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ORIGINAL ARTICLE

Association of Virulence Genes of Enterobacteriaceae and Biofilm Formation in Urinary Tract Infection at Sohag University Hospital

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ABSTRACT

Background: *Enterobacteriaceae are group of multiple organisms that causing different human infections including urinary tract infections. They harbor multiple virulence factors e.g. fimbria and pili that play an important role in attachment of the organism to uroepithelium which is the first step to infection. Attachment also forms the early steps in biofilm formation that covers the organisms with a shield protecting them from host immune system and from chemotherapeutic agents.* ***Objectives****: Determine some virulence factors of enterobacteriaceae, biofilm production ability and antibiotic resistance pattern of these bacteria.* ***Methodology:*** *Two hundreds urine samples were collected from different departments at sohag university hospital from patients complaining of UTI symptoms, cultured on MacConkey agar. Bacterial count was done and count ≥ 10^5/ml was considered significant bacteruiria. Identification, antibiotic susptability were done using VITEK II automated identification system. Phenotypic detection of biofilm was done by tissue culture plate method, molecular detection of biofilm and virulence genes by polymerase chain reaction.* ***Results****: Out of the 200 urine samples; 104 samples were positive for enterobacteriaceae, 50% of the isolates were enterobacter speceies, 30.8% were e.coli, 17.3% were k.peumonia and 1.9% were citrobacter.100% of the isolates were resistant to ampicillin, 94.2% were resistance to ampicillin sulbactam, 82.7% were resistance to trimethoprime/sulfamethoxazole. Tissue culture plate detected 10/104(9.6%) as high biofilm producers, 42/104(40.4%) as moderate, 10/104(9.6%) as weak and 42/104(40.4%) as non biofilm producers.pcr detected fimH gene in 34/104(32.7%), papC gene in18/104(17.3%) and BssS gene in 66/104(63.5%).* ***Conclusion****: Enterobacteriaceae group is an important cause of serious infections and has a great ability to form biofilm that enables them to cause recurrent, persistent and resistant infections. Infection control measure should be applied sharply to prevent spread of these virulent organisms.*

INTRODUCTION

The family Enterobacteriaceae is composed of a large number of closely related bacteria species that inhabit the large bowel of man and animals, soil, water, and decaying matter. Because of their normal habitat in man, they have often been referred to as the "enteric bacilli". The organisms of this family are responsible for the majority of nosocomial infections, causing urinary tract and wound infections, pneumonia, meningitis and septicemia. Enterobacteriaceae has several virulence factors such as flagella, production of urease enzyme, fimbria and pili some strains contain capsule [1].

Biofilms are accumulations of small colonies of bacteria surrounded by an extracellular polysaccharide matrix in which cell aggregations adhere to various surfaces, including medical devices and injured tissues. A hallmark of enterobacteriaceae is the formation of biofilm, which facilitates the persistence of these pathogenic isolates in the urinary tract and interferes with bacterial eradication. Biofilm infections are difficult to eradicate with antimicrobial treatment, and in vitro susceptibility tests show resistance of biofilm cells to killing. It is clear that a large number of biofilm-microorganisms are highly resistant to antimicrobial agents [2.] Biofilm formation in enterobacteriaceae requires a set of gene expressions facilitating its initiation, attachment, and subsequent maturation. A variety of virulence factors are involved in biofilm formation in *E. coli*, including hemolysin, fimbriae, lipopolysaccharides (LPS), secreted proteins, capsules, and iron-acquisition systems, which allow attachment and bacterial colonization in the mucosal epithelial cells lining the urinary tract, invading and further forming intracellular biofilm-like pods in uroepithelial cells. Three main virulence determinants of enterobacteriaceae isolates are involved in biofilm formation: type 1 fimbriae (fim), coded by the fim gene

cluster: the P-fimbriae (pap), coded by the pap (pyelonephritis-associated pili) gene; and biofilm genes[3].

