Virulence-Marker Distribution and Antibiotic Resistance in Enterococcus spp. Isolated from Tertiary Health Care Facility in Ekiti State, Nigeria

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Abstract

The virulence factors and antibiotic-resistance in enterococci isolated from the clinical samples and hospital environment were determined using standard microbiological methods. A total of 81 clinical samples and 35 environmental samples from a tertiary hospital in Ekiti State, Nigeria, were examined for the presence of Enterococcus spp. Species isolated were identified to include: Enterococcus faecalis (110), Enterococcus faecium (75), Enterococcus gallinarum (39), Enterococcus durans (37) and Enterococcus hirae (33). Enterococcus faecalis has the highest occurrence followed by E. faecium, while E. hirae had the least occurrence. The percentage prevalence of cytolysin (Cyt) was highest in E. gallinarum (74.4%) followed by E. faecalis (68.5%). A total of 49 (44.1%), 19 (48.7%) and 13 (17.3%) among E. faecalis, E. gallinarum and E. faecium, respectively, were positive for the combination of cytolysin (Cyt) and gelatinase (Gel). The presence of Gel with haemaglutinin (Hea) in the isolates was comparably lower than the Cyt and Gel combination. The occurrence of the three pathogenic factors is in this decreasing order: E. faecalis 20 (18.0%), E. faecium 8 (10.7%) and E. gallinarum 2 (5.1%). The susceptibility of isolates was tested against nine antibiotics. All the E. faecium isolates were resistant to cotrimoxazole, ampicillin and chloramphenicol while none was resistant to vancomycin. The highest resistance was observed against cotrimoxazole followed by erythromycin while the least was observed in vancomycin. The highest vancomycin resistance was found among E. faecalis (30.6%) followed by E. durans (18.2%). The resistance of Enterococcus spp. was minimal to vancomycin, ofloxacin and nitrofurantoin, in increasing order, among the tested antibiotics.

Keywords: Cytolysin, haemolysin, gelatinase, cotrimoxazole, chloramphenicol, ampicillin, vancomycin.

1. Introduction

Enterococci are non-spore forming, Gram-positive bacteria found mainly in the gastrointestinal tract of mammals and other warm-blooded animals (Aarestrup et al. 2002). Enterococci have natural ability to acquire, accumulate, and share genetic elements encoding virulence traits and antibiotic resistance. They frequently cause a variety of human infections. Not only the incidence of nosocomially acquired infections has dramatically increased but also the therapeutic failure due to increasing antimicrobial resistance of Enterococcus spp. Enterococci are naturally resistant to antibiotics (Murray 1990) while they acquire antibiotic resistance and spread this to other species (Kühn et al. 2003). Multiple antibiotic-resistant enterococci (MRE) are a significant challenge for therapeutic measures (Huycke et al. 2002; Portenier et al. 2003). Several virulence factors have been identified in enterococci, which among other include haemolysin, aggregation substance (Agg), enterococcal surface protein (Esp), gelatinase and serine protease (Franz et al. 1999; Busani et al. 2004; Gülhan et al. 2006).
Gelatinase (GelE) is an extracellular zinc metallo-endopeptidase secreted by enterococci (Koch et al. 2004). It has the ability to hydrolyze gelatin, casein, haemoglobin and other bioactive peptides. The gene (gelE) encoding GelE is located on the chromosome and is regulated in a cell-density-dependent manner (Lopes et al. 2006). The main role of gelatinase in enterococcal pathogenesis is to provide nutrients to the bacteria by degrading host tissue, although they also have some function in biofilm formation (Gilmore 2002; Mohamed and Huang 2007).

Agglutination of erythrocytes by bacteria is a convenient measure of adherence. It contributes to attachment to host cells (Kurl et al. 1989; Carvalho and Teixeira 1995) and was identified to be caused by thermostable compounds of proteineous and non-proteineous nature. Haemaggulitation-positive E. faecalis isolates produced identical results with all kinds of erythrocytes tested, suggesting that binding was unspecific or caused by the presence of different adhesins (Elsner et al. 2000).

Haemolysin is one of the virulence factors associated with enterococci, it is considered to be important as it enhances the severity of haemolytic activity and ability of the organism (Semedo et al. 2003). Cytolysin production is associated with a better ability to reach the blood stream and induces septicaemia and a fivefold increased risk of acutely terminal outcome in patients (Dupont et al. 1998).

