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Effect of Antimicrobial Agents on the Production and Release of Shiga Toxin by Enterotoxaemic *Escherichia coli* Isolates from Pigs

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ABSTRACT. Edema disease (ED) of pigs is an enterotoxaemic disease caused by enterotoxaemic *Escherichia coli* (ETEEC) infection. Antimicrobial therapy for pigs with ED is controversial because it may induce death of sickish piglets. In this study, we investigated the effects *in vitro* of 7 antimicrobial agents, ampicillin, gentamicin, colistin, bicozamycin, fosfomycin, sulfamethoxazole-trimethoprim and enrofloxacin, on the release and production of shiga toxin (Stx) 2e by ETEEC strains. We found that more Stx 2e accumulated in the bacterial cells than was released into supernatant. Associated with inhibition of cell wall synthesis, the exposure to ampicillin or fosfomycin increased the release of Stx 2e. The production levels of Stx 2e in all antimicrobial-treated cultures were equal to the level in the control or less than in the control. These results suggest that cell wall synthesis inhibitors, such as ampicillin and fosfomycin, may change for the worse in the signs in ETEEC infectious pigs. On the other hand, gentamicin, colistin, bicozamycin and enrofloxac in may be useful for the treatment of pigs with ED.

KEY WORDS: antimicrobia, edema disease, enterotoxaemic *Escherichia coli*, shiga toxin 2e, swine.

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Edema disease (ED) of the pig has been found throughout the world since ED was first reported in Ireland in 1938 [17]. ED has been a sporadically occurring disease found mainly in post-weaned piglets that causes systemic vascular damage as a result of intestinal infection with shiga toxin-producing *Escherichia coli* (STEC) [1, 2, 5]. The STEC is also called enterotoxaemic *Escherichia coli* (ETEEC) [14]. After ETEEC colonizes in the intestines, shiga toxin (Stx) 2e produced by the ETEEC is absorbed into the capillary vessels and then damages the endothelial cells [1, 2, 5]. The manifestations of ED include palpebral edema, neurological impairment, lateral recumbence and sudden death [1, 2]. The speed of onset and severity of clinical signs and gross pathological lesions are related to the dose of the toxin. Intervals between injection and onset of the disease vary from 7 to 28 hr, the mean time to death being from 24 to 42 hr [2]. Classical ED has been seen to occur sporadically and subside spontaneously [1, 2, 8, 11]. Recently, ED has tended to persist and spread, causing great economic damage to pig farmers [20, 21, 24].

Antimicrobial administration has been used as one of the most common treatments for the eradication of STEC from the intestines of humans and animals [12, 14, 16, 22]. Nevertheless, some antimicrobial agents have been reported to increase the release of Stxs from enterohemorrhagic *Escherichia coli* (EHEC) associated with enterohemorrhagic colitis and hemolytic uremic syndrome of humans *in vitro* [4, 10, 13, 22, 23]. In ED affected farms, deaths of piglets associated with antimicrobial treatment have increased in number [7, 24]. Until now, however, there has been no investigation to determine the effect of antimicrobial agents on the production and release of Stx 2e by ETEEC.

In this study, we determined the time-course of the number of viable bacteria and the Stx 2e production of O139 ETEEC strains isolated from pigs with ED in recent years. We also examined the effect of antimicrobial agents on Stx 2e production and release by ETEEC.

MATERIALS AND METHODS

Bacterial strains: O-serogroup 139 (O139) ETEEC MVH 269 and MVH 886 strains were used in this study. These strains were isolated from pigs with ED in 1999 and 2001, respectively [19]. We also used *E. coli* ATCC 23546, which was the O139 ETEEC reference strain isolated from pigs with ED in the 1950s [3].

