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ORIGINAL ARTICLE

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Influence of various antimicrobial agents on the intestinal flora in an intestinal MRSA-carrying rat model

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Abstract Using an intestinal methicillin-resistant *Staphylococcus aureus* (MRSA)-carrying rat model, we compared the influence of piperacillin (PIPC) on the intestinal flora to those of cefazolin (CEZ), cefmetazole (CMZ), and flomoxef (FMOX). The number of MRSA did not increase after PIPC and CEZ administrations compared with the nontreated group. However, it significantly increased in the cases of FMOX and CMZ administration ($P < 0.01$). In the FMOX- and CMZ-treated groups, the intestinal flora was severely disrupted and the recovery of the number of *Escherichia coli* and *Bacteroides* spp. cells was delayed. On the other hand, in the PIPC- and CEZ-treated groups, the rapid recovery of bacteria that composed the intestinal flora was observed. The C_{max}/MIC_{50} and C_{trough}/MIC_{50} ratios in *E. coli* and *Bacteroides* spp. in the case of FMOX and CMZ were relatively higher than those in the case of the PIPC- and CEZ-treated groups.

Key words MRSA · Animal model · Intestinal flora

Introduction

For surgery in the Department of Enterological Surgery, administration of antimicrobial agents such as cephems affects the intestinal flora, and proliferation of intestinal methicillin-resistant *Staphylococcus aureus* (MRSA) sometimes leads to enteritis when patients are MRSA carriers.[1] Before the 1980s, most bacteria isolated from patients with postoperative infection were gram-negative bacilli, and the common use of third-generation cephems had rapidly increased the incidence of MRSA infection. Thereafter, first-generation or second-generation cephems or penicillin antimicrobial agents have been used as a first-choice agent immediately after surgery; however, recently, antimicrobial agents have been selected in accordance with differences in the grade of contamination during surgery or purpose of administration, that is, prophylactic or therapeutic administration.[2]

To experimentally verify an increase in the number of MRSA bacteria in the intestinal tract related to administration of antimicrobial agents, previous studies used an intestinal MRSA-carrying rat model, and reported that a third-generation cephem, latamoxef, disordered the intestinal flora, inducing an increase in the MRSA count, and that a second-generation cephem, cefotiam, did not increase the MRSA count without disordering the intestinal flora.[3,4] On the other hand, the influence of penicillin on the intestinal flora was not yet studied. In this study, we investigated changes in the intestinal tract number of MRSA bacteria using a broad-spectrum penicillin, piperacillin (PIPC), and compared the results to those for cephems used for surgery in the Department of Enterological Surgery, cefazolin (CEZ), cefmetazole (CMZ), and flomoxef (FMOX).

Materials and methods

Preparation of an intestinal MRSA-carrying rat model

Seven-week-old male Wistar/ST rats were used to prepare an intestinal MRSA-carrying rat model. As an MRSA bacterial strain, *Staphylococcus aureus* F-3099 strain was used. This strain was isolated from Third Department of Surgery, Toho University School of Medicine, Tokyo, Japan and produced type II coagulase, enterotoxin type C, and toxic shock syndrome toxin 1 (TSST-1). The minimum inhibitory

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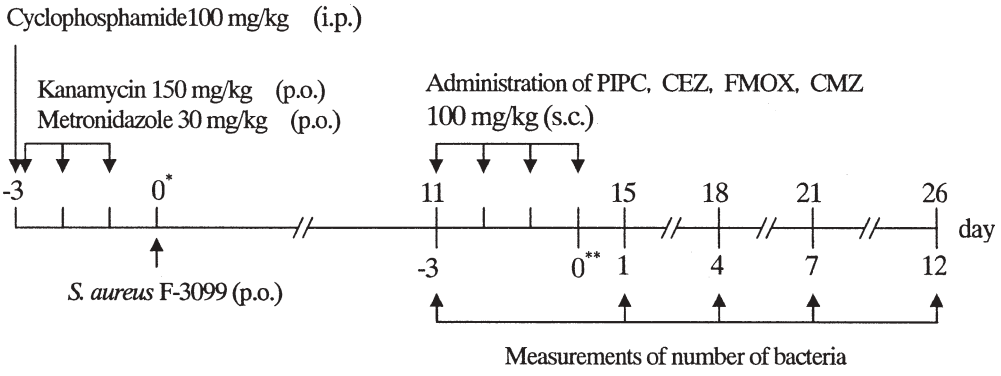

