Egyptian Journal of Medical Microbiology Volume 26 / No.2 / April 2017 37-45

ORIGINAL ARTICLE

Biofilm Formation and Antimicrobial Resistance Pattern of Uropathogenic *E. coli*: Comparison of Phenotypic and Molecular Methods

Amal F. Makled*, Eman H. Salem and Asmaa M. Elbrolosy
Department of Medical Microbiology and Immunology, Faculty of Medicine, Menoufia University, Egypt

Key words:
Uropathogenic Escherichia coli, biofilm, modified congo-red agar, microtitre plate pap, fimH, sfa

****Corresponding Author:***
Amal Makled
Associate Professor of Microbiology & Immunology
Faculty of Medicine,
Menoufiya and KAU
E-mail:
AmalMakled@msn.com;
Tel.: 01002810447

ABSTRACT

Background: *Biofilm structure is considered an important virulence factor and is associated with UPEC that is formed within the bladder and act as a reservoir for recurrent or/and persistent infection. Biofilm is formed from multiple adherent colonies surrounded by polysaccharide matrix that protects bacterial pathogens from an innate immune response and have major role in antibiotic resistance.* ***Objectives:*** *to determine biofilm production in UPEC phenotypically and genotypically by detection of the genes responsible for biofilm production (pap, fimH and sfa) and to assess their correlation with multi-drug resistance.* ***Methodology:*** *A total 278 catheterized and non-catheterized urine samples from clinically suspected UTI patients were processed by standard microbiological procedures. One hundred E. coli were isolated and analyzed for the production of biofilm by modified congo-red agar (MCRA) and microtitre plate (MTP) methods. Subsequently, the antimicrobial susceptibility test was performed by Kirby Bauer-disk diffusion method for both the biofilm and non-biofilm-producing E. coli strains. The presence of pap, fimH and sfa virulence genes was examined by conventional multiplex PCR assay.* ***Results:*** *One hundred UPEC were isolated from 278 catheterized and non-catheterized urine samples (35.97 %). A positive biofilm phenotype was detected in 75% and 68% on MCRA and MTP respectively. UPEC isolates in catheter-associated infection produced biofilm by MTP (73.0%) and MCRA (81.1%) methods. E. coli strains forming biofilm had significantly higher resistance to antibiotics than non-biofilm producer strains regarding tetracycline, cefotaxime, ciprofloxacin and amikacin. The frequency of detection of the virulence genes (fimH, Pap and sfa) among biofilm producing UPEC were 89.7 % vs. 81.3 %, 80.9 % vs. 73.3 % and 67.6 % vs. 61. 3 % as detected by MTP vs. MCRA respectively.* ***Conclusion:*** *Biofilm formation was more common in catheterized patients and there was an association between biofilm production and antimicrobial resistance. PCR was more reliable for the detection of biofilm forming UPEC as compared to MCRA and MTP methods. The adhesion gene fimH was the most common among those uropathogenic E. coli strains.*

INTRODUCTION

Urinary tract infections (UTIs) represent the most frequent bacterial infections encountered in both hospital and community settings. About 150 million people all over the world get infected with UTIs every year [1].

Although *E. coli* strains inhabit human bowel as part of normal flora, uropathogenic *E. coli* (UPEC) strains are capable of causing more than 80% of a significant UTIs that ranged from cystitis up to life-threatening sepsis. Many urovirulence mechanisms, including adhesins, α-hemolysin (Hly), cytotoxic necrotizing factor, fimbriae, aerobactin-mediated iron uptake, K1 capsular polysaccharide, and biofilm formation ultimately lead to tissue damage [2].

UPEC tends to form microcolonies in the mucosal lining of urinary bladder (biofilm) which make the organism more resistant to the host immune response and more virulent. Moreover, evolution of antibacterial drug resistance by enclosing them in an extracellular biochemical matrix occurs[3]. In UPEC, the initial process of attachment and initiation of infection is mediated by several adherence factors: 1) Type 1 fimbriae (*fim*H), coded by the *fim* H gene cluster, mediate UPEC attachment to the bladder epithelium by binding to mannose-containing glycoproteins and promote the early stages of biofilm formation on both biotic and abiotic surfaces. 2) P-fimbriae (*pap*), coded by the *pap* (pyelonephritis-associated pili) gene, which plays a critical role in colonization in kidneys [4,5]. 3) Group II capsule synthesis (*sfa)* that are considered to be the most important virulence factors of UPEC is involved in the induction of UTI and formation of biofilm [6].

Remarkably, UPEC is capable of forming biofilms on the abiotic surfaces of indwelling medical devices such as catheters, which lead to ‘‘Catheter-Associated Urinary Tract Infection’’ (CAUTI) in clinics. CAUTI is especially fatal in immunocompromised, debilitated and diabetic patients. Therefore, biofilm formation in UPEC is important for causing persistent colonization in bladder and kidneys, and in hospital settings [7].

Biofilms have major role in antibiotic resistance. They play a role in trapping of antibiotics, impairment of drugs and plasmid exchange [1]. Therefore, biofilms can lead to persistent infections of many pathogenic microbes. Moreover, they are important factors in nosocomial infections and medical conditions including indwelling medical device, dental plaque, upper respiratory tract infection and urogenital infection [8]. In this study, we aimed to detected biofilm-producing UPEC strains phenotypically and genotypically and assessed the relation between ability of biofilm production and antimicrobial drug resistance.

