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Distribution and Antimicrobial Susceptibility of *Clostridium* Species in Soil Contaminated with Domestic Livestock Feces of Korea

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Abstract Soil samples from five different areas in Korea were collected during 2001/02 and examined for presence of the genus *Clostridium*. Direct immuno-fluorescent assay (IFA) examination showed that *Clostridium septicum*, *Cl. novyi* and *Cl. chauvoei* were detected in the soil of specific areas in Korea. Sixteen species of *Clostridium* were isolated and cultivated from the soil samples. *Cl. perfringens* was detected in all sampling locations, while the other species were not. The *in vitro* activity of 14 antibiotic agents was determined against 421 clostridia isolated from the soil contaminated with animal feces in Korea. Trovafloxacin was effective against all isolates of the genus *Clostridium* except one isolate of *Cl. subterminale*, two of *Cl. tetani*, and three of *Cl novyi* with MIC_{50} 8–16 μg ml^{-1}. Thirteen species of *Clostridium* were resistant to vancomycin except for *Cl. perfringens*, *Cl. sporogenes*, and *Cl. subterminale*. Imipenem and trovafloxacin showed high antimicrobial activities (>95%) against all strains in the clostridia investigated. Therefore, antibiotic agents such as imipenem and trovafloxacin are the most suitable agents for polymicrobial infection as broad-spectrum monotherapy.

Key words: Korea, soil, *Clostridium*, antimicrobial susceptibility

Clostridium spp. are widely distributed in nature and are found in soil as well as in freshwater and marine sediments throughout the world [14]. The presence of the organism in soil has made it an important soil pathogen. Soil borne diseases may be considered a serious problem in extensive livestock production systems, despite the lack of up-to-date records and information on their epidemiology [18]. Moreover, clostridia are important causative agents of human pathogens and food poisonings associated with infections ranging from abscesses to serious life-threatening septicemia and have been identified as the main etiological agents in many clinically significant infections [2]. The symptoms related to food poisoning are caused by an enterotoxin produced by enterotoxigenic *Clostridium* species [19]. There are many reports of the incidence of enterotoxigenic *Clostridium perfringens* in samples taken from the gastrointestinal tract of both humans and other domestic animals as well as from soil [15, 23, 24, 25]. Soil and animal feces are considered to be the primary reservoir for direct contamination with these spoilage bacteria [1, 5, 11].

In the present study, a survey of the *Clostridium* species in soil was conducted in order to determine several species by using direct immuno-fluorescent assay (IFA) in different soil samples, to assess the antimicrobial susceptibility to various antibiotic agents, and to compare the *in vitro* activity of these agents.

Clostridium species were obtained from soil samples collected from livestock farming of cattle, pigs and poultry. A total 152 specimens were collected from five different parts of South Korea, during 2001–2002. The samples were collected from 9–11 locations. At each location, 50 g of soil was collected from a depth of 10–15 cm. Each sample was placed in a sterile plastic bag, transported to the laboratory in a chilled box, and analyzed on the day of collection.

Soil samples were analyzed in the laboratory with the following bacteriological methods for clostridia [4]. Fifty grams of soil was added to 50 ml of distilled water in a bottle. This was mixed thoroughly and then left for 2 h to settle at room temperature. Ten milliliters of the supernatant was centrifuged at 1,600 ×*g* for 10 min. Eight milliliters of brain heart infusion broth [BHIB; Oxioid, Hampshire, U.K.) brain heart infusion broth, 37.0 g; yeast extract, 5.0 g;

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supplement solution, 10.2 ml; resazurin (0.25% solution), 4.0 ml; and deionized water 1,000 ml] was added to the precipitate. The supplement solution was composed as follows: 0.5 g of cysteins·HCl·H_2O, 0.5 g, 10 ml of Hein solution, and 0.2 ml of vitamin K_1 solution. In addition, Hein solution included 50 mg of hemin, 10 ml of 1 N NaOH, and 100 ml of deionized water. 0.15 ml of vitamin K_1 was added to 30 ml of 95% ethanol, and this was used as vitamin K_1 solution. The resulting solution was subcultured in BHIB and incubated anaerobically in a BBL anaerobic jar (Difco, MD, U.S.A.) at 37°C. Direct immuno-fluorescent assay (IFA) examination was used to make an initial identification of samples reacting *Clostridium septicum*, *Cl. novyi*, and *Cl. chauvoei*. Because similar symptoms have been often confused with malignant edema, caused by *Clostridium septicum*, *Clostridium novyi*, or *Clostridium chauvoei*, and *Bacillus anthracis*, the causative agent of anthrax, each polyclonal antibody conjugated to fluorescent isothiocyanate was obtained from VMRD, Inc. (Washington, U.S.A.). The precultured BHIB was concentrated and smeared on a slide glass. After fixation for 20 min in aceton/metanol (75/25, v/v) at room temperature, slides were stained with the polyclonal antibody conjugates for 30 min at 37°C in a humid chamber.

