

## Transmission Electron Microscopy Study of Multidrug Resistant of *Enterococcus faecalis* and Amplification of vanA Gene in Clinical Samples

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### ABSTRACT

Vancomycin resistant *Enterococcus faecal* is a concern for both public health and medicine, as its importance is associated with serious multidrug resistant. It is an opportunistic pathogen which represents one of the agents of nosocomial infection in hospitalized patients. The aim of the present study was to determine the cell wall thickness by transmission electron microscopy and uncovering of resistant gene responsible gene for the vancomycin resistance *E. faecalis* strains isolated by clinical samples from Gulbarga region. Isolation and identification of *E. faecalis* of clinical isolates were done by using standard culturing and screening protocol. Antibiotic susceptibility and MIC were determined by using disc diffusion and broth dilution method. The thickness of cell wall of the stains was studied by using TEM and vanA gene was detected by Polymerase Chain reaction. In our study among 76 isolates of *E. faecalis*, 12 strains showed high resistance to gentamycin and vancomycin antibiotics. TEM-analysis showed cell wall and increased separate as compared to the normal *E. faecalis* 122 strain and standard culture *E. faecalis* NCIM 5625. The VREF 122 strain showed amplified product size of 352 pb of vanA gene. The study on TEM provides better evaluation in cell wall thickness treated with vancomycin antibiotic and PCR for the detection of resistant gene. These findings indicated the cell wall thickness is a characteristic phenotype of *E. faecalis* exposed to vancomycin antibiotic and PCR reveals the presence of the vanA gene. It is therefore believed that cell wall thickness and detection of resistance gene plays an important role in mechanism of action of Vancomycin antibiotic. Further these tools may find use in mark up application employing detection of resistance pattern in the bacteria.

**KEY WORDS:** E.FAECALIS, TEM, VANA GENE, MDR, VRE, CELL WALL THICKNESS.

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## INTRODUCTION

Enterococci are commensal organism that acts as opportunistic pathogens. Currently, they have been studied to be among leading pathogens of nosocomial infections, and thus they are a major international health burden (Dulber et al., 2020). Among the enterococci, *Enterococcus faecalis* cause 80-90 % of infections and it is a gram-positive commensal member of the gut microbiota of wide range organisms. With the advent of antibiotic therapy, it has emerged as multidrug resistant, hospital acquired pathogen (Daria et al., 2013). It majorly causes in humans such as bacteremia, endocarditis, intra-abdominal, pelvic infections and urinary tract infections. However, inappropriate vancomycin use has resulted in the emergence of Vancomycin resistant enterococci (VRE) and capably of transferring the resistance gene to another organism. VRE infection can be acquired through colonization with VRE or hospital environmental (Jatapat et al., 2019). Enterococci gladly accrue mutations and exogenous genes that confer additional resistance. They develop resistance to vancomycin by exchange of genetic material among themselves and or with another genera. The enterococci may acquire resistance through van associated genetic elements like *vanA*, *vanB*, *vanD*, *vanE*, *vanG*, *VanL* of which *vanA* and *vanB* are the most prevalent genotypes in clinical isolates (Addisu et al., 2020).

In Asian countries the prevalence was decreased may due to recent emergence of this resistance in this continent and only a handful of studies to document. The prevalence of VRE in India has been reported the highest was in New Delhi with 23%, Chandigarh 8% and lowest in Mumbai with 5.5% respectively and all of *vanB* phenotype study reported by Manimala et al., (2019). Transmission electron microscopy will insightful impact on understanding of bacteria and other microbial populations, it provides the 1000 fold improvement in resolution and allows to find the minor changes in topological and structure of bio-membrane organelles. (Shruthi and Vivek 2017). It is important to study the prevalence, pattern of resistance especially in this region of South India which has very few reports on *Enterococcus faecalis*. The present objective of the study to better understand the antimicrobial action and changes in cell wall thickness induced by vancomycin in *E. faecalis*.

## MATERIAL AND METHODS

**Isolation of *Enterococcus faecalis*:** The samples were examined for the presence of pathogenic. In the present study bile esculin azide agar was used for primary isolation of *E. faecalis*. Microscopic observation was done for confirmation and subculture again 3 to 4 times to isolate pure culture of *E. faecalis*. The single isolated colony was inoculated in BHI broth and incubated at 37°C for 18 hrs, glycerol (30%) were used for preparation of stock culture and stored in -80 °C for further use.

**Antimicrobial Susceptibility testing:** The standard disk diffusion method was used to perform antimicrobial susceptibility testing on Muller Hinton agar (Hi-Media, India) as recommended by the Clinical and Laboratory Standards Institute (CLSI 2007). The most commonly used antibiotics used for the tests were vancomycin, ampicillin, oxacillin, rifamycin, ciprofloxacin, tobramycin, gentamycin, teicoplanin and streptomycin.