METHDOLOGY

1. Collection of samples:

This study was conducted at the Microbiology and Immunology Department, Faculty of Medicine, Menoufia University Hospitals during the period from March 2015 to December 2016. Clinically suspected UTI patients (n=278), including catheterized (206) and non-catheterized (72) were studied and those receiving antibiotics were excluded. Urine samples were aseptically obtained. All urine sampled were counted to detect significant bacteriuria, cultured on blood agar, cysteine lactose electrolyte deficient (CLED) and MacConkey’s agar plates which were incubated at 37°C for 24 hours. The grown *E. coli* isolates were identified by colonial morphology, Gram staining, standard biochemical tests and API-20E System (Oxoid, England). Confirmed *E. coli* isolates were suspended in nutrient broth supplemented with 16% glycerol and stored frozen at -80^{0}C. They were tested for antimicrobial susceptibility and biofilm production[9].

2. Detection of biofilm formation and antibiotic susceptibility patterns:

All isolated *E. coli* strains were analyzed for the production of biofilm and antimicrobial susceptibility pattern.

a- Biofilm formation assay was determined by modified congo-red agar method (MCRA) and microtitre plate methods (MTP).

- *Modified Congo red Agar method (MCRA): E. coli* strains were inoculated on blood base agar-2 (BAB-2) (40gms/L BAB-2, 10 gms/L glucose and 1000 ml water), with 0.4 gms/L congo red stain and incubated at 37°C under aerobic conditions for 24 and 48 h. The positive *E. coli* isolate was indicated by black and dry crystalline colonies. Weak biofilm producers usually remained pink with the darkness at the center of colonies. Intermediate results were exhibited by the darkness of the colonies with the absence of dry crystalline colonies [10].
- *Microtitre plate (MTP) method: E. coli* strains grown overnight on trypticase soy agar (TSA) plates were subcultured in 10 ml of trypticase soy broth (TSB) with 5 % glucose, and were incubated at 37ºC for 24 h. The cultures were diluted and adjusted to 0.5 McFarland standard density. Individual wells of sterile 96 well-flat bottoms were inoculated with 200 µl of the diluted cultures. Negative control wells contained inoculated with sterile broth were included. The plates were incubated at 37ºC for 5 days. After incubation, contents of each well were removed by gentle tapping. The wells were washed with 200 µL of PBS (pH 7.4) four times to remove the planktonic bacteria. Biofilm formed by bacteria adherent to the wells were fixed by 300 µL of absolute methanol for 10 minutes and stained by 200 µL of crystal violet (2 %). The stain was solubilised by 160 µL of 33 % glacial acetic acid for 20 minutes and the optical density was measured with an ELISA reader at a wavelength of 570 nm. The experiment was performed in triplicate. The interpretation of biofilm production was done according to the criteria of Stepanovic et al [11].

b-Antimicrobial susceptibility test was done for all *E. coli* isolates by Kirby-Bauer disk diffusion method against different antimicrobial agents (Oxoid) as recommended by CLSI, 2015; ampicillin (10 µg), amikacin (30 µg), gentamycin (10 µg), cefotaxime (30 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), norfloxacin (10 µg), tetracycline (30 µg), nitrofurantoin (300 µg) and imepinum (10 µg) [12].

3. Detection of biofilm-associated genes:

- *DNA extraction:* Total genomic DNA was extracted from all preserved *E. coli* isolates using a DNA extraction kit (Sigma, USA) according to the Manufacturer’s instructions.
- *Conventional multiplex PCR reaction was performed to detect pap, fimH and sfa genes:* Specific primers for these genes were used for PCR amplification (Table 1).

a- Amplification of *pap* and *sfa* genes was done by the reaction mixtures containing 5 µL PCR buffer 10X, 1.25 mM Mgcl2, 150 µM dNTP (Fermentas), 1 µM of each primers F & R, 1.2 U Taq DNA polymerase (Fermentas) and 3 µL DNA template were incubated at 94°C for 1 min, followed by 30 cycles of 60 sec. at 94°C, 30 sec. at 63°C, 90 sec. at 72°C, and a final extension for 5 min at 72°C[13].

b- Amplification of *fim*H gene was done by the reaction mixtures containing 5 µL PCR buffer 10X, 2 mM $MgCl_2$, 200µM dNTP (Fermentas),

0.4 µM of each primer F & R, 3 U Taq DNA polymerase (Fermentas) and 3 µL DNA template were incubated at 94°C for 3 min, followed by 40 cycles of 60 sec. at 94°C, 70 sec. at 58°C, 70 sec. at 72°C, and a final extension for 6 min at 72°C[13]. The PCR programs were performed in a thermal cycler (Biometra-Germany).The amplified products were visualized on 1.5% agarose gel stained with ethidium bromide (Sigma, USA). A DNA ladder (100-1000bp) (Fermentas, Germany) was used to estimate allele sizes in base pairs (bp) [8].

