Original Article

Multiplex PCR to investigate virulence in uropathogenic Escherichia coli

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ABSTRACT

Background: Uropathogenic Escherichia coli (UPEC) is the most common cause of urinary tract infection (UTI) in both hospital and community settings. The recent emergence of multidrug-resistant Escherichia coli (E. coli) poses an alarming threat to health. The development of a novel antibiotic against resistant UPEC is a pressing need. Identification of virulent and resistant organisms is essential to target them specifically. **Materials and methods:** In this study, an established multiplex polymerase chain reaction assay was used to detect 29 virulence factor (VF) genes among 50 UPEC strains collected from patients from Saudi Arabia, and associations between virulence profiles and sequence types were observed. **Results:** fimH (mannose-specific adhesin of type 1 fimbriae: overall frequency, 92%), traT (serum resistance, 84%), fyuA (yersiniabactin, 64%), iutA (aerobactin, 64%), kpsMT II (capsule group II, 56%), and PAI (pathogenicity island marker, 48%) were the most frequent VF genes among 50 UPEC isolates. bmaE (M fimbriae), gafD (G fimbriae), nfaE (non-fimbrial adhesin), cdtB (cytolethal distending toxin), and kpsMT III (capsule group III) were not found in any strains tested. The most common sequence types were ST131 (14%), ST69 (10%), ST38 (10%), ST95 (8%), ST10 (8%), ST73 (8%), and ST127 (6%). Some isolates of ST127 and ST73 carried the highest number of VF genes (14), whereas ST131 isolates expressed fewer. **Conclusion:** These findings may help detect virulent E. coli strains and assess their resistance potential, with the aim of developing antibiotics targeting the most prevalent VF genes to treat resistant E. coli.

Keywords: Uropathogenic Escherichia coli, UTI, virulence factor, sequence type, multiplex PCR

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Introduction

Escherichia coli (E. coli) are facultative, gram-negative bacilli that are part of the normal flora of the human colon. E. coli is the leading cause of UTI, the most prevalent laboratory-confirmed infection in Europe.[1] UTI accounts for about 70-95% of community-acquired infections and 50% of hospital-acquired infections. Each year, UTI affects 150 million patients worldwide and results in 6 billion dollars in healthcare costs.[2] A serious complication of UTI caused by E. coli is bacteremia; in 2005, the Health Protection Agency estimated that 32.5 patients per 100,000 developed bacteremia. In England and Wales, E. coli is one of the most common causes of bacteremia.[3]

Virulence derives from the Latin word for poisonous. It refers to a microorganism's ability to produce pathologic effects in a particular host and results from the combined impact of diverse virulence factors (VFs). A number of VFs of UPEC have been identified. Fimbrial (P, S, and Type I fimbriae) and afimbrial adhesins are reported to be associated with UPEC strains. These adhesins bind to specific uroepithelial receptors in the host and enhance colonization of various areas of the urinary tract[4] Another definitive VF of UPEC includes the toxins - hemolysin, cytotoxic necrotizing factor 1, and secreted autotransporter toxins. These toxins facilitate host invasion and, most importantly, stimulate the host's inflammatory response.[5] Another group of VF, siderophores (such as the aerobactin system), plays a role in evading host defense mechanisms.[6] Capsules (such as group

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II and group III), another group of VF, have anti-phagocytic activity and decrease complement-mediated destruction. This group of VF also aids adherence to bladder cells.[7]

Nowadays, the continually increasing antibiotic resistance among UPEC strains is a major concern. A large surveillance study across Europe and North and South America suggested that 20-50% of UPEC isolates are resistant to frequently used antibiotics such as trimethoprim-sulfamethoxazole, fluoroquinolones, and β-lactams. This ultimately leads to an increased rate of failure of standard antibiotic treatment and increased use of second- and third-line therapies. Subsequently, multidrug-resistant UPEC emerges.[8] Hence, developing novel antibiotics against resistant UPEC is a pressing need.

A number of new VFs in E. coli have been identified over the years.[9,10] Further investigation of these UPEC VFs is necessary to elucidate their mechanisms of action and to develop targeted interventions against infection.[11] In recent years, advances in polymerase chain reaction (PCR) technology and the increased availability of VF gene-sequence data have facilitated the evaluation of VFs in UPEC.[12]

The population biology of UPEC remains unclear. UPEC exhibit specific virulence-associated traits, distinctive O antigens, and multidrug resistance.[13] The ability to cause disease depends on the VFs present within each lineage. For example, lineages with factors that promote adherence to the uroepithelium are more likely to cause UTI than strains lacking such factors.[14] Using multilocus sequence typing (MLST), one study examined the population biology of UPEC in the Northwest region of England over 3 years. Based on sequence typing, they identified eight sequence types (ST) of E. coli, with the following being more prevalent: ST173 (16.6%), ST131 (13.3%), ST69 (9%), ST95 (6.3%), ST10 (4.3%), ST127 (3.6%), ST14, and ST104.[15] It is generally acknowledged that multidrug-resistant clones possess low virulence. However, Croxall et al. found that ST131 E. coli has high virulence and potential multidrug resistance.[16]

Virulence is associated with phylogenetic group. UPEC strains originate mainly from group B2 and to a lesser extent from group D, whereas commensal *E. coli* belong to phylogenetic group A.[17,18]

In this study, 50 UTI samples were examined using an established multiplex PCR assay to detect 29 known UPEC-associated VF genes, grouped into five categories (adhesins, toxins, siderophores, capsule genes, and miscellaneous genes) (Table 1) [12]. VF profiles of the isolates were developed, and associations between VFs and sequence types were established. These investigations will enable us to determine sub-strain variation within clones and identify locally and globally important strains, with the aim of developing targeted therapies.

