

Sequencing Analysis of Pyelonephritis-Associated Pili Gene of Uro-Pathogenic *Escherichia coli* Isolated From Iraqi Patients-Baghdad

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Pyelonephritis-associated pili (Pap) fimbria considered as the main adhesive virulence factor that enable *Escherichia coli* (*E. coli*) to colonize in the urinary tract and resist the avoiding by the flow of urine. DNA adenine methyl-transferase gene (*Dam*) have a role in regulation of pap E expression and in bacterial DNA repair system and it could be targeted by antibiotics. Sixty Four isolates of *E. coli* from urine specimens were obtained from hospitalized and out-patients suffering from signs and symptoms of UTI. These isolates were identified molecularly as uropathic *E. coli* (UPEC) by detection of papE using PCR. Partial sequencing of pap E was done to study variation among isolates according this gene and its role in susceptibility to antibiotic. Also, *Dam* was detected using PCR. Detection of papE in *E. coli* strains revealed that 26/64(42.6%) were considered as UPEC. Analysis of nucleotide sequence changes from partial sequencing tree of pap E shown that there were three clads and UPEC included in clade B displayed the most nucleotide sequence changes. *Dam* was detected in 11/64 (17.1%) *E. coli* isolates. The study of multi-drug resistance(MDR) risk in association with the presence of pap E and *Dam* in UPEC revealed that *Dam* could be considered as etiological factored to developing MDR. In conclusion, *Dam* should be taken in consideration as one mechanism of MDR development in UPEC.

Keywords: Escherichia Coli; Multidrug Resistance; Pap E; Dam; Urinary Tract Infection.

Uropathogenic *Escherichia coli* (UPEC) is the most abundant causative bacteria of urinary tract infections (UTIs), worldwide accounting for 80–90% of all infections.¹ There are differences in prevalence of virulence factors among UPEC including the adhesins (Type 1, P, S, and F1C fimbriae), pore-forming hemolysin, toxins (RTX and CNF-1), sideophore and aerobactin, which exist in clusters, small virulence cassettes, or large blocks of genes in UPEC strains but, mainly, not observed in the genome of fecal isolates.^{2,3} Their expression and co-regulation depend on host response and environmental signals.⁴ Iraqi studies

from different cities referred to that the frequency of isolation of *E. coli* from urine of patients with UTI were ranged from 39%-73.6%.^{5,6,7,8}

The crucial step for starting of UTI is by adhesion of UPEC to mucosal cells that intermediated by P fimbriae and Type-1 fimbriae. Mannose-resistant pyelonephritis-associated pili fimbriae expressed from pap in about 80% of UPEC cause pyelonephritis.⁹ Genotyping of pap operon referred to its involve fimbriae structural subunits (pap A, C, D,E, F and G) to finish fimbrial growth and attach mature fimbriae to surface of host cell, pap H; and papB and papI, the divergently encoded



regulatory genes, within which the main promoter is located.¹⁰

These promoters expressed depending on the DNA methylation of the sequences GATC that located within the intergenic region. DNA methylation is essential epigenetic, post-replicative modification that is catalyzed by a group of enzymes as the DNA methyltransferases (MTases). It is key regulation mechanism of many cellular processes in prokaryotes and eukaryotes. Bacterial DNA methyl-transferases generate N4-methyl-cytosine, C5-methyl-cytosine, or N6-methyl-adenosine in GATC sequences, in an S-adenosyl-methionine-dependent reaction.^{11,12}

DNA adenine methylase (*Dam*) have important functions in DNA mismatch repair, regulation of transcription, and SOS response activation as part of the cell cycle.¹³ Methyl-directed mismatch repair is a regulatory process whereby it recognizes the biosynthetic error during the occurrence of the replication fork. The hemimethylated site differentiates of the DNA template strand and the DNA newly synthesized strand allowing protein MutS to bind to the site where the mismatch occurs and promotes the process of the recruitment of the addition repair proteins to form a ternary complex that initiate repair.^{13,14} Adherence is essential for UPEC strains, as it avoid removal of bacteria through micturition, and multiple phase-variable adhesins are exists to permit binding to different host tissues. The pap epigenetic switch activates through establishment of differential methylation patterns that regulate the expression phase (on or off) of the pap operon.^{15,16} Novel antibiotics drug that targets *Dam* can be intriguing as the enzymatic activity is a lack in human. Inhibiting *Dam* by DNA methyl-transferase inhibitors (DNMTi) can be detrimental to the bacterium. The inhibitors will reversely modify the deviating pattern of the DNA methylation by interfering the enzymatic activity of the DNMTs.^{17,18} The current study aimed to investigate if there is a relationship between sequence variation in pap E , presence of *Dam* and susceptibility to antibiotics in UPEC.

