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Original Article

Analysis of diarrheagenic potential of uropathogenic *Escherichia coli* isolates in Dhaka, Bangladesh

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Abstract

Introduction: Uropathogenic *Escherichia coli* (UPEC) strains are chiefly responsible for urinary tract infections (UTIs). The aim of the study was to observe the virulence properties of UPEC and to determine whether they carry virulence properties of diarrheagenic *E. coli*.

Methodology: Seventy-one pure cultures were collected from UTI patients. After biochemical identification, 56 UPEC strains were examined for biofilm formation capability, hemolytic activity, presence of UTI-associated virulence genes (*papC*, *fim*1, *afa*, *sfa*) and diarrhea-associated virulence genes (*estA*, *eltB*, *vt*1, *vt*2, *eaeA*, *aatA*, *ial*, *bfpA*) by multiplex polymerase chain reaction (PCR) assay.

Results: Among the 56 UPEC strains, 21 showed biofilm formation ability, and only 5 showed beta-hemolytic activity. In multiplex PCR, 42% were found positive for *papC* gene, 27% were *fim*1 positive, 11% were *afa* positive, and none were *sfa* positive. The diarrheagenic genes found were *vt*2, *ial*, *estA, eltB*, *bfpA*, and *aatA*, but only in seven isolates. Of these isolates, two were positive for *estA* and one was positive for *eltB*, characteristic genes also found in enterotoxigenic *E. coli*. One carried *vt*2, a gene characteristically found in enterohemorrhagic *E. coli*. Another one was characterized as enteropathogenic *E. coli* (EPEC), as it was carrying the EPEC gene *bfpA*. Another isolate was positive for *ial*, the characteristic gene found in enteroinvasive *E. coli*, and one isolate was found to harbor the *aatA* gene, a gene found in all enteroaggregative *E. coli*.

Conclusions: This study revealed that most UPEC strains were unique to uropathogenic virulence properties, and very few carried diarrheagenic properties.

Key words: diarrheagenic *E. coli*; ETEC; uropathogenic *E. coli*; UTI; virulence genes.

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Introduction

Urinary tract infections (UTIs) are one of the most common human infections. The development of UTIs depends on the anatomy of the tract, the integrity of the host's immunity, and the etiology of the infection [1]. UTIs are classified according to the site of infection: cystitis (the bladder), bacteriuria (the urine), or pyelonephritis (the kidneys) [2]. Successful establishment of infection by bacterial pathogens requires adhesion to host cells, colonization of tissues, and, in certain cases, cellular invasion followed by intracellular multiplication, dissemination to other tissues, or persistence [3]. A number of physiological factors such as sex, pregnancy, and diabetes can delineate the frequency, prevalence, and severity of UTIs [4].

Enteric bacteria are most commonly found as the etiologic agents of UTIs, with *E. coli* accounting for about 80% of UTI cases. Other pathogens associated with UTI include *Klebsiella* spp., *Enterobacter* spp., *Proteus* spp., *Pseudomonas* spp., *Staphylococcus saprophyticus*, and *Enterococcus* spp. [5].

Escherichia coli is a remarkably diverse organism. A group of virulotypes have been implicated in a wide range of human and animal diseases, from gastroenteritis to extraintestinal infections of the urinary tract, bloodstream, and central nervous system [6]. Each of the virulotypes is distinct with respect to the subset of genes harbored that are involved in the subversion of host responses and pathogenic modulation. More than eight pathovars have been extensively studied, which are broadly classified as either diarrheagenic *E. coli* or extraintestinal *E. coli* (ExPEC). Six pathovars, which include enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC; including *Shigella* like species), and diffusely adherent *E. coli* (DAEC) are diarrheagenic, and two pathovars, which include uropathogenic *E. coli*

(UPEC) and neonatal meningitis *E. coli* (NMEC) are the most common ExPEC isolates. UTI-causing *E. coli* isolates are broadly grouped in the ExPEC group and are commonly termed uropathogenic *E. coli* (UPEC) [7].

UPEC are well adapted to the challenge of moving from the intestinal tract to the urinary tract to establish themselves [8]. The ability to ascend the urinary tract reflects organ tropism defined by specific adhesins, evasion from innate immunity, and avoidance of clearance by urine flow [9]. Virulence factors of UPEC implicated in UTIs can be divided into two groups: the virulence factors associated with the bacterial surface (*e.g.*, type 1 fimbriae, P fimbriae, S fimbriae, and afimbrial adhesins), and those factors which are secreted and exported to the site of action [10]. Secreted toxins are important virulence factors in a variety of *E. coli*-mediated diseases; for uropathogenic *E. coli*, a lipoprotein called α-hemolysin associated with upper UTIs [11] and cytotoxic necrotizing factor 1 (CNF1) involved in kidney invasion are noteworthy [12]. Secreted autotransporter toxin [13] and the cytolethal distending toxins (CDT) could also be potential virulence factors in UPECs [14].

In this study, we examined a collection of UPEC strains for the presence of characteristic virulence properties of UPEC and determined whether these UPEC strains carry virulence factors characteristic of diarrheagenic *E. coli* (DEC) pathotypes.

