International Journal of Pharmaceutical Science and Practice.
Volume 2, Number 1 (2013) pp 1-10

http://www.ripublication.com

Enterococcus faecium and *Enterococcus faecalis*, the nosocomial pathogens with special reference to multi-drug resistance and phenotypic characterization

***Suddhanshu Bhardwaj, Kalyani Bhamre**
Jayashri Dhawale, Mahendra Patil and Sunil Divase

Jain R & D Laboratory, Agri Park, Jain Hills,
Jain Irrigation Systems Ltd, Jalgaon 425 001 (India)
bhardwaj.sudhanshu@jains.com , biogaslab@jains.com
micro@jains.com, biogaslab@jains.com, biogaslab@jains.com
Corresponding author bhardwaj.sudhanshu@jains.com

Abstract

Among *Enterococcus* genus, *Enterococcus faecium* and *Enterococcus faecalis* are the main causative agents for serious relevant nosocomial infections such as urinary tract infections (UTIs), endocarditis, bacteremia, intra-abdominal and intra-pelvic abscesses. enterococci, particularly *Enterococcus faecium*, always had a high level of intrinsic resistance of antimicrobial agents. The mainstay treatment of serious enterococcal infections was the synergistic effect of penicillin/ampicillin or vancomycin and an aminoglycoside. However, by the 2000's high level resistance i.e. minimum inhibitory concentration (MIC) ≥2000 µg/ml to gentamycin and other aminoglycoside was seen with increasing frequency. As enteroccoci are important nosocomial pathogen accounting for up to 10% of all infections among hospitalized patients. Clinical samples were collected from different medical colleges and hospitals. Isolation followed by preliminary screenings and antimicrobial activity was carried out using broad-spectrum antibiotics including β-lactum, aminoglycosides and glycopeptides. The isolated strains of *E. faecalis* showed 33.3% and 37.2% resistant to Penicillin G and kanamycin, respectively. However, *E. faecium* resistance ranged from 52.4 to 100% to various antimicrobials. Vancomycin re-resistance in *E. feacium* was not seen whereas, *E. faecalis* accounted up to 3.9%. In present study, 82.5% drug re-resistant *E. faecalis* and 66.7% drug re-resistant *E.*

faecium were isolated from all collected samples. In conclusion, multidrug resistant enterococci especially resistant to vancomycin and aminoglycosides have become a threat to patient's safety, making it a formidable nosocomial pathogen.

Introduction

Enterococci are Gram-positive, non-spore forming and facultative anaerobic cocci. They have important impact on human health due to their natural presence among gut microbiota and conversely their deleterious role in spoilage process of fruit juices and meat products[1,2,3]. Furthermore, among *Enterococcus* genus, *Enterococcus faecium* and *Enterococcus faecalis* are the main causative agents for serious relevant nosocomial infections such as urinary tract infections (UTIs), endocarditis, bacteremia, intra-abdominal and intra-pelvic abscesses[2,4,5,6]. Interestingly, many of these problems arises from the ability of enterococci to survive (i) in adverse conditions [temperature (10 to 45ºC), pH (9.6) and growth in NaCl (6.5%)], (ii) presence of several virulence determinants (cytolysin, gelatinase, aggregation substance, extracellular superoxide etc.) and (iii) possess both intrinsic as well as acquired antibiotic resistance trait (vancomycin, streptogramins, and cephalosporins)[1,7,8]. Risk factors including (i) indiscriminate use of antibiotics, (ii) prolonged hospital stay, (iii) severity of illness and (iv) immune-suppression are mainly responsible for nosocomial acquisition of drug resistant enteroccoci. This ultimately leads to environmental contamination and cross infections[9,10]. Enterococci with high level resistance to aminoglycosides (HLAR), β-lactamase production and glycopeptide resistance including vancomycin resistance are posing a therapeutic challenge not only for clinicians but also for healthcare institutions[9,10]. Recent studies have focused on enteroccoci due to their increasing role in nosocomial infections as well as their increasing antibiotic resistance[11].

Enterococci, particularly *Enterococcus faecium*, always had a high level of intrinsic resistance of antimicrobial agents[12,13]. The mainstay treatment of serious enterococcal infections was the synergistic effect of penicillin/ampicillin or vancomycin and an aminoglycoside. However, by the 2000's high level resistance i.e. minimum inhibitory concentration (MIC) ≥2000 µg/ml to gentamycin and other aminoglycoside was seen with increasing frequency[12,14]. In India high level of aminoglycoside, penicillin and vancomycin resistance have been illustrated in southern region of sub continent. However, no literature is available on the susceptibility pattern of this previously considered relatively non-virulent organism from central India. As enteroccoci are important nosocomial pathogen[11,15,16,17], accounting for up to 10% of all infections among hospitalized patients[18]. Hence, present study was undertaken to determine the antimicrobial resistance profile of enteroccoci by disc diffusion test (DDT) and MIC in various hospitals of Bhopal, M.P, India.

