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VIRULENCE GENOTYPE AND PHYLOGENETIC GROUPS IN RELATION TO CHINESE HERB RESISTANCE AMONG *ESCHERICHIA COLI* FROM PATIENTS WITH ACUTE PYELONEPHRITIS

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Abstract

Background: Clinical isolates of herb-resistant uropathogenic *E. coli* were isolated. It was possible that the virulence genotypes and phylogenetic background of *E. coli* differed between Chinese herb-resistant *E. coli* and -susceptible isolates. For this purpose, the prevalence of virulence factors (VFs) and phylogenetic background, with regard to Chinese herb resistance, among *E. coli* strains causing acute pyelonephritis from China were investigated.

Materials and Methods: *E. coli* isolates from patients with acute pyelonephritis were used in this study. Standard disc diffusion methodology was used to test the susceptibility of Chinese herbal concoction against *E. coli* strains. Multiplex PCR amplifications employed three markers (chuA, yjaA, and TSPE4.C2) to classify *E. coli* isolates into one of four phylogenetic groups (group A, B1, B2, or D). The isolates were also tested for 14 virulence-associated traits (VFs) of uropathogenic *E. coli*.

Results: A total of 115 *E. coli* strains were isolated. 79 (68.7%) were susceptible and 36 (31.3%) were resistant to the herbal concoction. 20.9% of the isolates encoded three or more of VFs for which they were screened, with 13.9% in susceptible isolates and 36.1% in resistant isolates. The key VFs (*fyuA* and/or *iutA* siderophores) present in >80% of isolates. The *papA* and *papC* adhesins were detected in the majority of resistant isolates (72.2% and 63.9% respectively). 78.5% of susceptible isolates belong to phylogenetic groups A, while 83.3% of resistant isolates belong to group B2.

Conclusion: *PapA* and *papC* are significant VFs with an essential role in contributing to Chinese herb-resistance. Chinese herb-resistance is associated with a shift towards more virulent strains and B2 phylogenetic group.

Key words: *Escherichia coli*; Virulence factors; Phylogenetic group; Chinese herb-resistance.

Introduction

Pyelonephritis is an important contributor to the overall health burden and costs attributable to urinary tract infection (UTI). Recently, managing pyelonephritis has been increasingly complicated by the increasing prevalence of resistance to antimicrobial agents among the causative microorganisms, most notably *Escherichia coli* (*E. coli*) (Jeon *et al.*, 2012). *E. coli* causes 80% of cases of acute pyelonephritis in women and 70% of cases in men (Czaja *et al.*, 2017).

Herbal medicine has been an integral part of traditional Chinese medicine (TCM) for more than 2000 years. Many herbal formulations have been developed and are used in the treatment of UTIs. Herbal formulation used in this study was proved to have antimicrobial and anti-adherent effects in our previous reseach (Tong *et al.*, 2011a; Tong *et al.*, 2011b). But clinical isolates of uropathogenic *E. coli* resistant to this herbal formulation have been isolated. It is possible that the virulence genotypes and phylogenetic background of *E. coli* differs between Chinese herb-resistant *E. coli* and -susceptible isolates. For this purpose, the prevalence of virulence factors (VFs) and phylogenetic background, with regard to Chinese herb resistance, among *E. coli* strains causing acute pyelonephritis from China were investigated.

Materials and Methods

Clinical isolates

The study was performed at the First Affilliated Hospital to Changchun University of Chinese Medicine between 2011 and 2012. Inclusion criteria were that patients with clinical symptoms of acute pyelonephritis: bacteriuria $\geq 10^5$/ml, fever ≥ 38.3□, costovertebral pain, and dysuria (Olesen *et al.*, 1994). Exclusion criteria were current treatment for urolithiasis or hydronephrosis, pregnancy, hemodialysis or peritoneal

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dialysis, a history of kidney transplantation or known presence of polycystic kidney disease.

Only one isolate per patient was included in our sample. All urine isolates in this study were processed at the Clinical Microbiology Laboratory in Changchun University of Chinese Medicine. The strains were isolated according to standard laboratory protocols (Murray *et al.*, 2003) and were isolated from>10^5 cfu. of a uropathogen per millilitre of midstream urine culture. After isolation, the bacteria were kept frozen at -70□ after the addition of 20% (v/v) glycerol and they were not subcultured 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 Violae Yedoensitis*) 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□for 30 mins 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. The extracts were clear brown, with relative weight 1.3020, viscosity 505 cP and pH value 5.6. Standard disc diffusion methodology (National Committee for Clinical Laboratory Standards, 2002) was used to test the concoction against *E. coli* strains. *E. coli* ATCC 25922 was the reference strain.

