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Detection of Virulence Factors and Antimicrobial Resistance in *Enterococci* Isolated from Urinary Tract Infection

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Abstract

Background: The genus *Enterococcus* is of increasing significance as a cause of nosocomial infections and this trend is exacerbated by the development of antibiotic resistance. **Objective:** The aims of this study was to find out the susceptibility pattern and the prevalence of virulence genes in *Enterococcus* strains isolated from urinary tract infection in Bangladesh. **Methodology:** This cross-sectional study was conducted in the Department of Microbiology at Dhaka Medical College, Dhaka from January 2015 to December 2015 for a period of one (01) years. All the admitted patients in the Dhaka Medical College & Hospital, Dhaka and Banghabandhu Sheikh Mujib Medical University, Dhaka who were presented with clinical features of urinary tract infection at any age with both sexes were included as study population. The antimicrobial susceptibility of the strains was determined using the disk diffusion method; vancomycin susceptibility pattern was detected by MIC method. PCR was performed for the detection of genus-species, *vanA*, *vanB* gene and potential virulence genes. **Result:** Out of 84 *Enterococci*, majority (71.42%) of the isolates were *E. faecalis* followed by 23.81% were *E. faecium*. Regarding antimicrobial susceptibility pattern, 100% isolated *Enterococci* were sensitive to vancomycin, linezolid and teicoplanin. Most of the *Enterococci* were resistant to ciprofloxacin and ceftriaxone (92.86%) followed by 88.10% to gentamicin and 85.71% to azithromycin and rifampicin. No VRE was identified and the range of MIC for vancomycin was 1-4 µg/ml. None of the *Enterococci* was positive for *vanA* and *vanB* genes. Out of total *E. faecalis* and *E. faecium*, 87.50% were positive for ebp and 77.5%, 72.5%, 55.0%, 45.0%, 40.0% and 10.0% of isolates were positive for gelE, esp, ace, cyl, asa and hyl respectively. **Conclusion:** Almost all the virulence genes were more prevalent in *E. faecalis* isolates. [*Bangladesh Journal of Infectious Diseases 2017;4(2):30-34*]

Keywords: Enterococcus; nosocomial infections; MIC; virulence factors

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Introduction

Enterococci are gram-positive bacteria which are normal inhabitants of gastrointestinal tracts of humans[1-2]. It is recognized that they cause serious infection such as endocarditis, UTI, septicemia, post-operative wound infection, meningitis[3-5]. The natural ability of *Enterococc*i to acquire, accumulate and share extra chromosomal elements encoding virulence traits or antibiotic resistance genes, inpart, explains their increasing importance as nosocomial pathogens[6-8]. It is believed that nosocomial *Enterococci* might have virulence elements that increase their ability to colonize hospitalized patients[7].

Several *Enterococcal* pathogenic factors have been identified including adhesions and secreted virulence factors. The most important adhesion factors are aggregation substance (asa), extracellular surface protein (esp), adhesion of collagen from *E. faecalis* (ace), endocarditis and biofilm associated pilli (ebp)[9-11]. As a virulence factor, aggregation substance increases bacterial adherence to renal tubular cells[12]. Extracellular surface protein (esp) contributes colonization and biofilm formation of *Enterococci* and leads to resistance to stresses and adhesion to eukaryotic cells such as those of endocarditis and UTI[13]. The ability of *Enterococci* to produce biofilms is fundamental in causing endodontic and urinary tract infections. The formation of pili by *Enterococci* is necessary for biofilm formation; the gene cluster associated with this is ebp (endocaditis and biofilm-associated pili). Another cell-surface protein present in *E. faecalis* is Ace (adheison of collagen from *E. faecalis*). This is a collagen-binding protein, belonging to the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) family. Ace may play a role in the pathogenesis of endocarditis[14].