In this study, the level of dissemination of virulence factors and antibiotic resistance in enterococci recovered from both clinical samples and hospital environment was determined in a tertiary health care setting in Ekiti State, Nigeria.

2. Materials and Methods

2.1 Isolation of Enterococci from Samples

The clinical and hospital environmental samples were collected by sterile cotton swabs moistened with sterile distilled water. The samples examined included stool (57), wound (15), high vaginal swab (9) and bed sheet swab (35). The samples were inoculated directly onto sterile plates of Bile Aesculin Azide Agar (Oxoid) and incubated at 37°C for 24 hours. Discrete colonies surrounded with dark hallow were picked and sub-cultured to get a pure culture. Isolates were identified by conventional standard methods described by Olutiola et al. (2000) and Schleifer and Kilpper-Bälz (1984).

2.2 Detection of the Pathogenic Factors

2.2.1 Detection of Gelatine Hydrolysis (GelE): The method of Su et al. (1991) was used with a slight modification to detect gelatinase production among the isolates. Briefly, nutrient agar supplemented with 0.4% by weight, of gelatin (BDH, Merck Chemicals Ltd., Nottingham, England, UK), with a final pH 7.2, was prepared and isolates were streaked on the plates and incubated for 48 hours at 37°C. The plates were observed for growth and subsequently flooded with 10 ml of a Frazier’s solution (mercuric chloride, 15.0 g in 20 ml of 37%v/v hydrochloric acid, made up to 100 ml with distilled water). The plates which showed area of opaque layer with zone of clearance around the colonies were taken as positive for gelatin hydrolysis.

2.2.2 Detection of Haemolysin Production: Brain heart infusion agar (Oxoid) supplemented with 5% human blood was used for the detection of haemolysin activity. Prepared plates were streaked with the isolates and incubated at 37°C for 24 hours. Haemolytic activity was observed as β-haemolysis surrounding bacterial colonies in the plates.

2.3 Haemaggulitation Test

Enterococcal isolates were cultivated in 10 ml of Brain Heart Infusion agar (Oxoid) for 24 hours at 37°C. The isolates were grown in peptone water, and thereafter concentrated by centrifugation at 3,500 rpm for 10 min at 4°C. The bacterial pellet was washed twice in 0.002 M phosphate buffered saline (PBS) (pH 6.8) and suspended in 5 ml of the same buffer. Red blood cells (RBCs) were obtained from human blood by centrifugation at 3,000 rpm for 10 min., washed and re-suspended in PBS containing 0.1% ethylenediaminetetraacetic acid (EDTA). Haemaggulitation tests were
carried out by mixing 10-μl bacterial suspension with 20 μl of 2% harvested human RBCs on a slide, being rotated gently, read and observed for agglutulation within 30 s according to Gülhan et al. (2006).

2.4 Antibiotic Sensitivity Testing

The isolates were grown at 37°C in Mueller-Hilton broth (Oxoid) for 18 hours and standardized according to the Clinical and Laboratory Standard Institute, Wayne, PA, USA (CLSI 2005). The susceptibility of the isolates was determined by the disc diffusion method as described by CLSI (2005). The following antibiotics (Oxoid) with their concentrations (in μg) were used: chloramphenicol (30), cotrimoxazole (25), amoxycilin-clavulanic acid (30), cefuroxime (30), ampicillin (10), erythromycin (15), nitrofurantoin (300), tetracycline (30), ofloxacin (40) and vancomycin (30).

3. Results

Three types of clinical samples were studied with one environmental sample in this study. The clinical samples included stool (n = 57), wound swab (n = 15) and high vaginal swab (n = 9), while 35 hospital bed sheets (environmental samples) were examined. Five species of the genus Enterococcus were isolated, characterized and identified (Table 1). The species isolated and identified include: E. faecalis (110), E. faecium (75), E. gallinarum (39), E. durans (37) and E. hirae (33). E. faecalis was most frequently isolated followed by E. faecium while E. hirae had the least occurrence.

