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Detection of *ermB* Mediating Erythromycin Resistance in Clinical Isolates of Enterococci

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ABSTRACT

Background: Enterococci are nosocomial pathogen. Their pathogenic potential has been attributed to their ability to resist antimicrobial drugs intrinsically, acquire and distribute antibiotic resistance determinants and adapt to changing environments. Linezolid resistance occurs by the mutations in the domain V of 23S rRNA, the presence of the *cfr* gene or mutations in the L3 and L4 ribosomal proteins. Erythromycin resistance is mainly due to target-site modification by the rRNAmethylating enzyme encoded by the *erm* gene or mediated by efflux pump mechanisms. This study aimed to detect and characterize the antimicrobial resistance mechanism to oxazolidinone (linezolid) and macrolide (erythromycin) among clinical isolates of Enterococci. Studies describing theresistance to erythromycin and linezolid are limited.

Objective: This study aimed to detect and characterize the antimicrobial resistance mechanismto oxazolidinone (linezolid) and macrolide (erythromycin) among clinical isolates of Enterococci.

Materials and Methods: A total number of 150 clinical isolates were included in this study. Susceptibility to various antibiotics was determined by disc diffusion. Minimum Inhibitory Concentration (MIC) was ascertained by agar dilution method for linezolid and erythromycin. Polymerase chain reaction was done to detect the genes, *ermB* and *cfr* that encode for erythromycin and linezolid respectively.

Results: Among 150 isolates, predominant were *Enterococcus faecalis* 116 (77%) followed by *Enterococcus faecium* 34 (23%). Of the 150 study isolates, the susceptibility to ampicillin was 64.6% (97/150), high level gentamicin 47% (70/150), linezolid (100%) and vancomycin (100%). Of the 90 exudative enterococcal isolates, only 13 were susceptible to erythromycin. Of the 77 erythromycin resistant isolates, *ermB* gene was detected in 42 and exhibited a high levelresistance with >32 µg/ml. The gene encoding for linezolid resistance, *cfr* was not detected in any of the study isolates.

Conclusion: High level resistance to erythromycin is mediated by *ermB*. Vancomycin and linezolid resistance were not encountered in the study. However, continuous monitoring of antimicrobial resistance of Enterococcus species is necessary to provide a guide for the appropriate selection of antibiotics for treatment and to implement preventive measures.

Key Words: *ermB; Enterococci*

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INTRODUCTION

Enterococci is an important nosocomial pathogen causing a variety of infections namely, urinary tract infections, bacteremia, infective endocarditis, intra-abdominal and pelvic infections. The most common species causing the majority of the infections are *Enterococcus faecalis* and *Enterococcus faecium.* Their pathogenic potential has been attributed to their ability to resist antimicrobial drugs intrinsically, acquire and distribute antibiotic resistance determinants and adapt to changing environments. Intrinsically they are resistant to common antibiotics like cephalosporins, penicillinase-

resistant penicillin, low-level aminoglycosides, clindamycin, sulfamethoxazole,and trimethoprim. Extrinsically they acquire resistance to high-level aminoglycoside, ampicillin, erythromycin, ciprofloxacin, linezolid, and vancomycin either through mutations or horizontal transfer of resistant genes.[1,2]

Linezolid, the first antimicrobial agent of the class of oxazolidinones was introduced to treat infections caused by multidrug-resistant aerobic Gram-positive bacteria, including vancomycin-resistant enterococci (VRE). It inhibits protein synthesis by binding the central loop of domain V in the 23S rRNA in bacterial ribosome. Linezolid resistance occurs by the mutations in the domain V of 23S rRNA, the presence of the *cfr* gene or mutations in the L3 and L4 ribosomal proteins. Besides resistance towards linezolid,*cfr* also confers resistance to phenicols, lincosamides, pleuromutilins, and streptogramin A antimicrobials (PhLOPSA phenotype).[3]

