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Antibiotic resistance profile in relation to virulence genes *fimH*, *hlyA* and *usp* of uropathogenic *E. coli* isolates in Lahore, Pakistan

Ahmed, N.[1], Zeshan, B.[1*], Naveed, M.[1], Afzal, M.[1] and Mohamed, M.[2]
[1]Faculty of Life Sciences, University of Central Punjab, Lahore, Pakistan
[2]Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, Malaysia
[*]Corresponding author e-mail: dr.basit@ucp.edu.pk
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Abstract. Uropathogenic *E. coli* (UPEC) is the major etiological agent of urinary tract infections. The objectives of this study were to evaluate *E. coli* isolates from these patients for the phenotypic pattern of antibiotic resistance and to detect the genes responsible for virulence namely *fimH*, *hlyA* and *usp*. A total of 110 *E. coli* isolates were studied and 30 antibiotics were applied for susceptibility testing. PCR detection of the genes *fimH*, *hlyA* and *usp* was done followed by sequencing and phylogenetic analysis. The results showed that the isolates were highly resistant to cephalaxin (100%) and cephradine (100%) but displayed high sensitivity to amikacin (96.27%), Imipenem (92.63%) and Meropenem (92.63%). The prevalence of *fimH*, *hlyA* and *usp* was 86%, 83% and 16%, respectively. The sequencing showed 99% similarity with previously reported sequences in NCBI GenBank database. The co-existence of multiple drug-resistant bodies and virulent genes has important implications for the treatment of patients with urinary tract infection. This study provides information about treating drug-resistant *E. coli* and the relationship of virulent genes with phenotypic resistance patterns.

INTRODUCTION

Escherichia coli (E. coli) is one of the important human pathogens. It is a gram-negative, non-spore forming straight rod, and is, a facultative anaerobe. *E. coli* is classified into three main groups based on pathogenicity and genetic diversity: intestinal pathogenic *E. coli* (enteric or diarrheagenic), extraintestinal pathogenic (ExPEC) and commensal (Anderson *et al.*, 2004; Giray *et al.*, 2012). Intestinal pathogenic *E. coli* are well characterized and very common cause of gastrointestinal tract infection. ExPEC are pathogenic *E. coli*, which are responsible for extra-intestinal diseases. One of the common extra-intestinal infections caused by *E. coli* is urinary tract infection (UTI), neonatal meningitis and sepsis. In addition, *E. coli* is also involved in various wound infections, intra-abdominal infections, osteomyelitis, nosocomial pneumonia and cellulites (Ahmed, 2018; Li *et al.*, 2018). Uropathogenic *E. coli* are the main cause of UTI worldwide (Arredondo-Garcia and Amabile-Cuevas, 2008). They cause symptomatic and asymptomatic infections.

Several properties of *E. coli* play a role in pathogenesis (Ahmed, 2018; Bauer *et al.*, 2002; Birosova *et al.*, 2004). Certain O serotypes that cause UTI's are characterised by pili with adhesin proteins which bind to a specific receptor located on the urinary epithelium. These binding sites consist of dimmers of galactose (Gal-Gal dimers). These pili are also called P fimbria or pyelonephritis associated pili (PAP) (Wu *et al.*, 2018). The motility of *E. coli* may help it to ascend the infection from ureters into

kidney and from the urethra into the bladder (Ahmed *et al*., 2018; Clements *et al.*, 2012).

Previous studies have reported various adhesin proteins (*afa*, *bmaE*, *fimH*, *papA*, *papC*, *papEF*, *papGI*, *papGII*, *papGIII* and *sfa)*, toxins (*cdtB* and *hlyA*), siderophores (*feoB*, *fyuA* and *iutA*), capsule synthesis proteins (*kpsMTII* and *kpsMTIII*) and uropathogenic-specific protein (*usp*) (Derakhshandeh *et al*., 2015).