Table 1: The oligonucleotide primers used for amplification of virulence genes of *E. coli* isolates using PCR assays [13,8].

Gene	*Primer name*	*Primer sequence (5'-3)*	*Size of product (bp)*
pap	*For*	GCAACAGCAACGCTGGTTGCATCAT	336
	Rev	AGAGAGAGCCACTCTTATACGGACA	
Sfa	*For*	CTCCGGAGAACTGGGTGCATCTTAC	410
	Rev	CGGAGGAGTAATTACAAACCTGGCA	
*fim*H	*For*	GAGAAGAGGTTTGATTTAACTTATTG	559
	Rev	AGAGCCGCTGTAGAACTGAGG	

4. Statistical analysis:

It was performed using a Statistical Package for Social Sciences (SPSS) version 22 (SPSS Inc., Chicago, USA). Chi-square ($\chi 2$) and Z tests were used for testing the difference in two proportions. EpiCalc 2000 was used to determine the sensitivity, specificity, positive and negative predictive values, and accuracy. Statistical significance was set at p value <0.05.

RESULTS

In this study, one hundred UPEC were isolated from 278 urine samples (35.97 %). 75 out of them (75%) displayed a positive biofilm phenotype under the optimized conditions on MCRA. While in MTP method, the positive biofilm phenotype strains were classified as strong (33 %), moderate (23 %) and weak (12%). About 25% and 32% of UPEC strains were non biofilm producers by MCRA and MTP methods respectively (table 2). Among 74 catheter-associated UTIs, 54 (73.0%) and 60 (81.1%) UPEC isolates produced biofilm by MTP method and MCRA respectively Catheterized-patients had significantly higher percentage of biofilm production as compared to non-catheterized patients (Table 3). Antibiotic susceptibility pattern was studied for all UPEC isolates. The multi-drug resistant patterns of the biofilm-producing and non-producing UPEC were shown in Table (4). All the biofilm-forming strains were markedly resistant to ampicillin and ceftriaxone (100 %), followed by tetracycline and ciprofloxacin (88.2%), cefotaxime (82.4%) and norfloxacin (79.4 %). UPEC strains forming biofilm were highly resistant to antibiotics as compared to non-biofilm producing strains regarding tetracycline, cefotaxime, ciprofloxacin and amikacin ($p<0.001$). Polymerase chain reaction showed that the virulence genes (*fim* H, *Pap* and *sfa*) among biofilm-producing UPEC by MTP and MCRA were 89.70% *vs.* 81.30%, 80.90% *vs.* 73.30% and 67.60% *vs.* 61.30% respectively (Fig. 1). Among the high biofilm producing UPEC isolates, *fim*H gene has the highest prevalence (93.9%) followed by *pap* and *sfa* genes (87.9%) and there was a significant association between biofilm production and presence of *fimH*, *pap* and *sfa* genes as shown in Table (5). Sensitivity, specificity, PPV, NPP and accuracy of MCRA *vs.* MTP methods in relation to PCR for detection of biofilm among UPEC isolates were 76.3% vs76.3%, 30% *vs.* 65%, 81.3% *vs.* 89.7%, 24% *vs.* 40.6% and 67% *vs.* 74% respectively (Table 6).

Table 2: Number and percent of biofilm formation in UPEC isolates by MCRA method and MTP test.

Biofilm formation in* E. coli *isolates (n=100)					
By MCRA		**By MTP**			
Positive	**Negative**	**Positive**			**Negative**
No. (%)	No. (%)	No. (%)			No. (%)
75 (75.0)	25 (25.0)	68 (68.0)			32 (32.0)
		Strong	**Moderate**	**Weak**	
		No. (%)	**No. (%)**	**No. (%)**	
		33 (33.0)	23 (23.0)	12 (12.0)	

Table 3: Biofilm-production in catheterized and non-catheterized patients.

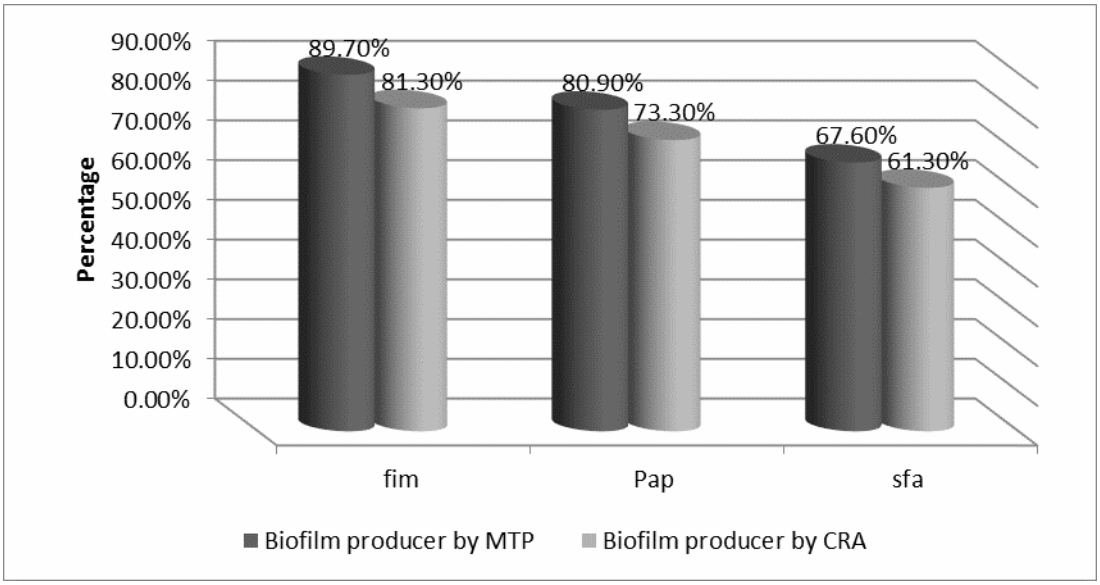
Patients	E. coli *isolates*	*MTP plate*		*MCRA*		χ^2 *test*	*p value*
		Biofilm producers	Non- biofilm producers	Biofilm-producers	Non -biofilm producers		
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)		
- Catheterized (n=206)	74 (35.9)	54 (73.0)	20 (27.0)	60 (81.1)	14 (18.9)	8.85	0.03*
- Non catheterized (n=72)	26 (36.1)	14 (53.9)	12 (46.1)	15 (57.7)	11 (42.3)		

