Antimicrobial Susceptibility and Molecular Mechanisms of Fosfomycin Resistance in Clinical Escherichia coli Isolates in Mainland China

Ya Li¹, Bo Zheng¹*, Yun Li¹, Sainan Zhu², Feng Xue¹, Jian Liu¹

¹ Institute of Clinical Pharmacology, Peking University First Hospital, Beijing, PR of China, ² Department of Biostatistics, Peking University First Hospital, Beijing, PR of China

* doctorzhengbo@163.com

Abstract

Escherichia coli is one of the most common pathogens in nosocomial and community-acquired infections in humans. Fosfomycin is a broad-spectrum antibiotic which inhibits peptidoglycan synthesis responsible for bacterial cell wall formation. Although low, the exact E. coli susceptibility to fosfomycin as well as the mechanisms of resistance in the population from Mainland China are mostly unknown. 1109 non-duplicate clinical E. coli strains isolated from urine, sputum, blood and pus samples in 20 widely dispersed tertiary hospitals from Mainland China were collected from July 2009 to June 2010, followed by determination of minimum inhibitory concentrations of fosfomycin. Detection of the murA, glpT, uhpT, fosA, fosA3 and fosC genes was performed in fosfomycin non-susceptible E. coli strains and conjugation experiments were employed to determine the mobility of fosA3 gene. In this study, 7.8% (86/1109) E. coli strains were fosfomycin non-susceptible. Amino acid substitutions in GlpT and MurA were found in six and four E. coli strains, respectively, while the uhpT gene was absent in eighteen E. coli strains. Twenty-nine isolates carried the transferable plasmid with the fosA3 gene at high frequencies of around 10⁻⁶ to 10⁻⁷ per donor cell in broth mating. The majority of isolates were susceptible to fosfomycin, showing that the drug is still viable in clinical applications. Also, the main mechanism of E. coli resistance in Mainland China was found to be due to the presence of the fosA3 gene.

Introduction

Escherichia coli is one of the most common pathogens in nosocomial and community-acquired infections in humans. Fosfomycin is a broad-spectrum antibacterial agent against Gram-positive and Gram-negative bacteria; it inhibits the UDP-N-acetylglucosamine enolpyruvyl transferase (MurA), responsible for the formation of UDP-GlcNAc-enolpyruvate in the biosynthesis of cell-wall peptidoglycans [1,2]. Fosfomycin enters the E. coli cell by using one of two transport systems: the glycerol-3-phosphate transport system (GlpT) or the hexose
phosphate transport system (UlpT) [3,4]. The prevalence of fosfomycin resistance in *E. coli* remains low [5–8], but several resistance mechanisms to fosfomycin have been reported. Mutations in the *glpT* or *uhpT* genes can decrease the uptake of fosfomycin via the transport systems and give rise to fosfomycin resistance [4,9,10]. Resistance against fosfomycin can also be conferred by mutations in the *murA* gene, resulting in either reduced affinity between MurA and fosfomycin or through the over-expression of MurA [11,12]. In addition, the presence of the plasmid-borne *fosA, fosA3* and *fosC2* genes, which encode fosfomycin-modifying enzymes, can inactivate the drug by catalyzing the covalent addition of glutathione to fosfomycin [13].

However, little is known about the rate of resistance or *E. coli* mechanisms of resistance to fosfomycin in Mainland China. Thus, here we investigated several fosfomycin resistance mutations and the mobility of the *fosA3* gene.

**Material and Methods**

**Bacterial strains**

A total of 1109 non-duplicate *E. coli* strains isolated from urine, sputum, blood and pus samples were collected as part of standard patient care from July 2009 to June 2010 in 20 widely dispersed tertiary hospitals. *E. coli* ATCC 25922 [14] was used for quality control for susceptibility testing and the sodium azide-resistant *E. coli* J53 [15] strain was used as a recipient in the mating experiment.

**Ethics**

The samples were collected from hospitals participating in the Ministry of Health National Antimicrobial Resistance Surveillance Net (Mohnarin) study. No ethical approval or informed consent was required due to the retrospective nature of the study. All patient identifiable information was removed from the samples before the authors received them for analysis. The authors were not involved in the treatment and had no direct contact with the patients.