Previous studies have shown that the adhesive subunit of type 1 fimbriae *(fimH)* is the most frequently found adhesion protein in isolates of UTI (Derakhshandeh *et al*., 2015; Mohamed *et al.*, 2018). A single nucleotide polymorphism (SNP) of the gene for *fimH* has been used as a screening tool for the epidemiological typing of UPEC. It has also been used in vaccine development and as a tool for the extension of rapid detection assays. It increases tropism for urinary tract receptors and mediates the attachment to the urothelial cell. The prevention of *E. coli* infections may be achieved by blocking bacterial attachment (Derakhshandeh *et al.*, 2015). A consistent distribution of *fimH* is observed in both drug responsive and un-responsive isolates.

Hemolysin *(hlyA)*, an extracellular cytolytic protein, lyses leukocytes, erythrocytes and renal tubular cells by the formation of pores in the cell membrane. About half of the UPEC isolates produced *hlyA* (Eto *et al*., 2007).

The present study was conducted to profile the antibiotic susceptibility of UPEC and to identify the genes which demonstrated virulence with antibiotic resistance, among uropathogenic *E. coli* isolates. The presence of virulence genes and a high rate of multi-drug resistance make them a serious and challenging health problem. It augments the need for public awareness on multi-drug resistance and the careful use of antibiotics.

MATERIALS AND METHODS

Sample collection and isolation of *E. coli*

A total of 479 samples of urine (427 urine and 52 from Foley catheter tips) were collected from 390 male, 89 female patients admitted to the Jinnah Hospital, Lahore from January, 2018 to July, 2018. The collected samples were immediately processed for the isolation, identification, antibiotic resistance profiling and molecular characterization of *fimH*, *hlyA* and *usp*.

Following collection, the samples were inoculated onto MacConkey and CLED agar. After 18 h of incubation at 37°C under aerobic conditions, a total of 110 isolates were selected. Of these, 102 were from urine samples and 8 were from Foley catheter tips. The selected colonies were identified by their colonial morphology, Gram staining and biochemical reactions.

Biochemical testing

The biochemical reactions used for the identification of *E. coli* were lactose fermentation, the oxidase, indole and citrate tests.

Antibiotic susceptibility testing

Following isolation and identification of *E. coli*, antibiotic susceptibility testing was performed by the disk diffusion method according to the protocol in the CLSI guidelines (2017). Inoculum density was standardized using McFarland standards. A total of 30 antibiotics were used for this study, as follows; amikacin (AMK), amoxicillin (AMX), ampicillin (AMP), cefaclor (CEC), cefepime (FEP), cefixime (CFM), cefoperazone (CFP), cefotaxime (CTX), ceftazidime (CZA), ceftriaxone (CRO), cefuroxime (CXM), cephalaxin (LEX), cephradine (RAD), ciprofloxacin (CIP), Doxycycline (DOX), fosfomycin

(FOF), gentamicin (GEN), imipenem (IPM), levofloxacin (LVX), meropenem (MEM), moxifloxacin (MXF), nalidixic Acid (NAL), nitrofurantoin (NIT), norfloxacin (NOR), ofloxacin (OFX), pipemedic Acid, sulbactum (SUL) tazobactum (TZB) tobramycin (TOB), and trime (TMP).

After the application of disks and incubation at 37°C for 48 h, each petri plate was checked for a confluent lawn of growth with the clear zones of inhibition. The isolates were marked as resistant or sensitive according to the diameter (mm) of inhibition zones for UPEC.

Molecular detection of virulence genes of *E. coli*

The positive isolates of UPEC were subjected to molecular identification. After DNA extraction by the CTAB (cetyltrimethylammonium bromide) method, the presence of DNA confirmed by horizontal electrophoresis containing 1% agarose gel in TAE (Tris-Acetate EDTA) buffer. The three virulence genes *fimH*, *hlyA* and *usp* genes were amplified by using specific primers that are mentioned in the protocol in Table 1. The PCR products were loaded on the agarose gel to visualize the amplified gene with 100bp and 1kb DNA ladder to confirm the size of PCR amplicon pair size of *fimH*, *hly* and *usp* genes. The specific DNA bands were purified using a gel elution kit protocol (Promega Technologies, USA).

Sequencing and phylogenetic analysis

Sequencing was performed using the commercial sequencing services of 1st Base (Singapore). Bioinformatics tools (NCBI-nBLAST) were used to determine the similarity index of the obtained sequences with already submitted sequences. The directly sequenced positive isolates were aligned to the reference sequences using BioEdit. MEGA-7 software was used to construct the phylogenetic tree.

