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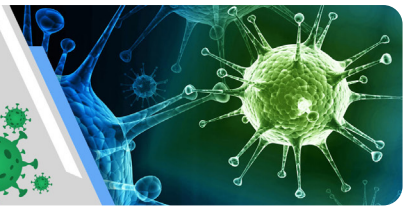

Phenotypic and Genotypic Virulence Factors and Their Association with Antibiotic Resistance in Clinical Isolates of *Enterococcus* Species in Bangladesh

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Abstract

Introduction: The emergence of drug resistant *Enterococcus* spp is now become an important public health threat as it is one of the leading causes of nosocomial infections. This study aimed to disclose the virulence factors and their encoding genes (*asa, gelE, esp, ebpR, hyl* gene for biofilm; *cylA* gene for hemolysis; *gelE* gene for gelatin hydrolysis) and observe their relations with antimicrobial resistance in *Enterococci*.

Methods: For this cross-sectional study, a total of 87 *Enterococci* isolated from different clinical samples (urine, blood, wound swab, pus and bile) were collected. Virulence factors were detected phenotypically by observing hemolysis, gelatin hydrolysis and biofilm formation by tissue culture plate method. For detection of virulence genes, conventional multiplex PCR was adopted for all genes except *ebpR* gene which was identified by single conventional PCR. Kirby Bauer disc diffusion method was used for antimicrobial susceptibility testing.

Results: Among the isolated *Enterococci* majority were *E. faecalis* (75%) followed by *E. faecium* (23%) and *E. raffinosus* (2%). About 52.3% of *E. faecalis* and 35% of *E. faecium* isolates were biofilm producers. Both in *E. faecalis* and in *E. faecium* significant association were found between biofilm formation and *asa, ebpR, esp* genes (p value<.05). Hemolysis was phenotypically observed in 30.8% isolates of *E. faecalis* and 20% isolates of *E. faecium*. A significant association was observed between *cylA* gene and hemolysin production in *E. faecalis.* Eighty five percent, 52.9% and 70.6% biofilm producing and 80%, 55% and 80% hemolysin producing isolates of *E. faecalis* were resistant to ciprofloxacin, ampicillin and gentamicin respectively which was statistically significant (p value <.05).

Conclusion: Antibiotic resistance was higher in biofilm and hemolysin producing isolates of both species. All biofilm producing isolates of *E. faecalis* and *E. faecium* were sensitive to vancomycin, linezolid, teicoplanin and fosfomycin.

Keywords: *Enterococci,* biofilm, hemolysin, gelatinase, virulence genes, PCR

Introduction

Enterococci are common residents of the gastrointestinal tracts of humans and animals which causes various community and hospital acquired infections such as urinary tract infections, bacteremia, endocarditis, meningitis and

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intra-abdominal infections [1]. *Enterococci* are considered as important nosocomial pathogens because they have the capacity to acquire and share extra chromosomal elements including antibiotic resistance genes or virulence traits. Virulence factors of *Enterococci* might have a role in increasing their capacity to colonize among hospitalized patients [2]. The ability of biofilm formation which protect the bacteria from host immune responses and antibiotics is one of the important virulent characteristics of *Enterococci* [3]. Numerous enterococcal virulence factors including secreted factors and adhesions such as Esp (extracellular surface protein), Asa 1 (aggregation substance) and Ebp (endocarditis and biofilm-associated pili) are related with the biofilm formation [4]. Asa1, a pheromone-inducible protein escalates bacterial attachment to renal tubular cells and Esp (cell wall-associated protein) helps in colonization, persistence and biofilms formation in the urinary system [5]. The expression of Ebp operon is regulated by *ebpR* (endocarditis and biofilm associated pilli regulator) gene and associated with pilli and biofilm formation in enterococcal species causing UTI [6, 7]. Biofilm formation is reduced in *ebp* mutant strains [6]. Several secreted virulence factors of *Enterococci* including CylA (cytolysin), GelEA (gelatinase) and Hyl (hyaluronidase) play a role in pathogenesis of *Enterococci*. Gelatinase (extracellular zinc-containing metalloproteinase) degrades host tissue, provides nutrients to the bacteria and helps in biofilm formation. Hyl acts on hyaluronic acid and facilitates the spread of *Enterococci* by damaging the host tissue with the help of its degradative enzyme [2]. Cytolysin (secreted toxin) is produced in response to pheromones and helps in the pathogenesis of *E. faecalis* by creating blood hemolysis [8]. Among *Enterococcus* species, *E. faecalis* is the most virulent and pathogenic strain which contains several effective mechanisms like highly transmissible plasmid for horizontal gene transfer that helps them to transmit virulence genes to other less virulent species as *E. faecium* [9]. Understanding the virulence factors of *Enterococci* may help to know the pathogenic process and antimicrobial resistance of this bacteria. So, this study profiled *Enterococci* isolates of clinical origin for their virulence factors, virulence encoding genes and their association with antibiotic resistance.

