Multidrug resistant Enterococci isolated from urine samples at a tertiary care hospital

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ABSTRACT

Enterococci are one of the important causes of nosocomial urinary tract infections. They are considered as difficult to treat pathogens, due to their intrinsic resistance to several antimicrobial agents and their tendency to acquire resistance. Hence it is essential to find their resistance pattern constantly to institute empirical therapy and as a measure of infection control in hospitals. This study was carried out in the tertiary care hospital at Melmaruvathur, Tamil Nadu to detect resistance pattern and virulence traits of various uropathogenic Enterococcus species. A total of 100 Enterococcus species were included in the study. Although E.faecalis (87%) was commonest species associated with UTI, there was lenience towards E.faecium (9%), necessitates species level identification in laboratory settings. More than half of the species were isolated as mixture of two (47%) or three bacteria (7%). E.faecalis was sensitive to Vancomycin (100%), Ofloxacin (61%), Ciprofloxacin (59%), erythromycin (46%), Amikacin (41%), tetracycline (36%) and HLGR (23%). Increased incidence of Multidrug resistance and association of mixed infections warrant culture and sensitivity for all urinary tract infections.

Key words: *Multidrug Resistant Enterococcus, Urinary tract infection, Enterococcus faecalis, Enterococcus faecium.*

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INTRODUCTION

Enterococci are part of normal faecal flora in humans, also colonizes oral cavity, genitourinary tract and skin particularly in the perianal area[1]. The main sites of colonization in the hospitalized patients are soft tissue wounds, ulcers and gastrointestinal tract[2]. They were traditionally regarded as low grade pathogens but have emerged as second leading cause of nosocomial infections and third most common cause of bacteremia[3].

Urinary tract infection is the most common cause of nosocomial infection among hospitalised patients[4]. The most frequent infections caused by enterococci are UTI[5]. Intra abdominal and intra pelvic abscesses or post surgery wound infections[5], & blood stream infections[3], are also commonly caused by Enterococcus next to UTIs.

Since the inception of separate genus Enterococcus, there are 23 species of enterococci with clinical significance[6], of which Enterococcus faecalis and Enterococcus faecium accounts up to 90% of clinical isolates belonging to this genus[7].

Enterococci are considered important difficult- to- treat pathogens, due to their intrinsic resistance to several antimicrobial agents and their propensity to acquire resistance. High level Aminoglycoside Resistant Enterococci (HLAR) were first reported in France in 1979 and since then have been isolated from all the continents[8]. Treatment of serious enterococcal infections requires the combination of an aminoglycoside with beta lactum drug such as ampicillin for synergistic bactericidal effect[9]. However, enterococci strains that show a high level aminoglycoside resistance (HLAR) phenotype would no longer be susceptible to aminoglycosides and could not be used for the combination therapy[10]. Increased occurrence of high level aminoglycoside resistance has necessitated routine testing of the HLAR in Enterococcal isolates.

Resistance to β lactum and related antibiotics in enterococci are either due to the altered penicillin binding proteins or chromosomally mediated β lactamases. They were first reported in early 1980s. Therefore it necessitates the higher dosage of β lactum and related drugs[1].So detection of β lactum resistant Enterococci also should be reported. Resistance to tetracycline, erythromycin and chloramphenicol has been common throughout the world for several decades. Isolation of Vancomycin Resistant Enterococci (VRE), has limited the therapeutic options and is associated with increased mortality, length of hospital stay, admission to the ICU, surgical procedures & cost [11].In enterococci vancomycin resistance has been acquired either by mutation or by receipt of foreign genetic material through the transfer of plasmid[12], and transposons[13].VRE is associated with the *Van A, Van B, Van D or Van E* gene cluster. *Van A* and *Van B* genes are acquired through the transfer of plasmids or transposons[14].In contrast, E.gallinarum and E.casseliflavus possess intrinsic, non transferable Vancomycin resistance encoded by *Van C1* and *Van C2 ligase* genes respectively[15]. These species rarely cause infections and are associated with transmission and

hospital outbreak. Hence, for infection control practices and prevention of person to person transmission detection of VRE and speciation is necessary.

