Antibiotic Susceptibility Pattern and Biofilm Formation in Clinical Isolates of Enterococcus spp.

Sridevi Shridhar and Biranthabail Dhanashree

Department of Microbiology, Kasturba Medical College, Mangalore, Manipal Academy of Higher Education, Manipal, India

Correspondence should be addressed to Biranthabail Dhanashree; dbiranthabail@yahoo.co.in

Received 15 January 2019; Accepted 5 February 2019; Published 3 March 2019

Enterococcus is a commensal in the intestine and is now emerging as a drug-resistant pathogen. It produces different virulence factors. Enterococcus surface protein (esp) is a virulence factor that helps in the adhesion, but its role in biofilm formation is still contradictory. Moreover, in many bacterial species, strong biofilm producer exhibits multidrug resistance. Hence, this study is done to know the antimicrobial susceptibility pattern of Enterococcus spp. and to correlate the drug resistance with biofilm production and esp gene. Enterococcal isolates were collected from various clinical specimens. Antibiotic susceptibility testing was done by disc diffusion, and biofilm production was performed by microtiter plate method. PCR was performed for detection of esp gene. Two E. faecium strains resistant to vancomycin and high-level aminoglycoside (HLAR) were non-biofilm-producers and did not harbor esp gene. However, other biofilm-producing E. faecium harbored esp gene, and this association was found to be statistically significant (p=0.024). It was observed that there was no significant association between biofilm formation and presence of esp gene in E. faecalis. Moreover, a significant correlation was not found between drug resistance and biofilm production in both Enterococcus species. Thus, biofilm formation is not always associated with the presence or absence of esp gene and or drug resistance in Enterococcus spp.

1. Introduction

Enterococci are normal flora of oral cavity, gut, and female genital tract of humans and are known to cause nosocomial infections [1–4]. E. faecalis is responsible for 80-90 percent and E. faecium 5-10 percent of the human enterococcal infections [5, 6]. Most frequent infections caused by Enterococcus spp. are urinary tract infections followed by intra-abdominal abscesses and bloodstream infections [7]. A high mortality rate of enterococcal infections is due to increasing resistance of the organism to β-lactam antibiotics, aminoglycosides, and glycocopeptides and inadequate response to the treatment [5, 8]. Pandemic spread of vancomycin-resistant Enterococcus (VRE) and acquisition of resistance to newer antimicrobials warrant continued surveillance and early detection of VRE along with Minimum Inhibitory Concentrations (MIC) [9].

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Biofilm protects Enterococci from host immune response and antibiotics. Biofilm-producing Enterococci cause recurrent, chronic, and antibiotic-resistant infections [10–12]. According to the National Institute of Health, 80% of infections are related to biofilm-forming microbes [13, 14]. Apart from biofilm-forming ability, Enterococcus spp. are known to produce various virulence factors [15]. Moreover, clinical isolates have been reported to harbor gene coding for esp virulence factor rather than the commensal strains [16]. Hence, the study was done to know the prevalence of drug resistance in clinical isolates of Enterococcus spp. and to find the association of drug resistance with biofilm formation and esp genes in this part of the country.

2. Materials and Methods

2.1. Isolation and Identification of Enterococcus spp. Enterococci isolated from clinical samples like pus, sputum, vaginal swab, and aspirates (n=150) received for routine culture at the Department of Microbiology, Kasturba Medical College (KMC) Mangalore, from December 2016 to June 2017 were included in the study. Institutional Ethics Committee, KMC Mangalore, India, has approved this study. All the media,
2.2. Antibiotic Susceptibility Test. Antimicrobial susceptibility to ampicillin (10 μg), penicillin (10 units), tetracycline (30 μg), erythromycin (15 μg), chloramphenicol (30 μg), vancomycin (30 μg), teicoplanin (30 μg), ciprofloxacin (5 μg), and nitrofurantoin (300 μg) was determined by Kirby-Bauer disk diffusion method [18] and interpreted as per CLSI guidelines [19]. The quality control strain used was ATCC E. faecalis 29212.

