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Drug Resistance and Molecular Characteristics of *Escherichia coli* Isolates Associated with Acute Pyelonephritis

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Abstract

Acute pyelonephritis (APN) as one of the most severe form of UTIs may result in significant morbidity. We aim to investigate the antimicrobial susceptibilities and genetic traits of *Escherichia coli* isolates associated with APN.

Totally, 64 APN *E. coli* isolates were analyzed for the antimicrobial susceptibilities, phylogenetic groups, resistance and virulence determinants, plasmid replicons, pulsed-field gel electrophoresis (PFGE), and Multi-locus sequence types (MLST).

High percentages of resistance (>65.0%) to ampicillin/sulbactam and levofloxacin were observed, imipenem and fosfomycin displayed good *in vitro* sensitivity (>93.0%). Most of the strains belonged to phylogenetic group D (50.6%) and B2 (21.6%), D strains were more resistant than B2 ones towards the cephalosporins tested ($p<0.05$). Thirty-six (56.3%) *bla*CTX-M, 3 (4.7%) *rmtB*, and 13 plasmid mediated quinolone resistance (PMQR) genes were identified. Plasmid replicon IncF (54/64, 84.4%) and virulence factors (VFs) *fimH* (57/64, 89.1%) was the most prevalent. PFGE and MLST displayed genetic diversity. Prevalence of *ompT*, *fdeC*, PAI, and *usp* were higher among B2 strains than that in D ones ($P<0.05$). Statistical associations between antimicrobial resistances and VFs were found.

This study provides new data on the molecular epidemiology and pathogenesis of *E. coli* isolates associated with APN.

Keywords: Acute pyelonephritis; Antimicrobial resistance; Phylogenetic groups; Virulence genes; Resistance determinants; *Escherichia coli*

Introduction

Acute pyelonephritis (APN) as an acute infection of the upper urinary tract is frequently caused by *Escherichia coli* (56–85%) when the bacteria in the bladder ascend the ureters and invade the kidneys, [1] with the possibility of causing significant morbidity and irreversible kidney damage. The genetic flexibility and the ability of *E. coli* to adapt to constantly changing environments allow the bacterium to acquire numerous antimicrobial resistance determinants such as extended-spectrum β-lactamases (ESBLs) and 16S rRNA methyltransferases (16S-RMTases) [2] and plasmid-mediated quinolone resistance (PMQR) genes are also frequently detected [3]. Thus, the production of these resistance determinants is a significant threat to patients with APN before the results of antibiotic susceptibility tests become available [1]. Moreover, mobile genetic elements such as plasmids have been reported to be associated with the dissemination of these resistance determinants among clinical strains [4]. However, limited data are available on the prevalence of ESBLs, PMQRs and 16S-RMTase and the distribution of plasmid replicons among *E. coli* isolates associated APN.

The pathogenicity of *E. coli* strains has also been reported to be strongly influenced by virulence factors (VFs) including adhesion molecules (e.g., P fimbriae), toxins (e.g., hemolysin and cytotoxic necrotizing factor), iron-acquisition systems (e.g., the aerobactin system), protectins (e.g., *kpsM* and *traT*), miscellaneous virulence determinants (e.g., *usp* and *malX*), cytolethal distending toxin, uropathogenesis-specific proteins, and the formation of biofilms [5,6] Furthermore, studies on *E. coli* strains isolated from Europe and USA have revealed that more VFs are distributed among phylogenetic group B2 and D strains than those among A and B1 strains [7,8]. However, such studies were rarely performed on *E. coli* strains associated with APN in China since the VFs and phylogenetic groups of such strains remain unclear.

In this study, we analyzed the molecular epidemiology and pathogenesis of *E. coli* isolates associated with APN. The relationship between distinct phylogenetic groups and resistance to antimicrobial agents as well as VFs in *E. coli* strains was analyzed. In addition, the association between antimicrobial resistance and the presence of VFs was also investigated.