Materials and methods

Collection of samples Clinical samples *viz.* urine, blood, pus, CSF, stool, fluids and aspirates were collected aseptically, from patients of Government Medical College, Udairam Ram memorial Hospital and Ayushman Hospital and Research Centre Bhopal, MP, India.

Processing in laboratory All samples were streaked on pre-incubated Mac-conkey's agar and blood agar plates within 5 hrs of sample collection and were kept under incubation at 30-35°C for 48 hrs. Colonies appeared were further confirmed by colony morphology on Mc Conkey's agar, Blood agar, Gram staining and Catalase test[18]. Confirmation and identification of the enterococcal isolates were carried out using Bile Esculin, mannitol and arginine hydrolysis test[18,19].

For comparison of the results, for positive controls were used such as (i) *Enterococcus faecalis* ATCC 29212, (ii) *E. coli* ATCC 25922 (mannitol fermenting and motile) and (iii) *Staphylococcus aureus* ATCC 25923 and negative control were (i) Group A *Streptococcus* and (ii) *Shigella dysenteriae* (mannitol non-fermentor non-motile).

Final confirmation of the enterococcal isolates were done by using Facklam and Collins scheme[20] as described in Table 1. Differentiation of enterococcal group was confirmed by tellurite reduction test.

Table 1. Facklam and Collins scheme for differentiation of enterococcal group.

Tests	Group		
	I	II	III
Mannitol fermentation	+	+	-
Arginine hydrolysis	-	+	+

Antibiotic susceptibility testing Each enterococcal isolates were tested by DDT[21] using Muller Hinton agar (MHA). Antibiotic disc were prepared using antibiotic stock solution[22,23]. Inoculum having bacterial count of 10^5cfu/ml was poured on MHA plates uniformly and antibiotic disc of different concentration were placed. These plates were kept for diffusion in refrigerator for 30 min and further incubated at 37°C for 24 hrs and examined for zone of inhibition. Zone was measured and results were interpreted as sensitive, intermediate and re-resistant according to Mendiratta et al.[24]

Based on the results of DDT, enterococcal isolates showing decreased susceptibility (intermediate) or resistance to each antibiotic were further subjected for determination of MIC by agar dilution method[24]. The MHA plates were incorporated with antibiotics with final concentration of 12.5, 25, 50, 100 and 200 µg/ml penicillin, 500, 1000 and 2000 µg/ml for each gentamycin, kanamycin and streptomycin and 2, 4, 8, 16, 32 µg/ml for vancomycin.

Inoculum of each selective isolate was prepared as for DDT giving a final concentration of 10^5cfu/ml. After incubation plates were examined for

sensitivity and re-resistance of isolates and MIC was determined.

Multi drug resistance (MDR) also studied using different combination of drugs and correlation of the resistance of drugs to enterococci with respect to infection/colonization was studied.

Results and discussion

A total of 150 isolates were obtained from 9024 clinical samples. Based on Facklam and Collin (2000) scheme and tellurite reduction test, 2 species of enterococci viz., 86% *E. faecalis* (129) and 14% *E. feacium* (21) were identified. Based on DDT, enterococcal isolates showing resistance or decreased susceptibility to various antimicrobials were identified as described in Table 2.

The isolated strains of *E. faecalis* showed 33.3 % and 37.2% resistant to Penicillin G and kanamycin, respectively. However, *E. faecium* resistance ranged from 52.4 to 100% to various antimicrobials. Vancomycin re-resistance in *E. feacium* was not seen whereas, *E. faecalis* accounted up to 3.9%.

MIC of antimicrobials by agar dilution method[24]

Enterococcal resistance to Penicillin G

The results of the MIC using Penicillin G showed only 1 isolate of enterococci was sensitive at concentration of ≤12.5 µg/ml which cannot be correlated with standards of Mendiratta et al.[24]. However, all enterococcal isolates represented significant relationship with DDT, having MIC ranging from 25 to ≥ 200 µg/ml. 38 strains of *E. faecalis* and 10 strains of *E. faecium* observed as HLPR (High level penicillin resistance) as per Table 3.

