

The Prevalence of Virulence Factors Among Uropathogenic *Escherichia coli* Strains Isolated From Different Hospitals in Kurdistan Region-Iraq

Narmin S. Merza*, Jaladet M. S. Jubrael

Scientific Research Centre, Faculty of Science, Dohuk University, Duhok, Iraq

Abstract

In this study, 150 isolates of Uropathogenic *Escherichia coli* (UPEC) strains have been collected from different major hospitals in Kurdistan region and showed high resistant rates to most tested antibiotics accounting for 98%. There was no isolate that had demonstrated sensitive to all antibiotics, but they manifested with a wide range of resistance to most tested antibiotics ranging from resistance to just two antibiotics (2%) to resistance to 15 out of 16 tested antibiotics (9.3%). Imipenem was found as the most potent at all other antimicrobial agents with the resistance rate (4.7%), whereas Tetracycline, Ampicillin, Amoxicillin and Amoxicillin / Clavulanic acid had the least effect on UPEC strains with resistant rates of 83.3%, 92.6%, 90.6% and 90% respectively. The resistance patterns of Chloramphenicol, Norfloxacin, Ciprofloxacin and Nalidixic acid were 30%, 48.6%, 52.6% and 78% respectively. Amikacin, Gentamicin, Trimethoprim and Trimethoprim/ sulfamethoxazole antibiotics also showed low effect on tested isolates with resistant rates 46%, 70.7%, 77.3% and 73.3%. Cephalosporins including; Cefixime, Cefotaxime, Ceftriaxone with resistance rates of 78%, 78% and 71.3% respectively. The results of the detection five virulence related genes including; (*cnf*, *hly*, *sfa*, *afa*, and *pai*) revealed that fifteen of these isolates accounting (10%) lacked any tested virulence markers. *pai* as a marker for presence of pathogenicity island was the most predominant marker among all other virulence markers accounting 110(73.3%) followed by *cnf* and *hly* accounting 64(42.7%) and 61(40.6%) respectively, while the prevalence of *sfa* gene is found with the rate 34(22.7%) and *afa* with 27(18%).

Keywords

Virulence Factors, Uropathogenic *Escherichia coli* (UPEC), Antibiotics

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1. Introduction

Escherichia coli considered as one of the most important opportunistic pathogen associated with the urinary tract infections (Blanco *et al.*, 1996). These Infections are usually occurring due to the movement of UPEC strains from the intestinal tract or in some cases from vagina to the urinary tract (Xie *et al.* 2006). UPEC was believed to exhibit a wide variety of virulence properties that help them to colonize host mucosal surfaces and circumvent host defenses to allow invasion of the normally sterile urinary tract (Moblely, 2000).

Adhesins are one of the most important virulence factor which allow these pathogens to attach to the urinary tract epithelium enabling them to resist the hydrodynamic forces of urine flow and also stimulate UPEC entry into host epithelial cells to promote the UPEC survival and establishment within the urinary tract (Bower *et al.*, 2005). These adhesins include; fimbrial (type 1, P, S, Dr) and a fimbrial adhesins (*afa*) which are considered as unique virulence traits of (UPEC) (Miyazaki *et al.*, 2002). Toxins are important virulence factors in UPEC strains which cause an inflammatory response and a possible pathway for urinary

* Corresponding author

E-mail address: Alzebary83@yahoo.com (N. S. Merza)

tract infections (UTI) symptoms. The most important secreted virulence factor of UPEC strains is a lipoprotein called α -haemolysin (HlyA) (encoded by *hlyA* gene) is a pore forming cytolysin and lyses erythrocytes (Wiles and Mulvey, 2013) thereby facilitating the release of nutrients and other factors like iron, that have a critical role in bacterial growth (Wiles *et al.*, 2008). It also mediated inflammation, tissue injury, impaired host defenses such these activities may contribute kidney damage seen in pyelonephritis (Slavchev *et al.*, 2008). It has been found that this enzyme encoded by 50% of UPEC strains responsible of pyelonephritis (Wiles and Mulvey, 2013). On the other hand Cytotoxic Necrotizing Factor 1 (CNF1) (encoded by *cnf* gene) is another most important toxin produced by one-third of all pyelonephritis strains and may also be involved in kidney invasion (Bien *et al.*, 2012). It has been found that CNF1 can promote apoptosis of bladder epithelial cells, possibly stimulating their exfoliation and enhancing bacterial access to underlying tissue and facilitating the dissemination and persistence of UPEC within the urinary tract (Wiles *et al.*, 2008).

