

Incidence of Diarrhea with Antibiotics and the Increase of Clostridia in Rabbits

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ABSTRACT. Rabbits were treated with a single intravenous injection of various antibiotics. More than 40 per cent of the animals showed diarrhea after being treated with sulbactam/cefoperazone, cefmetazole, clindamycin, piperacillin or aspoxicillin. *Clostridium difficile* was isolated from sulbactam/cefoperazone-treated diarrheic rabbits, with their cecal contents showing positive reaction in a latex agglutination test for *C. difficile* enterotoxin. However, 27 cefmetazole-induced diarrheic cases were not associated with *C. difficile*. Other enteropathogenic bacteria, such as *Campylobacter* spp., *Bacillus cereus*, enteropathogenic *Escherichia coli*, coagulase positive *Staphylococcus aureus*, *Salmonella* spp., *Vibrio* spp., *Clostridium perfringens* and *Clostridium spiroforme*, were not isolated from either of diarrheic rabbit. However, the counts of clostridia remarkably increased in the intestine of cefmetazole-associated diarrheic rabbits. This was ascribed to the overgrowth of *Clostridium innocuum* and *Clostridium sporogenes*. There were no remarkable differences in changes in other bacterial population between diarrheic and non-diarrheic rabbits. — **KEY WORDS:** antibiotic, clostridium, diarrhea, intestinal flora, rabbit.

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During clinical treatment, diarrhea has been observed to occur in association with various classes of antibiotics in human [1–3, 18, 22]. Although antibiotic-associated diarrhea has also been observed in animals [7, 8, 12, 14, 19], observations have been limited to relatively few antibiotics, and therefore, whether antibiotic-associated diarrhea takes place in animals is unknown for most antibiotics.

Drastic changes in intestinal microflora are regarded to be involved in the diarrhea caused by some antibiotics. In fact, clindamycin and lincomycin have been reported to cause pseudomembranous colitis associated with the increased population of *Clostridium difficile* in the intestinal flora of human [23] and animals [20]. Clindamycin has also been shown to cause diarrhea in rabbits accompanied by *Clostridium spiroforme*, some strains of which produced iota-like toxin [6]. However, there have been no studies on the mechanisms of diarrhea caused by other many antibiotics.

In order to get insight into antibiotic-associated diarrhea in rabbits in relation to changes in intestinal microflora, we studied the incidence of diarrhea in rabbits treated with many classes of antibiotics, as well as the association of *C. difficile* and changes of intestinal flora to diarrhea.

MATERIALS AND METHODS

Animals and administration of antibiotics: Four female Japanese white rabbits of 2–3 kg body weight were purchased from Funabashi farm (Funabashi, Japan). After being kept for 1 week, the animals were treated with a single intravenous injection of antibiotics. The administration dose of each antibiotic obeyed the Requirements for antibiotic products of Japan (Table 1) [21].

Microbiological methods: Isolation of *C. difficile* from

antibiotic-associated diarrheic rabbits: Diarrheic rabbits were killed by CO₂ asphyxiation. The cecal contents were aseptically collected, and inoculated on cycloserine cefoxitin fructose agar (OXOID, Hampshire) plates and then incubated at 37°C in an anaerobic steel-wool jar for 3 days for isolation of *C. difficile*.

Isolation of fecal and cecal bacteria from cefmetazole-treated rabbits: The method of bacteriological analysis of fecal and cecal flora in this study was essentially the same as that of Mitsuoka *et al.* [17]. Feces were collected from four rabbits before cefmetazole administration, on day 2 and 6 after the administration. At the time of occurrence of serious diarrhea, rabbits were killed by CO₂ asphyxiation and the cecal contents were aseptically collected. The feces and cecal contents were diluted in a 10-fold series (10⁻¹ to 10⁻⁸) with anaerobic diluent, and 50 ml of appropriate dilution was inoculated on the following media: glucose blood liver agar (Eiken, Tokyo) for anaerobes; modified Eggerth-Gagnon agar (Eiken) for anaerobes; trypticase soy blood agar (BBL Microbiology System, Cockeysville, Md., U.S.A.) for aerobes; bifidobacteria-selective agar for *Bifidobacterium* spp.; eubacteria-selective agar for *Eubacterium* spp.; neomycin brilliant-green taurocholate blood agar for *Bacteroides* spp.; neomycin nagler (NN) agar for lecithinase-positive *Clostridium* spp.; modified villanella-selective agar for *Veillonella* spp. and *Megasphaera* spp.; modified lactobacilli-selective agar for *Lactobacillus* spp.; triphenyltetrazolium chloride acidine-orange thallous sulfate esculin crystal-violet agar for *Enterococcus* spp. and *Streptococcus* spp.; phenylethylalcohol egg-yolk suspension agar for *Staphylococcus* spp.; potato dextrose agar (Difco Laboratories, Detroit, Mich., U.S.A.) for yeast and molds; deoxycholate hydrogen sulfide lactose (DHL) agar (Eiken) for enterobacteriaceae. Media for anaerobes were cultured at 37°C in anaerobic steel-wool

