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Antimicrobial susceptibility and mechanisms of fosfomycin resistance in extended-spectrum β-lactamase-producing *Escherichia coli* strains from urinary tract infections in Wenzhou, China

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ABSTRACT

Fosfomycin in combination with various antibiotics represents an excellent clinically efficacious regimen for the treatment of urinary tract infections (UTIs) caused by extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli*. Underlying mechanisms of fosfomycin resistance remain largely uncharacterised. To investigate the antibacterial efficacy of fosfomycin against ESBL-producing *E. coli*, 356 non-repetitive ESBL-producing *E. coli* clinical isolates were collected from urine specimens from patients with UTI in Wenzhou, China, from January 2011 to December 2015. Antimicrobial sensitivity testing indicated that 6.7% (24/356) of the ESBL-producing *E. coli* strains were resistant to fosfomycin. The *fosA3* gene encoding a fosfomycin-modifying enzyme was detected in 20 isolates by PCR and sequencing, alone or in combination with other ESBL determinants. Conjugation experiments and Southern blotting demonstrated that 70% (14/20) of the *fosA3*-positive isolates possessed transferable plasmids (ca. 54.2 kb) co-harbouring the ESBL resistance gene bla_{CTX-M} and the fosfomycin resistance gene *fosA3*. Among the four fosfomycin-resistant *fosA3*-negative *E. coli* isolates, three contained amino acid substitutions (Ile28Asn and Phe30Leu in MurA and Leu297Phe in GlpT). The results indicate that presence of the *fosA3* gene is the primary mechanism of fosfomycin resistance in ESBL-producing *E. coli* isolates in Wenzhou, China. In addition, a plasmid (ca. 54.2 kb) co-harbouring *fosA3* and bla_{CTX-M} genes is horizontally transferable. Furthermore, a low degree of homology in the fosfomycin-resistant *E. coli* was confirmed using multilocus sequence typing (MLST), suggesting that there is no obvious phenomenon of clonal dissemination.

1. Introduction

Escherichia coli is a major clinical concern due to its ability to cause a substantial proportion of urinary tract infections (UTIs) and it may be considered as an important vector for the increased acquired resistance among Gram-negative pathogens [1]. Over the past few decades, the increased acquired multidrug resistance rate of extended-spectrum β-lactamase (ESBL)-producing *E. coli* has imposed significant restrictions on appropriate and reliable antimicrobial therapeutic options [2].

Fosfomycin, a naturally occurring antimicrobial agent, exhibits broad-spectrum antibacterial activity against Gram-negative pathogens as it suppresses the peptidoglycan synthesis pathway, which is a major constituent of the bacterial cell wall [3]. Despite the development of antimicrobial resistance during anti-infective therapy, fosfomycin has regained a great deal of interest in recent years and has been increasingly used to treat infections caused by multidrug-resistant bacteria particularly under conditions of limited oxygen [4,5]. Consequently, as a potential and promising treatment alternative, fosfomycin has been broadly administered in combination with various antibiotics. Although it has been empirically and extensively used as an infectious therapy worldwide, fosfomycin resistance in pathogenic *E. coli* strains remains to be characterised at an extremely low incidence [6]. Several mechanisms of fosfomycin resistance have been proposed, including mutation or overexpression of the target enzyme MurA [7], defects in two transporters (GlpT

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and UhpT) [8] and the presence of a *fos* gene encoding a fosfomycin-modifying enzyme [6,9]. All of these factors are responsible for fosfomycin resistance. In the present study, we focused on modification of the fosfomycin target (MurA), mutations in transport system regulators (GlpT and UhpT) as well as the presence of genes encoding fosfomycin-modified enzymes (*fosA*) and regulatory genes (*uhpA*, *cyaA* and *pstI*) [10].

Production of a consistently high level of β-lactamases has been correlated with multiple antibiotic resistance, leaving few therapeutic options [11]. Currently, fosfomycin serves as a last-line antibiotic therapy against UTIs and severe infections. Oral treatment with fosfomycin tromethamine for UTIs caused by ESBL-producing *E. coli* exhibited good effectiveness and has rarely been found to develop co-resistance with other antimicrobial groups [12,13]. However, the antimicrobial susceptibility and molecular-level mechanistic data of fosfomycin resistance of ESBL-producing *E. coli* isolated from UTIs are currently not comprehensive in mainland China. Here we sought to evaluate the in vitro activity of fosfomycin against ESBL-producing *E. coli* isolated from urine samples and to investigate the prevalence and underlying mechanism of fosfomycin resistance of the *E. coli* isolates in Wenzhou, China.

2. Materials and methods

2.1. Bacterial strains

A total of 356 non-repetitive ESBL-producing *E. coli* strains isolated from urine specimens were collected and identified from January 2011 to December 2015 in the First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China). Initially, bacterial identification and antimicrobial susceptibility tests were conducted using a VITEK®2 system (bioMérieux, Lyons, France). Isolates were stored frozen at –80 °C with 30% glycerol. Sodium azide-resistant *E. coli* J53 served as a recipient in the conjugation experiments, and *E. coli* ATCC 25922 served as a control strain for antimicrobial susceptibility testing.