RESULTS

In the biochemical identification of *E. coli* the lactose fermentation and indole tests were positive while the oxidase and citrate tests were negative. The results of antibiotic susceptibility patterns of the isolates are reported in Table 2.

Molecular identification showed that 95 out of 110 isolates (86%) were positive for *fimH*, 92 (83%) for the *usp* gene and 18 (16%) for *hlyA*. The virulence genes were found to be predominantly present in female patients (Table 3). The antibiotic resistant isolates showed a high prevalence of virulence genes in the MDR strains of UPEC (Table 4).

Phylogenetic analysis of the sequences of the virulence genes revealed 99% similarities to that of the sequences of closely related strains in the NCBI (National Center for Biotechnology information) data bank (Table 5). The phylogenetic tree (Figure 3) that was generated using the neighbour-joining method shows relationship of strains with closely related species. Bootstrap values (>50%) expressed as percentage of 1000 replications, are indicated at the nodes.

Table 1. Nucleotide sequence of primer sets and annealing temperature for PCR amplifications

Genes	Primer Sequences (5'-3')	Annealing temperature for 1 min	Product Size (bp)	References
fimH	F: TGCAGAACGGATAAGCCGTGG R: GCAGTCACCTGCCCTCCGGTA	60°C	508	(Neamati *et al.*, 2015)
hlyA	F: CAATGCAGATGCAGATACCG R: CAGAGATGTCGTTGCAGCAG	62°C	432	(Idress *et al.*, 2010)
usp	F: ACATTCACGGCAAGCCTCAG R: AGCGAGTTCCTGGTGAAAGC	58°C	440	(Bauer *et al.*, 2002)

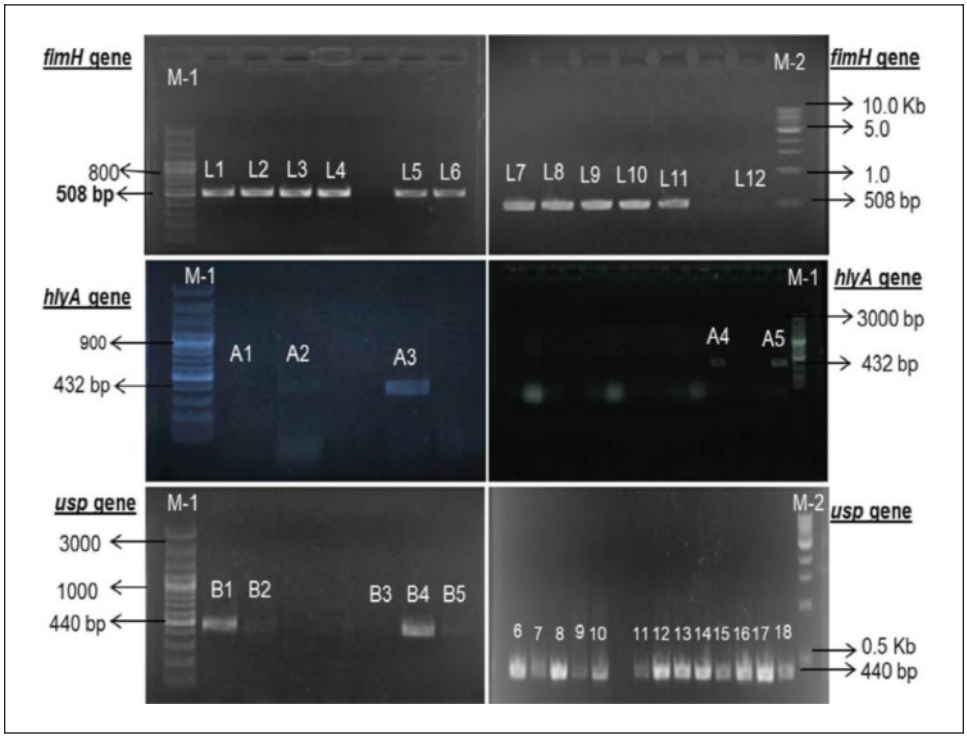

Figure 1. M1= 100bp DNA Ladder. M2= 1Kb DNA Ladder. L1=Positive control for *fimH* gene. L2-L11 shows positive samples for *fimH* gene. L12 shows negative control for *fimH* gene.
A1 is negative control and A5 shows positive control sample for *hlyA* gene. A2, A3 and A4 show positive samples for *hlyA* gene.
B1 is positive control for *usp* gene; B3 shows negative control while B2, B4, B5 and 6–18 show positive samples for *usp* gene.

