PREVALENCE, CHARACTERISATION AND DISTRIBUTION OF ENTEROCOCCUS SPECIES FROM VARIOUS CLINICAL SAMPLES IN A TERITARY CARE HOSPITAL IN CHENNAI, INDIA

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ABSTRACT

Introduction

Enterococci, though considered less virulent are notorious to cause various clinical infections like urinary tract infections, endocarditis, intraabdominal infections pelvic and neonatal infections. Enterococci tend to be the leading causes of nosocomial infections with E.faecium and E.faecalis accounting upto 90% of clinical isolates. Thus proper identification of enterococci to special level is crucial for management and prevention of these bacteria in any healthcare facility.

Aims and Objectives

To process several clinical samples obtained from various departments in our hospital, for the isolation of enterococcus spp.

To know the prevalence of enterococcal infections in our hospital and

To speciate the isolated enterococci from clinical samples

Materials and Methods

A total of 1164 samples were collected from the patients across all age groups. Clinical specimens such as urine, pus, blood, body tissues, peritoneal fluid and Endotracheal aspirate were included in our study. All the specimens brought to the laboratory were subjected to the following tests: A preliminary macroscopic and microscopic examination of specimens. Specimens were processed by inoculation onto 5% sheep blood agar, Macconkey agar, CHROM agar and Thioglycollate and Brain heart infusion broths . The inoculated media were incubated at 37 °c overnight and observed for growth. Preliminary identification of Enterococci was made on taking into account of their typical morphology on gram staining, Bile esculin test ,PYR hydrolysis ,Heat and salt tolerance tests. Further identification to species level was achieved with the battery of biochemical reactions, motility and pigment tests.
INTRODUCTION

Enterococci which constitute the normal flora of the gastro-intestinal tract, oral cavity and genitourinary tract of Humans and animals are relatively avirulent in healthy individuals but they are known to cause various clinical infections like urinary tract infections, endocarditis, intra-abdominal and pelvic infections [1-3]. Enterococci were originally classified as enteric gram positive cocci and later included in the genus *Streptococcus*. In the 1930s, with the establishment of Lancefield serological typing system, enterococci were classified as group D streptococci and were differentiated from non enterococcal group D streptococci such as *streptococcus bovis* by distinctive biochemical characteristics [4]. Ever since the inception of Enterococci as a separate Genus, there are at least 37 species classified into five groups based on biochemical characteristics but less than one third of these are associated with disease in humans [5-6]. *Enterococcus faecalis* and *enterococcus faecium* are the most common species, both accounting upto90% of clinical isolates. Other Enterococcal species found to cause human infections include *Enterococcus casseliflavus*, *Enterococcus raffinosus*, *Enterococcus hirae*, *Enterococcus gallinarum* and *Enterococcus mundtii* [7]. Nevertheless, the incidence of other species of Enterococci is often underestimated because of frequent misidentification. Hence proper identification to species level is essential for prompt management and prevention of the infections caused by enterococci. Keeping these in mind, the present study was conducted to know the prevalence and distribution of Enterococci from clinical samples with special reference to characterisation.

MATERIALS AND METHODS

The present study was conducted in the Department of Microbiology from April 2013 -March 2014. A total of 1164 samples were collected from patients and processed. Various clinical specimens such as urine, pus, blood, body tissues, peritoneal fluid and Endotracheal aspirate were included in our study.

Results

A total of 1164 samples were collected from the patients and processed for study purpose. Among these only 849 (72.93%) were culture positive and included *E.coli, S.aureus, K.pneumoniae, P.aeruginosa* and *Enterococcus* spp. Prevalence of enterococci in clinical samples were 128 (15.07%). Distribution of enterococci in clinical samples, The maximum number of Enterococcal isolates were from urine (54.68%). The rest of the isolates were from pus (32.81%), Blood (5.46%), Tissue (3.12%), Peritoneal fluid (3.12%) each. Endotracheal tube tip (0.78%). Species of enterococci, 97 isolates were identified as *E.faecalis* (75.78%) and 31 isolates (24.21%) as *E.faecium*.

