IJMCM
International Journal of Molecular and Cellular Medicine
p-ISSN: 2251-9637 o-ISSN: 2251-9645
Babol University of Medical Sciences
Cellular and Molecular Biology Research Center

# Emergence of OptrA Gene Mediated Linezolid Resistance among Enterococcus Faecium: A Pilot Study from a Tertiary Care Hospital, India

**Vandana Rani[1,2], Ajit Prakash[3], Mohammad Amin-ul Mannan[1,4], Priyanka Das[2] Hitha Haridas[2], Rajni Gaind[2*]**

*1. Department of Molecular Biology and Genetic Engineering, School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, Punjab, India.*

*2. Department of Microbiology, Vardhman Mahavir Medical College and Safdarjung Hospital, New Delhi, India.*

*3. Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599, United States of America.*

*4. Division of Infectious Disease, The Lundquist Institute, UCLA Harbor Medical Center, Los Angeles, California 90502, USA.*

**Article type:**
**Original Article**

**Received:**
2023.12.23
**Revised:**
2024.01.30
**Accepted:**
2024.03.05

**ABSTRACT**

*E. faecium* is the third most common cause of nosocomial infections. Linezolid (LNZ) is a reserve antibiotic recommended for infections caused by vancomycin resistant *E. faecium* (VREfm). The aim of the present study was to investigate the prevalence of *optrA* gene among linezolid resistant *E. faecium* (LREfm) and to study the molecular epidemiology using pulse field gel electrophoresis (PFGE). Clinically significant LREfm were identified and antimicrobial susceptibility was performed by disc diffusion. Minimum inhibitory concentration (MIC) of linezolid, vancomycin, daptomycin and quinupristin/dalfopristin was determined by E-test. PCR and PCR-RFPL were performed for the detection of *optrA/cfr* gene and G2576T mutation respectively. Molecular epidemiology was studied by PFGE. A total of 1081 clinically significant *Enterococci* species were isolated which included *E. faecium* 63.5% (n=687) and *E. faecalis* 36.5% (n=394). LREfm (30/687) were further studied. Multidrug resistance and **v**ancomycin resistance was 100% and 80%, respectively. Linezolid MIC range was 8-256µg/ml and the most common mechanism of resistance was *optrA* gene (83.3%) followed by G2576T mutation (33.3%). PFGE analysis demonstrated 4 major clones. The *optrA* gene mediated linezolid resistance was high and PFGE suggests resistance was emerging in the different background strains irrespective of resistance mechanism. Studies are required to investigate factors driving the emergence of linezolid resistance. The review suggests that this is the first report of *optrA*-mediated resistance in *E. faecium* from India.

**Keywords:** Linezolid, *E. faecium*, *optrA*, PFGE

***Cite this article:*** Rani V, *et al*. Emergence of *optrA* gene mediated linezolid resistance among *Enterococcus faecium*: A pilot study from a tertiary care hospital, India. *International Journal of Molecular and Cellular Medicine. 2023; 12(3): 242-256.* **DOI:** 10.22088/IJMCM.BUMS.12.3.242

*Corresponding: Rajni Gaind
Address: Department of Microbiology, Vardhman Mahavir Medical College and Safdarjung Hospital, New Delhi, India.
E-mail: rgaind5@hotmail.com





[ DOI: 10.22088/IJMCM.BUMS.12.3.242 ]

## Introduction

***E****nterococci* are commensal flora of the human gastrointestinal tract and are major causative agents of healthcare-associated infections (HAI) (1). The ability of *Enterococci* to survive under harsh conditions has contributed to its emergence as an important nosocomial pathogen. *E. faecium* and *E. faecalis* are the most pathogenic species. They are associated with serious infections like urinary tract infection (UTI), bacteremia, endocarditis, surgical site infections including burn wounds, gastrointestinal tract infections and infections associated with indwelling catheters and other implanted medical devices (2-4).

*Enterococci* show intrinsic resistance to various antimicrobial agents such as aminoglycosides, most cephalosporins, clindamycin, and trimethoprim-sulfamethoxazole (5). *Enterococci* are also capable of acquiring resistance either through mutation or by horizontal transfer of genetic elements resulting in resistance to several currently available antibiotics such as glycopeptides, quinolones and newer antibiotics like linezolid, daptomycin, and quinupristin-dalfopristin (6-8). Vancomycin resistance was first reported in 1980 from Europe and vancomycin-resistant *Enterococci* (VRE) have spread rapidly and increasing resistance is being reported globally. In a report published by the CDC in 2019, VRE caused approximately 54,500 infections and 5,400 deaths among hospitalized patients in the United States (9). In India, the prevalence of vancomycin resistance *Enterococci* was reported to be 14.9% as per the national surveillance report (ICMR, 2021) with vancomycin resistance being 6 times higher among *E. faecium* (25.4%) compared to *E. faecalis* (3.8%).

*E. faecalis* is associated with HAIs as it is more virulent than *E. faecium* but with modest levels of intrinsic and acquired antimicrobial resistance (10). However, there is an increasing trend for infections caused by *E. faecium*, mostly associated with the rise of vancomycin and β-lactam resistance. Recent data have suggested that Vancomycin-resistant *E. faecium* (VREfm) is widely distributed in hospitals around the world, with the prevalence varying according to geographical location (4). Treatment options for invasive VREfm infections are very limited, resulting in high mortality. The VREfm has also been listed as high priority pathogen by the World Health Organization (WHO) for which new antibiotics are urgently needed (11).

Linezolid was introduced in early 2000 as a novel therapeutic option for serious gram-positive infections, including multidrug-resistant organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE) (12). Linezolid binds with the central sphere part of domain V of the 23S rRNA to the 50S subunit of the bacterial ribosome and prevents the formation of an initiation complex (13)**.** Linezolid being a synthetic drug, there is a low risk of pre-existing or naturally occurring resistance (14) and it was predicted that resistance to linezolid would be rare. However, linezolid-resistant *Staphylococci* and *Enterococci* have been increasingly reported in recent years (15).

