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Antibiotic Resistance Patterns of *Enterococcus faecalis* and *E. faecium* Isolated from Infected Wounds. Incidence of High –level Resistance to Vancomycin and Aminoglycosides

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Abstract A total of fifty seven isolates of *Entercoccus faecalis* (N=42) and *E. faecium* (N=15) from infected wounds were screened for their resistance against 17 antibiotics including vancomycin. *E. faecalis* isolates demonstrated absolute resistance (100%) against eight antibiotics: chloramphenicol, nalidixic acid, gentamicin, streptomycin, tetracyclin, erythromycin, penicillin G and lincomycin. While isolates of *E. faecium* revealed absolute resistance (100%) to only three antibiotics: chloramphenicol, nalidixic acid, and lincomycin, yet they exhibited 80% resistance against gentamicin and streptomycin. Higher percentage of resistance against vancomycin was shown by *E. faecium* (53.3%) as compared to *Ent. faecalis* (47.6%). *E. faecalis* and *E. faecium* isolates were involved in 5 and 2 antibiotic resistance patterns (ARPs) respectively. Of the two species; 24.5% isolates (N=11, 26.2% *E. faecalis* and N=3, 20% *E. faecium*) were included in one ARP demonstrating resistance to all antibiotics under study.
High-level resistance to glycopeptides (HLRG) vancomycin (64 µg/ml) was higher among isolates of *E. faecium* (50%) as compared to *E. faecalis* (30%). Whereas high-level resistance to aminoglycosides (HLRA): gentamicin (500 µg/ml) and streptomycin (>1000 µg/ml) was higher among *E. faecalis* against gentamicin (80% VS 62.5%) and almost similar incidence was reported by the two species against streptomycin (60% and 62.5% respectively). Emergence of varied ARPs and high-level resistance to vancomycin and aminoglycosides among *E. faecalis* and *E. faecium* isolates will reduce to a great extent the therapeutic options against enterococcal infections. Hence, we emphasizes on the importance of performing susceptibility testing on all clinically significant isolates.

Keywords *Entercoccus faecalis*, *E. faecium*, Antibiotic resistance patterns, High level resistance to glycopeptides, High level resistance to aminoglycosides

Introduction

Enterococci are well adapted to survive in the gastrointestinal tract, being able to escape the action of most commonly used antibiotics against Gram-negative gut flora; accordingly enterococci become dominant under antibiotic pressure [1, 2]. Epidemiological data indicate that enterococci turned out to be important reservoirs for transmission of antibiotic resistance genes among different species of bacteria [3, 4]. Multidrug-resistant (MDR) enterococci survive in hospital environment and was isolated even from surfaces of a US hospital laundry facility [5, 6]; affecting mainly the severely ill and immunocompromised patients leading to invasive infections. Therefore, MDR enterococci become as important nosocomial pathogens in the surgical intensive care unit; septicemia, bacteremia, surgical site infections, burn infection, and infections related to the use of catheters and implanted devices [7, 8, 9, 10, 11, 12].

Among enterococci, the two species, *E. fecalis* and *E. faecium* are mainly responsible for the majority of hospital acquired human infections [13, 14]. Mendiratta, et al, 2008 [15]; Sanal, et al, 2013[16], Padmasini, et al, 2014 [17] and Chakraborty, et al 2015 [18] reported that *E. faecalis* is accounted for 80-90% of all clinical isolates in comparison with *E. faecium* (5-15%). Yet, blood culture isolates from septicemic patients [19] reported (53% VS 33%) and from infective endocarditis patients, almost similar incidence (48% VS 52%) was reported by both species [20] respectively. Nevertheless, Arias and Murray, 2012 [21] and Cattoir and Giard, 2014 [22] indicated that worldwide ratio of *E. faecalis*-*E. faecium* infections is currently changing in favor of *E. faecium* as an emergent and challenging nosocomial problem.

