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Characterization of Extended-Spectrum β-Lactamase Genes of *Shigella flexneri* Isolates With Fosfomycin Resistance From Patients in China

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Background: The emergence of fosfomycin resistance and extended-spectrum β-lactamase (ESBL) genes is a serious threat to public health and a new challenge in shigellosis treatment. The purpose of this study was to identify fosfomycin resistance and characterize β-lactamase genes in fos-carrying isolates of *Shigella flexneri* from patients in China.

Methods: A total of 263 *S. flexneri* isolates were collected from 34 hospitals in the Anhui Province of China during September 2012-September 2015 and screened for *fosA3, fosA,* and *fosC2* by PCR amplification and sequencing. The fos-carrying isolates were then screened for β-lactamase genes. The clonal relationships between *fosA3*-carrying isolates, the transmissibility of fosfomycin resistance, replicon types of plasmids carrying fosfomycin resistance genes and other associated resistance genes were investigated.

Results: Twenty-five of the 263 isolates (9.5%) showed resistance to fosfomycin, and 18 (6.8%) were positive for *fosA3*. None of the isolates was positive for *fosA* or *fosC2*. Seventeen of the isolates carrying *fosA3* (94%) were CTX-M producers (seven CTX-M-55, five CTX-M-14, and five CTX-M-123), while three (16.7%) were TEM producers (TEM-1).Sixteen (88.9%) *fosA3*-carrying isolates exhibited multi-drug resistance. The replicon types of the 13 *fosA3*-carrying plasmids were IncF (n=13), IncHI2 (n=3), IncII-Ir (n=2), and IncN (n=1).

Conclusions: Our results indicated that *fosA3* could spread through plasmids in *S. flexneri* isolates, along with the bla_{CTX-M} and bla_{TEM}, which facilitate its quick dispersal. To the best of our knowledge, this is the first report of CTX-M-123-type ESBLs in *S. flexneri* isolates from patients in China.

Key Words: ESBLs, Plasmids, *fosA3*, *Shigella flexneri*, Replicon

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INTRODUCTION

Shigellosis is still a major cause of morbidity and mortality worldwide, especially in developing countries, where 99% of the estimated 165 million annual cases occur. Children under five years of age are involved in more than a half of the cases and deaths [1]. In China, shigellosis is the top-ranked among gastrointestinal infectious illness with respect to the incidence. The predominant pathogen responsible for shigellosis is *Shigella flexneri* [2]. In recent decades, antimicrobial agents have been effective in alleviating the dysenteric syndrome associated with shigellosis. The growing prevalence of extended-spectrum β-lactamase (ESBL)-producing *S. flexneri* isolates in many countries has rekindled interest in fosfomycin (FOM) as a therapeutic agent [3,

4]. FOM is a naturally occurring antibacterial agent with a broad spectrum of action against both gram-positive and gram-negative bacteria. Despite its worldwide use in clinical practice for nearly four decades, FOM has remained effective against common uropathogens and has not given rise to clinically relevant resistant strains [3-5]. However, a FOM resistance gene, *fosA3*, was reported in *Escherichia coli* [6]. This gene was also detected in CTX-M-producing and multidrug-resistant *E. coli* and *Klebsiella pneumoniae* isolates [6-10]. It has been suggested that the increasing prevalence of *fosA3* was due to the dissemination of IncI and IncN plasmids, rather than the clonal expansion of specific strains. The purpose of this study was to identify FOM resistance and to characterize β-lactamase genes in fos-carrying *S. flexneri* isolates obtained from patients in China.

METHODS

1. Bacterial isolates

A total of 263 non-duplicate *S. flexneri* isolates were collected from 34 secondary level hospitals in the Anhui Province of China, between September 2012 and September 2015. The ages of the subjects ranged from six months to 78 yr. Individual isolates were identified by using standard microbiological and biochemical methods. All *Shigella* isolates were confirmed by using the API-20E system (bioMérieux, Marcy l' Étoile, France) and serotyped by using commercial antisera (Denka Seiken Co. Ltd., Tokyo, Japan). *E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603, and sodium azide-resistant *E. coli* J53 were stored at the Anhui Center for Surveillance of Bacterial Resistance (Hefei, Anhui, China).

The study was conducted in accordance with the guidelines of Declaration of Helsinki, the principles of Good Clinical Practice, and Chinese regulatory requirements, and was approved by the local Ethics Committees of the First Affiliated Hospital of Anhui Medical University (Hefei, China). All patients gave written informed consent.

2. PCR amplification

All the isolates were screened for *fosA3*, *fosA*, and *fosC2* genes by using methods described previously [11]. The fos-carrying isolates were screened for β-lactamase genes by using primer pairs described previously [12]. All the purified PCR products were sequenced on an ABI Prism 3730 sequencer (Applied Biosystems, Foster City, CA, USA). Sequence alignment was performed with the GenBank nucleotide database, using the nucleotide BLAST program.

3. Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of the following 11 antimicrobial agents were determined by using the agar dilution method, according to the CLSI guidelines (2015): FOM, cefotaxime (CTX), ceftriaxone (CRO), ceftazidime (CAZ), chloramphenicol (CHL), piperacillin-tazobactam (PTZ), ciprofloxacin (CIP), levofloxacin (LEV), amikacin (AMK), gentamicin (GEN), and imipenem (IMP) (all from the National Institutes for Food and Drug Control, Beijing, China). On the basis of the MICs, the *S. flexneri* isolates were classified as FOM-susceptible (MIC ≤ 64 mg/L), FOM-intermediate (64 mg/L < MIC < 256 mg/L), and FOM-resistant (MIC ≥ 256 mg/L). *E. coli* ATCC 25922 was used as the quality control strain. The susceptibility data of the *Shigella* isolates were accepted only if the MICs of the quality control strains tested in parallel were within the acceptable ranges set by the CLSI guidelines. Multi-drug resistance (MDR) was defined as resistance to three or more antimicrobial agents.

4. Conjugation experiments and transfer of drug resistance

Conjugation experiments were performed for all isolates that were positive for *fosA3*, with sodium azide-resistant *E. coli* J53 as the recipient. Transconjugants were selected on MacConkey agar plates supplemented with sodium azide (200 mg/L) (Sigma Chemical Co., St. Louis, MO, USA), FOM (100 mg/L), and glucose-6-phosphate (25 mg/L). Transconjugants were tested by using biochemical methods and verified by using the API-20E system to be *E. coli*. Plasmid DNA was extracted from donors and transconjugants by using the Qiagen Plasmid Purification kit (QIAGEN, Hilden, Germany). The transconjugants were then screened by using PCR for *fosA3* and β-lactamase genes, with the plasmid DNA as the template. The MICs of the following 11 antimicrobial agents were determined for the recipient, donors, and transconjugants: FOM, CTX, CRO, CAZ, CHL, PTZ, CIP, LEV, AMK, GEN, and IMP. All the assays were performed according to the protocols described in the previous section.

5. Plasmid replicon typing

Incompatibility groups were assigned by using PCR-based replicon typing analysis of the transconjugants, according to the protocol described by Carattoli *et al* [13].

RESULTS

1. Prevalence of plasmid-mediated fosfomycin resistance genes

Of the 263 *S. Flexneri* isolates, 25 (9.5%) showed resistance to

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FOM, and 18 (6.8%) were positive for *fosA3*. *fosC2* and *fosA* genes were not detected in any of the isolates. The antimicrobial resistance phenotypes of the *fosA3*-carrying isolates are shown in Table 1. All the isolates were resistant to FOM and CHL, while 16 of the 18 *fosA3*-carrying isolates (88.9%) exhibited MDR. The most prevalent antimicrobial resistance pattern was FOM-CHL-CTX-CRO. Seven (43.8%) and five (31.3%) of the MDR isolates were resistant to CIP and GEN, respectively, and all of them were susceptible to PTZ and IMP.

When screened for β-lactamase genes (Table 2), 17 (94.4%) of the *fosA3*-carrying isolates were CTX-M producers (CTX-M-55 [n=7], CTX-M-14 [n=5], and CTX-M-123 [n=5]), and three (16.7%) were TEM producers (TEM-1 [n=3]). In addition, the bla_{SHV} gene was not detected in any of these isolates. Of the 18 *fosA3*-carrying isolates, three were found to be co-carrying bla_{CTX-M} and bla_{TEM}.

Table 1. Antibiotic resistance patterns of 18 *fosA3*-carrying *Shigella flexneri* isolates from China

Antibiotic resistance pattern	N of isolates (%)
FOM-CHL	2 (11.1)
FOM-CHL-CTX-CRO	9 (50.0)
FOM-CHL-CTX-CRO-CIP	2 (11.1)
FOM-CHL-CTX-CRO-CIP-GEN	2 (11.1)
FOM-CHL-CTX-CRO-CIP-GEN-CAZ	2 (11.1)
FOM-CHL-CTX-CRO-CIP-GEN-CAZ-LVX-AMK	1 (5.6)

Abbreviations: FOM, fosfomycin; CHL, chloramphenicol; CTX, cefotaxime; CRO, ceftriaxone; CIP, ciprofloxacin; GEN, gentamicin; CAZ, ceftazidime; LVX, levofloxacin; AMK, amikacin.