Resistance to linezolid is commonly mediated by a point mutation in the central region of domain V of the 23S rRNA. The commonly reported mutation among *E. faecium* is G2576T (16). Moreover, mutations in the particular region of *L3, L4* and *L22* ribosomal proteins, although less significant, have also been associated with linezolid resistance (17). More recently, linezolid resistance has been reported through the acquisition of *cfr* gene that confers transferable resistance to oxazolidinone in *Enterococci* of both human and animal origin (18). Besides oxazolidinones, *cfr* also confers resistance to phenicol's, lincosamides,



[ DOI: 10.22088/IJMCM.BUMS.12.3.242 ]

pleuromutilin's and streptogramin A (19). In 2015, *optrA gene* (oxazolidinone phenicol transferable resistance), was first reported from China among linezolid-resistant *Enterococci* isolated from both human and animal sources (20). Since then, *optrA* mediated resistance has been reported worldwide including Poland, USA, Spain, India, Ireland, Czech Republic (21-26). *OptrA* gene encodes an adenosine triphosphate-binding cassette (ABC) transporter and carries the plasmid, which can be easily transferred among *Enterococcus* species (20). The plasmid-mediated resistance results in rapid dissemination between patients and bacteria during breaches in infection control practices (27). The reports of linezolid resistance are fast emerging and are a cause of serious concern (28, 29). Recently, Linezolid has been classified as a reserve antibiotic (WHO) (11).

Limited studies have investigated the prevalence of the *optrA* gene among linezolid-resistant *Enterococcus faecium* (LREfm). The present study aimed to determine the antibiotic susceptibility profile and the prevalence of the *optrA* gene among linezolid-resistant *Enterococcus faecium* (LREfm). The molecular epidemiology of LREF using PFGE was also studied.

## Materials and methods

### Study setting

The present study was conducted in the Department of Microbiology, VMMC & Safdarjung Hospital, New Delhi. This study was conducted over a period of one year (October 2019 to October 2020). Patients with suspected UTI, abdominal infections, Skin and soft tissue infections (SSTI), bacteremia, device and implant-associated infections were enrolled in the study. Informed consent was obtained from all enrolled patients. The principles outlined in the Declaration of Helsinki were followed for the study and data were kept anonymized. This study was approved by the institutional ethics committee (IEC/ VMMC/ SJH/ PROJECT/ 2020-10/CC-79).

The clinical samples were processed by standard methods for isolation of *Enterococcus* species. *Enterococcus* was identified by cultural characteristics, gram staining, and biochemical tests. Final identification was done by vitek 2 Compact (Biomerieux India, Pvt Ltd). Based on the antibiotic susceptibility profile, only LREfm was included in the study.

### Antibiotic susceptibility testing

Antimicrobial susceptibility testing was performed by the Kirby-Bauer disk diffusion method for ampicillin (10 µg), ciprofloxacin (5 µg), gentamycin (120 µg), vancomycin (30 µg), erythromycin (15 µg), chloramphenicol (30 µg) and tetracycline (30 µg). All discs were procured from HiMedia, Pvt Ltd, India. Additionally, nitrofurantoin (300 µg) was tested for urinary isolates. Simultaneously AST was also performed using vitek GPAST628 cards. Minimum inhibitory concentration (MIC) of linezolid, vancomycin, daptomycin and quinupristin/dalfopristin was performed by E-test (BioMérieux, France). Results were interpreted as per CLSI guidelines (Clinical Laboratory Standard Institute, USA, 2019) (30). *E. faecalis* ATCC 29212 was used as the control strain for all susceptibility assays. The clinical isolates of *S. haemolyticus* and *E. faecium* positive for *cfr* and *optrA* gene were previously confirmed by sequencing and were used as positive controls.



[ DOI: 10.22088/IJMCM.BUMS.12.3.242 ]

Multidrug-resistant (MDR) was defined as non-susceptible to ≥1 agent in ≥3 antimicrobial categories and extensively drug-resistant **(**XDR) was defined as non-susceptible to ≥1 agent in all but ≤2 categories (31).

**DNA extraction**

DNA was extracted by boiling preparation method (32). Briefly, 6-8 colonies from the overnight growth of *E. faecium* on nutrient agar were mixed in 200 µL of nuclease-free water. The mixture was vortexed for 15 s. The cell suspension was held in a heating block for 10 min at 99 ℃ for lysis of cells, followed by centrifugation at 12000 rpm for 5 min. The supernatant containing DNA was collected and used as the template for PCR. The quality of extracted DNA was checked by a Nanodrop spectrophotometer (Nanodrop 2000c Thermo Scientific). A ratio 260/280 = 1.8 indicates DNA is pure. DNA was stored at -80$^{o}$C for further use.

**Detection of Resistance genes**

PCR was performed for the amplification of *cfr* and *optrA* genes. Primer sequences (20, 33-34), amplicon size and annealing temperature are given in Table 1. Primers were procured from Eurofins Genomics (India Pvt. Ltd). All PCRs were performed in a 25 µL volume reaction by using 5 µL template DNA, 1x PCR buffer, 0.2 µM of forward and reverse primers, 200 µM of each deoxynucleotide triphosphate (dNTPs), 1.5 mM MgCl2 and 1U Taq DNA Polymerase. Amplification was done in a Thermal Cycler (Eppendorf Master cycler EPS thermo-module, Hamburg, Germany) with the following protocol: initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturing (94 °C, 30 s), annealing (as given in table 1, 30 s), and extension (72 °C, 30 s) and a final extension of 10 min at 72 °C. Amplified PCR products were subjected to electrophoresis using 1.5% agarose gel along with a 100bp ladder (Thermo Scientific, Lithuania).

**Table 1.** Primer sequences used in the study for the investigation of the mechanism of linezolid resistance.

| Target | Primer Sequences | Amplicon (bp) | Annealing temp | Ref. |
|---|---|---|---|---|
| 23S rRNA | FP-GCAGAAGGGAGCTTGACTGCGAG<br>RP-ACCCAGCAATGCCCTTGGCAG | 389 | 62 ℃ | 33 |
| *cfr* gene | FP- TGAAGTATAAAGCAGGTTGGGAGTCA<br>RP-ACCATATAATTGACCACAAGCAGC | 746 | 62 ℃ | 34 |
| *optrA* gene | FP-AGGTGGTCAGCGAACTAA<br>RP-ATCAACTGTTCCATTCA | 1395 | 50 ℃ | 20 |

**Molecular detection of G2576T mutation**

PCR-RFLP was performed for the detection of G2576T mutation in 23S rRNA. Amplified PCR product (389bp) of 23S rRNA gene was digested with the *NheI* restriction enzyme as described by Hong et al. (Hong et al., 2007) (35). After restriction treatment, the product was subjected to agarose gel electrophoresis. The presence of 244-bp and 145-bp fragments were indicative of G2576T mutation.

