A PROSPECTIVE STUDY OF ANTIBIOTIC RESISTANCE AND VIRULENCE FACTORS IN ENTEROCOCCI ISOLATED FROM PATIENTS WITH END STAGE RENAL DISEASE

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ABSTRACT

Introduction and objectives: Patients with end stage renal disease (ESRD) are at risk for colonization and infections with multidrug resistant (MDR) *enterococci,* which are a formidable challenge to treat. This study aims to speciate *enterococci* isolated from ESRD patients; determine their antibiogram and virulence factors.

Methods: 50 isolates of *enterococci* obtained from ESRD patients were studied. Rectal swabs were screened for gastrointestinal VRE colonisation. Sample collection, processing and antibiotic susceptibility testing were performed according to standard guidelines. Vancomycin resistant *enterococci* (VRE) were characterized based on vancomycin MIC as VanA, VanB and VanC phenotypes. Presence of *vanA* and *vanB* genes was confirmed by polymerase chain reaction. Haemolysin, gelatinase, beta-lactamase and biofilm production were detected using standard methods.

Results: out of the 50 isolates of *enterococci* 28(56%) were *E.faecalis*, 21(42%) *E.faecium* and 1(2%) was *E.gallinarum*. 12 isolates (*E.faecium* 33.33% and *E.faecalis* 17.85%) showed vancomycin resistance, of which 9 were VanA, 2 VanB and 1 VanD phenotype. 22(44%) isolates produced haemolysin, 16(32%) gelatinase, 19(38%) beta-lactamase and 12(24%) produced biofilms. 10/12(83.3%) patients with VRE infection showed gastrointestinal colonization with the same.

Interpretation & conclusion: Colonization and infection caused by MDR *enterococci* and VRE are a significant clinical problem in patients with ESRD. Judicious use of vancomycin, careful screening and infection control precautions should be practiced to limit the transmission of VRE.

Keywords: Enterococci, end stage renal disease, Vancomycin resistance, multidrug resistant Biofilm

1. INTRODUCTION

Enterococci are normal inhabitants of the intestinal tract of humans and animals with low intrinsic virulence. However they are emerging as important nosocomial pathogens due to the escalating antibiotic pressure. Enterococci having acquired resistance to high level aminoglycosides, erythromycin, tetracycline and more recently vancomycin are becoming more common. According to the data published by Centers for Disease Control and Preventions (CDC) National Nosocomial Infections Surveillance (NNIS) system on nosocomial infections in intensive care unit patients, the role of vancomycin resistant *enterococci* (VRE) increased from 0.5% in 1989 to 28.5% in 2003.[1] Approximately 85% to 90% of *enterococcal* infections in humans are caused by *Enterococcus faecalis* (*E.faecalis*) and 5-10% by *Enterococcus faecium* (*E.faecium*).[2] However in recent times *E.faecium* has become increasingly resistant to antimicrobial agents and has emerged as a major nosocomial pathogen.[3] The most frequent infections caused by *enterococci* are urinary tract infections followed by intra-abdominal and surgical wound infections, pelvic sepsis in which enterococci are usually part of a mixed flora of colonic organisms. The third most frequent infection is bacteremia, either primary bacteremia due to a source in the gastrointestinal tract or secondary to urinary tract and intra-abdominal infections or use of intravascular devices.[2] The characteristic of *enterococci* that makes them such formidable pathogens is their intrinsic resistance to a number of antimicrobial agents.[4] Enterococci exhibit low levels of intrinsic resistance to penicillins, cephalosporins, carbapenems, carbacephems, aminoglycosides and lincosamides They also have acquired genes to resist the action of glycopeptides such as vancomycin and teicoplanin.

