg), Doxycycline (30 µg), Minocycline (30 µg), Ciprofloxacin (30 µg), Levofloxacin (30 µg), Norfloxacin (30 µg), Erythromycin (15 µg), Fosfomycine (50 µg), Chloramphenicol (30 µg), Nitrofurantoin (300 µg), Rifampicin (5 µg) and Ampicillin (10 µg). The interpretation of the zone of inhibition was performed as per CLSI guidelines [8].

*Determination of Vancomycin Minimum inhibitory concentrations (MICs).* The enterococcal isolates resistant to vancomycin by disc diffusion method were further tested for minimum inhibitory concentrations. The MICs of vancomycin were determined by E.test using commercially available strips (MTS, Liofilchem, Italy). The interpretation of vancomycin MICs was carried out according to CLSI guidelines. The reference strains *E. faecium*, (ATCC 19434) and *E. faecalis*, (ATCC 19433) were used as control strains [8].

*Detection of antimicrobial resistance and virulence related genes.* Enterococcal genomic DNA was extracted from overnight culture by boiling method [9]. The vancomycin resistance associated genes *vanA*, *vanB*, and *vanD* and virulence related genes *(esp*, *ace*, *asa1*, *gelE*, and *cylA)* among *E. faecium* and *E. faecalis* were detected by using a series of PCR reactions as described earlier [10, 11].

*Statistical analysis.* The descriptive variables were expressed in percentages and frequencies. A Pearson test was used for correlation among the variables. The statistical analysis was done by SPSS (version 22) and a p-value of $< 0.05$ was considered statistically significant. Individual antibiotics sensitivity vs resistance percentages were cross tabulated among *E. faecalis* and *E. faecium* and the Odds ratio (OR) were determined.

## Results

*Characteristics of the study participant.* During the study period, 62.6% (n = 94) *E. faecalis*, 33.4% (n = 50) *E. faecium*, and 4% (n = 6) of other species were isolated. The distribution of the isolated strains from different specimens is shown in Table 1. The patient population of the isolated strains was 42.6% (n = 64) community-acquired whereas 57.4% (n = 86) were hospitalized. The majority, 56% (n = 84/150) of the isolates were recovered from patients who were > 50 years old and 58% (n = 49/84) of them were inpatients. Interestingly 6% (n = 9/150) of the total isolates were recovered from children (< 1 year) and 66.6% (n = 6/9) of them were inpatients.

*Antimicrobial susceptibility.* Antibiotic susceptibility was carried out and the predominant isolated strain *E. faecalis* showed the highest level of resistance, 95.7% (n = 90/94) to erythromycin, followed by ciprofloxacin 84% (n = 79/94), amoxicillin 66% (n = 62/94) and vancomycin 17% (n = 16/94). Low percentages of resistance were observed against linezolid as shown in Table 2. Among *E. faecium* isolates the resistance against erythromycin was 90% (n = 45/50), followed by ciprofloxacin and gentamicin 80% (n = 40/50 each), levofloxacin 76% (38/50), vancomycin and linezolid 16% (n = 8/50)

**Table 1. Distribution of isolated VRE and VSE strains among different specimens**

| Type of specimens, % (n) | VSE (n = 126), % (n) | VRE (n = 24), % (n) | *$\chi^2$ | p value |
|---|---|---|---|---|
| **Urine 52.7 (79)** | 53.2 (67) | 50 (12) | 0.059 | 0.000 |
| **Blood 28.7 (43)** | 28.6 (36) | 29.2 (7) | 0.013 | 0.00 |
| **Pus & Pus swab 10 (15)** | 9.5 (12) | 12.5 (3) | 0.178 | 0.010 |
| **Ascitic fluid 8 (12)** | 7.9 (10) | 8.3 (2) | 0.003 | 0.072 |
| **Tracheal secretions 0.6 (1)** | 0.8 (1) | Nil | 0.19 | 0.207 |

**Note.** VSE: Vancomycin sensitive Enterococci, VRE: Vancomycin resistant Enterococci, %: percentage, n = number; *$\chi^2$: The chi square was used to check the distribution of VRE and VSE among clinical specimens.

