Int.J.Curr.Microbiol.App.Sci (2017) *6*(7): 1160-1173

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 7 (2017) pp. 1160-1173**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.607.141

Phenotypic and Genotypic Characterization of *Enterococci* from Clinical Isolates in a Tertiary Care Hospital

Mohamadiya Rizwana*, S. Parvathi and B. Appalaraju

Department of Microbiology, PSG IMSandR, Coimbatore, Tamil Nadu, India
**Corresponding author*

A B S T R A C T

Enterococci mainly being commensals in human faeces are now considered as an important cause of nosocomial infections. Of the infections, the commonly observed are urinary tract infections, abdominal infections followed by bacteremia, endocarditis and meningitis rarely. Speciation of *Enterococcus* aids in effective management of the infections caused by them. The initial treatment for enterococcal infections has become challenging due to development of resistance. The resistance has been observed mainly against aminoglycosides due to the presence of bi-functional gene. VRE strains are mostly isolated from patients with recurrent bacteremia, endovascular infections leading to increase deaths in the patients. To isolate and speciate enterococcal isolates from clinical samples, study the antibiotic susceptibility pattern and the genotype associated with the aminoglycoside and vancomycin resistance. *Enterococcus* isolates obtained from various sections were characterized by conventional phenotypic methods. The antibiotic susceptibility was studied by disc diffusion method. The resistant strains were further confirmed by MIC using automated methods following which the genotypic analysis was done. The incidence of *Enterococci* was 2.5% in which around 86.4% were obtained as pure isolates. Among the species *E. faecalis* was found to be the maximum. The urinary isolates exhibited sensitivity of around 92.3% to nitrofurantoin and among the non - urinary isolates the maximum sensitivity was for linezolid. The molecular study showed van A to be most common gene with vancomycin resistance (71.42%), and bifunctional gene among the aminoglycoside resistance (96%). *Enterococcus faecalis* was the commonest species isolated. The maximum number of isolates was obtained from urinary samples. The increase incidence of resistance gene to high level gentamicin could result in failure to the synergy treatment of gentamicin in combination with penicillin group of antibiotics. The emergence of vancomycin resistant strains among the clinical isolates makes treatment options more challenging.

Keywords

Enterococci, Speciation, Antibiotic resistance, High level gentamicin, Vancomycin, Bifunctional gene.

Article Info

Accepted:
17 June 2017
Available Online:
10 July 2017

Introduction

Enterococci are gram positive organisms having the property to grow at 6.5% NaCl concentration, pH of 9.6 and hydrolyze bile esculin. Initially they were grouped under group D Streptococcus but later with the help of DNA studies they are characterized under the genus *Enterococcus* in 1960. They were considered as harmless pathogens formerly, but in the recent years have been emerging as the most important causative agents in nosocomial infections (Murray *et al.*, 1990).

Enterococci being the commonest micro flora of both humans and animals share their source

of origin. They have been isolated from variety foods including cheese, fish, sausages, beef and pork. Studies have been reported that *Enterococci* have been isolated from various clinical and environmental samples all over the world. Among the isolates obtained clinically it has been observed that *Enterococcus faecalis* has been the predominant species clinically (Lancefield *et al.,* 1933; Cetinkaya *et al.,* 2000).

Enterococcal infections have been referred to as tough, tenacious and troublesome infections (Marothi *et al.,* 2005).In the past years there has been an increase in the prevalence of enterococcal infections in hospitals and of particular concern is the emergence of antimicrobial resistant strains. Being second commonest organism behind the abdominal and pelvic infections, third in causing blood stream infections, cases have also been reported in causing CNS and neonatal infections.

Reports have been confirmed with their association with clinical conditions including respiratory tract infections, osteomyelitis and cellulitis (Fischer *et al.,* 2009). *Enterococci* were initially regarded as the disease causing agent in the early nineties. But now various reports have been established reporting them as the second commonest pathogen (Kuhn *et al.,* 2003).

Species identification becomes essential for investigating outbreaks in case of nosocomial infections and also susceptibility pattern varies accordingly which is also helpful in clinical management of infections due these agents (Gordon *et al.,* 1992).