Methodology

Sample collection

Mid-stream urine samples were collected from UTI-suspected patients who attended a private hospital in Dhaka. Samples were collected from both outpatients and inpatients. Patients who were hospitalized for less than 24 hours were considered to be outpatients, and patients who were admitted to the hospital and stayed overnight or for a certain period of time, usually several days or weeks, were considered to be inpatients. Information forms with patients' data (*e.g.*, age, sex, clinical history, and pre-hospitalization medication) were recorded and duly supplied to the microbiology laboratory of the concerned hospital. The physical appearance and microscopic observations of urine samples are recorded in Table 1. Approximately 10 µL of each freshly voided midstream urine samples was streaked by semi-quantitative streaking method onto UTI chrome agar (HiMedia, Mumbai, India). Bacterial count was measured after overnight incubation at 37°C. Bacterial colony characteristics were observed and preliminarily identified by comparing data from the manufacturer of chrome agar media. A total of 71 pure isolates from UTI chrome agar were collected from the hospital and processed for identification and further biochemical analysis.

Identification of isolates

On the basis of colony morphology using differential and selective media, MacConkey agar No.3 (MAC-3) and eosin methylene blue agar (EMB) (Oxoid, Basingstoke, UK), presumptively identified *E. coli* colonies were further processed for biochemical tests [15] (synthesis of oxidase, catalase and urease, motility test, utilization of citrate, fermentation of glucose and lactose, production of indole) for the identification of the isolates.

Observation of hemolytic activity

Pure discrete colonies from nutrient agar (Oxoid, Basingstoke, UK) plates were streaked onto sheep blood agar (blood agar base, Oxoid, Basingstoke, UK) supplemented with 5% sheep blood and incubated overnight at 37°C. Zones of hemolysis were observed against bright light; indication of β-hemolysis was determined by the presence of zone of complete lysis of erythrocyte around the colony and clearing of media [16].

Quantitative biofilm assay

Quantitative biofilm assay was measured with a slight modification of the method described by Naves *et al.* [17]. Overnight bacterial culture in Luria-Bertani (LB) broth (Oxoid, Basingstoke, UK) was centrifuged, washed, and re-suspended in autoclaved distilled water that had an optical density of approximately 0.6 at 655 nm. A 96-well round-bottom polystyrene microtiter plate was inoculated with 200 µL of the bacterial suspension in each well, in triplicate. The microtiter plate was incubated at 28°C for 30 minutes, 2 hours, 8 hours, 24 hours, 48 hours, and 72 hours without shaking and then washed with the autoclaved water. The plate was stained with 200 µL of 0.5% crystal violet (Oxoid, Basingstoke, UK) in each well and incubated for 30 minutes, followed by washing. The absorbance was read after the addition of 200 µL of 95% ethanol in each well by an enzyme-linked immunosorbent assay (ELISA) plate reader at 450 nm (Bio-Rad, Hercules, USA). The optical density of each strain was obtained by arithmetic mean of three wells, and this value was then compared with mean absorbance of negative control.

Table 1. Virulence properties of all biochemically positive *E. coli* isolates.

Isolate code	Age	Sex	Ip/Op	Pus cell (40 x)	RBC (40 x)	Yeast (40 x)	Biofilm formation capability	Hemolytic activity	UTI-associated *E. coli* virotypes				Diarrheagenic *E. coli* virotypes							
									*pap*C	*afa*	*sfa*	*fim* 1	*vt*1	*vt*2	*eaeA*	*ial*	*estA*	*eltB*	*bfpA*	*aatA*
DUM 3383605	61	M	OP	NUM	6-8	-	-	γ	+	-	-	-	-	-	-	-	-	-	-	
DUM 493783	82	F	IP	8-10	2-3	-	-	γ	+	-	-	-	-	-	-	-	-	-	-	-
DUM 6397244	51	F	OP	6-8	1-3	-	**Weak**	γ	-	-	-	+	-	+	-	-	-	-	-	-
DUM 8E178254	93	F	IP	NUM	2-3	-	-	γ	-	-	-	-	-	-	-	-	-	-	-	-
DUM 15394327	8	M	OP	1-3	6-8	-	-	γ	-	-	-	-	-	-	-	-	-	-	-	-
DUM 18397898	65	F	OP	4-6	0-2	-	-	β	+	-	-	+	-	-	-	-	-	-	-	-
DUM 19343159	56	F	IP	NUM	2-3	++	-	β	+	-	-	-	-	-	-	-	-	-	-	-
DUM 20396462	17	F	IP	NUM	8-10	-	-	γ	+	+	-	+	-	-	-	-	-	-	-	-
DUM 21396669	59	F	OP	NUM	2-3	-	-	γ	-	+	-	+	-	-	-	-	-	-	-	-
DUM 2222903	62	F	OP	4-6	1-2	-	-	β	-	-	-	-	-	-	-	-	-	-	-	-
DUM 23398445	39	F	OP	NUM	6-8	-	-	γ	+	-	-	+	-	-	-	-	-	-	-	-
DUM 26286615	75	M	IP	2-3	2-4	-	**Weak**	γ	-	-	-	-	-	-	-	-	-	-	-	-
DUM 28344871	1	M	OP	1-3	OCC	-	-	γ	-		-	-	-	-	-	-	-	-	-	-
DUM 29361422	51	F	OP	NUM	4-6	-	**Medium**	γ	+	-	-	+	-	-	-	-	-	-	-	-
DUM 30224082	37	F	OP	NUM	5-7	-	-	γ	-	-	-	+	-	-	-	+	-	-	-	-
DUM 32291185	62	F	OP	2-3	6-8	-	-	β	+	-	-	+	-	-	-	-	-	-	-	-
DUM 33399573	27	F	OP	NUM	2-3	-	-	γ	-	-	-	-	-	-	-	-	-	-	-	-
DUM 34355388	1	F	OP	6-8	OCC	-	-	γ	+	+	-	+	-	-	-	-	-	-	-	-
DUM 36284390	30	M	OP	NUM	2-4	-	**Week**	γ	+	-	-	-	-	-	-	-	-	-	-	-
DUM 38E383097	10 mo.	F	OP	2-3	NIL	-	-	γ	-	+	-	-	-	-	-	-	-	-	-	-
DUM 41357478	66	M	OP	NUM	2-4	-	**Weak**	γ	-	-	-	-	-	-	-	-	-	-	-	-
DUM 43400330	40	F	IP	NUM	8-10	++	**Weak**	γ	-	-	-	-	-	-	-	-	-	+	-	-
DUM 44400401	55	F	OP	NUM	1-2	-	**Strong**	γ	-	-	-	-	-	-	-	-	-	-	-	-
DUM 45400477	60	F	IP	NUM	4-6	-	**Medium**	γ	+	-	-	-	-	-	-	-	-	-	-	-
DUM 46408627	65	F	IP	NUM	10-12	-	-	γ	-	-	-	-	-	-	-	-	-	-	-	-
DUM 47149386	25	M	IP	3-5	1-3	-	-	γ	-	-	-	-	-	-	-	-	-	-	-	-
DUM 48400795	40	M	OP	NUM	10-12	-	**Weak**	γ	+	-	-	-	-	-	-	-	-	-	-	-
DUM 49396244	90	M	IP	6-8	5-7	-	**Weak**	γ	-	-	-	-	-	-	-	-	-	-	-	-
DUM 50390198	69	F	OP	NUM	1-3	-	**Weak**	γ	-	-	-	-	-	-	-	-	-	-	-	-
DUM 51381966	25	F	OP	NUM	2-3	-	**Medium**	γ	-	+	-	-	-	-	-	-	-	-	-	-
DUM 5438061	61	F	OP	NUM	2-4	-	**Medium**	γ	+	-	-	-	-	-	-	-	-	-	-	-
DUM 56401124	60	F	OP	8-10	1-2	-	**Strong**	γ	-	-	-	-	-	-	-	-	-	-	-	+
DUM 60401383	29	F	IP	NUM	NUM	-	**Weak**	γ	+	+	-	-	-	-	-	-	-	-	-	-