Conclusion

Precise identification of *Enterococcus* to species level is not only important to assess species specific antimicrobial resistance characteristics but also quintessential to know the epidemiological pattern of enterococcal infection and their significance in causation of human infections.

KEYWORDS: Enterococcus Species

Collection and transport of specimen

Data collection

Complete data with regard to patients like name, age, sex, hospital number, date, time of sample collection, nature of samples and clinical history were noted. All the specimens were collected under strict aseptic precautions. For urine collection, the patients were provided sterile universal container and clear instructions were given to provide clean catch midstream urine samples.

Pus and other aspirates were collected in a sterile syringe. Blood samples were collected after appropriate skin disinfection using 70% alcohol and 2% tincture of iodine. After drying up the vein was punctured and 5 - 10 ml of blood collected and inoculated into blood culture bottles.

The body tissues were aseptically collected from the patients and sent in a sterile, leak proof container. The specimen was ground digested and inoculated into culture broths.

Endotracheal aspirates were collected using endotracheal suction catheter tube and transported to microbiology laboratory for processing within 30 minutes. The collected samples were then processed.

Processing of samples

All the specimens brought to the laboratory were subjected to the following tests: A preliminary macroscopic and microscopic examination of specimens with exception of blood was done to look for pus cells and gram positive cocci arranged in pairs and short chains. The pus specimens were inoculated on 5% sheep blood agar, MacConkey agar and Thiglycollate broth. Semiquantitative culture for urine samples were done on 5% sheep blood agar and CHROM agar. The inoculated media were incubated at 37 °c overnight and observed for growth. A biphasic media using brain heart infusion broth and agar was used for isolating Enterococci from blood samples.

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Preliminary Identification of Enterococci [8-11]

Colony smear

A smear was prepared from the colonies and Gram staining was done with appropriate controls. The smear was examined for the presence of Gram positive cocci arranged in pairs and short chains.

Quality control

Positive - Staphylococcus aureus ATCC 25923

Negative -Escherichia coli ATCC 25922

Catalase Test

Catalase is an enzyme that decomposes hydrogen peroxide into oxygen and water. A small amount of culture to be tested was picked from a nutrient agar slope with a clean sterile, thin glass rod and inserted into 3% hydrogen peroxide solution held in a small, clean test tube. A positive catalase reaction was indicated by rapid and sustained bubbles or effervescence (nascent oxygen).

Bile Esculin Test

Esculin is a glycosidic coumarin derivative (6β Glucoside-7 Hydroxycoumarin). For this test esculin is incorporated into medium containing 4% bile salts or 40% bile. Esulin hydrolysis in the medium forms glucose and esculetin. Esculetin combines with Ferric citrate in the medium to form phenolic iron (black diffusible) complex. Two or three morphologically similar streptococcal colonies were inoculated on to the slant of bile esculin agar medium and incubated at 35°C in ambient air for 24-48 hours. Diffuse blackening of more than half of the agar slant indicates positive test.

Salt Tolerance Test

This test is useful for the presumptive identification of Group D Enterococci which have the ability to grow in high salt concentration (6.5% NaCl) in the medium. This test along with bile-esculin test distinguishes Enterococcus species from the group D streptococci (S.bovis and S.equinus). Group D streptococci - Bile esculin positive, salt tolerance negative; Enterococci - Bile esculin and salt tolerance positive. Two or three colonies were inoculated into 6.5% NaCl broth and incubated at 35°C in ambient air for 48 hours. A positive test is indicated by the presence of obvious bacterial growth.

Heat Tolerance Test

Enterococci tolerate temperature of 60°C for 30 minutes. An overnight broth culture of suspected colonies was streaked on blood agar plate. The same broth culture was heated for 30 minutes at 60°C. From this heated broth, another BAP is streaked. Both BAPs were incubated at 37°C for 24-48 hours. Growth in the second BAP indicates that the organism is heat tolerant.