Infections caused by enterococcal pathogens were initially being treated with the combined treatment of cell wall active agents as penicillins and aminoglycoside as the synergistic effect was effective. But now due to the development of antibiotic resistance as high level aminoglycoside resistance, beta lactamase production and also resistance to vancomycin have led to serious concern in management of these nosocomial pathogens (Mendiratta *et al.,* 2008). Vancomycin resistant *Enterococcus* was initially reported in 1980s, after which there was a increase in the incidence of its spread. The main phenotypes associated with its spread are Van A, Van B and Van C. Van A and Van B are mainly associated with *E. faecalis* and *E. faecium*. Van C is noted in *E. gallinarum* and *E. casseliflavus*. Among the phenotypes Van A is predominantly associated in the resistant strains.

VRE strains are mostly isolated from patients with recurrent bacteremia, endovascular infections leading to increase deaths in the patients. Vancomycin resistance and penicillin resistance are often seen co-existing which makes the treatment harder (Murray *et al.,* 1990).

Resistance development in *Enterococcus* against glycopeptide antibiotics gets transferred from them to other bacterial pathogens which include *Staphylococcus aureus, Streptococci, Listeria monocytogenes* (Gold *et al.,* 1996).

Thus due to the recent emergence in association with significant clinical infections and development of resistance, *Enterococci* have gained importance. Studies are thus required especially in tertiary care hospitals for appropriate isolation, identification and speciating them.

Antibiograms should also be followed up in accordance to the species. Emergence of VRE needs to be checked and limit its spread in hospital environment. CDC also stresses the control of VRE in hospitals by educating the staff in detecting VRE early, reporting them and action plan taken promptly.

Our study aims to isolate, speciate the *Enterococcus* in a simplified manner and also to study the drug sensitivity and resistance patterns among the isolates obtained.

Materials and Methods

The Institutional Human Ethical clearance was obtained before the commencement of the study.

Type of study

Experimental study

Confidentiality

Confidentiality of the reports were maintained

Sample size estimation

A total of 250 isolates of *Enterococci* obtained from outpatient and inpatient samples from blood section, miscellaneous and urine section were processed for the species identification, antimicrobial sensitivity followed by molecular study.

Specimen processing: Specimens from various sections including urine, pus and blood for cultures were collected and processed in the laboratory.

Pus/wound swabs

The swabs were streaked in blood agar and MacConkey agar and were incubated at 37^0C for 24 hours.

Urine

A loopful of the uncentrifuged specimen was examined as a wet mount for the presence of pus cells and bacteria. One loopful of specimen was inoculated onto blood agar and Mac Conkey agar. Plates were incubated at 37^0 C for 24 hours.

Growth in sheep blood agar

Following overnight incubation of the samples, colonies of *Enterococcus* were seen as grey colonies of about 0.5-1mm in diameter with alpha, beta or gamma hemolysis.

Growth in Mac Conkey agar

Colonies were about 0.5- 1mm in size with a smooth surface and convex margins, tiny magenta colored following 18-24 hour growth.

Catalase test

A drop of the catalase reagent (30% hydrogen peroxide) was added to the slide.

The growth was further applied to it with an applicator stick. Appearance of bubbles was taken as positive test.

Enterococcal colonies showed a negative test due to the absence of bubbles.

Gram stain

Smears made from the colony showed Gram positive cocci arranged singly, in pairs or in short chains.

Storage

If the isolate was catalase negative, gram staining showing the presence of gram positive cocci in pairs or short chains and growth in MacConkey agar as tiny lactose fermenting colonies, they were sub cultured and pure growth was stocked in Robertson's cooked meat medium in tubes.

The tubes were sealed and stored at 4-8^0C for 3-6 months. When the test was to be done they were further sub cultured.

Identification tests

Growth in media with pH – 9.6

Isolated colonies of *Enterococcus* was streaked in a media with a pH 9.6 and incubated at 37^0C overnight. The presence of growth was taken as positive.

Tolerance with 6.5% NaCl

The colonies were inoculated in a broth with 6.5% NaCl, and incubated overnight. The next day the tubes were checked for any increase in turbidity. This was further confirmed by sub culturing onto the agar plates. Growth confirmed the presence of organism. Whereas absence of turbidity indicated organism was unable to grow in this concentration.

Heat tolerance test

Enterococcus was inoculated in Todd-Hewitt broth. It was later incubated in water bath set at 45^oC overnight and 60^0 C for half an hour, and then sub cultured. *Enterococci* survived both the temperature showing positive reaction.

Bile esculin test

Colonies from 24 hour culture were inoculated in media containing 40% bile. Blackening of the medium confirmed positive reaction for *Enterococci* and non-*Enterococci*.

