

## Emergence of Fosfomycin-Resistant Isolates of Shiga-Like Toxin-Producing *Escherichia coli* O26

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We evaluated the susceptibilities of 129 Shiga-like toxin-producing *Escherichia coli* (STEC) isolates to various antibiotics. The numbers of isolates for which MICs were high ( $\geq 128$   $\mu\text{g/ml}$ ) were as follows: 5 for fosfomycin, 14 for ampicillin, 1 for cefaclor, 6 for kanamycin, 22 for tetracycline, and 2 for doxycycline. For two isolates of STEC O26 MICs of fosfomycin were high (1,024 and 512  $\mu\text{g/ml}$ , respectively). Conjugation experiments and glutathione *S*-transferase assays suggested that the fosfomycin resistance in these isolates was determined not by a plasmid but chromosomally. The amount of active intracellular fosfomycin in these STEC isolates was 100- to 200-fold less than that in *E. coli* C600 harboring pREFTT47B408 in the presence of either L- $\alpha$ -glycerophosphate or glucose-6-phosphate. Cloning, sequencing, and Northern blot analysis demonstrated that the transcriptional level of the *murA* gene encoding UDP-*N*-acetylglucosamine enolpyruvyl transferase in these isolates was greater than that in *E. coli* C600. Our results suggest that the fosfomycin resistance in these STEC isolates is due to concurrent effects of alteration of the *glpT* and/or *uhp* transport systems and of the enhanced transcription of the *murA* gene.

Fosfomycin, a broad-spectrum antibiotic, enters cells by active transport through the L- $\alpha$ -glycerophosphate ( $\alpha$ -GP) uptake system (GlpT) and the glucose-6-phosphate (G6P) uptake system (Uhp) and inhibits peptidoglycan biosynthesis through the inactivation of the enzyme UDP-*N*-acetylglucosamine (UDP-GlcNAc) enolpyruvyl transferase (8). Fosfomycin has been the drug of choice for pediatric gastrointestinal infections due to Shiga-like toxin-producing *Escherichia coli* (STEC) in Japan, and the early administration (within 3 days of onset) of fosfomycin is critical for the effective treatment of STEC infections (20).

It has been reported that chromosomally encoded fosfomycin-resistant strains had an impairment in fosfomycin uptake (7), a low-affinity UDP-GlcNAc enolpyruvyl transferase (22), or overproduction of the enzyme (11) in studies using *E. coli* E-15, B, Prl, K-12, or American Type Culture Collection strains. Studies have demonstrated that the fosfomycin resistance encoded by plasmids is due to an enzymatic modification of fosfomycin in some clinical isolates of *Serratia marcescens*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Staphylococcus epidermidis* (1, 3, 13, 15, 19). At present, however, little is known concerning the prevalence and mechanism of fosfomycin resistance, especially in clinical isolates. To characterize fosfomycin resistance epidemiologically and biologically in clinical isolates of STEC, we evaluated the susceptibilities of 129 STEC isolates to several oral antibiotics and determined the mechanism of fosfomycin resistance in the resistant isolates.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The strains and plasmids used in this study are listed in Table 1. Two *E. coli* O26 strains resistant to fosfomycin (NGY47 and NGY60) were selected from 129 strains of STEC isolated in 1996 and 1997 from different patients in Japan (Table 2). NGY47 and NGY60 were isolated from different patients who had visited different Nagoya city hospitals

from June to August 1997. The O and H antigen types of strains were determined with neutralizing antisera (Denka-Seiken, Tokyo, Japan). These STEC isolates included serotypes of O157 (80 strains), O26 (31 strains), O111 (11 strains), O103 (2 strains), O118 (2 strains), O1 (1 strain), O6 (1 strain), and O165 (1 strain). The H antigen types of NGY47 and NGY60 were nonmobile and H11, respectively. Bacteria were stored at  $-70^{\circ}\text{C}$  in Luria-Bertani (LB) broth (Difco Laboratories, Detroit, Mich.) containing 20% glycerol. Subsequently, bacteria were inoculated on LB agar plates and incubated at  $37^{\circ}\text{C}$  overnight.

