ASSESSMENT OF THE PREVALENCE, PHYLOGENY AND VIRULENCE OF LINEZOLID RESISTANT *ENTEROCOCCUS FAECIUM* IN HOSPITAL ACQUIRED INFECTIONS AND ENVIRONMENT

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

MICROBIOLOGY

By

VANDANA RANI
Registration Number: 11816269

Supervised By

DR. MOHAMMAD AMIN-UL MANNAN
Assistant Professor
Department of Molecular Biology and Genetic Engineering, School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, Punjab, India

Co-Supervised by

DR. RAJNI GAIND
Professor and Consultant
Department of Microbiology
Vardhman Mahavir Medical College and Safdarjung Hospital, New Delhi, India

LOVELY PROFESSIONAL UNIVERSITY, PUNJAB

2024

DECLARATION

I, hereby declared that the presented work in the thesis entitled "**Assessment of the Prevalence, Phylogeny and Virulence of Linezolid Resistant *Enterococcus Faecium* in Hospital Acquired Infections and Environment**" in fulfilment of degree of **Doctor of Philosophy (Ph. D.)** is outcome of research work carried out by me under the supervision Dr. Mohammad Amin-ul Mannan, working as assistant professor, in the Department of Molecular Biology and Genetic Engineering, School of Bioengineering and Biosciences of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigators. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

(Signature of Scholar)
Name of the scholar: Vandana Rani
Registration No.: 11816269
Department/school: School of Bioengineering and Biosciences
Lovely Professional University,
Punjab, India

CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled "**Assessment of the Prevalence, Phylogeny and Virulence of Linezolid Resistant *Enterococcus Faecium* in Hospital Acquired Infections and Environment**" submitted in fulfillment of the requirement for the reward of degree of **Doctor of Philosophy (Ph.D.)** in the **Microbiology**, is a research work carried out by **Vandana Rani**, **11816269**, is bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

(Signature of Supervisor)
Name of supervisor:
Dr. Mohammad Amin-ul Mannan
Assistant Professor
Department of Molecular Biology
and Genetic Engineering,
School of Bioengineering and Biosciences
LovelyProfessional University,
Phagwara, Punjab, India

(Signature of Co-Supervisor)
Name of Co-Supervisor:
Dr. Rajni Gaind
Consultant, Professor
Department of Microbiology
VMMC and Safdarjung Hospital
New Delhi, India

ABSTRACT

Enterococci are commensal flora of the gut and widely distributed in the environment. They are opportunistic pathogens causing infections in immunocompromised or hospitalised patients specially in ICUs with severe underlying disease. *Enterococci* show intrinsic resistance to various antibiotics such as aminoglycosides, most cephalosporins, clindamycin and trimethoprim-sulfamethoxazole. *Enterococcus faecium (E. faecium)* has rapidly evolved as a global nosocomial pathogen by successfully adapting to nosocomial environment by acquisition of acquired antibiotic resistance genes that enable evasion during antimicrobial therapy. Vancomycin resistance is increasingly being reported in *Enterococci.* Recent data suggest that vancomycin-resistant *E. faecium (*VREfm) is widely distributed in hospitals around the world. Treatment options for invasive VREfm infections are very limited, resulting in high mortality.

Linezolid is an oxazolidinone antibiotic**,** which was introduced in early 2000 as a novel therapeutic option for serious gram-positive infections, including methicillin resistant *Staph. aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE). Resistance to linezolid is commonly mediated by a point mutation in the central region of domain V of the 23S rRNA (G2576T). Moreover, resistance to linezolid can be caused by mutations in the particular region of *L3, L4* and *L22* ribosomal proteins and acquisition of acquired resistance genes (*cfr* and *optrA*).

Along with antibiotic resistance, virulence factors may have an impact on clinical outcomes. Several potential virulence factors contribute to the pathogenicity of the *E. faecium* isolates. Various methods have been used to study the molecular epidemiology of *E. faecium* like PFGE and MLST.

The reports of linezolid resistance are fast emerging and are a cause of concern. In India, sporadic cases of linezolid resistant *Enterococci* infections have been reported. However, there is paucity of studies to determine the prevalence, mechanisms of resistance and dissemination of linezolid resistance among *E. faecium* in India. The present study was undertaken to determine the prevalence of linezolid resistant *E. faecium* and characterize the various mechanisms associated with linezolid resistance. The risk factors and clinical outcomes of patient's infected with linezolid-resistant *E.*

faecium and the probable source of transmission of linezolid resistance within the hospital were also studied using molecular typing (PFGE and MLST).

This study was undertaken in the Department of Microbiology at Safdarjung Hospital and associated Vardhaman Mahavir Medical College, New Delhi. Linezolid-resistant *E. faecium* (LREfm) isolated from clinically significant samples and from patient's immediate environmental samples were included in the study. Identification of *E. faecium* was done by Vitek 2 automated system. Antimicrobial susceptibility was performed by disc diffusion method and MIC of linezolid, vancomycin, daptomycin and quinupristin/dalfopristin were determined by gradient E- test. RFLP- PCR and Sanger sequencing were performed for mutation in 23S rRNA. Mechanisms of linezolid resistance and virulence genes were studied by PCR. Genetic relatedness among LREfm was studied by pulse field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).

During the study period (January 2020 to June 2022) a total of 226 LREfm which includes 202 clinical and 24 environmental isolates were studied. Among the clinical isolates of LREfm majority of them were from urine (47.5%, n=96) followed by blood (43.6%, n=88) and pus (8.9%, n=18). The highest number of LREfm were isolated from patients admitted to ICUs followed by patients in non-ICU settings.

The risk factors significantly associated with increased risk of LREfm infection were site of infection UTI, use of carbapenem and linezolid therapy, use of central line, urinary catheter and ventilation. The hospital stays 8-14 days (<0.001) prior to infection and the mortality rate (p=0.003) were also significantly high among patients with LREfm infections.

The majority of LREfm were vancomycin resistant (≥80%). Linezolid MIC range was 8-256 µg/mL, vancomycin resistance was detected in ≥80% isolates. MDR and XDR were observed in 100% and 97.5% isolates respectively.

On the basis of phenotypic characteristics, *vanA* phenotype was observed in 48.6% (n=85) and 85.7% (n=18) among clinical and environmental isolates respectively. *VanB* phenotype was observed in 51.4% (n=90) and 14.3% (n=3) clinical and environmental isolates respectively. *VanA* and *vanB* genes were detected in 48.6%

(85/175) and 50.9% (89/175) clinical isolates and 85.7% (18/21) and 4.7% (1/21) environmental isolates respectively.

Among the clinical and environmental LREfm isolates, *cfr* gene was not detected. *OptrA* gene was the predominant mechanism of linezolid resistance (58.9% vs 75%) followed by point mutation at G2576T and C2610T in the domain V of the 23S rRNA (25.2% vs 33.3%) among clinical and environmental isolates. The MIC of linezolid among isolates with G2576T and C2610T point mutation in the domain V of the 23S rRNA was approximately 10fold higher (MIC50: 256 µg/mL) compared to isolates with no mutations (MIC50: 32 µg/mL). The MIC of these isolates (clinical) did not vary in the presence of *optrA* gene (MIC50: 256 µg/mL) or in the absence of *optrA* gene (MIC50: 256 µg/mL).

Among clinical and environmental LREfm isolates the most common virulence gene was *esp* (59.9% vs 70.8%) followed by *cyl* (25.7% vs 20.8%) and *hyl* (16.8% vs 25%). Virulence genes *gel* (1.5%) and *asa1* (0.5%) were detected rarely. Virulence genes detected in clinical isolates were more diverse as compared to environmental isolates. Among clinical LREfm presence of *esp* and *cyl* gene was significantly associated with the *vanB* gene (p=0.026).

A dendrogram of PFGE profiles of LREfm isolates (n=226) was constructed and a total of 20 clusters of related isolates (A1-A20) were observed. The clinical and environmental isolates of the same patient coexisted in the same clusters. Linezolid and vancomycin resistant isolates were detected in diverse background strains.

For MLST a total of 20 isolates representative of each PFGE cluster (A-1- A-20) were selected. Among 20 isolates, 14 were from the patient's clinical samples and 6 were from the patient's environmental samples. Overall, 6 different sequence types (STs) were characterized, Among the 6 sequence types, ST80 was the most frequent and was detected in 60%(n=12) isolates followed by ST761 (n=3), ST872 (n=2), only one isolate belongs to each ST1070 (n=1), ST409 (n=1), ST375 (n=1). All STs detected in the study belong to the CC17, which is a highly successful and high-risk clone for dissemination of antibiotic resistance in our hospital environment.

We can conclude that the MDR and XDR LREfm emerging in our hospital. The resistance is mediated primarily by *optrA* gene and mutations in 23S rRNA gene

(G2576T and C2610T). The study suggests that resistance is mediated partly by prior linezolid use and further spread through horizontal transmission of resistance strains facilitated by breach in infection prevention control (IPC) practices and *optrA* gene. As linezolid resistance is primarily detected in vancomycin resistant isolates, surveillance of MDR LREfm is needed to implement a dedicated stewardship programme to rationalize therapy for VRE and thus reduce linezolid consumption. Appropriate infection control policies are needed to prevent transmission of resistant strains.

ACKNOWLEDGEMENT

*I would like to express my sincere gratitude to my supervisor **Dr. Mohammad Amin-ul Mannan**, for his enthusiastic concern and his ever-vigilant help throughout the formation of this thesis to its completion. His constant guidance, encouragement and comfort have helped me in the difficult patches in this work. His untimely availability is sincerely appreciated.*

*Words cannot express my gratitude to my co-supervisor **Dr. Rajni Gaind**, Vardhman Mahavir Medical College & Safdarjung Hospital, New Delhi. I am deeply indebted for the help and support that she has provided from the initial infancy of this work to its final maturation and compilation. Her valuable opinion and monumental encouragement would be cherished. I would not have been able to complete this without her.*

She has played a major role in polishing my research skills. She taught me how good experimentation is done. I am truly blessed to be able to work with her.

*I extent my sincere thanks to **Prof. Neeta Raj Sharma**, Head of School of Bioengineering and Biosciences, **Dr. Ashish Vyas**, Head of the Department of Microbiology, **Dr. Umesh Gautam**, Head of Molecular Biology and Genetic Engineering. I also would like to express my sincere thanks to **Dr. Rattandeep Singh**, **Dr. Vikas Sharma** and all other faculty members of Microbiology department for their help and support.*

*I am sincerely thankful to **Dr. Ashok Mittal** (Chancellor) and **Dr. Lovi Raj Gupta** (pro-vice chancellor and Senior Dean) for giving me the opportunity to work in this reputed institute with all the required essential amenities.*

I am thankful to the Indian Council of Medical Research (ICMR), New Delhi, India for financial assistance in the form of Senior research fellowship during my research tenure.

I would also like to thank the Centre for Research Degree Programmes (CRDP) of LPU for always helping me throughout the session in documentation related work.

My heartfelt thanks to my fellow labmates, Ms. Kavita Sisodia and Ms. Samrin Sayed. My special thanks to my Ph.D. senior Dr. Amit Sharma for his help, suggestions and motivational support. They all have formed the core of my research time and we've all been there for sharing ideas and advice on lab-related things.

A big thank goes to my friends Ms. Nikki Rai, Mr. Rohit Kumar, Mr. Vishal Negi, Dr. Rashi Raizada, Dr. Priyanka Das and Dr. Hitha Haridas in VMMC and Safdarjung Hospital and Mr. Suryakant Ranjan and Mr. Nahid in LPU for their help and support.

My sincere thanks to Dr. Ajeet Singh for being a constant source of inspiration, for his valuable suggestions and his support even in the tough time of my Ph.D. tenure. I am truly blessed to have him.

I would also like to express my gratitude to the members of VMMC and Safdarjung Hospital who had directly or indirectly helped me during the course of this study.

Last but not the least, I would like to thanks to my family for their patience, endless love, support and positive energy that is always motivational for me.

Vandana Rani

Dedicated

To

My Family

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LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
ATCC	American Type Culture Collection
Bp	Base pair
CDC	Centre for Disease Control
CLSI	Clinical and Laboratory Standards Institute
CNS	Central Nervous System
DNA	Deoxyribose Nucleic Acid
dNTP	Deoxyribose Nucleotide Triphosphate
E. faecalis	*Enterococcus faecalis*
E. faecium	*Enterococcus faecium*
EDTA	Ethylenediaminetetraacetic Acid
ESBL	Extended Spectrum β-Lactamase
FDA	Food and Drug Administration
ICMR	Indian council of medical research
ICU	Intensive Care Units
kDa	Kilo Dalton
LREfm	Linezolid resistant *E. faecium*
LSEfm	Linezolid sensitive *E. faecium*
MDR	Multi Drug Resistant
MIC	Minimum Inhibitory Concentration
MLST	Multilocus Sequence Typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
NaCl	Sodium Chloride
PCR	Polymerase Chain Reaction
PDR	Pan Drug Resistant

PFGE	Pulsed Field Gel Electrophoresis
PICU	Paediatric Intensive Care Unit
RAPD	Random Amplification of Polymorphic DNA
RFLP	Restriction fragment length polymorphism
rpm	Revolutions Per Minute
rRNA	*Ribosomal Ribose Nucleic Acid*
SPSS	Statistical package for social sciences
ST	Sequence Type
TBE	Tris Borate EDTA
U	Unit
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
VMMC	Vardhman Mahavir Medical College
VRE	Vancomycin resistant *Enterococci*
VREfm	Vancomycin resistant *E. faecium*
WHO	World Health Organisation
XDR	Extensive Drug Resistant
µg	Micro gram
µl	Micro litre
µM	Micro molar

1. Introduction

Enterococci are commensal flora of the gut and isolated from both humans and animals. They can also colonize the female genital tract, oropharynx and the perineal region [Moellering *et al*., 2005]. They are gram-positive cocci and facultative anaerobes [Sava *et al*., 2010].

Enterococci are the 3rd most common cause of nosocomial infections after methicillin resistant *Staph. aureus* and extended spectrum beta lactamase producing *Enterobacterales* [Monegro *et al*., 2023]. The genus *Enterococcus,* includes 58 species, *E. faecium* and *E. faecalis* are the most pathogenic species and are associated with serious infections like urinary tract infection (UTI), bacteremia, endocarditis, surgical site infections, gastrointestinal tract infections and infection associated with indwelling catheters and other implanted medical devices [Levitus *et al*., 2023; Dubin *et al*., 2016; Zaheer *et al*., 2020]. In the past, *E. faecalis* was associated with 80-90% clinically significant *Enterococcal* infections and 5-10% were associated with *E. faecium*. However, recently the proportion of *E. faecium* infections has increased and exceeded the prevalence of *E. faecalis* [Moellering *et al*., 2005] [Seedat *et al*., 2006].

Enterococci show intrinsic resistance to several antimicrobial agents like aminoglycosides, clindamycin, most cephalosporins and trimethoprim-sulfamethoxazole [Huycke *et al*., 1998]. *Enterococci* are also capable of acquiring resistance either through mutation or by horizontal transfer of genetic elements [Patel *et al*., 2013; Guzman Prieto *et al*., 2016]. Currently, multi drug-resistant *E. faecium* has emerged as nosocomial pathogen and causes serious infections specially in immunocompromised patients. This may be due to acquisition of acquired ampicillin and glycopeptide resistant genes [Nilsson 2012; Jahansepas A *et al*., 2018].

The use of broad-spectrum antibiotics like cephalosporin among hospitalised patients predisposes to gut colonisation with MDR *E. faecium* [Hendrickx *et al*., 2013]. Other risk factors associated with acquisition of MDR *E. faecium* include contact with colonized patients and breach in infection prevention practices leading to cross transmission [Agudelo Higuita *et al*., 2014]. Recent literature suggests that resistance to linezolid is emerging in patients with recent linezolid exposure or in patients receiving linezolid for extended duration.

Vancomycin resistance was first reported in 1980 from Europe and vancomycin resistant *Enterococci* (VRE) have spread rapidly and increasing resistance is being reported globally [Kadri *et al*., 2019]. The main mechanism of vancomycin resistance is modification of the peptidoglycan synthesis pathway, specifically the substitution of D-Ala-D-Ala for either D-Ala-D-Lac or D-Ala-D-Ser. These alterations may lead to variable expressions of glycopeptide resistance [Courvalin, 2006]. 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%). World Health Organisation has classified vancomycin resistant *E. faecium* (VREfm) as high priority pathogen and linezolid is a reserve antibiotic for vancomycin-resistant isolates. Treatment options for invasive VREfm infections are very limited, resulting in high mortality [Asokan *et al*., 2019].

Linezolid was introduced in early 2000 as a novel therapeutic option for serious gram-positive infections, including MDR organisms such as methicillin resistant *Staph. aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE) [Asokan *et al*., 2019]. 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 initiation complex. Linezolid being a synthetic drug, there is a low risk of pre-existing or naturally occurring resistance. After the introduction of linezolid, it was predicted that resistance to linezolid would be rare. However, linezolid-resistant *Staphylococci* and *Enterococci* have been increasingly reported in recent years [Baits, 2000]. Moreover, majority of the reports of linezolid resistance among *Enterococci* are associated with concurrent resistance to vancomycin. Two programs monitoring infections with linezolid resistant strains: LEADER (in the USA) and ZAAPS (Zyyox ®Annual Appraisal of Potency and Spectrum Programme: worldwide) have also reported the emerging problem of simultaneous resistance to vancomycin and linezolid [Krawczyk *et al*., 2020; Levitus *et al*., 2023].

Resistance to linezolid is commonly mediated by a point mutation in the central region of domain V of the 23S rRNA [Prystowsky *et al*., 2001]. Moreover, mutations in the particular region of *L3, L4* and *L22* ribosomal proteins, although are less significant, have also been associated with linezolid resistance [Long *et al*., 2012].

Plasmid mediated linezolid resistance has also been reported such as *cfr* (*Staphylococci* homolog) and its derivatives, known as *cfr*(B) and *cfr*(D) variants have been detected in *Enterococcus* species [Guerin *et al*., 2020]. Additional research on the mechanism of resistance has shown linezolid resistance may emerge in the absence of mutation and lack of the *cfr* gene. *OptrA* and *poxtA genes*, have also recently emerged as linezolid resistance determinants. In 2015 *optrA* gene was first reported from China in linezolid resistant *Enterococci* from both human and animal sources [Wang *et al*., 2015]. Whereas in 2018, *poxtA* gene was first reported from Italy in a MRSA clinical isolate [Dejoies *et al*., 2021]

Along with antibiotic resistance, virulence factors may have an impact on clinical outcomes. Several potential virulence factors contribute to the pathogenicity of the *E. faecium* isolates [Coque *et al*., 1995; Alan P. Johnson, 1994; Libertin *et al*., 1992].

Various methods have been used to study the molecular epidemiology of *E. faecium.* PFGE had been successfully applied as a gold standard. In PFGE a slice of agarose containing chromosomal DNA after the digestion with appropriate restriction enzyme is subjected to the wells of agarose gel and the fragments are resolved into a pattern of discrete bands in the gel by an electrophoretic apparatus that switches the direction of current according to a predetermined pattern. The DNA restriction patterns of isolates are then compared to determine their relatedness [Tenover *et al*., 1995]. The need for global interlaboratory exchange of data limits the use of PFGE. Multilocus Sequence Typing (MLST) is a highly discriminative typing technique for bacterial pathogens using DNA sequences of seven housekeeping gene fragments and can be shared using an online database that can be accessed from any part of the world via the internet [Maiden *et al.*, 1998].

The reports of linezolid resistance are fast emerging and are a cause of concern. In India, sporadic cases of linezolid resistant *Enterococci* infections have been reported. However, there is paucity of studies to determine the prevalence, mechanisms of resistance and dissemination of linezolid resistance among *E. faecium* in India. The present study was undertaken to determine the prevalence of linezolid resistant *E. faecium*, characterize the various mechanism associated with linezolid resistance. The risk factors and clinical outcomes of patient's infected with linezolid-resistant *E.*

faecium and the probable source of transmission of linezolid resistance within the hospital were also studied using molecular typing (PFGE and MLST).

2. Review of Literature

2.1 The history of the genus *Enterococci*

The first historic communication of *Enterococcus* was in 1899, with the identification of a new gram-positive diplococcus which was isolated from the human faeces by ME Thiercelin and he named this bacteria enterocoque [Thiercelin and Jouhaud, 1903; Stiles & Holzapfel, 1997]. However, in 1906 based on its ability to build short or long chains, Andrewes and Harder renamed Thiercelin's 'enterocoque' as *Streptococcus faecalis*. The history of *Enterococci* due to these early links, cannot be considered separately from *Streptococcus* genus.

In 1933 Rebecca Lancefield coined the term *Enterococcus* and classified *Enterococci* as *Group D Streptococci* [Hancock *et al*, 2000]. *Group D Streptococci* included both *Enterococcal* as well as non-*Enterococcal* species like *Streptococcus bovis* and *Streptococcus equinus*. Many attempts have been made to distinguish *Enterococcus* species from *Streptococcus* species. Later on in 1984, it was clear that *Enterococci* belong to a separate genus *Enterococcus*. *Enterococcus faecalis* and *Enterococcus faecium* were the first species that were transferred to the new genus and renamed from *Streptococcus faecalis* and *Streptococcus faecium* respectively [Moellering *et al*, 2005].

2.2 Taxonomy of *Enterococcus*

In 1984 *E. faecium* and *E. faecalis* were first described by Schleifer and Kilpper-Ba lz in the genus *Enterococcus*. Prior to 1984, *Enterococci* were subdivided into different *Streptococcus* groups. *Streptococci* were classified into four groups by Sherman (1937): '*Enterococci* or faecal *Streptococci*, lactic or dairy *Streptococci*, *Viridans*, and pyogenous *Streptococci* [Klein, 2003]. The terms '*Viridans*' and '*Enterococci*' were later changed to oral and faecal *Streptococci*, respectively [Jones, 1978].

Enterococci belong to the phylum firmicutes with low G+C content. On the basis of phylogenetic analysis of 16S rRNA, it was revealed that genus *Enterococcus* closely related to the genera *Vagococcus* and *Tetragenococcus* in comparison to *Streptococcus* and *Lactococcus* [Facklam *et al*., 2002; Schleifer *et al*., 1984].

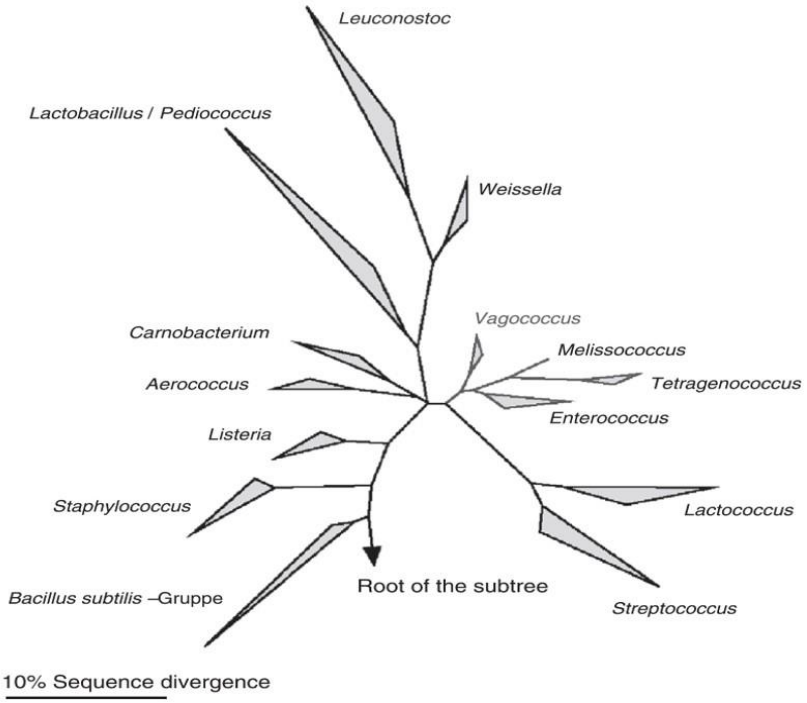


Figure 1: Phylogenetic dendrogram of 16S rRNA represents position of *Enterococcus* species [Image Adapted from Klein, (2003)].

Approximately 58 species are recognised in the genus *Enterococcus*, with *Enterococcus faecalis* and *Enterococcus faecium* being the most important and pathogenic to humans [García-Solache *et al*., 2019]. *Enterococci* are divided into 5 different groups based on fermentation of mannitol and hydrolysis of arginine.

2.3 Habitat of *Enterococci*

Enterococci are commensal gut flora of both humans and animals. *Enterococci* can also be isolated from the female genital tract, oropharynx and from the perineal region [Moellering *et al*., 2005]. They are gram-positive cocci and facultative anaerobic bacteria [Sava *et al*., 2010]. *Enterococci* can grow in a wide range of temperatures (ranging 5-65°C), pH (ranging 4.5-10) and at high NaCl concentration (6.5%), this environmental harshness is the main reason for the survival of *Enterococci* [Moellering *et al*, 2005; Vydra *et al*, 2002; Kainer *et al*, 2007].

2.4 Transmission of *Enterococci*

Enterococci are commensal flora of the gut and are abundantly found in faeces of humans and warm-blooded animals. Isolation of *Enterococci* from food and water is considered as a reliable sign of faecal contamination. In hospitals, poor implementation

of cleaning practices results in persistence of *Enterococci* in the environment [Moellering *et al*., 2005; Vydra J *et al*., 2002; Kainer *et al*., 2007; Hancock *et al*., 2000]. The sources of *Enterococci* in hospital environment are diverse and include colonised patients, contaminated medical equipment's, various environment surfaces and hands of healthcare workers. *Enterococci* are frequently isolated from high contact points such as bed rails, cuff of blood pressure machine, toilet seats and handles of doors. Isolation of *Enterococci* are excellent indicator of hospital environmental contamination [Arias & Murray, 2012]. Colonisation with MDR *Enterococci* may become endemic, when strict sterilization and disinfection practices are not implemented [Bonten *et al*., 1996]. The colonised healthcare workers and environmental sources may lead to the spread of *Enterococcal* infection via cross-contamination in the absence of infection prevention control practices [Bradley *et al*., 2002; Agudelo Higuita & Huycke 2014].

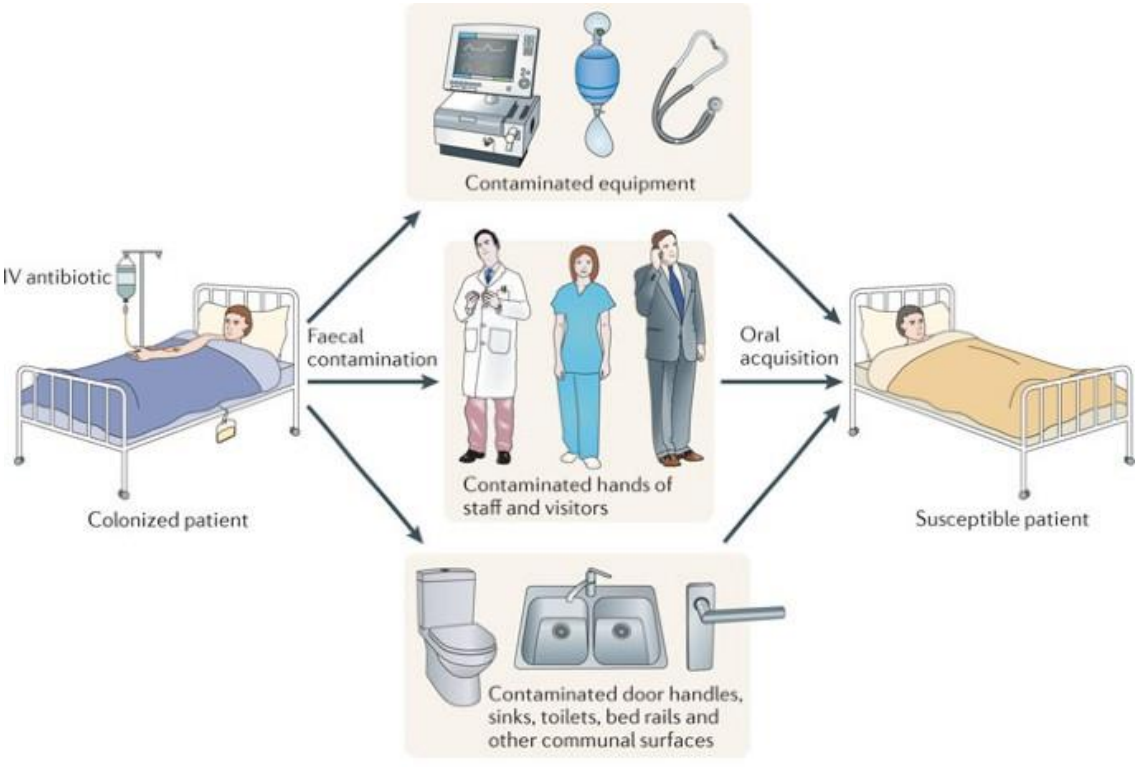


Figure 2: Route of transmission of MDR *Enterococci* [Image Adapted from Arias., Murray, B. (2012)].

2.5 Enterococci species and species of clinical significance

The genus *Enterococcus*, includes 58 species [García-Solache and LB Rice, 2019; Zaheer *et al*., 2020]. The most common and pathogenic species to humans are *E.*

faecium and *E. faecalis,* isolated from GIT. *E. hirae* is frequently isolated from farm animals. *E. mundtii* and *E. casseliflavus* are commonly isolated from plant sources [Dubin *et al*., 2016; Zaheer *et al*., 2020]. In the past, *E. faecalis* was associated with 80-90% clinically significant *Enterococcal* infections and 5-10% were associated with *E. faecium*. However, recently the proportion of *E. faecium* infections has increased and exceeded the prevalence of *E. faecalis* [Ruoff *et al*., 1990; Moellering *et al*., 2005; Seedat *et al*., 2006].

E. faecium is commensal in the human gastrointestinal tract but currently multi drug-resistant *E. faecium* has emerged as nosocomial pathogen and associated with serious infections specially in immunocompromised patients [Agudelo Higuita & Huycke, 2014]. Over the last two decades, *E. faecium* has rapidly evolved as a global nosocomial pathogen by successfully adapting to nosocomial environment. This may be due to the acquisition of ampicillin and glycopeptide resistant genes. However, resistance to antibiotics including ampicillin and glycopeptides is less common in *E. faecalis* [Werner *et al*., 2008; Jahansepas *et al*., 2018].

Currently, *Enterococci* are the 3rd most common cause of nosocomial infections after methicillin resistant *Staphylococcus aureus* (MRSA) and extended spectrum beta-lactamase producing *Enterobacterales* (ESBL-E) [Marra *et al*., 2006]. *Enterococci* are responsible for various infections in humans like bacteremia, pneumonia, skin & soft tissue infections, surgical site infections, and urinary tract infections resulting in significant morbidity and mortality [Arias *et al*., 2008; Bonora *et al*., 2006]. In neonatal intensive care units, infections with multidrug-resistant *Enterococci* are a serious health problem globally as it is associated with significant morbidity and mortality [Bonten *et al*., 2001]. Generally, infections are acquired by endogenous route in colonised patients. The pathogen is unlikely possess antimicrobial resistance beyond that intrinsic to the genus and are unlikely to spread among patients or infections may be acquired through cross transmission these isolates possess multiple antibiotic resistance traits and are capable for nosocomial infections [Huycke *et al*., 1998].

There is an increasing trend for infections caused by *E. faecium*, frequently associated with the rise of β-lactam and vancomycin resistance. Recent data suggest that vancomycin resistant *E. faecium (*VREfm) is widely distributed in hospitals all over the world with the prevalence varying according to geographical location [Levitus

et al., 2023]. Treatment options for invasive VREfm infections are very limited, resulting in high mortality. List of priority bacterial pathogens published by the World Health Organization (WHO) for which new antibiotics are urgently needed and VREfm is listed as the high-priority pathogen of antibiotic-resistant bacteria [Asokan *et al*., 2019].

2.6 Infections caused by *Enterococci*

E. faecium and *E. faecalis* are the most pathogenic species and are associated with serious infections like urinary tract infection (UTI), bacteremia, endocarditis, Intra-abdominal pelvic and soft tissue infection.

2.6.1 Urinary tract infection (UTI)

The most common infection caused by *Enterococci* is urinary tract infection. In elderly men, lower urinary tract infections such as cystitis, prostatitis, and epididymitis are most common and associated with upper urinary tract infections that can result in bacteremia more frequently. However, simple cystitis in young women caused by *Enterococci* is rare. About 15% of healthcare-related urinary tract infections in ICU patients are caused by *Enterococci* and VRE have emerged as important health care associated urinary tract infections among ICU patients [Agudelo Higuita & Huycke, 2014]. A survey of nosocomial infections was conducted between 2011-2014 in the US and it was observed that the overall 14% of the infections were caused by *Enterococci* and *E. faecium* were observed in 3.7% of cases, in these patients infections with vancomycin and ampicillin resistant *Enterococci* were observed in 80% and 40% respectively [Weiner *et al*., 2016]. In a multicentric retrospective study reported by Salm et al., (2022), a total of 102,736 male patients were screened for the UTI, *E. faecalis* were isolated from 16.5%. In a recent study it was reported that UTI due to *E. faecalis* was more common in men (8.8%) in comparison to women (1.8%) [Da Silva *et al*., 2022]. Reports from CDC and ISID suggest that among hospitalized patients 13% of the nosocomial infections present as UTIs and ranked as the 5^{th} most common infections [Nicastri & Leone, 2023].

Some species of *Enterococci* are capable to form biofilm. Biofilms are population of cells that are permanently adhered to a variety of biotic and abiotic

surfaces [Kristich *et al*., 2004]**.** Several studies have reported the association between urinary catheter and biofilm producing *Enterococci*.

2.6.2 Bacteremia

Gram-positive bacteria cause a high proportion of bloodstream infections, with *Enterococci* accounting for approximately 45% of the cases [Le Jeune *et al*., 2010]. Bacteremia is frequently associated with vancomycin-resistant *Enterococci*. These nosocomial infections occur as a result of the use of intravascular and urinary catheters. Infections with MDR *E. faecium* in the bloodstream is associated with increased mortality. Globally nosocomial bacteremia rate due to *Enterococci* is quite high, ranging between 25-50% [Fiore *et al*., 2019] [Pinholt *et al*., 2014]. In a review study of bloodstream infections, *Enterococci* were identified as the only Gram-positive pathogen associated with a high risk of mortality [Weinstein *et al*., 1983]. In 1980s, *Enterococci* were the sixth most common of healthcare-associated bacteremia [Agudelo Higuita & Huycke, 2014]. As per the recent study from Asia, it was reported that *Enterococci* species are 4th most common bacteria causing blood stream infections [Dai *et al*., 2022]. Previous report from Asia, showed that the *Enterococci* are 3rd most common gram-positive pathogens in community acquired blood stream infections [Zheng *et al*., 2017]. In addition, bacteremia due to *E. faecium* is associated with higher mortality rate compared to *E. faecalis* bacteremia [Noskin *et al*., 1995].

2.6.3 Endocarditis

Infective endocarditis (IE) is difficult to treat infections and *Enterococci* are the third leading cause of IE [Herrera-Hidalgo *et al*., 2023]. *Enterococci* are responsible for approximately 10-20% cases of infective endocarditis cases, the most common species associated with IE is *E. faecalis* followed by *E. faecium* [Giannitsioti *et al*., 2007; Herrera-Hidalgo *et al*., 2023]. The urinary and gastrointestinal tract are the initial source of bacteremia leading to endocarditis. Prosthetic valve *Enterococcal* endocarditis has become more common, possibly due to the increased use of these prostheses in adults (≥50 years), who are predisposed to *Enterococcal* bacteremia. The clinical presentation of *Enterococcal* endocarditis is usually a subacute infection characterized by heart failure rather than embolism; however, a rapidly progressive disease can also occur [Anderson *et al*., 2004; McDonald *et al*., 2005]. The mortality rate of *Enterococcal* endocarditis is lower than other forms of infective endocarditis, although mortality is

still significant. Currently, this is challenging to choose the antibiotic for the treatment of *Enterococcal* endocarditis caused by MDR isolates [Stevens & Edmond, 2005].

2.6.4 Intra-abdominal pelvic and soft tissue infection

In intra-abdominal, pelvic, and soft tissue infections *Enterococci* are frequently associated with polymicrobial infections. The importance of *Enterococci* in wounds and abscesses has been debated for a long time. However, in *Enterococcal* bacteremia, which is usually associated with intra-abdominal and pelvic abscesses and ulcers, Clinicians usually use the *Enterococcal* antibiotics for infections at these sites [Graninger *et al*., 1992; Patterson *et al*., 1995; Rajkumari *et al*., 2014]. The study reported by Agarwal et al., showed that the most common *Enterococci* isolated from the skin and soft tissue infections is *E. faecium* followed by *E. faecalis* [Agarwal *et al*., 2009]. In contrast, other studies from India and the US showed that *E. faecalis* is the most common isolates in *Enterococci* isolated from the skin and soft tissue infections [Fernandes *et al*., 2013; Sievert *et al*., 2013].

2.7 Virulence factors associated with *Enterococci*

Various virulence factors contribute to the pathogenicity of *Enterococcal* infections. These virulence factors are mainly associated with the adherence of bacteria to the host cells, invasion, modulation of host immune responses, and also induce toxicity. The major virulence factors of *Enterococci* are lytic enzymes gelatinase (*gelE*), serine proteases (*sprE*), aggregation substances (*asa1*), *Enterococcal* surface adhesin proteins (*esp*), sex pheromones and toxic cytolysin [Coque *et al*., 1995; Johnson 1994; Libertin *et al*., 1992].

2.7.1 Gelatinase (*gelE)* and serine protease (*sprE*)

Among various other virulence factors, gelatinase and serine protease have been reported as an important factor for the pathogenesis of various human diseases. Gelatinase (*gelE)* belongs to the family zinc-containing metalloproteinase enzymes. Gelatinase is an extracellular enzyme that can hydrolyze a wide range of substances such as gelatine, casein, collagen, human endothelin lactoglobulin, fibrinogen, and insulin [Mäkinen *et al*., 1989]. Other than hydrolyzing activity, its role has also been reported in association with biofilm formation [Hancock and Perego, 2004]. Expression of *gelE* and *sprE* genes in *Enterococci* are positively regulated by the *frs* locus quorum

sensing system. The *frs* locus contains four genes *fsrA, fsrB, fsrC* and *fsrD*. A signalling peptide sequence at the C-terminal of *fsrB* in coordination with *fsrA, fsrB* and *fsrD,* releases gelatinase biosynthesis activating pheromone (GBAP) peptide which accumulates and induces the expression of both *gelE* and *sprE* genes [Qin *et al*., 2000].

2.7.2 Aggregation substance (AS)

AS is a pheromones-mediated group of proteins, encoded by plasmid and required for bacterial adhesion that facilitates contact between donor and recipient bacterium to exchange plasmid. Recipient cells express binding substances on their surface which are required to interact with AS secreted by donor cells. Several in vitro studies have reported that AS mediates the interaction with a variety of eukaryotic cells including cultured intestinal epithelial cells [Olmsted *et al*., 1994] and cultured renal tubular cells [Kreft *et al*., 1992]. In addition to its conjugative transfer function, AS also promotes cell-cell adhesions, binding to host cells and ECM proteins such as collagen type I, thrombospondin, and fibronectin [Tendolkar *et al*., 2003]. This adhesion is facilitated by two RGD (Arginine, glycine, and aspartic acid) motifs by cell surface receptors called integrins.

2.7.3 *Enterococcal* surface adhesin proteins (*esp*)

Enterococcal surface adhesion proteins encode *esp* gene. Adhesion proteins are located on the bacterial surface. *Esp* and its variants were initially reported from the surface of gentamicin resistant *E. faecalis* and *E. faecium,* respectively [Shankar *et al*., 2002; Eaton *et al*., 2001]. Studies in animal models reported that surface proteins (*esp*) contribute to colonization and persistence of the bacterium in patients with urinary tract infection [Shankar *et al*., 2001]. Primary attachment to host tissues and biofilm formation is also associated with surface protein. In the case of *E. faecalis* surface protein (*esp)* is most commonly present in environmental isolates in contrast to *E. faecium,* where *esp* is frequently expressed in the nosocomial isolates [Willems *et al*., 2001] [Leavis *et al*., 2004]. Expression of *esp* is widely affected by various environmental factors such as oxygen availability, temperature, and moisture.

2.7.4 Sex Pheromones

Gram-positive bacteria have a specialized intercellular communication system called sex pheromones which is encoded 7 or 8 amino acids long hydrophobic peptides

chromosomally. The function of this signalling peptide is to communicate among the population to control the transfer of conjugative plasmid between the recipient and donor cells [Clewell & Weaver, 1989]. Sex pheromones increase the efficiency between the cells by many folds. Recipient cells that do not contain conjugative plasmid secrete pheromones and in response to this, donor cells secrete surface proteins which facilitate to make of close contact between donor and recipient cells. After acquisition of the plasmid, the recipient cell stops secreting this peptide but contribute to secrete pheromone which are specific for other plasmids not present in the bacteria.

2.7.5 Cytolysin

Enterococcal cytolysin is plasmid-encoded, also known as hemolysin, however few studies have reported, this toxin is chromosomally encoded as well [Ike & Clewell, 1992]. It is a large family of toxins secreted by pathogenic and non-pathogenic bacteria. Cytolysin enhances the virulence of *Enterococci*. Infections with isolates having this toxin are associated with increasing mortality. Production and activation of *Enterococcal* cytolysin is mediated by a unique two-peptide lytic system that involves a series of multistep processes. The two cytolytic genes $cylL_L$ and $cylL_S$ encode for $cylL_L$ (large subunit) and $cylL_S$ (small subunit) respectively. Precursor peptides of $cylL_L$ and $cylL_S$ are post-transcriptionally modified by *cylM*. After modification, these peptides are activated by proteolytic cleavage by *cylB* to generate $cylL_L$ and $cylL_S$. Further, six identical subunits are removed extracellularly by a serine protease *cylA* to generate $cylL_L$ and $cylL_S$. Active $cylL_L$ and $cylL_S$ subunits are toxic and mediate lysis of the host cells [Haas W et al 1999]. Cytolysin-producing enterococcus has a *cylI* gene to protect itself from the action of the toxin [Coburn *et al*., 1999]. The most common targets of cytolysin are PMNs, macrophages, erythrocytes, and gram-positive bacteria other than *Enterococci* [Basinger *et al*., 1968; Miyazaki *et al*., 1993].