Methods:

Bacterial strain

A total of 50 clinical isolates of E. coli collected between 2009 and 2012 from male and female patients with UTI, aged 3 weeks to 90 years, in Saudi Arabia (King Abdul Aziz Medical City, Riyadh) were studied. E. coli were isolated in pure culture from all samples at the Microbiology & Virology Unit of the University of Manchester. All strains were cultured on Columbia agar (CA; Oxoid) at 37 °C for 18 hours and then stored in suspension at -80 °C using Microbank beads (ProLab Diagnostics). Duplicate cultures were stored at -20 °C for routine use.

DNA extraction

DNA extractions of 50 UPEC isolates were performed using PrepMan Ultra (Applied Biosystems) sample preparation reagent following the manufacturer's instructions.

Multiplex PCR amplification

Fifty UPEC isolates were investigated for carriage of 29 extraintestinal pathogenic *E. coli* (ExPEC)-associated VF genes across five categories (Table 1) using an established multiplex PCR assay.12

Table 1. Categories of virulence factor genes.

Category	VF genes
Adhesin	*fimH* (mannose-specific adhesion of type 1 fimbriae)
	P fimbriae elements[*papAH, papC, papEF and papG* alleles (I,II,III)]
	sfaS (S fimbrial adhesin)
	focG (the putative F1C fimbrial adhesin)
	sfa/focDE (central region of sfaS and focG operons)
	afa/draBC (Dr antigen-specific adhesin operons)
	bmaE (blood group M-specific adhesion)
	nfaE (non-fimbrial adhesin)
	gafD (glucosamine-specific G fimbriae)
Toxin	*cnf1* (cytotoxic necrotizing factor)
	cdtB (cytolethal distending toxin)
	hlyA (α-haemolysin)
Siderophore	*fyuA* (yersiniabactin)
	iutA (aerobactin)
Capsule	*kpsMT* II
	kpsMT III
	K1
	K5
Miscellaneous	*rfc* (O antigen polymerase gene)
	cvaC (colicin V; multifunctional serum resistance-associated plasmids)
	traT (serum resistance associated)
	ibeA (invasion of brain endothelium)
	PAI/MalX, (a pathogenicity island marker (PAI) from the archetypal uropathogenic strain CFT073)

PCR amplification was performed in 25 µL reaction volumes containing 2 µL of 200 ng/µL chromosomal DNA template, 4 mM MgCl2, 0.8 mM each of 4 dNTPs, 0.6 µM of each primer (except papC, papG allele l, sfaS, afa/draBC, nfaE, cnf l, and kpsMT ll, which were used at 0.3 µM), and 2.5 units of AmpliTaq Gold in 1×PCR buffer (Bioline). Primer sequences followed those of a previous study (Johnson & Stell, 2000) (Table 2); the 29 primer pairs were grouped into 5 primer pools in order of decreasing amplicon size (bp) within each pool, as shown in Table 2 and Figure 1.

Table 2. The primer pools are arranged in decreasing order of amplicon size.

Pool	Gene(s)	Primer sequence (*5'- 3'*)	Size of product (bp)
	PAI	ggacatcctgttacagcgcgca tcgccaccaatcacagccgaac	930
	papAH	atggcagtggtgtcttttggtg cgtcccaccatacgtgctcttc	720
Pool 1	*fimH*	tgcagaacggataagccgtgg gcagtcacctgccctccggta	508
	kpsMT III	tcctcttgctactattccccctaggcgtatccatccctcctaac	392
	papEF	gcaacagcaacgctggttgcatcat agagagagccactcttatacggaca	336
	ibeA	aggcaggtgtgcgccgcgtac tggtgctccggcaaaccatgc	170
	fyuA	tgattaaccccgcgacgggaa cgcagtaggcacgatgttgta	880
	bmaE	atggcgctaacttgccatgctg agggggacatatagccccttc	507
	sfa/focDE	ctccggagaactgggtgcatcttaccggaggagtaattacaaacctggca	410
Pool 2	*iutA*	ggctggacatcatgggaactggcgtcgggaacgggtagaatcg	300
	papG allele III	ggcctgcaatggatttacctggccaccaaatgaccatgccagac	258
	kpsMT K1	tagcaaacgttctattggtgccatccagacgataagcatgagca	153
	hlyA	aacaaggataagcactgttctggctaccatataagcggtcattcccgtca	1177
	rfc	atccatcaggaggggactggaaaccataccaaccaatgcgag	788
Pool 3	*nfaE*	gcttactgattctgggatggacggtggccgagtcatatgcca	559
	papG allele I	tcgtgctcaggtccggaattttggcatcccccaacattatcg	461
	kpsMT II	gcgcatttgctgatactgttgcatccagacgataagcatgagca	272
	papC	gtggcagtatgagtaatgaccgttaatatcctttctgcagggatgcaata	200
	gafD	tgttggaccgtctcagggctcctcccggaactcgctgttact	952
	cvaC	cacacacaaacgggagctgttcttcccgcagcatagttccat	680
Pool 4	*cdtB*	aaatcaccaagaatcatccagttaaaatctcctgcaatcatccagttta	430
	focG	cagcacaggcagtggatacgagaatgtcgcctgcccattgct	360
	traT	ggtgtggtgcgatgagcacagcacggttcagccatccctgag	290
	papG allele II	gggatgagcgggcctttgatcgggcccccaagtaactcg	190
	papG I	R-tccagaaatagctcatgtaacccg	1190
	papG II, III	R-actatccggctccggataaaccat	1070
	papG	F-ctgtaattacggaagtgatttctg	
	afa/draBC	ggcagagggccggcaacaggccccgtaacgcgccagcatctc	559
Pool 5	*cnf1*	cattcagagtcctgccctcattattaagatggagtttcctatgcaggag	498
	sfaS	gtggatacgacgattactgtgccgccagcattccctgtattc	240
	kpsMT K5	cagtatcagcaatcgttctgtacatccagacgataagcatgagca	159

