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DETECTION AND CHARACTERISATION OF VANCOMYCIN RESISTANT ENTEROCOCCAL ISOLATES OF CLINICAL SAMPLES FROM A TERTIARY CARE HOSPITAL

Microbiology

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ABSTRACT

The emergence of Vancomycin resistant enterococci (VRE) has posed serious threats to the community because they exhibit multiple drug resistance, thus limiting the therapeutic options for the clinicians. As Vancomycin resistant enterococci (VRE) also have ampicillin resistance and high level aminoglycoside resistance, they are the most difficult to treat. The therapeutic options are limited by elimination of the synergy between aminoglycoside and the beta lactum drugs which is the treatment of choice for enterococcal infections which is of great concern. More antibiotic resistance makes these pathogens excellent survivors in hospital environment and cause nosocomial infections. A total of 142 enterococcal isolates from various clinical samples were identified to their species level and subjected to antimicrobial susceptibility testing to various antibiotics. Initial screening for Vancomycin resistance was done using the Vancomycin Screen Agar and the isolates showing resistance were subjected to Vancomycin and Teicoplanin MIC and later these isolates showing resistance were confirmed by genotypic methods for Vancomycin resistant genes.Total VRE isolates as per Vancomycin MIC value were 19 and the prevalence rate was 13.3% (19/142).In PCR assay, a total of 16 isolates including 13 E.faecium and 3 E.faecalis were found to be of Van B genotype and the remaining 3 isolates including 2 E.faecium and 1 E.faecalis were found to be of Van A genotype. In this study, the prevalence of Vancomycin resistance in Enterococcal species is 13.3% as per vancomycin MIC by Micro broth dilution technique. The phenotypic detection of Vancomycin resistance by MIC of Vancomycin and Teicoplanin correlates with the genotypic method of detection of Vancomycin resistant genes (VanA, VanB).

KEYWORDS

HLAR, Elimination Of Synergy, Correlation Of MIC Of Vancomycin And Teicoplanin To The Detection Of Vancomycin Resistant Genes , Regular Testing For VRE , Judicious Use Of Vancomycin

INTRODUCTION

Enterococci have emerged as an increasingly important cause of nosocomial infections. Urinary tract infections followed by intra abdominal and pelvic infections are the commonly encountered nosocomial infections caused by these organisms. They also cause bacteremia, surgical wound infections, endocarditis, neonatal sepsis and rarely meningitis (14). The major reason for the prolonged survival of these organisms in hospital environment is the intrinsic resistance to several antibiotics used commonly and their ability to acquire resistance to all currently available antibiotics either by gene transfer by plasmids and transposons or by mutation(25). The great concern about these organisms is the emergence of Vancomycin resistance. In this study, the prevalence of vancomycin resistance among the clinical enterococcal isolates has been assessed.

MATERIALS AND METHODS

Study Design: The present study was conducted in Government Rajaji Hospital, Madurai Medical College. The study period was from August 2013 to July 2014. Ethical committee clearance from the institution was obtained and informed written consent was received from the patients before collecting the specimens. A total of 482 clinical samples were collected from the patients admitted in various wards at Government Rajaji Hospital, Madurai.

Source And Sample Size

Urine samples,blood samples, pus and wound swab samples were collected from 482 patients admitted at Govt. Rajaji Hospital during the study period.

Inclusion Criteria

i) All age groups and both sexes were included.
ii) Patients admitted to various wards (ICU, CCU, Surgery, Medicine, Pediatrics, Urology) with signs and symptoms suggestive of impending infections such as post operative wound infection, wound infection following burns ,pyrexia of unknown origin, urinary tract infection, meningitis, endocarditis, intra abdominal abscesses and septicemia were included in this study.