**Antibiotics.** The antibiotics used were ampicillin, fosfomycin, and kanamycin (Meiji Seika Kaisha, Tokyo, Japan), azithromycin (Pfizer Pharmaceuticals, Tokyo, Japan), cefaclor (Shionogi Pharmaceutical, Osaka, Japan), chloramphenicol and doxycycline (Sankyo, Tokyo, Japan), gentamicin (Schering-Plough, Osaka, Japan), norfloxacin (Kyorin Pharmaceutical, Tokyo, Japan), and tetracycline and minocycline (Japan Lederle, Tokyo, Japan).

**Susceptibility testing.** MICs were determined by an agar dilution method as described by the National Committee for Clinical Laboratory Standards (14). Susceptibility testing was performed on Mueller-Hinton agar (Difco) in accordance with the manufacturer's instructions. MICs of fosfomycin were determined for all strains on Mueller-Hinton agar containing 50  $\mu\text{g}$  of G6P (Wako Pure Chemical Industries, Osaka, Japan) per ml.

**Glutathione *S*-transferase assay.** For preparation of cell extracts, the cells were ruptured by ultrasonication at  $4^{\circ}\text{C}$ . Cell debris was removed by centrifugation. Aliquots of 0.1 ml of fosfomycin (125  $\mu\text{g/ml}$ ), 0.1 ml of 40 mM glutathione, and 0.8 ml of crude extract (5 mg/ml) were mixed and allowed to react at  $37^{\circ}\text{C}$  for 1, 2, 3, 4, 6, and 24 h. The reaction was stopped by heating at  $100^{\circ}\text{C}$  for 90 s. After centrifugation, the residual potency of fosfomycin was determined by microbiological assay, as previously described (15). Control experiments were performed using *E. coli* JM83 harboring pUC19 or pMZY102 (Table 1).

**Determination of active intracellular fosfomycin levels.** Bacteria were grown in 20 ml of LB broth to an optical density at 590 nm of 0.2. Either  $\alpha$ -GP or G6P was added to a concentration of 2.5 mM, and the culture was incubated at  $37^{\circ}\text{C}$  for 90 min.  $\alpha$ -GP and G6P were used to exclude the presence of spontaneous subpopulations, which are impaired in fosfomycin uptake. The bacteria were then washed twice with fresh LB broth and finally resuspended in 1 ml of LB broth. This suspension was incubated for 3, 10, 20, 40, 60, and 90 min at  $37^{\circ}\text{C}$  in the presence of 2 mg of fosfomycin per ml. The bacteria were then collected by centrifugation and washed with hypertonic buffer (10 mM Tris, 0.5 mM  $\text{MgCl}_2$ , 150 mM NaCl, pH 7.3) to remove the antibiotic. Cells were resuspended in 5 ml of phosphate-buffered saline (pH 7.4) and disrupted by passage through a French press (SLM Instruments, Urbana, Ill.). The debris was discarded after centrifugation (100,000  $\times g$  for 15 min), and the antibiotic concentration in the supernatant was determined by microbiological assay, with *E. coli* XL1-Blue as the indicator strain (15). Control assays were carried out with an extract of each strain obtained by processing the cells immediately after fosfomycin addition (time zero). To quantify bacteria, aliquots (after the antibiotic had been removed) were serially diluted in phosphate-buffered saline, and 0.1 ml from each dilution was plated on LB agar. After overnight incubation at  $37^{\circ}\text{C}$ , the numbers of CFU/ml were counted.

