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Dissemination of the Fosfomycin Resistance Gene *fosA3* with CTX-M β-Lactamase Genes and *rmtB* Carried on IncFII Plasmids among *Escherichia coli* Isolates from Pets in China

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The presence and characterization of plasmid-mediated fosfomycin resistance determinants among *Escherichia coli* isolates collected from pets in China between 2006 and 2010 were investigated. Twenty-nine isolates (9.0%) were positive for *fosA3*, and all of them were CTX-M producers. The *fosA3* genes were flanked by IS*26* and were localized on F2:A−:B− plasmids or on very similar F33:A−:B− plasmids carrying both $bla_{CTX-M-65}$ and *rmtB*. These findings indicate that the *fosA3* gene may be coselected by antimicrobials other than fosfomycin.

Fosfomycin is a traditional antimicrobial agent with broad-spectrum bactericidal reactivity and good pharmacological properties. It used to be an alternative treatment for uncomplicated lower urinary tract infections which were caused by a wide variety of bacteria, including *Escherichia coli* (11, 19, 26). The recent growing prevalence of extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* and fluoroquinolone-resistant *E. coli* has rekindled interest in fosfomycin as a therapeutic agent in many countries (7, 8, 18). Despite its worldwide use in clinical practice for nearly 4 decades, fosfomycin remains effective against common uropathogens without giving rise to clinically significant resistance (3, 6, 7, 9, 12, 15, 20). The main type of resistance to fosfomycin appears to be chromosome mediated rather than plasmid mediated (17, 21, 23, 27). However, two novel plasmid-mediated fosfomycin-modifying enzymes, FosA3 and FosC2, were recently identified in CTX-M-producing *E. coli* in Japan (25). Transferable plasmids carrying *fosA3* or *fosC2* might accelerate the dissemination of fosfomycin resistance around the world.

Fosfomycin has been approved for clinical application for many years in China. However, information on the occurrence and characteristics of fosfomycin-resistant *E. coli* in China is scarce. In the present study, we intended to examine the prevalence of fosfomycin resistance and plasmid-borne fosfomycin resistance genes among *E. coli* isolates from companion animals. A total of 323 *E. coli* isolates were recovered from healthy (136 isolates) and diseased (187 isolates) pets (248 from dogs and 75 from cats) at 10 pet hospitals in Guangdong Province, China, between 2006 and 2010.

The MICs of fosfomycin were determined by the agar dilution method on Mueller-Hinton agar containing 25 μg/ml glucose 6-phosphate, according to guideline M100-S20 of the Clinical and Laboratory Standards Institute (CLSI) (5). Most of the strains (89.9%) studied were susceptible to fosfomycin, whereas 33 isolates (10.2%) showed resistance to fosfomycin (MIC > 256 μg/ml). The 33 isolates were screened for the plasmid-borne fosfomycin resistance genes *fosA3*, *fosC2*, and *fosA* by PCR amplification and sequencing with the primers and PCR conditions listed in Table 1. Twenty-nine isolates (9.0%) were positive for *fosA3* (Table 2). No *fosC2* or *fosA* gene was detected among these isolates. Phylogenetic grouping of FosA3 producers as previously described (4) revealed that these *E. coli* isolates belonged to three phylogenetic groups (A, B1, and D) (Table 2). Pulsed-field gel electrophoresis (PFGE) (10) was successfully performed on 26 FosA3 producers, and 21 different XbaI PFGE patterns were observed. This suggested that the dissemination of *fosA3* was not due to the clonal dissemination of *fosA3*-positive isolates. However, clonal expansion was observed between dogs and cats and between pet hospitals (Table 2). Moreover, three clonally related isolates (HN015, HN7A2, and HN109) which were grouped into phylogenetic group B1 were recovered from different animals and hospitals during 2008 and 2010 (Table 2). Multilocus sequence typing (MLST) analysis revealed that they all belonged to the same sequence type (clonal complex ST448) (data not shown).