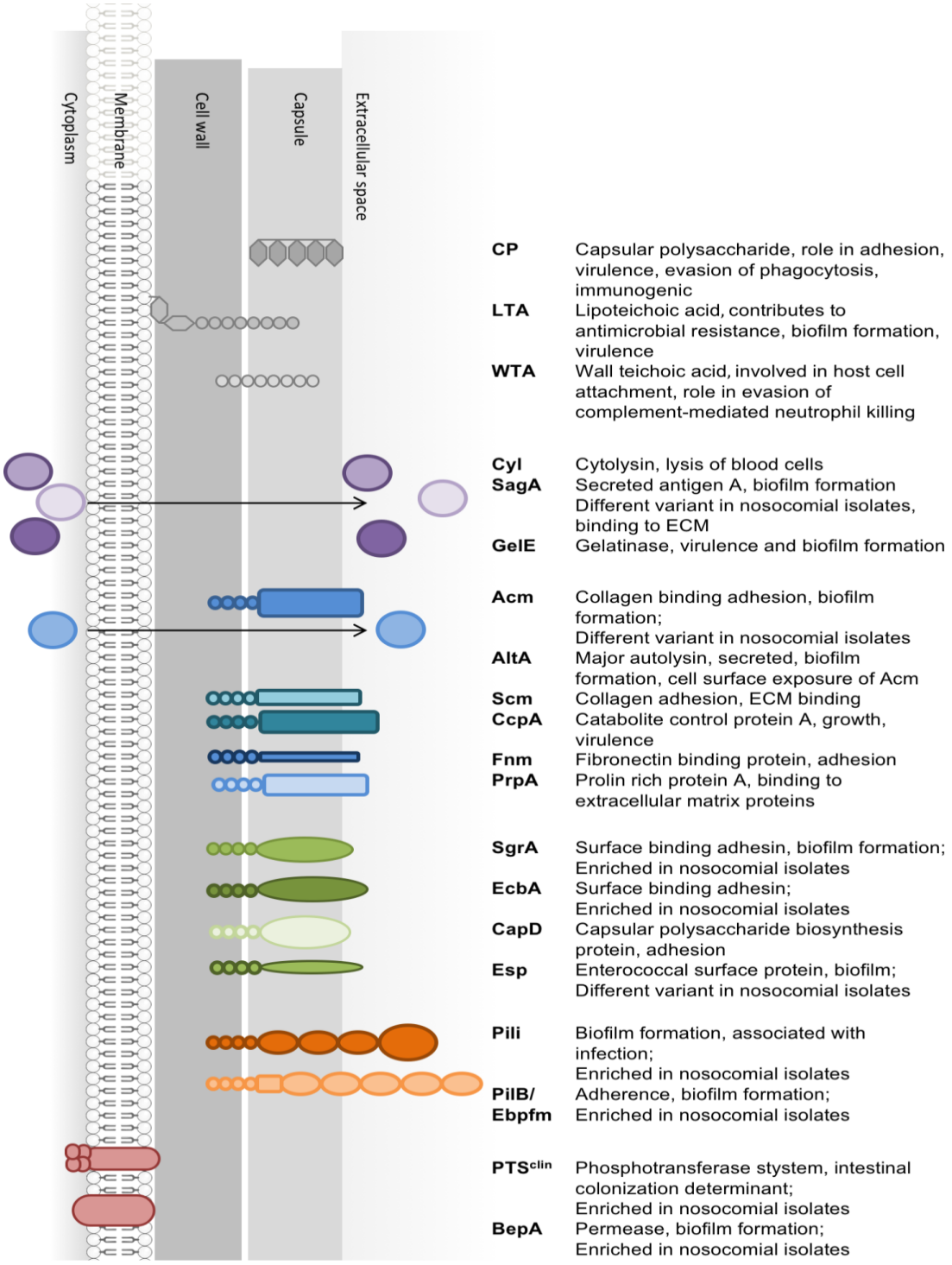


Figure 3: Overview of *Enterococci* virulence factors. Virulence factors associated with *Enterococci* including cell wall components, secreted virulence factors and membrane-bound virulence factors [Image Adapted from Gao *et al*., (2018)].

2.8 Mechanism of Antibiotic resistance

2.8.1 Intrinsic resistance

2.8.1.1 β-lactams (Ampicillin /penicillin)

β-lactams such as ampicillin and penicillin inhibit peptidoglycan synthesis. In *Enterococci* intrinsic resistance to β-lactams is associated with the expression of species specific low-affinity Penicillin binding proteins (PBPs). The most effective β-lactams for *Enterococci* are ampicillin and penicillin. Every *Enterococci* produces at least 5 PBPs. By genomic investigation of *E. faecium* and *E. faecalis*, six putative PBP genes have been identified, which include class A (*ponA, pbpF, pbpZ*) and class B (*pbp5, pbpA, pbpB*) with three types in each. *E. faecium* and *E. faecalis* express PBP5 with low affinity to β -lactam antibiotics [Gagetti *et al*., 2019; Miller *et al*., 2014]. MICs for penicillin are typically much higher for *E. faecalis* (2-8 µg/mL) and *E. faecium* (8-16 µg/mL) [Sifaoui *et al*., 2001] than MICs for *Streptococci* (≤0.6-2 µg/mL) and other related gram-positive bacteria that do not contain chromosomally encoded low affinity PBPs genes [Murray 1992]. *E. faecalis* is typically 10-100 times less susceptible to penicillin in comparison to *Streptococci*, while *E. faecium* is 4-16 times less susceptible than *E. faecalis* [Murray1990]. In *E. faecium* and *E. faecalis* another mechanism of β-lactam resistance (ampicillin) is mediated by β-lactamase enzyme that inactivates the antibiotic by hydrolysis of the β-lactam ring [Miller *et al*., 2014].

2.8.1.2 Cephalosporins

Intrinsic resistance to cephalosporin is well known trait of *Enterococci*, however the molecular basis of this phenotype is not clearly understood. This intrinsic resistance is associated with the decreased binding affinity to PBPs present in *Enterococci*, specifically to PBP5. As class B PBPs have only transpeptidase enzymatic activity and needs to associate with a glycosyltransferase to synthesize peptidoglycan. In vitro studies by Rice, *et al*., (2009) and Arbeloa *et al*., (2004) on both *E. faecalis* and *E. faecium*, the PBPs of class A PBPs (*PbpF, PonA* or *PbpZ*) were sequentially deleted individually or in combination to understand their impact on the resistant phenotype. It was observed that unlike *PonA* or *PbpF, PbpZ* alone was unable to produce the required trans-glycosylase activity to exhibit cephalosporin resistance in both species.

2.8.1.3 Aminoglycosides

Enterococci exhibit low level of intrinsic resistance to aminoglycosides and degree of resistance varies among various aminoglycosides with MICs ranging from 4μg/mL to 256μg/mL [Murray, 1990: George *et al*., 2001]**.** Intrinsic resistance results in low level resistance to aminoglycosides is associated with low uptake of aminoglycosides in *Enterococcal* species. Its metabolic pathway interferes with the proteins involved in the transport of electrons and restricts aminoglycoside absorption which leads to the intrinsic resistance of aminoglycosides at low levels [George *et al.,* 2001; Chow *et al.,* 2000]. An enzyme 6` acetyl transferase acetylase [AAC'6'-Ii] predominantly produced by *E. faecium* makes it intrinsic resistant to amikacin, netilmicin, kanamycin, and tobramycin [Sood *et al*., 2008]**.** Other additional intrinsic resistance mechanisms of aminoglycoside in *Enterococci* include ribosomal target alteration conferring resistance to kanamycin and tobramycin [Galimand *et al*., 2011]**.**

2.8.1.4 Lincosamides, Macrolides and Streptogramins

Lately resistance to macrolides and lincosamides among clinical isolates of gram-positive bacteria are being increasingly reported. Resistance to macrolides and lincosamides is mediated by drug inactivation, ribosomal modification and over expression of efflux pumps [Leclercq *et al.,* 2002]. In addition, *E. faecium* harbour the species-specific intrinsic gene *msrC* which confers the increased resistance to macrolide in comparison to other species of *Enterococci* [Kateete *et al*., 2019]**.** Ribosomal modification confers broad-spectrum resistance to lincosamides and macrolides, whereas inactivation of drug and over expression of efflux pumps leads to allowed susceptibility [Kateete *et al*., 2019]. Clinically significant *Enterococcal species* like *E. faecium, E. faecalis, E. avium, E. gallinarum* and *E. casseliflavus* harbour an intrinsic gene *Isa*, encodes a drug efflux pump which is structurally related to ABC-efflux pumps and confers intrinsic resistance to lincosamides and macrolides [Farha *et al*., 2020]. *E. faecalis* shows intrinsic resistance to quinupristin (streptogramin B class) [Kateete *et al.,* 2019], dalfopristin (streptogramin A class) and clindamycin (lincosamide) [Hallenbeck & LB Rice, 2012; Werner *et al*., 2002]**.**

2.8.1.5 Trimethoprim-sulfamethoxazole

Trimethoprim and sulfamethoxazole inhibit tetra-hydrofolate synthesis pathway. Thymidine and folic acid are required to carry out the various important cellular

functions like nucleic acid formation. The *p*-amino benzoic acid is a precursor of folate and majority of bacteria synthesize it as they are unable to take up exogenous folate from the environment. Trimethoprim and sulfamethoxazole prevent the production of dihydrofolate and its conversion to tetra-hydrofolate by inhibiting successive enzymes in this pathway. Trimethoprim and sulfamethoxazole are ineffective against *Enterococci in vivo* irrespective of the in-vitro susceptibility as organism have the ability to utilise folate from exogenous sources [Gholizadeh & Patrice Courvalin, 2002].

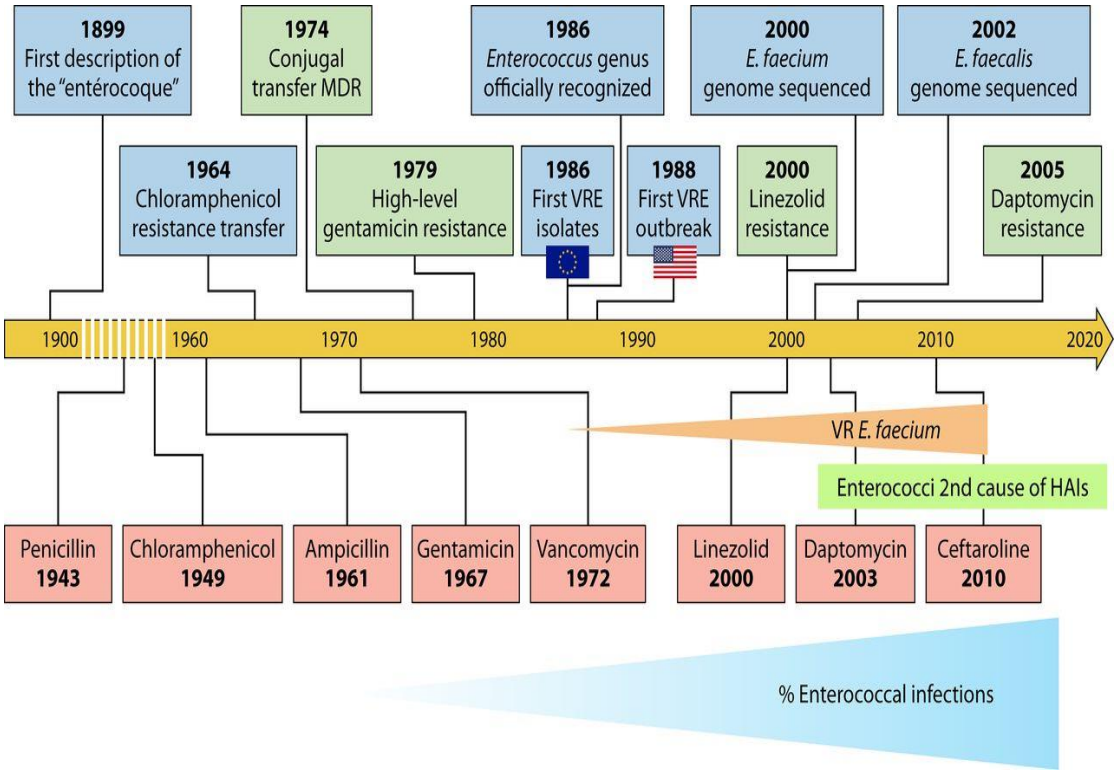


Figure 4: Timeline of relevant incidents in the history of *Enterococci* as human pathogens (sky blue rectangles), arrival of antibiotic resistance (light green rectangles), and antibiotic clinical introduction (orange rectangles) [Image adapted from García-Solache M, Rice LB. (2019)].

2.8.2 Acquired resistance in *Enterococci*

2.8.2.1 Glycopeptides

In gram positive bacteria, resistant to β-lactams, vancomycin and teicoplanin are frequently used glycopeptides for management [Gholizadeh & Patrice Courvalin, 2002]**.** Resistance to glycopeptide may be intrinsic or acquired through mobile elements. *Enterococci* isolates sometimes show resistance to both vancomycin and teicoplanin or sometimes only to vancomycin [Gholizadeh & Patrice Courvalin, 2002].

In 1986 vancomycin resistant *Enterococci* (VRE) were first reported after the 30 years of clinical use of vancomycin in Europe [Uttley *et al*., 1988; Rice, 2001]. Since then VRE have emerged as an important nosocomial pathogen [Gholizadeh & Patrice Courvalin, 2002].

Mode of action of glycopeptides

They are high in molecular weight and hydrophobic in nature. Due to these two properties, they are enabling to penetrate cytoplasmic membrane [Gholizadeh & Patrice Courvalin, 2002; Alduina *et al*., 2018]. Glycopeptides interact by 5 hydrogen bonds to the C-terminus of D-ala-D-ala residue of the pentapeptide precursors of peptidoglycan layer with high affinity, resulting in formation of a stable complex and blockage of the trans-glycosylation and transpeptidation reactions in cell wall synthesis. Consequently, the precursors accumulate inside the cell, losing integrity of cell wall resulting in cell death [Figure; 5] [Alduina *et al*., 2018].

2.8.2.1.1 Mechanism of glycopeptides resistance

In *Enterococci*, the main mechanism of glycopeptides resistance is modification of the peptidoglycan synthesis pathway, specifically the substitution of D-Ala-D-Ala for either D-Ala-D-Lac or D-Ala-D-Ser, these alterations may lead to variable expressions of glycopeptide resistance. It has been seen that the altered cell wall precursors D-Ala-D-Lac with high level of resistance (MIC>16 µg/mL) reduced the binding affinity of glycopeptide by around 1000- fold in comparison to the D-Ala-D-Ala (normal cell wall precursor), while the altered precursor D-Ala-D-Ser with low level of resistance (MIC of 8-16 µg/mL) reduce the binding affinity by seven-fold, when compared to the normal cell wall precursors D-Ala-D-Ala **[**Courvalin, 2006].

2.8.2.1.2 Phenotypic and Genotypic determinant of van resistance in *Enterococci*

On the bases of phenotypic and genotypic characteristic a total of nine types of vancomycin resistance operon have been reported among *Enterococci* namely, *vanA, vanB, van C, vanD, vanE, van G, van L, van M* and *van N* and are summarised in **table; 1.** Both vancomycin and teicoplanin shows inducible high level of resistance to *vanA* phenotype, while *vanB* shows variable levels of inducible resistance to vancomycin and susceptibility to teicoplanin. *VanC* show low level of intrinsic resistance in *E. gallinarum* (*van C1* genotype), *E. casseliflavus* (*vanC 2-4*), and *E. flavecens* [Leclerco

et al., 1992; Navarro Courvalin, 1994]. Resistance to vancomycin and teicoplanin varies among *vanD*-type *Enterococci* [Perichon & Courvalin, 2011]. *VanE* is defined as low level of resistance to vancomycin and sensitive to teicoplanin [Fines *et al*., 1999], *vanG* shows moderate level resistance to vancomycin [Depardieu *et al*., 2015]. Genetically and phenotypically *vanM* is similar to *vanA, vanB* and *vanD*, while *vanL* and *vanN* are similar to *vanC* type [Boyed *et al*., 2008; Lebreton *et al*., 2011; Xu *et al.*, 2010].

Among clinical isolates of *E. faecalis* and *E. faecium van A* and *van B* are the most frequently reported phenotypes [Kafil & Asgharzadeh, 2014]. The *vanA* operon is frequently associated with Tn1546 transposons, which contains nine polypeptides for different functional groups includes (a) orf1 and orf2 for transposition functions, (b) *vanR* and *vanS* for regulation of vancomycin resistance, (c) *vanH* and *vanA* for synthesis of dipeptide, (d) *vanX* and *vanY* for the peptidoglycan precursor hydrolysis, (e) the function of *vanZ* is still unknown [Arthur *et al*., 1997; Arthur *et al*., 1999]. Among them the necessary proteins for inducible resistance are *vanA, vanH, vanR, vanS* and *vanX*. *VanY* and *vanZ* are not necessary but the production of these proteins results in increased resistance to vancomycin and teicoplanin respectively [Arthur *et al*., 1996].

The normal *vanB* operon has a similar genetic foundation like *vanA* operon. Within *vanB* subtypes, high level vancomycin resistance designated as *vanB1-3*. Among *vanB* subtypes, however *vanB-2* is the most common genotypes globally. Acquisition or exchange of transposons such as Tn1547, Tn1549, and Tn5382 is required for the transfer of *vanB* resistance alleles. All gram-positive bacteria, including *VanB*-type *Enterococci*, include the conjugative *vanB* transposon Tn1549. This transposon is mostly found on chromosomes and is less common on plasmids.

2.8.2.1.3 Glycopeptides dependence

Some isolates of *Enterococci* (*vanA* and *vanB*-type) show glycopeptide dependency. These isolates are not only non-susceptible to glycopeptides, but they need glycopeptides for their growth. Clinical isolates of *Enterococci* that need vancomycin in the culture medium to grow have been isolated from patients who had prolonged vancomycin therapy. Both in vivo and in vitro methods have been used to produce mutants with a related phenotype. Vancomycin dependency is caused by inactivation of the chromosomal *ddl* gene. The mutations lead to synthesis of a truncated protein [Gholizadeh and Patrice Courvalin 2002]

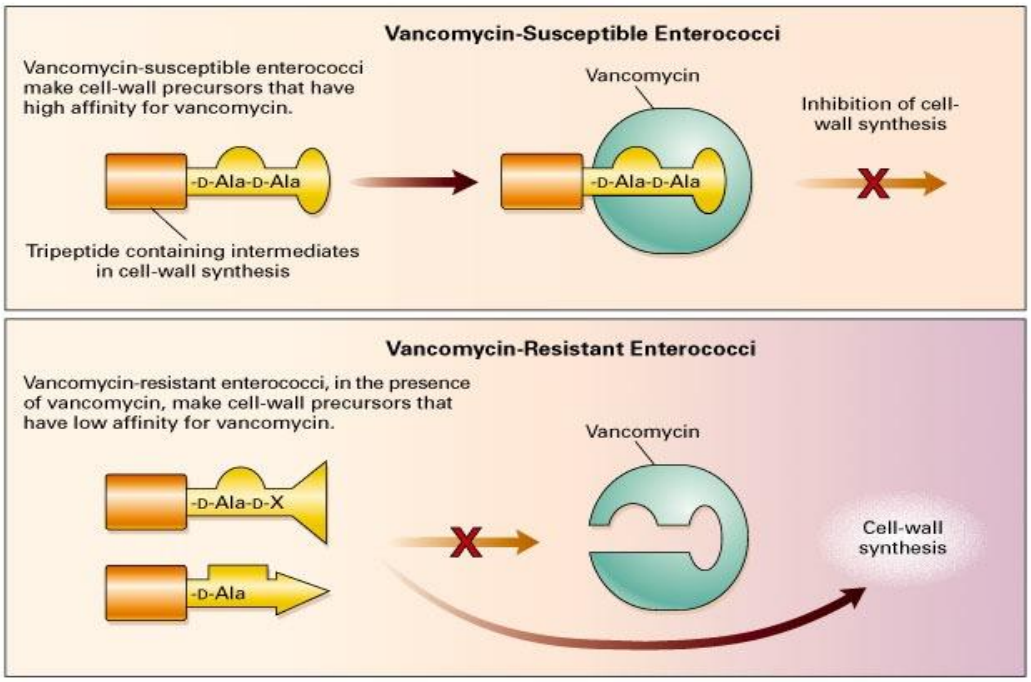


Figure 5: Schematic representation of mode of action of vancomycin and biosynthesis of peptidoglycan [Image Adapted from Alduina *et al*., (2018)].

Table 1: Glycopeptides resistance in *Enterococci*.

			MIC range (µg/mL)		
Resistance gene	Resistance type	Modified target	Vancomycin	Teicoplanin	Expression
vanA	Acquired	D-Ala-D-Lac	64-1000	16-512	Inducible
vanB	Acquired	D-Ala-D-Lac	4-1000	0.5-1	Inducible
vanC	Intrinsic	D-Ala-D-Ser	2-32	0.5-1	Constitutive or Inducible
vanD	Acquired	D-Ala-D-Lac	64-128	4-64	Constitutive or Inducible
vanE	Acquired	D-Ala-D-Ser	8-32	0.5	Inducible
vanG	Acquired	D-Ala-D-Ser	16	0.5	Inducible
vanL	Acquired	D-Ala-D-Ser	8	<8	Inducible
vanM	Unknown	D-Ala-D-Lac	>128	64->256	Inducible
vanN	Acquired		16	0.5	Constitutive

2.8.2.2 Fluoroquinolones

Quinolones have unique mode of action by inhibiting DNA gyrase and topoisomerase IV. In *Enterococci* resistance to quinolones can be intrinsic with low level or acquired

with high level [Lopez *et al*., 2011]**.** Fluoroquinolones have different primary targets in gram positive and gram-negative bacteria. In gram positive and gram negative bacteria's primary targets are Topoisomerase IV and DNA gyrase respectively [Arsene *et al*., 2007; Werner *et al*., 2010]. In gram positive bacteria resistance to fluoroquinolones is mediated by various mechanisms and include (a) Mutations present in target genes *gyrA* and *parC* that alter the binding affinity of drug in the quinolone resistance determining region (QRDR), this resistance frequently seen in *E. faecalis* and *E. faecium* [Werner *et al*., 2010; Yasufuku *et al.,* 2011]**,** (b)

Another mechanism of quinolone resistance is externalization of the fluoroquinolones through efflux pump, *emeA* and *norA* in *E. faecalis* and *E. faecium* respectively [Yasufuku *et al*., 2011]. (c) A 3rd mechanism of quinolones resistance frequently seen in *E. faecalis* mediated by *qnr* and encodes a sequence of pentapeptide repetitions for a protein similar to the plasmid-borne genes of quinolone resistance identified in *Enterobacterales*. The existence of this gene product is believed to protect DNA gyrase by limiting quinolone DNA binding and subsequent formation of the complex of quinolone-gyrase [Arsène *et al*., 2007].

2.8.2.3 Oxazolidinone

The 1st member of the oxazolidinone class is Linezolid. In 1978 oxazolidinones were first introduced to control plant diseases. In early 2000, linezolid was introduced as a novel therapeutic option for serious gram-positive infections, including MDR pathogens like methicillin resistant *Staph. aureus* (MRSA) and VRE [Ford *et al*., 2001].

Various reports have shown that linezolid has effective activity against some gram-negative anaerobes, anaerobic and aerobic gram-positive bacilli, anaerobic gram-positive cocci, nocardia species and mycobacteria species [Moellering, 2003 and Zurenko *et al*., 1996]. Reports also suggest that oxazolidinones, in clinically significant concentration, generally do not show activity against aerobic gram-negative bacteria like *E. coli* [Okusu *et al*., 1996].

Linezolid has various properties that were thought to make it less probable for developing drug resistance. Firstly, being a synthetic drug there is a minimal risk of pre-existing or naturally occurring resistance, which is observed in antibiotics produced by bacteria [Moellering, 2003]. Secondly, although the exact mechanism of action has

yet to be determined, several reports have shown that linezolid blocks ribosomal protein synthesis in bacteria [Eustice *et al*., 1988; Lin *et al*., 1997; Matassova *et al*., 1999 and Shinabarger *et al*., 1997]. However currently existing mechanisms of resistance to other agents acting on bacterial ribosomal subunit do not overlap with the linezolid resistance [Fines & Leclercq, 2000]. Lastly, linezolid specifically binds to the V domain of 23S rRNA of the large 50S ribosomal subunit, which contain multiple copies of genes (rRNA) in clinically significant organisms, for example, *E. faecalis* has 4 copies, while *E. faecium* and *S. aureus* have 5–6 copies [Klappenbach *et al*., 2001].

Linezolid resistance in clinical isolates of *Enterococci* is associated with mutation in central region of domain V of 23S rRNA gene and the most common mutation is G2576T [Prystowsky *et al*., 2001]. A *de novo* linezolid resistance is uncommon among *Enterococci*. However, the emergence of resistance to linezolid among clinical isolates of *Enterococci* from patients who had recently been exposed to linezolid therapy or in those who received it for extended periods is alarming [Seedat *et al*., 2006].

It is very clear from the published reports that recent or prolonged linezolid exposure is a crucial risk factor for the emergence of development of resistance among *Enterococci*. The majority of the reports of linezolid resistant *Enterococci* are associated with concurrent resistance to vancomycin [Scheetz *et al*., 2008; Pai *et al*., 2002].

2.8.2.3.1 Advantages of Linezolid

- Linezolid exhibits a broad spectrum of activity both *in-vitro* and *in-vivo*.
- The oral formulation of linezolid is 100% bio-available and is useful for to switch from iv to an oral therapy.
- Linezolid with its unique mode of action strongly inhibits the formation of preinitiation complex of protein synthesis which is achieved by selectively binding to the 50S ribosomal subunit. This is also prevents cross-resistance with any other antibiotics including vancomycin.

2.8.2.3.2 Mechanism of action of Linezolid

Linezolid binds to the central zone of domain V of 23S rRNA, a large subunit of 50S ribosome of bacteria via interactions and blocks preinitiation complexes formation of protein synthesis [Batts, 2000]. In some experiments of cross-linking it was observed that oxazolidinone binds to the A site of ribosomal subunit which probably get in the way with the placement of the aminoacyl-tRNA [Ament *et al*., 2002].

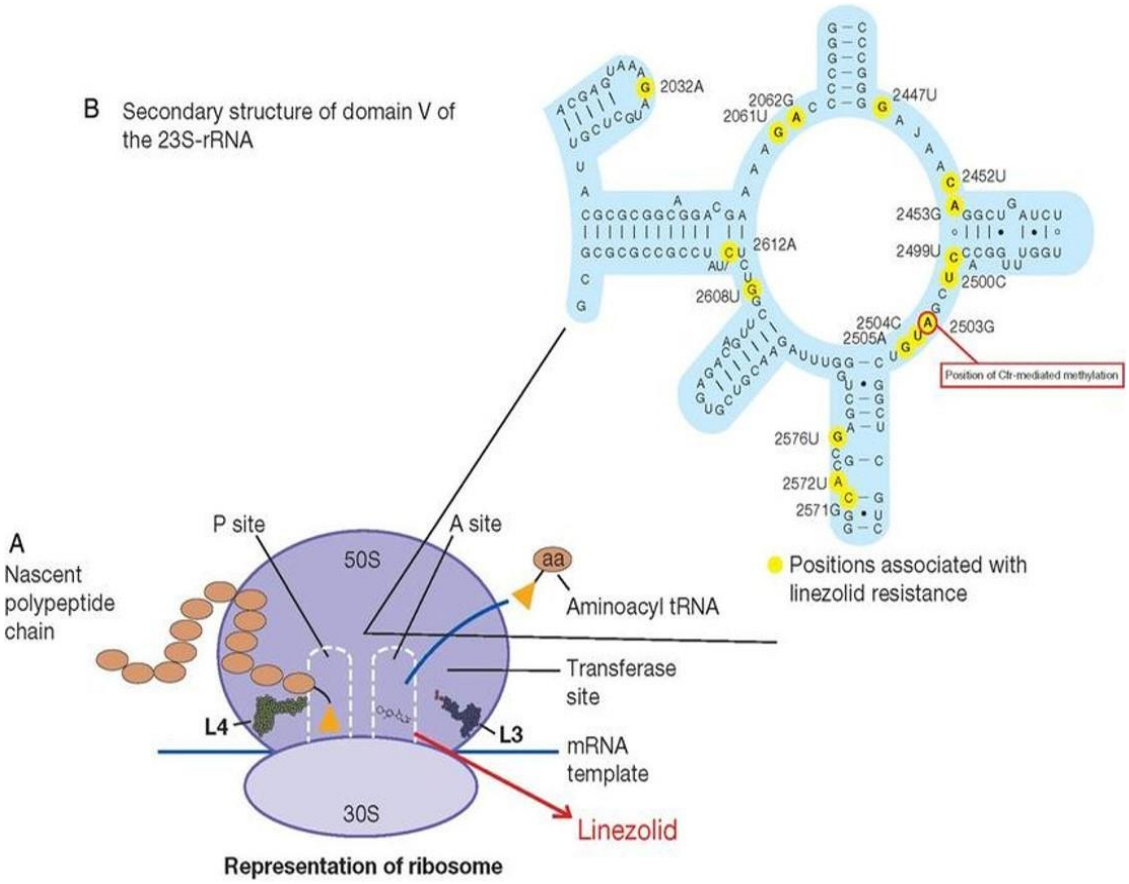


Figure 6: Schematic representation (A) Linezolid interferes with the positioning of aminoacyl-tRNA by interactions with the peptidyl-transferase center (PTC). Ribosomal proteins L3 and L4 associated with resistance are shown. (B) Representation of domain V of 23S rRNA showing mutations associated with linezolid resistance. Position A2503, which is the target of *cfr* methylation, is highlighted [Image Adapted from Munita, J. M., & Arias, C. A. (2016)].

2.8.2.3.3 Mechanisms of Linezolid Resistance

There are various mechanisms of linezolid resistance. As linezolid being a synthetic molecule, emergence of clinical resistance is rare due to a natural reservoir of genes.

2.8.2.3.4 Mutational resistance

a) Mutations in the domain V of the binding site of 23S rRNA

Over the decade, it has been seen that an increasing number of clinical isolates resistant to a group of antibiotics such as macrolide, lincosamide, streptogramin, ketolide, and oxazolidinone (MLSKO) with mutations in the domain V of 23S rRNA genes have emerged [Maravic, 2004]. Domain V is one of the most conserved region and it is also a very crucial part of the ribosomal peptidyl transferase center. Both Gram-positive and Gram-negative bacteria have these mutational changes with compromise the function of the 23S rRNA, resulting in the moderately decreased susceptibility to either one or more MLSKO group of antibiotics [Roberts, 2008]. Mutational changes in the central zone of domain V of 23S rRNA is the major cause of bacterial resistance to Linezolid. As almost all bacterial species have multiple gene copies of 23S rRNA, the development of linezolid resistance by mutation is less likely [Prystowsky *et al*., 2001].

Alteration in domain V have also been associated with the resistance to Linezolid in gram positive organism like *CONS, Staph. aureus, E. faecalis* and *E. faecium.* The most common mutation in clinical isolates of *Staphylococcus* species and *Enterococcus* species is G2576T [Hong *et al*., 2007; Saager *et al*., 2008]. Mutations other than G2576T include T2500A and G2447T in *S. aureus*, G2505A in *E. faecium*, C2534T in *S. epidermidis*, and G2513T, C2512T, and C2610G in *E. faecalis* [Kelly *et al*., 2008; Meka & Gold, 2004]. The various investigations have reported that linezolid MIC among *S. aureus* increased proportionally with number of copies in 23S rRNA gene with mutations, similarly in *E. faecium* isolates, an increased in the number of mutant copies of 23S rRNA, was shown to correlate with increasing MICs of linezolid [Arias *et al*., 2008; Ford *et al*., 2001].

b) The other mechanism of linezolid resistance increasingly being reported to mutation in L3, L4 and L22 ribosomal proteins of the peptide translocation center [Long & Vester, 2012].

2.8.2.3.5 Plasmid mediated resistance

a) *Cfr* gene mediated resistance: Plasmid mediated linezolid resistance mechanism such as *cfr* gene, which encodes methyltransferase that catalyses methylation of adenine at 2503 in the 23S rRNA gene. Acquisition of natural *cfr* gene confers resistance to florfenicol and chloramphenicol (phenicol) and clindamycin (lincosamide) has been described [Bender *et al*., 2018]. Linezolid resistance mediated by *cfr* gene was first reported in *E. faecalis* isolated from animal origin. The wild type *cfr* (*Staphylococci* homolog) and its derivatives, known as *cfr*(B) and *cfr*(D) variants, which are found on plasmids or within the bacterial chromosome, appeared in *Enterococcu*s spp. from both animal and human samples. Current studies suggest that the frequency of *E. faecium* isolates carrying the *cfr* gene are rising in both animal and human isolates [Long *et al*., 2006; Lee *et al*., 2017].

b) *OptrA* gene mediated resistance: Additional research on mechanism of resistance have shown linezolid resistance may emerge in absence of mutation and lack of the *cfr* gene. Acquisition of oxazolidinone and phenicol transferable resistance A (*optrA*) has emerged as another linezolid resistance determinant. This is the most common mechanism adopted by bacteria to develop antibiotic resistance. *OptrA* mediates resistance through target protection and encodes for an ATP-binding cassette (ABC)-F protein. The ABC-F family contains specified proteins which perform functions that confer resistance to a wide variety of clinically significant ribosome-targeting antibiotics It has also been reported that these proteins use a shield mechanism to protect ribosome and eliminate the drug away from their binding site [Sharkey *et al*., 2016]. *OptrA* gene was initially detected in *E. faecalis* of human origin, and subsequent studies have demonstrated *optrA* gene among isolates of *E. faecalis* and *E. faecium*, and more recently in *S. aureus*, *S. sciuri*, and *S. suis*. Surveillance studies from China suggest that *optrA* is more frequently reported in *Enterococci* from food-producing animals than humans, but also emerging in clinical isolates too [Wang *et al*., 2015; Tyson *et al*., 2018].

2.8.2.3.6 Use of Linezolid in critical care

In critically ill patients, severe infections are associated with high rates of morbidity and mortality and about half of the bloodstream infections in such patients, are caused by gram-positive bacteria [Gales *et al*., 2009]. A significant portion of gram-positive bacterial infections are caused by MDR isolates including MRSA and VRE, which are extremely prevalent in ICUs. For management of infections caused by vancomycin resistant *Enterococci* and *Staphylococci* specially in ICUs. The drug is beneficial in vitro and in vivo activity against the MRSA and VRE and is considered a useful antibiotic for the treatment of patients in the ICUs [Cepeda *et al*., 2004; McKenzie 2011; Khamesipour *et al*., 2015].

Table 2: Mechanism of action of antibiotics used for *Enterococci.*

Site of mechanism of action	Antibiotic class	Resistant type (Intrinsic, Acquired)
Inhibit Cell wall synthesis	• β-Lactams (Penicillins/Ampicillin) • Cephalosporins	Intrinsic
	• Glycopeptides	Intrinsic/Acquired
Inhibit Protein synthesis	**Bind to 30S Ribosomal Subunit** • Aminoglycosides **Bind to 50S Ribosomal Subunit** • Lincosamides • Macrolides • Streptogramins	Intrinsic
	Bind to 50S Ribosomal Subunit • Oxazolidinones	Acquired
Inhibit Nucleic acid synthesis	• Quinolones	Acquired
Inhibit Metabolic pathways	• Sulfonamides Trimethoprim	Intrinsic

2.9 Typing Methods for *Enterococci*

Various molecular techniques are used for the molecular typing of bacterial strains. Molecular techniques used for typing of *Enterococci* include Pulse Field Gel

Electrophoresis (PFGE), Random Amplification of Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and Multi Locus Sequence Typing (MLST).

2.9.1 Pulse Field Gel Electrophoresis (PFGE)

In 1982 PFGE was proposed by Schwartz as a molecular typing method to study diversity, In PFGE DNA molecules larger than 50 separated by applying an electric field to a gel matrix that changes direction on a regular basis.

As DNA molecule larger than 15-20kb migrate across the gel in a size-independent manner, the traditional gel electrophoresis technique was unable to successfully separate very large molecules of DNA, which led to the practise of pulsed field gel electrophoresis. PFGE is based on restriction digestion with rare cutting restriction enzymes of purified genomic DNA.

In PFGE a slice of agarose containing chromosomal DNA after the digestion with appropriate restriction enzyme is subjected to the wells of agarose gel and the fragments are resolved into a pattern of discrete bands in the gel by an electrophoretic apparatus that switches the direction of current according to a predetermined pattern. The DNA restriction patterns of isolates are then compared with one another to determine their relatedness [Tenover *et al*., 1995].

It is a time-consuming method that needs a high technical quality and requires trained technologist for both lab work and data analysis. Despite being labour intensive and need prior standardization, PFGE typing is still regarded as the "gold standard" for *Enterococcal* isolates typing, particularly for explaining suspected outbreak scenarios. The rare cutting restriction enzymes that have been shown to work well with PFGE for *Enterococci* are *sma*I and *apa*I [Kawanishi *et al*., 2005]. PFGE is reported to be a useful tool for the molecular epidemiology of *Enterococci* from a variety of sources including clinical samples [Patterson & Kelly, 1998].

2.9.2 Random Amplified Polymorphic DNA (RAPD)

RAPD is a PCR based DNA fingerprinting technique. RAPD employs arbitrary primers which consist of random sequences of 7-10 nucleotides in length that are not complimentary to any known sequence of the bacterial genome. Annealing of random

sequence primers to diverse regions in bacterial genome, generate complex patterns of PCR products. However, in interlaboratory results RAPD suffers from poor reproducibility because of consistent PCR amplification conditions, such as ramp speed in thermal cycler system. This method differs from classical PCR as in RAPD a single primer is used instead of two primers. RAPD PCR analysis is a well-accepted and reliable tool for the identification and molecular typing of *Enterococci* from food and clinical samples sources [Chiew & Hall, 1998; Mannu *et al*., 1999].

2.9.3 Amplified Fragment Length polymorphism (AFLP)

AFLP was first introduced in the early 1990s for bacterial isolate typing. AFLP is a restriction digestion and PCR amplification-based typing technique. AFLP consist three steps, in first step prior to PCR genomic DNA is digested into smaller fragments by restriction enzymes followed by ligation of double-stranded oligonucleotide adapters unique to the ends of the fragments formed. In AFLP for restriction treatment rare/frequent cutter restriction enzymes are used. During the PCR process, fluorescent dye is used to labelled these fragments. Capillary electrophoresis is used to separate labelled DNA fragments according to size and molecular charge [Wang *et al*., 2015]. AFLP was the first strategy to address the present understanding of *E. faecium* population biology. Data and subsequent fragment pattern analysis generated by AFLP, hospital strains of *E. faecium* form a unique subgroup (currently called C1) that can be distinguished from commensal (human/animal), environmental and probiotic/food strains. AFLP is a labour intensive and time taking than RAPD, but it is more accurately identify many polymorphic bands [Gerber *et al*, 2000]. AFLP was quickly replaced by alternative DNA-based methods with comparable or superior performance such as MLVA and MLST.

2.9.4 Multi-Locus Sequence Typing (MLST)

MLST is a technique that is used for the molecular typing of Bacteria. It was first introduced in the year 1998 for *Neisseria meningitidis*. MLST is based on the identification of genetic variation in the sequences of the housekeeping genes up to 400-500bp in length [Dingle *et al*., 2001]. MLST is used to measure the DNA sequence variations in a set of seven housekeeping genes and sequence variations identified within each gene are termed as alleles. Each allele is assigned with a number to produce

an isolate identifier or allelic profile. Sequence type (ST) is assigned based on allelic profile or the combination of alleles of every organism [Maiden *et al*., 1998].

The use of MLST revealed the existence of host-specific *E. faecium* genogroups and enabled the identification of a polyclonal subpopulation of *E. faecium* adapted from hospitals, particularly MLST ST17, which predominates in several geographic regions and is linked to the rise in *E. faecium* isolations globally. This hospital-adapted lineage is categorised as an example of the so-called HiRECC (high-risk *Enterococcal* complexes) and was originally known as the "C1 lineage" before being renamed "clonal complex-17" (CC17). Recent comparisons of the available genome sequences support the idea that the majority of commensal isolates do not belong to the hospital-associated lineage [34, 131]. Hospital-derived *E. faecalis* isolates have also been found to belong to two significant clonal complexes (referred to as CC2 and CC9). Various web-based servers have been developed those comprising *E. faecium* and *E. faecalis* MLST schemes with the possibilities for data exchange [www.mlst.net and www.pubMLST.org].

Table 3: Advantage, disadvantage and characteristics of molecular typing methods.

Molecular typing methods	Time	Cost	Reproducibility	Discrimination
PFGE	Time Taking	High	Average	Excellent
RAPD	Fast	Low	Poor	Good
AFLP	Fast	Average	Good	Excellent
MLST	Time taking	High	Excellent	-

2.10 Epidemiology of Linezolid resistant *Enterococcus*

2.10.1 International studies

Auckland et al., (UK, 2002), reported the first 3 cases of linezolid resistant *Enterococci* in the UK. They isolated the linezolid resistant *Enterococci* from three patients. All 3 patients were on prior linezolid therapy, among 3 LRE isolates 2 were *E. faecium* and 1 was *E. faecalis*. The linezolid MICs was 64 μg/mL for all 3 isolates. Linezolid resistance was mediated by mutation in 23S rRNA in all 3 isolates.

