Association of Iota-Like Toxin and *Clostridium spiroforme* with Both Spontaneous and Antibiotic-Associated Diarrhea and Colitis in Rabbits

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A helically coiled, anaerobic, gram-positive sporeforming bacillus, identified as *Clostridium spiroforme*, was isolated from the cecal contents of all of 27 rabbits with spontaneous diarrhea, at a mean concentration of $10^{6.0}$ spores per g of material. All of these rabbits also had a toxin present in their cecal contents that was neutralized by anti-*Clostridium perfringens* type E iota toxin, but not by other clostridial antitoxins. In addition, four rabbits with clindamycin-associated colitis were positive for *C. spiroforme* at a mean concentration of $10^{4.5}$. All of these animals also had iota-like toxin present. Iota-like toxin was not detected in the cecal contents of 72 healthy animals, although *C. spiroforme* was found in two of these animals at a mean concentration of $10^{6.0}$. *C. spiroforme* was shown to produce a toxin in vitro that was lethal to mice and caused dermonecrosis in guinea pigs. In all cases, this toxin was neutralized by anti-*C. perfringens* type E iota toxin.

There has been much recent interest in both spontaneous and antibiotic-induced enterotoxemia in rabbits (1, 5, 12, 16, 20, 22). Although a toxin that is specifically neutralized by anti-*Clostridium perfringens* type E iota toxin has been implicated, neither *C. perfringens* type E nor any organism with the ability to produce a cross-reacting toxin has been isolated.

The present communication describes the first isolation of a gram-positive, helically coiled, anaerobic bacterium, *Clostridium spiroforme*, from rabbits, its association with iota-like toxin-positive disease in these animals, and the in vitro production of a toxin by this organism that is neutralized by anti-*C. perfringens* type E iota toxin.

Coiled, gram-positive anaerobic bacteria were first reported from rat ceca by Bladen et al. (2). Since then, similar organisms have been described from the intestines of rats and mice (6, 7, 9, 11, 13, 14, 24), chickens (11), and humans (11, 19). They have also been isolated from a human abdominal abscess (21). This group of organisms had a checkered taxonomic history until Kaneuchi et al. (11) clarified their true position within the genus *Clostridium*.

Although previous workers (8; J. F. Prescott, Ph.D. thesis, University of Cambridge, 1977) have studied the cecal flora of rabbits in some depth, this work represents the first report of the isolation of this type of microorganism from

rabbits and represents the first isolation of iota-like toxin-producing organisms from diseased animals.

MATERIALS AND METHODS

Source of material. Samples of cecal contents from healthy and diseased rabbits were forwarded for analysis from four laboratories in the United Kingdom and one in the United States that housed rabbits for research purposes and from six commercial rabbit breeding farms in France.

All samples were individually sealed in small leak-proof containers which were packed in solid CO_2 in a polystyrene container. All samples were frozen at $-20°C$ immediately upon receipt.

Clindamycin treatment. Four young (1.0 to 1.5 kg) New Zealand White rabbits of 2-star quality (Report. The accredition and recognition schemes for suppliers of laboratory animals, 2nd ed. Carshalton, Surrey. Medical Research Council, 1974) from an external source (Bantin and Kingman Ltd., Grimston, Hull, England) were treated with clindamycin phosphate (Upjohn Ltd., Crawley, Sussex, England) at a concentration of 15 mg/kg per day for 3 days. The antibiotic was suspended in 2 ml of 0.9% sodium chloride and administered by stomach tube in two divided doses. One animal was sacrificed as soon as symptoms of the disease (scouring) were observed, and the other animals were sacrificed about 18 h after the onset of diarrhea.

