

CHINESE HERB-RESISTANCE AND ADHERENCE TO HUMAN UROEPITHELIAL CELLS OF
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*Email: tongyanqing@yahoo.com**Abstract**

Background: In order to define the virulence factors between Chinese herb-resistant uropathogenic *E. coli* and susceptible strains, the UPEC isolates were classified into two groups according to its resistance to Chinese herbs.

Materials and Methods: The susceptibility profile of strains was determined by disk diffusion method. PCR systems were used to detect genes encoding papC, Aer, hly and cnf1. Isolated human urothelial cells were incubated *in vitro* and investigated with light microscope immunohistochemistry. Adhesion of *E. coli* to urothelial cells was studied *in vitro*.

Results: The results showed that, among the 105 UPEC isolates, 18 were resistant to the herbal concoction. Cnf1 and papC occurred in $\geq 66.7\%$, of herb-resistant isolates, while, hly and Aer occurred in 22.2% and 27.8% of strains respectively. Only one gene (Cnf1) occurred in $>40\%$, of Herb-susceptible isolates. Other genes were also found in susceptible isolates: papC (20.7%), hly (11.5%), and Aer (6.9%). Light microscopy and immunochemical investigations demonstrated the normal pelvic transitional epithelial cells cultured. The adherence of strains in both groups increased in 30 min., and reached its peak at 60, (Susceptible *E. coli*) or 120 min., (Resistant *E. coli*). The adhesion of the susceptible bacteria to human uroepithelial cells was significantly lower compared with that of the resistant *E. coli* ($p < 0.05$).

Conclusion: These findings revealed that, Chinese herb-resistant uropathogenic *E. coli* isolates that are hemolytic, and have Aer, papC, hly, Cnf1 genes are more able to be uropathogenic and adherent.

Key words: *Escherichia coli*; Adherence; Virulence genes; Human uroepithelial cells; Chinese herb-resistance.

Introduction

Escherichia coli (*E. coli*) is the principal cause of urinary tract infections (UTIs) in both community, and hospital settings in China. Most UTIs are due to uropathogenic *E. coli* (UPEC) infections. As one of the variety of virulent factors, the adhesion of *E. coli* to uroepithelial cells is the first step in the development of UTIs (Beachey, 1981), enabling them to colonize the host.

Herbal medicine has been used in the treatment of UTIs over the years. Herbal formulation used in this study was proved to have anti-microbial and anti-adherent effect, in our previous research (Tong *et al.*, 2011a; Tong *et al.*, 2011b). But clinical isolates of uropathogenic *E. coli* resistance to this herbal formulation have been isolated. In order to define the virulence factors, and adherent ability between Chinese herb-resistant uropathogenic *E. coli* and susceptible strains in China, we compared four specific virulence genes expression and the bacterial adherence to human uroepithelial cells between these isolates.

Methods**Bacterial strains**

A total of 105 *E. coli* isolates from humans with community-acquired UTIs were used in this study. Isolation of *E. coli* strains was performed according to standard laboratory protocols and *E. coli* were isolated from, $>10^5$ cfu., of a uropathogen per milliliters of midstream

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urine culture. After isolation, the bacteria were kept frozen at, -70 °C after the addition of 20% (v/v) glycerol and they were not sub-cultured more than twice before the investigation.

Chinese herbs and susceptibility test

The Chinese herbs were prepared by the following ratio: Tong Cao (*Medulla Tetrapanacis*), 4 : Hua Shi (*Talcum*), 20 : Chi Shao (*Radix Paeoniae Rubrae*), 20 : Xiao Hui Xiang (*Fructus Foeniculi Vulgaris*), 30 : Rou Gui (*Cortex Cinnamomi*), 30 : Li Zhi He (*Semen Litchi*), 30 : Tian Kui Zi (*Radix Semiaquilegiae*), 30 : Zi Hua Di Ding (*Herba cum Rd Viola Yedoensis*), 30 : Qu Mai (*Herba Dianthi*), 40 : Ma Chi Xian (*Herba Portulacae*), 60 : Pu Gong Ying (*Herba taraxaci*), 60. The eleven crude drugs were mixed in 800ml water (100 °C for 30 min. twice), leaving 100ml liquor after decanting the mixture. The liquor was centrifuged, filtered and sterilized with the solution of 0.5g/ml as the drug concentration. Standard disc diffusion methodology (Maidment *et al.*, 2006), was used to test the concoction against *E. coli* strains. The minimal inhibitory concentration (MIC), of herbal solution for susceptible *E. coli* was determined as follows: Starting with the 0.5g/ml concentration, seven serial dilutions were prepared in LB agar (0.5g/ml, 0.2g/ml, 0.1g/ml, 0.05g/ml, 0.025g/ml, 0.0125g/ml, and 0.0g/ml). These concentrations were inoculated with bacterial strain. The MIC was the lowest concentration of herbal solution that prevented obvious turbidity after incubation for 18 hr at 37 °C.

