PREVALENCE, ANTIMICROBIAL RESISTANCE PATTERN AND VIRULENCE PROPERTIES OF \textit{E.\textit{faecalis}} ISOLATED FROM URINE SAMPLES IN NAMAKKAL AREA.

M. Suganthi\textsuperscript{1}, L. Ashok Kumar*\textsuperscript{1} and D. Jegadeesh Kumar\textsuperscript{2}

\textsuperscript{1}P.G.P. College of Arts and Science, Namakkal, Tamil Nadu, India.
\textsuperscript{2}Chromopark Research Centre, Namakkal, Tamil Nadu, India.

ABSTRACT

In this study, 30\% of \textit{E.\textit{faecalis}} was observed from 50 urine samples of different age groups. The highest occurrence was observed in female than male patients. \textit{E.\textit{faecalis}} was subjected to antibiotic susceptibility test with seven antibiotics, among them vancomycin and erythromycin showed highest antibiotic resistance and ciprofloxacin was active against most of the isolates. In this study, 12 types of antibiotic resistance patterns were observed. This study has been conducted to investigate the occurrence of virulence factors like biofilm (80\%), betalactamase (73.3\%) and gelatinase (53.3\%). In this study, two virulence genes were investigated in eleven clinical isolates of \textit{E.\textit{faecalis}} by using PCR technique. Among them, 64\% were harbored the cytolysis gene and 9\% were harbored the \textit{esp} gene. This study showed an emergence of VRE along with increased rates of multidrug-resistant enterococci in the area of the study. Furthermore, this study also attempted to determine if there is any significant difference in the biofilm positive isolates and biofilm negative isolates.

KEYWORDS: \textit{E.\textit{faecalis}}, \textit{Biofilm}, \textit{Betalactamase}, gelatinase, cytolysin.

INTRODUCTION

Urinary tract infection (UTI) is one of the most common infectious diseases seen in India. Totaling 150 million people are estimated to be diagnosed with UTI each year worldwide, which is costing the global economy in excess of 6 billion US dollars (Namratha, 2015). Urinary tract infection is a common in both men and women, but the incidence is quite high.
in females. It is due to the proximity of the genital tract and urethra (Devanand and Ramchandra, 2013).

This infection mostly caused by bacterial invasion on the lower and upper urinary tract. Numbers of bacterial species are responsible for the UT infection, especially *E. coli* account for 85% of the infection. In addition, *Enterococcus, Pseudomonas* and *Proteus* also cause the UTI (Ranganathan, 2014). The most common cause of the infection is unhygienic bathrooms where these bacteria are found; bacteria attach itself to the cell lining of the urinary tract and forms a protected film therefore, resistant to medication and this film are resistance to phagocytosis, making biofilms extremely difficult to eradicate the microbes from living hosts.

Enterococci are the most common causative agents of urinary tract infection. Recently, several studies have revealed increasing trends of resistance to many antimicrobial; especially Enterococci are intrinsically resistant to a wide range of antibiotics that most notably include beta-lactams and aminoglycosides (Mundy *et al.*, 2000). This phenomenon mainly occurred by the indiscriminate and inappropriate use of antibiotics for the treatment of the common bacterial infection. The bacterial species are able to survive after prolonged exposure to the antibiotics (Suganya *et al.*, 2014).

Acquired resistance to various antimicrobial agents and available antibiotics currently limits the therapeutic options. It is believed that nosocomial enterococci might have virulence elements that increase their ability to colonize hospitalized patients. The aims of this study were to investigate the susceptibility pattern of isolated *E. faecalis* from UTI and the prevalence of genes encoding gelatinase, enterococcal surface protein (*esp*), collagen adhesion. Many workers have tried to establish correlation between biofilm of *E. faecalis* and antimicrobial resistance, but paucity of literature exists in relation to UTIs caused by *E. faecalis*. Therefore, present studies aimed at gaining knowledge about the biofilm and type of virulence factors responsible for UTIs and their susceptibility patterns may help the clinicians choose the right empirical treatment.

