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Higher incidence of Pristinamycin resistance among *Enterococcus faecium* with $iMLS_B$/$cMLS_B$ phenotype

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Pristinamycin (quinupristin/dalfopristin) is recommended for the treatment of serious infections caused by *Enterococcus faecium*.Nevertheless,screening forpristinamycin (Q/D) susceptibility is not routinely performed. Decreased *in-vivo* bactericidal activity of quinupristin/dalfopristin is reported in *E. faecium* with $iMLS_B$ phenotype. Non-urinary clinical isolates of *E.faecalis*(n= 16) and *E.faecium*(n=9) were screened for inducible clindamycin resistance by D test and susceptibility to the standard antimicrobials by disc diffusion assay. High-level resistance to gentamicin and streptomycin(HLG^RHLS^R) was observed in 56% of the isolates. All the isolates were susceptible to vancomycin, linezolid and teicoplanin. Of the 25 isolates,64%,20%, 4% exhibited $cMLS_B$,$iMLS_B$phenotype and M type respectively. Three isolates(12%) belonged to an uncommon erythromycin-intermediately susceptible and clindamycin-resistant phenotype. All the *E. faecium*isolates with $iMLS_B$ phenotype were resistant to pristinamycin nevertheless, M type and $Ery^{IS}clin^R$ phenotypes were found to be susceptible to pristinamycin. Routine screening for inducible clindamycin resistance among *E.faecium*, would detecti MLS_B/$cMLS_B$ phenotypes thereby, predict the possible decrease *in-vivo* activity/ clinical inefficacy of pristinamycin.

Keywords: $cMLS_{B;}$ *Enterococcus faecium*; HLGR; HLSR; $iMLS_{B;}$ Pristinamycin.

Over the years, enterococci have developed resistance to virtually all antimicrobials currently used in clinical practice using diverse genetic strategies. Enterococci resistant to all the newer anti-gram positive antimicrobials have evolved as important nosocomial pathogens and their treatment has become aintimidating clinical challenge[1]. Treatment of enterococcal infections depends upon on a triad of factors –a) the species, b) the resistance pattern of the clinical isolate and c) the location and severity of the infection[2]. Enterococci exhibiting resistance to glycopeptides, fluroquinolones, erythromycin, high level resistance to aminoglycosides are continuing to be in the rise. Linezolid or a mixture of streptogramin B (quinupristin-Q) and A (dalfopristin-D) is effective against Glycopeptide resistant *E. faecium*. However, cross resistance

to Q/D in *E. faecium* with a MLS_B phenotype is also being reported[1]. Macrolide-lincosamide-streptogramin MLS antibiotics are chemically distinct but share a similar mode of action-inhibition of bacterial protein synthesis. Macrolides possess a 14, 15 or 16 membered lactone ring while the lincosamides (eg. clindamycin) are devoid of the lactone ring. In enterococci, acquired resistance to macrolides and lincosamides is reported to be established in one of the following ways, a) through modification of the 23s ribosomal target site,b) through efflux of the antibiotic and c) by inactivation of the antibiotic[3]. Methylation of the ribosomal target encoded by the *erm* (erythromycin ribosomal methylase) genes has led to the emergence of MLS_B resistant phenotype. The Erm protein dimethylates the adenine A2058 residue in the conserved region of the domain V, the peptidyl transferase centre of 23S *rRNA* and impairs the binding of erythromycin to its target. Overlapping of binding sites in the 23s*rRNA*contributes to broad cross resistance to Macrolides, Lincosamides and streptogramins B that is exhibited by the MLS_B phenotype[4]. Of the diverse classes of *erm* genes that have been reported so far, enterococci frequently express the *erm Bgenes.*Antibiotic susceptibility testing to determine resistance to erythromycin and clindamycin are not being carried out routinely. However, the presence of Erm methylases in clinical isolates of *E. faecium* is reported to decrease the *in vivo* bactericidal activity of Q/D and reduce the therapeutic efficacy when given as a monotherapy[1].This study was designed to determine the prevalence of inducible clindamycin resistance among the non-urinary enterococcal isolates.

MATERIALS AND METHODS

Twenty five non-repetitive clinical isolates (pus (n=19), fluid (n=5), blood(n=1)) of *Enterococcus* species were included in the study. Speciation was performed by standard biochemical tests and further confirmed by plating on Enterococcus Differential agar (HiMedia laboratories Pvt Ltd, India). Enterococci were screened for susceptibility to erythromycin (15µg), pristinamycin (Q/D) (15µg)(for *E. faecium* isolates only). All the enterococcal isolates were tested for possible inducible clindamycin resistance by disc approximation test. Briefly, erythromycin (15 µg/disc) and clindamycin (2 µg/disc) discs (HiMedia laboratories Pvt Ltd, India) were placed 15 mm apart on agar plates and were incubated at 37°C for 18 h. D-test positivity was identified by the D-type flattening of clindamycin zone towards the erythromycin disc. *S. aureus* ATCC 25923 was included as the standard control[5]. Also, susceptibility to standard antimicrobials (linezolid (30µg), teicoplanin(30µg), highlevel gentamicin (120µg) and high-level streptomycin (300µg)) were assessed by disc diffusion method (CLSI,2018)[5]. Susceptibility to vancomyc in was screened by vancomycin(6 µg/ml) agar screen method[5].