Note. F, forward primer; R, reverse primer.

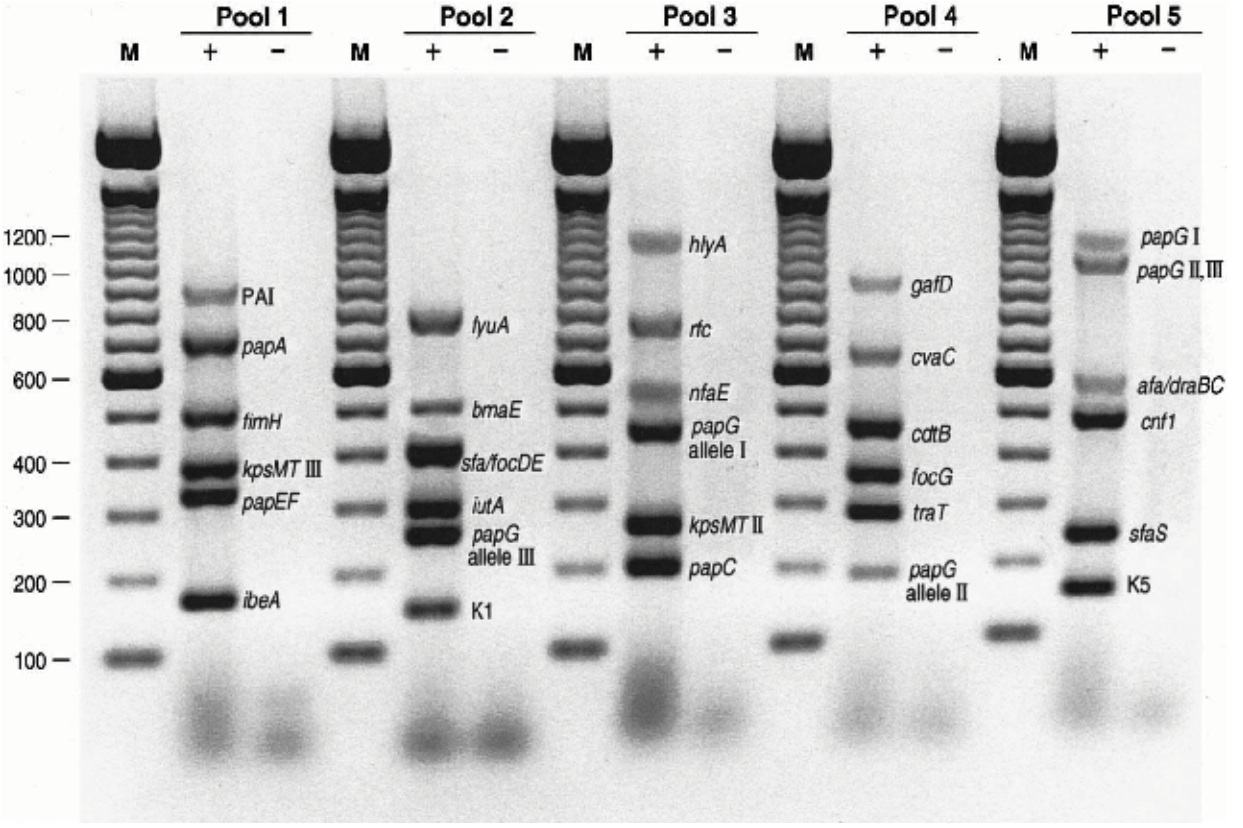

Figure 1. Agarose gel showing PCR products from multiplex PCR assay in 5 separate reactions using primer pools 1–5. M, molecular weight standard; +, positive control DNA; -, negative control DNA.12

PCR condition

The reactions were heated to 95 °C in an automated thermal cycler (PCR system 9700) for 12 min to activate AmpliTaq Gold. This was followed by 25 cycles of 30s at 94 °C, 30s at 63 °C, and 3 min at 68 °C with a final extension for 10 min at 72 °C.