**Pulse field gel electrophoresis (PFGE)**

PFGE was performed for LREFm using the protocol described by Saeedi *et al*., (36). Briefly, the genomic DNA was digested with *SmaI* (Thermo Fisher Lithuania) and the DNA fragments were



[ DOI: 10.22088/IJMCM.BUMS.12.3.242 ]

separated on 1% agarose gel, using CHEF Mapper System III (BioRad Laboratories, USA). *Enterococcus faecalis* ATCC 29212 was used as a reference control strain. PFGE patterns were further analysed by using InfoQuest[tm] FP Software v.5.4 (Bio-Rad Laboratories, USA).

## Results

### Prevalence of LREFm

During the study period (October 2019 to October 2020), a total of 1081 *Enterococci* species were isolated from clinical samples and included *E. faecium* (63.5%, n=687) and *E. faecalis* (36.5%, n=394). Among the *Enterococci* species, Vancomycin (MIC≥32µg/mL) and linezolid (MIC≥8µg/mL) resistance were detected in 155 (14.3%) and 30 (2.8%) isolates respectively. LNZ resistance was observed more frequently among VRE (15.4%, 24/155) and was rare among vancomycin susceptible (0.8%, 4/532) and this difference was statistically significant (P<0.001). There was a very good correlation between the results of the E-test and vitek for the detection of resistance to both vancomycin and linezolid. The distribution of linezolid and vancomycin resistance among *Enterococci* species, *E. faecium* and *E. faecalis* is given in Figure 1.

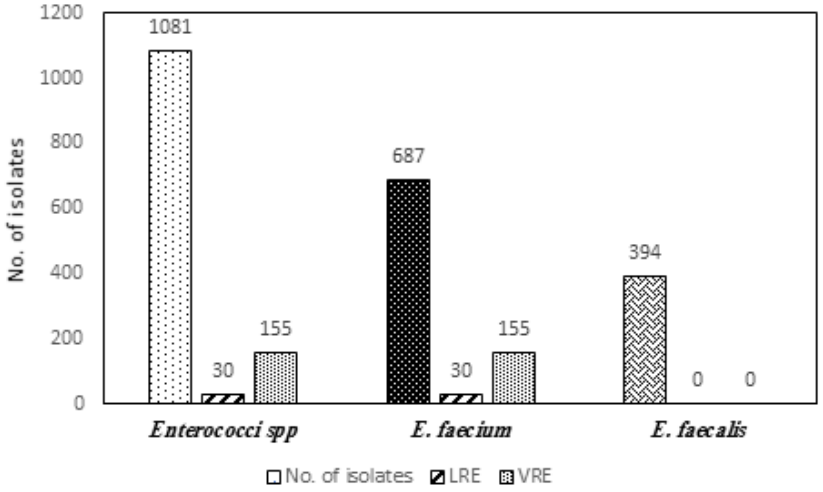


**Fig. 1**. Showing distribution of linezolid and vancomycin resistant isolates among *Enterococci* species, *E. faecium* and *E. faecalis.*

Linezolid and vancomycin resistance were observed only among *E. faecium* isolates (n= 687). The overall prevalence of LNZ and vancomycin resistance among *E. faecium* was 4.3%, (N=30) and 15.5% (n-155) respectively. A total of 30 clinically significant LREfm isolates were included in the study and further characterized. Only one isolate per patient was studied. The phenotypic and genotypic characteristics of 30 LREfm, including isolation site, resistance profile, MICs, resistance mechanism and pulsotypes are summarized in Table 2.

The majority of LREfm were isolated from UTI (56.7%, 17/30) followed by BSI (33.3%, 10/30), SSTI (10%,3/30). The highest number of LREfm was isolated from patients admitted to intensive care units (ICUs) (44%) followed by patients in non-ICU settings in the department of medicine (20%), paediatrics (13%), obstetrics & gynaecology (10%), dermatology (7%), oncology (3%), urology (3%). The distribution



[ DOI: 10.22088/IJMCM.BUMS.12.3.242 ]

of LREfm from various departments and clinical sites is summarised in Table 2. Blood isolates were predominantly from ICUs and urine isolates were from non-ICU patients (Table 3).

**Table 2.** Phenotypic and genotypic characteristics of LREfm isolates analysed in this study.

| Sample /location | Resistance profile | MIC(µg/mL) | | | | Resistance mechanism | | PFGE pulsotypes |
|---|---|---|---|---|---|---|---|---|
| | | LZ | VA | DP | QD | G2576T mutation | *optrA* | |
| 1-Urine Paediatrics | AMP CHL CIP ERY GEH LNZ NIT TCY VA | 8 | 256 | 0.75 | 32 | - | + | A 1 |
| 2-Urine Medicine | AMP CHL CIP ERY GEH LNZ NIT TCY VA | 32 | 256 | 0.5 | 32 | - | + | A 1 |
| 3-Urine Surgery | AMP CIP ERY GEH LNZ NIT TCY VA | 8 | 256 | 0.5 | 32 | - | + | A 1 |
| 4-Urine Medicine | AMP CHL CIP ERY GEH LNZ NIT TCY VA | 8 | 256 | 0.75 | 32 | - | + | A 3 |
| 5-Urine ICU | AMP CIP ERY GEH LNZ TCY VA | 24 | 256 | 1 | 32 | - | + | A 3 |
| 6-Blood ICU | AMP CHL CIP ERY LNZ TCY | 24 | 1 | 0.75 | 1.5 | - | + | A 1 |
| 7-Blood ICU | AMP CHL CIP ERY LNZ TCY | 8 | 1.5 | 0.75 | 1.5 | - | + | A 1 |
| 8-Urine Obstetrics &gynaecology | AMP CHL CIP ERY GEH LNZ NIT TCY VA | 8 | 256 | 0.75 | 32 | - | - | A 1 |
| 9-Urine Oncology | AMP CIP ERY GEH LNZ NIT TCY VA | 16 | 256 | 1 | 32 | - | - | A 1 |
| 10-Urine ICU | AMP CHL CIP ERY GEH LNZ NIT TCY | 12 | 1 | 1 | 1.5 | - | + | A 1 |
| 11-Urine Obstetrics &gynaecology | AMP CHL CIP ERY GEH LNZ NIT TCY VA | 24 | 256 | 0.75 | 32 | - | + | A 1 |
| 12-Urine Medicine | AMP CHL CIP ERY GEH LNZ NIT TCY VA | 12 | 256 | 2 | 32 | - | - | A 1 |
| 13-Pus Dermatology | AMP CHL CIP ERY GEH LNZ TCY VA | 8 | 256 | 0.75 | 32 | - | + | A 1 |
| 14-Pus Obstetrics &gynaecology | AMP CIP ERY GEH LNZ TCY VA | 16 | 256 | 0.5 | 32 | - | + | A 3 |
| 15-Blood ICU | AMP CIP ERY GEH LNZ VA | 8 | 256 | 0.25 | 32 | - | + | A 3 |
| 16-Blood ICU | AMP CHL CIP ERY GEH LNZ TCY VA | 32 | 256 | 3 | 32 | - | + | A 3 |
| 17-Blood ICU | AMP CHL CIP ERY GEH LNZ TCY VA | 8 | 256 | 0.75 | 32 | - | + | A 3 |



