Effects of fosfomycin on Shiga toxin-producing *Escherichia coli*: quantification of copy numbers of Shiga toxin-encoding 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 levels of released Shiga toxin (Stx), leading to the severe complication of haemolytic uraemic 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; IDSC, 2007). Furthermore, early administration of FOM, as well as a new quinolone, norfloxacin (NFLX), and kanamycin, 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 Stx 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 Stx proteins (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, Zhang *et al.* (2000) reported that an administration of the quinolone ciprofloxacin to mice infected with STEC harbouring *stx2* caused marked increases in faecal Stx2 and the 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 a 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 $1 \times$ MICs of FOM, NFLX, PAPM, CAZ and AZT for the strain were 1, 0.25, 0.25, 0.125 and 0.25 $\mu\text{g ml}^{-1}$, 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 $1 \times$ 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 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 (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 1 mM dNTP, 10 U 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 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 *stx1*, *stx2* and 16S rRNA were obtained with the corresponding full-length genes cloned into pGEM-T Easy (Promega Biosystems). The amounts of the *stx* genes and mRNA were normalized by 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 *stx* 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).

Table 1. Primers and probes used in this study

Primer/probe	Sequence (5' to 3')	Product size (bp)
<i>stx1Af</i>	TCTCGACTGCAAAGACGTATGTAG	118
<i>stx1Ar</i>	CCTGTGCCACTATCAATCATCAGTA	
<i>stx1Ap</i>	FAM-CGCTGAATGTCATTCGCTCTGCAATAGG-TAMRA	
<i>stx2Af</i>	GATGACACATTTACAGTGAAGGTTGA	
<i>stx2Ar</i>	TCACAGGTACTGGATTTGATTGTGA	125
<i>stx2Ap</i>	FAM-TACTGGACCAGTCGCTGGAATCTGCA-TAMRA	
<i>16S rRNAf</i>	GGATTAGATACCCTGGTAGTC	
<i>16S rRNAr</i>	TACCTTGTTACGACTT	
<i>16S rRNAp</i>	FAM-TGACGGGCGGTGTGTACAAGGC-TAMRA	728

FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

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. PAMP, 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 $1 \times$ (Tomita *et al.*, 2007), as well as with NFLX, PAMP, CAZ and AZT (data not shown). With PAMP, 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 PAMP 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-acetylglucosamine pyruvate transferase, which is involved in the early stage of cell wall synthesis and induces cell lysis (Kahan *et al.*, 1974). The PAMP carbapenem binds to penicillin-binding protein (PBP) 2, 1A and 1B, and induces spheroplast formation and cell lysis (Horii *et al.*, 1998; Jackson & Kropp, 1992). CAZ and AZT bind mainly to PBP 3, one of the septation proteins (Georgopapadakou *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 PAMP 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.

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 $1 \times$ 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.

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Fig. 2. Effects of antibiotics on the levels of *stx* DNA and mRNA expression. STEC cells were incubated without or with NFLX, FOM, PAMP, CAZ or AZT at $1 \times \text{MIC}$ 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 expression 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 SD of four samples from two separate experiments. White bars, *stx1* DNA; black bars, *stx1* mRNA; grey bars, *stx2* DNA; hatched bars, *stx2* mRNA. * $P \leq 0.05$ and ** $P \leq 0.01$, in comparison to levels in the control medium.

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