*significant difference

Table 4: Antibiotic resistance pattern of the biofilm-producing and non-producing UPEC as determined by MTP method.

Resistance to antimicrobial agent	*MTP method*				*Z test*	*P value*
	Biofilm producers (n=68)		Non biofilm producers (n=32)			
	No.	(%)	No.	(%)		
Ampicillin (AM)	68	(100.0)	32	(100.0)	-	-
Tetracycline (TE)	60	(88.2)	12	(37.5)	5.03	<0.001**
Cefotaxime (CF)	56	(82.4)	15	(46.9)	3.41	<0.001**
Ciprofloxacin (CP)	60	(88.2)	17	(53.1)	3.63	<0.001**
Norfloxacin (NOR)	54	(79.4)	21	(65.6)	1.24	0.22
Ceftriaxone (CRO)	68	(100.0)	32	(100.0)	-	-
Amikacin (AN)	42	(61.8)	11	(34.4)	2.35	0.02*
Imipenem (IMP)	25	(36.8)	5	(15.6)	1.92	0.06
Gentamicin (GM)	15	(22.1)	11	(34.4)	1.06	0.29
Nitrofurantoin (FM)	11	(16.2)	1	(3.1)	1.55	0.12

*significant difference **highly significant difference



Graph 1: Virulence genes (*fim* H, *Pap* and *sfa*) among biofilm-producing UPEC by MTP and MCRA. Polymerase chain reaction was used to detect the virulence genes (*fim H*, *Pap* and *sfa*) among biofilm-producing UPEC strains as determined by MTP and MCRA.

Table 5: Number and percent of presence of virulence genes in relation to biofilm production as determined by UPEC by MTP

Virulence gene	*UPEC* E. coli *(100)*				χ^2 *test*	*P value*
	High biofilm producers (n=33)	Moderate biofilm producers (n=23)	Weak biofilm producers (n=12)	Non biofilm producers (n=32)		
	No. (%)	No. (%)	No. (%)	No. (%)		
fim						
Positive	31 (93.9)	21 (91.3)	9 (75.0)	19 (59.4)	14.54	0.002*
Negative	2 (6.1)	2 (8.7)	3 (25.0)	13 (40.6)		
Pap						
Positive	29 (87.9)	18 (78.3)	8 (66.7)	18 (56.3)	8.83	0.03*
Negative	4 (12.1)	5 (21.7)	4 (33.3)	14 (43.7)		
sfa						
Positive	29 (87.9)	12 (52.2)	5 (41.7)	18 (56.3)	12.99	0.005*
Negative	4 (12.1)	11 (47.8)	7 (58.3)	14 (43.7)		

*Significant difference

Table 6: Sensitivity, specificity, PPV, NPP and accuracy of phenotypic methods in relation to PCR for detection of biofilm among UPEC isolates

Methods		*PCR*		*Sensitivity*	*Specificity*	*PPV*	*NPP*	*Accuracy*
		Positive (n=80)	Negative (n=20)					
	Positive (n= 75)	61	14					
MCRA	**Negative (n=25)**	19	6	76.3%	30%	81.3%	24%	67%
	Positive (n= 68)	61	7					
MTP	**Negative (n=32)**	19	13	76.3%	65%	89.7%	40.6%	74%

PPV =positive predictive value NPV =negative predictive value

Sensitivity, specificity, PPV, NPP and accuracy of MCRA *and* MTP methods were determined in relation to PCR as a golden test for detection of biofilm production among UPEC isolates.

