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Antimicrobials Effective for Inhibition of Enterohemorrhagic *Escherichia coli* Strains O26, O111, and O157 and Their Effects on Shiga Toxin Releases

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The susceptibilities of major enterohemorrhagic *Escherichia coli* (EHEC) strains to antimicrobial agents and the cytotoxicity of these agents were examined using a total of 38 strains of *E. coli* O26, O111, and O157, which are the major serogroups of EHEC. Among the 38 strains, 35, 36, and 36 were susceptible to amikacin, imipenem, and norfloxacin, respectively. These antimicrobial agents were further examined to determine their cytotoxicity on Vero cells as well as their effect on the release of Shiga toxins along with trimethoprim/sulfamethoxazole. Each of the *E. coli* O26, O111, and O157 strains containing both the *stx1* and *stx2* genes were grown in the absence or presence of these agents at 1/4 minimal inhibitory concentration for 6 h, 12 h, and 18 h. At the concentrations used in this study, none of the agents significantly altered cell count compared with the control group. The level of cytotoxicity in the imipenem group was lower at 12 h and 18 h than their respective controls. In contrast, the level of cytotoxicity in cultures treated with trimethoprim/sulfamethoxazole, norfloxacin, and amikacin was increased. The strains were also examined for the release of Shiga toxins 1 and 2 following treatment with the agents, which were measured by the reversed passive latex agglutination (RPLA) method. The RPLA assay showed a suppression of release of Shiga toxin 2 in the strain cultures containing imipenem. These results indicate that imipenem may be a safe and effective agent for inhibition of these bacteria, which has clinical implications for the treatment of EHEC infections.

Key words: EHEC, antibiotics, Shiga toxins, cytotoxicity

Enterohemorrhagic *Escherichia coli* (EHEC) is globally recognized as an important foodborne pathogen and is associated with bloody diarrhea, hemorrhagic colitis, and hemolytic–uremic syndrome (HUS) [1, 14]. Cattle have been implicated in the majority of foodborne EHEC outbreaks [6, 9]. EHEC infections are associated with the consumption of raw or undercooked meat and other foods contaminated with cattle feces [15]. The pathogenicity of EHEC is associated with a number of virulence factors, including Shiga toxins 1 and 2 (encoded by the *stx1* and *stx2* genes) [15]. Shiga toxins appear to play a major role in the pathogenesis of hemorrhagic colitis and hemolytic–uremic syndrome. Although *E. coli* strain O157 has been associated with the most important foodborne pathogen outbreaks throughout the world, other EHEC serotypes, particularly O26 and O111, have also emerged as significant causes of human disease [1, 2].

The risks involved in treating this disease with antimicrobial agents remains controversial. Indeed, the excessive release of Shiga toxins following treatment with antibiotics is one of the contributing factors to mortality and morbidity during infections with EHEC. Specifically, antimicrobial agents such as trimethoprim/sulfamethoxazole, ciprofloxacin, fosfomycin, and ceftazidime have been reported to increase the release of Shiga toxins from EHEC [17, 19]. Therefore, the release of Shiga toxins by these agents should be controlled, and maintaining a low-level release of Shiga toxins in EHEC infections might be important in preventing disease.

In this study, we investigated the susceptibility of several EHEC strains to antimicrobial agents, and examined the effects of these agents on cytotoxicity and the release of Shiga toxins from EHEC.

Materials and Methods

Bacterial Strains

A total of 38 strains of *E. coli* O26, O111, and O157 (3 human and 35 cattle strains) were used in this study. The human strains are EHEC O26:H28 (ATCC 25826), EHEC O111:H8 (ATCC 700840),

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and EHEC O157:H7 (ATCC 43894), and these were used for the cytotoxicity assay with Vero cells and antimicrobial susceptibility test. In addition, 10 EHEC O26, 8 EHEC O111, and 17 EHEC O157 isolates from feces of cattle, obtained between 2000 and 2007 in Korea, were also used for the antimicrobial susceptibility testing. The identification and serotyping of these EHEC strains have been previously well described [7, 8]. Genetic profiling for EHEC virulence markers such as *stx1, stx2*, *eae*, and *hly* was performed using PCR [7, 8]. All strains were positive for the *hly* and *eae* genes and contained at least one of the *stx1* or *stx2* genes.