Table 2. Antimicrobial resistance profile of 150 isolates of enterococci by DDT

Antimicrobials	NUMBER OF RERESISTANT ISOLATES					
	E. faecalis (n=129)			*E. faecium* (n=21)		
	Re-resistant	Decreased susceptibility	Total (%)	Re-resistant	Decreased susceptibility	Total (%)
β-lactams						
Penicillin	43	0	43 (33.3)	13	0	13 (61.9)
Ampicillin	12	0	12 (9.3)	11	0	11 (52.4)
Aminoglycosides						
Gentamycin	5	0	5 (3.9)	16	1	17 (81.0)
Kanamycin	47	1	48 (37.2)	21	0	21 (100)
Streptomycin	25	2	27(20.9)	17	1	18 (85.7)
Glycopeptides						
Vancomycin	1	4	5 (3.9)	0	0	0 (0.0)

Table 3. Enterococci resistant to Penicillin G by DDT and MIC

Enterococcal species (No.)	No. of isolates reresistant by DDT	MIC of Penicillin G (units)					
		≤12.5	25	50	100	200	>200
E. faecalis (129)	43	1	2	0	2	3	35
E. faecium (21)	13	0	2	1	0	1	9

Enterococcal resistance to aminoglycoside

MIC pattern of aminoglycosides for both types of bacterial isolates was found as per Table 4.

Table 4. MIC of aminoglycosides for enterococci, resistant to aminoglycosides high content disc by DDT

Aminoglycosides	No. of re-resistant isolates/ total isolates	MIC (µg/ml)			
		LLR	MLR	HLR	
		≤500 (%)	1000(%)	2000(%)	≥ 2000(%)
Genatamycin					
E. faecalis	5/129	2(1.6)	0	0	3(2.3)
E. faecium	17/21	2(9.5)	0	1(4.8)	14(66.7)
Kanamycin					
E. faecalis	48/129	6(4.7)	4(3.1)	15(11.6)	23(17.8)
E. faecium	21/21	1(4.8)	1(4.8)	5(23.8)	14(66.7)
Streptomycin					
E. faecalis	27/129	4(3.1)	0	3(2.3)	20(15.5)
E. faecium	18/21	4(19.0)	0	2(9.5)	12(57.1)

LLR: low level resistance, MLR: moderate level resistance, HLR: high level resistance.

Table 5. Concomitant high level penicillin and aminoglycosides resistance in 150 infections/colonization isolates of enterococci

Enterococcal species (No.)	Number of isolates				
	HLPR	HLAR			HLPR +HLAR
		HLGR	HLKR	HLSR	
E. faecalis (129)	38	03	38	23	14
E. faecium (21)	10	15	19	14	10
Total 150(%)	48 (32)	18 (12)	57 (38)	37 (24.7)	24 (16)

HLR: high level resistance, A: aminoglycoside, G: gentamycin, K: kanamycin, P: penicillin, S: streptomycin.

Enterococcal resistance to vancomycin

Table 6. MIC of vancomycin in enterococci re-resistant to vancomycin by DDT

Enterococcal species (No. of isolates)	No. of isolates with resistance or decreased susceptibility by DDT	No. of isolates with MIC of vancomycin (µg/ml)					
		≤ 2	4	8	16	32	≥32
E. faecalis (129)	5	2(I)	1(I)	1(I)	1(R)	-	--
E. faecium (21)	0	-	-	-	-	-	-

Includes four intermediate (I) and one re-resistant (R) isolate.

Five *E. faecalis* isolates were found to be reresistant to vancomycin by DDT However all isolates of *E. faecium* were found to be sensitive with sensitivity breakpoint of ≤4µg/ml[24]. Three isolates of *E. faecalis* that were intermediate by DDT were found sensitive with agar dilution test while 2 isolates that were intermediate and re-resistant by DDT had very low levels of resistance for vancomycin 8 and 16 µg/ml respectively.

Multiple drug resistance in Enterococci

Resistance to two or more drugs was encountered in both *E. faecalis* and *E. faecium* although 70 isolates (54.3%) of *E. faecalis* and 21(100%) of *E. faecium* showed multiple drug resistance.

Table 7. Pattern of Multiple Drug Resistance (R-pattern) in 150 infection/ colonization strains of enterococci

R-Pattern	Number of isolates	
	E. faecalis	*E. faecium*
Two drugs	4	0
Three drugs	16	3
Four drugs	9	7
Five drugs	5	3
Six drugs	0	8
Seven drugs	0	0

Correlating drug re-resistant enterococci with nature of infection / colonization it was observed that 85 (82.5%) drug re-resistant *E. faecalis* and 14 (66.7%) drug re-resistant *E. faecium* were isolated from significant enterococcal infections (Table 5).

Table 8. Correlation of drug reresistant enterococci with infection/colonization

Total number of drug re-resistant enterococci	Number of drug re-resistant Enterococci (%)		
	Enterococci from significant enterococcal infection (urine, blood and CSF)	Enterococci of doubtful significance (pus, vaginal swab, fluid, and aspirate)	Colonizing Enterococci (stool)
E. faecalis (103)	85 (82.5)	9 (8.7)	9 (8.7)
E. faecium (21)	14 (66.7)	4 (19.0)	3 (14.3)

DISCUSSION

A concomitant rise in prevalence of antimicrobial resistance is constantly observed as antimicrobial use continues to rise globally. In present study, enterococci were isolated from different clinical samples among which urinary enterococci accounted for 76.6% of total isolates. A faecal carriage of 12% of *E. faecium* was observed and approximately 10% isolates were non-faecal and non-urinary.