Because of prevalence and resistance to some antimicrobial agents such as ampicillin and trimethoprim-sulfamethoxazole which have been used as a first-line treatment for uncomplicated UTI, cephalosporines and quinolones have been used as alternative choice for this purpose (Hryniewicz *et al.*, 2001). However, the emergence of β -lactamases producing *E. coli* especially extended spectrum β -lactamases (ESBL) in both hospital and community settings create a challenge for microbiologists and clinicians due to difficulty in detection, reporting and treatment. (Luzzaro *et al.*, 2007). Moreover, multidrug resistant (MDR) UPEC has increased worldwide especially among extended-spectrum beta-lactamase (ESBL) producers

(Manges *et al.*, 2001). The aim of this study was to detection, analyze the prevalence of five virulence determinants (*cnf*, *hly*, *sfa*, *afa*, and *pai*) among UPEC collected from different hospitals in Kurdistan region-Iraq and examine their susceptibility to different antimicrobial agents to guide the initial empirical treatment.

2. Material and Methods

One hundred fifty (50 isolates from each province) of UPEC stains have been characterized and confirmed previously were collected from different settings including; at the Microbiology laboratories at the Rezgari, Teaching hospital and Azadi general hospitals in Erbil, Sulymani and Duhok provinces respectively.

I. Antimicrobial susceptibility testing

All isolates were subjected to antibiotic sensitivity testing by the disc diffusion method on Mueller-Hinton agar according to the National Committee for Clinical Laboratory Standards and Manual of Antimicrobial Susceptibility Testing guidelines (CLSI, 2007). Sixteen antibiotics have been used in this study, supplied by (Bioanalyse / Turkey) including; Amikacin (10mg), Gentamicin (30mg), Cefotaxime (10mg), Ceftriaxone (10mg), Cefexime (5mg), Chloramphenicol (30mg), Amoxicillin / Clavulanic acid (20/10mg), Ampicillin (10mg), Amoxicillin (25mg), Nalidixic acid (30mg), Ciprofloxacin (10mg), Norfloxacin (10mg), Trimethoprim (10mg), Trimethoprim / Sulfamethoxazole (75) μ g, Tetracycline (10mg) and Imepenem (10mg).

II. Oligonucleotides sequences:

The primers listed below have been used in this study Operon Incorporation (USA) Technologies.

Table (1). Represents primers used in this study.

Virulence Gene	Oligonucleotide sequence (5'–3') Forward and Reverse	Size of amplicons	Reference or source
Hly	F-AGATTCTGGGCATGTATCCT R- TTGCTTTGCAGACTGTAGTGT	565bp	Mladin et al., 2009
Cnf	F- AAGATGGAGTTTCCTATGCAGGAG R- CATTCAAGATCCTGCCCTCATTATT	498 bp	Chapman et al., 2006
Sfa	F-GTGGATACGACGATTACTGTG R-CCGCCAGCATTCCCTGTATTC	240bp	
Afa	F-GCTGGGCAGCAAAGTATAACTCTC R-CATCAAGCTGTTTGTTCGTCGCCCG	750bp	Le Bouguenec et al., 1992
Pai	F-GGACATCCTGGTACAGCGCGCA R-TCGCCACCAATCACAGCCGAAC	930bp	Oliveira et al., 2011

III. Extraction of genomic DNA and detection of virulence markers.

Genomic DNA has been extracted from 150 UPEC strains using commercial kit (DNP TM High yield DNA Purification). Five primers (*pai*, *hly*, *cnf*, *sfa*, *afa*) were used for the detection of virulence related genes including;

patogeneicity island, hemolysin, cytotoxic necrotizing factor-1, S-fimbrial adhesion, afimbrial adhesion, respectively among these isolates Table (2). The amplification reactions for each gene were carried out in 25 μ l volumes containing 2.5 μ l of 10XPCR buffer, 2.5 μ l dNTPs (0.2mM), 1 μ l of each primer including forward and reverse (10pmol/ μ l except for *hly* 30pmol/ μ l), 0.2 μ l *Taq* Polymerase (1unit) and 2 μ l (25-

50ng) of genomic DNA. The volume is completed to 25µl by adding 15.8µl of sterile de-ionized distilled water. The Amplification conditions of each gene were illustrated in

Table (2). After amplification, the presence of the PCR product was confirmed electrophoretically using 1.5% (w/v) of agarose in Tris/Borate/EDTA (TBE) Buffer.