**Table 2. Antimicrobial resistance among Enterococcus isolates, % (n)**

| Antibiotics | *E. faecalis* (n = 94) | *E. faecium* (n = 50) | p value | OR value | Other Enterococcus species (n = 6) | Total (n = 150) |
|---|---|---|---|---|---|---|
| **Penicillin** | 59.5 (56) | 68 (34) | 0.041 | 0.889 | 66.6 (4) | 62.6 (94) |
| **Ampicillin** | 57.4 (54) | 56 (28) | 0.048 | 1.333 | 66.6 (4) | 57.3 (86) |
| **Amoxicillin** | 66 (62) | 58 (29) | 0.014 | 1.472 | 33.3 (2) | 62 (93) |
| **Ciprofloxacin** | 84 (79) | 80 (40) | 0.042 | 1.28 | 83.3 (5) | 82.6 (124) |
| **Levofloxacin** | 68 (64) | 76 (38) | 0.037 | 0.726 | 50 (3) | 70 (105) |
| **Norfloxacin** | 55.3 (52) | 58 (29) | 0.047 | 0.965 | 66.6 (4) | 43.8 (85) |
| **Gentamicin** | 70 (66) | 80 (40) | 0.028 | 0.597 | 83.3 (5) | 74 (111) |
| **Minocycline** | 32 (30) | 22 (11) | 0.027 | 1.712 | 16.6 (1) | 28 (42) |
| **Doxycycline** | 29.7 (28) | 24 (12) | 0.04 | 1.193 | 16.6 (1) | 27.3 (41) |
| **Erythromycin** | 95.7 (90) | 90 (45) | 0.022 | 2.61 | 100 (6) | 94 (141) |
| **Teicoplanin** | 27.6 (26) | 22 (11) | 0.001 | 1.414 | 16.6 (1) | 25.3 (38) |
| **Rifampicin** | 61.7 (58) | 66 (33) | 0.038 | 0.968 | 33.3 (2) | 62 (93) |
| **Nitrofurantoin** | 42.5 (40) | 34 (17) | 0.039 | 1.572 | 33.3 (2) | 39.3 (59) |
| **Chloramphenicol** | 45.7 (43) | 38 (19) | 0.018 | 1.322 | 16.6 (1) | 42 (63) |
| **Fosfomycine** | 29 (27) | 30 (15) | 0.049 | 0.937 | 50 (3) | 30 (45) |
| **Vancomycin** | 17 (16) | 16 (8) | 0.024 | 1.023 | 0 (0) | 16 (24) |
| **Linezolid** | 3.1 (3) | 4 (2) | 0.05 | 0.757 | 0 (0) | 2.5 (5) |

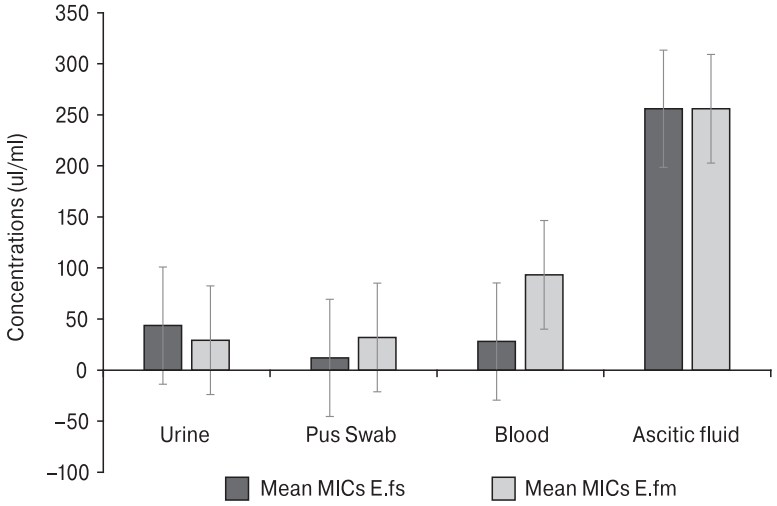


**Figure 1. Mean distribution of MICs of *E. faecalis* (E.fs) and *E. faecium* (E.fm) among various clinical specimens of patients attending a tertiary care hospital in Peshawar, Pakistan from January 2020 to February 2021**

and 4% (n = 2/50) respectively. Other species of enterococcus (other than *E. faecium* & *E. faecalis*) were resistant to erythromycin 100% (n = 6/6), followed by ciprofloxacin and gentamicin 83.3% (n = 5/6) each. No resistance was observed among other species against vancomycin and linezolid as shown in Table 2.