Antimicrobial resistance profile in *Enterococci*

In present study, resistance to penicillin observed in ⅓rd *E. faecalis* and ⅔rd *E. faecium* isolates (Table 2). Enterococcal isolates with MIC levels from 12.5 µg/ml up to ≥200 µg/ml were observed. Some of the isolates showed HLPR to ≥1000µg/ml of penicillin. Rather ampicillin is frequently used for treating a serious enterococcal infection. In present study, ampicillin resistance was less common in *E. faecalis* (9.3%) then in *E. faecium* it was observed to be 50%.

Out of the three aminoglycosides tested, resistance to kanamycin was most frequently observed accounting for ⅓rd *E. faecalis* and all of the *E. faecium* isolates (Table 4). However 80% of *E. faecium* were re-resistant to gentamycin and streptomycin , resistance to these aminoglycosides by *E. faecalis* was only 3.9% and 20.9% respectively at concentrations of 500, 1000, 2000 µg/ml. *E. faecium* thus found to have greater resistance than *E. faecalis*. Aminoglyciside resistance measured in terms of MIC in enterococci isolates was of low, moderate and high level (Table 4). More than 50% isolates of *E. faecium* and less than 20 % of *E. faecalis* showed HLAR (≥ 2000 µg/ml).

HLPR was encountered in 48 (32%) isolates of which 38 were of *E. faecalis* and 10 were of *E. faecium*. Among these 24 (16%) were also associated with HLAR. All of them were vancomycin sensitive. Thus only therapeutic option left for infection due to such isolates is vancomycin.

Vancomycin re-resistant enterococci (VRE), though very uncommon in our country were isolated in present study. Of 150 isolates studied four were found with decreased susceptibility and one with resistance to vancomycin (Table 6) in *E. faecalis*. All isolates in the present study had low level resistance to vancomycin (≤16 µg/ml) but high level vancomycin resistance was not observed.

It was observed that out of 129, 70 *E. faecalis* (54.3%) and all 21 (100%) *E. faecium* isolates were MDR (multiple drug resistance).

In present study, 82.5% drug re-resistant *E. faecalis* and 66.7% drug re-resistant *E. faecium* were isolated from all collected samples. This implies that drug re-resistant enterococci like other drug re-resistant pathogens are more invasive to various body tissues.

Conclusion

To sum up the present study, it has been shown that ⅓rd enterococcal isolates possessed HLPR, ½ of them were associated with HLAR, HLVR was also encountered. These isolates would have remained unrecognized by conventional susceptibility tests. The use of high content aminoglycoside disc for identification of HLAR enterococci and penicillin and vancomycin MIC test in DDT re-resistant enterococci is important for their early detection. Early identification can prevent treatment failure and control the spread of these organisms. Therapeutic significance of drug resistance offers challenge to therapy and opens the door for introduction of modified drug for clinical purpose.

In conclusion, multidrug resistant enterococci especially resistant to vancomycin and aminoglycosides have become a threat to patient's safety, making it a formidable nosocomial pathogen. The rising prevalence of antimicrobial resistance trait among *Enterococcus* spp. has critical outcome on health care system due to increasing in mortality as a result of existence of severe infections such as endocarditis without any effective antimicrobial therapeutic agents[5]. Hence, emergence of antimicrobial resistance, particularly multi-antibiotic resistant bacterial strains and shortage of newer antimicrobial agents with different mechanism of action from current antibiotics would be a serious problem in the near future and consequently development of novel alternative to conventional antibiotics is a necessity[3,13,22]. Implementation of effective infection control practices including use of gloves and gowns, scrupulous hand washing and correct non adherent practices are important contact precautions to reduce cross contamination by resistant organisms. Thus, multi-factorial control efforts can affect a decrease or at least prevent the spread of such strain in the hospital settings. With a long-term view toward new therapeutic approaches as well as optimal use of existing therapies, some researchers have begun examining in detail the interactions between enterococci and host[19]. A major obstacle is that enterococci also form part of the commensal flora. Since antibiotic use became widespread 50 years ago, bacteria have steadily and routinely developed resistance. Control of the emergence of resistance will depend on new approaches to prudent antibiotic use in hospitals and clinics, based in part on improved surveillance for MDR enterococci and

on better systems to encourage staff adherence to contact isolation procedures. Equally important will be development of new drugs with narrower spectra of activity aimed at known and potentially new targets and the evolution of market conditions that favor their use.

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