Prevalence of acquired fosfomycin resistance among extended-spectrum β-lactamase-producing Escherichia coli and Klebsiella pneumoniae clinical isolates in Korea and IS26-composite transposon surrounding fosA3

So-Young Lee1, Yeon-Joon Park1*, Jin Kyung Yu1, Seungwon Jung1, Yoonjoo Kim1, Seok Hoon Jeong2 and Yoshichika Arakawa3

1Department of Laboratory Medicine, College of Medicine, Catholic University of Korea, Seoul, Korea; 2Department of Laboratory Medicine, Yonsei University College of Medicine, Seoul, Korea; 3Department of Bacteriology, Nagoya University Graduate School of Medicine, Nagoya, Japan

*Corresponding author. Tel: +82-2-2258-1640; Fax: +82-2-2258-1719; E-mail: yjpk@catholic.ac.kr

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Objectives: To investigate the prevalence of plasmid-mediated fosfomycin resistance determinants among extended-spectrum β-lactamase (ESBL)-producing Escherichia coli and Klebsiella pneumoniae and their genetic environments.

Methods: A total of 347 non-duplicate ESBL-producing E. coli (165) and K. pneumoniae (182) were collected. The fosfomycin MICs were determined by the agar dilution method according to CLSI guidelines. PCR was used to detect the plasmid-encoded fosfomycin resistance genes (fosA, fosA3, fosB and fosC2). For isolates harbouring plasmid-encoded fosfomycin resistance genes, sequence types (STs) were determined. The transformation experiment was performed using E. coli TOPO10 (Invitrogen, USA) as a recipient strain. With the plasmids from the transformants, plasmid replicon typing was performed and the nucleotide sequences adjacent to fosA3 were determined.

Results: The susceptibility to fosfomycin was 92.9% in E. coli and 95.2% in K. pneumoniae. Of the 21 isolates non-susceptible to fosfomycin (8 E. coli and 13 K. pneumoniae), 7 (5 E. coli and 2 K. pneumoniae) isolates harboured fosA3 and all of them co-harbourted blaCTX-M-1group or blaCTX-M-9group. The STs of the isolates harbouring fosA3 were diverse (E. coli: ST1, ST1, ST533, ST2 and ST86; K. pneumoniae: ST11 and ST101). The plasmid replicon types of transformants co-harbouring blaCTX-M-1group and blaCTX-M-9group were IncF and IncN, respectively. By sequence analysis, we found the common feature that the fosA3 gene, connected to blaCTX-M via insertion sequences, was located between two IS26 elements oriented in the opposite direction, composing an IS26-composite transposon.

Conclusions: An IS26-composite transposon appears to be the main vehicle for dissemination of fosA3 in E. coli and K. pneumoniae of diverse clones.

Keywords: IncF, IncN, CTX-M

Introduction

The increasing rate of multidrug resistance in bacteria belonging to the family Enterobacteriaceae reduces the number of effective drugs that can be used. Fosfomycin, known for nearly four decades, has a unique mechanism of antimicrobial action that involves the inhibition of UDP-N-acetylglucosamine enolpyruvyl transferase (MurA), an enzyme that catalyses the first step in bacterial cell wall synthesis.1 It has a broad spectrum of antimicrobial activity against several Gram-negative and Gram-positive aerobic bacteria.2 Although fosfomycin resistance is mostly due to mutation in the chromosomal locus, including glpT, plasmid-mediated fosA3 and fosC2 were recently reported in CTX-M-producing Escherichia coli.3 In Korea, blaCTX-M is the
most frequent extended-spectrum β-lactamase (ESBL) in *E. coli* and the second most frequent in *Klebsiella pneumoniae*. Therefore we investigated the prevalence of plasmid-mediated fosfomycin resistance determinants among the ESBL-producing *E. coli* and *K. pneumoniae* and their genetic environments.