Materials and Methods

This cross-sectional study was conducted at the Department of Microbiology and Immunology, Bangabandhu Sheikh Mujib Medical University (BSMMU) over a period of one year from March 2019 to February 2020.

Bacteria isolates collection and identification

A total of 87 *Enterococci* isolated from different clinical samples (urine, blood, wound swab, pus and bile) were collected from the Laboratory of Microbiology and Immunology Department of BSMMU for this study. These samples were sent from different departments of BSMMU hospital for culture and sensitivity test. *Enterococci* were identified based on colony morphology on Chromogenic agar media and blood agar media, Gram staining method and standard biochemical test (catalase test, salt tolerance test, bile esculin test) [10]. Then identification of *Enterococci* species was done by fermentation of mannitol, sorbitol, raffinose, arabinose, utilization of pyruvate and arginine decarboxylase test [11].

Antibiotic susceptibility test by disc diffusion

For antimicrobial susceptibility testing of the isolated *Enterococci,* the Kirby-Bauer disk diffusion method was used using Mueller-Hinton agar (Himedia, India) and commercially available antibiotic discs (Biomaxima, Poland). Antibiotic discs of ampicillin (10 μg), ciprofloxacin (5 μg), cotrimoxazole (1.25/23.75 μg), nitrofurantoin (300 μg), gentamicin (120 μg), vancomycin (30 μg), linezolid (30 μg), teicoplanin (30 μg), fosfomycin (200 μg), and quinopristin-dalfopristin (15 μg) were used. The disc concentration and zone of inhibition were used as recommended by the Clinical Laboratory Standards Institute guideline (CLSI, 2019) [12]. *S. aureus* ATCC 25923 was used as a quality control strain.

Phenotypic identification of the virulence factors

Detection of hemolysis

Enterococci were inoculated on blood agar media to detect the hemolytic activity and observed the appearance of hemolytic zone around the colony after 24 hours of incubation at 37°C [13].

Gelatin hydrolysis activity

Production of gelatin was detected by inoculation of *Enterococci* on freshly prepared peptone yeast extract agar containing 4% gelatin and incubated at 37°C for 24 hours. Then cooled to room temperature for two hours. Gelatin hydrolysis was indicated by the appearance of turbid halo around the colonies [14].

Biofilm formation

Biofilm formation of *Enterococci* was performed by tissue culture plate method (TCP) as previously described by Toledo-Arana *et al* [15]. At first, the *Enterococci* were grown in Brain heart infusion broth (BHIB) (Becton Dickinson and company, USA) with 0.25% glucose which was incubated at 37°C overnight. Then the broth culture was diluted at a ratio of 1:40. Two hundred microliter of diluted culture suspension was inoculated in a sterile 96 well flat bottom polystyrene micro titer plate (Greiner Bio-One International, Kremsmunster, Austria). The positive control (*Klebsiella pneunoniae* ATCC 700603) and negative control (sterile BHIB-0.25% glucose) were also inoculated in the same way. Then the wells were washed three times with 200 µl of phosphate buffer saline

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(PBS) after overnight incubation at 37°C. The plate was air dried, fixed with 200 µl/well of 2% formalin at 4ºC for 1 hour. The plate was stained with 1% crystal violet for 15 min and to remove the excess stain plate was rinsed under running tap water. After that 200 µl ethanol acetone (80:20, v/v) was added in each well to solubilize crystal violet. Each assay was performed in triplicate and repeated three times. The optical density (OD) at 630 nm was measured using ELISA plate reader (Plate reader, model–A4, serial no.-1910, Das, Italy). The cut-off value (ODc) was calculated for each microtiter plate. ODc was of three standard deviations (SD) above the mean OD of the negative control: ODc = average OD of negative control + (3×SD of negative control). Final OD value of a tested strain was expressed as average OD value of the strain reduced by ODc value (OD = average OD of a strain - ODc). Negative value presented as zero, while any positive value indicates biofilm production [16].