*Vancomycin Minimum Inhibitory Concentrations.* Sixteen percent (n = 24) of the isolates (*E. faecium* and *E. faecalis*) were vancomycin-resistant. Whereas no vancomycin resistance was observed against other enterococcus species. The MIC values for vancomycin against *E. faecium* and *E. faecalis* remained higher and fell in the range of 32 μg/ml to 256 μg/ml as shown in Supplementary Table 1. Overall, the difference in vancomycin MIC values among *E. faecium* and *E. faecalis* was statistically not significant (p = 0.624). The mean distribution of MICs of *E. faecium* and *E. faecalis* is shown in Figure 1.

*Vancomycin-resistant phenotypes and virulence determinants.* The percentages of vancomycin-resistant phenotypes among *E. faecium* vs *E. faecalis* were as follows: *vanA*; 50% (n = 4) vs 19% (n = 3), *vanB*; 12.5% (n = 1) vs 62.5% (n = 10) and van D; 75% (n = 6) vs not detected. Overall MICs for *vanA*, *vanB*, and *vanD* positive isolates remained above 16 μg/ml (Supplementary Table 1).

A total of five different virulence factors were scrutinized among twenty-four VRE isolates. The prevalence of the virulence factors among *E. faecium* and *E. faecalis* is shown in Table 3. Overall no significant differences have been observed between *E. faecium* and *E. faecalis* virulence genes prevalence.

*Multi-drug resistant Enterococci.* The highest percentage, 96% (n = 144/150) of the enterococcus isolates had multi-drug resistant patterns. Overall, 94.4% (n = 136/144) of the isolates were resistant to > 5 tested antibiotics and 26.3% (n = 38/144) were resistant to > 10 tested antibiotics and mainly were penicillin, cephalosporin, monobactam, quinolone and aminoglycosides as shown in Supplementary Table 1.

**Table 3. Correlation of virulence gene and resistant phenotype among VRE isolated from urine, blood, pus and pus swab and ascitic fluid**

| Virulence Genes, % (n) | Urine (n = 12) | | Blood (n = 7) | | Pus (n = 3) | | Ascitic fluid (n = 2) | | Total (n = 24) |
|---|---|---|---|---|---|---|---|---|---|
| | *E. faecalis* (n = 9) | *E. faecium* (n = 3) | *E. faecalis* (n = 4) | *E. faecium* (n = 3) | *E. faecalis* (n = 2) | *E. faecium* (n = 1) | *E. faecalis* (n = 1) | *E. faecium* (n = 1) | |
| ***esp*** | 11.1 (1) | 33.3 (1) | 0 (0) | 66.6 (2) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 16.6 (4) |
| ***ace*** | 33.3 (3) | 33.3 (1) | 75 (3) | 0 (0) | 0 (0) | 0 (0) | 100 (1) | 0 (0) | 33.3 (8) |
| ***asa1*** | 88.8 (8) | 33.3 (1) | 100 (4) | 33.3 (1) | 100 (2) | 0 (0) | 100 (1) | 0 (0) | 70.8 (17) |
| ***gelE*** | 33.3 (3) | 0 (0) | 0 (0) | 66.6 (2) | 50 (1) | 0 (0) | 0 (0) | 0 (0) | 25 (6) |
| ***cylA*** | 11.1 (1) | 33.3 (1) | 25 (1) | 33.3 (1) | 100 (2) | 0 (0) | 0 (0) | 0 (0) | 25 (6) |
| **Resistant Phenotype, % (n)** | | | | | | | | | |
| ***vanA*** | 33.3 (3) | 33.3 (1) | 0 (0) | 66.6 (2) | 50 (1) | 0 (0) | 0 (0) | 0 (0) | 29.2 (7) |
| ***vanB*** | 44.4 (4) | 33.3 (1) | 75 (3) | 0 (0) | 0 (0) | 0 (0) | 100 (1) | 0 (0) | 37.5 (9) |
| ***vanD*** | 11.1 (1) | 66.6 (2) | 0 (0) | 66.6 (2) | 50 (1) | 100 (1) | 0 (0) | 100 (1) | 33.3 (8) |

## Discussion

The current study was carried out to investigate the growing importance of multi-drug-resistant enterococcal infections in a tertiary care hospital in Psehawar, Khyber Pakyhtunkhwa (KP), Pakistan.

The available collected clinical information confirmed the established risk factors for the acquisition of various enterococcal infections such as hospitalization, advanced age, and neonates which are parallel to the other reports [12, 13]. In our study majority of the enterococcal infections were observed in the ages above 50 years which is similar to the other reported studies [12, 13]. In the current study, the predominant species is *E. faecalis*. The same pattern has been observed among clinical isolates from other studies [11, 14]. It has been reported that majority of the enterococcal infections are caused by *E. faecalis* as compared to other enterococcal species. Furthermore, it has been reported that *E. faecalis* carries more virulence factors in comparison to other Enterococcal species; resulting in its higher pathogenicity [10].

