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Research Article

Prevalence of aac (6′)-Ie-aph (2″)-Ia gene and drug resistance pattern of Enterococcus isolated in a tertiary care hospital

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ABSTRACT

Background: Enterococci causes serious infection due to its higher ability to colonize and increasing resistance to various drugs. Mutation and plasmid mediated genetic exchange are the main reason for the high rate of acquisition of antibiotic resistance. The study was aimed to determine the antibiotic resistance profile of the enterococcal isolates from various clinical samples and to detect the presence of aac (6′) Ie-aph (2″) Ia gene in the isolates which show phenotypic high level gentamicin resistance.
Methods: Clinical enterococcal isolates from a tertiary care hospital in southern Delhi were subjected to antibiotic susceptibility testing. MIC for High level gentamicin was measured and the isolates were tested for presence of aac (6′) Ie-aph (2″) Ia gene by PCR.
Results: Out of the total 146 Enterococcal isolates, 112 were E. fecalis, 33 were E faecium and 1 was E gallinarum. 26.02% were resistant to High level gentamicin, and 15% were resistant to streptomycin. Vancomycin resistance was 5.4%. 11 E. fecalis and 25 E. faecium isolates showed presence of aac (6′) Ie-aph (2″) Ia gene.
Conclusions: High level antibiotic resistance among enterococci and the spread of vancomycin resistant is an issue of serious concern. Isolation rate of E. fecalis was much higher than E. faecium, but aac (6′) Ie-aph (2″) Ia gene was more prevalent in E.faecium. The study highlights spread of the gene aac (6′)-Ie-aph (2″)-Ia among the enterococcal isolates which can be easily transferred to other pathogenic gram positive cocci.

Keywords: Enterococci, HLGR, VRE

INTRODUCTION

The status of Enterococci has increased over the decades from being a minor nosocomial pathogen to a serious life threatening infection causing bacteria, mainly due to its higher ability to colonize and increasing resistance to various drugs. The organism is known to cause various infections ranging from urinary tract infection to endocarditis. Mutation and plasmid mediated genetic exchange are the main reason for the high rate of acquisition of antibiotic resistance.[1] Moreover increasing level of cephalosporin usage in hospitals helps enterococci to proliferate and spread, which are intrinsically resistant to cephalosporins.[2]

Enterococci are generally resistant to low level aminoglycosides due to impermeability of cell wall. Normally, gentamicin MIC values ranges from 4 to 64 mg/L.[3] Moreover E. faecium is intrinsically aminoglycoside-resistant due to the activity of a chromosomal acetyl-transferase enzyme that modifies the antibiotic.[4] The combination of a cell wall-active agent (Penicillin) with an aminoglycoside, however, provides a synergistic bactericidal effect that result in increased uptake of the aminoglycoside.[5] A combination of Penicillin and Gentamicin had been the mainstay of treatment of enterococcal infections for decades. But with the emergence of high level gentamicin resistance (HLGR), glycopeptides drug like vancomycin became the only alternative available.[6] The High level resistance to

Gentamicin (HLGR) in Enterococci (MICs ≥1000 mg/ml) was described in E. faecalis in 1979 and E. faecium in 1988. The gene encoding HLGR, aac (6′) Ie-aph (2″) Ia, was probably the result of the fusion of two genes encoding two aminoglycoside modifying enzymes (AMEs) into one gene encoding a very powerful bi-functional AME. This bestows resistance to nearly all aminoglycosides except streptomycin. Transposons and plasmids have spread this gene world-wide in both staphylococci and Enterococci.[7] Few other genes like aph (2′) Ib, aph (2′)-Ic, and aph (2′)-Id are also responsible for gentamicin resistance but their prevalence is lesser than the aac (6′) Ie-aph (2″) Ia gene.[8]

The present study aims to determine the antibiotic resistance profile of the enterococcal isolates from various clinical samples and to detect the presence of aac (6′) Ie-aph (2″) Ia gene in the isolates which show phenotypic high level gentamicin resistance.