Table 1. Incidence of diarrhea in rabbits after antibiotics injection and the relevance with *Clostridium difficile*

Antibiotic	(mg/kg)	Incidence of diarrhea ^{a)} (%)		Incidence of reaction in C.D. check D-1 kit ^{c)}	Isolation of <i>C. difficile</i>
Cephems					
sulbactam/cefoperazone	(25/25)	82/126	(65.1) ^{b)}	38/40 ^{d)}	3/3 ^{e)}
cefmetazole	(50)	86/173	(49.7) ^{b)}	0/36 ^{d)}	0/5
cefminox	(100)	11/28	(39.3) ^{b)}	2/3	—
cefoxitin	(50)	9/24	(37.5)	0.4	—
cefoperazone	(50)	25/67	(37.3) ^{b)}	2/5 ^{d)}	—
cefotetan	(50)	11/27	(37.0)	—	—
cefpiramide	(50)	92/264	(34.8) ^{b)}	1/3 ^{b)}	—
cefodizime	(100)	2/6	(33.3)	2/6 ^{d)}	—
cefuzonam	(60)	28/87	(32.2)	—	—
ceftriaxone	(60)	39/137	(28.5) ^{b)}	2/2	—
cefmenoxime	(60)	9/39	(23.1)	—	—
cefpimizole	(50)	19/104	(18.3)	—	—
cefbupreazone	(50)	16/120	(13.3)	—	—
cefotaxime	(100)	15/129	(11.6)	—	—
cefamandole	(200)	7/66	(10.6)	—	—
cefazolin	(50)	39/465	(8.4)	—	—
cefsulodin	(40)	12/151	(7.9)	—	—
ceftazidime	(50)	8/113	(7.1)	2/4	—
ceftizoxime	(50)	3/46	(6.5)	—	—
cefaloridine	(50)	3/55	(5.5)	—	—
cefotiam	(40)	4/92	(4.3)	—	—
cefapirin	(100)	16/290	(3.4)	—	—
cefalotin	(50)	4/155	(2.6)	—	—
ceftezole	(50)	0/22	(0)	—	—
cefuroxime	(50)	0/9	(0)	—	—
Oxacephem					
flomoxef	(200)	48/139	(34.5)	-	—
latamoxef	(200)	14/48	(29.2) ^{b)}	2/4 ^{d)}	—
Penicillins					
piperacillin	(40)	53/125	(42.4) ^{b)}	0/8 ^{d)}	0/2
aspoxicillin	(50)	31/77	(40.3) ^{b)}	1/2	—
cloxacillin	(25)	12/39	(30.8)	—	—
sulbenicillin	(100)	6/20	(30.0)	2/3 ^{b)}	—
carbenicillin	(100)	7/39	(17.9)	0/4	—
hetacillin	(25)	4/34	(11.8)	—	—
ticarcillin	(100)	2/33	(6.1)	—	—
ampicillin	(25)	12/206	(5.8)	—	—
Lincomycins					
clindamycin	(24)	19/42	(45.2) ^{b)}	0/2 ^{b)}	—
lincomycin	(5)	647/191	(35.1) ^{b)}	3/10 ^{d)}	—
Aminoglycosides					
bekanamycin	(10)	1/15	(6.7)	—	—
dibekacin	(10)	2/33	(6.1)	—	—
isepamicin	(10)	8/156	(5.1)	—	—
tobramycin	(10)	4/78	(5.1)	—	—
amikacin	(10)	8/192	(4.2)	0/3	—
gentamicin	(10)	2/68	(2.9)	—	—
netilmicin	(10)	8/299	(2.7)	—	—
sisomicin	(10)	2/75	(2.7)	—	—
ribostamycin	(10)	0/25	(0)	—	—
astromicin	(10)	0/15	(0)	—	—

a) Number of diarrheic rabbits/total number of antibiotic-treated rabbits. b) *Japanese Journal of Chemotherapy*, 39: 211–216, 1991. c) Number of positive rabbits/total number of rabbits tested. A part of diarrheic rabbits were used for examination. This kit were used for examination of *C. difficile* enterotoxin. d) Data combined with our previous data published in *Japanese Journal of Chemotherapy*, 39: 211–216, 1991. e) Number of *C. difficile* isolated rabbits/total number of rabbits tested. A part of diarrheic rabbits were used for examination. —; Not tested.

jars for 3 days. The media for aerobes were aerobically incubated at 37°C for 2 days.

