Original Paper

Some Virulence Factors Genes and Phylogenic Groups of Uropathogenic *Escherichia Coli* (UPEC) Isolated from Karbala Patients

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Abstract

Background: Uropathogenic *Escherichia coli* (UPEC) is most important causative agent of urinary tract infection (UTI). This disease is still big health problem among human population in spite of scientific progress.
Aims: aims of this study were to investigate the isolates of UPEC in patients with UTI, characterize the virulence factors and phylogenic groups among clinical isolates.
Materials and Methods: A total of 150 urine specimens were collected from patients with UTI after establishing the diagnosis via investigation and clinical diagnosis in Al-Hussein Hospital, Gynecology and Obstetrics Hospital, and Children Hospital in Karbala province during the period from November 2013 to April 2014. Thirteen stool samples from apparently healthy individuals as control group were included. All *E. coli* isolates were identified by standard methods. Molecular diagnosis and characterization for virulence and phylogenic genes were detected by Multiplex PCR technique in medical researches laboratory of medical college at Karbala University.
Results and Discussion: This study showed that 56/150 patients (37.3%) had UTI due to UPEC. The isolated UPEC were examined for the presence of the adhesion genes (*Papc*, *Afa* and *Sfa*) and the phylogeny groups' genes by Multiplex PCR assays. The primers encoding virulence genes were tested against to all the 56 UPEC and 13 stool isolates. It was found that 27(48.21%) urine isolates carried just one virulence factor, of them 16(28.57%) isolates carrying *papc*, 2(3.57%) isolates carrying *afa* and 9(16.07%) isolates carrying *sfa*. No isolates out of 56 had carried all the three genes. However, no virulence factor was found in 24 (42.85%) isolates. *Pap* gene was found mostly in 7 isolates among patients aged (1-10 years) and (21-30 years); while, the prevalence of *sfa* gene was found in 7 isolates (50%) among patients aged (31-40 years); however, two isolates have *afa* gene it was in one isolate in each age group (1-10 years) and (41-50 years).
The percentage of phylogenic groups of UPEC isolates belonging to B2 followed by D, A and B1, were 39.28%, 33.92%, 16.07% and 10.71% respectively; while in control group the results were A (53.84%), B2 (23.07%), B1 (15.38%) and D (7.69%).
Conclusions: Its concluded that the genes of virulence factors of UPEC isolates in patients with UTI were higher than in healthy persons. Significantly, *E. coli* strains responsible for UTI were far more likely to be members of phylogenic groups B2 or D than A or B1.
Key Words: Virulence factor genes, Phylogenic groups, UPEC and UTI.

Introduction

Urinary tract infections (UTIs) are among the most common infections that affect humans [(1)]. Fifty percent of all human population especially in women will experience at least one UTI in their lifetime and, of those, about 25% will have one or more recurrent infections [(2)]. In 90% of uncomplicated UTIs the most common bacterium is *Esherishia coli* [(3)]. *E. coli* strains causing disease outside the gastrointestinal tract have been named extraintestinal pathogenic *E .coli* (ExPEC). The uropathogenic *E .coli* (UPEC) is the most pathogen among ExPEC in humans

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[4]. UTI risk factors include extremes in age, female gender, bladder catheterizations, nephrostomy tubes, prior antibiotic administration, diabetes, mechanical obstructions, and anatomical abnormalities that promote urinary stasis (eg, neurogenic bladder, pregnancy, kidney stones). Women are at elevated risk due to the short distance between the anus and urethra, as well as a short urethra emptying the bladder [5].

The most important amongst these, probably, are the adhesins that help them to adhere to uroepithelium and this property was recognized decades ago [6]. These include type 1, S and P fimbriae, and adhesins like Dr. The type 1 fimbriae are widely prevalent and are probably involved in colonization of lower urinary tract [7]. Phylogenic analyses by multilocus enzyme electrophoresis have shown that *E. coli* strains fall into 4 groups: A, B1, B2, D. The nonpathogenic *E. coli* strains are typically from phylogenic groups A and B1. ExPEC, particularly UPEC strains, frequently are members of group B2 or group D and often exhibit specific O:K:H serotypes [8].