Hemolysis determination

UPEC isolates were assessed for hemolysis activity on blood agar plates (supplemented with 5% sheep blood), using standard methods (Maidment *et al.*, 2006).

Virulence genotyping

UPEC strains were investigated for 4, virulence-related genes by single PCR essays, as previously described (Forbes *et al.*, 1998; Johnson *et al.*, 2000). Targeted genes and their primer sequence for the amplification procedures are given in Table 1. The amplified products were visualized by standard gel electrophoresis of 15 µl of the final reaction mixture in 1.0% agarose gels in Triacetate/EDTA (TAE) buffer at 5 V cm⁻¹. The gels were stained with ethidium bromide (0.5 µg ml⁻¹) for 2 min, washed in distilled water, analyzed under UV light (300 nm), and photographed. DNA Ladder 100 bp was used as molecular size marker. All amplification procedures were repeated three times.

Tissues

The collection of surgical specimens was approved by the relevant Local Research Ethics Committees and had full patient consent. Ureter specimens obtained at the time of radical nephrectomy for renal cell carcinoma (a Chinese, male, age 56 years). Urothelial tissue samples were obtained at surgery. Tissue samples were transported at room temperature from surgery in Hanks' balanced salt solution (Shanghai Biocolor Bio-Science & Technology Company, China), containing 10mM HEPES, pH 7.6 and 20 kallikrein-inhibiting units (KIU)/ml of aprotinin (Shanghai Biocolor Bio-Science & Technology Company, China). The specimen was trimmed to remove any serosa, fat, or debris and dissected into 1-cm² pieces. The sample was placed into HBSS, containing 10 mM HEPES, pH 7.6, 20 KIU/ml Trasylol, and 0.1% (wt/vol.), EDTA and incubated at 4 °C overnight to release urothelial cell sheets.

Cell culture

The isolated urothelial cells were incubated in vitro as previously described (Southgate *et al.*, 1994; Southgate *et al.*, 2002). NHU cells were maintained in keratinocyte serum-free medium (KSFM), containing recombinant epidermal growth factor and bovine pituitary extract at the manufacturer's recommended concentrations (Invitrogen, Paisley, UK) and supplemented with 30 ng/ml cholera toxin to improve cell plating and attachment (Hutton *et al.*, 1993). KSFM fully supplemented with the aforementioned factors will be referred to as KSFM complete (KSFMc). The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂ in air. The medium was replaced every 24 h. Sections were investigated by light microscopy at ×400 magnification at day 5, 10, 15 and 20.

Light microscope immunohistochemistry

The seeded samples were investigated with immune histo-chemical methods at day 20. The samples were treated with 1% H₂O₂ for 5 min, washed with 0.1 M PBS and pre-incubated for 20 min., at room temperature with 0.1 M PBS (pH 7.4) added with 3% normal goat serum (NGS) and 0.5% Triton X-100. Then the samples were incubated in the primary antibody. The rabbit polyclonal antibody, which was raised in the rat against 21, amino acid peptide corresponding to the C-terminus of the rat capsaicin receptor protein (Zhongshan Goldenbridge Biotechnology Co. Ltd, China), was used at dilution 1:2000 in 0.1 M PBS (pH 7.4) added with 1.5% NGS and 0.5% Triton X-100. The antibody was applied overnight at 4 °C. This was followed by incubation with the biotinylated secondary antibody (dilution 1:100) for 1hr, at room temperature, rinsing in PBS, and by incubation with the avidin-biotin-complex (Zhongshan Goldenbridge Biotechnology Co. Ltd, China), for 30 min. Immuno-reactivity was detected at room temperature by addition of 3,3'-diaminobenzidine (Zhongshan Goldenbridge Biotechnology Co. Ltd, China), as a substrate, for 5-6 min. After washing in PBS containing 3% NGS and 0.5% Triton X-100, all samples were incubated with the primary antisera in moist chambers.