Antimicrobial Susceptibility Test

The susceptibilities of all 38 EHEC strains isolated from human and cattle to a panel of 10 different antimicrobial agents were examined using the minimal inhibitory concentration (MIC) assay as standardized by the Clinical and Laboratory Standards Institute [3]. The antimicrobial panel consisted of amikacin, cefazolin, cefoperazone, ciprofloxacin, gentamicin, imipenem, nalidixic acid, norfloxacin, ofloxacin, and trimethoprim/sulfamethoxazole. *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, and *Enterococcus faecalis* ATCC 29212 were used as the quality-control strains.

Bacterial Cultivation with Addition of sub-MICs of Antimicrobial Agents

Each of the human *E. coli* O26, O111, and O157 strains in log-phase growth was prepared (1×10^4 CFU/ml) in a brain heart infusion (Difco Laboratories, Detroit, MI, U.S.A.). Antimicrobial agents were then added to each tube containing an *E. coli* cell suspension to a final concentration of 1/4 MIC and were incubated at 37°C for 18 h. The control group was also prepared by incubating each of the *E. coli* isolates in the absence of the antimicrobial agents. Samples were collected at 6, 12, and 18 h after beginning the culture. The number of viable bacteria in each experimental group was counted and the remaining cell suspension was used for the Shiga toxin assay.

Vero Cell Cytotoxicity Assay

The cytotoxic effects of the antimicrobial agents on Vero cells were measured. For the toxin preparation, a 1.5-ml aliquot of the bacterial cultures was centrifuged (8,160 ×*g*, 3 min) and the resulting cell-free supernatants were stored in sterile tubes. The cell pellets were resuspended in 75 ml of a polymyxin B sulfate solution (2 mg/ml in PBS) and incubated in a shaker-incubator for 30 min to release cell-bound toxins. After centrifugation (8,160 ×*g*, 5 min), the supernatants were collected and combined with the original cell-free supernatants sterilized with 0.2-μm disc filters (Corning, NY, U.S.A.). The filtrates were used immediately or stored at −20°C until needed.

African green monkey kidney (Vero) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, U.S.A.) containing 5% fetal bovine serum. The cells were grown at 37°C in a 5% CO_2 atmosphere under saturating humidity. The cells (1×10^5) were seeded into 96-well plates. The toxin sample, which was diluted to one tenth of its original concentration with the medium, was added to each well and incubated under the same cell growth conditions described above; BHI medium was used as the control. Next, 50 μl of MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide; Sigma) solution (8 mg/ml) was added to each well and incubated for a further 1 h, after which the supernatant was removed and 100% ethanol was added to each well. The absorbance was then measured at 540 nm using a spectrophotometer. The level of cytotoxicity was calculated using the following formula: (1−absorbance of the sample/absorbance of the control)×100 [10].

Assay for Release of Shiga Toxins

Bacterial cultures of three human EHEC strains obtained from ATCC with or without the antimicrobial agents were collected at 6, 12, and 18 h and centrifuged at 4°C at 1,100 ×*g* for 15 min. The supernatants were sterilized through a 0.22-μm pore-size filter (Millipore, Bedford, MA, U.S.A.) and stored at −20°C until needed. Shiga toxins 1 and 2 activities were measured using the reversed passive latex agglutination method (RPLA) using anti-Shigatoxin 1 and anti-Shigatoxin 2 polyclonal antibodies. The kit used in this study was the VTEC-RPLA kit (Denka Seiken Co., Tokyo, Japan), and the test was performed according to the manufacturer's recommended protocol.