Enterococcal resistance to vancomycin (VRE) has emerged since 1990s; nearly 25% of the enterococcal bacteremic incidents were resistant to vancomycin [8]. VRE was demonstrated through a variety of mechanisms [23]. Currently in the United States, an estimated 30% of clinical *Enterococcus* isolates are resistant to glycopeptides [24]. VRE bacteremia was found to be associated with increased mortality of hospitalized patients [25, 26]. Also, hemodialysis and liver transplantation were factors associated with acquisition of VRE [20]. High-level acquired resistance to glycopeptides (HLGR) was first reported in United Kingdom since 1980s [27].

Besides, high level gentamicin resistance was first reported in *E. faecalis* at 1979 [3]. Soon later, Eliopoulos et al. 1988 [28] were the first to report the spread of high-level gentamicin resistance in *E. faecium* to other isolates and species of enterococci. Also, high-level aminoglycoside resistance (HLAR) in enterococcal septicemia was reported from a tertiary care hospital in east Delhi [19] and isolates of rectal swab and stool specimens from tertiary hospital isolates in China [29] where HLAR was detected in 119 (74.4%) of the isolates. During the last two decades, enterococci have become more resistant to ampicillin and have acquired a high level of resistance to aminoglycosides and glycopeptides [1, 15, 30, 31, 32].

Since resistance poses a therapeutic challenge to physicians who signify the difficulty in commencing effective treatment of serious enterococcal infections, the aim of the present study is to investigate incidence of multiple-antibiotic-resistant strains among *E. faecalis* and *E. faecium* isolated from infected wounds and their antibiotic resistant patterns with special emphasis on high levels resistance to glycopeptides and aminoglycosides.

Materials and Methods

Isolates

Fifty seven isolates of *E. facalis* (N=42) and *E. faecium* (N=15) from infected wounds were provided by Kithar, 2009 [33]. Identification of isolates was confirmed using conventional biochemical tests including: No catalase production, hydrolysis of pyrrolidonyl-ß-naphthylamide (PYR) and aesculin, and growth in the presence of 40% bile salts, 6.5% NaCl and in pH 9.6 (19, 34, 35].

Determination of antibiotic resistant patterns

Preparation of inoculum

Five colonies of each isolate grown overnight on Tryptic Soy agar (TSA) were inoculated into 5ml of Tryptic Soy broth (TSB) and incubated at 37C^{o} for 5-6h. Turbidity of suspensions was adjusted to achieve a final inoculum of 10^{6} CFU/ml.

Antibiotic sensitivity test

Sensitivity of *E. facalis* and *E. faecium* isolates was determined against 17 antibiotics by single disc diffusion method as described by Barry et al.1970 [36]. The following antibiotic discs supplied by Oxoid were used: chloramphenicol (CHL), 30; nalidixic acid (NAL), 30; tobramycin (TOB), 10; nitrofuration (NIT), 300; vancomycin (VAN), 30; gentamicin (GEN), 10; streptomycin (STR), 10; tetracycline (TET), 30; erythromycin (ERY), 15; rifampicin (RIF), 5; trimethoprim (TRI), 5; cefotaxime (CEF), 30; lincomycin (LIN), 15; cephalexin (CEP), 30; metranidazole (MET), 5; ampiclox (AMX), 30 and penicillin G (PEN), 10 IU. One ml inoculum of each isolate was spread on Mueller-Hinton agar plates in duplicates. After 15 min of absorption time at 37C^{o}, antibiotic discs were placed onto the plates. Plates were incubated overnight at 35C^{o}. Inhibition zones were measured by millimeters.

Screening for resistance to high- level glycopeptides and high-level aminoglycoside was performed by the agar screen method according to Clinical Laboratory Standard Institute (37).

Determination of high-level resistance to glycopeptides

Isolates of enterococci exhibited resistance to vancomycin, were subjected to minimum inhibitory concentrations (MICs) testing in the range of 4 to 64 µg/ml of vancomycin, and to 0.12 to 30 µg/ml teicoplanin (Rousell Laboratories). Mueller-Hinton agar was used as the test medium.