Multidrug resistant (MDR) *enterococci* exhibiting high level resistance to penicillin, glycopeptides, fluoroquinolones and aminoglycosides have emerged as an important cause of nosocomial infections and a formidable challenge to clinicians. This is probably related to the indiscriminate use third generation cephalosporins to which *enterococci* are naturally resistant [5].Patients with underlying malignancies, chronic renal disease on dialysis, transplant recipients and those with long term exposure to third generation cephalosporins and vancomycin are at an increased risk for development of *enterococcal* infections.[5] One of the first descriptions of vancomycin resistant *enterococci* (VRE) was in patients with end stage renal disease (ESRD) by Uttley et.al.[6] Risk factors for VRE colonization include host characteristics (immunosuppression, renal insufficiency and neutropenia), hospital factors (admission in an ICU or oncology ward) proximity to a VRE colonized patient, and extended duration of hospitalization and antimicrobial use.[7] VRE colonization independently increases a patient's risk of developing enterococcal infections, such as bloodstream infections (BSIs). Patients with end stage renal disease (ESRD) are susceptible to colonization and infections with MDR *enterococci,* because close proximity of patients in a hemodialysis unit for long duration provides an optimal setting for cross transmission of pathogens. Furthermore, dialysis patients also have contributory underlying co-morbid conditions, frequent hospitalizations and antibiotic exposures which predispose them to colonization with VRE.[6] Since VRE colonization is a critical risk factor, the optimal method for preventing clinical infections is to prevent colonization. Once a patient becomes colonized with VRE, it is important to recognize modifiable risk factors that contribute to the development of infection in a colonized patient.

Vancomycin is highly effective antibiotic against gram positive pathogens including Methicillin resistant *Staphylococci* (MRSA). It has an extended duration of action in patients with end stage renal disease. But unfortunately the increased use of vancomycin has been associated with a dramatic increase in hospital acquired infections with VRE and also with the danger of vancomycin resistance spreading to more virulent organisms like *S.aureus*. [8]

Rapid spread of VRE due to patient-to-patient transmission in health care settings is of concern because infections due to this organism remain difficult to treat, despite the availability of two new antibiotics tigecycline and linezolid. Since patients with ESRD play a prominent role in the emergence and spread of vancomycin resistance, it is important to understand the clinical epidemiology of VRE in this patient population. There is a paucity of data on the pattern of enterococcal infections among ESRD patients in India. Therefore this study was undertaken with an aim to evaluate antibiotic resistance among enterococci isolated from patients with ESRD, to study their virulence factors and identify the risk factors related to gastrointestinal VRE colonization in these patients.

2. MATERIALS AND METHODS:

This was a prospective study conducted from January 2008 to July 2009 in the microbiology department of St.John's medical college hospital, Bangalore which is a tertiary care referral center in south India. 50 consecutive isolates of *enterococci* isolated from clinical samples (exudates, urine, blood and body fluids) of patients with ESRD (only one isolate per person) were included in this study. Two rectal swabs were obtained from each patient with two weeks interval to screen for intestinal VRE colonization.[9] Collection and processing of samples was done according to the standard procedures.[10] Colonies morphologically resembling *enterococci* on blood agar and macConkey agar were presumptively identified based on gram stain and a negative catalase test. These isolates were further identified using bile aesculin agar, L-pyrrolidonyl-β-napthylamide (PYR) agar and growth in 6.5% NaCl. Species level identification of enterococci was done according to the conventional scheme of Facklam and Collins [11]. *E.faecalis* and *E.faecium* were differentiated using potassium tellurite reduction test, fermentation of L-arabinose and pyruvate utilization test.

Edward and Ewing's motility medium was used to identify motile strains of Enterococci. H_2S production was detected using lead acetate paper in peptone water. Fermentation of glucose, mannitol, arabinose, raffinose, sorbose, sorbitol, lactose, sucrose and melezitose was tested. Arginine decarboxylation was tested using Moeller's

decarboxylase basal broth with 1% arginine and amino acid free control. Sodium pyruvate utilization and hippurate hydrolysis was tested according to the recommended procedures. [12]
Antibiotic susceptibility was tested by Kirby Bauer disc diffusion method in accordance with Clinical and Laboratory Standards Institute (CLSI) recommendations.[13] Antibiotics tested included penicillin G (10U), amoxicillin (25µg), high level Gentamicin (120µg), tetracycline (30µg), ciprofloxacin (5µg), Vancomycin (30µg) and Teicoplanin (30 µg). MIC of vancomycin and teicoplanin was determined by agar dilution method and using E-test strips (AB BIODISK, Solna, Sweden). MIC values were interpreted in accordance with CLSI guidelines as follows: for vancomycin: (susceptible ≤ 4 µg/mL; intermediate, 8–16 µg/mL; and resistant, ≥32 µg/mL). For teicoplanin: (susceptible≤ 8 µg/mL; intermediate -16 µg/mL and resistant ≥32 µg/mL). E.faecalis ATCC 29212 was used for quality control. Isolates of VRE were defined phenotypically as Van A, Van B, Van C and Van D on the basis of their vancomycin MIC and susceptibility to teicoplanin.[11]

