Original Research Article

High-Level Aminoglycoside Resistance and Distribution of Aminoglycoside Resistant Genes among Clinical Isolates of Enterococcusin a Tertiary Care Hospital

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Abstract

Objectives: The aim of study was to determine the susceptibility of clinical isolates of Enterococcus species to high level aminoglycoside by MIC test and the presence of five different aminoglycoside modifying genes [AMEs]. **Methods:** Enterococci were isolated from various clinical samples. High level resistance to gentamicin and streptomycin was done by high potency disc diffusion method [HPDDM]. Screenings to both the antibiotics were done by agar screen method [ASM]. Minimum Inhibitory Concentration [MIC] was determined by Agar Dilution Method [ADM]. Multiplex PCR was used to detect the presence of AME genes. **Results:** 21.4% [24/112] and 25.8% [29/112] strains were resistant to gentamicin and streptomycin by ASM. A total of 32.2% [36/112] were found to be HLGR with MIC > 512µg/ml. 29 strains were found to show resistance to streptomycin with MIC i.e. ≥ 2048 µg/ml.aac(6′)-Ie-aph(2″)-Ia gene was found in 16.9% [19/112] of enterococcal isolates. Moreover, 4.5% (5/112) of the Non-HLAR strains with MIC [256 µg/ml] expressed aac(6′)-Ie-aph(2″)-Ia gene. Newer AME genes like aph(2″)-Ic&aph(2″)-Id were detected in 4.5% [5/112] and 5.4% [6/112] strains. The predominant virulence gene in HLAR was hyl gene [44.1%; 30/68]. **Conclusions:** The study concluded that the AMEs have disseminated amongst the non-faecalis non-faecium strains in this region. The number of aac(6")-Ie-aph(2")-Ia genes detected by PCR was less as compared to those detected by MIC test, it should be taken into consideration that due to the intrinsic limitations of any PCR assay, a negative result may not always signify the absence of a gene altogether in enterococcus.

Keywords: Aminoglycoside Modifying Enzymes, High Level Aminoglycoside Resistance, Non High-Level Aminoglycoside Resistant Strains.

Introduction

Enterococci are nosocomial pathogensand exhibit gentamicin resistance with MIC [500µg/mlto 1024µg/ml] by aac(6")-Ie-aph(2")-Ia. Recently, newer AME genes such as aph(2")-Ib, aph(2")-Ic, and aph(2")-Id have been detected as thoseconferring gentamicin resistance in enterococci. High level streptomycin and kanamycin resistance in enterococci are mediated by aph(3")-IIIa gene[1,2].

If the recently detected aminoglycoside resistance genes become more prevalent among clinical isolates, the approachfor detecting susceptibility to aminoglycoside synergism in enterococci will require modification.

Aims and objectives

The aim of the present study was to determine the high-level aminoglycoside resistance [HLAR] in enterococcal isolates by MIC test and the use of multiplex PCR to evaluate the presence of five different aminoglycoside modifying genes, in enterococcal isolates in this geographic region.

Materials &methods

Study Population

The study population included patients of both genders and all age groups attending the outpatient and inpatient departments of a tertiary care hospital in eastern Bihar, India. A total of 112 strains of enterococci were collected from samples selected at random and submitted to the Microbiology laboratory for culture and sensitivity. Ethical Clearance was obtained from Institutional Ethics Committee to carry out this study vide KMC/IEC/Dept Res/004/2019-2022 (Microbiology) dated 20/04/2019. The study was conducted during the period November 2019 to April 2021.

Isolation &Identification

One hundred and twelve enterococci were isolated from various clinical samples [urine, pus, blood, catheter tip]. All samples were inoculated on blood agar and McConkey's agar followed by overnight incubation at 37^{0}C.The isolates were identified to species level using standard procedures[3].

Antimicrobial Susceptibility Testing

Antibiotic susceptibility test was done by Kirby-Bauer disc diffusion method on Mueller-Hinton agar. Inoculum was prepared and adjusted to 0.5 Mc Farland's turbidity standard. Antibiotic discs were obtained from HiMedia, Mumbai, India[4].