METHODOLOGY

This study was done at Sohag University Hospital from October 2016 to March 2017 at Microbiology and Immunology Department to study biofilm producing bacteria in cases of UTI and some of their virulence factors. The study was carried out after getting approval from the Ethical Committee; written consent from all patients included in the study was taken prior to initiation of the study. Two hundred urine samples were collected from different departments in the hospital from patients complaining of symptoms of urinary tract infection under complete aseptic conditions and were sent to the laboratory.

Isolation and Identification of Microorganisms:

Samples were cultured on MacConkey agar (Oxoid.UK) , Quantitative urine culture to determine colony forming unit was done as described by Forbs et al.,[4] , colony count $>10^5$ cfu/mL indicating UTI. Identification of bacteria was done using (Vitek II bioMérieux, France) using identification cards GN for Gram negative, AST for antibiotic sensitivity testing.

Quantitative method for biofilm detection by using tissue culture plate method: [5]

The optical density (O.D.) was detected by ELISA reader (STAT FAX2100) at 630 nm was recorded and the results were interpreted according to table (1). The experiment was performed in triplicate and mean value was calculated.

Table 1: Classification of bacterial adherence by microtitre plate method

Mean OD value	Biofilm formation
< 0.060	Non
0.060 - <0.124	Weak
0.124-0.240	Moderate
≥0.240	High

Molecular detection of biofilm, virulence genes:

-DNA was extracted by boiling method [6]:

After an overnight pure growth on MacConkey agar, 3-5 colonies were suspended in 100 µl of sterile distilled water, incubated at 100 ºC for 10 minutes and centrifuged at 15000 rpm for 2 minutes. The supernatant was used in the gene detection by PCR.

DNA amplification for detection of BssS, fimH and papC genes was done as mentioned by Hassan et al.[5]

After amplification, 10µl of the PCR mixture was analyzed by agarose gel electrophoresis (2% agarose stained with ethidium bromide). The Gene Ruler 100 bp DNA ladder (Jena bioscience) was used as a DNA size marker visualization of bands was done using DNA documentation system.

Statistical analysis:

Statistical analysis was done by using spss version 22; Chi-square ($\chi 2$) test was used for comparison regarding qualitative variables, a 0.05 level was chosen as a level of significance in all statistical tests used in the study.

Results:

This study was done at Microbiology and Immunology Department in the period from October 2016 to march 2017. 200 urine samples collected from different departments in the hospital were cultured and colony count was detected.

Identification of the organism and antibiotic sensitivity testing was done using VITEK II. 104 samples were positive with colony forming unit >105 cfu/mL, 60/104(57.7%) were female, 44/104 (42.3%).

Table 2: Frequency of isolated enterobacteriaceae.

Bacteria	Frequency
Citrobacter koseri	**2 (1.9%)**
E. coli	**32 (30.8%)**
Enterobacter aerogenes	**12 (11.5%)**
Enterobacter cloacae	**36 (34.6%)**
Enterobacter cloacae complex	**4 (3.8%)**
K.pneumonia	**18 (17.3%)**

Enterobacter cloacae was the most frequent isolated organism 36/104(34.6%).