TABLE 1 Primers and PCR conditions used

Primer[a]	Sequence (5′–3′)	Size (bp)	Annealing temp (°C)
FosA3-F	GCGTCAAGCCTGGCATTT	282	57.5
FosA3-R	GCCGTCAGGGTCGAGAAA		
FosC2-F	TGGAGGCTACTTGGATTTG	217	50.5
FosC2-R	AGGCTACCGCTATGGATTT		
IS26-F	GCACGCATCACCTCAATACC	Unknown	56.7
FosA3-R2	TCATCCAGCGACAAGCACA		
FosA3-F2	GGGGCTGAGGTATGGAAAGA	Unknown	56.1
IS26-R	AGGAGATGCTGGCTGAACG		
FosA-F	ATCTGTGGGTCTGCCTGTCGT	271	59.5
FosA-R	ATGCCCGCATAGGGCTTCT		

[a] Primers were designed in this study.

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TABLE 2 Characterization of *fosA3*-carrying *Escherichia coli* isolates and plasmids

Isolate[a]	Date (yr.mo) of isolation	Pet hospital[j]	Origin[b]	Resistance phenotype[c]	Resistance gene(s)[d]	Phylogenetic group	PFGE type[e]	Plasmid			
								Distance (bp) downstream of *fosA3*[f]	FAB formula[g]	EcoRI RFLP[h]	Addiction system(s)
HN4E2	2008.5	PH1	Dog pharynx	CTX, AMI, CIP, CHL, TET	$bla_{CTX-M-65}$, *rmtB*	A	1a	536	F33:A−:B−	Ia	*pemKI*, *hok-sok*, *srnBC*
HN429	2008.4	PH2	Cat feces*	CTX, AMI, CIP, CHL, TET	$bla_{CTX-M-65}$, *rmtB*	A	1b	536	F33:A−:B−	Ia	*pemKI*, *hok-sok*, *srnBC*
HN2F2	2008.5	PH1	Cat feces	CTX, AMI, CIP, CHL, TET	$bla_{CTX-M-65}$, *rmtB*	B1	2	536	F33:A−:B−	Ia	*pemKI*, *hok-sok*, *srnBC*
HN7A8	2008.1	PH3	Dog feces	CTX, AMI, CHL, TET	$bla_{CTX-M-65}$, $bla_{CTX-M-55}$, *rmtB*	B1	2	536	F33:A−:B−	Ia	*pemKI*, *hok-sok*, *srnBC*
HN2B1	2007.12	PH1	Dog feces	CTX, AMI, CIP, CHL, TET	$bla_{CTX-M-65}$, *rmtB*	D	3	536	F33:A−:B−	Ia	*pemKI*, *hok-sok*, *srnBC*
HN3D12	2008.4	PH3	Cat feces	CTX, AMI, CIP, CHL, TET	$bla_{CTX-M-65}$, *rmtB*	A	4	536	F33:A−:B−	Ia	*pemKI*, *hok-sok*, *srnBC*, *ccdAB*
HN4B5	2008.1	PH3	Dog feces	CTX, AMI, CIP, CHL, TET	$bla_{CTX-M-65}$, *rmtB*	A	NT	536	F33:A−:B−	Ia	*pemKI*, *hok-sok*, *srnBC*, *ccdAB*
HN5E3	2008.4	PH1	Dog pus	CTX, AMI, CIP, CHL, TET	$bla_{CTX-M-65}$, *rmtB*	A	5	536	F33:A−:B−	Ib	*pemKI*, *hok-sok*, *srnBC*
HN4A1	2008.1	PH3	Dog feces*	CTX, AMI, CIP, TET	$bla_{CTX-M-65}$, *rmtB*	A	6	536	F33:A−:B−	Ia	*pemKI*, *hok-sok*, *srnBC*
HN127	2010.4	PH4	Dog feces*	CTX, AMI, CIP, CHL, TET	$bla_{CTX-M-65}$, *rmtB*	D	NT	536	F33:A−:B−	Ia	*pemKI*, *hok-sok*, *srnBC*