Materials and Methods

Bacterial isolates

Totally, 64 *E. coli* isolates were collected from 64 patients diagnosed as APN which was defined as the presence of two of the following syndromes: (a) axillary temperature ≥ 38.3°C or chills; (b) flank pain or costovertebral angle tenderness or pain on bimanual palpation of the kidney; and (c) mictional syndrome (including two or more of the following: dysuria, frequency, suprapubic pain or urgency), together with the presence of pyuria (a positive leukocyte esterase dipstick test result, subsequently confirmed by urinalysis with more than 10 leukocytes/mL in urine without centrifuging or more than 5 leukocytes per high-power field in centrifuged sediment) or a positive urine culture. The patients were admitted during 2012-2013, and Standard biochemical tests were used for the isolation and identification of *E. coli* strains obtained in clinical microbiology laboratories of Nanjing Drum Tower Hospital affiliated to Nanjing University, Jiangsu, China.

Antimicrobial susceptibility testing

The susceptibility of the 64 strains toward 16 antimicrobial agents were tested by the Kirby-Bauer's disk-diffusion method and the results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines [9]. The following antimicrobial agents (μg/disk) were used: ampicillin/sulbactam (SAM, 10/10 μg), cefuroxime (CXM, 30 μg), ceftazidime (CAZ, 30 μg), cefotaxime (CTX, 30 μg), cefepime (FEP, 30 μg), cefoxitin (FOX, 30 μg), aztreonam (ATM, 30 μg), imipenem (IPM, 10 μg), amikacin (AK, 30 μg), trimethoprim-sulfamethoxazole (SXT, 1.25/23.5 μg) levofloxacin (LEV, 5 μg), piperacillin (PRL, 100 μg), cefoperazone/sulbactam (SCF, 75/30 μg), ticarcillin/clavulanic acid (TLM, 75/10 μg), piperacillin/tazobactam (TZP, 100/30 μg) and fosfomycin (FOM, 200 μg). The disks were obtained from Oxoid Ltd. (Basingstoke, Hampshire, England). *E. coli* ATCC 25922 was used as the quality control in parallel.

Assignment of phylogenetic groups

The 64 strains were assigned to phylogenetic groups A, B1, B2, or D by using a triplex PCR-based strategy with specific primers for *chuA*, *yjaA*, and *TspE4. C2* determinants according to a previously described protocol [10].

Identification of resistance genes

The strains displaying non-susceptibility to cefotaxime or cefepime were screened for ESBLs encoding genes including *bla*CTX-M, *bla*TEM, and *bla*SHV genes according to the PCR protocol described previously [11]. The strains that survived on Luria-Bertani plates containing 0.125 μg/mL ciprofloxacin were screened for PMQR genes as previously described [12-14]. Strains resistant to amikacin were analyzed for the presence of 16S-RMTases [15]. PCR and sequencing (both strands) were used to analyze the genes, the nucleotide and deduced amino acid sequences were compared with those available in the GenBank database.

Plasmid incompatibility group

Plasmid replicon typing was performed in order to establish the range and diversity of plasmids amongst *E. coli* isolates associated with APN. Highly pure total DNA was prepared using the Wizard® Genomic DNA purification Kit (Promega) according to the manufacturer's procedure. Plasmids were typed using the PCR-based replicon typing method; 5 multiplex and 3 simple PCR amplification steps were used with specific primers and conditions, as described by Carattoli et al. [16].

Virulence genotyping

Multiplex PCR assays were performed with specific primers to check for the presence of 15 genes that have been reported to be associated with virulence in *E. coli* isolates: *iutA*, *ompT*, *fyuA*, *fdeC*, *fimH*, *traT*, *cvaC*, *pap*, *kpsMT*, *pAI*, *usp*, *aer*, *hlyA*, *cnf*, and *chuA* [17]. *chuA* was detected by triplex PCR as mentioned above.

Multi-locus sequence typing

The sequence types (STs) of 64 strains were analyzed by amplifying and sequencing 7 housekeeping genes, including *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA* according to the protocol available at **http://mlst.ucc.ie/mlst/dbs/Ecoli**.