In 2002, Woodford *et al*., studied 27 isolates of *Enterococci* for linezolid resistance and 16 were observed resistant with the MIC ≥8 μg/mL and 11 isolates were

susceptible with MIC ≤4 µg/mL, this study was performed for investigation of mutation in 23S rRNA genes by pyrosequencing with single nucleotide polymorphism conferring linezolid resistance in *Enterococci*. In this study, it was observed that the MICs of linezolid and the number of 23S rRNA gene copies having the mutation are correlated with each other.

A study conducted in 2006 in Verona (Italy) by Bonora *et al*., from January to December 2004, A total of 127 patients were screened. VRE and LRE colonisation was detected in 35 and 14 patients respectively. Linezolid resistance was observed only in VRE isolates. All Linezolid resistant isolates had the G2576T mutation in 23S rDNA. For molecular epidemiology PFGE and MLST was used. In this study a relatively high rate of linezolid resistant *E. faecium* was reported .

A case study was reported by Marra *et al*., in Virginia (2006**).** They reported a case of catheter colonization (central venous) by linezolid resistant *E. faecalis*.

Doern *et al*., (2016), studied linezolid resistance among *Staphylococci* and *Enterococci* by automated antimicrobial susceptibility testing system. A total of 27 isolates were observed resistant to linezolid includes *Enterococci* (n=11), 8 *Staphylococcus aureus* (n=8) and *Staphylococcus epidermidis (n=8).* Among 27, 23 were resistant and 4 were intermediate to linezolid. Mutations in 23S rRNA and *cfr* gene was detected in 16 and 3 isolates respectively. Mutations in the 23S rRNA gene were most common at 2576 and 2534 sites.

Similar to study by Bonora *et al*., In Tehran 200 clinical isolates were screened for vancomycin resistance, vancomycin resistant was detected in 17 isolates. Among 17 VRE isolates linezolid resistance was detected in 4 isolates. Among 4 LRE isolates 2 were reported as *E. faecium*, both isolates were recovered from blood samples. The linezolid MICs were between 16 and 32 µg/mL for LRE isolates [Yasliani *et al*., 2009].

Souli *et al*., (2009) conducted a point prevalence to study the faecal carriage and environmental colonisation of vancomycin and linezolid resistant *Enterococci*. In this study 6 isolates of vancomycin and linezolid resistant *Enterococci* were isolated (5 clinical and 1 environmental). All isolates were *E. faecium* and positive for *vanA* gene. The linezolid MIC for all 6 isolates was 12 µg/ml and having G2576T mutation in 23S

rRNA gene. PFGE was used for the molecular typing and results showed that the 4 clinical and environmental isolates were genetically related.

A study was conducted by Inkster *et al*., (2017) in UK, to investigate the colonization by vancomycin and linezolid resistant *E. faecium* harbouring the *cfr* gene in a nephrology unit. Over a 2-week period, five patients were colonised with linezolid resistant *E. faecium*. All isolates were positive for *cfr* and *vanA* gene.

In Poland a passive surveillance study was conducted by Gawryszewska *et al*., (2017) at 20 different polish hospitals. In this study there was a significant increase in linezolid resistant *Enterococci* from September 2008 to December 2015. For the molecular typing of LRE MLST, MLVA, VNTR were used. Among 50 linezolid resistant *Enterococci* were collected during the study period. Out of 50 LRE 41 were *E. faecium* (82%), 8 were *E. faecalis* (16%) and 1 isolate was identified as *E. avium*. G2576T mutation in 23S rRNA was the most common and detected in 94% isolates, no *cfr* gene mediated resistant was observed, *optrA* mediated resistant was identified in 2 isolates of *E. faecalis*. This study stated that the mutation acquired by clones of *Enterococci* is the main cause of increasing resistance to linezolid. The ST types associated with linezolid resistance were ST17, ST 78 for *E. faecium* whereas ST6 for *E. faecalis*. In contrast a study conducted by Krawczyk *et al*., in Poland (2020), nineteen linezolid resistant *Enterococci* were isolated from 18 patients. MIC of linezolid ranged between 32-256µg/ml. All isolates were positive for G2576T mutation in 23s rRNA, 14 isolates were positive for *cfr* gene.

Egan *et al*., (2020) studied 154 linezolid resistant *Enterococci* isolated from an Irish hospital during a period from June 2016 to august 2019. Linezolid resistant *Enterococci* were screened for the presence of *optrA/poxt* gene. In this study G2576T mutation in 23S rRNA and *optrA/poxt* gene were detected in 27% and 12% LRE isolates respectively.

In Barcelona (Spain) a study was conducted from May 2016 to April 2017 by Càmara *et al*., (2019), for investigation of linezolid resistance among 1640 *Enterococci*. The main purpose of this study was to investigate the presence of *optrA* gene. Screening of *optrA* and *cfr* gene was done by PCR. Whole-genome sequencing (WGS) was used to study Genetic relatedness, genes associated with linezolid resistance, virulence and

analysis of the genetic environment of the *optrA* gene. In this study linezolid MIC ≥ 4µg/ml was detected in only 6 isolates. Majority of isolates were positive for *optrA* gene 83% (5/6) isolates. In this study resistance mediated by mutations in 23S rRNA and *cfr* gene were not detected. Two different genotypes, ST585 and ST474 were detected by WGS.

Chua *et al*., (2021), reported 3 cases of infections with linezolid resistant *Enterococci* from Malaysia. Linezolid resistant *Enterococci* were isolated from 48-year-old female patient with advanced endometrioid adenocarcinoma, 70 years old male with acute epigastric pain and from a 20-year-old female, who was on chemotherapy for relapsed Pre-B acute lymphoblastic leukaemia and developed neutropenia.

From Czech Republic a study was conducted from 2009-2019 by Malisova *et al*., (2021) to investigate the resistance mechanisms in linezolid-resistant *Enterococci*. A total of 1442 *E. faecium* and *E. faecalis* isolates were investigated by the National Reference Laboratory for Antimicrobial surveillance. Linezolid resistance was observed in 115 of isolates includes *E. faecium* (n = 106), *E. faecalis* (n = 9). G2576T mutation in the 23S rRNA of domain V was detected in 93.4% (99/106) of *E. faecium* and 22.2% (2/9) of *E. faecalis* isolates. In *E. faecalis* isolates, *optrA* gene was the most common mechanism of linezolid resistance. All isolates were negative for *cfrB* or *poxtA* genes. In this study, seventeen sequence types (STs) were observed, including four new STs. clonal complex CC17 was detected in All *E. faecium* isolates.

A study from China (2022) was conducted from January 2014 – December 2018 by Yi et al., fifteen *E. faecium* isolates with linezolid MICs ranging from 4 to 16µg/ml were investigated. Linezolid resistance was detected in 66.7 % (10/15) of the isolates. *OptrA* and *poxtA* gene were detected in 46.7% (7/15) and 13.3% (2/15) of the isolates respectively. In one isolate Ser77Thr mutation in L22 ribosomal protein was detected in addition to *optrA* and *poxtA* gene. All isolates were negative for mutation in 23S rRNA gene, ribosomal protein L3/L4 or in and resistance mediated by *cfr* gene.

2.10.2 National studies

First case of linezolid resistance *Enterococci* from India (Kolkata) was reported by Kumar *et al*., in 2014. In this case report linezolid resistant *E. faecium* was isolated

from a patient with encephalopathy. Linezolid MIC was 1024µg/ml. This isolate was sensitive to vancomycin.

In 2015 a case was investigated by Rai *et al*. In this study linezolid resistant *E. faecium* was isolated from 80 years old male patient. The author also studied faecal carriage with linezolid resistant *Enterococci*, from this patient 2 isolates of *E. faecium* with MIC >4 µg/ml and ≥8 µg/ml were detected. This patient was also colonised with linezolid resistant *MRSA* with linezolid MIC for Linezolid ≥8 µg/ml.

In 2019 two cases of leukemia patients were reported by Kumar *et al*., from India (Delhi). In 1st case, linezolid resistant *E. faecalis* was isolated from nasal swab and in 2nd case linezolid resistant *E. faecalis* was isolated from the blood culture. Both isolates were observed resistant to vancomycin too.

A study was conducted from January 2018 to December 2019 by Sengupta *et al*., (2023) India, in this study 371 *Enterococci* were investigated, which includes 239 (64.4%) *E. faecalis*, 114(30.7%) *E. faecium* and others 17(4.6%) were *E. durans*, *E. casseliflavus*, *E. gallinarum*, and *E. avium*. Among these isolates resistance to vancomycin and linezolid were detected in 24 and 2 isolates respectively. Both linezolid resistant isolates had the G2576T mutation.

A study was conducted by Yadav and Agarwal, from June 2019 to May 2020, to investigate the antimicrobial resistance pattern of enterococcus isolates. A total of 145 isolates were studied, includes *E. faecalis* (n=73), *E faecium* (n=69), *E. durans* (n=2) and *E. gallinarum* (n=1). Majority of them were isolated from urine (86.2%) followed by pus (11.03%). Resistance to vancomycin and linezolid was detected in 9.6% (n=14) and 5.5% (n=8)) isolates respectively. Linezolid resistant *Enterococci* includes 6 *E. faecium* and 2 *E. faecalis* isolates.

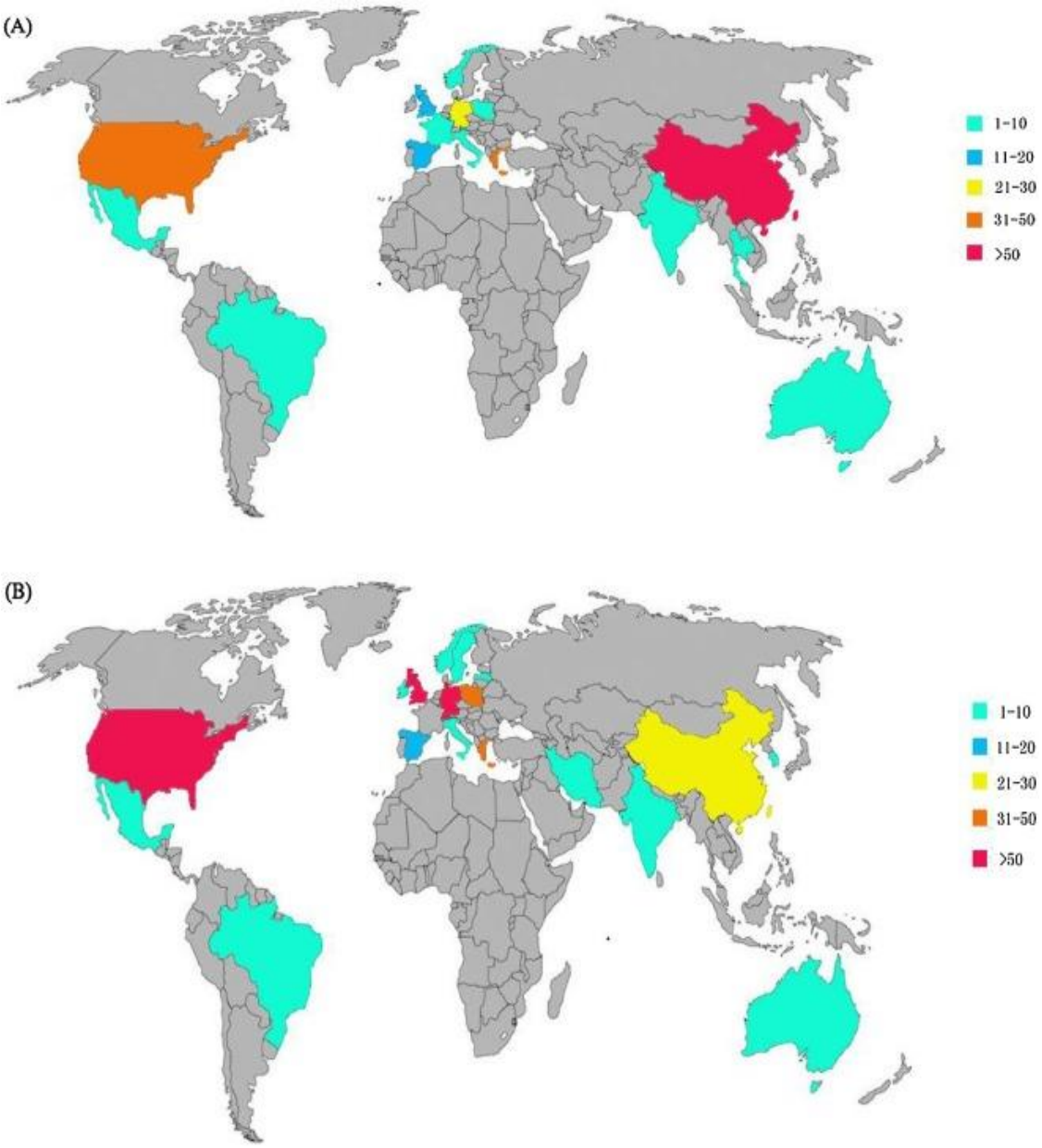


Figure 7: Distribution of linezolid resistance among linezolid resistant *Enterococci* geographically, (A) Linezolid resistant *E. faecalis* and (B) Linezolid resistant *E. faecium* respectively [Image Adapted from Bi, R., *et al*., (2018)].

Table 4: Worldwide reports performed on linezolid resistant *Enterococcus*.

Country	Year	Mechanism of resistance			
		Mutation at 23S r RNA	L3, L4 & L22 mutation	*cfr* gene	*optrA* gene
UK	2002	Not reported	Reported	Not reported	Not reported
UK	2002	Reported	Not reported	Not reported	Not reported
Italy	2006	Not reported	Reported	Not reported	Not reported
Virginia	2006	Not reported	Not reported	Not reported	Not reported
Iran	2009	Not reported	Not reported	Not reported	Not reported
Virginia	2016	Reported	Not reported	Reported	Not reported
Poland	2017	Reported	Not reported	Not reported	Reported
Poland	2020	Not reported	Reported	Not reported	Not reported
Ireland	2020	Not reported	Reported	Reported	Reported
UK	2017	Not reported	Not reported	Reported	Not reported
Spain	2018	Not reported	Not reported	Not reported	Reported
India	2014	Not reported	Not reported	Not reported	Not reported
India	2015	Not reported	Not reported	Not reported	Not reported
India	2019	Not reported	Not reported	Not reported	Not reported
Malaysia	2021	Not reported	Not reported	Not reported	Not reported
Czech Republic	2021	Reported	Not reported	Not reported	Reported
China	2022	Not reported	Mutation in L-22 was reported	Not reported	Reported

Research Gap (Lacunae in Existing Literatures)

- Linezolid is a last resort drug for the management of Vancomycin-resistant *Enterococcus* in hospitals. In India, rampant use of antibiotics leads to an alarming rise in resistance, especially against antibiotics viz., beta-lactams, carbapenems, glycopeptides and Linezolid.
- A study to determine the virulence factor and their contribution to Linezolid resistant were never attempted.
- Data regarding phylogenetic analysis and clonal distribution in India is not present for Linezolid resistant *Enterococcus faecium.*

3. Aim and objectives

Aim

The aim of the present study was to characterize the various mechanisms associated with linezolid resistance and to study the risk factors and clinical outcomes of patient's infected with linezolid-resistant *Enterococcus faecium* (LREfm).

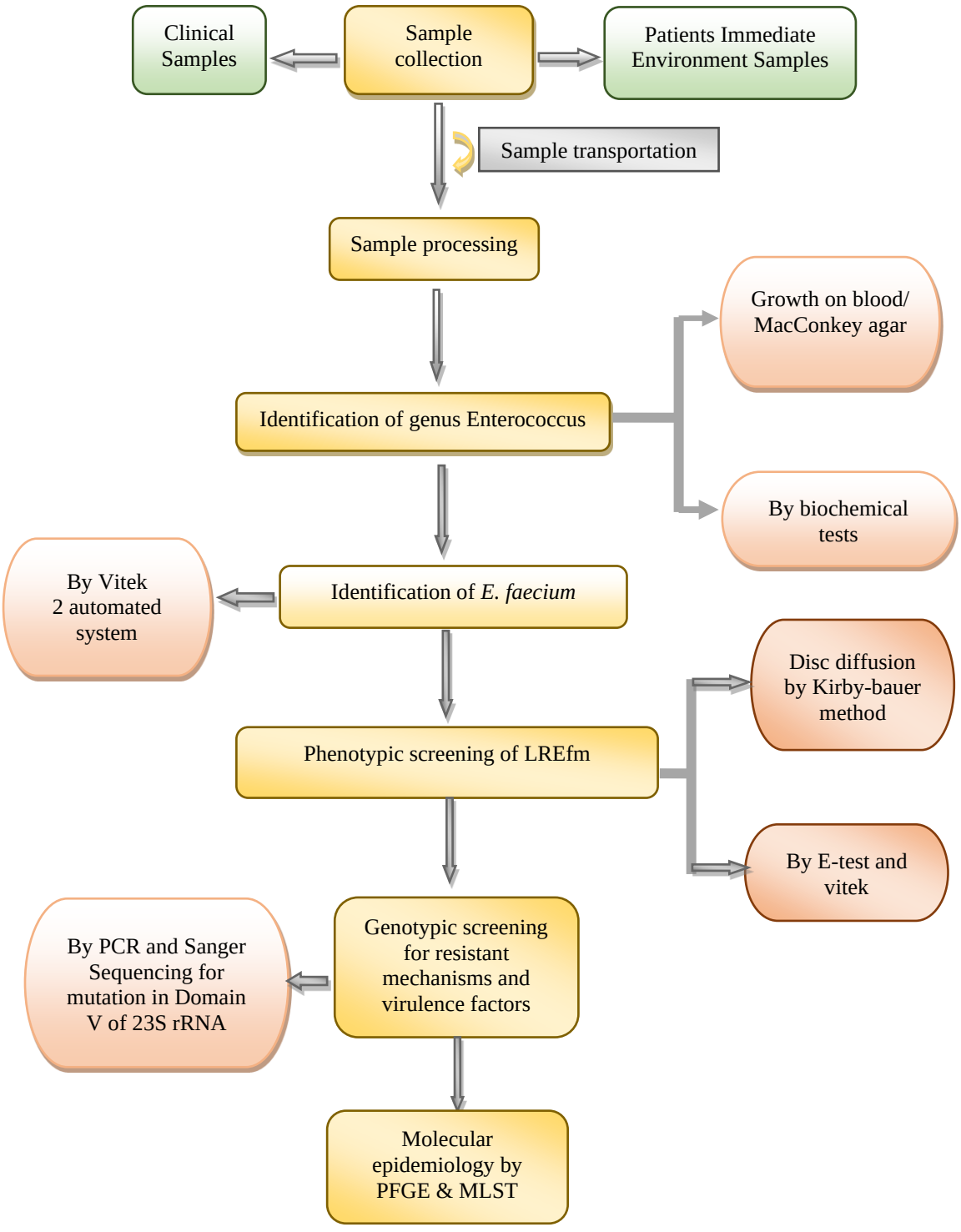
Objectives

1) To study the prevalence of linezolid resistance among *Enterococcus faecium* in hospital-acquired infections.
2) To study the risk factors and clinical outcomes of patient's infected with linezolid-resistant *Enterococcus faecium*.
3) To characterize various mechanisms of the linezolid resistance in *Enterococcus faecium* isolated from clinical samples and hospital environment.
4) To study virulence genes/factors associated with linezolid resistant *Enterococcus faecium*.
5) To study the molecular epidemiology of linezolid resistant *Enterococcus faecium* by Pulse field electrophoresis and Multi locus sequence type.

Hypothesis

Linezolid resistance among *Enterococcus faecium* is mediated by diverse resistance mechanisms and spreads through horizontal transmission of the strains or resistance genes.

STUDY FLOW CHART



4. Materials and Methods

4.1 Study setting

This study was conducted in the Department of Microbiology, Vardhman Mahavir Medical College & Safdarjung Hospital, New Delhi.

4.1.1 Study design

Prospective Observational Study.

4.1.2 Study period

Samples for the study were collected over a period of 30 months from Jan 2020 to June 2022.

4.2 Sample size

The prevalence of linezolid resistant *E. faecium* was 5% during the period 2018-19 in our hospital and was used to calculate the sample size for clinical isolates by using the following formulae **[Charan *et al*; 2013].**

$$n = z^2pq / d^2$$

n=number of samples required.

z = standard normal deviation which is related to the required confidence interval (Epidemiologically "z" has a constant value of 1.96)

P= Estimated prevalence (taken as 5%) (According to current prevalence)

q = 1- Estimated prevalence

d= Desired level of precision (3%)

Hence a total of 202 patients with clinically significant infections with isolates of linezolid resistant *E. faecium* (LREfm) and for risk factor analysis controls which included 200 patients with Linezolid susceptible *E. faecium* (LSEfm) infections were enrolled in the study. In addition, 24 isolates of linezolid resistant *E. faecium* recovered from patient's environment were also included in the study.

Ethical consideration

This study was approved by the institutional ethics committee (IEC/VMMC/SJH/PROJECT/2020-10/CC-79).

Institutional Reserch committee consideration

This study was approved by the Institutional Reserch committee (LPU/CRDP/PHD/2020025/000766)

Participants

Patients were enrolled after written consent and a predesigned proforma was used **(Annexure I).**

Cases and controls for risk factor analysis: Cases were defined as patients with infections with cultures positive for LREfm. For each case, one control patient was randomly selected from patients hospitalised at the same time in the same unit with a culture positive for LSEfm. Control patients were randomly selected from the same source population as the case patients to prevent the bias of relative risk while selecting patients with infection with susceptible bacteria [Anthony *et al.*, 2001].

4.3 Data collection for risk factors and outcomes

Patient demographic data was collected including dates of admission/discharge and outcome. Data recorded also included details of underlying diseases, prior use of antibiotics, intravenous/urinary catheters, mechanical ventilation and details of any invasive procedures. Duration of hospital stay prior to isolation of LREfm and LSEfm was also recorded.

Inclusion criteria

- Clinically significant isolates of linezolid resistant *E. faecium.*
- Only one isolate per patient was included.

Exclusion criteria

- Duplicate isolates of linezolid resistant *E. faecium* and isolates other than *E. faecium* were excluded.
- Patients hospitalized for <48 hours were excluded.

4.4 Samples

4.4.1 Clinical samples

Patients with clinically significant infections were enrolled and samples were processed in the Department of Microbiology. Samples received included urine, blood, pus aspirate and tissue. All samples were collected aseptically and processed by standard methods.

4.4.1.1 Sample collection

- **UTI**

  Patients with suspected UTI, 1-2 ounces of midstream urine was collected in a sterile container or from catheter port by aseptic technique in catheterized patients. Samples were transported immediately to the microbiology laboratory for processing.

- **Sepsis**

  Blood cultures (4-5 ml blood) were collected aseptically by the physician in Bactec blood culture bottles. After the collection, bottles were transported to the microbiology lab for processing in Bactec/alert 3D system (BioMerieux, France) which is an automated, continuous monitoring blood culture system for detection of bacteria.

- **Skin and soft tissue infections**

  Pus, aspirates and tissue were collected aseptically from patients with skin and soft tissue infections and processed after enrichment.

4.4.1.2 Processing of clinical samples

All samples were transported within an hour to the microbiology laboratory. The positive signaled Blood culture bottles were removed from the BACT/Alert system and processed immediately. All clinical samples (urine, blood and pus) were cultured on blood agar (5% sheep blood agar) and MacConkey agar plates and incubated for 18-24 hours at 37°C.

4.4.2 Environmental Samples

For the environmental samples, swabs were collected from the patient's immediate environment, patient's rectal swabs and swabs from the hands of healthcare workers to study the colonization. Samples of patient's immediate environment included swabs from the patient's bed, medical devices used for patient management and included ventilator, oxygen tubing, IV cannula, thermometer etc.

4.4.2.1 Sample collection

Samples from patient's environment

- Prior to sample collection hand hygiene was performed
- Prior to swabbing, each swab was moistened with sterile normal saline.
- Moist swabs were used to collect samples by swabbing the surface approximately 10 times in side-to-side horizontally, vertically, diagonally and lastly the swab was rolled over the outer border of the area.
- The area swabbed for large and small surfaces was approximately $10cm^2$ and $4cm^2$ respectively.

Samples from healthcare workers

- Prior to sample collection hand hygiene was performed.
- A sterile swab was moistened with sterile normal saline and the participant were instructed to have their 5 fingers aligned together.
- Moist swab was rubbed back and forth twice from the finger root to the finger end (the area was $30cm^2$).

Rectal swab collection:

- Samples were collected after written informed consent from the participants.
- For the specimen collection, the patient was positioned in the lithotomy or lying down position.
- The swab was inserted through the rectal sphincter 1-1.5 inches (2-3 cm) and gently rotated for 20-30 seconds.

- After the collection, swab was immediately transported in Amies media (amies media with charcoal) to the microbiology lab for processing.

4.4.2.2 Processing of Environmental Swabs

All environmental swabs were immediately transported to the microbiology laboratory after collection for processing. All swabs were processed after enrichment in the trypticase soy or brain-heart infusion broth and plated on blood and MacConkey agar plates.

4.5 Culture media used in the study

4.5.1 Blood agar

Commercially prepared sheep blood agar plates (HiMedia Laboratories Private Limited, Mumbai, Maharashtra, India) were used in the study.

4.5.2 MacConkey and Muller Hinton agar (MHA)

MacConkey and Muller Hinton agar plates were prepared in-house using dehydrated media as per the manufacturer's instructions (HiMedia Laboratories Private Limited, Mumbai, Maharashtra, India) (MacConkey agar M082) (Muller Hinton Agar M173ET).

4.5.3 Amines transport media

Commercially prepared media was used in the study (HiMedia Laboratories Pvt. Ltd, Mumbai, Maharashtra, India)

4.5.4 Storage of agar plates

Agar plates were prepared twice a week and stored at 2-8°C in the refrigerator in airtight bags until further use.

4.6 Identification of *Enterococcus*

4.6.1 Presumptive identification

Presumptive identification of enterococcus was based on growth characteristics on blood and MacConkey agar, gram staining and biochemical tests. Biochemical tests included catalase test, hydrolysis of bile esculin and growth in 6.5 % NaCl broth at 37°C.

4.6.2 Colony morphology on blood and MacConkey agar

After overnight incubation the colonies of *Enterococci* on blood agar are small with sizes ranging 1-3 mm in diameter, semi translucent with α, β and no haemolysis. On MacConkey agar (with bile), *Enterococci* appear as small, intensely colored, red-purple colonies (Figure; 8).

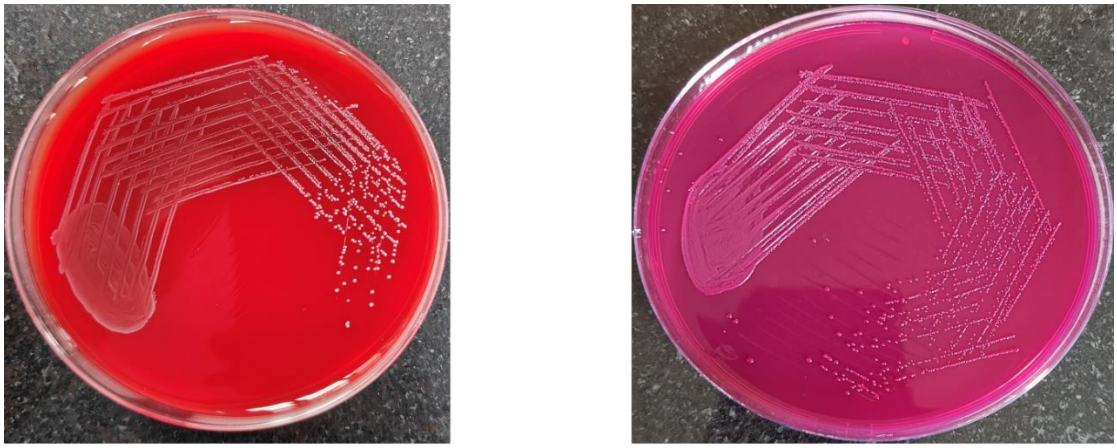


Figure 8: Colony morphology on (A) Blood and (B) MacConkey agar

4.6.3 Gram staining

Gram positive cocci arranged singly, in pairs or short chains were suggestive of *Enterococcus* (Figure; 9) [Gephart et al., 1981].

Procedure

- A thin smear was prepared on a grease free slide from a single colony. Smear was air dried, heat fixed and gram staining was performed as follows
    - 1% Crystal violet solution for 1 min.
    - Rinse with water
    - Add Gram's iodine for 1 min.
    - Rinse with water
    - Decolourised with acetone and rinsed with water.
    - Counterstained with 0.5% Saffranine solution for 30 sec and again rinsed with water and air dried. The smear was observed under oil immersion objective (100X).

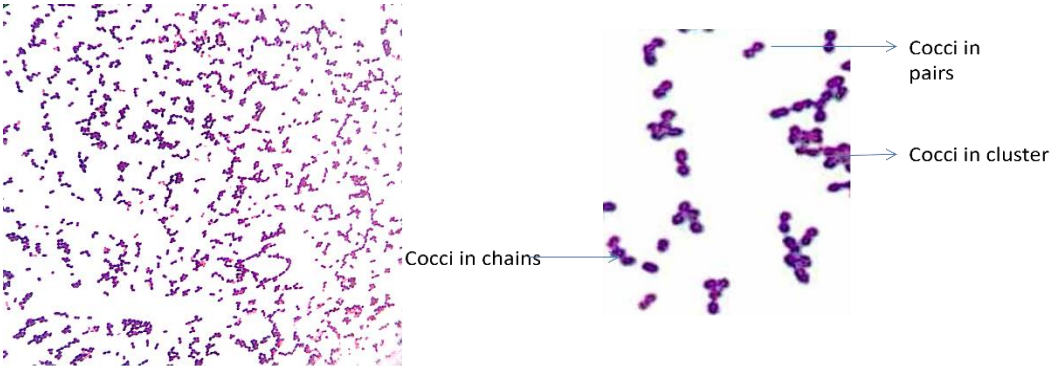


Figure 9: Gram's staining images of *E. faecium* under the 100X.

4.6.4 Biochemical Tests

4.6.4.1 Catalase test

This test demonstrates the presence of catalase and is used to differentiate *Enterococci* from *Staphylococcus* species (Figure 10).

Principle

The enzyme catalase mediates the breakdown of hydrogen peroxide into oxygen and water.

Procedure:

- Use a platinum loop or sterile wooden stick to transfer a small amount of colony growth to the surface of a clean, dry glass slide.
- A drop of catalase reagent (3% H_2O_2) was added on the glass slide.
- Observe for the evolution of oxygen bubbles.

Interpretation and controls

Organisms	Results expected
Staphylococcus aureus-ATCC 33592	Immediate evolution of oxygen bubbles within 10 seconds indicates a positive reaction.
Enterococcus faecalis- ATCC 29212	No evolution of oxygen bubbles indicates negative reaction.

Figure 10: Catalase test

4.6.4.2 Bile esculin hydrolysis

Bile esculin agar is a selective and differential medium used to presumptively identify *Enterococci* and *group D Streptococci* based on their ability to hydrolyse esculin (Figure; 11).

Principle

Gram positive bacteria other than *group D-Streptococci* or *Enterococci* are inhibited by the bile salt. Organisms capable to grow in the presence of 4% bile are able to hydrolyse esculin. The hydrolysis of esculin in the medium produces glucose and esculetin. Esculetin reacts with Fe^{3+} (ferric ions) in the medium and forms a phenolic iron complex that produces dark brown to black colour.

Procedure

- One to two colonies from an overnight culture were inoculated on bile esculin agar slant.
- Incubate at 35°-37°C in ambient air for 24 hours.
- Observe for growth and blackening of the medium.

Interpretation and Controls

Organisms	Results expected
Enterococcus species	Growth and blackening of the agar slant indicate positive reaction.
Streptococcus species	Growth and no blackening of medium indicates negative reaction.

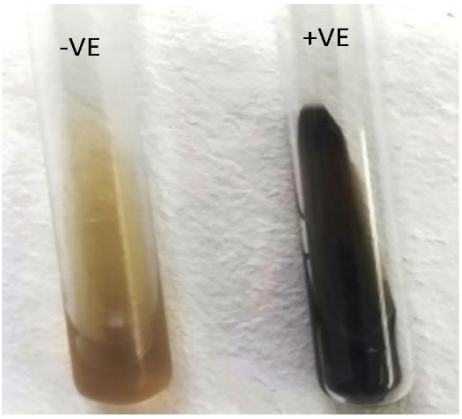


Figure 11: Bile esculin hydrolysis.

4.6.4.3 Growth in selective media

Growth in 6.5% NaCl broth at 37℃ was used for the identification of genus *Enterococcus* (Figure; 12).

Principle

Salt is a selective agent. Salt interferes with membrane permeability and osmotic equilibrium. A variety of bacteria are inhibited by the high salt concentration, but salt-tolerant bacteria such as *Enterococci* can grow in the presence of high salt concentration. The media with 6.5% NaCl, dextrose (a fermentable carbohydrate) and bromocresol purple (a colour indicator) is used. Organisms that can grow in high salinity medium use sugar and produce acid as a byproduct of their metabolism. When the pH drops, the indicator, bromocresol purple, changes from purple to yellow. This media helps to distinguish *Enterococci* from other *Streptococci*.

Procedure

- Inoculate a tube of 6.5% NaCl broth with 2-3 colonies to test organism (preferably from an overnight culture).
- Loosen the cap and incubate aerobically at 37°C for 24 hours.
- In case of negative result incubate up to 72 hours.

Interpretation and Controls

Organisms	Results expected
Enterococcus species	Presence of bacterial growth in the 6.5% NaCl medium, with or without a colour change indicates a positive reaction.
Streptococcus species	No growth after 72 hours indicates a negative result.

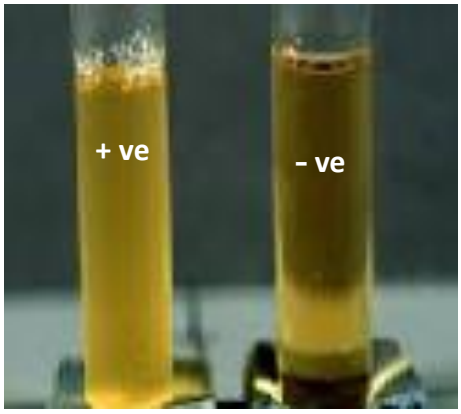


Figure 12: Growth in selective media 6.5% NaCl at 37℃.

4.6.4.4 Final identification of species

Final identification of *E. faecium* was done by the Vitek2 Compact (bioMérieux, Okhla industrial area, Delhi, India) **(Annexure II)**.

Procedure

Identification was performed by Vitek2 Compact (bioMérieux, Okhla industrial area, Delhi, India). Isolated colonies on 5% sheep blood agar were selected for preparation of inoculum as per the manufacturer's instruction. The turbidity of the suspension was adjusted to 0.5 McFarland by Densicheck plus (bioMérieux Okhla industrial area, Delhi, India). The turnaround time for identification by VITEK ® 2 Compact was 4-6 hours using VITEK® 2 GPID cards.

4.7 Strains preservation and maintenance

A single well isolated colony on nutrient agar plate was inoculated in sterile 750µl Brain-Heart Infusion (BHI) broth in a 1.8 ml cryovials. The vial was incubated at 37°C for 3-4 hours and 750 µl of sterile 60% glycerol was then added to the vial and vortexed to ensure even dispersal of glycerol. The vials were stored at -80º C till further use.

4.8 Antimicrobial Susceptibility Testing: (CLSI, M100-S31)

4.8.1 Kirby-Bauer disk diffusion method

Antimicrobial susceptibility testing was performed by Kirby-Bauer disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (M100-S31) for ampicillin (10µg), ciprofloxacin (5µg), gentamicin (120µg), erythromycin (15µg), chloramphenicol (30µg) and tetracycline (30µg). Additionally,

nitrofurantoin (300 µg) was tested for urinary isolates only. Minimum inhibitory concentration (MIC) of linezolid, vancomycin, daptomycin and quinupristin/dalfopristin was performed by gradient strips (Liofilchem® Diagnostics, Italy). *E. faecalis* ATCC® 29212 and *E. Faecium* BM4147 were used as controls.

Multidrug resistant (MDR) was defined as non-susceptible to ≥1 agent in ≥3 antimicrobial categories and extensively drug-resistant **(X**DR) was defined as non-susceptible to ≥1 agent in all but ≤2 categories. Pan drug resistance (PDR) was defined as non-susceptibility to all agents in all antimicrobial categories [Magiorakos *et al*., 2012].

4.8.1.1 Media

Antibiotic susceptibility was performed on MHA (Mueller-Hinton agar) (Ann II). All disc and media were procured from HiMedia Laboratories Pvt. Ltd, Mumbai, Maharashtra, India.

4.8.1.2 Inoculum

Four to five single isolated colonies from MHA incubated overnight were inoculated in peptone water and incubated at 37˚C for 20-30 mins. The turbidity of the growth obtained was adjusted to match the turbidity OD_{600} = 0.5 of the McFarland turbidity standards (HiMedia, Pvt, Ltd, India) resulting in suspension containing $1.5x10^8$cfu/ml.

4.8.1.3 Procedure

- A sterile cotton swab was dipped into broth containing bacterial suspension (as described above). Swab was squeezed along the sides of the tubes to remove the excess material. The pre-dried MHA plates were streaked all over the surface three times rotating the plate by an angle of approximately 60˚ after each application to ensure uniform distribution of inoculum. The inoculated plates were left for 5-10 minutes to allow excess moisture to be absorbed and antibiotic discs of known strength were applied aseptically with the help of sterile forceps equidistant at least 24 mm apart from each other and 15 mm from side of the plate with maximum 6 discs on the 90mm plates. Plates were incubated at 37°C for 18-24 hours and zone diameter were recorded as per the CLSI guidelines (M100-S31).

4.8.1.4 Measurement of Zone diameters and interpretation of antimicrobial susceptibility

The zone of inhibition is the point at which no growth is visible to the unaided eye. The zone diameters were measured manually using a scale graduated to 0.5mm with the plates held about 30cm from the eye at a 45-degree angle to identify sharp zone edges. The zone diameters were recorded in reflected light from front of the plate with the lid removed. The readings were taken by three independent observers. Mean of the three reading was taken and rounded to nearest whole number. Zone diameter was interpretated as per CLSI guidelines 2022 (M100-S31) and given in table; 5.

Readings were not recorded and test repeated if:

- Semi-confluent growth was observed
- Overlap zone diameter of two adjacent disc.
- Presence of fuzzy zones/zones showing distortion from circular edges.
- In case of double zones, or distinct colonies within zones, purity was checked and test was repeated.

Table 5: Break points of zone diameter (mm) used in the study for various antibiotics (CLSI 2021):

Antibiotic	Strength(µg)	Zone Diameter Interpretative Criteria (nearest whole mm)		
		Susceptible	Intermediate	Resistant
Ampicillin	10	≥17	-	≤16
Erythromycin	15	≥23	14-22	≤13
Ciprofloxacin	5	≥21	16-20	≤15
Tetracycline	30	≥19	15-18	≤14
Chloramphenicol	30	≥18	13-17	≤12
High level Gentamicin	120	-	-	-
Nitrofurantoin	300	≥17	15-16	≤14

4.9 Minimum Inhibitory Concentration (MIC) determination by gradient strips:

Method

Isolates were tested for **MIC** by using gradient strips for linezolid, vancomycin, teicoplanin, daptomycin and quinupristin/dalfopristin.

4.9.1 Procedure

MHA plates were inoculated as described in section **4.6.1.2**. Commercially available gradient strip of linezolid (0.016-256 µg/ml), vancomycin (0.016-256 µg/ml), daptomycin (0.016-256 µg/ml) and quinupristin/dalfopristin (0.016-256 µg/ml) (Liofilchem® Diagnostics, Italy) were applied aseptically to each plate using sterile forceps. The plates were then incubated at 35-37˚C for 18-24 hours. Results were recorded as per instruction of manufactures.

4.9.1.1 Reading of MIC

The MIC was determined as the point of interception of the zone of inhibition with the gradient strip visible to the unaided eye. The plates were read in reflected light, manually from the front of the plate with the lid removed. The readings were taken by three independent observers. Mean of the three reading was taken. Results were interpreted using break points prepared by CLSI (M100-S31) and given in table; 6.

Results were not recorded / repeated if:

- Semi-confluent growth was observed.
- In case of double zones, or distinct colonies within zones, purity was checked and test was repeated.

Table 6: MIC breakpoints (µg/ml) for reporting sensitive, intermediate and resistant (CLSI 2021) for linezolid, vancomycin, daptomycin and quinupristin/dalfopristin.

Antibiotic	Interpretative Criteria for MIC (µg/ml)		
	Susceptible	Intermediate	Resistant
Linezolid	≤2	4	≥8
Vancomycin	≤4	8-16	≥32
Teicoplanin	≤8	16	≥32
Daptomycin	-	-	≥8
Quinupristin/Dalfopristin	≤1	2	≥4

4.10 Phenotypic detection of linezolid resistance among *E. faecium*

Disc Diffusion

Disc diffusion testing using Linezolid (30µg) disc (Oxoid, USA) was performed as per CLSI guidelines 2021 (M100-S31). Breakpoints for resistant and sensitive were define as ≤ 20 mm and ≥ 23 mm respectively (Figure;13).

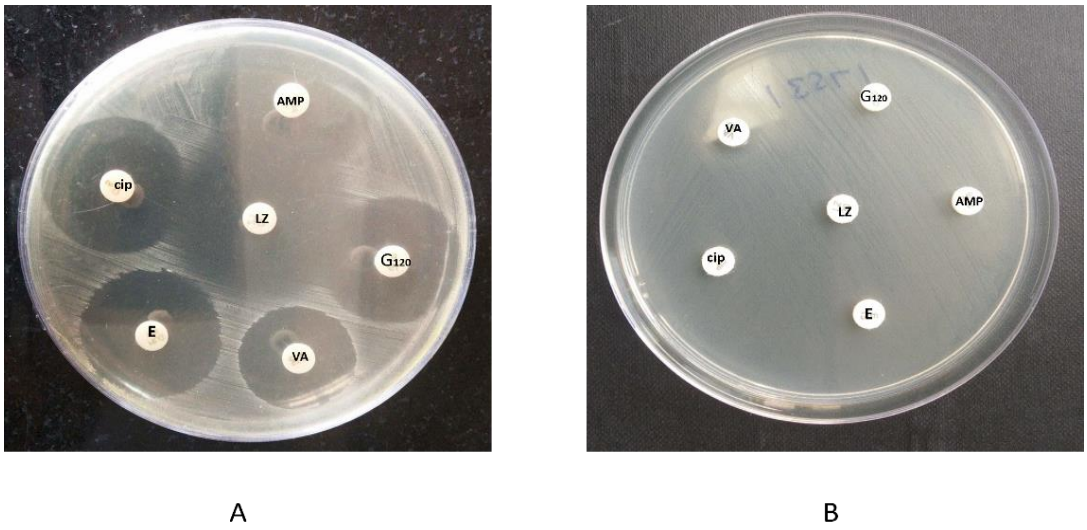


Figure 13: Antimicrobial susceptibility testing images, **(A)** AST of ATCC 29212 strain and **(B)** AST of linezolid resistant isolates B1102.

