DISTRIBUTION AND ANTIBIOTICS SUSCEPTIBILITY PATTERNS OF ENTEROCoccus SPP. FROM A SELECTED HOSPITAL IN INDIA

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ABSTRACT

Enterococci cause recurrent infections, especially among hospitalized patients. Their potential for resistance to multiple antibiotics and incumbent treatment failure constitutes a significant cause of morbidity and mortality. In this study, we aimed to determine the distribution of Enterococcus species from clinical samples and their antibiotic resistance profiles to supporting patients’ treatments based on informed-decision. We conducted a cross-sectional study at SRM Medical College Hospital, Tamil Nadu, India, from January to December 2014. Sixty Enterococcus isolates, from different clinical samples, were included in the study. The isolates were identified to species level based on sugar fermentation and biochemical reactions. The antibiotic susceptibility profile was determined using disk diffusion and agar dilution methods based on CLSI guidelines. The majority of Enterococcus isolates were recovered from urine samples (51.67%) and pus (38.33%). The predominant isolates were E. faecalis (55%) and E. faecium (33.30 %). Others were E. avium (3.3%) and 1.7 % each for E. durans and E. raffinosus. Overall, the isolates demonstrated the highest frequency of resistance to high-level gentamicin (33.30%), and one-third (33.30%) of the isolates were multidrug-resistant. Because the majority of the drug-resistant isolates were from urine and pus samples, we concluded that suspected cases of UTIs, wound infections, and sepsis need critical evaluation for possible enterococcal infection. Clinical use of gentamicin, among other antibiotics, shall be closely monitored while treating infections.

Keywords: Enterococcus, High-level gentamicin resistance, Multidrug resistance, Antibiotic Susceptibility, India.

INTRODUCTION

Enterococci are a natural component of human intestinal normal flora but are also important pathogens responsible for healthcare-associated and community-onset infections (Silverman et al., 1998). These infections present a serious problem in terms of medical and socio-economic costs as well as a significant cause in morbidity and mortality (EARSS, 2009).

Enterococci exceptionally cause disease in healthy individuals. The disease is mainly acquired endogenously and may disseminate via cross-infection among hospitalized patients (Mims et al., 1998). Under conditions where host’s resistance is compromised, or where the integrity of the gastrointestinal or genitourinary tract has been disrupted, for example by instrumentation, Enterococci can spread to normally sterile sites, causing urinary tract infections, bacteremia, sepsis, subacute bacterial endocarditis, biliary tract infection, or intra-abdominal abscesses (Richard et al., 2001; Mims et al., 1998).

Common sources of Enterococcus isolates are the penetrating injuries of the abdominal cavity, urinary tract infections, prostate infections, and infections of damaged or compromised skin, especially in burns and surgical wounds (Gary, 2011). With the inherent and continuous acquisition of resistance to multiple antibiotics, researchers recognize Enterococci as salient nosocomial pathogens that can be challenging to treat (Susan, 2013; Ibrahim, 2015).

Species of Enterococci implicated with human infections include E. faecalis, E. faecium Enterococcus avium, Enterococcus gallinarum, Enterococcus casseliflavus, Enterococcus durans, Enterococcus raffinosus and Enterococcus mundtii (Deviere et al., 1987; Gorbatch et al., 2001). The emergence of E. faecalis and E. faecium as species with significant medical importance paralleled the increased usage of glycopeptides and high-level aminoglycosides for the treatment of human infections (Guzman Prieto et al., 2016). Biotyping and antibiogram of Enterococci isolated from clinical specimens is an essential tool for epidemiologists and hospital policymakers to get useful information for hospital
antibiotic stewardship and overall treatment and prevention of the infections. The aim of this study is to biotype and evaluate antimicrobial susceptibility patterns of Enterococci isolated from clinical specimens in a tertiary care hospital in Tamil Nadu, India.