**Susceptibility testing**

Susceptibility to antimicrobial agents was determined using the agar dilution method according to the Clinical and Laboratory Standards Institute [14]. The following antimicrobial agents were tested: fosfomycin trometamol, piperacillin-tazobactam, cefuroxime, cefotaxime, cefepime, imipenem, amikacin, levofloxacin, and nitrofurantoin. Based on minimum inhibitory concentrations (MIC), *E. coli* were classified as fosfomycin-susceptible (MIC ≤64mg/L), fosfomycin-intermediate (MIC = 128mg/L) and fosfomycin-resistant (MIC ≥256 mg/L). Extended-spectrum beta-lactamase (ESBL) producing isolates were detected according to previously described methods [14]. The reference strain *E. coli* ATCC 25922 was used as the positive control. The spontaneous fosfomycin resistance rate for *E. coli* J53 strain determined in our research was <10⁻⁸.

**PCR amplification of genes and sequence analysis**

Detection of the *murA, glpT, uhpT, fosA, fosA3* and *fosC* genes was performed in fosfomycin non-susceptible *E. coli* strains (intermediate and resistant *E. coli* strains; MIC ≥128 mg/L), and the primers used are listed in Table 1 [16–18]. Sequence analysis was performed with a Dye primer and a Dye Terminator cycle sequencing kit (Applied Biosystems) and with a 310 gene analyzer (ABI Prism).
Conjugation experiments

The conjugation experiments were carried out to determine the mobility of fosA3 gene from azide-sensitive isolates as donors to azide-resistant E. coli J53 as the recipient. Overnight cultures of 0.05 ml of the donor and 0.45 ml of the recipient strains were added to 3 ml of fresh Mueller Hinton (MH) broth (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom), then incubated and gently stirred at 37°C for 12 hours. 0.1 ml of the mixtures were plated on MH agar (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) containing glucose-6-phosphate (25 mg/L), sodium azide (100 mg/L) and fosfomycin (Zambon Group, Milan, Italy) (32 mg/L) and incubated for 48 hours for selection. The conjugative transfer frequency was calculated as the ratio of the number of conjugants to the number of donors.

Statistical analyses

Statistical tests were performed using Social Sciences software for Windows Version 14.0 (SPSS, Inc., Chicago, IL, USA). Enumeration data were expressed as percentage values. The differences in susceptibility between the groups were compared using the Chi-square test or Fisher’s exact test. The differences between the groups were considered significant if the p-values were smaller than 0.05 (two-sided test). The Bonferroni method was used to adjust the significant levels (0.05/3 = 0.0167) in multiple comparisons between any two levels of the susceptibility outcome.

Results

1023/1109 (92.2%) of the E. coli isolates were susceptible to fosfomycin. The susceptibility rates of the isolates from urine, sputum, blood and pus samples were 95.0%, 87.1%, 93.3% and 93.3%, respectively. MIC<sub>50</sub> of the strains from different specimen types were all 0.25 mg/L, whilst the MIC<sub>90</sub> of the strains from urine, sputum, blood and pus samples were 4, 128, 16 and 32 mg/L, respectively (Table 2). The ESBL-positive rates were 67.2% (687/1023), 85.7% (30/35) and 90.2% (46/51) among fosfomycin-susceptible, intermediate and resistant isolates, respectively.

The antimicrobial resistance rates stratified by fosfomycin susceptibility categories are summarized in Table 3. Fosfomycin-resistant isolates were significantly more likely to be ESBL positive than were fosfomycin-susceptible isolates (P < 0.001). In addition, the resistance rates for piperacillin-tazobactam, cefuroxime, cefotaxime, amikacin and nitrofurantoin
were significantly higher in fosfomycin-resistant isolates than fosfomycin-susceptible isolates (P < 0.0167 for all comparisons).

Table 2. Fosfomycin susceptibility of E. coli strains from different samples by source.

| Sample source | No. of isolates | ESBL (%) | No. of isolates inhibited at fosfomycin MIC (mg/L) of | MIC₉₀₀₉ₐ₅₅ₐₜ (mg/L) | MIC₉₅₀ₙ₉₇₉₂₅₆ (mg/L) | S (%) | I (%) | R (%) |
|---------------|----------------|----------|-----------------------------------------------------|---------------------|------------------------|-------|-------|-------|
| urine         | 262            | 65.9     | 0.062 0.125 0.25 0.5 1 2 4 8 16 32 64 128 256 >256 | 0.25                | 0.25                   | 4     | 95.0  | 1.2   | 3.8   |
| sputum        | 264            | 77.9     | 7 59 116 34 15 4 4 1 4 4 1 3 8 2                | 0.25                | 0.25                   | 128   | 87.1  | 5.7   | 7.2   |
| blood         | 343            | 67.9     | 6 72 135 59 14 9 9 5 6 2 3 7 9 7                | 0.25                | 0.25                   | 16    | 93.3  | 2.0   | 4.7   |
| pus           | 240            | 63.3     | 5 46 90 38 19 5 4 3 3 6 5 10 3                | 0.25                | 0.25                   | 32    | 93.3  | 4.2   | 2.5   |
| Total         | 1109           | 68.8     | 7 59 116 34 15 4 4 1 4 4 1 3 8 2                | 0.25                | 0.25                   | 32    | 92.2  | 3.2   | 4.6   |