At the most basic epidemiological level, potential uropathogenic virulence factors have been identified by comparing the prevalence of a bacterial property of interest among urinary isolates with that among fecal strains from healthy control subjects [9]. Using phylogenetic grouping reported detailed analysis about phylogenetic background virulence attributes of UPEC strains isolates from uropsis and cystitis [10].

The aims of this study were to investigate the occurrence of UPEC in patients with UTIs, characterize their virulence factors and relevant genes, and phylogenic groups among clinical isolates of *E. coli* in Karbala human population.

Materials And Methods

Study patients:

One hundred and fifty patients were enrolled where they were suffering from UTI and had attended to AL-Hussein hospital, Gynecology and Obstetrics hospital and Pediatric hospital during the period extended from November 2013 to April 2014.

Specimen collection:

- **Urine Sample:** Mid-stream urine samples were collected from 150 UTI patients at morning in sterilized screw-capped container, a calibrated loopful urine samples had inoculated on the culture media (MacConkey agar, Blood agar, EMB agar) and incubated aerobically at 37C° for 18 hours.
- **Stool Sample:** Thirteen stool samples were collected from healthy persons (as control group) in sterile container. A loopful stool sample had inoculated on MacConkey agar and incubation at 37 C° for 18 hr.

Identification of UPEC isolates: *E. coli* isolates were identified to species level based on morphological properties, cultural characterization and standard biochemical tests [11, 12]. The identification was confirmed using API 20E strips (Himedia, India).

DNA extraction of UPEC isolates: Total DNA was extracted by boiling method according to manufacturer directions (Promega, USA). UPEC DNA templates were subjected to multiplex PCR (M-PCR) using 6 sets of primers targeting two groups of genes: the first group listed in Table-1 to detect the virulence genes and the second group listed in Table-2 to determine phylogenetic group of genes.

Detection of UPEC virulence genes by PCR: Genes of virulence factors including *Pap*C, *Afa* and *Sfa* were analyzed by the PCR assay. Assembling Multiplex PCR (M-PCR) materials were done according to the procedure of Promega Corporation (USA).

Detection of phylogenic groups by PCR: PCR was conducted to determine the phylogenetic grouping of the UPEC isolates by targeting two genes, *Chu*A,

*Yja*A and anonymous DNA fragment *TspE4.C2* [13].

Statistical analysis: Data were analyzed with SPSS (Statistical Packages for Social Sience) version-18. And chi-square test used to identify the correlation between the variables of this study.

Ethical approval and consent: The necessary ethical approval from all Karbala hospitals that included in the present study was obtained. Moreover, all subjects involved in this work were informed and the agreement was obtained from each one before the collection of samples.

Results

Identification of *E. coli* isolates: The present study includes one hundred fifty patients who had complaints related to urinary tract infection, of them ninety patients were females and sixty patients were males. Fifty six isolates of *Escherichia coli* have been isolated and identified according to morphological characterization and biochemical reactions. These 56 isolates were isolated from UTI patients; and 13 isolates of positive stool cultures were isolated from healthy people (control group).

Molecular detection of UPEC virulence genes: Virulence genes of adhesion factors in UPEC isolates *Pap*C, *Afa* and *Sfa* were analyzed by the multiplex PCR assay. Our results showed that the frequencies of these adhesion genes; *Pap*, *Sfa* and *Afa* among UPEC isolates were 22, 14 and 2 respectively. Out of 56 UPEC isolates, 27 isolates carried just one virulence factor, of them 16 isolates carrying *Pap*, 9 isolates carrying *Sfa* and 2 isolates carrying *Afa*. No isolate out of 56 had carried all the three genes. The *Pap* gene was commonly found in patients with age groups (1-10 years) and (21-30 years). *Sfa* gene was found among patients with age (31-40 years), while age group (1-10 years) has not this gene (0.0%).

Two isolates have *Afa* gene, it was among patient aged (1-10 years) and (41-50 years), while the other age groups were not founded this gene (0.0%) see Table-3.

To assess the association of virulence genes (*Pap*, *Afa* and *Sfa*) with each of gender and source of bacteria, statistical analysis by chi-square test was performed as in below in Table-4.