2.3. High-Level Aminoglycoside Resistance (HLAR) Detection. Detection of HLAR was performed with disks containing gentamicin (120 μg), and streptomycin (300 μg) by disk diffusion method. Results were read after incubation at 35°C for 24 h and after 48 h for streptomycin. A zone diameter of 6 mm indicates resistance, 7-9 mm shows that the results are inconclusive, and more than 10 mm suggests that the isolates are sensitive to aminoglycosides. Resistance by disk diffusion to gentamicin corresponds to MIC of >500 μg/ml, and susceptibility corresponds to MIC of <500 μg/ml. However, for high-level streptomycin, MIC of >1000 μg/ml by broth dilution and >2000 μg/ml by agar dilution method corresponds to a zone diameter of 6 mm by disk diffusion. MIC of ≤500 μg/ml by broth and ≤1000 μg/ml by agar dilution corresponds to 10 mm diameter by disk diffusion method [19].

2.4. Detection of MIC for Vancomycin. Enterococcal isolates were inoculated onto Muller Hinton Agar supplemented with 5% defibrinated sheep blood. Vancomycin E-strips (Ezy MIC™) were placed on the inoculated plates and incubated at 37°C in 5% CO₂ for 24 h. The MIC was read where the ellipse intersects the MIC scale on the strip. E. faecalis ATCC 29212 and ATCC 51299 were used as negative and positive controls, respectively. The MIC was determined by broth dilution and agar dilution method [19].

2.5. Biofilm Formation. All the clinical isolates were checked for biofilm production by the procedure used by Kafil and Mobarez (2013) and Triveda and Gomathi (2016) [20, 21]. Briefly, freshly subcultured strains of Enterococcus on blood agar plates were inoculated in 1 ml of Brain Heart Infusion (BHI) broth with 1% glucose and incubated at 37°C for 24 h. To 180 μl of fresh BHI medium, 20 μl of 24-hour-old bacterial growth was added, which corresponded to a turbidity of 0.5 McFarland standard. 200 μl of the suspension of the clinical isolates and the control strain (E. faecalis ATCC 29212) were inoculated into flat bottom microtiter plates in duplicates and incubated at 37°C in 5% CO₂ for 24 h. After incubation, the contents of the plate were removed, tapped, and washed three times with phosphate buffer saline. The biofilm was fixed by adding 150 μl of methanol for 20 min. It was air-dried for about 30 min in an inverted position and later stained with 0.1% crystal violet for 15 min. Excess stain was removed, and plates were washed with distilled water. 150 μl of 33% acetic acid was added in each well and kept for 30 min without shaking. The optical density (OD) was measured at 570 nm. Based on the OD values, the isolates were categorized as strong biofilm formers (OD₅₇₀ > 2), medium (OD₂₅₀ > 1 but < 2), weak (OD₅₀ > 0.5 but < 1), and non-biofilm-formers (OD₂₅₀ ≤ 0.5) [13].

2.6. Detection of the esp Gene by Polymerase Chain Reaction (PCR). All the isolates of E. faecalis and E. faecium were subjected to PCR for the detection of the esp gene. DNA extraction was done by boiling method. Briefly, three to four colonies of enterococcal isolates were suspended in 100 μl of distilled water. The bacterial cells were lysed by boiling for 10 minutes in a dry bath. The lysate was centrifuged briefly, and 2 μl of the supernatant was used as the DNA. PCR was done by using primers esp1 (5'- TTGCTAATGCTAGTCCACGACC-3') and esp2 (5'- CGTCAACACTTGGATTCGGCA-3'). Nuclease-free water and E. faecalis ATCC 29212 were used as esp negative and positive controls, respectively. PCR reaction mixture consisted of 200 μM of dNTP mixture and 2.5 U Taq polymerase with IX buffer and 25 mM MgCl₂, 0.2 μM of primers, and 1 μl of DNA. The PCR tubes containing master mix, primers, and DNA were amplified in a thermocycler (Bio Rad Inc., USA). PCR reaction conditions were initial denaturation at 95°C for 2 min, followed by 30 cycles of 94°C for 45 sec, 63°C for 45 sec, and 72°C for 1 min. Final extension was carried out at 72°C for 10 min. The amplified product was resolved by agarose gel electrophoresis using 1.5% agarose in 1X Tris-acetate EDTA (TAE) buffer. The gel was stained with 0.5 mg/ml ethidium bromide. Gels were visualized under UV transilluminator, and gel pictures were photographed using gel documentation system (Alpha View 1.3.0, Alpha Innotech Corporation Multi Image Light Cabinet) [15, 22].

2.7. Statistical Analysis. The data were tabulated and analyzed by statistical package SPSS ver11.0 (Chicago, IL, USA) to compare antibiotic resistance between different clinical isolates and biofilm production in the presence/absence of esp gene among Enterococcus spp. Chi-square test was used for categorical variables and P value < 0.05 was considered as significant.

