ORIGINAL ARTICLE

High-Level Aminoglycoside and Vancomycin Resistance in Enterococcus spp. Isolated from Hospital Acquired Infections, Ismailia, Egypt.

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ABSTRACT

Background: *Multidrug resistant Enterococci (MDR) are important hospital acquired infections (HAIs).* ***Objectives:*** *This study aimed to determine the genetic basis of resistance to aminoglycosides and vancomycin in enterococci strains isolated from hospitalized patients with HAIs.* ***Methodology:*** *The disc diffusion method was used to detect high-level aminoglycoside resistance (HLAR) by using gentamicin (120 µg) and streptomycin (300 µg) discs. Nested PCR was performed for the six-aminoglycoside resistance genes. Vancomycin resistance genes were discovered by conventional PCR.* ***Results:*** *In a total of 100 Enterococcus isolates, aac(6')-Ie-aph(2")-Ia gene was found in 30 strains, 25 of them were E.faecalis and the remaining were E.faecium. aph(3'). The presence of high levels of gentamycin and streptomycin resistance was more common in strains carrying aac(6')-1e-aph(2")-1a gene. We detected vanA gene in 9 isolates and van C in 15 isolates. vanB was not detected. E.faecium strains containing the vanA gene were shown to be resistant to vancomycin by MIC (MIC > 32 µg/mL), nitrofurantoin, ampicillin and penicillin (P<0.001). Van C was detected in 12 isolates of E.faecalis, 1 isolate of E.facium and 2 isolates of E.durans. E.faecalis carrying vanC gene was more resistant to aminoglycoside than vanC negative E.faecalis..* ***Conclusion:*** *E. faecalis has been seen to be more widespread than other Enterococcus species in our research. we found that the aac(6')-Ie-aph(2")-Ia and aph(3')-IIIa genes are more frequently found than other genes.*

INTRODUCTION

Multidrug resistant enterococci (MDR) are common pathogens that cause Health Care associated Infections (HAIs) in hospital settings[1] and are an increasingly common clinical issue for health care providers[2]. Infections caused by MDR enterococci can indeed be difficult to treat and has become a challenge to handle in the medical setting due to the widespread misuse of antimicrobial agents[3,4]

Enterococci are gram-positive cocci in pairs/chains that are facultatively anaerobic bacteria, reside in the gastrointestinal tract and usually function commensally with humans. They can, however, cause several infections, the most frequent of which is a urinary tract infection (UTI), intraabdominal infection, bacteremia, or endocarditis[4].

Resistance to many antibiotics were reported including aminoglycosides, B-lactams and glycopeptide[5]. Inherent antibiotic resistance, as well as the transmission of resistance genes through conjugative transposable elements and plasmid vector, play a significant role in the development MDR[6].

Changes in peptidoglycan formation that make up the bacterial cell wall leading to development of vancomycin resistance[7]. Vancomycin usually attaches to the D-Ala-D-Ala end of peptidoglycan protein precursors. As this end is modified to D-Ala-D-lactate resulting in development of resistance due to low affinity of the drug. This is encoded by genotypes that are alphabetically known as *vanA* to *vanG*, *vanM*, and *vanL*. The *vanA* and *vanB are* plasmid-based genotypes but *vanD* and *vanC* are chromosomal phenotypes[8].

Vancomycin Resistant Enterococci (VRE) develops resistance to aminoglycosides mostly due to the presence of 2'-phosphotransferase-6'-acetyltransferase enzyme, except in streptomycin resistance which caused by streptomycin adenyl-transferase production[9,10].

Despite these challenges, infection with VRE has been demonstrated to increase cost and mortality in comparison to vancomycin-susceptible strains[11,12].

Previous antimicrobial treatment is the most frequently examined risk factor for VRE infection. This process is likely to be due to changes in intestinal flora. In addition, those with serious underlying disorders, immunosuppressed or long-stay hospital patients are at higher risk[13].

Aminoglycosides alone are considered ineffective in treatment of these infections and are commonly paired with bacterial cell wall development inhibitors such as ampicillin or vancomycin[2]. Antibiotic therapy has been challenged by high-level aminoglycoside resistant (HLAR) and VRE[2,14].

Acquired genes that result in high levels of aminoglycoside resistance were reported resulting in high-level gentamicin resistance (HLGR, MIC 500 g/ml) and high-level resistance to streptomycin (HLSR, MIC ~2000 µg/ml) [15,16].