2. Conjugation experiments and plasmid analysis

The plasmids from 13 of the 18 *fosA3*-carrying isolates (72.2%) were transferred into the recipient *E. coli* J53Az^R by conjugation. The β-lactamase genes were co-transferred with the *fosA3* gene to the recipient hrough plasmids. All the transconjugants co-carried *fosA3* and bla_{CTX-M} (CTX-M-55 [n=6], CTX-M-14 [n=4], and CTX-M-123 [n=3]). One plasmid from a transconjugant co-carried *fosA3*, $bla_{CTX-M-55}$, and bla_{TEM-1}. The MICs of the 11 antibiotics for the recipient, donors, and transconjugantsare presentedin Table 3. All the clinical isolates and transconjugants were resistant to FOM, and their resistance to CTX and CRO was greater than that to CAZ. The MICs of CTX for the transconjugants increased from <0.0625 μg/mL to around 16–32 μg/mL.

Table 2. Distribution of the genotypes of ESBLs in 17 *fosA3*-carrying *Shigella flexneri* isolates from China

ESBL group	ESBL gene	*S. flexneri* (n=17)
CTX-M	CTX-M-55	6
	CTX-M-14	3
	CTX-M-123	5
CTX-M+TEM	CTX-M-55 + TEM-1	1
	CTX-M-14 + TEM-1	2

Abbreviation: ESBL, extended-spectrum β-lactamase.

Table 3. MIC_{50} and MIC_{90} of 11 antimicrobial agents for *Shigella flexneri* isolates and transconjugants

Antimicrobial agent	J53$_{AZ}^R$	*S. flexneri* isolates (n=13)				Transconjugants (n=13)			
		MIC range (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	R (%)	MIC range (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	R (%)
FOM	<4	1,024–>2,048	1,024	2,048	100	256–>2,048	256	512	100
CAZ	<0.25	1–32	8	16	38.5	1–16	4	8	7.7
CTX	<0.0625	16–>32	>32	>32	100	16–>32	>32	>32	100
CRO	<0.0625	16–>32	>32	>32	100	16–>32	>32	>32	100
CIP	<0.0625	2–16	2	8	46.2	0.125–2	0.125	2	0
LVX	<0.125	0.5–8	2	2	7.7	<0.125–2	<0.125	1	0
CHL	<0.5	32–128	64	128	100	4–128	8	64	23.1
GEN	<0.25	1–>128	8	>128	46.2	<0.25–64	4	8	7.7
AMK	<1	2–1024	8	128	38.5	2–256	2	4	7.7
PTZ	<1/4	1/4–16/4	8/4	16/4	0	<1/4–16/4	<1/4	8/4	0
IMP	<0.0625	<0.0625–0.5	<0.0625	0.5	0	<0.0625–<0.0625	<0.0625	<0.0625	0

Abbreviations: FOM, fosfomycin; CHL, chloramphenicol; CTX, cefotaxime; CRO,ceftriaxone; CIP, ciprofloxacin; GEN, gentamicin; CAZ, ceftazidime; LVX, levofloxacin; AMK, amikacin; PTZ, piperacillin-tazobactam; IMP, imipenem; J53AzR, sodiumazide-resistant *E. coli* J53; MIC, minimal inhibitory concentration; R, resistance.

Table 4. Distribution of the genotypes of replicons and β-lactamase genes in 13 *fosA3*-carrying transconjugants

Gene distribution	*Shigella flexneri* (n=13)
IncF + IncII-Ir + IncN + CTX-M-55 + TEM-1	1
IncF + IncII-Ir + IncHI2 + CTX-M-14	1
IncF + IncHI2 + CTX-M-123	2
IncF + CTX-M-14 + TEM-1	2
IncF + CTX-M-14	1
IncF + CTX-M-123	1
IncF + CTX-M-55	5

3. Plasmid replicon typing

The replicon types of the *fosA3*-carrying plasmids in the 13 transconjugants were IncF (n=13, 100%), IncHI2 (n=3, 23.1%), IncII-Ir (n=2, 15.4%), and IncN (n=1, 7.7%). Two transconjugants co-carried IncF and IncHI2, another one co-carried IncF, IncHI2, and IncII-Ir, and another one co-carried IncF, IncN, and IncII-Ir. The $bla_{CTX-M-55}$ gene was carried by IncF (n=6), IncII-Ir (n=1), and IncN (n=1) plasmids; the $bla_{CTX-M-14}$ gene was carried by IncF (n=4), IncII-Ir (n=1), and IncHI2 (n=1) plasmids; and the $bla_{CTX-M-123}$ gene was carried by IncF (n=3) and IncHI2 (n=2) plasmids. The bla_{TEM-1} gene was associated with the IncF plasmid (Table 4).