**DNA techniques.** The chromosomal DNA of *E. coli* NGY47 and NGY60 and plasmid DNA were extracted as described previously (16). Restriction endo-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Source or reference
<i>E. coli</i>		
C600	F <sup>-</sup> <i>lacY1 leuB6 supE44 thi-1 thr-1 tonA21</i>	Laboratory strain
CSH-2	<i>metB</i> F <sup>-</sup> Nal <sup>r</sup> Rif <sup>r</sup>	T. Sawai, Chiba University
JM83	F <sup>-</sup> <i>ara Δ(lac-proAB) rpsL</i> ( $\phi$ 80 <i>lacZΔM15</i> )	Laboratory strain
XL1-Blue	<i>endA1 gyrA96 hsdR17 lac recA1 relA1 supE44 thi-1 Tet<sup>r</sup></i>	Stratagene, La Jolla, Calif.
O26 NGY47	Clinical isolate, Fos <sup>r</sup> Tet <sup>r</sup>	This study
O26 NGY60	Clinical isolate, Fos <sup>r</sup>	This study
Plasmids		
pHSG398	High-copy-number vector, Cm <sup>r</sup>	24
pWKS130	Low-copy-number vector, Kan <sup>r</sup>	23
pBluescript SK II <sup>+</sup>	Vector for sequencing, Amp <sup>r</sup>	Stratagene
pUC19	Cloning vector, Amp <sup>r</sup>	24
pMZY102	Subclone carrying the <i>fosB</i> gene derived from fosfomycin-resistant <i>S. aureus</i> MF51 (vector, pUC19)	Unpublished data
pREFTT4701	Subclone carrying the <i>murA</i> gene of <i>E. coli</i> O26 NGY47 (vector, pHSG398)	This study
pREFTT6023	Subclone carrying the <i>murA</i> gene of <i>E. coli</i> O26 NGY60 (vector, pHSG398)	This study
pREFTT47B408	Subclone carrying the <i>murA</i> gene of <i>E. coli</i> O26 NGY47 (vector, pWKS130)	This study
pREFTT60K804	Subclone carrying the <i>murA</i> gene of <i>E. coli</i> O26 NGY60 (vector, pWKS130)	This study

nucleases and T4 DNA ligase were supplied by Takara Biomedicals (Ohtsu, Japan) and were used as recommended by the manufacturer.

To obtain clones containing the *murA* gene (encoding UDP-GlcNAc enolpyruvyl transferase) derived from STEC NGY47 and NGY60, partially *Sau3AI*-digested fragments of chromosomal DNA from these isolates were ligated into the *Bam*HI site of a high-copy-number vector, pHSG398. These recombinants were introduced into *E. coli* C600, and transformants resistant to both fosfomycin and chloramphenicol were isolated. A 3.9-kb derivative containing a 1.7-kb insert was termed pREFTT4701. The 1.7-kb fragment was transferred into the *Bam*HI site of a low-copy-number vector, pWKS130. This recombinant was introduced into *E. coli* C600 and was termed pREFTT47B408. Similarly, the construction of plasmids from chromosomal DNA of STEC NGY60 was performed. A 1.5-kb fragment digested partially by *Sau3AI* was ligated into the *Bam*HI sites of pHSG398 and pWKS130, and fosfomycin-resistant clones were isolated. These plasmids were termed pREFTT6023 and pREFTT60K804, respectively.

**Nucleotide sequencing and analysis.** To determine the nucleotide sequences of the inserts in pREFTT4701 and pREFTT6023, the DNA fragments were transferred into pBluescript SK II<sup>+</sup> and the recombinant was introduced into *E. coli* XL1-Blue. Deletion subclones for sequencing were constructed, by using exonuclease III, mung bean nuclease, and Klenow fragment in accordance with the manufacturer's instructions (deletion kit; Nippon Gene, Toyama, Japan), and the DNA fragments were sequenced, with the ABI PRISM dye primer cycle sequencing ready reaction kit with AmpliTaq DNA polymerase and -21M13 dye primers (Perkin-Elmer, Foster City, Calif.) and an automated DNA sequencing system (model 373A; Applied Biosystems, Foster City, Calif.), as previously described (6).

**Northern blot analysis.** As a hybridization probe, the 1.7-kb *Bam*HI fragment of pREFTT4701 was excised from a low-melting-temperature agarose gel (Nippon Gene) after electrophoresis and was labeled with digoxigenin-11-dUTP (random primer labeling kit; Boehringer, Mannheim, Germany). Total RNA of bacteria separated on agarose gels (Nippon Gene) was transferred to a Hybond N<sup>+</sup> nylon membrane (Amersham, Little Chalfont, Buckinghamshire, United Kingdom) (18). Total RNA was isolated as described by Kornblum et al. (10). Hybridization and immunological detection were performed with 1,2-dioxetane chemiluminescent enzyme substrate (CSPD) (Tropix, Bedford, Mass.) as the chemiluminescent substrate for alkaline phosphatase. Levels of *murA* expression were measured by the National Institutes of Health Image program, version 1.61, which is a public domain image processing and analysis program for the Macintosh (15a).