Exclusion Criteria

i) All other wound infections
ii) Abscesses other than intra abdominal abscess

Culture Identification

After 24 hrs of incubation at 37°c, plates were examined for the presence of growth and the organisms were identified by colony morphology on solid media, gram staining, biochemical reactions and other identification tests. Based on biochemical tests such as acid produced from arabinose, mannitol, pyruvate, Raffinose, Sorbitol, Sucrose, and Sorbose, NH_3 produced from arginine, aesculin hydrolysis, capability to grow in 6.5% sodium chloride (Nacl) broth, presence of pyrrolidonyl peptidase (PYRase) and leucine aminopeptidase (LAP), motility and pigment production, species of enterococci were indentified. E. faecalis was the most commonly encountered species. The next common isolate was E. faecium.

Antibiotics Susceptibility Testing

Preparation Of Inoculum

Morphologically similar 4 to 5 isolated colonies of Enterococci were taken from 24 hrs culture plate with the help of a sterile loop and transferred to a test tube containing sterile peptone water and incubated at 37° C for 4 hours. Then the turbidity was adjusted to 0.5 McFarland turbidity standards by using Wickerham chart. This inoculum was used for antibiotic susceptibility testing.

Antibiogram By Kirby – Bauer Disc Diffusion Method

According to CLSI guidelines (CLSI document M02&M07) the antibiotic susceptibility test was performed by using the Kirby – Bauer disc diffusion technique. Sheep blood agar was used for antibiotic susceptibility test.The inoculated plates were incubated aerobically at 37°C overnight. Zone size was interpreted using control strain Enterococcus faecium BM4147 under reflected light except for vancomycin which should be read through transmitted light. The interpretation as susceptible, intermediate and resistant was done according to the CLSI guidelines

Screening Test For High Level Aminoglycoside Resistance (HLAR)

The enterococcal isolates were screened for high level resistance to aminoglycosides using the high level gentamicin (HLG) – 120 µg and high level streptomycin (HLS) 300 µg on sheep blood agar by standard disc diffusion method as described above.The test also included E. faecalis ATCC 29212 as negative control and E. faecium BM 4147 as positive control.

Detection Of Vancomycin Resistance

Vancomycin screen agar (ie) brain heart infusion (BHI) agar containing 6 µg/ml of vancomycin was used for the presumptive identification of vancomycin resistance. Spot inoculation of 10µl of 0.5 McFarland turbidity standard bacterial suspension along with positive and negative control strains was done on to the agar surface and the plates were incubated for 24 hrs aerobically at 37°C. Growth of > 1 colony indicates presumptive vancomycin resistance. It should

be confirmed by determining the minimum inhibitory concentration (MIC) for vancomycin. This test included E. faecalis ATCC 29212 as negative control and E. faecium BM 4147 as positive control.

Vancomycin Mic By Broth Micro Dilution Method

As per the CLSI recommendations, the minimum inhibitory concentration of vancomycin for the Enterococcal isolates grown on Vancomycin screen agar was done.

Preparation Of Inoculum

Bacterial inoculum is prepared by direct colony suspension method and standardized with 0.5 McFarland standard. 1 in 100 dilution of this inoculum is prepared by adding 0.01ml of the inoculum to 0.9ml of Cation Adjusted Muller Hinton Broth (CAMHB). 10 µl of this diluted suspension is equivalent to the final recommended inoculum of 5 x 10^4 CFU/ml and it is dispensed per well.

Preparation Of Drug Concentrations Of Vancomycin

Stock solution of the drug is prepared as recommended in CLSI document on dilution testing. The vancomycin drug is dissolved in distilled water and the master dilution is prepared by diluting the required amount of drug in CAMHB. Serial doubling dilution of the master dilution was performed in CAMHB such as 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 2048 µg/ml. Then 100µl of serial doubling dilution of Vancomycin along with 10µl of bacterial suspension is dispensed in the corresponding wells with a growth control (well containing CAMHB and bacterial inoculum without Vancomycin) and sterility control (well containing CAMHB only) and the microtitre plate was incubated for 24 hrs at 37°C in an ambient air. The test included E. faecalis ATCC 29212 as negative control and E. faecium BM 4147 as positive control. Results were interpreted after reading the MIC of control strains. The MIC range of E. faecalis ATCC 29212 should fall within the range of 1-4µg/ml. The MIC of other isolates was interpreted as per CLSI guidelines.

