



SPECIES IDENTIFICATION OF ENTEROCOCCAL ISOLATES AND THEIR ANTIBIOGRAM IN RIMS, HOSPITAL, IMPHAL.

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ABSTRACT This study was conducted to isolate, identify, speciate enterococci from the various clinical specimens and study their antibiogram and also to determine the minimum inhibitory concentration of high level gentamicin and vancomycin. The present study was carried out in a bacteriology laboratory, Microbiology Department, RIMS Hospital. The total of 54 *Enterococcus species* were isolated from various clinical specimens such as urine, blood and pus. Urinary isolates were 43(81.62%), blood isolates were 7 (12.96%), pus isolates were 3 (5.55%). *E.faecalis* is a predominant species 33 (61.11%) followed by *E.faecium* 18 (33.33) and *E.gallinarum* 3 (5.55%). Antibiogram of the enterococcal isolates were, penicillin resistance 50 (92.5%), ciprofloxacin resistance 44 (81.8%), nitrofurantoin resistance 8 (14.8%) and HLG resistance 21(38.88%). All the isolates were sensitive to vancomycin and linezolid. MIC for HLG was determined by E-tests in which 21 isolates were $\geq 1024\mu\text{g/ml}$ and 33 isolates were $\leq 4\mu\text{g/ml}$. Determination of MIC for vancomycin was also performed by E-tests in which all the isolates were $\leq 4\mu\text{g/ml}$.

KEYWORDS : Enterococcus, Species, High Level Gentamicin, VRE.

Introduction

Enterococci were traditionally regarded as a low grade pathogen but have emerged as an increasingly important cause of nosocomial infection. The most frequent infections caused by enterococci are urinary tract infection (UTI) followed by intra-abdominal, intra-pelvic abscesses and post surgical wound infections and third most common infections caused by these organism is blood stream infection.¹ There are 12 medically important species causing Enterococcal infections namely, *E.avium*, *E.casseliflavus*, *E.durans*, *E.faecalis*, *E.faecium*, *E.gallinarum*, *E.hirae*, *E.maldoratus*, *E.mundtii*, *E.pseudoavium*, *E.raffinossus* and *E.solitarius*. Additional species such as *E.cecorum*, *E.columbae*, *E.saccharolyticus*, *E.dispar*, *E.sulpfureus*, *E.seriolicida* and *E.flavescens* have been proposed as addition to this list.²

Speciation of the enterococci is important due to differences in the antibiotic susceptibility pattern amongst them. Antibiogram need to be correlated with the species of enterococci and emergence of vancomycin resistant enterococci need to be monitored. Therefore this study was undertaken to determine enterococcal infections in RIMS hospital.

Aims and Objects

- To speciate the enterococcal isolates from various clinical specimen in RIMS hospital.
- To study the antibiogram of the enterococcal isolates.
- To study the resistance pattern of the enterococcal isolates to high level gentamicin and vancomycin.

Materials and Methods

A total of 5300 Clinical specimens include 2525 urine, 648 pus, 362 blood and 1765 others like throat swab and body fluids/aspirates were collected in appropriate sterile containers from both in-patients and out-patients from November 2013 to May 2015. The clinical specimens were processed in the bacteriology laboratory of Microbiology department, Regional Institute of Medical Sciences Hospital, Imphal, Manipur, India for isolation, identification and antibiotic sensitivity.

Processing of clinical specimens.

The collection and processing of samples were done according to the recommended standard procedures.

Genus level Identification

Presumptive identification of Enterococcus was done from the routine isolates based on the growth character, Gram staining and catalase reactions. Further genus level identification was confirmed by growth in 6.5% NaCl, heat tolerance test at 60°C for 30 minutes, bile aesculin hydrolysis test, PYR Test (Pyrolydonyl-β-naphthylamide)³.

Speciation of Enterococcal Isolates

Identification of isolates up to the species level was done by standard biochemical tests according to the conventional scheme of Facklam and Collins⁴.

Carbohydrate fermentation test

- The carbohydrate fermentation reactions was performed in brain heart infusion broth containing 1% sugar and bromocresol purple was used as an indicator.
- The following sugars were used mannitol, sorbitol, arabinose, sucrose, raffinose, Glucose, Lactose.

Antibiotic susceptibility testing

Antibiotic susceptibility were carried out by Kirby Bauer disc diffusion method as per CLSI recommendations⁵. The performance and reading of the tests were quality controlled using reference strains *E.faecalis* ATCC 29212.

