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High prevalence of fosfomycin resistance gene *fosA3* in $bla_{CTX\text{-}M}$-harbouring *Escherichia coli* from urine in a Chinese tertiary hospital during 2010–2014

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SUMMARY

Fosfomycin has become a therapeutic option in urinary tract infections. We identified 57 fosfomycin-resistant *Escherichia coli* from 465 urine-derived extended-spectrum β-lactamase (ESBL)-producing isolates from a Chinese hospital during 2010–2014. Of the 57 fosfomycin-resistant isolates, 51 (89·5%) carried *fosA3*, and one carried *fosA1*. Divergent pulsed-field gel electrophoresis profiles and multi-locus sequence typing results revealed high clonal diversity in the *fosA3*-positive isolates. Conjugation experiments showed that the *fosA3* genes from 50 isolates were transferable, with IncFII or IncI1 being the most prevalent types of plasmids. The high prevalence of *fosA3* was closely associated with that of $bla_{CTX\text{-}M}$. Horizontal transfer, rather than clonal expansion, might play a central role in dissemination. Such strains may constitute an important reservoir of *fosA3* and $bla_{CTX\text{-}M}$, which may well be readily disseminated to other potential human pathogens. Since most ESBL-producing *E. coli* have acquired resistance to fluoroquinolones worldwide, further spread of *fosA3* in such *E. coli* isolates should be monitored closely.

Key words: *Escherichia coli*, ESBL, fosfomycin, *fosA3*, urinary tract infection.

INTRODUCTION

In China, extended-spectrum β-lactamase (ESBL) production has been increasingly prevalent in strains of *Escherichia coli*, the major aetiological agent of urinary tract infections (UTIs) [1]. Options for effective antibiotic treatment of infections, including UTIs, are limited owing to the frequent occurrence of expanded-spectrum cephalosporin-resistant and carbapenem-resistant, Gram-negative bacteria of the family Enterobacteriaceae [2]. Use of older antibiotics such as fosfomycin has therefore been proposed as an alternative treatment of such infections [3].

Fosfomycin is an organic phosphonate agent that inhibits cell wall synthesis by irreversibly inhibiting MurA, which is responsible for the initial step of peptidoglycan biosynthesis [4]. Fosfomycin exhibits a broad spectrum of antimicrobial activity, including rapid bactericidal effects against several Gram-negative rods, particularly *E. coli*, and also has good activity against *Staphylococcus aureus* [4]. Successful

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treatment of infections, especially UTIs, with fosfomycin has been documented in Japan [5], and thus it is receiving renewed attention as an alternative agent for the treatment of UTIs caused by ESBL-producing *E. coli* [6].

To date, fosfomycin resistance in *E. coli* has primarily involved either reduced uptake of the drug due to mutations in chromosomally encoded transporters [7], or enzymatic inactivation by plasmid-mediated glutathione *S*-transferases (PMGST) such as FosA3, FosA4, and FosC2 [5, 8–11]. It has also been reported that fosfomycin-resistant isolates are more likely to be ESBL producers than fosfomycin-susceptible isolates [5]. However, information on the prevalence of plasmid-mediated fosfomycin resistance genes in ESBL-producing urinary *E. coli* strains is lacking, with only limited knowledge of the molecular characteristics and prevalence of *fosA3* and the ESBL genes bla_{CTX-M} in strains in China.

The purpose of this study was to examine the occurrence of fosfomycin-resistant *E. coli* in ESBL-producing, urinary *E. coli* isolates, and to identify the distribution of PMGST and ESBL determinants. Furthermore, the genetic relatedness in *fosA3*-positive strains, transferability of *fosA3,* and replicon types of *fosA3*-carrying plasmids were analysed.

MATERIALS AND METHODS

Bacterial isolates

A total of 821 non-repetitive urinary *E. coli* isolates were streaked from the Strain Library of the Department of Laboratory Medicine, Nanjing Drum Tower Hospital. ESBL production was confirmed phenotypically, using both cefotaxime and ceftazidime alone or in combination with clavulanic acid. The susceptibility of strains to fosfomycin was tested by the disk diffusion method described previously [12], using Mueller–Hinton agar plates (Oxoid, UK) containing 25 mg/l glucose-6-phosphate (G6P). *E. coli* ATCC25922 was used as the quality control strain in antimicrobial susceptibility testing.