The subcultured BHIB was incubated on agar plate: reinforced clostridia media (RCM, Merck), SEP (tryptose sulfate cyclosporine agar base with 3 mg polymyxin sulfate and 12 mg kanamycin disulfate per litter, Merck), beef liver medium (BL) for anaerobes with 7% horse blood (Nissui, Tokyo) and gelatin agar medium (GAM) with 7% horse blood (Nissui, Tokyo), for the differentiation of microorganisms of clinical significance based on proteolytic activity, were incubated in an AnaeroPack system (Mitsubishi Gas Co., Tokyo) for 3-5 days at 37°C. Typical *Clostridium* colonies were reisolated on GAM. Fluorocult-supplemented tryptose sulfite cycloserine (TSC; Merck) agar was also used for the presumptive identification, enumeration, and re-isolation of a single colony of clostridia. Identification was performed using the API kit 20 A (bioMérieux, Marcy IEtoile, France).

Minimum inhibition concentrations (MICs) of 16 species of *Clostridium* were determined using the National Committee for Clinical Laboratory Standards (NCCLS) agar dilution method [13]. For *Clostridium* spp., clostridia medium agar was composed as follows: 10 g of sodium L-lactate, 8 g of sodium acetate, 0.5 g of K_2HPO_4, 0.5 g of $(NH_4)_2SO_4 \cdot 7H_2O$, 0.5 g of sodium thioglycollate, 0.5 g of yeast extract, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.02 g of $FeSO_4 \cdot 7H_2O$, 100 μg of *p*-aminobenzoate 0.1 μg of biotin, and 1,000 ml of deionized water. Antibiotics were purchased from various companies such as: amoxicillin, ampicillin, cefoxitin, chloramphenicol, clindamycin, metronidazole, penicillin, piperacillin, and vancomycin from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.), imipenem from Merck Research Laboratories (Rahway, NJ, U.S.A.), cefpirome from Hoechst Marion Roussel (Milan, Italy), ciprofloxacin from Bayer (Leverkusen, Germany), trovafloxacin from Pfizer (Groton, CT, U.S.A.), and loracarbef from Eli Lilly (Indianapolis, IN, U.S.A.). The inocula were prepared by the direct suspension of colonies cultured on clostridia medium agar plates for 18 h at 37°C. Plates containing antibiotic dilutions were inoculated with 1×10^5 cfu ml^{-1} clostridia. MICs were investigated after 48 h of incubation at 37°C in an anaerobic environment generated by AnaeroGen gaspacks (Oxoid, Hampshire, U.K.). Approved and tentative NCCLS susceptible breakpoints [13] or preliminary breakpoints as suggested by the respective manufactures were employed. The breakpoint for loracarbef was not available.

The result of IFA is shown in Table 1. The presence of *Cl. septicum*, *Cl. novyi*, and *Cl. chauvoei* varies with sampling location. They were not detected in the samples from Kyungki-Do and Chungchung-Do, but they were detectable in the samples from Chonra-Do. In particular, it was found that a rate *of Cl. septicum* (26/30 location) in Chonra-Do was significantly higher than any other locations. The detectable frequency of *Cl. chauvoei* (13/33 locations) in the soil of Kyungsang-Do was higher than any other parts. In Chonra-Do, *Cl. chauvoei* was also detected. The distribution area of clostridia isolates was varied as shown in Table 2. *Cl. perfringens*, *Cl. sporogens*, *Cl. subterminale*, *Cl. sordellii*, *Cl. tetani*, *Cl. bifermentans*, *Cl, novyi*, *Cl. innocuum*, *Cl. barati*, *Cl. glycolicum*, *Cl. butyricum*, *Cl. limosum*, *Cl. difficile*, *Cl. beijerinckii*, *Cl. chauvoei*, and *Cl. septicum* were isolated from the soil

Table 1. Direct detection of *Cl. septicum*, *Cl. septicum*, and *Cl. chauvoei* using IFA in soil of Korea.

Sampling location	Total numbers of samples	No. (%) of sites detected		
		Cl. septicum	*Cl. novyi*	*Cl. chauvoei*
Kyungki-Do	30	0	0	0
Chungchung-Do	30	0	0	0
Kyungsang-Do	33	0	0	16 (48.5)
Kangwon-Do	29	5 (17.2)	6 (20.7)	0
Chonra-Do	30	26 (86.7)	14 (46.7)	9 (30.0)
Sum	152	31 (20.4)	20 (13.2)	25 (16.4)

Table 2. Distribution area of *Clostridium* species in Korea.

Species of Clostridium	Total no.	No. (%) of isolates from soil in				
		Kyungki-Do	Chungchung-Do	Kyungsang-Do	Kangwon-Do	Chonra-Do
Cl. perfringens	44	15 (29.4)	7 (11.7)	9 (10.4)	12 (17.1)	1 (0.6)
Cl. sporogenes	35	0	17 (28.3)	9 (10.4)	0	9 (5.9)
Cl. sordellii	26	19 (37.4)	3 (5.0)	0	0	4 (2.6)
Cl. subterminale	21	13 (25.5)	4 (6.7)	4 (4.6)	0	0
Cl. tetani	26	0	0	10 (11.5)	0	16 (10.5)
Cl. fermentans	33	3 (5.8)	6 (10.0)	6 (6.9)	8 (11.4)	10 (6.5)
Cl. novyi	31	0	0	6 (6.9)	9 (12.9)	16 (10.5)
Cl. innocuum	39	0	9 (15.0)	6 (6.9)	13 (18.6)	11 (7.2)
Cl. barati	9	0	9 (15.0)	0	0	0
Cl. glycolicum	5	0	5 (8.3)	0	0	0
Cl. butyricum	7	0	0	7 (8.0)	0	0
Cl. limosum	15	0	0	0	6 (8.6)	9 (5.9)
Cl. difficile	14	0	0	5 (5.7)	0	9 (5.9)
Cl. beijerinckii	3	0	0	0	3 (4.3)	0
Cl. septicum	41	0	0	0	7 (10.0)	34 (22.2)
Cl. chauvoei	30	0	0	19 (21.8)	0	11 (7.2)
Clostridium spp.	42	1 (1.9)	0	6 (6.9)	12 (17.1)	23 (15.0)
Sum	421	51	60	87	70	153