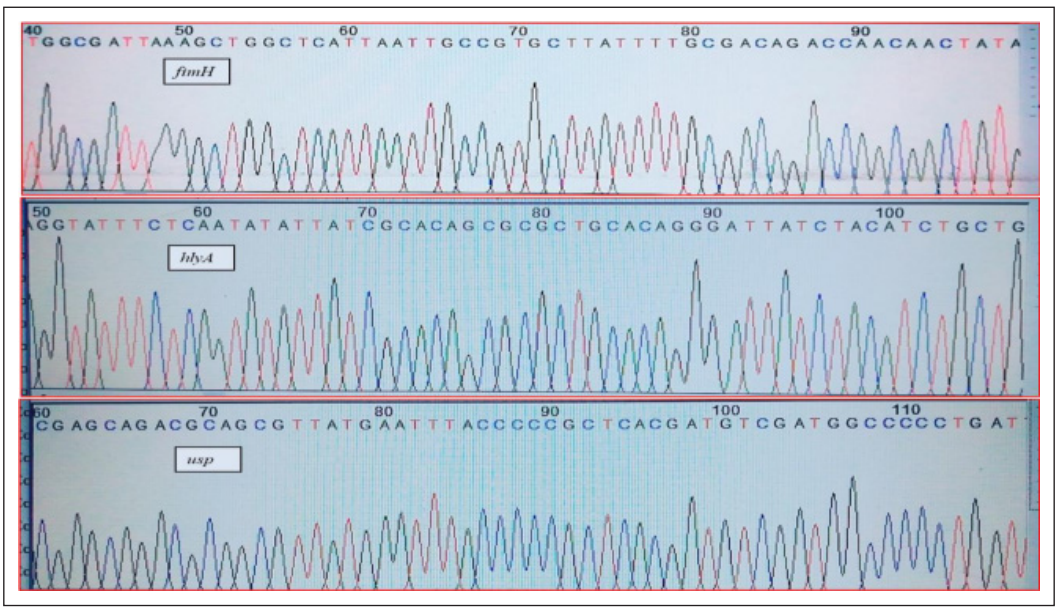

Figure 2. Chromatogram of *fimH*, *hlyA* and *usp* genes.

Table 2. Phenotypic resistance of *E. coli* isolates for various antibiotics

Antibiotics	Male		Female		Resistant %
	Sensitive	Resistant	Sensitive	Resistant	
AMX, AMP, CXM, CEC	5	23	13	69	83
FEP	7	21	22	60	73
CFP, CTX, CZA, CRO, CFM	7	21	18	64	77
LEX, RAD	0	28	0	82	100
IPM, MEM	24	4	78	4	7
AMK	26	2	80	2	3
GEN	16	12	48	34	42
TOB	10	18	41	41	53
DOX	7	21	14	68	63
NAL, Pipemedic acid	0	28	4	78	96
CIP, LVX, NOR, OFX, MXF	2	26	15	67	84
TMP	5	23	15	67	81
NIT	22	6	69	13	17
FOF, SUL	27	1	71	11	10
TZB	23	5	73	9	12

*Amoxicillin (AMX), Ampicillin (AMP), Cefepime (FEP), Cefoperazone (CFP), Cefotaxime (CTX), Cefuroxime (CXM), Ceftazidime (CZA), Ceftriaxone (CRO), Cephalaxin (LEX), Cephradine (RAD), Cefaclor (CEC), Cefixime (CFM), Meropenem (MEM), Imipenem (IPM), Gentamicin (GEN), Amikacin (AMK), Tobramycin (TOB), Doxycycline (DOX), Ciprofloxacin (CIP), Levofloxacin (LVX), Norfloxacin (NOR), Ofloxacin (OFX), Moxifloxacin (MXF), Trime (TMP), Nitrofurantoin (NIT), Fosfomycin (FOF), Nalidixic Acid (NAL), Sulbactum (SUL) and Tazobactum (TZB).