Genotypic detection of virulence genes by PCR

Extraction of DNA was performed by boiling method [17]. Conventional multiplex PCR was used to detect all virulence genes and single PCR was used to detect the *ebpR* gene using primer sets listed in Table 1. The PCR assay was done in a reaction mixture with total volume of 25 µl which contained 15 µl of master mix (HELINI, India), 0.15 µl Taq polymerase (Solis BioDyne Germany), 1 µl of forward and reverse primer each (10 pmol/µl), 2.85 µl of distilled water and 5 µl of undiluted extracted DNA. For all virulence genes the amplification condition was: pre-denaturation at 95 °C for 15 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C for 1 min and final extension at 72 °C for 10 min. In case of *ebpR* the amplification condition was: pre- denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 30 sec and extension at 72 °C for 50 sec and final extension at 72 °C for 10 min. The amplification products were electrophoresed on 1.5% agarose gel.

Statistical analysis

The qualitative data were expressed as frequency and percentages. The association between the biofilm formation, hemolysin production and their encoding genes with antibiotics resistance were assessed by the Pearson Chi-Square test using SPSS version 22. p values less than 0.05 were considered as statistically significant.

Results

Among the 87 *Enterococci* isolates, 75% (65) were *E. faecalis,* 23% (20) *E. faecium* and 2 % (2) *E. raffinosus*. Approximately 47% (41) *Enterococci* isolates were biofilm producers and 27.6 % (24) showed hemolysis. Among *E. faecalis* and *E. faecium* 52.3% (34) and 35% (7) of isolates were biofilm producers respectively. Twenty percent (4) and 30.8% (20) of isolates of *E. faecium* and *E. faecalis* showed hemolysis production respectively. Gelatin hydrolysis was not detected in any isolates [Table-2]. The association of single and combination of virulence factors encoding genes with phenotypic virulence factors *in E. faecalis* and *E. faecium* were shown in Table 3,4,5,6. The *asa, esp* and *ebpR* were found in 65.9%, 64.1% and 60.4% of biofilm producing *E. faecalis* (p value <.05). The combination of genes *asa, gelE, ebpR* and *asa, esp, gelE, ebpR* were also found in 87.5% & 86.4% of biofilm producing *E. faecalis* which were statistically significant [Table-3]. Hundred percent of *asa, gelE*, combination of *esp, hyl, ebpR* genes*;* 85.7% of *esp* gene and 50 % of *ebpR* gene were found in biofilm producing *E. faecium* isolates which were statistically significant (p value <.05) [Table-4]. Among 46 *cylA* positive *E. fecalis* isolates, 43.6% (20) were phenotypically hemolysin producers and all (19) *cylA* negative isolates were negative for hemolysis phenotypically which was statistically significant (p value 0.001) [Table-5]. The association of *cylA* gene and hemolysin production in *E. faecium* isolates were showed in Table 6. None of the hemolysin producing *E. faecium* isolate was positive for *cylA* gene. On the other hand, out of 20 *cylA* gene negative isolates, 20% (4) were hemolysin producers [Table-6].

Statistical analysis stipulated that there was a notable relationship between biofilm formation and antibiotic resistance in *E. faecalis*. Fifty three percent, 64.7%, 85.3% and 70.6% of biofilm producing *E. faecalis* isolates were resistant to ampicillin, cotrimoxazole, ciprofloxacin and gentamicin respectively (p< .05). Antibiotic resistance was higher in biofilm producing *E. faecium* but no significant association was found. All biofilm producing isolates of *E. faecalis* and *E. faecium* were sensitive to vancomycin, linezolid, teicoplanin and fosfomycin [Table-7, Table-8]. Eighty percent of hemolytic *E. faecalis* isolates were resistant to ciprofloxacin and gentamicin. Significant association was found in case of ampicillin, ciprofloxacin, nitrofurantoin and gentamicin resistance and hemolysin production in *E. faecalis* (p< .05). Hundred percent of hemolysin producing isolates of *E. faecium* were resistant to ampicillin, ciprofloxacin, gentamicin and 75% isolates were resistant to cotrimoxazole and nitrofurantoin but this association was not statistically significant [Table-9 and Table-10].