[ DOI: 10.22088/IJMCM.BUMS.12.3.242 ]

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 18-Urine Medicine | AMP CHL CIP ERY GEH LNZ NIT TCY VA | 24 | 256 | 4 | 32 | - | + | A 1 |
| 19-Pus Paediatrics | AMP CHL CIP ERY GEH LNZ TCY VA | 8 | 256 | 2 | 32 | - | + | A 3 |
| 20-Blood ICU | AMP CHL CIP ERY LNZ TCY VA | 48 | 256 | 1.5 | 32 | - | + | A 2 |
| 21-Urine ICU | AMP CHL CIP ERY GEH LNZ VA | 256 | 256 | 0.75 | 32 | + | + | A 1 |
| 22-Blood ICU | AMP CHL CIP ERY LNZ TCY VA | 256 | 256 | 1 | 32 | + | + | A 1 |
| 23-Urine ICU | AMP CHL CIP ERY GEH LNZ NIT TCY | 256 | 1 | 0.75 | 1.5 | + | + | A 3 |
| 24-Blood ICU | AMP CHL CIP ERY GEH LNZ TCY VA | 256 | 256 | 1.5 | 32 | + | + | A 3 |
| 25-Blood ICU | AMP CHL CIP ERY GEH LNZ TCY VA | 256 | 32 | 0.75 | 32 | + | + | A 2 |
| 26-Urine Paediatrics | AMP CHL CIP ERY GEH LNZ TCY | 256 | 1 | 0.25 | 1.5 | + | + | A 1 |
| 27-Urine Medicine | AMP CHL CIP ERY GEH LNZ NIT TCY | 256 | 1.5 | 0.75 | 1.5 | + | + | A 1 |
| 28-Urine ICU | AMP CHL CIP ERY GEH LNZ TCY VA | 256 | 256 | 0.75 | 32 | + | + | A 3 |
| 29-Blood ICU | AMP CHL CIP ERY GEH LNZ TCY VA | 256 | 128 | 0.75 | 32 | + | - | A 4 |
| 30-Urine ICU | AMP CHL CIP ERY GEH LNZ TCY VA | 256 | 256 | 0.75 | 32 | + | - | A 4 |

*AMP: Ampicillin, CHL: Chloramphenicol, CIP: Ciprofloxacin, ERY: Erythromycin, GEH: High level Gentamycin, LNZ: Linezolid, TCY: Tetracycline, VA: Vancomycin, NIT: Nitrofurantoin, Dap: Daptomycin, QDA: Quinupristin/dalfopristin. # No *cfr* gene was detected in any isolate.

**Table 3**. Isolation of LREF and its association with hospital locations (Non-ICU /ICUs) & site of infections.

| **Location** | **Sample type** | | | **Total** | **P value** |
|---|---|---|---|---|---|
| | Blood (%) | Pus (%) | Urine (%) | | |
| ICUs | 10 (71.4) | 0 (0) | 4(28.6) | 14 | P=0.001 |
| N-ICU | 0 (0) | 3(18.8) | 13(81.3) | 16 | P=0.001 |
| **Total** | 10 (33.3) | 3(10) | 17(56.7) | 30 | |

*N-ICU: Non-ICU patients

**Antimicrobial susceptibility profile**

The antibiotic susceptibility profile of LREfm (n=30) is summarised in Table 4. All LREfm isolates (n=30) were resistant to ampicillin, ciprofloxacin and erythromycin and, 90% (n=27) isolates were resistant to high-level gentamicin and tetracycline. Resistance to chloramphenicol, quinupristin/ dalfopristin and vancomycin was seen in 80% (n=24) isolates, similarly resistance to nitrofurantoin was observed in 70% of the urinary isolates (12/17) (Table 4). High resistance was observed to all antibiotics except daptomycin. The majority of the LREfm were MDR (100%) and XDR (93%). MIC range of linezolid and vancomycin ranged from 8-256 µg/mL and1-256 µg/mL respectively. Only 6 isolates were vancomycin susceptible with MIC range 1-1.5 µg/mL MIC of linezolid (8-256 µg/mL) did not vary significantly among VREfm and



[ DOI: 10.22088/IJMCM.BUMS.12.3.242 ]

VSEfm isolates (Table 4). All isolates were susceptible to daptomycin (MIC ranged 0.5-4 µg/mL). Resistance to quinupristin/ dalfopristin was high. It was observed that all isolates resistant to quinupristin/dalfopristin were also resistant to vancomycin and vice versa (Table 2).

**Table 4.** Antibiotic susceptibility pattern and minimum inhibitory concentration (µg/mL) of Daptomycin, Linezolid, Vancomycin and Quinupristin/dalfopristin for linezolid resistant *Enterococcus faecium* (n=30) by Disk diffusion and E-test method.

| Antibiotics | Break points as per CLSI | Resistant (%) | Sensitive (%) | MIC (µg/mL) | | |
|---|---|---|---|---|---|---|
| | | | | Range | 50 | 90 |
| Disk (µg) | | | | | | |
| Ampicillin (10µg) | ≥17-S<br>≤16-R | 30(100) | 0 | | | |
| Gentamicin-High (120µg) | - | 27(90) | 3(10) | | | |
| Ciprofloxacin (5µg) | ≥21-S<br>16-20-I<br>≤15-R | 30(100) | 0 | | | |
| Erythromycin (15µg) | ≥23-S<br>14-22-I<br>≤13-R | 30(100) | 0 | | | |
| Nitrofurantoin (300µg) | ≥17-S<br>15-16-I<br>≤14-R | 12(70) | 5(30) | | | |
| Chloramphenicol (30µg) | ≥18-S<br>13-17-I<br>≤12-R | 24(80) | 6(20) | | | |
| Tetracycline (30µg) | ≥19-S<br>15-18-I<br>≤14-R | 27(90) | 3(10) | | | |
| | | | | MIC(µg/mL) | | |
| Daptomycin | ≥8-R | 0(0) | 30(100) | 0.5-4 | 0.75 | 2 |
| Linezolid | ≥8-R<br>4-I<br>≤2-S | 30(100) | 0 | 8-256 | 32 | 256 |
| Vancomycin | ≤4-S<br>8-16-I<br>≥32-R | 24(80) | 6(20) | 1-256 | 256 | 256 |
| Quinupristin/dalfopristin | ≥4-R<br>2-I<br>≤1-S | 24(80) | 6(20) | 1.5-32 | 32 | 32 |