PYR Test

Hydrolysis of the substrate L-naphthylamide - β-naphthylamide by bacterial aminopeptidase enzyme releases free β-naphthylamide which is detected by the addition of \( N, N\)-dimethyloxacinamaldehyde. This detection reagent couples with the naphthylamide to form a red Schiff base.

The colonies were inoculated in PYR broth ( α-pyroliidinyl β - naphthylamide) and incubated at 37°C for 4 hours. A drop of PYR reagent (0.01% p-dimethyloxacinamaldehyde) was added to the broth. Positive test is indicated by the development of cherry red colour within a minute of reagent addition.

Identification of species [8-11]

Enterococci were further identified to species level using Facklam and Collins scheme where a battery of tests like sugar fermentation, arginine hydrolysis, pyruvate utilisation, potassium tellurite reduction, motility and pigment detection were performed.

Sugar Fermentation Tests

For identification of species, 1% of sugars (Glucose, arabinose, raffinose, mannitol, sorbitol, sucrose, lactose) in peptone water with Bromothymol blue (0.002%) as the indicator was used. To each tube of Sugars, 1-2 drops of 18-24 hour BHI broth culture was added and incubated at 37°C overnight.

Sugar Fermentation is indicated by the change of colour to yellow.

Arginine Dihydrolase Test

Moeller's decarboxylase basal broth with 1% Arginine along with an aminoacid free control were inoculated with the test strain. Both the tubes were overlaid with sterile liquid paraffin and incubated at 37°C overnight.

The colour of the indicator reverting back to original (purple) indicating arginine hydrolysis was considered as positive provided the control tube remains yellow indicating fermentation.

Pyrurate Utilization Test

This test is used to determine the ability of an organism to utilize pyruvate. This property aids in the differentiation between Enterococcus faecalis (positive) and Enterococcus faecium (negative).

Pyrurate broth with bromothymol blue indicator was lightly inoculated with an 18-24-hour culture of the organism from 5% sheep blood agar and incubated at 35°C in ambient air for 24-48 hours.
Positive result is indicated by colour change from green to yellow whereas negative reaction shows no colour change.

Potassium tellurite reduction Test

Certain species of reduce tellurite to metallic tellurium which imparts black colour to the colonies. *Enterococcus faecalis* reduces tellurite in contrast to *Enterococcus faecium* which does not. 0.04% Tellurite blood agar plates were streaked with overnight nutrient broth culture and incubated at 37 °C for 2 days.

Tellurite reduction is considered positive on appearance of black colonies within 3 days.

Motility (Hanging Drop preparation)

A drop of liquid culture was placed on the cover-slip. A 'hollow ground' slide with its concavity encircled by soft petroleum jelly was inverted over the cover-slip and quickly turned around. The hanging drop was examined first with a low-power objective and then with high-power.

Pigment production

Enterococcal isolates were streaked on Tryptose soy agar and incubated at 37 °C for 24-48 hours. A sterile cotton swab was used to pick up colonies and observed for any discolouration.

RESULTS

A total of 1164 samples were collected from the patients and processed for study purpose. Among these only 849 (72.93%) were culture positive and included *E.coli*, *S.aureus*, *K.pneumoniae*, *P.aeruginosa* and *Enterococci spp*. Prevalence of enterococci in clinical samples were 128 (15.07%) (Graph 1)

Distribution of enterococci in clinical samples, The maximum number of Enterococcal isolates were from urine (54.68%). The rest of the isolates were from pus (32.81%), Blood (5.46%), Tissue (3.12%), Peritoneal fluid (3.12%) each. Endotracheal tube tip (0.78%). (Graph 2)

Enterococci were isolated more from males compared to females, 76 (59.37%) of Enterococci were from males compared to 52 (40.62%) in females (Graph 3).

Age wise distribution of samples ,the youngest was 8 month old infant and oldest aged 80 years Majority of the isolates were from the patients aged between 61-80 years (Graph 4).

