

## Original Article

# ENTEROCOCCAL VIRULENCE DETERMINANTS IN URINARY TRACT INFECTION PATIENTS

Jabin Akhter,<sup>1</sup> Shaheda Anwar<sup>2</sup>, Sharmeen Ahmed<sup>2</sup>

<sup>1</sup>Chest Disease Clinic, Chittagong. <sup>2</sup>Department of Microbiology & Immunology Bangabandhu Sheikh Mujib Medical University

### Abstract

Urinary tract infection caused by *Enterococci* has become frequent occurrences in health care settings. Currently they emerged with increasing resistance to multiple antibiotics. Haemolysin, gelatinase and biofilm production are some markers that have been proposed as possible Enterococcal virulence factors. In view of the increasing importance of *Enterococcal* infection, the present study was designed to isolate and identify the *Enterococci* to the species level from urine of urinary tract infection patients and to investigate their possible virulence factors. Biofilm was detected on polystyrene microtitre plate to see the adherence of microorganism. Haemolysin production and gelatin hydrolysis detected by standard microbiological method. Fifty nine enterococcal isolates were speciated by conventional microbiological method and examined for their ability to form biofilm by microtitre plate assay. In this study, biofilm formations by *Enterococci* were found in 83.33% isolates from catheterized and 56.09% from non-catheterized patients. Among them, *E.faecalis* & 50% *E.faecium* produced biofilm. About 43.63% *E.faecalis* & 10% *E.faecium* produced haemolysin and only one isolate were found to be gelatinase positive. Frequency of virulence factors (VFs) in combination was observed in this study. Two VFs (haemolysin and biofilm) were observed in 27.11% in combination and 3 VFs (haemolysin biofilm and gelatinase) were present in 1.69% isolates. These results suggest that although there may not be an absolute role for individual virulence determinants in infectivity, combinations of factors may play a role in allowing a biofilm infection to be more resistant to therapy.

**Key words:** Enterococci, Biofilm.

### Introduction

*Enterococci*, recognized as opportunistic pathogens, are natural inhabitants of the oral cavity, normal intestinal microflora, and female genital tract of both human and animals. Recently they emerged as an important global cause of nosocomial infections, being increasingly associated with urinary tract infections, endocarditis, intra-abdominal and pelvic infections, catheter-related infections, surgical wound infections, and central nervous system infections because of the extent of its antimicrobial resistance<sup>1</sup>. Biofilm production, haemolysin and gelatinase are the potential virulence factors of Enterococci. Biofilm consist of a population of cells attached irreversibly on various biotic and abiotic surfaces

encased in a hydrated matrix of exopolymeric substances<sup>2</sup>. Biofilms are notoriously difficult to eradicate and are a source of many chronic infections. Bacteria in biofilms are resistant to phagocytosis, making biofilms extremely difficult to eradicate from living hosts<sup>3</sup>. The two most common *Enterococci* species are *Enterococcus faecalis* and *Enterococcus faecium* both are capable of producing biofilm. Bacteria in biofilms colonize a wide variety of medical devices, such as catheters, artificial cardiac pacemakers, prosthetic heart valves and orthopaedic appliances<sup>2</sup>. A mature biofilm can tolerate antibiotics at concentrations of 10-1000 times more than are required to kill planktonic bacteria. Infections due to *Enterococci* often prove difficult to treat due to high level resistance to multiple antibiotics as a result of both intrinsic and acquired mechanisms. The immense difficulties in treating serious Enterococcal infections underscore the importance of understanding virulence factors specially biofilm that may be targeted by alternative therapeutics. Haemolysin production is another potential virulence factor for *Enterococci*. Haemolysin may lyse

---

✉ **Corresponding author:**

Dr. Jabin Akhter

Medical Officer, Chest Disease Clinic, Chittagong.

Email : jabinakhter@yahoo.com

erythrocytes and release iron, which might enhance microbial growth *in vivo* and it also help in tissue destruction by cytotoxic effect<sup>4</sup>. Gelatinase is an extracellular zinc containing metalloproteinase can hydrolyze gelatin, collagen, fibrinogen, casein hemoglobin, certain *E. faecalis* sexpheromone related peptides and main role in Enterococcal pathogenesis is thought to be in providing nutrients to the bacteria by degrading host tissue and biofilm formation<sup>5</sup>. Therefore, the present study was undertaken on clinical isolates of *Enterococci* to determine the frequency of virulence factors.

