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## SIGNIFICANCE OF *CLOSTRIDIUM SPIROFORME* IN THE ENTERITIS-COMPLEX OF COMMERCIAL RABBITS

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### ABSTRACT

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Commercial rabbits showing clinical signs of enteritis-complex were examined for the presence of *Clostridium spiroforme* and its iota-like toxin. The bacterium was detected by Gram stain in 52.4% of 149 cecal samples and iota-like toxin in 7.4%. From 29 strains of *C. spiroforme* tested, 26 were toxigenic, originating from 24 of 29 rabbitries. In 13.4% of the samples, *C. spiroforme* was present as the only known disease agent. Gross and microscopic lesions were similar to those described in the literature. In the other samples, *C. spiroforme* was associated with attaching effacing *Escherichia coli* (29.5%), *Bacillus piliformis* (10.3%), rotaviruses (25.6%), coronavirus (2.6%), *Eimeria* spp. (44.9%) and cryptosporidia (6.4%). In 33.3% of *C. spiroforme*-containing samples, more than one of these agents was present. There was no significant difference between the presence of these organisms in *C. spiroforme*-positive and negative samples. On the basis of these results as well as that of previous data, we suggest that *C. spiroforme*-mediated diarrhea is favoured by maldigestion, initiated by infectious agents and/or nutritional factors.

### INTRODUCTION

Juvenile enteritis is the most important cause of losses in intensively raised slaughter rabbits. The highest mortality occurs at 5–8 weeks of age. A previous survey in commercial rabbitries showed the presence of one or more disease agents, such as attaching effacing *Escherichia coli* (AEEC), rotaviruses and coccidia in the gut of 71.5% of diarrheic rabbits examined (Peeters et al., 1984b). In most rabbitries more than one disease agent was present. This confirms the assumption of Whitney (1970) that the etiology of the disease is complex and that the term “enteritis-complex” for this type of juvenile enteritis is still valid.

In recent years several reports on the presence of *Clostridium perfringens*

Type E iota toxin in the ceca of diarrheic rabbits have been published, although workers failed to isolate *C. perfringens* Type E from the ceca (Katz et al., 1978; Patton et al., 1978; Lamont et al., 1979; Baskerville et al., 1980; Fernie and Eaton, 1980; Rehg and Pakes, 1982). In 1982, Carman and Borriello reported iota-like toxigenicity by *C. spiroforme* isolated from diarrheic rabbits. Since the recognition of *C. spiroforme* as the etiologic agent in iota enterotoxemia, simple isolation methods have been developed (Borriello and Carman, 1983); therefore we decided to study the significance of *C. spiroforme* in the enteritis-complex of commercial rabbits and its possible relationship with other pathogens.

#### MATERIALS AND METHODS

Between 1 July 1983 and 30 June 1984, 149 live weanling diarrheic rabbits were collected from 29 commercial rabbitries with between 60 and 400 does. In the rabbitries, New Zealand White or Dendermonde White rabbits were housed in wire cages and were fed ad libitum with a commercial pelleted ration containing 66 ppm robenidine as anticoccidial drug. Rabbits were weaned between 4 and 5 weeks of age. During each visit, one representative rabbit was taken from a cage of animals showing diarrhea for a maximum of 12 h without apparent signs of dehydration. None of the animals had been treated with antimicrobial drugs.

Within 20 min after killing the animals, specimens of duodenum, mid-jejunum, ileum, cecum, colon, heart, liver and kidney were fixed in 10% (v/v) formalin in phosphate-buffered saline and processed routinely for paraffin sections. Sections were cut at 5  $\mu$ m and stained with hematoxylin and eosin; some were stained with Warthin-Starry silver stain or with Gram's stain (Lee and Luna, 1960). Selected formalin-fixed samples were washed overnight in distilled water and fixed in 2.5% (w/v) cacodylate buffered glutaraldehyde (pH 7.6), post-fixed with 1% (w/v) osmium tetra-oxide and block-stained with 2% uranyl acetate (w/v). Blocks were embedded in epon 812. Ultra-thin sections were examined with a Philips 201 transmission electron microscope.

