豚および鶏糞便由来嫌気性菌の発育促進用ポリエーテル系抗生物質に対する感受性について

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Susceptibility of Fecal Anaerobic Bacteria from Pigs and Chickens to Five Polyether Antibiotics for Growth Promotion

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ABSTRACT. The minimun inhibitory concentrations (MIC) of 100 strains of anaerobic bacteria, isolated from the intestines of healthy pigs and chickens, for five polyether antibiotics including cationomycin (CNM), lysocellin (LSC), monension (MNS), portmicin (PRM) and salinomycin (SNM) were determined by an agar-dilution technique. CNM, MNS, PRM, and SNM, at a concentration of 6.25 μg/m*l*, were active against 41 to 56% of the *Bacteroides fragilis* group, whereas LSC showed less activity at the same concentration. All strains of genera *Fusobacterium, Selenomonas, Mitsuokella* and *Megasphasera* were resistant to the five polyether antibiotics. *Eubacterium* spp. were susceptible to the antimicrobial agents (MICs of 1.56 to 3.12 μg/m*l*) except for NMS. CNM and PRM inhibited all strains of *Bifidobacterium* spp. at lower concentrations, but LSC, MNS and SNM did not. CNM, PRM and SNM, at a concentration of 1.56 μg/m*l*, were active against all strains of *Lactobacillus* spp. whereas LSC and NMS did not inhibit them at the same concentration. *Clostridium perfringens* was also susceptible to five polyether antibiotics tested (MICs of 1.56 to 12.5 μg/m*l*). All strains of *B. fragilis* from pigs were resistant to the five polyether antibiotics but those from chickens were inhibited at lower concentrations.—KEY WRODS: chicken, intestinal bacterium, pig, polyether antibiotic, susceptibility.

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Antimicrobial agents have been widely used as food additives to improve growth and feed conversion in poultry, swine and cattle [31]. Although the mechanisms by which the feed additives promote growth of animals are unknown, the action of the agents may cause changes in the microbial populations within the intestinal tract [10, 31].

Polyether antibiotics are a diverse group of ionophores [27], most of which are produced by the genus *Streptomyces* [9, 13, 23]. Recently several new polyether antibiotics, produced by *Actinomycetes* other than *Streptomyces,* e.g. *Streptoverticillium* [18], *Nocardiopsis* [19], *Nocardia* [21], and *Actinomadura* [24], have been reported. It is known that polyether antimicrobial agents appear to reduce growth depressing toxins produced by intestinal bacteria, inhibit coccidial infection, and enhance the absorptive efficiency and utilization of nutrients by animals [16, 25, 28]. However, *in vitro* susceptibility of intestinal anaerobes from animals to polyether antibiotics has not been investigated.

The purpose of the present study was to provide information on the susceptibility of anaerobic bacteria isolated from the intestines of healthy pigs and chickens to five polyether antibiotics used for growth promotion.

MATERIALS AND METHODS

Bacteria tested. A total of 100 strains of anaerobes from the feces of healthy pigs and chickens shown in Table 1 was used. These animals were not exposed to the polyether antibiotics throughout the study, while pigs were exposed to virginiamycin. The isolated strains consisted of the following species: 27

Table 1. Bacterial strains tested

Species	No. of isolates	
	Pigs	Chickens
Bacteroides		
B. fragilis group[a)]	10	17
B. suis	13	
Bacteroides spp.	2	3
Fusobacterium		
F. necrophorum	1	1
Selenomonas		
Selenomonas spp.	2	
Mitsuokella		
M. multiacidus	1	
Eubacterium		
E. aerofaciens	4	3
Eubacterium spp.	3	2
Bifidobacterium		
B. pseudolongum	1	1
B. thermophilum	4	2
Propinoibacterium		
P. acnes	2	
Clostridium		
C. perfringens		3
Peptostreptococcus		
P. anaerobius	1	1
P. asaccharolyticus	4	
P. magnus	1	
P. productus	1	
Peptostreptococcus sp.	1	
Megasphaera		
M. elsdenii		1
Lactobacillus		
L. acidophillus	6	3
L. fermentum	1	1
L. salivarius	3	

a) Including *B. distasonis*, *B. fragilis*, *B. thetaiotaomicron*, and *B. vulgatus*.