PYR test

The colonies were inoculated in PYR broth and incubated for a period of 4 hours. Later 3 drops of PYR reagent was added to it and change of colour to red was confirmed positive indicating *Enterococci* whereas absence of colour change indicates negative test.

Motility test

Stab culture of the colony was done in a semi-solid media and motility was indicated by the spread of the organism into the media. The strains which did not diffuse into the media were considered non-motile. Tubes were incubated for up to 7 days for confirming motility.

Arginine test

A well isolated colony of *Enterococcus* from the culture was inoculated in Moeller's decarboxylase media with arginine as the amino acid and overlaid with mineral oil. After 24 hours of incubation the colour of the tube was changed to purple indicating a positive reaction. Yellow colour indicated only acid production only and no deamination.

Potassium tellurite reduction test

Colonies were streaked in media containing 0.004%potassium tellurite. *E. faecalis* formed black colonies with the reduction of tellurite to tellurium.

This test was used to differentiate *E. faecalis* from *E. faecium.*

Pyruvate test

When inoculated in a media containing pyruvate with bromothymol blue as indicator *E. faecalis* showed a colour change from green to yellow color after overnight incubation. Absence of colour change ruled out *E. faecalis.*

Pigment production

Ability to produce pigment was observed by a few species of *Enterococci*. This was tested by sub culturing colonies in nutrient agar.

Any color produced was visible and also further confirmed by touching the colony with a sterile swab.

Sugar fermentation tests

Species identification was further done by sugar utilization by inoculating the organism in peptone with 1% sugars with Andrade indicator. The sugars used were mannitol, arabinose, raffinose, sorbose, sorbitol, lactose and sucrose. Any change in colour from colourless to pink indicated the positive sugar fermentation test. Absence of colour change was taken as negative.

Antimicrobial susceptibility test: (CLSI 2015)

The 250 test isolates of *Enterococci* were checked for the antimicrobial susceptibility pattern using Kirby-Bauer disc diffusion method. Three or four pure colonies were inoculated in peptone water and incubated for about 3 hours. The turbidity of the growth was matched to 1 of Mc Farlands. A lawn culture of the broth with a sterile swab was done in Muller Hinton agar and the discs were placed at a distance of 24 mm.

About 6 discs were kept on each plate. The discs used were Penicillin (10U), Ampicillin (10µgm), Amoxicillin Clavulanic acid (20/10), Erythromycin (15µgm), Vancomycin (30µgm), High level gentamicin (120µgm), Linezolid (30µgm), Tetracycline (30µgm). For isolates from urine section Norfloxacin (10µgm) and Nitrofurantoin (300µgm) was kept. All discs were bought from HIMEDIA.

Zones of inhibition were measured and recorded and the organism was interpreted as sensitive or resistant as per the recommendations from CLSI guidelines. The quality Control used was *Enterococcus faecalis* ATCC 29212.

Screening for high level gentamicin resistance (CLSI 2015)

Screening media for high level gentamicin resistance was prepared. Screening for high level gentamicin resistance was done by agar dilution method. BHI agar with gentamicin at a concentration of 500µgm/ml was added and the plates were prepared. Inoculum of 10µL of 0.5 Mc Farland was spot inoculated. Presence of even 1 colony was interpreted as a resistant strain. These strains were collected for molecular studies.

Detection of beta lactamase (Marothi *et al.*, 2005)

The detection of beta lactamase test was done based on the chromogenic cephalosporin method. Nitrocefin disc was obtained commercially from BD. The disc was moistened with sterile saline and pure isolated colony was applied on it. The reaction was read within 5 min. Isolates producing beta lactamase produced a red color.

Molecular study of isolates

A representative sample of 50 high level gentamicin resistant isolates was selected for genomic analysis. The DNA was extracted using the QIAGEN kit. Followed by extraction, the isolates were analysed for the presence of Bifunctional resistant geneaac (6') le aph (2") la. The vancomycin resistant isolates were checked for the presence of van A gene simultaneously.

Detection of aac (6') le aph (2") la gene

The primers for HLGR with a base pair of 348 bp were amplified. The sequence was obtained from the studies done previously[11]. PCR Master mix: The master mix was prepared as follows: 2.5µl of PCR buffer,2.5µl of MgCl2, 2.5µl of DNTPs,

10.2µl of Mili Q H2O, 1µl of forward and reverse primers and 0.3µl of Taq Polymerase was added to eppend off. From the above mixture 18µl was taken and 5µl of the extracted DNA was added.