Gel electrophoresis

The PCR products were detected using a 2% (w/v) agarose gel (Promega), then stained with Gel Red (Biotium, USA) and photographed by an ultraviolet transilluminator (AlphaImager TM System 2200). To detect the amplicon sizes of the samples, PCR products were compared with 100-bp DNA (Bioline), which was run in a few lanes on the same gel.

A virulence score was calculated as the summation of positive virulence factors for each isolate. For example, sample 106 contains *fimH, fyuA,* and *traT*. The summation of virulence factor genes for the sample is 3. So, the virulence score for sample 106 is 3.

Statistical methods

Statistical analysis was performed using the Ordinary One-way ANOVA method in GraphPad Prism 6 Demo to determine associations among VFs. The P value indicates the significance of the association. A P value > 0.05 is non-significant (NS). A P value ≤ 0.05 indicates a significant association, and the level of significance correlates with the number of star marks ($P \leq 0.0001$ = ****; $P \leq 0.001$ = ***; $P \leq 0.01$ = **; $P \leq 0.05$ = *).

Results:

Prevalence of virulence factors (VFs)

Figure 2 and Table 4 show a wide range of 0% to 92% prevalence for 29 virulence genes across 50 UTI samples. More than 45% of isolates harbored fimH (92%), traT (84%), fyuA (64%), iutA (64%), kpsMT II (56%), PAI (48%), and papEF (46%), whereas papG I, bmaE, gafD, nfaE, cdtB, and kpsMT III were not found in any isolates. Among

the 5 categories of VF genes, siderophores were most prevalent, and toxins were least prevalent, while adhesins, capsules, and miscellaneous genes varied widely. Among the adhesins, type 1 fimbriae (92%) and P fimbrial elements were expressed in a higher number of isolates, whereas none of the other adhesins were found in more than 22% of isolates. Among the P fimbrial adhesins, papEF (46%) and papC (40%) were far more prevalent than pap allele I (4%) and pap allele III (8%). Of the toxins studied, cnf1 (26%) was more frequent than α-hemolysin (12%), while cdtB was not present in any samples. The siderophore gene yersiniabactin was as prevalent as aerobactin (64%). Of the capsules studied, kpsMT II and K5 were expressed in 56% and 44% of isolates, respectively, while kpsMT III was absent in all strains. Among the miscellaneous genes, traT was much more prevalent (84%) than the others, though PAI was present in 48% of strains, and the rest were present in no more than 10% (Figure 2, Table 4).

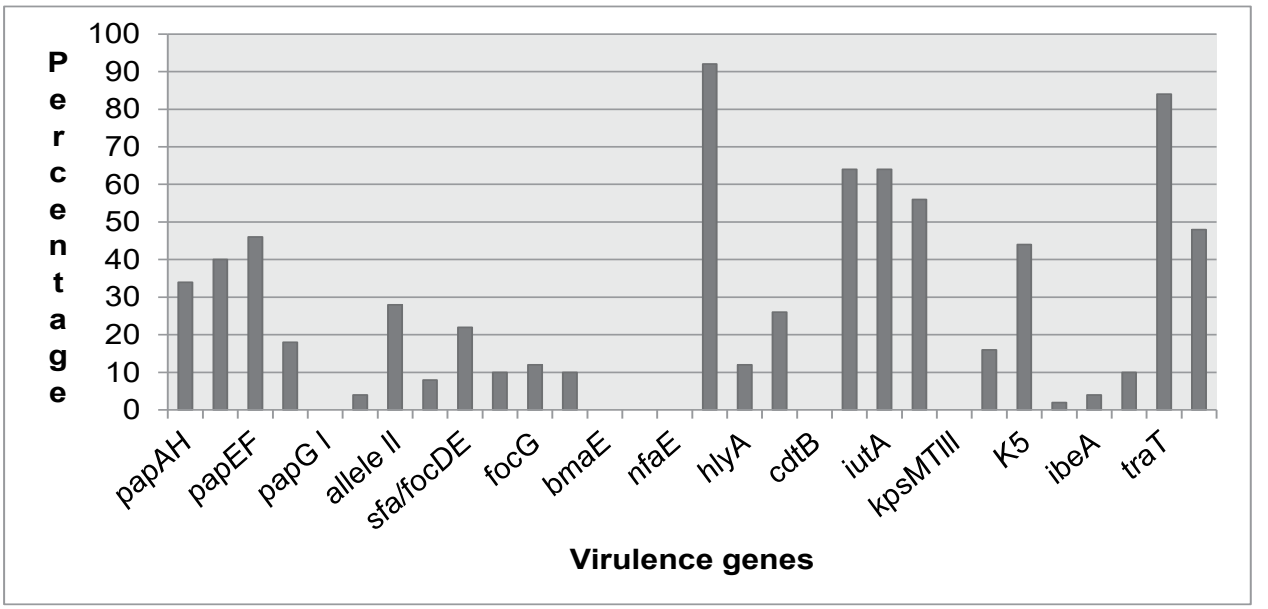

Figure 2. Percentage of 29 virulence genes of UPEC in 50 UTI isolates.