RESULTS AND DISCUSSION

Antimicrobial Susceptibility

The antimicrobial susceptibilities of the collected EHEC strains are summarized in Table 1. Among all 38 EHEC strains, the 35, 22, 32, 25, 24, 36, 33, 36, 22, and 26 strains were within the susceptible ranges of amikacin, cefazolin, cefoperazone, ciprofloxacin, gentamicin, imipenem, nalidixic acid, norfloxacin, ofloxacin, and trimethoprim/sulfamethoxazole, respectively. Furthermore, amongst all strains, 12 to 16 were within the resistant or intermediate susceptibility ranges of cefazolin, ciprofloxacin, gentamicin, ofloxacin, and trimethoprim/sulfamethoxazole.

A total of 32% to 42% of the isolates were resistant or of intermediate susceptibility to cefazolin, ciprofloxacin, gentamicin, ofloxacin, and trimethoprim/sulfamethoxazole. The occurrence of such resistant *E. coli* strains is related to the quantity of antibiotics, particularly penicillins and aminoglycosides, which are used in agriculture [16]. These results suggest that these types of drugs are not very effective against such bacterial strains. Importantly, given that these antimicrobial agents are added to the feed or water for therapeutic or preventive purpose, these antibiotic agents may select for resistant EHEC strains. Indeed, cattle are constantly exposed to such resistant bacterial strains in the environment, meaning that drug-resistant EHEC strains can lead to serious public health problems. This may complicate future therapeutic options that are being developed for treating human EHEC infections when these types of EHEC strains are transmitted to the human community. Nevertheless, the fact that the isolates used in this study were still highly susceptible to several antibiotics, such as amikacin, imipenem, and norfloxacin, is an encouraging finding for public health.

Effects of Antimicrobial Agents on Vero Cell Cytotoxicity and Release of Shiga Toxins

Amikacin, imipenem, and norfloxacin, which were relatively effective antimicrobial agents for the above-mentioned

Table 1. MICs for the 38 EHEC O26, O111, and O157 strains.

Antibiotic (BP[a])	Number of isolates for which the MIC (μg/ml) was as follows:									
	≤0.25	0.5	1	2	4	8	16	32	64	≥128
AN (≤16, ≥64)		13		6	3	3	10	3		
CFZ (≤8, ≥32)			2	3	5	12	11	2	3	
CFP (≤16, ≥64)			13	3	5	6	5	3	2	1
CIP (≤1, ≥4)	17	2	6	10	1	1	1			
GM (≤4, ≥16)		3	5	10	6	7	5	2		
IPM (≤4, ≥16)	18	1	3	4	10	2				
NAL (≤16, ≥32)				5	1	7	20	3	1	1
NOR (≤4, ≥16)	17	2	11	3	3	1	1			
OFX (≤2, ≥8)		1	12	9	13	2	1			
SXT[b] (≤2/38, ≥4/76)	4	8	7	7	6	6				

AN, Amikacin; CFZ, Cefazolin; CFP, Cefoperazone; CIP, Ciprofloxacin; GM, Gentamicin; IPM, Imipenem; NAL, Nalidixic acid; NOR, Norfloxacin; OFX, Ofloxacin; SXT, Trimethoprim/Sulfamethoxazole.

[a]Breakpoints for susceptible and resistant isolates.

[b]0.25=0.25/4.75; 0.5=0.5/9.5; 2=2/38; 4=4/76; 16=16/304.

isolates, were selected for further assays to determine their cytotoxicity to Vero cells and their effects on the release of Shiga toxins, along with trimethoprim/sulfamethoxazole. Each of the human *E. coli* O26, O111, and O157 strains containing both *stx1* and *stx2* genes was grown with or without amikacin, imipenem, norfloxacin, and trimethoprim/sulfamethoxazole at a 1/4 MIC for 6 h, 12 h, and 18 h. At the concentrations used, none of the agents significantly altered cell counts relative to the control group (Table 2). Furthermore, all the tests in the Vero cell cytotoxicity assay showed a cytotoxic effect after 6 h of incubation, which progressively increased until 18 h (Fig. 1). The level of cytotoxicity in the imipenem group was lower at 12 h and 18 h than those in any other groups including the respective controls. In contrast, trimethoprim/sulfamethoxazole, norfloxacin, and amikacin had a greater cytotoxic effect on the cultures than the controls.