3. Results

3.1. Distribution of Enterococcus spp. A total of 150 Enterococcus isolates were included in the study. Among these, 82 (58%) were E. faecalis and 63 (42%) were E. faecium. 56.3% of the E. faecalis and 69.9% of E. faecium were isolated from the males, whereas 43.7% of E. faecalis and 30.1% of E. faecium were isolated from female patients. Enterococcus spp. isolated from different clinical samples are shown in Table 1. Antibiotic susceptibility was performed on all 150 isolates. However, biofilm and eae gene detection was done on 137 isolates as 13 stored isolates were lost during recovery, which included
8 (4 *E. faecalis* and 4 *E. faecium*) urinary isolates and 5 (4 *E. faecalis* and 1 *E. faecium*) isolates from pus.

3.2. Antibiotic Susceptibility of *Enterococcus* spp. All the *E. faecalis* isolates were sensitive to vancomycin. Three strains of *E. faecium* were resistant to vancomycin by disk diffusion method. Among these, two isolates had a MIC of $\geq 32\mu g/ml$ and one MIC of $\geq 8\mu g/ml$. Thus, based on MIC, only two strains were vancomycin-resistant. One each of vancomycin-resistant *E. faecium* isolates was from pus and tissue. Resistance pattern of *Enterococcus* spp. to various antibiotics tested is shown in Table 2. It was observed that significantly higher number of *Enterococcus* spp. isolated from tissue and pus samples showed resistance to amikacin ($p=0.009$), amoxiclav ($p=0.002$), ampicillin ($p\leq 0.001$), high-level gentamicin ($p=0.004$), erythromycin ($p\leq 0.001$), penicillin ($p=0.006$), piperacillin-tazobactam ($p=0.005$), and vancomycin ($p=0.04$) when compared to enterococcal isolates from other samples. Moreover, a significant number of urinary enterococcal isolates showed resistance to ampicillin ($p=0.014$) when compared to isolates from other samples. The resistance of enterococcal isolates from blood and body fluid to imipenem was found to be statistically significant ($p=0.03$). It was observed that significant number of *E. faecalis* from all the clinical samples showed resistance to HLG ($p\leq 0.001$) and erythromycin ($p=0.009$).

### Table 1: *Enterococcus* spp. isolated from different clinical samples.

| Clinical specimen          | *E. faecalis* N (%) | *E. faecium* N (%) | Total *Enterococcus* spp. N (%) |
|----------------------------|---------------------|-------------------|--------------------------------|
| Tissue and pus             | 25 (56.8)           | 19 (43.1)         | 44 (29.3)                      |
| High vaginal swabs (HVS)   | 07 (63.6)           | 04 (36.3)         | 11 (7.3)                       |
| Bile                       | 03 (60)             | 02 (40)           | 05 (3.3)                       |
| Urine                      | 41 (27.3)           | 29 (41.4)         | 70 (46.6)                      |
| Blood and body fluids      | 09 (52.9)           | 08 (47)           | 17 (11.3)                      |
| Respiratory specimens      | 02 (66.6)           | 01 (33.3)         | 03 (2)                         |
| **Total**                  | **87 (58)**         | **63 (42)**       | **150**                        |

3.3. Biofilm Production. Among the 137 *Enterococcus* tested for biofilm production, five (2 *E. faecium* and 3 *E. faecalis*) urinary isolates and one *E. faecium* from pus and one *E. faecalis* from bronchoalveolar lavage (BAL) were strong biofilm producers. Among the three strong biofilm-producing urinary *E. faecalis* isolates, one was resistant to cotrimoxazole, and one was resistant to HLS and another strain to ampicillin. Strong biofilm-producing *E. faecalis* from BAL was sensitive to all the antibiotics tested. Strong biofilm-producing *E. faecium* from pus was found to be resistant to HLS. However, 107 (78.1%) isolates were non-biofilm-formers which included both sensitive and resistant strains of *E. faecalis* (n=65) and *E. faecium* (n=42).

3.4. Detection of esp Gene by PCR. PCR was performed on 137 isolates for the detection of esp genes, and 40 isolates (22 *E. faecalis* and 18 *E. faecium*) were positive for esp gene. Agarose gel picture of PCR showing *Enterococcus* spp. positive for esp gene is shown in Figure 1. The relation between biofilm and presence of esp gene among *Enterococcus* spp. is shown in Table 3. The occurrence of esp gene and biofilm production was not statistically significant in case of *E. faecalis* ($p=0.117$), while it was statistically significant in case of *E. faecium* ($p=0.024$).