### Material and methods

In this study the *Enterococci* were isolated from urine of suspected urinary tract infection patients of different age and sexes from outpatient and inpatient department of BSMMU, Dhaka. A total 1203 urine samples were tested for the isolation of *Enterococci*. All samples were initially cultured on chromogenic agar media (Difco laboratories, Detroit, USA). All suspected colonies were identified by standard microbiological methods including gram staining, catalase test and esculin hydrolysis test, growth in 6.5% NaCl and at pH 9.6<sup>6</sup>. *Enterococci* isolates were identified to the species level by using conventional physiological tests devised by Facklam and Collins<sup>7</sup> which are based on carbohydrate fermentation using 1% solution of following sugars: mannitol, sorbitol, arabinose raffinose, sorbose and pyruvate utilization in 1% pyruvate broth; arginine decarboxylation in Moellers decarboxylase broth<sup>6</sup>.

### Detection of virulence factors of isolated Enterococci :

**a) Detection of haemolysis:** The hemolytic activity of *Enterococci* was assessed on blood agar plates prepared with Mueller-Hinton agar containing defibrinated sheep blood. Hemolytic zone was observed around the colonies after incubation for 24 h at 37°C<sup>8</sup>.

**b) Gelatin hydrolysis activity:** Detected by inoculating the *Enterococci* on freshly prepared peptone yeast extract agar containing 4% gelatine & incubated at 37°C overnight and cooled to ambient temperature for two hours. The appearance of turbid halo or zone around the colonies was considered to be a positive indication of gelatinase<sup>8</sup>.

**c) Biofilm Assay:** The ability of the *Enterococci* isolates to form biofilm on an abiotic surface was described by Toledo-Arena *et al*<sup>9</sup>. Bacterial strains to be tested were grown in BHI broth containing 0.25% glucose and incubated overnight. Overnight broth cultures were diluted 1:20 in fresh BHI broth supplemented with glucose. 200µl of diluted strain was dispensed into triplicate wells in a single column of a sterile 96-well flat-bottom microtiter plate (Becton Dickinson) & incubated

at 37°C for 24 hours. Planktonic cells were discarded by tapping the microtitre plate, and the wells were washed three times with sterile phosphate-buffered saline (PBS). The plates were inverted and allowed to dry for 1 h at room temperature. 200 µl of 0.5% aqueous crystal violet solution was added to each well & allowed to stand for 15 min. The wells were subsequently washed thrice with sterile PBS to wash off the excess crystal violet. 200 µl of 80:20 (vol/vol) mixtures of ethyl alcohol and acetone was added to solubilized bound crystal violet. Absorbance of the extracted crystal violet was measured at 550 nm automatic microplate reader (ELX-800 Bio-Tek Instruments, Inc., Winooski, Vt.). For positive control *Staphylococcus epidermidis* and for negative control non-biofilm forming bacteria *Salmonella typhi* was used in each plate. All biofilm assays were repeated three times. The cut-off value (ODc) was established. ODc, defined as three standard deviations (SD) above the mean OD of the negative control. Final OD value of a tested strain was expressed as average OD value of the strain reduced by ODc value of the triplicate assays. Any OD value above the cutoff value was indicative of biofilm production<sup>8</sup>. In the quantitative assay for the biofilm production, the isolates were classified as strongly biofilm producing (strongly adherent), weakly adherent isolates and non-biofilm producers (non-adherent). [ Figure I]

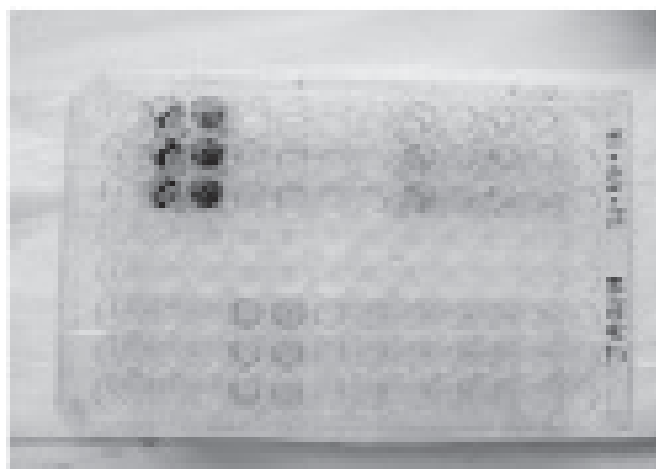


Figure I: Biofilm detection by microtitre plate assay

### Results

A total of 1203 urine samples collected from patients with suspected urinary tract infection Bacterial culture of urine from non-catheterized and catheterized patient yielded 666(55.36%) and 130 (76.92%) significant growth of different organisms respectively. Fifty-nine enterococci were isolated during the study period. Number of biofilm producing Enterococcal species isolated from catheterized and non-catheterized patients are depicted in table I. Among 42 *E. faecalis*, 22(52.38%) produced β-haemolysis and

2(4.76%) produced  $\alpha$ -haemolysis on sheep blood agar, Only one *E.faecium* produced  $\alpha$ -haemolysis and none other species found to produce haemolysin.