Minimum Inhibitory Concentration For Teicoplanin

The teicoplanin MIC was also tested in the same method as described above. The concentrations of Teicoplanin prepared were 0.125,0.5,1,2,4,8,16,32,64,128 µg/ml by dissolving the teicoplanin in Cation adjusted muller hinton broth (CAMHB) as described in the CLSI guidelines.The MIC results were interpreted by noting the minimum concentration of the drug showing complete inhibition of the growth of the organism in the same way as described for Vancomycin.

Molecular Method For The Detection Of Vancomycin Resistant Enterococci

Polymerase chain reaction (PCR) assay was done for the detection of Vancomycin resistance genes in Enterococci especially in E. faecalis and E. faecium by the PCR kit procured from Helini Biomolecules, Chennai. DNA was extracted by using Pure Fast R Bacterial Genomic DNA purification kit.2X PCR Master mix contained 2U of Taq polymerase, 10X Taq reaction buffer, 2mM Mgcl2, 1µl of 10mM dNTPs mix and PCR additives. Agarose gel electrophoresis was performed with agarose, 50X TAE buffer, 6X gel loading buffer and Ethidium bromide.

PRIMER

Van A Primers

Forward primer: 5'-TGCGCGGAATGGGAAAACGACA-3'
Reverse primer: 5'-CAGCCCGAAACAGCCTGCTCAA-3'
PCR Product size: 473bp

Van B Primers

Forward Primer: 5'-TCTTTGTGAAGCCGGCACGGTC-3'
Reverse Primer: 5'-AGCCGACCTCACAGCCCGAAAT-3'
PCR Product size: 147bp

PCR Amplication:

The PCR reactant mixture for each sample is prepared by adding 10µl of PCR master mix, 5µl of primer mix and 5µl of purified DNA of each sample to a total final volume of 20µl.

PCR Procedure:

20µl of the PCR reactant mixture was mixed gently, spin down briefly and placed into PCR machine. It was programmed as follows:
Initial Denaturation: 94ºC for 3 min followed by
Denaturation: 94ºC for 30sec / **Annealing:** 58ºC for 30sec / **Extension:** 72ºC for 30sec (35 cycles)
Final extension: 72º C for 5 min

Agarose Gel Electrophoresis:

2% agarose gel was prepared by mixing 2gm of agarose in 100ml of 1XTAE buffer. 8µl 6X Gel loading dye was added to each PCR vial and 5µl of PCR sample was loaded. After that run electrophoresis at 50V till the dye reaches three fourth distances .The bands were observed in Uvtransilluminator. 10µl of 100bp DNA Ladder (100bp,200bp,300bp, 400bp,500bp,600bp,700bp,800bp,900bp,1000bp) was used. The presence of VanA and VanB genes were indicated by the amplification of 473bp and 147 bp PCR products from the clinical isolates respectively.

RESULTS

Table - 1Specimen Wise Isolation Of Enterococcal Isolates

Specime-n N=142	No Of Entrococcal Isolates	Percentage
URINE	58	40.84%
BLOOD	32	22.53%
PUS	27	19.01%
WOUND SWAB	25	17.60%

Urine, blood, pus and wound swab samples collected from 482 cases admitted at Govt. Rajaji Hospital, Madurai were included in this study. Both sexes of all age groups were included. Among 482 samples, 463 showed growth and 19 samples showed no growth. Out of 482 samples, 174 from urine, 132 from blood, 89 from pus and 87 from wound swab. Among 482 samples, 142 were enterococi, 146 were other gram positive cocci and 175 were gram negative bacilli. Among the 142 enterococcal isolates, 58 were isolated from urine sample, 32 from blood sample, 27 from pus sample and 25 from wound swab sample. From the above table, it is observed that enterococci were isolated more from urine sample (40.84%) followed by blood sample. (22.53%).

Table-2 Distribution Of Enterococcal Species Among The Specimen.