DISCUSSION

To the best of our knowledge, this study is the first to characterize β-lactamase genes in *fosA3*-carrying *S. flexneri* isolates from patients in China. We showed that over 70% of FOM-resistant *Shigella* isolates harbored the *fosA3* gene, which indicated that this gene was a part of the main mechanism responsible for FOM resistance. Our results also showed that 88.9% of *fosA3*-carrying *Shigella* isolates showed resistance to all commonly used antimicrobial agents. The most frequent antimicrobial resistance pattern was FOM-CHL-CTX-CRO. These findings confirmed that these antimicrobial agents were inefficient for the empirical treatment of patients infected by *fosA3*-carrying *Shigella*. The *fosA3*-carrying *S. flexneri* isolates were observed to be susceptible to PTZ and IMP, indicating that these antibiotics could be the preferred treatment options in Anhui, China.

This study also showed that 94.4% of the *fosA3*-carrying *S. flexneri* isolates collected from patients produced ESBLs, which was consistent with previous studies that showed that *fosA3* was often found in CTX-M-producing *E. coli* isolates [6-10]. The production of ESBLs is the major defense mechanism of the *Shigella* species against third-generation cephalosporins. Different types of β-lactamase genes, belonging to the TEM, SHV, and CTX-M families, have been reported in *Shigella* species worldwide [14-17]. In China, there have been several reports of β-lactamase genes in *Shigella* isolates, which produced CTX-M-14, CTX-M-15, CTX-M-55, and TEM-1 [14, 18-20]. In the present study, CTX-M-55 was produced by 38.9% of the *fosA3*-carrying *S. flexneri* isolates, while CTX-M-14 was produced by 27.8%, CTX-M-123 by 27.8%, and TEM-1 by16.7% of the *fosA3*-carrying *S. flexneri* isolates. Thus, CTX-M-55 was the most prevalent ESBL produced by *fosA3*-carrying *S. flexneri* isolates in Anhui. CTX-M-123, which has been reported in *E. coli* isolates from animals in China, is a novel hybrid of the CTX-M-1 and CTX-M-9 group β-lactamases [21]. Notably, this is the first study to report CTX-M-123-type ESBLs in *S. flexneri* isolates from patients in China.

In this study, the *fosA3* and bla_{CTX-M} genes were co-carried by conjugative plasmids from multiple incompatible groups [6-10]. We also showed that all the plasmids in the transconjugants co-carried *fosA3* and bla_{CTX-M} genes, and all transconjugants contained determinants encoding resistance to FOM, CTX, and CRO. The production of *FosA3* and ESBLs, which were encoded on the conjugative plasmids, might explain the resistance to FOM and third-generation cephalosporins. Notably, one transconjugant co-carried *fosA3*, $bla_{CTX-M-55}$, and bla_{TEM-1}, and was resistant to FOM, CTX, CRO, and CAZ; it indicated that the plasmid-mediated *fosA3* and *ESBL* genes played important roles in transferring resistance, and should be closely monitored.

Plasmid replicons associated with the *fosA3* and bla_{CTX-M} genes are known to vary with geographical location. For example, *fosA3* and bla_{CTX-M} genes have been reported to be harbored by IncF replicons in Korea and the United States [7, 22], and by IncF, IncN, and IncI1 replicons in Japan and China [8, 9, 11]. In the present study, the plasmid replicons of the13 transconjugants, which co-carried *fosA3* and bla_{CTX-M}, were IncF (n=13, 100%), IncHI2 (n=3, 23.1%), IncII-Ir (n=2, 15.4%), and IncN (n=1, 7.7%); these results were consistent with the results for *E. coli* from most countries, suggesting that common mobilization events occurred in these bacteria.

In conclusion, this study showed that the prevalence of the plasmid-mediated *fosA3* gene in *S. flexneri* isolates with FOM resistance was very high among patients in China. Moreover, the high prevalence of *ESBL* genes in *fosA3*-carrying isolates presents a serious threat to public health in Anhui, China. CTX-M-123 was reported in *S. flexneri* isolates from patients for the first time in China. Conjugatable plasmids were responsible for the dissemination of *fosA3* and *ESBL* genes in *S. flexneri* iso-

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lates with high clonal diversity. Further studies will be needed to explore the mechanisms underlying the divergent evolution and the horizontal spread of plasmids that co-carried *fosA3* and *ESBL* genes in MDR strains. To prevent potential outbreaks of MDR *Shigella*, the antimicrobial resistance in *Shigella* isolates should be continuously monitored, and rational antibiotic treatment, based on antimicrobial susceptibility tests, should be provided.

Nucleotide sequence accession numbers

The sequence of CTX-M-123 in *S. flexneri* isolates from patients have been deposited in GenBank under the accession number KJ871006.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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