- The following antibiotic disc were used: penicillin (10units), ciprofloxacin (5μg), nitrofurantoin for urine (300μg), high level gentamicin (120), linezolid (30μg) and vancomycin (30μg).
- The minimum inhibitory concentration of high level gentamicin and vancomycin was determined by Epsilon test for all the enterococci isolates.

Ethical issue:

Informed consent taken from the respondents and confidential was maintained.

Ethical approval was sought from Institutional Ethics Committee, RIMS before the beginning of the study.

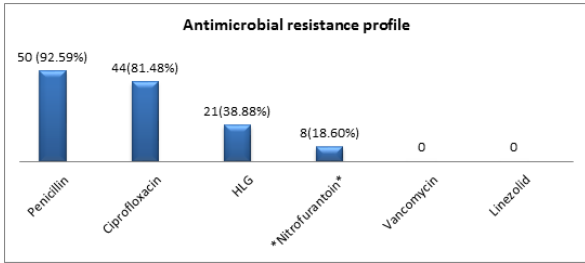
Results and observation

A total of 5300 clinical specimens which include 2525 urine, 648 pus, 362 blood and 1765 others from both inpatient and outpatient were processed during the study period of one year and six months. Among these 1600 (30%) were culture positive and 3700 (70%) were culture negative.

Out of 1600 culture positive, the most predominant organism isolated were *Escherichia coli* 723 (45.18%) followed by *Staphylococcus aureus* 304 (19%), *Klebsiella species* 160 (10%), Coagulase negative staphylococcus (CoNS) 132 (8.25%), *Pseudomonas species* 118 (7.37%), *Enterococcus species* 54 (3.37%), *Acinetobacter species* 42 (2.62%), *Proteus species* 34 (2.12%), Streptococcus species 21 (1.31%), Citrobacter species 9 (0.56%) and *Salmonella species* 3 (0.18%).

- Out of 54 isolates of *enterococci*, 44 (81.1%) isolates were from urine, 7 (12.96%) from blood and 3(5.55%) from pus.
- Out of 54 patients, 52 (96.2%) were ward patients and 2 (3.8%) were OPD patients.
- Out of 54 isolates, the most predominant isolates were *Enterococcus faecalis* 33 (61.12%) followed by *E.faecium* 18 (33.33%) and *E.gallinarum* 3 (5.55%).

Figure 1: Antimicrobial resistance pattern of enterococcal isolates (n=54).



* Nitrofurantoin is tested only in 43 urine sample.

Out of 54 isolates of enterococci, penicillin resistance were 50 (92.59%), ciprofloxacin resistance 44 (81.48%), high level gentamicin resistance 21 (38.88%), nitrofurantoin resistance 8 (18.60%) and all the isolates were sensitive to vancomycin and linezolid.

Table 1: Antimicrobial resistance pattern of *Enterococcus faecalis* (n=33)

Antibiotic	No. of resistance isolates	Percentage (%)
Penicillin	29	87.87
Ciprofloxacin	24	72.72
*Nitrofurantoin	3	10.34
High level gentamicin	5	15.15
Vancomycin	0	0
Linezolid	0	0

* Nitrofurantoin (n=29, tested in urine samples only)

Antimicrobial resistance pattern of *Enterococcus faecalis* shows that out of 33 isolates, 29(87.87%) isolates were resistant to penicillin, 24 (72.72%) were resistant to ciprofloxacin, 3 (10.34%) were resistant to nitrofurantoin, 5 (15.15%) were resistant to high level gentamicin and all the *Enterococcus faecalis* isolated in this study were sensitive to vancomycin and linezolid.

Table 2: Antimicrobial resistance pattern of *Enterococcus faecium* (n=18).

Antibiotic	No. of resistance isolates	Percentage (%)
Penicillin	18	100
Ciprofloxacin	17	94.44
Nitrofurantoin	4	33.33
High level gentamicin	12	66.66
Vancomycin	0	0
Linezolid	0	0

(*Nitrofurantoin* n=12, tested in urine samples only)

All the 18 isolates of *Enterococcus faecium* were 100% resistant to penicillin. Ciprofloxacin resistance was seen in 17 cases (94.44%), high level gentamicin resistance were seen in 12 cases (66.66%), nitrofurantoin resistance in 4 cases (33.33 %) and all the isolates of *Enterococcus faecium* in this study were sensitive to vancomycin and linezolid.

Table 3: Antimicrobial resistance pattern of *Enterococcus gallinarum* (n=3).

Antibiotic	No. of resistance isolates	Percentage (%)
Penicillin	3	100
Ciprofloxacin	3	100
Nitrofurantoin	1	33.33
High level gentamicin	3	100

Vancomycin	0	0
Linezolid	0	0

This table shows the antibiotic resistance pattern of *Enterococcus gallinarum*. All the 3 isolates of *Enterococcus gallinarum* were resistant to penicillin, ciprofloxacin and high level gentamicin. Only one isolate show resistance to nitrofurantoin (33.33%) and all the isolates were sensitive to vancomycin and linezolid.