MIC

MIC testing using a gradient strip (Liofilchem® Diagnostics, Italy) was performed. MIC ≥8 µg/ml was considered as resistant and ≤ 4µg/ml was considered as sensitive (CLSI, M100-S31) (Figure; 14).

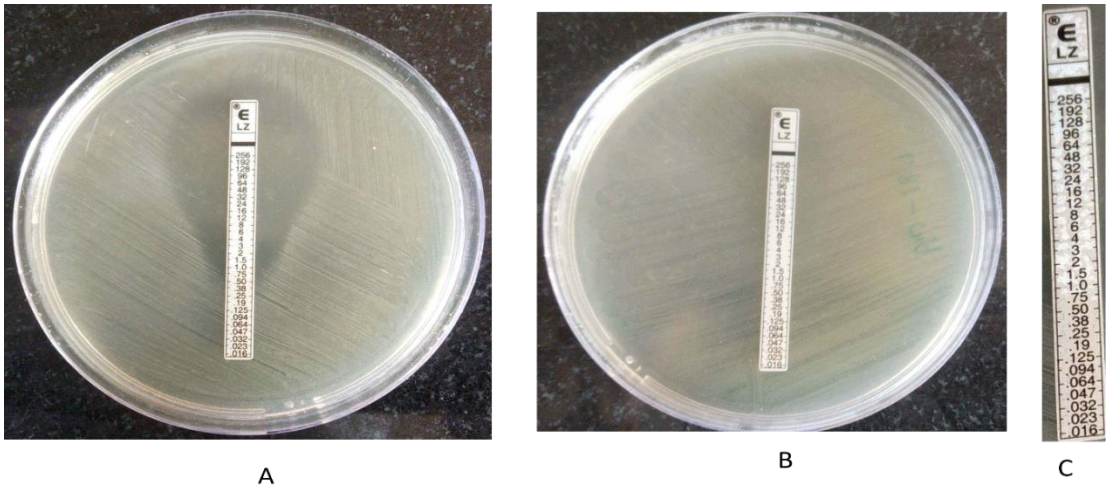


Figure 14: (A) Linezolid MIC of ATCC 29212 strain by E-test, (B) MIC of linezolid resistant strain by E-test and (C) Gradient of linezolid on E-test strip.

4.11 Molecular Characterization of vancomycin and linezolid resistant *E. faecium*

Molecular characterization of vancomycin and linezolid resistant *E. faecium* isolates was done by PCR.

4.11.1 DNA extraction

Genomic DNA was extracted by boiling preparation method [Dhasti *et al*., 2009]. Briefly, 8-10 colonies from the fresh MHA plate were mixed in 200 µL of nuclease free water in 1.5ml eppendorf tube. The mixture was vortexed for 15 sec. The cell suspension was held in a heating block for 10 min at 99°C for lysis of cells. After boiling the suspension was centrifuged at 12000 rpm for 5 min. Supernatant containing DNA was collected and used as the template for PCR. DNA was stored at -80ºC for further use.

4.11.2 Quantification of DNA

Quantification of the extracted DNA was done by using the nanodrop spectrophotometer (Nanodrop 2000c ThermoScientific).

Procedure

The pedestal was cleaned with tissue paper and distilled water before setting the blank. First blank was set in nanodrop spectrophotometer by placing 2µl of nuclease free water or elution buffer (in which DNA was eluted) onto the pedestal. After setting the blank, DNA concentration of test samples was determined using 2µl of eluted DNA. The DNA concentration and purity was noted for each sample. The UV absorbance spectrum of DNA exhibits maximum at 260 nm based on the aromatic ring structures of the DNA. A ratio 260/280 = 1.8 indicates DNA is pure. A ratio 260/280 below 1.8 indicates DNA was contaminated by proteins or aromatic compounds.

4.12 Molecular characterization of vancomycin resistant *E. faecium*

Identification of vancomycin resistant genotypes for clinical and environmental isolates were performed by using a multiplex PCR targeting *vanA* and *vanB* gene. Predesigned primers were used in this study and procured from Eurofins, India Pvt. Ltd. New Delhi, India. Details of primers are given in table; 7.

Table 7: Primer sequences used for amplification of *vanA* and *vanB* gene with amplicon size and annealing temperature.

Gene	Primers sequences	PCR Product (bp)	Reference
vanA	FP-AACAACTTACGCGGCACT RP-AAAGTGCGAAAAACCTTG	512	Getachew *et al*.,2012
vanB	FP-AAGCTATGCAAGCCATG RP-CCGACAATCAAATCATCCTC	536	

A multiplex PCR was performed in a 25µl volume reaction by using template DNA, 1× PCR buffer, 0.5µM each of forward and reverse primers, 200µM of each deoxy nucleoside triphosphate (dNTPs), 1.5mM MgCl2 and 1U Taq DNA Polymerase. Amplification was done in Gradient Thermal Cycler [Eppendorf Master Cycler EPS thermo-module, Hamburg, Germany]. PCR conditions used are summarized in table; 8.

Table 8: PCR conditions for amplification of *vanA* and *vanB* gene:

	Temperature	Time	Number of cycles
Initial denaturation	94°C	5 min	1
Denaturation **Annealing** **Extension**	94°C 55°C 72°C	30 sec 30 sec 45 sec	30
Final extension	72°C	10 min	1

4.12.1 Detection of amplicons by agarose gel electrophoresis

Agarose gel was prepared using weight/volume percentage solution. The 1.5% agarose gel was prepared in 0.5X Tris-borate ethylene diamine tetra acetic acid buffer (Sigma-Aldrich Pvt Ltd., India), containing 0.04 µg/ml of EtBr (Sigma-Aldrich Pvt Ltd., India) (**Annexure** III). After PCR amplification, 2 µl of loading dye was mixed with 8 µl PCR product and a total of 10 µl of the mixture was loaded into the respective wells of 1.5% agarose gel along with a 100 bp molecular size standard ladder (Thermofischer Scientific, Massachusetts, USA, catalog number-ER0591). The gel was run at 80 Volts for 60-90 mins. The amplicons were visualized by using an UV illuminator and photographed using Gel Doc™ (Bio-Rad, Hercules, California, USA).

4.13 Molecular characterization of linezolid resistance mechanism

Characterization of linezolid resistance mediated by G2576T point mutation in 23S rRNA and for the detection of *cfr* and *optrA* genes 3 different sets of PCRs was performed. The details of primers are given in table; 9. PCR was performed in a 25µl volume reaction by using template DNA, 1× PCR buffer, 0.5µM each of forward and reverse primers, 200µM of each deoxy nucleoside triphosphate (dNTPs), 1.5 mM MgCl2 and 1U Taq DNA Polymerase. Amplification was done in Gradient Thermal Cycler [Eppendorf Master Cycler EPS thermo-module, Hamburg, Germany]. PCR conditions has been given in table;10.

Table 9: Primer sequences with amplicon size used in this study for the investigation of linezolid resistance mechanisms.

Gene	Primer Sequences	PCR Product (bp)	References
23S rRNA	FP - GCAGAAGGGAGCTTGACTGCGAG RP – ACCCAGCAATGCCCTTGGCAG	389	Hong *et al.*, 2007
cfr	FP -TGAAGTATAAAGCAGGTTGGGAGTCA RP – ACCATATAATTGACCACAAGCAGC	746	Kehrenberg *et al.*, 2006
optrA	FP-AGGTGGTCAGCGAACTAA RP-ATCAACTGTTCCATTCA	1395	Wang *et al.*, 2015

Table 10: PCR conditions for amplification of 23S rRNA, detection of *cfr* and *optrA* gene

Steps	***23S rRNA* and *cfr gene***			***optrA* gene**		
	Temp.	**Time**	**No of cycles**	**Temp.**	**Time**	**No of cycles**
Initial denaturation	94°C	5 min	1	94°C	5 min	1
Denaturation **Annealing** **Extension**	94°C 62°C 72°C	30 sec 30 sec 45 sec	30	94°C 50°C 72°C	30 sec 45 sec 45 sec	30
Final extension	72°C	10 min	1	72°C	10 min	1

Detection of amplicons by agarose gel electrophoresis:

After PCR amplification, PCR amplified product was subjected to the agarose gel electrophoresis as described in section 4.10.1.

4.13.1 PCR – RFLP: Screening of G2576T mutation in *23S rRNA* gene

Amplified PCR product (389bp) was digested with the *NheI* restriction enzyme (Thermofisher scientific, Massachusetts, USA) as described by Hong *et al*., (2007). Restriction reaction was carried out at 37°C for 30 mins. The restriction mixture was used as follows:

5U of Enzyme (NheI)	**1µl**
10x Tango buffer	1.5µl
PCR Product	5µl
Nuclease free water	9.0µl

4.13.2 Analysis of Products after restriction treatment

After restriction treatment, the product was subjected to agarose gel electrophoresis. The presence of 244-bp and 145-bp fragments are indicative of G2576T mutation in domain V of *23s rRNA***.**

4.13.3 Purification of PCR product for 23S rRNA gene sequencing

The PCR amplicons were purified by QIAquick PCR purification kit (catalog No. 28104). PCR product was mixed in a ratio of 1:5 with PB buffer and 90 µl was added to the QIAquick column to bind DNA. Finally, the column was centrifuged at 12000 rpm for 1min and flow-through was discarded. The QIAquick column was placed back into the same tube and centrifuged once more in 2ml collection tube for 1min. to remove the residual wash buffer. Finally, the QIAquick column was placed in a clean 1.5ml microcentrifuge tube. Lastly the DNA was eluted by adding 50 µl buffer EB (10mM tris-CL, pH 8.5) to the centre of the QIAquick membrane and centrifuge the column for 1 min.

4.13.3.1 DNA Template quality

The quality of each purified PCR product was examined using agarose gel electrophoresis and DNA was seen as a single band.

4.13.3.2 DNA Template quantity

Quantification of purified template DNA was carried out for each sample by determining the absorbance of the sample at 260 nm in a spectrophotometer. This was

primarily done so as to use optimal amount of DNA template for the sequencing reaction. Each sequencing reaction was performed using the 60 to 80 ng of purified DNA.

4.11.4 Sanger Sequencing

The amplified fragments were sequenced (by outsourcing to Barcode biosciences, Dr. Shivarama Karanth Nagar, Bangalore). PCR sequencing reactions were performed in **Appliedbiosystems™ MiniAmp™ Plus** thermal cycler using **Big Dye™ Terminator V3.1** kit. Sequence reactions were performed as follows:

PCR Mix

Template	**2 µl (~50ng of DNA)**
Primer	1 µl (~2.5pmol)
Master Mix	7µl

After PCR amplification, the PCR amplified products were purified and proceeded for capillary electrophoresis and analysis in Genetic Analyzer, PCR amplified products sequenced with both forward and reverse primers in separate reactions.

4.13.4.1 Sequence analysis software

The sequences obtained were trimmed and aligned using BioEdit Sequence alignment editor software.

4.14 Detection of virulence genes/determinants

A multiplex PCR was performed for the detection of virulence genes *asa1, gelE, cylA, hyl* and *esp* gene. Two sets of multiplex PCRs (set I and set II) were performed depending on their annealing temperature. Predesigned primers were used and procured from IDT, Integrated DNA technologies (India Pvt. Ltd). Primers sequences for set I and set II along with amplicon sizes are given in table; 11. PCR conditions for set 1 and set 2 have been given in table; 12.

Table 11: Primer sequences and amplicon size for the detection of virulence genes.

Gene	Primer sequences	Amplicon size (bp)	References
asaI	FP-GCACGCTATTACGAACTATGA	375	Vankerckhoven *et al.*, 2004
	RP-TAAGAAAGAACATCACCACGA		
gelE	FP-ACCCCGTATCATTGGTTT	419	Lopes *et al*., 2006
	RP-ACGCATTGCTTTTCCATC		
cylA	FP-ACTCGGGGATTGATAGGC	688	Vankerckhoven *et al.*, 2004
	RP-GCTGCTAAAGCTGCGCTT		
esp	FP-AGATTTCATCTTTGATTCTTGC	510	Vankerckhoven *et al.*, 2004
	RP-AATTGATTCTTTAGCATCATCTGG		
hyl	FP-ACAGAAGAGCTGCTGCAGGAAATG	276	Vankerckhoven *et al.*, 2004
	RP-GACTGACGTCCAAGTTTCCAA		

Table 12: PCR condition for amplification of virulence gene *esp* and *hyl* (set 1) and for *asaI*, *gelE* and *cylA* gene (set 2).

	Set 1			Set 2		
	Temp.	**Time**	**No. of cycles**	**Temp.**	**Time**	**No. of cycles**
Initial denaturation	94°C	5 min	1	94°C	5 min	1
Denaturation **Annealing** **Extension**	94°C 56°C 72°C	30 sec 45 sec 45 sec	30	94°C 52°C 72°C	30 sec 30 sec 45 sec	30
Final extension	72°C	10 min	1	72°C	10 min	1

Detection of amplicons by gel electrophoresis

After PCR amplification, PCR amplified product was subjected to the agarose gel electrophoresis as described in section **4.12.1**

4.15 Molecular typing of linezolid resistant *E. faecium* by pulse field gel electrophoresis (PFGE)

Molecular epidemiology of linezolid resistant *E. faecium* isolates was studied by PFGE as described by Saeedi *et al*., (2002) with some modifications. The DNA was subjected to macro restriction analysis using the *smaI* restriction endonuclease (Thermo Fisher scientific, catalog number. ER0591).

4.15.1 Cell preparation

Pure colonies of LREfm grown on MHA with overnight incubation were used for cell preparation. Colonies were picked by using a sterile, cotton swab and suspended in 3ml of TE buffer. Turbidity of the suspension was adjusted to 8-10 McFarland and 1ml of cell suspension was centrifuged at 12000 rpm for 5 min in 1.5ml microcentrifuge tube. After centrifugation, the supernatant was aspirated and the pellets were dissolved in 150µl of TE buffer and kept in a water bath at 37°C for 10 min.

Composition of TE buffer:

Stock Solution	Working Solution
1 M Tris-HCL, pH-8.0	10mM Tris-HCL, pH-8.0
0.5M EDTA, pH-8.0	1mM EDTA, pH-8.0

4.15.2 Preparation of Plugs/blocks

For casting of plugs/blocks, disposable plug molds (Bio-Rad USA) were labelled corresponding to isolate ID. For plug preparation, 6µl of lysozyme (20mg/ml) (Sigma-Aldrich Pvt Ltd, India) was added to the 150µl of the cell suspension. Immediately after adding the lysozyme, 150µl of 1.2% (Wt/vol) Sea Kem Gold agarose in TE buffer (equilibrated at 55°C) was added. The solution was gently mixed and poured into the plug mould, two plugs were casted for each sample. After casting, plugs were allowed to set for 5 mins at room temperature followed by 10 min at 4°C for setting the plugs.

4.15.3 Lysis, deproteinization and washing of plugs

For lysis, plugs were removed from the plug molds and placed in 15 ml falcon tube containing 5 ml of EC lysis buffer followed by incubation at 37°C for 4 hrs. The EC lysis buffer was poured off and replaced with 2ml proteinase K buffer solution (20mg/ml) (Sigma-Aldrich Pvt Ltd, India) and incubated overnight at 55 °C with gentle shaking for deproteinization. Next day, proteinase K and buffer solution was poured off and the plugs were washed 2 times with sterile water at the interval of 20 mins with gentle shaking at room temperature followed by 3 additional washing with TE buffer. Plugs were stored in TE buffer at 4°C till required.

Composition of EC Lysis Buffer

Stock solution concentration	Working solution concentration
1 M Tris HCL, pH 8.0	6 mM Tris HCL, pH 8.0
5 M NaCl	1 M NaCl
0.5 M EDTA, pH 8.8	100 mM EDTA, pH 8.0
Brij-58	0.5% Brij-58
Sodium Deoxycholate	0.2% sodium Deoxycholate
Sodium Lauroylsarcosine	0.5% Sodium Lauroylsarcosine

4.15.4 Restriction enzyme treatment

Prior to treatment with restriction enzyme, plugs were washed with TE buffer with gentle shaking at 37°C. Washed plugs were removed from the tube. Approximately one-third of the plug was cut and transferred into a labelled 1.5 ml microcentrifuge tube containing 3µL of 30 units of *smaI* (Thermofisher scientific, Massachusetts, USA, catalog number-ER0591), 20µL of 10X restriction buffer, and 175µL of nuclease free water and further incubated at room temperature for 4 -6 hours.

4.15.5 Electrophoresis and interpretation

For electrophoresis, 1% pulse field grade agarose (Bio-Rad) was prepared in 0.5X TBE buffer (Tris-borate EDTA buffer). The gel was run on a CHEF Mapper (Bio-Rad Laboratories) at 14°C for 22 hours at 6.0v/cm with initial switch time of 5 seconds and final switch time of 35.5 seconds. Molecular size of bacterial plugs was determined and compared to Lambda PFG Ladder (New England Biolabs^{R} Ltd).

4.15.6 Staining and visualization of gel

After completion of run, the gel was stained with ethidium bromide (50µg/L) for 30 mins and destained with distilled water for 40 mins. For data analysis gels was photographed with the FOTO/Analyst Archiver system and saved as a Tiff file.

4.15.7 PFGE gel image analysis and dendrogram

Comparison and analysis of the PFGE patterns were performed with InfoQuest^{TM} FP Software v. 5.4 (Bio-Rad Laboratories, USA). PFGE patterns were compared on an unweighted pair-group method with agarose (UPGMA) dendrogram based on Dice

coefficients, where optimization and band position tolerance were set at 1%. A similarity coefficient of 75% was selected to define the patterns.

4.16 Multilocus Sequence Typing (MLST)

Molecular epidemiology of vancomycin and linezolid resistant *E. faecium* isolates was studied by MLST. Seven housekeeping genes including *adk* (adenylate kinase), *atpA* (ATP synthase, alpha subunit), *ddl* (d-alanine: d-alanine ligase), *gyd* (glyceraldehyde-3-phosphate dehydrogenase), *gdh* (glucose-6-phosphate dehydrogenase), *purK* (phosphoribosylaminoimidazol carboxylase ATPase subunit), and *pstS* (phosphate ATP-binding cassette transporter) were amplified by PCR using primers detailed in Table; 13.

Table 13: Primer sequences along with amplicon sizes used in the study for MLST.

Gene	Primer Sequences	Amplicon Size (Bp)	Reference
Adk	FP-GAACCTCATTTAATGGGG	530	http://efaecium.mlst.net/misc/info.asp
	RP-TGATGTTGATAGCCAGACG		
Atp	FP- TTCAAATGGCTCATACGG	556	
	RP-AGTTCACGATAAGCAACAGC		
Ddl	FP-GAGACATTGAATATGCCTTATG	560	
	RP- AAAAAGAAATCGCACCG		
Gdh	FP- GGCGCTAAAAGATATGGT	660	
	RP- CCAAGATTGGGCAACTTCGTCCCA		
Gyd	FP- CAAACTGCTTAGCTCCAATGGC	488	
	RP- CATTTCGTTGTCATACCAAGC		
purK	FP- CAGATTGGCACATTGAAAG	660	
	RP- TTCATTCACATATAGCCCG		
Pst	FP- TTGAGCCAAGTCGAAGC	630	
	RP- CGTGATCACGTTCTACTTCC		

PCR was performed in a 25µl volume reaction by using template DNA, 1× PCR buffer, 0.5 µM each of forward and reverse primers, 200µM of each deoxy nucleoside triphosphate (dNTPs), 1.5mM MgCl2 and 1U Taq DNA Polymerase. Amplification was done in Gradient Thermal Cycler [Eppendorf Master Cycler EPS thermo-module, Hamburg, Germany]. PCR conditions used are summarized in Table;14

Table 14: PCR conditions used for MLST.

Steps	Process	Temperature and Time	Number of Cycles
Step 1	Initial denaturation	94°C for 2minutes	1
Step 2	1. Denaturation 2. Annealing 3. Extension	94°C for 1minute 55°C for 1minute 72°C for 2minutes	30
Step 3	Final extension	72°C for 5minutes	1

Detection of amplicons by gel electrophoresis:

After PCR amplification, the PCR amplified products were subjected to agarose gel electrophoresis as described in section 4.12.1.

Purification of PCR product for sequencing: Purification of PCR amplified product was done as described in section 4.13.3.

Sequencing: The amplified PCR products were sequenced (by outsourcing to Barcode biosciences, Dr. Shivarama Karanth Nagar, Bangalore)) as described in section 4.11.4. The sequences obtained were trimmed using BioEdit Sequence alignment editor software. The assignment of alleles and sequence types was performed by the software available in the website www.pubmlst.org.

4.17 Statistical analysis

Data analysis was performed using SPSS Version 20.0 (SPSS, Chicago, USA). The categorical variables were presented as frequencies and percentages analysed by Wilcoxon-Mann-Whitney U Test, Chi-squared and Fisher's exact test for single-factor analysis. Variables that were significant in single factor analysis were entered into logistic regression model in multivariate analysis. All p values were two tailed and values <0.05 were taken as significant. Adjusted odds ratios (OR) and 95% confidence intervals (CIs) were calculated for significant variables. WHONET software (WHONET 5.6 to 2023) was used for analysis of MIC distribution and of the antimicrobial resistance profile.

4.18 Reference strains

S. No	ATCC Strains	Purpose	Source
1	*Enterococcus faecalis-ATCC 29212*	Control for AST and MIC	Procured from HiMedia Pvt. Ltd, India
2	*Enterococcus faecalis-ATCC 51299*	Positive control for *vanB* gene	
3	*Staphylococcus aureus-ATCC 33592*	Negative Control for biochemical tests	
4	*Enterococcus faecium-BM 4147*	Positive control for *vanA* gene	Kindly gifted by Dr. Beenu Dhawan, AIIMS, New Delhi
5	Inhouse positive controls: B1101, U109	As positive control for Linezolid resistance mechanisms and virulence genes	Retrieved from the departmental library of VMMC and SHJ, New Delhi

5. Results

Objective 1. To study the prevalence of Linezolid resistance among *Enterococcus faecium* in hospital-acquired infections.

5.1 Prevalence of LREfm

During the study period (January 2020 to June 2022) a total of 5069 isolates of *Enterococci* species were isolated from the clinically significant samples, the most frequent isolated species was *E. faecium* (n=3841, 75.7%) followed by *E. faecalis* (1204, 23.8%), *E. gallinarum* (n=14, 0.28%) and *E. casseliflavus* (n=10, 0.19%).

Linezolid and vancomycin resistance was observed only among *E. faecium* isolates. Vancomycin (MIC≥32µg/ml) and Linezolid (MIC≥8 µg/ml) resistance was detected in 720 (18.7%) and 202 (5.3%) of *E. faecium* isolates respectively. Linezolid resistance was observed more frequently among VRE (24.3%, 175/720) and was rare among vancomycin susceptible (0.9%, 27/3121) and this difference was statistically significant (P<0.0001). LREfm (n=202) from clinical samples were further studied for molecular characterisation. In addition, 24 isolates of linezolid resistant *E. faecium* recovered from patient's immediate environment were also enrolled to study transmission pathway and difference in virulence among clinical and environmental LREfm isolates.

5.1.1 Isolation of LREfm from various clinical specimens and locations (n=202)

Among 202 clinical isolates of LREfm, majority of LREfm were isolated from urine (n=96, 47.5%) followed by blood (n=88, 43.6%) and pus (n=18, 8.9%), details are summarized in table; 15 (Figure; 15). Highest number of LREfm were isolated from patient admitted to ICUs (48%) followed by patients in non-ICU setting in department of medicine (24.2%), paediatrics (6.9%), obstetrics & gynaecology (6.4%), surgery (5.9%), dermatology (1.9%), orthopaedics (1.9%), oncology (1.5%), urology (0.5%) HDU (0.5%). Distribution of LREfm from various departments and clinical sites is shown in table; 16. Blood isolates were predominantly from patient's admitted in ICUs and urine isolates were from patients in non-ICU settings. This difference was statistically significant [Table; 16] [Figure; 16].

Table 15: Isolation of LREfm from various clinical specimens.

Specimen Type	Number (n)	Percentage (%)	95% CI
Blood	88	43.6	36.7% - 50.7%
Pus	18	8.9	5.5% - 13.9%
Urine	96	47.5	40.5% - 54.6%

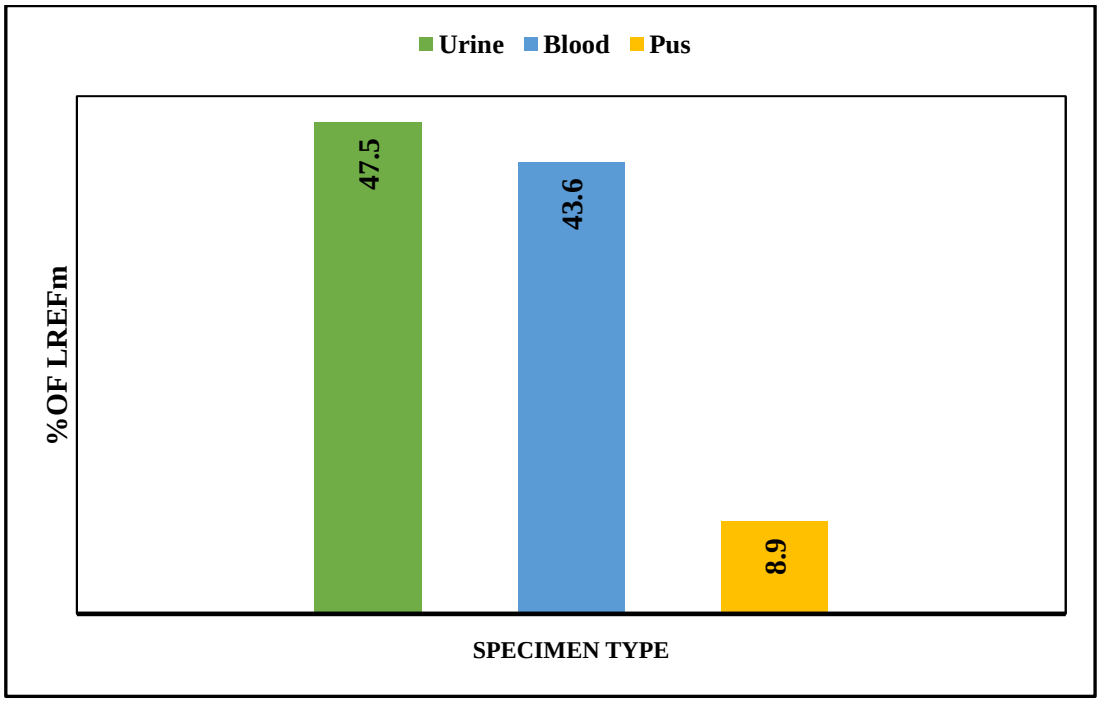


Figure 15: Isolation of LREfm from various clinical specimens.

Table 16: Isolation of LREfm from various clinical departments and specimens.

Location	Specimen Type				Chi-Squared Test	
	Blood n=88(%)	Pus n=18(%)	Urine n=96(%)	Total n=202(%)	χ^2	P Value
ICU	63 (71.6)	4 (22.2)	30 (31.2)	97 (48)	63.227	<0.001
HDU	0 (0)	0 (0)	1 (1)	1 (0.5)		
Medicine	15 (17.0)	4 (22.2)	30 (31.2)	49 (24.3)		
Nephrology	0 (0)	0 (0)	4 (4.2)	4 (2)		
Paediatric	4 (4.5)	2 (11.1)	8 (8.3)	14 (6.9)		
Genecology	2 (2.3)	1 (5.6)	10 (10.4)	13 (6.4)		
Surgery	3 (3.4)	4 (22.2)	3 (3.1)	10 (5)		
Dermatology	1 (1.1)	1 (5.6)	2 (2.1)	4 (2)		
Orthopaedics	0 (0)	2 (11.1)	2 (2.1)	4 (2)		
Oncology	0 (0)	0 (0)	3 (3.1)	3 (1.5)		
Urology	0 (0)	0 (0)	1 (1)	1 (0.5)		
Neurosurgery	0 (0)	0 (0)	2 (2.1)	2 (1)		

Note: Chi-squared test was used to explore the association between 'Specimen Type' and 'Location'. Blood isolates were predominantly from patient's admitted in ICUs and urine isolates were from patients in non-ICU settings ($\chi2 = 63.227$, $p = <0.001$).

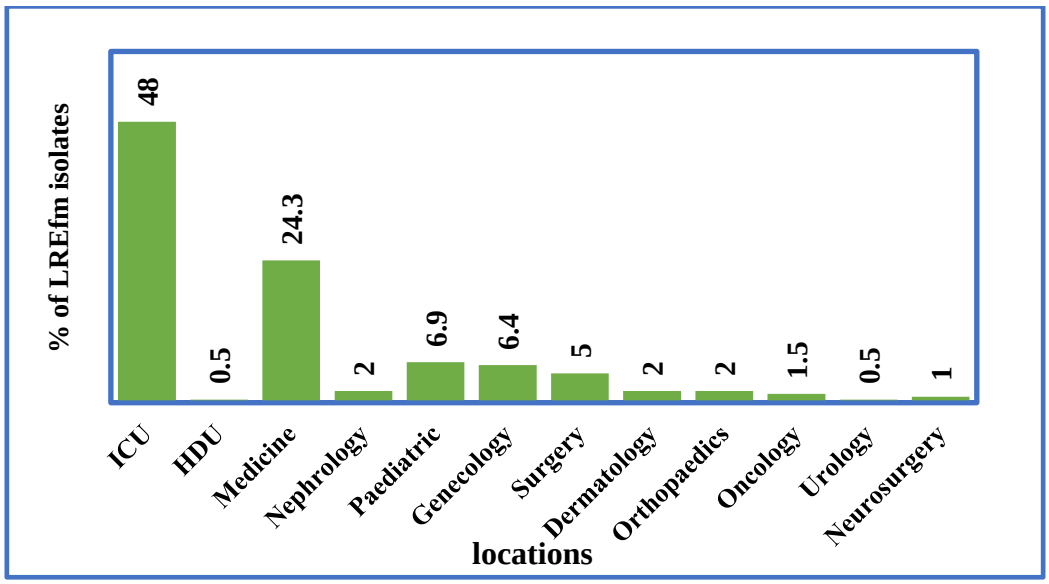


Figure 16: Isolation of LREfm from various clinical departments

Objective 2. To study the risk factors and clinical outcomes of patient's infected with Linezolid-resistant *Enterococcus faecium*.

To study risk factors, a total of 202 Cases (Study group) with clinically significant infections with linezolid resistant *E. faecium* and 200 patients with LSEfm infections (Controls) were enrolled in the study

5.2 Demographic data of Cases/study group (LREfm, n=202) and Controls (LSEfm, n=200)

Age of cases ranged between 28 days - 84 years with mean age (years) 38.78 ± 17.88. The age of controls ranged between 11 days to 82 years with mean age (years) 39.62 ± 18.83. Among cases and controls majority of patients were the females (111 vs 103). Details are summarised in table 1. There was no significant difference was observed in male/Female ratio and mean age of both groups. Demographic data of Cases (LREfm) and Controls (LSEfm) is given in table; 17

Table 17: Demographic data of Cases (LREfm, n=202) and Controls (LSEfm, n=200)

Parameters	Group	
	Cases LREfm (n = 202)	**Controls LSEfm (n = 200)**
Age (Years)	38.78 ± 17.88, 28 days - 84 years	39.62 ± 18.83, 11 days - 82 years
Gender		
Male	91 (45.0%)	97 (48.5%)
Female	111 (55.0%)	103 (51.5%)

5.2.1 Clinical characteristics and risk factors associated with Cases (LREfm) and Controls (LSEfm)

A. Cases/Study group (LREfm)

During the study period, LREfm were isolated from 202 patients, clinical data was available for 199 patients and is summarised in table; 18. In majority of patients the site of infection was abdominal (n=55, 27.1%), followed by urinary tract (n=44, 22.1%), respiratory tract (n=43, 21.6%), SSTI (n=29, 14.6%), CNS (n=19, 9.5%) and blood stream (n=9, 4.5%). Comorbidities were observed in 50.8% (101/199). The most

common comorbidity was hypertension (n=51, 25.6%), followed by T2DM (n=32, 16.1%), equal number of patients had T1DM and COPD (n=8, 4%) and only 2(1%) patient presented with chronic kidney disease (CKD). Overall, 91.5% (182/199) patients were on antibiotics with majority of patients receiving carbapenems (n=103, 51.8%) followed by linezolid (n=53, 26.6%), vancomycin (n=26, 13.1%). History of Invasive procedure, use of urinary catheter, central line and ventilation was observed in 35.6% (n=71), 83.8% (n=166), 46.2% (n=92) and 40.9% (n=81) patients respectively. The overall mortality rate was 31.7% (n=63).

Table 18: Clinical characteristics, risk factors and outcomes among Cases/Study group (Infections with LREfm, n=199)

Parameters	Frequency	Percentage (%)
Site of Infection:		
Respiratory	43	21.6
Abdominal	55	27.6
Soft Tissue	29	14.6
Urinary tract	44	22.1
Blood stream	9	4.5
CNS	19	9.5
Comorbidities:	**101**	**50.8**
T1DM	8	4.0
T2DM	32	16.1
HTN	51	25.6
COPD	8	4.0
CKD	2	1.0
On Antibiotic	**182**	**91.5**
Carbapenems (Meropenem/Imipenem)	103	51.8
Vancomycin	26	13.1
Linezolid	53	26.6
Invasive Procedure	**71**	**35.6**
Amputation	3	1.5
Burre Hole	1	0.5

Parameters	Frequency	Percentage (%)
Tracheostomy	21	10.6
Debridement Surgery	6	3.0
DJ Stunt	2	1.0
Drainage Tube	1	0.5
Laparotomy	9	4.5
ICD insertion	8	4.0
Ileostomy	1	0.5
Intercostal Drainage Tube	9	4.5
Intubated ET-Tube	1	0.5
Left Hemiarthroplasty	3	1.5
LSCS	2	1.0
Nephrostomy	1	0.5
TIPS	3	1.5
Invasive Devices		
On Central Line	92	46.2
Urinary Catheterization	166	83.8
Ventilations	81	40.9
Outcome		
Died	63	31.7
Discharged	128	64.3
LAMA	7	3.5
Referred	1	0.5

B. Controls (infection with LSEfm)

During the study period 200 patients with LSEfm were enrolled as the control group, details are summarised in table; 19. In majority of patients the site of infections was abdominal (n=54, 27%), followed by respiratory tract (n=52, 26%), SSTI (n=44, 22%), CNS (n=28, 14%), urinary tract (n=20, 10%) and blood stream (n=2, 1%). Comorbidities were observed in 30% (60/200). As observed in study group co-morbidities in the control group patients also included hypertension (n=28, 14%),

followed by T2DM (n=25, 12.5%), T1DM (n=4, 2%) CKD (n=2, 1%) and only one (0.5%) patient had the COPD. Overall, 40% (n=80) of the patients were on antibiotics, majority of patients were on carbapenems (n=52, 26%) followed by vancomycin (n=18, 9%) and linezolid (n=10, 5%). History of Invasive procedure, use of urinary catheter, central line and ventilation was observed in 27% (n=54), 21% (n=53), 21% (n=42) and 15.5% (n=51) respectively. The overall mortality rate was 19% (n=38).

Table 19: Clinical characteristics, risk factors and outcomes among Controls (Infections with LSEfm, n=200)

Parameters	Frequency	Percentage (%)
Site of Infection:		
Respiratory	52	26
Abdominal	54	27
Soft Tissue	44	22
Urinary tract	20	10
Blood stream	2	1
CNS	28	14
Comorbidities:	**60**	**30**
T1DM	4	2
T2DM	25	12.5
HTN	28	14
COPD	1	0.5
CKD	2	1
On Antibiotic	**80**	**40**
Carbapenems(meropenem/Imipenem)	52	26
Vancomycin	18	9
Linezolid	10	5
Invasive Procedure	**54**	**27**
Amputation	5	2.5
Burre Hole	0	0.0
Tracheostomy	13	6.5
Debridement Surgery	0	0.0

Parameters	Frequency	Percentage (%)
DJ Stunt	0	0.0
Drainage Tube	0	0.0
Laparotomy	24	12
ICD insertion	10	5
Ileostomy	0	0.0
Intercostal Drainage Tube	0	0.0
Intubated ET-Tube	0.	0.0
Left Hemiarthroplasty	0	0.0
LSCS	1	0.5
Nephrostomy	1	0.5
TIPS	0	0.0
Invasive Devices		
On Central Line	**42**	**21**
Urinary Catheterization	**53**	**26.5**
Ventilations	**51**	**15.5**
Outcome		
Died	**38**	**19**
Discharged	140	70
LAMA	16	8
Referred	6	3

5.2.1.1 Comparison of clinical characteristics, risk factors and outcomes between Cases/study group and Controls.

The comparison of risk factors and clinical characteristics of patients infected with LREfm and LSEfm are summarised in table; 20. By single factor analysis, it was observed that LREfm were more frequently isolated from the patients with UTI (p=<0.001) and BSI (p=0.032). The co-morbidities HTN (25.6% vs 14%, p=0.004) and COPD (4% vs0.5%, p=0.020) were significantly higher among Cases/study group patients compared to Controls. Invasive procedures like debridement surgery (p=0.015) and intercostal drainage (p=<0.001) were significantly higher among Cases compared to Controls. However, laparotomy (p=0.007) was significantly higher among

Controls with LSEfm infections. The other risk factors significantly associated with the LREfm infections were use of urinary catheter (p=<0.001), central line (p=<0.001) and mechanical ventilation (p=<0.001). Duration of hospital stay (from admission to time of infection) was 8-14 days for Cases (infection with LREfm) as compared to <7 days for Controls (infection with LSEfm) and this was statistically significant.

The prior use of carbapenems and linezolid among Cases and Controls was 51.8% vs 26% and 26.6% vs 5% respectively and was significantly higher among Cases (P =<0.001). The mortality rate (p=0.003) was also significantly higher among the Cases/study group (infections with LREfm).

Table 20: Comparison of clinical characteristics, risk factors and outcomes among Cases/study group (Infection with LREfm) and Controls (Infection with LSEfm).

Parameters	**Group**		**p value**
	Cases (LREfm) n = 199 (%)	**Controls (LSEfm) n = 200 (%)**	
Primary site of Infection:			
Abdominal	55 (27.6)	54 (27)	0.209
Respiratory	43 (21.6)	52 (26)	0.117
Skin and Soft Tissue	29 (14.6)	44 (22)	0.055
UTI	44 (22.1)	20 (10)	**<0.001**
BSI	9 (4.5)	2 (1)	**0.032**
CNS	19 (9.5)	28 (14)	0.168
Comorbidities			
T1DM	8 (4.0)	4 (2)	0.237
T2DM	32 (16.1)	25 (12.5)	0.307
HTN	51 (25.6)	28 (14)	**0.004**
COPD	8 (4.0)	1 (0.5)	**0.020**
CKD	2 (1.0)	2 (1)	1.000
Previous use of Antibiotics	**182 (91.5)**	**80 (40)**	**<0.001**
Carbapenems/Meropenem/ Imipenem	103 (51.8)	52 (26)	**<0.001**
Glycopeptide/Vancomycin	26 (13.1)	18 (9)	0.195
Linezolid	53 (26.6)	10 (5)	**<0.001**
Invasive Procedure	**81 (40.9)**	**60 (30)**	**0.023**

Parameters	Group		p value
	Cases (LREfm) n = 199 (%)	Controls (LSEfm) n = 200 (%)	
Amputation	3 (1.5)	5 (2.5)	0.724
Burre Hole	1 (0.5)	0 (0.0)	0.497
Tracheostomy	21 (10.6)	13 (6.5)	0.143
Debridement Surgery	6 (3.0)	0 (0.0)	**0.015**
DJ Stunt	2 (1.0)	0 (0.0)	0.247
Laparotomy	9 (4.5)	24 (12)	**0.007**
ICD insertion	8 (4.0)	10 (5)	0.645
Ileostomy	1 (0.5)	0 (0.0)	0.497
Intercostal Drainage Tube	11 (5.6)	0 (0.0)	**<0.001**
Left Hemiarthroplasty	3 (1.5)	0 (0.0)	0.122
LSCS	2 (1.0)	1 (0.5)	0.622
Nephrostomy	1 (0.5)	1 (0.5)	1.000
TIPS (transhepatic intrajugular portosystemic Shunt)	3 (1.5)	0 (0.0)	0.122
Invasive devices			
Central Line	92 (46.2)	42 (21)	**<0.001**
Urinary catheter	166 (83.8)	53 (26.5)	**<0.001**
Ventilation	81 (40.9)	51 (25.5)	**0.001**
Hospital Stay prior to infection			
≤7 Days	22 (11.1)	68 (34)	**<0.001**
8-14 Days	66 (33.2)	38 (19)	**<0.001**
>14 Days	111 (55.8)	94 (47)	
Outcome			
Died	63 (31.7)	38 (19)	**0.003**
Discharged	128 (64.3)	140 (70)	
LAMA	7 (3.5)	16 (8)	
Referred	1 (0.5)	6 (3)	

Note - Significant p<0.05 are shown in bold.