### 4. Discussion

*Enterococcus* is one of the significant pathogens affecting all age groups. *E. faecium* is more resistant than *E. faecalis*. Hence, speciation and antibiotic susceptibility testing are necessary to detect the emergence and changing pattern of drug resistance. Vancomycin-resistant *Enterococcus* is a significant cause of concern as this might share its resistance gene with other bacterial strains, causing crossover of gene rendering others resistant to vancomycin.

In the present study, out of 150 *Enterococcus* isolates, 58% were *E. faecalis* and 42% *E. faecium*. The rate of isolation of *Enterococcus* spp. was higher from urine (46.6%) and pus (29.3%), followed by blood and body fluids (11.3%), as shown in Table 1.
Table 2: Antibiotic resistance pattern of *E. faecalis* and *E. faecium*.

| Antibiotics tested     | Percentage resistance |
|------------------------|-----------------------|
|                        | *E. faecalis* N (%)   | *E. faecium* N (%) |
| Amikacin               | 62.2                  | 88.5                |
| Amoxiclav              | 15.2                  | 52.5                |
| Ampicillin             | 20.7                  | 58.7                |
| Erythromycin           | 40.7                  | 66.7                |
| High-level gentamicin  | 48.3                  | 65.1                |
| High-level streptomycin| 48.3                  | 71.4                |
| Imipenem               | 36                    | 59.5                |
| Meropenem              | 42                    | 59.5                |
| Nitrofurantoin         | 7.3                   | 24.1                |
| Piperacillin           | 39                    | 65.5                |
| Piperacillin-tazobactam| 25                    | 48.8                |
| Teicoplanin            | 8                     | 12.7                |
| Vancomycin             | 0                     | 3.17                |

Table 3: Relationship between biofilm and presence of *esp* gene among *Enterococcus* spp.

| Type of biofilm (OD$_{570}$) | *Enterococcus* spp. with *esp* gene | *Enterococcus* spp. without *esp* gene | Total *Enterococcus* spp. N (%) |
|------------------------------|-------------------------------------|---------------------------------------|---------------------------------|
|                              | *E. faecalis* N (%) | *E. faecium* N (%) | *E. faecalis* N (%) | *E. faecium* N (%) | |
| Strong (>2)                  | 3 (13.6)               | 3 (16.6)                | 1(1.7)                | 0 (0)                | 7 (5.1)  |
| Medium (1 to 2)              | 2 (9.0)                | 2 (11.1)                | 3 (5.2)               | 1 (2.5)              | 8 (5.8)  |
| Weak (0.5 to 1)              | 2 (9.0)                | 2 (11.1)                | 3 (5.2)               | 8 (20)               | 15 (10.9) |
| Non-biofilm-formers (≤0.5)   | 15 (68.1)              | 11 (61.1)               | 50 (87.7)             | 31 (77.5)            | 107 (78.1) |
| Total                        | 22 (16.1)              | 18 (13.1)               | 57 (41.60)            | 40 (42.3)            | 137 (100) |

In Table 1. Earlier studies from India and abroad report different rates of isolation of *Enterococcus* spp. from clinical samples, which ranged from 10 to 80% from urine, from 16 to 43% from pus, and from 3 to 36% from blood [4, 6, 23–25]. Isolation rate of *Enterococcus* spp. in the current study is at par with few of the earlier studies [6, 26]. Thus, our report and reports from earlier workers from India and abroad clearly indicate that variation in isolation rate depends on the geographical area and the clinical samples chosen in the study.

In our study, 73.1% and 53% of *E. faecalis* isolated from urine were resistant to HLG and HLS, respectively. Meanwhile the rate of resistance of urinary *E. faecium* was found to be 48.2% to amoxiclav, 65.5% each to HLG and piperacillin, and 68.9% to HLS. (Table 2). A similar earlier study on urinary *Enterococcus* isolates from India reported resistance for HLG (40%), piperacillin (54%), nitrofurantoin (11.5%), and vancomycin (8.5%) [25]. However, in our study, two (3.1%) of *E. faecium* strains were resistant and all the *E. faecalis* strains were sensitive to vancomycin. One each vancomycin-resistant *E. faecium* strain from pus and tissue were resistant to HLAR and sensitive to teicoplanin. In an Iranian study by Talebi et al., the resistance pattern was different from that of our research, where isolates were resistant to teicoplanin (3%) and vancomycin (9%), along with few other drugs [27]. This shows that resistance varies from region to region or from institution to institution in the same area. Hence, it is essential to know the antibiogram of the enterococcal isolates in an area to formulate antibiotic policy.