Erythromycin resistance is mainly due to target-site modification by the rRNA-methylating enzymeencoded by the *erm* gene, which causes methylation of 23S rRNA thus reducingthe ability of the macrolide to bind to the ribosome. Of the Erm genes, *ermB* gene is the most frequently reported.Also, export of antibiotics mediated by genes encoding efflux pumps namely the *mef* and *msr* are other mechanisms involved in macrolide resistance among Enterococci.[4]

Though many reports have been published on the incidence of erythromycin resistance in enterococci, molecular mechanism and genes encoding for resistance to erythromycin from India are limited.[5,6]Studies on linezolid resistance among Enterococci are scarce.[7,8]Hence the current study aimed to detect and characterize the antimicrobial resistance mechanism to oxazolidinone (linezolid) and macrolide (erythromycin) among clinical isolates of Enterococci.

MATERIAL AND METHODS

Study setting

This study was conducted in a 1600 bedded teaching hospital for a period of 1 year from August 2020 to February 2021. The study protocol was approved by the Institutional ethics committee (ref : CSP-MED/20/JAN/58/39)

Bacterial isolates

This study included 150 clinically significant, consecutive, and non-repetitive Enterococci isolated from hospitalised patients. The isolates were obtained from clinical specimens such as pus, blood and urine.The organisms were identified upto the species level using either conventional or automated methods (VITEK2 GP-card (bioMerieux, Marcy l'Etoile, France). Care was taken to differentiate commensal from pathogenic organisms in nonsterile sites by ascertaining their presence in Gram stain, growth of significant colony forming units in culture and correlation with clinical condition of the source patients.

Antimicrobial susceptibility testing:

Susceptibility to various classes of antibiotics was determined by the disc diffusion method in accordance with Clinical Laboratory Standard Institute guidelines(CLSI-M100-S30)[9]The antibiotics tested were ampicillin (10µg), high level gentamicin (120µg), erythromycin (15µg) (For isolates from Exudates), vancomycin (30µg) and linezolid (30µg). For urinary isolates susceptibility to nitrofurantoin (300µg) and ciprofloxacin (5µg)was tested.The antimicrobial agents were procured from Himedia Laboratories (Himedia, Mumbai, Maharashtra, India).

Minimal inhibitory concentration (MIC):

MIC of linezolid and erythromycin was determined by agar dilution methods and interpreted according to CLSI 2020 guidelines (CLSI-M100-S30)[9]The range tested was0.125-128µg/ml. Growth of the organism in media containing the highest concentration was noted as the MIC.

Detection of resistance encoding genes

Polymerase chain reaction (PCR) amplification was done for all the study isolates to detect the genes encoding resistance: erythromycin resistance (*ermB*) and linezolid resistance (*cfr*)

Template DNA preparation

A single bacterial colony was injected into Luria-Bertani broth (Himedia laboratories, Mumbai, Maharashtra, India) and incubated overnight at 37°C before being centrifuged for 10 minutes at 10,000 rpm. The pellet was re-suspended in 250 µl of Millipore water, heated for 10 minutes at 100°C, then cooled and centrifuged at 10,000 rpm for 10 minutes. The supernatant was used as a template for the DNA.

Polymerase chain reaction (PCR)

Simplex PCRs were carried out using the previously described primers and conditions for all the study isolates.[3, 10]The primers used for different sets of genes and the amplicon size are listed in [Table 1].Each reaction volume contained 2 µl of the DNA template added to the master mix which includes 10 pmol of the forward and reverse primers (Sigma-Aldrich, Missouri, United States), 10 Mm dNTPs (Takara, Shiga, Japan), 5U taq polymerase (Takara, Shiga,

Japan), and 10X buffer with MgCl2 (Takara, Shiga, Japan).The PCR product was then run on a 1.5 % agarose gel for detection of the amplified fragment Strains previously confirmed by PCR were sequenced and used as positive controls