Samples of cecal content were taken for parasitological, bacteriological and virological analyses. Smears were made for Gram's stain and other smears were stained with carbol-fuchsin for demonstration of cryptosporidia (Heine, 1981). Cecal content was examined using the salt-flotation-concentration technique for demonstration of coccidia and helminths. Numbers of parasites were counted by the McMaster egg counting technique (MAFF, 1977). Samples of cecal content were clarified by low speed centrifugation (8000  $\times$  g). The supernatants were negatively stained with 2% (w/v) uranyl acetate in bidistilled water and examined for the presence of viral particles by transmission electron microscopy.

The presence of *E. coli* in the duodenum, jejunum, ileum and cecum was evaluated after streaking plates of G2SN medium with intestinal contents

and incubating aerobically at 37°C for 18 h as described before (Peeters et al., 1984c). Coliform colonies were identified by the method of MacKenzie et al. (1948). Isolation of *C. spiroforme* from cecal contents was done according to Borriello and Carman (1983). When samples could not be examined immediately, they were stored at -20°C. Numbers of spores of *C. spiroforme* were evaluated after culture of 10-fold dilutions of cecal contents on Columbia base (Oxoid Ltd., London, England) 10% sheep blood agar plates. Strains were stored in Robertson's cooked meat medium (Southern Group Laboratories, Hithergreen, England) to await further identification. Presumptive *C. spiroforme*-isolates were identified by polyacrylamide gel electrophoresis (PAGE) of outer membrane proteins and compared with those of the reference strain of *C. spiroforme* NCTC 11493 (Cato et al., 1982). *C. spiroforme* isolates were tested for their ability to produce iota-like toxin in vitro by rocket immunoelectrophoresis (RIEF) of 48-h BHI peptone glucose salts culture filtrates against antiserum to *C. spiroforme* NCTC 11493 toxin (Carman et al., 1985). Cecal contents were analysed for the presence of iota-like toxin by intraperitoneal administration of cecal filtrates to mice and neutralization by *C. perfringens* antitoxins (Wellcome Research Laboratories, Beckenham, Kent, England) as described elsewhere (Sterne and Batty, 1975).

## RESULTS

In 44 rabbits, histology showed typical lesions for attaching effacing *E. coli* (AEEC) as described before (Peeters et al., 1984a, 1985b); these were moderate to large numbers of gram-negative organisms apparently attached to the epithelium of ileum, cecum and colon. Microvilli were effaced. The lamina propria beneath areas of attachment was infiltrated by polymorphonuclear leucocytes (PMNL). At necropsy, the cecal wall showed edema and cecal contents were foul smelling, watery and brown. Bacteriology of intestinal contents showed confluent growth of *E. coli* from at least the cecum of these animals.

Lesions of Tyzzer's disease were found in 14 animals. A detailed description of clinical signs, gross pathology and histopathological lesions is reported elsewhere (Peeters et al., 1985a). Briefly, lesions consisted of multifocal hepatic necrosis and patchy mucosal necrosis in the ileum, cecum and colon. Histology of the liver showed bundles of weakly-staining gram-negative and silver-positive rod-shaped bacilli in apparently viable hepatocytes bordering foci of necrosis. Transmission electron microscopy confirmed these organisms to have a similar ultrastructure to *Bacillus piliiformis*, the etiologic agent of Tyzzer's disease.

Transmission electron microscopy revealed the presence of rotaviruses, coronaviruses and parvoviruses in cecal contents of 40, two and one animal, respectively. Carbol-fuchsin stain and histology showed the presence of cryptosporidia in the intestinal tract of 12 animals. *Eimeria* spp. were found

in 64 animals: 19 cecal samples contained  $<10^3$  oocysts  $g^{-1}$ , 24 between  $10^3$  and  $10^4$ , 12 between  $10^4$  and  $10^5$  and nine samples  $>10^5$  oocysts  $g^{-1}$ .