MATERIALS AND METHODS

Study design and data collection

We conducted this descriptive cross-sectional study at the Microbiology Department, SRM Medical College Hospital and Research Centre, Kattankulathur, Tamil Nadu, India, from January to December 2014. Sixty (60) Enterococcus isolates were obtained from different clinical samples submitted to the Microbiology Laboratory of SRM Medical College Hospital. They included urine samples, pus samples, blood samples, bronchial wash, and gastric fluid. Ethical approval for the study (Approval No: 592/IEC/2014) was obtained from the Institutional Ethical Committee of SRM MCH & RC, Tamil Nadu, India, before the commencement of the study.

Isolation and identification of Enterococcus

Nutrient agar, MacConkey agar, and 5% Sheep Blood agar plates (all prepared from dehydrated powder, HiMedia, India) were used to isolate Enterococcus from blood, pus, and body fluids. Urine samples were inoculated on CLED (cysteine-lactose-electrolyte-deficient) agar, incubated overnight at 35°C, and sub-cultured on Nutrient agar. Enterococci were presumptively identified on Blood agar as non-hemolytic, 0.5-1mm size Streptococci-like colonies; on MacConkey agar as small dark red magenta colonies and CLED agar as small yellow colonies (due to fermentation of lactose). We confirmed the colonies as Enterococcus based on their biochemical reactions such as positive Gram staining, negative Catalase test, positive Bile Aesculin test, and ability to grow in 6.5% NaCl broth (Collee et al., 2012; Cheesbrough et al., 2009). The isolates were further identified to species level by specific sugar fermentation reactions according to standard protocols (Facklam et al., 1970; Facklam et al., 1989; Collee et al., 2012). We used microtitre 96-wells plates to test for acid production from various sugars (pyruvate, arabinose, mannitol, sorbitol, and sorbose) obtained from HiMedia India.

Antimicrobial Sensitivity Test

We determined the susceptibility to antimicrobial agents by the Kirby-Bauer disc diffusion method, according to Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2014). We used the following antibiotics discs (HiMedia, India): Ampicillin [AMP] (10 μg), Vancomycin [VAN] (30 μg), Teicoplanin [TEI] (30 μg), Amikacin [AK] (30 μg), Erythromycin [E] (15 μg), Tetracycline [TE] (30 μg), Linezolid [LZ] (15 μg), and Chloramphenicol [C] (30 μg). Besides, we used Nitrofurantoin disc [NIT] (300 μg) on isolates from urine samples. Further, we evaluated the high-aminoglycosides resistance using High-level Gentamicin disc [HLG] (120 μg) and Agar Dilution Method. We determined the high-level gentamicin resistance using agar dilution by spotting 10μL of 0.5 McFarland suspension of test strain on to the surface of Brain Heart Infusion Agar (BHIA) containing different concentrations of gentamicin. We regarded the presence of growth greater than one colony of the test strain in BHIA containing gentamicin at the concentration of 500 μg/mL as resistance (CLSI, 2014). We used E. fecalis (ATCC 29212) as a control strain.

Minimum Inhibitory Concentration (MIC) for strains that showed glycopeptides resistance (by disc diffusion method) was determined using Vancomycin and Teicoplanin Ezy MIC™ strips (HiMedia India). Each of the E-strips was placed on a separate lawn culture made from 0.5 McFarland turbidity of the test strain incubated overnight at 37°C.

Data analysis

Data obtained were analyzed by SPPS software version 22 (IBM, Chicago, IL, USA). The prevalence of Enterococcus species from clinical samples was expressed in simple proportions or percentages.

RESULTS

Sixty isolates recovered from clinical specimen were included in the current study; the majority from urine (51.67%, 31/60) followed by pus (38.33%, 23/60) samples [Table 1].