Due to lack of researches in our institute about vancomycin and aminoglycoside resistance, the current work designed to provide an overview on the frequency of HLAR and VRE strains in Suez Canal University Hospital (SCUH), Ismailia, Egypt.

METHODOLOGY

Collection of specimens

Various clinical specimens (wound, blood, urine, sputum, etc.) were collected; under aseptic conditions from patients admitted at Suez Canal University hospitals suffering from HAIs. Specimens were collected transported and processed in the Microbiology and Immunology Department, Faculty of Medicine, Suez Canal University.

Isolation and identification of Enterococcus spp.

Gram staining, colony morphology, catalase test, bile solubility, growth in sodium chloride, bile esculin test, and growth at 10°C and 45°C were done to identify Enterococcus isolates using standard protocols. Different biochemical tests based on sugar fermentation tests, pyruvate utilization and arginine decarboxylation were performed for species identification[17]. *E. faecalis* ATCC 29212 was used as a control reference strain.

A consent was taken from all patients to use their data in the current study. This study was approved by the Ethical Committee, Faculty of Medicine, Suez Canal University, Ismailia, Egypt.

Antimicrobial resistance patterns were detected by VITEK 2 Compact 5 Automated ID/AST System (bioMérieux, Inc., Durham, NC, USA). HLAR in enterococci was detected applying gentamicin (120 μg) and streptomycin (300 μg) discs (Becton Dickinson, Franklin Lakes, NJ, USA) on Muller Hinton agar medium[18]. Vancomycin Minimum Inhibitory Concentration (MIC) values were detected by agar diffusion method. *E. faecalis* ATCC 29212 (susceptible) and *E. faecalis* ATCC 51299 (resistant) were used.

Genotypic detection of vancomycin and HLAR genes:

DNA extraction:

Enterococcus DNA extraction was performed using Spin ABT Kit (Applied Biotechnology Co. Ltd, Egypt) from fresh subculture on Blood agar plates according to manufacturer's instructions. DNA concentration and the purity were assessed by a Nanodrop considering the yield should be more than 20 µg and the A260/A280 ratio should be between 1.7–1.9.

Detection of aminoglycoside resistance genes by nested PCR:

Nested PCR was done in a total reaction volume of 50 µl in Thermocycler (R Corbett research model RG 6000). We used primer sets for the six-aminoglycoside resistance genes (total of 12 primers) (Table 1)[19]. First, initial lysing step for 3 min at 94°C was adjusted; followed by 35 cycles of 40 s at 94°C, 40 s at 55°C, and 40 s at 72°C with a final extension step of 2 min at 72°C.

Table 1: Aminoglycoside resistance genes primers sequences in multiplex PCR

Aminoglycoside resistance gene	Product size (bp)	Primer sequence (5'-3')	Amount of each primer (pmol)
aac(6')-Ie-aph(2'')-Ia- F	369	CAG GAA TTT ATC GAA AAT GGT AGA AAA G	25 pmol
aac(6')-Ie-aph(2'')-Ia- R		CAC AAT CGA CTA AAG AGT ACC AAT C	
aac(6')-Ie-aph(2'')-Ia- F	348	CAG AGC CTT GGG AAG ATG AAG	
aac(6')-Ie-aph(2'')-Ia- R		CCT CGT GTA ATT CAT GTT CTG GC	
aph(2'')-Ib-F	867	CTT GGA CGC TGA GAT ATA TGA GCA C	25 pmol
aph(2'')-Ib-R		GTT TGT AGC AAT TCA GAA ACA CCC TT	
aph(2'')-Ic	444	CCA CAA TGA TAA TGA CTC AGT TCC C	3.5 pmol
aph(2'')-Ic		CCA CAG CTT CCG ATA GCA AGA G	
aph(2'')-Id	641	GTG GTT TTT ACA GGA ATG CCA TC	5 pmol
aph(2'')-Id		CCC TCT TCA TAC CAA TCC ATA TAA CC	
aph(3')-IIIa	523	GGC TAA AAT GAG AAT ATC ACC GG	3 pmol
aph(3')-IIIa		CTT TAA AAA ATC ATA CAG CTC GCG	
ant(4')-Ia	294	CAA ACT GCT AAA TCG GTA GAA GCC	2 pmol
ant(4')-Ia		GGA AAG TTG ACC AGA CAT TAC GAA CT	

Detection of Vancomycin resistance genes:

Genes for resistance were detected by conventional PCR. Primers used and PCR conditions were followed according to Aktas and colleagues[20] (Table 2)

Table 2: Vancomycin resistance genes primers sequences.