Table (2). Represents amplification conditions of PCR- based analysis for the detection of different virulence markers among *Escherichia coli* isolates using *afa*, *cnf*, *hyl*, *sfa*, and *pai* primers.

Primer	Initial denaturation	Denaturation	Annealing	Extension	Final Extension	Ref.
Afa	94 °C 5 min. 1 cycle	94°C 1 min 30 cycles	63°C 1 min	68°C 3 min	72°C 7 min. 1 cycle	Le Bouguenec et al., 1992
	95°C	94°C	68°C	68°C	72°C	
Cnf	3 min. 1 cycle	30 sec. 25 cycles	30 sec.	4 min.	10min. 1 cycle	Chapman et al., 2006
	94°C	94°C	55°C	72°C	72°C	
Hyl	4 min. 1 cycle	30 sec. 30 cycles	30 sec.	1min	5 min. 1 cycle	Mladin et al., 2009
	95°C	94°C	63°C	68°C	72°C	
Sfa	3 min 1 cycle	30 sec. 30 cycles	30 sec.	4 min.	10min. 1 cycle	Chapman et al., 2006
	94°C	94°C	63°C	72°C	72°C	
Pai	1 min 1 cycle	1 min 30 cycles	30 sec.	1.30 or 1.5 min.	5 min. 1 cycle	Oliveira et al., 2011
	94°C	94°C	63°C	72°C	72°C	

3. Results and Discussion

In this study, it was found that there was no isolate that had demonstrated sensitive to all antibiotics, but they manifested a wide range of resistance to most tested antibiotics. Imipenem antibiotic as one of carbapenems agents was found to be the most potent of all other antimicrobial agents with a resistance rate of 4.6%. Somehow similar rates have been reported and suggested that this rate should be considered since these agents are preferred in empiric therapy for serious bacterial infections caused by beta-lactam resistant bacteria (Paterson, 2006). Chloramphenicol may be considered as the second most effective antibiotic against these isolates with a resistant rate of 30%. The tested UPEC isolates were exhibited a high resistant to most tested antibiotics including; Ampicillin, Amoxicillin, Amoxicillin / Clavulanic acid, and Tetracycline with resistant rate of 92.6%, 90.6%, 90%, and 83.3%, respectively. Comparative studies including older antimicrobials are limited, but some of these agents remain useful for the treatment of selected patients. Amoxicillin or ampicillin remains the therapy of choice for susceptible enterococci (Nicolle, 2005) Trimethoprim and Trimethoprim/ sulfamethoxazole which traditionally considered as a front-line therapy for UTIs showed low effect on these isolates with resistance rate of 77.3% and 73.3%. The utility of these antibiotics have decreased in certain areas due to increasing resistance (Hilbert, 2011). The resistance pattern of tested isolates to Nalidixic acid, Ciprofloxacin and Norfloxacin was found in order; 78%, 52.6% and 48.6% respectively. Aminoglycoside agents including; Gentamicin and Amikacin antibiotics also showed low effect on tested isolates with resistant rates 70.7% and 46% respectively to these

antibiotics. These isolates also showed high resistant rates to the third generation of Cephalosporins including; Cefixime, Cefotaxime, Ceftriaxone with resistance rates of 78%, 78% and 71.3% respectively. The widespread use of third-generation cephalosporins as the driving force behind the emergence of ESBL-producing organisms has been shown in many studies (Paterson, 2006). It has been found that the genes that encode ESBLs are frequently found on the same plasmids as genes that encode resistance to aminoglycosides and trimethoprim- sulfamethoxazole (Yasufuku *et al.*, 2011). This means that ESBL-producing are commonly multidrug resistant, which poses a particular challenge for the treatment of nosocomial infections. Inappropriate empiric antimicrobial treatment for nosocomial l- or community- acquired infections has been reported to contribute to significantly greater mortality rates in the intensive care unit. Besides, inadequate antimicrobial treatment of infection was the most important independent determinant of hospital mortality (Paterson, 2006). Another important mechanism facilitating the increase in antimicrobial-resistant UTIs is the introduction and clonal expansion of competitive, resistant *E. coli* strains in the community (Nordstrom *et al.*, 2013).