Detection of HLAR in Enterococci by Disc-Diffusion and Agar Screen Methods

High level [120µg] gentamicin and streptomycin [300µg] disks were placed on the agar medium. Resistance was indicated by no zone; and susceptibility, by a zone of diameter 10 mm[4,5].

In agar-screen method, brain heart infusion agar BHIA [HiMedia, Mumbai, India] was supplemented with 500 µg/ml gentamicin and 2000 µg/ml of streptomycin. Presence of more than one colony or a

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haze of growth was read as resistant. The test was quality controlled using *E. faecalis* ATCC 29212 [susceptible] and *E. faecalis* ATCC 51299 [resistant].

Determination of Minimum Inhibitory Concentration by Agar Dilution Method

Agar dilution method was used to determine MIC, as per standard protocol. Brain-heart infusion agar [HiMedia, Mumbai] was supplemented with different concentrations of ampicillin,vancomycin, gentamicin and streptomycin. The minimum concentration of antibiotics, which inhibited bacterial growth, was considered MIC.*E. faecalis*ATCC 29212 was used as a negative control strain. Enterococci which had MICof ≥16µg/ml followed by ≥ 32µg/ml, ≥1048µg/ml and ≥2048 µgm/mlwere considered resistant to ampicillin, vancomycin, gentamicin and streptomycin respectively, and MIC of ≤ 8 µg/mlas susceptible to ampicillin followed by MIC of ≤4 µg/ml as susceptible to vancomycin and MIC of ≤ 510 µg/ml and ≤ 1000 µg/ml as susceptible to gentamicin and streptomycin, respectively[4].

DNA Extraction Method

Genomic DNA used as template for PCR amplification was prepared using conventional phenol-chloroform DNA extraction method[8]. Primers were obtained from Merck Specialities, Lucknow, India**[Table1]**[6].

PCR Assay for AMEs

Amplification was performed with PCR system and the cycling programs designed as per standard protocol. Each amplification product was resolved by electrophoresis with a 100-base pair molecular weight marker in a 1.2% agarose-Trisborate-EDTA gel stained with ethidiumbromide[0.5 µg/ml] and visualized under gel documentation system[6,7,8].

Statistical Analysis

Statistical analysis of data was done using online application available at the website link

http://www.physics.cbsju.edu/stats/contingency_NROW_NCOLUMN_form.html. P-values were calculated and P<0.05 was considered statistically significant, while P<0.001 was highly significant.

Observations

Out of the 112 enterococcal strains isolated from clinical samples, 37.5% [42/112] were identified as *E. faecalis*, 29.5% [33/112] and E. faecium, 13.4% [15/112]. Urinary tract infection [UTI] was found to be caused by 66.0% [74/112] isolates followed by 18.7% [21/112] isolates causing wound infection and 15.2% [17/112] isolates causing blood stream infection [BSI]. Of the urinary isolates, *E. faecalis*40.5% was the commonest isolates; E. faecium 33.3% [7/21] in case of wound infections was the main isolate.

All the 112Enterococcus strains were initially screened by ASM which revealed21.4%[24/112] and 25.8%[29/112] strains as resistant to gentamicin and streptomycin. However, by HPDDM15.2%[17/112] and 14.3%[16/112] strains were resistant to gentamicin and streptomycin and9.8% [11/112]strains showed combined resistance. Maximum resistance to gentamicin was seen in *E. dispar*. HLSR was most commonly seen in 20.0% strains each of *E. mundtii* [1/5], *E. solitarius* [1/5] &*E. dispar* [1/5] and 15.2% [5/33] strains of E. faecium exhibited combined resistance.

A total of 32.2% [36/112] were found to be HLGR with MIC [>5124µg/ml].MIC of various clinical strains showed 7 strains to have reduced susceptibility to gentamicini.e.MIC ≤512 µg/ml. Another 23Enterococcusstrainshad [MIC ≤ 1024 µg/ml].Two strains each of *E. faecalis* and E. faecium and 1 strain of *E. mundtii*&*E. gallinarum*had [MIC ≥2048 µg/ml][Table 2].

On the other hand, 29 strains were found to show resistance to streptomycin with MIC i.e., ≥ 2048 µg/ml. A total of 13 strains of *E. faecalis* followed by 11 strains of E. faeciumand1 strain each of *E. gallinarum*,*E. mundtii*, *E. pseudoavium*, *E. solitarius*&*E. dispar*were streptomycin resistant[Table 3].

