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Detection of virulence factors, phylogroups, serogroups and biofilm formation among *CTX-M-1* positive *Escherichia coli* isolated from patients with pyelonephritis.

Seyyed Khalil Shokouhi Mostafavi[1], Shahin Najar-Peerayeh[1*], Ashraf Mohabbati Mobarez[1], Mehdi Kardoust Parizi[2]

[1]Department of Bacteriology, Tarbiat Modares University, Tehran, Iran

[2]Department of Urology, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran

Abstract

Objectives: Urinary tract infections are among the most frequent infections caused by *Escherichia coli* isolates. The aims of this study were determination of antibiotic susceptibility and detection of virulence genes, phylogroups, serogroups and biofilm formation of *E. coli* isolates from patients with pyelonephritis.

Methods: A total of 20 *E. coli* isolates were isolated from pyelonephritis. The antimicrobial susceptibility test was performed for eleven antibiotic disks. The biofilm formation assay was performed with Microtitre Tissue Plate (MTP) assay. The *CTX-M1* gene and virulence genes including *fimH, fyuA, traT, iutA, papII, kpsMII, ompT, ibeA, sfa, iroN, iucD, afaC, papI* and *papIII* and also phylogroups and serogroups were detected with specific primers.

Results: In the biofilm assay, one isolate produced strong biofilm. Among virulence encoding genes, 19 (95%) isolates amplified the all *fimH, fyuA and traT* genes, followed by *iutA* (90%, n=18), *papII* (75%, n=17), *kpsMII* (60%, n=12), *ompT* (55%, n=11), *ibeA* (30%, n=6), *sfa* (20%, n=5), and *iroN*=*iucD*=*afaC*=15%, (n=3), but none for *papI* and *papIII*. The majority (50%, n=10) of *E. coli* isolates from pyelonephritis belonged to the phylogroup B2, followed by phylogroups D and A (each equal to 20%, n=4) and B1 (10%, n=2). Seroproups included O25 (20%, n=4), O1 (15%, n=3), O4=O18=O75 (each equal to 10%, n=2), and O2=O12=O15=O16=1 (0.05%).

Conclusion: The adhesive virulence factors play a critical role in the pathogenesis of pyelonephritis as their prevalence was high. Continued and vigilant surveillance is necessary to monitor the dissemination of antimicrobial resistance in uropathogens.

Keywords: *Escherichia coli*, Biofilms, Virulence, Phylogroups, Serogroups.

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Introduction

The etiologic agents causing pyelonephritis are narrow and the knowledge of their antimicrobial resistance profile is uncompleted. Urinary Tract Infection (UTI), is among the most frequent bacterial infections, and usually occurs by *E. coli* [1-4]. The pathogenesis of *E. coli* from UTI has been poorly understood despite profound study. *E. coli* strains mostly infect the host *via* entrance through gastrointestinal or vaginal routes. The virulence and also most prevalent clones of *E. coli* representing the infection within a host have not been fully elucidated and possibly the predominant strain tends to be the host's most prevalent fecal strain [3-6].

The ESBL-producing *E. coli* is isolated from pyelonephritis from hospitalized or out-patients have increasingly posed significant treatment challenges [4-6]. Resistance to the last resort antibiotics (such as carbapenems) is prone to the prolonged hospital stay (occurred in both healthcare and community settings), and history of misuse or overuse drug consumption which leads to increasing morbidity and mortality and costs in health care settings [7,8]. Following surveillance programs to track the increase in the antimicrobial resistance of certain pathogens to fulfil the appropriate strategies for their control is helpful in this regard.

In particular, the spread of ESBL-producing Enterobacteriacea is difficult to restrict at a wider international scale level, due to improper detection of ESBLs and disparity in their reporting [9]. The enhancement in the rate of Multi Drug Resistant (MDR) and ESBL-producing *E. coli* collected from patients and also environment throughout the globe is a great concern [10]. The aims of this study were detection of antibiotic resistance and virulence factors of *E. coli* isolates from pyelonephritis. Uropathogenic O25 *E. coli* pathogens belong to international clone ST131 which generally contain *CTX-M-1*

gene [11,12]. UPEC isolates are widespread in the globe and CTX-M-producing strains have been reported increasingly mainly due to the worldwide use of antibiotics. *TEM-1, OXA-1* and *aac (6')-Ib-cr* are other encoding genes carried by these strains [13,14]. The aims of this study were determination of antibiotic susceptibility and detection of virulence genes, phylogroups, serogroups and biofilm formation of *E. coli* isolates from patients with pyelonephritis.