The results in the present study may also come in agreement with other results reported in the regional and especially in developing countries to different antibiotics resistance. For example, in a study reported in Turkey by Nazik *et al.*, 2011 showed that the UPEC isolates displayed high resistance to amoxicillin-clavulanic acid 77%, Trimethoprim / Sulfamethoxazole 76%, Norfloxacin 70%, ciprofloxacin 68%, gentamicin 51% and all isolates were found susceptible to Imipenem. In another study recorded in Iran showed the resistance patterns of UPEC to Trimethoprim /

Sulfamethoxazole 75%, Tetracycline 72.8%, Nalidixic acid 60.7%, Norfloxacin 50.7%, Ciprofloxacin 47.6%, Gentamicin 33.6%, Chloramphenicol 20.7%, Amikacin 12.1% and Imipenem 1.4% (Rezaee *et al.*, 2011).

For many years, amoxicillin/ clavulanate, cephalixin, trimethoprim / sulfamethoxazole or fluoroquinolones (for example, ciprofloxacin) were used as first line treatment of uncomplicated UTI (Totsika *et al.*, 2012). However, resistance of *E. coli* to each of these antibiotics is now substantial and in many parts of the world these drugs can no longer be used as empiric therapy (Gupta *et al.*, 2011). In almost all UTI cases, empirical antimicrobial treatment is initiated before the laboratory results of the urine culture are available (Dash *et al.*, 2013). Misuse and self-medication in many countries including ours may be considered a major problem as antibiotics could be purchased without any prescription. Up to 95% of UTI cases are treated without bacteriological investigations (Warren *et al.*, 1999). There is a need to better define strategies to prevent emergence and more studies in this area are clearly required. Clinicians also must depend on more laboratory guidance, while laboratories must provide resistance pattern data for optimal patient management more rapidly. Many reports suggested that the resistance of *E. coli* strains to commonly used antimicrobial agents has made the clinical management of UTI complicated by increasing incidence of their infections (Van de *et al.*, 2008). Most of these studies agreed that there is a need to improve on infection control methods (Mukherjee *et al.*, 2013).

In this study all 150 UPEC isolates were subjected to PCR techniques to determine the prevalence rates of virulence related genes including; *cnf*, *hyl*, *sfa*, *afa*, and *pai* marker and their distribution in Erbil, Sulymani and Dohuk provinces.

The results of the tested isolates from Erbil province are revealed the high prevalence of *pai* marker accounting for 76% while *cnf* gene accounted for 54%; whereas *hyl* gene displayed among 50% of these isolates. The prevalence of *sfa* and *afa* was the lowest among these isolates with rates 14% and 12% respectively.

The prevalence of the five virulence related genes among UPEC isolates in Sulymani province displayed somehow similar pattern as those in Erbil province. *Pai* marker accounted for 74%, whereas 46% of these isolates exhibited *cnf* gene; 48% of these isolates displayed *hyl* gene, while the presence of *sfa* gene among these isolates accounted for 38% and *afa* was the lowest virulence genes prevalence among these isolates with a rate of 10%.

Some Sulymani isolates that produced *pai* amplicon samples were produced an extra band with a molecular weight of 650bp. Figure (1). The presence of extra band was also

reported in Duhok province among *E. coli* isolated from urine using the same primer for detection of *pai* as a marker for pathogenicity island (Rasol, 2013). This increases the likelihood of presence of more than one annealing site for this marker in different regions on the genomic DNA. This was supported by a study dealing with the sequencing of UPEC genome which has revealed a highly mosaic structure with numerous pathogenicity islands (PAIs) integrated at multiple sites in the genome (Hilbert, 2011).