samples and the percentage per total in each location depended on the locations. In this work, 16 species of *Clostridium* were isolated and cultivated from the soil samples. *Cl. perfringens* was detected in all the sampling locations, while the other species were not. The incidence of *Cl. perfringens* in the soil samples taken from livestock farming of cattle, pigs and poultry ranged from 0.6% to 29.4%. In the Okinawa Prefecture of Japan [9], the incidence of clostridia in soil was listed as 1.4-7.9% during 1988-1989. A retrospective surveillance study of soil-borne clostridial infections in cattle in Zambia, in the period 1985 to 1994, showed from 6.8% to 24.2%. With such a high incidence of *Cl. perfringens*, except in Chonra-Do, from 10.4% to 29.4%, the soil in Korea may be contaminated with this microorganism from the feces of domestic animals. In epidemiological investigation, the enumeration of toxic *Cl. perfringens* in the soil of domestic livestock farming rather than the total numbers of *Cl. perfringens* is important due to the fact that the symptoms associated with *Cl. perfringens* poisoning are caused by an enterotoxin producing *Cl. perfringens*. Therefore, relatively small cells of toxin producing *Cl. perfringens* isolated from domestic livestock farming may be enterotoxigenic [17, 19, 24]. This is a result of the species being ubiquitous in nature [14]. Among the isolated species such as *Cl. perfringens*, *Cl. novyi*, *Cl. fermentans*, *Cl. sordellii*, and *Cl. sporogenes* are considered soilborne clostridia and most commonly associated with gas gangrene-myonecrosis [1, 2, 6, 11, 18]. Krick and Ondendaal [8] reported that *Cl. chauvoei* was detected and survived with high frequency in soil in South Africa. One hundred and sixty five cases with malignant edema and 103 cases with blackwater were caused by soil-borne clostridia in Zambia during 1985-1994 [12]. In areas where *Cl. difficile* and *Cl. perfringens* have been detected, clinical cases have been documented. Therefore, this implies that the isolation of *Clostridium* species from soil could show the possibility of soilborn diseases in Korea and the analyses of soil sample locality are needed for economical clostridia vaccination.

After heat treatment, samples were sub-cultured anaerobically for 48 h. The incubation of the culture for 24-72 h at 35-37°C would be sufficient, but overnight incubation is often satisfactory for many clostridia in the appropriate media if growth is rapid and abundant [14]. For the isolation of *Cl. botulinum* producing botulinum nerotoxins that cause the neroparalytic illness in humans and animals [7], the soil samples were incubated at 30°C for 5 days [24]. However, we could not isolate *Cl. botulinum* from the soil in Korea due to soil alkalinity and non-aquatic environmental conditions. Yamakawa and Nakamura [26] reported that a major feature of the distribution of *Clostridium* spp. on a river of Japan was found to be the prevalence of *Cl. botulinum* in the whole river system. The incidence of *Cl. botulinum* has been affected by the aquatic environment in the United Kingdom [21, 22]. The results showed that in Britain the prevalence of *Cl. botulinum* in soils from sites associated for many years with animals is very much lower than in mud samples from British aquatic environments. Smith [20] investigated the obligate clostridia in soil from the U.S.A., and the most frequently isolated organisms were

Table 3. Antimicrobial susceptibility test of clostridia isolated from soil against various antibiotics.