Fig. 1. Preparation of an intestinal methicillin-resistant *Staphylococcus aureus* (MRSA)-carrying rat model and schedule of administration. *Upper schedule: "0" is the day when *S. aureus* F-3099 is orally inoculated; **lower schedule: "0" is the final day administered

concentration (MIC) of oxacillin (MPIPC; Sigma-Aldrich, St. Louis, MO, USA), piperacillin (PIPC; Toyama Chemical, Tokyo, Japan), cefazolin (CEZ; Fujisawa Pharmaceutical, Osaka, Japan), flomoxef (FMOX; Shionogi, Osaka, Japan), and cefmetazol (CMZ; Sankyo, Tokyo, Japan) against this strain were 100, 100, 100, 25, and 25 μg/ml, respectively. The method of preparing this model and the administration schedule of antimicrobial agents are shown in Fig. 1. Three days before infection, 100 mg/kg cyclophosphamide (Shionogi) was intraperitoneally administered to the rats, and from the same day, 150 mg/kg kanamycin (KM; Sigma-Aldrich) or 30 mg/kg metronidazole (MTN; Shionogi) was orally administered once a day for 3 days. The day after the end of administration of KM or MTN, approximately 10^9 colony-forming units (CFU)/rat of *S. aureus* F-3099 was orally inoculated. The day after inoculation of *S. aureus* F-3099 or later, the fecal numbers of viable MRSA, *Escherichia coli*, *Bacteroides* spp., *Enterococcus* spp., all aerobic bacteria, and also all anaerobic bacteria were measured and were regarded as the numbers of viable bacteria in the intestinal flora. Mannitol salt agar (Eiken Chemical, Tokyo, Japan) including oxacillin at 6.25 μg/ml for MRSA, MacConkey agar (Eiken) for *Escherichia coli*, EF agar (Nissui Seiyaku, Tokyo, Japan) for *Enterococcus* spp., Bacteroides agar (Nissui) for *Bacteroides* spp., brain heart infusion agar (Eiken) for all aerobic bacteria, and GAM agar modified (Nissui) for all anaerobic bacteria were used, respectively.

In rats that continuously excreted approximately 10^4 CFU/g feces of MRSA, administration of 100 mg/kg PIPC, CEZ, FMOX, or CMZ was started 11 days after bacterial inoculation, and these agents were subcutaneously administered once a day for 4 days. Fecal specimens were collected every day ($n = 4$), and changes in the fecal number of viable intestinal flora bacteria were investigated. To compare the number of MRSA bacteria between the groups treated with antimicrobial agents and the untreated group, the significance was tested using Dunnett's test (software; SAS system Ver. 8.2).

Confirmation of antimicrobial activity and β-lactamase activity

A portion of feces was collected from intestinal MRSA-carrying rats before administration of antimicrobial agents, and *Escherichia coli* and *Bacteroides* spp. were isolated. Then, 26 and 14 strains were randomly selected, respectively, and the MICs of PIPC, CEZ, FMOX, and CMZ were measured. MIC values were measured by the agar dilution method in accordance with the standard procedure established by the Japanese Society of Chemotherapy.[5,6] In addition, the presence or absence of β-lactamase production in these strains was confirmed using Identification Sticks β-Lactamase (Nitrocefin; Oxoid, Basingstohe, Hampshire, UK), and the percentage of β-lactamase-producing strains in feces was calculated.

