

Experimental infection with Cryptosporidium sp. in
conventionally-born suckling piglets and the effect
of treatment with neomycin on the course of infection

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1. INTRODUCTION

The protozoan parasite Cryptosporidium sp. was first described by Tyzzer (94) in 1907 in tame mice. Until the early 1970s, this protozoan did not gain much attention except being described in a few case reports in different animal species. Since 1976, however, Cryptosporidium sp. has been recognized to be a commonly found parasite in young animals, in which neonatal diarrhea was a problem (66,75), especially in beef herds and dairy calves. Currently, this organism is known to occur in more than twenty animal species.

Although some authors (28,50,85,91) consider Cryptosporidium sp. to be a commensal organism, it has been shown experimentally in gnotobiotic lambs (89), pigs (63) and calves (39) that this protozoan is a pathogenic intestinal parasite. In addition, this coccidium has been observed to cause infection and disease in the respiratory tract of humans (30,52,59), and birds (41,46,60,92), and infection in pigs (38,82).

Currently, cryptosporidiosis does not appear to be a problem in porcine intestinal or respiratory tract diseases. There are only a few reports (8,50,57,66) on naturally occurring porcine cryptosporidiosis. The pig, however, proves to be a susceptible animal for experimental infections (38,63,65,82,103,105). Thus, it is important to know more about cryptosporidiosis.

The objectives of this study are:

1. to monitor the course of experimental infection in naturally born, colostrum-fed pigs raised with the sow,

2. to evaluate and describe the parasitic distribution in the intestinal tract and to observe the response in the intestine and other mucosal surfaces by investigating animals on days 2, 4, 6, and 8 after experimental inoculation on day 3 of life,
3. to search for differences in mucosal response to parasitic infection in animals treated with neomycin and compare these to untreated animals to find out:
 - a) whether neomycin does impede the infection and can be used as a treatment, and
 - b) whether neomycin can predispose or contribute to an infection, since this drug has been shown to influence the intestinal morphology in humans.

2. LITERATURE REVIEW

2.1. History and parasitic characteristics of *Cryptosporidium* sp. and its life cycle

Cryptosporidium sp., a coccidian parasite, was first discovered in 1907 by Tyzzer in histopathological sections of gastric glands of tame mice. Thus, this parasite was named *Cryptosporidium muris* (94). In 1912, Tyzzer described a second species, *Cryptosporidium parvum*, which is smaller and was detected only in the small intestine of mice (93). He also found that the gastric parasite never affected the small intestine and vice versa (93). From 1912 on, it was generally accepted that each animal species could host a species specific *Cryptosporidium* sp. Many new species such as *C. meleagridis* (86), *C. anserinum* (78), *C. cuniculus* (44), *C. wrairi* (106), and *C. felis* (45) were named on the basis of oocysts found in feces of a specific host species. In 1980, Tzipori et al. (98) reported experimental transmission with homogenates of ileal contents from a calf infected with *Cryptosporidium* sp. to seven different animal species. From these results, the authors concluded that *Cryptosporidium* sp. is a single-species genus. Though similar results have been obtained by other authors (24,63,82), Current and Haynes (21) recently reported a strain occurring in chickens, which appeared to be more pathogenic for this species than in mammals. Similar observations have also been made with a guinea pig strain (Snodgrass, Moredun Research Institute, 408 Gilmerton Road, Edinburgh, Scotland, U.K., personal communication).

According to the classification of Levine et al. (56), the genus

Cryptosporidium belongs to the family Cryptosporidiidae, suborder Eimeriina, order Eucoocidia, subclass Coccidia, class Sporozoa and phylum Apicomplexa. The genera Eimeria and Isopora also belong to the suborder Eimeriina, but both are intracellular parasites which infect primarily the alimentary tract (95). Cryptosporidium sp., however, currently is thought to be "intracellular" (45) but "extra-cytoplasmic" (10).

The life cycle of Cryptosporidium sp. has been recently described by Iseki (45) and Current and Haynes (22). The mature oocysts are discharged in the feces. Sporozoites released from mature oocysts develop on mucosal surfaces. In general, these sporozoites parasitize the brush border of epithelial cells. They develop into trophozoites in which the nucleus divides into eight small nuclei through schizogony. Each mature schizont contains eight banana-shaped merozoites. Some authors (67,106) have shown that a second generation of schizonts develops before gametogony can occur, in which micro- and macro-gametocytes develop. A zygote is the result of fertilization between micro- and macro-gametes. Sporulated oocysts excreted in the feces or the small intestine are the infective stage. Current et al. (23,24) observed that thin-walled oocysts have to be considered as an autoinfective stage without leaving the host. Thick-walled oocysts passed with feces are infective in the environment. They do not require an intermediate host or outside host (45).