S-Sensitive, I-Intermediated, R-Resistant

**Mechanisms of resistance**

*OptrA* gene was detected in 83% of isolates (25/30) with the amplicon size 1395bp. For the detection of G2576T mutation in 23S rRNA, the 389bp PCR amplified products were digested with the *NheI* restriction enzyme, the presence of 244-bp and 145-bp fragments indicative of G2576T mutation was



[ DOI: 10.22088/IJMCM.BUMS.12.3.242 ]

observed in 33.3% (10/30) isolates. Both *optrA* and G2576T mutation were detected in 8 isolates and only G2576T mutation was detected in 2 isolates. The *cfr* gene was not detected. No resistance mechanism was observed in 3 isolates, details are given in Table 4.

**Molecular epidemiology by PFGE**

To study the molecular epidemiology of the LREfm, PFGE was performed. A dendrogram of PFGE profiles of LREfm isolates (n=30) was constructed (Figure 2) and four clusters of related isolates (A1, A2, A3, A4) were observed. Further analysis of PFGE data revealed that the majority of isolates were clustered in A1 (n=16) followed by cluster A3 (n=10) and 2 isolates each in cluster A2 and cluster A4. The majority of isolates in Cluster A1 included isolates from patients with UTI (n=10) followed by BSI (n-5) and SSI

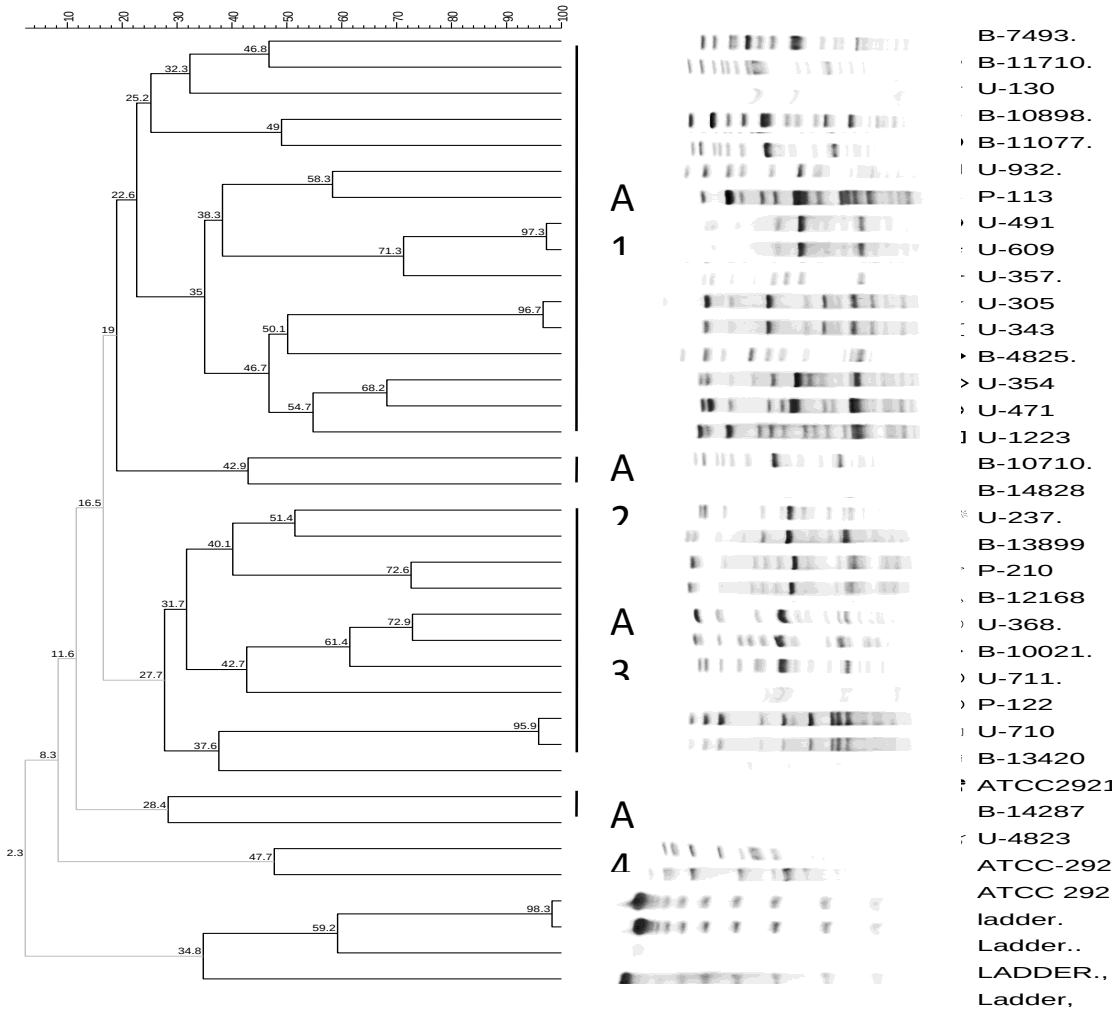


**Fig. 2**. Dendrogram of PFGE profiles of LREfm (n=30) isolates.

(n=1). Cluster A2 included isolates from BSI only, cluster A3 (n=10) included an even number of isolates from urine and BSI (n=4) and 2 isolates of SSTI. Cluster A4 had an even number of isolates of UTI and BSI (Figure 2). The majority of vancomycin susceptible LREfm isolates (5/6) was associated with cluster A1.



[ DOI: 10.22088/IJMCM.BUMS.12.3.242 ]

Isolates positive for the *optrA* gene were present in Cluster A1, A2, and A3, While Isolates with point mutation G2576T were present in all 4 clusters. Phenotypic and genotypic characteristics of LREfm (n=30) with pulsotypes are given in Table 2.

## Discussion

*Enterococci* are commensal flora of the human gastrointestinal tract and are associated with various clinical infections like BSI, SSTI, UTI and meningitis with high morbidity and mortality (1-3). Recent data have suggested that VRE is widely distributed in hospitals around the world, with the prevalence varying according to geographical location (37). Currently, *E. faecium* is emerging as an important nosocomial pathogen and has acquired resistance to different classes of antimicrobials. MDR *E. faecium* is common and is frequently associated with nosocomial infections with increased mortality rates (30, 38).

