Effects of fosfomycin on Shiga toxin-producing *Escherichia coli*: quantification of copy numbers of Shiga toxin genes and their expression levels using real-time PCR

Antibiotic therapy for infection with Shiga toxin-producing *Escherichia coli* (STEC) is not generally recommended, because it is thought to increase released levels of Shiga toxin (Stx), leading to a severe complication of hemolytic uremic syndrome (HUS) (Tarr et al., 2005). However, the incidence of HUS in children with STEC infection was found not to be higher in Japan than in other countries, despite the use of antibiotics such as fosfomycin (FOM) (Ikeda et al., 1999; IASR, 2007). Furthermore, early administration of FOM as well as a new quinolone, norfloxacin (NFLX), and kanamysin was recommended in the 1997 guidelines for medical treatment of STEC O157 infection issued by the Ministry of Health and Welfare of Japan. Hence, it remains controversial whether the use of antibiotics for STEC infection is effective or harmful (Wong et al., 2000; Bennish et al., 2006; Panos et al., 2006).

The production of Stxs proteins is considered to be closely related to the copy numbers and expression levels of stx genes. For example, quinolones have been demonstrated to induce stx prophages, which are associated with the production of Stxs (Kimmitt et al., 1999; Matsushiro et al., 1999). On the other hand, FOM and gentamicin do not induce the expression of stx2 (Kimmitt et al., 2000). In accordance with those *in vitro* results, Zang et al. (2000) reported that an administration of the quinolone ciprofloxacin to mice infected with STEC harboring stx2 caused marked increases in fecal Stx2 and mortality rate, whereas administration of FOM did not have those effects. The results of those studies suggest that the risk of sequelae associated with antibiotic therapy for STEC infection is closely related to the antibacterial mechanism of each agent and its effects on the stx genes. Herein, we conducted the quantitative study of the effects of FOM on the copy numbers and mRNA expression levels of the stx genes, by measuring the amounts in *E. coli* O157:H7 with real-time PCR. In addition, we compared the effects of FOM with those of NFLX and three other β-lactams.
FOM was obtained from Meiji Seika, NFLX from Kyorin-Pharm, panipenem (PAPM) from Sankyo, ceftazidime (CAZ) from Eisai, and aztreonam (AZT) from Tanabe Seiyaku. MICs for the bacterial strain were established using a micro-dilution method. The 1x MICs of FOM, NFLX, PAPM, CAZ and AZT for the strain were 1.00, 0.25, 0.25, 0.125, and 0.25 µg ml\(^{-1}\), respectively.

The *E. coli* clinical strain O157:H7 Iwate M1-2, which produces Stx1 and Stx2, was isolated during an STEC outbreak in the Morioka area of Japan in 1996 (Ohara-Nemoto *et al.*, 1997: Ichinohe *et al.*, 1999), and the *stx2* gene was maintained as a prophage (Ohara-Nemoto *et al.*, 1997). For the present study, a single colony was aerobically cultured at 37 °C in Luria-Bertani broth overnight, then diluted 1:20 with fresh medium and further cultured at 37 °C in the presence of antibiotics at 1x MIC. After 1, 4, 8 and 12 h of cultivation, the bacterial cells were harvested by centrifugation and then immediately stored at -80 °C.

On the following day of cultivation, genomic DNA and total RNA were purified as described previously (Ohara-Nemoto *et al.*, 2002; Sasaki *et al.*, 2001). A total of 1 µg of RNA was mixed with 4 µM oligo(dT)\(_{8-12}\) and incubated at 70 °C for 10 min, and then put on ice. Reverse transcription (RT) reactions were performed at 37 °C for 1 h to generate cDNA in a 20 µl reaction mixture consisting of 50 mM Tris/HCl (pH8.3), 75 mM KCl, 3 mM MgCl\(_2\), 1 mM dNTP, 10 U of RNasin and 2 U Superscript II reverse transcriptase (Invitrogen). Primers and probes for the genes of Stx1 subunit A and Stx 2 subunit A (Jackson *et al.*, 1987), as well as of 16S rRNA, were designed using the Primer Express software package (Applied Biosystems) (Table 1). A total of 5 ng genomic DNA was used for real-time PCR analysis of the copy numbers of the genes, and 0.25 µl of the RT products was used for analysis of the expression levels of their mRNA. The amplification reaction was performed with 25 µl of TaqMan Universal PCR mix (Applied Biosystems) as a reaction mixture, with the following cycling parameters: 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The reactions were performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Standard curves for *stxl*, *stx2* and 16S rRNA were obtained with the corresponding full-length genes cloned into pGEM-T Easy
The amounts of the stx genes and mRNA were normalized by the copy numbers of the copy numbers of the 16S rRNA gene for each sample, then compared with the control without antibiotics. Statistical analysis was performed using a Mann-Whitney U-test.

STEC was incubated with NFLX and FOM, then the copy numbers and mRNA levels of the stx genes were determined. NFLX significantly increased the copy numbers of the two stxs genes at 4 h after antibiotic treatment (Fig. 1a). In particular, stx2 DNA was dramatically increased by up to three orders of magnitude as compared to without the antibiotic. In accordance with the increase in DNA copy numbers, mRNA levels of stx1 and stx2 reached the maximum at 4 h, and the amounts were 40 times and 3-4 orders of magnitude higher, respectively, than those of the controls. In contrast, the effects of FOM were substantially distinct to those of NFLX. After 1 h of treatment with FOM, a slight increase in DNA copy numbers and stx1 and stx2 mRNA levels were observed, after which those values significantly declined to the final time point of 12 h (Fig. 1b).