Table 3: Antibiotic sensitivy pattern of enterobactericeae,

Antibiotics	Frequency	
Ampicillin	**No**	**%**
Resistant (%)	**104**	**(100%)**
Gentamycin(CN)		
Sensitive (%)	**82**	**(79%)**
Resistant (%)	**22**	**(21%)**
Ciprofloxacin		
Sensitive (%)	**80**	**(77%)**
Resistant (%)	**24**	**(23%)**
Tigecyclin		
Sensitive (%)	**94**	**(90.4%)**
Resistant (%)	**10**	**(9.6%)**
Moxifloxacin		
Sensitive (%)	**80**	**(77%)**
Resistant (%)	**24**	**(23%)**
Nitrofurantoin		
Sensitive (%)	**56**	**(53.9%)**
Resistant (%)	**20**	**(19.2%)**
Intermediate (%)	**24**	**(26.9%)**
Trimethoprim/Sulfamethoxazole		
Sensitive (%)	**18**	**(17.3%)**
Resistant (%)	**86**	**(82.7%)**
Cefazolin(CN)		
Sensitive (%)	**6**	**(5.7%)**
Resistant (%)	**98**	**(94.3%)**
Ceftriaxon		
Sensitive (%)	**28**	**(26.9%)**
Resistant (%)	**76**	**(73.1%)**
Cefepime		
Sensitive (%)	**66**	**(63.5%)**
Resistant (%)	**36**	**(34.6%)**
Intermediate (%)	**2**	**(1.9%)**
Aztreonam		
Sensitive (%)	**48**	**(46.2%)**
Resistant (%)	**32**	**(30.8%)**
Intermediate (%)	**24**	**(23%)**
Etrapenem		
Sensitive (%)	**88**	**(84.6%)**
Resistant (%)	**16**	**(15.4%)**
Imipenem		
Sensitive (%)	**86**	**(82.7%)**
Resistant (%)	**16**	**(15.4%)**
Intermediate (%)	**2**	**(1.9%)**
Meropenem		
Sensitive (%)	**88**	**(84.6%)**
Resistant (%)	**16**	**(15.4%)**
Amikacin		
Sensitive (%)	**102**	**(98.1%)**
Resistant (%)	**2**	**(1.9%)**
Tobramycin		
Sensitive (%)	**78**	**(75%)**
Resistant (%)	**24**	**(23.1%)**
Intermediate (%)	**2**	**(1.9%)**
Ampicillin Sulbactam		
Sensitive (%)	**4**	**(3.9%)**
Resistant (%)	**98**	**(94.2%)**
Intermediate (%)	**2**	**(1.9%)**

All the isolates were 100% resistant to ampicillin, 94.2% were resistance to ampicillin/sulbactam and cefazolin, 82.7% were resistance to Trimethoprim/Sulfamethoxazole.

Table 4: Quantitative detection of biofilm by tissue culture plate.

Bacteria	High	Moderate	Week	Non
Citrobacter koseri	**0 (0.0%)**	**0 (0%)**	**2 (100%)**	**0 (0%)**
E. coli	**2 (6.3%)**	**12 (37.5%)**	**0 (0%)**	**18(56.3)**
Enterobacter aerogenes	**2 (16.7%)**	**6 (50%)**	**4 (33.3%)**	**0 (0%)**
Enterobacter cloacae	**2 (5.6%)**	**8(22.2%)**	**4(11.1%)**	**22 (61.1%)**
Enterobacter cloacae complex	**0 (0%)**	**4 (100%)**	**0 (0%)**	**0 (0%)**
K.pneumonia	**4 (22.2%)**	**12 (66.7%)**	**0 (0%)**	**2 (11.1%)**

Table 4 shows that 10/104 (9.6%) were high biofilm producers, 42/104 (40.4%) were moderate, 10/104 (9.6%) were weak and 42/104(40.4%) were non biofilm producers.

Fig. 1: Tissue culture plate method.

Table 5: Molecular detection of fimH gene.

Bacteria	FimH gene	
	Positive(N=34)	Negative (N=70)
Citrobacter koseri	**0 (0.0%)**	**2 (100.0%)**
E. coli	**22 (68.7%)**	**10 (31.3%)**
Enterobacter aerogenes	**6 (50.0%)**	**6 (50.0%)**
Enterobacter cloacae	**2 (5.6%)**	**34 (94.4%)**
Enterobacter cloacae complex	**0 (0%)**	**4 (100%)**
K.pneumonia	**4 (22.2%)**	**14 (77.8%)**

E.coli was the most frequent organism harboring FimH gene (68.7%).

Table 6: Frequency of BssS gene in different bacteria.