Table 1 (continued). Virulence properties of all biochemically positive *E. coli* isolates.

Isolate code	Age	Sex	Ip/Op	Pus cell (40 x)	RBC (40 x)	Yeast (40 x)	Biofilm formation capability	Hemolytic activity	UTI-associated *E. coli* virotypes				Diarrheagenic *E. coli* virotypes							
									*pap*C	*afa*	*sfa*	*fim* 1	*vt*1	*vt*2	*eaeA*	*ial*	*estA*	*eltB*	*bfpA*	*aatA*
DUM 6172747	48	F	IP	NUM	1-3	-	-	γ	-	-	-	-	-	-	-	-	+	-	-	-
DUM 62382265	26	M	OP	NUM	8-10	-	-	γ	+	-	-	+		-	-	-	-	-	-	-
DUM 63236990	46	F	IP	6-8	OCC	-	-	γ	-	-	-	-	-	-	-	-	-	-	-	-
DUM 65400845	42	F	IP	NUM	1-2	-	**Week**	γ	+	-	-	+	-		-	-	-	-	-	-
DUM 66205238	51	F	OP	NUM	2-3	-	-	γ	+	-	-	-	-	-	-		-	-	-	-
DUM 68401996	23	F	OP	NUM	NUM	-	-	γ	+	-	-	+	-	-	-	-	-	-	-	-
DUM 70402007	45	F	IP	NUM	6-8	-	-	γ	-	-	-	-	-	-	-	-	-	-	-	-
DUM 71175237	6	M	OP	2-3	4-6	-	-	γ	-	-	-	-	-	-	-	-	-	-	-	-
DUM 72309768	37	F	OP	NUM	2-4	-	**Weak**	γ	+	-	-	-	-	-	-	-	-	-	-	-
DUM 73399414	74	F	OP	5-7	1-3	-	-	γ	-	-	-	+	-	-	-	-	-	-	-	-
DUM 74402268	71	F	OP	NUM	6-8	-	**Weak**	γ	+	-	-	+	-	-	-	-	+	-	-	-
DUM 75401960	43	F	IP	2-4	NUM	-	-	γ	-	-	-	+	-	-	-	-	-	-	-	-
DUM 76229821	72	F	OP	NUM	6-8	-	-	γ	-	-	-	-	-	-	-	-	-	-	-	-
DUM 78376065	43	M	OP	NUM	10-12	-	**Medium**	β	+	-	-	-	-	-	-	-	-	-	-	-
DUM 80398564	61	F	OP	1-3	NUM	-	-	γ	-	-	-	-	-	-	-	-	-	-	-	-
DUM 81360474	82	F	IP	2-3	8-10	+++	-	γ	-	-	-	-	-	-	-	-	-	-	-	-
DUM 82400477	60	F	OP	NUM	8-10	-	-	γ	+	-	-	-	-	-	-	-	-	-	-	-
DUM 84264733	104	M	IP	NUM	1-2	-	**Weak**	γ	-	-	-	-	-	-	-	-	-	-	-	-
DUM 85351969	52	M	OP	8-10	0-2	-	-	γ	-	-	-	-	-	-	-	-	-	-	-	-
DUM 86402588	13	F	IP	10-12	NUM	++	-	γ	+	-	-	-	-	-	-	-	-	-	-	-
DUM 87387931	79	F	OP	NUM	1-2	-	-	γ	-	-	-	-	-	-	-	-	-	-	-	-
DUM 88307455	31	F	OP	NUM	1-2	-	-	γ	-	-	-	-	-	-	-	-	-	-	+	-
DUM 90243405	79	M	IP	4-6	8-10	-	**Weak**	γ	+	-	-	-	-	-	-	-	-	-	-	-
Total	**56**						**21**	**5**	**24**	**6**	**0**	**15**	**0**	**1**	**0**	**1**	**2**	**1**	**1**	**1**

UTI: urinary tract infection; M: male; F: female; IP: inpatients; OP: outpatients; NUM: too numerous to count.