Strain typing by pulsed-field gel electrophoresis (PFGE)

All the 64 strains associated with APN were analyzed for genetic relatedness by using *XbaI* according to the U.S. CDC PulseNet protocol [18]. Briefly, electrophoresis was performed with the switch time from 2.2 to 54.2 s at a gradient of 6 V/cm and an included angle of 120° for 19 h after the bacterial chromosome was digested by *XbaI*. The analysis of the PFGE profiles was performed by Bionumerics software v6.5 (Applied Maths, Sint-Martens-Latem, Belgium) using the Dice similarity coefficient on the basis of the unweighted-pair group method using average linkages (UPGMA), with a 1.5% band tolerance. And genetic relatedness was observed with the cutoff lines at 85% and 69%.

Statistical analysis

The statistical analysis was performed by using SPSS software version 20 (SPSS Inc., Chicago, IL, USA). Proportions were compared using the chi-squared test or, when the expected numbers were <5, Fisher's exact test (two-tailed). $P<0.05$ was considered statically significant.

Results

Antimicrobial resistance of *E. coli* isolates associated with acute pyelonephritis

The *E. coli* isolates exhibited the highest resistance rate toward SAM (59/64, 92.6%) followed by LEV (42/64, 65.6%), CXM (34/64, 53.1%), SXT (35/64, 54.7%), PRL (33/64, 51.6%), CTX (31/64, 48.4%), TLM (31/64, 48.4%), and FEP (21/64, 32.8%) TZP (28/64, 43.8%). By comparison, the resistance rates were relatively lower toward AK (19/64, 29.7%), CAZ (14/64, 21.9%), FOX (8/64, 12.5%), and SCF (7/64, 10.9%). Good *in vitro* sensitivity towards IPM (2/64, 3.1%) and FOM (4/64, 6.3%) was observed.

Assignment of phylogenetic groups

Among the 64 *E. coli* isolates tested, 30 (50.6%) strains were assigned to phylogenetic group D, 19 (21.6%) to B2, 8 (12.5%) to A, and 7 (8.5%) to B1. As shown in Table 1, the D strains exhibited higher resistances than the B2 strains did toward CTX (50.0% vs. 10.5%,

P=0.012), PRL (63.3% vs 21.1%, P=0.009), FEP (60.0% vs 26.3%, P=0.021), and ATM (63.3% vs 15.8%, P=0.003).

Prevalence of antimicrobial resistance determinants

On the whole, 36 (56.3%) *bla*CTX-M including 18 *bla*CTX-M-14, 13 *bla*CTX-M-15, 3 *bla*CTX-M-27, and 1 *bla*CTX-M-55 as well as 1 *bla*CTX-M-3 were identified; 37(57.8%) *bla*TEM-1 including 36 *bla*TEM-1 and 1 *bla*TEM-104 as well as 1 (1.6%) *bla*SHV-1 were also detected.

Among the 16S-RMTase-encoding genes, 3 (4.7%) *rmt*B genes were identified in the 19 isolates resistant to amikacin.

The PMQR genes were identified in the following isolates: 6 (9.4%) *aac(6')-Ib-cr*, 3 (4.7%) *qnr* (1 *qnr*B6 and 1 *qnrS1*1 as well as 1 *qnrS*2) and 4 efflux pumps genes including 3 (4.7%) *oqxAB* and 1 (1.7%) *qepA*.

