Studies on a Canine Intestinal Spirochete
I. Its Isolation, Cultivation and Ultrastructure

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ABSTRACT

The microscopic examination of colonic scrapings from 12 of 18 normal dogs revealed the presence of a spirochete. From two such dogs a spirochete was isolated and successfully cultured. Although similar in outward appearance to the porcine spirochete of swine dysentery the canine spirochete possessed ultrastructural properties which distinguished it from the porcine agent. This canine agent is thought to constitute a separate species and should not be confused with the agent associated with swine dysentery.

RÉSUMÉ

L'examen microscopique de grattages de la muqueuse du côlon de 12 chiens normaux, sur un total de 18, révéla la présence d'un spirochète. Chez deux de ces chiens, on isola un spirochète et on réussit à le cultiver. En dépit de sa ressemblance morphologique au spirochète de la dysenterie porcine, le spirochète canin possédait des propriétés ultrastructurales permettant de le différencier du spirochète du porc. On pense que le spirochète canin représenterait une espèce particulière; il faut par conséquent éviter de le confondre avec celui qu'on retrouve, lors de dysenterie porcine.

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INTRODUCTION

There is considerable evidence to indicate that spirochetes not only constitute part of the dog's normal flora (10, 15) but may also be responsible for dysentery in this species (4, 8, 16). Unfortunately, because of the failure to isolate such agents and conduct transmission studies, their true nature, whether harmless saprophytes or enteric pathogens, remains unclear.

In recent years, a spirochete referred to as *Treponema hyodysenteriae* has been found to be an etiologic factor in swine dysentery (5, 12). Investigators using fluorescent antibody tests have observed spirochetes in the feces of diarrheic dogs which reacted with serum to *T. hyodysenteriae* (4, 16) and speculated that the organism observed in the dog might be the same as or related to that associated with swine dysentery.

This report describes the successful isolation and cultivation of a canine intestinal spirochete from the colon and compares it to *T. hyodysenteriae* in terms of general morphology, ultrastructure, motility and cultural properties.

MATERIALS AND METHODS

Dogs

Eighteen healthy normal dogs of mixed breed and age were used.

Specimens

Two inches of colon, distal to the cecum, were removed from euthanized dogs and placed in sterile petri dishes. After removal of fecal material mucosal scrapings were taken with a glass microscope slide. Smears were prepared, stained with crystal violet and examined by light microscopy for the presence of spirochetes. Isolation attempts were carried out only on material from two of the dogs in which the agent appeared most numerous.

Solid Medium

The composition of the solid medium is presented in Table I and was prereduced (BBL Gas Pak) for at least 24 hours before use.

Liquid Medium

The liquid medium, also prereduced for at least 24 hours before use (BBL Gas Pak), consisted of trypticase-soy broth supplemented with 10% fetal calf serum, 2% laked bovine defibrinated blood and 1.5% bovine rumen fluid.

Isolation Technique

Forty ml of trypticase-soy broth was boiled in a centrifuge tube for ten min to remove excess oxygen and then cooled. Two grams of mucosa scrapings were emulsified in the broth and centrifuged to remove debris at 2500 rpm in a table-top centrifuge for 15-20 min (Model HN IEC, Boston, Mass.). Ten ml of the supernatant fluid was then mixed with 1 ml of sterile fetal calf serum and the mixture was passed through a Millipore HA filter (0.45 μm syringe type fitted with prefilter). Approximately 0.3 ml of the filtrate was streaked onto each agar plate of prereduced anaerobic medium. The cultures were incubated anaerobically at 37°C (BBL Gas Pak) for five to seven days before examination. To minimize contact with oxygen the cultures were opened inside a plastic chamber containing an atmosphere of CO_2. Once pure cultures were obtained the use of the chamber was discontinued, although the organisms were never exposed to the air for more than 15 min during subculturing.