Primers for aac (6')Ie- aph (2")la with base pair of 348 bp (Vakulenko *et al.*, 2003):

F (5'CAGAGCCTTGGGAAGATGAAG3')
R (5'CCTCGTGTAATTCATGTTCTGGC3')

PCR program

The program consisted of initial denaturation step at 95^0C for 10 minutes followed by 30 cycles of denaturation at 94^0C for 300 seconds, annealing at 56^0C for 1 minute and extension at 72^0C for a period of 1 minute. The program had a holding temperature of 72^0C for 10 minutes.

Analysis

PCR products were analyzed by running gel electrophoresis with 1.5% agarose gel in Tris Boric acid buffer. The samples along with the controls obtained by running ladder were analysed.

The gel was stained with ethidium bromide and the bands were obtained were visualized under UV light and also in automated GEL DOC viewer.

Detection of vanA gene

The primers with a base pair of 732bp for detection of vanA were obtained from the studies done previously.

Primers for van A with base pair of 732bp (Dutka-Malen *et al.*, 1995):

F (5' GGGAAAACGACAATTGC-3')
R (5'GTACAATGCGGCCGTCGTTA-3')

PCR program

This program had a denaturation step at 95^0C for 5 minutes followed by 30 cycles of DNA denaturation at 95^0C for 1 minute, annealing at 54^0C for 45 seconds and primer extension at 72^0C for 45 seconds. This was followed by a holding temperature of 72^0C for 10 minutes.

Analysis

PCR products were analyzed by running gel electrophoresis with 1.5% agarose gel in Tris Boric acid buffer. The samples along with the controls obtained by running ladder were analysed. The gel was stained with ethidium bromide and the bands were obtained were visualized under UV light and in automated GEL DOC viewer.

Results and Discussion

All the 250 isolates of *Enterococcus* from various clinical samples including urine, pus, wound swab, blood and CSF were processed.

The maximum number of isolates was obtained from urine section (184) followed by wound swabs (24), blood section (14), 4 from peritoneal samples, 1 from CSF (Figure 1). The majority of the isolates were obtained as pure growth (216) and 34 was obtained as mixed from the samples processed. Figure 2 depicts the speciation of enterococcal isolates in which *E. faecalis* was the predominant species (157), succeeded by *E. faecium* (79), *E. raffinosus* (7), and *E. durans* (5) and *E. casseliflavus* (2).

Illustration 1 shows the genus specific biochemical tests used in the identification.

The antibiotic susceptibility pattern of the enterococcal isolates by disc diffusion method revealed that out of the 250 isolates, about 69.2% were ampicillin sensitive, 49.2% were

high level gentamicin sensitive, 89.3% were sensitive to erythromycin. The maximum number of isolates was found to be sensitive to vancomycin (97.2%) in our study (Table 1). Among the urinary isolates maximum sensitivity was noticed to nitrofurantoin (92.3%) (Table 2) and norfloxacin (83.1%).

The maximum sensitivity was observed for linezolid followed by vancomycin, teicoplanin, ampicillin and high level gentamicin. Among the non-urinary isolates erythromycin sensitivity was observed to be the highest.

The chromogenic nitrocefin disc was used to screen the isolates for the presence of beta lactamase. In our study none of the isolates were found to be positive for beta lactamase.

Figure 3 shows the presence of Bifunctional gene aac (6')1eaph(2")1a among the high level gentamicin resistant strains. Out of the 50 randomly selected resistant strains for genotype study, 48 had the gene.

Illustration 2 shows the presence of bands of size 348 bp during the gel electrophoresis.

In our study 7 out of the 250 isolates were found to be resistant to vancomycin. The genotypic analysis was done among these resistant strains using the van A gene. The results showed that 5 of the 7 strains were found to be positive for van A gene (Figure 4).

Illustration 3 shows the presence of van A gene of base pair 732, confirming its presence among the resistant strains.

Enterococcus has been noted to cause a majority of urinary tract infections. This being the most common followed by bacteremia, sepsis and even case reports of meningitis has been reported. In our study out of 250 samples studied the majority of the isolates were obtained from urinary tract infections (73.6%) followed by wound swabs (9.6%) and blood (5.6%). This correlates well with other studies in which *Enterococci* was obtained from urine isolates maximally (Karmarkar *et al.,* 2004, Mathur *et al.,* 2003). According to the studies *Enterococci* are considered as the commonest urinary pathogen justifying the increase in the rate of isolation from urinary samples (Murray *et al.,* 1990).