Secreted pathogenic factors of *Enterococci* with a value in pathogenesis are cytolysin (cyl), gelatinase (gelE) and hyaloronidase (hyl)[15-17]. Gelatinase is an extracellular zinc-containing metalloproteinase, which has a role in providing nutrients for the bacteria by degrading host tissue. It has some functions in biofilm formation as well[5,17]. Cyl has beta haemolytic properties in human and is bactericidal against other gram-positive bacteria. Hyl acts on hyaluronic acid and mainly consist of degradative enzymes which are associated with tissue damage and facilitates spread of *Enterococci* and their toxins through host tissue[13]. These virulence factors may play a role in promoting persistence of *Enterococci.*

Few studies on species detection and drug susceptibility of *Enterococci* have been carried out in Bangladesh. But the virulence factors of *Enterococci* have not been identified yet in Bangladesh. Therefore, this study was designed to observe different virulence factors with their antimicrobial resistance pattern.

Methodology

This cross-sectional study was conducted in the Department of Microbiology at Dhaka Medical College, Dhaka from January 2015 to December 2015 for a period of one (01) years. All the admitted patients in the Dhaka Medical College & Hospital, Dhaka and Banghabandhu Sheikh Mujib Medical University, Dhaka who were presented with clinical features of urinary tract infection at any age with both sexes were selected as study population. Urine samples were collected aseptically from patients attending outpatient and inpatient departments of Dhaka Medical College, Dhaka and Bangabanbhu Sheikh Mujib Medical University, Dhaka inoculated on blood agar and MacConkey's agar media and was incubated at 37^0 C for overnight. Suspected enterococci colonies were identified by Gram stain and other biochemical test. The enterococci stains were cultured in tryptica soya broth in microcentrifuse tubes, after overnight incubation, microcentrifuse tubes were centrifused at 5000 x g and the deposite were store at -30^0 c for DNA extraction. Species and virulence factors were detected by PCR (Table 1).

Table 1: Selected primers with annealing temperature used in this study

Gene	Oligonucleotide sequence (5´to 3´)	Annealing temperature	Product size (bp)
asa1	GCACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA	56˚c	375
gelE	TATGACAATGCTTTTTGGGAT AGATGCACCCGAAATAATATA	56˚c	212
cyl	ACTCGGGGATTGATAGGC	56ºc	688

	GCTGCTAAAGCTGCGCTT		
Esp	AGATTTCATCTTTGATTCTTGG AATTGATTCTTTAGCATCTGG	56ºc	510
Hyl	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCCAA	56ºc	276
Ebp	AAAAATGATTCGGCTCCAGAA TGCCAGATTCGCTCTCAAAG	52ºc	101
Ace	GGAGAGTCAAATCAAGTACGTTGGTT TGTTGACCACTTCCTTGTCGAT	58ºc	101
E. faecalis	ATCAAGTACAGTTAGTCTTTATTAG ACGATTCAAAGCTAACTGAATCAGT	58ºc	941
E. faecium	TTGAGGCAGACCAGATTGACG TATGACAGCGACTCCGATTCC	65ºc	658
vanA	CATGAATAGAATAAAAGTTGCAATA CCCCTTTAACGCTAATACGATCAA	54ºc	1030
vanB	GTGACAAACCGGAGGCGAGGA CCGCCATCCTCCTGCAAAAAA	65ºc	433

Antibiotic Susceptibility Testing

The antimicrobial susceptibility of the isolates was determined using disk diffusion method and the minimum inhibitory concentration (MIC) of vancomycin was determined using the agar dilution method based on CLSI 2015 guidelines. Staphylococcus aureus ATCC 25923 was used as quality control stain.

Genomic PCR

DNA extraction: Three hundred microlitre distilled water was mixed with bacterial pellet and was vortexed until mixed well. The microcentrifuse tube was kept in block heater (DAIHA Scientific, Seoul, Korea) at 100^0C for 10 minutes for boiling. After boiling the tube was immediately kept on ice. Then the tube was centrifuged at 4^0C at 14000 x g for 6 minutes. Finally supernatant was taken using micropipette and was used as template DNA for PCR. This DNA was kept at -20^0C for future use.