ESBL—extended spectrum β-lactamases; MIC—minimum inhibitory concentration; MIC₉₀₀₉ₐ₅₅₅ₐₜ—minimum concentration that inhibits 50% of the growth; MIC₉₅₀ₙ₉₇₉₂₅₆—minimum concentration that inhibits 90% of the growth; S—susceptibility; I—intermediate; R—resistance

doi:10.1371/journal.pone.0135269.t002

In 86 fosfomycin non-susceptible E. coli strains, amino-acid substitutions Val389Ile, Asp390Ala, Gln59Lys and Glu139Lys were found in MurA of E. coli strains J621, E261, E374 and R162, respectively. In addition, amino-acid substitutions Ile4Val and Gly84Asp were found in GlpT of E. coli strains H039 and R538, respectively. In strain Q446, the GlpT protein was truncated due to deletion of nucleotide 401A. Amino acid substitutions Thr144Pro and Pro173Ser were found in the GlpT of strains R046 and P305. Amino acid substitutions Ala12-Val and Gln437Cys were found in GlpT of strains T229 and T297. The uhpT gene was absent from 18 of the test strains. 69 E. coli isolates were positive for fosA₃, yet no fosC₂ or fosA genes were detected among these isolates (Table 4). The fosA₃ gene was able to transfer at frequencies varying from 1.1×10⁻⁷ to 9.9×10⁻⁶ between the donor and recipient in 29 isolates tested.

Table 3. Antimicrobial resistance of Escherichia coli stratified by fosfomycin susceptibility.

| Agenta | % resistant | Fos-S (n = 1023) | Fos-I (n = 35) | Fos-R (n = 51) | pᵇ |
|--------|-------------|------------------|----------------|----------------|-----|
| TZP    | 3.8         | 11.4             | 13.7**         |                | 0.002 |
| CXM    | 71.1        | 94.3*            | 98.0**         | <0.001         |     |
| CTX    | 70.0        | 94.3*            | 98.0**         | <0.001         |     |
| FEP    | 34.1        | 48.6             | 49.0           | 0.023          |     |
| IMP    | 0.2         | 0.0              | 2.0            | 0.215          |     |
| AMK    | 4.2         | 37.1*            | 35.3**         | <0.001         |     |
| LVX    | 58.7        | 68.6             | 60.8           | 0.494          |     |
| TET    | 77.4        | 77.1             | 90.2           | 0.098          |     |
| NIT    | 1.4         | 14.3*            | 11.8**         | <0.001         |     |
| ESBL positive | 67.2     | 85.7             | 90.2**        | <0.001         |     |

Fos-S, fosfomycin—susceptible (MIC ≤64 mg/L); Fos-I, fosfomycin—intermediate (MIC = 128 mg/L); Fos-R, fosfomycin-resistant (MIC ≥256 mg/L); ESBL—extended spectrum β-lactamases.

aDrug abbreviations: FOS, fosfomycin trometamol; TZP, piperacillin-tazobactam; CXM, cefuroxime; CTX, cefotaxime; FEP, cefepime; IMP, imipenem; AMK, amikacin; LVX, levofloxacin; TET, tetracycline; NIT, nitrofurantoin.

bP value for comparison of resistance rates between fosfomycin-susceptible and fosfomycin-resistant isolates.

*compared to FOS-S, P < 0.0167
**compared to FOS-R, P < 0.0167

doi:10.1371/journal.pone.0135269.t003
Discussion

Fosfomycin has been introduced in clinical practice for about 30 years. However, this antibacterial agent is still not commonly used in China, and the fosfomycin resistance rate in clinical E. coli isolates remains very low [18]. In this study, only 7.8% (86/1109) of E. coli isolates were fosfomycin non-susceptible with a high ESBL-positive rate and levofloxacin-resistant rate. Among the fosfomycin-resistant isolates, the ESBL-positive rates were higher than in fosfomycin-susceptible isolates.