All the samples were counterstained with haematoxylin for nuclei labelling.

In vitro adhesion of E. coli to human urothelial cells

5 ml of the bacterial suspension was added to a Leighton tube containing a cover slip with attached urothelial cells, and incubated for 1 hr, with gentle shaking at 37 °C. The cover slip was removed from the Leighton tube and washed twice with PBS to remove bacteria that had not adhered. The urothelial cells were fixed for 15 min., in methanol, washed twice again with PBS, and stained for 20 min., with 30% filtered Giemsa stain (Giemsas-L6sung; Merck, Darmstadt, Germany). After two washings with distilled water, the cover slip was dried in the air and mounted upside down on a glass slide. To determine the changes of adherence by herbal solution, the assay was carried out in the presence of herbal solution (MIC). Adherence was examined with a light microscope at ×400, magnification. 100, urothelial cells were selected randomly. The number of cells with bacterial adhesion and total number of adherent bacteria were counted. The adherence index and adherence ratio were calculated at time of 15, 30, 60, 120 and 180 min:

Adherence index = number of adherent bacteria/100;

Adherence ratio (%) = (number of cells with bacterial adhesion/100) ×100%.

Table 1: Primers used for the amplification study

Gene	Amplicon size (bp)	Primer sequence
Aer	209	5' end: 5'-CCAAGGGTCTGTGGCGACA-3'
		3' end: 5'-TTTCACCGGTAACAGGATTG-3'
papC	328	5' end: 5'-GACGGCTGTACTGCAGGGTGTGGCG-3'
		3' end: 5'-ATATCCTTTCTGCAGGGATGCAATA-3'
hly	1177	5' end: 5'-AACAAGGATAAGCACTGTTCTGGCT-3'
		3' end: 5'-ACCATATAAGCGGTCATCCCGTCA-3'
CnfI	498	5' end: 5'-AAGATGGAGTTTCCTATGCAGGAG-3'
		3' end: 5'-CATTCAGAGTCCTGCCCTCATTATT-3'

Table 2: Prevalence of virulence genes in UPEC isolates

Gene	Herb-resistant isolates		Herb- susceptible isolates	
	No. of isolates	Percentage	No. of isolates	Percentage
Aer	5	27.8	6	6.9
papC	12	66.7	18	20.7
hly	4	22.2	10	11.5
CnfI	15	83.3	35	40.2

Table 3: Adherence assay of *E. coli*; a $p < 0.05$ compared with resistant *E. coli*

Time (min)	Resistant <i>E. coli</i>		Susceptible <i>E. coli</i>	
	Adherence ratio (%)	Adherence index	Adherence ratio (%)	Adherence index
15	17.3 ± 2.1	4.4 ± 0.5	8.3 ± 0.8 ^a	2.9 ± 0.2 ^a
30	54.8 ± 5.1	17.8 ± 1.6	28.7 ± 2.9 ^a	10.2 ± 1.1 ^a
60	58.3 ± 5.9	19.2 ± 1.8	29.4 ± 3.0 ^a	11.3 ± 1.3 ^a
120	63.5 ± 6.7	22.4 ± 2.1	28.9 ± 2.4 ^a	10.9 ± 1.2 ^a
180	60.1 ± 5.8	20.3 ± 2.0	26.7 ± 2.5 ^a	9.8 ± 0.9 ^a

Results

Susceptibility test

Among the 105 UPEC isolates, 18 (17.4 %) was resistant to the herbal concoction.

The MIC value of herbal solution was 0.1g/ml for susceptible *E. coli*. Among the 87, susceptible strains, 6 (6.9%) caused clearing of blood agar around areas of bacterial growth. Among the 18, resistant isolates, the number is 3 (16.7%).

Table 2 summarizes the content of virulence genes for UPEC isolates. The genes occurred in the majority of herb-resistant isolates, with Cnf1 and papC occurring in ≥66.7% of isolates, except for hly, and Aer, which occurred in 22.2% and 27.8% of strains respectively. Only one gene (Cnf1) occurred in >40%, of herb-susceptible isolates. Other genes were also found in susceptible isolates: papC (20.7%), hly (11.5%), and Aer (6.9%). Figure 1 shows the representative examples of the comparative PCR analysis of herb-resistant and herb-susceptible strains.