Specimen n=142	E.faecalis	E.faecium	E.Raffinosus	E.sulfurous	Total
Urine	34(23.94%)	19(13.38%)	4(2.82%)	1(0.7%)	58
Blood	18(12.68%)	12(8.45%)	2(1.4%)	0.0	32
Pus	16(11.26%)	8(5.63%)	2(1.4%)	1(0.7%)	27
Wound swab	18(12.68%)	6(4.22%)	0.0	1(0.7%)	25
Total	86(60.56%)	45(31.69%)	8(5.63%)	3(2.11%)	142

E. faecalis 86 (60.56%) was the predominant species followed by E. faecium, 45 (31.69%). Other enterococcal species isolated were E. raffinosus from urine, blood and pus and E. sulfureus from urine, pus and wound swab.

Antibiotic Susceptibility Pattern Of Enterococcus Species By Kirby Bauer Disc Diffusion Method

According to the CLSI guidelines, results were interpreted by measuring the zone of inhibition of growth around each discs. Most of the E. faecalis were sensitive to ampicillin (84%), high level streptomycin (66%), high level gentamicin (53%) teicoplanin (91%) and Vancomycin (95%). They were resistant to ciprofloxacin (76%) and doxycycline (79%). Antimicrobial susceptibility pattern of E. faecium showed resistance to ampicillin (73%), ciprofloxacin (82%), doxycycline (62%) and high level gentamicin (64%) and sensitivity to high level streptomycin (78%) Teicoplanin (93%) and vancomycin (67%). E. faecium showed more antimicrobial resistance than E. faecalis. 5% of E. faecalis and 33% of E. faecium isolates were vancomycin resistant. The total high level aminoglycoside resistance (HLAR) observed was 69%. 19% isolates showed resistance to both high level streptomycin and high level gentamicin. 21% isolates showed only resistance to HLS and 30% isolates showed only resistance to HLG.

Table - 3Mic Values Of Vancomycin For The Vre Isolate

ENTROCOCCAL ISOLATES			E.faecium	E.faecalis	Total
VRE Isolates			15	4	19
Vancomycin MIC Value µg/ml	Intermediate 8 -16 µg/ml	8 µg/ml	-	-	-
		16 µg/ml	1	1	2
	Resistant > 32 µg/ml	32µg/ml	2	1	3
		64µg/ml	4	2	6

		128µg/ml	6	-	6
		256µg/ml	1	-	1
		512µg/ml	1	-	1
TOTAL			15	4	19

Out of 19 VRE isolates, 2 isolate fall within the intermediate range of 8-16 µg/ml and remaining 17 isolates fall within the resistant range of 32 – 512 µg/ml , Interpreted as per CLSI guidelines.

Table-4 Mic Value Of Teicoplanin For The Vre Isolates

ENTROCOCCAL ISOLATES			E.faecium	E.faecalis	Total
VRE Isolates			15	4	19
Teicoplanin MIC Value µg/ml	Susceptible	0.5µg/ml	7	2	9
		1µg/ml	6	1	7
		2µg/ml	0	0	0
		4µg/ml	0	-	0
		8µg/ml	-	-	-
	Intermediate 16 µg/ml		-	1	1
Resistant ≥32 µg/ml			2	-	2
TOTAL			15	4	19

As per CLSI guidelines, results were interpreted. Out of 19 VRE isolates the Teicoplanin MIC of 16 isolates fall within the susceptible range (0.5 – 1 µg/ml), 1 isolates fall within intermediate range (16µg/ml) and 2 isolates fall within resistant range (> 32 µg / ml).

Table-5 Distribution Of Van A And Van B Genotypes In The Vre Isolates By Pcr Assay

ENTEROCOCCAL ISOLATES		E.faecium	E.faecalis	Total
TOTAL VRE ISOLATES		15	4	19
Van A GENO TYPE	PRESENT	2(13.33%)	1(25%)	3(15.79%)
	ABSENT	13	3	16(84.21%)
Van B GENO TYPE	PRESENT	13 (86.67%)	3 (75%)	16 (84.21%)
	ABSENT	2	1	3(15.79%)

Out of 15 E. faecium VRE isolates, 2 were Van A genotype positive and 13 were Van B genotype positive. Out of 4 E. faecalis VRE isolates, 1 showed Van A genotype and 3 isolates showed Van B genotype. Van A and Van B genotype isolates showed bands at 473bp and 147bp in polymerase chain reaction (PCR) assay.