## Materials and methods

A total of 347 non-duplicate, ESBL-producing *E. coli* (165 isolates) and *K. pneumoniae* (182 isolates) were collected at 25 hospitals in Korea from June to July 2009.

The fosfomycin MICs were determined by the agar dilution method according to the CLSI guidelines. The presence of plasmid-encoded fosfomycin resistance genes (*fosA*, *fosB*, *fosA3* and *fosC2*) were determined by PCR as described previously, and the PCR products were sequenced on a 3730 DNA analyser (Applied Biosystems, Foster City, CA, USA). For isolates resistant to fosfomycin, but having no plasmid-mediated fosfomycin resistance determinants, we investigated the chromosomal mutation in *glpF* using primers reported previously for *E. coli* and *K. pneumoniae* using KgpT-f, 5'-TTAAAGCCCGCAGCATCAA-3' and KgpT-r, 5'-ATCA TCACCATGAAGCCGCC-3'. The sequence types (STs) of *E. coli* and *K. pneumoniae* isolates harbouring plasmid-encoded fosfomycin resistance genes were determined by analysing the eight housekeeping genes (*dnaB*, *icdA*, *pabB*, *polB*, *putP*, *tpaB*, *trpB* and *uidA*) and seven housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *pboB* and *tonB*), respectively, and they were compared with the multilocus sequence typing (MLST) databases available at http://www.pasteur.fr/recherche/genopole/PGP/ mlst/EColi.html and http://www.pasteur.fr/recherche/genopole/PGP/mlst/Kpneumoniae.html.

The transferability of resistance was studied by transformation experiments using *E. coli* TOPO10 (Invitrogen, USA) as a recipient strain. The transformants were selected on LB agar plates supplemented with sodium azide (150 mg/L), fosfomycin (40 mg/L) and glucose-6-phosphate (25 mg/L). The conjugation transformants were selected on LB agar plates supplemented with fosfomycin (40 mg/L) and glucose-6-phosphate (25 mg/L). The conjugation experiments were also performed with azide-resistant *E. coli* JS3 as a recipient strain by the filter mating method. Transconjugants were selected on LB agar plates supplemented with sodium azide (150 mg/L), fosfomycin (40 mg/L) and glucose-6-phosphate (25 mg/L). The plasmid DNAs digested with EcoRV and PvuII (New England Biolabs, Beverly, MA, USA) for IncF and IncN, respectively, to demonstrate the transferability of resistance.

### Table 1. Characteristics of the seven isolates harbouring *fosA3* and their transformants

<table>
<thead>
<tr>
<th>Strain</th>
<th>FOS MIC (mg/L)</th>
<th>CTX MIC (mg/L)</th>
<th>Plasmid profile</th>
<th>Replicon type</th>
<th>CTX-M type</th>
<th>CTX-M type (transformant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.coli</td>
<td>ECO021</td>
<td>&gt;256</td>
<td>ST1</td>
<td>IncF</td>
<td>CTX-M-14</td>
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<tr>
<td>E.coli</td>
<td>ECO096</td>
<td>&gt;256</td>
<td>ST1</td>
<td>IncF</td>
<td>CTX-M-14</td>
<td>CTX-M-14</td>
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<tr>
<td>E.coli</td>
<td>ECO110</td>
<td>&gt;256</td>
<td>ST2</td>
<td>IncF</td>
<td>CTX-M-14</td>
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<tr>
<td>E.coli</td>
<td>ECO144</td>
<td>&gt;256</td>
<td>ST3</td>
<td>IncF</td>
<td>CTX-M-14</td>
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<tr>
<td>E.coli</td>
<td>ECO248</td>
<td>&gt;256</td>
<td>ST1</td>
<td>IncN</td>
<td>CTX-M-14</td>
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<tr>
<td>E.coli</td>
<td>KPN229</td>
<td>&gt;256</td>
<td>ST1</td>
<td>IncN</td>
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<tr>
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FOS, fosfomycin; CTX, cefotaxime.