Phylogenetic analysis and virulence genotyping

Multiplex PCR amplifications employed three markers: (i) chuA, (ii) yjaA, and (iii) TSPE4.C2. Use of these markers allowed the classification of the *E. coli* isolates into one of four phylogenetic groups (group A, B1, B2, or D) by use of a dichotomous decision tree (Clermont *et al.*, 2002). The isolates were also tested for 14 virulence-associated traits (VFs) of uropathogenic *E. coli*. All testing was done with appropriate positive and negative controls included in each run. Boiled whole-cell lysates (400 ml) from each isolate were used as DNA template and amplification was carried out in a 25 µl reaction mixture containing 4 mM $MgCl_2$, 600 µM each dNTP, 10 µl 5 × Green GoTaq Flexi buffer (Promega), 2.5 U GoTaq Flexi DNA polymerase (Promega), 0.5 µM each primer and 5 µl whole-cell lysate. Reactions were performed in a GeneAmp PCR System 9700 (Applied Biosystems). DNA sequencing was used to confirm the identity of the amplified PCR products. Initially, the nucleotide sequence of an amplified PCR product of each virulence gene was determined using the Maxam-Gilbert (chemical degradation) method. The DNA sequence was submitted to the Basic Local Alignment Search Tool (BLAST) nucleotide resource at the National Center for Biotechnology Information for identification. Once a PCR product for an individual virulence determinant was confirmed, the DNA from this isolate was used as a positive control for all subsequent PCRs. An aggregate virulence score was calculated as the sum of all VFs for which the isolates tested positive, with adjustment for multiple detection of the *pap*, *foc*, and *kps* operons.

Statistical methods

The statistical analysis of observed differences was done using Fisher's exact test. $P \leq 0.05$ was considered to be significant.

Results

A total of 115 *E. coli* isolates from patients with acute pyelonephritis were used in this study. Of the 115 isolates, 79 (68.7%) were susceptible and 36 (31.3%) were resistant to the herbal concoction.

Virulence genotypes

Among the 79 susceptible strains, eleven of the 14 VFs were detected, with the overall prevalences ranging from 1.3% (*kpsMT II*) to 84.9% (*iutA*). The exception was the *focG*, *kpsMT III* and *ibeA*, which was not detected in any isolate. Ten VFs were detected in the 36 resistant isolates, ranging from 2.7% (*fimH*) to 83.3% (*fyuA*), with *focG*, *kpsMT II*, *kpsMT III* and *ibeA* undetected. In total, 20.9% of the isolates encoded three or more of VFs for which they were screened, with 13.9% in susceptible isolates and 36.1% in resistant isolates. The key VFs detected were

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iron acquisition systems (*fyuA* and/or *iutA* siderophores), present in >80% of isolates, and *cnf1* toxin, present in 73.4% (susceptible) and 75% (resistant) of isolates. The *papA* and *papC* adhesins were detected in the majority of resistant isolates (72.2% and 63.9% respectively). Resistant *E. coli* isolates possessed an average of 4.8 VFs (range,0 to 9 VFs), which was 4.5 VFs (range,0 to 8 VFs) in susceptible isolates (Table 1).

Table 1: Distribution of VFs and phylogenetic groups in relation to Chinese herb-resistance

	No. of associated VFs (%)	
	Susceptible n=79	Resistant n=36
Adhesins		
papA	43 (54.47) [a]	26 (72.2)
papC	35 (44.3) [a]	23 (63.9)
papEF	26 (32.9)	14 (38.9)
bmaE	5 (6.4)	3 (8.3)
fimH	2 (2.5)	1 (2.7)
focG	0	0
Toxins		
hlyA	29 (36.7)	11 (33.3)
cnf1	58 (73.4)	27 (75.0)
Siderophores		
fyuA	65 (82.2)	30 (83.3)
iutA	67 (84.9)	29 (80.6)
Protectins		
traT	27 (34.2)	10 (27.8)
kpsMT II	1 (1.3)	0
kpsMT III	0	0
Invasins		
ibeA	0	0
Average VF score	4.5	4.8
Multivirulent (≥three genes)	11 (13.9) [a]	13 (36.1)
Phylogenetic group		
A	62 (78.5) [b]	5 (13.9)
B1	0	0
B2	14 (17.7) [b]	30 (83.3)
D	3 (3.8)	1 (2.8)

a. $p<0.05$, compared with Resistant *E. coli;* b. $p<0.01$, compared with Resistant *E. coli*

Phylogenetic background

All isolates were successfully categorized (Table 1). In susceptible isolates, the numbers belonging to phylogenetic groups A, B1, B2, and D were 62 (78.5), 0, 14 (17.7), and 3 (3.8%), respectively. The average VF scores were higher in group A (5.1) than among those in groups B1, B2 and D (0, 3.8, and 4.0, respectively).

In resistant isolates, the numbers of groups A, B1, B2, and D were 5 (13.9), 0, 30 (83.3) , and 1 (2.8%). In group B2, the average VF scores were higher (4.9) than those in groups A, B1, and D (4.1,0, and 3.9, respectively).