Vancomycin-resistant *E. faecium* (VREfm) has been classified as high high-priority pathogen (WHO) and linezolid is a reserve antibiotic for the management of VRE isolates (4, 11). The first report of linezolid resistance was reported during a nosocomial outbreak of *E. faecium*. Isolates were resistant to both vancomycin and linezolid (39). Interestingly, in our study too, vancomycin (14.3%) and linezolid (2.8%) resistance were detected only among *E. faecium* isolates.

Beata Krawczyk et al. studied 18 isolates of LREfm and reported high resistance to ampicillin (89.9%), and ciprofloxacin (94.3%), however resistance to high-level gentamicin, chloramphenicol & tetracycline was 49.1%, 31.6% and 5.3% respectively (40). In the present study, a high percentage of resistance was observed to all antibiotics except daptomycin, more than 70% of isolates were resistant to ampicillin, ciprofloxacin, erythromycin, high-level gentamicin, tetracycline, chloramphenicol, vancomycin, and quinupristin/dalfopristin. Resistance to nitrofurantoin was observed in 70% (12/17) of urine isolates.

In our study, we observed that the resistance to linezolid among *E. faecium* (n=687) is relatively uncommon (30/687, 4.4%) compared to vancomycin (155/687, 22.6%). However, the important finding of our study was that linezolid resistance was frequently associated with vancomycin resistance. The majority of the LREfm were also vancomycin-resistant (24/30, 80%) and the majority were isolated from ICUs, where the burden of infections caused by VRE is high, these findings suggest that the use of linezolid for such infections may be one of the factors driving the emergence of LREfm. However, in this study, 6 patients with BSI and UTI were infected with vancomycin susceptible LREfm suggesting that vancomycin susceptibility does not exclude linezolid resistance. Similar to our findings,two programs monitoring infections with linezolid-resistant strains: LEADER (in USA) and ZAAPS (Zyyox ®Annual Appraisal of Potency and Spectrum Program: worldwide) have also reported the emerging problem of simultaneous resistance to vancomycin and linezolid (40).

Interestingly in our study, vancomycin resistance co-existed with resistance to quinupristin/ dalfopristin. All vancomycin susceptible linezolid resistant isolates (n=6) were susceptible to quinupristin/ dalfopristin too. This together has important implications as there are limited options for the management of linezolid and vancomycin-resistant *E. faecium*. In our study, daptomycin was the only antibiotic that can be used as a therapeutic option for the management of VREfm and LREfm**.**



[ DOI: 10.22088/IJMCM.BUMS.12.3.242 ]

In our study, MDR and XDR were observed in 100% & 93% of isolates respectively. The hospital setting is particularly responsible for selective pressure and facilitates the formation of MDR including LREfm and VREfm. This is likely to be driven by excessive use of cephalosporins, fluoroquinolones, aminoglycosides and glycopeptides. Other risk factors include prolonged hospitalization, contact with colonized patients and breach in infection prevention practices leading to cross-transmission.

Linezolid was introduced in early 2000 as a novel therapeutic option for serious gram-positive infections. Linezolid-resistant *Staphylococci* and *Enterococci* have been increasingly reported in recent years (14). Linezolid binds to rRNA, specifically to domain V of the 23S rRNA of the 50S ribosomal subunit, which is encoded by genes (rDNA) present in 6 copies in *E. faecium* (39). Resistance to linezolid is commonly mediated by a point mutation in the central region of domain V of the 23S rRNA (G2576T) (16). There is a correlation between the number of rDNA gene copies carrying the G2576T mutation and the phenotypic level of resistance to linezolid. Resistant to linezolid can also be caused by mutations in the particular region of *L3, L4,* and *L22* ribosomal proteins and through the acquisition of resistance genes (*cfr*) (18, 20). However, the acquired resistance mechanism is of great clinical and epidemiological importance. Recent studies have suggested that the frequency of isolates carrying the *cfr* gene are rising in animal-origin isolates (33, 41-43). Additional research on *Enterococci* that lack the *cfr* gene but still exhibit LNZ resistance has shown the importance of the oxazolidinone and phenicol transferable resistance A (*OptrA*) as another LND resistance determinant. *OptrA* gene was initially detected in *E. faecalis* of human origin, and subsequent studies have reported *optrA* gene in isolates of *E. faecium*, as well as most recently in *S. aureus, S. sciuri*, and *S. suis* (20, 44).

Interestingly in our study, all linezolid-resistant *E. faecium* were negative for *cfr* gene and the main mechanism of linezolid resistance was *optrA* gene (83.33%) followed by point mutation G2576T in the domain V of the 23S rRNA (33.33%). The MIC of linezolid was >256 µg/ml for the isolates with G2576T mutation irrespective of the presence or absence of *optrA* gene. As all isolates with G2576T demonstrate high MIC we did not investigate for any mutated copies of the 23Sr RNA gene. Previous studies have reported mutated gene dosage effects on linezolid MIC.

The MIC of linezolid for isolates, positive for *optrA* gene alone, varied from 8-128 µg/ml, suggesting the role of other mechanisms of resistance that may be contributing to these variations in the MIC and need to be investigated. In three LREfm isolates, no linezolid resistance mechanism was detected and needs further investigation.

Resistance mediated by *optrA* has been reported worldwide including in China, Poland, Spain, USA, Ireland, Czech Republic (42-45). In India, two cases of LREfm were investigated by WGS and were harbouring the *optrA* gene and G2576T point mutation in 23S rRNA (46). Linezolid resistance mechanisms vary with geographical regions. Studies from European countries have reported linezolid resistance is predominantly mediated by a mutation in domain V of the 23S rRNA, however, reports from China suggest that the predominant mechanism of resistance was *optrA* gene. Surveillance studies from China suggest that *optrA* is more frequently reported in *Enterococci* from food-producing animals than humans (20), but is now emerging in clinical isolates also. Another report from China shows the high prevalence of *optrA* gene (46.6%) in clinical human isolates of *E. faecium* (44). However, in our study, although the predominant



[ DOI: 10.22088/IJMCM.BUMS.12.3.242 ]

mechanism of linezolid resistance was *optrA* gene (83.33%), significant resistance was also mediated by point mutation G2576T in the domain V of the 23S rRNA (33.33%). Limiting linezolid use may partly curtail the spread of resistance, as the G2576T resistance mutation can arise in pathogens due to prolonged drug exposure. The *optrA* resistance gene is capable of horizontal transfer as they are often associated with mobile genetic elements.