Among the various species, in our study *E. faecalis* was the most common isolate accounting for 62.8% followed by *E. faecium, E. durans, E. raffinosus* and *E. casseleflavus.* Similar findings were reported from other studies done in Bangalore in which *E.faecalis* was 59%, *E. faecium* was 38 % (Golia *et al.,* 2014).

Enterococci show intrinsic resistance to cotrimoxazole, clindamycin, cephalosporins and normal aminoglycosides. Thus these antibiotics are not tested (Mendiratta *et al.,* 2008).

The antibiotic susceptibility pattern of the isolates in our study showed the following:

The sensitivity to linezolid was maximum (98.8%) followed by vancomycin (97.2%), erythromycin (89.3%), teicoplanin (75.2%), ampicillin (69.2%), high level gentamicin (49.2%) and penicillin (25%).

Among the urinary isolates maximum sensitivity was noted in Nitrofurantoin (92.3%) followed by Norfloxacin (83.15%). Studies done in North India showed a similar susceptibility pattern with increased resistance to penicillin and aminoglycosides. Also the sensitivity percentage to linezolid and vancomycin was higher compared to other antibiotics (Mulla *et al.,* 2012).

Fig.1 Distribution of *Enterococcus* isolates among various samples (N=250)

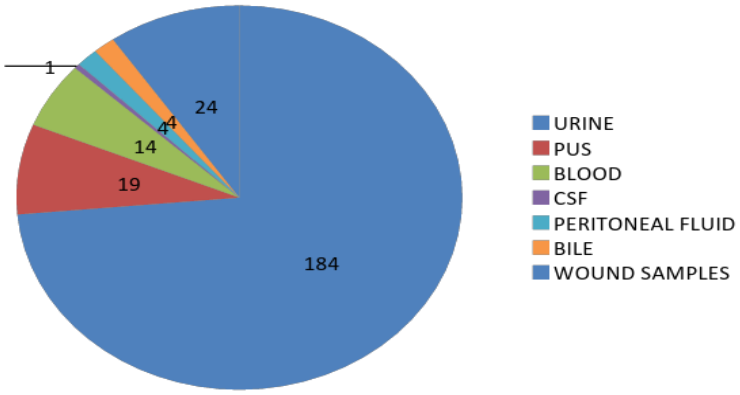
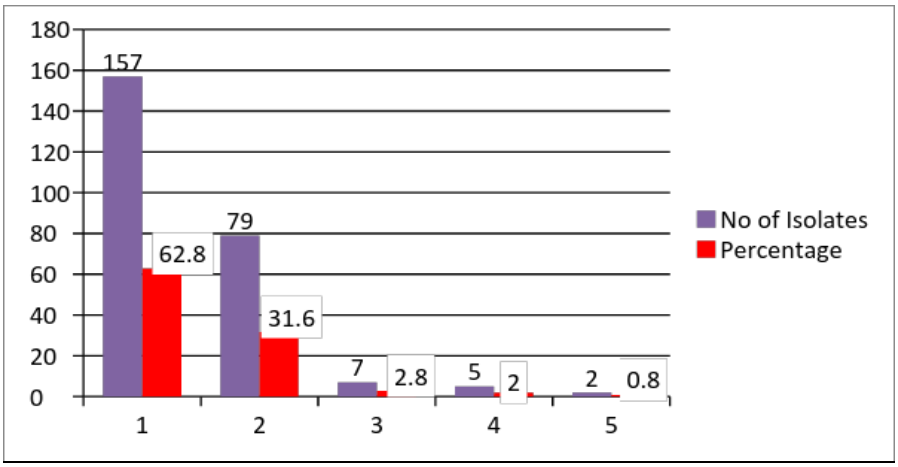