MATERIAL AND METHODS

Bacterial Isolation

Sixty Five isolates of *E. coli* from urine

specimens were obtained from hospitalized and out-patients suffering from signs and symptoms of UTI recruited to Department of Microbiology Lab, AL-Imamein Medical City Hospital, Baghdad, Iraq, from March 2018 to October 2018. Also, 2 *E. coli* isolates from wound infections and 2 *E. coli* isolates from chest fluid were obtained. These isolates were cultured on MacConkey agar and identified using API20E Epi 20 system and/or VITEK2 Vitic system. Information such as age, gender, antibiotic susceptibility of included patients were obtained from consent form of each patient.

Antimicrobial Susceptibility Testing

The antibiotic susceptibility test of included isolates were done using disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.¹⁹ The following antibiotics were included: amoxicillin-clavulanic acid (AMC), ciprofloxacin (CIP), gentamicin (GE), nitrofurantoin (NIT), trimethoprim-sulfamethoxazole (SXT), cefpodoxime (CPD), cephalothin (CLT), tetracycline (TE), rifampin (RP) and ticarcillin (TIM). The definitions of multidrug resistant (MDR), extended drug resistant (XDR) and pan drug resistant (PDR) isolates were as per standardized by European Centre for Disease Control (ECDC) and Centre for Disease Control & Prevention (CDC).²⁰

Molecular Identification of *E. coli*

Genomic DNA was extracted from *E. coli* isolates using WIZARD Genomic DNA Extraction Kit following manufacturer instructions (Promega, USA). Concentration and purity of extracted DNA of each sample were measured using Nanodrop (AcT Gene NAS-99, USA). Primers specific for the lacY (lactose permease gene) and phoA (bacterial alkaline phosphatase

Table 1. Age distribution of patients infected with *E. coli* positive to papE UPEC

Age group (year)	Male	Female
10-Jan	1	–
20-Oct	–	5
20-30	–	1
30-40	2	2
40-50	1	3
50-60	–	5
60 and more	3	3
Total	7	19

gene) were used for molecular identification of *E. coli* following Das Mitra et al., 2015 with modifications.²¹

The reaction was prepared in 25 μ L total volume. The concentration of primer was 0.15 μ M for lacY, and 0.6 μ M for phoA (Alpha DNA, Canada), 1X PCR buffer (Promega, USA), 1.5 U of Taq DNA polymerase (Promega, USA), 200 mM dNTPs (Promega, USA). Then, DNA (100 ng) was added. The cycling conditions were as follows: 94 °C for 5 min followed by 35 cycles of 94 °C for 30 sec, 52°C for 1 min and 72 °C for 1 min and final extension at 72°C for 10 min using Thermal Cycler (Eppendorf master cycler, Germany). The amplified products were analyzed using agarose gel 1.5%. Extracted DNA from *Pseudomonas aeruginosa* identified using VITEK2 VITIC system and molecularly from previous study,²² and extracted DNA from *Klebsiella pneumoniae* (identified using VITIC system in this study) were used as negative control to evaluate primer specificity. Two bands with molecular size 289bp and 468bp will be seen if the DNA was from *E. coli*. The appearance of amplicon with 468bp only or no amplified products will be referred to bacteria other than *E. coli*.