Over the time, the bacteria acquired resistance to anti-enterococcal antibiotics such as glycopeptides, ampicillin, and aminoglycosides. This might contribute to the increased prevalence of *E. faecalis* infections. However, recently certain studies have reported a relative shift in favor of *E. faecium* [2, 12, 15]. In our study high level of resistance to *E. faecalis* has been observed against erythromycin, ciprofloxacin, gentamicin, and ampicillin which is in accordance with the previous studies [16, 17]. The high level of resistance to enterococcal strains against gentamicin is a major concern as this might limit the option of combination therapy (Cell wall inhibitor antibiotics like ampicillin or vancomycin plus aminoglycosides such as gentamicin) which could be considered essential for the treatment of severe infections. Linezolid which was available for the first time in the year 2000 has been considered an alternate drug of choice for treating VRE infection. This is active against both *E. faecalis* and *E. faecium* [16]. In our study, the resistance of linezolid against *E. faecium* and *E. faecalis* was 4% and 3.1% respectively.

Surprisingly, in the current study, 96% of the enterococcus isolates were multi-drug resistant which is parallel with the previous report from Iran [18]. In our country, the treatment for the infections associated with MDR enterococci is complicated due to extensive misuse of antibiotics. Furthermore, the acquisition of antimicrobial resistance and its dissemination through plasmid and conjugative transposons play an important role in the progression of MDR enterococci [18].

The prevalence of VRE in the current study was 16% which is slightly raised from the results reported from Germany, Iran, and Italy; 11.2%, 9.4%, and 9% respectively [19]. However, the prevalence of VRE varies in different regions and a high frequency of VRE has been reported in the UK: (14.5%), Saudi Arabia: (17.3%), and Turkey: (80.2%) (14, 20, 21). The MICs of vancomycin in the majority cases for both *E. faecium* and *E. faecalis* fell in the range of 32 μg/ml to 256 μg/ml. The emergence of VRE in enterococci is considered one of the influential factor of enterococcal nosocomial infections [10]. The increased prevalence of VRE in Pakistan is a serious concern, *esp*ecially for the treatment of multi-drug resistant Gram-positive infections.

In the current study, we observed various percentages of *vanA*, *vanB*, and *vanD* phenotypes among VRE isolates. A study conducted in Iran reported that all VRE isolates were *vanA* phenotype [22]. One possible explanation for this variation might be the presence of other resistance genes such as *vanB* and *vanD* in the current study and the presence of other resistance mechanisms including thick cell wall production etc. However, some studies have reported variations in van phenotypes which are following our findings [2, 19, 23].

The observed prevalence of the *ace* gene among *E. faecalis* and *E. faecium* were 44% and 12.5% respectively. In other studies, the reported prevalence of *ace* was 42% and 39% respectively [11, 24]. Previously, it was hypothesized that *ace* gene products facilitate bacterial binding to the root dentin canal. Furthermore, they found out a significant correlation between the intact gene presence and subsequent attachment to dentin by *E. faecalis* [25]. Thus the presence of the *ace* gene in enterococcus species might be considered as an important virulence factor [11, 25]. Moreover, the frequency of *gelE* gene (25%) almost remained the same in both species. Gelatinase is a zinc metalloprotease with hydrolytic ability [16]. The observed frequency is slightly higher from the previous report which was 16% [26]. The percentages of *cylA*, *asa1*, and *esp* genes among *E. faecalis* and *E. faecium* were 12.5% vs 25%, 94% vs 25%, and 6% vs 37.5% respectively. Previously no *cylA* gene was detected in any isolates of *E. faecium* and low prevalence of *asa1* (2%) and *esp* (17.5%) were reported [2, 27]. Other studies reported a high frequency of *esp* gene among clinical isolates of vancomycin resistant *E. faecium* in comparison to fecal isolates. This increased prevalence of the *esp* gene in clinical isolates might indicate its role in enterococcal pathogenesis [2, 28]. The *asa1* gene-encoded aggregation substances facilitate binding to the host epithelium and during conjugation mediate bacterial aggregation and participate in plasmid exchange [16].