2.2. Cryptosporidiosis in different animal species

Since Tyzzer in 1907 first described Cryptosporidium sp., the parasite has been reported in a variety of more than twenty warm- and cold-blooded vertebrates. It has been shown to inhabit humans (4, 10,17,24,30,54,69,62,68,87), macaques (19,53,108), rabbit (44), mice (37,84), rats (79), raccoons (16), squirrels (91), guinea pigs (106), dogs (31,85), cats (45,77), pigs (8,38,50,57,63,66,82,103,105), calves (3,5,70,71,74,75,81,104), lambs (2,6,7,89,97,100), goats (24, 102), deer (99), gazella (28), horses (34,90), quails (92), turkeys (36,41,86), chickens (25,29,46), peacock chicks (60), parrots (26), geese (78), finch (32), snakes (13), and fish (42). It has not been determined whether this organism is a pathogen in all species listed above.

The first recognized case of human cryptosporidiosis was described in a three-year-old child in 1976 by Nime et al. (68). Acute self-limiting enteritis resulted from Cryptosporidium sp. in this case. In 1984, Alpert et al. (1) reported an outbreak of cryptosporidial infection in a day-care center. The Cryptosporidium sp. oocysts were found in eleven of the seventeen children with clinical signs. Hunt et al. (43) investigated 867 patients presenting gastrointestinal symptoms and found 43 (5%) infected with Cryptosporidium sp., which was the second-most common enteric pathogen identified. Respiratory cryptosporidiosis associated with shortness of breath (59) and cough (30,59) has been reported in association with various immune defects,

predominantly in patients suffering from the acquired immune deficiency syndrome (AIDS) (9,20,30,59,67,72,73). These patients were usually presented with persistent, intractable watery diarrhea with enormous fluid losses, as well as mild epigastric cramping pain, nausea, vomiting and anorexia (67). Immunological abnormalities with cryptosporidial infection were also described in foals (34,90), macaques (108) and mice (37). The Arabian foals with combined immunodeficiency were infected with Cryptosporidium sp. and developed diarrhea. Premature thymic atrophy with cryptosporidiosis was reported in two macaques which had diarrhea. In none of these, the immunological disorders were clearly defined. Heine et al. (37) found that nude mice infected on day six of life suffered from more persistent cryptosporidial infection than white mice infected at the same age, and that some of the nude but none of the white mice died of the infection.

Pancieria et al., in 1971, first described cryptosporidiosis in the bovine species (70). They reported that an eight-month-old calf with cryptosporidiosis had diarrhea and debilitation. Since then, many case reports and descriptions of bovine cryptosporidiosis have been published (2,5,71,74,74,81,104).

Cryptosporidiosis in swine was first mentioned in the literature by Morin et al. (66) in 1976, complicating a case of transmissible gastroenteritis. Only four reports of naturally-occurring cryptosporidiosis in pigs were described from 1976 to 1982 (8,50,57,66). No clinical significance could be attributed to the presence of the organism in these reports. Porcine cryptosporidiosis was studied in

experimental infections including transmission either from humans to pigs (65) or from calves to pigs (38,63,82,103,105). These experimental results indicated that cryptosporidial transmission does not require a second or an intermediate host and that the organism is pathogenic for pigs. Experimentally-infected animals develop diarrhea and emesis. Respiratory cryptosporidiosis in pigs has been detected after oral (82) or tracheal (38) inoculation with the organism. However, no clinical signs of the respiratory disorder were described.

Besides humans and pigs, an infection of the respiratory system was also reported in chickens (25,46), turkeys (41), quails (92) and a peacock chick (60). The clinical signs were respiratory distress (25,92), sneezing (60,92), gurgling respiration and coughing (60). Death losses were often caused by this infection.

2.3. Transmission of cryptosporidiosis

The main route of cryptosporidial transmission is considered to be fecal-oral. This route is an important method for spread of the infection among homosexual men (9). The fecal-oral route has been successfully demonstrated in numerous experiments (37,63,79,89,103). Many cases of human cryptosporidiosis are thought to have resulted from contact with infected calves (4,24,72,83). Since respiratory infection with Cryptosporidium sp. has been observed in birds (25,41,46,60,92), an airborne route of transmission is suspected. An unusual finding was neonatal cryptosporidiosis in a gazella which died approximately 24 hours after birth without clinical signs of di-

arrhea (28). Transplacental transmission was considered in this report, but this is rather unlikely because of the size of the organism and its propensity to settle on surfaces and not to invade tissues.