Two E. faecium isolates and one E. faecalis isolates showed high level resistance to both Vancomycin and Teicoplanin and were of Van A genotype. Thirteen E.faecium isolates and three E. faecalis isolates showed variable level resistance to Vancomycin and susceptible to Teicoplanin and were of Van B genotype.

Table-6 The Comparison Of Mic Values Of Vancomycin And Teicoplanin Of Vre Isolates With Van A And Van B Genotypes.

MIC	Van A GENO TYPE	Van B GENO TYPE	Total
VAN MIC ≥ 64µg/ml TEICO MIC>16µg/ml	3	--	3
VAN MIC ≥ 8µg/ml TEICO MIC >0.5-1µg/ml	--	16	16
TOTAL	3(15.79%)	16(84.21%)	19

Hence, there is 100% correlation between phenotypic classification of Vancomycin resistance by Vancomycin and Teicoplanin MIC and genotypic detection of Van A and Van B resistance type by PCR assay.

DISCUSSION

The great concern about enterococci is the emergence of Vancomycin resistance. VRE, especially E. faecium has emerged as an important nosocomial pathogen and represents a serious threat to patients with impaired host defences (2). The glycopeptide (Vancomycin) resistance genes commonly encountered are as follows.

Van A It is an inducible high level resistance to both Vancomycin (MIC 64-1000µg/ml) and Teicoplanin (MIC 16-512 µg/ml).It is mediated by transposon Tn 1546.

Van B It is an acquired inducible, variable level resistance to Vancomycin (MIC 8 – 1000 µg/ml) but susceptible to Teicoplanin (MIC 0.5 – 1µg/ml). The gene is located in plasmid and mediated by *transposons Tn 1547, Tn1549, Tn 5382.*

Van C exhibits intrinsic, low level resistance to Vancomycin (MIC 2-32µg/ml) and susceptible to Teicoplanin (MIC 0.5-1 µg/mi) .

Van Dconstitutively expressed, chromosome mediated, moderate level resistant to Vancomycin (MIC 64- 128 µg/ml) and susceptible / resistant to teicoplanin (MIC 4 – 64 µg/ml) .

Van E, Van G and Van L – located in chromosome results in inducible intermediate level resistance to vancomycin (MIC 8 – 32 µg/ml), susceptible to teicoplanin (MIC 0.5-1µg/ml).

In the present study, out of 482 samples processed, 142 (29.46%) were enterococcal species. Other organisms isolated were 146 (30.29%) other gram positive cocci and 175 (36.3%) gram negative bacilli. Among 142 clinical enterococcal isolates, 58 (40.84%) were from urine, 32 (22.53%) from blood, 27 (19.01%) from pus and 25 (17.60%) from wound swab samples. The results correlated with the previous study by Baragundi MC et al who observed that out of 120 enterococcal isolates, 50 were isolated from Urine sample, 35 were from blood sample, 20 were from pus sample and 15 were from wound swab samples (4). In both studies, isolation rate of enterococci were more from urine samples followed by blood samples. The present study detected 86 (60.56%) E. faecalis, 45 (31.69%) E. faecium, 8 (5.63%) E. raffinosus and 3(2.11%) E. sulfurous from various clinical samples. A prospective study conducted by Seema sood et al showed 42.90% E.faecium and 40% E.faecalis as the predominant isolates. In blood culture, E.faecium was the commonest isolate. In pus and urine samples, E.faecalis was predominant (19).