Materials and Methods

Antibiotic susceptibility testing

From March 2016 through January 2017, a total of 20 *E. coli* isolates were isolated from pyelonephritis. The antimicrobial susceptibility test was performed according to the instructions by Clinical and Laboratory Standards Institute (CLSI) version 2016. Eleven antibiotic disks including ampicillin (10 µg), cefazolin (30 µg), amoxicillin-clavulanic acid (20/10 µg), ceftazidime (25 µg), cefotaxime (30 µg), co-trimoxazole (25 µg), ciprofloxacin (5 µg), fosfomycin (50 µg), imipenem (10 µg), gentamicin (10 µg) and nitrofurantoin (100 µg) were tested [15].

Detection of CTX-M-1 and virulence genes by PCR

The *CTX-M-1* and virulence genes including *fimH, fyuA, traT, iutA, kpsMII, papII, ompT, ibeA, sfa, iroN, iucD, afaC, papI* and *papIII* were detected with specific primers shown in Table 1.

Detection of phylogroups and serogroups

The specific primers for the amplification of genes for detection of phylogroups and serogroups have been shown in Table 2.

Biofilm formation

The biofilm formation assay was performed with Microtitre Tissue Plate (MTP) assay. Briefly, bacterial isolates were cultured in Luria-Bertani broth for an overnight and next diluted 1:100 in saline and 20 µl of this was inoculated in 180 µl LB broth in the 96-well plate in foursome and incubated for an overnight. The wells of plate were washed with sterile water and then the crystal violet was added and kept in ambient temperature for 15 min. the wells were washed and methanol was added for the fixation and left to dried. Next, the ethanol was added and the solvent biofilm opacity was measured at OD=540 nm and 620 nm (negative control) with ELISA reader [16].

Data analysis

Data was analysed using SPSS software version 20, with ANOVA test and t-test and 95% confidence interval ($p<0.05$ being significant result).

Results

Patients' demographic data

Twenty patients included 14 females and 6 males with the mean ages of 54 and 66 years, respectively and mostly because of urinary tract infection (70%, n=14), followed by kidney impair (15%, n=3), leukemia (10%, n=2) and bladder stone (5%, n=1). They were all inpatients in the hospital wards including 6 from urology, 7 from emergency and 7 from each of kidney implantation, blood emergency, nephrology, internal ICU, and internal general wards.

The antibiotic susceptibility test

All 20 *CTX-M-1* positive *E. coli* isolates from pyelonephritis were susceptible to imipenem, meropenem and fosfomycin and all of them were resistant to ceftazidime, amoxicillin and erythromycin (Table 3).

The virulence genes

Among virulence encoding genes, 19 (95%) isolates amplified the all *fimH, fyuA* and *traT* genes, followed by *iutA* (90%, n=18), *papII* (75%, n=17), *kpsMII* (60%, n=12), *ompT* (55%, n=11), *ibeA* (30%, n=6), *sfa* (20%, n=5), and *iroN=iucD=afaC*=15%, (n=3), but none for *papI* and *papIII*.

The phylogenetic grouping and serogrouping

The majority (50%, n=10) of *E. coli* isolates from pyelonephritis belonged to the phylogroup B2, followed by phylogroups D and A (each equal to 20%, n=4) and B1 (10%, n=2) which is shown in Table 4. Serogroups included O25 (20%, n=4), O1 (15%, n=3), O4=O18=O75 (each equal to 10%, n=2), and O2=O12=O15=O16=1 (0.05%), but two of them were non-type able with this method. Among 10 isolates belonged to B2 phylogroup, all were *iutA*, *tratT* and *fimH* positive and 9 of them were *fyuA* and moreover the major serogroups in this phylogroup were O75 (n=2), followed by one isolate belonging to each O12, O18, O25, O4, O1 and O15, and two isolates were not typed by this method. There was no significant relation between phylogroups and serogroups among UPEC isolates. Among four isolates belonging to each phylogroups A and D, serogroups, O25 and O1 (n=2, 50%) were mostly detected respectively (Table 4).

Biofilm formation

In the biofilm assay, one isolate produced strong biofilm, four isolates produced moderate and 8 isolates weak biofilm, but 7 isolates produced no biofilm (Table 5).