Isolation of clostridia. Cecal contents were removed aseptically postmortem from rabbits within 30 min of their deaths, which were caused either by presumptive

clostridial enterotoxemia (a finding based on fecal soiling of the tail fur and diarrhea [5]) or, in the case of healthy animals, by sacrifice. Material was stored at −20°C until processed. For processing, approximately 1 g of thawed and well-mixed cecal material was transferred to 9 ml of dilution salts (10). The actual weight of the sample was calculated from the increased weight of the first dilution tube. The sample was mixed by vigorous shaking, and a 10-fold dilution series was prepared. Separate 1-ml aliquots from each dilution were either heated at 80°C for 10 min or exposed to an equal volume of absolute ethanol (yielding a final concentration of 50% ethanol) for 1 h at room temperature (15). Both procedures were used for the selection of clostridial spores. Samples (0.1 ml) of these treated dilutions were spread onto the surface of Columbia base (Oxoid Ltd., London, England) 10% blood agar plates, and egg yolk agar (25). All plates were prereduced before use by leaving them in an anaerobic atmosphere at room temperature for 24 h. Seeded plates were incubated anaerobically (80% nitrogen, 10% carbon dioxide, 10% hydrogen) at 37°C in an anaerobe jar. The anaerobic atmosphere was generated by the use of a gas-kit system (Oxoid). Plates were examined at 2, 3, and 5 days. All different colonial types were Gram stained and recorded. Presumptive *C. spiroforme* could be identified and enumerated due to their characteristic cellular morphology (see Fig. 1). At least three presumptive *C. spiroforme* isolates from each specimen were kept in Robertson cooked meat medium (RCM) (Southern Group Laboratories, Hithergreen, England) for further identification. This procedure also allowed for the screening of *Clostridium difficile* (3).

Identification criteria. A full range of biochemical tests were performed, including gas-liquid chromatographic analysis of the volatile fatty acids produced after incubation for 3 days at 37°C in chopped meat-carbohydrate (10), and the isolates were identified by the criteria of Kaneuchi et al. (11).

Toxin detection. Cecal contents were analyzed for the presence of toxin by administration of cecal filtrates to mice as described elsewhere (23), with the exception that material to be tested was administered intraperitoneally and not intravenously (16, 20). *C.*

spiroforme isolates were tested for their ability to produce toxin in vitro. Eight isolates selected at random from diseased animals and both strains isolated from the "healthy" animals were grown anaerobically at 37°C for 18 h in both chopped meat-glucose medium (10) and RCM. Cells were collected by centrifugation and discarded, and the resultant supernatants were filtered through 0.22-μm-pore filters. A 0.5-ml sample of the filtrate was mixed with 0.3 ml of a 1% aqueous solution of Casamino Acids (diluent) (23). Each of a pair of AKR 3-star quality mice (15 to 20 g) were injected intraperitoneally with 0.4 ml of the prepared filtrate. In addition, 0.3 ml of similarly prepared culture filtrate was injected intradermally into the backs of guinea pigs as described previously by Sterne and Batty (23), and the animals were screened for dermonecrosis. Neutralization of the toxic effects was performed by mixing 0.5 ml of the filtrate with 0.2 ml of diluent and 0.1 ml of antitoxin or with 0.1 ml of normal serum. The antitoxins used were polyvalent gas gangrene antitoxin and individual antitoxins to *C. perfringens* types A, C, D, and E, *Clostridium novyii* types A and B, and *Clostridium haemolyticum* (Wellcome Research Laboratories, Beckenham, Kent, England). The mixture was allowed to stand at room temperature for 30 min before the animals were challenged as described above. In both animal systems, sterile RCM or chopped meat-glucose and filtrates of cecal contents from diseased rabbits were used as negative and positive controls, respectively.