Bacterial cultures grown with or without the antimicrobial agents and collected at 6, 12, and 18 h were tested for Shiga toxins 1 and 2 activities, as measured by the reversed passive latex agglutination method using anti-Shigatoxin 1 and anti-Shigatoxin 2 antibodies. The RPLA assay showed that the releases of Shiga toxin 1 in the supernatants of all cultures were much lower than that of Shiga toxin 2 (Figs. 2 and 3). In addition, the levels of Shiga toxin 1 in the supernatants of the cultures treated with the antimicrobial agents did not change significantly compared with the controls. However, a suppressive effect on the release of Shiga toxin 2 was observed in cultures containing imipenem. In contrast, the levels of Shiga toxin 2 in the supernatants of the cultures treated with trimethoprim/sulfamethoxazole, norfloxacin, and amikacin were higher than those of the controls, particularly at 12 h and 18 h incubations (Fig. 3).

The use of antimicrobial agents in EHEC infection is controversial because their use is believed to increase the risk of severer diseases [11, 12]. Certain antibiotics induce Shiga toxin-encoding bacteriophages, which lead to increased expression of the Shiga toxin genes. In addition, some antibiotics may also cause bacterial lysis, which can further increase the levels of free Shiga toxin in the intestinal tract [18, 20]. Overuse or misuse of these types of antibiotics in the veterinary medicine can induce and increase the Shiga toxin productions, which may result in causing severe diseases in animals. The Vero cell cytotoxicity assay in this study showed that the levels of

Table 2. Viable bacterial counts ($\log_{10}$ CFU/ml) of EHEC strains treated with norfloxacin, imipenem, amikacin, and trimethoprim/sulfamethoxazole at 1/4 MIC.

	E. coli O26			*E. coli* O111			*E. coli* O157		
	6 h	12 h	18 h	6 h	12 h	18 h	6 h	12 h	18 h
Control	6.71	8.52	8.61	6.66	8.34	8.57	7.15	8.41	8.55
Norfloxacin	6.89	8.32	8.54	7.02	8.21	8.45	6.35	8.23	8.49
Imipenem	7.11	8.47	8.53	6.43	8.56	8.60	6.68	8.33	8.61
Amikacin	6.52	8.61	8.59	6.89	8.62	8.65	6.59	8.12	8.48
Trimethoprim/Sulfamethoxazole	7.02	8.56	8.66	7.21	8.45	8.50	6.88	8.45	8.53