The isolates were tested for the presence of virulence factors. The factors examined were cytolysin (Cyt), gelatinase (Gel) and haemaglutinin (Hae). The results are shown in Table 2. The occurrence of the virulence factor was very prominent in E. faecalis, E. faecium and E. gallinarum. Cytolysin (Cyt) was most observed in E. gallinarum (74.4%) followed by E. faecalis (68.5%). For Gel, E. faecium had the highest occurrence (64.0%) followed by E. gallinarum (61.5%) while E. hirae (24.2%) had the least among the identified species. A total of 49 (44.1%), 19 (48.7%) and 13 (17.3%) among E. faecalis, E. gallinarum and E. faecium, respectively, were positive for the combination of Cyt and Gel. The number of isolates with combinations of Gel and Hea was comparably lower than the Cyt and Gel combination. The detection of the three virulence factors is in this decreasing order: E. faecalis 20 (18.0%), E. faecium 8 (10.7%) and E. gallinarum 2 (5.1%)

Nine antibiotics were tested against the isolates to determine their susceptibilities (Table 3). All the E. faecium strains were resistant to cotrimoxazole, ampicillin and chloramphenicol, and none were resistant to vancomycin. Ofloxacin was effective against E. faecalis with a percentage resistance of 27.9% while the highest resistance was observed against cotrimoxazole. Vancomycin and nitrofurantoin, in decreasing order, had better inhibitory effects on the pathogens. Enterococcus gallinarum was the most susceptible among the species. Based on percentage resistance of the isolates, the least effective antibiotics are: cotrimoxazole > erythromycin > ampicillin > chloramphenicol > amoxycilin-clavulanic acid.

4. Discussion

Among the enterococci isolates recovered in this study, E. faecalis and E. faecium occurred in high percentage (94.9% and 64.7%, respectively). Similar trend has been reported by Faholahzadeh et al. (2006), Baragundi et al. (2010) and Olawale et al. (2011). However, Faholahzadeh et al. (2006) and Olawale et al. (2011) did not isolate E. durans and E. hirae. This points out the increased clinical importance of Enterococcus species other than E. faecalis and E. faecium. Fourteen of the isolates were not characterized beyond the generic level.

The virulence traits found in the isolates have been considered as possible factors described to play important roles in making enterococci potential pathogens (Mäkinen et al. 1989; Eaton and Gasson 2001; Toledo-Arana et al. 2001).
Table 1. Distribution of *Enterococcus* species isolated from clinical and environmental samples.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Stool samples n = 57 (%)</th>
<th>Wound swab n = 15 (%)</th>
<th>High vaginal n = 9 (%)</th>
<th>Hospital bed sheet n = 35 (%)</th>
<th>Total n = 116 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. faecalis</em></td>
<td>56 (98.3)</td>
<td>15 (100.0)</td>
<td>6 (66.7)</td>
<td>33 (94.3)</td>
<td>110 (94.8)</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>43 (75.4)</td>
<td>10 (66.7)</td>
<td>1 (11.1)</td>
<td>21 (60.0)</td>
<td>75 (64.7)</td>
</tr>
<tr>
<td><em>E. gallinarum</em></td>
<td>21 (36.8)</td>
<td>2 (13.3)</td>
<td>0</td>
<td>16 (45.7)</td>
<td>39 (33.6)</td>
</tr>
<tr>
<td><em>E. durans</em></td>
<td>14 (24.56)</td>
<td>4 (26.7)</td>
<td>2 (22.2)</td>
<td>17 (48.6)</td>
<td>37 (31.9)</td>
</tr>
<tr>
<td><em>E. hirae</em></td>
<td>10 (17.5)</td>
<td>8 (53.3)</td>
<td>1 (11.1)</td>
<td>14 (40.0)</td>
<td>33 (28.5)</td>
</tr>
<tr>
<td><em>Enterococcus</em> spp</td>
<td>5 (8.8)</td>
<td>6 (40.0)</td>
<td>1 (11.1)</td>
<td>2 (5.7)</td>
<td>14 (12.1)</td>
</tr>
</tbody>
</table>

Table 2. Incidence of virulence factors in *Enterococcus* species isolated from clinical and environmental samples.