Genes	Primers (5'-3')	Product, base pairs (bp)
Van A F	CAT GAA TAG AAT AAA AGT TGC AAT A	1030
Van A R	CCC CTT TAA CGC TAA TAC GAT CAA	
Van B F	GTG ACA AAC CGG AGG CGA GGA	433
Van B R	CCG CCA TCC TCC TGC AAA AAA	
Van C F	GAA AGA CAA CAG GAA GAC CGC	796
Van C R	ATC GCA TCA CAA GCA CCA ATC	

Demonstration of PCR products

The PCR products were investigated in a 2% (w/v) agarose gel in 1X Tris Borate EDTA (TBE) (Wisent, Canada). Ethidium bromide-stained DNA amplicons were visualized under UV light and photographed using gel documentation system (syngen G: box, UK). DNA marker with defined molecular weights in the range 100-1500 bp was used.

Data Management and Statistical Analysis

SPSS (version 22 for windows) was used to conduct all statistical analyses. Statistical significance was defined as a p value of less than 0.05.

RESULTS

One hundred non copied Enterococcus isolates were collected from patients with HAIs admitted to SCUH, Ismailia, Egypt. The mean ± SD of age was 52 years± 1.56, with a range of 15 to 86 years. Elderly patients (≥ 65 years) were the most susceptible groups for enterococcal infection (P<0.001). Most strains (42%) were detected from urine, 33% from wound,15% from abscesses and 6% from blood and 4% from body fluid samples (peritoneal and ascetic fluid).

Four different species were characterized by biochemical reactions. Most of strains (62%) were *E. faecalis*, (30%) were *E. faecium*, (6%) were *E.durans* and only (2%) were *E.avium*.

Generally, most strains were susceptible to teicoplanin (95%), linezolid (90%), ampicillin and penicillin (76% for each) and nitrofurantoin (68%). Susceptibility to fluoroquinolones was detected in levofloxacin (41%) and ciprofloxacin (39%). HLGR was identified in 38% of isolates, most of them were from urine (24/38), wounds (13/38) and blood (1/38). HLSR was identified in 59% and were detected from urine (25/59), wound (17/59), abscesses (8/59), blood (6/59) and body fluid (3/59). Meanwhile, we found 23% of strains were resistant to both gentamycin and streptomycin.

All strains were susceptible to vancomycin by disc diffusion method. VRE was considered if MIC ≥32. MIC was ranged from 0,5 to 256 µg/mL with 15 isolates were found to be resistant to vancomycin. Most of them (12 strains) were *E.faecalis* and three of them were *E. faecium*

Regarding the species level of enterococci, *E.faecalis* were noticed to be more resistant to tetracycline, gentamycin and streptomycin (P<0.05), and more susceptible to nitrofurantoin, ampicillin, penicillin, levofloxacin, erythromycin and linezolid (P<0.05). Meanwhile, *E. faecium* strains were observed to be more resistant to ampicillin, penicillin and nitrofurantoin (p<0.01), and more susceptible to tetracycline than the other isolates (P<0.001).

Recognition of aminoglycoside resistance was performed by nested PCR to detect 7 genes for resistance. *aac(6')-Ie-aph(2")-Ia* gene (348 bp) was found in 30 strains, 25 strains of them were *E.faecalis* and the leftover was *E.faecium*. *aph(3')-IIIa* gene (623 bp) was detected in 10 strains which were *E.faecalis*. All strains with *aph(3')-IIIa* gene was positive for *aac(6')-Ie-aph(2")-Ia* gene. We didn't detect *aph(2")-Ib* (867bp), *aph(2")-Ic* gene(444bp) , or *aph(2")-Id* (641 bp), *ant(4')-Ia* gene (294bp). HLGR and HLSR were found more frequently in isolates with *aac(6')-1e-aph(2")-1a* gene (P<0.001) (Figure 1).