Fig.2 Speciation of *Enterococcus* from clinical isolates N=250



E.faecalis	1
E.faecium	2
E.raffinosus	3
E.durans	4
E.casseliflavus	5

Fig.3 Detection of the presence of aac (6') le aph (2") la gene N = 50

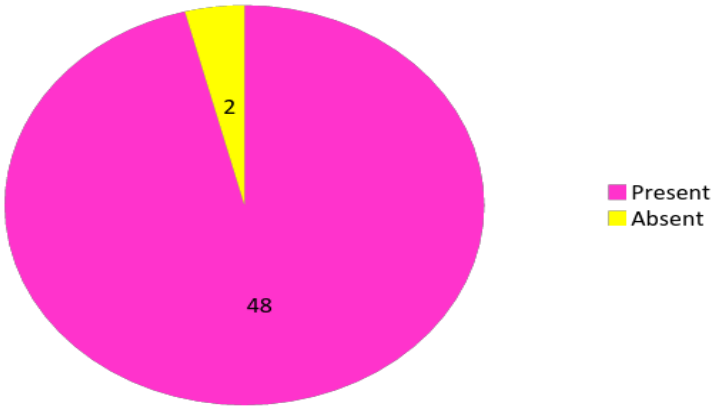


Fig.4 Detection of van A gene among the VRE isolates (N=7)

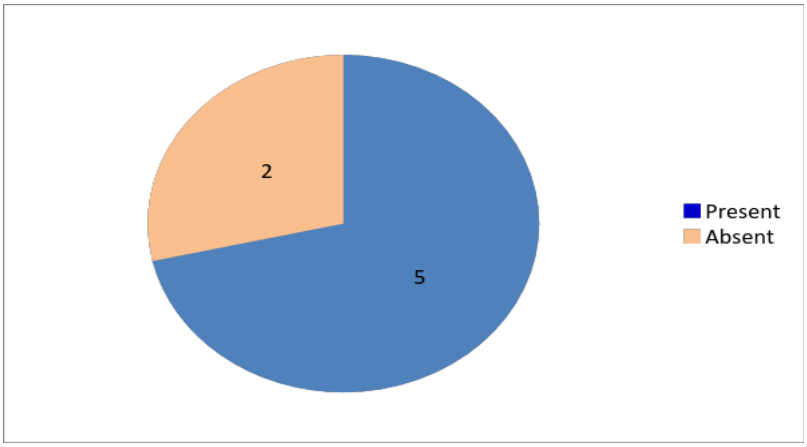


Table.1 Antibiotic susceptibility pattern of Enterococcal isolates

N = 250

RESISTANCE	SENSITIVE	ANTIBIOTICS
77	173	AMPICILLIN
30.8	69.2	%
127	123	HIGH LEVEL GENTAMICIN
50.8	49.2	%
7	243	VANCOMYCIN
2.8	97.2	%
3	247	LINEZOLID
1.2	98.8	%
62	188	TEICOPLANIN
24.8	75.2	%

Table.2 For urinary isolates

N = 184

RESISTANCE	SENSITIVE	ANTIBIOTICS
31	153	NORFLOX
16.85	83.15	%
14	170	NITROFURANTOIN
7.61	92.39	%
37	147	TETRACYCLINE
20.11	79.89	%

