

Original Research Article

Detection of various virulence factors in high level aminoglycoside resistance and vancomycin resistant enterococci isolates of uropathogenic Enterococci

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ABSTRACT

Background: Enterococci are common commensal organism of enteric tract and act as opportunistic pathogen and may cause infection in community as well as in hospitalised individuals. In present study association of several types of virulence factors like haemolysin, gelatinase and biofilm formation have been studied among HLAR and Vancomycin resistant *Enterococci* (VRE) isolates of enterococci among UTI patients.

Methods: The samples were collected from all hospitalized and OPD patients of MBS Hospital, JK Lone Hospital and NMC Hospital. Government Medical College, Kota, Rajasthan, India. A total of 360 isolates of enterococcus were collected during the period of 2 years from April 2016 to April 2018 in microbiology laboratory, Department of Microbiology, Government Medical College, Kota, Rajasthan, India. All virulence factors were detected by phenotypic methods and MIC values were detected for high level gentamicin and vancomycin.

Results: Among all enterococcal isolates most common factor was biofilm production 191 (53.05%) followed by haemolysin 131 (36.38%) and gelatinase production 72 (20%). Total resistant (MIC > 500 µg/ml) isolates for gentamicin was 194 (89.4%). In agar dilution 14 (11.2%) isolates were found sensitive, 61 (48.8%) isolates were found intermediate and 50 (40%) isolates were found to be resistant for vancomycin. HLAR and VRE was maximum associated with haemolysin + bio-film followed by gelatinase+biofilm, haemolysin+gelatinase+bio- film and least with haemolysin + gelatinase.

Conclusions: In present study enterococcus show significant production of biofilm and other virulence factors. With production of biofilm they become more resistant to routinely used concentration of antibiotics posing threat for treatment failure. A continuous monitoring is needed particularly for resistance to aminoglycoside and vancomycin to stop their institutional spread. Judicial use of antibiotics should be encouraged both in community as well as in institutions.

Keywords: Enterococcus, HLAR, Uropathogenic, Virulence factors, VRE

INTRODUCTION

Enterococci are common commensal organism of enteric tract and act as opportunistic pathogen and may cause infection in community as well as in hospitalized

individuals.^{1,2} Among all kind of infection, UTI is the most common infection caused by enterococci specially in patients with underlying structural abnormality and patient undergone urologic manipulation. In present study several types of virulence factors like hemolysin,

gelatinase and biofilm formation have been detected in enterococcus. Antibiotic treatment regimens for enterococci usually include Aminopenicillins such as ampicillin for uncomplicated infections, and a combination of an aminopenicillins with gentamicin or streptomycin in severe infections. In life threatening infection vancomycin is considered to be drug of choice but now a day's resistance against vancomycin has also developed. In present study association of various virulence factors among HLAR and vancomycin resistant enterococci (VRE) isolates were studied in isolates of enterococci among UTI patients.

METHODS

The samples were collected from all hospitalized and OPD patients of MBS Hospital, JK Lone hospital and NMC Hospital. Government Medical College, Kota, Rajasthan, India. A total of 360 isolates of enterococcus were collected during the period of 2 years from April 2016 to April 2018 in microbiology laboratory, department of microbiology, government medical college, Kota, Rajasthan, India. The isolates were maintained in 10% (v/v) skimmed milk solution with 10% glycerol (v/v) in distilled water and stored at -20°C.³

Detection. of virulence factors

Various virulence factors demonstrated are hemolysin production, bio-film production, gelatinase production (phenotypic method).

Hemolysin (cytolysin) production

It was determined by plating the enterococcal isolates onto Todd-Hewitt agar (Himedia laboratories, Mumbai, India) supplemented with 10% human defibrinated blood. A clear zone of β hemolysis around the bacterial colonies indicate the production of hemolysin.⁴

Bio-film production

Bio film production was detected by microtiter plate method.