Associations between virulence factors (VFs)

Figure 3 shows that VFs were associated with one another. The *papG* gene was found to be associated with *papC* ($P \leq .0001$) (Figure 3). However, *papG* allele III was correlated with *papEF* ($P \leq .0001$). *fyuA* and *iutA* were expressed in cnf1-positive strains. *kpsMT II was* positively associated with *papG* ($P \leq .0001$), *papG* allele III ($P \leq .0001$), *sfaS* ($P \leq .0001$), *focG* ($P \leq .0001$), and *hlyA* ($P \leq .0001$), whereas K1 was associated with *iutA* ($P \leq .0001$) and *kpsMT* II ($P \leq .0001$), and K5 was correlated with *hlyA.rfc* and *ibeA* showed a negative association with *papEF, fyuA, iutA, kpsMT* II, and K5. *cvaC* was also negatively associated with *fyuA, iutA,* and *kpsMT* II. Though *traT* had a positive correlation with *papAH, papC, papEF, papG, papG* allele II, *papG* allele III, *sfa/focDE, sfaS, focG, afa/draBC, hlyA, cnf1,* K1, K5, *rfc, ibeA,* and *cvaC,* PAI was significantly associated with *papG* allele III, *sfaS,* and *hlyA* (Figure 3).

Figure 3. Statistical analysis of associations between virulence factors (VFs). *P* values are shown only where the *P* value reflects statistical significance; Dashes indicate non-significance ($P \geq 0.05$). The VFs with frequencies 0% and 92% are not shown. II and III indicate *papG* alleles II and III, respectively; NA indicates not applicable (comparison with self).

Prevalence of sequence types (STs)

Sequence type (ST) was determined by use of an MLST method for *E. coli* specified at the University College Cork *E. coli* MLST web site (http://mlst.ucc.ie/mlst/dbs/Ecoli).15 The 50 UPEC isolates tested were assigned to 20 STs. Interestingly, a novel ST was found in this study (sample number 147), though the virulence score of this sample was low (2). The most common STs were ST131 (n=7; 14% of isolates), ST69 (5; 10%), ST38 (5; 10%), ST95 (4; 8%), ST10 (4; 8%), ST73 (4; 8%), and ST127 (3; 6%) (Table 3). These seven STs accounted for > 60% of the isolates.

	papAH	*papC*	*papEF*	*papG*	II	III	*sfa/focDE*	*sfaS*	*focG*	*afa/draBC*	*hlyA*	*cnf1*	*fyuA*	*iutA*	*kpsMT*II	K1	K5	*rfc*	*ibeA*	*cvaC*	*traT*
papAH	NA																				
papC	-	NA																			
papEF	-	-	NA																		
papG	≤.001	≤.0001	≤.0001	NA																	
II	-	-	-	-	NA																
III	-	≤.01	≤.0001	-	-	NA															
sfa/focDE	-	-	-	-	-	-	NA														
sfaS	-	≤.01	≤.001	-	-	-	-	NA													
focG	-	≤.05	≤.001	-	-	-	-	-	NA												
afa/draBC	-	≤.01	≤.001	-	-	-	-	-	-	NA											
hlyA	-	≤.05	≤.001	-	-	-	-	-	-	-	NA										
cnf1	-	-	-	-	-	-	-	-	-	-	-	NA									
fyuA	≤.01	-	-	≤.0001	≤.001	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001	NA								
iutA	≤.01	-	-	≤.0001	≤.001	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001	-	NA							
*kpsMT*II	-	-	-	≤.0001	≤.05	≤.0001	≤.001	≤.0001	≤.0001	≤.0001	≤.0001	≤.01	-	-	NA						
K1	-	-	≤.01	-	-	-	-	-	-	-	-	-	≤.0001	≤.0001	≤.0001	NA					
K5	-	-	-	-	-	≤.001	-	≤.001	≤.01	≤.001	≤.0001	-	-	-	-	≤.05	NA				
rfc	≤.01	≤.0001	≤.0001	-	-	-	-	-	-	-	-	-	≤.0001	≤.0001	≤.0001	-	≤.0001	NA		-	
ibeA	≤.01	≤.001	≤.0001	-	-	-	-	-	-	-	-	-	≤.0001	≤.0001	≤.0001	-	≤.0001	-	NA	-	
cvaC	-	≤.01	≤.001	-	-	-	-	-	-	-	-	-	≤.0001	≤.0001	≤.0001	-	≤.001	-	-	NA	
traT	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001	-	-	≤.05	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001	NA
PAI	-	-	-	≤.01	-	≤.0001	-	≤.0001	≤.001	≤.0001	≤.0001	-	-	-	-	≤.01	-	≤.0001	≤.0001	≤.0001	≤.001

Table 3. Most common sequence types (STs) with virulence scores.

Research Accession No.	STs	Virulence Score
SA 135	10	5
SA 140	10	6
SA 141	10	3
SA 158	10	5
SA 109	38	3
SA 133	38	7
SA 154	38	6
SA 157	38	5
SA 160	38	7
SA 114	69	7
SA 117	69	5
SA 137	69	12
SA 148	69	9
SA 149	69	11
SA 113	73	14
SA 123	73	14
SA 144	73	13
SA 146	73	14
SA 115	95	11
SA 121	95	11
SA 143	95	10
SA 156	95	13
SA 126	127	12
SA 151	127	13
SA 153	127	14
SA 120	131	7
SA 127	131	7
SA 129	131	6
SA 130	131	7
SA 134	131	7
SA 136	131	5
SA 145	131	5

Association between sequence types (STs) and virulence factors (VFs)

Table 4 shows the association between STs and VFs. ST131 was positively correlated with *kpsMT* II but negatively correlated with *kpsMT* III, because among the 7 ST131 samples, 5 carried *kpsMT* II and none expressed *kpsMT* III. Similarly, it was noted that ST131, ST10, and ST38 had low levels of pap element carriage. ST131 did not show a marked association with toxins but did show a strong association with siderophores. *traT* and PAI were associated with ST131 (Table 4).