Detection of genes for fosfomycin resistance and ESBL production

Genes reported to be involved in fosfomycin resistance in Enterobacteriaceae, including *fosA, fosB, fosC*, and *fosX*, as well as the subtypes *fosA1, fosA2, fosA3, fosA4,* and *fosC2,* were detected by PCR and DNA sequencing analyses according to previously described protocols [5, 8–11]. The presence of *bla* genes for ESBL production (bla_{CTX-M}, bla_{TEM}, bla_{SHV}) was assessed in each of the 57 ESBL-positive strains, following a previously described protocol [13].

Phylogenetic grouping

Phylogenetic grouping of fosfomycin-resistant *E. coli* isolates was conducted via triplex PCR, using six primers in a single reaction [14]. The amplification of three DNA markers (*chuA, yjaA*, TSPE4.C2) generated fragments of 279, 211, and 152 bp, respectively. This allowed *E. coli* isolates to be classified into the phylogenetic groups A, B1, B2, or D. *E. coli* strains ECOR 20 (*yjaA* positive), ECOR 48 (*chuA* positive), ECOR 58 (TSPE4.C2 positive), and ECOR 62 (*chuA*, *yjaA*, and TSPE4.C2 positive) were used as the positive controls, and *E. coli* strain ECOR 4 was used as the negative control. All controls were kindly provided by Statens Serum Institute, Denmark.

Genetic relatedness by pulsed-field gel electrophoresis (PFGE)

The *fosA3*-positive *E. coli* isolates were characterized by PFGE using the CHEF Mapper System (Bio-Rad Laboratories, USA) as described previously [15]. Briefly, the chromosomal DNA of *E. coli* isolates was subjected to digestion with *Xba*I for 2 h at 37 °C. Electrophoresis was conducted at 6·0 V/cm and 14 °C for 19 h with an angle of 120°. The switch time was increased from 2·2 s to 54·2 s at a gradient of 6 V/cm. *Salmonella enterica* serovar Braenderup HP812 (kindly provided by the Centers for Disease Control and Prevention, USA) was used in parallel as a molecular weight standard. The results were analysed and interpreted using Bionumerics software v. 6.5 (Applied Maths, Belgium). The Dice similarity coefficient on the basis of the unweighted-pair group method using average linkages (UPGMA) with a 1·5% band tolerance was used. Furthermore, cut-off lines at 80% were used to analyse genetic relatedness.

Multi-locus sequence typing (MLST)

The *fosA3*-positive *E. coli* isolates were assessed for sequence types (STs) according to the MLST scheme developed for *E. coli* by the University College Cork (http://mlst.ucc.ie/mlst/dbs/Ecoli). Briefly, the housekeeping genes *adk, fumC, gyrB, icd, mdh, purA*, and *recA* were analysed using the primer sequences and

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amplification conditions available at http://mlst.warwick.ac.uk/mlst/.

Conjugation experiments

Conjugation experiments were performed using azide-resistant *E. coli* J53 as a recipient strain by the broth mating method. Trans-conjugants were selected on trypticase soy agar plates supplemented with 150 mg/l sodium azide, 40 mg/l fosfomycin, and 25 mg/l G6P. The presence of *fos* genes in phenotypically selected ESBL producers harbouring bla_{TEM}, bla_{SHV}, or bla_{CTX-M} was assessed by PCR as described previously [16].

PCR-based replicon typing

DNA was extracted from 51 trans-conjugants, and main plasmid incompatibility groups, including F, FIA, FIB, FIC, HI1, HI2, I1-Ic, L/M, N, P, W, T, A/C, K, B/O, X, Y, and FII, were determined using the PCR-based replicon typing scheme, as described by Carattoli *et al.* [17].