The antimicrobial susceptibility pattern of E. faecium showed 73% ampicillin resistance. Agarwal J et al have reported significantly higher resistance to ampicillin among E. faecium isolates which was similar to our study (1) and they have documented multidrug resistant enterococci similar to our finding (8,23).E. faecium strains as compared to E.faecalis display a higher degree of drug resistance to multiple antibiotics including ampicillin,gentamycin,ciprofloxacin, vancomycin and teicoplanin (7,14).The total high level aminoglycoside resistance (HLAR) observed was 69%. This finding is similar to the report by Kapoor et al and in their study they reported 66% of total HLAR(9). 63% of E. faecium and 82% of E. faecalis showed total HLAR in our study. In Mohanty S et al study, 81% of E. faecium and 72% of E. faecalis isolates exhibited HLAR (13). In this study, 21% of resistance to high level streptomycin only and 30% of resistance to high level gentamicin only were reported(6).

Among 142 enterococcal isolates, 19 were identified as vancomycin resistant on vancomycin screen agar(8). These isolates were subjected to detection of minimum inhibitory concentration for vancomycin and teicoplanin as a confirmatory test.In broth microdilution technique, 2 isolates showed vancomycin MIC in intermediate range (16 µg / ml) and remaining 17 isolates showed vancomycin MIC in resistant range (> 32 – 512 µg / ml) (3). For teicoplanin, 16 isolates showed susceptible range of 0.5-1 µg/ml, 1 isolate showed intermediate range of 16 µg /ml and 2 isolates showed resistant range of > 32 µg/ml. About 13 E. faecium isolates and 3 E. faecalis isolates showed variable range of resistance to vancomycin (MIC 16-512µg/ml) and susceptible to Teicoplanin (0.5 – 1 µg/ml) and are of Van B phenotype. 2 isolates of E. faecium and 1 isolate of E. faecalis showed high level resistance to both vancomycin (MIC > 64 µg/ml) and Teicoplanin (MIC > 16 µg/ml) and are of Van A phenotype(11). In this study, VanB phenotype was more prevalent than VanA phenotype. This result was similar to the study conducted by Taneja N et al, Kapoor et al and Karmarker MG et al and all these studies shown higher isolation of VanB phenotype than VanA phenotype (9, 10 and 21). All the 19 VRE isolates were subjected to polymerase chain reaction (PCR) which showed 13 E. faecium and 3 E. faecalis were of Van B genotype and 2 E. faecium and 1 E. faecalis were of Van A genotype (5). The present study shown higher isolation rate of VanB genotype 84.2% (16/19) than VanA genotype 15.7% (3/19) This result was similar to the study by Nelson et al who documented that 97% isolates were Van B positive genotype and the remaining isolates were Van A genotype(14,18).

The prevalence of VRE in this study was 13.3% which was higher than the results shown by Baragundi et al, Taneja N et al, Kapoor et al ,they

reported 7.5%, 5.5% and 8% of VRE respectively (3,9,21) and was lower than the study of Karmarker MG et al, they reported 23% VRE (10). The present study showed 100% correlation between phenotypic classification of Vancomycin resistance by detecting Vancomycin and Teicoplanin MIC and genotypic detection of Van A and Van B resistance type of VRE by PCR assay (5, 12).

Commonly used antibiotics that achieve high gastro intestinal concentrations but are inactive against enterococci such as the cephalosporins, ticarcillin and vancomycin favour colonization with high levels of VRE(20).Increased use of Vancomycin for the treatment of methicillin resistance in Staphylococcus aureus and Coagulase negative staphylococci probably contributing to the emergence of Vancomycin resistant enterococci(VRE).In European countries, the use of avoparcin in animal husbandry was responsible for the emergence of VRE. Montecalvo et al showed that adult patients hospitalized on an oncology unit showed persistent gastrointestinal colonization with VRE for minimum of 7 weeks in the most of the patients studied (15,16). In a study comparing the prognosis of patients with Vancomycin sensitive versus Vancomycin resistant enterococcal bacteremia, clinical failure was higher for patients with VRE bacteremia (60%) versus VSE bacteremia (40%) ($p<0.001$).

CONCLUSION

Judicious use of Vancomycin , rapid isolation of patients suspected to have VRE infection and regular testing of all Enterococcal isolates for Vancomycin resistance is recommended for the prevention of nosocomial transmission of VRE (17).In molecular method limited settings ,phenotypic detection of Vancomycin resistance by Vancomycin and Teicoplanin MIC can be recommended (2,22).

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