Measurement of the small intestine content concentrations of antimicrobial agents

A single dose of PIPC, CEZ, FMOX, or CMZ at 100 mg/kg was subcutaneously administered to intestinal MRSA-carrying rats ($n = 4$). Subsequently, approximately 1 g intestinal contents was collected from the small intestine of these rats 0.5, 1, 3, 6, and 24 h (C_{trough}) after administration, mixed with an equivalent volume of methanol, and centrifuged. The supernatant was used for the determination of drug concentration by high-performance liquid chromatography (HPLC). In all agents, a STR-ODSII column was used. As the mobile phase, a mixture, CH_3CN: 1 M CH_3COOH: 1 M CH_3COONa: H_2O, at ratios of 210:5:20:765, 100:5:20:875, 150:5:20:825, and 130:5:20:845, were used to measure PIPC, CEZ, FMOX, and CMZ levels, respectively. PIPC was detected at an absorption wavelength of 220 nm, whereas CEZ, FMOX, and CMZ were detected at an absorption wavelength of 254 nm.

Histopathological observation of intestine

In intestinal MRSA-carrying rat model, on the 11th day after MRSA infection, the cecum was removed. After fixation with 10% phosphate-buffered formalin and staining with hematoxylin and eosin, histopathological observation was performed.

Results

Influence of antimicrobial agents on the intestinal flora

One of the histopathological findings in the cecum of a rat on the 11th day after MRSA infection is shown in Fig. 2. Marked thickness of the internal cecal wall and severe hemorrhage were observed in the intestine of the MRSA-infected rat. Edema of the muscular layer was also observed.

Changes in the fecal numbers of viable MRSA, *E. coli*, *Bacteroides* spp., *Enterococcus* spp., all aerobic bacteria, and all anaerobic bacteria between day −3 and day 12 (the day when administration of antimicrobial agents was completed, day 0) are shown in Fig. 3. The day after the end of administration, the numbers of viable bacteria of all bacterial species were decreased in the FMOX- or CMZ-treated groups. Four days after the end of administration, the numbers of *Enterococcus* spp., all aerobic bacteria, and all anaerobic bacteria returned to the pretreatment values, but the number of *E. coli* was not different from that of the day after the end of administration in the FMOX- or CMZ-treated groups. In addition, the number of *Bacteroides* spp. did not completely return to the pretreatment value, and improvement in the numbers of the two kinds of bacteria was delayed. Simultaneously, the number of MRSA bacteria was approximately 10^9 CFU/g feces and was significantly larger than that in the untreated group ($P < 0.01$).

In the PIPC-treated group, the numbers of viable bacteria of all bacterial species were transiently decreased the day after the end of administration, as observed in the FMOX- or CMZ-treated groups. However, there was no delay in the improvement of the numbers of *E. coli* or *Bacteroides* spp., and there was also no significant increase in the number of viable MRSA. In the CEZ-treated group, the numbers of viable *E. coli* and *Bacteroides* spp. were decreased to about 1/100 of the pretreatment values; however, there was no delay in the improvement of the numbers of these bacteria, and there was also no significant increase in the number of viable MRSA.

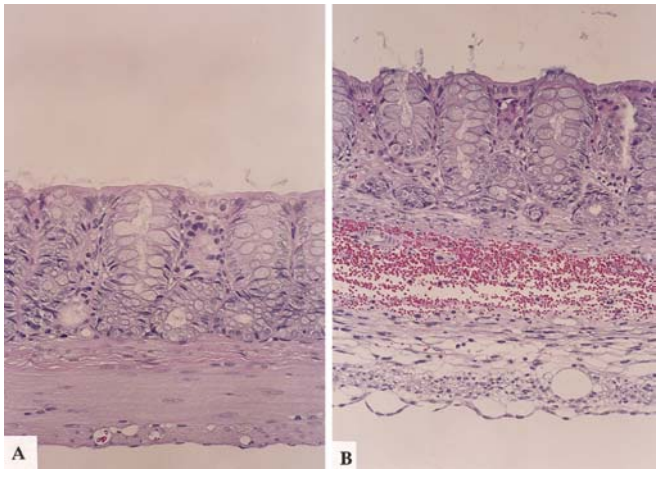

Fig. 2A,B. Histopathological findings of cecum. **A** Normal rat; **B** MRSA-carrying rat 11 days after inoculation