strains of the *Bacteroides fragilis* group (13 of *B. fragilis,* 8 of *B. vulgatus,* 5 of *B. distasonis,* 1 of *B. thetaiotaomicron*), 13 of *B. suis,* 3 of the *B. buccae-oris* group, 2 of *B. pyogenes,* 2 of *Selenomonas* spp., 1 of *Fuso-bacterium necrophorum,* 2 of *F. varium,* 1 of *Mitsuokella multiacidus,* 7 of *Eubacterium aerofaciens,* 1 of *E. moniliforme,* 1 of *E. limosum,* 3 of *Eubacterium* spp., 6 of *Bifidobacterium thermophilum,* 2 of *B. pseudolongum,* 2 of *Propionibacterium acnes,* 3 of *Clostridium perfringens,* 4 of *Peptostreptococcus asaccharolyticus,* 2 of *P. anaerobius,* 1 of *P. magnus,* 1 of *P. productus,* 1 of *Peptostreptococcus* sp., 1 of *Megasphaera elsdenii,* 9 of *Lactobacillus acidophilus,* 3 of *L. salivarius* and 2 of *L. fermentum.* Three species of *B. thermophilum* P2–91, *C. perfringens* ATCC 13124, and *L. acidophilus* ATCC 4356 were used as standard strains. The strains were maintained on Eggerth-Gagnon liver agar slants [22] filled with CO_2 gas and stored at 4°C. Transfers were usually made at 1-month intervals.

Antibiotics. The polyether antibiotics tested were cationomycin (CNM), lysocellin (LSC), monensin (MNS), portmicin (PRM), and salinomycin (SNM). They were supplied by Kaken Pharmaceutical Co., Ltd., Tokyo.

Antimicrobial susceptibility. The agar dilution method was used. Briefly, antimicrobial soltuions were freshly prepared and added to *Brucella* agar (Difco, Detroit) supplemented with 5% defibrinated horse blood. The inoculum, a 24-hour culture in peptone-yeast extract-Fildes solution-glucose [14] broth to match McFarland standard No. 5, was dispensed using an automatic replicating system (Sakuma Co. Ltd., Tokyo) to deliver approximately 10^8 colony-forming units. Control plates without drugs were inoculated before and after each set of drug-containing plates. Final concentrations of antibiotics tested ranged from 0.05 to 100 $\mu g/ml$. Plates were incubated in an anaerobic steel-wool jar [26] filled with 85% nitrogen gas, 10% carbon dioxide and 5% hydrogen gas at 37°C for 48 hrs. The lowest antimicrobial concentration that produced no visible bacterial growth was considered as the minimum inhibitory concentration (MIC).

RESULTS

The MIC values of five polyether antibio-

Table 2. Susceptibility of fecal anaerobic bacteria from pigs and chickens to five polyether antibiotics for growth promotion

Group and species	No. of strains tested	Cumulative percentage MIC at increasing antibiotic concentrations[a)]											
		≦0.05	0.1	0.2	0.39	0.78	1.56	3.12	6.25	12.5	25	50	≧100
Cationomycin													
Bacteroides	(45)		4.4	6.7		15.6		17.8	44.4	48.9	68.9	73.3	100
B. fragilis group	(27)					14.8		18.5	51.9	55.6	63.0	66.7	100
B. suis	(13)		15.4	23.1					30.8	38.5	84.6	92.3	100
Other *Bacteroides* spp.	(5)								20.0		60.0		100
Fusobacterium spp.	(3)												100
Selenomonas spp.	(2)												100
Mitsuokella multiacidus	(1)												100
Eubacterium spp.	(12)			23.1	92.3	100							
Bifidobacterium spp.	(8)			14.3	28.6	85.7	100						
Propinoibacterium acnes	(2)				50.0	100							
Clostridium perfringens	(3)				33.3	66.7			100				
Peptostreptococcus spp.	(9)		33.3		88.9	100							
Megasphaera elsdenii	(1)												100
Lactobacillus spp.	(14)			14.3	57.1	85.7	100						
Lysocellin													
Bacteroides	(45)		2.2	4.4	6.6				22.2	48.9	64.4	82.2	100
B. fragilis group	(27)								14.8	48.1	59.3	70.4	100
B. suis	(13)		7.7	15.4	23.1				46.2	69.2	76.9	100	
Other *Bacteroides* spp.	(5)										60.0	100	
Fusobacterium spp.	(3)												100
Selenomonas spp.	(2)												100
Mitsuokella multiacidus	(1)												100
Eubacterium spp.	(12)				7.9	30.8	61.5	100					
Bifidobacterium spp.	(8)						25.0	50.0			87.5		100
Propinoibacterium acnes	(2)					50.0	100						
Clostridium perfringens	(3)						33.3	66.7		100			
Peptostreptococcus spp.	(9)			33.3	55.6	100							
Megasphaera elsdenii	(1)												100
Lactobacillus spp.	(14)					21.4	42.9	92.9	100				
Monensin													
Bacteroides	(45)	2.2		6.7		8.9		26.7	40.0	55.6		100	
B. fragilis group	(27)			3.7				22.2	40.7	55.6		100	
B. suis	(13)	7.7		15.4		23.1		38.5	46.2	53.8		100	
Other *Bacteroides* spp.	(5)							20.0		60.0		100	
Fusobacterium spp.	(3)											100	
Selenomonas spp.	(2)											100	
Mitsuokella multiacidus	(1)											100	
Eubacterium spp.	(12)							30.8	46.2	84.6	92.3	100	
Bifidobacterium spp.	(8)							12.5		37.5		100	
Propinoibacterium acnes	(2)							100			100		
Clostridium perfringens	(3)							33.3		66.7			
Peptostreptococcus spp.	(9)		33.3			55.6	100						
Megasphaera elsdenii	(1)											100	
Lactobacillus spp.	(14)							14.3	28.6	100			