Detection of pap E

The presence of pap E in UPEC isolates was detected using conventional PCR.²³ Master mix was prepared in 25 μ L total volume containing 1X PCR buffer, 10 pmol of forward and reverse primers, 200 μ M of dNTP, 1.25U of Taq DNA polymerase. Then, 100ng DNA was added. Two extracted DNA samples from each of *E. coli* isolated from wound infection and chest fluid, respectively, were used as negative control for the presence of pap E. PCR conditions were as following: 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 52°C for 1.5 min, 72°C for 3 min and final extension at 72°C for 10 minutes using Thermal Cycler (Eppendorf master cycler, Germany). PCR products were electrophoresed on agarose gels 1.5%. Appearance of amplicon with molecular size 336bp referred to presence of pap E.

DNA Sequencing of pap E

The amplicons of pap E from 21 selected strains of UPEC were sent for Sanger sequencing using automated DNA sequencer ABI 3730XL (Macrogen Corporation, Korea). The results were analyzed using Genious software. The sequences

of each fragment were trimmed to a uniform length that corresponded with the region used to identify the target gene. Sequences were compared with standard strain MH455215 using online BLAST software (<http://www.ncbi.nlm.nih.gov/BLAST/>). Partial sequencing tree of fimbrial adaptor papE and fimbrial adaptor papF of 13 *E. coli* isolates was constructed.

Nucleotide Sequence Accession numbers

The partial sequencing alignment of pap E of 5 included strains were compared with the sequences of previously published strains from DNA GenBank sequences using Basic Local Alignment Search Tool (BLAST) search and deposited in GeneBank under accession numbers: LC479519.1 of strain MR-1, LC479520.1 of strain MR-2, LC479521.1 of strain MR-14, LC479522.1 of strain MR-18 and LC479523.1 of strain MR-21.

Detection of DNA Adenine Methyl-Transferase Gene in UPEC Isolates

Extracted DNA from 26 UPEC was screened for *Dam* using conventional PCR. PCR condition was modified as the following: 95°C for 5 min followed by 35 cycles of 95°C for 1 min, 55°C for 1.5 min, 72°C for 1.5 min and final extension of 72°C for 10 min using Thermal Cycler (Eppendorf master cycler, Germany). Products of PCR were electrophoresed on agarose gels 0.8%. Appearance of amplicon with molecular size of 1071bp referred to presence of *Dam*.

RESULTS

Patients Demography

The mean age of included patients was 38.11 \pm 22.02 ranging from (1-78) years with median 42.5 (36.5) years. Male 24/64 (37.5 %) to female 40/64 (62.5 %) ratio 1:1.7.

Prevalence of pap E among UPEC Isolates

Of the obtained 65 isolates, 64 (98.4%) were approved to be *E. coli* depending on molecular identification of lacY and phoA using PCR. Detection of papE in *E. coli* strains using PCR revealed that 26/64 (42.6%) were UPEC (Fig.1). Prevalence of UPEC was showed to be the highest among the female patients within age group (10-20) years and (50-60) years (Table1).

Analysis of pap E Sequencing in Studied Strains

PCR amplified products of papE from 21 strains were partially sequenced, only 13 strains

gave successful sequencing analysis of target gene which revealed three clades labeled as A, B and C (Fig. 2). The correlation between studied strains according to analysis of genetic distance based on partial sequencing of *pap E* showed that strain 17 (clade A) has 100% similarity with standard strain, while clade B, which included strains (1,2,5,14,18,21) and clade C which included strains (4,7,8,10,12,13,16) have (99%) similarity with standard strain.

From clade B, strains 1, 2 and 21 considered as sister group. Also, strains 5, 14 and 18 considered as sister group. From clade C, strains 12, 4, 7, 8, 10, 13, 16 have similarity of 100%. The main clusters that were similar were conserved.

Analysis of nucleotide sequence changes from partial sequencing of *pap E* showed that UPEC included in clade B displayed the most nucleotide sequence changes as substitution result in synonymous and non-synonymous mutation (Table 2).