In our results, the virulence genes of UPEC adhesion factors such as *Papc*, *Afa* and *Sfa* were detected by Agarose-Electrophoresis technique; shown in Figure-1, Figure-2 and Figure-3.

Table 1. Primers sequences of Virulence genes

Genes	Primer sequences(5́-3́)		Product size (bp)	References
PapC	F	GACGGCTGTACTGCAGGGTGTGGCG	328	Soto *et al.*,2011 (9)
	R	ATATCCTTTCTGCAGGGATGCAATA		
Sfa	F	CTCCGGAGAACTGGGTGCATCTTAC	410	Soto *et al.*, 2011 (9)
	R	CGGAGGAGTAATTACAAACCTGGCA		
Afa	F	CGGCTTTTCTGCTGAACTGGCAGGC	672	Soto *et al.*, 2011 (9)
	R	CCGTCAGCCCCCACGGCAGACC		

Table 2. Primers sequences of phylogenic group markers

Genes	Primer Sequence(5'-3)		Product Size(bp)	Reference
ChuA	F	GACGAACCAACGGTCAGGAT	279	Clermont *et al.*, 2000(14)
	R	TGCCGCCAGTACCAAAGACA		
Yja	F	TGAAGTGTCAGGAGACGCTG	211	Clermont *et al.*, 2000
	R	ATGGAGAATGCGTTCCTCAAC		
TspE4.C2	F	GAGTAATGTCGGGGCATTCA	152	Bonacorsi *et al.*, 2000(15)
	R	CGCGCCAACAAAGTATTACG		

Molecular Detection of phylogenic groups of UPEC: In the present study among of 56 UPEC isolates frequencies of (A, B1, B2 and D) were 9 (16.07%), 6 (10.71%), 22 (39.82%) and 19 (33.92%) respectively in patients with UTI while the results of control group were 7 (53.84%), 2 (15.38%), 3 (23.07%) and 1(7.69%) respectively among 13 control isolates. Details are given in Table-5, and Figure-4, Figure-5 and Figure-6.

The phylogenetic grouping of UPEC isolates was made on the basis of the presence of specific PCR-amplified fragments as follows: group A (*chu* A - , *yja* A +/-, *TspE4C2* -), group B1 (*chu* A -, *yja* A+/-, *TspE4C2* +), group B2 (*chu* A+, *yja* A +, *TspE4C2* +/–) and group D (*chu* A+, *yja* A -, *TspE4C2* -/+), each group showed two different patterns as shown in Table-6.

When the relationship of phylogenicity and virulence was studied, the prevalence of virulence genes was showed in Table-7. *Pap* C was found higher in phylogenetic group B2 isolates, followed in group D. *Sfa* gene was prevalent in phylogenetic group B2. *Afa* gene was absent in groups B2 and B1 isolates.

To explain the correlation between each phylogenic group of UPEC isolates and gender of UTI patients, chi-square test was performed for interpretation of our results as in Table-8.

The relationship between phylogenic groups of *E. coli* isolates in UTI patients and in health group, see below Table-9.

Agarose gel electrophoresis is used for study and detection of phylogenic group genes in local isolates of *E.coli* isolated from patients with UTI and from healthy persons, and grouping these isolates into major standard groups of genotyping (genes: *chu* A, *yja* A and DNA fragment *TSPE4.C2*).

The phylogenic group genes of UPEC isolates such as *chu A, Yja A* and *Tspe4.c2* were detected by Agarose-electrophoreses technique; shown in Figure-4.

Discussion

In this study, 56 UPEC were isolated from 150 patients in Karbala city, 43 (76.79%) infections occur in female patients and 13 (23.21%) infections in male patients. Similar observation regarding the relative occurrence of the gender groups has been documented in Hilla/Iraq [20], and other developing countries as well as developed countries by several workers [10, 13]. The reasons for the high prevalence of the UTIs in females can be due to the anatomical structure of the urogenital tract having short urethra, presence of normal flora in vagina, menstrual cycle and pregnancy.

Table 3. Prevalence of virulence genes among study population according to age groups.