Fig. 1: Congo red agar plate showing biofilm formation. A) Red colored colonies-negative for biofilm; B) Dry crystalline colonies-positive for biofilm

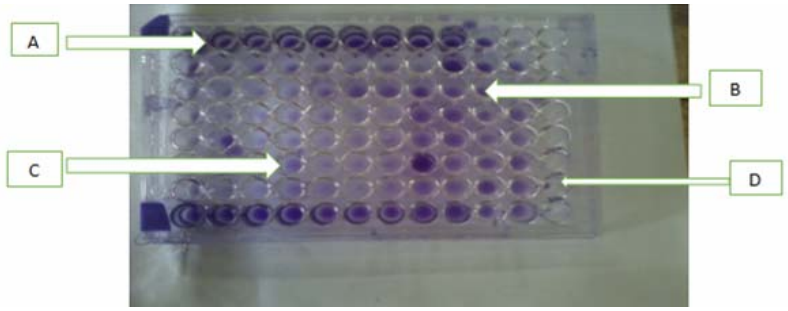


Fig. 2: Biofilm by microtitre plate. A) Strong biofilm producer; B) Moderate biofilm roducer; C) Week biofilm producer; D) Negative biofilm producer

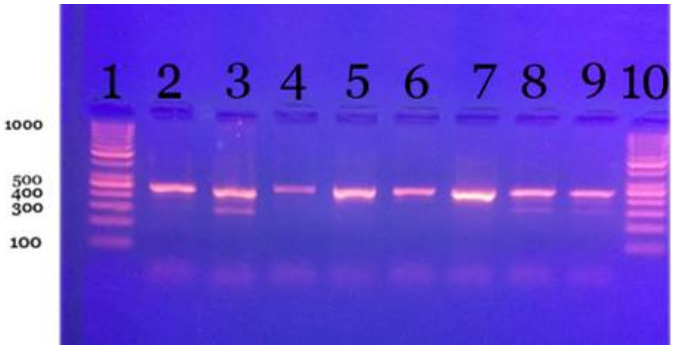


Fig. 3: Agarose gel electrophoresis for the multiplex PCR amplified products of *E .coli pap* and *sfa* genes. Lane 1 and lane 10: DNA molecular size marker (1000 bp). Lanes 2, 3, 4, 5, 6, 7, 8 and 9 represent positive *pap* gene (336 bp). Lane 3 and 8 represent positive *sfa* gene (410 bp)

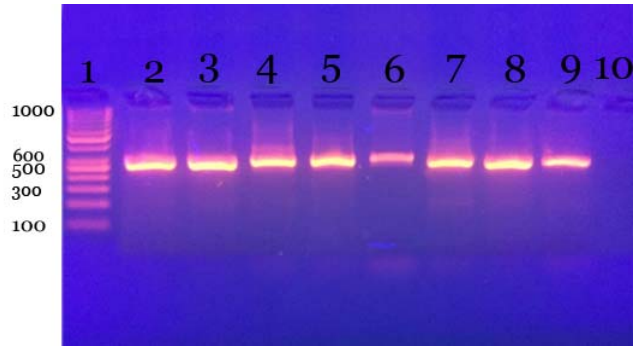


Fig. 4: Agarose gel electrophoresis for the PCR amplified products of *E.coli fim* gene. Lane 1: DNA molecular size marker (1000 bp). Lanes 2, 3, 4, 5, 6, 7, 8 and 9 represent positive *fim* gene (559 bp). Lane 10 represents negative sample.

DISCUSSION

Escherichia coli is the major pathogen associated with UTIs in humans. The virulence factors of the infecting strains and the susceptibility of the host, especially if there is an associated urological anomaly, are the leading causes for infections [14]. The higher ability of the UPEC to form biofilm makes the treatment more difficult, and increasing the mortality and severity of the infections [15].

In this study, 100 UPEC were isolated from 278 catheterized and non-catheterized urine samples (35.97%). Nearer results were reported by Tadepalli et al[16] who isolated 137 UPEC from 520 urine samples (26.3%), while higher rates were reported by Ponnusamy et al[17] (60.24%) and Gosh et al [1] (50%). Difference in the results may be due to regional differences in hygiene status and variable resistance to antibiotics. The targeted community in this study was selected from one specific geographical area. This may explain the difference in results compared to other studies [5].

The ability of bacteria to form biofilms on medical devices, e.g. catheters, is believed to be a major role in the development of nosocomial infections, including catheter-associated urinary tract infections [18]. This fact was noted in this study where, 74 and 26 UPEC were isolated from catheterized and non-catheterized patients respectively with statistically significant difference (p value <0.05). A similar result was reported by Ghosh et al.[1] who found that *E. coli* isolated from catheterized patients were higher than those from non-catheterized patients.

Among the virulence factors, adhesion of *E. coli* to the uroepithelium is a basic factor that protects the bacteria from urinary discharge and promotes their ability to multiply and invade renal tissue[19]. In the present study, 75% of UPEC displayed a positive biofilm phenotype under the optimized conditions on MCRA. While in MTP method, only 68% of UPEC displayed a positive biofilm phenotype (33% strong, 23% moderate and 12% weak). On the other hand, 25% and 32% of UPEC strains were non-biofilm producers by MCRA and MTP methods respectively. Tabasia et al[20] detected biofilm formation in 85.3% of UPEC

isolates and classified them into four groups, strong biofilm producers (17.3%), moderate biofilm producers (18.6%), weakly biofilm producers (49.4%), and non-biofilm producers (14.7%). Tadepalli et al [16] found that 48 (35%) were biofilm producers and 89 (65%) were biofilm non- producers by MTP method. Tajbakhsh et al [21] detect biofilm in 61.53 % of the *E. coli* examined by MCRA. Higher results were reported by Fattahi et al [5] who detected biofilm in 100% of isolates (48.6% strong, 11.4% moderate and 40% weak biofilm producers) by MTP method. Also, Ponnusamy et al [17] detected biofilm in 100% of isolates under the optimized conditions on MCRA. Those authors classified their isolates as highly positive (23%), moderate positive (37%) and weakly positive (40%). On the other hand, the biofilm positive phenotype strains were also classified as highly positive (6%), moderate positive (80%) and weakly positive (14%) using the MTP method.