Colonies of enterococci which grown overnight on blood agar were inoculated in trypticase soy broth (TSB) (Himedia laboratories, Mumbai, India) with 2% sucrose and incubated at 37°C overnight. This overnight growth was then diluted 1:100 in the TSB with sucrose. 200 μ l of this diluted inoculum was added onto sterile flat-bottomed polystyrene microtitre plates (LAXBRO Taurus Bio Medical aids Pvt. Ltd, Pune, India). The first well was used as control (without bacterial inoculum). The plates were then incubated aerobically at 37°C for 48 hours. The wells were then gently washed with PBS (phosphate buffer saline) (pH 7.2) to remove non adherent planktonic cells. The adherent bio films were then fixed by using 2% sodium acetate for 20minutes. Then the plates were dried and stained with 0.1% safranin for 20minutes. following

washing five times as previously described the absorbance was read at 490nm with an ELISA microtitre plate reader. Bio-film formation was considered to be high when the absorbance was >0.2 OD, moderate when it was between 0.20 and 0.10 and weak/absent when <0.10.^{5,6}

Gelatinase production

Todd-Hewitt agar (Hi media laboratories, Mumbai, India) with 30 g of gelatin per liter was used. A spot inoculation was made from colonies on the gelatin agar plates and the plates were then incubated overnight (18hours) at 37°C. Hydrolysis was determined by screening for the appearance of a turbid halo around the colony after the cooling the plates at 4°C for 5 hours rs.⁴

Detection of high-level aminoglycoside resistance (HLAR)

First all the isolates were tested by disc diffusion test (Kirby Bauer) using 120 μ g gentamicin disc. The isolates which show intermediate zone (7-9 mm) and resistance zone (<6 mm) to 120 μ g disc were further tested for MIC by E-strip method.^{7,8} MIC determination were done by using gentamicin Ezy MICTM (HiMedia) strip containing gradient concentration from 0.064 to 1024 mcg/ml. MIC >500 mcg/ml is considered as HLAR.⁹

Detection of vancomycin resistance

Vancomycin resistance was tested by vancomycin screen agar method. On brain heart infusion agar (Hi media laboratories, Mumbai, India) containing 6 μ g/ml Vancomycin. Plates were inoculated with 10 μ l spots of bacterial suspension after adjusting the turbidity with McFarland 0.5 standard with a micropipettor in order to achieve 10⁶ CFU per spot. Plates were read at 18, 24, and 48hours respectively. Growth was interpreted as positive, if confluent growth occurs, weak if hazy (that is difficult to read) or colonies <10, and as negative if no growth occurred after 48 hours. The test strain was considered resistant if it is positive, weak or more than one colony is present on-screen agar.¹⁰

RESULTS

Most common species found in present study was *Enterococcus faecalis* 222 (61.66%) followed by *E. faecium* 108 (30%), *E. durans* 22 (6.11%) and *E. avium* 8 (2.22%) (Table 1).

Distribution of various virulence factors among urinary isolates of enterococcus

Among all enterococcal isolates most common factor was biofilm production 191 (53.05%) followed by hemolysin 131 (36.38%) and gelatinase production 72 (20%) (Table 2).

Table 1: Distribution of various enterococcal species among total isolates.

Species	Numbers	% (N=360)
<i>E. faecalis</i>	222	61.66
<i>E. faecium</i>	108	30
<i>E. durans</i>	22	6.11
<i>E. avium</i>	8	2.22
Total	360	

Table 2: Distribution of various virulence factors among urinary isolates of enterococcus.

Species	Gelatinase	Haemolysin	Biofilm
<i>E. faecalis</i> (N=222)	42(18.91%)	78 (35.13%)	122 (54.95%)
<i>E. faecium</i> (N=108)	23 (21.29%)	40 (37.03%)	56 (51.85%)
<i>E. durans</i> (N=22)	6 (27.27%)	9 (40.9%)	9 (40.9%)
<i>E. avium</i> (N=8)	1 (12.5%)	4 (50%)	4 (50%)
Total (N=360)	72 (20%)	131(36.38%)	191(53.05%)

Distribution of combined virulence factors shared by urinary isolates of enterococcus

In present study most commonly shared virulence factor by a isolate was production of biofilm and hemolysin 50 (43.86%), 28 isolates (24.56%) showed combined production of biofilm + gelatinase, 24 isolates (21.05%) showed combined production of all the three virulence factors hemolysin + gelatinase + biofilm, and 12 isolates (10.52%) showed combined production of hemolysin + gelatinase production which is least combined virulence factor found (Table 3).

Table 3: Distribution of combined virulence factors shared by urinary isolates of enterococcus.

Combination of virulence factor	No. of isolates	Percentage N=114
Hemolysin + Gelatinase	12	10.52
Hemolysin + Biofilm	50	43.86
Gelatinase + Biofilm	28	24.56
Hemolysin + Gelatinase +Biofilm	24	21.05
Total	114	

Antibiotic sensitivity pattern among all enterococcus by Kirby -Bauer method

Ampicillin, piperacillin, ciprofloxacin, norfloxacin, nitrofurantoin, gentamicin, vancomycin and linezolid antibiotic discs were tested by Kirby-Bauer disc diffusion method. Linezolid was most effective among all followed by vancomycin nitrofurantoin, ampicillin, piperacillin,

ciprofloxacin, norfloxacin. High level gentamicin was tested for HLAR detection, this was sensitive to 143(39.72 %) and resistance for 217 (60.27%) isolates (Figure1).