Table 1. *The specific primers used for detection of virulence genes.*

Primer	Sequence (5' to 3')	Amplicon (bp)
CTX-M1	F-GGTTAAAAAATCACTGCGTC	863
	R-TTGGTGACGATTTTCGCCGC	

fimH	F: TGCAGAACGGATAAGCCGTGG	508
	R: CTCCGGAGAACTGGGTGCATCTTAC	
fyuA	F: GCGACGGGAAGCGATGACTTA	774
	R: CGCAGTAGGCACGATGTTGTA	
traT	F: GCGCATTTGCTGATACTGTTG	429
	R: CATCCAGACGATAAGCATGAGCA	
iutA	F: GCGCGTAGCCGATGAAAT	302
	R: CACTGAAAACAAGATTGAT	
kpsMII	F: AAGTCAAAGCAGGGGTTGCCCG	668
	R: GACGCCGACATTAAGACGCAG	
ompT	F: ATCTAGCCGAAGAAGGAGGC	559
	R: CCCGGGTCATAGTGTTCATC	
ibeA	F: AGGCAGGTGTGCGCCGCGTAC	170
	R: TGGTGCTCCGGCAAACCATGC	

sfa	F: CTCCGGAGAACTGGGTGCATCTTAC	408
	R: CGGAGGAGTAATTACAAACCTGGCA	
iroN	F: AAGTCAAAGCAGGGGTTGCCCG	668
	R: GACGCCGACATTAAGACGCAG	
iucD	F: TACCGGATTGTCATATGCAGACCGT	602
	R: AATATCTTCCTCCAGTCCGGAGAAG	
afaC	F: TAAGGAAGTGAAGGAGCGTG	802
	R: CCAGTAACTGTCCGTGACA	
papGI	F: TCGTGCTCAGGTCCGGAATTT	461
	R: TGGCATCCCCCAACATTATCG	
papGII	F: GGGATGAGCGGGCCTTTGAT	190
	R: CGGGCCCCCAAGTAACTCG	
papGIII	F: GGCCTGCAATGGATTTACCTGG	258
	R: CCACCAAATGACCATGCCAGAC	

Table 2. *The specific primer for the detection of serogroups.*

Target gene	Sequence (5' to 3')	Amplicon Size, bp	Optimal annealing temperature (°C)
rfbO1	F: ATACCGACGACGCCGATCTG	189	59
	R: CCAGAAATACACTTGGAGAC		
rfbO2	F: ATACCGACGACGCCGATCTG	274	59
	R: GTGACTATTTCGTTACAAGC		
rfbO18	F: ATACCGACGACGCCGATCTG	360	59
	R: GAAGATGGCTATAATGGTTG		
rfbO16	F: ATACCGACGACGCCGATCTG	450	59
	R: GGATCATTTATGCTGGTACG		
rfbO6	F: ATACCGACGACGCCGATCTG	584	59
	R: AAATGAGCGCCCACCATTAC		
rfbO7	F: ATACCGACGACGCCGATCTG	722	59
	R: CGAAGATCATCCACGATCCG		
rfbO4	F: ATACCGACGACGCCGATCTG	193	67
	R: AGGGGCCATTTGACCCACTC		
rfbO12	F: ATACCGACGACGCCGATCTG	239	59
	R: GTGTCAAATGCCTGTCACCG		
rfbO25	F: ATACCGACGACGCCGATCTG	313	59
	R: GAGATCCAAAAACAGTTTGTG		
rfbO75	F: ATACCGACGACGCCGATCTG	419	58
	R: GTAATAATGCTTGCGAAACC		
rfbO15	F: ATACCGACGACGCCGATCTG	536	59
	R: TGATAATGACCAACTCGACG		

rfbO157	F: ATACCGACGACGCCGATCTG	672	59
	R: TACGACAGAGAGTGTCTGAG		
chuA	F: GACGAACCAACGGTCAGGAT	279	54
	R: TGCCGCCAGTACCAAAGACA		
yjaA	F: TGAAGTGTCAGGAGACGCTG	211	54
	R: ATGGAGAATGCGTTCCTCAAC		
tspE4C2	F: GAGTAATGTCGGGGCATTCA	152	54
	R: CGCGCCAACAAAGTATTACG		

Table 3. *The antibiotic susceptibility of 20 E. coli isolates from pyelonephritis.*

Antibiotic	**FO**	**PTZ**	**CAZ**	**CTX**	**AMX**	**CZ**	**AMC**	**IMI**	**FM**	**GM**	**CIP**	**MEN**	**SXT**
Susceptible no (%)	20 (100)	19 (95)	2 (10)	-	-	-	2 (10)	20 (100)	20 (100)	7 (35)	5 (25)	20 (100)	15 (75)
Resistant No (%)	-	1 (5)	18 (90)	20 (100)	20 (100)	20 (100)	18 (80)	-	-	13 (65)	15 (75)	-	5 (25)

Amx: Amoxicillin (10 µg), AMC: Amoxicillin-Clavulanic Acid (20/10 µg), CZ: Cefazolin (30 µg), CTX: Cefotaxime (30 µg), CAZ: Ceftazidime (30 µg), CIP: Ciprofloxacin (5 µg), SXT: Co-trimoxazole (25 µg), FO: Fosfomycin (50 µg), GM: Gentamicin (10 µg), IMI: Imipenem (10 µg), and FM: Nitrofurantoin (100 µg).