Organism (n)	Antibiotics agent	Susceptibility at breakpoint (%)[a]	MIC (μg ml^{-1})		
			MIC_{50}	MIC_{90}	Range
Cl. perfringens (44)	Amoxicillin	100	≤0.06	0.125	≤0.06-0.125
	Ampicillin	100	0.125	0.25	≤0.06-0.25
	Cefoxitin	100	0.5	1	≤0.25-2
	Cefpirome	100	1	2	≤0.05-4
	Chloramphenicol	100	1	4	1-4
	Ciprofloxacin	100	1	2	0.25-4
	Clindamycin	86	0.125	2	≤0.06-8
	Imipenem	100	0.125	0.25	≤0.06-0.25
	Loracarbef	-	1	2	≤0.06-2
	Metronidazole	100	0.06	1	0.06-4
	Penicillin	100	0.125	1	≤0.06-2
	Piperacillin	100	1	16	≤0.06-32
	Trovafloxacin	100	0.125	0.25	≤0.06-0.5
	Vancomycin	100	0.5	1	≤0.125-1
Cl. sporogenes (35)	Amoxicillin	100	0.125	0.25	≤0.06-0.25
	Ampicillin	100	0.125	0.25	≤0.06-0.5
	Cefoxitin	100	≤0.05	≤0.05	≤0.06
	Cefpirome	100	1	4	≤0.06-4
	Chloramphenicol	100	2	4	0.5-4
	Ciprofloxacin	100	0.5	1	0.25-1
	Clindamycin	83	1	2	≤0.06-64
	Imipenem	100	≤0.06	≤0.06	≤0.06
	Loracarbef	-	1	4	0.125-4
	Metronidazole	89	0.5	>64	0.5->64
	Penicillin	100	≤0.06	≤0.06	≤0.06-0.5
	Piperacillin	100	1	2	0.06-2
	Trovafloxacin	100	0.125	0.5	≤0.06-1
	Vancomycin	100	0.25	1	0.25-1
Cl. sordellii (26)	Amoxicillin	92	0.125	1	≤0.06-64
	Ampicillin	96	0.125	1	≤0.06-32
	Cefoxitin	100	0.25	2	≤0.06-8
	Cefpirome	85	1	16	≤0.06-64
	Chloramphenicol	100	1	2	0.5-4
	Ciprofloxacin	92	1	2	0.125-8
	Clindamycin	96	0.125	4	≤0.06-4
	Imipenem	100	≤0.06	0.25	≤0.06-2
	Loracarbef	-	1	8	≤0.06-64
	Metronidazole	100	≤0.06	1	≤0.06-2
	Penicillin	85	≤0.06	1	≤0.06-8
	Piperacillin	92	0.5	8	0.125-32
	Trovafloxacin	100	0.25	1	≤0.06-1
	Vancomycin	91	0.25	0.5	≤0.06-4
Cl. subterminale (21)	Amoxicillin	71	0.25	32	≤0.06-64
	Ampicillin	81	0.125	16	≤0.06-64
	Cefoxitin	100	0.5	8	≤0.06-16
	Cefpirome	91	0.5	16	0.25-32
	Chloramphenicol	100	1	1	≤0.06-2
	Ciprofloxacin	100	1	1	≤0.06-2
	Clindamycin	86	≤0.06	16	≤0.06-32
	Imipenem	100	≤0.06	2	≤0.06-4
	Loracarbef	-	0.5	1	≤0.125-128
	Metronidazole	100	0.5	1	≤0.06-1
	Penicillin	86	≤0.06	8	≤0.06-16
	Piperacillin	81	0.25	32	≤0.06->128
	Trovafloxacin	95	≤0.06	2	≤0.06-8
	Vancomycin	100	0.25	0.5	0.125-1

Table 3. Continued.

Organism (n)	Antibiotic agent	Susceptibility at breakpoint (%)[a]	MIC (μg ml^{-1})		
			MIC_{50}	MIC_{90}	Range
Cl. tetani (26)	Amoxicillin	23	32	>128	2->128
	Ampicillin	31	32	>128	2->128
	Cefoxitin	69	16	32	4-64
	Cefpirome	19	64	>129	16->128
	Chloramphenicol	100	2	4	0.5-8
	Ciprofloxacin	12	4	128	1-128
	Clindamycin	69	4	>128	≤0.06->128
	Imipenem	100	0.25	1	0.25-2
	Loracarbef	-	>128	>128	16->128
	Metronidazole	100	0.25	4	≤0.06-4
	Penicillin	100	≤0.06	2	≤0.06-4
	Piperacillin	62	32	>128	8->128
	Trovafloxacin	92	0.125	2	≤0.06-8
	Vancomycin	8	32	128	8->128
Cl. fermentans (33)	Amoxicillin	27	16	-	1->128
	Ampicillin	46	16	-	≤0.5->128
	Cefoxitin	82	8	32	≤0.5-64
	Cefpirome	61	16	>128	2->128
	Chloramphenicol	100	1	4	≤0.06-8
	Ciprofloxacin	52	4	-	2->128
	Clindamycin	49	1	-	1->128
	Imipenem	100	0.25	1	≤0.06-2
	Loracarbef	-	>128	-	4->128
	Metronidazole	100	0.125	2	≤0.06-4
	Penicillin	70	4	64	1->128
	Piperacillin	61	8	64	2->128
	Trovafloxacin	100	0.25	1	≤0.05-2
	Vancomycin	46	16	-	4->128
Cl. novyi (31)	Amoxicillin	65	8	32	0.5->128
	Ampicillin	100	≤0.06	1	≤0.06-2
	Cefoxitin	74	4	32	≤0.5-64
	Cefpirome	84	8	32	≤0.5-64
	Chloramphenicol	100	2	4	1-8
	Ciprofloxacin	100	1	4	≤0.06-8
	Clindamycin	84	1	4	≤0.06->128
	Imipenem	100	0.25	1	≤0.06-2
	Loracarbef	-	>128	>128	16->128
	Metronidazole	100	0.125	1	≤0.06-2
	Penicillin	100	=0.06	1	≤0.06-2
	Piperacillin	84	4	32	≤0.6-64
	Trovafloxacin	90	0.125	0.5	≤0.06-1
	Vancomycin	17	16	64	4->128
Cl. innocuum (39)	Amoxicillin	85	2	16	≤0.5-64
	Ampicillin	74	2	32	≤0.5->128
	Cefoxitin	100	4	32	≤0.06-64
	Cefpirome	56	16	-	2->128
	Chloramphenicol	100	1	8	≤0.06-8
	Ciprofloxacin	46	8	-	0.25->128
	Clindamycin	100	≤0.06	1	≤0.06-1
	Imipenem	100	≤0.06	2	≤0.06-4
	Loracarbef	-	16	-	2->128
	Metronidazole	100	1	2	≤0.06-4
	Penicillin	100	≤0.06	1	≤0.06-2
	Piperacillin	100	0.125	4	≤0.06-8
	Trovafloxacin	100	0.125	2	≤0.06-2
	Vancomycin	36	8	64	2->128

Table 3. Continued.