Virulence genes (Type and No.)	**Age group (year) %**				
	1-10	**11-20**	**21-30**	**31-40**	**41-50**
Pap(22)	7(31.8)	1(4.5)	7(31.8)	6(27.2)	1(4.5)
Sfa(14)	0(0.0)	1(7.1)	4(28.6)	7(50)	2(14.3)
Afa(2)	1(50)	0(0.0)	0(0.0)	0(0.0)	1(50)

Table 4. Prevalence of virulence genes according to their relationship to the gender and source of bacteria.

Virulence Genes	Gender		P value	Hospital		P value
	Male	Female		Outpatient	Inpatient	
Pap positive	7(31.81%)	15(68.18%)	0.183	2(9.09%)	20(90.9%)	0.224
Sfa positive	3(21.4%)	11(78.6%)	0.585	4(28.6%)	10(71.4%)	0.147
Afa positive	0(0.0%)	2(100%)	0.586	0(0.0%)	2(100%)	0.702

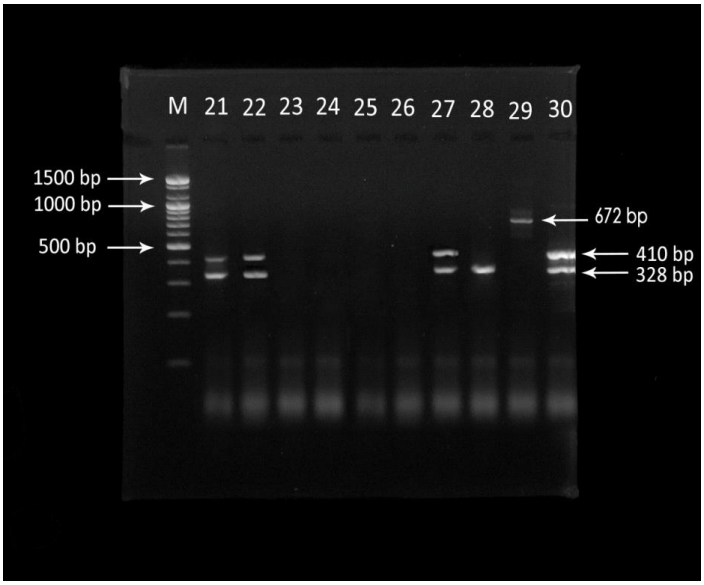

Figure 1. Agarose gel electrophoresis of UPEC virulence genes (*papc*, *sfa* and *afa*) genes detected by multiplex PCR in 56 isolates of *E. coli* isolated from people with UTI. **Lane** (M), DNA molecular size marker (100-bp ladder). **Lanes** (21), (22), (27) and (30) show positive results with *papc* and *sfa* virulence factors genes. **Lanes** (23), (24), (25) and (26) show negative results with all virulence genes. **Lane** (29) *E. coli* shows positive result with *afa* gene (672bp) only.