Antimicrobial activity against *E. coli* and *Bacteroides* spp. isolated from the feces of intestinal MRSA-carrying rats

The MIC_{50} and MIC ranges of PIPC, CEZ, FMOX, and CMZ against 26 strains of *E. coli* and 14 strains of *Bacteroides* spp. isolated from the feces of intestinal MRSA-carrying rats before administration of antimicrobial agents are shown in Table 1. The MIC_{50} values of PIPC, CEZ, FMOX, and CMZ against *E. coli* were 1.56, 1.56, 0.10, and 1.56 μg/ml, respectively. These results show that the antimicrobial activity of FMOX is 16 times higher than that of other antimicrobial agents. Furthermore, the MIC_{50} values of PIPC, CEZ, FMOX, and CMZ against *Bacteroides* spp. were 25, 25, 0.39, and 3.13 μg/ml, respectively, which also show that the antimicrobial activity of FMOX is 64 times higher than that of PIPC or CEZ and that it is 8 times higher than that of CMZ.

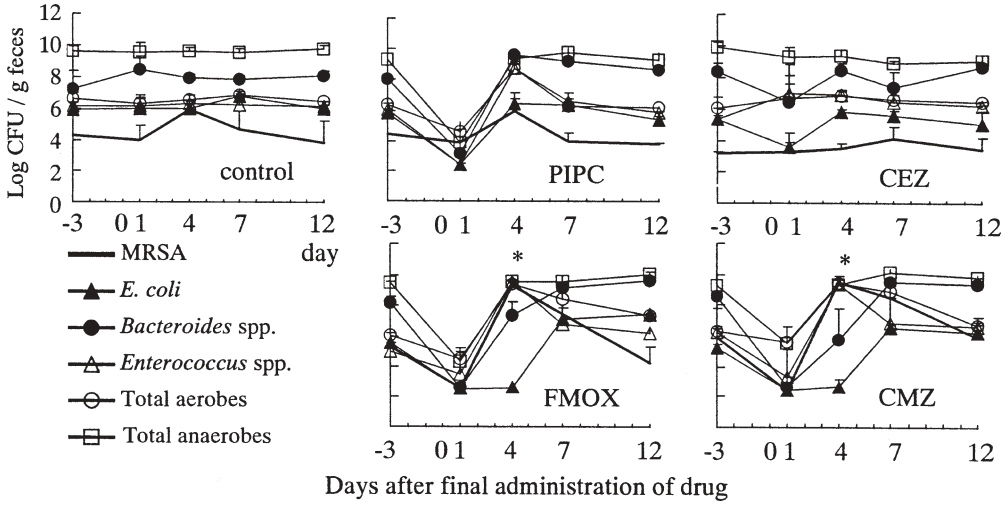

Fig. 3. Changes of intestinal flora in MRSA-carrying rats feces before and after administration of drugs. Mean ± SD ($n = 4$): *$P < 0.01$ vs. colony-forming units (CFU) of MRSA in control by Dunnett's test. PIPC, piperacillin; CEZ, cefazolin; FMOX, flomoxef; CMZ, cefmetazole

Production of β-lactamase was observed in 18 (69.2%) of the 26 *E. coli* strains and in all of the 14 *Bacteroides* spp. strains (100%) isolated from fecal specimens.

Ratios of the small intestinal content concentrations of various antimicrobial agents to their MICs

The maximal small intestinal content concentration (C_{max}), time to C_{max} (T_{max}), and the concentration 24 h after administration (C_{trough}) of drugs after single-dose subcutaneous administration of 100 mg/kg of PIPC, CEZ, FMOX, or CMZ to intestinal MRSA-carrying rats are shown in Table 2. The C_{max} of CMZ was 3.5×10^3 μg/g, being highest among the C_{max} values of the agents used in this study. The C_{trough} was 3.6×10^1 μg/g. The C_{max} of CEZ was 6.4×10^2 μg/g, being the lowest among the C_{max} values of the agents used in this study; however, this agent in small intestinal contents disappeared most slowly among the agents used in this study, and the C_{trough} value was 7.1×10^1 μg/g. PIPC and FMOX showed similar changes in concentration, and C_{max} values were 1.8×10^3 and 2.2×10^3 μg/g, respectively, whereas the C_{trough} values were 4.8 μg/g and 8.3 μg/g, respectively.