In this study, the majority of biofilm producing UPEC were isolated from catheterized patients (73.0% and 81.1% by MTP and MCRA respectively). SarojGolia et al [22] found that 89.5% of biofilm-producing bacteria were isolated form catheterized patients. Also, Donlan and Costerton[23] reported an association between biofilm-production and urinary catheters.

Antibiotic susceptibility pattern for all UPEC isolates was studied in our study. All the biofilm-forming UPEC strains were resistant to ampicillin and ceftriaxone (100 %). UPEC strains forming biofilm were highly resistant to tetracycline (88.2%), ciprofloxacin (88.2%) cefotaxime (82.4%), and amikacin (61.8%) as compared to non-biofilm producing strains with a statically significant difference ($p<0.001$). Similar findings were reported by Asadi et al [24] and Tabasia et al [20]. These results can be explained by increased consumption of these antibiotics empirically, self-medication, dissemination of resistant strains in hospital settings and using different combinations of antibiotics. These factors may result in varying degrees of resistance among the biofilm-producing uropathogenic *E.coli*[25]**.** Ponnusamy et al [17] demonstrated that 100 %, 86%, 84%, 83%, 75% and 70% of biofilm-producing *E. coli* were resistant to chloramphenicol and amoxicillin-clavulanate, gentamicin and cefotaxime, ceftazidime, co-trimoxazole and piperacillin/tazobactam, tetracycline and amikacin respectively. Tabasia et al [20] noted that among the antibiotics tested, ampicillin resistance prevalence was the highest (77.6%), followed by tetracycline (60.3%), amoxicillin (59%), cotrimoxazole (58.3%), and piperacillin (55.8%). Tadepalli et al [16] found that antibiotic resistance of biofilm producing *E. coli* was significantly higher than that of biofilm-nonproducing *E. coli* ($p<0.05$) except in case of amikacin, nitrofurantoin and cephalexin

In the current study, 83.8%, 77.9% and 63.2% of the biofilm-forming UPEC strains were sensitive to nitrofurantoin, gentamicin and imipenem respectively. Zaki and Elewa [28] detected the highest susceptibility rate to amikacin (79.1%) and Cefazolin (68.1%), and Gosh et al [1] detected that 94% of nitrofurantoin appeared to be useful and could be considered as a choice for treating uncomplicated lower urinary tract infections, while aminoglycosides appeared to be the best choice for complicated infections.

Microorganisms growing in a biofilm are intrinsically resistant to many antibiotics increasing the antibiotic resistance up to 1000 folds and high antimicrobial concentrations are required to inactivate organisms growing in a biofilm [26]. This may be attributed to the insufficient concentrations of antibiotics reaching some areas of the biofilms and to the metabolic inactivity of the bacteria located at the base of the biofilms [27].

This study showed that the virulence genes (*fim H*, *Pap* and *sfa*) among the biofilm-producing UPEC as detected by MTP and MCRA were 89.7 % *vs.* 81.3 %, 80.9 % *vs.* 73.3 % and 67.6% *vs.* 61.3 % respectively. Zaki and Elewa [28] detected a higher frequency of adhesion genes [*fimH* (65.9%), *pap* (63.7%) and *sfa* (56%)] as compared with the rest of the studied genes. Among the high biofilm-producing UPEC isolates, *fimH* gene had the highest prevalence (93.9%) followed by and *pap* and *sfa* genes (87.9%). Similar studies had established that *fimH* was the most frequent in isolates from a variety of forms of UTI [29-31]. In the current study, biofilm production was significantly associated with *fimH*, *pap* and *sfa* virulence genes ($p<0.05$). Similar results were reported by Tajbakhsh et al [21] who found that biofilm production was significantly associated with *fim*H, *pap*, *afa* and *sfa* virulence genes ($p<0.05$), Fattahi et al [5] noted high prevalence of *fimA* (94%) and *papC* (43%) genes among their isolates.