Not determined; transformation failed. Not determined; the *korA* gene of the IncN plasmid was not amplified.
The nucleotide sequences of the genetic environments of \textit{fosA3} from ECO021TF, ECO096TF and ECO141TF have been deposited in the GenBank database under the numbers JQ343849, JQ343850 and JQ343851, respectively.

**Results and discussion**

The susceptibility to fosfomycin was 92.9\% in \textit{E. coli} and 95.2\% in \textit{K. pneumoniae}. Of the 21 isolates non-susceptible to fosfomycin (8 \textit{E. coli} and 13 \textit{K. pneumoniae}), 7 (5 \textit{E. coli} and 2 \textit{K. pneumoniae}) isolates harboured \textit{fosA3} and all of them co-harboured ESBLs from the \textit{bla}_{CTX-M-1} group or \textit{bla}_{CTX-M-9} group. None harboured other plasmid-mediated fosfomycin resistance determinants. By sequencing analysis of the \textit{glpT} in three \textit{E. coli} isolates (ECO150, ECO215 and ECO243) resistant to fosfomycin, ECO150 lacked \textit{glpT} gene, ECO215 was found to harbour two amino acid substitutions (Leu297Phe and Glu448Lys) and ECO243 had one amino acid substitution (Glu448Lys). However, because this study is focused on the acquired fosfomycin resistance mechanism, we did not elucidate if these mutations played a role in developing fosfomycin resistance. Of the 11 \textit{K. pneumoniae} isolates, no mutation in the \textit{glpT} gene was found. We presume the specific activity of fosfomycin glutathione-S-transferase or reduced permeability might be associated with the fosfomycin resistance in these isolates.\textsuperscript{13}

The fosfomycin resistance rate in Korean isolates of ESBL-producing \textit{E. coli} and \textit{K. pneumoniae} was low (4.2\% and 5.5\%, respectively), and this result was similar to that from Japan, where 96.4\% of the CTX-M-producing \textit{E. coli} were susceptible to fosfomycin.\textsuperscript{3} However, the prevalence of \textit{fosA3} among the

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**Figure 1.** Schematic representation of the \textit{fosA3} environment. (a) Genetic environment of the IncN-type plasmid of ECO21TF. (b) Genetic environment of the IncFII:2-type plasmid of ECO96TF. (c) Genetic environment of the IncFII:33-type plasmid of ECO141TF. Arrows indicate open reading frames, grey arrows indicate transposase genes and filled bars indicate inverted repeats of \textit{IS}\textsubscript{26}.
fosfomycin-resistant isolates was different between the two species: it was high (62.5%) for E. coli, but low (15.4%) for K. pneumoniae. Considering that all the isolates harbouring fosA3 co-harbourred bla<sub>CTX-M</sub>, this difference might be derived from the fact that the dominant type of ESBL in E. coli and K. pneumoniae in Korea is bla<sub>CTX-M</sub> and bla<sub>SHV</sub>, respectively.<sup>14,15</sup>

The clones of the isolates harbouring fosA3, determined by MLST, were diverse: the STs of the five E. coli isolates were ST1, ST11, ST533, ST2 and ST86 and those of the two K. pneumoniae isolates were ST111 and ST101. Moreover, in the two E. coli isolates belonging to the same ST, the plasmid Inc types were different.