Preparations for Electron Microscopy

Five to seven days' growth was washed from the surface of agar plates using chilled phosphate buffered 0.85% saline, pH 7.2. Some samples of this suspension were treated with 1% sodium desoxycholate or 0.5% 7X detergent (Linbro Chemical Co., New Haven, Conn.) for 30-60 seconds or were exposed to ultrasonic disruption for two or three seconds in order to reveal internal components such as axial fibrils and their points of attachment.

Staining of the spirochetes was accomplished by using 2% phosphotunstic acid, pH 4.5 or 1% ammonium molybdate, pH 5.0. The specimens were placed on copper grids with carbon membranes and examined in a JEOLCO Jem 6A or a JEOLCO Jem 100C electron microscope with the first scope operating at 50Kv and the latter at 60Kv. Pictures were taken on Kodak electron microscope sheet film 4489.

RESULTS

Examination of Colonic Mucosal Scrapings

Spirochetes were found in 12 of 18 healthy dogs examined. Although spirochetes were usually present in moderate numbers, approximately one organism per microscopic field, two dogs in particular had very large numbers of spirochetes in what appeared to be almost pure cultures (Fig. 2). The same organisms observed in the mucosal scrapings, while present, were rarely seen in the fecal contents from these dogs.

Morphology of the Canine Spirochete

The examination of fixed and stained slides of mucosal scrapings revealed a thin, flexible organism possessing one to three loose coils but on occasion also appeared as a slightly curved or straight rod with pointed ends (Fig. 2).

Motility

When observed in wet mounts of mucosal scrapings by darkfield illumination the

TABLE I. Composition of Anaerobic Medium

Ingredients and Source	Quantity
Brain-heart infusion broth (DIFCO)	12 g
Trypticase-soy broth (BBL)	8 g
Yeast extract (BBL)	4 g
Thioglycollate broth (DIFCO)	4 g
L-cysteine (NBCO)	0.4 g
$NaHCO_3$	0.4 g
Agar (DIFCO)	17 g
Distilled H_2O	1 liter
Sterile bovine defibrinated blood[a]	5%
Sterile bovine rumen fluid[a]	3%

[a]Added after autoclaving and cooling to 50° C

spirochete exhibited only a very rapid corkscrew type of motility. In preparations prepared from cultures, however, this corkscrew motility was considerably slower. In addition, three other types of motility were noted: (a) a slow graceful motility which resembled the movement of a rope fixed at both ends and swung in an arc, (b) a serpentine swimming type of motility and (c) a lashing about without movement in any one direction, accompanied by an alternate constriction and relaxation of its coils.

Growth on Solid Media

On the medium described, this organism appeared within 48 hours as a thin haze on the agar surface and occasionally formed small, round, white-tan colonies 0.5-1.0 mm in diameter after five days of incubation. A slightly diffuse, incomplete hemolysis generally could be seen after 72 hours. Sparse growth occurred on brain-heart infusion blood agar, with the formation of clear pinpoint colonies after seven days of incubation. The organism was routinely transferred every seven to eight days on to fresh prereduced media as the viability of such culture declined rapidly after ten days.

Growth in Liquid Medium

In order to grow and maintain this spirochete in broth it was necessary to subculture young, actively growing cultures every four to five days. For the initial inoculation a block of agar approximately two sq cm in area which contained confluent growth of the spirochete was used to inoculate the broth. For serial passage of the organism an inoculum of at least 0.3 ml from a four to five day old broth culture was necessary. The organism grew well within 72 hours resulting in a hazy turbidity. If a wet mount from a culture over five days of age was examined, tangled masses of motile spirochetes could be observed, as well as many spirochetal spheres and organisms with bulb-like swellings at the end of the cell.

