In vitro bactericidal activity of antimicrobial agents against enterohaemorrhagic Escherichia coli

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Objectives: In vitro bactericidal activity of four antimicrobial agents was determined against nine strains of enterohaemorrhagic E. coli.

Methods: Pulsed-field gel electrophoresis was carried out with the Bio-Rad Gene Path system. Each antimicrobial agent was added to logarithmic phase of enterohaemorrhagic E. coli (four strains of E. coli O157:H7, two of E. coli O26, two of E. coli O111, and one of E. coli O165) in broth to obtain a concentration of 10 or 50 mg/L, and viable cells were counted after 1, 2, 6 and 24 h.

Results: All nine strains were confirmed to differ in their DNA pattern by pulsed-field gel electrophoresis. Norfloxacin at concentrations of 10 and 50 mg/L had bactericidal effects on all nine strains of enterohaemorrhagic E. coli. However, cefoperazone, kanamycin and fosfomycin had no bactericidal effects on some strains. In particular, after addition of 10 mg/L fosfomycin or kanamycin, four of the nine strains showed proliferation.

Conclusions: Norfloxacin had marked bactericidal effects on enterohaemorrhagic E. coli. This information could be of value in planning randomized clinical trials of antimicrobial agents as treatment for enterohaemorrhagic E. coli infection.

Keywords: killing kinetics, norfloxacin, cefoperazone, kanamycin, fosfomycin

Introduction

The role of antimicrobial agents in the management of enterohaemorrhagic E. coli infection remains under debate.1–13 Bactericidal agents, however, may be necessary for the treatment of life-threatening enterohaemorrhagic E. coli infections.12 There is also evidence that subinhibitory concentrations of some antimicrobial agents may stimulate verotoxin production.14 In Japan, fosfomycin is the most commonly used agent because of its minimal side effect profile; fluoroquinolones and cephem antibiotics are also used.15 Studies have evaluated the minimum inhibitory concentrations (MICs) of these agents, but there are few data about bactericidal activity and the most effective drug for this infection is still unknown.16–22 Therefore, to determine the most effective agent for enterohaemorrhagic E. coli, we evaluated the in vitro bactericidal activity of various antimicrobial agents against this bacterium.

Materials and methods

Bacterial strains

Four strains of E. coli O157:H7, two of E. coli O26, two of E. coli O111, and one of E. coli O165 were used. The four E. coli O157:H7 strains had been isolated in outbreaks in Japan: one strain in an outbreak in Osaka Prefecture (12,680 patients) in 1996 (E. coli O157 Osaka),15,23 one in an outbreak in Iwate Prefecture (220 patients) in 1996 (E. coli O157 Iwate), one in an outbreak in Saitama Prefecture (67 patients) in 2001 (E. coli O157 Saitama), and one in an outbreak in Yamaguchi Prefecture (26 patients) in 2001 (E. coli O157 Yamaguchi). The two strains of E. coli O26 (E. coli O26-1 and E. coli O26-2), two strains of E. coli O111 (E. coli O111-1 and E. coli O111-2), and one strain of E. coli O165 had been isolated from five patients with sporadic infection in Yamaguchi Prefecture, Japan in 2001.
Killing test

Each drug was added to 5 mL of Sensitivity Test broth (Nissui Pharm., Tokyo) containing log-phase inocula (final concentration, approximately 5 x 10^8 cfu/mL) to obtain a drug concentration of 10 or 50 mg/L. Viable cells were counted after incubation at 37°C for 1, 2, 6 and 24 h. The evaluated agents were norfloxacin (Kyorin Pharm., Tokyo), cefoperazone sodium (Pfizer Pharm., Inc., USA), kanamycin sulphate (Meiji Seika Co., Tokyo), and fosfomycin sodium (Meiji Seika Co., Tokyo). In fosfomycin killing tests, 25 mg/L of glucose-6-phosphate (Oriental Yeast Co., Ltd., Tokyo) was also used. The two agents were combined because in vitro susceptibility tests for fosfomycin have been standardized by adding 25 mg/L of glucose-6-phosphate, and glucose-6-phosphate is present physiologically wherever glycolysis takes place. The three agents excluding norfloxacin were provided in the form of a freeze-dried amorphous powder.