Organism (n)	Antibiotic agent	Susceptibility at breakpoint (%)[a]	MIC (μg ml^{-1})		
			MIC_{50}	MIC_{90}	Range
Cl. barati (9)[b]	Amoxicillin	67	≤0.5	-	≤0.5->128
	Ampicillin	44	16	-	≤0.5->128
	Cefoxitin	89	1	-	0.5-32
	Cefpirome	56	2	-	2->128
	Chloramphenicol	100	≤0.06	-	≤0.06-1
	Ciprofloxacin	56	2	-	1-8
	Clindamycin	89	≤0.06	-	≤0.06-4
	Imipenem	100	≤0.06	-	≤0.06-0.5
	Loracarbef	-	32	-	1->128
	Metronidazole	100	0.125	-	≤0.06-2
	Penicillin	100	≤0.06	-	≤0.06-1
	Piperacillin	89	0.5	-	0.125-64
	Trovafloxacin	100	0.5	-	≤0.06-1
	Vancomycin	67	4	-	1->128
Cl. glycolicum (5)[b]	Amoxicillin	80	2	-	≤0.5->128
	Ampicillin	100	0.5	-	≤0.06-2
	Cefoxitin	100	1	-	≤0.125-16
	Cefpirome	80	16	-	8-128
	Chloramphenicol	100	0.125	-	≤0.06-4
	Ciprofloxacin	100	1	-	≤0.06-8
	Clindamycin	100	≤0.06	-	≤0.06-0.125
	Imipenem	100	≤0.06	-	≤0.06-8
	Loracarbef	-	4	-	≤0.06->128
	Metronidazole	80	≤0.06	-	≤0.06-4
	Penicillin	100	≤0.06	-	≤0.06-1
	Piperacillin	80	2	-	1-64
	Trovafloxacin	100	0.5	-	≤0.5-1
	Vancomycin	20	32	-	8->128
Cl. butyricum (7)[b]	Amoxicillin	57	≤0.06	-	≤0.06-128
	Ampicillin	86	≤0.06	-	≤0.06-128
	Cefoxitin	100	≤0.06	-	≤0.06-2
	Cefpirome	71	≤0.5	-	≤0.06-32
	Chloramphenicol	100	2	-	1-4
	Ciprofloxacin	100	1	-	0.25-1
	Clindamycin	86	≤0.06	-	≤0.06-32
	Imipenem	100	≤0.06	-	≤0.06-0.25
	Loracarbef	-	1	-	≤0.06-8
	Metronidazole	86	≤0.06	-	≤0.06-0.25
	Penicillin	100	≤0.06	-	≤0.06-1
	Piperacillin	86	≤0.5	-	≤0.5-16
	Trovafloxacin	100	0.125	-	≤0.06-0.5
	Vancomycin	86	1	4	≤0.06-4
Cl. limosum (15)	Amoxicillin	27	16	>128	≤0.06->128
	Ampicillin	40	64	>128	2->128
	Cefoxitin	67	8	>128	≤0.06->128
	Cefpirome	27	16	>128	≤0.06->128
	Chloramphenicol	100	0.5	2	≤0.06-4
	Ciprofloxacin	73	1	4	≤0.06-8
	Clindamycin	80	≤0.06	16	≤0.06-16
	Imipenem	40	32	>128	1->128
	Loracarbef	-	>128	>128	≤0.06->128
	Metronidazole	100	0.25	1	≤0.06-4
	Penicillin	80	≤0.06	8	≤0.06-16
	Piperacillin	60	32	>128	≤0.06->128
	Trovafloxacin	100	≤0.05	1	≤0.06-2
	Vancomycin	13	>128	>128	16->128

Table 3. Continued.