Antimicrobial agents	Prevalence of resistant strains, no. (Column %)				P value	
	A(N=8)	B1(N=7)	B2(N=19)	D(N=30)	D vs B2	D vs A& B1
SAM	8(100)	7(100.0)	17(89.5)	27(90.0)	1.000	0.540
AK	2(25.0)	2(28.6)	5(26.3)	10(33.3)	0.604	0.909
CAZ	3(37.5)	1(14.3)	1(5.3)	9(30.0)	0.084	1.000
CXM	6(75.0)	4(57.1)	11(57.9)	23(76.7)	0.165	0.475
FEP	2(25.0)	2(28.6)	2(10.5)	15(50.0)	0.012	0.240
FOX	1(12.5)	1(14.3)	0(0.0)	6(20.0)	0.069	0.890
IPM	1(12.5)	0(0.0)	0(0.0)	1(3.3)	1.000	1.000
SCF	2(25.0)	0(0.0)	1(5.3)	4(13.3)	0.671	1.000
TZP	2(25.0)	2(28.6)	10(32.6)	10(33.3)	0.181	0.909
PRL	7(87.5)	3(42.9)	4(21.1)	19(63.3)	0.009	0.826
CTX	6(75.4)	2(28.6)	5(26.3)	18(60.0)	0.021	0.670
TIM	2(25.0)	2(28.6)	12(63.2)	15(50.0)	0.367	0.240
LEV	5(62.5)	6(85.7)	12(63.2)	19(63.3)	0.990	0.737
SXT	4(50.0)	4(57.1)	10(52.6)	17(56.7)	0.782	0.832
ATM	4(50.0)	4(57.1)	3(15.8)	19(63.3)	0.003	0.519
FOM	1(12.5)	0(0.0)	1(5.3)	2(6.67)	1.000	1.000

SAM, ampicillin/sulbactam; CXM, cefuroxime; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; FOX, cefoxitin; ATM, aztreonam; IPM, imipenem; AK, amikacin; SXT, trimethoprim-sulfamethoxazole; LEV, levofloxacin; PRL, piperacilin; SCF, cefoperazone/sulbactam; TLM, ticacillin/clavulanic acid; TZP, piperacilin /tazobactam; FOM, fosfomycin.

Table 1: Association between resistance to antimicrobial agents and phylogenetic groups of *Escherichia coli* isolates associated with acute pyelonephritis.

Plasmid replicons

On the whole, 60/64 (93.8%) strains contained plasmid replicons and IncF was the most prevalent replicon (54/64, 84.4%), followed by IncI1 (32/64, 50.0%); 3 IncP (4.7%), 1 IncN (1.6%), and 1 IncA/C (1.6%) replicons were also detected. Notably, 30 strain (46.9%) carried 2 plasmid replicons.

The distributions of plasmid replicons among the group B2 *E. coli* isolates (18/19, 94.7%) was higher than that among group D isolates (19/30, 63.3%) (*P*=0.032). Resistance to SAM (*P*=0.027) and AK (*P*=0.026) were obviously higher among IncF positive strains than IncF negative ones. Significant association between the distribution of IncI1 and resistance to CAZ (P=0.034), CXM (*P*=0.035), and CTX (*P*=0.024) were found.

In addition, the distribution of *traT* was higher among the IncF replicon-positive strains than that among IncF replicon-negative strains: (87.0% vs 0.0%, *P*=0.000). Whereas, the distribution of PAIs was higher among the IncF replicon-negative strains than among IncF replicon-positive strains: (40.0% vs 7.4%, *P*=0.019). The distribution of all the virulence genes were independent of IncI1 replicons.

Distribution of virulence genes

Multiple VFs were identified among the strains examined in our study. All the strains carried at least 1 adhesion-associated gene with the *fimH* (57/64, 89.1%) and *fdeC* (45/64, 70.3%) being the most prevalent genes, followed by *pap* (14/64, 21.9%). *traT*, which encodes a protectin, was detected in 47 (73.4%) strains, and the iron uptake-

associated genes *fyuA* (42/64, 65.6%), *iutA* (41/64, 64.1%), and *kpsMT* (34/64, 53.1%) were also identified. *chuA* was also found among 49 (76.6%) out of 64 strains. By comparison, other virulence genes such as *ompT* (28/64, 44.3%), *usp* (15/64, 20.5%), *PAIs* (8/64, 15.9%), and *cvaC* (2/64, 3.4%) were less common, and *aer, hlyA*, and *cnf* were not detected among our strains. We also found that 57 (89.1%) strains carried ≥ 3 VFs.