**Table I: Biofilm formation of Enterococcus species isolated from urine of non-catheterized and catheterized UTI patients**

Enterococcus species	SNo. of biofilm positive isolates / Total isolates in urine		No. of total biofilm positive isolates / total isolates
	Mid stream urine	Catheter urine	
<i>E.faecalis</i>	21/32(65.6)	09/10(90)	30/42(71.42)
<i>E.faecium</i>	02/06(33.33)	03/04( 75)	05/10(50)
<i>E. avium</i>	-/01	-	-/01
<i>E. raff</i>	- /01	-	-/01
Unidentified species	-/01	03/04(75)	03/05(60)
Total	23/41(56.09)	15/18(83.33)	38/59(64.40)

# Parenthesis indicates in percentage

**Table II : Frequency of virulence factors (VFs) in Enterococci isolated from UTI patients**

Combination of VFs	Haemolysin production	Gelatin hydrolysis	Biofilm production	Total isolates
No VFs	-	-	-	11(18.64)
1 VF	-	-	+	22(37.28)
2 VFs	+	-	+	15(25.42)
3 VFs	+	+	+	1(1.69)
Total	26	01	38	59

**Discussion**

Biofilm attached irreversibly on various biotic and abiotic surfaces results in persistent infections and resistant to antimicrobial therapy. Therefore it is important to understand the virulence factors specially biofilm formation and the antimicrobial sensitivity pattern of enterococci in UTIs<sup>10</sup>.

The reported frequency of Enterococcal urinary tract infection is variable in the different studies. In the present study, prevalence of enterococcal urinary tract infection was 8.44% which almost correlate with the findings of Barros *et al*<sup>11</sup>. Thirteen point eighty four percent (13.84%) *Enterococci* were isolated from patient on indwelling urethral catheter. The finding is similar to a study conducted in U.S. acute-care hospitals where the incidence of *Enterococci* causing nosocomial catheter-associated urinary tract infections was 16% & 13% in hospital wide & ICU setup respectively<sup>12</sup>. In this study majority of the isolates were *E.faecalis* 42(71.18%) found both in catheterized & non-catheterized patients, while *E.faecium* accounted for 10(16.94%) & was more frequent in catheterized urine. *E.avium* 1(2.43%) & *E.raff* 1(2.43%) were found only in non-catheterized urine.

There are various methods for biofilm detection<sup>13, 14, 15</sup>. In this study we evaluated 59 isolates from urine samples for

their ability to form biofilms by tissue culture plate(TCP) method. In our study we found that the majority of biofilm producing bacteria was form catheterised patients (83.33%) and 56.09% from non-catheterized patients. Among them 71.42% *E.faecalis* & 50% *E.faecium* produced biofilm. Biofilm positive *Enterococci* were more frequently found in catheter urine than in non-catheter urine (P<0.05). Similarly, Donlan<sup>16</sup> reported in his study the association of biofilm producing bacteria with urinary catheter. Rakhshanda *et al*<sup>17</sup> stated in a study that biofilm production by uropathogen was detected in 75% of the isolates from UTI. *Enterococci* are the one of the causative organism of UTI & biofilm formation allows the strains to persist for a long time in the genitourinary tract and interfere with bacterial eradication. Survival advantages conferred by the biofilm community include resistance to phagocytosis, and to antimicrobial agents. Biofilm formation is indirect evidence of adhesiveness & microtitre plate assay is the indirect way to measure adhesion of *Enterococci*. In this study, biofilm production by *Enterococci* isolates suggests that they are involved in colonization of the urinary tract by adhering to uroepithelial cells and thereby responsible for infection. Microtitre plates made of polystyrene were found to be most sensitive, accurate & reproducible screening method for detection of biofilm formation in clinical isolates.

In our study we performed tissue culture plate method by addition of 2% sucrose to BHI broth. Sugar supplementations are essential for biofilm formation. This was reported by studies conducted by Mathur *et al*<sup>18</sup>. In modified TCP method extended incubation for 24 hrs could lead to a better discrimination between moderate and non-biofilm producing isolates<sup>18</sup>.