Table 2. Continued.

Group of species	No. of strains tested	Cumulative percentage MIC at increasing antibiotic concentrations[a]											
		≦0.05	0.1	0.2	0.39	0.78	1.56	3.12	6.25	12.5	25	50	≧100
Portmicin													
Bacteroides	(45)	2.2		6.6	11.1		13.3	28.9	35.6	46.7	75.6	80.0	100
B. fragilis group	(27)	3.7		7.4			11.1	37.0	40.7	51.9	66.7	70.4	100
B. suis	(13)			7.7	23.1				30.8	38.5	92.3		100
Other *Bacteroides* spp.	(5)								20.0	60.0		80.0	100
Fusobacterium spp.	(3)												100
Selenomonas spp.	(2)												100
Mitsuokella multiacidus	(1)												100
Eubacterium spp.	(12)				30.8	61.5	100						
Bifidobacterium spp.	(8)			25.0			87.5		100				
Propinoibacterium acnes	(2)					100							
Clostridium perfringens	(3)						33.3		100				
Peptostreptococcus spp.	(9)		33.3		88.9	100							
Megasphaera elsdenii	(1)												100
Lactobacillus spp.	(14)			7.1	21.4	42.9	100						
Salinomycin													
Bacteroides	(45)	2.2	4.4	8.9		11.1	17.8	37.8	62.2	80.0	82.2	86.7	100
B. fragilis group	(27)					7.4	18.5	29.6	55.6		77.8	88.9	100
B. suis	(13)		7.7	23.1				46.2	76.9	92.3	100		
Other *Bacteroides* spp.	(5)							60.0		100			
Fusobacterium spp.	(3)												100
Selenomonas spp.	(2)												100
Mitsuokella multiacidus	(1)												100
Eubacterium spp.	(12)			7.7	61.5	92.3				100			
Bifidobacterium spp.	(8)				25.0	37.5	50.0			87.5			100
Propinoibacterium acnes	(2)				100								
Clostridium perfringens	(3)				33.3	66.7				100			
Peptostreptococcus spp.	(9)		33.3	55.6	88.9		100						
Megasphaera elsdenii	(1)												100
Lactobacillus spp.	(14)		7.1	42.9	92.9	100							

a) μg/m*l*.