Organism (n)	Antibiotic agnet	Susceptibility at breakpoint (%)[a]	MIC (μg ml^{-1})		
			MIC_{50}	MIC_{90}	Range
Cl. difficile (14)	Amoxicillin	79	≤0.06	>128	≤0.06->128
	Ampicillin	79	≤0.06	>128	≤0.06->128
	Cefoxitin	86	≤0.06	64	≤0.06-64
	Cefpirome	86	≤0.06	>128	≤0.06->128
	Chloramphenicol	93	≤0.06	16	≤0.06-64
	Ciprofloxacin	93	≤0.06	32	≤0.06-32
	Clindamycin	100	0.25	1	0.12-1
	Imipenem	86	≤0.05	>128	≤0.06->128
	Loracarbef	-	≤0.06	>128	≤0.06->128
	Metronidazole	100	0.25	0.5	≤0.05-1
	Penicillin	100	0.125	0.25	≤0.06-0.5
	Piperacillin	86	≤0.06	32	≤0.06-64
	Trovafloxacin	100	0.125	0.5	≤0.06-1
	Vancomycin	21	32	>128	≤8->128
Cl. beijerinckii (3)[c]	Amoxicillin	-	-	-	0.125; >128; >128
	Ampicillin	-	-	-	0.25; >128; 16
	Cefoxitin	-	-	-	1; >128; 8
	Cefpirome	-	-	-	32; >128; >128
	Chloramphenicol	-	-	-	8; 2; 16
	Ciprofloxacin	-	-	-	0.5; 4; 8
	Clindamycin	-	-	-	8; 4; 4
	Imipenem	-	-	-	8; >128; >128
	Loracarbef		-	-	0.5; >128; >128
	Metronidazole	-	-	-	0.125; 0.25; 0.25
	Penicillin	-	-	-	0.5; 0.5; 2
	Piperacillin	-	-	-	1; 8; >128
	Trovafloxacin	-	-	-	1; 2; 2
	Vancomycin	-	-	-	128; 128; 128
Cl. septicum (41)	Amoxicillin	100	≤0.06	0.25	≤0.06-1
	Ampicillin	100	≤0.06	0.5	≤0.06-1
	Cefoxitin	100	≤0.06	0.25	≤0.06-0.25
	Cefpirome	100	0.125	1	≤0.06-1
	Chloramphenicol	100	1	2	≤0.06-2
	Ciprofloxacin	100	0.5	1	0.25-1
	Clindamycin	100	≤0.06	0.125	≤0.06-0.25
	Imipenem	100	≤0.06	0.125	≤0.05
	Loracarbef	-	0.125	0.5	0.125-1
	Metronidazole	100	≤0.06	0.25	≤0.06-0.25
	Penicillin	100	≤0.06	0.5	≤0.06-1
	Piperacillin	100	≤0.06	0.5	≤0.06-1
	Trovafloxacin	100	0.125	1	≤0.06-2
	Vancomycin	35	8	>128	1->128
Cl. chauvoei (30)	Amoxicillin	93	1	4	≤0.06->128
	Ampicillin	93	1	8	≤0.06->128
	Cefoxitin	97	8	16	≤0.06->128
	Cefpirome	93	8	16	≤0.06->128
	Chloramphenicol	100	1	2	0.125-2
	Ciprofloxacin	100	0.125	0.125	≤0.06-0.125
	Clindamycin	100	≤0.06	0.125	≤0.06-0.125
	Imipenem	100	0.25	1	≤0.06-2
	Loracarbef	-	8	16	0.125m>128
	Metronidazole	100	0.5	2	0.125-4
	Penicillin	100	0.125	1	≤0.06-2
	Piperacillin	40	16	128	≤0.06->128
	Trovafloxacin	100	0.25	0.5	≤0.06-2
	Vancomycin	0	128	>128	64->128

Table 3. Continued.

Organism (n)	Antibiotic agent	Susceptibility at breakpoint (%)[a]	MIC (μg ml^{-1})		
			MIC_{50}	MIC_{90}	Range
Clostridium spp. (42)	Amoxicillin	100	0.25	1	≤0.06-2
	Ampicillin	100	0.25	1	≤0.06-2
	Cefoxitin	93	1	4	≤0.06->128
	Cefpirome	76	1	128	≤0.06->128
	Chloramphenicol	93	2	4	≤0.06-16
	Ciprofloxacin	64	1	8	≤0.06-16
	Clindamycin	69	0.5	32	≤0.06->128
	Imipenem	98	0.25	2	≤0.06-8
	Loracarbef	-	2	16	0.5-16
	Metronidazole	100	0.125	1	≤0.06-2
	Penicillin	83	0.125	1	≤0.06-2
	Piperacillin	100	1	16	≤0.06-32
	Trovafloxacin	100	0.25	0.5	≤0.06-2
	Vancomycin	93	0.5	4	≤0.06-8

[a]Susceptibility breakpoints (μg ml^{-1}): amoxicillin, 4; ampicillin, 8; cefoxitin, 16; cefpirome, 16; chloramphenicol, 8; ciprofloxacin, 2; clindamycin, 2; imipenem, 4; metronidazole, 8; penicillin, 0.5; piperacillin, 32; trovafloxacin, 2; vancomycin, 4; loracarbef (not available).
[b]No MIC_{90}s were examined if the number of isolates was <10.
[c]No MIC_{50}s or MIC_{90}s are examined if the number of isolates testes was ≤4. Individual MIC values fro each strain are reported in the MIC range column.

Cl. subterminate, *Cl. sordelii*, *Cl. Sporogenes*, *Cl. indolis*, *Cl. bifermentans*, *Cl. mangenoti*, and *Cl. perfringens*. However, *Cl. botulinum* was demonstrated in one to six soil samples because the soil was neutral to alkaline in reaction (average pH 7.9) and low in organic matter content (1.4%). In Korea, river flooding usually occurs during the rainy season and most farmers in these areas use the river as their main source of water. Thus, understanding the relationship between the aquatic environment and soil-borne diseases caused by *Cl. botulinum* can be important for the control of the organism. And, further study is needed for the isolation of *Cl. botulium*.

Table 4. Comparative antibiotic activities against 421 clostridia isolated from soil in Korea.