Fosfomycin inactivates the MurA enzyme by binding to the active site of its Cys-115 residue [19,20]. The amino acid Asp369Asn and Leu370Ile substitutions have been reported in fosfomycin-resistant E. coli isolates MSC17327 and MSC17323. Inspection of the crystal structure of E. coli MurA complexed with fosfomycin does not suggest an obvious role for Asp-369 and Leu-370 in the protein-inhibitor interaction [16]. In our study, the MurA substitutions of Val389Ile, Asp390Ala, Gln59Lys and Glu139Lys were found in four E. coli strains. However, further investigations are needed to find out whether the substitutions contribute to fosfomycin resistance.
Fosfomycin is transported into cells via two pathways: the glycerol-3-phosphate or the hexose phosphate transport systems. Several studies have reported *E. coli* fosfomycin resistance due to the defects in GlpT or UhpT [9,13,16]. In this study, mutations in the *g.lpT* gene were found in six *E. coli* strains, all of which resulted in amino acid substitutions in GlpT. In addition, single nucleotide deletion in the *g.lpT* gene, which would lead to a truncation of the GlpT sequence, was also detected.

Alteration in the chemical structure of fosfomycin by FosA3, a protein encoded by the *fosA3* gene, was previously reported in three *E. coli* strains in Japan in 2010 for the first time [13]. In our study, over 80% of fosfomycin non-susceptible *E. coli* strains (69/86) harbored the *fosA3* gene, which indicated that it is the main mechanism responsible for fosfomycin resistance in Mainland China. The *fosA3* gene identified in 42% isolates (29/69) can transfer between different *E. coli* strains. Previous research has suggested that the *fosA3* gene is encoded on a conjugated plasmid [21,22]. As the mobility of this gene may accelerate the dissemination of fosfomycin resistance around the world, future research is warranted to confirm this for *E. coli*.

**Acknowledgments**

Editorial support from Mihai Surducan (XPE Pharma & Science on behalf of BC-Biostat) is gratefully acknowledged.

**Author Contributions**

Conceived and designed the experiments: BZ SNZ. Performed the experiments: Ya Li Yun Li FX JL. Analyzed the data: SNZ. Contributed reagents/materials/analysis tools: Ya Li Yun Li FX JL. Wrote the paper: Ya Li BZ Yun Li FX SNZ JL.

**References**

1. Kahan FM, Kahan JS, Cassidy PJ, Kropp H. The mechanism of action of fosfomycin (phosphonomycin). Ann N Y Acad Sci. 1974; 235: 364–386. PMID:4605290
2. Patel SS, Balfour JA, Bryson HM. Fosfomycin tromethamine. A review of its antibacterial activity, pharmacokinetic properties and therapeutic efficacy as a single-dose oral treatment for acute uncomplicated lower urinary tract infections. Drugs. 1997; 53: 637–656. PMID:9098664
3. Tsuruoka T, Miyata A, Yamada Y. Two kinds of mutants defective in multiple carbohydrate utilization isolated from in vitro fosfomycin-resistant strains of Escherichia coli K-12. J Antibiot (Tokyo). 1978; 31: 192–201.
4. Kadner RJ, Winkler HH. Isolation and characterization of mutations affecting the transport of hexose phosphates in Escherichia coli. J Bacteriol. 1973; 113: 895–900. PMID:4347928
5. Kahlmeter G, Poulsen HO. Antimicrobial susceptibility of Escherichia coli from community-acquired urinary tract infections in Europe: the ECO.SENS study revisited. Int J Antimicrob Agents. 2012; 39: 45–51. doi:10.1016/j.ijantimicag.2011.09.013 PMID: 22055529
6. Falagas ME, Kastoris AC, Kapaskelis AM, Karageorgopoulos DE. Fosfomycin for the treatment of multidrug-resistant, including extended-spectrum beta-lactamase producing, Enterobacteriaceae infections: a systematic review. Lancet Infect Dis. 2010; 10: 43–50. doi:10.1016/S1473-3099(09)70325-1 PMID:20129148
7. Knoottnerus BJ, Nys S, Ter Riet G, Donker G, Geerlings SE, Stobberingh E. Fosfomycin tromethamine as second agent for the treatment of acute, uncomplicated urinary tract infections in adult female patients in The Netherlands? J Antimicrob Chemother. 2008; 62: 356–359. doi:10.1093/jac/dkn177 PMID:18424789
8. Kahlmeter G, Eco.Sens. An international survey of the antimicrobial susceptibility of pathogens from uncomplicated urinary tract infections: the ECO.SENS Project. J Antimicrob Chemother. 2003; 51: 69–76. PMID:12493789
9. Nilsson Al, Berg OG, Aspevall O, Kahlmeter G, Andersson DI. Biological costs and mechanisms of fosfomycin resistance in Escherichia coli. Antimicrob Agents Chemother. 2003; 47: 2850–2858. PMID:12936984
10. Tsuruoka T, Yamada Y. Characterization of spontaneous fosfomycin (phosphonomycin)-resistant cells of Escherichia coli B in vitro. J Antibiot (Tokyo). 1975; 28: 906–911.