The aac(6′)-Ie-aph(2″)-Ia gene was found in 16.9%[19/112] of enterococcal isolates that were resistant to gentamicin with MIC [≥ 512µg/ml]. Of the36 HLGR strains identified by MIC method, only 19 strains carried aac(6′)-Ie-aph(2″)-Ia gene and remaining 17 strains that were HLGR didnot carry any gene. Amongst the 19 strains that expressed aac (6′)-Ie-aph(2″)-Iagene, 21.0% were of E. faecium, 20.0%strains were *E. solitarius* and *E. dispar* each, 19.0% of *E. faecalis*, 14.3% of *E. pseudoavium* and 6.6% strains of *E. gallinarum*[Table 3].

PCR analysis showed the presence of gentamicin resistance genes even in susceptible strains. 4.5% [5/112] of the Enterococcus strains that was susceptible to gentamicin with MIC ≥ 256 µg/mlwere found to express aph (2'')-Ic gene. Of these strains, 9.5% [4/42] were *E. faecalis* and 3.0% [1/33] were E. faecium[Table 3].

25.8% [29/112]of the strains expressed streptomycin resistance genes aph(3′)-IIIa both by phenotypic and genotypic methods. Among the strains carrying aph(3′)-IIIa gene, 30.9% were of *E. faecalis* followed by 30.3% of E. faecium, 20.0% each of *E. mundtii*, *E. solitarius*&*E. dispar*, 14.3% of *E. pseudoavium*and13.3% of *E. gallinarum*[Table 3].

Of the 9.8% [11/112] HLAR isolates which were resistant to both gentamicin [MIC ≥ 1024 µg/ml] and streptomycin [MIC ≥ 2048 µg/ml] by MIC, 3.6% [4/112]isolates carried both aac(6′)-Ie-aph(2″)-Ia and aph(3′)-IIIa genes and the remaining 0.9%[1/112] isolates had one or the other of the genes aac(6′)-Ie-aph(2′)-Iaandaph(3′)-IIIa[Table3].

The presence of both aac(6′)-Ie-aph(2″)-Iaandaph(3′)-IIIa were seenin 7.1% [3/42]of *E. faecalis* and 6.6% [1/15]of *E. gallinarum*. Overall, 4.5% [5/112] strains were found to express the aph(2″)-Ic the commonest being, 14.3% [1/7] of *E. pseudoavium*, *E. faecalis* 4.8% [2/42], *E. gallinarum* 6.6%[1/15] and E. faecium 3.0%[1/33]. 5.4% [6/112] strains expressed the aph(2″)-Id gene of which 20.0% [1/5] was *E. solitarius* followed by *E. faecalis* 7.2% [3/42] and E. faecium 6.1%[2/33] [Table 3].

The predominant virulence gene in HLAR &Non-HLAR isolates was hyl gene, being, 44.1% [30/68] and 50.0% [22/44] respectively. In contrast, the gene that was detected the least was cyl A, being 19.1% [13/68] and 20.5% [20.5%], respectively. None of the values were statistically significant.The differences in the presence of different virulence markers like esp [32.4% vs 20.5%] and asa1 [39.7% vs 25.0%] in HLAR and Non-HLAR isolates was statistically insignificant [P= 0.169 & P = 0.108] [Table 4].

Enteococcus faecium showed maximum resistance to penicillin and ciprofloxacin [90.0% each] followed by piperacillin [81.8%]. *E. faecalis* showed maximum resistance to penicillin [92.9%] and ampicillin & imipenem [73.8% each]. Amongst the non-faecalis non-faecium strains, both *E. solitarius* and *E. pseudoavium* showed 100% resistance to penicillin. Vancomycin resistance was seen in 18.2% E. faecium followed by 16.7% *E. faecalis* and 6.7% *E. gallinarum*[Table 5].