Determination of high-level resistance to aminoglycosides

Isolates of enterococci displayed resistance to gentamicin and streptomycin were examined to determine high-level resistance to aminoglycosides as described by Sahm and Torres, 1988 [38] and Forward et al. 1990 [30]. Mueller-Hinton agar plates were supplemented with gentamicin (500 and 1000 µg/ml) and streptomycin (>1000 µg/ml).

Plates were spot inoculated with cultures of tested isolates grown overnight on TSA, turbidity adjusted suspensions was equivalent to a 0.5 McFarland standard.

Inoculated plates were incubated at $35C^{o}$ and examined for evidence of growth after 24 h. indicating vancomycin and gentamicin resistance and for 48 hours for streptomycin resistance.

Results and Discussion

E. faecalis and *E. faecium* isolates from infected wounds demonstrated resistance against 17 antibiotics ranging from 26% - 100% and 20% - 100% respectively. *E. faecalis* exhibited absolute resistance (100%) against eight antibiotics: chloramphenicol, nalidixic acid, gentamicin, streptomycin, tetracyclin , erythromycin, penicillin G and lincomycin. Whereas, isolates of *E. faecium* were absolutely resistant (100%) to only three antibiotics, yet they exhibited 80% resistance against gentamicin and streptomycin. This result is in accordance with Mendiratta et al, 2008 [15] and Sanal et al, 2013 [16] studies from central and south India. In contrast, Jain et al, 2011 [19] reported that *E. faecium* is the leading cause, comprising 67% of multidrug-resistant enterococcal infection among isolates from patients with septicemia from a tertiary care hospital in east Delhi. The mechanisms underlying antibiotic resistance in enterococci may be intrinsic to the species or acquired through mutation of intrinsic genes or horizontal exchange of genetic material encoding resistance determinants [1, 2] through the transfer of plasmids and transposons [39].

On the other hand, the present study clarifies higher percentage of VR among *E. faecium* isolates (53.3%) than *E. faecalis* (47.6%). These percentages were higher than that reported by isolates from infective endocarditis (30%) in the United States [24] who emphasized occurrence of higher mortality and prolonged bacteremia with VR *E. faecium* than VR *Ent. faecalis*. Also, in German hospitals, Klare et al, 2005 [40] reported that incidence of VR in *E. faecium* has increased up to 14% by the second half of 2003 and the first half of 2004 and attributed this increase to the occurrence and spread of epidemic-virulent ampicillin / vancomycin-resistant strains. Furthermore, Arias and Murray, 2008 [21] reported that glycopeptide resistance in enterococcal infections are attributed to *E. faecium* and claimed that glycopeptide and β-lactam resistance becomes a common feature of the majority of *E. faecium* hospital isolates; which could be attributed to high genome plasticity of *E. faecium* [22]. In contrast, although lower incidence, but proportion of *E. faecalis* (22%) was much higher than *E. faecalis* (6%) as reported in Iranian hospital [32]. Because VRE can survive in the environment for prolonged periods (>1 week), it can contaminate almost any surface, and can be passed from one patient to another by health care workers [41]. Emergence of (VRE) is worrisome as enterococci may play a crucial role in merging resistance to vancomycin to *Staphylococcus aureus* isolates [23, 39]. On the contrary, all enterococci isolates from clinical samples[18] and blood cultures [19] in India, were found sensitive to vancomycin.