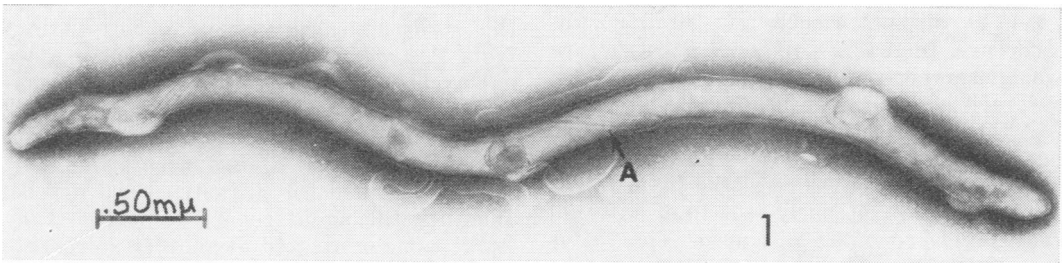
Ultrastructure

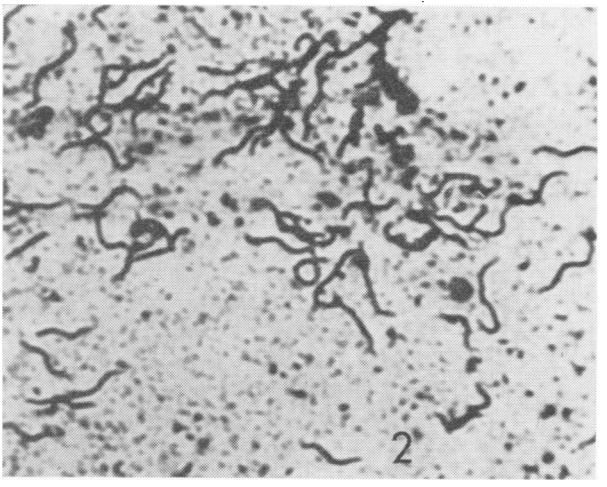
The spirochete possessed blunt ends and was found to be from 0.18-0.20 μm in diameter and 5.0-7.0 μm in length (Fig. 1). It possessed six flagellar filaments (axial fibrils) approximately 15 nm in diameter which were attached to a row of subterminally placed insertion discs 60 nm in diameter (Figs. 4, 5). The outer cell envelope (OCE) of the organism was close fitting about the protoplasmic cylinder and possessed a latticework-like substructure most evident at the end of the cell (Fig. 3).

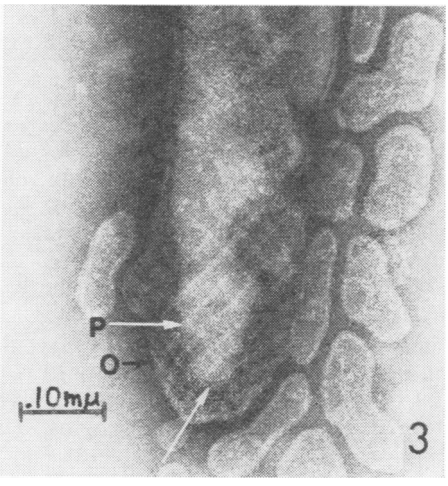
DISCUSSION

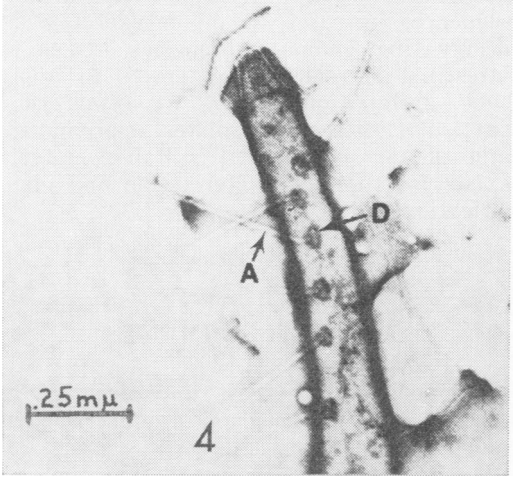
Spirochetes have been observed in the intestine of dogs (4, 8, 13, 15) and other animals (14) and man (6) for many years. Only recently, however, has the cultivation of such organisms from dogs been successful. In some instances the spirochetes in the dog (4, 8, 16) were thought to be associated with dysentery but there is no experimental evidence indicative of any etiological relationship.