Time-course of Stx 2e production by ETEEC: ETEEC were grown at 37°C overnight with shaking in brain heart infusion broth (BHI broth, Difco, GA, U.S.A.), and the bacterial solution was diluted 1:20 with the broth. The bacterial suspensions (approximately 10^8 colony-forming units (cfu)/m*l*) were cultured at 37°C for 3 hr. These pre-cultured organisms were centrifuged at 3,500 rpm for 10 min, and the precipitate was resuspended in BHI broth to make approximately 10^4 cfu/m*l* and then cultured at 37°C with shaking. The number of viable cells was counted by the agar diluted method. The culture was centrifuged at 3,500 rpm for 15 min, and the supernatant was recovered as samples of released Stx 2e. The pellets, suspended in BHI broth with equal amounts of removal supernatant, were disrupted for 1 min (4 times) by the cell disrupter (SONIFIRE, CELL DISRUPTOR 185, Branson, MO, U.S.A.) in cold water with ice. The cell-disrupted suspension was used for the samples of the production of Stx 2e in the cell body. These samples

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were filtrated through 0.20-μm pore-size membrane filters and then tested for cytotoxic activity in a Vero cell assay system. Individual samples were collected at 0, 2, 4, 6, 8, 12, 16, 20, 24 and 36 hr post-incubation (pi). The Stx 2e cytotoxic activities were determined by Vero cell assay.

Effect of antimicrobial agents on ETEEC growth and the release/production of Stx 2e: The minimum inhibitory concentration (MIC) against the ETEEC MVH 269 strain was determined by agar dilution methods according to NCCLS (the National Committee for Clinical Laboratory Standards, 2002) guidelines [15]. The following seven antimicrobial agents were used in this study: ampicillin (ABPC), gentamicin (GM), colistin (CL), fosfomycin (FOM), sulfamethoxazole-trimethoprim (5:1, ST) (Wako Pure Chemicals Co., Osaka, Japan), bicozamycin (BCM, Fujisawa Pharmaceutical Co., Osaka, Japan) and enrofloxacin (ERFX, ICN Biomedicals Inc., CA, U.S.A.). Each antimicrobial agent at a final concentration of $\times$ 1 MIC or $\times$ 50 MIC was added to the cell suspension, and the mixture was then incubated at 37°C for 4 hr with shaking. The number of viable cells and Vero cytotoxic activities of release/production of Stx 2e were measured at 0 and 4 hr pi. The examination was repeated three times, and the results are expressed as the mean ± standard deviation. Statistical significance was determined by Student's *t*-test. The control culture without an antimicrobial agent was incubated at 37°C for 4 hr.

Vero cytotoxicity assay: Vero cells maintained in Eagle's MEM (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) with 1% newborn calf serum (Gibco BRL Co., New Zealand) were grown as monolaylers in 96-well microtiter tissue culture trays. The specimens of Stx 2e were 2-fold diluted in the cell tray and incubated at 37°C in 5% CO_2 for 72 hr. After that, the residual Vero cells were stained with crystal violet and eluted with 1% Sodium Lauryl Sulfate Solution (Nacalai Tesque, Inc., Kyoto, Japan) to measure the absorbance (EASY READER EAR400, Slt-Labinstrumants, Austria). The 50% cytotoxic dose (CD_{50}) corresponded to the dilution required to kill 50% of the Vero cells [9].

RESULTS

Time-course of Stx 2e production: The number of viable bacteria of all strains reached 9.0 $\log_{10}$ cfu/m*l* at 6 hr pi and remained at that level (Fig. 1A). The Stx 2e in the supernatant of each strain was detected from 6–8 hr pi, which was almost the same time needed to reach the growth curve plateau. The highest Vero cytotoxicity (CD_{50}) of MVH 269, MVH 886 and ATCC 23546 was 129 (36 hr pi), 175 (36 hr pi) and 15 (20 hr pi), respectively (Fig. 1A). The Stx 2e in the bacterial body of each strain was detected from 4–6 hr pi, which was, as above, almost the same time needed to reach the growth curve plateau. The highest CD_{50} of MVH 269, MVH 886 and ATCC 23546 was 14,950 (24 hr pi), 26,726 (36 hr pi) and 711 (12 hr pi), respectively (Fig. 1B). The cell disruption rates were more than 98 percent, so almost all of the Stx 2e in the cell bodies was recovered. The cytotoxic activities of Stx 2e in the bacterial cells were higher than those in the supernatant. The Stx 2e cytotoxicities of MVH 269 and MVH 886 strains were higher than that of ATCC 23546 strain.