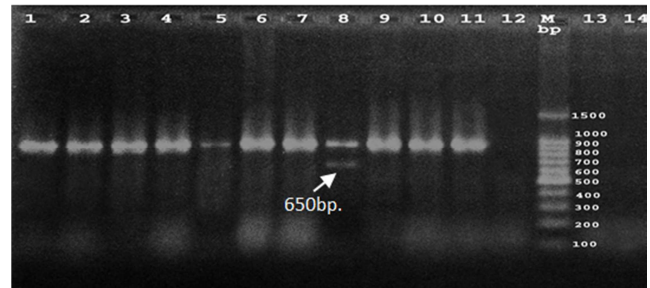


Figure (1). Represents PCR amplification of UPEC strains isolated from Sulymani Province produced *pai* amplicon with molecular weight 930bp. Sample (8) produced an extra band with m.wt. 650bp. Electrophoresis was performed on (1.5%) agarose gel and run with 3V/Cm, for 2hours. Lanes M contained DNA molecular weight marker (100bp).

the results of the prevalence virulence related genes among UPEC isolates collected from Duhok province also displayed a high rate of *pai* marker accounting for 70% whereas *cnf* gene exhibited with a rate of 28%. These isolates also displayed *hyl* gene accounting for 24%, while *afa* gene accounted for 32%. Finally, *sfa* gene was found to be the lowest virulence gene with a rate of (16%).

From the overall results of virulence related genes prevalence among UPEC isolates collected from three provinces in Kurdistan Region, it is found that only 10% of these isolates lacked the presence of any of the tested virulence markers.

The results of isolates that harbored these markers are summarized in Table (3) From these results, it became clear that *pai* was the most predominant marker among all other virulence related genes accounting for 73.3% followed by *cnf* and *hyl* accounting for 42.7% and 40.6% respectively, while the prevalence of *sfa* gene was found with rate 22.7% and *afa* in rate 18%.

Table (3). Represents the prevalence of virulence markers among UPEC strains collected from Erbil, Sulymani and Duhok provinces.

Province	Virulence markers				
	<i>Pai</i>	<i>Cnf</i>	<i>hyl</i> (%)	<i>sfa</i> (%)	<i>Afa</i> (%)
Erbil	38 (76)	27(54)	25(50)	7(14)	6(12)
Sulymani	37 (74)	23(46)	24(48)	19(38)	5(10)
Duhok	35(70)	14(28)	12(24)	8(16)	16(32)
Total	110(73.3)	64(42.6)	61(40.6)	34(22.6)	27(18)

These rates are in agreement with a number of related published studies for example, Oliveira *et al.*, 2011 results who also found that 10% of UPEC strains lacked these virulence related genes. In another study conducted in Duhok province, it was shown that the prevalence of virulence genes among *E. coli* isolated from urine exhibited *cnf* with rate 67%, while *pai* marker with a rate of 48%, *sfa* 40.4% and *afa* 38% (Rasol, 2013). Karimian *et al.*, 2012 also determined the virulence factors of *E. coli* isolated from urine and found that their prevalence rates were also different and as following; *cnfI*, *hlyA*, *afa* genes was, 79.67% 50.4%, 50.4%, 8.13% respectively.

The high prevalence of *pai* as a marker for (PAIs) which may carry urovirulence genes among UPEC strains in these results was also consistent with other recent studies. For example, Brzuszkiewicz *et al.* 2006 results showed that the genomic differences between UPEC strains were mainly restricted to large (PAIs). Navidinia *et al.*, 2012 in another study reported that (PAIs) were enriched among UPEC isolates and confirmed the prevalence of eight PAI markers in *E. coli* strains isolated from the urine of children with UTI.

The high incidence of both *hlyA*, *cnfI* genes corresponding with incidence of *pai* marker obtained in this study may reflect the fact that there was a high association between these genes which are often linked and known to carry PAIs. Similar studies have been reported reflecting this type of association (Smith *et al.*, 2008 and Starčić-Erjavec *et al.*, 2008). Furthermore, Hacker and Kaper, 2000 suggested that *sfa* genes are also present on PAI in UPEC strains. Thus; PAIs may contribute to urovirulence and may potentially serve as targets for interventions.

The results of this study (Table 3) also revealed that there were differences in the prevalence rates of these genes among three provinces. The differences in prevalence of UPEC virulence genes due to geographic region was also reported by Rasol., 2013 in Duhok province and by Karimian *et al.*, 2012 in Iran who suggested that the climate of each regions, customs, food diets, the levels of public health, hospital's health may be considered as factors that attribute to the presence of variety in the prevalence rates of virulence genes of UPEC strains among different regions.

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