The ratios of the small intestinal content C_{max} or C_{trough} of each antimicrobial agent to MIC_{50} against *E. coli* or *Bacteroides* spp. are also shown in Table 2. The C_{max}/MIC_{50} ratios of PIPC and CEZ for *E. coli* were 1200 and 410, respectively, and were approximately 1/18 and 1/54 in comparison to that of FMOX (22000), respectively. The C_{trough}/MIC_{50} ratios of PIPC and CEZ were 3.1 and 46, respectively, and were approximately 1/27 and 1/1.8 in comparison to that of FMOX (83), respectively.

Furthermore, the C_{max}/MIC_{50} ratios of PIPC and CEZ for *Bacteroides* spp. were 72 and 26, respectively, and were approximately 1/78 and 1/220 in comparison to that of FMOX (5600), respectively. On the other hand, the C_{trough}/MIC_{50} ratios of PIPC and CEZ were 0.19 and 2.8 and were approximately 1/110 and 1/7.5 in comparison to that of FMOX (21), respectively.

The C_{max}/MIC_{50} and C_{trough}/MIC_{50} ratios of FMOX were markedly higher than those of PIPC and CEZ. The C_{max}/MIC_{50} and C_{trough}/MIC_{50} ratios of CMZ were also higher than those of PIPC and CEZ, as demonstrated for FMOX.

Table 1. Minimum inhibitory concentration (MIC) for *Escherichia coli* and *Bacteroides* spp. in feces before administration

Agents	MIC (μg/ml)			
	E. coli[a]		*Bacteroides* spp.[b]	
	Range	MIC_{50}	Range	MIC_{50}
PIPC	1.56–3.13	1.56	12.5–25	25
CEZ	0.78–1.56	1.56	25–100	25
FMOX	0.05–0.10	0.10	0.39–0.78	0.39
CMZ	0.78–1.56	1.56	3.13–6.25	3.13

PIPC, piperacillin; CEZ, cefazolin; FMOX, flomoxef; CMZ, cefmetazole
[a] 26 strains, β-lactamase producing strain: 69.2%
[b] 14 strains, β-lactamase producing strain: 100.0%

Discussion

Infection caused by MRSA has been increasing in Japan since the late 1980s.[7–10] Enterocolitis resulting from MRSA, one of the serious postoperative complications, was noted because this infection commonly accompanies a rapid aggravation and, if there is a delay in the start of treatment, then the outcome can sometimes be fatal.[11,12] For this reason, the usual regimen of antibiotics for the prevention of postoperative infection has become a serious issue.[13] Yoshida demonstrated by means of basic studies that the alteration of the intestinal flora caused by some antibiotics resulted in the proliferation of MRSA in the intestine and that there was a causative relationship between some antibiotics and MRSA proliferation.[14] However, it has not been studied whether CEZ, CTM, PIPC, FMOX, and CMZ, which are commonly used clinically for the prevention of postoperative infection in surgery, have an effect on the proliferation of MRSA in the intestine. Therefore, in the present study, using an intestinal MRSA-carrying rat model, we investigated changes in the intestinal tract number of MRSA bacteria using a broad-spectrum penicillin, PIPC, and compared the results to those for cephems such as CEZ, CMZ, and FMOX.

A transient decrease in the number of enteric bacteria including *E. coli* and *Bacteroides* spp. was confirmed in the PIPC-treated group; however, no bacterial species showed more than a 4-day delay in the improvement of the number of bacteria, and a significant increase in the number of

Table 2. C_{max}, T_{max}, and C_{trough} of antibacterial agents and ratio of drug concentration in intestinal contents and MIC for *E. coli* and *Bacteroides* spp.