RESULTS

C. spiroforme was isolated from the ceca of all 27 rabbits with disease at a mean concentration of $10^{6.0}$/g (wet weight) of cecal contents. This organism was only detected in the cecal contents of 2 of 72 healthy control animals in concentrations of $10^{5.0}$ and $10^{6.3}$ (Table 1). *C. spiroforme* was also isolated at a mean concentration of $10^{4.5}$ from the four animals with clindamycin-associated colitis (Table 1). These animals also had iota-like toxin present. Neither *C. perfringens* type E nor *C. difficile* was isolated from

TABLE 1. Presence of *C. spiroforme* and iota-like toxin in rabbits

Source of animals studied	Status of animal (no.)	No. with iota toxin	*C. spiroforme*	
			No. positive	Mean log₁₀ no. of spores/g cecal material (range)
United Kingdom laboratories (4)	Spontaneous disease (12)	12	12	6.2 (2.3–7.0)
	Clindamycin-associated disease (4)	4	4	4.5 (2.3–5.2)
	Healthy animals (20)	0	0	
North American laboratory	Spontaneous disease (1)	1	1	7.0
	Healthy animals (4)	0	0	
European rabbit farms (6)	Spontaneous disease (14)	14	14	5.7 (3.0–6.3)
	Healthy animals (48)	0	2	6.0 (5.0–6.3)
Total	Diseased (31)	31	31	5.8 (2.3–7.0)
	Healthy (72)	0	2	6.0 (5.0–6.3)

TABLE 2. Characteristics of *C. spiroforme* isolated from rabbits and those described by Kaneuchi et al. (10)[a]

Characteristics	This report (6 strains)	Kaneuchi et al. (8 strains)	Type strain (NCTC 11211)
Acid from:			
Esculin	−	−w	−
Cellobiose	−	+−	−
Dulcitol	−	−	−
Galactose	−	−	−
Glucose	+	+	+
Lactose	+	+	+
Maltose	−	−	−
Mannitol	−	−	−
Melibiose	−	−	−
Raffinose	−	−	−
Salicin	−	+−	−
Starch	−	−	−
Sucrose	+	+	+
Trehalose	−	−	−
Esculin hydrolysis	−	+−	−
Gelatin hydrolysis	−	−	−
Meat digestion	−	NR	NR
Indole	−	−	−
Nitrate	−	−	−
Catalase	−	−	−
Lecithinase	−	−	−
Lipase	−	−	−
Hemolysis	−	−	−
Motility	−	−	−
Urease	−	−	−
Spore formation	Terminal	Terminal (6/8)	+
Products of fermentation	Acetic acid	Acetic acid	Acetic acid

[a] +, Reaction positive; −, reaction negative; w, reaction weak; +−, −w, first reaction most frequent, 10 to 40% of strains had second reaction; NR, not recorded.

any of the healthy or diseased animals. The numbers of *C. spiroforme* present were probably much higher than those reported here, as all the figures quoted are based on recovery of spores.

All of the animals screened for the presence of a toxin specifically neutralized by anti-*C. perfringens* type E iota toxin and shown to be positive had diarrhea and postmortem features consistent with enterotoxemia (5) and harbored *C. spiroforme* (Table 1). Of the *C. spiroforme*-positive animals, 31 were toxin positive and 2 were toxin negative. Of the *C. spiroforme*-negative animals, none were toxin positive and 70 were toxin negative. Eight strains of *C. spiroforme* isolated from diseased rabbits were tested for production of toxin. All strains produced a toxin in vitro that was lethal to mice and caused dermonecrosis in guinea pigs. All of these effects were neutralized by anti-*C. perfringens* type E

iota toxin but not by normal serum or any of the other antitoxins used. The strains tested only produced toxin when grown in chopped meat-glucose medium. Toxin could not be detected in the filtrates of cultures grown in RCM. The National Collection of Type Cultures strain of *C. spiroforme* (NCTC 11211) consistently failed to produce toxin in either medium, raising the possibility that nontoxigenic variants exist. Results of biochemical and cultural tests performed on the isolates are presented in Table 2. Cells measured 0.5 by 2.0 to 6.0 μm in the coiled state and showed various amounts of coiling (Fig. 1). Coiling was retained despite 15 subcultures of two of the isolates.