Few virulence factors have been identified like haemolysin, gelatinase, aggregation substances, surface protein & biofilm formation[16].Haemolysin increases the virulence of E.faecalis in infection models of different animal species. Gelatinase producing strains resulted in more severe clinical findings in experimental endocarditis model[17].Agg, is a surface protein encoded by sex-pheromone responsive plasmids, increases the number of bacteria adhering to renal and intestinal epithelial cells, suggesting agg is important for colonisation and translocation of host tissues by E. Faecalis[18].Enterococcal (esp)surface protein was found in E.faecalis, strain that caused multiple infections within a hospital ward. A variant *Esp* gene was also found in Vancomycin Resistant Enterococcus faecium spreading in hospitals[19].

This study was aimed to determine the prevalence of multi drug resistant uropathogenic Enterococcus, speciation and changing trends of isolation along with their virulence characterization.

MATERIALS AND METHODS

The study was conducted in the department of microbiology, Melmaruvathur Adhiparasakthi Institute of Medical Sciences and Research, Tamil Nadu, India from January 2012 to January 2014. A total of 100 Enterococcal isolates were included in the study and clinical data were collected from patients after obtaining informed written consent.

Methodology

Identification of *Enterococcus* was done using the following parameters (i) Colony morphology on blood agar, Cystine Lactose Electrolyte Deficient agar and Mac Conkey agar[20], (ii) Gram's stain (iii) Catalase (iv) Bile Esculin (v) heat resistance (vi) Salt tolerance. Then speciation was performed by sugar fermentation, pyruvate fermentation, motility and reduction of tellurite in tellurite blood agar plate. All the tests were carried out and interpreted as described by Facklam and Collins[21]. Determination of virulence factors such as haemolysis and gelatinase were carried out by appropriate tests [22].

Resistance profile

Antibiotic Susceptibility Pattern: Antimicrobial susceptibility test was performed by Kirby-Bauer disc diffusion method. The peptone water culture standardized to 0.5 McFarland opacity was used for surface seeding on Mueller Hinton agar. After plates were dried, antibiotic discs were placed over the medium and incubated at 37° C for 24 hours. Then the results were recorded[23] for the following antibiotic discs: Vancomycin (30µg), Erythromycin (15 µg), Amoxycillin (10 µg), Penicillin (10 µg), Amikacin (30 µg), High level gentamycin (120 µg), Ciprofloxacin (5 µg), Chloramphenicol (30 µg), Tetracycline (30 µg). E.fecalis ATCC 29212 was used as a control strain for disc diffusion tests [23].

High level Gentamicin resistance (HLGR):Minimum inhibitory concentration of Gentamicin was detected for the 31 isolates which showed resistance to high level gentamycin discs (120µg). The bacterial inoculums of 1.5 X 10^8 cfu/ml was prepared with brain heart infusion broth, stock solution of gentamycin 5120 µg /ml was prepared with distilled water, to which 9 ml of overnight culture of brain heart infusion broth, was added. Using the two fold dilutions up to 1 µg /ml was done. One control culture broth without antibiotic was also kept. After overnight incubation at 37°C with ambient air, the tubes were examined. The point, at which there was no visible growth, was taken as the minimum inhibitory concentration of gentamycin[24].

Production of beta lactamase: Employing iodometric method, production of beta lactamase in Enterococci was determined. β-lactamases are enzymes produced by microbes to destroy β-lactum ring, thereby showing resistance to the β-lactum and related antibiotic. Resistance to β-lactum antibiotic could also be by altered penicillin binding proteins.

1% soluble starch solution was prepared by dissolving starch at 100°C. Iodine reagent containing 2.03 g iodine and 5.32g potassium iodide in 100ml distilled water was also prepared. From an overnight culture of the test organism, a heavy suspension was made containing 10 ^{9} cfu/ml in 100 mm sodium phosphate buffer at pH 7.3 containing penicillin at 6 g/litre. A positive and negative control for β-lactamase producers was included in a microtitre plate with 0.1 ml aliquots into the wells. The bacterial suspension in BHI broth 100µl was added into the wells incubated for 1 hour at 37°C. Then two drops of 1% starch solution were added to each well followed by a drop of iodine. If blue colour is lost within 10 minutes, the presence of β-lactamase was inferred. If blue colour persists, cultures were considered negative for β-lactamase production. Since all the isolates showed resistance to penicillin and cephalosporin by agar disc diffusion method of Kirby Bauer, β-lactamase production was determined for all the isolates [25].