At day 5, cell growth was observed around the sample pieces. At day 10, the regular pattern of urothelial cells can be noticed. Most of the cells were spindle shaped. At day 15, cells lush, densely covered the surface of the medium. At day 20, urothelial cells in KSFM grew as closely packed cobblestone-like colonies (Figure 2).

Light microscopy demonstrated cell structures were observed as well in specimens. Hematoxylin staining confirmed that all the specimens were normal and no dysplasia, or tumoral cells were recorded. Representative results are shown in Fig.3. Immuno-labelling intensity in all cells was similar. No difference in the immuno-labelling was recorded. The labelling was often slightly granular. No difference in labelling intensity was recorded among all cells (Figure 3).

Light microscopy and immunochemical investigations demonstrated the normal pelvic transitional epithelial cells cultured.

The role of herbal solution on the adhesion to human uroepithelial cells was evaluated. The adherence of strains in both groups increased at 30 min time point and reached the peak at 60 (Susceptible *E. coli*) or 120 min (Resistant *E. coli*) time point. The adhesion of the susceptible bacteria to human uroepithelial cells was significantly lower compared with that of the resistant *E. coli* (Table 3).

Discussion

The convenience of use of Chinese herbs is well recognized. However, increased prescription of Chinese herbs for common infections such as UTIs will facilitate the emergence of resistance, and promote the emergence of Chinese herb-resistant strains. Although clinical strains of UPEC resistance to Chinese herbs have been isolated in our previous study, the adherent ability and expression of virulence genes between resistant and susceptible isolates have not to our knowledge been compared before. Since Chinese herbs are currently used to UTIs, we feel that it is appropriate to develop a stronger understanding of the interaction between Chinese herb-resistance and changes in virulence factor expression.

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In this study, we have compared the adherent ability and genetic profiles of resistant and susceptible strains.

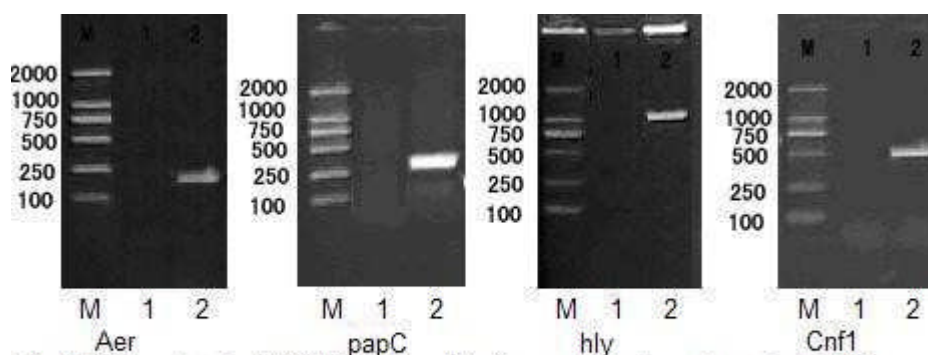


Fig.1 PCR analysis of UPEC strains. Strains were analyzed by single-PCR approaches. The herb-resistant strains were positive for Aer, papC, hly and Cnf1. M, Marker; 1, herb-susceptible strains; 2, herb-resistant strains.