In its morphology, motility, growth and close association with the colonic mucosa, the canine agent reported here was similar to the swine spirochete *T. hyodysenteriae* (5). Although outwardly similar, the canine spirochete was smaller than *T. hyodysenteriae* and was less hemolytic on media containing blood. It is at the ultrastructural level however that differences between the canine spirochete described and *T. hyodysenteriae* were most evident. Not only did the canine organism possess fewer flagellar filaments (axial fibrils) than reported for *T. hyodysenteriae* (5) but the outer cell envelope of the canine organism had a characteristic latticework-









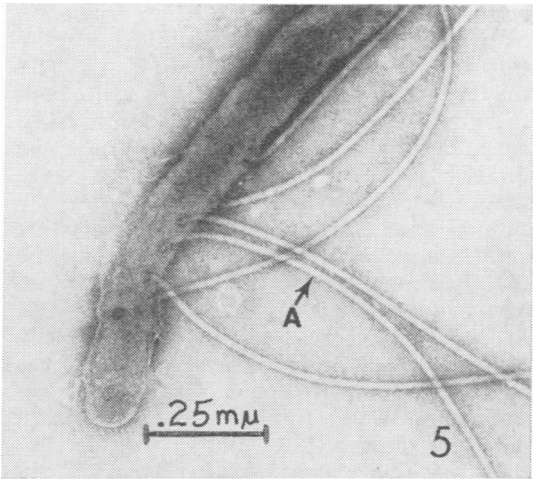


Fig. 1. Ammonium molybdate (AM) stain of the entire cell. Arrow indicates flagellar filament (A). X28,946.

Fig. 2. Organism as observed from colonic mucosal scraping. Fontana's silver stain. X2000.

Fig. 3. Organism from culture after exposure to sonic disruption for two to three seconds. Note latticework substructure (O) separated from protoplasmic cylinder (P). Also note the periodic striations at the tip of the protoplasmic cylinder (arrow). AM. X94,210.

Fig. 4. Organism from culture treated with sodium desoxycholate for 60 seconds. Note flagellar filaments (A) and insertion discs (D). PTA. X55,263.

Fig. 5. Organism from culture treated with 7 X for 30 seconds. Note six flagellar filaments (A). AM. X60,000.

like structure (Fig. 3). By comparison a pathogenic strain of *T. hyodysenteriae* B-78 (11) did not reveal any similar substructure. Instead a polygonal substructure was observed similar to that described in several treponemas by Hovind-Hougen (7).

Although the OCE of both the canine agent and *T. hyodysenteriae* was rather close fitting the envelope of the swine agent stained more intensely so that internal features were often obscured. The latter spirochete was also more resistant to the action of detergents and required a longer exposure time for partial disintegration of the OCE to occur.

In the light of two previous report (3, 5) the number of flagellar filaments (axial fibrils) possessed by *T. hyodysenteriae* needs additional clarification. In our observations *T. hyodysenteriae* had at least 12 flagellar filaments. These results, while differing from the first report (5), are in substantial agreement with a more recent article (3).

Several explanations could account for these differences: (a) the cultures used in the early reports (3) may have contained a mixture of spirochetes, (b) the number of flagellar filaments is not a constant characteristic of this organism or (c) the number of flagellar filaments may be influenced or determined by the cultural methods and techniques used. If the latter two explanations are true then the number of flagellar filaments observed as a means of identification would be limited.