In the present study, 43.63% *E.faecalis* & 10% *E.faecium* produced haemolysin and only one isolate were found to be gelatinase positive. In the recent years, many studies notes the prevalence of haemolysin as 13-60%<sup>19</sup>. Less geletinase producing isolates were observed in this study. The gelatinolytic activity of enterococcal isolates varies with environmental conditions related to temperature, pH, divalent cations and carbon sources. These findings suggest that variation in such conditions could have impact on the virulence of this opportunistic pathogen associated with infections. The role of virulence factors of *Enterococci* in pathogenesis was not observed in this study. It is likely that VFs in organism play redundant or overlapping role such that the absence of single VF has only subtle effects<sup>20</sup>.

The very frequent presence of virulence determinants in combinations suggests possible synergistic activity in causation of infection.

**References:**

1. Murray, B. E. & Weinstock, G. M. Enterococci: new aspects of an old organism. *Proc Assoc Am Physicians* 1999; 111: 328-334.
2. Costerton, J. W., Stewart, P. S. & Greenberg, E. P. Bacterial biofilms: a common cause of persistent infections. *Science* 1999; 284: 1318-1322.
3. Lewis K. Riddle of biofilm resistance. *Antimicrob Agents Chemother* 2001; 45: 999-1007.
4. Coburn PS and Gilmore MS. The *Enterococcus faecalis* cytolyisin: a novel toxin active against eukaryote and prokaryotic cells. *Cell Microbiol*; 2003; 5: 661-669
5. Murray BE. The life and time of enterococci. *Clin Microbiol Rev.* 1990; 3:46-65.
6. Colle JG, Duguid JP, Fraser AG, Marimon BP, Simmons A. Laboratory strategy in the diagnosis of infective syndromes. *Mackie and McCartney Practical Medical Microbiology*. 14th Edition. Churchill Livingstone Inc: London; 1996, 53-94
7. Facklam RR and Collins MD. Identification of *Enterococcus* Species Isolated from Human Infections by a conventional test scheme. *J of Clin Microbiol.* 1989; 27: 731-734
8. Upadhaya G, Umapathy BL and Ravikumar KL. Comparative study for the presence of Enterococcal virulence factors, gelatinase, haemolysin and biofilm among clinical and commensal isolates of *Enterococcus faecalis*. *Journal of Clinical Microbiology.* 2010; 27: 731-734
9. Toledo-Arena A, Valle J, Solano C, Arrizubieta MJ, Cucarella C, Lamata M, *et al.*, The Enterococcal surface protein, Esp, is involved in *Enterococcus faecalis* biofilm formation. *Appl and Environmental Microbiol.* 2001; 67: 4538
10. Segreti J Efficacy of current agents used in the treatment of Grampositive infections and the consequences of resistance. *Clin Microbiol Infect.* 2005; 11: 29- 35.
11. Barros M, Martinelli R and Rocha H. Enterococcal Urinary Tract Infections in a University Hospital: Clinical Studies. *The Brazilian Journal of Infectious Diseases.* 2009; 13(4): 294-296.
12. Office of public health, Louisiana Dept of health & hospitals, 2008
13. Maki DG. Nosocomial bacteremia. An epidemiologic overview. *Am J Med.* 1981; 70 : 719-32.
14. Christensen GD, Simpson WA, Bisno AL, Beachey EH Adherence of slime producing strains of *Staphylococcus epidermidis* to smooth surfaces. *Infect Immune.* 1982; 37: 318 -26.
15. Freeman DJ, Falkiner FR and Keane CT. New method for detecting slime production by coagulase negative staphylococci. *Journal of Clinical Pathology.* 1989; 42(8): 872-874
16. Donlan RM. Biofilms and device associated infections. *Emerg. infect Dis.* 2001; 7: 277-281.
17. Rakhshanda B, Mubashir A, Ghulam R Urinary tract infection in diabetic patients and Biofilm formation of Uropathogens. *Infectious Diseases Journal of Pakistan.* 2008; 17(1):7-9
18. Mathur T Singhal S Khan S. Uphadta Fatma T Rattam A Detection of Biofilm formation among clinical isolates of Staphylococci : An evaluation of three different screening methods *Indian J Med. Microbiol.* 2006; 24:25-29
19. Coque T, Patterson J, Steckelberg J *et al.* Incidence of haemo-lysin, gelatinase and aggregation substance among Enterococci isolated from patients with endocarditis and other infections and from feces of hospitalized and community-based persons. *J Infect Dis.* 1995; 171: 1223-9
20. Pires-Boucas PD, Izumi E, Furlaneto-Maia L, Sturion L and Suzart S. Effects of environmental and nutritional factors on gelatinolytic activity by *Enterococcus faecalis* strains isolated from clinical resources. *African Journal of Microbiology Research.* 2010; 4(10): 969-976