ORIGINAL PAPER

Uropathogenic *Escherichia coli* Isolates with Different Virulence Genes Content Exhibit Similar Pathologic Influence on Vero Cells

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Abstract

Uropathogenic *Escherichia coli* are the major causative agent of urinary tract infection – they may simultaneously express a number of virulence factors to cause disease. The aim of this study was to investigate the relation between virulence factors content of fifteen UPEC isolates and their pathogenic potential. The isolates belonged to the five serotypes O78:K80, O114:K90, O142:K86, O164 and O157. Nine of the virulence factors have been explored, *ibeA*, *pap*, *sfa/foc*, *cnf1*, *hly*, *fyuA*, *pil*, *ompT* and *traT*. Virulence factors profiling of the isolates revealed a different content ranging from 22% to 100% of the virulence genes explored. The pathogenic capacity of all fifteen isolates when tested on Vero cells showed that the cytotoxicity for all tested strains on Vero cells was approximately equal and enhanced after growth in syncase broth, leading mainly to cell lysis. The toxic effects reduced slightly after heat treatment of the toxin, and greatly after formalin detoxification, but not all the deleterious effect was abolished. Endotoxin also has cytotoxic effects on Vero cells, but longer time is needed for cytolysis which is greatly diminished with formalin treatment. In conclusion, our study revealed that pathogenic strains of UPEC can exert their pathogenic effect on live cells or system with limited virulence factors gene content.

Introduction

Urinary tract infections (UTI) are probably the most common bacterial infections. Bacteria responsible for UTI often originate from the faecal and perineal flora. Under normal circumstances, these bacteria are cleared from the urinary system by effective protective mechanisms. If, however, they overcome these mechanisms, they can colonize the lower urinary tract. Subsequent progress is determined by the host susceptibility and bacterial virulent factors (Kaper *et al.*, 2004; Wullt, 2002).

Several *in vitro* and in vivo models of infection have provided insights into the progression of uropathogenic *Escherichia coli* (UPEC)-induced UTI (Justice *et al.*, 2004). The unique ability to colonize different sites and to cause a UTI is potentially due, at least in part, to the versatile genome of UPEC, which are able to remodel their genetic repertoire by acquiring and losing virulence attributes.

A number of virulence determinants facilitate the ability of UPEC to colonize the urinary tract and exert cytopathic effects, including type 1 fimbriae, P fimbriae, Dr adhesins, hemolysin, cytotoxic necrotizing factor 1, flagella, capsule polysaccharide, lipopolysaccharide O antigen, and iron transport systems (Hagan and Mobley, 2007; Slavchev *et al.*, 2009). The distribution of virulence properties can also vary depending on host characteristics and type of infection. It has been found that these virulent genes are the distinguishing factor between pathogenic and non-pathogenic strains of *E. coli* (Bisi-Johnson *et al.*, 2011). According to the conventional notion pathogens can develop from commensals by acquisition of virulence-associated genes located for example on pathogenicity islands or plasmids, and the commensal-to-pathogen shift in *E. coli* is bi-directional (Klemm *et al.*, 2007).

The question is how many virulent genes are sufficient for UPEC to produce pathogenic properties? To give insight for answering this question, we studied many UPEC strains freshly isolated from patients with urinary tract infection after complete cultural, biochemical and serological identification, explored them

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for virulence factor genes and studied the pathogenic effect of their cell-free culture supernatant in vitro on tissue culture of Vero cells (green monkey kidney cells).

Experimental

Materials and Methods

Sample collection. A total of 15 UPEC isolates obtained in counts of > 10^5 cfu/ml and in pure growth, from routine urine cultures (William and Parasuraman, 2001) of urinary tract infected patients presenting to the urology department in Ismailia university hospital, Suez canal university, Egypt. Identification of isolates was done using standard microbiological techniques (Cheesbrough, 1993). Serotyping for each pathogenic strain was performed by standard techniques as described by Ørskov and Ørskov (1984) using a commercial kit of Denka Seiken Co. LTD.