Species of enterococci , *E.fcacalis* constituted 97/128 (75.78%) and *E.fcacium* 31/128 (24.21%) (Graph 5)

*Enterococcus faecalis* isolates fermented most sugars, and also showed positive reactions for pyruvate utilization, arginine hydrolysis and tellurite reduction tests while *enterococcus faecium* isolates showed negative biochemical reactions.(Table 1)

GRAPH 1: Distribution Of Culture Isolates

128 non repetitive clinically significant isolates of *Enterococci* were considered for the study.
GRAPH 2: Distribution Of Enterococci In Clinical Samples

<table>
<thead>
<tr>
<th>Site</th>
<th>No. of Isolates</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>70</td>
<td>54.68</td>
</tr>
<tr>
<td>Pus</td>
<td>42</td>
<td>32.81</td>
</tr>
<tr>
<td>Blood</td>
<td>7</td>
<td>5.46</td>
</tr>
<tr>
<td>Tissue</td>
<td>4</td>
<td>3.12</td>
</tr>
<tr>
<td>P.Fluid</td>
<td>4</td>
<td>3.12</td>
</tr>
<tr>
<td>ET tube tip</td>
<td>1</td>
<td>0.78</td>
</tr>
</tbody>
</table>

GRAPH 3: Gender Wise Distribution Of Patients With Enterococcal Infections

Males - 76 (59.37%) : Females - 52 (40.62%)

GRAPH 4: Age Wise Distribution Of Patients With Enterococcal Infections

<table>
<thead>
<tr>
<th>Age Group</th>
<th>No of persons</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-20</td>
<td>18</td>
<td>14.06</td>
</tr>
<tr>
<td>21-40</td>
<td>26</td>
<td>20.31</td>
</tr>
<tr>
<td>41-60</td>
<td>35</td>
<td>27.34</td>
</tr>
<tr>
<td>61-80</td>
<td>49</td>
<td>38.28</td>
</tr>
</tbody>
</table>
Enterococci cause a plethora of urinary tract infections, wound infections, intra-abdominal infections, bacteremia, septicemia and endocarditis particularly in hospitalised patients. Species identification of Enterococci has gained importance over the last decade. Enterococci are being isolated more frequently from clinical specimens and are gaining upper hand in causing nosocomial infections. Hence the present study was aimed on the isolation, identification and speciation of Enterococci. During the past 12 months of the present study Enterococci were isolated from several clinical specimens with the prevalence rate of 15.07%. The wide variety of infectious material from which enterococci was isolated was similar the observations made by several authors [13].

In our study, out of a total of 1164 samples submitted to bacterial culture 849 (72.93%) specimens were culture positive. The isolates included Escherichia coli 220 (25.91%) followed by Staphylococcus aureus (19.19%), Klebsiella species (17.19%), Pseudomonas sp (15.90%), Enterococcus spp 128 (15.07%), Candida spp (2.82%)

### BIOCHEMICAL TESTS

<table>
<thead>
<tr>
<th>Species</th>
<th>E. faecalis (97)</th>
<th>E. faecium (31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid Production from:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>97 100</td>
<td>31 100</td>
</tr>
<tr>
<td>Mannitol</td>
<td>97 100</td>
<td>29 93.54</td>
</tr>
<tr>
<td>Arabinose</td>
<td>0 0</td>
<td>31 100</td>
</tr>
<tr>
<td>Raffinose</td>
<td>0 0</td>
<td>9 29.03</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>91 94</td>
<td>13 41.93</td>
</tr>
<tr>
<td>Sucrose</td>
<td>97 100</td>
<td>29 93.54</td>
</tr>
<tr>
<td>Lactose</td>
<td>97 100</td>
<td>31 100</td>
</tr>
<tr>
<td>Trehalose</td>
<td>97 100</td>
<td>31 100</td>
</tr>
<tr>
<td>Inulin</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>Pyruvate Utilization</td>
<td>97 100</td>
<td>- -</td>
</tr>
<tr>
<td>Arginine Hydrolysis</td>
<td>97 100</td>
<td>- -</td>
</tr>
<tr>
<td>Tellurite reduction</td>
<td>94 97</td>
<td>- -</td>
</tr>
<tr>
<td>Motility</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>Pigmentation</td>
<td>- -</td>
<td>- -</td>
</tr>
</tbody>
</table>