In multivariate analysis [table; 21], independent predictor for infection with LREfm were compared to infections with LSEfm. Factors significantly associated with increased risk of LREfm infection were site of infection UTI (OR 5.87, 95% CI 2.59-13.29, p=<0.001), use of carbapenem (OR 2.85 95% CI 1.62-5.02, p=<0.001) and

linezolid (OR 10.13 95% CI 4.13-24.82, p=<0.001), use of central line (OR 5.54 95% CI 2.35-13.09, p=<0.001), urinary catheter (OR 0.29 95% CI 0.12-0.70, p=<0.001) and ventilation (OR 14.87 95% CI 7.86-28.11, p=<0.007).

Table 21: Multivariate analysis of risk factors associated with Cases (Infections with LREfm) and Controls (Infections with LSEfm).

Dependent: Group	Cases (LREfm) (n = 199)	Controls (LSEfm) (n=200)	OR (95%CI)	p value
Hospitalization prior to infection				
≤7 Days	22 (11.1)	68 (34)	1.80 (0.87-3.78)	p=0.116
8-14 Days	66 (33.2)	38 (19)	**5.89 (2.63-13.77)**	**p<0.001**
>14 Days	111 (55.8)	94 (47)	1.44 (0.25-12.27)	p=0.704
Comorbidities:				
HTN	51 (64.6)	28 (35.4)	0.94 (0.46-1.95)	p=0.865
COPD	8 (88.9)	1 (11.1)	10.33 (1.07-279.03)	p=0.089
Previous use of Antibiotics				
Carbapenems/Meropenem/Imipenem	103 (66.5)	52 (33.5)	**2.90 (1.63-5.22)**	**p<0.001**
Glycopeptide/Vancomycin	26 (59.1)	18 (40.9)	2.34 (0.93-6.03)	p=0.073
Linezolid	53 (84.1)	10 (15.9)	**9.81 (4.11-25.66)**	**p<0.001**
Any Invasive Procedure	81 (57.4)	60 (42.6)	0.94 (0.49-1.77)	p=0.846
Central Line	92 (68.7)	42 (31.3)	**5.60 (2.36-13.78)**	**p<0.001**
Urinary catheter	166 (75.8)	53 (24.2)	**15.13 (8.05-29.89)**	**p<0.001**
Ventilation	81 (61.4)	51 (38.6)	**0.29 (0.12-0.70)**	**p=0.007**

Note - Significant p <0.05 values are shown in bold.

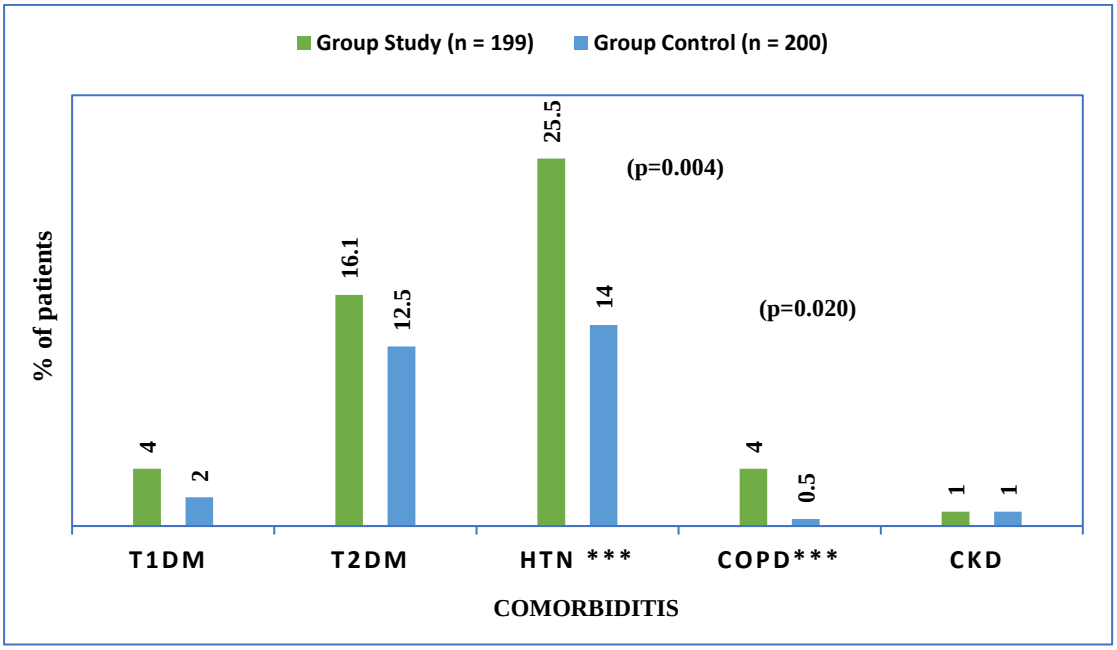


Figure 17: Comparison of comorbidities among Cases/study group and Controls.

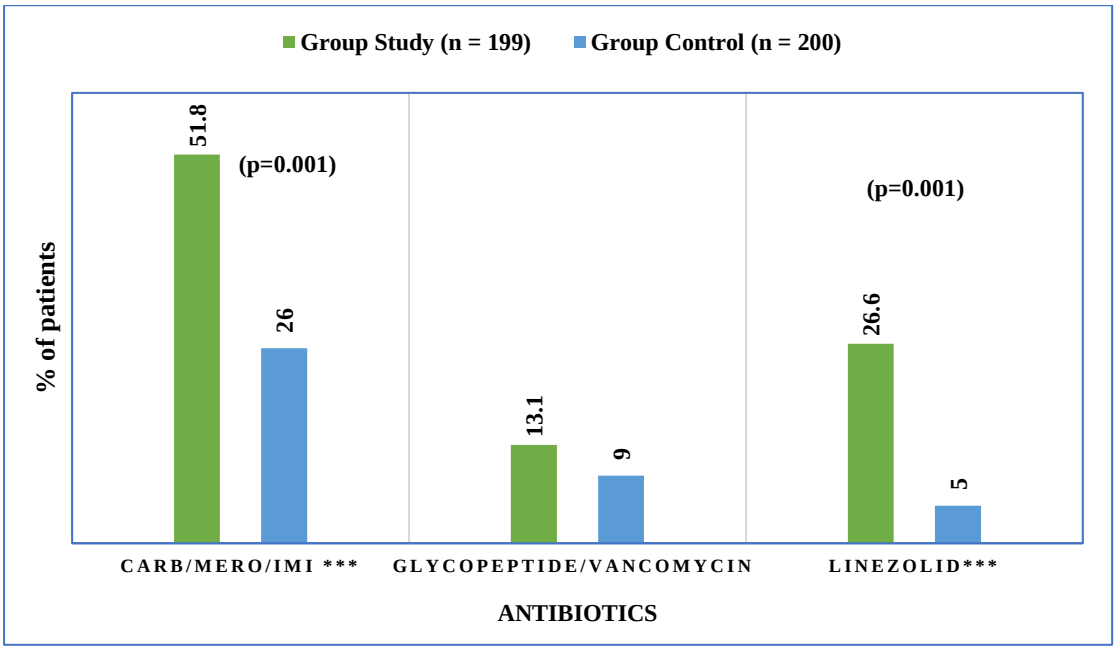


Figure 18: Comparison of use of antibiotics during hospital stay among Cases/study group and controls.

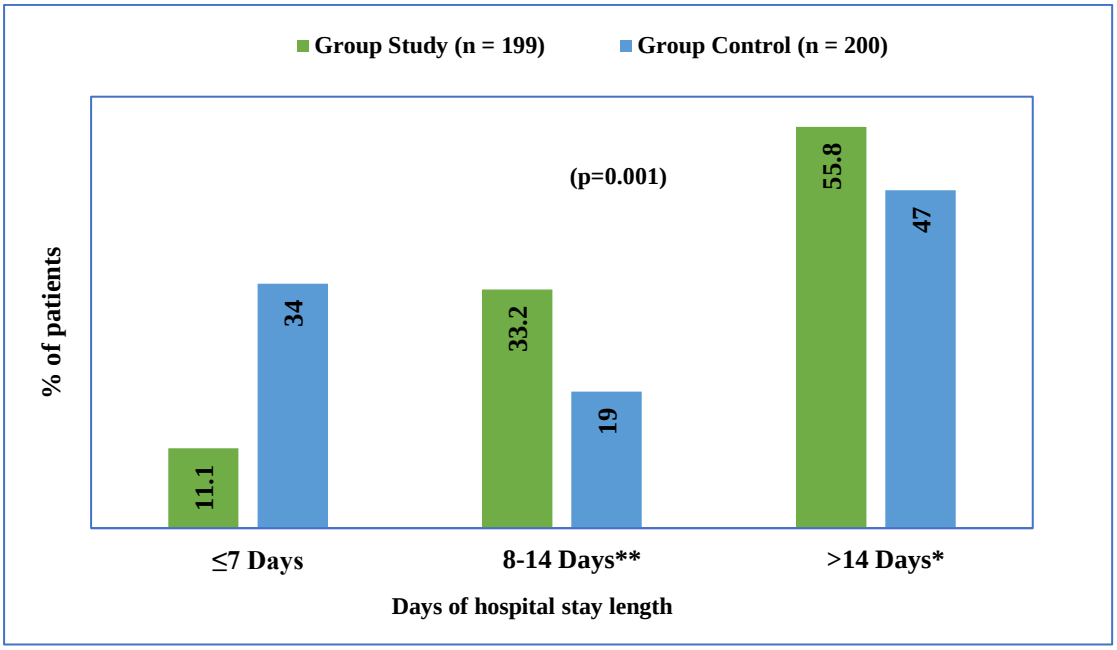


Figure 19: Comparison of length of hospital stay (days) prior to isolation of LREfm or LSEfm among Cases/study group and controls respectively.

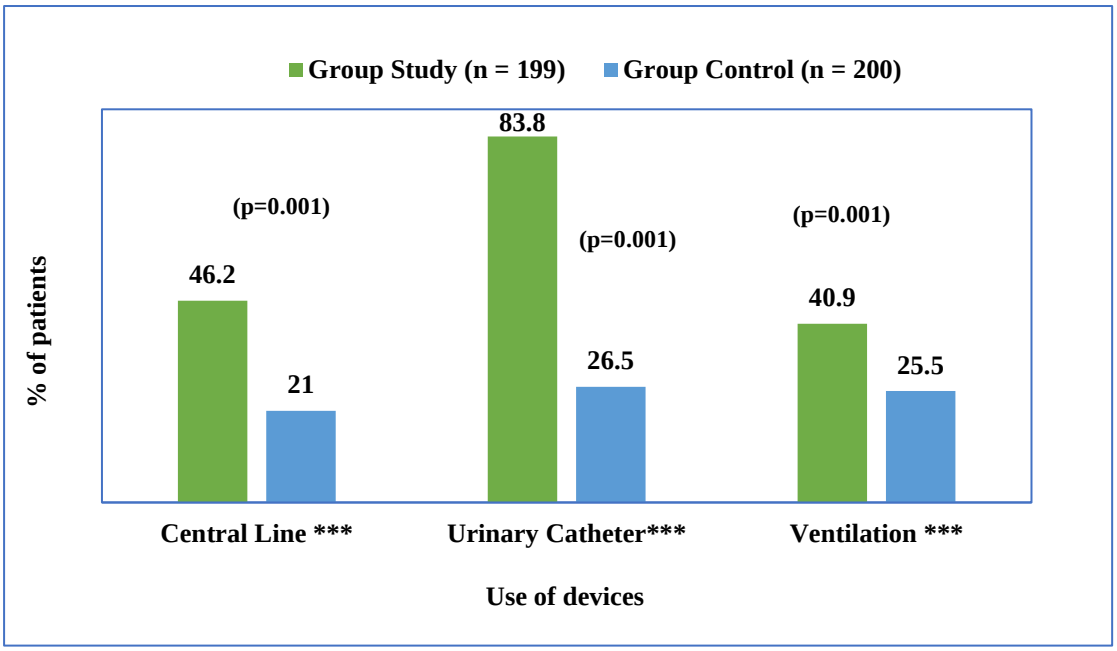


Figure 20: Use of invasive devices among Cases/study group and Control.

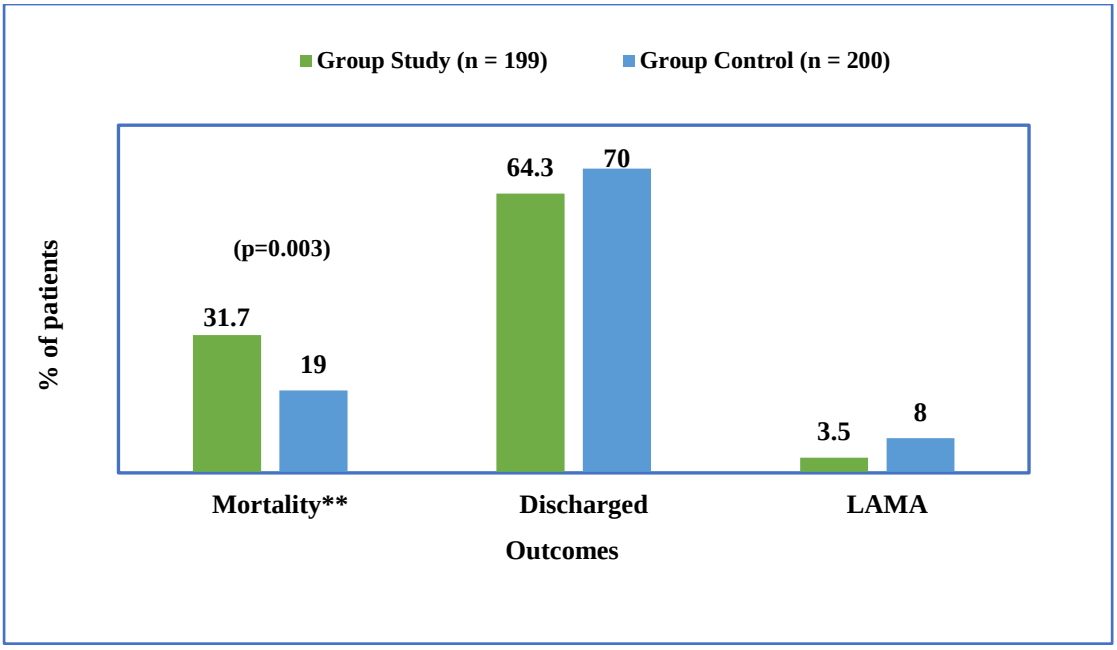


Figure 21: Outcomes among Cases/study group and Controls.

5.3 Antimicrobial susceptibility profile of clinical (n=202) and Environmental LREfm isolates (n=24) by Disk Diffusion

The antibiotic susceptibility profiles of clinical LREfm (n=202) and environmental LREfm (n=24) isolates are summarised in table; 22. All clinical and environmental isolates were resistant to ampicillin and erythromycin. Overall resistance to ciprofloxacin (99.5% vs 100%), high level gentamicin (95% vs 75%), tetracycline (83.2% vs 87.5%) and chloramphenicol (62.4% vs 75%) was high among both clinical and environmental isolates respectively. Resistance to nitrofurantoin was observed in 69.8% of the urinary isolates (12/96). Resistance to high level gentamicin was significantly high among clinical isolates (p=0.004). MDR, XDR and PDR were observed in 100%, 97.5% and 31.2% isolates respectively.

5.3.1 Antimicrobial susceptibility profile of Clinical (n=202) and Environmental LREfm isolates (n=24) by E-test (Teicoplanin, Vancomycin, Linezolid, Quinupristin/dalfopristin and Daptomycin)

Among clinical and environmental isolates resistance to vancomycin was high (86.6% vs 87.5%). Resistance to teicoplanin was significantly (p=0.017) higher among environmental isolates (75%) compared to clinical isolates (42.1%). Resistance to quinupristin/dalfopristin was significantly (p=0.001) higher among clinical isolates (91.1%) in comparison to environmental isolates (70.8%). Excluding one clinical

isolate (MIC 8 µg/ml) all clinical and environmental isolates were susceptible to daptomycin. Details are summarized in table; 23.

MIC of teicoplanin

Teicoplanin MIC ranged from 0.125 - 256µg/ml for clinical isolates and 0.5 - 256 µg/ml for environmental isolates. MIC50 and MIC90 for clinical isolates were 1 and 64 respectively. For environmental isolates MIC50 and MIC90 were 128µg/ml and 256µg/ml respectively.

MIC of vancomycin:

Vancomycin MIC ranged from 0.19 – 256 µg/ml for clinical isolates and 1-256 µg/ml for environmental LREfm isolates. There was no difference in MIC50 and MIC90 for clinical and for environmental isolates. MIC50 and MIC 90 were 128µg/ml and 256µg/ml respectively.

MIC of linezolid

Linezolid MIC ranged from 6-256 µg/ml for both clinical and environmental LREfm isolates. MIC50 and MIC90 for clinical isolates were 32µg/ml and 256µg/ml respectively. For environmental isolates MIC50 and MIC90 were 64µg/ml and 256µg/ml respectively.

MIC of daptomycin

MIC of Daptomycin ranged from 0.064 - 8 µg/ml and 0.2 - 4 µg/ml for clinical and environmental LREfm isolates respectively. MIC50 for clinical and environmental isolates was 1.5µg/ml, MIC90 for clinical isolates was 3µg/ml and for environmental isolates was 2µg/ml.

MIC of quinupristin/dalfopristin

MIC of quinupristin/dalfopristin ranged from 0.38 – 32 µg/ml and 0.5 - 32 µg/ml for clinical and environmental isolates respectively. MIC50 and MIC90 were 2-fold and 3-fold higher respectively for clinical isolates in compared to environmental isolates.

Table 22: Antibiotic susceptibility pattern by disc diffusion for Clinical (n=202) and Environmental (n=24) LREfm isolates.

Antibiotics	Resistant n (%)		Sensitive n (%)	
	Clinical	Environmental	Clinical	Environmental
AMP	202 (100)	24(100)	0(0)	0(0)
GEH	192 (95)	18(75)	10(5)	6(25)
CIP	201(99.5)	24(100)	1(0.5)	0(0)
ERY	202 (100)	24(100)	0(0)	0(0)
NIT	67(69.8)		29(30.2)	
CHL	126(62.4)	18(75)	76(37.6)	6(25)
TET	168(83.2)	21(87.5)	34(16.8)	3(12.5)

Note: AMP - Ampicillin, GEH – High level Gentamicin, CIP – Ciprofloxacin, ERY- Erythromycin, NIT- Nitrofurantoin, CHL- Chloramphenicol, TET- Tetracycline

Table 23: Antimicrobial susceptibility pattern based on MIC (E test) for select antibiotics for Clinical (n=202) and Environmental (n=24) LREfm isolates.

Antibiotics	MIC (µg/ml)						AST				P value
	Range		50		90		C		E		
	C	E	C	E	C	E	R n (%)	S n (%)	R n (%)	S n (%)	
TEIC	0.125 - 256	0.5 – 256	1	128	64	256	85 (42.1)	117 (57.9)	18 (75)	6 (25)	**0.017**
VAN	0.19 – 256	0.38 – 256	256	256	256	256	175 (86.6)	27 (13.4)	21 (87.5)	3 (12.5)	0.723
DAP	0.064-8	0.25 – 4	1.5	1.5	3	2	201 (99.5)	1 (0.05)	24 (100)	0 (0)	0.362
LNZ	6-256	6 – 256	32	64	256	256	202 (100)	0 (0)	24 (100)	0 (0)	0.360
QDA	0.38 – 32	0.5 – 32	8	4	32	12	184 (91.1)	18 (8.9)	17 (70.8)	7 (29.1)	**<0.001**

Note: C- Clinical, E- Environmental, R- Resistant, S-Sensitive. DAP- Daptomycin, LNZ – Linezolid, VAN-Vancomycin, TEIC- Teicoplanin, QDA quinupristin/dalfopristin.

5.4 Phenotypic detection of *vanA* and *vanB* phenotypes

A total of 30 LREfm isolates were vancomycin susceptible which includes 27 clinical and 3 environmental isolates with MIC range 0.19-6 µg/ml. Phenotypic detection of

vanA and *vanB* was determined based on MIC (E-test) of teicoplanin and vancomycin. The *vanA* phenotype was observed in 48.6%(n=85) and 85.7% (n=18) among clinical and environmental isolates respectively. The vancomycin and teicoplanin MICs range was 64- 256µg/ml and 8-256µg/ml respectively. Whereas *vanB* phenotype was observed in 51.4%(n=90) and 14.3%(n=3) clinical and environmental isolates respectively. The vancomycin and teicoplanin MICs range was 16-256µg/ml and 0.38-1.5µg/ml respectively, details are given in table; 24.

Table 24: Distribution of *vanA* and *vanB* phenotypes based on MIC of vancomycin and teicoplanin among clinical and environmental LREfm isolates.

Phenotypes (Definitions)	**Clinical isolates (n=175)**	**Environmental isolates (n=21)**
vanA **Vancomycin MIC range 64- 256µg/ml** **Teicoplanin MIC range 8-256µg/ml**	85 (48.6%)	18 (85.7%)
vanB **Vancomycin MIC range 16-256µg/ml** **Teicoplanin MIC range 0.38-1.5µg/ml**	90 (51.4%)	3 (14.3%)

Objective 3. To characterize various mechanisms of the vancomycin and Linezolid resistance among *E. faecium* isolated from clinical and environmental samples.

5.5 Mechanisms of vancomycin resistance

PCR was performed to characterize vancomycin resistance mechanisms mediated by *vanA* and *vanB* gene (Figure; 22). The *vanA* and *vanB* genes were detected in 48.6% (85/175) and 50.9% (89/175) clinical isolates and 85.7% (18/21) and 4.7% (1/21) environmental isolates respectively. The *vanA* and *vanB* genes were equally distributed among clinical isolates, however *vanA* gene was more frequently detected compared to *vanB* gene among environmental isolates [table; 25]. The effect of resistance mechanisms on vancomycin and teicoplanin MICs were also studied and details are given in table; 26.

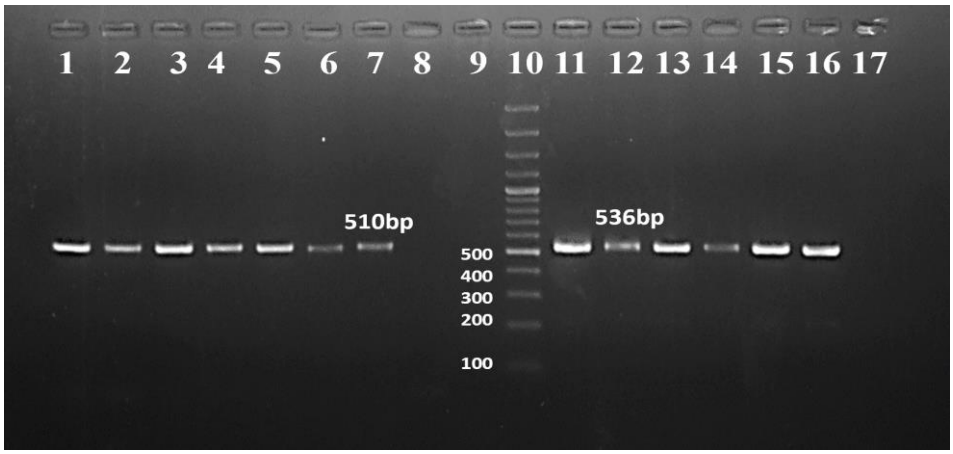


Figure 22: PCR amplified product of *vanA* and *vanB* genes. Total length of PCR product for *vanA* and *vanB* gene is 510 bp and 536 bp respectively, resolved on 1.5% agarose gel. Lane 1 shows positive control of *vanA* gene, lane 11 shows positive control of *vanB* gene, lane 8 and 17 shows negative control. DNA ladder: 100bp.

Table 25: Distribution of *vanA* and *vanB* genes among LREfm and VREfm isolated from clinical and environmental isolates.

Gene	Clinical isolates N=175(%)	Environmental isolates N=21(%)
vanA	85(48.6)	18(85.7)
vanB	89(50.9)	1(4.7)
No gene was detected	1(0.57)	2(9.5)

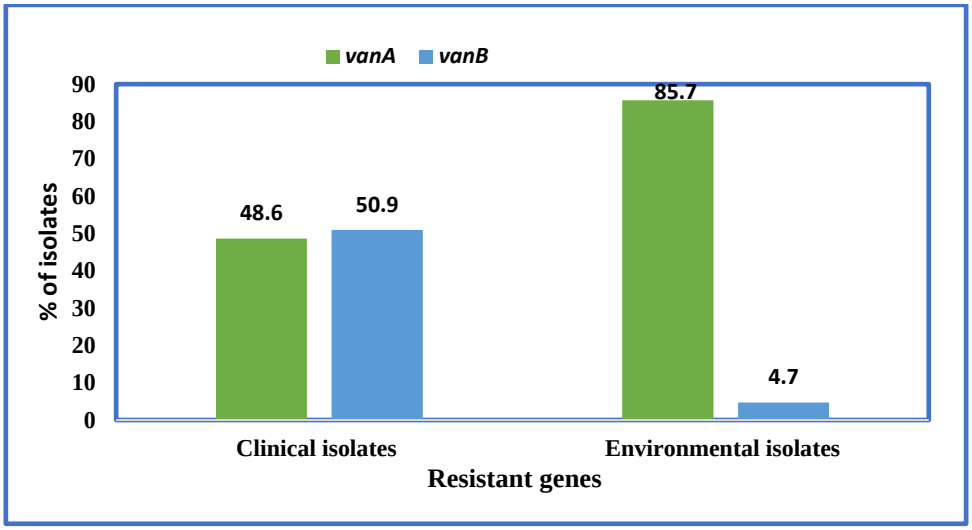


Figure 23: Distribution of *vanA* and *vanB* genes among LREfm and VREfm isolated from clinical and environmental isolates.

5.5.1 Effect of resistance mechanisms (*vanA* and *vanB*) on vancomycin and teicoplanin MIC

Isolates with *vanA* gene, vancomycin and teicoplanin MICs ranged from 64-256µg/ml and 8-256µg/ml among clinical isolates and 256-256µg/ml and 32-256µg/ml among environmental isolates respectively. The isolates with *vanB* gene, teicoplanin MIC (0.38 - 1.5 µg/ml) was in susceptible range. Among clinical and environmental isolates vancomycin MIC50 (256µg/ml) did not vary with *vanA* and *vanB* gene. Details are given in table; 26.

Table 26: Effect of resistance mechanisms on vancomycin and teicoplanin MICs.

	Vancomycin MIC (µg/ml)				Teicoplanin MIC (µg/ml)			
	MIC 50	MIC 50	Geo. Mean	MIC range	MIC 50	MIC 50	Geo. Mean	MIC Range
Clinical isolates								
***vanA* gene (n=85)**	256	256	251.9	64 – 256	32	256	43.8	8 – 256
***vanB* gene (n=89)**	256	256	242.3	16 – 256	1	1	0.8	0.38 - 1.5
Environmental isolates								
***vanA* gene (n=18)**	256	256	256	256 – 256	128	256	83.8	32 – 256
***vanB* gene (n=1)**	256	256	256	256 – 256	1	1	1	1-1

5.5.2 Co-relation of phenotypic and genotypic mechanisms of vancomycin resistance among clinical and environmental isolates

A very good corelation was observed between phenotypic and genotypic characterization. However, one clinical and 2 environmental isolates with *vanB* phenotype no vancomycin resistance mechanism was detected.

5.6 Mechanisms of linezolid resistance

PCR was performed to characterize the mechanism of linezolid resistance among 226 LREfm isolates which included 202 clinical and 24 environmental isolates.

5.6.1 Chromosomal mediated resistance

5.6.1.1 PCR-RFLP Screening of G2576T mutation in 23S rRNA

Amplified PCR product (389bp) of 23Sr RNA was digested with the *nheI* restriction enzyme and after restriction digestion product was subjected to agarose gel electrophoresis. The presence of 244-bp and 145-bp fragments was indicative of G2576T mutation (Figure; 24). In this study overall, point mutation G2576T was observed in 26.1% (59/226) of LREfm isolates.

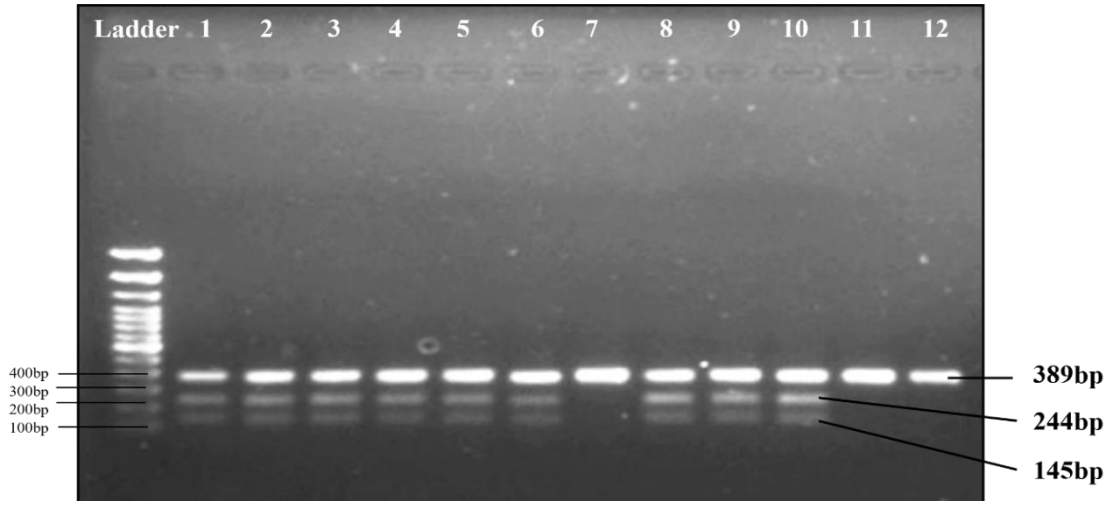


Figure 24: NheI digestion of PCR amplified product from domain V of 23S rRNA gene of *E. faecium* shows the presence of G2576T mutation. Lane 1: positive control. Lane 12: negative control. Lane 2-6, 8-10 show the presence of 244-bp and 145-bp bands indicate G2576T mutation. Lane 7 and 11 show the presence of a single 389bp band indicate the absence of G2576T mutation. Resolved on 2% agarose gel. Ladder: 100bp.

5.6.1.2 Sanger sequencing

Sanger sequencing was performed to the confirm the presence of point mutation G2576T and for mutations other than G2576T. In the present study a very good corelation was observed between sanger sequencing and PCR-RFLP for the detection of G2576T mutation. By sanger sequencing G2576T point mutation was confirmed in 26.1% (59/226 LREfm isolates). Sequencing confirmed that G2576T mutation co-existed with C2610T mutation in domain V of the 23S rRNA. Both mutations coexisted in the same isolates (Figure; 25 and 26).

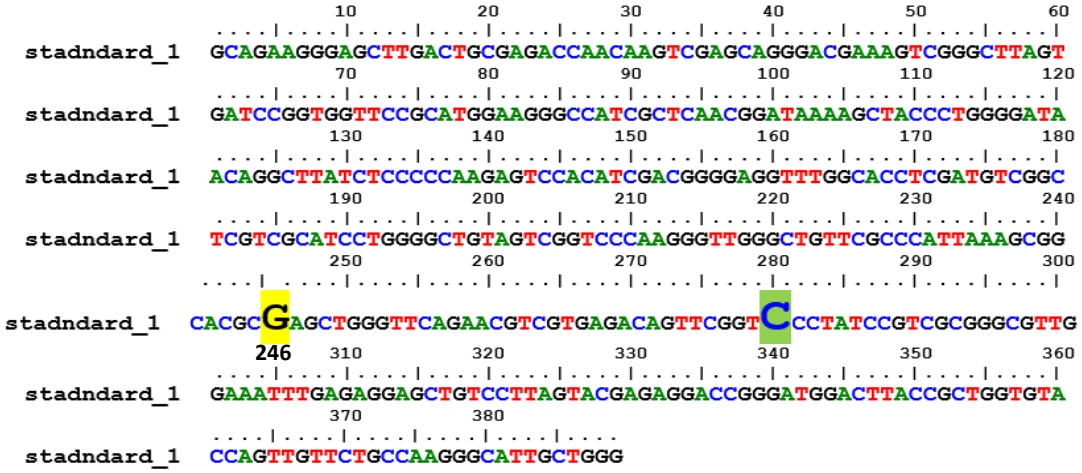


Figure 25: Shows sequence of 389 bp amplified product of Domain V of 23S rRNA gene of linezolid sensitive strain, at position 246 base is G, indicate the absence of G2576T mutation and at position 280 base is C indicate the absence of C2610T mutation.

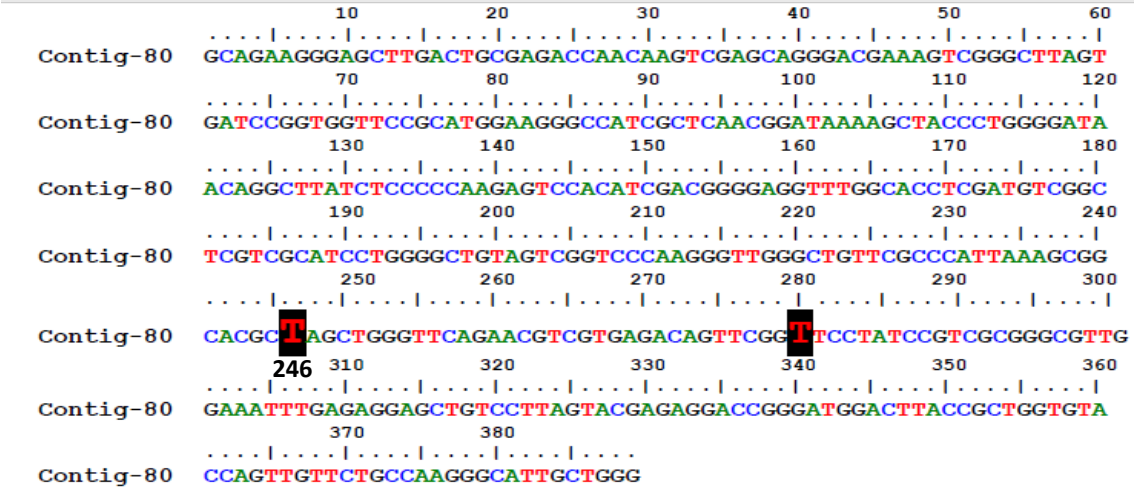


Figure 26: Shows sequence of 389 bp amplified product of Domain V of 23S rRNA gene of linezolid resistant *E. faecium.* At position 246 base is T, indicate the presence of G2576T mutation and at position 280 base is T indicate the presence of C2610T mutation.

5.6.2 Plasmid mediated Resistance

Among clinical and environmental LREfm isolates *cfr* gene was not detected (Figure; 27). Overall *optrA* gene was detected in 60.1% (137/226), with PCR yielding an amplicon of 1395bp (Figure; 28).

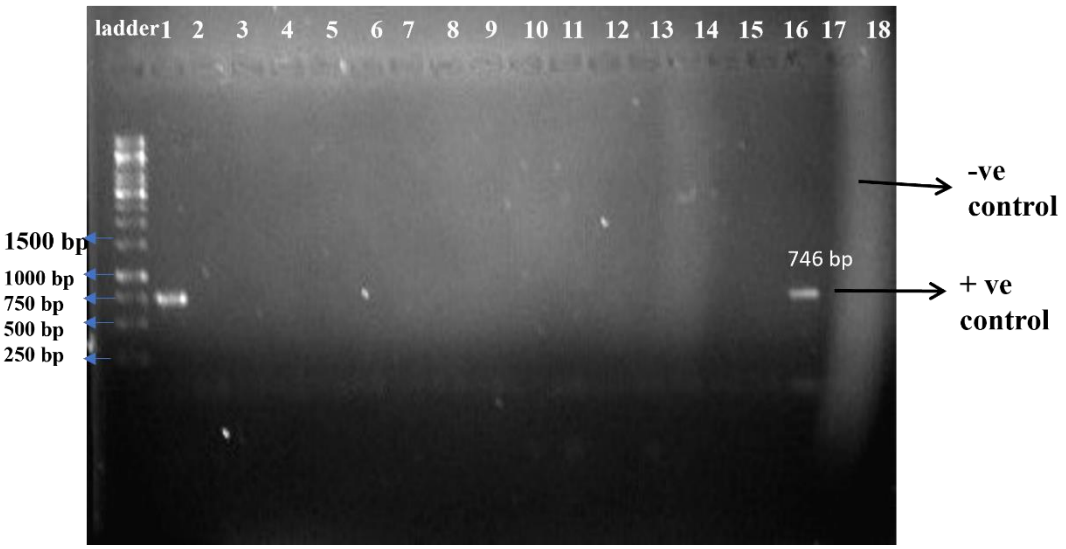


Figure 27:- Amplified PCR products of *cfr* gene. Amplicon size is 746 bp for *cfr* gene resolved on 2% agarose gel. Lane 2-16 shows: LZ-R isolates, lane 1 and 17 shows positive control and lane 18 shows negative control. DNA ladder: 1kb.

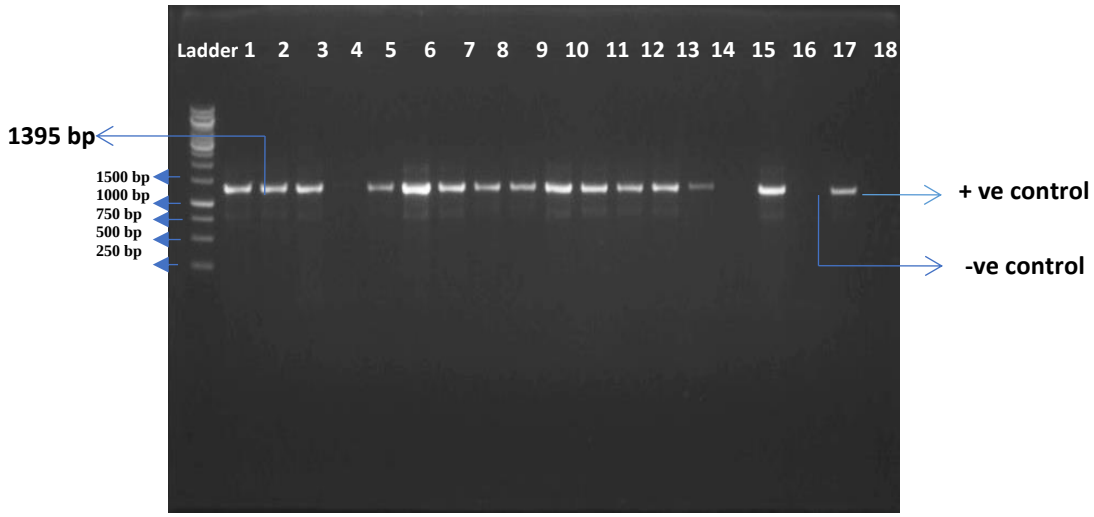


Figure 28: Amplified PCR products of *optrA*. Amplicon size is 1395 bp for *optrA* gene resolved on 1.5% agarose *gel*. Lane 1-16 indicates: LZ-R isolates. Lane 4 and 15 indicates absence of *optrA* gene, lane 17 indicate: negative control and lane 18 indicate: positive control. DNA ladder: 1kb.

5.6.3 Resistance mechanisms among clinical isolates (n=202)

Among clinical LREfm isolates *cfr* gene was not detected. *OptrA* gene was predominant mechanism of linezolid resistance and detected in 58.9% (119/202) isolates followed by point mutations at G2576T and C2610T in the domain V of the 23S rRNA and detected in 25.2% (51/202) LREfm isolates. Among isolates with G2576T and C2610T mutations, 42 were also positive for *optrA* and 9 were negative for *optrA*.

5.6.4 Mechanisms of resistance among environmental isolates (n=24)

Similar to clinical isolates all environmental LREfm isolates (n=24) were negative for *cfr* gene. *OptrA* gene was predominant mechanism of linezolid resistance and detected in 75%% (18/24) isolates. Point mutations G2576T and C2610T in the domain V of the 23S rRNA were detected in 33.3% (8/24) isolates. All isolates with G2576T and C2610T mutations were also positive for the *optrA* gene.

5.6.5 Effect of resistance mechanisms on Linezolid MIC among clinical and environmental isolates

The effect of resistance mechanisms on linezolid MIC was also studied and details are given in table; 27. The MIC range, MIC50 and MIC90 of linezolid among clinical and environmental isolates with *optrA* gene alone did not vary. The MIC range of these isolates varied from 6 -128 µg/ml.

The MIC of linezolid among clinical isolates with G2576T and C2610T point mutations in the domain V of the 23S rRNA was approximately 10fold higher (MIC50: 256 µg/ml) compared to isolates with no mutations (MIC50 32µg/ml). The MIC of these isolates (clinical) did not vary in the presence of *optrA* gene (MIC50 was 256µg/ml) or in the absence of *optrA* gene (MIC50 256µg/ml). However, among environmental isolates the MIC of linezolid was 5fold higher (MIC50: 256 µg/ml) compared to isolates with no mutations (MIC50: 48µg/ml) in the presence of *optrA* gene. *OptrA* negative and point mutation (G2576T and G2610T) positive phenotype was not observed among environmental isolates. Among clinical isolates, no resistance mechanism was observed in 74 isolates, with MIC50, MIC90 and geometric mean of 32, 48 and 17.6 respectively. Similarly, among environmental isolates no resistance mechanism was observed in 6 isolates with MIC50, MIC90 and geometric mean of 8, 32 and 10.9 respectively.

Table 27: Effect of linezolid resistance mechanisms on MIC (µg/ml) of linezolid (Clinical and environmental isolates).

	MIC50	MIC90	Geom. Mean	MIC range
Clinical isolates				
***optrA* pos and mutations (G2576T and C2610T) neg (n=77)**	24	96	24.4	6 – 128
***optrA* pos and mutations (G2576T and C2610T) pos (n=42)**	256	256	240.3	256 – 256
***optrA* neg and mutations (G2576T and C2610T) pos (n=9)**	256	256	256	256– 256
***optrA* neg and mutations (G2576T and C2610T) neg (n=74)**	32	48	17.6	6 – 128
Environmental isolates				
***optrA* pos and mutations (G2576T and C2610T) neg (n=10)**	48	64	35.7	8 – 128
***optrA* pos and mutations (G2576T and C2610T) pos (n=8)**	256	256	256	256 – 256
***optrA* neg and mutations (G2576T and C2610T) neg (n=6)**	8	32	10.9	6-32

Objective 4. To study virulence genes/factors associated with Linezolid resistant *E. faecium*.