DISCUSSION

A number of workers have looked at enterotoxemia in rabbits with a view to delineating its etiology (1, 5, 20) and as a model of antibiotic-associated gastrointestinal disorders in man (12, 16, 22). These workers noted an overall increase in total clostridia (12) and the presence of a toxin in the cecum which was neutralized by anti-*C. perfringens* type E iota toxin (16). The presence of iota-like toxin was also noted in this study of the naturally occurring disease. In addition, this work reports the isolation from rabbits with disease of an organism conforming to the characteristics of *C. spiroforme*. The observation that this organism was isolated from all cases of iota-like toxin-positive disease and rarely from healthy animals confirms our earlier observations on a small number of animals (4). It is important to note that both of the animals in the
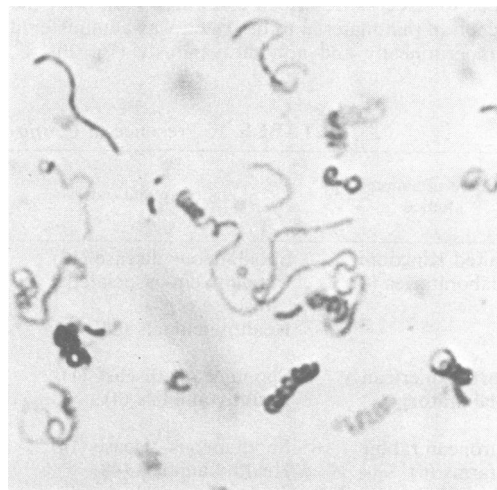

FIG. 1. Morphological appearance of *C. spiroforme*. Gram stain from a surface colony after 48 h of incubation (×1,600).

healthy control group that carried *C. spiroforme* had cecal contents that were more like those of diseased animals than healthy animals, and it is possible that these animals may have had early disease. In addition to this association of *C. spiroforme* with disease and the presence of iota-like toxin in cecal contents is the demonstration of the production of a toxin by this organism in vitro that is neutralized by anti-*C. perfringens* type E iota toxin. We feel that the isolation of *C. spiroforme* from all of the rabbits with either spontaneous or antibiotic-associated disease and its absence in healthy rabbits, the concordance between presence of this organism and iota-like toxin in the cecum (see above), and the in vitro production of iota-like toxin by *C. spiroforme* strongly implicate *C. spiroforme* as the etiological agent of this disease. These observations are strengthened by the absence of *C. perfringens* type E and *C. difficile* and the observation that the association of *C. spiroforme* with disease was noted in animals from a variety of sources both in Europe and the United States and was not restricted to one colony.

It is not known whether the disease is a true infection or is due to an overgrowth of small numbers of *C. spiroforme* normally resident in the gut. The fact that one of the rabbit colonies studied (United Kingdom laboratory) was derived by caesarian section and then barrier maintained (5) and was seeded with a "normal" gut flora which did not include *C. spiroforme* implied that the organism was acquired from the environment and was able to establish itself in the gastrointestinal tracts of these rabbits with an artificial gut flora. One would assume that in the antibiotic-associated disease the antibiotic disrupts the normal flora, creating an environment conducive to the establishment of exogenously acquired *C. spiroforme*. This sort of process has been shown in other antibiotic- and clostridia-associated diseases. For example, in the hamster model of antibiotic-associated, *C. difficile*-mediated ileocecitis, it has been shown that the animal has to be exposed to the organism after administration of antibiotic to induce disease (17).

Since a specific toxin-producing microorganism has now been implicated in the disease, it should be possible to attempt treatment of diseased animals with specific antimicrobial agents, such as vancomycin, or with antitoxin. By analogy with the use of vaccination for the prevention of pig-bel (18), which is a disease of the human gut caused by the toxins of *C. perfringens* type C, it may prove possible to prevent iota-like toxin-associated disease of rabbits by the development of an appropriate vaccine.

It is also of interest that *C. spiroforme* will induce disease in guinea pigs that have been treated with clindamycin (R. J. Carman, unpublished data). This finding raises the possibility that *C. spiroforme* may be associated with disease in other animal species and suggests that its possible role in human gastrointestinal disorders should be investigated.

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