The correlation between the virulence factors gene (*fim* H, *Pap* and *sfa*) and biofilm-positive strains was examined in our study. UPEC biofilm-producing strains were 81.3% as determined by MCRA and 89.7% as determined by MTP. This result may indicate that MTP method was better for screening of biofilm formation as virulence marker in drug-resistant UPEC isolates than MCRA. Tajbakhsh et al [21] detected 67 positive biofilm genes among 80 isolates forming biofilm by CRA (83.75%). These results highlight a crucial role of these virulence genes in *E. coli.* Similarly, Fattahi et al[5] demonstrated a significant association between virulence gene expression involved in bacterial attachment and biofilm formation, indicating that biofilm-forming bacteria are more pathogenic than the planktonic form in urinary tract infections. Moreover, biofilm formation may cause increased rate of bacterial virulence as well as the severity of disease, making biofilm-based UTI very difficult to cure. We determined the sensitivity, specificity, PPV, NPP and accuracy of MCRA *vs.* MTP methods in relation to PCR as a standard method for detection of biofilms among UPEC

isolates. They were 76.3% *vs*76.3%, 30% *vs.* 65%, 81.3% *vs.* 89.7%, 24% *vs.* 40.6% and 67% *vs.* 74% respectively. Similar results were reported by Hassan et al [32]; as the sensitivity, specificity, PPV, NPP and accuracy of MCRA vs. MTP methods for detection of biofilm were 11% *vs*. 73%, 92% *vs.* 92.5%, 73% *vs.* 94%, 37% *vs.* 66% and 41% *vs.* 80% respectively, and de Castro Melo et al [33] who detect sensitivity, specificity, PPV and NPP of MCRA *vs.* MTP methods in relation to PCR for detection of biofilm as follows; 89% *vs.* 100%, 100% *vs.* 25%, 100% *vs.* 96% and 28.6% *vs.* 100% respectively.

CONCLUSIONS & RECOMMENDATION

In conclusion, UTIs, caused by biofilm producing *E. coli*, may promote colonization and increased the incidence rate of UTI's. Biofilm formation was more common in catheterized patients and there was an association between biofilm production and antimicrobial resistance. PCR was more reliable method for detection of biofilm-forming UPEC as compared to MCRA and MTP methods. The adhesion gene *fim*H was the most common among those uropathogenic *E. coli* strains. However, further studies are required to identify the relation between other UPEC virulence factors responsible for UTIs and biofilm formation to consider possible prevention measures and means. Nitrofurantoin, gentamycin and imipenem may be more valuable for treatment of UTI patients. More restricted antibiotic policy and antibiotic-coated catheters and standard guidelines on care of catheter to reduce occurrence, chronicity and recurrence of UTIs.

REFERENCES

1. Ghosh P, Saha S, Mitra G, Gupta I, Nandi P, Kar M, Banerjee D. (2016): Evaluation of different virulence factors and antibiogram of uropathogenic *Escherichia coli* (UPECe) isolated in a tertiary care centre. IJMDS, 5 (1). www.ijmds.org.
2. Slavchev G, Pisareva E and Markova N. (2009): Virulence of uropathogenic *Escherichia coli.* J Cult Collect, (6):3-9.
3. Singh SK, Seema K and Gupta M Detection of AmpC-lactamase and adherence factors immuropathogenic *Escherichia* coli isolated from aged patients. Microbial Pathogenesis; 2016; 100: 293-298
4. Melican K, Sandoval RM, Kader A, Josefsson L, Tanner GA, Molitoris B and Richter-Dahlfors A. (2011). Uropathogenic *Escherichia coli* P and Type 1 fimbriae act in synergy in a living host to facilitate renal colonization leading to nephron obstruction. PLoS Pathog; 7: e1001298.
5. Fattahi S, Kafil HS, Nahai MR, Asgharzadeh M, Nori R, Aghazadeh M. Relationship of biofilm formation and different virulence genes in uropathogenic *Escherichia coli* isolates from Northwest Iran. GMS Hygiene Infect Cont. 2015, 10, ISSN 2196-5226
6. Bergsten G, Wullta B, Svanborg C. *Escherichia coli*, fimbriae, bacterial persistence and host response induction in the human urinary tract. Int J Med Microbiol; 2005, 295: 487-502.
7. Chenoweth CE and Saint S. Urinary tract infections. Infect Dis Clin North Am; 2011, 25:103-117.
8. Tajbakhsh E, Ahmadi P, Abedpour-Dehkordi E, Arbab-Soleimani N and Khamesipour F. Biofilm formation, antimicrobial susceptibility, serogroups and virulence genes of uropathogenic *E. coli* isolated from clinical samples in Iran. Antimicrob Resist Infection Contr; 2016, 5:11
9. Cheesbrough M, District laboratory practice in tropical countries. 2000, Part 2. Chapter 7. Page 105-115. Cambridge University press, UK.
10. Mariana NS, Salman SA, Neela V and Zamberi S. Evaluation of modified Congo red agar for detection of biofilm produced by clinical isolates of methicillin–resistant *S. aureus*. Afr J Microbiol Res; 2009, 3(6): 330-338.
11. Stepanović S, Vuković D and Hola V. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci," Acta Pathologica Microbiologica Scandinavica; 2007, 115(8):891-899
12. Clinical and Laboratory Standard Institute. Performance standards for antimicrobial susceptibility testing: Twentieth Informational Supplement; 2015, CLSI document Ml00-S25.Wayne, PA.
13. Momtaz H, Karimian A, Madani M, Dehkordi F, Ranjbar R, Sarshar M Soud N.Urophathogenic *Escherichia coli* in Iran serogroup distributions, virulence factors and antimicrobial resistance properties. Ann Clin Microbiol Antimicrob; 2013, 12:1-8
14. Sanchez CJ Jr1, Mende K, Beckius ML, Akers KS, Romano DR, Wenke JC, Murray CK. Biofilm formation by clinical isolates and the implications in chronic infections. BMC Infect Dis; 2013, 29 (13):47.
15. Subramanian P, Umadevi S, Kumar S and Stephen S. Determination of correlation between biofilm and extended spectrum β lactamases producers of Enterobacteriaceae. SchoRes J.; 2012, 2:2-6.
16. Tadepalli S, Prudhivi S, Babu Myneni R and Rao S. Biofilm formation in uropathogenic *Escherichia coli* isolates and its association with extended

spectrum betalactamase production and drug resistance. Saudi J. Pathol. Microbiol; 2016, 1:260-64