Viable cells were counted by a membrane filter filtration method to prevent antimicrobial agent carry-over. The broth (0.5 mL) was filtered through a 0.22 μm membrane filter (diameter, 5 cm; Nippon Becton Dickinson Co., Japan), and sterile saline (100 mL) was filtered a total of four times. Subsequently, this membrane filter was placed in a bottle containing 10 mL sterile physiological saline, and this bottle was ultrasonicated (Sine Sonic 100, Ikemoto Rikagaku Co., Japan) at 37 kHz for 10 min. Each sample of physiological saline from this bottle was filtered 10^-1 to 10^-3-fold in sterile saline; two aliquots (0.5 mL each) of 10^-1 to 10^-3-fold dilution and of an undiluted sample were plated on two trypticase soy agar plates (Nippon Becton Dickinson Co., Japan). Viable cells were counted after 24 h incubation at 37°C. The experiments were carried out twice, and the mean values were calculated.

The MICs were also determined for the nine strains of enterohaemorrhagic E. coli after 18 h incubation at 37°C by dilution on Sensitivity Test agar (Nissui Pharm., Tokyo). Twofold serial dilutions ranging from 128 to 0.008 mg/L were employed. The inocula (~10^5 cfu/spot) were plated using a multipoint inoculator (Sakuma Co., Japan). MICs were defined as the lowest concentration of agent inhibiting visible growth.

Pulsed-field gel electrophoresis

The preparation of high molecular weight chromosomal DNA was carried out according to Murray et al. Digestion was carried out by placing a small slice of an agarose plug in 200 μL of reaction buffer with 30 U of XbaI. Pulsed-field gel electrophoresis was carried out with the Bio-Rad Gene Path system in a 1% agarose gel in 0.5 x TBE buffer at 14°C with a linear ramp time of 4 to 50 s over a period of 20 h. Thereafter, the gels were stained with ethidium bromide and photographed.

Data analysis

The effects on the viability of nine strains of enterohaemorrhagic E. coli were compared among control (without drugs), norfloxacin, cefoperazone, kanamycin and fosfomycin by the Kruskal–Wallis test.

Results

Although the pulsed-field gel electrophoresis results are not shown, all nine strains used were confirmed to differ in their DNA pattern. Table 1 shows the MICs of four agents against the nine strains of enterohaemorrhagic E. coli. Figure 1 and Table 2 show log viable cells after addition of each antimicrobial agent (10 mg/L) to the nine enterohaemorrhagic E. coli strains (the minimum level of detection was 20 cfu/mL). Norfloxacin had bactericidal effects on all strains. However, after addition of cefoperazone, kanamycin and fosfomycin, one, four and four strains, respectively, showed proliferation (Figure 1). Figure 2 and Table 3 show log viable cells after addition of each antimicrobial agent (50 mg/L) to the nine strains of enterohaemorrhagic E. coli. Norfloxacin and kanamycin had bactericidal effects on all strains. However, after addition of cefoperazone and fosfomycin, one and two strains, respectively, showed proliferation (Figure 2). Among the four antimicrobial agents, norfloxacin had the most marked bactericidal effects.

Discussion

Bactericidal agents may be necessary in enterohaemorrhagic E. coli infection. There is also evidence that subinhibitory concentrations of some antimicrobial agents may stimulate verotoxin production. Therefore, we evaluated the bactericidal activity of four antimicrobial agents (norfloxacin as a fluoroquinolone, cefoperazone as a bile excreting-type cepham antibiotic, kanamycin as an aminoglycoside antibiotic, and fosfomycin widely used for this infection in Japan) against four strains of E. coli O157:H7 that caused outbreaks and a total of five strains of E. coli O26, E. coli O111 and E. coli O165. The concentrations of the agents used were determined to be 10 and 50 mg/L. Though there were no data on the intestinal concentrations of the agents, we selected 10 and 50 mg/L, considering

Table 1. MICs (mg/L) of four antimicrobial agents against nine strains of enterohaemorrhagic E. coli

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>O157-Osaka</th>
<th>O157-Iwate</th>
<th>O157-Saitama</th>
<th>O157-Yamaguchi</th>
<th>O26-1</th>
<th>O26-2</th>
<th>O111-1</th>
<th>O111-2</th>
<th>O165</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norfloxacin</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
<td>0.06</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>0.25</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
<td>0.5</td>
<td>1</td>
<td>0.12</td>
<td>0.25</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Fosfomycin*</td>
<td>8</td>
<td>0.25</td>
<td>0.25</td>
<td>0.5</td>
<td>0.25</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>4</td>
</tr>
</tbody>
</table>