Discussion

In Traditional Chinese Medicine (TCM), many herbal formulations were developed and used in the treatment of UTI. In the formulation

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studied in this report, Ma Chi Xian, as the king, can kill insects and relieve internal heat. Ministers include Tian Kui Zi, Zi Hua Di Ding, Pu Gong Ying and Qu Mai. The four ministers enter the small intestine and bladder meridians respectively, with the effects of diuresis and detoxification. Xiao Hui Xiang, Rou Gui, and Li Zhi He are the assistants. They warm the yang of lower energizer, get rid of cold and dampness, promote the circulation of qi, expel the pathogenic factors. Chi Shao, Tong Cao and Hua Shi are the guides. They tend to go downward and clear the stasis sluggish.

E. coli is the leading cause of Gram-negative UTIs. Antimicrobial resistance among uropathogens causing UTIs is increasing (Bonadio *et al.*, 2001). Few data are available on the risk factors for the development of Chinese herb-resistance of the uropathogens. In this study, we investigated the differencess of virulence genotypes, phylogenetic background between Chinese herb-susceptible *E. coli* and -resistant ones isolated from humans with acute pyelonephritis by midstream urine culture.

Many VFs have been reported in uropathogenic *E. coli* and are frequently associated with UTIs. In the present study, we reported similar findings, with multivirulent uropathogenic *E. coli* isolates being detected significantly more in Chinese herb-resistant isolates than in -susceptible ones. Of the 14 VFs examined here, we found only *papA* and *papC* (P fimbriae) significantly associated with Chinese herb-resistant *E. coli* isolated compared with -susceptible ones.

As iron is limiting, iron acquisition systems are important VFs. Of the studied siderophoreencoding genes, *fyuA* was found in as many as 82.2% of the tested susceptible strains and 83.3% of resistant strains. *iutA* was present in 84.9% of the susceptible isolates and 80.6% of the resistant ones. The prevalence was high among the investigated strains, compared to 79% reported by Houdouin *et al.* (Houdouin *et al.*, 2006), while all other studies reported lower percentages (Johnson *et al.*, 2005; Soto *et al.*, 2007). Such differences might be due to differences in association with host characteristics. Previous studies indicated that aerobactin-positive *E. coli* strains were more common in isolates from patients with acute pyelonephritis (Jacobson *et al.*, 1988; Brauner *et al.*, 1988). In this study, the virulence gene *iutA* (aerobactin) was proved to be the key VF and detected at almost equal rates among Chinese herb-resistant and -susceptible *E. coli* (80.6% vs 84.9%).

P fimbriae are encoded by a “pilus associated with pyelonephritis” *pap* operon which can be carried on one or more mobile genetic elements. It was demonstrated that the chromosomal determinants for P fimbriae (Johnson *et al.*, 1988) is conserved in antibiotic susceptible uropathogenic *E. coli* strains. Previous studies indicated that *papC, fyuA* and *aer* (Bingen-Bidois *et al.*, 2002) represent the minimal prerequisite for bacterial passage and infection of the bloodstream. Among the tested VFs in our study, *papA* and *papC* (encoding P fimbriae) exhibited a statistically significant higher prevalence among -resistant strains which indicated that resistance to this Chinese herbal concoction was significantly associated with *papA* and *papC*. Chinese herb-resistant *E. coli* isolates that have *papA* and *papC* should be more able to be uropathogenic. Furthermore, resistance itself may be a virulence factor that allows for the survival of a bacterium within the urinary tract of patients.

The -resistant strains also exhibited a statistically significant higher prevalence of multivirulence than -susceptible strains. A slightly higher VF score, albeit not statistically significant, was determined among -resistant isolates (4.8), compared to -susceptible isolates (4.5). The underlying basis for the association of resistance to Chinese herbal concoction and virulence is unclear. It is possible that a high presence of certain VFs precedes resistance or that high presence of these VFs is followed by acquisition of resistances.

E. coli strains can be assigned to one of the four main phylogenetic groups: A, B1, D1 and B2 (Herzer *et al.*, 1990). Some previous studies have shown that bacteraemia is caused by *E. coli* strains predominantly from phylogroups B2 followed by D, A and B1 (Johnson *et al.*, 2002). Several investigators have reported that VF-containing uropathogenic *E. coli* isolates cluster mainly in phylogeny group B2 (Boyd *et al.*, 1998). In the present study, for Chinese herb-resistant *E. coli*, VFs clustered significantly in group B2 (83.3%). However, for Chinese herb-susceptible isolates, VFs were found at a higher proportion in group A (78.5%) than in group B2 (17.7%) or D (3.8%). The marked phylogenetic differences noted between -resistant and -susceptible isolates suggest that these groups may represent, in part, distinct bacterial populations. Thus, the resistant isolates are not simply mutant derivatives of the susceptible isolates. Their relative lack of VFs does not necessarily represent loss of VFs in exchange for resistance. Our finding of a significant association of Chinese herb-resistant *E. coli* with group B2, strongly support the notion that Chinese herb-resistance is caused by more virulent uropathogenic *E. coli*.

In conclusion, our study indicates that *papA* and *papC* are significant VFs with an essential role in contributing to Chinese herb-resistance. Chinese herb-resistance is associated with a shift towards more virulent strains and B2 phylogenetic group. The basis for the striking concentration of Chinese herb-resistance within phylogenetic group B2 remains to be determined.

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