Discussion

The increased incidence of drug resistant enterococcal infections in recent years suggests that several virulence factors might have a role in accelerating the pathogenesis and antibiotic resistance of this bacteria. So, it is very important to detect the virulence factors with their encoding genes and their association with antimicrobial resistance in clinical

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setting for epidemiological surveillance, formulation of local antibiogram, treatment guideline, infection control and preventive measures. Among 87 *Enterococci* isolates, 75% were *E. faecalis* followed *E. faecium* (23%) and *E. raffinosus* (2%). Biofilm-producing *Enterococci* are responsible for recurrent, chronic and antibiotic-resistant infection [19]. In biofilm producing bacteria the matrix component of biofilm reduces the penetration of antibiotic, presence of persister cells and the horizontal gene transfer of antibiotic resistance genes leads to antibiotic resistance [20, 21]. Approximately 47.1% of *Enterococci* isolates were biofilm producers which is almost similar to the findings (32.4%) reported in a study of Eastern India [22]. On the contrary in another study, lower percentage (21.9%) of biofilm producing isolates were reported where less virulent *E. faecium* was the predominant isolates [23].

In our study, 52.3% of *E. faecalis* and 35.0% of *E. faecium* isolates were biofilm producers. Similarly in a study of China, 47.2% of biofilm producing *E. faecalis* isolates were observed [24]. Some study reported that *E. faecium* produces more biofilms than *E. faecalis* [5]. The conflict may be due to type of sample, number of *E. faecium* isolates and geographic dissimilarities. *Enterococci* harbors plasmid pAD1 which encodes cytolysin and helps in the transfer of virulence factors and antibiotic resistance genes [8, 25]. In this study about 27.6% of hemolysin producing isolates of *Enterococci* were observed. Similarly, 34.2% of hemolysin producing *Enterococci* were reported by other studies [22]. Fifty eight percent of hemolysin producing *Enterococci* was reported in a study of West Iran which may be due to regional difference [26]. The role of gelatinase in enterococcal infection is to formation of biofilm and supply of nutrients to the bacteria [2]. None of the *Enterococcus* species was positive for gelatin hydrolysis and similar findings were also reported by studies done in Egypt. Many *gelE* positive isolates failed to secrete gelatinase which may be the cause of this findings [27]. Transmembrane protein, FsrB regulates the production of gelatinase by *gelE* gene which is controlled by locus *fsr* and deletions within locus *fsr* produce mutants that do not synthesize gelatinase [28]. Approximately 20% of gelatinase producing *Enterococci* isolates were reported in a study of Kolkata which may be due to infection with different strains of *Enterococcus* in that region [29].

In this study, 100.0 % of biofilm producing *E. faecium* and 65.9% of biofilm producing *E. faecalis* isolates had *asa* gene. Abdel *et al* also found the association of *asa* gene with biofilm formation but opposite findings were reported by Fallah *et al* [30, 5]. The *esp* gene was observed in 64.1% and 85.7% of biofilm producing *E. faecalis* and *E. faecium* respectively which was statistically significant (p value < .05). A correlation between the *esp* gene and biofilm formation was also observed by studies done in Saudi Arabia [30]. Some other studies suggest that the *esp* gene is not mandatory for the biofilm formation in *E. faecium* and *E. faecalis* [31]. Hundred percent and 53.8% of biofilm forming *E. faecium* and *E. faecalis* isolates had *gelE* gene where the association was statistically significant for *E. faecium* (p value < .05)**.** Association of *gelE* gene with production of biofilm in *Enterococci* specially in *E. faecium* were reported by some studies but other study suggests that gelatinase was not necessary for biofilm production [32, 33].

Fifty percent and 60.4% of biofilm producing *E. faecium* and *E. faecalis* isolates had *ebpR* gene which was statistically significant (p <.05). Higher percentages (93.6%) of biofilm producing *Enterococcus* having *ebp* gene is also reported [4].

All biofilm producing *E. faecium* and *E. faecalis* isolates of this study had multiple biofilms forming genes. Combination of *asa, gelE, ebpR* genes and *asa, esp, gelE, ebpR* genes had significant relationship with biofilm formation (p <.05) in *E. faecalis*. We also found statistically significant (p < .05) association of combination of *esp, ebpR* and *esp, hyl, ebpR* genes with biofilm formation in *E. faecium*. This was supported by other studies where they found that single biofilm associated gene (*esp, asa, gelE, ebpR*) was not enough for biofilm development in *Enterococci* [5, 34]. On the contrary, other study reported that single biofilm forming gene was related with biofilm production [32]. Production of biofilm is a complicated process and relies on multiple factors in *Enterococcus* strains which may be the reason of different findings of the biofilm encoding genes with biofilm formation [35].