Of the seven isolates harbouring fosA3, it was successfully transferred both by transformation and conjugation in six isolates. In one isolate (ECO110), where fosA3 was not transferred by either transformation or conjugation, the location of fosA3 was determined to be on the plasmid by S1 nuclease digestion and hybridization with fosA3 and the 16S rRNA gene. bla<sub>CTX-M</sub> was always co-transferred to the recipient E. coli strain, and the replicons types of the transformants co-harbouring the bla<sub>CTX-M</sub>-1 group (bla<sub>CTX-M</sub>-3 or bla<sub>CTX-M</sub>-53) and bla<sub>CTX-M</sub>-9 group (bla<sub>CTX-M</sub>-1,4) were IncFII and IncN, respectively (Table 1). bla<sub>CTX-M-14</sub> is one of the most dominant plasmid-borne ESBLs in E. coli and it shows a worldwide distribution,<sup>16</sup> but its presence on an IncN-type plasmid has not been reported. Considering that the IncN-type plasmids are involved in the transmission of various resistance determinants (VIM-1, KPC-2, CTX-M-1 and NDM-1),<sup>17</sup> this is a worrisome phenomenon. By plasmid profile analysis, the two FII2 plasmids from E. coli and the two IncN-type plasmids from K. pneumoniae showed highly similar patterns (Table 1).

As neither ISEcp1 nor ISCR1, the mobile elements for the co-transferred bla<sub>CTX-M</sub>-3 were not detected, we determined the sequences of DNA adjacent to fosA3. We found the common feature that the fosA3 gene, connected to bla<sub>CTX-M</sub> via insertion sequences, was located between two IS26 elements oriented in the opposite direction, composing an IS26-composite transposon. In IncFII-type plasmids, there was another IS26 connecting bla<sub>TEM</sub> (Figure 1).

In the upstream structure of fosA3 of an IncN-type plasmid, the genetic support of bla<sub>CTX-M-14</sub> consisted of an upstream truncated ISEcp1 element and a downstream truncated IS903. Except for the truncation in ISEcp1 in ECO211F in this study, this structure is similar to the structure found in IncFII and in other plasmids linked to the spread of CTX-M-14 in China, the UK and Spain.<sup>18</sup> A similar structure was also found in Korean isolates of E. coli harbouring bla<sub>CTX-M-14</sub>, on an IncF-type plasmid.<sup>19</sup>

In IncFII-type plasmids, the structure consisted of IS26, truncated ISEcp1, bla<sub>CTX-M</sub>-3 and orf<sub>477</sub> upstream of fosA3. This structure containing truncated ISEcp1 instead of an intact copy of ISEcp1 has been detected in IncN-type and IncI1-type plasmids carrying bla<sub>CTX-M-1</sub>-group from German E. coli isolates.<sup>20</sup> Downstream of fosA3 there was also a common feature. There was a sequence showing 78% identity to part of the chromosomal nucleotide sequence of K. pneumoniae 342 (CP000964). This sequence similarity was also reported in E. coli co-harbouring fosA3 and bla<sub>CTX-M</sub>-3 in Japan.<sup>9</sup> As Wachino et al.<sup>3</sup> have indicated, considering the fact that the homology region in the chromosome of K. pneumoniae is close to the fosA gene, our finding supports their speculation that fosA3 might have originated from fosA of K. pneumoniae.

We also determined the full susceptibility profiles to a panel of antibiotics in order to obtain an indication of resistance determinants, which may also be encoded on the fosA3-encoding plasmids. Resistance to ampicillin, cefalotin, cefuroxime, cefotaxime and piperacillin was found in all six parental strains and their transformants, but resistance to trimethoprim/sulfamethoxazole, ciprofloxacin and gentamicin, which was observed in three, four and two of the six parental strains, respectively, was not observed in transformants. In conclusion, the fosfomycin resistance rate in ESBL-producing E. coli and K. pneumoniae was low (4.2% and 5.5%, respectively), but up to 62.5% and 15.4% of the fosfomycin-resistant E. coli and K. pneumoniae isolates, respectively, harboured plasmid-mediated fosA3. The fosA3 genes were always co-harbourred with bla<sub>CTX-M</sub> on an IS26-composite transposon in IncN- and IncFII-type plasmids, and were distributed among various clones of E. coli and K. pneumoniae. Taking into account the emerging importance of IS26 in the spread of bla<sub>ESBL</sub> genes, and the wide dissemination of IncN- and IncF-type plasmids, fosfomycin should be used cautiously.

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Transparency declarations

None to declare.

References