<table>
<thead>
<tr>
<th>Type of Sample</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>31 (51.67)</td>
</tr>
<tr>
<td>Pus</td>
<td>23 (38.33)</td>
</tr>
<tr>
<td>Bronchial wash</td>
<td>3 (5.00)</td>
</tr>
<tr>
<td>Gastric fluid</td>
<td>1 (1.67)</td>
</tr>
<tr>
<td>Blood</td>
<td>1 (1.67)</td>
</tr>
<tr>
<td>Post-op drain</td>
<td>1 (1.67)</td>
</tr>
<tr>
<td>Total</td>
<td>60 (100)</td>
</tr>
</tbody>
</table>
Table 2 shows the distribution of clinical isolates of *Enterococcus* recovered from clinical specimens by the respective wards. The majority of the samples were from surgical wards (46.3%, 25/60) and ICUs (24.97%, 15/60).

<table>
<thead>
<tr>
<th></th>
<th>Medical wards No. (%)</th>
<th>Surgical wards No. (%)</th>
<th>Obs &amp; Gyne No. (%)</th>
<th>ICUs No. (%)</th>
<th>Pediatric ward No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>7 (11.69%)</td>
<td>11 (18.37%)</td>
<td>2 (3.3%)</td>
<td>10 (16.7%)</td>
<td>1 (1.7)</td>
</tr>
<tr>
<td>Blood</td>
<td></td>
<td></td>
<td></td>
<td>1 (1.7)</td>
<td></td>
</tr>
<tr>
<td>Bronchial wash</td>
<td>1 (1.7)</td>
<td>1 (1.7)</td>
<td></td>
<td>1 (1.7)</td>
<td></td>
</tr>
<tr>
<td>Pus</td>
<td>3 (5%)</td>
<td>14 (23.38%)</td>
<td>2 (3.3%)</td>
<td>4 (6.6%)</td>
<td></td>
</tr>
<tr>
<td>Post-op drain</td>
<td>1 (1.7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastric Fluid</td>
<td>1 (1.7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>12 (20%)</strong></td>
<td><strong>27 (45%)</strong></td>
<td><strong>4 (6.7%)</strong></td>
<td><strong>16 (26.7%)</strong></td>
<td><strong>1 (1.7%)</strong></td>
</tr>
</tbody>
</table>

Obs&Gyne = Obstetrics and Gynecology ward, ICUs = Intensive Care Units, Post-op drain = Post-operative drain, No. = Numbers

Table 3 shows the distribution of *Enterococcus* species in various clinical specimens. The predominant isolates identified were *Enterococcus fecalis* (55%) followed by *E. fectium* (33.3%). We could not identify three of the isolates (5%) to species level based on the sugars and the biochemical characteristics.

**Table 3: Distribution of *Enterococcus* species isolated from various clinical samples**

<table>
<thead>
<tr>
<th>Number of isolates from clinical samples (%)</th>
<th>Urine</th>
<th>Pus</th>
<th>Bronchial Wash</th>
<th>Gastric fluid</th>
<th>Blood</th>
<th>Post-operative drain</th>
<th>Total Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. fecalis</em></td>
<td>18 (30)</td>
<td>13 (21.7)</td>
<td>1 (1.7)</td>
<td>1 (1.7)</td>
<td>-</td>
<td>-</td>
<td>33/60 (55%)</td>
</tr>
<tr>
<td><em>E. fectium</em></td>
<td>12 (20)</td>
<td>5 (8.3)</td>
<td>1 (1.7)</td>
<td>-</td>
<td>1 (1.7)</td>
<td>1 (1.7)</td>
<td>20/60 (33.3%)</td>
</tr>
<tr>
<td><em>E. avium</em></td>
<td>1 (1.7)</td>
<td>1 (1.7)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2/60 (3.3%)</td>
</tr>
<tr>
<td><em>E. durans</em></td>
<td>-</td>
<td>1 (1.7)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/60 (1.7%)</td>
</tr>
<tr>
<td><em>E. raffinosus</em></td>
<td>-</td>
<td>1 (1.7)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/60 (1.7%)</td>
</tr>
<tr>
<td><em>E. spp</em></td>
<td>1 (1.7)</td>
<td>1 (1.7)</td>
<td>1 (1.7)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3/60 (5%)</td>
</tr>
</tbody>
</table>