Table 2. Primers PCR and conditions for the detection of genes associated with adhesion of uropathogenic *E. coli*.

Target genes	Primer Sequences	PCR conditions (time and temperature)			Amplified product size (bp)	Ref
		Denaturation	Annealing	Extension		
papC	F: 5'- GACGGCTGTACTGCAGGGTGTG-3' R: 5'- ATATCCTTTCTGCAGGGATGCA-3'	94ºC for 1 minute	65ºC for 1 minute	72ºC for 1 minute	328	[41]
afa	F: 5'- GCTGGGCAGCAAACTGATAACT-3' R: 5'- CATCAAGCTGTTTGTTCGTCCG-3'	94ºC for 1 minute	65ºC for 1 minute	72ºC for 1 minute	750	
*fim*1	F: 5'- CGACGCATCTTCCTCATTCTTC-3' R: 5'- ATTGGTTCCGTTATTCAGGGTT-3'	94ºC for 2 minutes	65ºC for 1 minute	72ºC for 1 minute	700-750	
sfa	F: 5'- CTCCGGAGAACTGGGTGCATCT-3' R: 5'- CGGAGGAGTAATTACAAACCTG-3'	94ºC for 2 minutes	65ºC for 1 minute	72ºC for 1 minute	410	[42]

The interpretation of biofilm formation assay was measured based on the formula BF = AB – CW, where BF is biofilm formation, AB is stained attached bacteria, and CW is stained control wells. The following classification was used for the determination of biofilm formation: no biofilm production when BF < 0.100; weak biofilm production when 0.100 ≤ BF ≤ 0.199; moderate biofilm production when 0.2 ≤ BF ≤ 0.299; and strong biofilm production when BF ≥ 0.300 [17].

Molecular characterization of E. coli

For extraction of DNA, the strains were grown on 6.0 mL of LB broth. After overnight growth, cells were harvested from the broth and subjected to alkaline cell lysis followed by the phenol-chloroform extraction method [18]. The DNA was stored at -20°C for subsequent polymerase chain reaction (PCR) analysis.

Genes of *fim*1 (type 1 fimbriae), *papC* (P fimbriae), *afa* (afimbrial adhesins), and *sfa* (S fimbriae) are known to be important determinants of pathogenesis for UPEC to establish initial adhesion for infection in the urinary tract. The bacterial isolates were screened for the presence of these genes by multiplex PCR; the sequence of oligonucleotide primers and PCR conditions are listed in Table 2. Detection of virulence marker genes associated with diarrheagenic *E. coli* was performed by multiplex PCR using eight primer pairs (Table 3) targeting genes *eaeA* (intimin of EHEC and EPEC strains), *bfpA* (bundle-forming pilus of EPEC strains), *vt*1 and/or *vt*2 (Shiga toxins 1 and 2 of EHEC strains), *eltB* and/or *estA* (enterotoxins of ETEC strains), *ial* (invasion-associated locus in EIEC and *Shigella*), and *aatA* (*Eco*RI-*Pst*I DNA fragment of pCVD432 of EAEC). The multiplex PCR reaction was segregated into three different sets, choosing primer pairs generating PCR products of different sizes distinguishable by agarose gel electrophoresis. A list of the oligonucleotide primers used in the assay is included in Table 3. PCR was performed in a 20 µL reaction mixture containing 1 µL of template DNA, 0.2 µL of 2 U/µL DNA polymerase (DyNAzyme, Thermo-Fisher Scientific, Waltham, USA,), 2 µL of 10 × buffer for DyNAzyme, 0.4 µL of a mixture of deoxynucleoside triphosphates (25 mM of each), and 0.5 µL of 25 µM solution of each primer (Sigma-Aldrich, Munich, Germany). The thermocycler conditions (Peltier Thermal Cycler, MJ Research,

Table 3. Oligonucleotide primers used in the multiplex PCR assay for the detection of genes associated with diarrheagenic *E. coli* virulotypes.

	Primer	Target gene	Primer sequence	Amp size (bp)	Ref
Set 1	**ST**	*estA*	F: 5'-GCTAAACCAGTA$^{G}_{A}$GGTCTTCAAAA-3' R: 5'-CCCGGTACA$^{G}_{A}$GCAGGATTACAACA-3'	147	[43]
	LT	*eltB*	F: 5'-TCTCTATGTGCATACGGAGC-3' R: 5'-CCATACTGATTGCCGCAAT-3'	322	[44]
Set 2	**bfpA**	*bfpA*	F: 5'-TTCTTGGTGCTTGCGTGTCTTTT-3' R: 5'-TTTTGTTTGTTGTATCTTTGTAA-3'	367	[45]
	EA	*aatA*	F: 5'-CTGGCGAAAGACTGTATCAT-3' R: 5'-CAATGTATAGAAATCCGCTGTT-3'	630	[46]
	SHIG	*ial*	F: 5'-CTGGTAGGTATGGTGAGG-3' R: 5'-CCAGGCCAACAATTATTTCC-3'	320	[47]
Set 3	**VT1**	*vt1*	F: 5'-GAAGAGTCCGTGGGATTACG-3' R: 5'-AGCGATGCAGCTATTAATAA-3'	130	[48]
	VT2	*vt2*	F: 5'-ACCGTTTTTCAGATTTT$^{G}_{A}$CACATA-3' R: 5'-TACACAGGAGCAGTTTCAGACAGT-3'	298	[49]
	Eae	*eaeA*	F: 5'-CACACGAATAAACTGACTAAAATG-3' R: 5'-AAAAACGCTGACCCGCACCTAAAT-3'	376	[50]

Waltham, USA) were as follows: of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute for 30 cycles, with an initial denaturation at 96°C for 4 minutes and a final 7-minute extension at 72°C. The details of positive controls for each gene used in the study are included in Supplementary Table 1-S.