Ethical statement

All procedures were performed in compliance with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

RESULTS

Susceptibility of ESBL-producing strains to fosfomycin

In total, 465 out of 821 *E. coli* isolates were found to be ESBL producers. Antimicrobial susceptibility testing revealed that fosfomycin exhibited good antibacterial activity towards ESBL-producing urinary *E. coli* strains, demonstrating effectiveness against 87·7% (408/465) of strains. The average fosfomycin resistance rate of ESBL-producing *E. coli* associated with UTIs was about 10% over the 5 years (2010–2014).

Prevalence of plasmid-mediated fosfomycin resistance genes and ESBL genes in fosfomycin-resistant *E. coli*

Molecular analysis showed that 89·5% (51/57) of the fosfomycin-resistant isolates were positive for *fosA3*, whereas only one was *fosA1*-positive; other fosfomycin resistance determinants were not identified. Fifty-five isolates were also bla_{CTX-M}-positive, 26 harboured $bla_{CTX-M-15}$, 22 $bla_{CTX-M-14}$, 4 $bla_{CTX-M-3}$, and three harboured $bla_{CTX-M-123}$ (Fig. 1). In addition, 17 isolates carried bla_{TEM} variants (14 $bla_{TEM-104}$ and three bla_{TEM-1b}) and 13 carried bla_{SHV} variants (nine bla_{SHV-12} and four bla_{SHV-11}) (Fig. 1).

Phylogenetic groups

Of the 57 fosfomycin-resistant isolates in phylogenetic groups, 19 were classified as group D, 18 group A, 12 group B1, and eight group B2.

Genetic relatedness of *fosA3*-positive isolates determined by PFGE and MLST

The 50 strains harbouring *fosA3* exhibited 44 different PFGE profiles and one strain was not typable. MLST revealed 37 STs and major STs were ST410 ($n = 4$ strains), ST10 ($n = 4$), ST405 ($n = 3$), ST156 ($n = 3$), and ST964 ($n = 3$), which together comprised 33·3% of the strains analysed. Similar or identical PFGE profiles were observed within ST10 clones, ST156, ST354, ST405, ST964, and ST2309. This level of genetic diversity indicates that most of the *fosA3*-carrying isolates were clonally unrelated (Fig. 1).

Transferability and replicon typing of *fosA3* plasmids

Conjugation assays revealed that the *fosA3* genes were transmissible. Moreover, bla_{CTX-M} and bla_{TEM} genes were able to be transferred simultaneously, indicating genetic linkage between *fosA3* and bla_{CTX-M}. Plasmids carrying *fosA3* from 50 isolates were successfully transferred by conjugation. These 50 plasmids consisted of 39 that were replicon type IncFII, nine that were IncI1, four that were IncN, two that were IncA/C, and one that was IncP. In addition, plasmids from four isolates were fused, containing both the IncN and IncFII replication origins.

DISCUSSION

Fosfomycin has been extensively used in several European countries since 1988 for the treatment of uncomplicated UTIs [4], but it was not approved for clinical use in China until recently. This is the first investigation of the prevalence of fosfomycin resistance (*fos*) genes in ESBL-producing urinary *E. coli* isolates in mainland China.