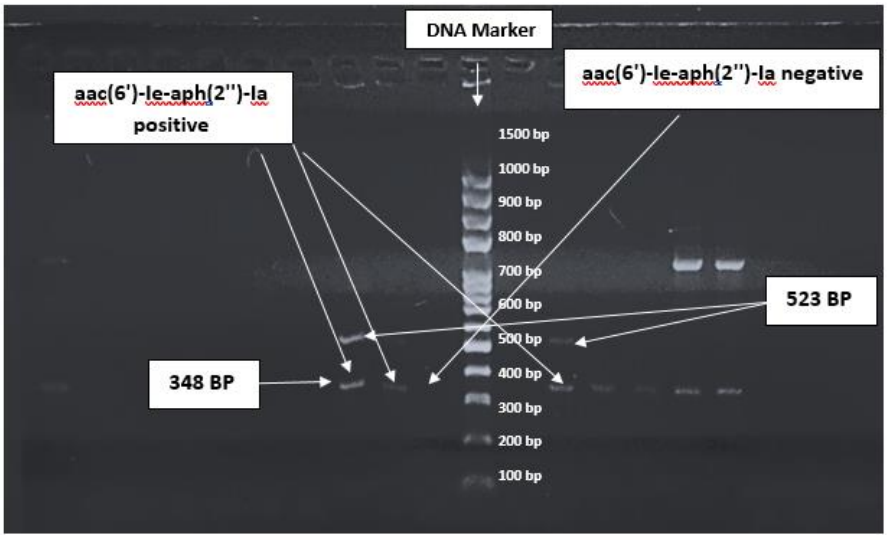

Fig. 1: Amplification of Aminoglycoside resistance genes by nested PCR. *aac(6')-Ie-aph(2'')-Ia* gene (348bp) and *aph(3')-IIIa* gene (523 bp) were detected. Other genes were negative.

We detected both *vanA* (1030 bp) and *van C* (796 bp) genes in 9 and 15 isolates respectively. All isolates with *vanA* gene were *E.faecium* and were observed to be resistant to vancomycin by MIC (MIC > 32 µg/mL), nitrofurantoin, ampicillin and penicillin (P<0.001). *Van C* was identified in *E.faecalis* (12 isolates), *E.faecium* (one isolate) and *E. durans* (Two isolates). *E.faecalis* carrying *vanC* gene was more resistant to aminoglycoside than *vanC* negative *E.faecalis*. We discovered that 6 isolates were carrying both *aac(6')-1e-aph(2'')-1a* gene and *vanA* gene (Figures 2A and 2B).