## RESULTS

**Antibiotic susceptibility.** The results of susceptibility testing are summarized in Table 2. These STEC isolates were generally susceptible to gentamicin, minocycline, norfloxacin, and azithromycin. The numbers of isolates for which MICs were high ( $\geq 128$   $\mu$ g/ml) were as follows: 5 for fosfomycin, 14 for ampicillin, 1 for cefaclor, 6 for kanamycin, 22 for tetracycline, and 2 for doxycycline. STEC O26 NGY47 and NGY60 were especially highly resistant to fosfomycin (1,024 and 512  $\mu$ g/ml, respectively).

To investigate the mechanism of fosfomycin resistance in

NGY47 and NGY60, further experiments were performed. Since previous studies had shown that high-level resistance to fosfomycin was due to a plasmid-mediated fosfomycin-glutathione *S*-transferase (1, 3, 13, 15, 19), we measured the activity of this enzyme in these STEC isolates. After treatment with the crude extracts of both NGY47 and NGY60 in the presence of glutathione, the residual potency of fosfomycin was not reduced, whereas intracellular fosfomycin was reduced in *E. coli* JM83 harboring pMZY102 encoding the *fosB* gene (data not shown). Moreover, the transfer of fosfomycin resistance from these isolates to *E. coli* CSH-2 by conjugation was unsuccessful. These results suggested no involvement of fosfomycin-glutathione *S*-transferase in the fosfomycin resistance of NGY47 and NGY60.

**Accumulation of active intracellular fosfomycin.** Other possible mechanisms of fosfomycin resistance include impairment of fosfomycin uptake (7), a low-affinity UDP-GlcNAc enolpyruvyl transferase (22), or overproduction of this enzyme encoded by the *murA* gene (11). A time course study of fosfomycin uptake into cells was undertaken for NGY47, NGY60, and *E. coli* C600 harboring pREFTT47B408 in the presence of either  $\alpha$ -GP or G6P (Fig. 1). NGY47 and NGY60 accumulated 100- to 200-fold less fosfomycin than C600 harboring pREFTT47B408, which rapidly incorporated fosfomycin at the examined concentration. Because the MIC of fosfomycin for

TABLE 2. Antibiotic susceptibilities of 129 STEC isolates

Antibiotic	Range	MIC ( $\mu$ g/ml)	
		% Inhibition	
		50	90
Fosfomycin <sup>a</sup>	0.5– $\geq 512$	8	32
Ampicillin	4– $\geq 512$	4	128
Cefaclor	1–128	4	16
Gentamicin	0.125–2	1	2
Kanamycin	1– $\geq 512$	4	8
Tetracycline	0.5–256	2	2
Doxycycline	0.5–128	2	64
Minocycline	0.5–32	2	4
Norfloxacin	$\leq 0.064$ –1	0.125	0.125
Azithromycin	2–8	4	8
Chloramphenicol	1–64	4	8

<sup>a</sup> Determined on Mueller-Hinton agar containing 50  $\mu$ g of G6P per ml.