Virulence factor profiling with PCR. DNA was extracted from UPEC isolates cultured on Luria broth, with spin column according to the instructions of the manufacturer Bioer co. China. Amplification was performed in a 25 μl reaction mixture containing 8 μl of DNA template, 0.1 μl of each of nine primers (0.1–0.2 μM) from Bio Basic Inc., Canada and 8 μl of ready to go master mix 1X (containing thermostable DNA polymerase, dNTPs, reaction buffer with $(NH_4)_2SO_4$, $MgCl_2$ and Triton X-100, stabilizers) from Jena Bioscience co., Germany. PCR tubes were filled to 25 μl volume with PCR-grade water. The reaction was carried out in a PCR thermal cycler (Techgen co., USA) according to Takahashi *et al.* with modification in the following schedule: preheating at 94°C for 10 min followed by 32 cycles of denaturation at 94°C for 1 min, annealing at 60°C or 55°C, and extension at 72°C for 2 min, with a final extension at 72°C for 7 min (Table I). The PCR products were electrophoresed on 2% agarose gels, then stained with ethidium bromide, and examined under UV transillumination (Takahashi *et al.*, 2006).

Tissue culture preparation (Vero cells). Vero cells were supplied as 90% confluent monolayer sheet in growth medium with 10% new born calf serum. The confluent monolayer sheet was trypsinized with trypsin-versin 0.25% to separate the individual cells. The cells were distributed in tissue culture microtiter plate in which each well contained 10^3 cell/ml and were cultivated at 37°C in a 5% CO_2 atmosphere until the monolayer was confluent.

Exotoxin production. The selected strains were seeded in nutrient and syncase broth and then incubated for 24 h at 37°C in rotary shaker. Cultures were later centrifuged at 1800 xg for 30 min. The supernatants were then filtered through 0.22 mm Millipore filters. Two parts of exotoxins from syncase broth for each isolates were treated with heat and formalin.

Endotoxin production. Endotoxin was obtained following the procedure of Clugston and Nielson, in which bacterial suspension was harvested in PBS, and the toxin obtained after ten cycles of freezing and thawing (Culgston and Nielsen, 1974). One part of endotoxin for each isolate was formalin treated.

Toxigenicity test in cell cultures. Toxin preparations were added to the cell culture in maintenance medium consisting of MEM supplemented with 2% fetal bovine serum (Caprioli *et al.*, 1983; Slavchev *et al.*, 2009). The assays were incubated for 24 h at 37°C. The control consisted of cell monolayer containing maintenance media, PBS, nutrient broth, syncase broth or 0.05% formalin alone after staining, cells were examined with inverted microscope (Olympus 1X70-S8F2, Olympus Optical Co., LTD. Japan).

Results

Serotypes. The 14 UPEC isolates belonged to 5 serogroups, while one of the isolate had no detectable reaction when available somatic and capsular antigens reagent were tested. This strain was identified as untypable. Five of these isolates have antigenic formula O78:K80 which represented 33% of the isolates and four isolates have antigenic formula O114:K90 (27% of the isolates). However, two of the isolates were with antigenic formula O142:K86 (13% of the isolates). Another two of the isolates were O164 antigen (13% of the isolates) and one isolate with formula O157 somatic antigen (7% of the isolates) and neither of them with unidentified capsular antigen (Table II).

Virulent factor gene profiles of UPEC isolates. Nine virulent factor genes were explored for all fifteen UPEC isolates. Of the fifteen strains obtained only one (strain 11) carried all tested virulent gene factors. However, two strains showed the presence of 78% of tested genes, and other two UPEC strains showed 67% of the nine virulent genes. Four strains showed evidence for pathogenicity by detection of the most virulence gene factors, which represent 56% of the tested genes. Among the rest of the UPEC strains, four strains showed the presence of 44% of virulence gene factors, and two strains showed fewer virulence gene, *i.e.* 22% and 33%, respectively.

The distribution patterns of virulent factor genes of our isolated UPEC strains are presented in Table II. The high prevalence one is traT gene, with 100% frequency within tested strains, followed by fyuA which was 93%. The third most prevalent gene was ompT gene with incidence of 87%. Genes with lower incidence among

Table I

Virulence factors genes primer sets used for PCR.