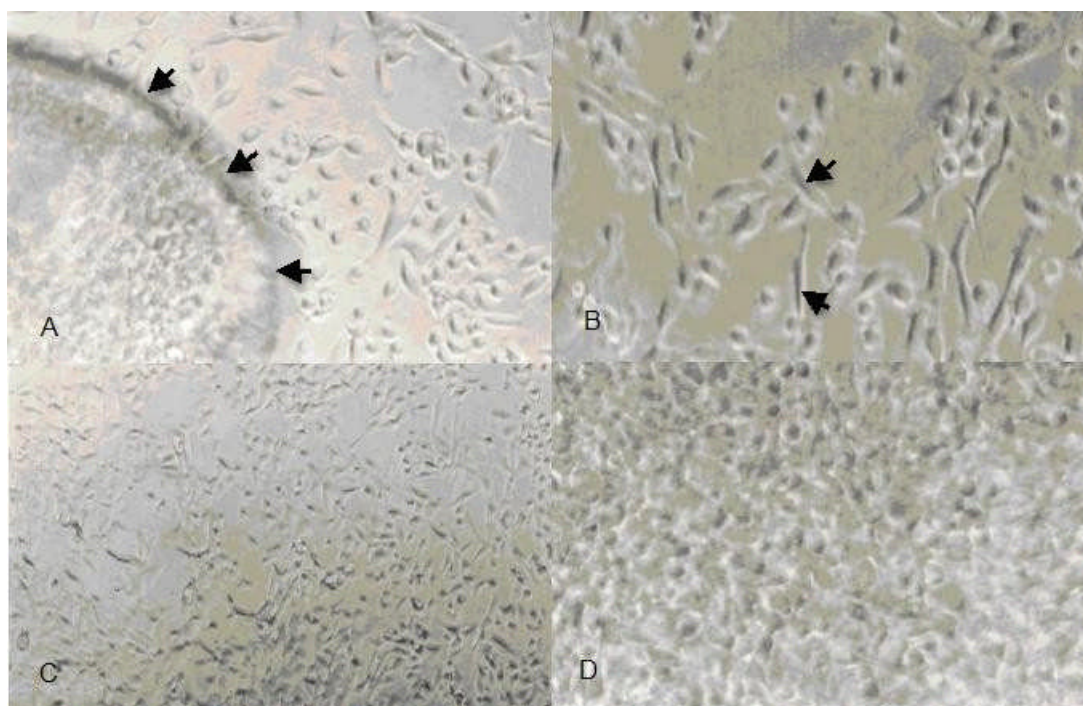


Fig.2 Cell culture. A. day 5. Cell growth around the sample piece (Arrow). B. day 10. Cells are spindle shaped (Arrow). C. day 15. Cells densely covered the surface of the medium. D. day 20. Cells grew and exhibited an epithelial cobblestone appearance.

In this research, the urothelial cells were derived from Chinese people, in whom, the surface of urothelial cells are almost with P-blood group antigens. Hence, *E. coli* with P fimbriae will show the combined effects of adhesion with these antigens. Thus, human urothelial cells obtained are suitable for adhesion study. Normal human urothelial cells are close to the micro-environment in vivo and can truly reflect the process of UTIs.

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The data presented in this study reveal a considerable difference between Chinese herb-resistant and herb-susceptible uropathogenic *E. coli* clinical isolated. The increase in adhesion of resistant *E. coli* culturing in herbal solution can theoretically be caused by several mechanisms, such as the maintenance of fimbriae, which need further investigations.

The data presented in this study also reveal a considerable difference between Chinese herb-resistant and herb-susceptible UPEC, clinical isolated. This study shows that Chinese herb-resistant UPEC isolates have a higher prevalence of carrying virulence genes than susceptible isolates.

The adhesin gene, *papC*, presents frequency of 66.7% in resistant isolates and 20.7% in susceptible ones. Since the binding to the host cells is a key factor for development of infection, the expression of adhesin confers advantages to pathogens (Antão *et al.*, 2009). *hly*, which contained in a higher number of resistant strains, is associated with higher clinical severity in UTI patients (Marrs *et al.*, 2005). *hly* can inactivate the serine/threonine kinase Akt, which plays an essential role in host cell cycle progression, metabolism, survival, and inflammatory signaling pathways (Wiles *et al.*, 2008a).

Production of toxins, such as *cnf1*, causes tissue damage, facilitating bacterial dissemination, releasing of host nutrients, and may also modulate host signaling pathways (Wiles *et al.*, 2008b; Smith *et al.*, 2008). The frequency of *cnf1* expression is extremely higher in resistant strains, which indicate higher degree of virulence.

In summary, Chinese herb-resistant uropathogenic *E. coli* isolates that are haemolytic and have *Aer*, *papC*, *hly*, *Cnf1* genes are more able to be uro-pathogenic and adherent. Furthermore, the resistance itself may be a virulence factor that allows for the survival of a bacterium within the urinary tract of patients.

As Chinese herbs use in UTIs drives up the frequency of Chinese herb-resistant isolates of UPEC, clinicians will see a modification in the patterns of UTIs accompanied by changes in the expression of specific UPEC virulence factors.

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