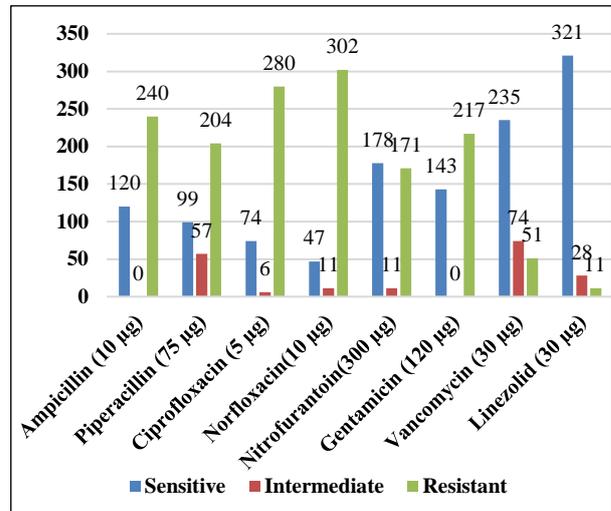


Figure 1: Antibiotic sensitivity pattern among all enterococcus by Kirby -Bauer method.

E. faecium isolates show maximum percent resistance for aminoglycoside 92 (85.18%), followed by *E. faecalis* 120 (54.05%), *E. avium* 2 (25%) and *E. durans* 3 (13.63%) (total 217 out of 360) where the N value was 108, 222, 8, and 22 respectively.

Gentamicin MIC value by E test among resistant isolates by disc diffusion test

There were 217 isolates resistant to gentamicin (120µg) disc by Kirby-Bauer disc diffusion method were further evaluated for their MIC value by gentamicin ezy MICTM strip (HLG) (0.064-1024mcg/ml) Hi-media. The MIC value is depicted in (Table 4).

Table 4: Gentamicin MIC value by E test among resistant isolates of disc diffusion test.

MIC value µg/ml	Number of isolates (%) N=217
384	23 (10.59)
512	30 (13.82)
768	34 (15.66)
≥1024	130 (59.9)
Total	217

Among 217 isolate 23 (10.59%) showed 384µg/ml MIC, 30 (13.82%) isolates showed 512µg/ml MIC, 34 (15.66%) isolates showed 768µg/ml MIC and 130 (59.9%) maximum isolates showed ≥1024µg/ml MIC value. By this method 23 (10.59%) isolates was susceptible (MIC <500 µg/ml) for synergistic action with cell wall active agents. So, the total resistant

(MIC>500µg/ml) isolates for gentamicin was 194 (89.4 %)

Vancomycin susceptibility on vancomycin screening agar

After disc diffusion susceptibility testing isolates which showed intermediate and resistance to vancomycin (Figure 1). (74+51=125) were further tested on vancomycin screen agar and in that 14 (11.2%) isolates were found sensitive and 111 (88.8%) isolates were vancomycin resistant enterococci (VRE). Among all species maximum percentage of vancomycin resistance was shown by *E. avium* 100% followed by *E. faecium*

89.47%, *E. faecalis* 88.8% and least was in *E. durans* 50% (Table 5).

Vancomycin MIC value of resistant isolates of enterococci confirmed in screen agar test

After screening on vancomycin screen agar, the 125 isolates were further evaluated for MIC value by agar dilution method (Table 6). Isolates which showed MIC of 2-4µg/ml, 8-16 and ≥32 were considered as sensitive, Intermediate and resistant respectively to vancomycin. In agar dilution 14 (11.2%) isolates were found sensitive, 61 (48.8%) isolates were found intermediate and 50 (40%) isolates were found to be resistant for vancomycin.

Table 5: Vancomycin susceptibility on vancomycin screening agar.

Vancomycin 6µg/ml	<i>E. faecalis</i> N=63	<i>E. faecium</i> N=57	<i>E. durans</i> N=2	<i>E. avium</i> N=3	Total N=125
Sensitive	7 (11.1%)	6 (10.52%)	1 (50%)	0	14 (11.2%)
Resistant	56 (88.8%)	51 (89.47%)	1 (50%)	3 (100%)	111 (88.8%)
Total	63	57	2	3	125

Table 6: MIC of resistant isolates of enterococci confirmed in screen agar test.