From Table 3, it can be observed that the highest virulence score (14) was observed in some isolates of ST73 and ST127, whereas some ST410, ST662, and the new ST isolates had the lowest virulence score of 2. The virulence scores of ST131, ST10, and ST38 were not more than seven.

In general, the virulence scores of ST73 isolates were (Table 3), and these scores were strongly associated with pap elements, cnf1, kpsMT II, K5, and PAI (Table 4). ST127 isolates had a virulence score of 12 (Table 3). These ST isolates were strongly associated with papG allele III, sfa/focDE, sfaS, kpsMT II, K5, and PAI, but showed a negative association with rfc, ibeA, cvaC, K1, and iutA (Table 4). ST69 showed some association with siderophores and toxins, though the association with toxins was very weak (Table 4). The virulence scores of ST95 strains were high (≥10) (Table 3), and ST95 had a positive association with iutA, kpsMT II, K1, traT, and PAI. However, ST95 isolates lacked any toxin genes (Table 4).

Table 4. Percentage of virulence genes and association of sequence types with virulence factors (VFs)

	VF	No 50	ST69n=5	ST73n=4	ST95n=4	ST127n=3	ST131n=7
papAH	*papAH*	17 (34%)	4	4	3	3	0
papC	*papC*	20 (40%)	5	3	3	3	0
papEF	*papEF*	23 (46%)	5	3	3	3	0
papG ll, lll	*papG* ll, lll	9 (18%)	3	2	1	2	0
papG l	*papG* l	0 (0%)	0	0	0	0	0
allele l	allele l	2 (4%)	0	0	0	0	0
allele ll	allele ll	14 (28%)	4	3	3	0	0
allele lll	allele lll	4 (8%)	0	1	0	3	0
sfa/focDE	sfa/focDE	11 (22%)	0	4	1	3	0
sfaS	*sfaS*	5 (10%)	0	1	1	3	0
focG	*focG*	6 (12%)	0	4	0	0	0
afa/draBC	*afa/draBC*	5 (10%)	0	0	0	0	1
bmaE	*bmaE*	0 (0%)	0	0	0	0	0

	VF	No 50	ST69n=5	ST73n=4	ST95n=4	ST127n=3	ST131n=7
gafD	gafD	0 (0%)	0	0	0	0	0
nfaE	*nfaE*	0 (0%)	0	0	0	0	0
fimH	*fimH*	46 (92%)	5	4	4	3	7
hlyA	*hlyA*	6 (12%)	1	2	0	2	0
cnf1	*cnf1*	13 (26%)	1	4	0	2	1
cdtB	cdtB	0 (0%)	0	0	0	0	0
fyuA	*fyuA*	32 (64%)	4	2	1	1	7
iutA	*iutA*	32 (64%)	3	3	4	0	6
kpsMT ll	*kpsMT* ll	28 (56%)	2	4	4	3	5
kpsMT lll	*kpsMT* lll	0 (0%)	0	0	0	0	0
K1	K1	8 (16%)	0	0	4	0	0
K5	K5	22 (44%)	2	4	1	3	5
rfc	*rfc*	1 (2%)	0	0	0	0	0
ibeA	*ibeA*	2 (4%)	0	0	1	0	0
cvaC	*cvaC*	5 (10%)	0	0	3	0	0
traT	*traT*	42 (84%)	5	3	4	2	5
PAI	PAI	24 (48%)	0	4	4	3	7

Discussion:

In this experiment, *fimH, traT, fyuA, iutA, kpsMT II, and* PAI were most commonly observed among the 29 VFs identified in the 50 UPEC isolates, suggesting they may be more responsible for UTI pathogenesis.

In this study, the fimH gene, which encodes the type 1 fimbrial adhesin, was found in almost all tested strains. Similar findings have been reported in other studies. Using the protocol described in this study, one group analyzed 29 VF genes in UPEC among 75 urosepsis isolates and found fimH in all isolates.[12] In addition to facilitating binding to the uroepithelial receptor, type 1 fimbriae of UPEC have recently been shown to decrease ureteral contractility, leading to upward spread of infection to the kidneys.[19]

In the samples, traT was highly prevalent. It may be a strong virulence factor gene that causes urinary tract infection (UTI). One study found that traT is more common in UPEC than in commensal E. coli, which do not cause disease. In that study, the traT gene was detected in 58% of E. coli isolates and was significantly more common among extraintestinal than among fecal isolates.[20] fyuA is also an effective virulence factor. The high prevalence of fyuA observed in this study is consistent with another study, which found that 80% of E. coli blood culture isolates from Germany carry the fyuA-irp gene cluster, whereas less than one-third of fecal E. coli strains possess it.[21]