METHODS

A total of 146 Enterococcus isolates obtained from various clinical samples sent to Microbiology laboratory, HAH Centenary Hospital, Jamia Hamdard, New Delhi, for culture and sensitivity from Jan 2014 to Jan 2015 were considered for this study. All samples were cultured onto 5% Sheep blood agar and MacConkey Agar (Hi-Media, Mumbai), incubated in presence of 5 - 10% CO_2 at 37°C for 16 – 48 hour.[9] All small/ pinpoint, cream or white, smooth, entire, alpha, beta or non-hemolytic (on blood agar) and lactose fermenting colonies (on MacConkey agar), catalase negative and gram positive cocci which appears singly, in pairs or in small chains on gram Staining were considered for further processing.[9] Enterococci were identified by growth on Bile -esculin azide medium, growth on Brain heart infusion (BHI) broth with 6.5% NaCl and Bacitracin resistance.

Antimicrobial susceptibility testing for Enterococci was performed by Kirby Bauer disc diffusion method (KBDDM) as recommended by Clinical Laboratory Standards Institute (CLSI) using Amoxicillin (10 µg), Penicillin (10 units/disc), Norfloxacin (10µg), Erythromycin (15µg), high level Gentamicin (120µg), Streptomycin (300 µg) Vancomycin (30 µg), Teicoplanin (30 µg), Linezolid (30µg), ciprofloxacin (5 µg), levofloxacin (5µg), Ampicillin- clavulanic acid (20/10 µg) and Nitrofurantoin (300 µg), tetracycline (30 µg), chloramphenicol (30 µg).[10] A zone size of less than ≤6 mm was considered resistant for high level gentamicin and streptomycin, indicating a MIC of >500 µg/ml and >2000 µg/ml respectively. A Reference strain, Enterococcus faecalis ATCC 29212 was used as control.

The species determination and confirmation of high level gentamicin resistance was done by VITEK-2 system, Biomerieux, France. MIC of HLG was also measured with Gentamicin Ezy MIC Strip (HLG) (Hi Media, Mumbai).

Total DNA was extracted from isolates as previously described.[11] Briefly, the strains was grown overnight at 35°C on Sheep Blood agar, BHI agar or Mueller Hinton agar (MHA). Three to five colonies of each sample was taken and suspended in 1 ml of molecular grade water. The suspension was heated to 1000C for 15 minute and centrifuged at 15,000g for 10 min. The supernatant was used as a template.

Amplification of the aac (6′)-Ie-aph(2″)-Ia gene was done using the primer sequence F:CAGAGCCTTGGGAAGATGAAG, R:CCTCGTGTAATTCATGTTCTGGC[12] and master mix consisting of: 1X PCR buffer, 3.5 mM $MgCl_2$, 2.5 U Taq DNA polymerase, 0.2 mM dNTP Mix and 3µL of DNA template (10 µg/mL). DNA amplification was carried out in a PCR thermocycler (2720 thermal cycler, Applied Biosystems) with the following thermal cycling profile: an initial denaturation step at 94^0C for 10 min, followed by 25 cycles of amplification (94^0C for 60 s, 55^0C for 60 s, and 72^0C for 60 s), and an extension at 72^0C for 5 min.

The amplified products were then subjected to electrophoresis on a 1.5% agarose gel stained with ethidium bromide. A 100-bp DNA ladder was run in each gel, and the presence of 348 bp band was considered positive for presence of aac (6′)-Ie-aph (2″)-IA gene. The results were documented using Gel Doc system (Bio-Rad, USA). Data generated was analyzed using Statistical Package for Social Sciences (SPSS). Chi square was used to detect statistically significant correlation among variables. Significance is defined as $P \leq 0.05$.

RESULTS

Out of the total 146 Enterococcal isolates, 112 were E. fecalis, 33 were E faecium and 1 was E gallinarum. The most common site of infection by Enterococci was urine (82%) followed by pus/wound (10.3 %). Figure 1 shows the number of isolates from common body sites. Out of 146 isolates 38 (26.02%) were resistant to High level gentamicin, and 22 (15%) were resistant to streptomycin. 62% were resistant to penicillin and 81 % were resistant to erythromycin. Table 1 depicts the resistance pattern of the isolates towards various antibiotics.