VF gene	Product size (bp)	Annealing Temp. °C	Reverse sequence	Forward sequence	Primer used	Reference
ibeA	170	55	5'-TGGTGCTCCGGCAAAACCATGC-3'	5'-AGGGCAGGTGTGCGCCGCGTAC-3'	**ibe f/r**	Johnson & Stell, 2000
pap	336	60	5'-AGAGAGAGCCACTCTTATACGGACA-3'	5'-GCAACAGCAACGCTGGTTGCATCAT-3'	**pap 3/4**	Yamamoto *et al*., 1995
sfa/foc	410	60	5'-CGGAGGAGTAATTACAAACCTGGCA-3'	5'-CTCCGGAGAAACTGGGTGCATCTTAC-3'	**sfa/foc 1/2**	Yamamoto *et al*., 1995
cnf1	498	60	5'-CATTCAGAGTCCTGCCCTCATTATT-3'	5'-AAGATGGAGTTTCCTATGCAGGAG-3'	**cnf 1/2**	Yamamoto *et al*., 1995
hly	1,177	60	5'-ACCATATAAGCGGTCATTCCCGTCA-3'	5'-AACAAGGATAAGCACTGTTCTGGCT-3'	**hly 1/2**	Yamamoto *et al*., 1995
FyuA	880	55	5'-CGCAGTAGGCACGACGATGTTGTA-3'	5'-TGATTAACCCCGGACGCGGGAA-3'	**FyuA f/r**	Johnson & Stell, 2000
pil	207	60	5'-ATAACACGCGCGCCATAAGCC-3'	5'-CATTCGCCTGTAAAACCGCC-3'	**fimH f/r**	Tseng *et al*., 2001
ompT	559	60	5'-CCCGGGTCATAGTGTTCATC-3'	5'-ATCTAGCCGAAGAAGGAGGC-3'	**ompT-f/r**	Johnson *et al*., 2000
traT	290	60	5'-CACGGTTCAGCCATCCCTGAG-3'	5'-GGTGTGGTGCGATGAGCACAG-3'	**traT f/r**	Johnson & Stell, 2000

Table II

Distribution of virulent genes within UPEC serotypes and gene's incidence rate.

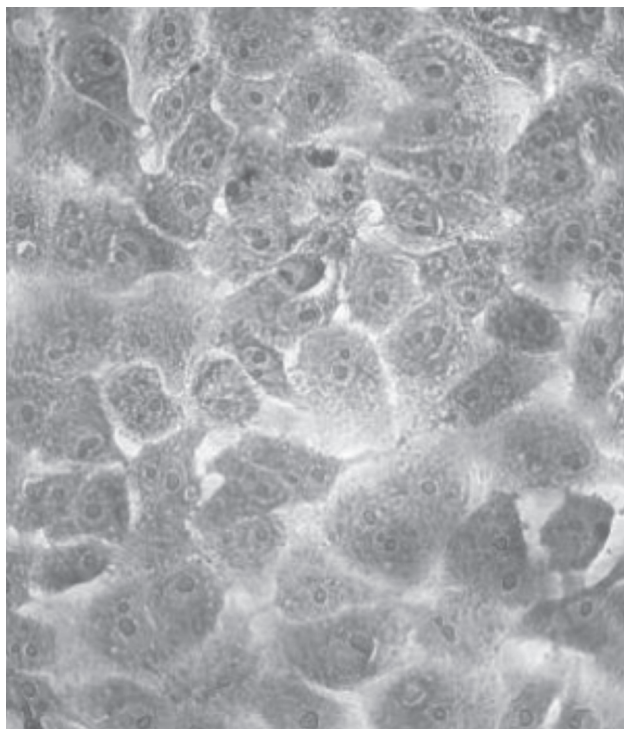| Strain No.	UPEC serotypes	Virulence genes (VG)									VG content (%)
		Pil	Pap	sfa/foc	cnf1	hly	ompT	traT	fyuA	ibeA	
1	O78 K80	+	-	-	-	-	+	+	+	-	44
2	O142 K86	+	-	-	-	-	-	+	-	-	22
3	O114 K90	+	-	-	-	-	+	+	+	-	44
4	O142 K86	+	-	-	-	+	+	+	+	-	56
5	O114 K90	-	-	+	-	-	+	+	+	+	56
6	O78 K80	+	+	+	-	-	+	+	+	+	78
7	O164 k Untypable	+	-	+	+	-	+	+	+	-	67
8	O157 k Untypable	+	-	+	+	-	+	+	+	-	67
9	O78 K80	+	-	-	+	-	+	+	+	-	56
10	O114 K90	+	-	+	-	-	-	+	+	-	44
11	O78 K80	+	+	+	+	+	+	+	+	+	100
12	O164 k Untypable	+	-	-	-	-	+	+	+	-	44
13	O114 K90	-	-	+	+	-	+	+	+	-	56
14	Untypable	+	-	+	+	-	+	+	+	+	78
15	O78 K80	-	-	-	-	-	+	+	+	-	33
Gene's incidence (%)		80	13	53	40	13	87	100	93	27	