Table 4. *The phylogroups, serogroups and virulence factors of UPEC in this study.*

Isolate	Phylogroup	Serotype	*papI*	*papII*	*papIII*	*ompT*	*sfa*	*iroN*	*iucD*	*fyuA*	*afaC*	*fimH*	*ibeA*	*traT*	*kpsMII*	*iutA*	Biofilm
1	D	O1	-	+	-	+	-	-	-	+	-	+	-	+	+	+	M
2	A	Nd	-	+	-	+	-	-	-	+	-	+	-	+	-	-	N
3	B2	O18	-	-	-	-	-	-	-	+	-	+	+	+	-	+	M
4	B2	Nd	-	-	-	-	-	-	-	-	+	+	+	+	-	+	N
5	B1	O25	-	+	-	+	-	-	+	+	-	+	-	+	+	+	S
6	A	O25	-	+	-	-	-	-	-	+	-	+	+	+	-	+	W
7	B2	O12	-	+	-	-	+	-	-	+	-	+	-	+	-	+	N
8	B2	O15	-	+	-	-	-	-	-	+	-	+	-	+	-	+	W
9	A	O2	-	+	-	-	+	-	-	+	-	-	+	+	-	+	N
10	B2	O1	-	-	-	+	-	-	-	+	-	+	-	+	+	+	W
11	D	O18	-	+	-	-	+	+	-	+	-	+	-	+	+	-	W
12	B2	O75	-	+	-	+	+	+	-	+	-	+	+	+	+	+	W
13	B2	O4	-	+	-	+	+	-	-	+	+	+	-	+	+	+	W
14	A	O16	-	+	-	+	-	-	-	+	-	+	-	+	+	+	N
15	B1	O25	-	+	-	-	-	-	-	+	-	+	-	+	+	+	N
16	B2	O75	-	+	-	+	-	-	+	+	-	+	+	+	+	+	W
17	B2	O25	-	+	-	+	-	-	+	+	-	+	-	+	+	+	M
18	B2	Nd	-	+	-	+	-	-	-	+	-	+	-	+	-	+	N
19	D	O4	-	+	-	-	-	-	-	+	+	+	-	+	+	+	N
20	D	O1	-	+	-	+	-	+	-	+	-	+	-	-	+	+	W

In this table Nd: Non detect; S: Strong; M: Moderate; W: Weak; N: Negative.

Table 5. *The biofilm formation among UPEC isolates, ODc: OD of control.*

Biofilm level	OD cut off	No (%)
Strong	OD>4 × ODc	1 (5)
Moderate	2 × ODc<OD ≤ 4 × ODc	4 (20)
Weak	ODc<OD ≤ 2 × ODc	8 (40)
No biofilm	OD ≤ 0.08324	7 (35)

Discussion

Healthcare associated UTI is associated with higher therapy failure and thus patients should be more under care to avoid morbidity [17,18]. In this study, three patients had kidney failure which developed pyelonephritis with the CTX-M1 producing UPEC (ANOVA test, p>0.05). Furthermore, 20/95 of CTX-M1 producing UPEC isolates had caused this type of infection which was included in this study. Previous studies have exhibited that the rate of ESBL-producing *E. coli* has increased in Iran [19-21]. *CTX-M-1* producing isolates are predominant in the Middle East region, Eastern Europe, western areas of Russia, India, United States and Australia. We found that potential risk factors for spread of pyelonephritis caused by *CTX-M-1* producing *E. coli* were urinary tract infection (70%, n=14), followed by kidney impair (15%, n=3), leukemia (10%, n=2) and bladder stone (5%, n=1) which are approximately similar to risk factors from previous surveys including prior use of antimicrobials and beta-lactams, prior UTI and hospitalization caused by ceftriaxone-resistant organisms [22,23]. In this study, the rate of fluoroquinolones resistance among *E. coli* isolates from pyelonephritis was 75% which is a concern. The *CTX-M-1* enzyme is the predominant encoding gene responsible for ESBL production by *E. coli* isolates worldwide. There is no previous study for the assessment of *CTX-M-1* producing *E. coli* from pyelonephritis in Iran.