2.1 Detection of virulence factors: Haemolysin and gelatinase detection was done using Todd Hewitt agar supplemented with human O group blood and gelatin respectively.[14] Beta-lactamase production was detected using iodometric and clover leaf methods.[15] Biofilm production was demonstrated using test tube method suggested by Baldassarri et.al.[16]

2.2 Polymerase chain reaction: Uniplex PCR was performed on all the 12 VRE to detect the presence of vanA and vanB genes. The following protocol was used:

2.3 DNA template preparation was done as outlined below: Fresh cultures of the test organism and the control strains were suspended in 500µl of normal saline (0.8%) and vortexed to get a uniform suspension. The cells were lysed by heating them at 100°C for 10 min and cellular debris were removed by centrifugation at 8000 rpm for 5 min. The supernatant was used as a source of template for amplification. Uniplex PCR amplification for the simultaneous detection of vanA and vanB genes were carried out on a Thermal Cycler 9700 instrument (Applied Biosystems, Norwalk, USA).

2.4 PCR Master Mix: Master mix for the PCR was prepared as follows: 2.5µl of PCR buffer, 2.5µl of $MgCl_2$, 2.5µl of DNTPs, 10.2µl of MiliQ H_2O, 1µl of each of forward and reverse primers and 0.3µl of Taq Polymerase. 18µl of the master mix was dispensed in individual amplification tubes and 5µl of the extracted DNA was added in the corresponding tubes, the total volume being 25µl. The primers used for amplification of *vanA* at a concentration of 20picomolar each were *vanA* F(5'GGGAAAACGACAATTGC-3') and *vanA* R (5'GTACAATGCGGCCGTCGTTA-3') which amplified a 732bp amplicon. The primers used for the amplification of *vanB* at a concentration of 20picomolar each were *vanB* F(5'ATGGGAAGCCGATAGTC-3') and *vanB* R(5'GATTTCGTTCCTCGACC-3') which amplified a 635bp amplicon. *E.faecium* BM4147 was used as a control strain for *vanA* and *E.faecalis* V583 was used as the control strain for v*anB*. PCR program consisted of an initial denaturation step at 94°C for 2 min, followed by 30 cycles of DNA denaturation at 94°C for 1min, primer annealing at 54°C for 1 min and primer extension at 72°C for 1.5 min with a holding temperature of 72°C for 5 min. After the last cycle the PCR products were stored at 4°C. PCR products were analyzed by electrophoresis with 1.5% agarose gel in TBE buffer. The gel was stained with ethidium bromide (75µl in 500ml of distilled water) and the PCR products were visualized with UV light.

Rectal swabs were screened for the presence of gastrointestinal VRE colonization. A patient was considered colonized with VRE, when culture of at least one of the two rectal swabs yielded VRE with MIC and virulence pattern similar to the VRE obtained from any other site. Rectal swabs were plated on Vanco screen agar which is selective for VRE (i.e mueller hinton agar with 6µg/mL vancomycin). Vancomycin MIC of rectal enterococcal isolates was confirmed by E-test. Medical records of patients with ESRD from whom enterococci were isolated in significant numbers were retrospectively reviewed for data pertaining to demographics, clinical findings, underlying medical problems, surgical procedures, use of invasive devices, treatment with antimicrobial agents and outcome to ascertain their role in colonization.

2.5 Statistical analysis: Risk factors associated with VRE colonization were evaluated by univariate analysis, using the chi-square test and Fisher exact test for categorical variables and the Student's t test for continuous variables. The tests were two-tailed and the significance level was set at $p < 0.05$.