2.4. Diagnosis and current knowledge of treatment and disinfection of cryptosporidiosis

In 1978, Pohlenz et al. (75) described that the Giemsa stain is useful for detection of Cryptosporidium sp. in fecal samples or in scrapings of intestinal mucosa. To avoid confusion with yeasts possibly present in the intestine, a modified acid-fast stain was introduced (40). Different stages of the life cycle are seen as long banana-shaped organisms or as round 2.5-6 μm in diameter structures, containing distinct internal granules. The parasite is generally surrounded by a halo. Specimens inoculated with auramine revealed nonspecific fluorescence comparable to that obtained with *Mycobacterium paratuberculosis* (Pohlenz, Department of Veterinary Pathology, ISU, Ames, IA, personal communication). To determine a serologic titer against Cryptosporidium sp., Tzipori and Campbell (101) used the indirect immunofluorescence techniques on frozen sections from an infected intestine. A direct immunofluorescence test with hyper-immune serum has been employed to localize the parasite on the large and small intestinal surface (Pohlenz, Department of Veterinary Pathology, ISU, Ames, IA, personal communication). Histologic sections from animals necropsied immediately after euthanasia were used in this study. This method is reliable only when tissues are fixed before epithelial cells slough in the early phase of autolysis (75). Colonic cryptosporidiosis in man is diagnosed by biopsy samples prepared for histologic and elec-

tron microscopic examination. In most reports describing experimental work in cryptosporidiosis, electron microscopy, scanning electron microscopy and histology are employed. For quantitative analysis of fecal material, sugar flotation is recommended for work in diagnostic and experimental parasitology. To better detect the relatively small parasites, a Nomarski interference contrast microscope is often used (74).

Currently, no treatment against this parasitic infection is available. More than 40 different drugs, including antibiotics, sulfa drugs, coccidiostats and antihelminthics have been shown to be ineffective (67, 95).

Cryptosporidium sp. is highly resistant to most chemical and physical treatments. Ten percent formal saline and 5% ammonia were shown to be effective (15). Concentrated bleach diluted with equal amounts of water (50%) was used to effectively clean animal housing. Ethylenoxide is currently employed to disinfect isolators used in experiments with gnotobiotic calves (Pohlenz, Department of Veterinary Pathology, ISU, Ames, IA, personal communication). The parasite was shown to be infectious after storage at 4°C for 3-6 months (84). Sporocysts kept for 6 months at 5°C and heated for 15 minutes at 50°C or frozen for 24 hours at -18°C were not infective for mice (11).

2.5. Lesions and pathogenesis

The lesions caused by Cryptosporidium sp. are similar in all species. The predominant alterations occur in the intestine and respiratory tract. Both organ systems are more severely affected in mammals

suffering from immunosuppression (30,52). The intestinal lesions are best known from experimental infections and field cases in calves and lambs, but are similar in dogs and cats.

Cryptosporidium sp. organisms in calves, lambs, dogs and cats appear to be confined to the small and large intestine (5,31,45,71,75,77,89,100). The morphologic changes including villous atrophy and fusion of villi, mononuclear cell infiltration and presence of cuboidal to squamous lining epithelial cells on the surface of the intestinal mucosa are characteristic findings. In addition, especially in calves and lambs, combined infections with Cryptosporidium sp. and viruses or Escherichia coli have been documented (64,66).

Equine cryptosporidiosis is known to complicate adenoviral infection. The parasite was found in the stomach, small and large intestine, pancreatic ducts, bile ducts and common bile duct (90). Atrophy and fusion of villi, dilated crypts and purulent enteritis in the small intestine were described in one of the case reports (34).

In humans, Cryptosporidium sp. has been found in the tonsils (10), pharynx, esophagus, stomach, duodenum, jejunum, ileum, appendix, colon, rectum (67), gall bladder (72,73), as well as in the respiratory tract (30,59). The intestinal lesions associated with the organism included villous atrophy, distended crypt lumens and mild to moderate mononuclear cell infiltrates. Active bronchiolitis, interstitial pneumonia (59) and epithelial metaplasia with chronic bronchitis (30) were diagnosed in respiratory cryptosporidiosis. In monkeys, Cryptosporidium sp. was

described in the common bile duct, intraheptic and pancreatic ducts (53), and small and large intestine. The histologic lesions were similar to those described in humans.