**MATERIALS AND METHODS**

**Isolation of urine isolates**

The urine samples were collected in sterile condition and cultured on sheep blood agar and chromogenic agar then incubated at 37°C. After 48 hrs, observed the colony morphology and
colour. The suspected colony was subjected to a gram staining and biochemical test up to genus and species level wherever applicable.

**Susceptibility testing**
All the isolates were subjected for antimicrobial susceptibility testing and this was performed as per Kirby-Bauer disc diffusion method as per CLSI guidelines 2013. Antimicrobial discs used were vancomycin (30µg), ampicillin (10 µg), tetracyclin (30µg), gentamicin (20µg), erythromycin (15µg), ciprofloxacin (15µg) and chloramphenicol (30µg) (Himedia, India).

**Slime Production Assay**
Brain heart infusion agar supplemented with 5% sucrose and Congo red (0.08 g/l) was prepared and autoclaved at 121°C for 15 minutes. The isolates were inoculated and incubated the plates aerobically for 24 to 48 hours. Biofilm production was indicated by black colonies with a dry crystalline consistency, whereas biofilm non-producers remain pink, though occasional darkening at the center of the colony was observed (Vijayalakshmi et al., 2013).

**Assay for β-lactamase Production**
The β-lactamase production was assayed using the method of Lateef (2004). The broth culture of the test organism was spot inoculated on to starch agar containing penicillin and then incubated overnight at 37°C. The plates were then flooded with freshly prepared phosphate buffered saline containing potassium iodide, iodine. The presence of clear, colourless zones around the bacterial growth is an indication of β-lactamase production.

**Gelatinase assay**
Gelatinase production was assessed using Luria-Bertani (LB) agar containing gelatin (30 g/L). All isolates were grown overnight on Brain Heart Infusion (Himedia, India) agar plates at 37°C. One loop of each of the colonies was inoculated onto LB agar containing gelatin. After inoculation, the plates were incubated overnight at 37°C and then cooled for 5 h at 4°C. The colonies which give an inference of a turbid halo were considered to be positive for gelatinase production (Kanemitsu et al., 2001).

**Amplification of Cytolysin and Surface protein (esp) from Enterococcus faecalis by PCR**

**DNA preparation**
DNA samples were prepared from bacterial strains using standard protocols (Un-Ho Jin et al., 2004). The bacterial cells are heated with Tris, EDTA and SDS solution. Then nucleic
acid extracted by Phenol: chloroform: isoamylalcohol (24:25:1). Finally the DNA was precipitated by 70% ethanol.

Polymerase chain reaction (PCR) was performed according to Hasani (2004); Zhou Hia (2013) method with some modification. Amplification was performed in 20 μl reaction mixture consisting of 1μl genomic DNA, 10 μl of 2X Master Mix (Promega), 20 pm (0.5μl) of each primer for Cytolysin and Surface protein (esp) and make up to 201μl with molecular grade water. Reactions were performed in a thermal cycler (Genei, India) with an initial denaturation step of 10 min at 95°C; 30 cycles of 60sat 94°C, 60s at 57°C, and 60s at 72°C; and a final extension step of 10 min at 72°C. PCR products were analyzed using gel electrophoresis on a 1.5% agarose gel with 0.5× Tris-borate-EDTA buffer. The gels stained with ethidium bromide were photographed under UV illumination.

RESULTS
A total of 50 samples was collected from pregnant women, where clinically evident patients of UTI. Among them, 15 (30%) samples contaminated with Enterococcus faecalis. The isolates were obtained by selective media and standard biochemical tests. Females 32.0% were found to be more prone to Enterococcal infection as compared to males 28%. The 40% of prevalence were seen in the 4 age groups. The age wise distribution of the result was depicted in Fig.1.

![Fig. 1 Age wise distribution result of Prevalence of E. faecalis in urine sample](image)

The distribution of antimicrobial resistance patterns of isolated enterococci is summarized in Fig.2. The results show that the majority of isolates were resistant to vancomycin (80%),
erythromycin (73.3%), ampicillin and tetracycline (67%). Among urinary isolates least sensitivity was observed with gentamycin (33.3%). Among the different age groups the highest antibiotic resistance was observed in 21 to 30 and followed by 11 to 20 age groups. In this study, 12 types of antibiotic resistance patterns were observed the result was tabulated in table 2.