Species of enterococci, *E. faecalis* constituted 97/128 (75.78%) and *E. faecium* 31/128 (24.21%)

**DISCUSSION**

Enterococci cause a plethora of urinary tract infections, wound infections, intra-abdominal infections, bacteremia, septicemia and endocarditis particularly in hospitalised patients. Species identification of Enterococci has gained importance over the last decade. Enterococci are being isolated more frequently from clinical specimens and are gaining upper hand in causing nosocomial infections. Hence the present study was aimed on the isolation, identification and speciation of Enterococci. During the past 12 months of the present study Enterococci were isolated from several clinical specimens with the prevalence rate of 15.07%. The wide variety of infectious material from which enterococci was isolated was similar the observations made by several authors [13].

In our study, out of a total of 1164 samples submitted to bacterial culture 849 (72.93%) specimens were culture positive. The isolates included Escherichia coli 220 (25.91%) followed by Staphylococcus aureus (19.19%), Klebsiella species (17.19%), Pseudomonas sp (15.90%), Enterococcus spp 128 (15.07%), Candida spp (2.82%)
Proteus and Acinetobacter (< 2%) each, Enterobacter and Citrobacter (< 1%) each.

This prevalence of Enterococci in clinical samples is similar to the study conducted by Sunilkumar et al who reported a prevalence rate of 15.46%.[14]. But, Parvathy et al reported a very low prevalence rate of 3.38% Enterococci, despite including samples from fistulae and stool.[15].

This study showed that majority of the patients with Enterococcal infections were males (59.37%). Likewise, in a study by Saraswathy et al, 74% of the patients with Enterococcal infections were males.[16]. This can perhaps be due to the reflection of the fact that more importance and priority is given to the health of males in our society.

In the present study with respect to different clinical specimens positive for Enterococci, 70 (56.48%) were from urine samples, 42 (32.81%) were from pus, 7 (5.46%) and 4 (3.12%) were from blood and tissue samples, 2 (1.56%) were each from peritoneal fluid and peritoneal drain tip. 1 (0.78%) was endotracheal aspirate. Previous studies have also shown urinary tract as the most common site of infection followed by infections of soft tissue, blood and body fluids.[18-20]. However in few studies enterococcal isolates from pus remained high as compared to urine isolates.[16]

Our study shows that the highest prevalence (38.28%) was seen in the age group of 61-80 years, followed by 41-60 years group with 27.34% and 20.31% among 21-40 years old. Enterococci were least isolated in the 0-20 years age group which constituted only 14.06% of the total Enterococci isolated. In a study by Bose et al. the maximum number of patients affected belonged to the age group of 21 – 30 years followed by 31 – 40 years.[17]. This might reflect the role of co-morbid conditions and other risk factors associated with Enterococcal infections.

In our study, E. faecalis (75.78%) was the predominant species followed by E. faecium (24.21%) in accordance with other studies by of Garry cotter et al., simonson et al., and Mohammad rahbar et al.[23-25]. Predominance of E. faecalis in endogenous flora of the body could be the reason behind its high proportion among hospital isolates.[26]. On the contrary, Baragundimahesh et al reported E. faecium as the predominant species (47.50%)[27]. Historically, the ratio of infections due to E. faecalis to those due to other species is approximately 10:1 which plummeted in the recent years.[28].

CONCLUSION

Definitive identification of Enterococci at species level is mandatory not only to assess species specific antimicrobial resistance characteristics but also reveal the epidemiological pattern of enterococcal infections and their clinical significance in human infections.

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