Furthermore, using ANOVA test analysis, among virulence genes, 19 (95%) isolates amplified the all *fimH, fyuA* and *traT* genes (p<0.001), followed by *iutA* (90%, n=18) (p<0.001), *papII* (75%, n=17) (p=0012), *kpsMII* (60%, n=12), *ompT* (55%, n=11), *ibeA* (30%, n=6), *sfa* (20%, n=5), and *iroN=iucD=afaC*=15%, (n=3), but none for *papI* and *papIII*. It was found that among *fimH, fyuA* and *traT* positive isolates, 14/19, 12/19 and 13/19 of them were resistant to ciprofloxacin, respectively. Four moderate biofilm producers in this study amplified the all *fimH, fyuA* and *traT* genes and three amplified *iutA, papII, ompT, kpsMII, sfa* and *ibeA* genes. This result exhibits the role of adhesion genes in the ability of UPEC isolates for biofilm formation. Moreover, 11/19, 13/19 and 12/19 of them were resistant to aminoglycosides. Furthermore, 11/18 and 12/18 of iutA positive isolates were fluoroquinolones and aminoglycoside resistant UPEC. Similarly, a high number of UPEC isolates from South Korea were fyuA positive [24]. Another study in Mexico showed that *kpsMII* and *fimH* genes were high among UPEC isolates similar to this study [25]. In Tunisia, 68% and 41% of UPEC were fimH and pap positive [26]. In Jahrom city, the prevalence of *papG, afaC, sfa, fimH, ibeA*, and *iucD* were 53.3%, 51.7%, 53.3%, 56.7%, 20%, and 73.3%, respectively which were mostly similar to findings of the present survey [27]. There was a difference between children and adults regarding the prevalence of *hlyA, kpsMII* and *ibeA* among UPEC [28], but this was concluded in our study. Momtaz in Shahrekord found that *fimH* virulence gene was detected in 86.17% of UPEC isolates, and 2.43% of them belonged to the O1, O2, O6, O7 and O16 serogroups [29]. A great number of *E. coli* virulence factors are encoded by Pathogenicity Islands (PIs) and are known as "Pathogen Associated Molecular Patterns" (PAMPs) [30,31], however the exact prevalence or role of adhesive virulence factors in the pathogenesis of pyelonephritis is not obvious. It was shown that extra-intestinal isolates were more efficient colonizers of the digestive tract compared to non-pathogenic strains [32].

In this study, among 10 isolates belonged to B2 phylogroup, all were *iutA, traT* and *fimH* positive and 9 of them were *fyuA* positive and moreover the major serogroups in this phylogroup were O75 (n=2), followed by one isolate belonging to each O12, O18, O25, O4, O1 and O15, and two isolates were not typed by this method. There was no significant relation between phylogroups and serogroups among UPEC isolates. Among four isolates belonging to each phylogroups A and D, serogroups, O25 and O1 (n=2, 50%) were mostly detected respectively. therefore, it is proposed that more isolates are needed to decide surely about the relationships between phylogroups and serogroups of UPEC isolates. Furthermore, one strong biofilm producer was detected in this study and was belonged to the phylogroup B1 and also contained *papII, ompT, iucD, fyuA, fimH, traT, kpsMII* and *iutA* genes and exhibited alpha-hemolysis. Moreover, three moderate-biofilm producing isolates were belonged to the D (n=1) and B2 (n=2) phylogroups. In this study, the phylogroups B2 was predominant among *E. coli* isolates from pyelonephritis as exhibited in previous studies [33-35]. From our results and scarce previous studies it is concluded that the adhesive factors have an important role in the colonization, biofilm formation and dissemination of strains in the body. There a limitation in this study where we did not assess the expression level of the adhesive or other virulence factors related to the pyelonephritis. Several drawbacks in this study include small sample size and studied patients, and lack of gene expression assay for detected virulence factors.

Conclusion

The antibiotic resistance rate was high among isolates of *E. coli* from pyelonephritis. The *fimH, fyuA* and *traT* genes

($p<0.001$), followed by *iutA* (90%, n=18) ($p<0.001$), *papII* (75%, n=17) (p=0012) and *kpsMII* (60%, n=12) adhesive virulence factors was highly detected, possibly exhibiting their role in the pathogenesis of pyelonephritis as their prevalence was high. Furthermore, fluoroquinolones and aminoglycoside resistance was highly determined exhibiting a threat due to the presence of pathogenic and drug-resistant UPEC in pyelonephritis. Continued and vigilant surveillance is necessary for the monitoring the spread of antimicrobial resistance among uropathogens.

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*Correspondence to

Shahin Najar-Peerayeh

Department of Bacteriology

Tarbiat Modares University

Iran