To isolate pathogenic bacteria the following media were used: modified Skirrow agar (Nissui, Tokyo) for *Campylobacter* spp.; NaCl glycine kim goepfert agar (Nissui) for *Bacillus cereus*; thiosulfate citrate bile salt sucrose agar (TCBS, Nissui) for *Vibrio* spp.; DHL agar was used for selection of *Salmonella* and enterotoxin-producing *Escherichia coli*; NN agar for *Clostridium perfringens*.

Isolates were grouped to genus according to colonial and cellular morphology, Gram-stain reaction, spore formation, and aerobic growth. Clostridia were identified at the species level by fermentation test, esculin hydrolysis, gas formation, indole production, nitrate reduction, H₂S production, gelatin liquefaction, motility, and production of volatile fatty acid, lactic acid and succinic acid. These tests were based on the Virginia Polytechnic Institute method [11].

Examination of toxin productibility: The cecal contents of diarrheic rabbits were applied on a kit of latex agglutination test for *C. difficile* enterotoxin (C. D. check D-1 kit, Mitsubishi Chemical Industries, Tokyo). *E. coli* strains isolated for DHL agar were examined for production of heat-stable and heat-labile enterotoxins by using an enzyme-linked immunosorbent assay (Colist EIA, Denka, Tokyo) and a reversed passive hemagglutination test (VET-RPLA, Denka), respectively. Isolates from TCBS agar were examined for production of enterotoxin of *Vibrio cholerae* by VET-RPLA (Denka). *Staphylococcus* spp. isolates were examined for coagulase production.

RESULTS

Table 1 shows the incidence of diarrhea in rabbits given different classes of antibiotics. Diarrhea occurred in high incidence with sulbactam/cefoperazone, cefmetazole, clindamycin, piperacillin or aspoxicillin treatment. Cefminox, cefoxitin, cefoperazone, cefpiramide, cefodizime, latamoxef, ceftriaxone, sulbenicillin, carbenicillin and

lincomycin caused medium incidence of diarrhea. The other antibiotics such as amikacin, ceftazidime, isepamicin, netilmicin, ampicillin, cefapirin, cefalotin, cefazolin and cefsulodin, caused very low incidence of diarrhea.

C. difficile was isolated in 6 to 7 colony forming units (log₁₀, CFU) per g of cecal contents from the rabbits showing diarrhea after sulbactam/cefoperazone treatment, but not isolated from those after either cefmetazole or piperacillin treatment (Table 1). Consistent with this observation, the reaction of the cecal contents in C. D. check D-1 kit was positive in most sulbactam/cefoperazone-treated rabbits, whereas it was negative in either cefmetazole- or piperacillin-treated rabbits (Table 1). In cefmetazole-treated diarrheic rabbits, hemorrhage was noted on the surface of the cecum.

In order to study cefmetazole-induced alteration of intestinal microflora, the antibiotic was given to four rabbits of which cecal or fecal microflora were examined. Two out of the four rabbits showed serious diarrhea on day 3 or 4 after cefmetazole treatment, but the other two showed no diarrhea for more than 20 days after the antibiotic treatment.

Table 2 shows the counts of fecal and cecal bacteria of rabbits with cefmetazole treatment. Changes in counts of fecal and cecal bacteria observed in all the rabbits after cefmetazole treatment were as follows: The counts of bacteroidaceae decreased on day 2 after the antibiotic treatment and then increased: Those of streptococci increased after the treatment. Changes in the counts of enterobacteriaceae differed among four rabbits. Clostridia were detected in the two diarrheic rabbits in 6.9 and 3.2 (log₁₀, CFU/g of feces), but not in the non-diarrheic rabbits, before cefmetazole treatment. The counts (log₁₀, CFU/g of feces) of clostridia increased after the treatment in the two diarrheic rabbits: The count of clostridia including *Clostridium innocuum* was 10.1 on day 3 in rabbit A; The count of clostridia including *C. innocuum* and *Clostridium sporogenes* was 10.7 on day 4 in rabbit B.