The trachea (25,41), bronchi (41), small intestine (86), caecum (29,46), large intestine (78), cloacal coprodium (26), bursa of Fabricus (28,36,46), nasal cavity (46,92), larynx (46), infraorbital sinus (36,46), nasal sinus (60), conjunctival (60) and salivary and esophageal glands (92) of birds have been demonstrated to be infected with Cryptosporidium sp. Hyperplasia of lining epithelium, heterophils and lymphocytes infiltrating the lamina propria and the presence of the organisms attached to the surface were the consistent histological findings.

Cryptosporidium sp. organisms have been found in the small intestine of a rabbit (44), stomach of mice (94), small intestine (37,79, 84,106) of guinea pigs, mice and rats and large intestine (37,79,84) of mice and rats. The lesions were similar to those described previously.

Intestinal cryptosporidiosis has also been described in raccoons (16), gazella (28), and squirrel (81). Histologically, only raccoons had villous atrophy with mononuclear cells and eosinophils in the lamina propria of the small intestine.

Hypertrophic gastritis associated with Cryptosporidium sp. was diagnosed in snakes (13). Fish (42) infected with Cryptosporidium sp. did not have morphological changes in affected intestinal villi.

Morin et al. (66) found Cryptosporidium sp. in the lower jejunum

of two pigs and the organism was thought to be a complicating factor in an infection with transmissible gastroenteritis virus. Bergeland (8) reported the organisms on the surface of unaffected villi in one pig. Cryptosporidium sp. in two other natural infections were found in the small intestine (57) and large intestine (59). The microscopic lesions in the large intestine consisted of cellular debris, sloughed epithelial cells and degenerated leukocytes in the crypts, increased numbers of mitotic figures and mononuclear cells infiltrating the lamina propria. By electron microscopy, the organisms phagocytized by leukocytes (50) were demonstrated. Moon and Bemrick (63), in transmission experiments where pigs were infected with Cryptosporidium sp. taken from infected calves, observed villous atrophy and cuboidal and basophilic epithelium in the ileum and large intestine. Mononuclear cells and neutrophils were the major inflammatory cells in the affected intestine. Similar histologic lesions were also reported in transmission experiments where pigs were infected with Cryptosporidium sp. originating from humans. Schloemer (82), in an experiment of fecal-oral transmission with Cryptosporidium sp. from calf to pigs showed the organisms in the stomach, ileum, colon, and respiratory tract. Tzipori et al. (103) described stunting and fusion of villi and crypt hyperplasia in the small intestine infected with Cryptosporidium sp. In experimental tracheal and conjunctival infections with this organism, Heine et al. (38) described irregular and disrupted ciliated borders, squamous changes and perivascular infiltrates by lymphocytes

and macrophages in the affected tracheal epithelium. The affected conjunctival epithelium was cuboidal with mixed superficial inflammatory cell infiltrates.

The pathogenesis of cryptosporidial infection is still incompletely understood (20,33). Some studies in lambs and guinea pigs indicated a decrease in intracellular enzyme activity, e.g., sucrase, lactase (95), alkaline and acid phosphatase, glucose-6-phosphatase, succinic dehydrogenase, diphosphopyridine and triphosphopyridine diaphorases, and monoamine oxidase (49). The decreased levels may partly explain malabsorption and diarrhea in sick animals (51). Impaired digestion, malabsorption and diarrhea may be caused by morphological changes of microvilli including loss or degeneration (33,74). The liberation of parasitic metabolites and toxins derived from parasites during their development have been discussed (62,96). A possible hypersensitivity reaction to the parasitic antigen (95) has been considered to be an important factor in the pathogenesis of this disease. Like other enteric diseases, atrophy and fusion of villi may apparently decrease absorptive surface. Pohlenz et al. (74) and Lefkowitz et al. (54) found increased clustering of lysosomes in parasitized cells in calves (74) and humans (54). The proximity of the lysosomes to the surface of Cryptosporidium sp. suggested that they had some degree of correspondence to the parasitic infection. Abnormal accumulation of lysosomes in the epithelial cells may reflect failure of the phagocytic mechanism of the host (54).