E.Test for MIC determination of vancomycin among the various isolates of enterococcal species shows that all the isolates were sensitive to vancomycin i.e MIC of vancomycin ≤ 4 μ g/ml.

Discussion

In this study, a total of 5300 clinical samples were processed, of which 54 isolates were identified as enterococcal strains. The prevalence of enterococcal infection in this study is 1.01%. The prevalence of enterococcal infection in other Indian studies were, 1.49% from tertiary hospital of western India.⁶ 2.3% from tertiary hospital of south India⁷ and 1.16% from rural hospital of central India.⁸

In United States the most common infection cause by enterococci was UTI (84%).⁹ Many authors in India also reported that enterococci were mostly isolated from urine samples.^{6,8,10,11.}

In United state enterococci are third most common pathogen causing blood stream infections (BSIs).¹² While *E. faecalis* remains the predominant species, *E. faecium* isolates are increasing in proportion. The trend is particularly true for blood isolates where the ratio of *E. faecalis* to *E. faecium* has decreased from 3.7: 1 in 1996 to 1.9:1 in 1999.¹³ This microbiologic shift is due to emergence of VRE and *E. faecium* being the dominant species identified among VRE.

Mohanty S et al¹⁴ conducted a prospective study on enterococcal infections in tertiary care hospital of north India showed that, *E. faecium* (42.90%) and *E. faecalis* (40.00%) constituted the major isolates of which *E. faecium* was the predominant isolates from blood culture. In retrospective study conducted by Rajkumari N et al¹⁵ on magnitude of enterococcal bacteremia in south Asian country, the prevalence of BSIs caused by enterococci was 1.07%. Thus, in case of BSIs caused by enterococci, the finding of this present study is consistent with the finding of other Indian authors.

Out of 54 isolates of enterococci in this study, from various clinical samples, 3 were isolated from pus samples which account for 5.5% of all enterococcal isolates. MM Salem et al¹⁶ and Modi GB et al¹¹ reported that enterococcal isolates from pus sample were 6.7 % and 7.2 %. Enterococci causing surgical site infection (SSI) in the present study is very low as compared with the finding of Suchitra JB et al¹⁷ in which enterococci causing SSI account for 4.3% in a tertiary care hospital of south India.

In this study, most of enterococci were isolated from ward (96.20%) and this finding is consistent with the finding of Modi GB et al¹¹ in which 97.60 % of enterococcal isolates were from ward patients and 2.40 % from OPD.

Speciation of the enterococci in this study is done by conventional methods according to the scheme of Facklam and Collins.³ The most predominant species isolated were *E.faecalis* (61.11%) followed by *E.faecium* (33.33%) and *E.gallinarum* (5.55%). Historically, the rate of infections due to *E. faecalis* to that of other enterococcus species was approximately 10:1 but now the rate is decreasing because of higher resistance to commonly used antibiotic among the non-faecalis group. Thus in this present study the ratio of *E.faecalis* to non-faecalis is 1.57:1. Most of the Indian authors have reported that either *E.faecalis* or *E.faecium* was the predominant isolates.^(6,7,8,11)

The antibiogram of the present study shows that, penicillin resistance in 50 cases (92.50%), ciprofloxacin resistance in 44 cases (81.48%), high level gentamicin resistance in 21 cases (38.80%), nitrofurantoin resistance in 8 cases (18.60%). All the isolates were sensitive to vancomycin and linezolid. Correct speciation is important since there is variation in antibiotic resistance with different species.

In the present study, 92.59% of isolates were resistant to penicillin, of which *E. faecalis* showed 87.87% penicillin resistance and *E. faecium* and *E. gallinarum* were 100% resistant to penicillin. However, Mathur

et al¹⁸ reported 66% isolates were resistant to penicillin. Kapoor et al¹⁹ reported that 72% of the strains were resistant to penicillin.

Enterococci are intrinsically resistant to most beta-lactam antibiotics because of low affinity penicillin binding proteins (PBP), which enable them to synthesize cell wall components even in the presence of modest concentration of most beta-lactam antibiotics.²⁰

In this study, ciprofloxacin resistance *E.faecalis* was 72.72%, *E.faecium* 94.44% and *E.gallinarum* 100%. Overall resistance being 81.80%. A similar finding was observed by Jada SK et al²¹ in which ciprofloxacin resistance accounted for 82%.