Detection of virulence factors

Gelatinase production: Gelatine breakdown by gelatinase can be demonstrated by incorporating it in a buffered nutrient agar, growing the culture on it and then flooding the medium with mercuric chloride solution that differentially precipitates gelatine or its breakdown products. Gelatin agar was seeded with the culture and incubated at 37°C for 48 hours. Then plates were flooded with tannic acid, which causes an opacity around the colonies, clearance in the medium [22].

β haemolysis detection: Enterococci are usually non haemolytic but sometimes α or β haemolytic. β

haemolytic strains are considered as virulent strains. Hence the property of β haemolysis was detected by using 5% human blood/ equine/ or rabbit blood agar medium. Colonies were inoculated on 5% human blood agar plate, incubated at 37°C for 24-48 hours. Then the plates were examined for β-haemolysis[22].

DISCUSSION

Since *Enterococcus* species has emerged as one of the leading nosocomial pathogen and important cause of UTI, it is important to know the changing trends of the *Enterococcus* infections and their antimicrobial susceptibility pattern. In our study, about 100 *Enterococcal* isolates were recovered from urine specimen.

Out of 100 isolates, 69% were from maleand 31% were from female patients. About 11% of isolates were recovered from patients below 20 years of age, of which 27% were obtained from neonates. Kafayat et al 2011 found *enterococcal* infections in the age group of 20-29 years constitute the largest proportion (42.4%) followed by age group of 30-39 years (23.7%) [26]. About 64%*enterococcal* isolates were recovered from hospitalized patients, having more contribution from urology patients (51%) (Table-2). This was supported by a survey done by CDC on nosocomial infections, in which *Enterococcus* accounted for 13.9% infections, being next to Escherichia coli as a causative agent of hospital acquired urinary tract infections [27].

Although the recent studies stated that there is an increase in isolation of *E.faecium* and other *enterococcal* species [28], In our study,*E fecalis* (87%) constitute the major isolate, followed by *E.faecium*(9%), *E.durans*(3%) and *E.raffinosus* (1%). Similar findings were shown by Facklam et al study [29]. *Enterococcus* were isolated in pure form (46%), recovered with other organisms as mixture of two organisms (47%) or three organisms (7%). Enterococcus was commonly associated with *E.coli* (46.3%), *Klebsiella* (16.7%) and *Candida* (11%). Enterococcal isolates were recovered as mixture of three organisms along with Coagulase Negative Staphylococcus & Candida. (Table 3).

In this study, gelatinase was produced by 36% of enteroccal isolates. Fifty two enterococcal isolates were gamma haemolytic and 27 & 21 were β and alpha haemolytic respectively.About 13% of isolates produced both gelatinase and beta haemolysin (Table 4). Hancock et al from California reported that inactivation of *gelE* geneencoding metalloprotease, gelatinase was found to prevent biofilm formation, suggesting that this enzyme is a unique target for therapeutic interventions in Enterococcal endocarditis [30]. A study by Vittal Prakash et al 2002 showed 2 of 44 *E.faecalis* and 1 of 4 *E.faecium* produced β haemolysin [31]. In contrast, haemolysin was produced by 82 % and gelatinase by 40.6 % of the isolates in a study by Sanal C et al 2013[32]. In Giridhara Upadhyaya PM et al study, Seventy-eight (39%) clinical isolates were gelatinase producing and 33 (16.5%) clinical isolates produced haemolysin [33].