11. Marquardt JL, Siegele DA, Kolter R, Walsh CT. Cloning and sequencing of Escherichia coli murZ and purification of its product, a UDP-N-acetylgalacosamine enolpyruvyl transferase. J Bacteriol. 1992; 174:5748–5752. PMID: 1512209

12. Venkateswaran PS, Wu HC. Isolation and characterization of a phosphonomycin-resistant mutant of Escherichia coli K-12. J Bacteriol. 1972; 110: 935–944. PMID: 4555418

13. Wachino J, Yamane K, Suzuki S, Kimura K, Arakawa Y. Prevalence of fosfomycin resistance among CTX-M-producing Escherichia coli clinical isolates in Japan and identification of novel plasmid-mediated fosfomycin-modifying enzymes. Antimicrob Agents Chemother. 2010; 54: 3061–3064. doi: 10.1128/AAC.01834-09 PMID: 20404116

14. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; M100-S24. Wayne, PA: Clinical and Laboratory Standards Institute; 2014.

15. Yi H, Cho YJ, Yong D, Chun J. Genome Sequence of Escherichia coli J53, a Reference Strain for Genetic Studies. J Bacteriol. 2012; 194: 3742–3743. doi: 10.1128/JB.00641-12 PMID: 22740669

16. Takahata S, Ida T, Hiraishi T, Sakakibara S, Maebashi K, Terada S, et al. Molecular mechanisms of fosfomycin resistance in clinical isolates of Escherichia coli. Int J Antimicrob Agents 2010; 35: 333–337. doi: 10.1016/j.ijantimicag.2009.11.011 PMID: 20071153

17. Hou J, Huang X, Deng Y, He L, Yang T, Zeng Z, et al. Dissemination of the fosfomycin resistance gene fosA3 with CTX-M beta-lactamase genes and rmtB carried on IncFII plasmids among Escherichia coli isolates from pets in China. Antimicrob Agents Chemother. 2012; 56: 2135–2138. doi: 10.1128/AAC.05104-11 PMID: 22232290

18. Lai B, Zheng B, Yun Li, Zhu S, and Tong Z. In vitro susceptibility of Escherichia coli strains isolated from urine samples obtained in mainland China to fosfomycin trometamol and other antibiotics: A 9-year surveillance study (2004–2012). BMC Infect Dis. 2014; 14: 66–69. doi: 10.1186/1471-2334-14-66 PMID: 24502648

19. Zhu JY, Yang Y, Han H, Betzi S, Olesen SH, Marsilio F, et al. Functional consequence of covalent reaction of phosphoenolpyruvate with UDP-N-acetylgalactosamine 1-carboxyvinyltransferase (MurA). J Biol Chem. 2012; 287: 12657–12667. doi: 10.1074/jbc.M112.342725 PMID: 22378791

20. Kim DH, Lees WJ, Kempsell KE, Lane WS, Duncan K, Walsh CT. Characterization of a Cys115 to Asp substitution in the Escherichia coli cell wall biosynthetic enzyme UDP-GlcNAc enolpyruvyl transferase (MurA) that confers resistance to inactivation by the antibiotic fosfomycin. Biochemistry. 1996; 35: 4923–4928. PMID: 8664284

21. Hou J, Yang X, Zeng Z, Lv L, Yang T, Lin D, Liu JH. Detection of the plasmid-encoded fosfomycin resistance gene fosA3 in Escherichia coli of food-animal origin. J Antimicrob Chemother. 2013; 68: 766–770. doi: 10.1093/jac/dks465 PMID: 23190765

22. Hou J, Huang X, Deng Y, He L, Yang T, Zeng Z, Chen Z, Liu JH. Dissemination of the fosfomycin resistance gene fosA3 with CTX-M beta-lactamase genes and rmtB carried on IncFII plasmids among Escherichia coli isolates from pets in China. Antimicrob Agents Chemother. 2012; 56: 2135–2138. doi: 10.1128/AAC.05104-11 PMID: 22232290