High level gentamicin resistance (HLGR) in this study is 37.03% by Kirby Bauer disc diffusion method but E-test detected 38.8% HLGR in which *E.faecalis* contributing 18.2%, *E.faecium* 66.6% and *E.gallinarum* 100% respectively. This finding is comparable to a study by Bhatt P et al²² in which 32% isolates were found to be HLGR by Kirby Bauer disc diffusion method as compared to 39% isolates by E-test method of which, 62% of HLGR was seen in *E.faecium* as compared to 39.3% in *E. faecalis*. This indicates that E-test method is a better method to confirm HLGR among enterococci because disc diffusion method may not detect borderline resistance. These results are in concurrence with the results of other Indian studies.^{7,8} Enterococci exhibit intrinsically low level resistance to all aminoglycoside (MIC 8 to 256 µg/ml) which is due to low uptake of these agents. However, aminoglycoside uptake is enhanced when enterococci are exposed to beta-lactam.²⁰ Enterococci which develop HLGR will not be effective in synergistic therapy (gentamicin and penicillin).

Nitrofurantoin resistance in this study is 18.60% of which *E.faecalis* showed 10.71% and *E.faecium* and *E.gallinarum* showed 33.33% each. Shrihari N et al²³ conducted a similar study on enterococci, in which all the enterococcal isolates were sensitive to Nitrofurantoin. Zhanel et al²⁴ conducted a study on nitrofurantoin and concluded that none of the 300 isolates of enterococci tested were resistant to nitrofurantoin (MICs ≥ 128 µg/ml) including vancomycin resistant isolates of *E. faecium* with the *vanA* or *vanB* genotype and vancomycin-resistant *E. gallinarum* isolates with *vanC* genotypes. That is why nitrofurantoin is being used increasingly at present to treat vancomycin-resistant enterococci (VRE) nosocomial urinary tract infections (i.e. catheter-associated bacteria). It is the preferred oral antibiotic for nosocomial VSE or VRE catheter-associated bacteriuria.²⁵

VRE was not detected in this study. All the enterococcal isolates were sensitive to vancomycin by disc diffusion method and E.Test for MIC determination of vancomycin showed MIC value of all the isolates were ≤ 4 µg/ml. Similarly some authors conducted a study on enterococci from various clinical samples in northern India¹⁴ and central India⁸ where all the isolates were sensitive to vancomycin. In India, at All India Institute of Medical Sciences, New Delhi, five isolates of *E.faecalis* were found to be resistant to vancomycin by the disc diffusion and agar screen methods. On PCR, four had VanA genotype and one had VanB genotype.¹⁸ In another study from New Delhi¹⁹, Chandigarh³ and Mumbai²⁶ VRE were seen in 8%, 5.5% and 23% respectively and all being VanB genotype.

Outbreaks of VRE should be dealt by isolation of patients and hand washing, antibiotic pressure should be reduced by restricting the clinical use of broad-spectrum cephalosporins, quinolones and glycopeptides.²⁷ On the other hand vancomycin dependent Enterococcus are also emerging, two cases were reported by Swann RA and Bhattacharya S.²⁸ Awareness of existing such strains is also equally important especially in the context of long term vancomycin therapy.

Conclusion

This study has shown that enterococci can cause urinary tract infection, blood stream infection and surgical site infection. Routine media such as 5% sheep blood agar and MacConkey agar are well suited for the recovery of enterococci from the clinical specimens. Further speciation of enterococcal isolates is important due to difference in the antibiotic susceptibility pattern amongst them.

Antimicrobial susceptibility pattern of different species of enterococci in this study revealed that, *E.faecium* were more resistant to antibiotics

(penicillin 100%, ciprofloxacin 94.44%, nitrofurantoin 33.33% and high level gentamicin 66.66%) than that of *E.faecalis* (penicillin 87.87%, ciprofloxacin 72.72%, nitrofurantoin 10.34% and high level gentamicin 15.15%). On the other hand, all the isolates of *E.gallinarum* were found resistant to penicillin, ciprofloxacin and high level gentamicin (HLG).

Routine antibiotic sensitivity testing by disc diffusion method may not be able to identify many of the strains with intermediate sensitivity to both high level gentamicin and vancomycin. Therefore, E-test was performed for confirmation of high level gentamicin resistance (HLGR) and vancomycin resistant enterococci (VRE).

The treatment of choice for enterococcal infections is usually the synergistic combination of penicillin or glycopeptides with an aminoglycoside but high level gentamicin resistant (HLGR) caused resistance to this synergism between gentamicin and penicillin. Vancomycin is considered as last line of defense against enterococcal infections. However, in this study VRE is not detected.

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