Bacteria	BassS gene	
	Positive(N=66)	Negative (N=38)
Citrobacter koseri	**2 (100%)**	**0 (0.0%)**
E. coli	**30 (93.7%)**	**2 (6.3%)**
Enterobacter aerogenes	**8(66.7%)**	**4 (33.3%)**
Enterobacter cloacae	**14 (38.9%)**	**22 (61.1%)**
Enterobacter cloacae complex	**2 (50%)**	**2 (50%)**
K.pneumonia	**10 (55.6%)**	**8 (44.4%)**

Eschericia.coli was the most frequent organism harboring BssS gene 30 (93.7%).

Table 7: Frequency of PapC gene in different bacteria.

Bacteria	PapC gene	
	Positive(N=18)	Negative (N=86)
Citrobacter koseri	**0 (0%)**	**2 (100%)**
E. coli	**18 (56.3%)**	**14 (43.7%)**
Enterobacter aerogenes	**0 (0%)**	**12 (100%)**
Enterobacter cloacae	**0 (0%)**	**36(100%)**
Enterobacter cloacae complex	**0 (0%)**	**4 ((100%))**
K.pneumonia	**0 (0%)**	**18 ((100%)**

Table 7 shows that only 56.3% of *E.coli* contain the gene.

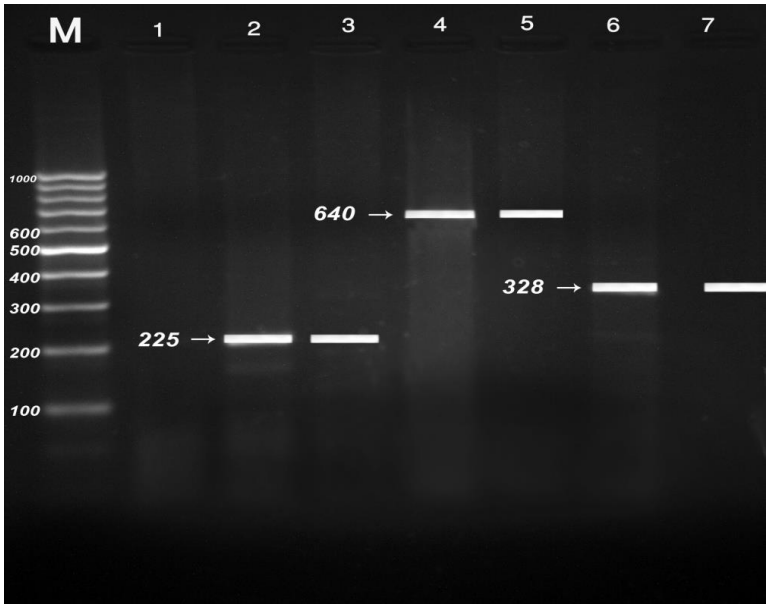

Fig. 3: Gel electrophoresis showing DNA ladder from 100-1000 bp in lane M.
-Lane 1: negative control
-Lane2, 3: Amplified product of BssS gene size 225 bp in *E.coli*.
-Lane 4, 5: Amplified products of Fim H gene size 640 bp in *Enterobacter cloacae*.
-Lane 6, 7: The amplified product of PapC gene size 328 bp in *E.coli*.

Table 8: Relation between the presence of biofilm gene (BssS) plus genes of adhesion (FimH, PapC) and degree of biofilm by TCP.

Genes	TCP test				P- value
	Non	Weak	Moderate	Strong	
Bss S gene	**18(52.9%)**	**4 (11.8%)**	**12(35.3%)**	**0 (0.0%)**	**0.004 ***
Fim H gene	**2(100%)**	**0 (0.0%)**	**0 (0.0%)**	**0 (0.0%)**	
Bss S gene + Fim H gene	**2(14.3%)**	**2 (14.3%)**	**2(14.3%)**	**8(57.1%)**	
Bss S gene + Fim H gene + Pap C gene	**4(22.2%)**	**0 (0.0 %)**	**12(66.7%)**	**2(11.1%)**	
Negative	**18 (50%)**	**0 (0.0 %)**	**18 (50%)**	**0 (0.0 %)**	