Table.1: Primers and their Sequences for Aminoglycoside Resistance Genes & Virulence Markers used in Multiplex PCR

Genes	Primer sequences (5'-3')	Size of PCR product (bp)
aac(6")-Ie-aph(2")-Ia	F: CAGGAATTTATCGAAAATGGTAGAAAAG R: CACAATCGACTAAAGAGTACCAATC	369
aph(2")-Ib	F: CTTGGACGCTGAGATATATGAGCAC R: GTTTGTAGCAATTCAGAAACACCCTT	867
aph(2")-Ic	F: CCACAATGATAATGACTCAGTTCCC R: CCACAGCTTCCGATAGCAAGAG	444
aph(2")-Id	F: GTGGTTTTTACAGGAATGCCATC R: CCCTCTTCATACCAATCCATATAACC	641

aph(3")-IIIa	F: GGCTAAAATGAGAATATCACCGG R: CTTTAAAAAATCATACAGCTCGCG	523
asa1	ASA11- GCACGCTATTACGAACTATGA ASA12- TAAGAAAGAACATCACCACGA	375
cyl A	CYT I- ACTCGGGGATTGATAGGC CYT IIb- GCTGCTAAAGCTGCGCTT	
gel E	GEL 11- TATGACAATGCTTTTTGGGAT GEL 12- AGATGCACCCGAAATAATATA	100
Esp	ESP 14F- AGATTTCATCTTTGATTCTTGG ESP 12R- AATTGATTCTTTAGCATCTGG	570
Hyl	HYL n1- ACAGAAGAGCTGCAGGAAATG HYL n2- GACTGACGTCCAAGTTTCCAA	200

Table 2: Results of High-Level Aminoglycoside Resistance by MIC Test among Enterococcus Species

Clinical	MIC of gentamicin [µg/ml]									MIC of streptomycin [µg/ml]								
	≤ 8	16	32	64	128	256	512	1024	≥2048	≤ 8	16	32	64	128	256	512	1024	≥2048
E. faecalis[n=42]	0	2	2	9	1	11	4	11	2	0	0	0	6	2	0	7	14	13
E. faecium [n=33]	0	0	3	8	4	7	1	8	2	0	0	0	2	0	0	6	14	11
E. mundtii [n=5]	1	0	0	2	1	0	0	0	1	0	0	0	2	2	0	0	0	1
E. solitaries [n= 5]	0	2	1	1	0	0	0	1	0	0	0	0	2	2	0	0	0	1
E. pseudoavium [n= 7]	0	0	1	1	4	0	1	0	0	0	0	0	3	3	0	0	0	1
E. gallinarum [n=15]	8	0	3	0	0	0	0	3	1	0	0	0	0	5	3	0	6	1
E. dispar [n=5]	1	2	1	0	0	0	1	0	0	0	0	0	0	0	2	1	1	1
Total =112	10	6	11	21	10	18	7	23	6	0	0	0	15	14	5	14	35	29

Table 3: Results of High-Level Aminoglycoside Resistance and Distribution of Aminoglycoside Modifying Enzyme Encoding Genes Among Enterococcus Species