5.7 Distribution of Virulence genes/factors among clinical LREfm isolates (n=202)

In the present study virulence genes were studied by multiplex PCR (Figure 29,30, 31) and were detected in 76.2%% (n=154) of LREfm isolates. The *esp* was the most common virulence gene and was detected in 59.9% (n=121) isolates followed by *cylA* (25.7% (n=52), *hyl* 16.8% (n=34). Virulence genes detected rarely were *gelE* 1.5% (n=3) and *asa1* 0.5% (n=1). Single virulence gene was detected in 76.2%(n=154), 2 virulence genes were detected in 13.9% (n=28) isolates. In one isolate (0.5%), 3 virulence genes were detected. Virulence genes detected in clinical isolates were more diverse. The combinations of virulence genes detected are summarised in table;36. Among clinical isolates genes detected singly were *esp* (47.5%, n=96) followed by *cylA* (16.8%, n=34) *hyl* (10.9%, n=22) and *gelE* (0.9%, n=2), details are given in table; 28 and figure; 32.

Table 28: Distribution of virulence genes among clinical isolates of LREfm (N=202).

Virulence gene	No. of isolates (%)
Esp	96 (47.5)
cylA	34(16.8)
Hyl	22(10.9)
gelE	2(0.9)
cylA + esp	14(6.9)
esp + hyl	8(3.9)
cylA+ hyl	4(2)
esp + gelE	1(0.5)
esp + asa1	1(0.5)
hyl + esp + cylA	1(0.5)

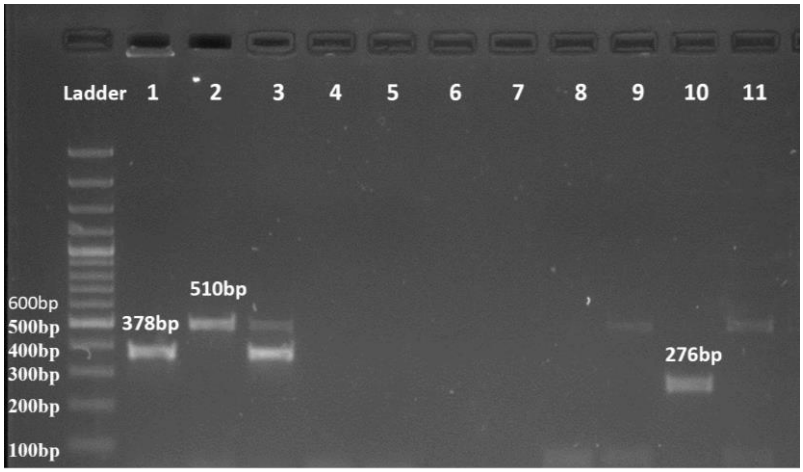


Figure 29: PCR amplified products of *esp, asaI* and *hyl* genes. Total length of PCR product of *esp, asaI* and *hyl* are 510bp, 378bp and 276bp respectively, resolved on 1.5% agarose gel. Lane 1, 2 & 10 showing positive control of *asa1*, *esp* and *hyl* genes respectively. Lane 3 shows negative control, DNA ladder: 100bp.

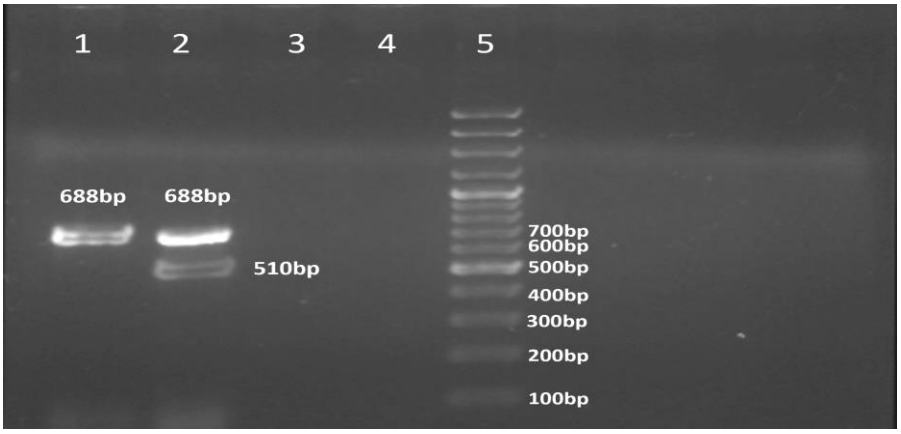


Figure 30: PCR amplified product of *cylA* gene. Total length of PCR product is 688bp, resolved on 1.2% agarose gel. Lane 1 shows positive control of *cylA* gene. Lane 3 shows negative control and lane 5 shows DNA ladder: 100bp.

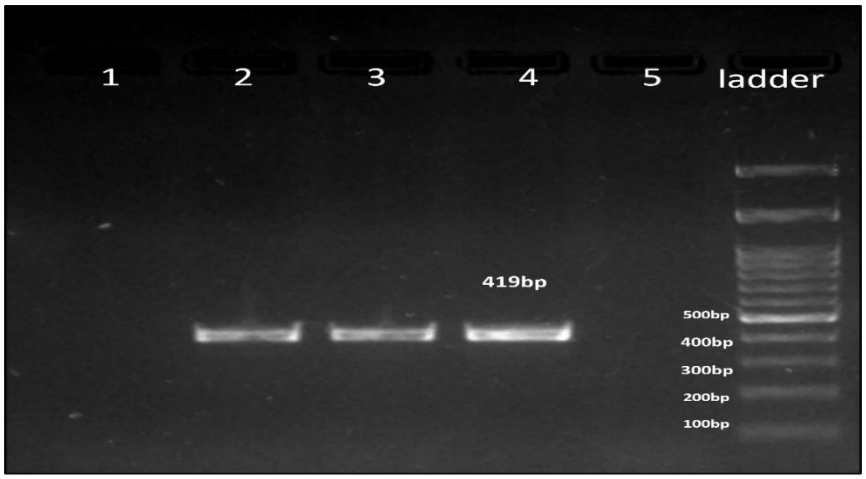


Figure 31: PCR amplified product of *gelE* gene. Total length of PCR product is 419bp, resolved on 1.5% agarose gel. Lane 4 showing positive control of *gelE* gene. Lane 5 shows negative control. DNA ladder: 100bp.

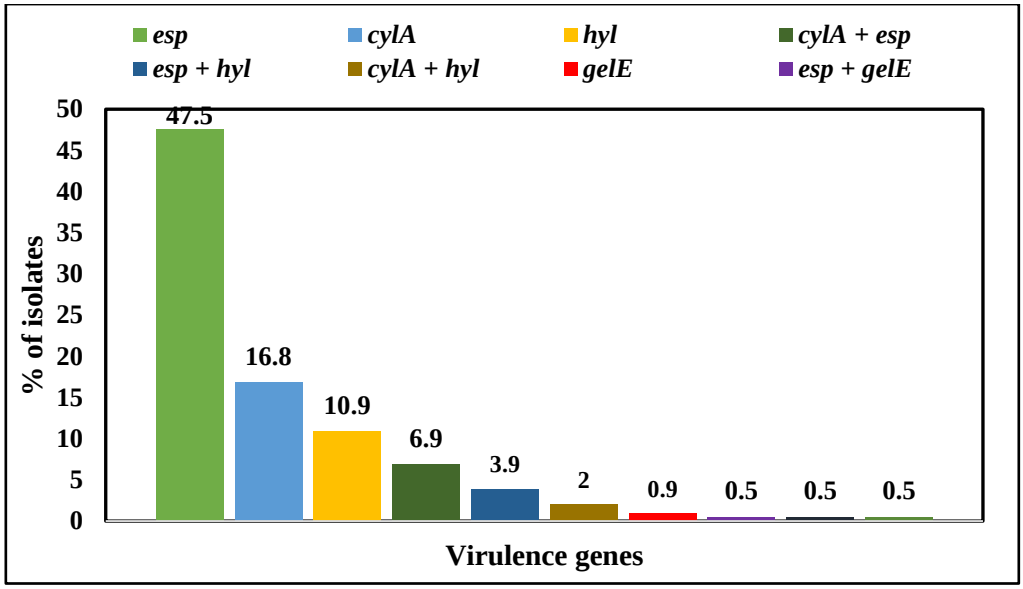


Figure 32: Distribution of virulence genes among clinical isolates of LREfm (N =202)

5.7.1 Association of *esp* gene with resistance mechanisms and other virulence genes (n=121) among clinical isolates

Majority of isolates with *esp* gene (n=121) were isolated from urine (47.9%, n=58) followed by blood (43%, n=52) and pus (9.1%, n=11). No significant association was observed between *esp* and specimen type. The presence of *esp* gene was significantly associated with the presence of *vanB* gene (p=0.026). The absence of *esp* gene was significantly associated with mutation (G2576T and C2610T) (p=0.005), *hyl* (p= <0.001) and *cylA* gene (p= <0.001), details are summarised in table; 29.

Table 29: Association of *esp* gene with clinical specimens, resistance mechanisms and other virulence genes (n=121) among clinical LREfm isolates.

Parameters	Virulence gene: *esp*		p value
	Positive n = 121(%)	**Negative n = 81(%)**	
Specimen Type			0.977[2]
Blood	52 (43.0)	36 (44.4)	
Pus	11 (9.1)	7 (8.6)	
Urine	58 (47.9)	38 (46.9)	
vanA	45 (37.2)	40 (49.4)	0.085[2]
vanB ***	61 (50.4)	28 (34.6)	**0.026[2]**

G2576T and C2610T* mutations**	22 (18.2)	29 (35.8)	**0.005[2]**
optrA	65 (53.7)	54 (66.7)	0.067[2]
hyl **	9 (7.4)	25 (30.9)	**<0.001[2]**
gelE	1 (0.8)	2 (2.5)	0.565[3]
asa1	1 (0.8)	0 (0.0)	1.000[3]
cylA ***	15 (12.4)	37 (45.7)	**<0.001[2]**

*****Significant at p<0.05, 1: Wilcoxon-Mann-Whitney U Test, 2: Chi-Squared Test, 3: Fisher's Exact Test**

5.7.2 Association of *cyl* gene (n=52) with clinical specimen, resistance mechanisms and other virulence genes among clinical isolates

Similar to *esp* gene, no significant association was observed between presence of *cylA* gene and specimen type. *CylA* virulence gene (n=52) was equally distributed among isolates from blood and urine 44.2% (n=23) followed by pus 11.5% (n=6). The presence of *cyAl* gene was significantly associated with isolates with mutation (G2576T and C2610T) (p=0.001). The absence of *cylA* gene was significantly associated with the absence of *esp* (p= <0.001) and *hyl* (p= <0.041) virulence genes and, details are summarised in table; 30.

Table 30: Association of *cylA* gene (n=52) with clinical specimen, resistance mechanisms and other virulence genes among clinical isolates.

Parameters	**Virulence gene: *cyl***		**p value**
	Positive n = 52(%)	**Negative n = 150(%)**	
Specimen Type			0.701[2]
Blood	23 (44.2)	65 (43.3)	
Pus	6 (11.5)	12 (8.0)	
Urine	23 (44.2)	73 (48.7)	
vanA	27 (51.9)	58 (38.7)	0.095[2]
vanB	20 (38.5)	69 (46.0)	0.345[2]
G2576T and C2610T*mutations**	22 (42.3)	29 (19.3)	**0.001[2]**

Parameters	Virulence gene: *cyl*		p value
	Positive n = 52(%)	Negative n = 150(%)	
optrA	34 (65.4)	85 (56.7)	0.271[2]
esp***	15 (28.8)	106 (70.7)	**<0.001[2]**
hyl***	4 (7.7)	30 (20.0)	**0.041[2]**
gelE	0 (0.0)	3 (2.0)	0.571[3]
asa1	0 (0.0)	1 (0.7)	1.000[3]

*****Significant at p<0.05, 1: Wilcoxon-Mann-Whitney U Test, 2: Chi-Squared Test, 3: Fisher's Exact Test.**

5.7.3 Association of *hyl* gene (n=34) with clinical specimens, resistance mechanisms and other virulence genes among clinical isolates

Majority of *hyl* gene positive isolates (n=34) were from blood (55.9%, n=19) followed by urine (38.2%, n=13) and pus (5.9%, n=2). No significant association was observed between presence of *hyl* gene and specimen type, other virulence genes and resistance mechanism. It was observed that absence of *hyl* gene were significantly associated with the absence of *esp* (p= <0.001) and *cylA* gene (p= <0.041), details are summarised in table; 31.

Table 31: Association of *hyl* gene (n=34) with resistance mechanisms and other virulence genes among clinical isolates (N=202).

Parameters	*hyl* gene		p value
	Positive n = 34(%)	Negative n = 168(%)	
Specimen Type			0.274[2]
Blood	19 (55.9)	69 (41.1)	
Pus	2 (5.9)	16 (9.5)	
Urine	13 (38.2)	83 (49.4)	
vanA	15 (44.1)	70 (41.7)	0.792[2]
vanB	16 (47.1)	73 (43.5)	0.699[2]
G2576T and C2610T mutations	8 (23.5)	43 (25.6)	0.800[2]

Parameters	*hyl* gene		p value
	Positive n = 34(%)	**Negative n = 168(%)**	
optrA	20 (58.8)	99 (58.9)	0.991[2]
esp* **	9 (26.5)	112 (66.7)	**<0.001[2]**
gelE	0 (0.0)	3 (1.8)	1.000[3]
asa1	0 (0.0)	1 (0.6)	1.000[3]
cylA* **	4 (11.8)	48 (28.6)	**0.041[2]**

*****Significant at p<0.05, 1: Wilcoxon-Mann-Whitney U Test, 2: Chi-Squared Test, 3: Fisher's Exact Test**

5.7.4 Association of *gelE* and *asa1* gene with resistance mechanisms and other virulence genes among clinical isolates

As the number of isolates with virulence genes *gelE* and *asa1* were limited to three and one respectively, statistical analysis was not performed.

5.8 Distribution of virulence genes among environmental LREfm (n=24) isolates

Among environmental isolates virulence genes were detected in 75% (n=18) isolates, *esp* was the predominant virulence gene and detected in 70.8% (n=17) isolates followed by *hyl* 25% (n=6), *cylA* 20.8% (n=5). Single virulence was detected in 75% (n=18) and among 5 isolates (20.8%) two genes were detected. The combinations of virulence genes detected are given in table 36. The virulence genes among environmental isolates were less diverse compared to clinical isolates [table; 36] [figure; 33]. All isolates were negative for *asa1* and *gel* genes details are given in table; 32.

Table 32: Distribution of virulence genes among environmental isolates of LREfm (N=24).

Genes	No. of isolates (%)
esp	15 (62.5)
cylA	2 (8.3)
hyl	1 (4.1)
hyl* + *cylA	5 (20.8)
esp* + *hyl	2 (8.3)

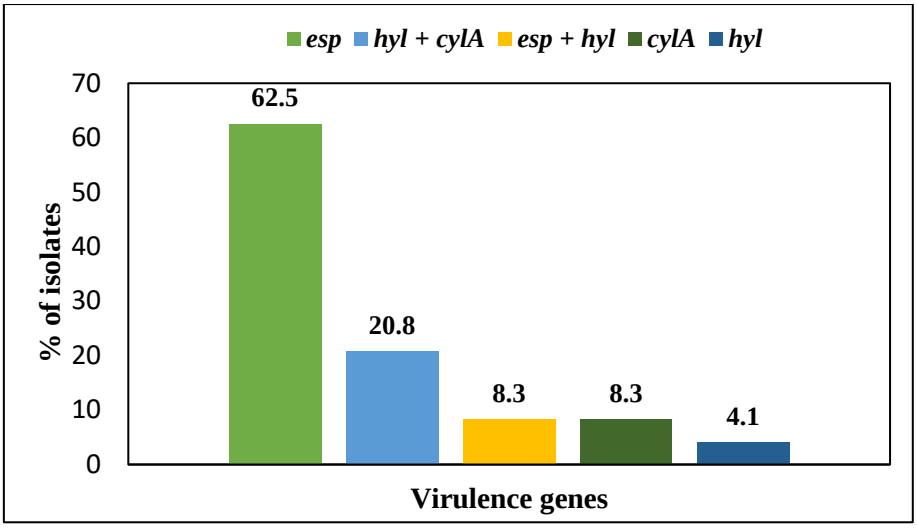


Figure 33: Distribution of virulence genes among environmental isolates of LREfm (N=24).

5.8.1 Association of *esp* gene (n=17) with resistance mechanisms and other virulence genes among environmental isolates

Among environmental isolates, it was observed that the absence of *esp* gene was significantly associated with absence of mutation (G2576T and C2610T) associated with resistance (p=0.021), virulence gene *hyl* (p= <0.038), and *cylA* gene (p= <0.001), details are summarised in table; 33.

Table 33: Association of *esp* gene (n=17) with resistance mechanisms and other virulence genes among environmental isolates.

Parameters	Virulence Gene: *esp*		p value
	Positive n = 17(%)	**Negative n = 7(%)**	
vanA	14 (82.4)	4 (57.1)	0.307[2]
vanB	0 (0.0)	1 (14.3)	0.292[2]
G2576T and C2610T mutations* **	3 (17.6)	5 (71.4)	**0.021[2]**
optrA	12 (70.6)	6 (85.7)	0.629[2]
hyl* **	2 (11.8)	4 (57.1)	**0.038[2]**
gel	0 (0.0)	0 (0.0)	1.000[1]
asa1	0 (0.0)	0 (0.0)	1.000[1]
cyl* **	0 (0.0)	5 (71.4)	**<0.001[2]**

****Significant at p<0.05, 1: Wilcoxon-Mann-Whitney U Test, 2: Chi-Squared Test.**

5.8.2 Association of *hyl* (n=6) gene with resistance mechanisms and other virulence genes among environmental isolates

Among environmental isolates absence of *hyl* gene significantly associated with the absence of *esp* (p= <0.038) gene. No significant association was observed in the presence or absence of *hyl* gene with resistance mechanisms (vancomycin and linezolid) and virulence genes *gel, asa1* and *cylA,* details are summarised in table; 34.

Table 34: Association of *hyl* (n=6) gene with resistance mechanisms and other virulence genes among environmental isolates.

Parameters	Virulence gene: *hyl*		p value
	Positive n = 6(%)	Negative n = 18(%)	
vanA	5 (83.3)	13 (72.2)	1.000[2]
vanB	1 (16.7)	0 (0.0)	0.250[2]
G2576T and C2610T	4 (66.7)	4 (22.2)	0.129[2]
optrA	5 (83.3)	13 (72.2)	1.000[2]
esp***	2 (33.3)	15 (83.3)	**0.038[2]**
gelE	0 (0.0)	0 (0.0)	1.000[1]
asa1	0 (0.0)	0 (0.0)	1.000[1]
cylA	3 (50.0)	2 (11.1)	0.078[2]

***Significant at p<0.05, 1: Wilcoxon-Mann-Whitney U Test, 2: Chi-Squared Test.**

5.8.3 Association of *cylA* gene (n=5) with resistance mechanisms and other virulence genes among environmental isolates.

Among environmental isolates the presence of *cylA* gene was significantly` associated with the linezolid resistant mechanism (G2576T and C2610T mutation). The absence of *cyl* gene was significantly associated with the absence of *esp* gene (p=<0.001), details are summarised in table; 35.

Table 35: Association of *cylA* gene (n=5) with resistance mechanisms and other virulence genes among environmental isolates.

Parameters	Virulence Gene: *cylA*		p value
	Positive n = 5(%)	Negative n = 19(%)	
vanA	3 (60.0)	15 (78.9)	0.568[2]
vanB	0 (0.0)	1 (5.3)	1.000[2]
G2576T and C2610T mutations* **	4 (80.0)	4 (21.1)	**0.028[2]**
optrA	5 (100.0)	13 (68.4)	0.280[2]
esp* **	0 (0.0)	17 (89.5)	**<0.001[2]**
hyl	3 (60.0)	3 (15.8)	0.078[2]
gelE	0 (0.0)	0 (0.0)	1.000[1]
asa1	0 (0.0)	0 (0.0)	1.000[1]

*****Significant at p<0.05, 1: Wilcoxon-Mann-Whitney U Test, 2: Chi-Squared Test.**

5.9 Association of virulence genes among clinical (n=202) and environmental (n=24) LREfm isolates: There was no significant association of various virulence genes was observed between clinical and environmental isolates of LREfm, except for combination of *cylA* and *hyl* which was significantly high in environmental isolates (p=0.00001). Details are summarised in table; 36.

Table 36: Association of virulence genes among clinical (n=202) and environmental (n=24) LREfm isolates

Virulence gene	Clinical isolates N (%)	Environmental isolates N (%)	P value
esp	96(47.5)	15(62.5)	0.08
cylA	34(16.8)	2(8.3)	0.14
hyl	22(10.9)	1(4.1)	0.15
gelE	2(0.9)	0 (0)	0.3
esp + hyl	8(3.9)	2(8.3)	0.16
cylA + hyl	4(2)	5(20.8)	**0.00001**
esp + gelE	1(0.5)	0 (0)	0.36
cylA + esp	14(6.9)	0 (0)	0.09
esp + asa1	1(0.5)	0 (0)	0.36
hyl + esp + cylA	1(0.5)	0 (0)	0.36

*****Significant at p<0.05**

Objective 5. To study the molecular epidemiology of Linezolid resistant *Enterococcus faecium* by PFGE and MLST.

5.10 Molecular epidemiology by PFGE

After the digestion of genomic DNA with *smaI* enzyme (figure; 34), the molecular epidemiology of clinical (n=202) and environmental (n=24) LREfm was studied by PFGE. Dendrogram of PFGE profiles of LREfm isolates (n=226) was constructed [Figure; 35] and a total of 20 clusters of related isolates (A1-A20) were observed. Further analysis of PFGE data revealed that majority of isolates were clustered in A-3 (n=11), A-4 (n=27), A-5 (n=15), A-6 (n=19), A-7 (n=10), A-8 (n=17), A-11 (n=16), A-12 (n=14), A-14 (n=19), A-16 (n=12), A-17 (n=15) and A-20 (n=12). Clinical and environmental isolates were distributed in 10 clusters (A-2, A-4, A-5, A-6, A-7, A-8, A-11, A-14, A-16, A-17). The clinical and environmental isolates of same patient coexisted in same clusters (A-5, A-6, A-17). LREfm isolates isolated from BSI only were clustered in cluster A-13, isolates from BSI and UTI were clustered in cluster A-1, A-9, A-15, A-19. Isolates from all 3 clinical presentation BSI, UTI and SSTI were clustered in cluster A-3, A-10, A12, A-14, A-18, A-20. Vancomycin resistance was detected in diverse background strains and observed in all clusters (A1-A20) with *vanA* and *vanB* gene. Isolates resistant to both linezolid and vancomycin were observed in all clusters; however, the majority of the vancomycin susceptible LREfm isolates were clustered in all clusters except A-2, A-10, A-15, A-18, A-19.

It was also observed that isolates with both resistance mechanisms (G2576T and C2610T mutation and *optrA* gene) were emerging in different clusters except cluster A-13, A-15, A-19. Isolates positive only for *optrA* gene were clustered in A-15 and A-19. Isolates were negative for both resistance mechanisms investigated in the study were also clustered in all clusters. Isolates with virulence gene *esp* were clustered in all clusters. Distribution of pulsotypes among LREfm isolates from BSI, SSTI, UTI and patient's environment is shown in table; 37.

Cluster A-1 (n=4) includes isolates from BSI (n=3) and UTI (n=1). Vancomycin resistance was detected in 50% (n=2) isolates with *vanA* (n=1) and *vanB* gene (n=1) and vancomycin susceptible isolates (n=2) were present in this cluster. Cluster A-1, includes 2 isolates with both resistance mechanisms mutations (G2576T and C2610T)

and *optrA* gene and 2 isolates with no resistance mechanism investigated in the study. This cluster also included isolates with virulence gene *esp* (n=2), *hyl* (n=1) and *cyl* (n=1).

Cluster A-2 (n=7) includes clinical isolates from BSI (n=1), UTI (n=5) and environment (n=1). All isolates were vancomycin resistant and included isolates with *vanA* (n=4) and *vanB* gene (n=3). This cluster includes 2 isolates with only mutations (G2576T and C2610T), 3 isolates with only *optrA* gene and 2 isolates with no resistance mechanism investigated in the study. This cluster also included isolates with virulence gene *esp* (n=6).

Cluster A-3 (n=11) includes clinical isolates from BSI (n=1), SSTI (n=1) and UTI (n=7). Vancomycin resistance was observed in 8 isolates with *vanA* (n=7) and *vanB* gene (n=1). This cluster includes 3 vancomycin susceptible isolates. The linezolid resistant isolates include 2 isolates with both resistance mechanisms (mutations and *optrA* gene), 5 isolates with only *optrA* gene and 4 isolates with no resistance mechanism investigated in the study. The virulence genes in this cluster include *esp* (n=7), *cyl* (n=4) and *hyl* (n=1).

Cluster A-4 (n=27) includes clinical isolates from BSI (n=7), UTI (n=19) and isolates from environment (n=1). Vancomycin resistance was observed in 88.9% (n=24) isolates with *vanA* (n=8) and *vanB* gene (n=16). This cluster also includes the vancomycin susceptible isolates (n=3). The linezolid resistant isolates include 1 isolate with mutations only, 6 isolates with both resistance mechanisms (mutations and *optrA*), 9 isolates with only *optrA* gene and 11 isolates with no resistance mechanism investigated in the study. This cluster includes virulence genes *esp* (n=18), *hyl* (n=3), *cyl* (n=6) and *gel* (n=1).

Cluster A-5 (n=15) includes isolates only from BSI (n=7) and patients' environment (n=8). Highest number of patients environment isolates belongs to this cluster. Vancomycin resistance was observed in 93.3% (n=14) isolates with *vanA* (n=13) and *vanB* gene (n=1) and 6.7% (n=1) isolates were vancomycin susceptible. This cluster includes 6 isolates with both resistance mechanisms (mutations and *optrA* gene), 7 isolates with only *optrA* gene and 2 isolates with no resistance mechanism investigated in the study. The virulence genes include *esp* (n=3), *hyl* (n=2) and *cyl* (n=3).

Cluster A-6 (n=19) includes isolates from BSI (n=10), SSTI (n=2), UTI (n=3) and environment (n=4). Second highest number of patients environmental isolates belongs to this cluster. Four isolates were vancomycin susceptible and vancomycin resistance was observed in 78.9% (n=15) isolates with *vanA* (n=9) and *vanB* gene (n=6). This cluster includes 5 isolates with both resistance mechanisms (mutations and *optrA* gene), 6 isolates with only *optrA* gene and 8 isolates with no resistance mechanism investigated in the study. The virulence genes include *esp* (n=7), *hyl* (n=3), *cyl* (n=3) and *gel* (n=1).

Cluster A-7 (n=10) includes isolates from BSI (n=4), UTI (n=4) and environment (n=2). One isolate was vancomycin susceptible and vancomycin resistance was detected in 90% (n=9) isolates with *vanA* (n=7) and *vanB* gene (n=2). This cluster includes 1 isolate with both resistance mechanisms (mutations and *optrA* gene), 6 isolates with only *optrA* gene and 3 isolates with no resistance mechanism investigated in the study. The virulence genes include *esp* (n=5), *hyl* (n=2) and *cyl* (n=1).

Cluster A-8 (n=17) includes isolates from BSI (n=7), UTI (n=9) and environment (n=1). Two isolates were vancomycin susceptible and vancomycin resistance was detected in 88.2% (n=15) isolates with *vanA* (n=6) and *vanB* gene (n=9). This cluster includes 4 isolates with both resistance mechanisms (mutations and *optrA* gene), 5 isolates with only *optrA* gene and 8 isolates with no resistance mechanism investigated in the study. The virulence genes include *esp* (n=8), *hyl* (n=4) and *cyl* (n=3).

Cluster A-9 (n=7) includes isolates from BSI (n=5) and UTI (n=2). Vancomycin resistance was detected in 71.4%% (n=5) isolates with *vanA* (n=2) and *vanB* gene (n=3). In this cluster two isolates were vancomycin susceptible. This cluster includes 1 isolate with both resistance mechanisms (mutations and *optrA* gene), 3 isolates with only *optrA* gene and 3 isolates with no resistance mechanism investigated in the study. The virulence genes include *esp* (n=3), *hyl* (n=3) and *cyl* (n=2).

Cluster A-10 (n=6) includes isolates from BSI (n=2), SSTI (n=2) and UTI (n=2). All isolates were resistant to vancomycin with *vanA* (n=1) and *vanB* gene (n=5). This cluster includes 1 isolate with mutations only, 3 isolates with both resistance mechanisms (mutations and *optrA* gene) and 2 isolates with no resistance mechanism investigated in the study. The virulence gene includes *esp* (n=4) and *cyl* (n=2).

Cluster A-11 (n=16) includes isolates from BSI (n=4), SSTI (n=3), UTI (n=8) and environment (n=1). Vancomycin resistance was detected in 81.2% (n=13) isolates with *vanA* (n=8) and *vanB* gene (n=5). Three isolates were vancomycin susceptible. This cluster includes 2 isolates with both resistance mechanisms (mutations and *optrA* gene), 6 isolates with only *optrA* gene and 8 isolates with no resistance mechanism investigated in the study. The virulence gene includes *esp* (n=10), *hyl* (n=4) and *cyl* (n=2).

Cluster A-12 (n=14) includes isolates from BSI (n=7), SSTI (n=2) and UTI (n=5). Vancomycin resistance was detected in 85.7% (n=12) isolates with *vanA* (n=8) and *vanB* gene (n=4). In this cluster two isolates were vancomycin susceptible. This cluster includes 2 isolates with both resistance mechanisms (mutations and *optrA* gene), 8 isolates with only *optrA* gene and 4 isolates with no resistance mechanism investigated in the study. The virulence genes include *esp* (n=7), *cyl* (n=5), *hyl* (n=2) and *asa1* (n=1).

Cluster A-13 (n=3) includes isolates from only from BSI (n=3). Vancomycin resistance was detected in only one isolate with *vanA* and two isolates were vancomycin susceptible. In this cluster no linezolid resistance mechanism was observed. The virulence genes include only *esp* (n=1).

Cluster A-14 (n=19) includes isolates from BSI (n=5), SSTI (n=3), UTI (n=10) and environment (n=1, 5.3%). Vancomycin resistance was detected in 94.7% (n=18) isolates with *vanA* (n=7) and *vanB* gene (n=18). This cluster includes 6 isolates with both resistance mechanisms (mutations and *optrA* gene), 8 isolates with only *optrA* gene and 5 isolates with no resistance mechanism investigated in the study. The virulence genes include *esp* (n=11), *cyl* (n=3) and *hyl* (n=2).

Cluster A-15 (n=3) includes isolates from BSI (n=3) and UTI (n=1). All 3 isolates were resistant to vancomycin with *vanB* gene (n=3). This cluster includes the linezolid resistant isolates with *optrA* gene alone (n=3). The virulence genes include *esp* (n=2), *hyl* (n=2) and *cyl* (n=1).

Cluster A-16 (n=12) includes isolates from BSI (n=2), UTI (n=8) and environment (n=2). Vancomycin resistance was detected in 91.7% (n=11) isolates with *vanA* (n=7) and *vanB* gene (n=4). This cluster includes 1 isolate with mutations alone, 1 isolate with both resistance mechanisms (mutations and *optrA* gene), 5 isolates with only *optrA* gene

and 5 isolates with no resistance mechanism investigated in the study. The virulence genes include *esp* (n=6) and *cyl* (n=5).

Cluster A-17 (n=15) includes isolates from BSI (n=6), SSTI (n=2), UTI (n=4) and environment (n=3). Vancomycin resistance was detected in 86.7% (n=13) isolates with *vanA* (n=7) and *vanB* gene (n=5). Two isolates were vancomycin susceptible. This cluster includes 2 isolates with mutation only, 2 isolates with both resistance mechanisms (mutations and *optrA* gene), 7 isolates with only *optrA* gene and 4 isolates with no resistance mechanism investigated in the study. The virulence genes include *esp* (n=7), *hyl* (n=3) and *cyl* (n=3).

Cluster A-18 (n=7) includes isolates from BSI (n=2), SSTI (n=2) and UTI (n=3). In this cluster all vancomycin resistant isolates with *vanA* (n=1) and *vanB* gene (n=6). This cluster includes 2 isolates with mutations alone, 4 isolates with *optrA* gene alone and only1 isolates with no resistance mechanism investigated in the study. Often carried virulence gene *esp* (n=6).

Cluster A-19 (n=2) carried even number of isolates from BSI and UTI, both isolates were vancomycin resistant and only isolate was with *vanA* (n=1). This cluster includes the linezolid resistant isolates with *optrA* gene (n=2) only and only one isolate carried the virulence gene *cyl*.

Cluster A-20 (n=12) includes isolates from BSI (n=7), SSTI (n=1) and UTI (n=4). Vancomycin resistance was detected in 91.7% (n=11) isolates with *vanA* (n=5) and *vanB* gene (n=6). Only one isolate was vancomycin susceptible. This cluster includes 4 isolates with both resistance mechanisms (mutations and *optrA* gene), 3 isolates with only *optrA* gene and 5 isolates with no resistance mechanism investigated in the study. The virulence gene includes *esp* (n=9), *cyl* (n=5) and *hyl* (n=1).

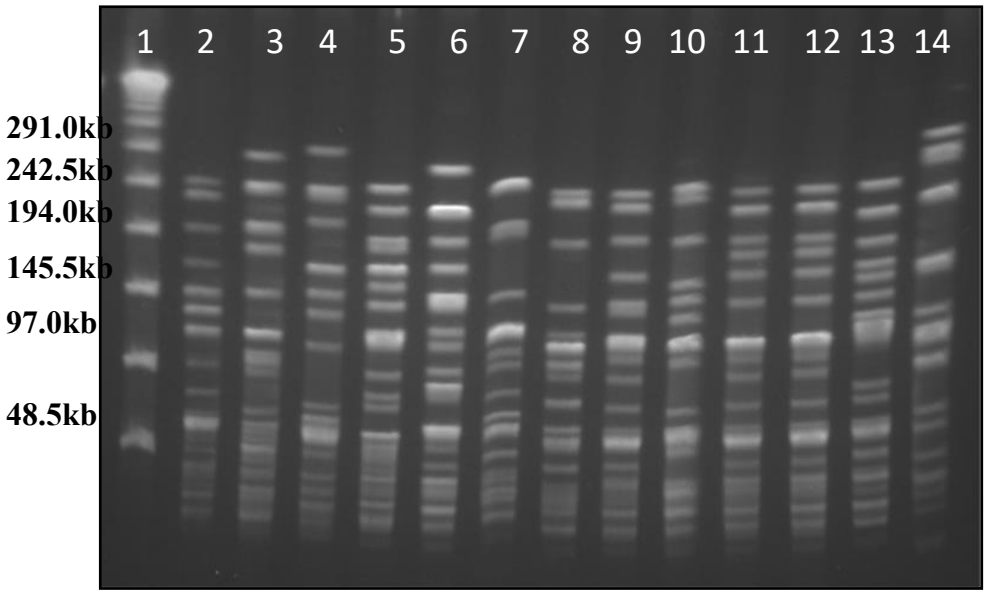
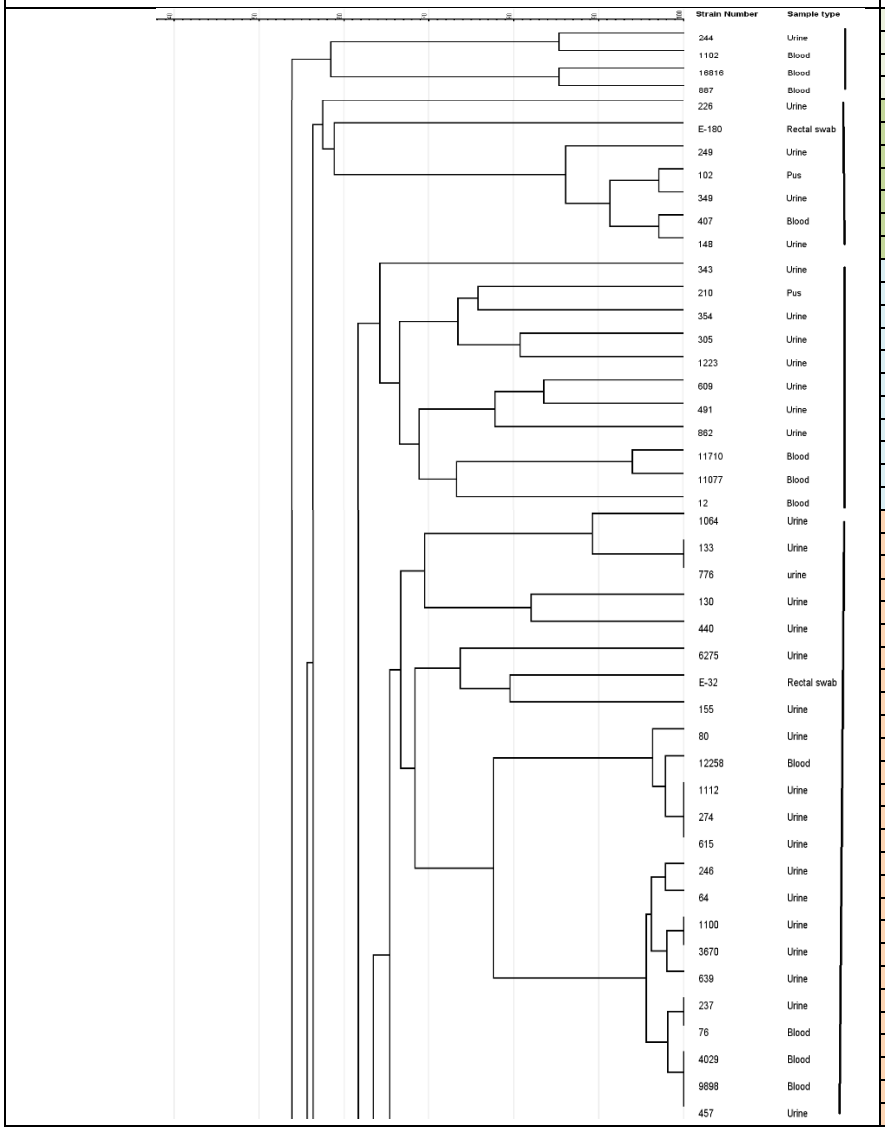


Figure 34: PFGE image of linezolid resistant *E. faecium*. Lane 1 shows ladder, lane 2-14 show linezolid resistant *E. faecium* strain which were digested by SmaI restriction enzyme.

Table 37: The characteristics of 20 Pulsotypes of 226 LREfm isolates based on site of isolation, vancomycin and linezolid resistance mechanisms and virulence genes.