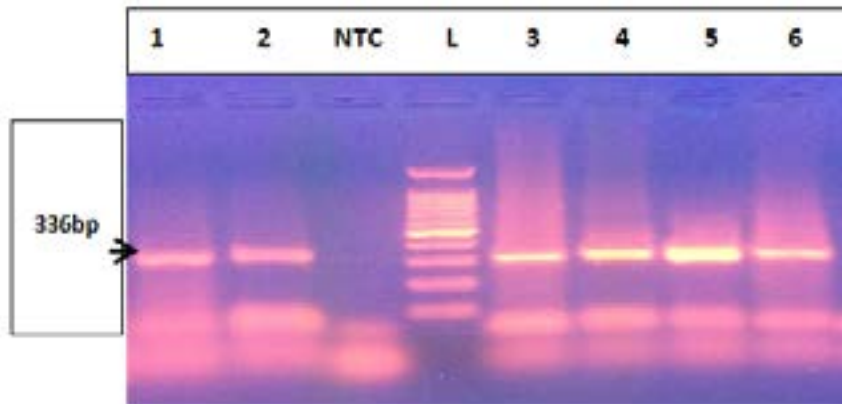


Fig. 1. Detection of *pap E* in *E. coli* isolates from urine specimens. Lane (1-6): PCR amplified products of extracted DNA from isolates 2, 11, 14, 16, 18 and 21, respectively, positive for *pap E* (336bp). Lane NTC: no template control. Lane L: DNA ladder (100bp). Electrophoresis was done in agarose gel 1.5% at (5V/cm) for 60 min

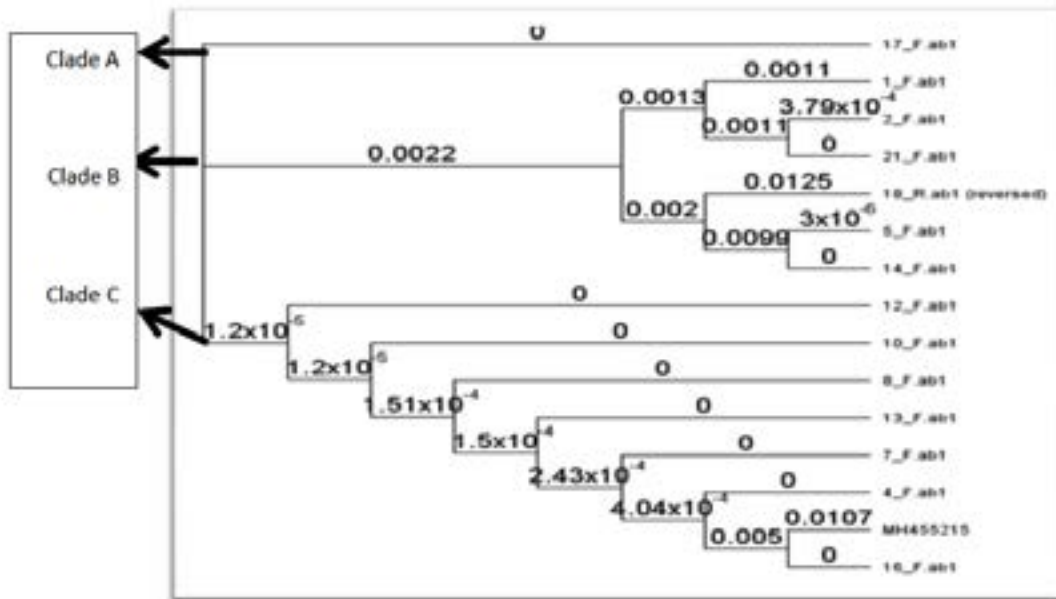


Fig. 2. Partial sequencing tree of *pap E*. The analysis involved 13 isolates and (one standard strain MH455215 sequences from the NCBI GenBank database). All positions containing gaps and missing data were eliminated. The number on the branches referred to genetic distance between sub-clusters

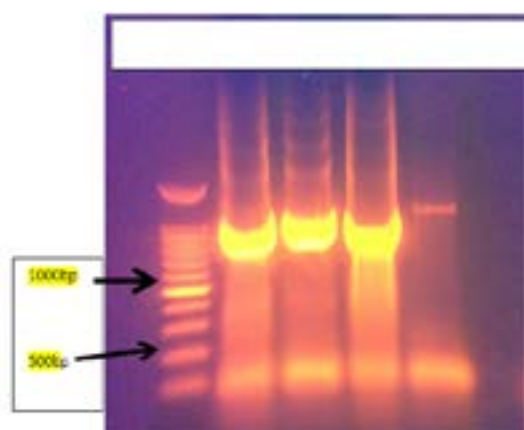


Fig. 3. Detection of *Dam* in *E. coli* isolates from urine specimens .Lane (1-4): PCR amplified produces of *Dam* from isolates 2, 19,21and 22, respectively (1071 bp). Lane NTC: no template control. Lane L: DNA ladder (100bp). Electrophoresis was done in 0.8% agarose gel at (5V/cm) for 60 min