Vancomycin resistance enterococci (VRE) was seen in 8 (5.4 %) cases. 6 were E. faecium and 2 were E. fecalis. 4 VRE showed MIC ≥ 32µg/ml, 1 had MIC ≥ 64µg/ml whereas 3 showed MIC≥128µg/ml. 6 out of 8 VRE were also resistant to Teicoplanin. Seven out of the eight VRE isolates were HLGR. Among the 38 HLGR Enterococci, 27 were E. faecium (71%) and 11 were E. fecalis (28%). Hence the number of HLGR among E. faecium (81%) was significantly higher than of the E. fecalis isolates (10%) ($p<0.05$).

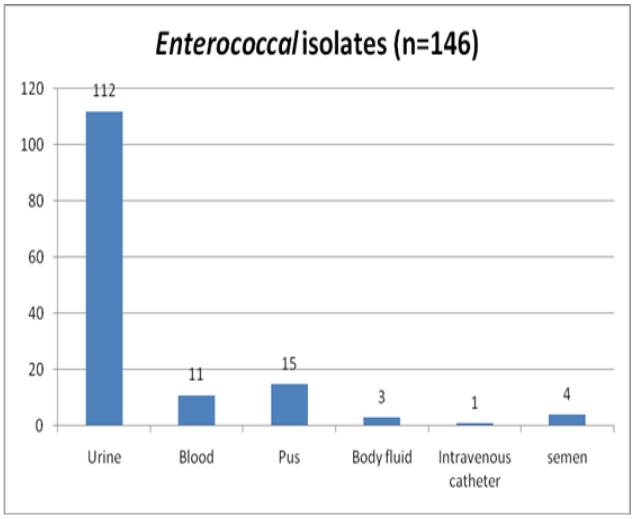

Figure 1: Enterococcus isolated from various body sites.

High level streptomycin resistance (HLSR) was seen in 9 E. fecalis isolates. All of these 9 isolates were also resistant to high level gentamicin. Among the E. faecium 11 were both HLSR and HLGR and 2 were only HLSR. Table 2 depicts the distribution of HLAR.

Table 1: Percentage resistance of Enterococcus to various antibiotics.

Antibiotic	Resistance %
Amoxycillin (10 µg)	46.7%
Penicillin(10 units/disc)	62%
Norfloxacin (10µg)	75.6%
Erythromycin (15µg)	81.8%
high level Gentamicin (120µg)	26.02%
Streptomycin (300 µg)	15%
Vancomycin (30 µg)	5.4%
Teicoplanin (30 µg)	11.2%
Linezolid(30µg)	00
ciprofloxacin(5 µg)	55%
levoflocxacin (5µg)	51.7%
Ampicillin- clavulanic acid (20/10 µg)	14%
Nitrofurantoin (300 µg)	14.2%
tetracycline (30 µg)	30%
chloramphenicol (30 µg)	27%

Out of the 38 HLG isolates, 36 had gentamicin MIC ≥512 µg/mL and 2 showed Gentamicin MIC ≥768 µg/mL. The high level aminoglycoside resistant (HLAR) isolates showed varying degree of resistance to different antibiotics. High degree of resistance was seen towards β-lactam antibiotics (penicillin and amoxicillin). All the HLAR E. fecalis and 96.2% E. faecium were resistant to erythromycin.

Table 2: HLAR Enterococci.

Enterococcus species	HLGR	HLSR	HLGR + HLSR
E. faecalis(n=112)	11(09.8%)	09(08%)	09(08%)
E. faecium(n=33)	27(81.8%)	13(39.3%)	11(33.3%)
Total (n=145)	38(26.2%)	22(15%)	20(13.7%)

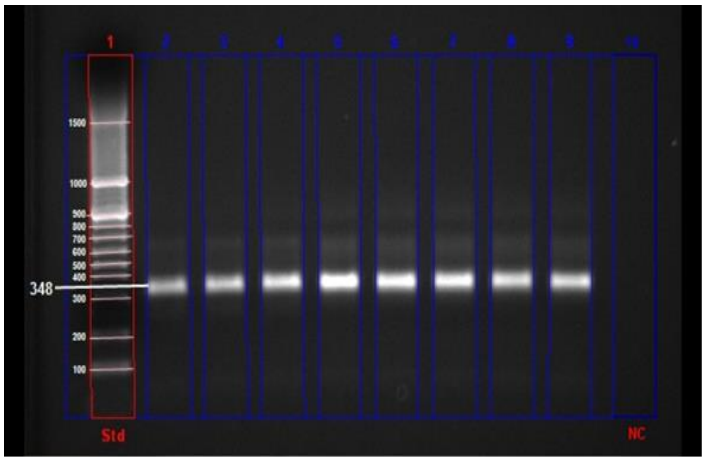

Figure 2: Amplified gentamicin resistant gene from HLGR isolates (1- DNA ladder, 2 to 9- positive isolates (348 bp), 10- negative control).