Table 1 – Primers used in PCR

Gene	Primer sequence (5′ → 3′)	Amplicon size (bp)
cfr	5'-TGAAGTATAAAGCAGGTTGGGAGTCA 3' 5'-ACCATATAATTGACCACAAGCAGC -3'	850
ermB	5'-GAAAAGGTACTCAACCAAATA 3 5'- AGTAACGGTACTTAAATTGTTTAC 3'	639

DNA sequencing:

The DNA of *ermB* positive isolates was extracted using the Pure Link Genomic DNA Kit (Invitrogen) according to the instructions included with the kit and submitted to automated DNA sequencing (ABI 3100, Genetic Analyser, Applied Biosystems, Foster city, CA, USA). The aligned sequences were evaluated using the Bioedit sequence tool, and nucleotide sequence similarities were found using the BLAST programme. The sequence was submitted to Genbank and the accession number given was OL539552

RESULTS

Among 150 isolates,predominant were *Enterococcus faecalis*116 (77%) followed by *Enterococcus faecium*34 (23%). Majority of the study isolates were obtained from exudative specimens which accounted to 60% (90) followed by 31% (46) from urineand 9% (14) from blood.

Of the 150 study isolates, the susceptibility to ampicillin was 64.6 %. (97/150),high level gentamicin 47% (70/150), linezolid (100%) and vancomycin (100%).Among the 45 urinary isolates, 43 (95.5%) isolates weresusceptible to nitrofurantoin and 57.7% (26/45) to ciprofloxacin. Of the 90 exudative isolates,85.5% (13/90) were susceptible to erythromycin.

MIC determination:

All the study isolates were susceptible to linezolid with their MIC ranging from the 0.5-2µg/ml.Of the 90 study isolates, only 13 were susceptible to erythromycin and had MIC within the susceptible range of ≤0.5µg/ml. Seventy-seven isolates exhibited resistance to erythromycin with MIC ranging from 8-128µg/ml. Intermediate range MIC of 1-4µg/ml was not observed in any of the study isolates

PCR for *ermB* and *cfr*gene*:*

ErmB gene was detected in 42 isolates whichincluded, 32 *E.faecalis* and 10 *E.faecium*. The *ermB* positive isolatesexhibited a high level of MIC to erythromycin ranging from 32µg/ml to 128µg/ml. *ErmB* gene was not detected in 35 isolates which were resistant to erythromycin with MIC ranging 8µg/ml to 16µg/ml.

The gene encoding for linezolid resistance, *cfr* was also not detected in any of the study isolates.

DISCUSSION

Enterococcus faecalis and *Enterococcus faecium* are responsible for the majority of enterococcal infections in humans and these species are resistant to multiple antimicrobial agents such as vancomycin (vancomycin resistant enterococci; VRE), aminoglycosides (the high-level gentamicin resistant; HLGR), macrolides (erythromycin), oxazolidinones (linezolid) and tetracyclines. The present study aimed at detecting and characterising the mechanism of resistance to erythromycin and linezolid.

Erythromycin resistance among enterococci is associated with the presence of erythromycin resistance methylase (*erm*) genes, such as *ermA, ermB,* and *ermC.* More than 40 *erm* genes have been discovered. The predominant *erm* gene in erythromycin resistant isolates of enterococci is the *ermB*gene, that encodes for the ribosomal RNA methylase which results in methylation of 23S rRNA reducing the ability of macrolide for ribosome binding. This modification of the ribosomal target causes crossed resistance to macrolide, lincosamide, streptogramin (MLS) group of antibiotics. *ErmA* and *erm C*are occasionally reported.[4]

In the present study, 85.5% (77/90) of the study isolates exhibited resistance to erythromycin. The results of the disc diffusion test and MIC determination were concordant. Fiftytwo isolates had MIC >8 µg/ ml among which 25 isolates had high level MIC of 128 g/ml. This is in concordance with studies from North and South India where the prevalence of resistanceto erythromycin was 85% and 80.6% respectively.[6,10] In China, the prevalence of erythromycinresistance was 89%.[13] In two different studies from Iran the prevalence was 69% and 87%. [14,15]In all these studies, decreased effect of erythromycin on enterococci was reported to be due to the wide- spread use of macrolideantibiotics.[6,11-14]