tics, including CNM, LSC, MNS, PRM, and SNM, for a total of 100 strains of anaerobic bacteria isolated from the intestine of healthy pigs and chickens are shown in Table 2. Within *Bacteroides* spp., 41, 41, 52, and 56% of the *B. fragilis* group tested were inhibited by NMS, PRM, CNM, and SNM, respectively, at MIC of 6.25 μg/m*l*, whereas LSC showed less activity on these microorganisms at the same concentration. *B. suis* from pig feces had an MIC pattern with two peaks except for MNS, 23% at MIC of 0.2 to 0.4 μg/m*l* and 77% at MIC of 6.25 to 100 μg/m*l*. All strains belonging to genera *Fusobacterium, Selenomonas, Mitsuokella,* and *Megasphaera* were resistant to the five polyether antibiotics tested. With the exception of NMS, all *Eubacterium* spp., which are a beneficial in animal production, were inhibited by CNM and PRM at a concentration ranging from 1.56 to 6.25 μg/m*l*. LSC, MNS, and SNM, however, were less active against the microorgainsms at the same concentration. *Propinoibacterium acnes* was inhibited by all antibiotics at 0.78 to 3.12 μg/m*l*. *C. perfringens* which produces

Table 3. Susceptibility of fecal *Bacteroides fragilis* from pigs and chickens to five polyether antibiotics for growth promotion

Antibiotic	Source	MIC (μg/ml)[a)			
		Range	MIC_{50}	MIC_{75}	MIC_{90}
Cationomycin	Pigs	25 −≧100	≧100	≧100	≧100
	Chickens[c)]	0.78− 12.5	6.25	6.25	12.5
Lysocellin	Pigs	12.5−≧100	≧100	≧100	≧100
	Chickens	6.25−50	12.5	12.5	50
Monensin	Pigs	12.5−≧100	≧100	≧100	≧100
	Chickens	3.12−12.5	0.25	12.5	12.5
Portmicin	Pigs	12.5−≧100	≧100	≧100	≧100
	Chickens	0.2 −12.5	3.12	12.5	12.5
Salinomycin	Pigs	6.25−≧100	50	≧100	≧100
	Chickens	1.56−12.5	6.25	12.5	12.5

a) Concentration of anti-microbial agents at showing growth inhibition of over 50%, 75%, or 90%.
b) 8 strains was tested.
c) 5 strains was tested.

growth depressing toxins was inhibited by the polyether antibiotics tested at a concentration ranging from 1.56 to 12.5 μg/ml. All strains of *Peptostreptococcus* spp. were susceptible to the polyether antimicrobial agents, with the exception of CNM, at MIC of about 1.56 μg/ml. *Lactobacillus* spp. which predominate in the intestinal microflora of healthy pigs and chickens were inhibited by CNM, PRM, and SNM at MIC of 1.56 μg/ml, whereas these microorganisms revealed susceptibility to LSC and NMS at a concentration ranging from 6.25 to 12.5 μg/ml.

Table 3 shows the susceptibility of *B. fragilis* isolated from the intestines of healthy pigs and chickens to five polyether antibiotics used for growth promotion. All strains of *B. fragilis* from pigs were resistant to the polyether antibiotics but those from chickens were inhibited by these antibiotics at a concentration ranging from 0.2 to 12.5 μg/ml.

DISCUSSION

It is widely known that polyether antibiotics exhibit both antimicrobial and anticoccidial activities [5, 6, 15]. Polyether antimicrobial agents, including NMS and SNM, are added to feeds as growth promotors in Japan, but CNM, LSC and PRM are not used in this way. In the near future, however, three polyether antibiotics may be permitted for use as a food additives.

The criteria of susceptibility as used here with the growth promoting agents are tentative because criteria for the evaluation of susceptibility and resistance have never been established. Dutta and Devriese [7, 8] reported that *C. perfringens* strains isolated from the feces of chickens, cows and pigs were inhibited by NMS at MIC of 4 μg/ml and this MIC was established as the susceptible value. *In vitro* sensitivity levels of the polyether antibiotics against the intestinal isolates with growth-depressing activity may be interpreted as indicating sensitivity to the growth promotor. It is of interest to determine what effects, if any, polyether antibiotics exert on the intestinal microflora. Further studies are required to determine the effects of polyether antibiotics on the composition of intestinal microflora in the animals.

The existence of growth-depressing activity which can be influenced by antimicrobial agents used for growth promotion has been noted mainly among the gram-positive intestinal flora, e.g. *Clostridium perfringens* [20], *Streptococcus faecalis* [11, 12], *S. faecium* and *S. faecalis* subspecies *liquefaciens* [1]. In the present study, five polyether antibiotics were active *in vitro* against most of the species belonging to the genera *Eubacterium, Bifidobacterium, Propionibacterium, Clostridium, Peptostreptococcus,* and *Lactobacillus*. However, they did not act uniformly and their activity spectra differed makedly.