Antibiotic agent	Susceptibility at breakpoint (%)[a]	MIC (μg ml^{-1})		
		MIC_{50}	MIC_{90}	Range
Amoxicillin	77	0.5	32	≤0.06->128
Ampicillin	81	0.5	32	≤0.06->128
Cefoxitin	92	1	16	≤0.06-64
Cefpirome	76	2	>128	≤0.06->128
Chloramphenicol	99	0.125	4	≤0.06-32
Ciprofloxacin	78	1	8	≤0.06->128
Clindamycin	82	0.125	4	≤0.06->128
Imipenem	99	0.125	2	≤0.06-8
Loracarbef	-	4	>128	0.25->128
Metronidazole	98	0.5	2	≤0.06->122
Penicillin	93	0.125	1	≤0.06->128
Piperacillin	84	1	128	≤0.06->128
Trovafloxacin	99	0.25	1	≤0.06->128
Vancomycin	57	1	>128	≤0.06->128

[a]Susceptibility breakpoints (μg ml^{-1}): amoxicillin, 4; ampicillin, 8; cefoxitin, 16; cefpirome, 16; chloramphenicol, 8; ciprofloxacin, 2; clindamycin, 2; imipenem, 4; metronidazole, 8; penicillin, 0.5; piperacillin, 32; trovafloxacin, 2; vancomycin, 4; loracarbef (not available).

The results of the antimicrobial susceptibility tests for the different *Clostridium* species investigated are shown in Table 3, and comparative antibiotic activity against all the isolates are given in Table 4. *Cl. sordellii*, *Cl. subterminale*, and *Cl. limosum* exhibited resistance to penicillin. While most isolates of *Cl. perfringens* and *Cl. sporogenes* were susceptible to cefpirome, *Cl. tetani* and *Cl. limosum* were not. The overall majority of *Clostridium* species strains investigated was sensitive to imipenem except *Cl. limosum*. In particular, *Cl. limosum* was resistant to most antibiotics except for chloramphenicol, metronidazole, and trovafloxacin. However, in most isolates of *Clostridium*, MICs of metronidazole were close to the susceptibility breakpoint for *Cl. perfringens* strains. Only one isolate of *Cl. sporogenes* was resistant to metronidazole with MIC_{50} 64 μg ml^{-1} (Table 3). Trovafloxacin was effective against all isolates of the genus *Clostridium* except one isolate of *Cl. subterminale*, two of *Cl. tetani*, and three of *Cl. novyi* with MIC_{50} 8–16 μg ml^{-1}. Thirteen species of *Clostridium* were resistant to vancomycin except for *Cl. perfringens*, *Cl. sporogenes*, and *Cl. subterminale*. All the isolates of *Cl. chauvoei* were resistant to vancomycin with MIC_{50} 128 μg ml^{-1}. In Table 4, choramphenicol, imipenem, metronidazole, and trovafloxacin showed high resistance (>95%) to all the investigated strains in clostridia. Moreover, cefoxitin and penicillin were shown to be effective antibiotic agents. Among the pathogenic microbes examined, only 82% were susceptible to clindamycin and 92% cefoxitin. Nevertheless, 239 isolates

of *Clostridium* were resistant against vancomycin. Vancomycin resistance was first noted in USA [3] and then vancomycin resistant strains were reported in many European countries [10]. There is evidence that vancomycin-resistant isolates are becoming common in Korea [16]. Certainly, the isolate with the greatest clinical importance is the pathogenic clostridia isolated from soil in Korea. Amoxicillin was effective against *Cl. perfringens*, *Cl. sporogenes*, *Cl. sordellii* and *Cl. chauvoei*, and intermediate resistance was maintained by *Cl. subterminale*, *Cl. innocuum* and *Cl. difficile*. The results of the present study emphasize the need for an antimicrobial susceptibility test for anaerobic pathogenic bacteria, which transmit soilborne disease. It also indicates that antibiotic agents such as the imipenem and trovafloxacin are of value for the treatment of polymicrobial infection. To maintain effective treatment against anaerobic pathogenic bacteria, it is urgent to monitor susceptibility data because antimicrobial resistance is constantly emerging, and investigation of the potential of new antibiotic agents is necessary.