The frequency of resistance to single and multiple antibiotics among the clinical *Enterococcus* isolates is shown in Figure 1, while the distribution of antibiotic resistance by different *Enterococcus* species is presented in Table 4. Out of the sixty isolates in our study, 63.3% showed resistance to at least one antibiotic. One-third (33.3%) of the isolates in our study were multidrug-resistant (Fig 1). Resistance to high-level gentamicin (HLG) was the most noticeable (33.33%) among the isolates examined (Table 4). Figure 2 shows the distribution of *Enterococcus* species that were resistant to HLG. Also, a ward-wise distribution of the antibiotic-resistant *Enterococcus* isolates was shown in Figure 3.
Figure 1: Frequency of resistance to multiple antibiotics among the clinical Enterococcus isolates
Sensitive = No resistance recorded, Resist ≥ 1 antib = resistant to one group of antibiotics, Resist ≥ 2 antib = resistant to two
groups of antibiotics, Resist ≥3 antib = resistant to at least three groups of antibiotics (MDR).

Table 4: Antibiotic resistance profiles of Enterococcus isolates from clinical samples

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>E. fecalis (n=33)</th>
<th>E. feccium (n=20)</th>
<th>E. avium (n=2)</th>
<th>E. durans (n=1)</th>
<th>E. raffinosus (n=1)</th>
<th>E. spp (n=3)</th>
<th>Total n= 60</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S n (%)</td>
<td>R n (%)</td>
<td>S n (%)</td>
<td>R n (%)</td>
<td>S n (%)</td>
<td>R n (%)</td>
<td>S n (%)</td>
</tr>
<tr>
<td>LZ</td>
<td>31 (51.67)</td>
<td>2 (3.33)</td>
<td>19 (31.67)</td>
<td>2 (3.33)</td>
<td>0 (0)</td>
<td>1 (1.67)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>NIT</td>
<td>28 (46.67)</td>
<td>5 (8.33)</td>
<td>14 (23.33)</td>
<td>2 (3.33)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>AMP</td>
<td>27 (45.00)</td>
<td>6 (10.00)</td>
<td>13 (21.67)</td>
<td>1 (1.67)</td>
<td>1 (1.67)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>HLG</td>
<td>26 (43.33)</td>
<td>7 (11.67)</td>
<td>8 (13.33)</td>
<td>12 (20.00)</td>
<td>2 (3.33)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>VAN</td>
<td>33 (55.00)</td>
<td>0 (0)</td>
<td>19 (31.67)</td>
<td>2 (3.33)</td>
<td>0 (0)</td>
<td>1 (1.67)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>TEI</td>
<td>33 (55.00)</td>
<td>0 (0)</td>
<td>18 (30.00)</td>
<td>2 (3.33)</td>
<td>0 (0)</td>
<td>1 (1.67)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>E</td>
<td>27 (45.00)</td>
<td>6 (10.00)</td>
<td>13 (21.67)</td>
<td>1 (1.67)</td>
<td>0 (0)</td>
<td>2 (3.33)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>AK</td>
<td>29 (48.33)</td>
<td>4 (6.67)</td>
<td>14 (23.33)</td>
<td>1 (1.67)</td>
<td>6 (10.00)</td>
<td>1 (1.67)</td>
<td>1 (1.67)</td>
</tr>
<tr>
<td>C</td>
<td>33 (55.00)</td>
<td>0 (0)</td>
<td>19 (31.67)</td>
<td>2 (3.33)</td>
<td>0 (0)</td>
<td>1 (1.67)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>TE</td>
<td>31 (51.67)</td>
<td>2 (3.33)</td>
<td>16 (26.67)</td>
<td>4 (6.67)</td>
<td>1 (1.67)</td>
<td>1 (1.67)</td>
<td>1 (1.67)</td>
</tr>
</tbody>
</table>

Ampicillin = [AMP], Vancomycin = [VAN], Teicoplanin = [TEI], Amikacin = [AK], Erythromycin = [E], Tetracycline = [TE], Linezolid = [LZ], Chloramphenicol = [C], Nitrofurantoin = [NIT], High-level Gentamicin = [HLG]. S= sensitive; R= resistant; n= number of the isolate.
Plate 1: BHIA plates used to determine Minimum Inhibitory Concentration (MIC) of the HLG resistant isolates by agar dilution method.