Fig. 1. Normal Vero cell monolayer after incubation for 24 hours with sterile broth. 300X
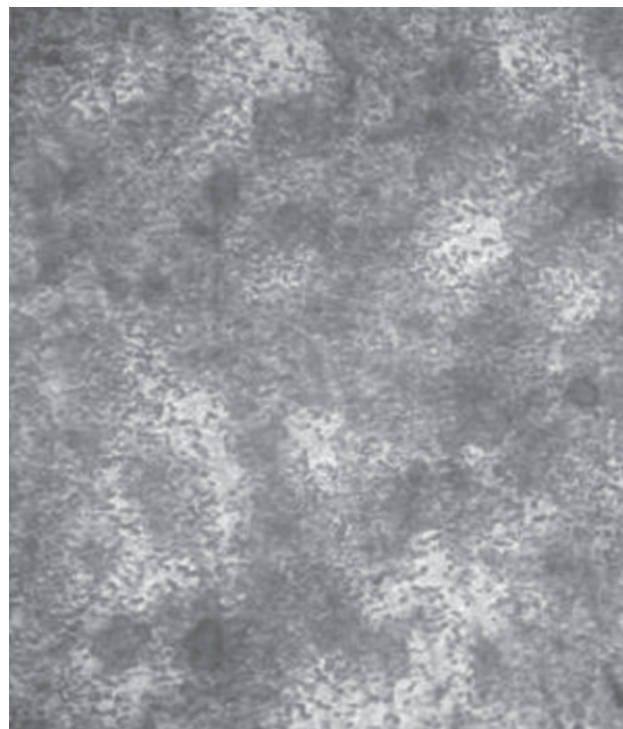

Fig. 2. Complete Vero cell lysis with debris after incubation with exotoxin of UPEC isolate prepared in syncase broth for 24 hours. 300X

the detected virulent gene factors were 27% for ibeA and 13% for both hly and pap.

Cytotoxic effects of UPEC on Vero cells. Cytopathic effects were observed in 15 culture filtrates and endotoxin preparations of UPEC strains. After incubation there were changes from monolayer spindle-shaped cells characteristic of normal Vero cells to abnormal shapes and arrangements, in addition to complete lysis which represented the major cytopathological effect (Fig. 1 and 2). These effects are expressed as percentage and cytopathological scores for comparison.

Percent of lysed cells on nutrient broth compared to syncase broth showed that significant higher numbers of lysed cells were observed in syncase broth as untreated exotoxin. However, treated filtrates, for all strains, showed significantly less virulence compared to the same syncase broth growth medium of untreated filtrate. Formalin treated-filtrates showed less pathologic effect compared to untreated filtrates, obtained from cells grown in syncase broth, or to heat-treated filtrates. All UPEC strains showed 100% cell lysed after incubation for 24 hrs except for strains 2 and 9 which showed 80% lysis when grown in syncase broth. Generally, we can conclude that cytopathic effects were varied among the UPEC strains when grown in nutrient broth but less virulent effect when compared to those recorded in syncase broth or heat-treated filtrates.