Results

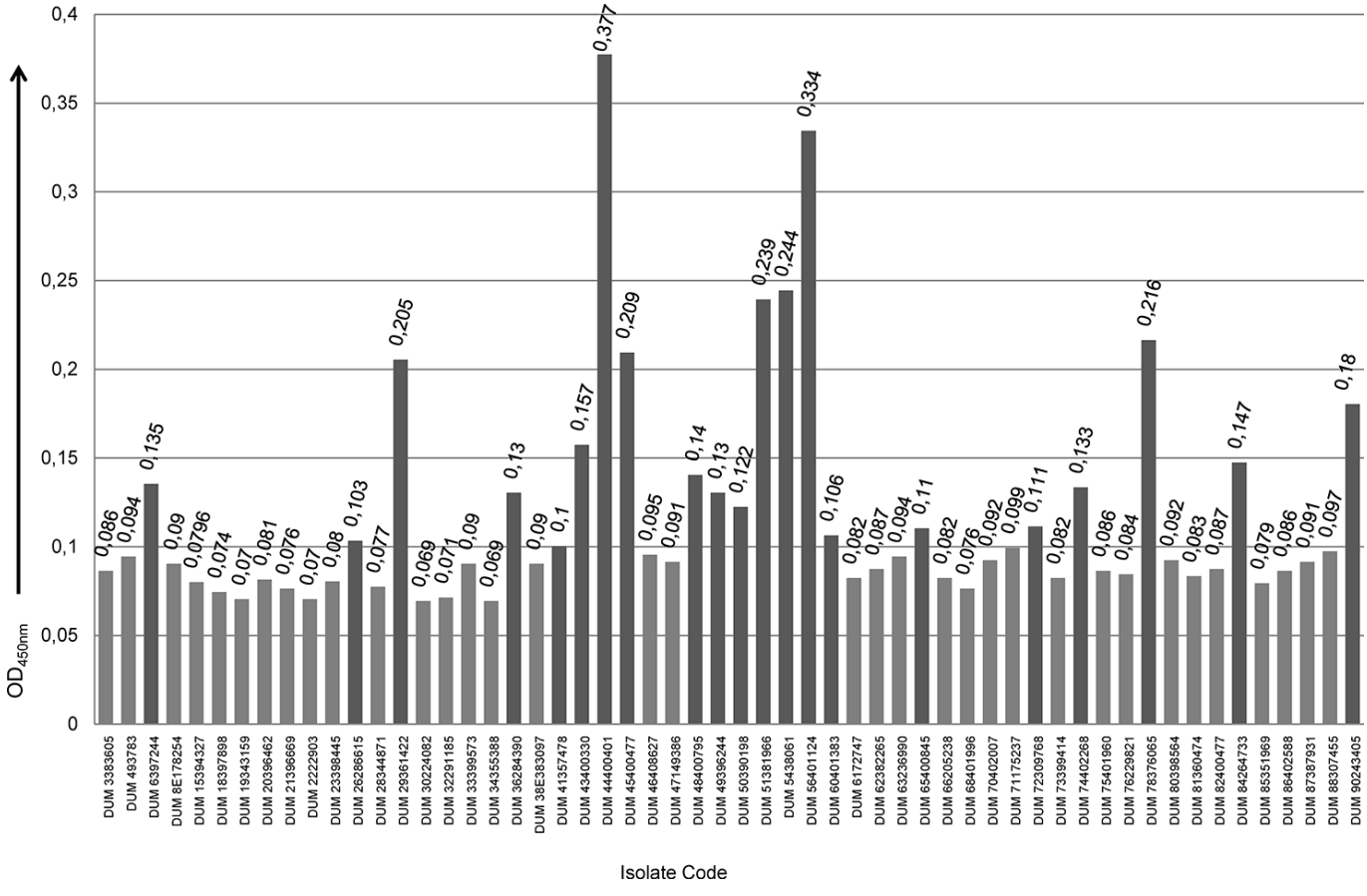
A total of 71 pure cultures representing 71 UTI patients were collected randomly from a hospital situated in Dhaka, Bangladesh, of which 26 were inpatients and 45 were outpatients. UTI was confirmed in these patients by positive urine culture with about 10^5 cfu/mL. Most of the patients (66%) were female, indicating a higher occurrence of UTIs among females.

Among the 71 isolates, 56 isolates were identified as *E. coli* according to colony morphology on the differential and selective media and biochemical tests performed in this study. Of these 56 *E. coli* strains, 62.5% were obtained from outpatients and 37.5% were from inpatients.

Biofilm formation is a prominent test for screening colonization efficiency onto the epithelial surface of the urinary tract, which is also a driving factor for UPEC strains to get established in the urinary tract. Among the tested isolates, almost 38% (21/56) were found to be capable of biofilm formation (Figure 1). Of these, 14 isolates were found to be weak biofilm producers, 5 were moderate biofilm producers, and 2 were strong biofilm producers. In the test for hemolytic activity, a few (5/56) isolates showed zone of β-hemolysis around the colony.