Antibacterial agent	C_{max} (T_{max}) (μg/g, (h))	C_{trough} (μg/g)	*E. coli*		*Bacteroides* spp.	
			C_{max}/MIC_{50}	C_{trough}/MIC_{50}	C_{max}/MIC_{50}	C_{trough}/MIC_{50}
PIPC	1.8×10^3, (3)	4.8×10^0	1200	3.1	72	0.19
CEZ	6.4×10^2, (3)	7.1×10^1	410	46	26	2.8
FMOX	2.2×10^3, (3)	8.3×10^0	22000	83	5600	21
CMZ	3.5×10^3, (1)	3.6×10^1	2200	23	1100	12

C_{max}, mean of maximum concentration of small intestinal content after drug administration ($n = 4$); T_{max}, time to C_{max}; C_{trough}, mean of small intestinal content concentration at 24 h after drug administration ($n = 4$)

MRSA bacteria was not induced, differing from the FMOX-treated group. In the CEZ-treated group, decreases in the numbers of *E. coli* and *Bacteroides* spp. were noted; however, no bacterial species showed more than a 4-day delay in improvement of the number of bacteria, and a significant increase in the number of MRSA bacteria was not induced, as observed in the PIPC-treated group. In the CMZ-treated group, a decrease in the number of enteric bacteria including *E. coli* and *Bacteroides* spp. was observed; however, after the decrease in the number of enteric bacteria, a significant increase in the number of MRSA bacteria was observed with improvement in the intestinal flora, as observed in the FMOX-treated group. Furthermore, improvement in the number of *E. coli* bacteria required 4 days or more, and improvement in the number of *Bacteroides* spp. bacteria was delayed compared to that in both the PIPC- and CEZ-treated groups.

When the PIPC- and CEZ-treated groups were compared to the FMOX- and CMZ-treated groups, the significant increase in the number of MRSA bacteria in the latter may have been related to the delay in the improvement of the numbers of viable *E. coli* and *Bacteroides* spp. bacteria. Furthermore, the C_{max}/MIC_{50} and C_{trough}/MIC_{50} ratios of FMOX and CMZ, which resulted in a delay in the improvement of the numbers of *E. coli* and *Bacteroides* spp. bacteria, were higher than those of PIPC and CEZ. It was suggested that maintaining a high C_{max}/MIC_{50} and C_{trough}/MIC_{50} ratio of antimicrobial agents for a long duration inhibited additional proliferation of *E. coli* and *Bacteroides* spp. over a long period. In terms of the mode of action of β-lactam with time dependency, it was considered that high C_{trough}/MIC_{50} ratios for *E. coli* and *Bacteroides* were especially important for the proliferation of MRSA in the small intestinal tract.

Kusachi et al. used CEZ and CTM as antibiotics for the prevention of postoperative infections in elective surgery during the period between 1990 and 1997 when the incidence of postoperative infections caused by MRSA in Japan had been increasing dramatically, and consequently has achieved successful control of postoperative MRSA infection, the incidence having decreased to below 1% in all cases of digestive tract surgery performed.[15] The present basic study carried out by us corresponded with these clinical outcomes, and demonstrated a decrease in the isolation ratio of MRSA clinically. In the *Guideline for Prevention of Surgical Site Infection, 1999* presented by the Centers for Disease Control and Prevention (CDC), CEZ is generally recommended and viewed as the surgical antimicrobial prophylaxis (AMP) agent of first choice for both clean operations and clean-contaminated operations.[16] On the grounds that the antibacterial spectrum of PIPC, which includes many gram-negative rods, enterococci, and anaerobes, is broader than that of CEZ, it was considered that PIPC was also a useful antibiotic because PIPC did not induce proliferation of MRSA in the intestinal tract, with less influence on the intestinal flora, as well as CEZ.

In conclusion, PIPC had less influence on the intestinal flora including *E. coli* and *Bacteroides* than FMOX and CMZ in the intestinal MRSA-carrying rat model, as demonstrated for CEZ, suggesting that PIPC does not induce proliferation of MRSA in the intestinal tract.

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