Pulsotypes (n=226)	Blood n=88 (%)	Pus n=18(%)	Urine (n=96) (%)	Env (n=24) (%)	VRE(n=196) (%)	VSE (n=30) (%)	*vanA* (85C +18E) (%)	*vanB* (89C+1E) (%)	G2576T & C2610T (%)	*optrA* n (%)	*esp* n (%)	*hyl* n (%)	*cyl* n (%)	*gel* n (%)	*asa1* n (%)	STs
A-1 (n=4)	3 (75)	0 (0)	1 (25)	0 (0)	2 (50)	2 (50)	1 (25)	1 (25)	2 (50)	2 (50)	2 (50)	1 (25)	1 (25)	0 (0)	0 (0)	ST 80
A-2 (n=7)	1 (14.3)	0 (0)	5 (71.4)	1 (14.3)	7 (100)	0 (0)	4 (57.1)	3 (42.9)	2 (28.6)	3 (42.9)	6 (85.7)	0 (0)	0 (0)	0 (0)	0 (0)	ST 872
A-3 (n=11)	3 (27.2)	1 (9.1)	7 (63.6)	0 (0)	8 (72.7)	3 (27.2)	7 (63.6)	1 (9.1)	2 (18.2)	7 (63.6)	7 (63.6)	1 (9.1)	4 (36.3)	0 (0)	0 (0)	ST 80
A-4 (n=27)	7 (25.9)	0 (0)	19 (70.4)	1 (3.7)	24 (88.9)	3 (11.1)	8 (29.6)	16 (59.3)	8 (29.6)	17 (62.9)	18 (66.7)	3 (11.1)	6 (22.2)	1 (3.7)	0 (0)	ST 80
A-5 (n=15)	7 (46.7)	0 (0)	0 (0)	8 (53.3)	14 (93.3)	1 (6.6)	13 (86.7)	1 (6.6)	6 (40)	13 (86.7)	3 (20)	2 (13.3)	3 (20)	0 (0)	0 (0)	ST 80
A-6 (n=19)	10 (52.6)	2 (10.5)	3 (15.8)	4 (21.1)	15 (78.9)	4 (21.1)	9 (47.3)	6 (31.6)	5 (26.3)	11 (57.9)	7 (36.8)	3 (15.8)	3 (15.8)	1 (5.2)	0 (0)	ST 872
A-7 (n=10)	4 (40)	0 (0)	4 (40)	2 (20)	9 (90)	1 (10)	7 (70)	2 (20)	1 (10)	6 (60)	5 (50)	2 (20)	1 (10)	0 (0)	0 (0)	ST 375
A-8 (n=17)	7 (41.2)	0 (0)	9 (52.9)	1 (5.8)	15 (88.2)	2 (11.7)	6 (35.2)	9 (52.9)	4 (23.5)	9 (52.9)	8 (47.1)	4 (23.5)	3 (17.6)	0 (0)	0 (0)	ST 80
A-9 (n=7)	5 (71.4)	0 (0)	2 (28.6)	0 (0)	5 (71.4)	2 (28.6)	2 (28.6)	3 (42.8)	1 (14.3)	4 (57.1)	3 (42.8)	3 (42.8)	2 (28.6)	0 (0)	0 (0)	ST 761
A-10 (n=6)	2 (33.3)	2 (33.3)	2 (33.3)	0 (0)	6 (100)	0 (0)	1 (16.6)	5 (83.3)	4 (66.6)	3 (50)	4 (66.6)	0 (0)	2 (33.3)	0 (0)	0 (0)	ST 761
A-11 (n=16)	4 (25)	3 (18.6)	8 (50)	1 (6.2)	13 (81.2)	3 (18.6)	8 (50)	5 (31.2)	2 (12.5)	10 (62.5)	10 (62.5)	4 (25)	2 (12.5)	0 (0)	0 (0)	ST 80
A-12 (n=14)	7 (50)	2 (14.3)	5 (35.7)	0 (0)	12 (85.7)	2 (14.2)	8 (57.1)	4 (28.6)	2 (14.3)	9 (64.3)	7 (50)	2 (14.3)	5 (35.7)	0 (0)	1 (7.1)	ST 80

Pulsotypes (n=226)	Blood n=88 (%)	Pus n=18(%)	Urine (n=96) (%)	Env (n=24) (%)	VRE(n=196) (%)	VSE (n=30) (%)	*vanA* (85C +18E) (%)	*vanB* (89C+1E) (%)	G2576T & C2610T (%)	*optrA* n (%)	*esp* n (%)	*hyl* n (%)	*cyl* n (%)	*gel* n (%)	*asa1* n (%)	STs
A-13 (n=3)	3 (100)	0 (0)	0 (0)	0 (0)	1 (33.3)	2 (66.7)	1 (33.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (33.3)	0 (0)	0 (0)	ST 1070
A-14 (n=19)	5 (26.3)	3 (15.9)	10 (52.6)	1 (5.3)	18 (94.7)	1 (5.3)	7 (36.8)	11 (57.9)	6 (31.6)	13 (68.4)	11 (57.9)	2 (10.5)	3 (15.9)	0 (0)	0 (0)	ST 80
A-15 (n=3)	2 (66.7)	0 (0)	1 (33.3)	0 (0)	3 (100)	0 (0)	0 (0)	3 (100)	0 (0)	3 (100)	2 (66.6)	2 (66.6)	1 (33.3)	0 (0)	0 (0)	ST 761
A-16 (n=12)	2 (16.7)	0 (0)	8 (66.7)	2 (16.7)	11 (91.7)	1 (8.3)	7 (58.3)	4 (33.3)	4 (33.3)	6 (50)	6 (50)	0 (0)	5 (41.7)	0 (0)	0 (0)	ST 409
A-17 (n=15)	6 (40)	2 (13.3)	4 (26.7)	3 (20)	13 (86.7)	2 (13.3)	7 (46.7)	5 (33.3)	4 (26.7)	9 (60)	7 (46.7)	3 (20)	3 (20)	0 (0)	0 (0)	ST 80
A-18 (n=7)	2 (28.6)	2 (28.6)	3 (42.9)	0 (0)	7 (100)	0 (0)	1 (14.3)	6 (85.7)	2 (28.6)	4 (57.1)	6 (85.7)	1 (14.3)	1 (14.3)	0 (0)	0 (0)	ST 80
A-19 (n=2)	1 (50)	0 (0)	1 (50)	0 (0)	2 (100)	0 (0)	1 (50)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	1 (50)	0 (0)	0 (0)	ST 80
A-20 (n=12)	7 (58.3)	1 (8.3)	4 (33.3)	0 (0)	11 (91.7)	1 (8.3)	5 (41.7)	6 (50)	4 (33.3)	7 (58.3)	9 (75)	1 (8.3)	5 (41.7)	1 (8.3)	0 (0)	ST 80



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A-1	Neg	Neg	Pos	Pos	Pos	Neg	Pos	Neg	Neg	Neg
A-1	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg
A-2	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg
A-2	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg
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A-3	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg
A-3	Pos	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Pos
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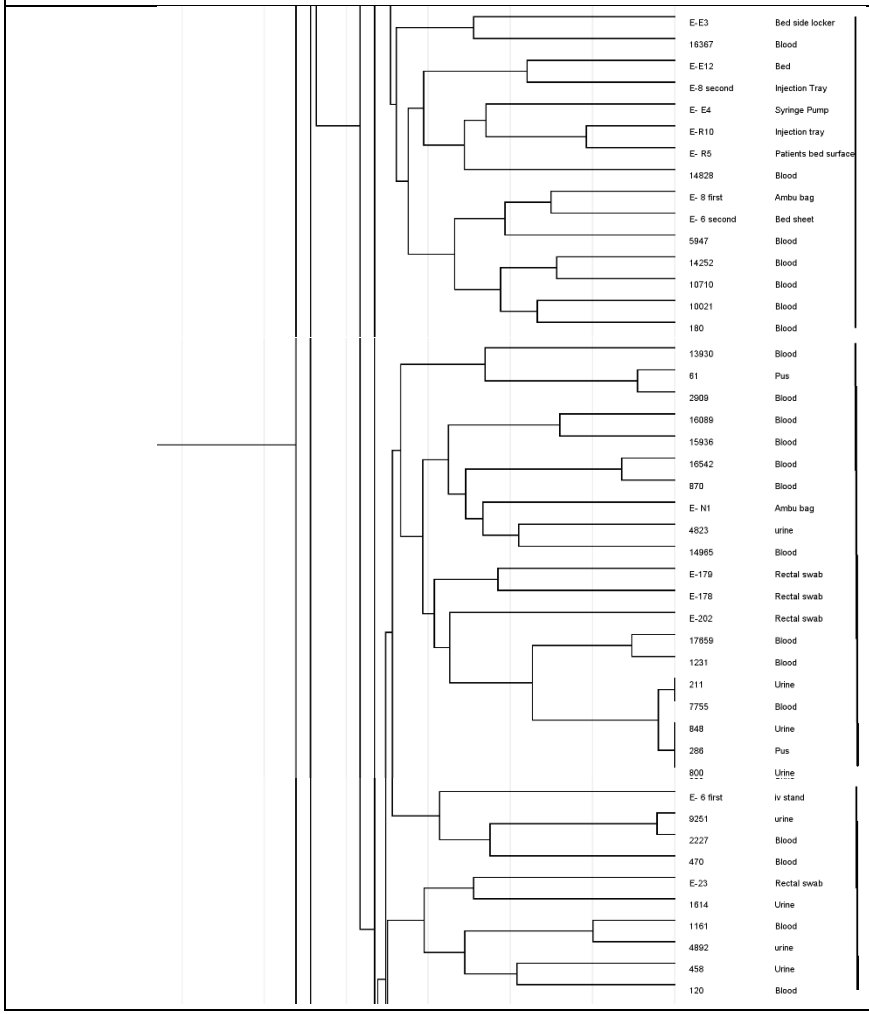

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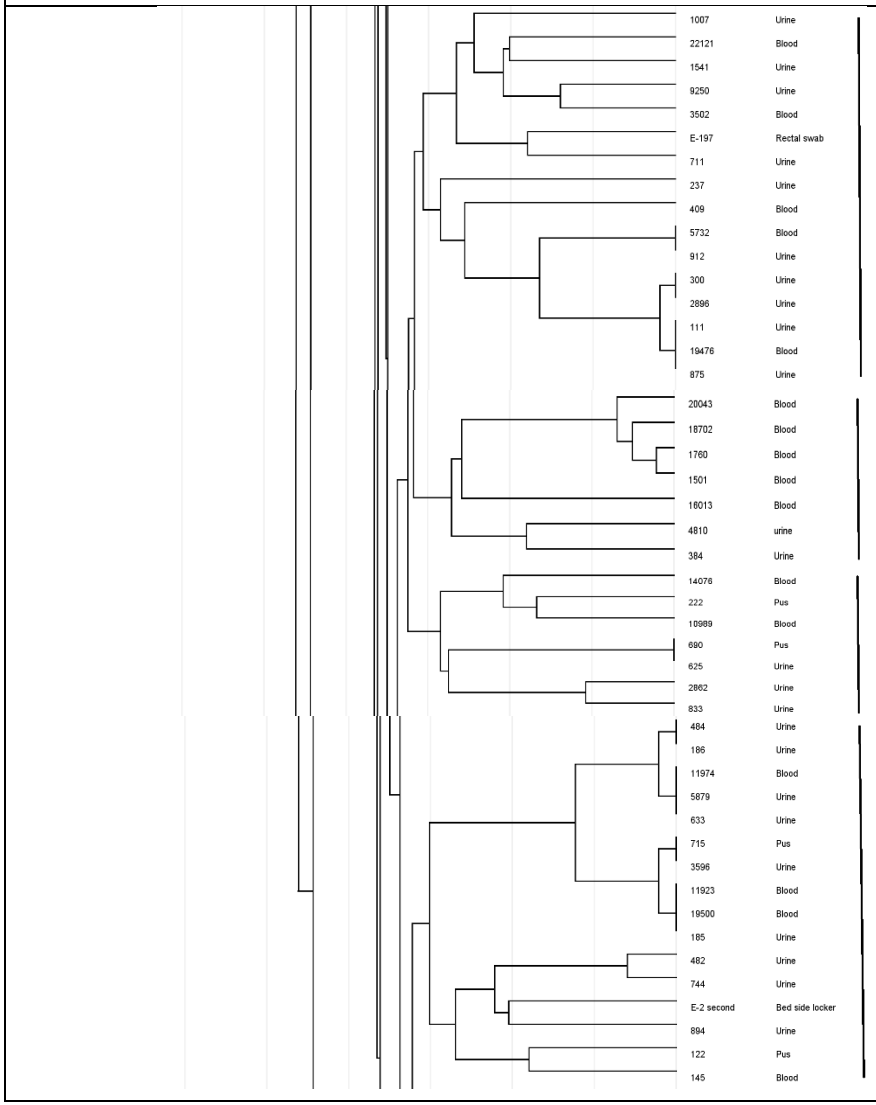

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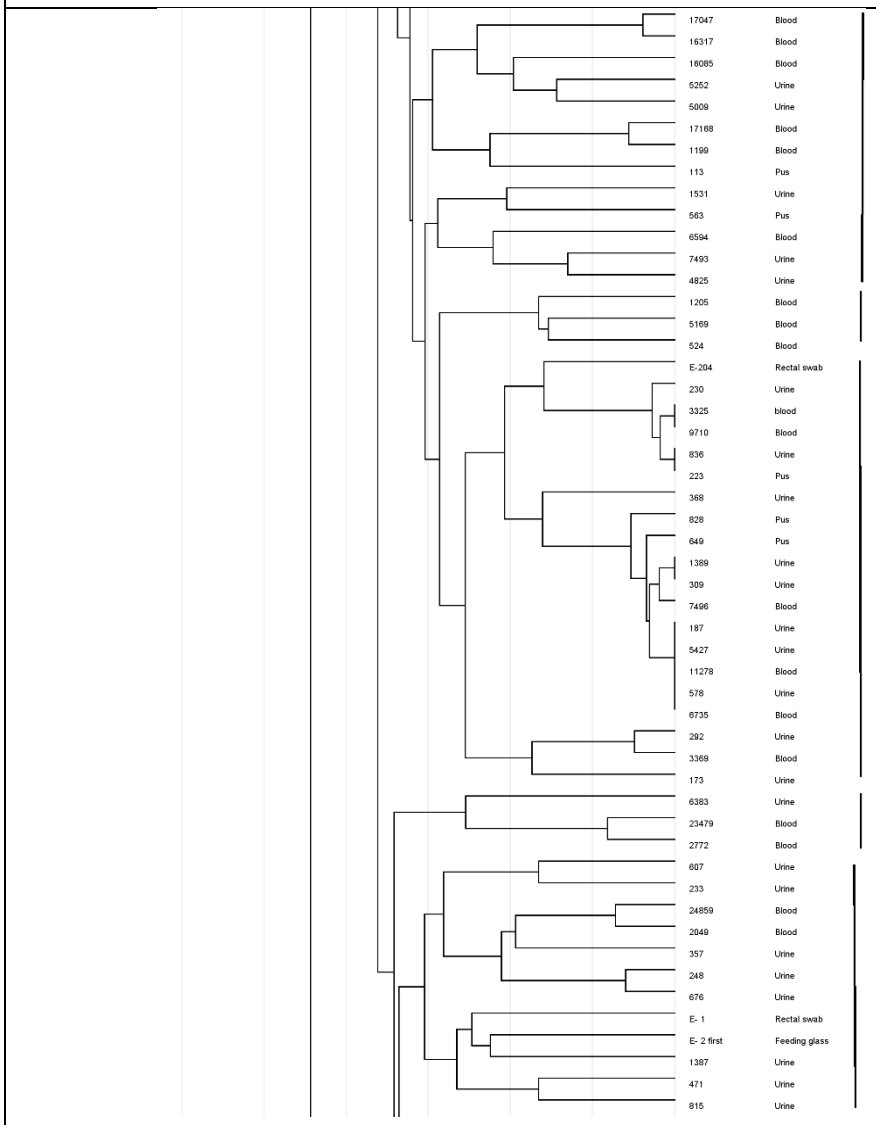

Pulso type	*vanA*	*vanB*	*G2576T*	*C2610T*	*optrA*	*esp*	*hyl*	*gel*	*asa1*	*cyl*
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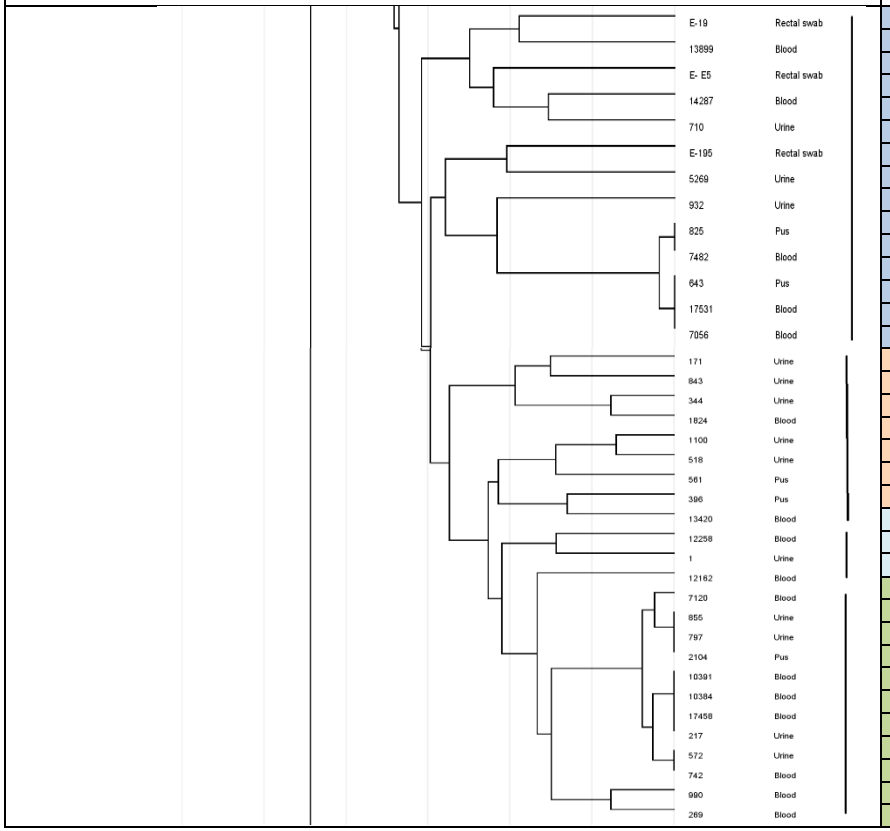

Pulso type	*vanA*	*vanB*	*G2576T*	*C2610T*	*optrA*	*esp*	*hyl*	*gel*	*asa1*	*cyl*
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A-20	Neg	Pos	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg

Figure 35: Dendrogram of PFGE profiles of LREfm isolates

5.11 Molecular epidemiology by MLST (Multi locus sequence typing)

MLST typing was performed for selected LREfm isolates on the basis of PFGE pattern. Amplification of seven housekeeping genes is given in figure; 36. Among the 20 clusters detected in PFGE, A total of 20 isolates were selected representative each PFGE clusters (A-1- A-20). Among 20 isolates, 14 were from patient's clinical samples and 6 were from patient's environmental samples. Overall, 6 different sequence types (STs) were characterized, Among the 6 sequence types ST80 was the most frequent (n=12, 60%) followed by ST761 (n=3) and ST872 (n=2). The ST1070 (n=1), ST409 (n=1), ST375 (n=1) were also detected. All 20 ST belongs to the CC17 (where CC stands for clonal complex).

ST 80: Among the 20 isolates, 60% (n=12) isolates belong to the ST80 including 9 clinical and 3 patient's environments. ST80 belong to the PFGE cluster A-1, A-3, A-4, A-5, A-8, A-11, A-12, A-14, A-17, A-18, A-19 and A-20.

ST872: Similar to ST80, In the present study ST872 belong to the both sources clinical and environmental. ST872 belongs to the cluster A-2 and A-6.

ST761 and ST1070: These STs belong to the clinical sources. ST761 belongs PFGE cluster A-9, A-10 and A-15, whereas ST1070 belongs to the cluster A-13.

ST375 and ST409: Even number of environmental isolates belong to the ST375 (n=1) and ST409 (n=1). ST 375 and ST409 belongs to cluster A-7 and A-16 respectively.

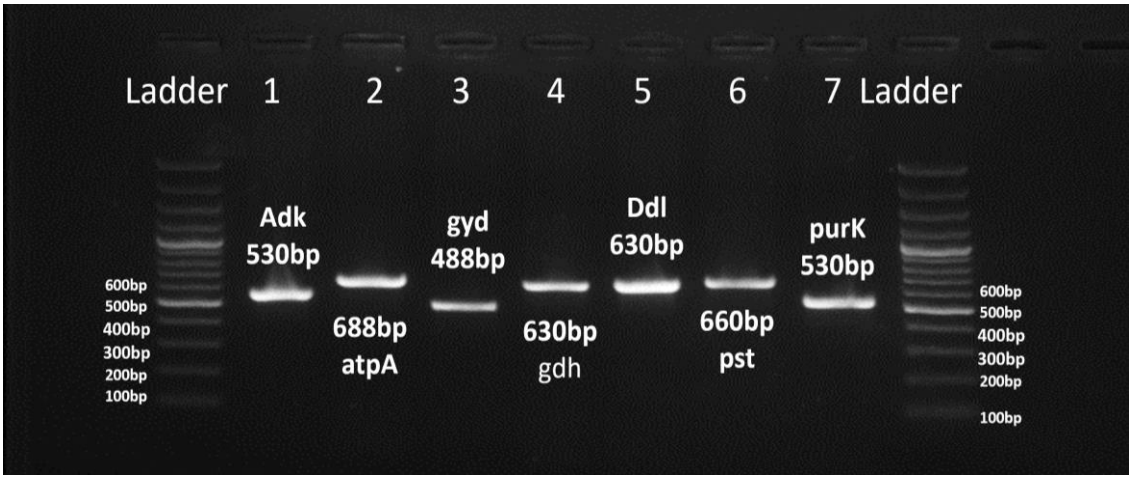

Figure 36: Amplification of seven housekeeping genes (adk, atpA, ddl, gdh, gyd, pst and purK).

Table 38: Allelic profile of clinical LREfm isolates

S. No.	*atp*	*Ddl*	*gdh*	*purK*	*Gyd*	*pstS*	*adk*	ST type	Clonal
1	70	1	1	1	12	1	1	761	CC17
2	9	1	1	1	12	1	1	80	CC17
3	70	1	1	1	12	8	1	761	CC17
4	70	1	1	1	12	1	1	761	CC17
5	9	1	1	1	12	1	1	80	CC17
6	9	1	1	1	12	1	1	80	CC17
7	9	1	1	1	12	1	1	80	CC17
8	9	1	1	1	12	1	1	80	CC17
9	15	1	1	1	12	1	3	1070	CC17
10	9	1	1	1	12	1	1	80	CC17
11	9	1	1	1	12	1	1	80	CC17
12	9	1	1	1	12	6	1	872	CC17
13	9	1	1	1	12	1	1	80	CC17
14	9	1	1	1	12	1	1	80	CC17

Table 39: Allelic profile of environmental LREfm isolates

S. no.	*atp*	*Ddl*	*Gdh*	*purK*	*Gyd*	*pstS*	*adk*	ST type	Clonal
1	9	1	1	1	12	1	1	80	CC17
2	7	1	1	1	12	1	3	409	CC17
3	9	3	1	1	12	1	1	375	CC17
4	9	1	1	1	12	1	1	80	CC17
5	9	1	1	1	12	1	1	80	CC17
6	9	1	1	1	12	6	1	872	CC17

6. Discussion

Enterococci are commensal flora of the gut and isolated from both human and animals. They can also colonize the female genital tract, oropharynx and the perineal region [Moellering *et al*., 2005]. They are gram-positive cocci and facultative anaerobes [Sava *et al*., 2010]. *Enterococci* are the 3rd most common cause of nosocomial infections after methicillin resistant *Staphylococcus aureus* (MRSA) and extended spectrum beta lactamase producing *Enterobacterales* (ESBL-E) [Marra *et al*., 2006].

The genus *Enterococcus*, includes 58 species [García-Solache *et al*., 2019; Zaheer *et al*., 2020]. *E. faecium* and *E. faecalis* are the most pathogenic species and are associated with serious infections like urinary tract infection (UTI), GIT infections, bacteremia, endocarditis, surgical site infections (burn and wound) and infection associated with indwelling catheters and other implanted medical devices [Levitus *et al*., 2023; Zaheer *et al*., 2020].

In the past, 80-90% of the clinically significant *Enterococcal* infections were reported to be caused by *E. faecalis* and 5-10% were associated with *E. faecium*. However, recently the proportion of infections caused by *E. faecium* have increased and exceeded the reported prevalence of *E. faecalis* [Ruoff *et al*., 1990; Moellering *et al*., 2005; Seedat *et al*., 2006].

As per the ICMR AMR surveillance report (2021), which included data from 20 regional centre and 4 tertiary care hospitals, a total of 95,728 culture positive isolates were studied. Among the culture positive isolates, 5.9% (n=5647) were *Enterococci* species. Among the *Enterococcus* species, *E. faecium* (42.89%) and *E. faecalis* (42.02%) were the predominant species. *E. faecium* was more frequently isolated from the CSF (4.6 %) followed by urine (4.2%), SSTI (4.4%) and blood (3.7%) while *E. faecalis* was more frequently isolated from urine (4.5%) followed by CSF (3.5%) and blood (2.5%).

Prevalence of *E. faecium*

In the present study, a total of 5069 isolates of *Enterococci* species were isolated from the clinically significant samples. The most frequently isolated species was *E. faecium* (n=3841, 75.7%) followed by *E. faecalis* (1204, 23.8%), *E. gallinarum* (n=14, 0.28%)

and *E. casseliflavus* (n=10, 0.19%). Our findings are similar to other studies from India. Telkar *et al*., from Karnataka, reported predominance of *E. faecium* (70%) among 50 isolates of *Enterococci* from BSI. Jain *et al*., from New Delhi (2011), also reported *E. faecium* (53%) as the predominant species among 110 isolates of *Enterococci* from BSI. Similarly, studies from Iran and Italy reported *E. faecium* as the predominant species followed by *E. faecalis* [Sattari-Maraji *et al*., 2019; Azza *et al*., 2013]. In contrast a study from Italy, investigated the species distribution among 3236 clinical isolates of *Enterococci*, *E. faecalis* (82.2%) was the predominant species followed by *E. faecium* (17.8%) [Boccella *et al*., 2021]. The report of ICMR AMR surveillance showed that *E. faecium* was most commonly isolated from the patients admitted to the ICU (3.1%) followed by non-ICU settings and out patients' units (OPD); whereas, *E. faecalis* was commonly isolated from the OPD (2.8%) followed by the non-ICU settings and the ICU. Similarly in our study majority of *E. faecium* were isolated from the patients admitted to the ICUs followed by non-ICU settings.

These studies suggest that the distribution of *E. faecium* and *E. faecalis* varies not only between types of clinical infections but also varies geographically and within the institution.

In India, sporadic cases of linezolid resistant *Enterococci* infections have been reported. However, there is paucity of studies to determine the prevalence, mechanisms of resistance and dissemination of linezolid resistance among *E. faecium* in India. The present study was undertaken to determine the prevalence of linezolid resistant *E. faecium*, characterize the various mechanism associated with linezolid resistance and the risk factors and clinical outcomes of patient's infected with linezolid-resistant *E. faecium*. The probable source of transmission of linezolid resistance with in the hospital were studied by molecular typing.

Prevalence of linezolid resistance

Recent data suggest that vancomycin-resistant *E. faecium (*VREfm) is widely distributed in hospitals around the world, with the prevalence varying according to geographical location [Levitus *et al*., 2023]. WHO has classified vancomycin resistant *E. faecium* (VREfm) as high priority pathogen and linezolid as a reserve antibiotic for the management of VRE isolates [Asokan *et al*., 2019].

As per national surveillance report by ICMR (2021), prevalence of vancomycin resistance was 14.9% among *Enterococci* (*E. faecalis* and *E. faecium*), however, the vancomycin resistance rate was 6 times higher in *E. faecium* (25.4%) compared to *E. faecalis* (3.8%).

The first report of linezolid-resistance was reported during a nosocomial outbreak of *E. faecium*. Isolates were resistant to both vancomycin and linezolid [Dobbs *et al*., 2006]. Reports of linezolid resistance from European countries show a low prevalence (1%) [Bender *et al*., 2018]**.** In India, the reports of linezolid resistance among *E. faecium* are scarce and are limited to case reports [Kumar *et al.,* 2014; Rai *et at*., 2015; Kumar *et al*., 2019**].** A study from India, investigated 961 *Enterococci*, among them 14% (n=136) were resistant to both vancomycin and linezolid, however in this study the identification of *Enterococci* to species level was not undertaken [S Jain *et al*., 2023]. To the best of our knowledge, this is first report from India on prevalence of linezolid resistance among clinical isolates of *E. faecium.* This study also includes 24 isolates of linezolid resistant *E. faecium* recovered from immediate patient's environment and to investigate the probable source of transmission**.**

In the present study vancomycin and linezolid resistance was observed only among *E. faecium* isolates (n=3841) and was detected in 720 (18.7%) and 202 (5.3%) respectively. Linezolid resistance was observed more frequently among VREfm (24.3%, 175/720) and was rare among vancomycin susceptible (0.9%, 27/3121) isolates and this difference was statistically significant (p=0.0001). In the present study majority of LREfm (56.66%) were isolated from UTI (47.5%), followed by BSI (43.6%) and (8.9%) from SSTI. However, LREfm isolated from BSI were predominantly from patients admitted to the ICUs and LREfm isolated from UTI were from non-ICU settings. This difference was statistically significant.

Present study demonstrated the high frequency of LREfm in patients with UTI in our hospital. The invasive devices, especially indwelling catheter might facilitate the development of LREfm UTI infection in hospital setting.

Risk factors associated with LREfm infections

Various studies have reported the risk factors for developing a nosocomial infection due to *Enterococci*. The risk factors include prolonged hospitalization, serious

underlying disease, prior surgery, renal insufficiency, immunosuppression, neutropenia, invasive devices and ICU stay [Moellering *et al*., 2005]. The epidemiology and risk factors for LREfm infections are scare, poorly defined and existing studies have not consistently linked prior linezolid use as a risk factor for infections with linezolid resistant *Enterococci*. The true incidence of emergence of resistance during therapy is not known as majority of the studies have not investigated isolation of linezolid susceptible isolates prior to therapy for comparison with linezolid resistance isolates [McGregor *et al*., 2013; Pai *et al*., 2002].

The present study describes the association of several risk factors with development of LREfm infections. After applying logistic regression model, use of carbapenems and linezolid were one of the most significant risk factors for infections caused by LREfm compared to LSEfm. Use of broad-spectrum antibiotics like carbapenems may facilitate infection and colonization with resistant *Enterococci* by depleting the commensal flora including gram negative bacteria and anaerobes. Similarly, Chen *et al*., (2018) and Gudiol *et al*., (2013) have also reported carbapenems use as risk factor for infection with linezolid resistant *E. faecalis* and *E. faecium* bacteremia.

In our study linezolid use was an independent risk factor for emergence of linezolid resistant *E. faecium.* Similarly, Krawczyk *et al*., (2020) have also reported linezolid use including prolonged use as an independent risk factor for infections with LREfm. Various studies have reported that the median days of linezolid exposure varied from of 9 days to 15days [Smith *et al*., 2018; Olearo *et al*., 2021]. In contrast study by Pogue *et al*., (2007) did not identify prior linezolid use as a significant risk factor for infections with linezolid resistant *Enterococci*.

Recent studies reported by Ramos *et al*., and Zaheer *et al*., have provided evidence that increased isolation of LREfm can be related to nosocomial transmission events. Smith *et al*., reported that the risk factors associated with the linezolid resistant *Enterococcal* infections are invasive procedures, neutropenia and immunosuppression, Whereas Krawczyk *et al*., reported that the length of hospital stay prior to infection with linezolid resistant *Enterococci*, use of urinary catheter, prior surgery, antibiotics during hospital stay (cephalosporin, meropenem, clindamycin, linezolid, amikacin and colistin) and comorbidity (HTN) are the important risk factors associated with linezolid

resistant *E. faecalis* infections. Similarly, in our study hospital stay (8-14 days) prior to isolation of LREfm, invasive devices like use of urinary catheter, central line and ventilation were also identified independent risk factors associated with LREfm infections. It is well known that indwelling urinary catheters, central line and ventilation along with increased hospital stay may facilitate dissemination of resistant bacteria including LREfm. Although linezolid use was also an independent risk factor, in the present study only 26.6% of the cases has received linezolid. Based on these data it may be inferred that spread of LREfm may be driven by two mechanisms: De-novo emergence of linezolid resistance during therapy and subsequent nosocomial spread facilitated by invasive procedures or devices and increased hospital stay due to breach in infection control practices. This is strongly supported by detection of LREfm in patients with no exposure to linezolid (73.4%).

In a recent study by Zou et al., the reported mortality rate in patients infected with linezolid resistant *E. faecalis* was relatively low (7.1%), which was attributed to small number of blood stream infections in the study group. In contrast the present study investigated infections associated with LREfm and mortality was high (31.7%) and cases presented with diverse clinical infections involving skin and soft tissue, abdominal, blood stream, CNS and urinary tract. The difference in mortality may also be attributed to differences in pathogen profile, as *E. faecium* is intrinsically more resistant to antibiotics and associated with nosocomial infections.

Antimicrobial susceptibility: A total of 226 LREfm which included 202 clinical and 24 environmental isolates were studied for antimicrobial susceptibility and molecular characterization.

Enterococci show Intrinsic resistance to various antimicrobial agents like aminoglycosides, β-lactams, cephalosporins and trimethoprim-sulfamethoxazole [Gales *et al*., 2009]. Although the most prevalent species associated with HAIs is *E. faecalis*, which is more virulent than *E. faecium* but with modest levels of intrinsic and acquired antimicrobial resistance. However, there is an increasing trend for infections caused by *E. faecium*, mostly associated with the rise of β-lactam and vancomycin resistance [Werner *et al*., 2008] [Jahansepas *et al*., 2018]**.** Infections caused by multidrug resistant *E. faecium* can be difficult to treat with limited therapeutic options. According to a National Healthcare Safety Network report, the majority of device-

associated infections (associated with central lines, urinary drainage catheters, and ventilators) were caused by MDR *E. faecium*, vancomycin- and ampicillin-resistance was 80% and 90.4% respectively [Hidron *et al*., 2008].

A recent study from Poland, investigated 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 [Krawczyk *et al*., 2020]. A study on 67 clinical isolates of *E. faecium* from Iran, reported resistance to quinupristin/dalfopristin among 70% (n=47) isolates [Nasaj *et al*., 2016]. In contrast a study from China reported resistance to quinupristin/dalfopristin in 1% isolates among 911 clinical isolates of *E. faecium* [Wang *et al*., 2016].

In the present study high resistance was observed to all antibiotics except daptomycin. More than 95% clinical and environmental isolates were resistant to ampicillin, ciprofloxacin and erythromycin. Resistance to high level gentamicin and quinupristin/dalfopristin was significantly high in clinical isolates, Whereas, resistance to chloramphenicol and teicoplanin was high in environmental isolates. Resistance to nitrofurantoin was observed in 69.8% of the urinary isolates (67/96). We can conclude that the variation in the antimicrobial susceptibility pattern of clinical and environmental isolates may be attributed to the less sample size of environmental isolates.

In an earlier study from Slovenia by Golob *et al*., 73.3% isolates of *E. faecium* were reported as MDR. In our study MDR and XDR was observed in 100% and 97.5% isolates respectively. Multidrug resistant (MDR) was defined as non-susceptible to ≥1 agent in ≥3 antimicrobial categories and extensively drug-resistant **(X**DR) was defined as non-susceptible to ≥1 agent in all but ≤2 categories [Magiorakos *et al*., 2012].

Linezolid resistance is emerging and increasingly being reported among VRE world-wide. Two programmes monitoring infections with linezolid resistant *Enterococci*: LEADER (in USA) and ZAAPS (Zyyox ®Annual Appraisal of Potency and Spectrum Programme: worldwide) have also reported the emerging problem of simultaneous resistance to vancomycin and linezolid [Krawczyk *et al*., 2020]. In the present study majority of the LREfm isolates were also vancomycin resistant (85%).

These findings suggest that increased use of linezolid for management of VRE may be driving emergence of linezolid resistance. In hospitals where VREfm and LREfm coexist, management may be challenging as therapeutic options are limited. The limited therapeutic options currently available for infections with LREfm include daptomycin and tigecycline. Various studies have shown limited success with these antibiotics [Greene *et al*., 2018; Shukla *et al*., 2016]. Although daptomycin possesses in- vitro bactericidal activity against VRE, observational data suggests that it is as effective or inferior to linezolid [Shukla *et al*., 2016].

A dedicated antimicrobial stewardship programme intended to regulate linezolid use is critical to curtail infections with LREfm. In a Spanish hospital a focused antimicrobial stewardship programme resulted in significant reduction of linezolid resistant *E. faecalis* [García-Martínez *et al*., 2016]. In addition, implementation of strict infection control policy is important to prevent the cross transmission of resistant bacteria including LREfm.

Phenotypic and genotypic glycopeptide resistance

In gram positive bacteria, resistance to glycopeptide may be intrinsic or acquired through mobile elements**.** In 1986 vancomycin resistant *Enterococci* (VRE) were first reported after the 30 years of clinical use of vancomycin in Europe [Uttley *et al*., 1988; Rice 2001]. Since then VRE have emerged as an important nosocomial pathogen [Gholizadeh Y and Patrice Courvalin, 2002]. *Enterococci* isolates have varied resistance to both vancomycin and teicoplanin [Gholizadeh Y and Patrice Courvalin, 2002]. In *Enterococci*, the main mechanism of glycopeptides resistance is modification of the peptidoglycan synthesis pathway, specifically the substitution of D-Ala-D-Ala for either D-Ala-D-Lac or D-Ala-D-Ser, these alterations may lead to variable expressions of glycopeptide resistance [Xu *et al*., 2010]. The *vanA* and *vanB* are most common. *vanA* phenotype results in high level inducible resistance to both vancomycin **(MIC: 64- 256 µg/mL)** and teicoplanin (**(MIC: 8-256 µg/mL**), while *vanB* shows variable levels of inducible resistance to vancomycin (**(MIC: 16-256 µg/mL**) and susceptibility to teicoplanin (**(MIC: 0.38-1.5 µg/mL**).

In the present study based on MICs of vancomycin and teicoplanin *vanA* phenotype was observed in 48.6%(n=85) and 85.7% (n=18) of clinical and environmental isolates respectively. While *vanB* phenotype was observed in

51.4%(n=90) and 14.3%(n=3) clinical and environmental isolates respectively. By genotypic characterization, among clinical isolates *vanA* and *vanB* genes were equally distributed (48.6% vs 50.9%). Whereas in environmental isolates *vanA* gene was predominantly detected in 85.7% (18/21) and *vanB* was detected only in 4.7% (1/21). The difference in distribution of *vanA* and *vanB* among clinical and environmental isolates may be attributed to the limited number of environmental isolates.

In the present study a very good corelation was observed between phenotypic and genotypic characterization. However, in one clinical and 2 environmental isolates with *vanB* phenotype (based on MIC) no investigated vancomycin resistance mechanism was detected [table; 24]. This needs further investigation to identify the other known and novel vancomycin resistance mechanisms. On the bases of phenotypic and genotypic characteristic a total of nine types of vancomycin resistance operon have been reported among *Enterococci* namely, *vanA, vanB, van C, vanD, vanE, van G, van L, van M*, and *van N* [Boyd *et al*., 2008; Lebreton *et al*., 2011; Xu et al., 2010]**.** As per literature survey only *vanD* genotype have reported in *E. faecium* isolates with vancomycin and teicoplanin MICs 64 μg/ml and 4 μg/ml respectively.

In our study vancomycin resistance was mediated by either *vanA* or *vanB* gene. These finding are supported by study from Saudi Arabia, where 15 VRE which include 14 VREfm and one *E. casseliflavus* were investigated. Among 14 VREfm, *vanA* and *vanB* gene were detected in 57.1% (n=8) and 42.8% (n=6) isolates respectively [Kankalil George *et al*., 2021]. Another supporting study from Iran investigated 43 VREfm, among the 43 isolates, *vanA* gene was detected in 55.8% (n=24) isolates and *vanB* was detected in 44.2%(n=19) isolates [Kafil & Asgharzadeh 2014].

In contrast study from Iraq, reported the predominance of *vanA* gene among vancomycin resistant *E. faecium* [Salem-Bekhit *et al*., 2012]. However, a study from Turkey by Gozalan *et al*., (2015) reported predominance of *vanA* gene among 55 VREfm isolates.

Mechanisms of Linezolid resistance

Alteration in domain V of 23S rRNA is associated with the resistance to Linezolid in gram positive organism like *CONS, S. aureus, E. faecalis* and *E. faecium*. The most common mutation in clinical isolates of *Staphylococcus* species and *Enterococcus*

species is G2576T [Hong *et al*., 2007; Saager *et al*., 2008; Prystowsky *et al*., 2001]. Other mutations reported in various studies include T2500A and G2447T in *S. aureus*, G2505A in *E. faecium*, C2534T in *S. epidermidis*, and G2513T, C2512T, G2614 T, C2384T and C2610G in *E. faecalis* [Kelly *et al*., 2008; Meka & Gold *et al*., 2004; Mittal *et al*., 2019].

Antimicrobial surveillance from LEADER and ZAAPS programs showed that the predominant mechanisms of linezolid resistance among *Enterococci* were mutations in the 23S rRNA and were detected in 83.9% (392/467) of the isolates [Bi *et al.*, 2018]. Similarly report from Germany also suggest that G2576T mutation in the domain V of the 23S rRNA was the most common resistance mechanism of linezolid resistance among *E. faecium* and was detected in 92.4% of the isolates [Klare *et al*., 2015]. Moreover, mutations in the particular region of *L3*, *L4* and *L22* ribosomal proteins, although are less commonly reported, have also been associated with linezolid resistance [Long *et al*., 2012].

Plasmid mediated linezolid resistance have also been reported such as *cfr* gene, which encodes methyltransferase that catalyses methylation of adenine at 2503 in the 23S rRNA gene of the large ribosomal subunit has been described [Marshall *et al*., 2002]. Acquisition of natural *cfr* gene confers resistance to florfenicol, chloramphenicol (phenicol) and clindamycin (lincosamide) has been described [Bender *et al*., 2018]. This mechanism of resistance was first reported in *E. faecalis* of animal origin. The wild type *cfr* (*Staphylococci* homolog) and its derivatives, known as *cfr*(B) and *cfr*(D) variants, which are encoded on plasmids or integrated within the chromosome, have been detected in Enterococcus spp. from both animal and human samples. Current studies suggest that the frequency of *E. faecium* isolates carrying the *cfr* gene are rising in both animal and human isolates [Fioriti *et al*., 2021; Guerin *et al*., 2020; Almeida *et al*., 2020].

Additional research on mechanism of resistance have shown linezolid resistance may emerge in absence of mutation and *cfr* gene. Acquisition of oxazolidinone and phenicol transferable resistance A (*optrA*) has emerged as another linezolid resistance determinant. The ABC-F family contains specified proteins which perform functions that confer resistance to a wide variety of clinically significant ribosome-targeting antibiotics. It has also been reported that these proteins use a shield

mechanism to protect ribosome [Sharkey *et al*., 2016]. *OptrA* gene was initially detected in *E. faecalis* of human origin, and subsequent studies have reported *optrA* gene among isolates of *E. faecium*, as well as most recently in *S. aureus, S. sciuri*, and *S. suis*. Surveillance studies from China suggest that *optrA* is more frequently reported among *Enterococci* from food-producing animals [Wang *et al*., 2015], but has now emerged in clinical isolates from humans too. Another report from China shows the high prevalence of *optrA* gene (46.6%) in clinical human isolates of *E. faecium* [Yi *et al*., 2022].

Interestingly in our study, all LREfm were negative for *cfr* gene. Among clinical and environmental LREfm isolates the main mechanism of linezolid resistance was *optrA* gene (58.9% vs 75%) followed by point mutation G2576T in the domain V of the 23S rRNA (25.2% vs 33.3%), in these isolates C2610T mutation also coexisted. Based on the resistance mechanism isolates were divided into four groups [Table; 28]. In our study clinical and environmental isolates with G2576T and C2610T mutation, had the higher MIC of linezolid (>256 µg/mL) irrespective of the presence or absence of *optrA* gene. Gram positive bacteria have multiple copies of 23S rRNA gene, *E. faecalis* has 4 copies, while *E. faecium* and *S. aureus* have 5–6 copies [Klappenbach *et al*., 2001]. Various studies have reported that linezolid MIC among *S. aureus* increased proportionally with number of copies in 23S rRNA gene with mutations, similarly in *E. faecium* isolates, an increase in the number of mutant copies of 23S rRNA, was shown to correlate with increasing MICs of linezolid [Arias *et al*., 2008; Ford *et al*., 2001; Saager *et al*., 2008]. As MIC of >256 µg/mL was detected in all isolates with G2576T and C2610T mutations irrespective of *optrA* gene it may be inferred the presence of two mutations or mutations in multiple copies may have contributed to the higher MIC observed in these isolates. This study has not studied the mutation in multiple copies of 23S rRNA, further investigations with WGS help to understand the role of novel mutations or mutations in multiple copies of 23Sr RNA. As all isolates with G2576T and C2610T demonstrate high MIC we did not investigate for number of mutated copies of the 23S rRNA gene.