In the present study, the data from PFGE suggest that linezolid resistance was emerging in diverse clones that were isolated from patients with diverse clinical presentations and hospitalized in different clinical departments. Association of linezolid and vancomycin resistance was observed in all clusters; however, the majority of the vancomycin susceptible LREfm isolates were clustered to cluster A1 (Figure 1). The majority of LREfm were also clustered in cluster A1, suggesting that linezolid resistance is emerging within the hospital and likely driven spread by a breach in infection prevention and control strategies. It was also observed that isolates with both resistance mechanisms, point mutation G2576T in the domain V of the 23S rRNA and *optrA* gene were emerging in different clusters. These findings suggest that resistance was emerging in different background strains with likely horizontal gene transfer. Another explanation for the diversity of LREfm is their commensal origin from the gut of patients. Further studies are required to investigate factors driving the emergence of linezolid resistance in different background strains.

The strength of our study is that we investigated both mutational and plasmid-mediated resistance. PFGE was used to study the dissemination of resistance isolates in the hospital. The limitation of our study is that the other mechanism of resistance was not investigated and mutation G2576T in the domain V of the 23S rRNA was not characterized for heterogeneity, which needs to be investigated to study the molecular epidemiology of LREfm.

In this study, linezolid resistance was observed in 2.8% (30/1081) of *Enterococci* and all isolates were *E. faecium. OptrA* gene was the predominant mechanism and was detected in 83.3% of LREfm isolates, one third of isolates had the G2576T mutation in domain V of 23S rRNA. The association of linezolid and vancomycin resistance is a cause of concern as it limits the therapeutic options for the management of VRE. The data from PFGE suggest that linezolid resistance was emerging in diverse clones. As the *optrA* gene is frequently associated with plasmids, it has clinical implications and results in rapid dissemination between patients and species of bacteria, hence implementations of infection control practices are important to prevent further spread in the hospital. The literature review suggests that this is the first report on molecular characterization and epidemiology of linezolid-resistant *E. faecium* from a tertiary care centre, India.

**List of abbreviations**

LNZ- Linezolid, LREfm- Linezolid Resistant *E. faecium*, VSEfm- Vancomycin sensitive *E. faecium*, VREfm- Vancomycin resistant *E. faecium*, VRE- Vancomycin resistant *Enterococci*, MIC- Minimum Inhibitory Concentration, PFGE- Pulse field gel electrophoresis, UTI- Urinary tract infection, BSI- Blood sepsis infection, SSTI- Skin and Soft tissue infection, ICMR- Indian Council of Medical Research.

**Acknowledgement**

This study was supported by the Indian Council of Medical Research Government of India. (Scheme no. AMR/Adhoc/238/2020-ECD-II).



[ DOI: 10.22088/IJMCM.BUMS.12.3.242 ]

**Authors' contributions**

Rajni Gaind: Conceived and designed the experiment, Vandana Rani: Experimental Work, Rajni Ganid, Vandana Rani: Manuscript writing and manuscript drafting, Rajni Gaind, Vandana Rani, Mohammad Amin-ul Mannan, Ajit Prakash: Data analysis, Priyanka Das, Hitha Haridas: provided data. All authors have read and approved the manuscript.

## References

1. Agudelo Higuita NI, Huycke MM. Enterococcal Disease, Epidemiology, and Implications for Treatment. In: Gilmore MS, Clewell DB, Ike Y, et al., editors. Enterococci: From Commensals to Leading Causes of Drug Resistant Infection. Boston2014.

2. Arias CA, Reyes J, Zuniga M, et al. Multicentre surveillance of antimicrobial resistance in enterococci and staphylococci from Colombian hospitals, 2001-2002. J Antimicrob Chemother 2003;51:59-68.

3. Bonora MG, Solbiati M, Stepan E, et al. Emergence of linezolid resistance in the vancomycin-resistant Enterococcus faecium multilocus sequence typing C1 epidemic lineage. J Clin Microbiol 2006;44:1153-5.

4. Cetinkaya Y, Falk P, Mayhall CG. Vancomycin-resistant enterococci. Clin Microbiol Rev 2000;13:686-707.

5. Huycke MM, Sahm DF, Gilmore MS. Multiple-drug resistant enterococci: the nature of the problem and an agenda for the future. Emerg Infect Dis 1998;4:239-49.

6. Zirakzadeh A, Patel R. Vancomycin-resistant enterococci: colonization, infection, detection, and treatment. Mayo Clin Proc 2006;81:529-36.

7. Patel SN, Memari N, Shahinas D, et al. Linezolid resistance in Enterococcus faecium isolated in Ontario, Canada. Diagn Microbiol Infect Dis 2013;77:350-3.

8. Guzman Prieto AM, van Schaik W, Rogers MR, et al. Global Emergence and Dissemination of Enterococci as Nosocomial Pathogens: Attack of the Clones? Front Microbiol 2016;7:788.

9. Kadri SS. Key Takeaways From the U.S. CDC's 2019 Antibiotic Resistance Threats Report for Frontline Providers. Crit Care Med 2020;48:939-45.

10. Sharifi Y, Hasani A, Ghotaslou R, et al. Survey of Virulence Determinants among Vancomycin Resistant Enterococcus faecalis and Enterococcus faecium Isolated from Clinical Specimens of Hospitalized Patients of North west of Iran. Open Microbiol J 2012;6:34-9.

11. Asokan GV, Ramadhan T, Ahmed E, et al. WHO Global Priority Pathogens List: A Bibliometric Analysis of Medline-PubMed for Knowledge Mobilization to Infection Prevention and Control Practices in Bahrain. Oman Med J 2019;34:184-93.

12. Ford CW, Zurenko GE, Barbachyn MR. The discovery of linezolid, the first oxazolidinone antibacterial agent. Curr Drug Targets Infect Disord 2001;1:181-99.

13. Batts DH. Linezolid--a new option for treating gram-positive infections. Oncology (Williston Park) 2000;14:23-9.

14. Robert C, Moellering J. Enterococcus species, Streptococcus bovis and Leuconostoc species. Principles and practice of infectious diseases. 6th ed. New York: Churchill Livingstone; 2005. p. 2411-21.

15. Doern CD, Park JY, Gallegos M, et al. Investigation of Linezolid Resistance in Staphylococci and Enterococci. J Clin Microbiol 2016;54:1289-94.

16. Prystowsky J, Siddiqui F, Chosay J, et al. Resistance to linezolid: characterization of mutations in rRNA and comparison of their occurrences in vancomycin-resistant enterococci. Antimicrob Agents Chemother 2001;45:2154-6.



