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Short communication

The detection of fosfomycin resistance genes in *Enterobacteriaceae* from pets and their owners

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A B S T R A C T

The aim of this study was to investigate the prevalence of fosfomycin resistance and molecular characteristic of fosfomycin-resistant strains isolated from companion animals and their owners. A total of 171 samples collected from pets and pet owners in a Chinese veterinary teaching hospital were screened for the presence of phenotype and genotype of fosfomycin-resistance by selective media containing fosfomycin and PCR & sequencing. Among 171 samples tested, nineteen isolates were resistant to fosfomycin. Sixteen and three of these fosfomycin-resistant isolates were positive for *fosA3* and *fosA* genes, respectively. The *fosA3* gene was detected both in chromosomes and plasmids in bacteria. All of the *fosA3* gene-positive isolates except one were CTX-M producers and nearly half (7/16) of them also harbored the *rmtB* gene. The *fosA3* gene-carrying plasmids, which were readily transferrable to recipient *E. coli* J53 by conjugation, conferred resistance to multiple antimicrobial agents. Genetic structures were IS*26*-385bp-*fosA3*-1810bp-IS*26* (n = 11) and IS*26*-385bp-*fosA3*-588bp-IS*26* (n = 5). Molecular typing indicated that two *fosA3*-positive isolates from dogs were genetically identical to the isolates from the pet owners. Our results indicated that active transmission of *fosA3*-mediated fosfomycin resistance has occurred among *Enterobacteriaceae* isolated from pets and their owners by both horizontal transfer and clonal expansion.

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1. Introduction

Fosfomycin is a phosphonic acid derivative with broad-spectrum of antimicrobial activity. It is bactericidal against most *Escherichia coli* isolates, as well as other *Enterobacteriaceae* members (Patel et al., 1997; Raz, 2012). Fosfomycin may provide a valuable alternative option for the treatment of cystitis caused by extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* (Falagas et al., 2010). Although fosfomycin has been used for clinical treatment globally for long times, the overall fosfomycin resistant rate is relatively low in *E. coli* strains (1%–3%) (Raz, 2012). To date, several transferable fosfomycin-resistant genes have been described, such as *fosA*,*fosA3*, *fosC2*, and *fosK* (Kitanaka et al., 2014; Lee et al., 2012; Wachino et al., 2010). Multiple studies indicated that fosfomycin resistance is mediated mainly by the plasmid-encoded *fosA3* gene, and this gene has been mostly described in *E. coli* strains from various origins, including humans, pets, and livestock (Chan et al., 2014; Ho et al., 2013b,c; Hou et al., 2012, 2013; Li et al., 2015; Tseng et al., 2015; Wachino et al., 2010). In this study, we investigated the prevalence of fosfomycin resistance in *Enterobacteriaceae* from pets and their owners. The *fosA3* gene was detected in various *Enterobacteriaceae* species including *E. coli*, *Citrobacter freundii*, *E. fergusonii* and *Proteus mirabilis* from companion animals. Interestingly, molecular typing result indicated that *E. coli* and *E. fergusonii* isolates from dogs have the identical PFGE type with the strains associated with dog owners, respectively, which provides the direct evidence about the spread of fosfomycin resistant strains between companion animals and their owners.

2. Materials and methods

2.1. Bacterial isolates

A total of 171 samples were collected from companion animals (dogs, n = 148; cats, n = 2) and pet owners (n = 21) at the Veterinary Teaching Hospital of China Agricultural University during March to June, 2013. All the samples were taken with the pet owners'

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permission when the pets were sent by their owners to the Veterinary Teaching Hospital for therapy or health care. All the samples were streaked on Mueller-Hinton (MH) agar (MHA; Sigma-Aldrich, MO, USA) plates supplemented with 128 μg/mL fosfomycin and 25 μg/mL glucose-6-phosphate (Sigma) after preculture in MH broth. The addition of glucose-6-phosphate could enhance the activity of fosfomycin.

2.2. PCR screening

The strains grown on MH agar plates containing 128 μg/mL of fosfomycin and 25 μg/mL of glucose-6-phosphate were screened for the presence of the fosfomycin resistance genes (*fosA*, *fosA3*, *fosC2*, *fosK*) by PCR amplification. The PCR experiments were performed according to the conditions described previously (Hou et al., 2012). Subsequently, all isolates were identified using 16 S rRNA gene sequencing (Weisburg et al., 1991). The *fosA3* gene often coexists with the extended-spectrum β-lactamase gene bla_{CTX-M} and aminoglycosides resistance gene *rmtB* (Hou et al., 2012); therefore, the *fosA3*-positive isolates were also screened for these genes using PCR.

2.3. Antimicrobial susceptibility testing

For the *fosA3*-positive isolates and transconjugants, the minimum inhibitory concentrations (MICs) of fosfomycin, ampicillin, cefazolin, ciprofloxacin, gentamicin, amikacin, kanamycin, tetracycline, florfenicol, and chloramphenicol were determined using agar dilution method according to the recommendations of the documents M100-S (2016) of the Clinical and Laboratory Standards Institute (CLSI). The reference strain *E. coli* ATCC25922 was used as a quality control.