In the current study, all the 90 isolates were subjected to PCR for detecting the presence of *ermB*gene irrespective of their susceptibility to erythromycin.Among the 77 erythromycin resistant isolates, the *ermB*gene was detected in 42 constituting 55.5%. The *ermB* positive isolates exhibited high level resistance to erythromycin with MIC ranging from 32 µg/ ml to 128 µg/ ml.

A study from Egypt found that 92.2% of resistance to erythromycinwas mainly mediated by *ermB*.[15]Another study from Spain found that, *ermB* was detected in 39 of 40 (97.5%) erythromycin resistant Enterococcus isolates with MICs>128 µg /ml and one isolate carried *ermA* gene. [9]Both the above studies concluded that in enterococci,*erm* methylase gene were responsible for high level erythromycin resistance and the MICs were ≥32µg/ml which was in agreement with the present study. Similar observation has been published from Iran with *ermB*gene being the most common (77.7%) mediator of high levelerythromycin resistance, followed by *ermA* (15%) and *ermC* (8.3%).[16]

In the present study *ermB* gene was not detected in 35erythromycin resistant isolates.The resistance mechanism operative in these isolates could be dueto presence of other erm genes such as *ermA*, *ermC*,*ermD*, *ermE*, *ermF*, *ermG*, *ermQ* or the macrolide efflux pump (*msrA* or *mefA*).[4]Sincethese isolates exhibited low level resistance to erythromycin and had an MIC between 8 µg/ml and 16 µg/ml, it may be assumed that the resistance is probably mediated by efflux pump. The abovementioned genes were not looked for in this study.Hence, further studies including these genes should be done to detect macrolide resistance mechanism.

Resistance to linezolid is mediated by either mutational mechanisms or by gene acquisition. The major mechanism of linezolid resistance in enterococci is a mutation in domain V of the23S rRNA and the G2576T substitution is the most frequent. Mutations in the ribosomal proteins L3 and L4 has also been described. Additionally, plasmid borne transferable genes have been implicated and to date, upto five acquired linezolid resistance genes have been described among Enterococcus spp.: *cfr*, *optrA*, and *poxtA*.[3]In India, the first report of linezolid resistance in Enterococcusfecium exhibiting MIC of >256 µg/ml was published by Smit Kumar et al, in 2014 at Kolkata in the blood culture isolate of a 72- year female patient who had no previous reported history of linezolid medication.[7]More recently, a study from South India described the coexistence of *optrA*gene and G2592T mutation in domainV of 23SrRNAin two isolates of *Enterococcus fecium*.[8]In the present study, all isolates were susceptible to linezolid and the*cfr*gene was also not detected in any isolate.This observation is in concordance with the studies done in India and worldwide.[17,18,19]The results of the present study suggested that resistance to linezolid might be slow to emerge as resistance was not observed in any isolates. However, considering its extensive usage, continuous monitoring of emergence of resistance is necessary.

In the present study, MIC was determined by agar dilution method for erythromycin, and linezolid. The results of MIC by agar dilution method and Kirby-Bauer disc diffusion method were comparable and there was no discrepancy. Hence, disc diffusion technique can be used as a reliable method to determine the susceptibility of enterococci to erythromycin and linezolid in a clinical laboratory

Conclusion

High level resistance to erythromycin is mediated by *ermB* in 54.5% of erythromycin resistant isolates. Further studies are required to detect the presence of other *erm*genes and the effluxpump mediated mechanisms.Vancomycin and linezolid resistance were not encountered in the study. However, continuous monitoringof antimicrobial resistance of Enterococcus species is necessary to provide a guide for the appropriate selection of antibiotics for treatment and to implement preventive measures.

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