Table 1. Antibiotic resistance patterns of E. faecalis

<table>
<thead>
<tr>
<th>S.no</th>
<th>Antibiotic</th>
<th>No.of isolates</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>E</td>
<td>1/15</td>
<td>6.6</td>
</tr>
<tr>
<td>2</td>
<td>Va,E</td>
<td>1/15</td>
<td>6.6</td>
</tr>
<tr>
<td>3</td>
<td>E,Ch</td>
<td>2/15</td>
<td>13.3</td>
</tr>
<tr>
<td>4</td>
<td>Va,A,T,E</td>
<td>1/15</td>
<td>6.6</td>
</tr>
<tr>
<td>5</td>
<td>Va,A,E,Ch</td>
<td>1/15</td>
<td>6.6</td>
</tr>
<tr>
<td>6</td>
<td>Va,T,E,Ch</td>
<td>1/15</td>
<td>6.6</td>
</tr>
<tr>
<td>7</td>
<td>Va,A,T,G,C</td>
<td>1/15</td>
<td>6.6</td>
</tr>
<tr>
<td>8</td>
<td>Va,A,T,G,E</td>
<td>2/15</td>
<td>13.3</td>
</tr>
<tr>
<td>9</td>
<td>Va,A,T,C,Ch</td>
<td>2/15</td>
<td>13.3</td>
</tr>
<tr>
<td>10</td>
<td>Va,A,T,G,C,Ch</td>
<td>1/15</td>
<td>6.6</td>
</tr>
<tr>
<td>11</td>
<td>Va,A,T,E,C,Ch</td>
<td>1/15</td>
<td>6.6</td>
</tr>
<tr>
<td>12</td>
<td>Va,A,T,G,E,C,Ch</td>
<td>1/15</td>
<td>6.6</td>
</tr>
</tbody>
</table>

All isolates were subjected to biofilm formation with Congo red plate method. Among the 15 isolates, 12 (80%) of isolates produce the biofilm formation. Out of 12 isolates, 66.6% of isolates as strong biofilm producers followed by moderate and weak positive was 16.6%. The hundred percentages of strong producers were observed in 11 to 20 age group peoples. Isolates screened for biofilm formation by plate assay method are shown in (Fig.3).
Betalactamase producing isolates were determined by the starch agar plate method using betalactam antibiotic. Totally, 73.3% of isolates were betalactamase producers. Mostly all age groups of isolates were betalactamase producers. Gelatinase activity was observed by gelatin containing plate method. Among the 15 isolates, 53.3% of isolates was gelatinase producers. Most of the vancomycin resistance and biofilm producing isolates had the capacity for the gelatin degradation. Statistical analysis showed a significant relationship of biofilm formation with antibiotic resistance. Most of the biofilm producers had highest antibiotic resistance and virulence factors.

The presence of virulence genes was investigated from the highest antibiotic resistance isolates. Among the 11 isolates, 7 (64%) of were harbored the cytolysis gene and single isolate had esp gene. Most of the highest antibiotic resistance isolates had cytolysis gene. The results were shown in Fig.3.

**DISCUSSION**

It is generally known that *E. coli* are the most common pathogen in both complicated and uncomplicated UTI. However, other bacteria, such as *Enterococcus* species, *Pseudomonas aeruginosa*, and *Candida* species, are more common in complicated UTI than in uncomplicated UTI. Among the *Enterococcus* species, the *E. faecalis* strains are found more frequently in urine from patients with complicated UTI than is the *E. faecium* strains (Matsumoto et al., 2008; Lee, 2011). In this current study, 30% of *E. faecalis* were obtained from different age group peoples from around Namakkal area. The urine samples were collected from five types of age group peoples, among them the highest range of prevalence were observed in 21 to 30 age group. In our current studies, highest incidence were observed in female (32%) urine samples followed by male (28%). Many investigators in India also showed that women were the usual victims of the urinary pathogens (Asad, 2006; Nasreen, 2006; Nancy, 2012). In recently, Anjana et al., (2013) also observed the highest incidence of *Enterococcus* spp from female than male. Our report was correlated with the above findings. Several multi-resistant enterococci strains obtained in this study. The highest resistance was observed against to vancomycin (80%), ampicillin and aetracycline (67%). In 2014, Shamshad et al was observed the 97% of VRE from Pakistan. The second highest resistance was seen against erythromycin, which is in agreement with other studies carried out in India (Sanal, 2013). The emergence of VRE (Vancomycin resistance Enterococci) strains at the turn of the 20th century has generated major concern among clinicians (Cetinkaya et al,
2000) particularly in the last two decades; virtually these strains have emerged in nosocomial infections of hospitalized patients.