Prevalence of *Dam* among UPEC Isolates

Dam was detected in 11/64 (17.1%)*E. coli* isolates (Fig. 3). The association between the presence of pap E and *Dam* in studied isolates shown that 10/64(15.6%) isolates contain both genes (Table 3).

Antimicrobial Susceptibility Test

Of the UPEC isolates, 13/26 (50%) isolates were considered as MDR, that acquired non-susceptibility to at least one antibiotic in three or more antimicrobial categories (Table S1).

Association between pap E clades of UPEC and MDR

Studying the association between the pap E clades of UPEC isolates and MDR was shown that there was statically not significant (Table 4).

Correlation between Antibiotic Susceptibility, MDR and the Existence of pap E and *Dam*

The correlation between the presence of papE and /or *Dam* and antibiotic susceptibility

Table 2. The sequencing analysis of partial sequencing tree of *pap E*

Clade type of <i>pap E</i>	Strain no.	Nucleotide change	Amino-acid change	Type of nucleotides change
A	17	N	N	N
		GTC/GTT	Val-Val	Substitution (synonymous)
B	1	TGG/TAA	Val-Val	Substitution(synonymous)
		GTC/GTT	Val-Val	Substitution(synonymous)
	2	TGG/TAT	Trp-Tyr	Substitution(non-synonymous)
		GAA/GAG	Glu-Glu	Substitution(synonymous)
	5	ATC/GTA	Lie-Val	Substitution (non-synonymous)
		GAA/GAG	Glu-Glu	Substitution(synonymous)
	14	ATC/GTA	Lie-Val	Substitution(non-synonymous)
		TTC	Phe	Insertion-frame shift
	18	GCT/ATT	Ala-Lie	Substitution(non-synonymous)
		GTC/GTT	Val-Val	Substitution(synonymous)
21	ATC/GTC	Lie-Val	Substitution(non-synonymous)	
	GTC/GTT	Val-Val	Substitution(synonymous)	
C	4	TTC	Phe	Insertion-frame shift
		N	N	N
	7	N	N	N
		N	N	N
	8	N	N	N
		N	N	N
	10	N	N	N
		N	N	N
12	N	N	N	
	N	N	N	
13	N	N	N	
	N	N	N	
16	N	N	N	
	N	N	N	

-N: no change in nucleotides, no change in amino acid; Val: valine ; Trp: tryptophan; Tyr: tyrosine; Glu: glutamine; Lei: leucine; Phe: phenylalanine ; Ala: alanine.

revealed that there was a statistically significant correlation between resistance to tetracycline and rifampin and the presence of both *papE* and *Dam*, a statistically significant correlation between resistance to tetracycline and the presence of *papE* and a high statistically significant correlation between resistance to tetracycline and the presence of *Dam* (Table S2). The study of MDR risk in association with the presence of *papE* and *Dam* in UPEC revealed that *Dam* could be considered as etiological factored to developing MDR (Table 5).

DISCUSSION

Colonization of *E. coli* strains within the urinary tract and occurrence of UTI begin with binding of bacteria to epithelial surface.^{25,26} In the current study, 26/64(42.6%) of *E. coli* were

confirmed to be UPEC according to molecular detection of *papE*. There is a diversity in the frequency of *papE* gene among UPEC strains across the globe and within the same geographical region. Iraqi studies at 2015 and 2017 referred to that *papE* was detected in 58/112 (51.785%) *E. coli* and 31/43(72.09%) *E. coli*, respectively, isolated from urine of patients with UTI.^{27,28} Iranian studies were revealed that there were variations in *papE* gene prevalence in UPEC isolated from different locations including 83.63%, 16.6% and 20.5%.^{29,30,31} Indian study at 2018 found that the prevalence of *papE* was 72/350(20.5%) isolates.²⁶ This variation result from UPEC use a variety of adhesions to attach to the bladder urothelium if the usual adhesions are not expressed. However, there is always the possibility of occurring mutations in *papE* result in missing of its detection. A positive PCR product confirm the detection of the gene, but a negative result does not insure absence of the gene.