When exotoxins obtained from Syncase broth were treated with heat, the complete lysis of cells decreased

to approximately 50–70% lysis with debris. The remaining cells showed some distortions of the cytoplasm with conserved nuclei and clumped chromatin but still even a short rim of cytoplasm around. When treating the UPEC exotoxins obtained from syncase broth with formalin, the cell lysis decreased to about 10–20% in all UPEC strains and the monolayer kept its general architecture. Few cells show slight enlargement, and some showed loss of nuclear or cytoplasmic membrane. Few cells showed with cell dendrites.

The cytopathological effect of untreated UPEC endotoxins on Vero cells is shown in Fig. 3. It's mainly effects were pathological changes with no cell lysis. The Vero cell monolayer sheet with most strains lead to lost their communication and makes the cells to be separated with shrinking. Most cells were distorted without distinction between nucleus and cytoplasm. Most of the cytoplasm was taken up by vacuolation. When the cell lysate was treated with formalin the most pathological changes disappeared.

Discussion

Pathogenesis is a multi-factorial process which depends on the immune status of the host, the nature of the species or strain and the number of organisms in the initial exposure. The pathogenic mechanisms of urinary tract infection have already been investigated
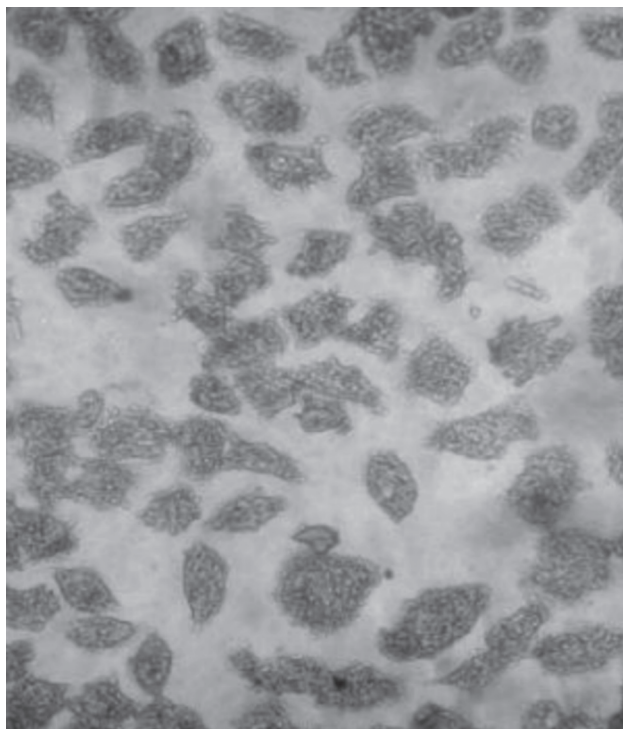
Fig. 3. Vero cell shrinkage after incubation with untreated endotoxin of UPEC isolate for 24 hours, no discrimination between nucleus and cytoplasm. 300X

in detail. UPEC strains initiate infection by binding to the superficial bladder epithelial cells that line the luminal surface of the bladder. This adherence prevents the pathogen from being washed out by the urine flow. Subsequently, bladder cells internalize the UPEC, a process that is considered an escape mechanism to protect the bacteria from the host immune system. However, internalization causes exfoliation of the superficial bladder cells harboring internalized bacteria into the urine. Before clearance, intracellular bacteria start to replicate and induce host cytokine responses, leading to the symptoms of UTI (Marquis *et al.*, 1995; Schilling *et al.*, 2001; Mulvey, 2002; Schmidt and Hensel, 2004). UPEC strains express a collection of strain-specific anti-host weapons called virulence markers (that include virulence factors), these markers specified bacteria to be pathogenic as mentioned later.

The five serogroups were classified toxigenic *E. coli* either O78 and O114, pathogenic *E. coli* O142, O157 or O114 and invasive *E. coli* as O164 according to the classification described by Scheutz and Strockbine (Scheutz and Strockbine, 2001). Most of them were isolated previously from urine cultures of UTI patients (Olesen *et al.*, 1994; Gonzalez *et al.*, 1997; Klapproth *et al.*, 2000; Abullah and Al-Moslih, 2005; Ananias and Yano, 2008). *E. coli* O157 which is mainly enterohaemorrhagic pathotype, isolated here from urine may be a transmission from the intestine, as in India there was a urinary Gram negative isolate, which studied by

sequencing and phylogenetic analysis showed 100% similarity to reference strain *E. coli* O157 (Nandy *et al.*, 2007). Other evidence for the suspected uropathogenicity of this strain was the sharing of 41.8% of genes not found in K12 (non-pathogenic strain) with the prototype CFT073 UPEC (Landraud *et al.*, 2004).