2.6. Influence of neomycin on the intestinal mucosa

As reported by Brander et al. (12), neomycin was first discovered by Waksman and Lechevalier in 1949. It is a broad-spectrum, aminoglycoside antibiotic destroying gram-negative and gram-positive bacteria, as well as some actinomyces (12). The antibiotic is poorly absorbed from a noninflamed gastrointestinal tract. In calves and piglets, good results are noted in the treatment of scours with neomycin (12). Thus, this antibiotic is frequently used. Malabsorption syndrome has been recognized to be associated with large oral doses of neomycin in humans (27,47,48), but not animals. The common findings were steatorrhea, azotorrhea (47,48), reduced disaccharidase (14), lactase deficiency (69,80) and interference with absorption (47,48). However, these changes returned to normal after withdrawal of the drug (47,48). Morphological changes in the small intestine were blunting (14) or cubbing of villi (48), edema and increased mononuclear cells (14,48). Scattered ballooning and fragmentation of villi were found by electron microscopy. Loss of villi or disruption of glycocalyx were found in some areas. These lesions suggested that early toxic action of neomycin on small intestine is possible (14).

3. MATERIALS AND METHODS

3.1. Experimental animals

Four litters of piglets originating from different sows (A,B,C,D) were used in this experiment. The sows were raised at the National Animal Disease Center¹ (NADC) and individually moved into isolation pens shortly before farrowing. All piglets were fed colostrum ad libitum after birth and housed with the sow. The piglets were marked by ear notches and prophylactically supplied with an iron injection.

3.2. Experimental design

Litters from sow C and sow D were treated orally once daily for 5 days with neomycin (Biosol)² 1 ml per piglet per day, 200 mg neomycin sulfate (equivalent to 140 mg neomycin) starting on day 1 of life. Two piglets of each litter (A,B,C,D) were killed on day 3 and the remaining piglets were inoculated orally with a solution of 5 ml containing 10^6 Cryptosporidium sp. oocysts per piglet by gavage on the same day. Two littermates from each litter were necropsied on days 2, 4, 6 and 8 post-inoculation (PI).

3.3. Preparation of 10^6 Cryptosporidium sp. inoculum

The inoculum was prepared as described by Heine et al. (39). Briefly, the procedure was as follows: Feces from a calf infected with Cryptosporidium sp. were suspended in 2 volumes of 2.5% potas-

¹ NADC, USDA, Ames, Iowa.

² Biosol liquid, Upjohn Company, Kalamazoo, MI.

sium dichromate solution and stored at 4°C. For quantification of the oocysts, 10 ml of the feces-dichromate solution was centrifuged at 1000 xg for five minutes. The supernate was discarded, the pellet was washed once in tap water and the volume of the pellet noted. A volume of Kinyoun's carbol-fuchsin equal to the volume of the pellet was added and thoroughly mixed. One ml of this suspension was placed on a microscope slide and a thin, even smear was made with a dissecting needle. The smear was allowed to dry and was then covered with immersion oil. The number of oocysts in a known percentage of the smear was determined and from that the number of oocysts per milliliter of fecal dichromate was calculated. A sample of this suspension was mixed with peracetic acid diluted to a final concentration of 3%. This was allowed to stand at room temperature (25°C) for 30 minutes; the suspension was then centrifuged for 10 minutes at 500 xg and the pellet washed three times in sterile phosphate buffered saline (PBS). The suspension was then diluted to a final concentration of 2×10^5 oocysts per ml and stored in a sterile flask at 4°C until used. The inoculum was cultured in brain heart infusion broth³ (BHI) to exclude contamination with bacteria.

3.4. Clinical observations and fecal examinations

General appearance of all piglets and sows were checked twice daily during the experiment. Fecal samples were taken each morning by

³Difco Laboratories, Detroit, MI.

cotton swabs from each piglet starting on day 1 and continuing until the day of necropsy. Fecal smears were made on two glass slides. Pasty or solid feces were diluted with adequate amounts of Ringer's solution, watery feces were smeared directly. The smears were stained with Giemsa stain (75) and modified Ziehl-Neelsen stain (40), and examined for Cryptosporidium sp. with a light microscope. Selected samples from the animals with diarrhea were negatively stained (61) and investigated with the electron microscope to exclude possible virus contamination.

3.5. Necropsy

Piglets were euthanized by intravenous injection of sodium pentobarbital, weighed and necropsied. Gross findings were recorded. The following tissues were collected and immediately fixed in 4% neutral buffered formalin: nasal mucosa, mid-trachea, main-bronchus on each side along with associated lung tissue, mid-esophagus, pars proventricularis and pylorus of the stomach, duodenum (Duo.) with crosscut through pancreas, jejunum 100 cm (Jej. 100), 200 cm (Jej. 200) distal to duodenal site, 5 cm, 15 cm and 120 cm proximal to ileocecal junction (5PICJ, 15PICJ and 120PICJ), ileocecal junction, cecum (mid of corpus), proximal and spiral colon at ansa centralis, liver, gall bladder, kidney with pelvis, urinary bladder and conjunctiva. All intestinal tissues except ileocecal entrance and cecum were treated prior to collection with the following method. A 3-5 cm intestinal segment was ligated at both ends, intralumenally instilled with formalin and removed. A second piece of the intestine was fixed after stapling onto a piece of

plastic to avoid shrinking. Both tissues were immersed in the jar of formalin.