Increased antibiotic resistance in bacteria causing UTI has complicated the treatment of such infections. In the current study, 13/26(50%) UPEC isolates were MDR. Iraqi study referred to that of the 62 *E. coli* isolated from urine, (69.4%) were showed MDR.³² Iraqi stud was found that 44% of UPEC were resist to ciprofloxacin and these isolates have mutation in *gyrA* that identified using sequencing of this gene.³³ Other Iraqi study

Table 3. Frequency distribution and prevalence rate of *papE* and *Dam*

Characteristic	N=64	(%)
Pap E -/ Dam -	37	57.8
Pap E +/- Dam -	16	25
Pap E -/ Dam +	1	1.6
Pap E +/- Dam +	10	15.6
Pap E+	26	40.6
Dam +	11	17.2

Table 4. UPEC *papE* clads in association with MDR.

Clad Type	Total	MDR +	MDR -	P
A	1	0	1	0.462 FNS
B	6	4	2	0.592 FNS
C	6	3	3	1.000 FNS
Total	13	7	6	—

F: Fischer exact test; NS: not significant at $p \leq 0.05$

Table 5. Multidrug resistance risk in association with *papE* and *Dam*

Gene	MDR + n = 28		MDR - n = 36		P	OR	95 % CI
	n	%	n	%			
EF							
<i>papE</i>	13	46.4	13	36.1	0.404 CNS	1.53	0.56 -4.20
<i>Dam</i>	8	28.6	3	8.3	0.047 FS	4.40	1.04 -18.54

n: number of cases; OR: Odds ratio; CI: confidence interval; EF: etiologic fraction; C: Chi-square test; F: Fischer exact test; NS: not significant at $P \leq 0.05$

at 2018 found that of 42 UPEC, 37(88.09%) were found to be MDR³⁴. Iranian study at 2015 found that 111/150(74%) of UPEC strains showed MDR phenotype.³¹

In the current study, the etiological factor of *Dam* in MDR isolates was 0.56. That referred to the possibility of considered the presents of *Dam* as one of predisposing factor for resistance. A study was done to determine the effect of the bacterial epigenome in antibiotic stress survival to describe genomic methylation kinetics using single-molecule real-time sequencing and they find that without adenine methylation at GATC sites, *E. coli* growth under antibiotic stress is extremely reduced. The explanation for the role of *Dam* in antibiotic resistance is that during drug stress, the adenine methylome persist stable but without GATC methylation, methyl-dependent mismatch repair is repressed and that cause toxic DNA breaks in bacteria. In drug-resistant and pathogenic *E. coli* strains, *Dam* insufficiency reduce responsiveness to antibiotics such as β -lactam and quinolone classes.³⁵ A study referred to that there was an 8-fold decrease in resistance for amoxicillin/clavulanic acid, gentamicin and trimethoprim/sulfamethoxazole and 4-fold decrease in resistance for ciprofloxacin in *Dam* deleted mutated UPEC strains.³⁶

Current studies are focus on the detection of *Dam*,^{17,37} but some *E. coli* strains could be have DNA cytosine methyltransferase (Dcm) that methylates the second cytosine in the sequence 5'CCWGG 3'.^{13,38} Also, it was referred to that *Dam* could be recognized and methylate cytosine in sequences other than GATC.³⁹

CONCLUSION

The study of multi-drug resistance(MDR) risk in association with the presence of pap E and *Dam* in UPEC revealed that *Dam* may be considered as etiological factored to developing MDR. *Dam* should be taken in consideration as one mechanism of MDR prediction development in UPEC. Farther studies included a large sample size required to sport the role of pap and *Dam* in responsiveness to antibiotic.

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Not applicable.

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