Figure 2: Distribution of High-level gentamicin (HLG) resistance among clinical Enterococcus isolates.
DISCUSSION

The current study revealed that the majority of the Enterococcus isolates were from urine (51.67%, 31/60), followed by pus (38.33%, 23/60) samples. Enterococci are the normal resident of gastrointestinal tracts. The proximity of urethra and anus in the perineum might account for the high number of Enterococcus isolates from urine samples. The highest number of isolates from urine samples were also reported in previous studies (Praveen et al., 2012; Fernandes et al., 2013; Sharma et al., 2013; Tamanna et al., 2013; Golia et al., 2014; Padmasini et al., 2014) but this was not in agreement with reports from other countries where most of the isolates were from blood (Acharya et al., 2003) and pus (Salem-Bekhit et al., 2012). Similarly, isolates from pus were ranked second in some studies (Fernandes et al., 2013; Acharya et al., 2003).

In this study, the majority of the samples were from surgical wards (46.3%, 25/60) and ICUs (24.97%, 15/60), as reported in other parts of India (Jain et al., 2011) and Saudi Arabia (Salem-Bekhit et al., 2012) were mainly from ICUs alone.

Out of the sixty isolates, the predominant isolates identified were Enterococcus fecalis (55%) followed by E. fecium (33.3%). E. fecalis has been the predominant Enterococcus species reported in various studies in India (Fernandes et al., 2013; Miskeen et al., 2002; Rahangdale et al., 2008; Deshpande et al., 2013; Palanisamy et al., 2013; Padmasini et al., 2013; Sharma et al., 2013; and Desai et al., 2001). Comparable results reported from other parts of the world include Europe (EARSS, 2009; Fisher et al., 2009), USA (Silverman et al., 1998; Madani et al., 1999), France (Monstravers et al., 2009), Kingdom of Saudi Arabia (Salem-Bekhit et al., 2012), Nepal (Acharya et al., 2003) and Nigeria (Olawale et al., 2011). The dominance of E. fecalis among the isolates might be related to the fact that E. fecalis is as well the predominant luminal and gut mucosal microbiota than the rest of the species (Jandhyala et al., 2015). However, E. fecium was reported as predominant isolates from the blood of bacteraemic patients by Jain et al. (2011) and Randhawa et al. (2003). The distribution of uncommon (non-faecalis non-fecium) Enterococcus species varies throughout the world. Our study reported E. avium in 3.3% of the isolates, 1.7% each for E. durans and E. raffinosus. Studies in India (Desai et al., 2001; Praven et al., 2012; Sharma et al., 2013; Padmasini et al., 2014) reported the distribution of E. avium between 0.94% and 9.4% meanwhile; lesser proportions were reported from other parts of the world (Madani et al., 1999). Similar to our findings, the distribution of E. durans in India and other parts of the world was between 0.6 and 4%. Similarly, our finding corroborates that of previous studies on the distribution of E. raffinosus (Sharma et al., 2013; Jain, 2011; Desai et al., 2011). The frequency of uncommon Enterococci in our study was 12%, which was comparable to that reported from some parts of India Fernandes et al. (2013) and Desai et al. (2011) but not in agreement with the findings of Despande et al. (2013) who did not report a single isolate of non-faecalis, non-fecium enterococci among clinical samples. Moreover, we could not identify three (5%) of the isolates in our studies to the species level based on the sugar and biochemical tests used. One remarkable feature of enterococci is their resistance to a wide range of antibiotics, making efficient treatment of enterococcal infections highly challenging. The majority (63.33%) of isolates in our studies showed resistance to at least one antibiotic and were mainly recovered from pus. This result was comparable to those reported by other studies (Despande et al., 2013; Acharya et al., 2003) but another study (Lall et al., 2014) demonstrated that the majority of the drug-resistant isolates were recovered from urine samples. Resistance to high-level gentamicin (HLG) was the most noticeable (33.33%) among the isolates examined in this study. This finding corroborates the outcome of other studies from within India where HLG resistance among clinical Enterococcus
isolates was between 30% to 70% (Fernandes et al., 2013; Despande et al., 2013; Palanisamy et al., 2013; Padmasini et al., 2013; Sharma et al., 2013;) and other parts of the world (Salem-Bakht et al., 2012; Tamanna et al., 2013; Acharya et al., 2003). The majority of the HLG resistant isolates were E. fecium (60%) and E. fecaIis (35%) [figure 2]; 65% of the HLG resistant isolates were also resistant to ampicillin. The highlights mentioned above depicts the limited therapeutic options in treating enterococcal infections in the study centre. Resistance to at least three different groups of antibiotics is termed multidrug resistance (MDR). One-third (33.3%) of the isolates in our study were multidrug-resistant (shown in figure 1). MDR is a common phenomenon among clinical Enterococcus isolates; it’s been reported in various studies around the world (Despande et al., 2013; Acharya et al., 2003; Jain et al., 2011; Madani et al., 1999; Lall et al., 2014). In the current study, E. fecium showed more resistance to antibiotics compared to E. fecaIis. Similar results were reported in other studies (Despande et al., 2013; Palanisamy et al., 2013; Jain et al., 2011; Madani et al., 1999). Isolates from ICUs showed the highest frequency (43%) of antibiotic resistance (figure 3), and the majority were from urine samples. This fact might not be surprising, because patients in ICUs are usually on the catheter, relatively immune-compromised, and prone to multiple antibiotic therapies. Similarly, reports from Saudi Arabia (Salem-Bakht et al., 2013) showed that isolates from ICUs exhibited the highest frequency of antibiotic resistance. However, in Nepal, the highest antibiotic resistance was reported among isolates from a surgical ward (Acharya et al., 2003). Among the HLG resistant isolates, one isolate was susceptible when evaluated for MICs by agar dilution method, as shown in Plate 1; other isolates confirmed to be resistant. Among the twenty HLG resistant isolates, the majority were E. fecium (60%) and E. fecaIis (35%).