3.6. Histopathology

Tissues fixed in formalin for at least 24 hours were trimmed, processed by routine paraffin techniques, sectioned at 5 μ m, stained with Haematoxylin and Eosin (H&E) and examined microscopically. In some cases, Giemsa stain on tissues (58) was employed to better differentiate the Cryptosporidium sp. organism. From H&E sections, the mitotic figures were counted. Twenty straight-cut crypts were evaluated and the number of mitotic figures per twenty crypts was recorded as the mitotic index. Additionally, the number of Cryptosporidium sp. was estimated and graded from the whole flat fixed histologic section as follows: + (average of <5 organisms present at villous surface), ++ (10-15 organisms), +++ (15-20 organisms) and ++++ (>20 organisms). In sections where necessary, +-++, ++-+++, and +++-++++ grading was used.

3.7. Morphometric evaluation of villous length, crypt depth and ratio of villous length per crypt depth

Wet tissues were used to measure villous length (vl), crypt depth (cd) and to determine the ratio of villous length per crypt depth (vl/cd ratio). To standardize the measurements taken at a rectangular cut surface, a vibratome 1000⁴ was employed. The sections were prepared as follows: a flat segment of formalin-fixed small intestine was trimmed

⁴Lancer [®] Vibratome 1000, A Brunswick Company, St. Louis, MO.

with a razor blade into a piece of approximately 2 x 2 mm in size. After this, one of the four cut surfaces was mounted on the specimen mounting, in which a small piece of glass taken from a slide had been mounted as an intermediate plate. Industrial adhesive⁵ was used for mounting. When after 2-3 minutes the adhesive was dry enough, the block with the specimen was transferred into the vibratome tank and clamped with the specimen vise. A single-edge sectioning blade was mounted into the blade holder and the angle to be sectioned was adjusted 15° with a blade angle indicator. The vibratome tank was then filled with distilled water. After this, the sectioning procedures were followed according to Lancer Vibratome 1000 Operator's Manual.⁶ At least three sections (60-80 µm in thickness) were cut and retrieved from the tank with a fine tissue forceps and were delivered to a standard microscope slide, on which one drop of distilled water had already been placed. A droplet of 1% methylene blue solution was then added and mixed by shaking gently for ten seconds. The excessive staining solution was absorbed with filter paper. Finally, the tissue was covered directly with a coverslip and immediately examined under the light microscope and measured using a graticule in 10x eyepiece. Villi were selected only when present in entire length with an intact villous architecture from the base of villi to the tips. Crypts were chosen

⁵Loctite Quick Set 404 Industrial Adhesive, Loctite Corporation, Newington, CT.

⁶Lancer[®] Vibratome 1000 Operator's Manual, A Brunswick Company, St. Louis, MO.

only when they were visible from the lamina muscularis to the point they opened at the base of villi (Figure 1). Twenty villous lengths and ten crypt depths which fulfilled these criteria described above were selected from each preparation and then measured. V1/cd ratio was defined as the mean value of twenty villous lengths divided by the mean value of ten crypt depths.