Initial attachment, colonization, and development of UTIs is partially determined by the presence of adhesive molecules on the bacterial surface. Among the 56 *E. coli* isolates, 24 were found to harbor the *papC* gene, a common adhesive gene for P fimbriae of uropathogenic *E. coli* strains. These P fimbriae can recognize kidney glycosphingolipids carrying Gal *α* (1–4) Gal determinant on renal epithelia via adhesion [7]. The attachment of P fimbriae to this receptor leads to the release of ceramide that acts as an agonist of toll-like receptor 4 (TLR4), a receptor involved in activation of the immune cell response [19]. This, in turn, leads to the development of local inflammation and pain associated with UTIs [20]. Fimbrial adhesion gene *fim*1 was found in 15 isolates and afimbrial adhesion gene, *afa*, was found in 6 of the isolates. No isolates were

Figure 1. Biofilm formation of each strain and the absorbance after subtracted from control.



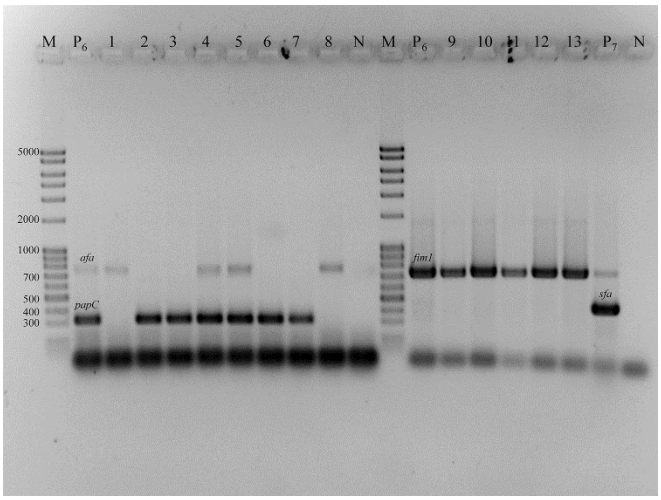
found to carry the *sfa* adhesin gene (Figure 2). Whereas the *papC* gene was found more frequently in inpatients (57%) than in outpatients (34%), the genes *afa* and type 1 were found more frequently in outpatients (11.4% and 34%, respectively) than in inpatients (9.5% and 14.3%, respectively).

Multiplex PCR assay was performed targeting eight genes (*estA*, *eltB*, *vt*1, *vt*2, *eaeA*, *bfpA*, *ial*, and *aatA*) usually harbored by five different diarrheagenic virotypes (ETEC, EHEC, EPEC, EIEC, EAEC), to assess the diarrheagenic potential of the UPEC isolates. Only seven isolates were found to harbor these diarrheagenic genes (Table 1). Among these seven isolates, two isolates were found to carry the *estA* gene, of which one (DUM 74402268) was also positive for *papC* and *fim*1 genes. The *estA* and *eltB* genes are characteristic of ETEC, and *papC* is characteristic of UPEC. The findings suggest that the isolate DUM 74402268 is either a UPEC strain containing the properties of ETEC or an ETEC strain possessing the properties of the UPEC pathotype. One isolate (DUM 43400330) was found to be positive for the *eltB* gene, but negative for all the four uropathogenic genes; thus, DUM 43400330 containing the *eltB* gene could putatively be characterized as ETEC. One isolate, DUM 6397244, found to be positive for *vt*2 and characterized as EHEC, was also positive for the *fim*1 gene. Another isolate (DUM 30224082) positive for the *ial* gene and characterized as EIEC was found to carry the *fim*1 gene as well. The isolate DUM 88307455, found to be positive for the *bfpA* gene and characterized as EPEC, was negative for all the other genes. Isolate DUM 56401124, found to harbor the *aatA* gene but negative for all four genes involved in uropathogenesis, was characterized as EAEC (Figure 3). Two ETEC strains, one containing only the *estA* gene and the other containing only the *eltB* gene, were obtained from inpatients. The other five were found in outpatients.

Discussion

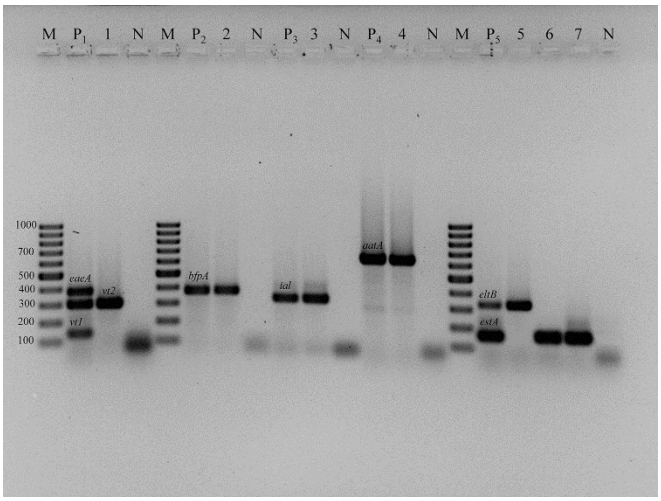
We identified and characterized the virulence and possible diarrheagenic potential of UPEC isolates obtained from patients who had UTIs. Among the 71 isolates collected from the patients, 56 (78%) were identified as *E. coli.* Though the patients' samples were randomly selected, 47 (65.2%) were from female patients and the rest (24; 34.8%) were from male patients, a result consistent with previous studies showing that UTIs are more common in women than in men and that many women experience persistent infection [21,22]. Women have a shorter urethra than men, and the urethral opening is relatively close to the anus in women; these are the probable causes of fecal-perineal-urethral contamination and development of UTIs in women [23]. There is also a strong association between anatomical and functional alterations of bladder emptying and recurrent UTIs in postmenopausal women [24]. In this study, around 47% of women were 55 years of age or older; the average

Figure 2. Agarose gel electrophoresis showing PCR amplification product of uropathogenic virulent genes.



