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Original Articles

Relationship between the biofilm formation & virulence genes of enterococci isolated from urinary tract infection

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Abstract

Objective: The genus *Enterococcus* is of increasing significance as a cause of nosocomial infections and this trend is exacerbated by the development of antibiotic resistance. The aims of this study were to investigate the possible associations between virulence profiles and biofilm formation in 56biofilm producing *Enterococcus* strains isolated from urinary tract infection in Bangladesh. **Materials & Method**: Biofilm of isolated *Enterococci* was detected by tissue culture plate method (TCP). PCR was done for detection of virulence genes. **Results:** Among the virulence genes, esp and ebp positive biofilm forming isolates were significantly higher (P =.0001) than esp and ebp negatives isolates. **Conclusion**: The purpose of this study was to show different virulence genes profiles, to establish possible relationship between virulence factors and biofilm formation.

Key words: Biofilm, Virulence factors.

Introduction

Enterococci are gram-positive bacteria which are normal inhabitants of gastrointestinal tracts of humans.[6,20] It is recognized that they cause serious infection such as endocarditis, UTI, septicemia, post-operative wound infection, meningitis.[3,13,14,25] It is believed that nosocomial *Enterococci* might have virulence elements that increase their ability to colonize hospitalized patients.[12] 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] As a virulence factor, aggregation substance increases bacterial adherence to renal tubular cells.[11] 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.[2,23] The ability of *Enterococci* to produce biofilms is fundamental in causing endodontic and urinary tract infections, as well as endocarditis. The formation of pili by *Enterococci* is necessary for biofilm formation, the gene cluster associated with this being 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.[17] Secreted pathogenic factors of *Enterococci* with a value in pathogenesis are cytolysin (cyl), gelatinase

(gelE), hyaloronidase (hyl).[16] 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.[10,19] 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.[10] Biofilm are defined as microbial derived sessile communities characterized by the cells that are irreversibly attached to a substratum or to each other. They are embedded in a matrix of extracellular polymeric substance they are produced, and exhibit an altered phenotype with respect to growth rate and gene transcription.[7] The adherence and production of a biofilm by *E. faecalis* and *E. faecium* on different biomaterials have been demonstrated, and the capacity of *Enterococci* to bind to various medical devices, such as ureteral stents, intravascular catheters,[21] biliary stents,[8] silicone gastrostomy devices,[5] has been associated with the ability of *Enterococci* to produce biofilm.[1,18.19.24] Several studies investigated the role of these virulence factors in biofilm formation by *Enterococci*.[21,22] But others suggest that these genes do not seem to be necessary for the production of biofilm in *enterococci*. The purpose of this study was to investigate the relationship between biofilm formation and virulence genes of *Enterococci.*

Materials and Methods

Urine samples were collected aseptically from patients attending outpatient and inpatient departments of Dhaka medical college and Bangabanbhu Sheikh Mujib Medical University, inoculated on blood agar and MacConkey· s agar media and 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 -30^0c 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 GCTGCTAAAGCTGCGCTT	56ºc	688
Esp	AGATTTCATCTTTGATTCTTGG AATTGATTCTTTAGCATCTGG	56ºc	510
Hyl	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCCAA	56ºc	276
Ebp	AAAAATGATTCGGCTCCAGAA TGCCAGATTCGCTCTCAAAG	52ºc	101
Ace	GGAGAGTCAAATCAAGTACGTTGGTT TGTTGACCACTTCCTTGTCGAT	58ºc	101

Genomic PCR and 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^{o}C for 10 minutes for boiling. After boiling the tube was immediately kept on ice. Then the tube was centrifuged at 4^{o}C 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^{o}C 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^{o}C for 10 minutes followed by 36 cycles of denaturation at 94^{o}C for 1 minute, annealing at 58^{o}C for 45 seconds, extension at 72^{o}C for 2 minutes with final extension at 72^{o}C 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 ethilium 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).