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References

1. Bell, R. G. 1997. Distribution and sources of microbial contamination of beef carcasses. *J. Appl. Microbiol.* **82:** 292-300.
2. Gyobu, Y. 1978. Distribution of *Clostridium perfringens* in the environment and the enterotoxin production by isolated strains. *J. Food Hyg. Soc. Jap.* **19:** 236-241.
3. Handwerger, S., B. Raucher, D. Altarac, J. Monka, S. Marchione, K. Y. Sigh, B. E. Murray, J. Wolff, and B. Walters. Nosocomial outbreak due to *Enterococcus faecium* highly resistant to vancomycin, penicillin, and gentamycin. *Clin. Infect. Dis.* **16:** 750-755.
4. Hatheway, C. L. and E. A. Johnson. 1998. Clostridium, the spore-bearing anaerobes. *In* A. Balowa and B. G. Duerden (ed.), *Topley and Wilsons principle of microbial infections.* Avon: The Bath Press, London, U.K.
5. Hwang, Y.-H. and H.-S. Lee. 2002. Antibacterial activity of *Pinus densiflora* leaf-derived components toward human intestinal bacteria. *J. Microbiol. Biotechnol.* **12:** 610-616.
6. Kim, J.-D., H.-W. An, J.-H. Yoon, Y.-H. Park, F. Kawai, C.-M. Jung, and K.-H. Kang. 2002. Identification of *Clostridium perfringens* AB&J and its uptake of bromophenol blue. *J. Microbiol. Biotechnol.* **12:** 544-552.
7. Kim, J. S., H. Y. Seong, M. W. Kim, J. S. Ku, and S.-Y. Choi. 2003. Effects of minor arginyl tRNA and isoleucyl tRNA on the expression of *Clostridium botulinum* neurotoxin light chain in *Escherichia coli*. *J. Microbiol. Biotechnol.* **13:** 287-291.
8. Krick, N. P. J. and M. W. Ondendaal. 1994. *Clostridium chauvoei* infections. *In* J. A. W. Coeter, G. W. Thomson, and R. C. Yustin (eds.), *Infectious disease of livestock with special reference to Southern Africa.* Oxford University Press, Oxford, U.K.
9. Kobayashi, T., K. Watanabe, and Ueno, K. 1992. Distribution of *Clostridium botulinum* and *Clostridium tetani* in Okinawa Prefecture. *Kansenshogaku Zasshi.* **66:** 1639-1644.
10. Martin, C., M. G. Cormicam, and Ronald N. Jones. 1996. Emerging resistance in *Enterococcus* sp. *Med. J. Aust.* **164:** 116-120.
11. McEvoy, J. M., A. M. Doherty, and M. Finnerty. 2000. The relationship between hide cleanliness and bacterial numbers on beef carcasses at a commercial abattoir. *Lett. Appl. Microbiol.* **30:** 390-395.
12. Munang'andu, H. M., P. M. Muyoyeta, A. S. Mweene, and H. Kida. 1996. Bovine clostridial infection in Zambia (1985-1994). *Jap. J. Vet. Res.* **44:** 175-178.
13. NCCLS. 1993. Methods for antimicrobial susceptibility testing of anaerobic bacteria. Approved standard M11-A3, National Committee for Clinical Laboratory Standards Villanova, PA, U.S.A.
14. Onderdonk, A. B. and A. D. Allen. 1994. *Clostridium. In* P. R. Murray, E. J. Baron, E. J. Pfaller, F. C. Tenover, and R. H. Yolken (eds.), *Manual of clinical microbiology.* ASM Press, Washington, D.C., U.S.A.
15. Park, J.-H., M. Kwon, D.-J. Kwon, J.-Y. Yoo, and Y.-J. Koo. 1996. Effect of soybean extract on growth and metabolism of *Clostridium perfringens* and some human intestinal bacteria. *Food Biotechnol.* **5:** 220-225.
16. Rhee, K.-H., K.-H. Choi, C.-J. Kim, and C.-H. Kim. 2001. Identification of *Streptomyces* sp. AMLK-335 producing antibiotic substance inhibitory to vacomycin-resistance Enterococci. *J. Microbiol. Biotechnol.* **11:** 467-474.
17. Saito, M. 1990. Production of enterotoxin by *Clostridium perfringens* derived from humans, animals, and the natural environment in Japan. *J. Food. Protect.* **53:** 115-118.
18. Siefert, H. S. H., K. Bader, J. Cyplik, S. J. Gonazalez, F. Roth, M. Z. Salinas, and U. Sukop. 1996. Environment, incidence, etiology, epizootiology, and immnuoprophylaxis of soil-borne disease in northeast Mexico. *J. Vet. Med. B* **43:** 593-606.
19. Skjelkvale, R. and T. Umemura. 1977. Experimental diarrhea in human volunteers following oral administration of *Clostridium perfringens* enterotoxin. *J. Appl. Bacteriol.* **47:** 329-339.
20. Smith, G. R. 1975. Common mesophilic anaerobes, including *Clostridium botulinum* and *Clostridium tetani*, in 21 soil specimens. *Appl. Microbiol.* **29:** 590-594.
21. Smith, G. R. and C. J. Morison. 1975. *Clostridium botulinum* in the lakes and waterways of London. *J. Hyg.* **75:** 371-379.

22. Smith, G. R., R. A. Milligan, and C. J. Morison. 1978. *Clostridium botulinum* in aquatic environments in Great Britain and Ireland. *J. Hyg.* **80:** 431-436.
23. Sunagawa, H., M. Umemura, K. Kameyama, K. Takeshi, H. Mitamura, and Y. Ando. 1985. Incidence of *Clostridium perfringens* in swine, cattle and chicken, and enterotoxin production and spore germination of the isolates. *Rep. Hokkaido Inst. Publ. Health* **35:** 22-25.
24. Tschirdewahn, B., S. Notermans, K. Wernars, and F. Unterman. 1991. The presence of enterotoxigenic *Clostridium perfringens* strains in faces of various animals. *Int. J. Food Microbiol.* **14:** 175-178.
25. Van Mamme-Jongsten, M., K. Wernars, and S. Notermans. 1989. Cloning and sequencing of *Clostridium perfringens* enterotoxin gene. *Antonie van Leeuwenhoek* **56:** 181-190.
26. Yamakawa, K. and S. Nakamura. 1992. Prevalence of *Clostridium botulinum* type E and coexistence of *Closrisium botulinum* nonproteolytic type B in the river soil of Japan. *Microbiol. Immunol.* **36:** 583-591.