Spiral bacteria like *C. spiroforme*, which Borriello and

Table 2. Change of fecal bacteria in rabbits after injection of cefmetazole

Bacterial group	Rabbit Day after injection	Occurrence of diarrhea											
		+						—					
		A		B ^{a)}		C		D					
		0 ^{b)}	2	3 ^{c)}	0	4 ^{c)}	0	2	6	0	2	6	
Bacteroidaceae		8.3 ^{d)}	5.2	9.8	8.0	9.2	8.6	7.2	9.9	9.0	6.6	8.3	
Bifidobacteria		7.8	5.8	0	0	0	0	0	0	6.1	0	0	
Lactobacilli		6.6	5.5	0	3.7	0	0	0	0	0	0	0	
Clostridia		6.9	0	10.1 ^{e)}	3.2	10.7 ^{f)}	0	0	0	0	0	5.2	
Peptococci		0	0	6.5	0	0	0	4.1	0	8.7	7.0	0	
Enterobacteriaceae		5.8	6.9	2.3	9.7	0	9.4	0	2.5	3.9	9.9	4.8	
Streptococci		5.8	6.1	9.3	3.2	9.5	3.8	7.7	7.6	4.0	9.7	8.2	

a) The feces were not collected on day 2 after cefmetazole treatment because of constipation.

b) On day 1 before injection of cefmetazole (50 mg/kg body weight).

c) The cecal contents were collected since the feces were not collected for severe diarrhea.

d) Bacteria counts (log, CFU/g of feces).

e) *Clostridium innocuum* (9.5) and others (not classified).

f) *Clostridium innocuum* (10.5), *Clostridium sporogenes* (9.5) and others (not classified).

Carman [5] isolated from antibiotic-treated rabbits, were not isolated from the feces and cecal contents, nor detected in direct smear of the cecal contents in diarrheic rabbits. Other pathogenic bacteria such as *Salmonellae* spp., *V. cholerae*, *Campylobacter* spp., *B. cereus*, *Staphylococcus aureus* and enteropathogenic *E. coli*. were not isolated from either rabbit.

DISCUSSION

We observed large differences in the incidence of diarrhea among the rabbits given different antibiotics, presumably reflecting the difference in the fate of the antibiotics in animal body.

C. difficile has been isolated from and *C. perfringens* iota toxin has been found in the cecal contents of rabbits showing clindamycin-associated colitis [20]. We examined the *C. difficile* enterotoxin in cecal contents by using C. D. check D-1 kit. Animals showing diarrhea after treatment with lincomycin, 8 antibiotics of cepheims or 2 antibiotics of penicillins showed positive reaction in the kit. Especially sulbactam/cefoperazone caused the *C. difficile* enterotoxin-positive reaction in a large population of diarrheic rabbits (95 per cent and 38 rabbits), from which *C. difficile* was isolated. On the other hand, no toxin-positive reaction was found in, nor was *C. difficile* isolated from, either diarrheic rabbit given cefmetazole, suggesting that *C. difficile* may not be involved in cefmetazole-associated diarrhea in rabbits.

The rate of the toxin-positive reaction in the sulbactam/cefoperazone-associated and cefmetazole-associated diarrheic rabbits was near to the rate of *C. difficile* isolation. The C. D. check D-1 kit has been shown to cross react to other bacterial products than the *C. difficile* enterotoxin [4, 16], but the present result may indicate that the method using this kit is useful for screening the presence of *C. difficile* in the intestinal contents of rabbits.

Cefmetazole has a wide antibacterial spectrum [10, 15] including staphylococci, bacteroidaceae, *E. coli*, klebsiellae and other some gram negative rods in human. Enterobacter are resistant and streptococci are less susceptible to cefmetazole [10]. This study showed that bacteroidaceae in feces of rabbits were decreased by cefmetazole as has been observed in human. On the other hand, streptococci in feces of the rabbits, however, were not decreased by cefmetazole, differing from the case in human.

The change of clostridial counts in these rabbits showed that cefmetazole once suppressed the growth of clostridia as well as that of some other intestinal bacteria, followed by an increase in clostridia to a population higher than that before the antibiotic treatment, presumably because of the disappearance of cefmetazole from the intestinal lumen. On the other hand, such a high population of clostridia was not seen in the rabbit showing no diarrhea. Thus, the result indicates that the increase in counts of clostridia other than *C. difficile* is associated with diarrhea induced by cefmetazole.

C. innocuum is not known to be pathogenic, but was isolated from human with infectious disease in the intestinal tract [9]. Also *C. sporogenes* was isolated from human [13] and domestic animal [24] with infectious diseases, but the role of these species in any disease is unclear. It is needed to investigate whether these species are involved in cefmetazole-associated diarrhea.

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