Lane M: DNA molecular size marker (100 bp extended DNA ladder, Carl Roth GmbH & Co., Karlsruhe, Germany); lanes P_6, P_7: positive control for *papC*+*afa*+*type1* & *sfa*+*fim*1, respectively; lanes 1-13: PCR results of *E. coli* strains; lane N: negative control for PCR reaction.

Figure 3. Agarose gel electrophoresis showing PCR amplification product of diarrheagenic virulent genes.



Lane M: DNA molecular size marker (100 bp DNA ladder, Thermo Scientific, Waltham, USA); lanes P_1, P_2, P_3, P_4, P_5: positive control for *vt*1+*vt*2+*eaeA*, *bfpA*, *ial*, *aatA*, *eltB*+*estA*, respectively; lanes 1, 2, 3, 4, 5, 6, 7: PCR results of *E. coli* strains; lane N: negative control for PCR reaction.

age of menopause in women in Asia is around 48 years [25].

For establishment of UTIs, uroepithelial adherence is critical. Strains of uropathogenic *E. coli* possess an impressive repertoire of adhesion molecules that enable the bacteria to aggregate and adhere to the cellular surfaces of the urinary tract [26]. Among the UTI-specific virulence-associated genes detected in this study by sets of multiplex PCR assay, *papC*, considered to be the second common virulence factor of UPEC [27], was found in 42% of isolates. *E. coli* can cause ascending UTIs when it has the challenge of ascending from the intestinal tract to establish an infection in the bladder (cystitis) and can proceed from the bladder via the ureters to the kidneys to cause pyelonephritis with the possibility of causing irreversible kidney damage and death [28].

Descending UTIs occur when the bacteria cause infection in kidney via blood or lymph nodes [28]. Though pyelonephritis is generally a descending infection, the presence of P fimbriae plays an important role in the pathogenesis of ascending UTIs and pyelonephritis in humans [27]. Moreover, the samples from which these strains were collected were urine, so it was difficult to say whether these *papC*-positive strains were responsible for ascending or descending UTIs. It was not confirmed, therefore, whether the patients had infection in their kidneys. If the infection was descending, which means the bacteria entered the bladder from blood via infecting kidney, this would suggest that the patients were already suffering from pyelonephritis. But if the bacteria ascended from intestinal tract to lower urinary tract (the bladder) [2] via fecal-perineal-urethral route and caused infection there, that means the patients had not yet developed upper UTIs, but were at risk of developing upper UTI or pyelonephritis, as the bacteria possess the *papC* gene. It is more appropriate, therefore, to infer that these *papC*-positive strains have the ability to cause infection in the upper urinary tract (the kidneys) [2].

Martinez *et al*. [29] and Schembri *et al*. [30] showed that in murine UTI models, the type 1 fimbriae promote bacterial survival to stimulate mucosal inflammation and to enhance invasion and grow as a biofilm [29,30]. The type 1 fimbriae bind to the mannosylated glycoproteins uroplakin Ia and IIIa (UPIIIa) in the urothelial layer via the adhesin subunit FimH, which is located at the fimbrial tip. The interaction results in molecular phosphorylation events that are required for stimulation of signaling pathways involved in invasion and apoptosis. These may also contribute to the elevation of intracellular Ca2+ levels in urothelial cells [29]. In clinical and experimental findings, it has been suggested that *E. coli* strains carrying *afa* adhesins are involved in UTIs and have properties potentially supporting the establishment of chronic or recurrent infection [31]. In this study, the gene *afa* was found in 6 (11%) and *fim*1 was found in 15 (27%) isolates. Though hemolysin is an effective toxin for UPEC and is usually associated with pyelonephritis [11], the occurrence of hemolytic activity was found to be limited in this study to only 5 (9%) isolates showing beta hemolysis on blood agar plate. Of these 5 isolates tested for the presence of the selected virulence genes, 2 carried both the *papC* and *fim*1 gene, 2 possesed only the *papC* gene, and 1 isolate was negative for all the other virulence genes examined in the study. Jakobsson *et al*. [32] reported permanent renal scarring as a common complication following hemolytic *E. coli* infection [32], which may be independent of bacterial adherence properties [33]. In this study, we observed that among the 5 strains showing beta-hemolytic activity, 4 showed no evidence of biofilm formation *in vitro* assays (Table 1).

Melican *et al*. [34] defined previously unknown synergistic functions of both P fimbriae and type 1 fimbriae that facilitate bacterial colonization under dynamic *in vivo* conditions. P fimbriae have been shown to enhance early colonization of the tubular epithelium, while the type 1 fimbriae mediate colonization of the center of the tubule via a mechanism that involves inter-bacterial binding and biofilm formation. The heterogeneous bacterial community within the tubule subsequently affects renal filtration, leading to total obstruction of the nephron. The obstruction contributes to the full pathophysiology of pyelonephritis [34]. In our study, 10 isolates (18%) were found to be positive for both *fim*1 (gene for type 1 fimbriae) and *papC* (a gene of P fimbriae). Formation of biofilm protects bacteria from hydrodynamic wash in the urinary tract, from phagocytosis and other host defense mechanisms, and from antibiotic chemotherapy [35], which contributes to the persistence of bacteria. Several isolates (21/56) in this study were found to be capable of biofilm formation. Thirteen of the isolates that exhibited biofilm formation ability also possessed at least one of the four uropathogenic adhesin genes. Among the thirteen isolates, seven were found to carry the *papC* gene alone, three were found to harbor both *papC* and *fim*1, and one was positive for *papC* and *afa* genes. One isolate was positive for the *afa* gene alone and one was carrying *fim*1 alone; the remaining eight isolates were carrying none of these uropathogenic adhesion genes, or there may have been some other