Method of Detection of Biofilm:

Biofilm assays by Tissue Culture Plate Method (TCP)

The microorganisms are grown in polystyrene tissue culture plates for 24 hours then after washing, fixed with sodium acetate *(2%)* and stained with crystal violet (0.1% w/v). Biofilm formation is detected by measuring optical density with ELISA reader.[4]

The organisms were grown overnight in brain heart infusion broth (BHIB) with 0.25% glucose at 37°C. The culture was diluted 1:40 in TSB 0.25% glucose, and 200 µl of this cell suspension was used to inoculate sterile 96 well polystyrene microtiter plates. After 24h at 37^{0}C, wells were gently washed three times with 200 µl of phosphate buffered saline (PBS), dried in an inverted position, and stained with 0.1 % crystal violet for 15 min. The wells were rinsed again, and the crystal violet was solubilized in 200 µl of acetone (80:20, vol/vol). The optical density at 595nm (OD_{595}) was determined using a microplate reader. Each assay was performed in triplicate and repeated three times.[24]

Calculation of OD values (Stepanovic *et al*., 2007):

OD value was calculated by using the following method. The average OD values were calculated for all tested strains and negative controls, since all tests were performed in triplicate and repeated three times. Second, the cut off value (ODc) was

established. It was defined as three standards (SD) above the mean OD of the control: ODc = average OD of negative controls + (3 X 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). ODc value was calculated for each micro titer plate separately. If a negative value is obtained, it should be present as zero, while any positive value indicates biofilm.

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

Among 56 biofilm producing *Enterococci*, 85.71% had ebp, 78.57% had esp, 53.57% had gelE gene, 57.14% had asa1, 35.71% had ace gene. None of the biofilm producer had hyl gene. Biofilm formation was significantly higher in ebp and esp positive isolates than in ebp and esp negative isolates (P = .0001) (table 2).

Table: 2: Relationship between biofilm formation and virulence genes in isolated Enterococci (N= 56).

Virulence factors	Number of biofilm formation	Percentage of biofilm formation	P value
Esp positive	44	78.57	0.0001
Esp negative	12	21.43	
Asa1 positive	32	57.14	0.4
Asa1 negative	24	42.86	
Ebp positive	48	85.71	0.0001
Ebp negative	8	14.19	
Cyl positive	8	14.19	0.0001
Cyl negative	48	85.71	
GelE positive	30	53.57	> 0.05
GelE negative	26	46.43	
Ace positive	20	35.71	0.05
Ace negative	36	64.29	
Hyl positive	0	0.00	< 0.05
Hyl negative	56	100.00	

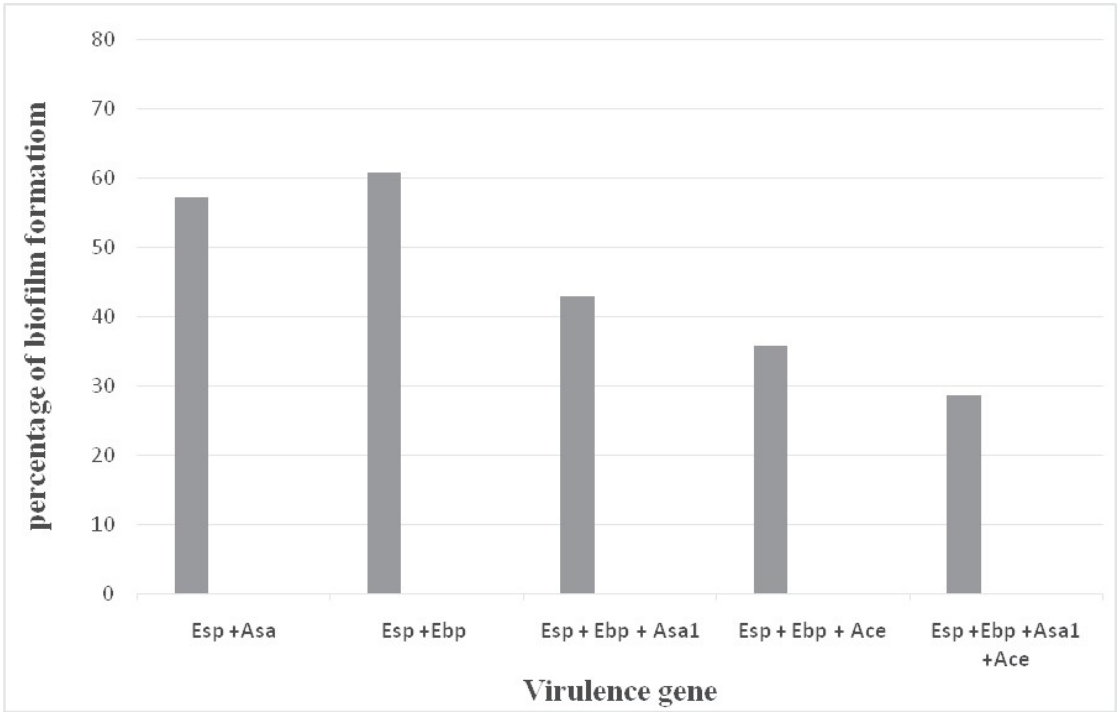

Figure: 1: Biofilm forming capacities of isolated Enterococci according to association of esp gene with other colonization genes.