Determination of minimal inhibitory concentration (MIC's) of Vancomycin and Gentamycin by broth dilution method: MIC for antibiotic (i.e, Gentamycin and Vancomycin) concentration ranging from 6-256 µg/ml and 512 to 1026µg/ml respectively were prepared with BHI broth and used to test each isolate and final concentration of each antibiotic were 10 mg/ml.

**Transmission Electron Microscopy:** TEM was performed for the morphological characterization of the cell wall ultra-structure of GREF and VREF isolates. The protocol for the preparation and examination was followed as described earlier (Hanaki et al., 1998). The overnight culture was centrifuge at 8000 rpm for 10 mins 4°C, the supernatant fluids were discarded, and the cell pellets were washed twice with 50 mM potassium phosphate buffer (pH 7.0). Bacteria were then fixed in 3 % glutaraldehyde (in 0.1 M sodium phosphate buffer, pH 7.2). Then followed bacteria were fixed with 3% glutaraldehyde for overnight at 4°C. For high contrast amplification, the bacterial cells were treated with 2 % uranyl acetate in 95 % alcohol for 1 h at 20° C in the dark. Infiltration is done with help of araldite propylene oxide (1:1) solution for five times. Cells were subjected to dehydration with 70 % ethanol for 18 hrs in acrylic resin. The ultra-microtome was used to prepare ultra-thin section, finally stained with lead citrate and uranyl acetate and examined under Transmission electron (Techn G2 Spirit TEM, at 80 KV).

**Plasmid DNA Analysis:** The plasmid isolation kit (Bangalore gene pvt.ltd) were used for the extraction of Plasmid DNA from vancomycin resistant isolates by user manual of kit, further it was analyzed in 0.8% agarose gel electrophoresis containing 5 µg/mL of ethidium bromide at 3.5 V/ cm for 4 h in a Tris EDTA buffer (TAE). Molecular markers were used as a size reference for molecular determinations.

**vanA Gene Amplification:** Plasmid DNA from vancomycin resistant enterococci isolated by the plasmid isolation kit (Bangalore gene pvt. ltd) were subjected to amplification assays employing the oligonucleotide forward primer (5'-GGGAAAACGACAATTGC-3') and reverse primer (5'-GTACAATGCGGC CGTTA-3') and amplification conditions as described by (Dutka et al., 1995). The PCR reaction consisted of Initial denaturation at 94 °C for 10 mins, followed by 35 cycles of denaturation to 94°C for 30 secs, annealing to 47 °C for 45 secs, then extension to 72°C for 30 secs. Followed by final extension at 72 °C for 10 mins. The amplified products were resolved by electrophoresis on a 1% Agarose-TAE gel containing gel red.

## RESULTS AND DISCUSSION

### Bacterial Isolates and Antimicrobial Susceptibility Test:

In the present study, strains of *E. faecalis* were isolated from clinical samples. A total of 250 samples were collected, among them 122 have showed positive for different clinical isolates. The *E. faecalis* 76 (62.29 %) was the predominant isolates from urine, pus, CSF and blood samples. The *E. faecalis* isolates were Gram positive. The biochemical characteristics for the all isolates were positive for tellurite reduction and arginine hydrolysis, arabinose, raffinose and mannitol expect for catalase sorbitol and lactose. The results of the susceptibility tests are carried out by disc diffusion method for 76 *E. faecalis* strains showed resistant to the different antibiotic like vancomycin (77.63%), gentamycin (64.47%) and oxacillin (55.26%) antibiotics, and were multi drug resistant. The isolates were found sensitive to rifamycin (61.84%), teicoplanin (55.26%) streptomycin 52.63%) and tobramycin (51.13%).

Figure 1: Transmission electron micrographs of *E. faecalis* (A) Control Isolate *E. faecalis* EF122 (B) Control of *E. faecalis* EF122 Septum; (C) NCIM 5205 *E. faecalis* Std Strain; (D) NCIM 5205 *E. faecalis* Std. Strain septum magnification at X68, 000.