Semi-circular Gram-positive *C. spiroforme*-like organisms were demonstrated in smears of 78 cecal samples from diarrheic rabbits out of a total of 149 examined. When organisms were abundantly present, helically coiled forms were also seen. Organisms presenting as loosely-coiled rods were cultured anaerobically from 46 of these samples on Columbia sheep blood agar. Between  $1 \times 10^2$  and  $2 \times 10^6$  spores of these organisms were present per gram (wet weight) of cecal contents. Numbers of *C. spiroforme* present were probably higher, as figures are based on recovery of spores. A total of 32 *C. spiroforme*-like strains was isolated and stored in standard Robertson's cooked meat medium. Presumptive *C. spiroforme* strains with typical loosely-coiled morphology as seen by Gram stain could not be separated from contaminants in samples containing  $<10^3$  spores  $g^{-1}$ . PAGE confirmed identity between outer membrane proteins of 25 isolates and those of the reference strain of *C. spiroforme* NCTC 11493. Four strains grew insufficiently and were not tested. Rocket immuno-electrophoresis of 48-h BHI peptone glucose salts culture filtrates showed the presence of iota-like toxins in 26 cultures, including the cultures from the four strains which could not be tested by PAGE. So, it can be assumed that 29 isolates of 32 were in fact *C. spiroforme*. Three of the 29 isolates were not toxigenic. Toxic strains originated from 24 of the 29 rabbitries visited. Neutralization trials with iota-anti-toxins in mice showed that 11 out of 78 cecal filtrates of *C. spiroforme*-positive samples contained enough iota-like toxin to kill mice.

Table I shows the presence of parasites, bacteria and viruses in cecal samples which were positive or negative for *C. spiroforme* as judged by

TABLE I

Occurrence of disease agents in cecal samples from diarrheic rabbits with or without *C. spiroforme* (assessed by Gram's stain)

Pathogens other than <i>C. spiroforme</i>	<i>C. spiroforme</i>	
	Present	Absent
Not detected or absent	20	14
<i>E. coli</i> (AEEC)	23	21
<i>B. piliformis</i>	8	6
Coronavirus	2	0
Parvovirus	0	1
Rotavirus	20	20
<i>Eimeria</i> spp.	35	29
<i>Cryptosporidium</i> sp.	5	7
Number of animals	78	71
Number of rabbitries	24	28

Gram's stain. There was no significant difference between the presence of these organisms in *C. spiroforme*-positive or negative samples. *C. spiroforme* was associated with AEEC in 23 of 44 cases (52%), rotaviruses in 20 of 40 cases (50%), *B. piliformis* in eight of 14 cases (57%), cryptosporidia in five of 12 cases (42%) and coccidia in 35 of 64 cases (55%). In 26 of 78 samples, more than one of these agents was present. In 20 samples, only *C. spiroforme* was established as potential pathogen by Gram's stain. From 12 of these 20 samples, *C. spiroforme* was cultured: all 12 strains were toxigenic. Three of the 20 cecal samples contained enough toxin to kill mice.

In 115 of 149 rabbits examined more than one pathogen was established. This makes evaluation of gross and microscopic lesions difficult, so only lesions in the 20 rabbits showing *C. spiroforme* as the only possible pathogenic agent can be evaluated. The perineum and hind legs of these 20 rabbits were extensively soiled by liquid feces. At necropsy of these animals, the small intestine was dilated and contained a colourless to light yellowish liquid. In two animals, the small intestine was moderately congested. Cecal contents were watery (16/20). In animals killed in extremis, cecal contents were blood-stained (3/20): blood-stained cecal contents filtrates were toxic to mice. The cecal wall was thickened in five animals and showed paint-brush hemorrhages on the serosa of two animals; in the latter the proximal colon was congested. Mesenteric lymph nodes were moderately to severely swollen. Kidneys were pale and swollen in eight animals. Similar lesions were also established in animals with mixed infections. However, hemorrhages were only found in *C. spiroforme*-positive rabbits.