HN053	2009.7	PH1	Dog feces*	CTX, AMI, CIP, TET	$bla_{CTX-M-65}$, *rmtB*	A	7	536	F33:A−:B−	Ia	*pemKI*, *hok-sok*, *srnBC*
HN131	2010.5	PH4	Dog feces	CTX, AMI, CIP, TET	$bla_{CTX-M-65}$, *rmtB*	A	8	536	F33:A−:B−	Ic	*pemKI*, *hok-sok*, *srnBC*
HN212	2010.1	PH1	Dog feces	CTX, AMI, CIP, TET	$bla_{CTX-M-65}$, *rmtB*	A	9	1,758	F33:A−:B−	VI	*pemKI*, *srnBC*
HN1E1	2009.5	PH1	Dog feces	CTX, AMI, CIP, CHL, TET	$bla_{CTX-M-65}$, *rmtB*	B1	10	1,758	F2:A−:B−	IVc	*pemKI*, *hok-sok*
HN015	2008.1	PH4	Cat feces*	CTX, AMI, CIP, TET	$bla_{CTX-M-3}$, *rmtB*	B1	11	1,758	F2:A−:B−	ND[i]	*pemKI*, *hok-sok*
HN7A2	2008.1	PH4	Dog sneeze	CTX, AMI, CIP, TET	$bla_{CTX-M-3}$, *rmtB*	B1	11	1,758	F2:A−:B−	IVa	*pemKI*, *hok-sok*
HN109	2010.5	PH3	Cat feces	CTX, AMI, CIP, TET	$bla_{CTX-M-3}$, *rmtB*	B1	11	1,758	F2:A−:B−	ND	*pemKI*, *hok-sok*
HNC50	2006.12	PH1	Dog feces	CTX, AMI, CIP, TET	$bla_{CTX-M-3}$, $bla_{CTX-M-65}$, *armA*	D	NT	1,758	F2:A−:B−	ND	*pemKI*
HNC1	2006.9	PH1	Cat feces	CTX, AMI, CIP, TET	$bla_{CTX-M-3}$, *rmtB*	A	12	536	Unknown	V	*pemKI*
HN225	2010.2	PH1	Cat feces	CTX, AMI, CIP, CHL, TET	$bla_{CTX-M-27}$, *rmtB*	D	13	1,758	Unknown	ND	*pemKI*, *hok-sok*
HN357	2010.4	PH1	Dog feces	CTX, AMI, CIP, CHL, TET	$bla_{CTX-M-27}$, *rmtB*	D	13	1,758	ND	ND	ND
HN1D5	2008.2	PH1	Dog feces	CTX, AMI, CIP, CHL, TET	$bla_{CTX-M-65}$, *rmtB*	A	14	536	F2:A−:B−	IIa	*pemKI*, *hok-sok*
HN2E7	2008.5	PH1	Dog feces	CTX, AMI, CIP, CHL, TET	$bla_{CTX-M-65}$, *rmtB*	A	15	536	F2:A−:B−	IVb	*pemKI*, *hok-sok*
HN3C6	2008.3	PH1	Dog feces	CTX, AMI, CIP, TET	$bla_{CTX-M-14}$, *rmtB*	D	16	536	F2:A−:B−	IIb	*pemKI*, *hok-sok*
HN5A12	2008.3	PH1	Dog feces	CTX, AMI, CIP, CHL, TET	$bla_{CTX-M-27}$, *armA*	D	17	536	Unknown	IIIa	*pemKI*
HN061	2009.7	PH5	Dog feces	CTX, AMI, CIP, CHL, TET	$bla_{CTX-M-14}$, *rmtB*	D	18	536	ND	ND	ND
HN3B9	2009.4	PH1	Dog feces	CTX, CIP, CHL, TET	$bla_{CTX-M-65}$	A	19	370	F2:A−:B−	IIIb	*pemKI*, *hok-sok*
HN3E7	2008.2	PH4	Cat feces*	CTX, CHL, TET	$bla_{CTX-M-3}$	D	20	370	F2:A−:B−	IVb	*pemKI*, *hok-sok*
HN4F8	2008.4	PH3	Dog feces	CTX, CIP, CHL, TET	$bla_{CTX-M-14}$	A	21	370	Unknown	ND	None