Table 3. Relationship between the presences of virulence genes of uropathogenic *E. coli* and gender

	Gene					
	fimH		*Usp*		*hlyA*	
	+ve	-ve	+ve	-ve	+ve	-ve
Male (%)	53.57	46.42	42.85	57.14	7.14	92.85
Female (%)	97.56	2.43	96.34	3.65	19.51	80.48
Total (*n*)	95	15	92	20	18	92

DISCUSSION

We studied UPEC isolates and their sensitivity to the antibiotics that are, frequently in treatment. The UPEC isolates showed a high resistance to cephalaxin (100%) and cephradine (100%) but were highly sensitive to amikacin (96.27%), imipenem (92.63%) and meropenem (92.63%). Studies from India (Mukherjee *et al*., 2013), Mexico (Arredondo-Garcia and Amabile-Cuevas, 2008) and Iran (Neamati *et al*., 2015) highlighted the low resistance to nitrofurantoin and high resistance to ampicillin. Considering the relative antibiotic resistance rate, AMC, IMP and MEM can be recommend to treat the UTIs. In Mongolia, fluoroquinolones and cephalosporin are used for the treatment of UTIs and other infections. Therefore, ampicillin, ceftazidime, and ciprofloxacin resistance rates may be elevated due to the wide usage of these antibiotics.

In this study, UPEC strains also showed high sensitivity to nitrofurantoin, fosfomycin and sulbactum. Sensitivity to nitrofurantoin may be the result of the lower frequency of the use of this drug. Fluoroquinolones, including ciprofloxacin, cannot be recommended as first-line antibiotics for

Table 4. Relationship in the presence of virulence genes and antimicrobial susceptibility testing

Antibiotics		*fimH*		*hlyA*		*usp*	
		Percentage					
		+ve	-ve	+ve	-ve	+ve	-ve
AMX, AMP, CXM, CEC	Sensitive	50	50	66.66	33.33	66.66	33.33
	Resistant	93.47	6.52	6.52	93.47	86.95	13.04
FEP	Sensitive	48.27	51.72	10.34	89.65	62.06	37.93
	Resistant	87.65	12.34	18.51	81.48	91.35	8.64
CFP, CTX, CZA, CRO	Sensitive	76	24	24	76	68	32
	Resistant	89.41	10.58	14.11	85.88	88.23	11.76
LEX, RAD	Sensitive	0	0	0	0	0	0
	Resistant	86.36	13.63	16.36	83.63	83.63	16.36
CFM	Sensitive	56	44	16.66	83.33	75	25
	Resistant	95.29	4.70	16.47	83.52	87.05	12.94
TOB	Sensitive	82.35	17.64	11.76	88.23	66.66	33.33
	Resistant	89.83	10.16	20.33	79.66	96.66	3.33
DOX	Sensitive	55	45	5	95	30	70
	Resistant	93.33	6.66	18.88	81.11	95.55	4.44
NAL, Pipemedic acid	Sensitive	0	100	0	100	25	75
	Resistant	89.62	10.37	16.98	83.01	85.84	14.15
CIP, LVX, NOR, OFX, MXF	Sensitive	72.22	27.77	16.66	83.33	72.22	27.77
	Resistant	89.24	10.75	16.12	83.87	84.94	15.05
TMP	Sensitive	70	30	10	90	55	45
	Resistant	93.40	5.49	17.77	82.22	90	10

Table 5. Relation of virulent genes with closely related taxa identified by using the nBlast in *E. coli* isolated from patients with urinary tract infection in Lahore, Pakistan