Mixing of master mix and primer with DNA template: Amplification was performed in a final reaction volume of 25 µl. Each PCR tube contained 2 ml of extracted DNA, 12.5 µl master mix-PCR buffer, dNTP, Taq polymerase enzyme, $MgCl_2$ and loaded dye (Promega Corporation, USA), 2µl extracted DNA from *Enterococcus spp.* was mixed in 12.5 µl master mix together with 4 µl primer (forward and reverse). Volume of the reaction mixture was adjusted by adding 6.5 µl filtered deionized water (nuclease free). After a brief vortex, the tubes were centrifuged in a micro centrifuge for few seconds.

Amplification through thermal cycler: PCR assays were performed in a DNA thermal cycler (Eppendorf AG, Master cycler gradient, Hamburg, Germany). Each PCR run was comprised of preheat at 94^0C for 10 minutes followed by 36 cycles of denaturation at 94^0C for 1 minute, annealing at 58^0C for 45 seconds, extension at 72^0C for 2 minutes with final extension at 72^0C for 10 minutes.

Gel electrophoresis and visualization: Amplified products were run on to horizontal gel electrophoresis in 1.5% agarose (Bethesda Research Laboratories) in 1X TBE buffer at room temperature at 100 volt (50 mA) for 30 minutes. Five µl amplified DNA mixed with tracking dye was then loaded into an individual well of the gel. One hundred bp DNA molecular size markers were loaded into well at the middle or at two sides of the gel for comparing with the base pair of identified band. DNA bands were detected by staining with ethidium bromide (0.5 µl/ ml) for 30 minutes at room temperature and then destained with distilled water for 15 minutes. Photographs were taken using digital camera with UV trans-illuminator (Gel Doc, Major science, Taiwan).

Statistical Analysis: The results of the study were recorded systematically. Data analysis was done by using 'Microsoft Office Excel 2013' program and using Z test. P value ≤ 0.05 was taken as minimal level of significance and p value <0.001 was taken as highly significant.

Results

In total, 60 (71.43%) *E. faecalis* and 20 (23.81%) *E. faecium* were isolated from urine specimens. Two (4.76%) *Enterococci* were unidentified by biochemical test. Also in PCR results 2 samples had no clear bonds. Antibiotic susceptibility testing by the disk diffusion showed high resistance to ciprofloxacin and ceftriaxone (92.86%) followed by gentamicin (88.10%), azithromycin and rifampicin (85.71%), amikacin (66.67%). All isolates were sensitive to vancomycin, linezolid and teicoplanin. The agar dilution method indicates that all isolates were vancomycin sensitive (Table 2).

Table: 2: Antimicrobial susceptibility pattern *of Enterococci* (n=84)

Antimicrobial Agent	Sensitive n (%)	Resistant n (%)
Amikacin	28 (33.33)	56 (66.67)
Gentamicin	10 (11.90)	74 (88.10)
Ciprofloxacin	6 (7.14)	78 (92.86)
Azithromycin	12 (14.29)	72(85.71)
Ceftriaxone	6 (7.14)	78 (92.86)
Imipenem	60 (71.43)	24 (28.57)
Rifampicin	12 (14.29)	72 (85.71)
Piperacillin-tazobactam	82 (97.62)	2 (2.38)
Teicoplanin	84(100.00)	0(0.00)
Vancomycin	84(100.00)	0 (0.00)
Linezolid	84(100.00)	0 (0.00)

Out of 84 isolates, 87.0% were positive for ebp and 77.5%, 72.5%, 55.0%, 45.0%, 40.0% and 10.0% were positive for gelE, esp, ace, cyl, asa1 and hyl respectively (table 3). Ace (P = 0.05), cyl (P = 0.05) and gelE gene (P = < 0.05) were significantly more in *E. faecalis* than *E. faecium*. Hyl gene was significantly higher in *E. faecium* than *E. faecalis* (P = 0.01) (Table 3).