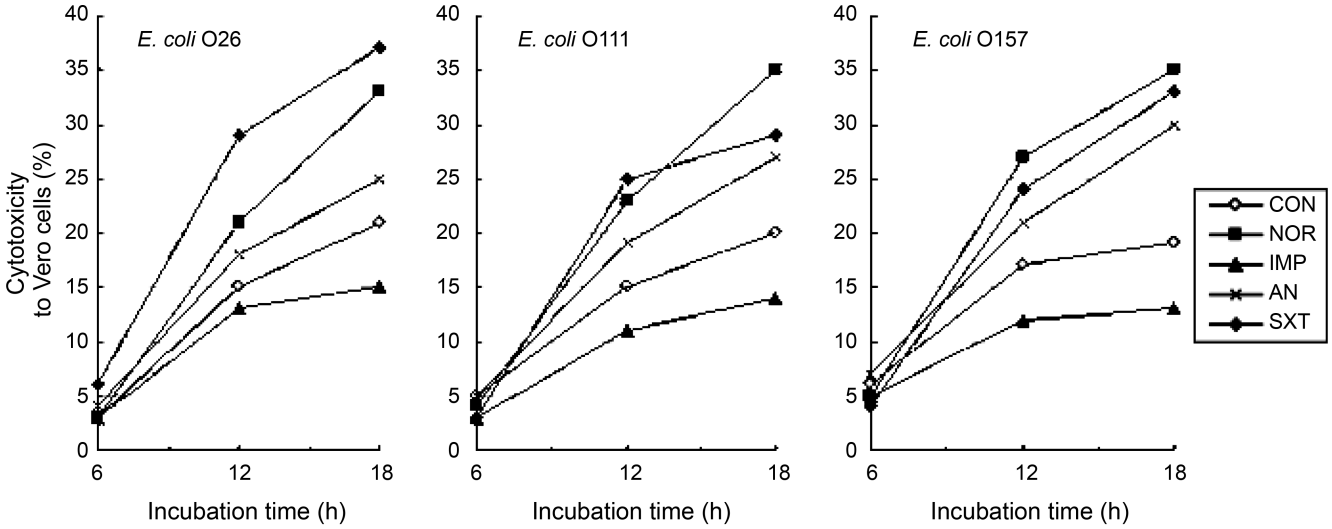

Fig. 1. Effects of the antimicrobial agents on toxin production of the EHEC strains as measured by Vero cell cytotoxicity.
CON, untreated control; NOR, norfloxacin; IMP, imipenem; AN, amikacin; SXT, trimethoprim/sulfamethoxazole.

cytotoxicity in the culture groups treated with trimethoprim/sulfamethoxazole, norfloxacin, and amikacin were higher than the respective controls. In addition, the RPLA assay also showed that the levels of the Shiga toxin 2 in the supernatants of the cultures treated with these antibiotics were higher than those of the controls, indicating that the induction of a higher level of cytotoxicity was related to an increased release of Shiga toxin 2 from the cultures treated with the antimicrobial agents. In previous studies, trimethoprimsulfamethoxazole, and fluoroquinolones such as cirprofloxacin and norfloxacin, could increase the yield of Shiga toxins, which are associated with complications such as HUS [4, 5]. In contrast, the cytotoxic effects and the release of Shiga toxin 2 in the cultures treated with imipenem were suppressed compared with the controls, suggesting that imipenem may be a potentially effective and safe agent for treating EHEC infections and that might reduce the incidence of EHEC-associated diseases. Imipenem is a member of the carbapenem antibiotic subclass and changes the morphology of EHEC into a spheroplast [13]. Thus, the release of lower Shiga toxin production may be associated with the alteration of the cell structure. However, the precise mechanism by which imipenem suppresses the production of Shiga toxin remains unclear. Further studies