Table 8 shows that 35.3% of isolates containing BssS only were moderate biofilm, 57.1% of isolates containing FimH genes and BssS gene were strong biofilm forming but 66.7% of isolates containg 3 genes were moderate biofilm**.**

DISCUSSION

Urinary tract infection is a serious problem with increasing morbidity and mortality worldwide, multiple organisms are implicated in the pathogenesis of UTI especially enterobacteriaceae that normally habitat the large intestine of humans. Pathogenic strains posses special virulence factors that enable bacteria to cause diseases.

In our study 200 urine sample were collected, 104 strains were positive for enterobacteriaceae; 60/104 were isolated from females and 44/104 were isolated from males. Similar results were shown by kashef et al.[8]. Kamat US et al in their study noted females are more prone to develop UTIs, probably due to their anatomical physiological changes like short urethra, being near to the anus, dilatation of the urethra and the stasis urine during pregnancy[9].

The most frequent isolated organism was enterobacter spp. (34.6%) followed by *E.coli*(30.8%), *k.pneumonia* (17.3%) and citrobacter (1.9%). These finding are not similar to Hassan et al.[7] who reported that *E.coli* was the most frequent organism in his study, this may be due different sample size and difference in population characteristics in both studies. 100% of the isolates were resistant to ampicillin, 94.2% were resistance to ampicillin/sulbactam and cefazolin, 82.7% were resistance to Trimethoprim/Sulfamethoxazole, the most effective antibiotics against enterobacteriaceae are amikacin(98.1%), tigecyclin(90.4%), meropenem(84.6%) and imipinem(82.7%) similar to that found by kabir et al.[10].

In our study fimH gene was detected in 34/104(33%) of enterobacteriaceae bacteria, *E.coli* was the organism harbouring higher number of fimH 22/32 (69%), which is similar to the result of Elahe Tajbakhsh et al.[11] who detected 75% of fimH gene in isolates of *E.coli* and less to that of Plinio et al.[12] who detected 93.3% of fimH gene in *E.coli.*

The fimH gene, which encodes type 1 fimbriae, is thought to be an important factor enhancing adhesion, invasion and biofilm growth [13], Strains expressing this gene in addition to biofilm gene show more ability to form biofilm in vitro with a significant difference between strong, weak biofilm producing bacteria (p value 0.04). This result is different from that of plinio et al.[12] who reported that no significant difference was seen between strong and weak biofilm producers.

PapC gene was detected in 18/104(17%) all the 18 positive were *E.coli* similar to the findings detected by G L Paniagua-Contreras et al.[14] who detected 65/194(33%) of papC gene in uropathogenic *E.coli*.

By correlating the degree of biofilm production by tissue culture plate with the presence of the virulence genes fimH, papC genes we found an association between strong biofilm formation and the presence of these genes. Biofilm production was significantly associated with fimH, papC virulence genes ($P < 0.05$) as reported by Elahe Tajbakhsh et al [11]. Recently it has been reported that a close association between a higher potential to form biofilm and presence of some urovirulence genes [15, 16] Consistently, the most important correlation detected in our work was that papC, fimH were more prevalent in the strong biofilm producers this result is similar to that reported by plinio et al.[12]. Tissue culture plate detected 62 biofilm positive strains. 42 isolates are containing BssS gene and 20 negative for the gene with a sensitivity of 63.6% and a significant p value 0.05 this result is lower than that reported by Hassan et al.[7] who detected 100% correlation between phenotypic and genotypic methods in biofilm detection.

CONCLUSION

Urinary tract infection is a great problem caused by multiple organism especially enterobacteriaceae. They have a capacity to form biofilm in the urinary tract that makes treatment difficult to be achieved. Prevention of infection is the corner stone could be done by application of infection control policy and wise description of antibiotics to prevent spread of resistant strains.

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