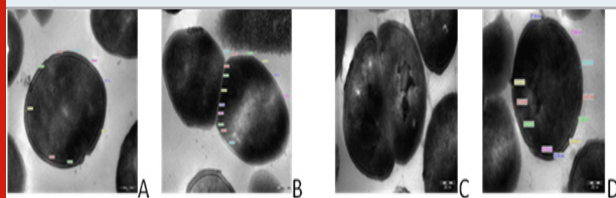
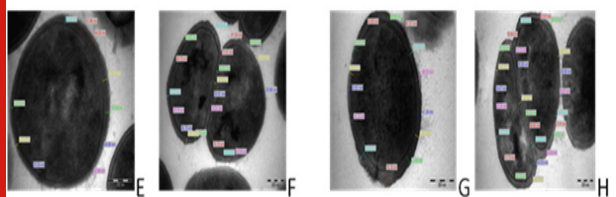


Figure 2. Transmission electron micrographs of *E. faecalis* isolates (E) Gentamycin treated *E. faecalis* EF122 isolate; (F) Gentamycin treated *E. faecalis* EF 122 isolate of Septum; (G) Vancomycin treated *E. faecalis* EF 122 isolate; (H) Vancomycin Treated *E. faecalis* EF122 isolate septum magnification at X68,000



**Determination of MIC's in *E. faecalis* isolates:** MIC's for gentamycin among 76 isolated 12 *E. faecalis* high resistance to gentamycin and Vancomycin among them 5 isolates showed  $\geq 1024$   $\mu\text{g/ml}$  and 5 isolates had MIC of  $\geq 512$   $\mu\text{g/ml}$  and 2 strains showed 256  $\mu\text{g/ml}$ . The vancomycin MIC for 8 isolates showed  $\geq 64$   $\mu\text{g/ml}$  and 4 isolates had  $\geq 128$   $\mu\text{g/ml}$  of the total 12 isolates were tested as shown in the Table.1.

**Transmission Electron microscopy:** TEM was employed to examine the cell morphology (especially cell wall thickness) of *E. faecalis* in in-vitro using gentamycin and vancomycin. Using broth dilution method, *E. faecalis* (GREF 122 and VREF 122) was adapted during MIC study and further sub cultured and stored at  $-80^{\circ}\text{C}$  (expressed high MICs,  $\geq 1024$   $\mu\text{g/ml}$  and 128  $\mu\text{g/ml}$ ). To ensure the resistance mechanism of *E. faecalis* to gentamycin and vancomycin, the TEM was carried out along with control isolates (EF122 and NCIM 5025). The isolates showing baseline resistance of 16  $\mu\text{g/ml}$  MIC were subjected to chemical fixation prior to examination.

Figure 3: PCR amplification of vanA gene Lane M- 100bp ladder and Lane-1 amplified Product vanA gene of *E. faecalis* EF122

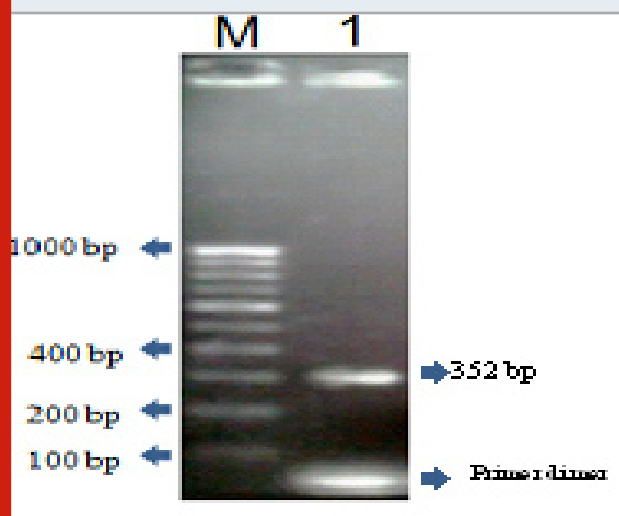


Table 1. MICs determination of Gentamycin and Vancomycin resistance of *E. faecalis* Isolates

Isolates	Sample	MIC $\mu\text{g/ml}$	
		Gentamycin	Vancomycin
121	Urine	1024	128
122	Urine	1024	128
123	Urine	1024	128
124	Urine	1024	64
125	Urine	1024	64
136	Blood	512	64
137	Blood	512	64
138	Blood	512	64
139	Blood	512	64
140	Blood	512	64
141	CSF	256	128
142	Pus	256	64

TEM photographs showed that there was significant alteration in the thickness of the bacterial cell wall. The control strain EF 122 showed the cell wall thickness of 30.598 nm and septum of 18.902 nm with X68,000

magnification as shown in Fig.1 a & b. The standard strain NCIM *E. faecalis* 5025 showed 30.832 nm cell wall thickness and septum of 24.358 nm with X68, 000 magnifications as shown in Fig.1 c & d. The isolates when exposed to gentamycin the cell wall and septum thickness was increased in size of 40.164 nm and 35.770 nm respectively at magnification at X68, 000 as show in Fig. 2 e & f. Similarly, the cell wall and septum thickness was increased higher in size of 47.842 nm and 46.358 nm respectively for vancomycin antibiotic as shown in Fig. 2 g & h.