HLGR/HLSR Phenotypes by MIC test	Distribution of High-Level Aminoglycoside resistance in E. species							
	***E. faecalis*, n= 42**	***E. faecium*, n= 33**	***E. gallinarum*, n=15**	***E. mundtii*, n=5**	***E. pseudoavium*, n=7**	***E. solitarius*, n=5**	***E. dispar*, n=5**	**Total n=112**
HLGR [512 µg - ≥ 2048 µg/ml]	17 [40.5%]	11 [33.3%]	4 [26.6%]	1 [20.0%]	1 [14.3%]	1 [20.0%]	1 [20.0%]	36 [32.2%]
HLSR [≥ 2048 µg/ml]	13 [30.9%]	10 [30.3%]	2 [13.3%]	1 [20.0%]	1 [14.3%]	1 [20.0%]	1 [20.0%]	29 [25.8%]
HLAR [≥ 1024µg for gentamicin +≥ 2048µg/ml for streptomycin]	6 [14.3%]	4 [12.1%]	1 [6.6%]	0	0	0	0	11 [9.8%]
Detection of gentamicin and streptomycin resistant genes by PCR	***E. faecalis*, n= 42**	***E. faecium*, n= 33**	***E. gallinarum*, n=15**	***E. mundtii*, n=5**	***E. pseudoavium*, n=7**	***E. solitarius*, n=5**	***E. dispar*, n=5**	**Total n=112**
Aac(6')-Ie-aph[2'']-Iain strains with MIC [512 µg/ml - ≥ 2048 µg/ml]	8 [19.0%]	7 [21.2%]	1 [6.6%]	0	1 [14.3%]	1 [20.0%]	1 [20.0%]	19 [16.9%]
aph(3')-IIIa in strainswith [≥ 2048 µg]	13 [30.9%]	10 [30.3%]	2 [13.3%]	1 [20.0%]	1 [14.3%]	1 [20.0%]	1 [20.0%]	29 [25.8%]
aac(6')-Ie-aph(2'')-Ia + aph(3')-IIIa in strains with MIC [≥ 1024 µg/ml for gentamicin +≥ 2048µg/ml for streptomycin]	3 [7.1%]	0	1 [6.6%]	0	0	0	0	4 [3.6%]
aac(6')-Ie-aph(2'')-Ia in strains with MIC [256 µg]	4 [9.5%]	1 [3.0%]	0	0	0	0	0	5 [4.5%]
aph(2'')-Ib	0	0	0	0	0	0	0	0
aph(2'')-Ic	2 [4.8%]	1 [3.0%]	1 [6.6%]	0	1 [14.3%]	0	0	5 [4.5%]
aph(2'')-Id	3 [7.2%]	2 [6.1%]	0	0	0	1 [20.0%]	0	6 [5.4%]

Table 4: Presence of Virulence Genes in HLAR and Non-HLAR Strains

Genes of Virulence Markers	HLAR [HLGR+ HLSR=68]	Non-HLAR Strains [n=44]
gelE*	25 [36.7%]	16 [36.3%]
cyl A**	13 [19.1%]	9 [20.5%]
esp***	22 [32.4%]	9 [20.5%]
hyl****	30 [44.1%]	22 [50.0%]
asa1*****	27 [39.7%]	11 [25.0%]
* P = 0.966; **P = 0.862; ***P = 0.169, ****P=0.106, *****P=0.108		

Table 5: Antibiotic Resistance Pattern of Clinical Isolates

Antibiotics	*E. faecalis* n=42	E. faecium n=33	*E. gallinarum* n=15	*E. mundtii* n=5	*E. solitarius* n=5	*E. dispar* n=5	*E. pseudoavium* n=7
Ampicillin	31 [73.8%]	18 [54.6%]	3 [20.0%]	1 [20.0%]	1 [20.0%]	1 [20.0%]	2 [28.6%]
Penicillin	39 [92.9%]	30 [90.0%]	9 [60.0%]	4 [80.0%]	5 [100%]	1 [20.0%]	7 [100%]
Piperacillin	38 [41.8%]	27 [81.8%]	5 [33.3%]	1 [20.0%]	1 [20.0%]	1 [20.0%]	1 [14.3%]
Tetracycline	26 [61.9%]	21 [63.6%]	4 [26.7%]	0	0	0	2 [28.6%]
Erythromycin	22 [52.4%]	19 [57.6%]	2 [13.3%]	0	0	0	2 [28.6%]
Ciprofloxacin	29 [69.1%]	30 [90.0%]	6 [40.0%]	2 [20.0%]	2 [20.0%]	2 [40.0%]	2 [28.6%]
Imipenem	31 [73.8%]	18 [54.6%]	3 [20.0%]	1 [20.0%]	1 [20.0%]	1 [20.0%]	2 [28.6%]
Teicoplanin	2 [4.8%]	1 [3.0%]	0	0	0	0	0
Vancomycin	7 [16.7%]	6 [18.2%]	1 [6.7%]	0	0	0	0
Linezolid	3 [7.1%]	1 [3.0%]	0	0	0	0	0
Nitrofurantoin was used for urinary isolates. Of the 91 isolates, 39.6% showed complete resistance, 5.5% showed intermediate resistance and 53.0% showed sensitivity							

Discussions

The major aim of the study was to identify the recently detected aminoglycoside resistance genes i.e.aph(2')-Ib, aph(200)-Icand aph(2')-Id besides the gentamicin and streptomycin resistant genes by multiplex PCR, because, if these genes become more prevalent among clinical isolates, the approach for detecting susceptibility to aminoglycoside synergism in enterococci will require modification[6,7].