Conclusively, our study reported that *E. faecalis* was most prevalent among other enterococcus species. The majority of the isolates were multi-drug resistant and the highest percentages of resistance were observed against erythromycin, ampicillin, aminoglycosides, and vancomycin. The VRE isolates carried antimicrobial resistance and virulence-related genes and the most common glycopeptides-resistant phenotypes were *vanA, B, D* among VRE enterococcus isolates. Furthermore, due to this increased prevalence of MDR enterococci in clinical isolates, appro-

priate control measures and surveillance are essential to control the transmission and emergence of these isolates in hospitals.

## Additional information

**Author contributions.** This study was designed and supervised by Ihsan Ali and Abdul Jabbar. Jamshid Ullah, Atif Aziz, Inam Ullah, Muhammad Umair, Aman Ullah and Hanif Ullah carried out bench work and assembled the data. Matiullah, Mutiullah, Abdul Jabbar and Ihsan Ali performed analysis, interpretation and drafted the manuscript. The final manuscript were read and approved by all authors.

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**Авторы:**

**Улла Д.**, магистр философии, преподаватель, кафедра медицинских лабораторных технологий, факультет фундаментальных и прикладных наук, Университет Харипура, КПК, Пакистан;
**Азиз А.**, магистр философии, преподаватель, кафедра медицинских лабораторных технологий, факультет фундаментальных и прикладных наук, Университет Харипура, КПК, Пакистан;
**Улла А.**, магистр философии, преподаватель, кафедра медицинских лабораторных технологий, факультет фундаментальных и прикладных наук, Университет Харипура, КПК, Пакистан
**Улла И.**, магистр философии, преподаватель, кафедра медицинских лабораторных технологий, факультет фундаментальных и прикладных наук, Университет Харипура, КПК, Пакистан;
**Джаббар А.**, ассистент, кафедра медицинских лабораторных технологий, факультет фундаментальных и прикладных наук, Университет Харипура, КПК, Пакистан;
**Умейр М.**, магистр философии, преподаватель, Институт парамедицинских наук (IPMS), Хайберский медицинский университет, Пешавар, КПК, Пакистан;
**Улла Мути**, ассистент, Институт патологии и диагностической медицины (IPDM), Хайберский медицинский университет, Пешавар, КПК, Пакистан;
**Улла Х.**, магистр философии, преподаватель, Группа Сайду клинической больницы, Сайду-Шариф, Сват, КПК, Пакистан;
**Улла Мати**, магистр философии, преподаватель, Институт парамедицинских наук (IPMS), Хайберский медицинский университет, Пешавар, КПК, Пакистан.
**Али И.**, доцент кафедры медицинских лабораторных технологий, Институт парамедицинских наук (IPMS), Хайберский медицинский университет, Пешавар, КПК, Пакистан.

**Authors:**

**Ullah J.**, MPhil Scholar, Department of Medical Laboratory Technology, Faculty of Basic & Applied Sciences, The University of Haripur, KPK, Pakistan;
**Aziz A.**, MPhil Lecturer, Department of Medical Laboratory Technology, Faculty of Basic & Applied Sciences, The University of Haripur, KPK, Pakistan;
**Ullah A.**, MPhil Scholar, Department of Medical Laboratory Technology, Faculty of Basic & Applied Sciences, The University of Haripur, KPK, Pakistan;
**Ullah I.**, MPhil Scholar, Department of Medical Laboratory Technology, Faculty of Basic & Applied Sciences, The University of Haripur, KPK, Pakistan;
**Jabbar A.**, Assistant Professor, Department of Medical Laboratory Technology, Faculty of Basic & Applied Sciences, The University of Haripur, KPK, Pakistan;
**Umair M.**, MPhil Scholar, Institute of Paramedical Sciences (IPMS), Khyber Medical University, Peshawar, KPK, Pakistan;
**Ullah Muti**, Assistant Professor, Institute of Pathology and Diagnostic Medicine (IPDM), Khyber Medical University, Peshawar, KPK, Pakistan;
**Ullah H.**, MBBS, Trainee Medical Officer, Saidu Group of Teaching Hospitals Saidu Sharif, Swat, Pakistan;
**Ullah Mati**, MPhil Lecturer, Institute of Paramedical Sciences (IPMS), Khyber Medical University, Peshawar, KPK, Pakistan.
**Ali I.**, Assistant Professor in the Department of Medical Laboratory Technology, Institute of Paramedical Sciences (IPMS), Khyber Medical University, Peshawar, KP, Pakistan.

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