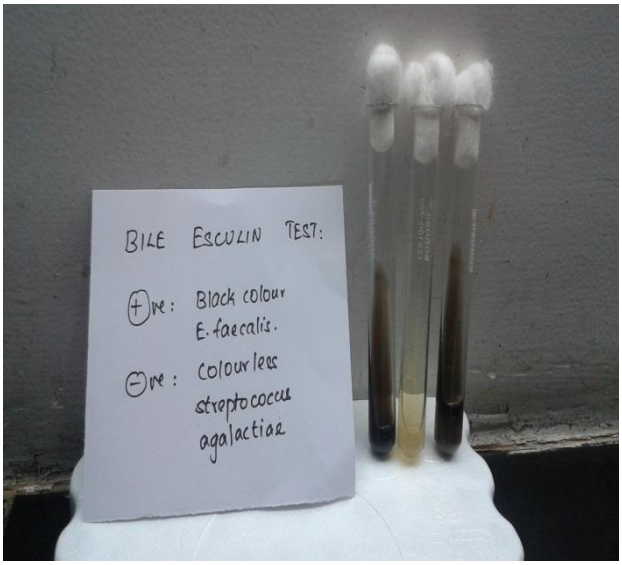
Table.3 For non urinary isolates

N = 66

RESISTANCE	SENSITIVE	ANTIBIOTICS
49	17	PENICILLIN
74.25	25.75	%
7	59	ERYTHROMYCIN
10.7	89.3	%

Illustration.1 Genus specific test

A) Bile-Esculin Test



B) PYR Test

C) 6.5% NaCl test

Illustration.2 PCR showing aac(6')-aph(2") BAND OF SIZE 348 bp

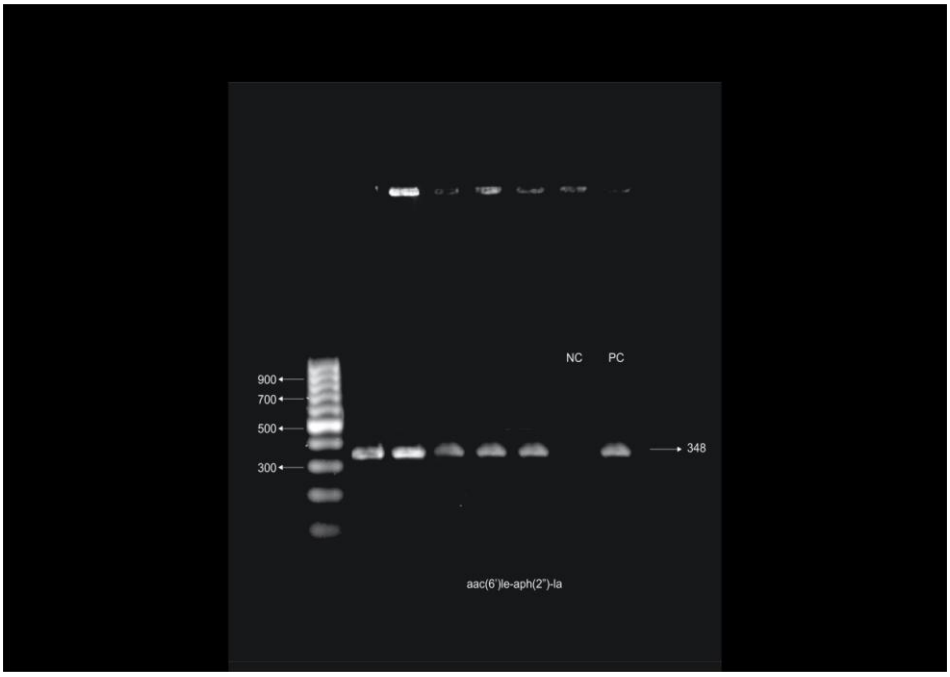
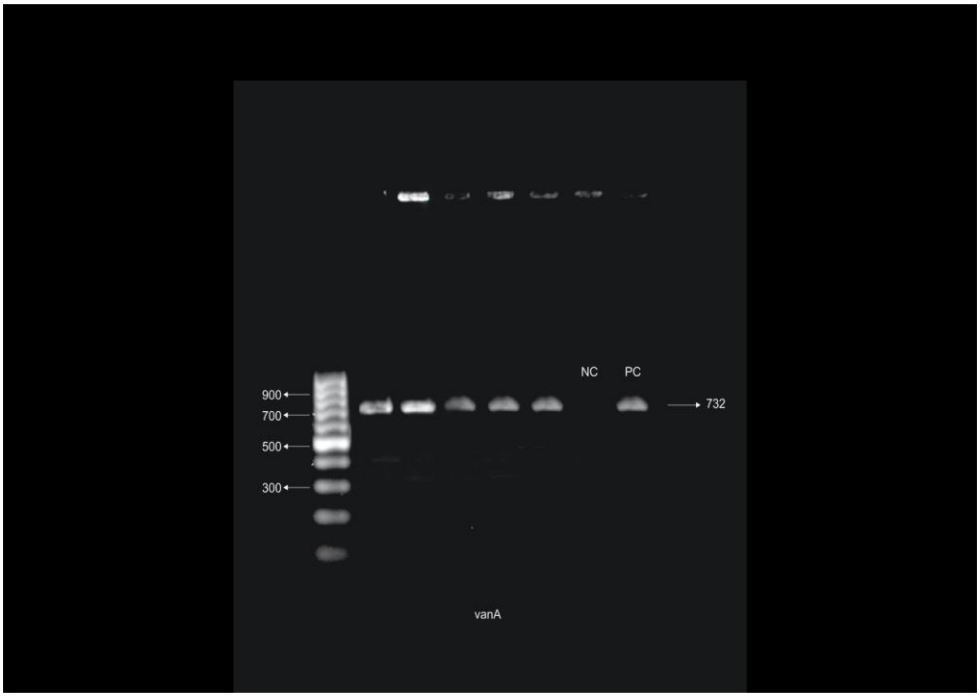


Illustration.3 PCR showing van A gene of band size 732bp



Another study showed the percentage of high level gentamicin resistance to be 68% (Randhawa *et al.*, 2004). Reports from South India showed the resistance percentage of high level aminoglycoside to be more than 50% (Sreeja *et al.*, 2012).