Resistance Patterns

Six antibiotic resistance patterns (ARP) were demonstrated; *E. faecalis* (N=26, 62%) and *E. faecium* (N=5. 33.3%) isolates have participated into 5 and 2 ARPs respectively (Table 2). This variation indicates spread of antibiotic-resistant strains between patients which agrees with Kuzucu et al, 2005 [31] who indicated high clonal diversity among the isolates. Besides, Feizabadi et al, 2003 [42] indicated that *E. faecalis* isolates recovered from patients in Tehran were genetically diverse. This signifies that treatment of enterococcal infections should depends upon the

resistance patterns [1]. Nevertheless, Emaneini et al, 2016 [32] claimed that most of strains have an identical or very similar antibiotic and gene resistance pattern. This could be applicable to the sole ARP emerged in the present study, where 24.5% isolates of both species (N=11, 26.2% *E. faecalis* and N=3, 20 % *E. faecium*) were involved into one ARP demonstrating resistance to all antibiotics under study (RD: 17).

Table 1: Incidence of resistance against 17 antibiotics of *Enterococcus faecalis and E. faecium* isolated from infected wounds

	Number of resistant isolates (%)	
Source	***E. faecalis***	***E. faecium***
No. of isolate	42	15
Chloramphenicol	42 (100)	15 (100)
Nalidixic acid	42 (100)	15 (100)
Tobramycin	39 (92.9)	14 (93.3)
Nitrofuratoin	27 (64.3)	6 (40)
Vancomycin	20 (47.6)	8 (53.3)
Gentamicin	42 (100)	12 (80)
Streptomycin	42 (100)	12 (80)
Tetracycline	42 (100)	14 (93.3)
Erythromycin	42 (100)	13 (86.7)
Rifampicin	15 (59.5)	7 (46.7)
Penicillin	42 (100)	12 (80)
Trimethoprim	11 (26.2)	7 (46.7)
Cefotaxime	33 (78.6)	9 (60)
Lincomycin	42 (100)	15 (100)
Cephalexin	35 (83.6)	11 (73.3)
Metronidazole	16 (38)	6 (50)
Ampiclox	13 (31)	3 (20)
Number of antibiotics all isolates are resistant to (%)	8(47)	3 (17.6)

High-Level Resistance to Vancomycin and Aminoglycosides

Two phenotypes of glycopeptide resistance by enterococci have been distinguished by early 1990s [43]: those that are resistant to high levels of vancomycin and teicoplanin and those that are inducibly resistant to low levels of vancomycin and susceptible to teicoplanin. Both phenotypes were recorded in the present study (Table 3). HLRG among isolates of *E. faecium* against vancomycin was (50%) as compared to *E. faecalis* (30%); lower and almost similar incidence against teicoplanin (25% VS 20%). Karmarkar et al, 2004 [44] reported lower incidence of resistance against vancomycin among clinical isolates (23%) and none were resistant to teicoplanin. However, HLGR among the enterococcal isolates in Sanal et al, 2013 [16] study was 53% and 38.46% against vancomycin and teicoplanin respectively, with no significant difference between *E. faecalis* and *E. faecium* isolates. HLRG isolates against: teicoplanin and vancomycin could be due to the synthesis of a novel cytoplasmic peptidoglycan precursor [45].

On the other hand, incidence of HLRA was higher among *E. faecalis* against gentamicin (80%) as compared to *E. faecium* (62.5%) and almost similar incidence was reported by the two species against streptomycin (60% VS 62.5%) respectively. These incidences were higher than that reported by isolates from patients with septicemia at tertiary care hospital in east Delhi[19] and at tertiary care hospital in Kolkata, Eastern India [18], and from rectal swab and stool specimens from tertiary hospital isolates (58.8%, and 50%) in China [29]. Besides, HLAR for gentamicin and streptomycin antibiotics was found to be even lower (42.7% and 29.8%) in Padmasini et al, 2014 [17] study on clinical isolates from India who detected and identified aminoglycoside modifying enzyme encoding genes (AME) by multiplex PCR.