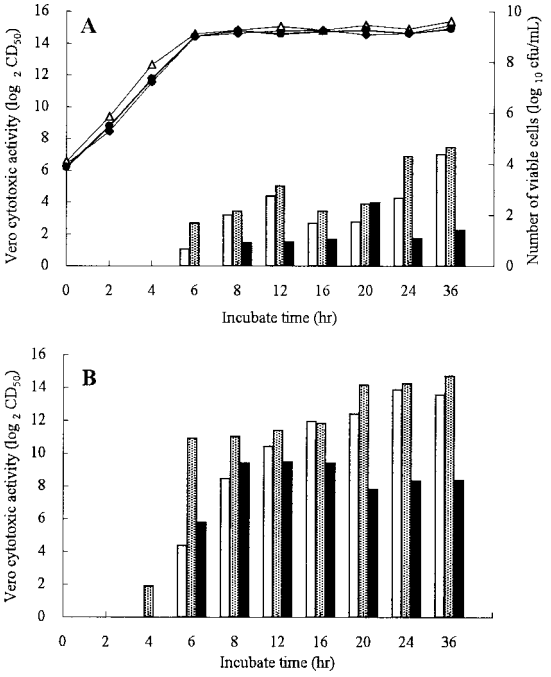

Fig. 1. The time-course of ETEEC growth and Stx 2e production of ETEEC. A: The time-course of ETEEC growth and release of Stx 2e by ETEEC. B: The time-course of Stx 2e in the ETEEC cells. Viable cell number of MVH 269 (–◆–), MVH 886 (–△–) and ATCC 23546 (–●–). Vero cytotoxic activities ($\log_2$ CD_{50}) of MVH 269 (□), MVH 886 (▩) and ATCC 23546 (■).

Effects of antimicrobial agents on ETEEC growth and release/production of Stx 2e: The MICs of each antimicrobial agent against ETEEC MVH 269 were 256 mg/*l* for ABPC, 16 mg/*l* for BCM and FOM, 0.5 mg/*l* for ST, 0.25 mg/*l* for GM and CL, and 0.125 mg/*l* for ERFX.

The numbers of viable cells in the control without antimicrobials were 8.32 ± 0.09 $\log_{10}$ cfu/m*l* (0 hr pi) and 9.34 ± 0.12 $\log_{10}$ cfu/m*l* (4 hr pi) (Fig. 2). The released Stx 2e cytotoxic activities were 3.19 ± 0.8 (0 hr pi) and 54.7 ± 16.9 (4 hr pi) (Fig. 2). The production of Stx 2e cytotoxic activities were 70.3 ± 30.9 (0 hr pi) and 724.8 ± 126.0 (4 hr pi) (Fig. 2).

The number of viable cells decreased dramatically as the ABPC concentration increased. The amount of Stx 2e released into the supernatants increased in volume compared with the control. The productions of Stx 2e were less than that of the control (Fig. 2A).

While $\times$ 1 MIC GM could not inhibit the bacterial growth, $\times$ 50 MIC GM did reduce the number of viable cells to 10 percent of the pre-culture level. The Stx 2e release in the $\times$ 50 MIC and release/production in the $\times$ 1 MIC were not different from the control volume, but the Stx 2e produc-