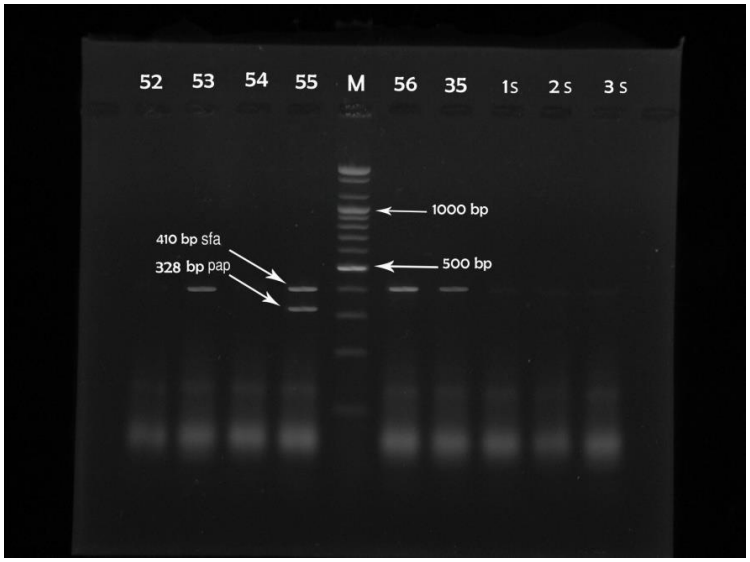

Figure 2. Agarose gel electrophoresis of UPEC virulence factor genes (*papc* 328bp, *sfa* 410bp, *afa* 672bp) genes detected by multiplex PCR in isolates of *E. coli* isolated from people with UTI and from control group. **Lane** (M), DNA molecular size marker (100-bp ladder). **Lanes** (52), (54), (1), (2) and (3) show negative results with all virulence factors genes. **Lanes** (53), (56) and (35) show positive results with sfa virulence gene only. **Lane** (55) *E. coli* shows positive result with pap and sfa genes (328 bp) and (410 bp) respectively. 1, 2 and 3 represent stool samples from healthy people as control group.

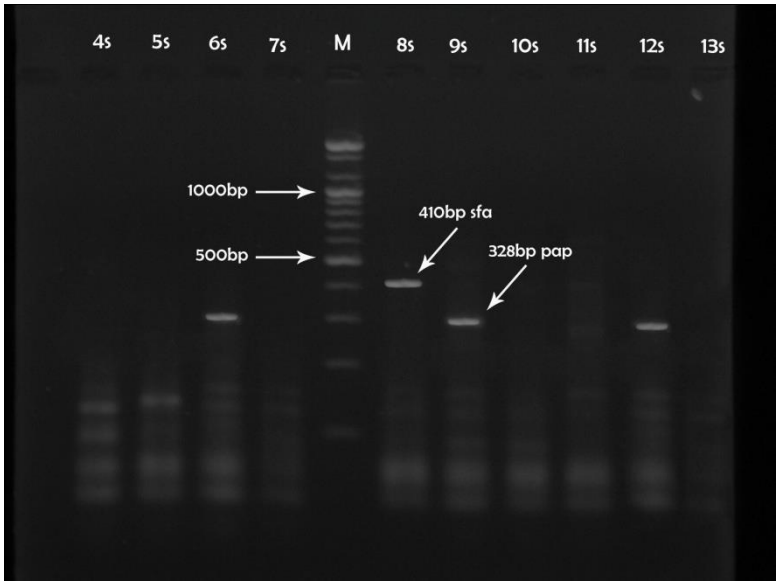

Figure 3. Agarose gel electrophoresis of UPEC virulence factor genes (*papc* 328 bp, *sfa* 410 bp, *afa* 672 bp) genes detected by multiplex PCR in 13 isolates of *E. coli* isolated from stool of healthy people as control group. **Lane** (M), DNA molecular size marker (100-bp ladder). **Lanes** (4s), (5s), (7s) and (10s), (11s) and (13s) show negative results with all virulence factors genes. **Lanes** (6s), (9s) and (12s) show positive results pap (328bp) virulence gene. **Lane** (8s) *E. coli* shows positive result with sfa gene only.

Table 5. Distributions of phylogenic groups among UPEC and stool isolates

Phylogenic groups	No. of UPEC isolates	% of UPEC	No. of stool isolates	% of stool isolates
A	9	16.07%	7	53.84%
B1	6	10.71%	2	15.38%
B2	22	39.28%	3	23.07%
D	19	33.92%	1	7.69%
Total	56	100%	13	100%

Table 6. Phylogenetic distribution of uropathogenic *E. coli* isolates

Phylogenetic Groups	No. of UPEC isolate N=56	Distribution according to gene (groupings no)	*Chu*A gene	*Yja*A gene	TspE4.C2 Fragment
Group A	9 (16.07%)	4	-	+	-
		5	-	-	-
Group B1	6 (10.71%)	4	-	-	+
		2	-	+	+
Group B2	22 (39.28%)	19	+	+	+
			+	+	-
Group D	19 (33.92%)	12	+	-	-
		7	+	-	+

Table 7. Prevalence of virulence related genes in various phylogenetic groups of uropathogenic *E. coli* isolates.

Phylogenic groups	No. of isolates with genetic markers	Distribution of virulence genes		
		Pap C	*Sfa*	*Afa*
Group A	5/9 (55.5%)	2	2	1
Group B1	4/6 (66.6%)	2	2	0
Group B2	19/22 (86.3%)	11	8	0
Group D	12 /19(63.1%)	9	2	1

Table 8. Cross tabulation of gender with each of phylogenic group.