One isolate (1.7%), of E. fecium, recovered from blood was resistant to both vancomycin and Teicoplanin. The MIC of the isolate was determined by HiMediaEzy MIC strips (HiMedia India) and was found to be above 256µg/mL for both vancomycin and Teicoplanin; shows that the isolate was of the VanA phenotype. A low frequency of vancomycin-resistant Enterococcus (VRE) of VanA phenotype exhibiting a high level of vancomycin resistance above 256µg/mL was also reported in India (Maradia et al., 2017). In addition to glycopeptides, the isolate was also resistant to Ampicillin, HLG, Erythromycin but susceptible to Linezolid and chloramphenicol. Resistance to vancomycin is relatively low throughout India (Fernandes et al., 2013; Despande et al., 2013; Golia et al., 2014; Randhawa et al., 2003; Maradia et al., 2017). Vancomycin-resistant Enterococcus (VRE) infections pose serious challenges to clinicians because they are usually susceptible to a limited number of antibiotics including Linezolid, making them barely untreatable.

CONCLUSION

The distribution and antibiotic resistance of Enterococcus isolates in urine and pus is higher than in any other clinical sample examined in the health facilities; suspected cases of UTI, wound infections, and sepsis need critical evaluation for possible enterococcal infection. One-third of the isolates were multidrug-resistant and also resistant to HLG and ampicillin. Confirmed susceptibility to antibiotics shall be available before prescription against enterococcal infections for judicial drug use. Clinical use of gentamicin, among other antibiotics, should be closely monitored while treating infections.

Limitations of the study
Limited resources hinder our ability to investigate the molecular basis for identification and antibiotic resistance among the studied Enterococci.

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