Serial ID	Gene	GenBank Accession Number	Closely Related Taxa Identified	Sequence Identity (%)	Sequence query coverage (%)
1	*fimH*	CP025747.1	*E. coli strain ML35*	99	97
2	*fimH*	CP023364.1	*E. coli strain 144*	99	97
3	*fimH*	CP026399.1	*E. coli strain ECONIH4*	99	97
4	*fimH*	CP011915.1	*E. coli strain PSUO2*	99	97
5	*fimH*	CP021935.1	*E. coli strain AR_0055*	99	96
6	*hlyA*	CP009072.1	*E. coli ATCC 25922*	99	97
7	*hlyA*	CP018991.1	*E. coli strain Ecol_AZ146*	99	97
8	*hlyA*	CP018991.1	*E. coli strain Ecol_AZ146*	99	97
9	*hlyA*	CP009072.1	*E. coli ATCC 25922*	99	97
10	*hlyA*	CP018991.1	*E. coli strain Ecol_AZ146*	99	97
11	*usp*	CP024720.1	*E. coli isolate NQ3*	99	97
12	*usp*	CP024720.1	*E. coli isolate NQ3*	99	97
13	*usp*	CP024720.1	*E. coli isolate NQ3*	99	97
14	*usp*	CP012379.1	*E. coli strain PAR*	99	98

* The mentioned GenBank Accession Numbers are of those species that shows resemblance with our sequences.

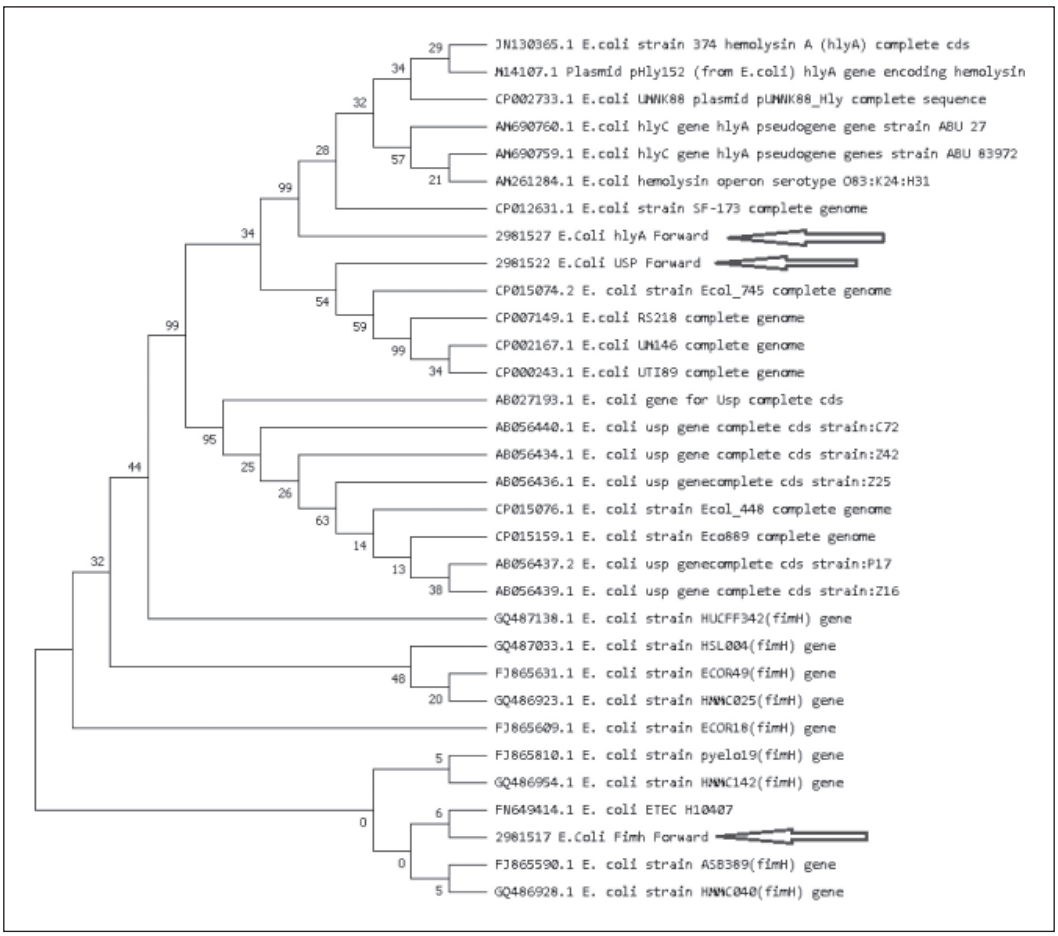

Figure 3. Phylogenetic tree showing inter-relationship of genes with closely related species. The tree was generated using the neighbour-joining method. Bootstrap values (>50%) expressed as percentage of 1000 replications, are indicated at the nodes.