3. RESULTS

50 isolates of *enterococci* were isolated from various clinical samples of patients with ESRD as shown in **Table 1**. 28(56%) were identified as *E.faecalis*, 21(42%) as *E.faecium* and 1(2%) as *E.gallinarum*. Antibiotic resistance pattern of these *enterococcal* isolates is shown in **Figure 1**. 12 isolates of *enterococci* were resistant to vancomycin by E-test. [7 *E.faecium* (33.33%) and 5 *E.faecalis* (17.85%)]. Based on vancomycin MIC values 9 VRE isolates were of VanA phenotype, 2 were VanB and 1 was of VanD phenotype. **Figure 2** shows the presence of vanA/ vanB genes among VRE isolates detected by PCR. Production of virulence factors such as haemolysin, gelatinase, beta lactamase and biofilm by these isolates is summarized in **Figure 3**. Rectal swabs from 10 patients with VRE infections yielded *enterococci* with antibiogram and virulence factors identical to those isolated from their clinical samples with a colonization rate of 83.3%

Uni-variate risk analysis identified the following predisposing risk factors for VRE colonization among patients with ESRD: previous treatment with glycopeptides, third generation cephalosporins, metronidazole, long-term hospitalization and immunosuppressive therapy with cyclosporin A, tacrolimus, corticosteroids etc. in renal transplant recipients. The rate of VRE carriage was higher among the 27 patients who were treated with vancomycin in the previous 24 months than among the 23 patients without a history of vancomycin pre treatment (33.33% vs 4.34%). The odds ratio for VRE carriage after vancomycin treatment was 2.1. One of the 10 VRE carriers had not received any glycopeptide therapy during the previous 3 years. VRE carriers had a history of hospitalization for significantly longer duration during the previous year (median, 46 days) than the non-colonized patients (median, 9 days) (P=0.009). Other risk factors such as previous administration of 3rd generation cephalosporins, metranidazole, renal transplant, indwelling urinary catheter, immunosuppression etc. were present in patients colonized with VRE.

4. DISCUSSION

As to our knowledge this is the first reported study from India evaluating the antibiotic resistance and virulence factors among *enterococci* isolated from patients with ESRD. This study reveals that MDR *enterococci* are a significant problem in this group of patients as 12 (24%) of enterococci isolated from patients with ESRD were vancomycin resistant. The prevalence of VRE in this study was higher compared to previous studies in dialysis patients. VRE prevalence was 9.5% at the center affiliated with the University of Maryland hospital [17], 9% among 111 dialysis patients near New York City [18], 6.0% at the Vanderbilt University Medical Center [19], 8.1% at Johns Hopkins and 4.5% in a study performed across seven haemodialysis centers in USA in 2001. Using a highly sensitive broth enrichment technique, VRE were found in 13.8% of patients hospitalized on the renal service of the University Hospital in Belgium [22] and 14% of dialysis outpatients at 29 dialysis centers in Belgium.

An important observation in this study was the relatively higher proportion of *E.faecium* causing infection, demonstrating a shift towards more resistant species of *enterococci*. Many of these isolates have multiple virulence factors which enhance their potential as pathogens. Colonization of the gastrointestinal tract is an important predisposing risk factor for infection. Vancomycin and third generation cephalosporin usage has been identified as a major risk factor for colonisation and infection with VRE. [17] Medical records of 9/10 patients with VRE colonization revealed prior exposure to vancomycin in the past 24 months. Unrestricted and rapid increase in the use of vancomycin in the last two decades has been implicated in the selection of VRE. The center for disease control and prevention has specifically advised that empiric antibiotic protocols should be reviewed to reduce reliance on vancomycin. Antibiotic selective pressure exerted by extensive use of 3rd generation cephalosporins and metronidazole has been reported to predispose to VRE colonization and infection. In this study prior exposure to third generation cephalosporins

and metronidazole in the past 12 months was noted in 3 and 2 colonised patients respectively. Other risk factors such as length of hospital stay and admission to the intensive care unit, severe illness (malignancy, neutropenia, transplant, renal failure etc.), abdominal and cardiothoracic surgery and presence of an indwelling urinary catheter or a venous catheter also predispose to colonization with VRE.

Kidney transplant patients may be prone to developing high rates of VRE colonization and infection due to frequent use of antibiotics, particularly vancomycin both before and after transplantation. Multiple outbreaks VRE infections have been described due to person to person transmission, contact with contaminated hospital environment [18] and contaminated shared medical equipment. A patient colonised with VRE heavily contaminates the surrounding environment as these organisms persist on dry surfaces for weeks to months [23,24] and may not be adequately removed by routine cleaning procedures. Early accurate detection of VRE by periodic screening can be a useful tool to control the spread of VRE.