Resistance was less against Chloramphenicol and Ampicillin- clavulanic acid. No resistance was seen against Linezolid. (Table: 3) The difference in resistance pattern of HLAR E. fecalis and E. faecium was statistically insignificant (P>0.05).

Table 3: Resistance pattern of HLAR E. faecalis and E. faecium to other antibiotics.

Antibiotic	HLAR(n=38)	
	E. faecalis (n=11)	E. faecium (n=27)
Penicillin*	90.9%	88.8%
Amoxicillin	72.2%	81.4%
Erythromycin	100%	96.2%
Ciprofloxacin	63.6%	70.3%
Tetracycline	54.5%	59.2%
Chloramphenicol	45.4%	37%
Norfloxacin	72.7%	74%
Linezolid	-	-
Levofloxacin	54.5%	59.2%
Ampicillin-clavulanic acid	36.3%	33.3%

The electrophoresis of the amplified product revealed presence of aac (6′)-Ie-aph (2″)-IA gene with 348 bp in 36 out of 38 HLGR isolates (Figure 2). Among these 11 were E. fecalis and 25 were E. faecium. The 2 E. faecium isolates which didn't show presence of aac (6′)-Ie-aph (2″)-Ia gene, were both positive for HLGR and HLSR.

Moreover one of the isolate negative for the gene, had gentamicin MIC ≥768 µg/mL.

DISCUSSION

The prevalence of Enterococcal infection has increased with time in India and throughout the world. The organism which was related to mainly endocarditis once is now isolated from nearly every infective condition.[13] Worldwide, the most common site of infection is urinary tract, followed by abscesses and blood stream.[14] The isolation pattern was similar in the present study. Comparable findings were seen in another study conducted in Amritsar by Oberoi et al in Amritsar in 2009.[15] The isolation rate of E. fecalis (76%) was much higher than E. faecium (22%), which is comparable to findings in many Indian studies.[16,17] But few studies, like one conducted by Karmakar et al in Mumbai reported much higher isolation rate of E. faecium than that of E. fecalis.[18]

The antibiotics sensitivity profile is not much different from other studies conducted around India. The 62% resistance towards penicillin was in accordance to the result obtained by Gupta et al in a study conducted in another North Indian city, Chandigarh.[17] The present study showed high level of resistance towards erythromycin which is similar to the findings of Mathur et al.[19] There was no resistance identified towards Linezolid similar to most studies around the world.[17,20,21,22]

High level aminoglycoside resistance is mediated by few aminoglycoside modifying enzymes. The most common is the 6'-acetyltransferase-2'-phosphotransferase which confers resistance not only to gentamicin, but also to kanamycin, Tobramycin, Amikacin and netilmicin (not to streptomycin).[23] 3' phosphotransferase confers resistance to kanamycin and Amikacin but not to gentamicin, where as 6' adenyl transferase causes resistance only to streptomycin.[23] In the present study the percentage of HLGR was just above 26% which is comparatively lower than other Indian studies.[16,24,25] Among the individual species the percentage of HLGR isolates was much higher in E. faecium (81.8%) than in E. fecalis (9.8%). The finding was significant statistically ($p<0.05$) and comparable to other studies.[16,17]

Most of the HLSR strains isolated in the present study were also HLGR, except two which showed resistance only to streptomycin. Both of these isolates were E. faecium. This can be attributed to the fact that High-level streptomycin resistance may be due to mutation of a ribosomal protein or due to the production of enzyme streptomycin adenyltransferase due to the genes Ant(6)-Ia or Ant(3'')-Ia.[26] These strains while being resistant to streptomycin, can remain susceptible to gentamicin. There was no statistically significant difference in the incidence of combined HLGR and HLSR strains among the HLAR isolates of E. fecalis and E . faecium.