[a] Isolates from which the *fosA3* gene was transferred to the recipient by conjugation or transformation (isolates HN7A8, HN3D12, HN109, and HN225) are underlined.
[b] Healthy pets are indicated by asterisks.
[c] All isolates and all transconjugants and transformants were resistant to fosfomycin. Resistance phenotypes transferred to the recipient by conjugation are underlined. CTX, cefotaxime; AMK, amikacin; CIP, ciprofloxacin; CHL, chloramphenicol; TET, tetracycline.
[d] Genes that were transferred by conjugation or transformation, as determined by PCR, are underlined.
[e] PFGE types (1, 2, 3, etc.) were assigned by visual inspection of the macrorestriction profile. Patterns that differed by fewer than six bands were considered to represent subtypes within the main group (1a, 1b, etc.). NT, nontypeable.
[f] The size of the spacer region between the 3′ end of *fosA3* and IS*26*.
[g] Allele numbers were assigned by submitting the amplicon sequence to the Multilocus Sequence Typing database (www.pubmlst.org/plasmid).
[h] RFLP patterns that differed by only a few bands (1 to 3) were assigned to the same RFLP profile.
[i] ND, not determined.
[j] PH1 to PH5, pet hospitals 1 to 5, respectively.

MICs of cefotaxime, amikacin, tetracycline, chloramphenicol, and ciprofloxacin were determined by the agar dilution method, and the results were interpreted according to the CLSI breakpoints (5). It revealed that all *fosA3*-positive isolates were resistant or intermediate to cefotaxime, while 26 and 27 isolates showed resistance to amikacin and ciprofloxacin, respectively. The occurrence of *rmtB*, *armA*, and bla_{CTX-M} among these *fosA3*-positive isolates was determined by PCR amplification and sequencing as previously described (2, 22). All of the *fosA3*-positive isolates were CTX-M producers, and 16 of them produced CTX-M-65 (Table

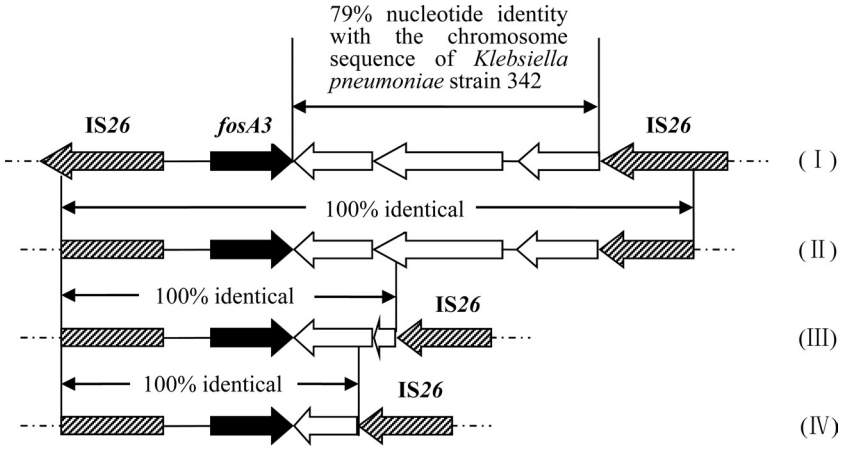

FIG 1 Comparison of regions flanking *fosA3*. (I)*Escherichia coli* 08-642 from Japan (23). (II) The size of the spacer region between the 3′ end of *fosA3* and IS*26* is 1,758 bp (GenBank accession no. JF411006). (III) The size of the spacer region between the 3′ end of *fosA3* and IS*26* is 536 bp (GenBank accession no. JF411007). (IV) The size of the spacer region between the 3′ end of *fosA3* and IS*26* is 370 bp (GenBank accession no. JF411008).

2). In addition, 24 and 2 of them carried *rmtB* and *armA*, respectively. Details for all *fosA3*-positive isolates are shown in Table 2. Specific primers were designed according to reported surrounding structures to determine the genetic environment of the *fosA3* gene (Table 1). The results showed that all *fosA3* genes were flanked by IS*26*, which was similar to the genetic environment of the first-reported *fosA3* gene (25). All *fosA3* genes were located 316 bp downstream of IS*26*. However, the size of the spacer region between the 3′ end of *fosA3* and IS*26* varied (536, 1,758, and 370 bp) (Table 2 and Fig. 1). Moreover, the 1,758-bp region had 79% nucleotide identity with a part of the chromosome sequence of *Klebsiella pneumoniae* strain 342 and was 100% identical to the sequence downstream of *fosA3* in *E. coli* 08-642, an isolate from Japan (Fig. 1) (25).