E.faecalis isolates were sensitive to Vancomycin (100%), Ofloxacin (61%), Ciprofloxacin (59%), Erythromycin (46%), Amikacin (41%), Tetracycline (36%) and HLG(77%). *E.faecium* and *E.durans* showed 100% resistance to Ciprofloxacin, one of the commonest antibiotic used to treat urinary tract infection. About 59% of *E.fecalis* was found to be resistant to one of the commonly used antibiotic Amikacin. Fifteen isolates showed intermediate sensitive to Vancomycin by Kirby-Bauer disc diffusion method. All became sensitive to Vancomycin by E-test strip. 67% enterococcal isolates showed resistance to amoxicillin and β-lactamase was produced by 45% isolates. HLGR (120µg/ml) by disk diffusion method was observed in 31% isolates. HLGR was referred as MIC > 500µg/l [34],was seen in 23% isolates. (Table - 5).Sreeja S et al 2012 observed 45% resistance to ampicillin, 50% to ciprofloxacin and 47% to high level gentamicin among *Enterococcus* isolates [35]. A study by Kapoor et al 2005 observed 66% HLAR [36], Vittal P Prakash et al 2005 observed 43.5% HLGR [37]. Betalactamse by iodometric method was detected in(27%) isolates (Table-6).

Although E.faecalis (87%) was common species identified in clinical specimens, there was a deviation towards E.faecium isolation (9%). Further, there is an increased isolation of uncommon *Enterococcal* species and multidrug resistant *Enterococcus* with special reference to β lactamase producers (27%), HLGR (23%). Thus, definite identification of *Enterococci* at species level is mandatory to assess their variable sensitivity pattern and treat accordingly. Since nearly half of the *Enterococcal* isolates were identified as a mixed bacterial growth, ultimate care should be taken before choosing empirical antibiotic therapy.

Table 1: Basic data of patients with Enterococcus infection

Variables		No. of Enterococcal isolates (%)
Sex	Male	69 (69%)
	Female	31 (31%)
OP/IP	Inpatient	64 (64%)
	Outpatient	36 (36%)
Age	<20 years	11 (11%)
	20-40	46 (46%)
	40-60	22 (22%)
	60-80	21 (21%)

Table 2: Ward wise distribution of Enterococcus species (no.:100)

Ward	% of isolates
Urology	69
Medicine	8
Nephrology	6
Neonatal ward	3
Plastic surgery	1
ICU	7
Surgery	2
OG	4

Table 3: Pattern of Enterococcus isolation in culture

Pattern of mixture	Name of the Isolates	No. of isolates	Percentage
Enterococcus pure culture (46)		46	100
Mixture of two organisms (47)	Enterococcus+E. Coli	25	53.2
	Enterococcus+Klebsiella species	9	19
	Enterococcus+Candida species	6	12.7
	Enterococcus+ Staphylococcus aureus	2	4.3
	Enterococcus+ CONS	5	10.6
Mixture of three organisms (7)	Enterococcus+ CONS+ Candida species	7	100

Table 4: Haemolytic property and Gelatinase production by Enterococcus (total no.:100)

Haemolysis	Type	No. of isolates (%)
	Alpha	21 (21%)
	Beta	27 (27%)
	Gamma	52 (52%)
Gelatinase		36 (36%)

Table 5: Antimicrobial susceptibility pattern of Enterococcus (no.:100)

Drug	E faecalis (87)		E faecium (9)		E durans (3)		E raffinosus (1)	
	S(%)	R(%)	S(%)	R(%)	S(%)	R(%)	S(%)	R(%)
Erythromycin	40	47	2	7	1	2	1	0
Amoxycillin	62	25	6	3	1	2	0	1
Ciprofloxacin	51	36	0	9	0	3	1	0
Ofloxacin	53	34	6	3	1	2	1	0
Tetracyclin	31	56	7	2	1	2	1	0
Chloramphenicol	34	53	7	2	3	0	1	0
Amikacin	48	39	4	5	2	1	1	0
High level Gentamicin	57	30	8	1	3	0	1	0
Vancomycin	87	0	9	0	3	0	1	0

Table 6: Incidence of β-lactamase production in Enterococcus species (no.:100)

Production of beta lactamase	No. Of isolates (%)
β lactamase producers	27 (27%)
Non β lactamase producers	73 (73%)

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