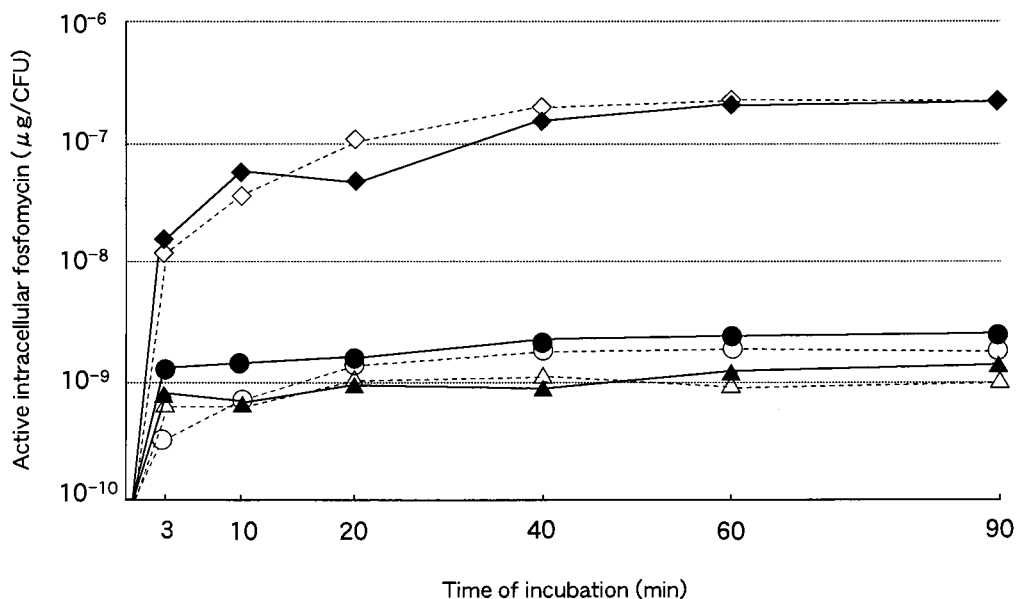


FIG. 1. The amount of active intracellular fosfomycin over the time course (micrograms per CFU). The fosfomycin uptake into cells was compared in NGY47, NGY60, and C600 harboring pREFTT47B408 in the presence of either α-GP or G6P. △, NGY47 (α-GP); ▲, NGY47 (G6P); ○, NGY60 (α-GP); ●, NGY60 (G6P); ◇, C600 harboring pREFTT47B408 (α-GP); ◆, C600 harboring pREFTT47B408 (G6P).

C600 harboring pREFTT47B408 was only 32 µg/ml, the poor uptake in NGY47 and NGY60 is thought to contribute to the high fosfomycin resistance. It should be noted that the time course response of fosfomycin accumulation in the presence of α-GP was similar to that in the presence of G6P.

**Analysis of the *murA* gene.** The *murA* genes of NGY47 and NGY60 were cloned, and the plasmids containing them were termed pREFTT4701 and pREFTT6023, respectively. A 1.7-kb insert of pREFTT4701 and a 1.5-kb insert of pREFTT6023 were mapped with various restriction enzymes. Their low-copy-number vector derivatives were termed pREFTT47B408 and pREFTT60K804, respectively.

The nucleotide sequences of the 1,692-bp *Bam*HI fragment of pREFTT4701 and 1,455-bp *Bam*HI-*Sma*I fragment of pREFTT6023 were determined. Both sequenced regions contained a 1,260-bp open reading frame with a putative promoter region that encodes a 419-amino-acid protein. These open reading frames showed good homology with the *murA* (*murZ*) gene encoding UDP-GlcNAc enolpyruvyl transferase (98.9 and 98.7% at the nucleotide level, respectively) of *E. coli* AB1157 (11). The homology at the amino acid level of MurA of NGY47 and NGY60 with that of AB1157 MurA (MurZ) was 100 and 99.8%, respectively. Lys11 of AB1157 MurA (MurZ) was substituted for Glu11 in NGY60.

**Expression of the *murA* gene.** To determine the involvement of the *murA* gene in resistance to fosfomycin, we performed Northern blotting on total RNA extracted from NGY47, C600 harboring pREFTT4701 or pREFTT47B408, and C600. As Fig. 2 shows, the transcriptional level of the *murA* gene was enhanced in NGY47, compared with C600. The *murA* mRNA level in C600 harboring pREFTT4701 was much higher than that in C600 harboring pREFTT47B408. The *murA* mRNA level of NGY47 appeared to be similar to that of C600 harboring pREFTT47B408. The quantitative estimates of the mRNA level by the National Institutes of Health Image program were 79.6, 174.0, 70.1, and 32.9 for NGY47, C600 harboring pREFTT4701, C600 harboring pREFTT47B408, and C600, respectively. To characterize the relationship be-

tween the increase in the *murA* mRNA level and MIC of fosfomycin, MICs for these strains were determined. MICs for NGY47, C600 harboring pREFTT4701, C600 harboring pREFTT47B408, and C600 were 1,024, 1,024, 32, and 2 µg/ml, respectively (Fig. 2). These results suggest that the increase in the *murA* mRNA expression is correlated with the MIC of fosfomycin. Therefore, the enhanced transcriptional level of the *murA* gene in NGY47 confers, at least partly, resistance to fosfomycin.

Comparable results were obtained when Northern blotting was performed on total RNA from NGY60 and C600 harboring pREFTT6023 or pREFTT60K804 (data not shown). MICs for C600 harboring pREFTT6023 and pREFTT60K804 were 1,024 and 32 µg/ml, respectively.