The MIC of linezolid among clinical and environmental isolates, positive for *optrA* gene alone, varied from 8-128 µg/mL with geometric mean 24.4 µg/mL and 35.7 µg/mL respectively. A study from China has investigated the variation in MIC

associated with variants of *optrA* gene. Chai *et al*., (2019) identified the 19 variants of *optrA* gene by WGS and linezolid MIC of ≥8 µg/mL for isolates with *optrA* gene and its variants. As *optrA* gene is associated with the lower MIC of linezolid. However, the variation in MIC of 8-128 µg/mL among isolates with *optrA* gene alone suggest the role of other mechanisms of resistance which may be contributing to these variations in the MIC and need to be further investigation. Resistance mediated by *optrA* have been reported worldwide including China, Poland, Spain, USA, Ireland, Czech Republic. In India, as per ICMR surveillance report (2021), *optrA* gene reported in two cases of LREfm by WGS [Bakthavatchalam *et al*., 2021].

In the present study a significant number of LREfm, which included 74 (36.6%) clinical and 6 (25%) environmental isolates, no investigated linezolid resistance mechanism widely reported in literature and investigated in the study (Mutation in 23S rRNA, resistant gene *cfr* and *optrA* gene) was detected. This needs further investigation to identify the other known and novel resistance mechanisms. Our findings are supported by study performed by Lee Sae-Mi *et al*., (2017), The authors studied the linezolid resistance mechanisms including mutation in 23S rRNA, L3, L4, L22 ribosomal protein and presence of *cfr*, *cfrB* and *optrA* gene among 43 LRE (34 *E. faecium* and 9 *E. faecalis*). The *optrA* gene was detected in 3 *E. faecium* and 13 *E. faecalis*, whereas G2576T mutation was detected only in 10 LREfm isolates, only one isolate harboured the mutation in L22 ribosomal protein. No resistance mechanism was detected in 22.2% (2/9) of *E. faecalis* and 67.6% (23/34) *E. faecium* resistant to linezolid.

Linezolid resistance mechanisms vary with geographical regions. Studies from European countries have reported linezolid resistance is predominantly mediated by mutation in domain V of the 23S rRNA, however reports from China and other developing countries 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 [Wang *et al*., 2015], but now is emerging in clinical isolates too. Another report from China shows the high prevalence of *optrA* gene (46.6%) in clinical human isolates of *E. faecium* [Cai *et al*., 2019]. However, in our study although the predominant mechanism of linezolid resistance was

optrA gene, significant resistance was also mediated by point mutation G2576T and C2610T in the domain V of the 23S rRNA.

Based on our data it may be inferred that linezolid resistance is driven by two different mechanisms: (i) De-novo emergence of linezolid resistance during therapy and subsequent nosocomial spread facilitated by increased hospital stays and invasive procedures or devices due to breach in infection control practices. (ii) As the *optrA* gene is frequently associated with plasmids, it spread through rapid dissemination between species of bacteria. This is supported by isolation of *cfr* and *optrA* genes among various species of *CONS* (*S. sciuri* and *S. suis*) and *S. aureus* [Wang *et al*., 2015]. Hence implementations of infection control practices are important to prevent further spread of linezolid resistance in the hospital.

Virulence genes

Another objective of the study was to identify the virulence factors associated with clinical LREfm isolates. Virulence factors along with antibiotic resistance may have an impact on clinical outcomes. Various virulence factors contribute to the pathogenicity of the pathogen. As a result, infection associated with isolates containing virulence factors may be more severe than infections without such virulence factors. Virulence factors can be acquired through phages, plasmids, transposons, or pathogenicity islands. Virulence factors are mainly associated with the adherence of bacteria to the host cells, invasion, modulation of host immune r*esp*onses, and also induce toxicity [Krawczyk *et al*., 2020].

In the present study number of clinical and environmental LREfm isolates with virulence genes did not vary (76.2%% and 75% respectively). However, virulence genes detected in clinical isolates were more diverse as compared to environmental isolates (Table; 37). The *gelE* and *asaI* genes were not detected among environmental isolates. Among clinical and environmental LREfm isolates *esp* was the most common virulence gene (59.9% vs 70.8%) followed by *cyl* (25.7% vs 20.8%), *hyl* (16.8% vs 25%). A study conducted by Aung *et al*., (2023), investigated virulence genes among 54 *E. faecium* isolates and the *esp* gene (48.1%) was the predominant virulence gene followed by *hyl* gene (11.1%). In contrast Shokoohizadeh *et al*., (2018), investigated 21 *E. faecium* and reported that *asa1*(43%) was most common virulence gene followed by *gel* (33%), *esp* (19%) and *hyl* (4.7%).

Esp is a surface protein that affects biofilm formation ability of *E. faecium*. Studies in animal models have reported that surface proteins (*esp*) contribute to colonization and persistence of the bacterium in patient with urinary tract infection [Shankar *et al*., 2001]. Whereas *cylA*- enhances the virulence of *Enterococci* by secreting the toxins. Infections with isolates having this toxin are associated with higher mortality, *hyl*- encodes hyaluronidase and facilitate spread of the organism in the body. Among the clinical isolates of LREfm virulence genes rarely detected in the study included *gelE* (1.5%) and *asa1* (0.5%). Gelatinase (*gelE*) is an extracellular enzyme that can hydrolyze a wide range of substances such as gelatine, casein, collagen, human endothelin lactoglobulin, fibrinogen, and insulin [Mäkinen *et al*., 1989]. Other than hydrolyzing activity, its role has also been reported in association with biofilm formation [Hancock and Perego, 2004]. *Asa1*-encoded collagen-binding protein that allows *Enterococci* to colonise [Coque *et al*., 1995; Johnson 1994; Libertin *et al*., 1992].

In this study, among clinical isolates of LREfm, presence of *esp* and *cyl* gene was significantly associated with the *vanB* gene (p=0.026), G2576T and C2610T mutation (p=0.001) respectively. Whereas, among environmental isolates the presence of *cyl* gene was significantly associated with the G2576T and C2610T mutation (p=0.001).

In the present study, *esp* was the predominant virulence gene and was associated with the vancomycin resistance. Similarly, studies from UK and Italy reported, *esp* as a most common virulence gene among *E. faecium* isolates [Vankerckhoven *et al*., 2004]. Studies from European countries have reported an increase in the prevalence of *esp* gene associated with vancomycin resistance mediated by *vanB* gene among *E. faecium* [Aung *et al*., 2023].

Considering these results, we can conclude that the presence of *esp* may enhance the ability of *E. faecium* isolates to acquire additional antibiotic resistance genes, and there is an association between *esp* and resistance mechanisms of vancomycin and linezolid. A possible explanation for the association between *esp* and resistance mechanisms might be the higher conjugation frequencies observed in isolates that are positive for *esp* compared with those that were negative for *esp* genes. In our study association of virulence genes with mortality was not observed.

Given the increasing importance of enterococcus as nosocomial pathogens, the identification of virulence factors associated with the disease severity has become an important subject of concern. The development of other methods to control infections, such as preventing biofilm formation or inhibiting the action of other virulence factors, may provide an alternative method of therapy, especially considering that antimicrobial treatment is challenging for MDR *E. faecium*.

Molecular epidemiology

The molecular epidemiology was studied by PFGE and MLST. This is the first report from India regarding the sequence types of LREfm. Molecular epidemiological studies of VRE outbreaks in US using PFGE showed the spread of single clones within and between hospitals [Handwerger *et al*., 1993; Boyce, 1994]. Similarly using of PFGE, the presence of polyclonal VRE in a single hospital indicated the simultaneous spread of several VRE clones [Bonten *et al*., 1996].

PFGE typing is still regarded as the "gold standard" for *Enterococcal* strain typing. The rare cutting restriction enzyme that has been shown to work well with PFGE for *Enterococci* is *smaI* [Turabelidze *et al*., 2000]. PFGE is reported to be a useful tool for the molecular epidemiology of *Enterococci* from a variety of sources including clinical samples [Patterson & Kelly, 1998].

In the present study, the molecular epidemiology of clinical (n=202) and environmental isolates (n=24) of LREfm was studied by PFGE. Dendrogram of PFGE profiles of LREfm isolates (n=226) was constructed [Figure; 33] and a total of 20 clusters of related isolates (A1-A20) were observed.

In our study, the data from PFGE suggest that linezolid resistance was emerging in diverse clones that were isolated from patients with diverse clinical presentation hospitalized in different clinical departments and from the patient’s environment. Coexistence of clinical and environmental isolates of same patient in same the clusters (A-5, A-6, A-17) suggest spread of LREfm isolates via cross transmission.

Vancomycin resistance was detected in diverse background strains and observed in all clusters with *vanA* and *vanB* gene. Similar to vancomycin resistant isolates vancomycin susceptible LREfm were also observed in all clusters. Isolates

resistant to both linezolid and vancomycin were observed in all clusters. It was also observed that isolates with both resistance mechanisms, point mutation G2576T and C2610T in the domain V of the 23S rRNA and *optrA* gene were emerging in different clusters. Isolates with virulence gene *esp* were also observed in all clusters.

PFGE suggest that vancomycin and linezolid resistance was emerging in diverse clones. Another explanation for diversity of LREfm is their commensal origin from the gut of patients. The ability of *Enterococcus* to persist in the environment and the clonal relationship observed among clinical and environmental isolates strongly point towards cross transmission of *LREfm* from the environment to the patients or vice- versa and also horizontal transmission of resistance genes. 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.

MLST

Multi-locus Sequence Typing (MLST) offers the possibility to transfer typing data from laboratory to laboratory or compare results via the internet, thus providing a powerful tool for global epidemiologic studies, as well as for studies of the population biology of bacterial species which was lacking in the other typing methods. Population genomics and genetics revealed that *E. faecium* belongs to two different subpopulations. The first subpopulation consists of normal flora of gastrointestinal tract, which are rarely involved in clinical infection. The second subpopulation belongs to hospital-associated *E. faecium* lineages, which cause nosocomial outbreaks and opportunistic infections in hospitalised patients. Two decades ago, using amplified fragment length polymorphism (AFLP) these unique subpopulations were discovered [Pinholt *et al*., 2014]. Later on, these unique subpopulation of *E. faecium* were confirmed and further described by MLST and whole genome sequencing (WGS) [Zhou *et al.,* 2020]. These two groups are currently known as clades A and B. *E. faecium* were further divided into 3 different clades: A1, A2 and B. The successful hospital acquired lineages are members of clade A1. This hospital-adapted lineage is categorised as the "clonal complex-17" (CC17). Clade A2 belongs to the human and animal commensals isolates. Whereas, clade B belongs to community associated isolates [Lebreton *et al*., 2013].

It was assumed that acquisition of insertion sequence (IS) elements helped CC17 to increase its genome plasticity to facilitate adaption in a hospital environment [leavis et al., 2007]. In MLST data, the registered isolates of *E. faecium* belongs to five major clonal complexes (CC) in the order of their size: CC17, CC9, CC22, CC5 and CC94 [Getachew *et al*., 2013]. The most common ST type found in *E. faecium* belongs to the clonal complex CC17 (92% of the isolates) (The source: **pubmlst.org**).

In the present study, a total of 20 isolates were selected to represent each of PFGE clusters (A-1- A-20). Among 20 isolates, 14 were from patient's clinical samples and 6 were from patient's environmental samples. Overall, 6 different sequence types (STs) were characterized.

As per reports the clonal complex-17 lineage is r*esp*onsible for the spread of linezolid and vancomycin resistance in hospitals worldwide. In 1997, ST80 was initially described in the blood of an Israeli patient (The source: **pubmlst.org**). Since then, it has been reported from all over the world [refe].

In 2019 a study from Sweden**,** investigated 188 VREfm by WGS and reported that ST80 was predominant ST and detected in 60% VREfm followed by ST761, ST17, ST117, and ST1495 [Fang *et al*., 2019]. In 2021 a study from New Delhi, investigating 39 isolates of VREfm, also reported that ST80 was the most common sequence type among VREfm isolates followed by ST17, ST117, ST132, and ST280, all belonging to CC17 [Rao *et al*., 2021].

Similarly in the present study ST80 was the predominant sequence type followed by ST782 (among clinical and environmental LREfm). ST761 and ST1070 were observed only among the clinical isolates and ST375 and ST409 were observed among the environmental isolates. All ST belongs to the CC17 (where CC stands for clonal complex). This hospital-adapted lineage is categorised as highly successful and high-risk *Enterococcal* clone for dissemination of antibiotic resistance in the hospital environment. In the present study it was observed that CC17 is associated with the MDR LREfm isolates circulating in our hospital.

Strength of the Study

The strength of this study is that it was a largest prospective study and have investigated prevalence and risk factors for LREfm infections. The study also investigates mutational and plasmid-mediated resistance and correlated the linezolid MIC with the resistance mechanism. PFGE and MLST were used to study the disseminations of resistance isolates in the hospital.

Limitations of the Study

The limitation of this study is that the other mechanisms of resistance were not investigated. Mutation G2576T in the domain V of the 23S rRNA was not characterized for heterogeneity, which needs to be investigated further by whole genome sequencing.

7. Summary

- This study entitled **"Assessment of the Prevalence, Phylogeny and Virulence of Linezolid Resistant *E. Faecium* in Hospital Acquired Infections and Environment**" was undertaken in the Department of Microbiology at Safdarjung Hospital and associated Vardhaman Mahavir Medical College, New Delhi.
- The aim of the present study was to characterize the various mechanisms associated with Linezolid resistance and to study the risk factors and clinical outcomes of patients infected with Linezolid-resistant *E. faecium.*
- During the study period (January 2020 to June 2022) a total of 5069 isolates of *Enterococci* species were isolated from the clinically significant samples, the most frequent isolated species was *E. faecium* (n=3841, 75.7%) followed by *E. faecalis* (1204, 23.8%), *E. gallinarum* (n=14, 0.28%) and *E. casseliflavus* (n=10, 0.19%).
- Linezolid and vancomycin resistance were observed only among *E. faecium* isolates. Vancomycin and linezolid resistance were detected in 720 (18.7%) and 202 (5.3%) of *E. faecium* isolates respectively. In addition, 24 isolates of linezolid resistant *E. faecium* recovered from patient's environment
- Among 202 clinical isolates of LREfm majority of them were from urine (47.5%, n=96) followed by blood (43.6%, n=88) and pus (8.9%, n=18). Highest number of LREfm were isolated from patient admitted to ICUs followed by patients in non-ICU setting.
- To study risk factors, a total of 202 Cases (Study group) with clinically significant infections with linezolid resistant *E. faecium* and 200 patients with LSEfm infections (Controls) were enrolled in the study.
- Among 202 patients with LREfm infections, the age of patients was ranged from 28 days - 84 years with mean age (years) 38.78 ± 17.88. Among them 45% (n=91) were male and 55% (n=111) were Female. The age of patients with LSEfm was ranged from 11 days - 82 years with mean age (years) 39.62 (18.83). Among them 48.5% (n=91) were male and 51.5% (n=103) were female.
- Among 202 Cases, clinical data was available for 199 patients. By comparing Cases/study group and Controls, it was observed that among site of infections significantly associated were UTI (p=<0.001) and BSI (p=0.032). Other risk factors associated with LREfm infections (Cases) were comorbidities (HTN (p=0.004) and

COPD (p=0.020)), invasive procedure (Debridement surgery (p=0.015), Intercostal drainage tube (p=0.002) and Intubation tube (p=<0.001)). In contrast invasive procedure laparotomy (p=0.007) was significantly high among controls. It was observed that the use of urinary catheter (p=<0.001), central line (p=<0.001), ventilation (p=<0.001) and mortality rate (p=0.003) was also significantly high among the cases.

- It was also observed that prior hospital stay length ≤7days was significantly high among the Controls, whereas 8-14 days was significantly high among the Cases (p=<0.001).
- In multivariate factors significantly associated with increased risk of LREfm infection were site of infection UTI (OR 5.87, 95% CI 2.59-13.29, p=<0.001), use of carbapenem (OR 2.85 95% CI 1.62-5.02, p=<0.001) and linezolid (OR 10.13 95% CI 4.13-24.82, p=<0.001) therapy, use of central line (OR 5.54 95% CI 2.35-13.09, p=<0.001), urinary catheter (OR 0.29 95% CI 0.12-0.70, p=<0.001) and ventilation (OR 14.87 95% CI 7.86-28.11, p=<0.006).
- Antimicrobial susceptibility was performed for the 226 LREfm, which includes 202 clinical and 24 environmental LREfm isolates.
- All clinical and environmental isolates were resistant to ampicillin and erythromycin. Overall resistance to ciprofloxacin (99.5% vs 100%), tetracycline (83.2% vs 87.5%) and chloramphenicol (62.4% vs 75%) was high among both clinical and environmental isolates respectively. Resistance to nitrofurantoin was observed in 69.8% of the urinary isolates (12/96). Resistance to high level gentamicin was significantly high among clinical isolates (p=0.004). All clinical and environmental LREfm were MDR.
- Among clinical and environmental isolates resistance to vancomycin was high (86.6% vs 87.5%). Resistance to teicoplanin was significantly (p=0.017) high among environmental isolates (66.6%) compared to clinical isolates (44.1%). Resistance to quinupristin/dalfopristin was significantly (p=0.001) high in clinical isolates (91.1%) in comparison to environmental isolates (70.8%). Excluding one clinical isolate (MIC 8 µg/ml) all clinical and environmental isolates were susceptible to daptomycin.
- Phenotypic detection of *vanA* and *vanB* was determined by teicoplanin and vancomycin E-test. On the basis of phenotypic characteristics, *vanA* phenotype was observed in 48.6%(n=85) and 85.7% (n=18) among clinical and environmental isolates

respectively. *VanB* phenotype was observed in 51.4%(n=90) and 14.3%(n=3) clinical and environmental isolates respectively.

- *VanA* and *vanB* genes were detected in 48.6% (85/175) and 50.9% (89/175) clinical isolates and 85.7% (18/21) and 4.7% (1/21) environmental isolates respectively.
- With *vanA* gene, vancomycin and teicoplanin MIC were ranged from 64-256 µg/mL and 8-256 µg/mL among clinical isolates and 256-256 µg/mL and 32-256 µg/mL among environmental isolates respectively.
- Among clinical and environmental LREfm isolates *cfr* gene was not detected. *OptrA* gene was predominant mechanism of linezolid resistance (58.9% vs 75%%) followed by point mutation at G2576T and C2610T in the domain V of the 23S rRNA (25.2% vs 33.3%) among clinical and environmental isolates.
- The MIC of linezolid among isolates with G2576T and C2610T point mutation in the domain V of the 23S rRNA was approximately 10fold higher (MIC50: 256 µg/mL) compared to isolates with no mutations (MIC50 32 µg/mL). The MIC of these isolates (clinical) did not vary in the presence of *optrA* gene (MIC50 was 256 µg/mL) or in the absence of *optrA* gene (MIC50 256 µg/mL).
- Among clinical LREfm isolates the most common virulence gene was *esp* (59.9%) followed by *cyl* (25.7%) and *hyl* (16.8%). Virulence genes *gel* (1.5%) and *asa1* (0.5%) were detected rarely. Among clinical isolates singly detected virulence genes were *esp* (47.5%) followed by *hyl* (10.9%), *gel* (0.9%) and *cyl* (16.8%).
- Among clinical LREfm presence of *esp* and *cyl* gene was significantly associated with the *vanB* gene (p=0.026) and point mutation (G2576T and C2610T) (p=0.001) respectively.
- Similar to clinical isolates among environmental LREfm isolates *esp* was the predominant virulence gene (70.8%) followed by *hyl* (25%), *cyl* (20.8%).
- Among environmental isolates singly detected virulence genes were *esp* (62.5%) followed by *cyl* (8.3%) and *hyl* (4.1%).
- As of clinical LREfm isolates among environmental isolates the presence of *cyl* gene was significantly associated with the G2576T and C2610T mutation (p=0.001).

- Virulence genes detected in clinical isolates were more diverse in compared to environmental isolates. Among combination of virulence only combination of *cyl* gene with *hyl* (p=0.00001) was significantly high among environmental isolates.
- The molecular epidemiology of clinical (n=202) and environmental (n=24) LREfm was studied by PFGE and MLST.
- Dendrogram of PFGE profiles of LREfm isolates (n=226) was constructed and a total of 20 clusters of related isolates (A1-A20) were observed.
- Isolates from both sources clinical and environmental were detected in 10 clusters. The clinical and environmental isolates of the same patient coexisted in the same clusters.
- Vancomycin resistance was detected in diverse background strains and observed in all clusters with *vanA* and *vanB* genes.
- It was also observed that isolates with both resistance mechanisms, G2576T mutation and *optrA* gene were emerging in different clusters except cluster A-13, A-15, A-19. Isolates negative for both resistance mechanisms were also clustered were also clustered in all clusters. Isolates with virulence gene *esp* were clustered in all clusters.
- MLST typing was performed on the basis of PFGE pattern. Among the 20 clusters detected in PFGE, A total of 20 isolates were selected representative each PFGE cluster (A-1- A-20). Among 20 isolates, 14 were from patient's clinical samples and 6 were from patient's environmental samples. Overall, 6 different sequence types (STs) were characterized, Among the 6 sequence types ST80 was the most frequent and detected was in 60%(n=12) isolates followed by ST761 (n=3), ST872 (n=2), only one isolates belongs to each ST1070 (n=1), ST409 (n=1), ST375 (n=1).
- The two major STs viz., ST80 and ST761 belong to both sources' isolates (clinical and environmental). All STs belonging to the CC17 are highly successful and high-risk clones for dissemination of antibiotic resistance in our hospital environment.

8. Conclusion

In the present study linezolid resistance was observed in 5.3% (202/3841) of clinically significant *E. faecium* isolates. The majority of LREfm (>80%) were also resistant to vancomycin. Vancomycin resistance was mediated by *vanA* and *vanB* gene. The association of linezolid and vancomycin resistance is a cause of concern as it limits the therapeutic options for the management of VRE. All LREfm were MDR, XDR and PDR were observed in 97.5% and 31.2% isolates respectively. In the present study daptomycin was the only therapeutic option for the management of infections with MDR LREfm. Considering the limited therapeutic options, spread of LREfm is an emerging threat in healthcare facilities. Surveillance of MDR LREfm is needed to implement a dedicated stewardship programme to rationalize therapy for VRE.

A total of 226 LREfm, which includes 202 clinical and 24 environmental were characterised for resistance mechanisms widely reported in literature. In the present study *optrA* gene was the predominant mechanism of linezolid resistance among LREfm isolates, one third of isolates had the G2576T and C2610T mutations in domain V of 23S rRNA. Isolates with G2576T and C2610T mutation, had the higher MIC of linezolid (>256 µg/mL) irrespective of the presence or absence of *optrA* gene. Linezolid MIC among isolates with *optrA* gene varied from 8-128 µg/ml. A significant number of LREfm, which included 74 (36.6%) clinical and 6 (25%) environmental isolates, no resistance mechanism investigated in the study was detected. Further investigations are required to identify the other known and novel resistance mechanisms and to understand the effect of other resistance mechanism on MIC of linezolid.

In our study prior use of carbapenem and linezolid were identified as an independent risk factor. Another independent risk factors were hospital stay (8-14 days) prior to isolation of LREfm, invasive procedures like use of urinary catheter, central line and ventilation. It is well known that indwelling urinary catheters, central line and ventilation along with increased hospital stay may facilitate dissemination of resistant bacteria including LREfm.

Based on our data it may be inferred that linezolid resistance is driven by two different mechanisms: (i) De-novo emergence of linezolid resistance during therapy and subsequent nosocomial spread facilitated by increased hospital stays and use of

invasive procedures or devices, due to breach in infection control practices during hospital stay. (ii) 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.

In the present study, *esp* was the predominant virulence gene and was associated with the vancomycin resistance. Considering these results, we can conclude that the presence of virulence genes may enhance the ability of *E. faecium* isolates to acquire additional antibiotic resistance genes.

The data from PFGE suggest that vancomycin and linezolid resistance was emerging in diverse clones that were isolated from patients with diverse clinical presentation hospitalized in different clinical departments and from the patient's environment. Coexistence of clinical and environmental isolates of same patient in same clusters suggest spread of LREfm isolates via cross transmission. Isolates with mutation in 23S rRNA were also clustered in diverse clones. Emergence of resistance mediated by mutation in 23S rRNA in diverse clones may be driven by prior use linezolid during hospital stay. Another explanation for diversity of LREfm is their commensal origin from the gut of patients. The ability of *Enterococcus* to persist in the environment and the clonal relationship observed among clinical and environmental isolates strongly point towards cross transmission of LREfm from the environment to the patients or vice- versa and also horizontal transmission of resistance genes.

CC17 is reported to be a hospital-adapted lineage worldwide and categorised as high-risk *Enterococcal* clone for dissemination of antibiotic resistance in the hospital environment. In the present study 6 different sequence types (STs) were detected. All STs belonged to the CC17. On the basis of MLST data, we can conclude that MDR LREfm isolates are circulating in our hospital.

The study highlights that the MDR and XDR LREfm emerging in our hospital. The resistance is mediated primarily by *optrA* gene and mutations in 23S (G2576T and C2610T). The study suggest that resistance is mediated partly by prior linezolid use and further spread through horizontal transmission of resistance strains facilitated by breach in IPC practices and optrA gene. As linezolid resistance is primarily detected in

vancomycin resistant isolates, surveillance of MDR LREfm is needed to implement a dedicated stewardship programme to rationalize therapy for VRE and thus reduce linezolid consumption. Appropriate infection control policies are needed to preventing transmission of resistant strains.

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Annexure I

S. No.			
1.	**Serial No./Lab No**	**MRD No./ICU No.**	
DETAILS OF THE PATIENT			
2	**Patient Name/ Age/ Gender**		
3	**Contact No./ Address**		
4	**Date of admission in hospital**		
5	**Date of admission in ICU**		
6	**Primary Diagnosis:**		
7	**Chief Complain with duration**	**Fever**	
		Breathlessness (RR)	
		Tachycardia (HR)	
		BP	
		Ant other	
8	**Any past history of disease/ treatment? (Diabetic/ Hypertension/any other clinical condition)**		
9	**Invasive procedure?**		
10	**Is the patient on central line?**	Y / N	
11	**Any catheterization?**		
12	**Is the patient on ventilation?**	Y / N	
13	**Is patient on antibiotics, if yes mention**		
Site of infection			
Respiratory infection/VAP	Y / N	**Blood steam catheter**	Y / N
UTI	Y / N	**Endocarditic**	Y / N
Acute abdominal infection	Y / N	**CNS infection**	Y / N

Skin/soft tissue	Y / N	**Bone/ Joint**	Y / N

Annexure II

Vitek-2 compact system (bioMérieux, Inc. France) for bacterial identification and antibiotic MIC (minimum inhibitory concentration) estimation:

- The isolated colonies were picked up from fresh cultures using a sterile inoculation nichrome wire loop and emulsified in 3ml of sterile saline (in clear polystyrene tube 1).
- The densicheck (bioMérieux, Inc. France) was used to measure the turbidity of bacterial suspension and adjusted to 0.50-0.63 McFarland standard.
- For antimicrobial testing, 248µl of bacterial suspension was transferred to adjacent test tube 2 containing 3ml of sterile saline.
- GP cards were placed for the identification of gram-positive cocci (GPC). P628 were placed for AST of GPC.
- All ID and AST cards were procured from bioMérieux, Inc. France.
- The cassettes were placed in VITEK® 2 Compact system (bioMérieux, Inc. France) and procedure was followed as per the manufacturer's instruction. The final result was received in approximately 10 hours or less.

Annexure III

Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyzed the amplified PCR product.

- **Preparation of Agarose Gel**
    - Agarose gel (1.5%) was prepared using weight/volume percentage and accordingly the agarose powder (Bio-Rad Laboratory, California, USA) was added in 1X TAE (Tris-Acetate-EDTA) buffer (Thermo Scientific, USA).
    - The solution was mixed and heated intermittently (in microwave) until the agarose powder dissolved completely.
    - The agar was allowed to cool till lukewarm temperature and ethidium bromide (EtBr) [Bio-Rad Laboratory, California, USA] at a concentration of 0.5µg/ml was added to the melted agarose.
    - The melted agarose was mixed well and poured into the gel casting apparatus fitted with comb and allowed to set the agarose at room temperature for 30-40 minutes.
    - When the agarose gel got solidified, the comb was removed and the agarose gel was transferred into electrophoresis tank filled with 1x TAE running buffer.

List of Publications

Related to the thesis work:

- **Rani, V**., Aye, N. K., Saksena, R., Dabi, K. C., Mannan, M. A., & Gaind, R. (2024). Risk factors and outcome associated with the acquisition of MDR linezolid-resistant Enterococcus faecium: a report from tertiary care centre. *European journal of clinical microbiology & infectious diseases:*, *43*(4), 767–775.
- **Rani V**, Prakash A, Mannan M A, Das P, Haridas H, Gaind R. (2024). Emergence of OptrA Gene Mediated Linezolid Resistance among Enterococcus Faecium: A Pilot Study from a Tertiary Care Hospital, India. *Int J Mol Cell Med*. 12 (3)

Related to other projects:

- Gajanand Mittal, Vasundhra Bhandari, Rajni Gaind, **Vandana Rani**, Shimpi Chopra, Reetika Dawar, Raman Sardana, P K Verma. (2019). Linezolid resistant coagulase negative staphylococci (LRCoNS) with novel mutations causing blood stream infections (BSI) India. *BMC infectious diseases*. BMC Infect Dis. 2019 Aug 14;19(1):717.
- Manisha Jain , Amit Sharma, M K Sen , **Vandana Rani** , Rajni Gaind , J C Suri. (2019). Phenotypic and molecular characterization of Acinetobacter baumannii isolates causing lower respiratory infections among ICU patients. *Microbial pathogenesis*. Volume 128,75-81, ISSN 0882-4010
- Sylvester Agha Ibemgbo, Rajni Nyodu, Sakshi Chaudhary, Dileep Kumar Verma , Kritika Dixit, Kaustuv Nayak, **Vandana Rani**, Rajni Gaind, Anmol Chandele, Sujatha Sunil. (2022). Short communication: Virological and B cell profiles of chikungunya and Dengue virus co-infections in Delhi during 2017-2019. *Virus research.* 15;320:198888.

Accepted Manuscript

- Anupam Kr. Anveshi*, **Vandana Rani*,** Anusha Bhatnagar, Moninder Narang, Rajni Gaind. (2024). Prevalence of Panton-Valentine Leukocidin and Toxic Shock Syndrome Toxin-1 genes in *Staphylococcus aureus* nasal carriage among Health care workers in a tertiary care hospital. *JIDC*

  *The first and second authors contributed equally.

Presentations in Conferences

- Presented a poster in an international conference on Clinical and Applied Microbiology (ICCAM 2023) conducted by Bioleagues (27^{th} - 28^{th} April 2023). Title **"Plasmid mediated linezolid resistance among Enterococcus faecium: Phenotypic characterization & molecular epidemiology".**
- Presented a poster in an international conference conducted by federation of Infectious societies (8^{th} - 9^{th} Nov. 2021). Title **"High Prevalence of *optr-A* gene among Linezolid resistant *Enterococcus faecium*: First report from India"** at the 2nd International Conference on Trends in Cell and Molecular.
- Presented a poster entitled **"Impact of resistance mechanism on Linezolid MIC among E. faecium isolated from clinical and environmental samples"** at 2^{nd} Chapter Meet of IAMM Delhi Chapter (22^{nd} July 2023).

Symposium & Workshop Attended

- Attended a symposium & workshop on ***whole genome sequencing*** at the molecular laboratory, Kasturba hospital for infectious diseases, Mumbai, India, from 26^{th} -28^{th} April 2022.
- Attended a short-term course on material characterization: analysis and interpretation" organized by Central Instrumentation Facility, DRD, **LPU**. From 23^{rd} - 28^{th} August, 2021.
- Attended an international webinar on emerging trends in Biotechnology, organized by the Dept. of Biotechnology, Panchhunga University College and Mizoram University from 24^{th} -26^{th} June 2020.

European Journal of Clinical Microbiology & Infectious Diseases
https://doi.org/10.1007/s10096-024-04784-0

ORIGINAL ARTICLE

Risk factors and outcome associated with the acquisition of MDR linezolid-resistant *Enterococcus faecium*: a report from tertiary care centre

Vandana Rani[1,2] · N. Kitoi Aye[1] · Rushika Saksena[1] · Kailash Chandra Dabi[1] · Mohammad Amin-ul Mannan[2,3] · Rajni Gaind[1]

Received: 7 January 2024 / Accepted: 9 February 2024

Abstract

Objective The aim of the study was to determine the resistance profile of linezolid-resistant *Enterococcus faecium* (LREfm) and to investigate risk factors and outcomes associated with LREfm infections.

Material and methods A prospective case-control study was undertaken (2019 to 2022) and included 202 patients with LREfm infections (cases) and 200 controls with LSEfm infections. Clinical data was prospectively collected and analysed for risk factors and outcomes. Antimicrobial susceptibility was performed, and resistance profile was studied using WHOnet.

Results Risk factors associated with LREfm infection were site of infection UTI (OR 5.87, 95% CI 2.59–13.29, $p \leq 0.001$), prior use of carbapenem (OR 2.85 95% CI 1.62–5.02, $p \leq 0.001$) and linezolid (OR 10.13, 95% CI 4.13–24.82, $p \leq 0.001$), use of central line (OR 5.54, 95% CI 2.35–13.09, $p \leq 0.001$), urinary catheter (OR 0.29, 95% CI 0.12–0.70, $p \leq 0.001$) and ventilation (OR 14.87, 95% CI 7.86–28.11, $p \leq 0.007$). The hospital stay 8–14 days (< 0.001) prior to infection and the mortality rate ($p = 0.003$) were also significantly high among patients with LREfm infections. Linezolid and vancomycin resistance coexisted; further, MDR, XDR and PDR phenotypes were significantly higher among LREfm.

Conclusion This study provided insight into epidemiology of MDR LREfm in a setting where linezolid use is high. The main drivers of infections with LREfm are multiple, including use of carbapenems and linezolid. Invasive procedures and increased hospital stay facilitate spread through breach in infection control practises. As therapeutic options are limited, ongoing surveillance of LREfm and VRE is critical to guide appropriate use of linezolid and infection control policies.

Keywords *E. faecium* · VRE · LREfm · Linezolid

Abbreviations

LREfm	Linezolid-resistant *E. faecium*
LSEfm	Linezolid-sensitive *E. faecium*
VREfm	Vancomycin-resistant *E. faecium*
VRE	Vancomycin-resistant enterococci
MIC	Minimum inhibitory concentration
UTI	Urinary tract infection
BSI	Blood sepsis infection
SSTI	Skin and soft tissue infection
MDR	Multidrug resistant
T1DM	Type 1 diabetes
T2DM	Type 2 diabetes
HTN	Hypertension
COPD	Chronic obstructive pulmonary disease
CKD	Chronic kidney disease

✉ Mohammad Amin-ul Mannan
mannan.phd@gmail.com

✉ Rajni Gaind
rgaind5@hotmail.com

[1] Department of Microbiology, Vardhman Mahavir Medical College and Safdarjung Hospital, New Delhi 110029, India

[2] Department of Molecular Biology and Genetic Engineering, School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, Punjab 144422, India

[3] Division of Infectious Disease, The Lundquist Institute, UCLA Harbor Medical Center, Los Angeles, CA 90502, USA

Introduction

Generally, enterococci are commensal flora of the gut and widely distributed in environment. They are opportunistic pathogens causing infections in immunocompromised or hospitalised patients specially in ICUs with severe

Published online: 19 February 2024

IJMCM
International Journal of Molecular and Cellular Medicine
p-ISSN: 2251-0637 e-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 vancomycin resistance was 100% and 80%, respectively. Linezolid MIC range was 8-256 µg/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

Mittal et al. BMC Infectious Diseases (2019) 19:717
https://doi.org/10.1186/s12879-019-4368-6

BMC Infectious Diseases

RESEARCH ARTICLE Open Access

Linezolid resistant coagulase negative staphylococci (LRCoNS) with novel mutations causing blood stream infections (BSI) in India

Gajanand Mittal[1†], Vasundhra Bhandari[2†], Rajni Gaind[1*], Vandana Rani[1], Shimpi Chopra[1], Reetika Dawar[3], Raman Sardana[3] and P. K. Verma[1]

Abstract

Background: Coagulase-negative Staphylococci (CoNS) have emerged as a major causative agent of blood-stream infections (BSI). Linezolid (LZD) is currently used for treating glycopeptide and methicillin-resistant staphylococci. It is important to understand the resistance mechanism and probable transmission of LZD resistant (LR) CoNS within the hospital.

Methods: Clinically significant LRCoNS from patients with BSI were characterized using MALDI-TOF and 16S *rRNA* gene sequence analysis. Antimicrobial susceptibility and MIC of vancomycin and LZD were determined. LZD resistance mechanisms using PCR for the *cfr* gene and mutation in the V domain of the 23S *rRNA* gene were studied.

Results: The MIC of LZD ranged from 8 to 32 μg/ml. LR was observed in three different CoNS species from diverse locations within the hospital. The *cfr* gene was identified in all the isolates. Sequence analysis of V domain region of 23S rRNA gene confirmed mutation in single copy among 12/15 isolates with novel mutations: G2614 T and C2384T. All infections were nosocomially acquired and LZD resistance was emerging in the absence of prior LZD use. Horizontal spread of resistant isolates and *cfr gene* among diverse species were the probable mechanisms of transmission.

Conclusion: The study highlights the novel mutations associated with LRCoNS and the importance of surveillance & transmission pathway within the hospital. It also systematically discusses the published information on LRCoNS.

Keywords: Novel mutation, Linezolid resistance, CoNS, cfr, *S. arlettae*, India

Background

Coagulase-negative Staphylococci (CoNS) are normal commensals of the skin and mucous membranes and have emerged as the important cause of hospital-acquired infections [1, 2]. They are the most common cause of healthcare-associated blood stream infection (BSI) for many years, partly because of an increase in the number of hospitalized immuno-compromised patients, the increased use of indwelling medical devices, such as central venous catheters and other prosthetic implants [1]. The clinical significance of species other than *S. epidermidis* has been increasingly recognized in recent years [2]. As the pathogenic significance increases, it becomes important to learn about the epidemiology and pathogenic potential of individual species [2]. Species identification is also important as certain species like *S. epidermidis* and *S. haemolyticus* are resistant to multiple antibiotics [2]. Routine species identification may thus better define the clinical spectrum of disease caused by CoNS. Currently, there is a paucity of data on the clinically significant CoNS species as conventional identification

* Correspondence: rgaind5@hotmail.com
Gajanand Mittal and Vasundhra Bhandari are Shared Authorship
[1]Vardhman Mahavir Medical College & Safdarjung Hospital, New Delhi 110029, India
Full list of author information is available at the end of the article

ScienceDirect

Microbial Pathogenesis

Volume 128, March 2019, Pages 75-81

Phenotypic and molecular characterization of *Acinetobacter baumannii* isolates causing lower respiratory infections among ICU patients

Manisha Jain [a 1], Amit Sharma [a 1], M.K. Sen [b], Vandana Rani [a], Rajni Gaind [a], J.C. Suri [b]

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https://doi.org/10.1016/j.micpath.2018.12.023

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Abstract

Background

Multi-drug resistant Acinetobacter *baumannii* has emerged as important nosocomial pathogen associated with various infections including lower respiratory tract. Limited therapeutic options contribute to increased morbidity and mortality. Acinetobacter baumannii has the ability to persist in the environment for prolonged periods. Breach in infection control practices increases the chances of cross transmission between patients and inter/intraspecies transmission of resistance elements. The present prospective work was conducted among patients with lower respiratory tract infections (LRTI) in the intensive care unit (ICU) to study the etiology with special reference to Acinetobacter *baumannii* and the role of immediate patient environment in the ICU as possible source of infection. Acinetobacter baumannii were characterized for antimicrobial susceptibility, mechanism of

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Virus Research

Volume 320, 15 October 2022, 198888

Short communication

Short communication: Virological and B cell profiles of chikungunya and Dengue virus co-infections in Delhi during 2017–2019

Sylvester Agha Ibemgbo [a 1], Rajni Nyodu [b 1], Sakshi Chaudhary [a], Dileep Kumar Verma [a], Kritika Dixit [b], Kaustuv Nayak [b], Vandana Rani [c], Rajni Gaind [c], Anmol Chandele [b], Sujatha Sunil [a]

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https://doi.org/10.1016/j.virusres.2022.198888

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Highlights

- We report that co-infections of DENV-CHIKV occurred at a frequency of 6.7% in the transmission cycle.
- While DENV-3 is now frequently detected in the Indian subcontinent, we do not see a serotype bias in the patients that are co-infected with ESCA strain of CHIKV.
- The effector B cell response (plasmablasts) observed are specific to both infecting viruses indicating no overt bias.

KEY TO MASTER CHART

No- Serial Number

LID- Lab ID

Loc- location

A- ampicillin

Ci- ciprofloxacin

G- gentamycin

Va- Vancomycin

E-Erythromycin

TE- Tetracycline

Teic- teicoplanin

Ch- chlroamphenicol

NF- Nitrofurantoin

LZ- linezolid

QDA- quinpristin

Dap- Daptomycin

Mcef- monocef

NOR- norfloxacin

Mem- meropenem

IMP- imipenem

CL- colistin

PTZ- piperacillin tazobactam

Mi- minocycline

Ak-Amikacin

AMC- Amoxclav

Sp- Specimen type

BL- blood

U-urine

P- pus