Table 3: The proportion of virulence genes among isolated *E. faecalis* (n=60) *and E. faecium* (n=20)

Virulence genes	*E. faecalis* n (%)	*E. faecium* n (%)	Total n (%)	P value
Ace	8(63.33)	6 (30.00)	44(55.00)	0.05
Esp	44(73.33)	14(70.00)	58(72.50)	>0.05
Ebp	54(90.00)	16(80.00)	70(87.50)	>0.05
Asa1	26(43.33)	6 (30.00)	32(40.00)	>0.05
Hyl	0(0.00)	8 (40.00)	8(10.00)	0.01
Cyl	32(53.33)	4 (20.00)	36(45.00)	0.05
gelE	56(93.33)	6 (30.00)	62(77.50)	<0.05

E. faecium were more antibiotic resistant than *E. faecalis*. By PCR, all (100.0%) *Enterococci* were negative *for vanA and vanB* genes.

Discussion

In this study, it was found that *Enterococci* were resistant to most of the antimicrobial agents, possibly, these reflect miss-using of antibiotics. The proportion of *Enterococci* showing high level of resistance to gentamicin (88.1%), ciprofloxacin (92.86%), azithromycin (85.71%), ceftriaxone (92.86%). The findings of present study coincide with the results of previous studies[18-23].

In the present study, no *Enterococcus* was found resistant to vancomycin, linezolid and teicoplanin. Likewise, previous studies no VRE was detected[12,15,16]. Considering these results, the absence of vancomycin, linezolid and teicoplanin resistance among *Enterococci* strain permits the use of these antibiotics to treat the patients.

According to results from the present study, almost all virulence genes were higher in *E. faecalis* than *E. faecium*. The ace gene (codes for collagen-binding protein) has been detected in high frequency in *E. faecalis* than *E. faecium* (p=0.05) that is in agreement with previous study[20]. The present study revealed the prevalence of the esp gene was same (p≥0.05) in *E. faecalois* and *E. faecium.* In agreement with our research, other studies revealed same prevelance of esp gene for causing UTI[11]. In contrast, a study showed the higher prevalence of this gene in *E. faecium* isolates from urine samples.

In this study, ebp (coding endocarditis and biofilm associated pilli) was present in almost all of *E. faecalis* (90%) and *E. faecium* (80%). A high incidence of this ebp gene in both *Enterococci* was reported in previous study[11].Three MSCRAMMs (EF0089, EF2505, and EF1093 (renamed EbpA, EbpB and EbpC) produce immunogenic and pleomorphic pili[19] which have a role in UTI[22]. In the present study, the asa1 (encodes aggregation substance) was 40.0% in the isolates which is consistent with previous studies. In total, the rate of asa1 gene in urine isolates did not indicate an association between the presence of asa1 and emergence of UTI and these results are similar to the finding of other studies[9].

Gene cyl in *E. faecalis* isolates were significantly higher (p =0.05) than *E. faecium* and the findings of the present study co-inside with the results of

previous study where 54.7% *E. faecalis* and 6.9% *E. faecium* had cyl gene[11]. In this study, gelE (codes for gelatinase is an extracellular zinc metalloendopeptidase) enriched in *E. faecalis* in comparison with *E. faecium* and may involve in the creation of a urinary tract infection ($p < 0.05$). Likewise, previous studies in *E. faecalis* demonstrate the presence of the gene in high incidence among their isolates[20]. The main role of gelatinase in *Enterococcal* pathogenesis is thought to be in providing nutrients to the bacteria by degrading host tissue, although they also have some function in biofilm formation[5,17]. In this study, the high prevalence of gelE gene in *E. faecalis* indicated an association of the presence of gelE and emergence of UTI.

Conclusion

This present study demonstrated that though *Enterococci* were multidrug resistance in Bangladesh but yet now all *Enterococci* are sensitive to vancomycin, linezolid and teicoplanin. So, these drug now should be the choice for treatment of UTI caused by *Enterococci.*

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