At present it is not known whether the canine spirochete is of any pathogenic significance since the organism can be found in moderate to relatively large numbers on the colonic mucosa of normal dogs. A study by Leach *et al* (10) found loosely coiled spirochetes in large numbers of the colonic crypts of normal dogs and occasionally inside of goblet cells. It has been noted in the rat (10) and in the pig (1) that the administration of a laxative can often cause large numbers of spirochetes to appear in the stools. In a preliminary study we conducted with three dogs this phenomenon was also observed (unpublished data). This may indicate that the detection of spirochetes in the stools of diarrheic dogs is due to a flushing action within the gut and may be of no etiologic significance. However, it is also possible that spirochetes observed in some dysenteric dogs (8) were pathogenic variants or a different species.

The spirochete observed in the dogs of this study appeared to be a species distinct from *T. hyodysenteriae*. Although this does not exclude the possibility of *T. hyodysenteriae* being found in dogs it should be noted that the canine spirochete may easily be confused with *T. hyodysenteriae* if it is not examined for ultrastructural details. Whether the agent reported in this study is the same or related to another spirochete isolated from a dog (9) is not known.

This canine agent conceivably might be capable of colonizing a variety of animal species and be more ubiquitous than presently believed. In one experiment (unreported data) the canine spirochete successfully established itself in the colon of a gnotobiotic pig, indicating the possibility that such agents could colonize swine. This observation might explain the presence in some pigs and dogs of weakly hemolytic, nonpathogenic spirochetes which some investigators have also called *T. hyodysenteriae* (9).

The significance of intestinal spirochetes in different animal species needs to be determined and the basis for classifying and differentiating intestinal spirochetes needs considerable refinement. As a consequence, restraint should be exercised in designating genus and species names to intestinal spirochetes. Attempts to apply such designations as *T. hyodysenteriae*, particularly to canine isolates, is premature and should wait until suitable criteria exists for their characterization and identification (11).

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BOOK REVIEW

ANIMAL DISEASE MONITORING. *Edited by D. G. Ingram, W. R. Mitchell and S. W. Martin. Published by Charles C. Thomas, Springfield, Illinois. 1975. 215 pages. Price $21.50.*

Animal Disease Monitoring is a proceedings of an international symposium held at the University of Guelph in July 1974. The book contains 24 chapters representing papers on theory and practice of animal disease surveillance and reporting, and on the storage and retrieval of animal disease data.

The symposium covered a wide variety of topics including on-the-farm monitoring by veterinary practitioners, surveillance of diseases which are the object of disease control programs, the U.S. National Cancer Institutes, Veterinary Medical Data Program, and reports on the surveillance spinoff from the socialized veterinary medical programs utilized in some of the Canadian provinces. In addition, there is a section on the utilization of diagnostic laboratories for disease surveillance and sections on some well-planned specific disease monitoring systems such as the Neoplasm Registry in Alemeda-Costa Condra counties in California.

Most of the authors deal with their specific monitoring procedures independently and there is little effort to compare the advantages and disadvantages of various approaches. However, it is likely that those attending the symposium acquired a great deal of such information in informal discussions.

Because the papers were mostly presented by epidemiologists and administrators, the book emphasizes the manipulation, use, and interpretation of animal monitoring data and makes very little reference to the problems of the individuals generating the data. In a sense, then, the symposium largely overlooks the axiom that the persons generating data are the key to the success of any retrieval or surveillance system.

The whole area of animal disease monitoring is ridden with problems. These surface over and over again in the individual papers. Some of these include accuracy of diagnosis and entry, completeness of reporting, promptness and completeness of retrieval and the problem of converting raw data into a useable and interpretable form. Anyone considering establishing a system of animal disease records or a morbidity or mortality reporting program on any scale would do well to read this *entire* book and then seek out individual authors for personal consultation. While the book is certainly not intended for veterinary practitioners, it is a must for regulatory veterinarians, academicians or any others interested in morbidity and mortality reporting, animal disease surveillance, monitoring the progress of eradication and control programs, or storage and retrieval of clinical veterinary data. — *R. F. Kahrs.*