The high prevalence of *iutA* identified here is similar to that reported by another group, which found that the *iutA* virulence gene is present in 80% of *E. coli* strains from patients with urosepsis. They demonstrated that a positive UPEC not only causes disease in compromised patients with low immunity but also causes urosepsis in non-compromised patients with satisfactory immunity. So, *iutA* may have high potential to increase the severity of infection.12 Like this study, the prevalence of *kpsMT* II is higher in a previous study carried out on *E. coli* isolates from four patients with severe extraintestinal *E. coli* infections.[22] A study was carried out on 160 isolates of *E. coli* from urine and blood and identified a distinct PAI in the highly virulent pyelonephritogenic *E. coli* CFT073.[23] The high prevalence of the PAI marker in our study is consistent with that study, which may suggest that PAIs have a high virulence property.

In this study, a notable observation was that some UPEC samples carried a small number of VFs (low virulence score). For example, UTI sample number 138 expressed only 2 VFs – fimH and iutA. Similarly, UTI sample number 106 expressed only 3 VFs: fimH, traT, and fyuA. In most cases, UPEC with a low virulence score carried more prevalent VFs, and in some cases, the combination of VFs across different samples was similar. Does this mean that more prevalent VFs have greater potential to cause infection than others? Do VFs act additively when

present together? Further investigation is needed to address these issues. According to a study by Cusumano et al., FimH is critical for colonization, invasion, and the formation of intracellular bacterial communities (IBCs) in uroepithelium, and these processes can be significantly reduced by novel biaryl mannoside FimH inhibitors. Thus, acute, chronic, and recurrent UTIs can be controlled.[24]

Abe et al. examined 225 UPEC strains to compare the virulence properties of diarrheagenic E. coli (DEC) and UPEC pathotypes. 6.9% of UPEC strains harbor aggregative adherence fimbriae type I, II, and III of enteroaggregative E. coli (EAEC). This result suggests that the most prevalent VF-harboring UPEC are not only potential uropathogens but also express EAEC properties associated with diarrhea.[25]

Taken together, these data suggest that the most prevalent VFs (*fimH, traT, fyuA, iutA, kpsMT II, and* PAI) have more ability to potentiate infection than other VFs. Hence, these can serve as useful targets for interventions.

In this experiment, the prevalence of bmaE, nfaE, cdtB, and kpsMT III was 0%. In addition, several VFs - sfa/focDE, sfaS, focG, afa/draBC, hlyA, K1, rfc, ibeA, and cvaC - were found in only a small minority (< 20%) of strains. These VFs may not play an effective role in pathogenesis. Johnson and Stell also found a very low prevalence of these VFs. They reported that the prevalence of bmaE, gafD, cdtB, and kpsMT III was 5%, 1%, 8%, and 1%, respectively, among 75 urosepsis samples.[12] Hence, VFs with lower prevalence would not be ideal targets for developing an antibiotic for the treatment of UTI. However, Zdziarski et al. characterized the genotypes of selected virulence factors in E. coli strains that cause asymptomatic bacteriuria and are related to UPEC strains. They identified that foc, afa/draBC, hlyA, and rfc could have played a contributory pathogenic role.[26] Therefore, more studies of all these low-prevalence VFs are required.

There is a relationship between the prevalence of VFs and antibiotic susceptibility. Kawamura-Sato et al. studied 312 UPEC strains collected from patients in 7 hospitals in Japan. They showed that 113 isolates were quinolone-resistant and 49 were fluoroquinolone-resistant. These resistant strains exhibited a significantly lower prevalence of the virulence genes papA, hlyA, and cnf1 than the susceptible strains.[11] Another study found that the prevalence of adhesin genes is lower in ESBL-producing UPEC than in non-ESBL-producing strains.[27] Therefore, an extension of this study could be conducted to determine the susceptibility profiles of the same samples and correlate them with the prevalence of VF genes found.

Qin *et al.* showed a correlation between the prevalence of VFs and a patient's disease condition. They demonstrated that *afa* is present in 36% of UPEC isolates from recurrent lower urinary tract infections, whereas *papG* is significantly more prevalent in the acute pyelonephritis group (71%).[27] So, to analyze this type of correlation, future work can be done.

In the present study, the most common STs were ST131, ST69, ST38, ST95, ST10, ST73, and ST127. This finding suggests that these STs may be more virulent or more likely to cause disease than other STs. Lau et al. analyzed 88 cephalosporin-resistant E. coli isolates by MLST and identified 22 different STs. The predominant STs were ST131 (59%), ST73 (6.8%), and ST95 (6.8%) 18 Qin et al. reported that seven STs (ST131, ST69, ST405, ST10, ST393, ST95, and ST38) accounted for 69% of the UPEC strains tested, with ST131 the most prevalent (19%). [27]

In this study, ST131 strains had lower virulence scores than other STs. ST131 samples were deficient in fimbrial adhesins but possessed non-fimbrial adhesins (the Dr family of adhesins). sfa/focDE, papC, and papG allele III were not found in ST131 isolates. ST131 carries virulence-associated genes (VAGs) similar to those reported in highly virulent ST131 isolates from Spain, the UK, and the USA.[12,16,28]