Cytolysin which is responsible for hemolytic activity has a key role in the extremity of human infections [36]. Hemolytic activity was observed phenotypically in 30.8% isolates of *E. faecalis* whereas *cylA* was present in 43.5% isolates which was statistically significant (p value 0.001). Similar findings were also reported in another study where they showed 41% of *Enterococci* carried the *cylA* gene and hemolytic activity was observed in 38% of *cylA* positive isolates [34]. This lack of phenotypic/genotypic expression of cytolysin might propose the mislay genes in the *cyl* operon [34]. The *cylA* gene was absent in all (4) hemolysin producing isolates of *E. faecium*. The cytolysin structural gene was always detected in β-hemolytic *E. faecalis* while absent in *E. faecium* [37]. So, the hemolysis of *E. faecium* must be occured by another cytotoxic component. In a study of South Korea, *cylA* gene negative *E. faecium* isolates with hemolytic activity were detected which suggests possible function of other genes in hemolytic activity [38].

Treatment of biofilm forming *Enterococci* are difficult because they are more antibiotic resistant [24]. Regarding antibiotic resistance in *E. faecalis,* higher resistance was observed in biofilm producers compared to non-biofilm

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producers. Resistance to some antibiotics including ampicillin (52.9% vs. 12.9%), cotrimoxazole (64.7% vs. 16.1%), ciprofloxacin (85.3% vs. 35.5%), gentamicin (70.6% vs. 29.0%) were significantly higher in biofilm producing *E. faecalis* than non-biofilm producers. Similar results were reported by Fallah *et al* and they found significantly higher resistance among biofilm positive isolates [5]. Biofilm producing *E. faecium* isolates were 100.0% resistant to ampicillin, ciprofloxacin, gentamicin followed by 71.4% resistant to cotrimoxazole and nitrofurantoin. Biofilm producing *E. faecium* strains were more antibiotic resistant [39]. Hemolysin producing *E. faecalis* isolates of this study were more antibiotic resistant than non-hemolysin producing isolates. Resistance to some antibiotics including ampicillin (55% vs. 24.4%), ciprofloxacin (80% vs. 53.3%), gentamicin (80% vs. 37.8%), nitrofurantoin (30% vs. 4.4%) were significantly higher in hemolysin producer than non-hemolysin producers.

Resistance to various antibiotics including ampicillin (100% vs. 87.5%), cotrimoxazole (75% vs. 43.8%), ciprofloxacin (100% vs. 87.5%), gentamicin (100% vs. 93.8%) were higher in hemolysin producer than non-hemolysin producers of *E. faecium.* Hemolysin producing isolates were more antibiotic resistant than non-hemolysin producing isolates but no significant association was observed and similar findings were also reported by Jankoska *et al* [40]. Higher number of resistances were also seen in non-hemolysin and non-biofilm producing isolates of *E. faecium*. The reason may be due to *E. faecium* is more drug resistant.

Conclusion

This study reveals the ability of *Enterococci* to develop biofilm formation and hemolysin production. The biofilm encoding genes *ebpR, asa, esp, gelE* were found in biofilm producing isolates of both species and hemolysin encoding gene *cylA* in hemolysin producing *E. faecalis* isolates which suggests the potential link between these genes in biofilm formation and hemolysin production. Vancomycin, linezolid, fosfomycin and teicoplanin remains the most effective antimicrobial agents in hemolysin and biofilm producing isolates of *E. faecalis* and *E. faecium*.

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Recommendations

Further nationalwide large scale study should be explored to know the prevalence of *Enterococcal* infection in Bangladesh.

Ethical Approval: The study was ethically approved by Institutional Review Board (IRB) of Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh [NO. BSMMU/2019/8188, Date- 29/07/2019]

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

Author's Contribution

Abu Naser Ibne Sattar was responsible for the conception of the study. Abu Naser Ibne Sattar, Rehana Razzak Khan, Fatima Afroz, Md. Nahidul Islam, Sharmin Chowdhury, Tahani Momotaz and Mohammad Tanvir Sarwar participated in its design and coordination. Tahani Momotaz was chief investigator and responsible for the acquisition, analysis and interpretation of the data. Mohammad Tanvir Sarwar, Abu Naser Ibne Sattar, Fatima Afroz and Rehana Razzak Khan reviewed the results and statistical analyses. Tahani Momotaz and Fatima Afroz drafted the manuscript and all the authors contributed substantially to its revision. All authors met ICMJE authorship criteria and have read and approved the final manuscript.

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