Resistance to erythromycin was shown by a higher number of *E. faecium* strains (p<0.002) as they are intrinsically resistant to macrolides, lincosamides, and streptogramin B (MLSB phenotype). Cross-resistance to all macrolides arises from modification of the 23S rRNA target (except linezolid resistance) by a variety of methylase genes, commonly *ermB*. Hence, macrolides and lincosamides are not used to treat enterococcal infections, even if *E. faecalis* and *E. faecium* are susceptible to quinupristin-dalfopristin in vitro [28]. In the present study, erythromycin was tested for its susceptibility just to know the resistance pattern and not to use for treatment.

In this study, 21.9% of enterococcal isolates produced biofilm, which included 27.5% *E. faecium* and 17.7% *E. faecalis*. A study from Tamil Nadu [29] showed 68% isolates to be biofilm formers. The study used isolates from diverse clinical samples and detected biofilm formation by three different methods: microtiter plate method, tube method, and Congo red method. In our study, we have used only microtiter plate method. Thus, the method used for the detection of biofilm and origin of the isolate will influence the biofilm formation.
Among the biofilm-producing Enterococcus faecalis (n=14) isolates, 13 were resistant to HLAIR, two were resistant to teicoplanin, and one was sensitive to all the antibiotics except amikacin. Among the biofilm-producing Enterococcus faecium (n=16) isolates, 14 isolates were resistant to HLAIR. However, all the biofilm-producing Enterococcus faecalis and Enterococcus faecium were susceptible to vancomycin. Thus, vancomycin-resistant Enterococcus faecalis was non-biofilm-producer. In the present study, 29.2% of the isolates carried esp gene (Figure 1), while the rest did not. In a study done by Kafel et al. [20, 30], 75% of the Enterococcus isolates producing biofilm carried the esp gene. A survey by Toledo-Arana et al. [15] reported biofilm production by 46.5% of esp gene carrying Enterococcus faecalis. However, there are no reports from India to show a clear relation between presence of esp gene and biofilm production.

In our study, an association of biofilm and presence of esp gene as depicted in Table 3 was not significant among Enterococcus faecalis (p=0.117), while for Enterococcus faecium it was statistically significant (p=0.024). A study by Kafel et al. [24] showed no significant association between biofilm formation and the presence or absence of esp gene (p>0.05). However, studies from Iran by Kafel et al. [30] and Toledo-Arana et al. [15] shows that presence of esp gene in urinary drug-resistant Enterococcus isolates led to strong biofilm formation and a firm adherence to host cells. However, in our study, only ten urinary Enterococcus faecalis and nine Enterococcus faecium were biofilm-producers, and five each had esp gene. Of the 19 biofilm-producing urinary Enterococcus spp., 14 showed HLAIR resistance and two were resistant to teicoplanin. Moreover, vancomycin-resistant Enterococcus faecium did not harbor esp gene and did not produce biofilm. Thus, biofilm production, presence of esp genes, and drug resistance were not interrelated in the present study.

A study by Dale et al. (2015) has shown evidence of Enterococcus faecalis genetic determinants mediating antibiotic resistance within biofilms [30]. The same research also suggests that Enterococcus faecalis employs biofilm-specific mechanisms and not the simple extracellular matrix diffusion barriers to keep antibiotics away from their targets. Since our study period was only of four months’ duration, we targeted for esp gene alone. However, further research is required to study more virulence factors and correlation of the same with biofilm production and antibiotic resistance. In conclusion, biofilm formation is not always associated with the presence of esp gene or drug resistance. Emergence of VRE, HLAIR, and resistance to teicoplanin has left us with very few therapeutic options for enterococcal infection.

Data Availability
The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethical Approval
All procedures performed in this study were in accordance with the ethical standards of the Kasturba Medical College Ethical committee.

Consent
For this type of study, formal consent was not required.

Conflicts of Interest
The authors declare that they have no conflicts of interest.

Acknowledgments
The authors acknowledge the Department of Microbiology, Kasturba Medical College, Mangalore, Manipal Academy of Higher Education, Manipal, for giving them the opportunity to conduct the study.

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