The detection of beta lactamase production by the chromogenic nitrocefin disc method showed that none of the isolates had beta lactamase enzyme. Studies done in Delhi and in Maharashtra also showed similar results (Jain *et al.,* 2011; Mendiratta *et al.,* 2008). The resistance to beta lactams though common, the mechanism could be due to alteration in penicillin binding protein PBP 5 which is more common than beta lactamase production (Murray *et al.,* 1992).

The molecular study done to detect the presence of the resistant gene confirmed the presence of the bifunctional gene aac (6') le-aph (2") la of base pair 348 to be present in 48 (96%) out of the 50 randomly selected resistant strains.

Studies done in Chennai have showed the presence of this gene in 38.2% of the isolates (Padmasini *et al.,* 2014) whereas there are other studies in which all the resistant isolates possessed this bifunctional gene (Hasani *et al.,* 2012). The resistance to high level gentamicin indicates resistance to all the aminoglycosides except streptomycin (CLSI 2015). The combination effect of aminoglycoside with beta lactam antibiotic is not helpful in the treatment of patients possessing such resistant strains.

Out of the 250 isolates the vancomycin resistant isolates obtained in our study was 2.8%. Studies have shown the prevalence of vancomycin resistance to be less in India ranging around 1.7-20% (Sreeja *et al.,* 2012).

Molecular studies revealed the presence of van A gene in 5 (71.42 %) isolates. Studies have also been reported showing van A to be the most common genotype isolated among the vancomycin resistant strains (Praharaj *et al.,* 2013)

Out of the seven isolates, five were *E. faecium* and two were *E. casseliflavus*. The remaining two isolates were *E. casseliflavus* which would have exhibited intrinsic resistance to vancomycin with van C gene (Praharaj *et al.,* 2013).

The incidence of *Enterococci* from the clinical isolates in our study was 2.5%.

The incidence of the pure isolates was 86.4% in comparison to the mixed isolates which was found to be 13.6%.

Among the isolates of *Enterococci*, the predominant species was *E. faecalis* (62.8%) followed by *E. faecium* (31.6%), *E. raffinosus* (2.8%), *E. durans* (2%) and *E. casseliflavus* (0.8%).

The antibiotic sensitivity pattern of the urinary isolates showed 92.3% sensitive to nitrofurantoin and 83.15% sensitive to norfloxacin. Among the non-urinary isolates maximum sensitivity was observed in linezolid which was 98.8% followed by vancomycin (97.2%), erythromycin (89.3%), teicoplanin (75.2%), ampicillin (69.2), high level gentamicin sensitivity (HLG) (49.2%) and pencillin (25%) (Table 3).

The vancomycin resistance was found to be 2.8% in *E. faecium*.

All the isolates were found to be beta lactamase negative by chromogenic (nitrocefin) method proving the alteration in PBP5 to be the more common mechanism involved in penicillin resistance.

The molecular characterization of HLGR(high level gentamicin resistant) strains, 50 isolates selected randomly out of 127, showed the presence of bifunctional gene aac(6')le-aph(2")la to be around 96%. (Primers of base pair 348 - F (5'CAGAGCCTTGGGAAGATGAAG3') R (5'CCTCGTGTAATTCATGTTCTGGC3')

The presence of van A gene was found to be 71.42% in our study.

Acknowledgements

I thank Dr. S. Ramalingam and Dr. B. Appalaraju for permitting me to carry out the work in the Department of Microbiology, PSGIMSandR, Coimbatore. I acknowledge the kind help rendered by the supervisor Dr. Parvathi.S. for having guided at every level.

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How to cite this article:

Mohamadiya Rizwana, S. Parvathi and Appalaraju, B. 2017. Phenotypic and Genotypic Characterization of *Enterococci* from Clinical Isolates in a Tertiary Care Hospital. *Int.J.Curr.Microbiol.App.Sci.* 6(7): 1160-1173. doi: https://doi.org/10.20546/ijcmas.2017.607.141