RESULTS

Of the 25 non-urinary enterococcal isolates that were included in this study, 16 (64%) were identified as *E. faecalis* and 9 (36%) as *E. faecium*. High-level resistance to gentamicin and streptomycin (HLG^RHLS^R) was observed in

Table 1. Antibiotic susceptibility pattern of the non-urinary enterococcal isolates

Phenotype	$iMLS_B$	$cMLS_B$	M type	$Ery^{IS}clin^R$
E. faecalis(n=16)	0(0%)	14(87.5%)	0(0%)	2(12.5%)
E. faecium(n=9)	5(55.6%)*	2(22.2%)*	1(11.1%)†	1(11.1%)†
Total (n=25)	5(20%)	16(64%)	1(4%)	3(12%)
Phenotype	HLG^RHLS^R	HLG^RHLS^S	HLG^SHLS^R	HLG^SHLS^S
E. faecalis(n=16)	10(62.5%)	0(0%)	2(12.5%)	4(25%)
E. faecium(n=9)	4(44.4%)	5(55.6%)	0(0%)	0(0%)
Total (n=25)	14 (56%)	5(20%)	2(8%)	4(16%)

*Resistant toPristinamycin.
†Susceptible to Pristinamycin.

14/25 (56%, *E. faecalis* = 10, *E. faecium* = 4) isolates while, 5(20%), 2(8%) of the enterococci exhibited $HLG^{R}HLS^{S}$ and $HLG^{S}HLS^{R}$ phenotype respectively. However, none of the study isolates were found to be resistant to vancomycin, linezolid and teicoplanin.

Among the 25 enterococcal isolates, 22(88%) and 3(12%) isolates exhibited resistance and intermediate susceptibility to erythromycin. When screened for D-test positivity, 16(64%) exhibited $cMLS_B$ phenotype (constitutively resistant to clindamycin), while, D-type flattening of clindamycin zone in the presence of the inducer, erythromycin was exhibited by 5(20%) isolates and were scored as inducible clindamycin resistance-$iMLS_B$ phenotype. It is noteworthy, that all the isolates with $iMLS_B$ phenotype were *E. faecium* while, none of the *E. faecalis* isolates exhibited inducible clindamycin resistance (Fisher's exact two tailed, $p = 0.000049$).One isolate of *E. faecium* belonged to the M type(Table 1).Erythromycin-intermediately susceptible and clindamycin-resistant ($Ery^{IS}clin^{R}$) phenotype was exhibited by 1/9 (11.1%) and 2/16 (12.5%) of the*E. faecium* and *E. faecalis* isolates respectively (Fisher's exact two tailed, $p = 1$)(Table 1). Among the *E. faecium* isolates with $iMLS_B$ phenotype(n=5), 3 (60%) (pus=2, fluid=1), 2(40%) (pus=1, fluid=1)exhibited the $HLG^{R}HLS^{R}$ and $HLG^{R}HLS^{S}$ phenotype respectively.

Of the 9 *E. faecium* isolates tested for pristinamycin resistance, the isolate with M phenotype(n=1) was susceptible, while all the $iMLS_B$(n=5),$cMLS_B$(n=2) resistance phenotypes exhibited co-resistance to Pristinamycin. Of note the susceptibility to pristinamycin exhibited by the uncommon $Ery^{IS}clin^{R}$phenotype of *E. faecium*(n=1).

DISCUSSION

Linezolid or a mixture of streptogramin B (quinupristin-Q) and A (dalfopristin-D) is reported to be effective against glycopeptide resistant *E. faecium, as well* synergistic combinations of a cell wall active agent, such as a β-lactam(ampicillin) or glycopeptide(vancomycin) plus an aminoglycoside (gentamicin) is recommended for the treatment of serious enterococcal infections[6]. Hence, we screened for the susceptibility pattern of the isolates against linezolid, vancomycin, high-level gentamicin and high-level streptomycin and pristinamycin.

Previous Indian reports have documented vancomycin resistance rates of 1 – 24%[7-11]. However, none of this study isolates were resistant to vancomycin, this corroborates with other studies[12-14].The absence of VRE isolates in our study could be attributed to the restricted vancomycin usage practices in our setting. High level aminoglycoside resistance observed among 76% of the study isolates (56%, $HLG^{R}HLS^{R}$, 12%, $HLG^{R}HLS^{S}$ and 8%, $HLG^{S}HLS^{R}$) eliminates the synergistic bactericidal effect with beta lactams/glycopeptides. This is in line with the previous Indian studies which report an increased dissemination of aminoglycoside resistance genes in our setting[15-18].