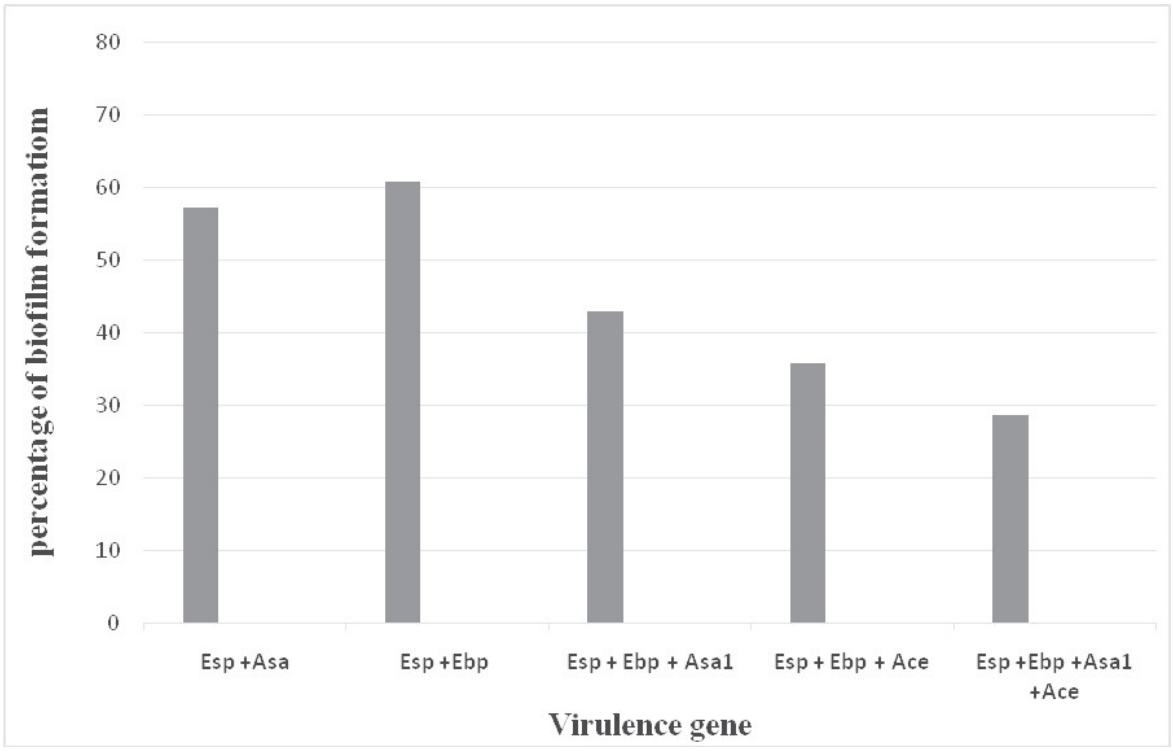

Figure: 2: Biofilm forming capacities of isolated Enterococci according to association of esp gene with other colonization genes.