17. Ponnusamy P, Natarajan V and Sevanan M.(2012). In vitro biofilm formation by uropathogenic *Escherichia coli* and their antimicrobial susceptibility pattern. Asian Pacific Trop Med. Mar;5(3):210-213
18. Schroll C, Barken KB, Krogfelt KA, Struve C. Role of type 1 and type 3 fimbriae in *Klebsiella pneumoniae* biofilm formation. BMC Microbiol. 2010, 10:179. DOI: 10.1186/1471-2180-10-179.
19. Soto SM, Zuniga S, Ulleryd P, Vila J. Acquisition of pathogenicity island in an *Escherichia coli* clinical isolates causing febrile urinary tract infection. EUR J Clin Microbiol Infect Dis; 2011, 10:1258–62.
20. Tabasia M, Karama MR, Habibia M, Yekaninejadb MS, Bouzaria S. Phenotypic assays to determine virulence factors of uropathogenic *Escherichia coli* (UPEC) isolates and their correlation with antibiotic resistance pattern. Osong Public Health and Research Perspectives; 2015, Volume 6, Issue 4, Pages 261-268 (http://dx.doi.org/10.1016/j.phrp.2015.08.002).
21. Tajbakhsh E, Ahmadi P, Abedpour-Dehkordi E, Arbab-Soleimani N, Khamesipour F. Biofilm formation, antimicrobial susceptibility, serogroups and virulence genes of uropathogenic *E. coli* isolated from clinical samples in Iran. Antimicrob Resist Infect Control; 2016, 5: 5: 11.
22. SarojGolia, Hittinahalli V, Karjigi S and Reddy M. Correlation between biofilm formation of uropathogenic *E. coli* and its antibiotic resistance pattern J Evolution Med Dental Sci.Volume1/ Issue3/ Page 166.
23. Donlan RM, Costerton W. Biofilms Survival mechanisms of clinically relevant microorganisms. Clin Microbiol; 2002, 15(2):167-93.
24. Asadi S, Kargar M, Solhjoo K, Najafi A and Ghorbani-Dalini S. The association of virulence determinants of uropathogenic *escherichia coli* with antibiotic resistance. Jundishapur J Microb: 2014, 7 (5) pp. 1-5
25. Johnson JR, Johnston B, Kuskowski MA, Colodner R, Raz R. Spontaneous conversion to quinolone and fluoroquinolone resistance among wildtype *Escherichia coli* isolates in relation to phylogenetic background and virulence genotype. Antimicrob. Agents Chemother; 2005, 49(11):4739–4744.
26. Stewart PS Costerton JW. Antibiotic resistance of bacteria in biofilms. Lancet; 2001, 358:135-8.
27. Soto SM. Importance of biofilms in urinary tract infections: new therapeutic approaches. Adv Biol. 2014(2014):543974.
28. Zaki ME and Elewa A. Evaluation of uropathogenic virulence genes in *Escherichia coli* isolated from children with urinary tract infection. Int J Advanced Res; 2015, 3(3) 165-173
29. Cheng CH, Tsau YK, Kuo CY, Su LH and Lin TY. Comparison of extended virulence genotypes for bacteria isolated from pediatric patients with urosepsis, acute pyelonephritis, and acute lobar nephronia. Pediatr Infect Dis J; 2010, 29:736-40
30. Wang MC, Tseng CC, Wu AB, Lin WH, Teng CH, Yan JJ and Wu JJ. Bacterial characteristics and glycemic control in diabetic patients with Escherichia coli urinary tract infection. J Microbiol Immunol Infect; 2013, 46:24-9.
31. Reisner A, Maierl M, Jörger M, Krause R, Berger D, Haid A, Tesic D and Zechner EL. Type 1 fimbriae contribute to catheter-associated urinary tract infections caused by *Escherichia coli.* J Bacteriol; 2014, 196(5):931-9.
32. Hassan A, Usman J, Kaleem F, Omair M, Khalid A, Iqbal M. (2011): Evaluation of different detection methods of biofilm formation in the clinical isolates. Braz J Infect Dis. 2011 Jul-Aug; 15(4):305-11.
33. de Castro Melo P, Ferreira LM, Filho AN, Zafalon LF, Vicente HG, de Souza V; 2013, Comparison of methods for the detection of biofilm formation by *Staphylococcus aureus* isolated from bovine subclinical mastitis Braz J Microbiol; 44(1): 119-124.