As shown in Table 2, *ompT, fdeC*, PAI, and *usp* were more prevalent among B2 strains than among D strains ($P < 0.05$), whereas the distribution of *iutA*, *ompT*, *fyuA*, *fdeC*, *traT*, and *kpsMT* was higher in group D than that in groups A and B1 ($P < 0.05$).

Virulence genes	Prevalence of gene, no. (Column %)				P value	
	A(N=8)	B1(N=7)	B2(N=19)	D(N=30)	D vs B2	D vs A& B1
iutA	3(37.5)	2(28.6)	15(78.9)	21(70.0)	0.719	0.043
ompT	0(0.0)	2(28.6)	14(73.7)	12(40.0)	0.021	0.139
fyuA	1(12.5)	1(14.3)	16(84.2)	24(80.0)	1.000	0.000
fdeC	6(75.0)	6(85.7)	17(89.5)	16(53.3)	0.021	0.158
fimH	6(75.0)	6(85.7)	18(94.7)	27(90.0)	0.956	0.642
traT	4(50.0)	4(57.1)	12(63.2)	27(90.0)	0.056	0.016
cvaC	0(0.0)	0(0.0)	1(5.3)	1(3.3)	1.000	1.000
kpsMT	0(0.0)	1(14.3)	12(63.2)	21(70.0)	0.619	0.000
pap	0(0.0)	1(14.3)	7(36.8)	6(20.0)	0.913	0.647
PAI	0(0.0)	0(0.0)	7(36.8)	1(3.3)	0.007	1.000
usp	0(0.0)	0(0.0)	15(78.9)	0(0.0)	0.000	1.000
chuA	0(0.0)	0(0.0)	19(100.0)	30(100.0)	ND	ND
ND: not determined.						

Table 2: Phylogenetic group distribution of virulence-associated genes among 64 *Escherichia coli* isolates associated with acute pyelonephritis.

Additionally, the distribution of *ompT* and *kpsMT* was higher among levofloxacin resistant strains than that among levofloxacin susceptible strains: (72.7% vs 38.1%, 72.7% vs 42.9%, P0.05). There were statistically significant associations between *traT* gene and resistances to PRL, (P=0.033), and ATM, (P=0.0011). Such associations were also found between usp gene and resistances to CXM, (P=0.023), TZP (P=0.041), PRL, (P=0.002), and ATM (P=0.007) as well as between *chuA* gene and CTX (P=0.035). Significant associations between *iutA* gene and resistances to TZP, (P=0.001), and TLM, (P=0.048) was observed, in addition to the associations between *ompT* gene and PRL (P=0.025) and AK (P=0.008) as well as between *fdeC* gene and CXM (P=0.046).

MLST

MLST analysis identified 16 different STs with ST131 (10, 15.6%), ST38 (8, 12.5%), ST69 (8, 12.5%), ST648 (8, 12.5%), ST405 (6, 9.38%)ST10 (n=5) being the major STs which accounted for 70.3% (45/64) of the isolates. In addition, ST73 (n=3), ST14 (n=2), ST155 (n=2), ST393 (n=2), ST394 (n=2), ST410 (n=2), ST12, ST31, ST95, ST101, ST393, ST522 were also found, indicating a diverse lineages.

PFGE

PFGE displayed that 64 *E. coli* isolates associated with APN showed a genetic diversity, indicating that they were not clonally related.

Discussion

The goal of this study was to assess the antimicrobial susceptibility *E. coli* associated with APN, and to explore the correlations between antimicrobial resistance and genetic traits including plasmid replicons, virulence factor possession, as well as *E. coli* phylogenetic group.

The results showed that, in contrast to the low resistance rates displayed by UPEC isolated from patients in USA and Europe, [19,20] the strains examined in our study exhibited a high frequency of resistance toward multiple antimicrobial agents, which agrees with previous reports from China [21] and is similar to that has been reported in India [22]. This seriously limits the choice of treatments for UTIs and poses a serious challenge to public health. Such a high level of resistance might result from the high proportion of *bla*ESBLs identified in our collection, indicating that APN caused by ESBL-producing *E. coli* are a great concern. Fortunately, imipenem and fosfomycin still can be available because of the good *in vitro* sensitivity. Furthermore, the prevalence of *rmtB* and PMQR genes in our isolates was lower than that in MDR *E. coli* isolates [23]. However, the particular concern is that the presence of these determinants might facilitate the development of multi-drug resistant strains under the selection pressure imposed by the antimicrobial agents used frequently in hospitals.