[ DOI: 10.22088/IJMCM.BUMS.12.3.242 ]

17. Long KS, Vester B. Resistance to linezolid caused by modifications at its binding site on the ribosome. Antimicrob Agents Chemother 2012;56:603-12.

18. Shen J, Wang Y, Schwarz S. Presence and dissemination of the multiresistance gene cfr in Gram-positive and Gram-negative bacteria. J Antimicrob Chemother 2013;68:1697-706.

19. Long KS, Poehlsgaard J, Kehrenberg C, et al. The Cfr rRNA methyltransferase confers resistance to Phenicols, Lincosamides, Oxazolidinones, Pleuromutilins, and Streptogramin A antibiotics. Antimicrob Agents Chemother 2006;50:2500-5.

20. Wang Y, Lv Y, Cai J, et al. A novel gene, optrA, that confers transferable resistance to oxazolidinones and phenicols and its presence in Enterococcus faecalis and Enterococcus faecium of human and animal origin. J Antimicrob Chemother 2015; 70:2182-90.

21. Gawryszewska I, Zabicka D, Hryniewicz W, et al. Linezolid-resistant enterococci in Polish hospitals: species, clonality and determinants of linezolid resistance. Eur J Clin Microbiol Infect Dis 2017;36:1279-86.

22. Tyson GH, Sabo JL, Hoffmann M, et al. Novel linezolid resistance plasmids in Enterococcus from food animals in the USA. J Antimicrob Chemother 2018;73:3254-8.

23. Camara J, Camoez M, Tubau F, et al. Detection of the Novel optrA Gene Among Linezolid-Resistant Enterococci in Barcelona, Spain. Microb Drug Resist 2019;25:87-93.

24. Bakthavatchalam YD, Vasudevan K, Babu P, et al. Genomic insights of optrA-carrying linezolid-resistant Enterococcus faecium using hybrid assembly: first report from India. J Glob Antimicrob Resist 2021;25:331-6.

25. Egan SA, Shore AC, O'Connell B, et al. Linezolid resistance in Enterococcus faecium and Enterococcus faecalis from hospitalized patients in Ireland: high prevalence of the MDR genes optrA and poxtA in isolates with diverse genetic backgrounds. J Antimicrob Chemother 2020;75:1704-11.

26. Malisova L, Jakubu V, Pomorska K, et al. Spread of Linezolid-Resistant Enterococcus spp. in Human Clinical Isolates in the Czech Republic. Antibiotics (Basel) 2021;10.

27. Kalawat U, Sharma KK, Reddy S. Linezolid-resistant Staphylococcus spp. at a tertiary care hospital of Andhra Pradesh. Indian J Med Microbiol 2011;29:314-5.

28. Bradley SJ, Kaufmann ME, Happy C, et al. The epidemiology of glycopeptide-resistant enterococci on a haematology unit--analysis by pulsed-field gel electrophoresis. Epidemiol Infect 2002;129:57-64.

29. Courvalin P. Vancomycin resistance in gram-positive cocci. Clin Infect Dis 2006;42 Suppl 1:S25-34.

30. Wayne PA. Clinical and laboratory standards institute. Performance standards for antimicrobial susceptibility testing. 29th informational supplement M100-S29. USA2011.

31. Magiorakos AP, Srinivasan A, Carey RB, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect 2012;18:268-81.

32. Englen MD, Kelley LC. A rapid DNA isolation procedure for the identification of Campylobacter jejuni by the polymerase chain reaction. Lett Appl Microbiol 2000;31:421-6.

33. Marshall SH, Donskey CJ, Hutton-Thomas R, et al. Gene dosage and linezolid resistance in Enterococcus faecium and Enterococcus faecalis. Antimicrob Agents Chemother 2002;46:3334-6.

34. Kehrenberg C, Schwarz S. Distribution of florfenicol resistance genes fexA and cfr among chloramphenicol-resistant Staphylococcus isolates. Antimicrob Agents Chemother 2006;50:1156-63.

35. Hong T, Li X, Wang J, et al. Sequential linezolid-resistant Staphylococcus epidermidis isolates with G2576T mutation. J Clin Microbiol 2007;45:3277-80.



[ DOI: 10.22088/IJMCM.BUMS.12.3.242 ]

36. Saeedi B, Hallgren A, Jonasson J, et al. Modified pulsed-field gel electrophoresis protocol for typing of enterococci. APMIS 2002;110:869-74.

37. Bender JK, Cattoir V, Hegstad K, et al. Update on prevalence and mechanisms of resistance to linezolid, tigecycline and daptomycin in enterococci in Europe: Towards a common nomenclature. Drug Resist Updat 2018;40:25-39.

38. Miller WR, Munita JM, Arias CA. Mechanisms of antibiotic resistance in enterococci. Expert Rev Anti Infect Ther 2014;12:1221-36.

39. Dobbs TE, Patel M, Waites KB, et al. Nosocomial spread of Enterococcus faecium resistant to vancomycin and linezolid in a tertiary care medical center. J Clin Microbiol 2006;44:3368-70.

40. Krawczyk B, Wysocka M, Kotlowski R, et al. Linezolid-resistant Enterococcus faecium strains isolated from one hospital in Poland -commensals or hospital-adapted pathogens? PLoS One 2020;15:e0233504.

41. Fioriti S, Morroni G, Coccitto SN, et al. Detection of Oxazolidinone Resistance Genes and Characterization of Genetic Environments in Enterococci of Swine Origin, Italy. Microorganisms 2020;8.

42. Guerin F, Sassi M, Dejoies L, et al. Molecular and functional analysis of the novel cfr(D) linezolid resistance gene identified in Enterococcus faecium. J Antimicrob Chemother 2020;75:1699-703.

43. Almeida LM, Gaca A, Bispo PM, et al. Coexistence of the Oxazolidinone Resistance-Associated Genes cfr and optrA in Enterococcus faecalis From a Healthy Piglet in Brazil. Front Public Health 2020;8:518.

44. Yi M, Zou J, Zhao J, et al. Emergence of optrA-Mediated Linezolid Resistance in Enterococcus faecium: A Molecular Investigation in a Tertiary Hospital of Southwest China from 2014-2018. Infect Drug Resist 2022;15:13-20.

45. Cai J, Schwarz S, Chi D, et al. Faecal carriage of optrA-positive enterococci in asymptomatic healthy humans in Hangzhou, China. Clin Microbiol Infect 2019;25:630 e1- e6.



[ DOI: 10.22088/IJMCM.BUMS.12.3.242 ]