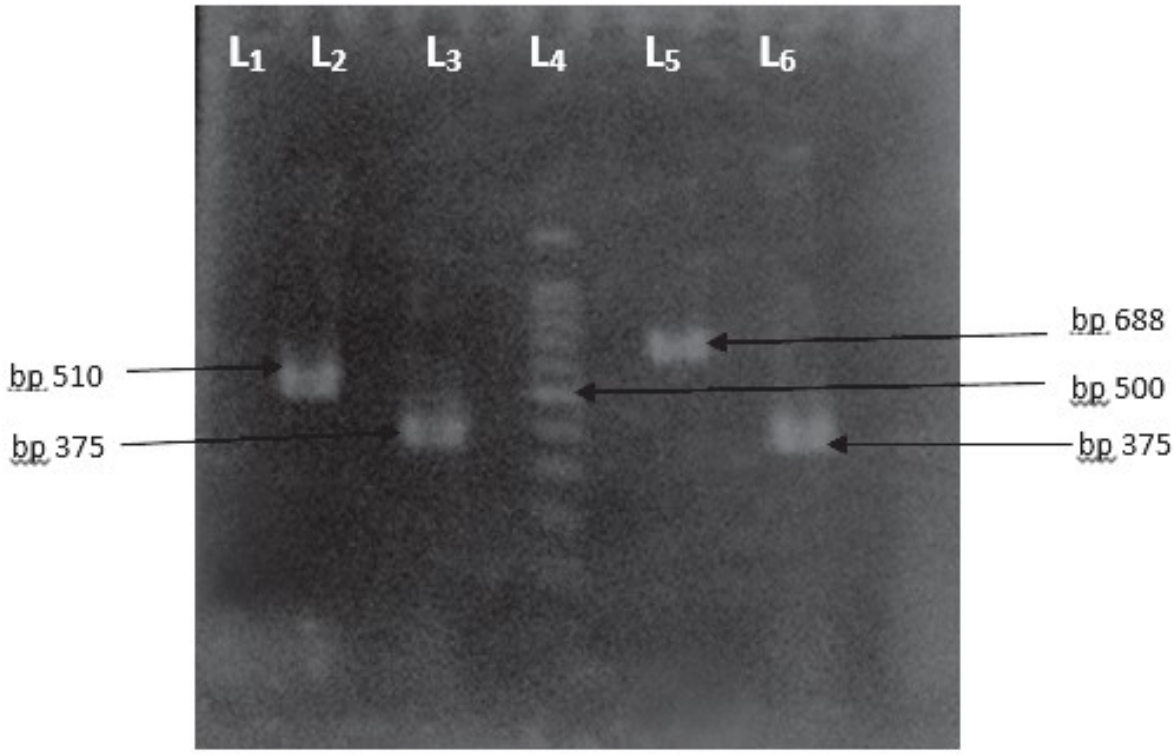

Figure 3: Photograph of gel electrophoresis of amplified DNA of 510 bp for esp gene (lane 2), DNA of 375 bp for asa1 gene (lane 3& 6), Hundred base pair DNA ladder (lane 4), DNA of 688 bp for cyt gene (lane 5). Negative control Staphylococcus aureus ATCC 25923 (lane 1).

Discussion

In the present study, 78.57% biofilm producing *Enterococci* had esp gene and it was significantly higher (p=0.0001) than esp negative isolates. This data was similar to the results of previous study where it was reported that 75% biofilm producers had esp gene.[15] Consistent with this findings, among 200strains of *Enterococcus*, a high correlation between the presence of esp gene and ability of a given strains to produce a biofilm.[24] In contrast, some studies demonstrated that esp was not require to form biofilm but that it's presence was associated with higher amount of biofilm.[19] In this study, 85.71% biofilm producing *Enterococci* had ebp gene and it was significantly higher (p=0.0001) than ebp negative isolates. This data was similar to the results of previous study where it was reported that 93.36% biofilm producers had ebp gene.[15]The formation of pili by *Enterococci* is necessary for biofilm formation, the gene cluster associated with this being ebp (endocaditis and biofilm-associated pili).[9]

Conclusion

Results of the present study showed that the presence of esp and ebp gene in isolates was associated with the higher biofilm formation of urinary tract isolates. This indicated that *Enterococci* isolates that had accumulated biofilm producing genes were apt to form persistent biofilms in the urinary tract.