Table 2: Frequencies of antibiotic resistance patterns of *Enterococcus faecalis and E. faecium* from infected wounds

E. faecalis **N= 42 (%)**	***E. faecium*** **N= 15 (%)**	**Antibiotic Resistance Patterns**	**Resistance Determinants**
Wound infection	Surgical wound infection	*CHL,NAL,TOB,NIT,VAN,GEN,STR,TET,ERY, RIF,PEN,TRI,CEF,LIN,CEP,MET,AMX	17
11 (26.2)	3 (20)	CHL,NAL,TOB,NIT,VAN,GEN,STR,TET,ERY, RIF,PEN,TRI,CEF,LIN,CEP,MET	16
-	2 (13.3)	CHL,NAL,TOB,NIT,VAN,GEN,STR,TET,ERY,RIF, PEN,CEF,LIN,CEP	14
4 (9.5)	-	CHL,NAL,TOB,NIT,GEN.STR,TET,ERY,PEN,CEF, LIN,CEP	12
5 (11.9)	-	CHL,NAL,TOB,GEN.STR,TET,ERY,PEN, CEF,LIN,CEP	11
3 (7.1)	-	CHL,NAL,TOB,GEN.STR,TET,ERY,PEN,LIN	9
3 (7.1)	-		
26 (62)	5 (33.3)		

N: Number of resistant isolates. *****CHL, Chloramphenicol; NAL, Nalidixic acid; TOB, Tobramycin; NIT, Nitrofuratoin; VAN, Vancomycin; GEN, Gentamicin;
STR, Streptomycin; TET, Tetracycline; ERY, Erythromycin, RIF, Rifampicin; PEN, Penicillin G; TRI, Trimethoprim; CEF, Cefotaxime, LIN, Lincomycin;
CEP, Cephalexin; MET, Metronidazole, AMX, Ampiclox

Table 3: Incidence of high –level resistance to vancomycin and aminoglycoside (gentamicin, streptomycin) among resistant strains of *Enterococcus faecalis* and *E. faecium* isolated from infected wounds

Enterococcus **Species**	**Vancomycin MIC: ≥ 64 μg / ml**	**Teicoplanin**	**30μg**	**Gentamicin ≥ 500 μg / ml**	**Streptomycin > 1000 μg /ml.**
E. faecalis N=20 (%)	6 (30)	4 (20)		16 (80)	12 (60)
E. faecium N= 8 (%)	4 (50)	2 (25)		5 (62.5)	5 (62.5)

Nevertheless, Sanal et al, 2013 [16] reported HLR for streptomycine in 49.3% of the isolates, with *E. faecium* showing greater resistance (59%) as compared to *E. faecalis* (49%) and HLR for gentamicin was almost similar in both species. Mendiratta et al, 2008 [15] reported much higher incidence of HLAR among *E. faecium* isolates (95.5%) than *E. faecalis* (37.5%). Also, Emaneini et al ,2016 [32] found that more than 96.2% of isolates were HLR for gentamicin; they described AMEs genes encoding aminoglycoside modifying enzymes and vancomycin-resistance targeted by multiplex-PCR reaction. Feizabadi et al, 2004 [46] indicated that gentamicin resistance was associated with conjugative plasmids (>70 kb) in most strains. Previous studies on HLAR have been done almost exclusively on *E. faecalis.*. High-Level resistance to gentamicin and/or streptomycin indicates that an enterococcal isolate will not be killed by the synergistic action of a penicillin or glycopeptide combined with that aminoglycoside [47] .

Conclusions

The present study illustrates high prevalence of resistance to multiple antibiotics, HLRG and HLAR among *E. faecalis* and *E. faecium* isolated from infected wounds. This will limit, to a great extent, the therapeutic options against most enterococcal infections. Hence, routine screening for vancomycin and aminoglycoside resistance among clinical and hospital environment isolates, active surveillance for VRE in intensive care units and surgery wards turn out to be of vast importance for evaluating the degree of the resistance problem: emergence, spread, and patterns of resistance. Also, developing strict control measures for rapid detection of such strains in addition to sensible use of vancomycin.

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