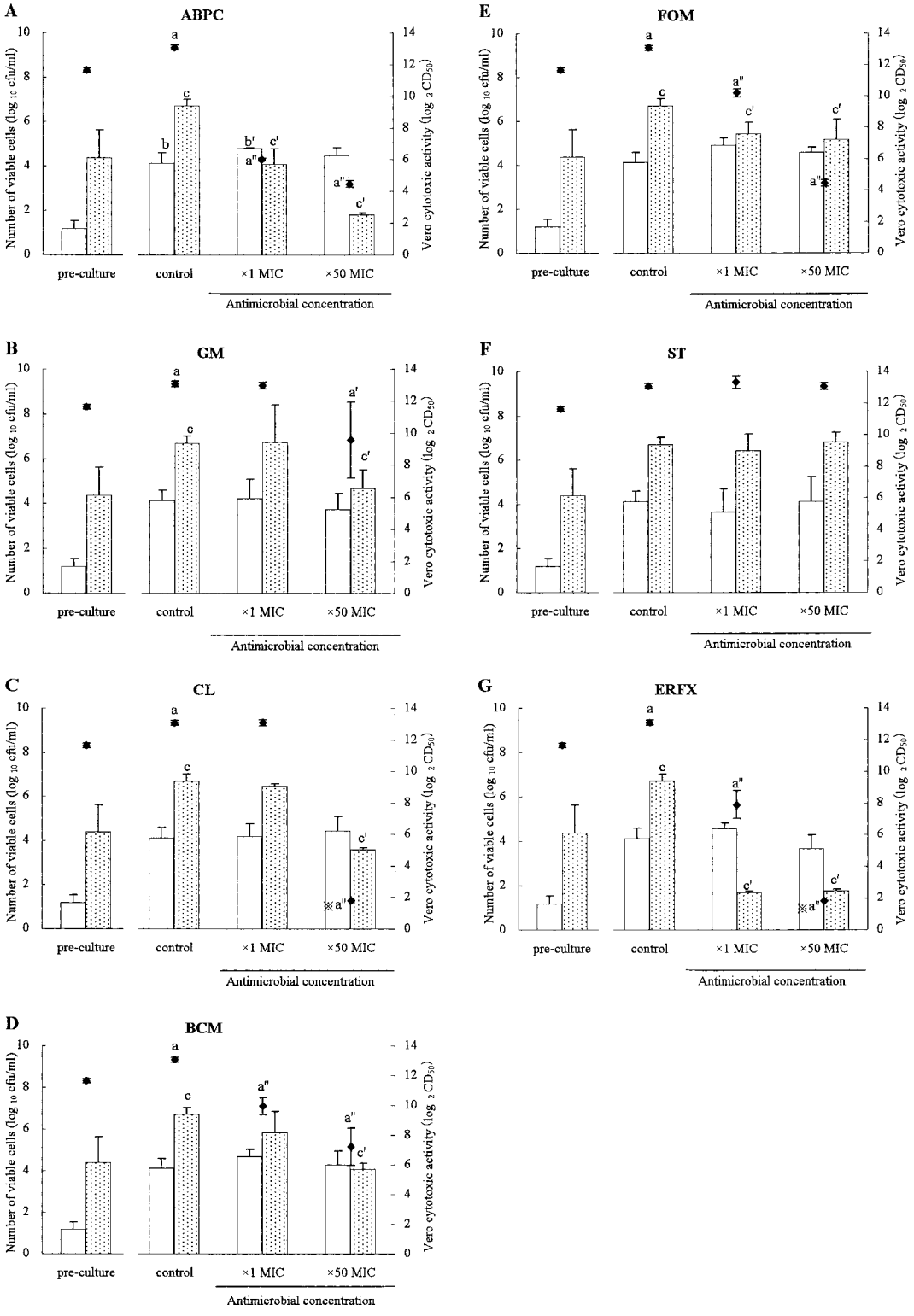

Fig. 2. Effect of antimicrobial agents on ETEEC growth and the release/production of Stx 2e by ETEEC MVH 269. (A) ABPC, (B) GM, (C) CL, (D) BCM, (E) FOM, (F) ST, (G) ERFX. Control means cultured without antimicrobial agents. Number of viable cells ($\log_{10}$ cfu/m*l*) (◆). ※: The number of viable cells is less than the detection limit (1.3 $\log_{10}$ cfu/m*l*). Vero cytotoxic activities of release of Stx 2e ($\log_2$ CD_{50}) (□) and accumulation of Stx 2e ($\log_2$ CD_{50}) (⬚). The error bars represent the standard deviations of three values. a-a" ($p<0.01$), a-a', b-b' and c-c' ($p<0.05$).

tion in the × 50 MIC decreased in volume (Fig. 2B).

Although × 1 MIC CL could not inhibit the bacterial growth, × 50 MIC CL did reduce the number of viable cells to less than the detection limit (1.3 $\log_{10}$ cfu/m*l*). The Stx 2e releases were not different from that of the control. The Stx 2e production was not different from the control at × 1 MIC CL either; but there was an obvious decrease in volume at × 50 MIC CL (Fig. 2C).