mechanism by which they were able to form the biofilm that results in infection in the urinary tract. In addition, Pruss *et al*. [36] showed that the expression of hemolysin and type 1 fimbriae was significantly associated with biofilm production. Type 1 fimbriae, which enhance adhesion to host epithelial cells, have been found to be important in the initial steps of biofilm formation [36]. In our study, the *fim*1 gene (type 1 fimbriae) was found only in six biofilm-forming strains, and only one strain was found to be positive for both hemolytic activity and biofilm formation.

Other virulence-associated genes, more frequently found in diarrheagenic pathotypes, were detected in only seven isolates by three sets of multiplex PCR using eight different primer pairs targeting *vt*1, *vt*2, *ial*, *eaeA*, *aatA*, *estA*, *eltB*, and *bfpA* genes. More than 85% of the UPEC strains carried none of these eight diarrhea-associated genes, although there is little information on the role of these genes in uropathogenesis. This study shows evidence that diarrheagenic and uropathogenic strains may share common virulence properties. The isolates we examined may have the capacity of causing both intestinal and extraintestinal infection. Three isolates were detected as having the properties of ETEC, among which one also carried two important uropathogenic genes, *papC* and *fim*1 (Table 1). Though the product of the *estA* gene has no role in urinary tract infections, the presence of these three significant genes (*papC*, *fim*1, and *estA*) in a single isolate indicate its potential capability to cause infection in both the intestinal and urinary tracts. The *eltB* gene possessing the ETEC strain, the *aatA* gene containing the EAEC strain, and the *vt*2 gene-positive EHEC strain, though negative for all other uropathogenic genes, were capable of biofilm formation, which may explain their ability to colonize the urinary tract, leading to infection. Another two *E. coli* strains (DUM 88307455 and DUM 30224082) found to be carrying a diarrheagenic gene and negative for all other uropathogenic virulent properties (Table 1), made it necessary to find out how they developed into infection in the urinary tract. Again, the isolates found to harbor a diarrheagenic gene, whether they developed into ascending or descending UTIs, could not be deduced. As these strains were found to carry a diarrheagenic gene, the possibility of ascending UTI was higher in this respect. This study raises the possibility that some UPEC may have acquired DEC markers, becoming a potential cause of diarrhea, or that some diarrheal *E. coli* strains may have acquired properties of UPEC by means of horizontal gene transfer or some other mode, which requires further study. Though the percentages of in-and outpatients regarding the presence of particular virulence gene were enumerated, no significant relationship was observed between the patients' hospital residency and the prevalence of virulence genes.

Our study had a few similarities and differences with other studies on UPEC. A study in Iran found that 9.4% of the 138 UPEC strains were carrying *astA*, a gene characteristic of the EAEC pathotype [37]. Abe *et al.* [38] found 16 UPEC strains (7.1%) positive for the *aatA* gene sequence, a typical gene of the EAEC pathotype [38]. In Copenhagen, Denmark, a study on an UTI outbreak detected the presence of EAEC virulence genes in UPEC strains and demonstrated that in the presence of adhesin genes of EAEC, outbreak UTI strains exhibited increased adherence to human bladder epithelial cells compared to prototype UPEC strains [39]. In Germany, a study found that of 265 *E. coli* strains collected from urine samples, 28 (10.6%) were positive for one or more known diarrheagenic pathogenic *E. coli* virulence genes, and among these 28 isolates, 23 were found to carry the *astA* gene of EAEC [40]. In all these studies on *E. coli* strains isolated from urine samples of UTI patients, EAEC characteristic virulence genes were observed more than other diarrheal *E. coli* pathotypes. In our study, conducted in Bangladesh, we found that 12.5% of the UPEC strains possessed diarrheagenic genes of different pathotypes.

Twenty-five percent of the isolates were found to be negative for all the 12 genes assessed and also showed negative in hemolysis activity and biofilm formation assay. Interestingly, most of them were isolated from elderly patients, which supports the hypothesis that the commensal *E. coli* may also cause UTIs, especially in elderly patients who may have suppressed immunity, or that these strains possess some other virulence properties which were not included in this study.

Conclusions

A proportion of the isolates in this study were found to carry properties of both UPEC and DEC. These isolates have expanded their site of infection, gaining the ability to cause infection in both the intestinal and urinary tracts, and have thus become of great clinical importance in our setting.

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Conflict of interests: No conflict of interests is declared.

Annex – Supplementary Items

Supplementary Table 1. Positive controls for pathogenic genes.

No.	Isolate codes	Genes	GenBank accession No.
P1	mduc1Lvve	*vt1*	KY319038
		vt2	KY221829
		eaeA	KY073237
P2	mduc5sB	*bfpA*	KY221831
P3	mduc7i	*ial*	KY221830
P4	mdueR1Pc	*aatA*	KY243935
P5	mduc1iLS	*estA*	KY221833
		eltB	KY221832
P6	mduc20PAT	*papC*	KY243934
		afa	KY290889
P7	mdues7s	*fim1*	KY319036
		sfa	KY319037