Clonal expansion of ESBL-producing *E. coli* ST131 has led to the worldwide dissemination of CTX-M-15. It shows higher resistance to the frequently used antibiotics ciprofloxacin, trimethoprim, ampicillin, and cefotaxime than non-ST131 strains.[16] Totsika *et al*. studied ST131, which has limited treatment options and causes recurrent UTI. They develop a potential of *fimH* inhibitor as an alternative option which prevents acute bladder infection and treats chronic cystitis against globally disseminated multidrug-resistant *E. coli* ST131.[8]

In this experiment, the ST131 samples did not appear to harbor a specific set of VF genes, and those VFs common to other UPEC were not significantly higher in ST131 than in the other STs. This suggests

that the key to increased virulence potential and the rising incidence of ST131 cannot be generalized to a particular set of VAGs. However, Gibreel et al. reported an interesting finding: high metabolic potential may contribute to the virulence of ST131. Metabolic profiling of ST131 isolates using the Vitek2 Advanced Expert System (AES) showed that ST131 was significantly associated with eight tests, including peptidase, decarboxylase, and alkalization activity assays, which contribute to virulence.[15] Therefore, further study is needed to identify the cause of the virulence of ST131.

In this experiment, the prevalence of ST127 was lower but had a high virulence score. One research group reported that this ST had not been previously identified.[18] Gibreel et al. mentioned that ST127 has emerged recently. They characterized 300 UPEC isolates from the Northwest region of England by MLST. They showed that the prevalence of ST127 is only 3.6%, but it has a high virulence score.[15] Another group assessed the molecular epidemiology and possession of VAGs in 121 UPEC isolates from Nottingham University Hospital. They identified that ST127 exhibits a higher level of VAGs (9) than other STs screened, but a low level of antibiotic resistance.[16] Due to having a high virulence score, ST127 may cause a rapidly progressing disease. This suggests that ST127 may be an emerging, potentially significant clone that warrants detailed surveillance.

In this study, both the prevalence and the virulence score of ST95 were high. So, it can be said that ST95 is highly virulent. ST95 isolates carry *pap* genes encoding P-type fimbriae, which may enhance colonization of human epithelial cells. Johnson and Russo observed that this sequence type contains members of avian pathogenic *E. coli* (APEC), human UPEC, and neonatal meningitis *E. coli* (NMEC).[29] The *pap* genes facilitate colonization in avian epithelial cells as well. These characteristics may create opportunities for zoonotic transmission.[30] Adams-Sapper *et al. analyzed* 249 *E. coli* samples from patients with bloodstream infection (BSI) from a San Francisco public hospital. They detected ST95 (18%) as one of the most common STs tested. Like ST131, a large proportion (90%) of ST95 strains is considered to represent community-onset infection. In addition, they confirmed that dissemination in community settings is possible with drug-susceptible ST95 strains.[31]

ST69 isolates were also highly virulent, as they were among the most commonly found STs, and their virulence scores ranged from 7 to 11 in this study. This observation is not new. Croxall et al. found that ST69 accounts for 9% of E. coli isolates.[16] Tartof et al. studied 45 E. coli isolates and found that the ST69 complex accounts for 22 isolates. These 22 isolates belong to UPEC and bacteremic E. coli strains and, by ERIC2 PCR, are classified as clonal group A (CgA).[32] Novais et al. showed that ST69 has high virulence potential (range 9-15). The ST69 isolates are enriched for the pap allele, kpsMT II, and K5 in the samples tested. Based on the similarity of the XbaI restriction profiles, ST69 strains are divided into two clusters. Of them, Cluster I exhibits a common virulence gene profile (80%): fimH-iha-iutA-kpsMTII-K5-traT-sat-ompT-papA-papEF-papGII-papC, which is associated with hospital- or community-acquired infection. Cluster I is highly transmissible and has been circulating among different continents since 1999. All ST69 isolates tested are resistant to streptomycin and trimethoprim-sulfamethoxazole and frequently to tetracycline and chloramphenicol, but the isolates of ST69 do not produce ESBL.[33]

In this study, the prevalence of ST10 was high. Another research group found a similar result. They found that ST131 and ST10 account for 19% and 20% of UPEC isolates, respectively.[27]

As far as I am aware, there are no such large-scale studies on UPEC in the Middle East. In the present study, UTI samples from Saudi Arabia were investigated to determine UPEC virulence. From the above discussion, it is observed that the results of this study show a strong correlation with VF data from other regions of the world, indicating that these clones have also disseminated into the Middle East.

In summary, the results of this study were consistent with earlier reports. The multiplex PCR assay detected 29 extraintestinal E. coli VF genes. Among UTI samples tested, fimH, traT, fyuA, iutA, kpsMT II, and PAI were highly prevalent. Hence, these VF genes may be useful targets for the development of novel treatments. This study also clearly shows that the most prevalent STs among 50 UPEC strains screened were ST131, ST69, ST38, ST95, ST10, ST73, and ST127. Strains with the most prevalent VFs and the most common ST isolates were more virulent than others. To define UPEC virulence, further studies are required on antibiotic susceptibility, phylogenetic typing, serotyping, disease severity, mode of onset, and host immunization status. The findings of this

study, using multiplex PCR, provide background information for further studies on the epidemiology of traditional and newer VFs in extraintestinal infections caused by E. coli.

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