The decrease in viable cells depended on the BCM concentration. The Stx 2e releases did not change in association with BCM addition. The Stx 2e productions in the × 50 MIC BCM were less than the control volume (Fig. 2D).

The viable cells were decreased as the FOM concentration increased. The Stx 2e released on the addition of FOM increased in volume. The production of Stx 2e decreased (Fig. 2E).

When ST was added, there was no significant difference in the cell growth or the release/production of Stx 2e compared with the control (Fig. 2F).

The viable cells were decreased depending on the ERFX concentration. The Stx 2e releases were not significantly different from that of the control; but the productions of Stx 2e were greatly decreased (Fig. 2G).

DISCUSSION

Growth curves of ETEEC cells did not differ among the tested strains, including the reference strain. The release and accumulation of Stx 2e were detected soon after ETEEC growth reached the plateau phase (4–8 hr pi). There was a higher level of accumulated Stx 2e in the ETEEC cells than the released Stx 2e, which suggests that ETEEC retained most of the Stx 2e in the cell body. More Stx 2e was released and stored by wild ETEEC strains than that of the reference strain. In the case of EHEC O157, the amount of released Stx 1 was the same as that of accumulated Stx 1, and the amount of released Stx 2 was more than that of accumulated Stx 2 [23]. As mentioned above, Stx 2e by ETEEC might have a similar mode of action as that of Stx 1 by EHEC. It is possible that Stx 2e produced by ETEEC in the growth phase was stocked in the cell body, and then the accumulated Stx 2e was released and/or leaked to the supernatant.

It was reported that ABPC and FOM, which inhibit the cell wall synthesis of bacteria, induce the release of Stx from EHEC [6, 18, 22]. In the present study, we confirmed the finding of increased Stx 2e release associated with ABPC and FOM addition. The result suggested that ABPC and FOM were possible causes of worsened ETEEC infectious piglets.

It was proposed that Kanamycin be classified as an aminoglycoside, and likewise GM, which was useful for human EHEC infection, because the released Stx was inhibited to a low level [6, 18]. BCM decreased the number of EHEC O157:H7 and inhibited production of Stx 1 and Stx 2 by EHEC [10]. BCM was also an effective agent for the eradication of STEC in calves and for the treatment for ED-infected pigs [12, 24]. In this study, × 50 MIC of GM, CL and BCM demonstrated a bactericide effect and inhibited Stx 2e production. Furthermore, orally administered GM, CL and BCM could not be easily absorbed from the gut, which meant these drugs could be kept at a constant concentration in the enteric tract. These drugs have a propensity to act more effectively against ETEEC colonized in the intestine. It was suggested that GM, CL and BCM were useful for ED treatment.

It was reported that subinhibitory concentrations of norfloxacin, classified as the new quinolones, promoted the Stxs production from EHEC O157 [6, 13, 23]. In contrast, × 1 and × 50 MIC of ERFX as a new quinolone suppressed the production of Stx 2e from ETEEC O139 in the present study. Actually, opposite reactions to treatment with new quinolones have been reported; in one case an accidental death occurred due to treatment with new quinolones for pigs with ED [7], and in another case ERFX and orbifloxacin were useful for treatment of pigs infected with ED [20]. It is likely that the effects on ETEEC differ among the new quinolones.

From these results, when the inhibitors of cell wall synthesis were used for the treatment of piglets sick with ED, Stx 2e accumulated in the bacterial cells was released, and it was suggested that enterotoxaemia was promoted. GM, CL, BCM and ERFX might be useful for the treatment of pigs with ED, but the new quinolones, which have a broad antibacterial spectrum, might cause microbial substitution, in which case ERFX should be chosen as a secondary drug. In conclusion, ETEEC accumulate Stx 2e in the bacterial cells, and it is important to be careful of not only the antimicrobial susceptibility but also the function of antibiotics when choosing antibiotics to use for the treatment of piglets sick with ED. Furthermore, because the amount of Stx released from EHEC O157 following exposure to subinhibitory concentrations of antimicrobial agents was different among strains [4], we will investigate the effect of antimicrobial agents on more ETEEC isolates.

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