Interpretation	Vancomycin MIC value	<i>E. faecalis</i> N=63	<i>E. faecium</i> N=57	<i>E. Durans</i> N=2	<i>E. avium</i> N=3	Total N=125
S	2	6	1	1	0	8
	4	1	5	0	0	6
I	8	28	15	1	1	45
	16	9	7	0	0	16
R	32	4	3	0	0	7
	64	13	20	0	2	35
	128	2	6	0	0	8
Total		63	57	2	3	125

Table 7: Correlation of HLAR and VRE with virulence factors.

Virulence factors	HLAR (N=194) (MIC >500 µg/ml)	VRE (N=50) (MIC ≥32 µg/ ml)	P value
Haemolysin	26 (13.4%)	10 (20%)	0.2408
Gelatinase	5 (2.57%)	1 (2%)	0.8142
Biofilm	53 (27.31%)	13 (26%)	0.8514
Haemolysin + Gelatinase	3 (1.54%)	1 (2%)	0.8218
Haemolysin + Biofilm	25 (12.88%)	7 (14%)	0.8353
Gelatinase + Biofilm	17 (8.76%)	6 (12%)	0.4849
Haemolysin + Gelatinase + Biofilm	13 (6.7%)	5 (10%)	0.4262
Total	142	43	

Correlation of HLAR and VRE with virulence factors

A correlation between HLAR enterococci and VRE was done with various virulence factors produced by them (Table 7).

Among single virulence factor producing strains maximum HLAR and VRE was associated with production of biofilm. Second most associated virulence factor associated with HLAR and VRE was hemolysin production and least with gelatinase.

Statistically there were no significant correlation between virulence factors and HLAR and VRE isolates in present study ($P>0.05$). Among combined virulence factors producing strains HLAR and VRE was maximum associated with Hemolysin + bio-film followed by gelatinase + biofilm, hemolysin + gelatinase + bio-film and least with hemolysin + gelatinase.

In present study bio- film was prominent virulence factor which is associated with drug resistance.

DISCUSSION

Enterococcus is a most important cause of urinary tract infection (UTI) caused by gram positive bacteria. In present study urinary isolates of enterococci were studied for various virulence markers along with antibiotic susceptibility pattern specially for high level gentamicin resistance and vancomycin resistance were studied, which are very useful antibiotics for treatment of complicated enterococcal infections.

The resistance mechanism as describe for aminoglycosides among *E. faecalis* is also common in *E. faecium* but additionally *E. faecium* possess a chromosomally encoded 6'-acetyltransferase enzyme (AAC(6')-Ii) which is capable to modify tobramycin, sisomicin, kanamycin and netilmicin.^{11,12} Due to this fact, only two aminoglycosides (gentamicin and streptomycin) can be reliably used in clinical practice (for synergism with β -lactams) as these compounds are not readily affected by intrinsic enzymes produced by enterococci 11. A bifunctional modifying enzyme AAC (6')-Ie/ APH (2')-Ia that possesses both 6'-acetyltransferase and 2'-phosphotransferase activities is responsible for high-level resistance to Gentamicin which also confers resistance to all aminoglycosides except streptomycin.^{11,13,14}

In present study enterococcus show significant production of biofilm and other virulence factors like hemolysin and gelatinase. With production of biofilm they become more resistant to routinely used concentration of antibiotics posing threat for treatment failure.

By observing the various parameters of present study, it can be concluded that enterococci are now emerging as a potential pathogen, particularly among hospitalized patients. *E. faecalis* and *E. faecium* found to be the most prevalent species which confer resistance to various groups of antibiotics. In our institution Enterococcus isolates was more resistant to fluoroquinolones, aminoglycosides and β -lactams agents like Ampicillin & Piperacillin. This may be due to selection pressure of these antibiotics in our set up. Early detection of enterococcal species and resistance to Aminoglycoside and vancomycin can be helpful in limiting the morbidity in hospital set up. A continuous monitoring is needed particularly for resistance to Aminoglycoside and Vancomycin to stop their institutional spread. Judicial use

of antibiotics should be encouraged both in community as well as in institutions to limit the development of drug resistance, similarly an approach is to be included in treatment of patients to limit or decrease the production of Biofilm and other virulence factors like hemolysin and gelatinase as these factors may also play an important role in pathogenesis and development of drug resistance.

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Conflict of interest: None declared

Ethical approval: The study was approved by the Institutional Ethics Committee

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