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Comprehensive expression analysis of pathogenicity genes in uropathogenic *Escherichia coli* strains

Gloria Luz Paniagua-Contreras, Tania Hernández-Jaimes, Eric Monroy-Pérez, Felipe Vaca-Paniagua, Clara Díaz-Velásquez, Alina Uribe-García, Sergio Vaca*

Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, Av. de Los Barrios 1, Los Reyes Iztacala, Tlalnepantla 54090, Edo. de México, Mexico

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ABSTRACT

In this study, we investigated distinct expression patterns of genes encoding iron-acquisition systems, adhesins, protectins, and toxins in human uroepithelial cells infected with 194 uropathogenic *Escherichia coli* (UPEC) strains *in vitro*. We assessed the association of these genes with antibiotic resistance genes in this group of UPEC strains, previously characterised by polymerase chain reaction (PCR). Strains were isolated from patients with urinary tract infections (UTIs) from Unidad Médica Familiar de Salud Pública, located in Estado de México, México. Antibiotic resistance genes were identified by PCR, and the expression of virulence genes was detected by reverse-transcriptase-PCR after *in vitro* infection of cultured A431 human keratinocytes derived from a vulvar epidermoid carcinoma. The most frequently expressed virulence genotypes among the investigated UPEC strains included *usp* (68%), *iha* (64.9%), *kpsMT* (61.3%), *fim* (58.2%), *irp2* (48.4), *papC* (33.5%), *set* (31.4%) and *astA* (30.9%), whereas the most frequently detected antibiotic resistance genes were *tet(A)* (34%), *sul1* (31.4%) and *TEM* (26.3%). Furthermore, the most abundant pattern of gene expression (*irp2/fim/iha/kpsMT/usp*), associated with 8 different combinations of antibiotic resistance genotypes, was exhibited by 28 strains (14.4%). Taken together, these results indicate collective participation of distinct virulence UPEC genotypes during *in vitro* infection of cultured human epithelial cells, suggesting their potential involvement in UTI pathogenesis.

1. Introduction

Uropathogenic *Escherichia coli* (UPEC) is responsible for the majority of community acquired urinary tract infections (UTIs) [1,2]. The pathogenicity of UPEC strains is due to their wide range of virulence factors, including genes related to iron-acquisition systems (*iroN, irp*2, and *iuc*), adhesins (*fim, afa*I, *sfa*, *iha*, *tsh*, *papC*, and *papG*I, -II, and -III), protectins (*kpsMT, ompT*, and *iss*), and genes encoding toxins (*cnf1, hlyA, set, astA, vat, usp,* and *cva/cvi*) [3,4]. These virulence factors contribute to bacterial host colonisation and invasion, biofilm formation, tissue damage, stimulation of the inflammatory response, evasion of the immune response, and ascent to the bladder and kidney [5,6].

The increasing numbers of strains that are resistant to common antibiotics has recently complicated the treatment of UTIs caused by UPEC [7,8]. *E. coli* strains resistant to four or more unrelated families of antibiotics have been reported [9,10], and their emergence is considered a serious health concern [11]. Virulence genotypes associated with antibiotic resistance phenotypes have been recently studied in distinct serogroups of *E. coli* strains, isolated from patients with UTI [12–14]. However, the expression of these virulence markers among UPEC strains has been scarcely studied, or studied using a limited number of UPEC strains, focusing on the expression of only one trait [15]. For this reason, we implemented an *in vitro* model of infection of human epithelial cells with 194 UPEC strains isolated from UTIs, to determine the distinct patterns of expression of the bacterial virulence genes that encode iron-acquisition systems, adhesins, protectins, and toxins. Furthermore, we investigated association of these virulence genes with patterns of antibiotic resistance.

* Corresponding author.
E-mail address: vacasergio@gmail.com (S. Vaca).

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2. Material and methods

2.1. Bacterial strains

A total of 194 *E. coli* strains previously characterised by conventional polymerase chain reaction (PCR) [14], were used for reverse-transcriptase (RT)-PCR-based expression analysis of genes encoding iron-acquisition systems, adhesins, protectins, and toxins, as well as for genes associated with antibiotic resistance. Each one of the 194 UPEC strains was isolated from one UTI patient.

2.2. DNA extraction

DNA extraction was performed according to the boiling method [14]. Bacteria were grown in brain-heart-infusion broth (Becton Dickinson, México City, México) at 37 °C overnight. The overnight culture (1.5 mL) was pelleted, the supernatant discarded, and the cell pellet suspended in 200 μL sterile water. The resuspended pellet was then incubated at 100 °C for 10 min and centrifuged at 10,000 × *g* for 5 min. The pellet was discarded and the DNA present in the supernatant was stored at −20 °C until use.

2.3. Detection of antibiotic resistance genes

The primers and PCR conditions used for detection of genes associated with resistance to sulfonamides (*sul1*), streptomycin (*aadA1*), chloramphenicol (*cat1* and *cmlA*), β-lactams (*tem*), tetracycline [*tet(A)* and *tet(B)*] and trimethoprim (*dfrA1*) are listed in Table 1.