Hospital Infection Control Practices Advisory Committee (HICPAC) of the CDC has formulated recommendations for preventing the spread of vancomycin resistance.[25] This multipronged approach includes judicious use of antimicrobials especially of vancomycin to limit selection for vancomycin resistant strains, education programs for medical and paramedical staff to inform them regarding the epidemiology of VRE, its impact on patient care and the importance of infection control procedures, periodic surveillance for early detection of VRE carriage and effective isolation of carriers. Microbiology laboratory must be able to identify enterococci, detect vancomycin resistance and characterise the strain.

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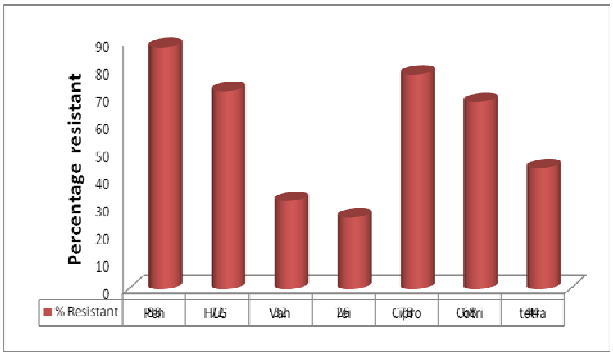
Table 1: Clinical samples from which enterococci were isolated

Sample	No. of isolates	Percentage
Urine	32	64 %
• Catheter samples • Mid stream samples	5 27	10% 24%
Blood	9	18 %
Peritoneal fluid	6	12 %
Pus	3	6 %
TOTAL	50	100%

Figure1: Antibiogram of Enterococci isolated from patients with chronic renal disease



Pen=Penicillin G 10 units, HLG=high level Gentamicin 120mcg, Van=Vancomycin 30mcg, Tei=Teicoplanin 30mcg, Cipro= ciprofloxacin 5mcg, Cotri= cotrimoxazole 1.25/23.75mcg, tetra=tetracycline 30mcg

Figure 2: Uniplex PCR for vanA and vanB detection

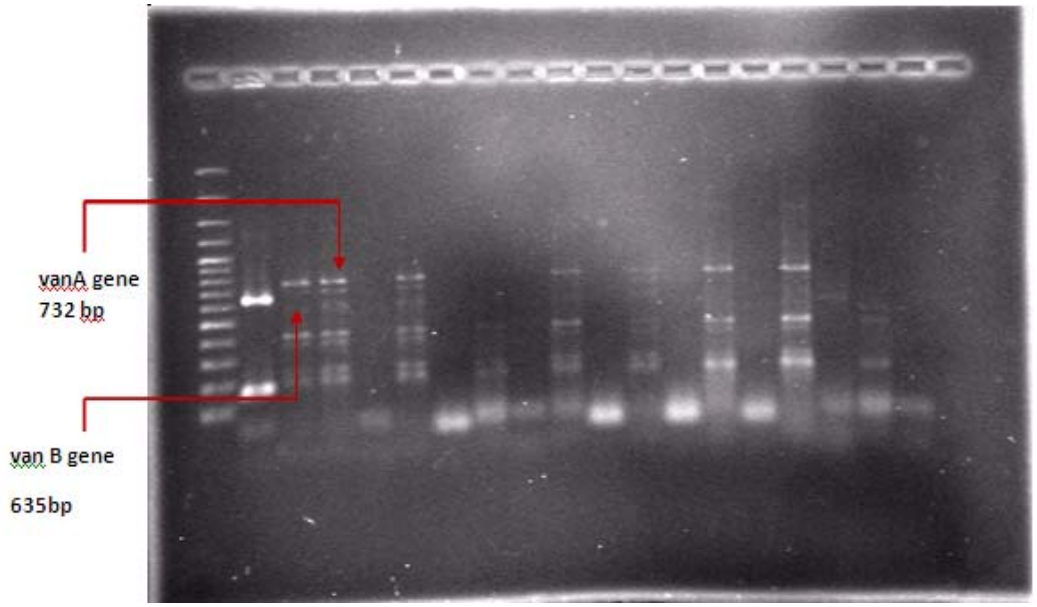


Figure 3: Virulence factors in Enterococcal isolates