Conjugation was carried out to determine the transferability of *fosA3* genes with *E. coli* C600 (high level resistance to streptomycin) as the recipient (2). Transconjugants were selected on MacConkey agar plates containing fosfomycin (100 μg/ml) and streptomycin (2,000 μg/ml) for counterselection. When plasmid cotransfer occurred, a transformation experiment was carried out. Transformants were selected in LB agar plates containing 200 μg/ml fosfomycin by using *E. coli* DH5α as the recipient. Antimicrobial susceptibility testing was conducted for transconjugants and transformants, and the transfer of the resistance gene was confirmed by PCR as described above. The *fosA3* genes were successfully transferred to the recipients from 27 donors by conjugation or transformation (Table 2). The 23 transconjugants and 4 transformants all showed extraordinarily high-level resistance to fosfomycin (Table 2). In addition, $bla_{\text{CTX-M}}$ and *rmtB* genes were cotransferred to the recipients with *fosA3* from 25 and 18 donors, respectively. Plasmids were assigned to incompatibility groups by PCR-based replicon typing (1). Replicon sequence typing was used to characterize the IncFII plasmids (24). F33:A−:B− and F2:A−:B− were identified in 13 and 10 plasmids carrying *fosA3*, respectively. F33:A−:B− plasmids also contained $bla_{\text{CTX-M-65}}$ and *rmtB* and had nearly identical sizes and EcoRI digestion profiles (Table 1 and Fig. 2). Southern blot hybridization was performed on EcoRI digestion fragments of 12 F33:A−:B− plasmids with a digoxigenin-labeled probe specific for *fosA3*. It showed that *fosA3* was located on the same-size band (>15 kb, the largest digestion fragment) in 9 isolates (Fig. 2), demonstrating the presence of an epidemic plasmid responsible for the dissemination of *fosA3*. However, the predominance of the F33:A−:B− plasmid type was unexpected, since the pets were epidemiologically unrelated and samples had been obtained in different periods at four different hospitals between 2007 and 2010. To better understand the successful dissemination of these IncFII plasmids carrying *fosA3*, plasmid addiction systems were determined using primers described by Mnif et al. (16). *pemKI* ($n = 26$), *hok-sok* ($n = 22$), and *srnBC* ($n = 13$) were the most frequently represented systems, and almost all F33:A−:B− plasmids carried these three addiction systems (Table 2).

The occurrence of fosfomycin resistance in *E. coli* from human and pet animal isolates is still rare in many countries (<5%) (3, 6, 7, 9, 12, 13, 15, 20). However, in this study, a higher prevalence of fosfomycin resistance mainly mediated by FosA3 was observed in *E. coli* isolates recovered from pets during 2006 and 2010, although none of the pets had received fosfomycin treatment. The

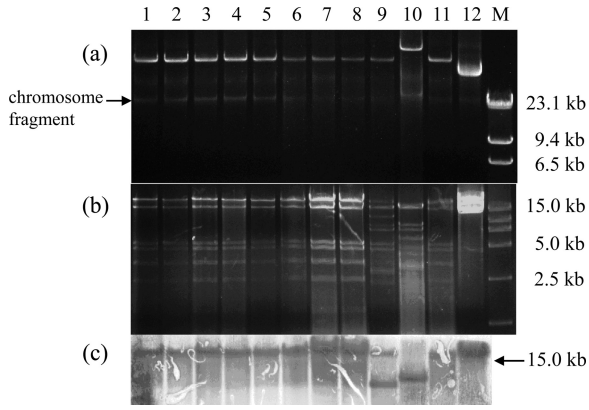

FIG 2 Analysis of F33:A−:B− plasmids carrying *fosA3*. Lanes 1 to 12, HN429, HN7A8, HN2B1, HN3D12, HN4B5, HN4A1, HN127, HN053, HN5E3, HN212, HN4E2, and HN131; lane M, λHindIII and DL15000 marker. (a) Plasmid profiles of transconjugants and transformants carrying F33:A−:B− plasmid. (b) EcoRI restriction digestion profiles of F33:A−:B− plasmids. (c) Southern blot hybridization of EcoRI-digested plasmids with a digoxigenin-labeled *fosA3*-specific probe.

association with other resistance determinants has likely favored the dissemination and maintenance of *fosA3*, since the additional resistance genes, such as bla_{CTX-M} and *rmtB*, allow coselection of *fosA3* by cephalosporins and/or aminoglycosides (especially amikacin and gentamicin), which have been frequently used for pet therapy in China (14).

In conclusion, the dissemination of the *fosA3* gene, which is closely associated with bla_{CTX-M} and *rmtB*, is mainly driven by horizontal transfer of F33:A−:B− and F2:A−:B− plasmids rather than clonal expansion. Since pets are able to acquire multidrug-resistant pathogens and transmit them to humans due to their close contact, the presence of these resistance bacteria and plasmids in pets may become a public health concern. Effective antimicrobial policies in veterinary hospitals should be developed in China.

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in GenBank under the accession numbers JF411006, JF411007, and JF411008.

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