Histology showed diffuse infiltration of the liver by PMNL in six animals and some miliary necrosis in one rabbit. Renal tubuli were dilated and showed mild epithelial degeneration in eight animals. Intestinal lesions were most pronounced in the distal ileum, cecum and proximal colon. They varied from accumulation of basophilic debris in enterocytes to necrosis and desquamation of epithelial cells. In the ileum and to a lesser degree in the jejunum and duodenum, villus length was reduced. In the three cases with toxic cecal contents, ileal, cecal and colonic mucosa were partly denuded. Where the mucosa was still intact, it was covered by cuboidal epithelial cells. The ileal mucosa was almost completely flat. The lumen was filled with a proteinaceous fluid containing cellular debris, blood cells and fibrin. Edema of cecal propria and submucosa was present in seven animals. Hemorrhages were regularly seen. Propria and submucosa were infiltrated by PMNL and round cells.

## DISCUSSION

The data from this survey indicate that *C. spiroforme* occurs commonly in diarrheic rabbits: 52% of diarrheic rabbits were carriers, 90% of the isolated strains were toxigenic and 83% of the rabbitries sampled were

positive. Gross and microscopic lesions were similar to those described in the literature (Carman and Evans, 1984). However, *C. spiroforme* was not the only factor involved in juvenile enteritis.

A previous survey (Peeters et al., 1984b) showed that different pathogenic agents can be detected in rabbits affected by juvenile enteritis: sometimes only one agent is present, sometimes different agents occur together or succeed each other in time. In each of the rabbitries sampled, more than one agent was present. This was confirmed by the present results (Table I).

Pathogenic agents may alter digestion and absorption of nutrients by destruction of epithelial cells and villous atrophy. The resulting maldigestion changes the composition of the substrate available to the intestinal flora which may be disrupted. This may change intestinal ecology from one that exerts resistance to colonization by *C. spiroforme* to one that is permissive to colonization, resulting in proliferation of *C. spiroforme* and consequent production of iota-like toxin after a lag phase of 8–18 h (Carman et al., 1984). This hypothesis may be supported by following findings: *C. spiroforme* is seldom present in healthy rabbits (Borriello and Carman, 1983), although the organism is present in most rabbitries; in 78 cases of *C. spiroforme*-associated diarrhea, *C. spiroforme* was present as the only known infectious agent in only 26%; experimental infection with *C. spiroforme* only causes diarrhea if the infection coincides with a stress factor such as weaning (Carman and Borriello, 1984) or with the administration of some antibiotics (Lamont et al., 1979; Rehg and Pakes, 1982; Borriello and Carman, 1983). Both these conditions are reputed to modify intestinal flora.

Because toxigenic strains of *C. spiroforme* are present in a high number of rabbitries, factors influencing the intestinal proliferation of *C. spiroforme* and toxin production should be investigated further. More research on preventive measures is also needed.

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#### REFERENCES

- Baskerville, M., Wood, M. and Seamer, J.H., 1980. *Clostridium perfringens*. Type E enterotoxaemia in rabbits. *Vet. Rec.*, 107: 18–19.
- Borriello, S.P. and Carman, R.J., 1983. Association of toxigenic *Clostridium spiroforme* with iota toxin positive enterotoxaemia in rabbits. *J. Clin. Microbiol.*, 17: 414–418.
- Carman, R.J. and Borriello, S.P., 1982. *Clostridium spiroforme* isolated from rabbits with diarrhoea. *Vet. Rec.*, 11: 461–462.