Items	Categories	Gender		X^2	*P value*
		Males	Females		
Phylogenic groups	A	2	7	0.40	0.94
	B1	2	4		
	B2	5	17		
	D	4	15		

Table 9. Cross tabulation of patients and controls with each of phylogenic groups.

Items	Categories	Patients	Controls	X^2	P value
Phylogenic groups	A	9	7	9.96	0.02
	B1	6	2		
	B2	22	3		
	D	19	1		

Note: * 0.05 significant correlations.

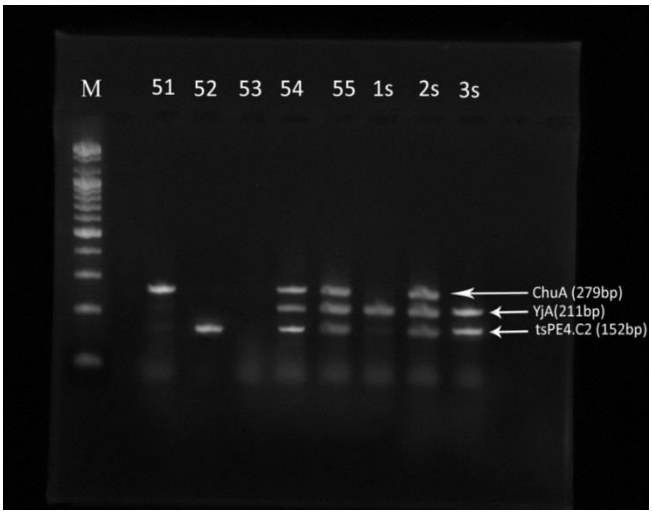

Figure 4. Agarose gel electrophoresis of UPEC phylogenic group genes (*chu* A, *yja* A and DNA fragment *TSPE4.C2*) detected by multiplex PCR in 56 *E. coli* isolated from people with UTI and 13 *E. coli* isolated from stool of healthy people. **Lane**: (M), DNA molecular size marker (100-bp ladder).**Lanes:** (1s) and (53) group. Aisolates showing amplification product of *yja*A (211bp) and negative result with all products of phylogenic groups respectively. **Lane:** (52) and (3s) group B1 isolate showing amplification product of *Tspe4.C2* (152bp) and *yja*A (211bp) and *TSPE4.C2* (152bp) respectively. **Lanes:** (51) group D isolate showing amplification product of *chu*A and *Tspe4.C2* (279bp and 152 bp). **Lanes:** (54), (55) and (2s) group B2 isolates showing amplification products of *chu*A, *yja*A and *Tspe4.C2* (279 bp, 211 bp and 152bp).

Virulence genotypes of UPEC:

In area of present study because there is no genotyping or phylogenic grouping tested available. Therefore, the main goal of present study was to document the association of UPEC isolates with urinary infection in patients of all ages in Karbala city, based on PCR identification of some of their intrinsic virulence factor and phylogenic groups. The findings of molecular detection (Table-3) of UPEC virulence genes of adherence(*Pap*C, *Afa*, and *Sfa*) in present study differs from Mohajeri and his colleagues study findings in which shows no such virulence factors in their population [13]. Other studies show some similarity, and at the same time some differences, in their results to this study regarding to the presence of the genes frequencies findings. Tarchouna and his colleagues' study [16] find that 41%, 34% and 20% of isolates were of *Pap*, *Sfa*, and *Afa*, respectively; and the analysis of the urovirulence gene distribution in *E. coli* did not allow the determination of a clear correlation between a determined gene and the complexity of the UTI. Other study [17] shows the prevalence of genes coding for fimbrial adhesive systems was 32.0%, 19.0% and 11.0% for *Pap*, *Sfa* and *Afa* respectively, and it found a small increase in the percentage of *afa* PCR-positive strains in isolates from patients

with pyelonephritis (13.3%), compared with those associated with cystitis (9.4%).