2.4. Infection of cultured A431 cells with UPEC strains

To promote the expression of UPEC virulence genes, an *in vitro* model of infection was implemented using the A431 cell line derived from a vulvar epidermoid carcinoma (ATCC, Manassas, VA, USA). A total of 2×10^6 *E. coli* cells, suspended in 50 μL phosphate-buffered saline, were inoculated onto the surface of the A431 cultured cells and incubated at 37 °C for 72 h under 5% CO_2 atmosphere and saturated humidity. Change of cell medium (SkinEthic, Lyon, France) was made every 24 h with micropipettes and using sterile tips.

2.5. E. coli RNA purification and reverse transcription

E. coli cells were harvested from inoculated A431 cultures and suspended in 1 mL RNA Protect Bacteria Reagent (Qiagen, Hilden, Germany). The sample was vortexed for 30 s, followed by incubation at room temperature (26–28 °C) for 5 min. After centrifugation at 10,000 × *g* for 10 min, the cells were resuspended in 200 μL TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8]) containing 1 mg/mL lysozyme. The sample was vortexed for 10 s and incubated at room temperature for 5 min. Automated purification of total RNA was performed using the robotic workstation QIAcube (Qiagen) and the RNeasy Mini Kit (Qiagen), including DNase treatment, according to manufacturer instructions. The concentration and purity of total RNA were analysed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). cDNA synthesis was performed with the QuantiTec reverse-transcription kit (Qiagen), according to manufacturer instructions.

2.6. Real-time PCR amplification of virulence genes from UPEC strains

The primers used for real-time PCR amplification of virulence genes encoding iron-acquisition systems (*iroN* [iron], *irp2* [iron-repressible protein], and *iuc* [aerobactin]), adhesins (*fim* [type-1 fimbriae], *afaI* [afimbrial adhesin I], *sfa* [S fimbriae], *iha* [non-haemagglutinating adhesin], *tsh* [temperature-sensitive haemagglutinin], *papC, papG* allele I, *papG* allele II, and *papG* allele III [pilus associated with pyelonephritis]), protectins (*kpsMT* [K-antigen], *ompT* [outer-membrane protease T], and *iss* [increased serum-survival protein]), and toxins (*cnf1* [cytotoxic necrotising factor 1], *hlyA* [haemolysin], *set* [*Shigella* enterotoxin 1], *astA* [enteroaggregative heat-stable toxin], and *vat* [vacuolating autotransporter toxin]) are listed in Table 2 and have been described previously [20]. The Rotor-Gene SYBR Green PCR kit (Qiagen) was used for real-time PCR experiments. A final volume of 25 μL was used for each reaction, including 12.5 μL SYBR Green master mix, 1 μL of 1 μM forward primer, 1 μL of 1 μM reverse primer, 2 μL cDNA (20 ng), and 8.5 μL RNase-free water. The amplification conditions used were 95 °C for 5 min, followed by 95 °C for 5 s, and 40 cycles of annealing/extension at 60 °C for 10 s, using a Corbett Rotor Gene 6000 (Qiagen). For each assay, the *E. coli* strain ATCC 11,775 was used as a positive control, and the *Staphylococcus epidermidis* strain ATCC 35,984 was used as a negative control.

2.7. Statistical analysis

The frequency of gene expression in the UPEC strains was analysed by the chi-squared test. A $p < 0.05$ was considered significant.

Table 1
Primers used for detection of antimicrobial resistance genes in the UPEC strains.

Antimicrobial agent	Resistance gene	Primer sequence (5′–3′)	Product size (bp)	References
Sulfonamides	*sul1*	(F) TTCGGCATTCTGAATCTCAC (R) ATGATCTAACCCTCGGTCTC	822	[16]
Streptomycin	*aadA1*	(F) TATCCAGCTAAGCGCGAACT (R) ATTTGCCGACTACCTTGGTC	447	
Chloramphenicol	*cat1*	(F) AGTTGCTCAATGTACCTATAACC (R) TTGTAATTCATTAAGCATTCTGCC	547	
	cmlA	(F) CCGCCACGGTGTTGTTGTTATC (R) CACCTTGCCTGCCCATCATTAG	698	
β-lactams	*TEM* variants including TEM-1 and TEM-2	(F) CATTTCCGTGTCGCCCTTATTC (R) CGTTCATCCATAGTTGCCTGAC	800	[17]
Tetracycline	*tet(A)*	(F) GGTTCACTCGAACGACGTCA (R) CTGTCCGACAAGTTGCATGA	577	[18]
	tet(B)	(F) CCTCAGCTTCTCAACGCGTG (R) GCACCTTGCTGATGACTCTT	634	
Trimethoprim	*dfrA1*	(F) GGAGTGCCAAAGGTGAACAGC (R) GAGGCGAAGTCTTGGGTAAAAAC	367	[19]