References

1. Baldassarri L, Cecchini R, Bertuccini L, Ammendolia MG, Losi F, Arciola CR, et al. *Enterococcus* spp. Produces slime and survives in rat peritoneal macrophages. Med Microbial Immunol, 2001; 190: 113-120.
2. Borgmann S, Niklas DM, Klare I, Zabel LT, Buchenau P, Autenrieth IB, et al. Two episodes of vancomycin-resistant *Enterococcus faecium* outbreaks caused by two genetically different clones in a newborn intensive care unit. Int J Hyg Environ Health, 2004; 207:386-9.
3. Cetinkaya Y, Falk P, Mayhall CG. Vancomycin-resistant *Enterococci*. Clin Microbiol Rev, 2000; 13: 686-707.
4. Christensen GD, Simpson WA, Younger JA, Baddour LM, Barrett FF, Melton DM, et al. Adherence of coagulase negative *Staphylococci* to plastic tissue cultures: a quantitative model for the adherence of *Staphylococci* to medical device. J Clin Microbiol, 1985; 22(6): 996-1006.
5. Dautle MP, Wilkinson TR & Gauderer MW. Isolation and identification of biofilm microorganisms from silicone gastrostomy devices. J Pediatr, 2003; 38: 216-220.
6. Desai PJ, Pandit D, Mathur M, Gogate A Prevalence, identification and distribution of various species of *Enterococci* isolated from clinical specimens with special reference to urinary tract infection in catheterized patients. Indian J Med Microbiol, 2001; 19: 132-137.
7. Donlan RM, Costerton W. Biofilms: Survival mechanisms of clinically relevant Microorganisms. Clin microbial Rev, 2002; 15(2): 167-93.
8. Dowidar N, Moesgaard F. & Matzen P. Clogging and other complications of endoscopic biliary endoprostheses. Scand J Gastroenterol, 1991; 26: 1132-1136.
9. Fisher K, Philips C. The ecology, epidemiology and virulence of *Enterococcus.* Microbiology 2009; 155:1749-57.
10. Gilmore MS. Coburn PS. Nallapareddy SR. Murray BE. *Enterococcal* virulence. In: Gilmore MS, editors. The *Enterococcl*: pathogenesis, molecular biology and antibiotic resistance, Washington DC: ASM Press; 2002: pp.301-354.
11. Guzman C, Pruzzo AC, Lipira G, and Calegari L. Rote of adherence in pathogeness of *Enterococcus faecalis* urinary tract infection and endocarditis. Infect Immun, 1989; 57: 1834-1838.
12. Hällgren A, Claesson C, Saeedi B, Monstein HJ, Hanberger H, Nilsson LE. Molecular detection of aggregation substance, enterococcal surface protein, and cytolysin genes and in vitro adhesion to urinary catheters of *Enterococcus faecalis* and *E. faecium* of clinical origin. Int J Med Microbiol, 2009; 299:323-32.
13. Huycke MM, Sahm DF & Gilmore MS. Multiple-drug resistant *Enterococci*: The nature of the problem and an agenda for the future. Emerg Infect Dis, 1998; 4: 239-249.
14. Jones RN, Sader HS, Erwin ME, Anderson SC. *Enterococcus* Study group. Emerging multiple resistant *Enterococci*. Diagn Microbiol Infect Dis, 1995; 21: 95-100.
15. Kafil HS, Mobarez AM. Assessment of biofilm formation by *enterococci* isolates from urinary tract infections with different virulence profiles. Journal of Kind Saud University-Science, 2015; 27: 312-317.
16. Kayaoglu G, Orstavik D. Virulence factors of *Enterococcus faecalis*: Relationship to

endodontic disease. Crit Rev Oral Biol Med, 2004; 15: 308-20.

17. Koch S, Hufnagel M, Theilacker C, Huebner J. *Enterococcal* infections: Host response, therapeutic, and prophylactic possibilities. Vaccine, 2004; 22:822-30.
18. Mohamed JA, Singh KV, Huang W, Teng F and Murray BE. Influence of clinical origin and of various genes on biofilm formation by Enterococcus faecalis. In: Program Abstracts of the 43rd Annual Interscience Conference on Antimicrobial Agents and Chemotherapy, abstract B-821, Washington DC: ASM press, 2003; p 52.
19. Mohamed JA, Huang W, Nallapareddy SR, Teng & Murray BE. Influence of origin of isolates, especially endocarditis isolates, and various genes on biofilm formation by *Enterococcus faecalis*. Infect Immun, 2004; 72: 3658-3663.
20. Murray BE. The life and times of the *Enterococci*. Clin Microbiol Rev, 1990; 3: 46-65.
21. Sandoe JA, Witherden IR, Cove JH, Heritage J & Wilcox MH. Correlation between *enterococcal* biofilm formation in vitro and medical-device-related infection potential in vivo. J Med Microbiol, 2003; 52: 547-550.
22. Shankar V, Baghdayan AS, Huycke MM, Lindahi G, and Gilmore MS. Infection-derived *Enterococcus faecalis* strains are entiched in esp, a gene encoding a novel surface protein. Infect Immun, 1999; 67: 193-200.
23. Shepard BD, Gilmore MS. Differential expression of virulence-related genes in *Enterococcus faecalis* in response to biological cues in serum and urine. Infect Immun 2002; 70: 4344-52.
24. Toledo-Arana A, Valle J, Solano C, Arrizubieta MJ, Cucarella C, Lamata M, Amorena B, Leiva J, Penades JR and Lasa I. The *Enterococcal* Surface Protein, ESP, Is Involved in *Enterococcus faecalis* Biofilm Formation. Appl Environ Microbiol, 2001; 67(10): 4538-4545.
25. Woodford N, Morrison A. Johnson P, Briant V, George RC, Cookson B Application of DNA probes for rRNA and *vanA* genes to investigation of a nosocomial cluster of vancomycin-resistant *Enterococci.* J Clin Microbiol, 1993; 31: 653-658.