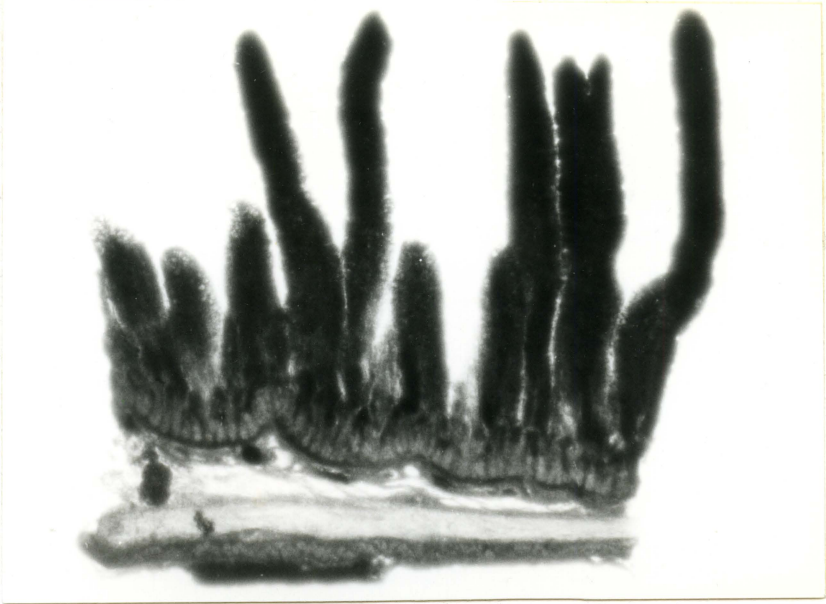
3.8. Statistical analyses

Split-plot design (88) and least significant difference (LSD) analysis (18) were used for the analysis of mitotic index, villous length, crypt depth and v1/cd ratio. There were only four piglets left for the investigation on day 8 PI, one (B9) out of the untreated group and three (C9, D9 and D10) out of the group treated with neomycin. For adequate data balance, the statistical analysis was restricted to animals killed in days 0, 2, 4 and 6 PI.

Figure 1. Sections cut by a vibratome, 1% methylene blue stain

- a) (20x) Villi selected only in entire length with an intact villous architecture from the base of a villus to the tip
- b) (140x) Crypts chosen only visible from the lamina muscularis to the point they opened at the base of villi

a)



b)



4. RESULTS

4.1. Clinical observations and fecal examinations

There were 36 experimental piglets: eight from sow A(1-8), nine from sow B(1-9), nine from sow C(1-9) and ten from sow D(1-10).

Sow C temporally refused food for two days after parturition. Piglets C5 and C8 had mild diarrhea and other littermates had yellow pasty feces after birth. Mild diarrhea was present in piglets D1, D3, and D8 which still had a good appetite when they were three days old and prior to inoculation of Cryptosporidium sp. B4 had lameness of the left foreleg due to accidental trauma, but the piglet ate well. The remaining sows and piglets were not suffering from any obvious disease.

None of the fecal smears taken prior to inoculation were positive for Cryptosporidium sp. The samples taken from piglets with diarrhea before and after inoculation with 10^6 Cryptosporidium sp. oocysts were negative for viral infections detectable by the negative staining procedure.

Vomited stomach contents containing undigested milk were seen in litter B during days 3 PI to 5 PI, in litter C on days 4 PI and 5 PI, and in litter D on day 4 PI. Vomiting was not identified individually. Diarrhea and shedding of oocysts (Figure 2) are shown in Table 1. Shedding of oocysts started on day 4 PI in four animals, was seen in two animals on day 5 PI and in six animals on day 6 PI. Oocysts were

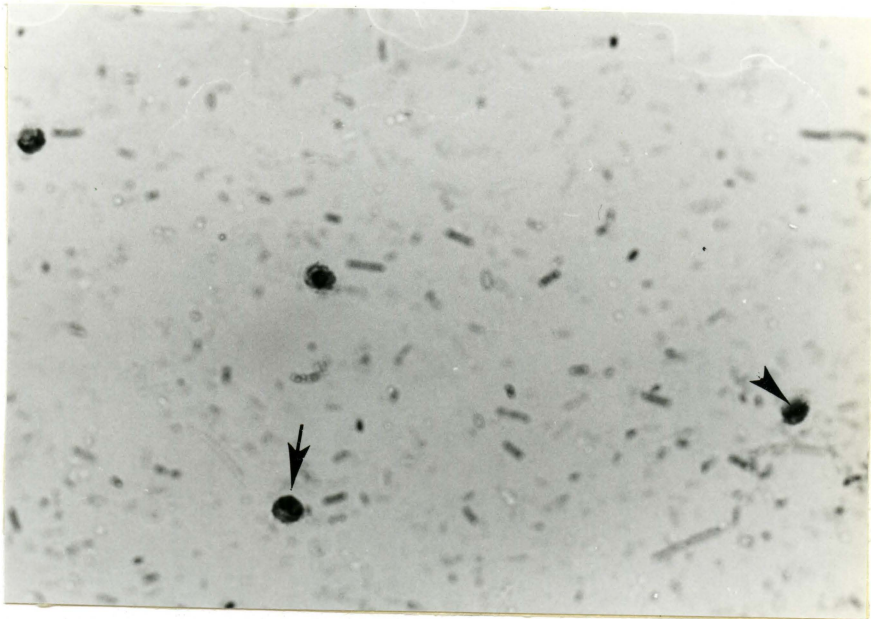


Figure 2. Fecal smear, day 4 PI, Giemsa stained

Cryptosporidium sp. oocysts: Notice the eccentric granular material (arrowhead) in a round organism surrounded by a halo (long arrowhead) (1000x)