- Carman, R.J. and Borriello, S.P., 1984. Infectious nature of *Clostridium spiroforme*-mediated rabbit enterotoxaemia. *Vet. Microbiol.*, 9: 497-502.
- Carman, R.J. and Evans, R.H., 1984. Experimental and spontaneous clostridial enteropathies of laboratory and free living lagomorphs. *Lab. Anim. Sci.*, 34: 443-452.
- Carman, R.J., Borriello, S.P. and Waynforth, H.B., 1984. Microbiological changes during experimental *Clostridium spiroforme* mediated diarrhoea (iota enterotoxaemia) of rabbits. In: J. Archibald (Editor), *Proc. VIIIth ICLAS/CALAS Scientific Symposium*, Vancouver, Gustav Fischer Verlag, Stuttgart.
- Carman, R.J., Van Tassell, R.L. and Wilkins, T.D., 1985. Production of iota toxin by *Clostridium spiroforme*: a requirement for divalent cations. *FEMS Microbiol. Lett.*, submitted.
- Cato, E.P., Hash, D.E., Holdeman, L.V. and Moore, W.E.C., 1982. Electrophoretic study of *Clostridium species*. *J. Clin. Microbiol.*, 15: 688-702.
- Fernie, D.S. and Eaton, P., 1980. The demonstration of a toxin resembling *Clostridium perfringens* iota toxin in rabbits. *FEMS Microbiol. Lett.*, 8: 33-35.
- Heine, J., 1981. Eine einfache Nachweismethode für Kryptosporidien im Kot. *Zentralbl. Veterinaermed., Reihe B*, 29: 324-327.
- Katz, L., Lamont, J.T., Trier, J.S., Sonnenblick, E.B., Rothman, S.W., Broitman, S.A. and Rieth, S., 1978. Experimental clindamycin associated colitis in rabbits. Evidence for toxin-mediated mucosal damage. *Gastroenterology*, 74: 246-252.
- Lamont, J.T., Sonnenblick, E.B. and Rothman, S., 1979. Role of clostridial toxin in the pathogenesis of clindamycin colitis in rabbits. *Gastroenterology*, 76: 356-361.
- Lee, G. and Luna, H.T., (Editors), 1960. *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*. McGraw-Hill Book Company, New York, 258 pp.
- MacKenzie, E.F.W., Taylor, E.W. and Gilbert, W.E., 1948. Recent experiences in the rapid identification of *Bacterium coli* type I. *J. Gen. Microbiol.*, 200: 197-204.
- MAFF, 1977. Technical Bulletin No. 18. *Manual of Veterinary Parasitological Laboratory Techniques*. Ministry of Agriculture, Fisheries and Food, London, 129 pp.
- Patton, N.M., Holmes, H.T., Riggs, R.J. and Cheeke, P.R., 1978. Enterotoxemia in rabbits. *Lab. Anim. Sci.*, 28: 536-540.
- Peeters, J.E., Geeroms, R. and Glorieux, B., 1984a. Experimental *Escherichia coli* enteropathy in weanling rabbits: clinical manifestations and pathological findings. *J. Comp. Pathol.*, 94: 521-528.
- Peeters, J.E., Pohl, P. and Charlier, G., 1984b. Infectious agents associated with diarrhoea in commercial rabbits: a field study. *Ann. Rech. Vét.*, 15: 335-340.
- Peeters, J.E., Pohl, P., Okerman, L. and Devriese, L.A., 1984c. Pathogenic properties of *Escherichia coli* strains isolated from diarrheic commercial rabbits. *J. Clin. Microbiol.*, 20: 34-39.
- Peeters, J.E., Charlier, G., Halen, P., Geeroms, R. and Raeymaekers, R., 1985a. Naturally-occurring Tyzzer's disease (*Bacillus piliformis* infection) in commercial rabbits: a clinical and pathological study. *Ann. Rech. Vét.*, 16: 69-79.
- Peeters, J.E., Charlier, G.J. and Raeymaekers, R., 1985b. Scanning and transmission electron microscopy of attaching effacing *Escherichia coli* in weanling rabbits. *Vet. Pathol.*, 22: 54-59.
- Rehg, J.E. and Pakes, S.P., 1982. Implication of *Clostridium difficile* and *Clostridium perfringens* iota toxins in experimental lincomycin-associated colitis of rabbits. *Lab. Anim. Sci.*, 32: 253-257.
- Sterne, M. and Batty, I., 1975. *Pathogenic Clostridia*, Butterworth and Co. Ltd., London, p. 79-84.
- Whitney, J.C., 1970. Some aspects of the enteritis complex of rabbits. In: W.D. Tavernor (Editor), *Nutrition and Diseases in Experimental Animals*. Baillière, Tindall and Cassell, London, pp. 122-131.