Also table-3 shows findings of distribution of the above virulence genes in UPEC isolates according to age-groups of UTI patients. The three genes of adherence showed that it was distributed irregularly on the isolates of UPEC were isolated from patients with urinary tract infection in the study area in accordance with age of patients, and this result may resemble a result of a study conducted in Iran; Which shows that the *Pap* gene encodes for p-fimbriae that allows bacteria to adhere to epithelial surfaces and protect them against urine lavage, thus allowing them to ascend further to even cause a serious disease [13].

The biometric analysis of correlation between gender of patients and source of infection (Table-4) show no significant correlation of each of the virulence genes among males and females; χ^2 (1, N =56) = 1.505, P = 0.183; χ^2 (1, N =56) = 0.33, P = 0.585, χ^2 (1, N =56) = 0.627, P = 0.586 for *Pap, Sfa and Afa* respectively; which mean virulence genes were equally prevalence among males and females. In regard to the source of bacterial infection, no significant difference was found with the distribution of virulence genes; χ^2 (1, N =56) = 1.309, P = 0.224; χ^2 (1, N =56) = 2.162, P = 0.147, χ^2 (1, N =56) = 3.310, P = 0.702 for *Pap, Sfa* and *Afa* respectively; which means virulence genes were equally prevalence among inpatients and outpatients.

Phylogenic groups of UPEC:

Based on the presence/absence of these three genetic markers (two genes; *Chu*A and *Yja*A; and anonymous DNA fragment *TspE4.C2*), an *E. coli* isolates could be assigned to one of the main phylo-groups, A, B1, B2 or D (Table-5 and Table-6). The findings of phylogenic groups in our study are in agreement with several previous investigations have indicated that most of the isolates belonged to B2 followed by D and B1 groups [18, 19]. In contrast, the study by Abdul-Razzaq and Abdul-Lateef, (2011) in Hilla, Iraq who reported A and B1 phylogenetic groups were the most prevalent in the different clinical isolates [20] also Piatti *et al*. (2008) found the same results that phylogenetic groups A and B1 were the most prevalent in the isolates, respectively(10). In other studies the results were B2 (50%), D (12%) and (19%) each of A and B1(21), while in Mexic study [8] showed 36% of studied isolates belonged to B2, 28.7% to A, 27.8% to D, and 8.4% to B1. Significantly, strains responsible for extra-intestinal infection were far more likely to be members of phylo-groups B2 or D than A or B1 [23]. The clinical significance of these observations suggested that a simple method of assigning isolates to a phylo-group would be of value. This led to the development and validation of a PCR assay to detect the genes *Chu* A and *Yja* A and a DNA fragment *TspE4.C2* [22]. This triplex PCR phylo-group assignment has been used extensively as a simple and inexpensive method for assigning an *E. coli* isolate to a phylo-group and has provided further evidence that strains of the various phylo-groups differ in their phenotypic and genotypic characteristics, their ecological niche, life history traits and ability to cause disease[24,25].

The prevalence of all studied virulence genes of UPEC among members of phylogenic groups was showed in (Table-7). *Pap* and *Sfa* were prevalent in group B2 of UPEC isolates and *Afa* gene was absent in group B1 and group B2, which indicate different distribution of virulence genes among clinical isolates of various phylogenetic groups of this organism.

Additionally, a chi-square analysis was show no significant correlation between gender of patients with each of phylogenic groups (Table-8), X^2 (3, N=56)=0.40, P=0.94 . That means phylogenic groups were equally prevalence among males and females. While the evaluation of relationship between phylogenic groups among patients and controls (Table-9) by chi-square analysis was show a significant correlation between them $\chi 2$ (3, N =56) = 9.96, P = 0.02. That means the prevalence of phylogenic groups differed among patients and controls.

Conclusions

From the results carried out in this study, it's concluded that the virulence factors' genes of UPEC isolates in patients with UTI were higher than in healthy persons. Significantly, *E. coli* strains responsible for extra-intestinal infection were far more likely to be members of phylogenic groups B2 or D than A or B1.

Recommendations

The molecular methods must be using in detection of genes responsible for other virulence factors and for ESBL resistance among UPEC local isolates, further genotyping and phylogenic grouping should be using for other strains of *E. coli* that responsible for diarrhea and UTI in area of present study.

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