Our test result showed *Enterococcus faecalis* [37.5%] was the main isolate followed by Enterococcus faecium [29.5%]. Among the unusual species *Enterococcus gallinarum* [13.4%], followed by *Enterococcus pseudoavium* [6.3%] and *Enterococcus mundtii*, *Enterococcus solitarius* and *Enterococcus dispar*[4.5% each] were the major isolates. Astudy from Rohtak showed the distribution of common and unusual species of enterococcus species from different clinical samples. They identified *Enterococcus faecalis* 72.3% [180/260] to be the predominant species, followed by Enterococcus faecium 17.3% [45/260], unspeciated Enterococcus 4.6% [12/260], *Enterococcus raffinosus* 2.3% [8/260], *Enterococcus durans* 2.3% [6/260], *Enterococcus casseliflavus* 1.9% [5/260], and *Enterococcus dispar* 1.5% [4/260][9].

21.4% & 25.8% strains were resistant to gentamicin and streptomycin by ASM whereas high potency disk diffusion test found 15.2% & 14.3% strains to be resistant to gentamicin and streptomycin. Similar findings were seen in other studies where ASM could detect 27.8% [50/180] strains to be resistant to gentamicin, whereas, only 25.6% [46/180] strains showed resistance by HPDDM. It is possible that disc-diffusion method may not detect borderline resistance in Enterococcus. The result of the present study indicates that ASM must be used to confirm HLAR in enterococci.

Out of the 36 HLGR identified by MIC method, only 19 strains carried aac(6′)-Ie-aph(2″)-Ia gene.17 strains did not carry the above gene and thus would be falsely deemed to be resistant to gentamicin. Moreover, 7.2% *E. faecalis* and 6.1% *E. faecium* were simultaneously found to express aac(6′)-Ie-aph(2″)-Ia gene and aph(2")-Idgene. Report says that such strains retainsusceptibility to ampicillin-amikacin synergism.Similar findings were seen in another study, where, out of 76 strains of HLGR identified by MIC method, only 52 strains [68.4%] carried aac(6′)-Ie-aph(2″)-Ia gene. 31.57% [24/76] of the isolates did not carry any of the genes[10].

Similarly, 25.9% [29/112] of the strains expressed streptomycin resistance genes aph(3′)-IIIa both by phenotypic and genotypic methods. Of the corresponding genes expressed by HLSR, 30.9% were *E. faecalis* followed by 30.3% of *E. faecium*, 14.3% of *E. pseudoavium*, 13.3% of *E. gallinarum* and 20.0% each of *E. mundtii*, *E. solitarius*&*E. dispar*. HLSR [MIC 1024 µg/ml] in enterococci could be due to a single mutation in ribosomal protein or enzymatic inactivation by AMEs encoded by aph(3')-IIIa genes. Other authors detected the HLSR gene in 32% of isolates, which is higher than in our studies[11]. Li *et al*, and Ramin*et al* reported a high rate of prevalence of HLSR gene, being 56% and 49%. The differences in the detection rate could possibly be due to the horizontal transfer of the resistance factors, since HLAR genes are located on plasmid and conjugative transposons[12]. Findings of other studies show that in agar dilution tests, a total of 13 [15%] Enterococcus faecium and 15 [17%] *Enterococcus faecalis*isolates were highly resistant to streptomycin [MIC ≥ 2,000 µg/ml]. Out of 85 Enterococcusfaecium isolates, 14 [16%] strains and out of 90 *Enterococcus faecalis*isolates, 12 [13%] strains were intermediate resistant to gentamicin [MIC≥500 µg/ml]. High level resistances to streptomycin and gentamicin were found in 8 [9%] strains of Enterococcus faecium and 4 [4%] strains of *Enterococcus faecalis*[13].