Decuypere *et al.* [4] and Vervaeke *et al.* [30] found that virginiamycin and spiramycin suppress growth of lactobacilli and streptococci in the small intestines of pigs, thereby influencing carbohydrate metabolism in a way apparently beneficial to the host. In this study, *Lactobacillus* spp. were inhibited by polyether antimicrobial agents with the exception of MNS at MIC ranging from 0.2 to 6.25 μg/m*l*. Thus, effects of polyether antibiotics on lactobacilli were expected.

It is widely known that pathogenicity of *Eimeria tenella* is promoted by the presence of *C. perfringens* in the intestine of chickens [3, 17, 29]. Our previous study [2] demonstrated that SNM and MNS inhibited *C. perfringens* isolated from broiler intestines at lower concentrations. One of three strains of *C. perfringens* tested in this study was resistant to SNM and MNS at higher concentrations. The existence of polyether antibiotic-resistant *C. perfringens* strains may soon be increased by the use of polyether antimicrobial agents.

On the other hand, gram-negative anaerobic bacteria were less affected by five polyether antibiotics. Although the strain tested was a few, the genera *Fusobacterium, Mitsuokella, Selenomonas,* and *Megasphaera* were not inhibited at concentrations of more than 100 μg/m*l*. Some isolates of the *B. fragilis* group, however, did not show a uniform susceptibility and revealed different activity spectra. CNM and SNM exhibited less activity on the *B. fragilis* group with an MIC_{50} of 6.25 μg/m*l*, MIC_{75} ranging from 12.5 to 25.0 μg/m*l*, and MIC_{90} of more than 100 μg/m*l*. In addition, the concentrations at which the *B. fragilis* group were resistant to LSC, MNS, and PRM were markedly higher than those for CNM and SNM. Although the polyether antibiotics are characterized by lower activity against anaerobic gram-negative bacilli as described previously [9, 23], the *B. fragilis* group, which predominates in the intestines of animals, had different activity spectra.

In the present study the susceptibilities to five polyether antibiotics were noted to vary with the animal source of *B. fragilis*. All strains of *B. fragilis* isolated from chickens, but not all of the *B. fragilis* strains isolated from pigs were sensitive to the five antimicrobial agents. The pigs and chickens, from which *B. fragilis* was isolated, had not been exposed to the polyether antibiotics. Although the pigs were exposed to virginiamycin, the reason why the isolates revealed different susceptibilities is unknown. Further studies are required to confirm which anaerobic bacteria have accquired or natural resistance to polyehter antibiotics.

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要　　約

豚および鶏糞便由来嫌気性菌の発育促進用ポリエーテル系抗生物質に対する感受性について：辨野義己[1)]・遠藤希三子[1)]・白神伸江[1)]・光岡知足[1,2)]（[1)]理化学研究所・[2)]東京大学農学部実験動物学教室）——発育促進用ポリエーテル系抗生物質，カチオノマイシン（CNM），ライソセリン（LSC），モネンシン（MNS），ポートミシン（PRM）およびサリノマイシン（SNM）に対する健康豚および鶏糞便由来嫌気性菌100菌株の最小発育阻止濃度（MIC）を寒天希釈法によって測定したところ，*Bacteroides fragilis* グループに属する菌株の41～56％はCNM, MNS, PRMおよびSNMの6.25μg/m*l* の濃度で抑制されたが，LSCには同一濃度で抑制されなかった．*Fusobacterium*, *Selenomonas*, *Mitsuokella* および *Megasphaera* の全供試菌株はポリエーテル系抗性物質に耐性を示した．*Eubacterium* はMNSを除く抗生物質に1.56～3.12μg/m*l* の濃度で抑制された．*Bifidobacterium* の全菌株はCNMおよびPRMに対して低濃度で抑制されたが，LSC，MNSおよびSNMには弱い耐性を示した．また，*Clostridium perfringens* は全供試薬剤に1.56～12.5μg/m*l* で抑制され，*Lactobacillus* はCNM，PRMおよびSNMの1.56μg/m*l* で抑制されたが，LSCおよびMNSに対して12.5μg/m*l* で抑制された．豚糞便由来 *B. fragilis* はポリエーテル系抗生物質に対して耐性を示したが，鶏糞便由来 *B. fragilis* は低濃度で抑制された．