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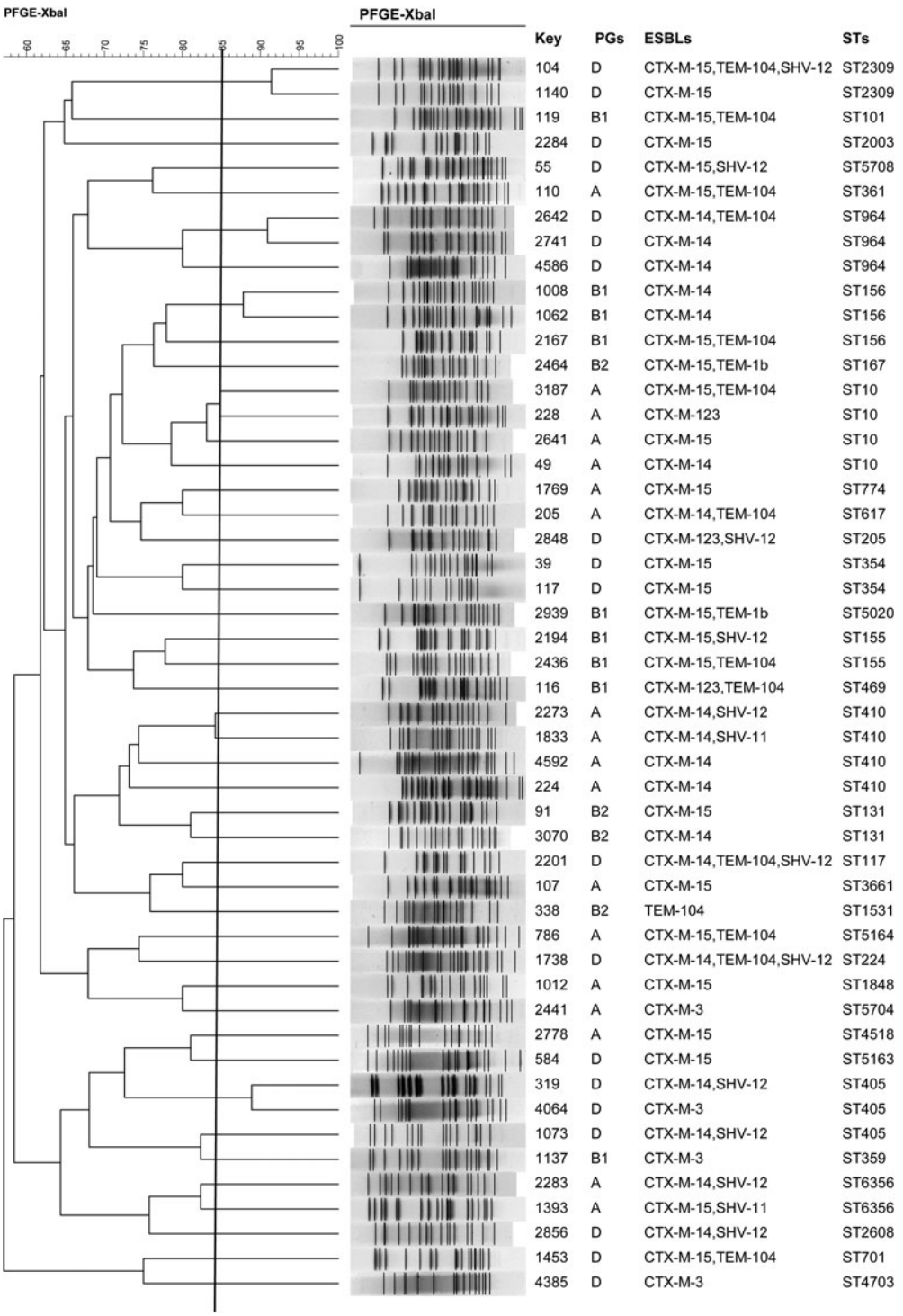

Fig. 1. Dendrogram of PFGE profiles of 50 *fosA3*-carrying *E. coli* isolates, phylogentic groups (PGs), extended-spectrum *β*-lactamases (ESBLs), and sequence types (STs).

The strains in our study displayed a rate of resistance to fosfomycin of about 10%, which is higher than that previously reported [18]. However, our data indicate that fosfomycin should still be considered for the treatment of patients with infections due to ESBL-producing *E. coli* in China if they exhibit high resistance rates to other commonly used antimicrobial agents, including cephalosporins and fluoroquinolones [19]. This is because a previous study reported that fosfomycin retains its activity against

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both Gram-positive and Gram-negative multiple-drug-resistant (MDR) and extremely-drug-resistant (XDR) bacteria [4]. To date, fosfomycin has not been used for clinical treatment in our hospital, and so we speculate that the observed resistance may be co-selected by antimicrobials other than fosfomycin.

Our study found that $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-14}}$ were the main ESBL-encoding genes detected in fosfomycin-resistant urinary *E. coli* strains. This is in line with the results of previous studies investigating the global prevalence of *bla* genes in ESBL producers [20]. It should be noted that we also detected $bla_{\text{CTX-M-123}}$, which has been identified as a novel hybrid of the $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-9}}$ β-lactamases recovered from *E. coli* isolates in China [21]. In parallel, there was a high prevalence of the $bla_{\text{TEM-104}}$ variant in our study which to the best of our knowledge, has been identified in a MDR avian pathogenic *E. coli* strain isolated from septicaemic broilers in Egypt [22]. This is therefore the first report of TEM-104 variants in clinical urinary *E. coli* isolates in China.