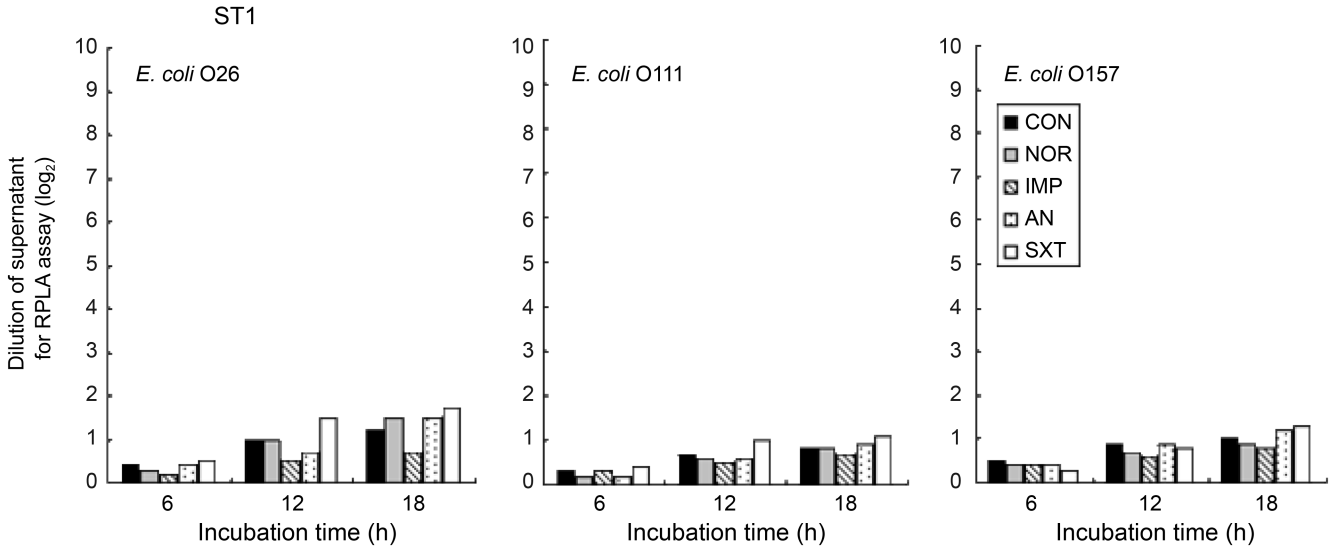

Fig. 2. Effects of the antimicrobial agents on the release of Shiga toxin 1 (ST1) from the EHEC as measured by reversed passive latex agglutination.
CON, untreated control; NOR, norfloxacin; IMP, imipenem; AN, amikacin; SXT, trimethoprim/sulfamethoxazole.

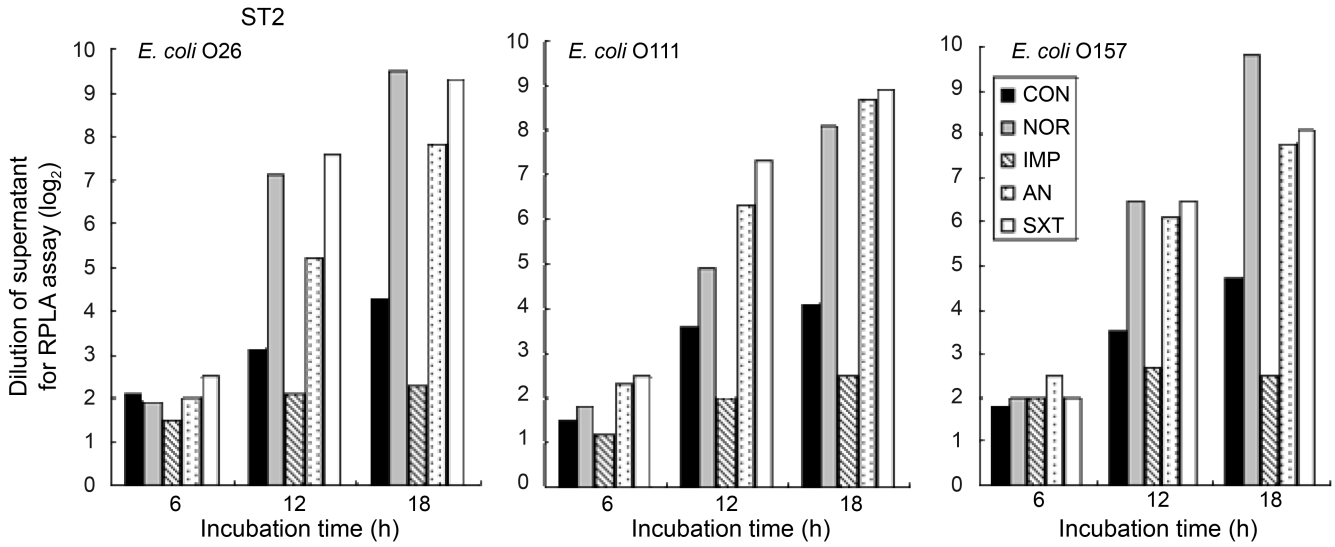

Fig. 3. Effects of the antimicrobial agents on the release of Shiga toxin 2 (ST2) from the EHEC as measured by reversed passive latex agglutination.

CON, untreated control; NOR, norfloxacin; IMP, imipenem; AN, amikacin; SXT, trimethoprim/sulfamethoxazole.

are needed to clarify the mechanism by which this antimicrobial agent suppresses Shiga toxin production.

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