Since ampicillin is the drug of choice in the treatment of enterococcal infections, the relatively high resistance of isolates in this study to ampicillin is of great concern. In this study, vancomycin resistant isolates had highest other antibiotic resistance compared to vancomycin sensitive isolates. A similar rising trend of enterococcal resistance to various antibiotics was noted in another Indian study by Shinde et al (2012) in a tertiary care hospital in Mumbai.

*Enterococcus* is resistant to several first-choice antibiotics for the treatment of UTI. These isolates treated difficult because of producing biofilm. Biofilm is defined as a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface. This was associated with the production of exopolysaccharide matrix and biofilm leads to chronic or persistent infection. It’s a serious global threat and a challenge to health care professionals (Saravana Murugan et al., 2011). During the production of biofilm, isolates was expressing several virulence factors and an increased resistance against phagocytosis and other host defense mechanisms (Nancy et al., 2012).

Enterococci are not highly toxigenic, nor highly invasive, but they cause human infections. Some markers have been proposed as possible virulence factors in enterococci. Gelatinase, enterococcal surface protein (*esp*) and aggregation substance are the most frequently mentioned virulence determinant (Coque et al., 1995; Mundy et al., 2000; Coburn et al., 2005). In this study, 53.3% of gelatinase producers were observed from urine isolates of *E. faecalis*. In 2013, Praharaj Ira et al., was observed 19% of Gelatinase producers from India. Gelatinase elaborated by some *Enterococcus* isolates has been identified to be an extracellular zinc-endopeptidase capable of hydrolyzing gelatin. The role of gelatinase in enterococcal infection is in providing nutrition to the bacteria by degradation of host tissues. Beta-lactamases are bacterial enzymes that inactivate betalactam antibiotics, which inactivate all the penicillins and cephalosporins. In this study, iodometric method was used for the detection of betalactamase producers. In this study, 73.3% of the betalactamase producers were observed from urine samples. The positive result was indicated by a zone of clearance observed around the colony on starch agar plate. To our knowledge this is the first report of the detection of enterococci producing betalactamase by iodometric method. The previous study of Mario et al., (2012) was observed the beta lactamase producers by nitrocefin.
(chromogenic cephalosporin) method. It also easy to perform and detects most known betalactamases. The increasing incidence of enterococcal infections in recent years suggests that the acquisition of certain virulence factors might play a role in increasing the pathogenesis of these organisms. The present study aimed to determine the virulence gene by PCR method. Among the 11 isolates, 7 (64%) of were harbored the cytolysis gene. Most of the highest antibiotic resistance isolates had cytolysis gene. In case of esp gene, which gene was observed from single isolate of *E. faecalis*. An earlier study of Shankar, 2001 has been demonstrated a role for esp gene product for *E. faecalis* isolates causing UTI. Eaton and Gasson (2001) described a higher percentage of cyl genes among clinical strains compared with food strains. Production of cytolysin appears to be a major risk factor associated with pathogenic enterococci. Although a majority of the clinical isolates of enterococci tested was found to be positive for cytolysin in our study, contrary to some other studies. We conclude that biofilm production in clinical isolates of *E. faecalis* are an important pathogenic factor in UT infection. Based on our statistical analysis, biofilm formation linked to other virulence factors. Most of the biofilm isolates harboring potential virulence factors and multidrug resistance compare than non biofilm producing isolates. Currently, there is more urgency to define the reservoirs for colonization and the routes of transmission of enterococci since only few therapeutic options exist for treatment of VRE infections. Further molecular studies may have to be conducted to establish the basis of MDR and government should make a substantial effort to establish an antibiotic policy for the country.

REFERENCE