2.2. Antimicrobial susceptibility profiling

Minimum inhibitory concentrations (MICs) of fosfomycin, ceftazidime, cefotaxime, ceftriaxone, ertapenem, imipenem, amikacin, gentamicin, levofloxacin, ciprofloxacin, and nitrofurantoin were determined by the agar dilution method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines [14]. The MIC of fosfomycin was determined using supplementation with 25 mg/L glucose-6-phosphate (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China). According to the MIC breakpoint for testing and reporting of *E. coli* urinary tract isolates recommended by the CLSI in 2015 [14], *E. coli* strains were classified as fosfomycin-susceptible (MIC ≤ 64 mg/L), intermediate (MIC = 128 mg/L) or fosfomycin-resistant (MIC ≥ 256 mg/L).

2.3. Molecular detection of extended-spectrum β-lactamases and confirmatory tests

Based on the CLSI guidelines [14], *E. coli* strains were screened for ESBL production using a VITEK®2 system, and positive strains were confirmed by the double disk test using cefotaxime or ceftazidime with and without clavulanic acid. An increase of ≥5 mm in inhibitory zone diameter in the presence of clavulanic acid indicated the presence of an ESBL. *Klebsiella pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 served as positive and negative controls, respectively.

For each resistant ESBL-producing isolate, DNA was extracted from fresh bacterial colonies using an AxyPrep™ Bacterial Genomic DNA Miniprep Kit (Axygen Scientific, Union City, CA). PCR assays were performed on a Veriti™ 96-well Thermal Cycler (Bio-Rad, Hercules, CA) using primers as previously described [15,16] to screen for bla_{SHV}, bla_{TEM} and bla_{CTX-M} genes. PCR products were sequenced and nucleotide sequences were compared by BLAST to verify the ESBL phenotype.

Table 1
Primers used for PCR amplification of fosfomycin resistance genes.

Amplified gene	Sequence	Amplicon size (bp)
fosA	F: 5′-ATCTGTGGGTCTGCCTGTCGT-3′ R: 5′-ATGCCCGCATAGGGCTTCT-3′	271
fosA3	F: 5′-GCGTCAAGCCTGGCATTT-3′ R: 5′-GCCGTCAGGGTCGAGAAA-3′	282
fosB	F: 5′-CAGAGATATTTTAGGGGCTGACA-3′ R: 5′-CTCAATCTATCTTCTAAACTTCCTG-3′	312
fosB2	F: 5′-CCTGGCCGAGAAAGAGATGAG-3′ R: 5′-AACCGGTTTTGCAAAGTGCC-3′	101
fosC	F: 5′-CCTTGCTCACTGGGGATCTG-3′ R: 5′-TACAAGACCCGACGCACTTC-3′	112
fosC2	F: 5′-TGGAGGCTACTTGGATTTG-3′ R: 5′-AGGCTACCGCTATGGATTT-3′	217
fosX	F: 5′-TGTCCCTCACCTTCGACTCT-3′ R: 5′-TTGCTGGTCTGTGGATTTGC-3′	131
murA	F: 5′-AAACAGCAGACGGTCTATGG-3′ R: 5′-CCATGAGTTTATCGACAGAACG-3′	1541
glpT	F: 5′-GCGAGTCGCGAGTTTTCATTG-3′ R: 5′-GGCAAATATCCACTGGCACC-3′	1785
uhpT	F: 5′-TTTTTGAACGCCCAGACACC-3′ R: 5′-AGTCAGGGGCTATTTGATGG-3′	1668
uhpA	F: 5′-GATCGCGGTGTTTTTTCAG-3′ R: 5′-GATACTCCACAGGCAAAACC-3′	770
pstI	F: 5′-GAAAGCGGTTGAACATCTGG-3′ R: 5′-TCCTTCTTGTCGTCGGAAAC-3′	1907
cyaA	F: 5′-AACCAGGCGCGAAAAGTGG-3′ R: 5′-ACCTTCTGGGATTTGCTGG-3′	2771

2.4. Molecular detection of fosfomycin resistance genes

Plasmid-encoded fosfomycin resistance genes (*fosA*, *fosA3*, *fosB*, *fosB2*, *fosC*, *fosC2* and *fosX*) were detected and characterised by PCR. Moreover, *murA*, *glpT*, *uhpT*, *uhpA*, *cyaA* and *pstI* were also amplified to screen for mutations in the 24 fosfomycin-resistant isolates. Twenty randomly selected fosfomycin-susceptible isolates served as controls. Primers for the aforementioned resistance genes are listed in Table 1 [17–19]. Positive PCR products were sequenced by Beijing Genomics Institute Technology Co. Ltd. (Shanghai, China). Nucleotide sequences were compared using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Genotyping of fosfomycin-resistant isolates

Molecular typing of fosfomycin-resistant strains was carried out by multilocus sequence typing (MLST). The sequences of eight housekeeping loci (*dinB*, *icdA*, *pabB*, *polB*, *putP*, *trpA*, *trpB* and *uidA*) were amplified and sequence types (STs) were assigned using the database available at the *E. coli* MLST website (http://bigsdb.web.pasteur.fr/ecoli/ecoli.html).

2.6. Conjugation experiments

Conjugation experiments were implemented using Luria–Bertani mating experiments with sodium azide-resistant *E. coli* J53 as the recipient strain. Transconjugants were selected on Mueller–Hinton agar (Oxoid Ltd., Basingstoke, UK) supplemented with 100 μg/mL sodium azide (Huifengda Chemical Co., Ltd., Jinan, Shandong, China) and 32 μg/mL fosfomycin (National Institute for the Control of Pharmaceutical and Biological Products). Resistance gene transfer from the *fosA3*-positive donor strains was verified by PCR and antimicrobial susceptibility testing. MICs of antimicrobial agents for