It has been previously reported that *fosA3* is the most prevalent PMGST in *E. coli* isolates of both clinical and non-clinical (healthy persons, companion and food animals) origins in several Asian countries (China, South Korea, Japan) [5, 23–27]. Thus, the high prevalence of *fosA3* found here is consistent with these reports, and confirms that *fosA3* is the primary mechanism of fosfomycin resistance in mainland China. Moreover, all but one of the 51 *fosA3*-positive isolates in our study were CTX-M producers, suggesting a high degree of association between the two resistance determinants. Indeed, the high transferability of these two genes via plasmids with identical replicon types further indicates that the two genes may be simultaneously disseminated by plasmids [23, 26]. The implication of this is that there is a high risk for their widespread dissemination and suggests a critical need for close monitoring of such strains.

UTI-causing *E. coli* isolates have been closely associated with phylogroups D and B2 in China [27]. D was the main phylogroup in the ESBL-producing urinary *E. coli* isolates in this study, consistent with previous reports [27, 28]. This indicates that group D may contribute more to MDR and UTI infections in China than other phylogroups. Phylogroups A and B1, however, were more common in our study than B2. Since phylogroups A and B1 have been reported in animal or human commensal *E. coli* strains [29, 30], this provides evidence that animals may be the source of some UTI-causing *E. coli* isolates [31].

Clonal diversity in *fosA3*- and $bla_{\text{CTX-M}}$-harbouring *E. coli* from humans, as revealed by both PFGE and MLST, indicates that the spread of *fosA3* in ESBL-producing *E. coli* is not attributable to clonal transfer of FosA3 producers in patients. In addition, MLST results suggest that several clonal strains involved in the dissemination of $bla_{\text{CTX-M}}$-positive *E. coli*, such as ST450, also carry *fosA3* [23]. IncFII, IncI1, and IncN plasmids carrying *fosA3* as well as $bla_{\text{CTX-M}}$ β-lactamase genes have previously been reported in *E. coli* from chickens, pets, livestock, and other animals in China [26, 32, 33]. Furthermore, *fosA3* and $bla_{\text{KPC-2}}$ genes were found to be able to spread together worldwide through IncP plasmid transfer [34, 35]. The high transferability of plasmids carrying *fosA3* and multiple replicons found here provide further evidence of the high potential for transfer of fosfomycin resistance gene *fosA3*. Recently, *fosA3* has been found on an epidemic plasmid carrying $bla_{\text{CTX-M-65}}$ and *rmtB* [36], Of particular concern, the gene has also been identified on a novel IncR-F33:A-:B- plasmid harbouring $bla_{\text{KPC-2}}$, $bla_{\text{CTX-M-65}}$, $bla_{\text{SHV-12}}$, and *rmtB* that was isolated from an epidemic *Klebsiella pneumoniae* ST11 strain in China [37]. Therefore, close monitoring and continued surveillance of patterns of fosfomycin resistance are necessary in order to prevent further dissemination of *fosA3* genes.

One limitation of this study stems from the fact that only ESBL producers in the 821 *E. coli* urinary isolates were screened for fosfomycin-resistance genes based on the strong association between the presence of *fosA3* and that of bla_{CTX} [23, 26]. However, the high prevalence of *fosA3* demonstrates the rapid spread of fosfomycin resistance in this region.

In summary, the high prevalence of fosfomycin resistance observed in ESBL-producing urinary *E. coli* isolates recovered during 2010–2014 is mainly attributed to the widespread occurrence of plasmid-mediated *fosA3* genes. The dissemination of the *fosA3* gene is closely associated with that of $bla_{\text{CTX-M}}$. Rather than clonal expansion of *fosA3*-harbouring *E. coli* lineages, horizontal transfer of plasmid-mediated mobile elements carrying *fosA3* played a central role in the spread of *E. coli* harbouring both *fosA3* and $bla_{\text{CTX-M}}$ in our hospital.

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DECLARATION OF INTEREST

None.

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