#### Plasmid DNA analysis and *vanA* gene amplification:

Figure 3. Reports the plasmid profile of human clinical isolates. The two isolates 121 and 122 are independently of their origin had more than one plasmid with different molecular weight. Among the observed plasmid, those ranging from 2 strains, more than one plasmid species were detected in isolates. Among the observed plasmids, those ranging approximately from 21 and 19 kbp in molecular size. (Not data shown) shows an example of amplification of the 352 pb fragment in plasmid DNA of isolates, confirming the affiliation to the *vanA*

Table 2. Averaged means values of Cell wall thickness of the isolates

Name of the isolates	Measurement of thickness magnification at X 68,000 nm	
	Cell wall	Septum
Control Strain EF 122 Standard Culture	30.598 nm	18.902 nm
<i>E. faecalis</i> NCIM 5025	30.832 nm	24.358 nm
Antibiotic treated EF 122 (Gentamycin) Strain	40.164 nm	35.770 nm
Antibiotic treated EF 122 (Vancomycin) Strain	47.842 nm	46.358 nm

gene cluster. This result also demonstrates the extra chromosomal location of the glycopeptide resistance determinants. The amplified PCR product was sequenced and submitted to NCBI (Acc .No JN791694).

Drug resistant infectious provides opportunities to address the associated clinical and public health burden on individuals, health systems, and society, (Olaniyi et al., 2020). Worldwide there is a trend in increasing of vancomycin resistant enterococci ever since first described in 1987, although the epidemiology of these microorganism varies widely in different geographical areas (Hosseini and Mohammad 2014). *E. faecalis* has recently evolved from a generally a virulent commensal into an multi-drug resistant healthcare-associated pathogen causing difficult-to-treat infections. Therefore, studies of *E. faecalis* resistance have increased. The most species distribution of the enterococcus species was *E. faecalis* with (81.72%) isolated, followed by *E. faecium* (12.9%) as reported by Jahnabi et al., 2016. In recent study they have been

reported has (64%) of *E. faecalis* and *E. faecium* (36%) in Chennai, India (Alexander et al., 2020).

In our studies *E. faecalis* occurrence was (62.29%) the difference may be due to geographical variation. Vancomycin resistance was high with numbers of screened isolates were less 28/31 (90.62%) reported in North Indian tertiary care hospital (Hibs et al., 2020). In the year 2016 a study showed high rate (35.3%) of VRE carriage as compared to studies that have estimated comparatively low VRE colonization on admission to the ICU from Europe (2.7%), US (12.3%), South America (7%) and other Asian countries (5.3%) (Prakhar et al., 2016). In our study, about 77.64 % of *E. faecalis* strains showed high resistant to vancomycin by disk diffusion test and MIC of 12 strains as showed  $\geq 64$  to  $\geq 128$   $\mu\text{g/ml}$  for vancomycin respectively. The last therapeutic resort for enterococci was vancomycin.

Transmission electron microscopy has been invaluable tool in imaging bacteria and their components. The first report to show cell wall thickening was in *S. aureus* following treatment with acriflavine. Beta-lactam antibiotics which inhibit cell wall synthesis cause marked thickening of the cross wall in *Staphylococci*. MRSA has showed that vancomycin is affinity trapped inside the peptidoglycan layers by false targets, resulting in peripheral wall thickening (Mako and Jun 2009). In present study the isolate vancomycin resistant *E. faecalis* EF122 showed lesser thickness of cell wall and septum compared standard strain NCIM *E. faecalis* 5025 when grown in the medium without vancomycin. In contrast, when the isolate exposed to 16  $\mu\text{g/ml}$  of vancomycin and gentamycin increased thickness of cell wall was 9.564 nm and 16.566 nm against gentamycin and vancomycin, whereas in case of septum 26.868 nm and 27.456 nm with gentamycin and vancomycin respectively.

In our study PCR proved helpful in detecting the van genotypes present in this geographic region. The study reveals an expression of *vanA* gene in the *E. faecalis* strain with 352bp fragment in the plasmid DNA and it confirms isolates the affiliation to the *vanA* gene cluster and the extra chromosomal location of the glycopeptide-resistance determinants. The *vanA* type resistance is dominant in the United States and Europe whereas *vanB* type resistance is more frequent in Australia and Southeast Asia, (Nerfis and Baris 2020). However, VRE strains carrying *vanB* or both *vanA/vanB* have reported in various countries (Coombs et al., 2014, Marcade et al., 2014, Papagiannitsis et al., 2017). In India the most of strains of VRE has showed the *vanA* genotype (Hiba et al., 2020). In our study, the increase in the rate of prevalence of *E. faecalis* and emergence of multidrug resistance among them (Ajay et al., 2012), highlights the significance of rapid and accurate in identification of enterococci to the species level for imitating appropriate therapeutic regimen, and reemphasizes the importance of the implementation of appropriate infection control measures to limit the nosocomial spread of these unusual species in any nosocomial.

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**Conflict of Interest:** None

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