the treatment of UTIs. However, these antibiotics are generally used in empirical therapies. Our study showed a resistance of ciprofloxacin to UPEC isolates, of 84.6%. These results are higher than those of (Giray *et al.*, 2012) and (Russo and Johnson, 2003) who reported a resistance of *E. coli* to ciprofloxacin of 47% and 62.3%, respectively.

Strains which carried the virulence genes were more resistant to antibiotics (Oliveira *et al.*, 2011; Schwartz *et al.*, 2013). The results of this study demonstrated high frequency of *fimH* gene (86%) in UPEC isolates and suggests a role in causing UTI in Pakistani patients. Females had a higher rate of *fimH* than males (Table 3). Most strains of uropathogenic *Escherichia coli* (UPEC) encode filamentous adhesive organelles called type 1 pili. We have determined that the type 1 pilus adhesin, FimH, mediates not only bacterial adherence, but also invasion of human bladder epithelial cells. This colonization was upregulated by fimbrial adhesion which recognizes α1 and β3 integrins (Hannan *et al.*, 2012). It is well documented that the specific adherence of mucosal inflammation and the increased induction are the most likely underlying mechanisms availed by P fimbriae to increase the virulence of UPEC. The results of the present study which showed the prevalence of *fimH* among the UPEC isolates was similar to that of with the other studies (Eto *et al.*, 2007).

The marked association between the *fimH* gene and gender may be due to difference in physiological and anatomical structure of urinary tract in male and female (Eto *et al*., 2007).

The prevalence of the *hlyA* gene in present study was 16%, and similar to the results of (Mukherjee *et al*., 2013) but lower than the 47% obtained by (Jalali *et al*., 2015). There was a clear association between the presence of hemolysin and tissue damage. The prevalence of these virulence genes varies on the basis of clinical representation, geographical distribution and phylogenetically (Basu and Mukherjee, 2018; Kaper *et al*., 2004).

The prevalence of the *usp* gene in this study was 83% as against the 63.7% that shown by (Bauer *et al*., 2002). The isolates that were negative for *usp*, *hlyA* and *fimH* could be a part of the normal flora. It is also possible that they could be gene mutations of the corresponding operon. On the other hand, a positive PCR usually confirms the presence of the virulence genes (Tarchouna *et al*., 2013; Usein *et al*., 2001).

A high incidence of multidrug resistant strains was observed in these isolates. The UPEC isolates showed 100% multidrug resistant phenotypes and demonstrated resistance to three or more of the antibiotics that were tested. Similar results were obtained by other studies (Hickling *et al*., 2015; Mukherjee *et al*., 2013). For example, the rate of multidrug resistance in UPEC isolates was 92.5% in India (Mukherjee *et al*., 2013), while that for Iran was 82.1% (Neamati *et al*., 2015).

CONCLUSION

The virulence genes *fimH*, *hlyA* and *usp* have a prominent role in antibiotic resistance in UPEC. This co-existence of multiple drug resistance and the virulence genes is an alarming situation. Further research is needed to find the relationship between virulence and antibiotic resistance genes.

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Author contribution

Mr. Naveed Ahmed collected all samples and performed the wet lab work, reviewed the literature and drafted the initial manuscript. Dr. Muhammad Naveed and Maizan Mohamad helped in sequencing the samples, guided this work and prepared the figures. Dr. Basit zeshan and Dr. Muhammad Afzal supervised this work and oversaw the preparation and revision of the manuscript which all the authors have read and approved.

Conflict of interest

The authors do not have any conflict of interest with any of the research work done by other colleagues/authors.

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