Since the maximal difference in effects of FOM and NFLX on the induction of stx genes was observed at 4 h after treatment, the effects of three other β-lactams, i.e. PAPM, CAZ and AZT, were examined under the same conditions (Fig. 2). In previous experiments, bacteria were markedly killed after 4 h of incubation with FOM at an MIC concentration of 1x (Tomita et al., 2007), as well as with NFLX, PAPM, CAZ and AZT (data not shown). With PAPM, the DNA copy numbers and mRNA levels were markedly decreased in the same manner as with FOM, whereas CAZ only slightly reduced the DNA copy numbers and scarcely changed mRNA levels, and AZT had little effect on the levels of the stx genes and their expression. These results clearly demonstrated that the effects on the levels of stx genes and their expression were substantially different among the tested antibiotics.

Results of quantitative real-time PCR analysis in the present study showed that the effects of antibiotics on the stx genes and mRNA levels were quite different, as FOM and PAPM significantly decreased the DNA copy numbers and mRNA expression levels, while CAZ and AZT had little effect on those. In contrast, apparent induction of those genes and their
mRNA expression were demonstrated with NFLX, in accordance with other reports (Kimmitt et al., 1999; Matsushiro et al., 1999). The effect of NFLX, which inhibits DNA gyrase, is considered to occur via an SOS response that induces prophages in bacterial cells (Kimmitt, et al., 2000). Thus, it was speculated that production of phage particles including the stx2 gene markedly occurred in the strain following NFLX treatment, causing a significant expression of stx2 mRNA.

FOM, as well as the three other antibiotics that did not induce the stx genes, inhibit cell wall synthesis, thus the present results could be reasonably explained by the inhibitory mechanisms of these agents. FOM specifically inhibits UDP-N-acetyl-glucosamine-pyruvate transferase, which is involved in the early stage of cell wall synthesis and induces cell lysis (Kahan et al., 1974). The PAPM carbapenem binds to penicillin-binding protein (PBP) 2, 1A, and 1B, and induces formation of spheroplast and cell lysis (Horii et al., 1998, Jackson and Kropp, 1992). CAZ and AZT bind mainly to PBP 3, one of the septation proteins (Georgopapodakou et al., 1982), after which lysis does not occur rapidly, though elongation of the bacterial cells is induced. The effects on the levels of the stx genes and mRNA seemed to correlate with bacterial cell lysis induced by these antibiotics. Treatment with either FOM or PAPM may cause rapid cell lysis of STEC cells, which is not accompanied by amplification of the stx genes present either in prophage or other forms. Therefore, we think that an early administration of antibiotics for STEC infection should be carefully reconsidered from the aspect of the bactericidal effects of the antibiotics. Taking into consideration these effects, FOM therapy may be valid for STEC infection and may not increase the risk of HUS.

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Figure Legends

Fig. 1. Effects of NFLX and FOM on the levels of stx DNA and mRNA expression. STEC cells were cultured with (a) NFLX or (b) FOM at 1x MIC at 37 °C. Genomic DNA and total RNA were purified from bacterial cells harvested at 0 (white bars), 1 (grey bars), 4 (hatched bars), 8 (spotted bars) or 12 h (black bars) after incubation with the antibiotics. The numbers of gene copies of stx1 and stx2 were determined using genomic DNA (5 ng DNA per reaction), and stx1 and stx2 mRNA was determined using cDNA (0.25 µl RT product per reaction) with quantitative real-time PCR. Each copy number was standardized by the genomic copies for the 16S rRNA gene. The data are shown as the mean of triplicates. Similar results were obtained from three separate experiments.

Fig. 2. Effects of antibiotics on the levels of stx DNA and mRNA expression. STEC cells were incubated without or with NFLX, FOM, PAPM, CAZ or AZT at 1x MIC at 37 °C. After 4 h, the bacterial cells were harvested, and genomic DNA and total RNA were purified. The numbers of gene copies of stx1 and stx2 were determined using genomic DNA, and stx1 and stx2 mRNA were determined using cDNA with quantitative real-time PCR. Each copy number was standardized by the genomic copies for the 16S rRNA gene. Data are shown as the mean plus standard deviation of four samples from two separate experiments. White bars, stx1 DNA; black bars, stx1 mRNA; grey bars, stx2 DNA; hatched bars, stx2 mRNA. **P≤0.01 and *P≤0.05, in comparison to levels in the control medium.
Table 1. Primers and probes used in this study

<table>
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<tr>
<th>Primer/probe</th>
<th>Sequence (5’ to 3’)</th>
<th>Product size (bp)</th>
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<tr>
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</tr>
<tr>
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<tr>
<td>stx1Ap</td>
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<tr>
<td>stx2Ap</td>
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<td>16S rRNAp</td>
<td>6FAM-TGACGGGGCGGTGTGTAAGGC-TAMRA</td>
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FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.