2.4. Conjugation experiments and plasmid analysis

Conjugation experiments were performed to determine the transferability of the *fosA3* gene according to the method described previously (Hou et al., 2013). The *fosA3*-positive isolates served as the donors, while *E. coli* J53 (sodium azide resistant) was used as the recipient strain. Transconjugants were selected on MH agar supplemented with sodium azide (200 μg/mL) and fosfomycin (128 μg/mL). *fosA3* genes in the transconjugants were identified by PCR and confirmed by DNA sequencing. Transconjugants were also confirmed by pulse field gel electrophoresis (PFGE). Antimicrobial susceptibility testing of the donors, transconjugants, and the recipient strains was conducted.

The plasmid sizes were determined by S1 nuclease PFGE, and the location of the *fosA3* gene was conducted by Southern blotting according to the manufacturer's instruction (Roche Diagnostics GmbH, Germany). Briefly, S1 nuclease PFGE was used to estimate the plasmid sizes and the resultant agarose gels were subjected to depurination, denaturation and neutralization before the DNA was transferred to a nylon membrane. Hybridization was conducted using a *fosA3*-specific DIG-labeled probe, and the signals from the bands were visualized using a nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP) color detection kit (Roche Diagnostics, Mannheim, Germany).

2.5. Pulsed-field gel electrophoresis (PFGE)

PFGE was performed using CHEF-DR III apparatus (Bio-Rad Laboratories, Hercules, CA), according to the PulseNet protocol for *E. coli* (http://www.cdc.gov/pulsenet/PDF/ecoli-shigella-salmonella-pfge-protocol-508c.pdf). *Salmonella* H9812 was used as the reference marker. The genomic DNA of performed strains was digested with *XbaI* (Takara, Dalian, China) at 37 °C for 2 h. The running conditions were as follows: 0.5 × Tris-borate-EDTA-1% SeaKem Gold agarose at 14 °C for 20 h with initial and final switch times of 6.75 s and 38.35 s, respectively. PFGE results were analyzed using the InfoQuest FP software version 4.5 (Bio-Rad Laboratories, Hercules, CA). The dendrograms were constructed from the PFGE data by UPGMA (unweighted pair group method with arithmetic average) with the Dice coefficient.

2.6. Analysis of the genetic environment of fosA3 gene

The genetic environment of the *fosA3* gene was investigated by PCR mapping and DNA sequencing. The sequences of the primers designed to amplify the regions up- and down-stream of the *fosA3* genes are shown in Table S1. The PCR was performed with the following cycling conditions: heat denaturation at 95 °C for 5 min, 32 cycles at 95 °C for 30 s, 56 °C for 30s, 72 °C for 2 min, and a final extension at 72 °C for 10 min.

3. Results and discussion

3.1. Identification of fosA3-positive isolates from pets and their owners

Among the 19 (19/171, 11.1%) fosfomycin-resistant *Enterobacteriaceae* isolates, 16 isolates (16/171, 9.4%) were identified as *fosA3*-positive, including 8 *E. coli*, 4 *P. mirabilis*, 3 *E. fergusonii* and 1 *C. freundii* (Table 1). Of these, 14 (14/150 = 9.3%) were obtained from pets and 2 (2/21 = 9.5%) from pet owners (Table 1). The remaining fosfomycin-resistant isolates, namely, *Enterobacter aerogenes* (n = 1), *Klebsiella oxytoca* (n = 1) and *K. pneumonia* (n = 1) carried the *fosA* gene. The two *fosA3*-positive isolates, 84N-2 (*E. fergusonii*) and 103A-2 (*E. coli*) were isolated from pet owners.

Four different bacterial species carrying the *fosA3* gene were isolated from pets and its owners, in combination with the high incidence of *fosA3* gene (84.2%) in fosfomycin-resistant isolates, suggesting the high prevalence of *fosA3* gene in fosfomycin-resistant *Enterobacteriaceae* strains in the pets and their owners in the current study. It is consistent with a previous study that higher prevalence of fosfomycin resistance (10.2%) was conferred mainly by the *fosA3* gene in *E. coli* strains from pets in China (Hou et al., 2012), which is much higher than that from humans and animals in other countries (1–3%) (Falagas et al., 2010; Johnson et al., 2013).

3.2. Antimicrobial resistance patterns

The details of the *fosA3*-carrying isolates, including their antimicrobial resistance patterns, are shown in Table 1. All of the 16 *fosA3*-positive *Enterobacteriaceae* isolates were resistant to fosfomycin, ampicillin and cefazolin, while the majority of them were also resistant to gentamicin (50%), kanamycin (75%), amikacin (68.8%), tetracycline (87.5%), chloramphenicol (68.8%), florfenicol (93.8%) and ciprofloxacin (81.3%). Our data showed that all of the *fosA3*-carrying *Enterobacteriaceae* isolates were identified as multidrug resistant isolates (resistant to three or more classes of antimicrobial agents) (Schwarz et al., 2010), which will further limit clinical therapeutic options for these pets. Moreover, antibiotic usage records in this teaching hospital revealed cephalosporins, aminoglycosides and penicillins had been used for the treatment of bacterial infections in some of the pets, while the molecular characterization showed that 15 *fosA3*-positive isolates were CTX-M producers, and seven isolates carried *rmt*B (data not shown), the coexistence of these genes with the *fosA3* gene will allow for the persistence and coselection of the *fosA3* gene under the selective pressure imposed by the usage of antibiotics other than fosfomycin.

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