The majority (88%) of the enterococcal isolates tested were resistant to erythromycin. This corroborates with other Indian studies that have reported erythromycin resistance rates in the range of16.9% - 87% among the non-urinary enterococcal isolates[10,13,18].In our study, majority of the *E. faecalis* (87.5%) and *E. faecium* (77.8%) isolates were resistant to erythromycin. This is in line with the previous Indian studies which have reported a higher prevalence of erythromycin resistance in both *E. faecalis*(81%, 64.3%, 91%) and *E. faecium*(90.1%, 66.7%, 86%)[7, 8, 19].

Previous Indian studies have not screened / reported clindamycin resistance in enterococci, except for a single report on inducible clindamycin resistance among *E. faecalis* by Dubey & Pathy, (2015)[20].Here in, we report for the first time inducible clindamycin resistance among *E. faecium* in India. Majority (64%) of the isolates in this study were constitutively resistant to clindamycin while, 20% exhibited $iMLS_B$ phenotype. Inducible clindamycin (i.e)$iMLS_B$ resistant phenotype was exhibited only by *E. faecium* but not *E. faecalis.* Also, this is the first Indian report of enterococcal isolates (*E. faecalis* (n=2), *E. faecium* (n=1)) with an uncommon $Ery^{IS}clin^{R}$ phenotype. However, Bozdogan *et al*, (1999) had reported an uncommon erythromycin-susceptible and clindamycin-resistant phenotype of *E. faecium* HM1025[21].

Screening for constitutive/inducible clindamycin resistance is not being routinely carried out owing to 2 reasons, a). There are no

interpretive criteria for disc susceptibility testing for clindamycin against Enterococci, b) CLSI guidelines has cautioned (Warning) that reporting of in vitro susceptibility to clindamycin as follows "clindamycin may appear active in vitro but are not active clinically and hence should not be reported as susceptible"[5].

Linezolid and pristinamycin(Q/D - streptogramin B/A combination) are the two antibiotics proved to be clinically efficacious and have been approved by the FDA for the treatment of glycopeptide resistant enterococcal (GRE) infections[22, 23]. Q/D is clinically effective against *E. faecium*, but not *E. faecalis* due to the intrinsic presence of a chromosomal gene *lsa* (for lincosamide and streptogramin A resistance), which encodes a putative protein with an ATP-binding cassette(ABC) motif of transporter proteins[2, 24]. Several investigators have documented the promising clinical efficacy of Q/D against *E. faecium*(especially, vancomycin resistant) infections as intrinsic resistance to streptogramins has not been reported in *E. faecium*. The CLSI guidelines, also recommends the susceptibility testing of Q/Dagainst vancomycin resistant *E. faecium*[5].

Theoretically, the streptogramin A, dalfopristin should remain active even in strains with MLS_B phenotype as they exhibit resistance only to MLS-B antibiotics conferred by the *erm* genes that modifies their 23s *rRNA* target.Nevertheless, an animal study has cautioned the clinical implementation of Q/D as the activity of Q/D could be influenced by MLS_B phenotype. Indeed, Fantin *et al*. has documented this phenomenon in a rabbit endocarditis model wherein reduced *in vivo* activity of Q/D was observed in enterococci possessing inducible MLS_B resistance. This has been attributed to the incomplete penetration of dalfopristin in the valvular vegetation[25]. Hence, we screened for resistance to pristinamycin among the *E. faecium*. In our study, all the $iMLS_B$(n=5),$cMLS_B$(n=2) resistancephenotypes of *E. faecium* were found to be resistant to pristinamycin.Of note the susceptibility to pristinamycin exhibited by the M phenotype and the uncommon $Ery^{IS}clin^R$phenotype of *E. faecium*.

The higher prevalence of HLAR and the absence of VRE in this study reflects the antibiotic usage practices in our setting. Nevertheless, the combination therapy of an aminoglycoside and a cell wall active agent (â-lactam / glycopeptide) is void due to the higher incidence of HLAR. Though the current CLSI guidelines, recommends the screening of quinupristin/dalfopristin for vancomycin resistant *E. faecium,* pristinamycin (Q/D) susceptibility is not routinely screened for in many of the laboratories. Nevertheless, D test is routinely performed for other grampositive cocci, which could also be adopted for Enterococci. Macrolides and lincosamides are not considered as therapeutic alternatives in insidious enterococcal infections, still our observation emphasises the need for screening inducible clindamycin resistance for prompt detection of $iMLS_B$/$cMLS_B$phenotypes as these strains could predict the decreased in vitro and *in vivo* bactericidal activity of Pristinamycin (Q/D). Our results negate the possible role of this combination in the treatment of *E. faecium*strains with $iMLS_B$/$cMLS_B$ phenotype.

CONCLUSION

The combination of quinupristin/ dalfopristin is recommended for vancomycin resistant *E. faecium*. Nevertheless,screening forpristinamycin (Q/D) susceptibility is not routinely performed.Screening inducible clindamycin resistance by D test could be adopted as the prompt detection of $iMLS_B$/$cMLS_B$ phenotypes of *E. faecium* by D test could predict the decreasedin vitro and *in vivo* bactericidal activity of Pristinamycin (Q/D).

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