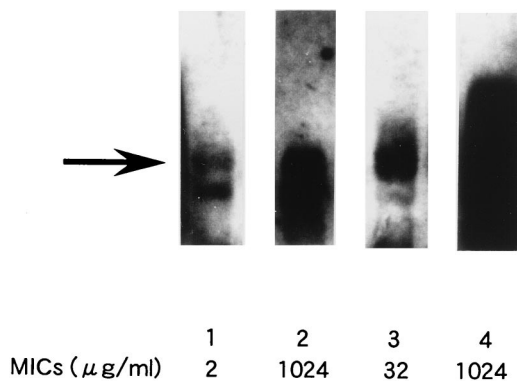


FIG. 2. Northern blot analysis of the *murA* transcript in NGY47, C600 harboring pREFTT4701 or pREFTT47B408, and C600. Lane 1, C600; lane 2, NGY47; lane 3, C600 harboring pREFTT47B408 (vector, pWKS130); lane 4, C600 harboring pREFTT4701 (vector, pHSG398). An arrow indicates the position of the *murA* transcript. The MIC of fosfomycin for each strain is shown under its lane number.



## DISCUSSION

In this study, we characterized fosfomycin resistance in two clinical isolates of STEC O26. The emergence of fosfomycin-resistant STEC isolates has become a significant problem in antibiotic therapy for STEC infections (20). To date, fosfomycin resistance has been studied in some strains (1, 5, 7, 8, 11, 12). Although no STEC strains showing high resistance to fosfomycin have been reported (21), we found that two STEC isolates whose H types were different, NGY47 and NGY60, exhibited high resistance to fosfomycin, among 129 clinical isolates. Therefore, it is of great value to characterize the fosfomycin resistance of STEC isolates for antibiotic therapy.

Previous reports have shown that fosfomycin resistance in *E. coli* was conferred by chromosomal genes such as *glpT*, *uhp*, *murA* (*murZ*), and *fsr* (5, 8, 11, 12), whereas an inactivating enzyme, fosfomycin-glutathione *S*-transferase, mediated by a plasmid (1, 3, 13, 15, 19) has not been reported in *E. coli*. Our results also indicated that the presence of the inactivating enzyme in NGY47 and NGY60 was unlikely because of the failures of both the detection of glutathione *S*-transferase activity and the conjugational transfer of the fosfomycin resistance. Moreover, our results suggested that these fosfomycin-resistant STEC isolates had presumably altered *glpT* and/or *uhp* transport systems.

It has been reported that the mutation of the covalently attaching site of fosfomycin, Cys115, in MurA (*MurZ*) or overexpression of the *murA* (*murZ*) gene conferred fosfomycin resistance (2, 11, 12, 17). MurA of NGY47 and NGY60 exhibited 100% identity with *E. coli* MurA (*MurZ*), and Cys115 was conserved, suggesting that the fosfomycin resistance in these strains was not caused by a mutation of MurA. Northern blot analysis suggested that enhanced transcription of the *murA* gene in these strains was also involved in fosfomycin resistance.

Our results were consistent with previous observations. In clinical isolates, some studies have demonstrated that the mechanism of chromosomally encoded fosfomycin resistance mainly involved reduced permeability of the cell membrane (1, 15). Arca et al. (1) evaluated genetically and biologically a number of fosfomycin-resistant clinical isolates, including *E. coli*, *Staphylococcus aureus*, and *Morganella morganii*. They demonstrated that the fosfomycin resistance conferred by plasmids constituted little of the overall resistance to fosfomycin, which was caused by an alteration of the transport system. The results reported here, however, demonstrated that alterations of at least two chromosomal determinants conferred high resistance to fosfomycin in STEC. To the best of our knowledge, fosfomycin resistance owing to concurrent alterations has not previously been reported. Although the frequency of these mutations in STEC is not known, our results suggest that more fosfomycin-resistant isolates will emerge spontaneously without the acquisition of a plasmid in the future.

Additionally, we evaluated the susceptibilities of STEC isolates to various oral antibiotics. In previous studies, the emergence of STEC strains resistant to ampicillin, gentamicin, tetracycline, streptomycin, sulfisoxazole, and/or trimethoprim-sulfamethoxazole has been reported (4, 9, 21). Our results showed the presence of STEC isolates for which MICs of fosfomycin, ampicillin, cefaclor, kanamycin, tetracycline, and/or doxycycline were high, whereas all STEC isolates were susceptible to gentamicin, minocycline, norfloxacin, and azithromycin. The presence of STEC strains resistant to some oral antibiotics, therefore, should be taken into account in planning therapy for STEC infections.

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