*With 25 mg/L glucose-6-phosphate added.
that the intestinal concentrations of agents may be lower than their faecal or biliary concentrations. As for the oral administration of 400 mg norfloxacin to five volunteers, its faecal concentrations were reported to be 48–605 μg/g after 12–24 h.\textsuperscript{27} Concerning cefoperazone, within 65 min after intravenous injection of 1 g to four patients, the maximum concentrations were reported to be 373.4–3100 μg/mL in common duct bile and 6.8–680 μg/mL in gallbladder bile.\textsuperscript{28} After oral administration of 6.0 g kanamycin, assays of faeces from four patients revealed concentrations of 9300–17 500 μg/g in the faeces collected during the first 24 h after initiation of therapy.\textsuperscript{29} In addition, 3 h after oral administration of 30–80 mg/kg per day fosfomycin to 14 patients, its faecal concentrations were reported to be 27.7–5807 μg/g (mean, 853 μg/g).\textsuperscript{30} 

Norfloxacin had bactericidal effects on all the nine strains, showing the highest bactericidal activity among the four agents.

**Figure 1.** Bactericidal effects of antimicrobial agents (10 mg/L) on nine strains of enterohaemorrhagic *E. coli* (37°C); open circle, norfloxacin; open triangle, cefoperazone; open square, kanamycin; inverted open triangle, fosfomycin with 25 mg/L glucose-6-phosphate; closed circle, control.
Some studies have shown a significant decrease in the incidence of haemolytic uraemic syndrome (HUS) after administration of appropriate antimicrobial agents such as fluoroquinolones for *E. coli* O157:H7 infection, or a significant decrease in the mortality rate after administration of a fluoroquinolone for this infection in a mouse model. Therefore, fluoroquinolones such as norfloxacin may be useful for treating enterohaemorrhagic *E. coli* infection. On the other hand, cefoperazone, kanamycin and fosfomycin had no bactericidal effects on some strains. In particular, fosfomycin’s low bactericidal activity suggests that it may not be the optimal agent for treatment of enterohaemorrhagic *E. coli* infection.

Figure 1. (continued)

Figure 2. Bactericidal effects of antimicrobial agents (50 mg/L) on nine strains of enterohaemorrhagic *E. coli* (37°C); open circle, norfloxacin; open triangle, cefoperazone; open square, kanamycin; inverted open triangle, fosfomycin with 25 mg/L glucose-6-phosphate; closed circle, control.
Only a small number of strains of enterohaemorrhagic *E. coli* were studied, but they were chosen to include representative strains from large outbreaks. In addition, pulsed-field gel electrophoresis confirmed that the study strains were all distinct. Our preliminary study has shown that the MICs for these nine strains of enterohaemorrhagic *E. coli* were low (less than or equal to 8 mg/L) for all antimicrobial agents studied, however the bactericidal activity differed markedly, with norfloxacin showing the highest level of activity. This possible activity of norfloxacin against enterohaemorrhagic *E. coli* may be of value in planning future clinical trials of antimicrobial agents for the management of enterohaemorrhagic *E. coli* infection.

Figure 2. *(continued)*
Table 2. Bactericidal effects of antimicrobial agents (10 mg/L) on nine strains of enterohaemorrhagic E. coli (logarithm of viable counts; 37°C)

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Time of exposure (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>1.71 ± 1.07*</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>1.79 ± 1.09*</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>5.37 ± 0.39</td>
</tr>
<tr>
<td>Fosfomycin*</td>
<td>2.08 ± 0.40</td>
</tr>
<tr>
<td>Without drugs (control)</td>
<td>6.62 ± 0.12</td>
</tr>
</tbody>
</table>

*With 25 mg/L glucose-6-phosphate. *Significant difference from the control (P < 0.05).

Table 3. Bactericidal effects of antimicrobial agents (50 mg/L) on nine strains of enterohaemorrhagic E. coli (logarithm of viable counts; 37°C)

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Time of exposure (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>1.86 ± 0.96*</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>1.90 ± 0.69*</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>5.84 ± 0.44</td>
</tr>
<tr>
<td>Fosfomycin*</td>
<td>2.16 ± 0.58</td>
</tr>
<tr>
<td>Without drugs (control)</td>
<td>6.64 ± 0.44</td>
</tr>
</tbody>
</table>

*With 25 mg/L glucose-6-phosphate. *Significant difference from the control (P < 0.05).

Acknowledgements

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References

In vitro susceptibilities of enterohaemorrhagic E. coli