Newer aminoglycoside resistance genes, such asaph(2")-Ic and aph(2")-Id, also found to encode high level resistance to gentamicin, were detected in our study isolates. Aph(2")-Ib is the newest gene identified for HLGR.This does not code for resistance to amikacin and streptomycin. The aph(2")-Id gene is responsible for production of enzyme aminoglycoside phosphotransferase which modifies gentamicin, tobramycin, kanamycin, netilmicin and dibekacin. These strains are sensitive to amikacin and streptomycin but have MIC≥2000 µg/ml for other aminoglycosides. Our study results showed the presence of aph(2″)-Id gene in 5.4% strainsandaph(2″)-Icgene in 4.5% of strains. 20.0% [1/5] of *E. solitarius*, 7.2% [3/42] of *E. faecalis*, 6.1% [2/33] ofE.faeciumwere found to express aph(2″)-Id gene with MIC [512-2048µg/ml). Moreover, these HLGR strains were deficient in aac(6′)-Ie-aph(2″)-Iagene. PCR if not done for such strains would be deemed to be resistant to amikacin too. Contrasting results were reported by other authors who did not find aph(2')-Id gene in the *E. faecalis* and E. faecium strains[14].

4.5% [5/112] of the Enterococcus strains susceptible to gentamicin with MIC ≥ 256 µg/ml were found to express aac(6′)-Ie-aph(2″)-Iagene. Enterococci with gentamicin MIC 256–384 µg/ml is mistakenly considered susceptible to ampicillin-gentamicin synergism, when they are actually resistant to it[15]. Such strains would be falsely deemed to be susceptible to all aminoglycosides inspite of carrying aac(6")-Ieaph (2")-Ia gene[16].

The differences in the presence of esp, asa1 &cylAgenes in HLAR and non-HLAR were insignificant[P=0.169, 0.108, 0.862] in our

study.In a study from China, 10 [33.3%] carried gelE; 8 [26.7%] carried efaA; 7 [23.3%] carried esp; 7 [23.3%] carried cylA; and 1 [3.3%] carried ace. The agggene was not detected in any of the isolates. Moreover, the authors determined the AME gene profiles and distributions of virulence determinants among HLGR or HLSR isolates, and found no significant correlations[15].

In another study by Li *et al*, espwas the most frequently identified virulence gene [50.6% of isolates], followed by hyl [28.8%][17]. These findings differed from the results of Zou *et al*., who showed that gelE was the most prevalent virulence gene, and that hyl and cylA were not detected,while Vankerckhoven, *et al*. detected esp in 65% of isolates, and gelE and cylA were not detected.[8,18] Comparison of virulence genes amongst HLAR and HLAS strains showed that only esp was significantly more prevalent in HLAR isolates than in HLAS isolates. Other than hyl and esp, all tested virulence genes, including ace, cylA, efaA, gelE, and asa1, were significantly more prevalent in *Enterococcus faecalis* compared with Enterococcus faecium. These differences indicate that virulence genes are present at different levels between human and animal isolates, and that with the passage of time, Enterococcus species have acquired an increasing number of virulence genes[18].

Findings of other authors showed that most frequent virulence gene was ace [88.6%],followedby esp [67.1%], PAI [45.5%]and sprE [41.7 %]. The frequency of ace,cylA and esp genes among *Enterococcus faecalis* isolates was significantly higher than Enterococcus faecium [$P < 0.05$]. All *Enterococcus faecalis* isolates carried at least one virulence gene. However, gelE, and cylA genes were not detected in Enterococcus faecium isolates[19].

The trends in antimicrobial susceptibility vary within, as well as between, countries and continents depending on various factors, which include the characteristics of the healthcare facility, infection control practices and antimicrobial use. The antibiotic resistance pattern of various clinical isolates shows that the E. faecium showed maximum resistance to penicillin and ciprofloxacin [90.0% each] followed by piperacillin [81.8%]. *E. faecalis* showed maximum resistance to penicillin [92.9%] and ampicillin & imipenem [73.8% each], and the unusual species of Enterococcus showed 100% resistance to penicillin. Similar findings were seen by other authors[20].

Conclusion

This study concluded that the AMEs have disseminated amongst the non-faecalisnon-faecium strains in this region. The number of aac(6")-Ie-aph(2")-Ia genes detected by PCR was less as compared to MIC test.It should be taken into consideration that due to the intrinsic limitations of any PCR assay, a negative result may not always signify the absence of a gene altogether in Enterococcus.

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