As reported previously [2], strains of phylogenetic group D were the most prevalent strains in our study. Notably, the group D strains in our

study were more resistant toward cephalosporin than were group B2 strains. To the best of our knowledge, this observation has not been reported previously. Nevertheless, the B2 strains exhibited a higher virulence potential than the D strains did, albeit both of these phylogenetic groups were more virulent than A and B1 strains. This is in accordance with the results of multiple studies conducted in Europe [7-8].

The replicon type that was most highly represented among our strains was IncF, which is consistent with previously reported results [24]. This finding suggests that plasmid replicon IncF may be well-adapted to *E. coli* strains. Furthermore, the IncI1 replicons identified here were also frequently detected in our previous study [25], which might result from the negligible fitness costs imposed by the IncI1 plasmid replicon on its *E. coli* host, as demonstrated *in vitro* by Fischer et al. [26]. The other replicons detected in this study have also been reported previously in *E. coli* associated with UTIs [27]. Additionally, the associations between the prevalence of IncI1 and resistance to cephalosporins and between the prevalence of IncF and resistance to AK and SAM observed in our study indicate that plasmid IncF and IncI1 played an important role in the resistance development. Thus, the high occurrence of plasmid replicons, especially IncF and IncI1 in our study poses a serious challenge to clinical treatment based on their extensive horizontal gene transfer potential for resistance determinants between clinical strains under the selection pressure imposed by antimicrobial agents.

Novel epidemiological information pertinent to the molecular characteristics of pyelonephritis *E. coli* VFs is provided by our study which showed a high prevalence of fimbriae-associated adherence, indicating that adhesion of uropathogenic *E. coli* onto the epithelial cells is the first event leading to APN. Of note, the frequency of the *pap* gene in our study is near to the one reported by Firoozeh et al. [28] showing the important role of P fimbriae in the development of pyelonephritis. One of the most striking findings in our study was the high prevalence of multiple iron acquisition systems (heme/hemoglobin gene cluster, aerobactin, and yersiniabactin), suggesting the importance of iron for the survival of UPEC in the urinary tract.

In contrast to the most commonly accepted view that resistance to quinolones is linked to a loss of VFs, [29] we found statistical associations between distribution of VFs *ompT*, *kpsMT* and levofloxacin resistant strains. The group II capsule (*kpsMT*) and outer membrane protease T (*ompT*) have been frequently reported to be associated with urinary tract infections [30]. However, the correlations of these 2 VFs with resistance to floroquinolones has not been reported previously. Notably, we found that the prevalence of *traT* was associated with the production of ESBLs. This could be attributed to the occurrence of this VF and ESBL-encoding genes on the same mobile elements, which was further suggested by the association between virulence genes *traT* and the *IncF* replicon. In addition, positive associations observed between the VFs and the resistance phenotypes suggest that some *E. coli* isolates associated with APN have been the reservoirs for antimicrobial resistance and virulence determinants.

Both of the PFGE and MLST in our study revealed a genetic diversity of the *E. coli* isolates studied, and the major STs identified has also been frequently reported to be involved in the urinary tract infections [31].

In conclusion, we found that *E. coli* isolates associated with APN showed a high frequency of resistance toward multiple antimicrobial agents routinely used during clinical treatment except imipenem and fosfomycin. Most of the isolates belonged to phylogroup D followed by B2. Phylogenetic group D strains were more resistant and less virulent than were B2 strains. A high proportion of the strains examined in this study contained plasmid replicons which may potentially facilitate the dissemination of virulence and resistance in the hospital setting. Our study brings new insight into relationships between the antimicrobial resistance, plasmid replicons, VFs, as well as phylogenetic groups in *E. coli* isolates associated with APN.

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