The HLGR isolates showed relatively higher resistance to other antibiotics. Resistance to penicillin and amoxicillin was nearly 90% and 80% respectively. In Enterococcus the production of Beta-lactamase enzyme occur due to gene encoded on a transferable plasmid which also carries high-level gentamicin resistance. Hence HLGR strains may have higher resistance towards the β-lactum group. Higher resistance was shown towards other drugs like erythromycin and ciprofloxacin. The study conducted by Mendiratta et al[16] showed similar result. The resistance towards choramphenicol was relatively lower when compared to other studies. Even though HLGR stains E faecium was found to be more multi-drug resistant than that of E. fecalis, the difference was statistically insignificant.

A combined treatment of penicillin and gentamicin had been used against enterococcal infections over the years. But with the emergence of high level aminoglycoside resistance (HLAR), vancomycin became the only alternative available.[4] Presently there is steep increase in vancomycin-resistant Enterococci (VRE) strains in clinical isolates throughout the world. The situation is much complicated now by the fact that Enterococci have developed a number of mechanisms for the transfer of resistance genes.[27] The basic mechanism of Vancomycin resistance in Enterococci is the formation of peptidoglycan receptors with reduced glycopeptide affinity. This results in decreased binding of Vancomycin and decreased inhibition of cell wall synthesis. Six glycopeptide-resistant enterococcal phenotypes VanA, VanB, VanC, VanD, VanE, and VanG, are known. Recently, new gene clusters encoding for vancomycin resistance have been discovered (vanL, vanM,and vanN).[28] They can usually be distinguished on the basis of the level, inducibility, and transferability of resistance to vancomycin and teicoplanin. VanA and VanB, are most relevant clinically. Most Indian studies show presence of Van A and Van B phenotype.[15, 29] Taneja et al reported Van C phenotype along with Van B.[30] In the present study the 6 out 8 VRE isolates appeared to be of Van A phenotype (resistant to both vancomycin and teicoplanin) and the other 2 were of Van B phenotype (resistant to vancomycin but sensitive to teicoplanin). But the finding needs to be further substantiated by molecular characterization of the isolates for vancomycin resistant gene.

The molecular analysis in the present study showed presence of the gene aac(6′)-Ie-aph(2″)-Ia in 36 out of 38 HLGR strains. The gene has been recognized to be the part of transposon Tn5281. The association with the transposon aids in the rapid spread of the gene.[31] The gene has been found present in nearly every HLGR isolates identified in various studies conducted in India[16] and worldwide.[32] In the study conducted by Tsai et al the aac (6′)-Ie-aph (2″)-Ia was found in 79% isolates.[33] Other genes responsible for gentamicin resistance like aph (2")-Ib, aph (2'')-Ic, and aph(2)-Id were detected in 5%, 1.6% and 14% isolates respectively. Aph (2") - Ib is the newest

gene identified for HLGR .This does not codes for resistance to amikacin and streptomycin. In Enterococci with aph (2'')-Ic gene, the gentamicin MIC is 256–384mg/mL and is mistakenly considered susceptible to ampicillin-gentamicin synergism when they are actually resistant to it. The aph (2")-Id gene is responsible for production of enzyme aminoglycoside phosphotransferase. The strains are sensitive to Amikacin and streptomycin but have MIC≥2000 µg/ml for other aminoglycosides.[26] The present study should be further extended for detection of these genes in the HLGR isolates. The presence of aac (6′)-Ie-aph (2″)-Ia in the present study much higher in E. faecium (75%) than E. fecalis (10%) and the finding was statistically significant. There were two E. faecium isolates with only high level streptomycin resistance but sensitive to high level gentamicin. This may be due to the presence of gene ant (3")-Ia or ant (6")-Ia and should be further looked into.

CONCLUSION

The present study underlines the spread of the gene aac (6′)-Ie-aph (2″)-Ia among the enterococcal isolates which can be easily transferred to other pathogenic gram positive cocci. Even though the incidence of HLGR strains was comparatively lesser than other similar studies, the threat of multi drug resistant enterococcal infection cannot be ignored. Moreover the identification of VRE strains makes the situation much more severe. This emphasizes on the need for continuous monitoring of aminoglycoside resistance and also the need to identify newer and effective aminoglycosides.

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