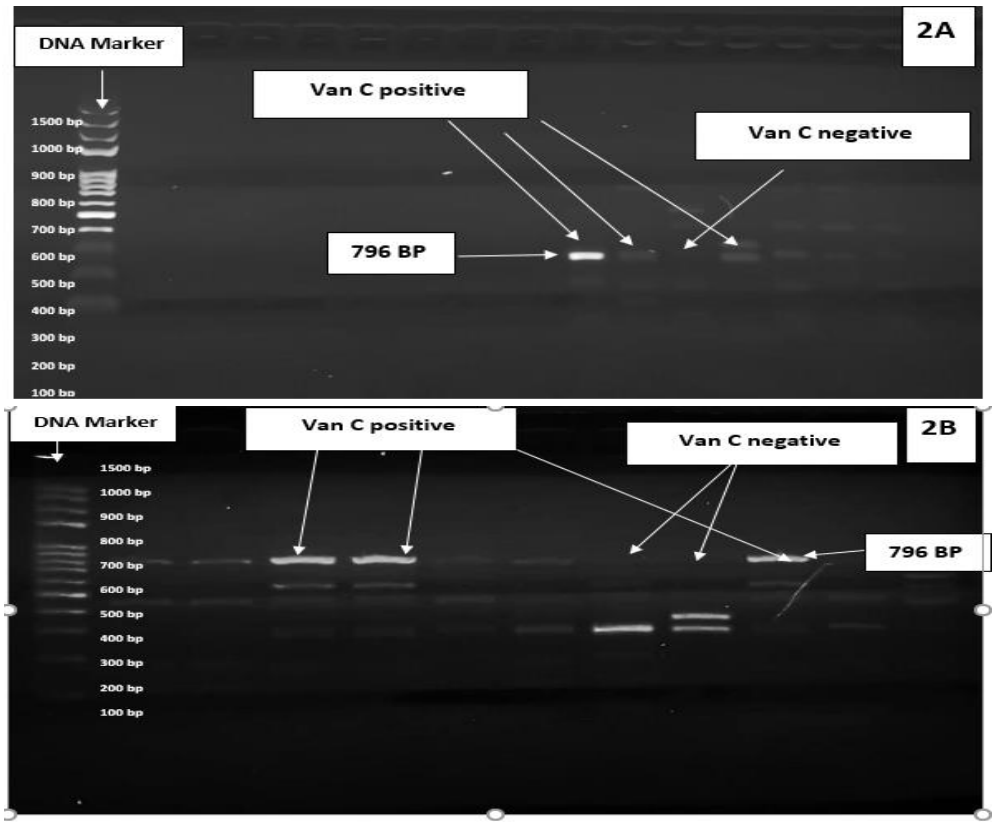

Fig. 2: *VanC* gene (796 bp) detection in Enterococci isolates.

DISCUSSION

Enterococci have grown in importance as HAIs in recent years, owing to their intrinsic resistance to antibiotics (cephalosporins), living in extreme environments and capacity to adhere to indwelling medical devices[21].

In the present work, out of the total 100 Enterococcus isolates from various clinical samples, maximum numbers of Enterococcus isolates were from urine (42%). Similar observations were noticed in different studies[22,23].

The frequency of antibiotic susceptibility was as follows: ciprofloxacin (39%), ampicillin and penicillin (76% for each) and nitrofurantoin (68%). Linezolid and vancomycin were found sensitive in 90% and 85% isolates. Other studies show prevalence of antibiotic resistance as ciprofloxacin (25%), penicillin (66.67%), and nitrofurantoin in urinary isolates (24.3%)[24]. Linezolid and vancomycin were found sensitive in 99% and 95% isolates of enterococcus.

We detected HLGR in 38% of strains, while HLSR was discovered in 59% of strains. In another research, the frequency of HLGR was 29% of the studied isolates, instead HLSR was reported in 35% of the same isolates [25]. In addition, other studies reported high incidence of HLGR[16,26].

E.faecalis were noticed to have more resistant to tetracycline, gentamycin and streptomycin and more susceptible to nitrofurantoin, ampicillin, penicillin, levofloxacin, erythromycin and linezolid. On the other hand, *E. faecium* were noticed to have more resistant to ampicillin, penicillin and nitrofurantoin, and more susceptible to tetracycline than the other strains.

Genes for aminoglycoside resistance were observed with a higher frequency in *E. faecalis* than in *E. faecium*, which is coherent to previous studies[2,27]. We found that *aac (6')-Ie-aph (2")-Ia* was the greatest widespread resistance gene detected in 30 strains followed by *aph(3')-IIIa* gene was found in 10 strains. These results were matched with numerous studies[26,28]. Another research reported less frequency of *aac (6')-Ie-aph (2")-Ia* while ant (3″)-III was more frequent[29].

Due to its tendency for acquiring and transmitting genes, VRE are considered one of the prominent causes of HAIs and a major public health concern according to previous reports[26,28,30].

In the present research, *Van C* was revealed in 12 isolates of *E.faecalis*, 1 isolate of *E.facium* and 2 isolates of *E. durans*. In difference to our study, VRE was reported in 2% of *E. faecalis* and 60% of *E. faecium* isolates[31]. Our study detected *vanA* gene in 9 isolates and *vanB* in 15 isolates. Like our results, Cekin et al. did not discovered *vanB* among enterococci[32].

One of the key causes contributing to the rising frequency of VRE and HLAR is a lack of effective antibiotic therapy and infection control actions to prevent MDR enterococci from spreading. For clinicians, such stresses represent a risk of therapeutic failure. Laboratories may need to provide precise antibiotic resistance patterns for enterococci.

CONCLUSION

E. faecalis was noticed to be more frequent Enterococcus species in this work. Resistance to gentamicin and streptomycin antibiotics were detected phenotypically although the absence of resistance genes. This could be attributable to the expression of gene is not involved in this study analysis.

We noticed that the *aac(6')-Ie-aph(2")-Ia and aph(3')-IIIa* genes were more frequently detected than other genes, and that both genes coexist.

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This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

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