Cytotoxic effect of UPEC toxin on Vero cells using different media and different treatment is a good and applicable effect study for pathogenicity extent of UPEC in vitro, which yielded a wide array of cytopathological effects worthy to be studied.

Using Syncase broth in the production of exotoxin, it was striking because of great difference and severity in cytopathological effects compared with that produced upon using nutrient broth. This may attribute to the increased production of toxins with casamino acids, which is the major constituent of syncase broth with low carbohydrate and iron. Increased toxin up to three fold with this media was proved with recombinant cholera toxin previously (Osek *et al.*, 1995). These toxins caused a complete Vero cell lysis, and this strong effect persists with small concentrations of toxins.

The toxins produced by UPEC here were thought to be more than one type because some of these Verotoxins (toxic to Vero cells) was detoxified with heat while others detoxified with formalin, the last one was still effective even after formalinization, in contrast with known Vero toxin and Shiga toxin, which were inactivated by heat (Caprioli *et al.*, 1983; Carbonell *et al.*, 1997; Speirs *et al.*, 1997). Vero cells cytopathic effect of treated toxin in non lysed cells presented as enlarged cells, cell membrane change and little nuclear membrane change.

Freeze-thaw lysate affects cell as a whole leading to inability to distinguishing the cytoplasm and nucleus. These may be due to cell preparation for apoptosis, vacuolation and loss of continuation between cells present may enhance this suggestion. This effect was negated after endotoxin formalinization.

In essence, the ability of pathogenic bacteria to cause disease in a susceptible host is determined by multiple virulent factors acting individually or together at different stages of infection, so bacteria equipped with virulent genes coded for these VFs. Several of these virulent genes are involved in increasing the pathogen's fitness and adaptability.

Some researchers described the use of VFs genes content for the prediction of UTI status. Johnson *et al.*, (2005) found that the predictors of pyelonephritis included three traditionally recognized uropathogenic traits adhesins genes (*pap*, *afa/dra*, and *sfa/foc*), and meningitis-associated trait (*ibeA*). Vranes *et al.* noted that the strains isolated from patients with acute pyelonephritis were found to mostly express all five or four virulence markers (O-serogroup, adhesin

type, motility, production of hemolysin, and the amount of capsular polysaccharide antigen) tested, while the less virulent strains were detected in the group of patients with chronic pyelonephritis. The lowest virulence was observed among the strains isolated in the group of patients with asymptomatic bacteriuria (Vranes *et al.*, 2001).

To what extent can these VFs bring about the net pathological effect? We investigated VFs genes or so-called pathogenicity genes content of the isolates. It was obvious that for all isolates with a different content of VFs genes, all were produce a destructive pathogenic effect (complete cell lysis) on Vero cells equally, *i.e.* no significant difference between those isolates with high VFs gene content and those with low VFs gene.

In vivo other conditions affect the environment of infection. As one can see, cytotoxins, secretion systems, fimbriae and others can be virulent factors in some conditions, colonization factors in other conditions and symbiosis factors in yet other conditions. Pathogenic and commensal microorganisms appear to employ similar or even identical molecular mechanisms to express their pathogenic or symbiotic potential (Hentschel *et al.*, 2000). In particular, both pathogenic and symbiotic bacteria must actively manipulate the host immune system to make it possible for them to colonize the body. Any microorganisms will be pathogenic or commensal in a given context, under given conditions. It is the interplay between the context and the intrinsic features of a microbe that make it pathogenic or safe (Swiatczak *et al.*, 2011). The major conclusion of our study is that pathogenic strains of UPEC can exert their pathogenic effect on living cells or systems with a few amount of virulent factors gene content in a given condition with a given context of host factors and molecules in the environment.

Disclosures

The authors have no financial conflicts of interest.

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