<table>
<thead>
<tr>
<th>Pathogenic Factors</th>
<th>Enterococci Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>*E. faecalis n = 111 (%)</td>
</tr>
<tr>
<td>Cyt</td>
<td>76 (68.5)</td>
</tr>
<tr>
<td>Gel</td>
<td>71 (64.0)</td>
</tr>
<tr>
<td>Hae</td>
<td>40 (36.0)</td>
</tr>
<tr>
<td>Cyt+Gel</td>
<td>49 (44.1)</td>
</tr>
<tr>
<td>Cyt+Hea</td>
<td>25 (22.5)</td>
</tr>
<tr>
<td>Gel+Hea</td>
<td>30 (27.0)</td>
</tr>
<tr>
<td>Cyt+Gel+Hea</td>
<td>20 (18.0)</td>
</tr>
</tbody>
</table>

Table 3. Antibiotic susceptibility pattern of *Enterococcus* species isolated from clinical and environmental samples.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Enterococci</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>*E. faecalis n (%)</td>
</tr>
<tr>
<td>COT</td>
<td>98 (88.3)</td>
</tr>
<tr>
<td>AMP</td>
<td>89 (80.2)</td>
</tr>
<tr>
<td>CHL</td>
<td>92 (82.9)</td>
</tr>
<tr>
<td>TET</td>
<td>71 (64.0)</td>
</tr>
<tr>
<td>CEF</td>
<td>80 (72.1)</td>
</tr>
<tr>
<td>AMOX/CLAV</td>
<td>78 (70.3)</td>
</tr>
<tr>
<td>ERY</td>
<td>77 (69.4)</td>
</tr>
<tr>
<td>NIT</td>
<td>62 (55.9)</td>
</tr>
<tr>
<td>OFL</td>
<td>31 (27.9)</td>
</tr>
<tr>
<td>VAN</td>
<td>34 (30.6)</td>
</tr>
</tbody>
</table>
Haemolysin is a plasmid-encoded toxin produced by beta-haemolytic *E. faecalis*. Its lysis erythrocytes, polymorphonuclear neutrophils (PMN) and macrophages kill bacterial cells and may lead to reduced phagocytosis (Ike *et al.* 1992). The enterococcal infections caused due to the potential virulence factors are difficult to treat (Huycke and Gilmore 1997). Mundy *et al.* (2000), Ghoshal *et al.* (2006) and Agrawal *et al.* (2009) have reported earlier the spread of antimicrobial resistance and virulence markers among clinical isolates. The wide spread of the virulence-marker-borne strains may be as a result of evolution (Eaton and Gasson 2001) or exchange of genetic materials between the enterococci in the ecosystem (Dunny and Clewell 1975).

In this study, some strains of *E. faecium* isolates were positive for gelatinase production. However, Kanemitsu *et al.* (2001) has reported earlier *E. faecium* isolates to possess gelatinase. In this study, there was a reasonable distribution of virulence markers in *Enterococcus* species isolated from clinical and environmental samples. This supports the earlier reports that virulence markers are common traits among genus *Enterococcus* from clinical samples (Jett *et al.* 1994; Semedo *et al.* 2003; Macovei *et al.* 2009) and the environment (Coque *et al.* 1995; 1996). These virulence factors permit adherence to host cells and extracellular matrix, facilitate tissue invasion, affect immunomodulation and cause toxin-mediated damage. (Kristich *et al.* 2004; Ahmadova *et al.* 2013).

All the *E. faecium* strains were resistant to cotrimozazole, ampicillin and chloramphenicol while none were resistant to vancomycin. This report is in agreement with the findings of Chayakul *et al.* (2007) and Rudy *et al.* (2004) who reported no resistance to vancomycin among *E. faecium*. The majority of the vancomycin-resistant enterococci (VRE) was found among *E. faecalis* (30.6%) followed by *E. durans* (18.1%) which is similar to the report of Faholahzadeh *et al.* (2006). To the best knowledge of the authors, this is the first report of vancomycin resistance among strains of *E. durans* in Nigeria. The case of vancomycin resistance (30.6%) among the *E. faecalis* strains in this study shows that the epidemiology of enterococci is changing in southwestern Nigeria. Olawale *et al.* (2011) reported 42.9% vancomycin resistance among enterococci while David (2010) reported 17.4% resistance to vancomycin among *E. faecalis* isolates. The vancomycin-resistant enterococci probably represent the most serious challenge among many microbes with antibiotic resistance as a source of human clinical infections. Enterococci have the ability to transfer plasmids to both closely and distantly related Gram-positive bacteria (Clewell 1993; Bohle *et al.* 2011). In addition, the recent appearance and increase of antibiotic-resistant and, notably, vancomycin-resistant enterococci poses a serious clinical problem. Resistance to antibiotics commonly leads to a failure of treatment with other antimicrobials. Most enterococcal isolates in this study possessed at least one virulence factor and were also resistant to various antimicrobials. This may be an explanation for their dominance in nosocomial infections.

### 5. References


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