Table 1. Clinical signs and shedding of oocysts of the piglets orally inoculated with 10^6 Cryptosporidium sp. oocysts

Piglet number	Day of necropsy (PI) ^a	Treatment	Diarrhea (on days, PI)	Shedding of oocysts (starting on day PI)
A ₁	0	u ^b	- ^c	-
A ₂	0	u	-	-
B ₁	0	u	-	-
B ₂	0	u	-	-
C ₁	0	T ^d	-	-
C ₂	0	T	-	-
D ₁	0	T	0	-
D ₂	0	T	-	-
A ₃	2	u	-	-
A ₄	2	u	-	-
B ₃	2	u	-	-
B ₄	2	u	-	-
C ₃	2	T	-	-
C ₄	2	T	3	-
D ₃	2	T	0-2	-
D ₄	2	T	-	-

^aPI: post-inoculation.

^bu: untreated with neomycin.

^c-: negative.

^dT: treated with neomycin.

Table 1. continued

Piglet number	Day of necropsy (PI)	Treatment	Diarrhea (on days, PI)	Shedding of oocysts (starting on day PI)
A ₅	4	u	-	-
A ₆	4	u	-	-
B ₅	4	u	4	+ ^e (4 PI)
B ₆	4	u	-	-
C ₅	4	T	-1 ^f	-
C ₆	4	T	3	-
D ₅	4	T	1-4	-
D ₆	4	T	2-4	+ (4 PI)
A ₇	6	u	-	+ (6 PI)
A ₈	6	u	-	+ (6 PI)
B ₇	6	u	-	+ (6 PI)
B ₈	6	u	6	+ (4 PI)
C ₇	6	T	-	+ (6 PI)
C ₈	6	T	-2, ^f +2,3,6	+ (6 PI)
D ₇	6	T	3-6	+ (6 PI)
D ₈	6	T	0-1	+ (4 PI)
B ₉	8	u	5-6	+ (5 PI)
C ₉	8	T	4-5	+ (5 PI)
D ₉	8	T	2	-
D ₁₀	8	T	-	-

^e+: positive.

^f-1 PI, -2 PI: 1 day, 2 days prior to inoculation.

not detected in the fecal samples of two piglets (D9 and D10) which were killed on day 8 PI. A total of 20 piglets were killed on days 4, 6 and 8 PI. Seven out of 20 (35%) (B5, D6, B8, C8, D7, B9 and C9) had diarrhea with oocysts present in the feces. Four out of 20 (20%) (C5, C6, D5 and D9) had diarrhea but oocysts were not found. Five out of 20 (25%) (A7, A8, B7, C7 and D8) had no diarrhea but oocysts were present. Four out of 20 (20%) (A5, A6, B6 and D10) had neither diarrhea nor oocysts in the feces. There were no significant differences when clinical signs and shedding of oocysts in the group treated with neomycin were compared with the untreated group.

4.2. Morphologic lesions

4.2.1. Macroscopic findings Enlarged mesenteric lymph nodes were seen in two piglets, B5 and C9. Piglet B5 also had a flaccid intestine. Melanosis in lung and spleen were grossly diagnosed in piglet D2. No gross findings were observed in any of the organs of the remaining experimental piglets.

4.2.2. Microscopic findings Neither Cryptosporidium sp. organisms nor lesions were present in the trachea, lung, pancreatic ducts, bile ducts, gall bladder, kidney, urinary bladder or conjunctiva of any experimental piglet. Mild hyperkeratosis associated with bacterial colonization was a regular finding in the esophageal mucosa of these piglets. A few scattered neutrophils were present in the keratinized layer and the lamina propria of the esophagus in some piglets. In general, the esophageal lesions were more prominent after inoculation than before.

Cryptosporidium sp. was not found in any part of the digestive tract in animals killed before inoculation. The distribution of the organisms on the villous surface after inoculation is listed in Figure 3. The parasites were present in the histologic sections of different intestinal sites in seven piglets on day 2 PI, in all eight piglets on days 4 and 6 PI and all four piglets on day 8 PI. On day 2 PI, Cryptosporidium sp. organisms were detected in all but one piglet (A3). The parasites were found in two piglets in Duo., three piglets in Jej. 100, seven piglets in Jej. 200 and three piglets in 120PICJ. The organisms were not detected in 15- and 5PICJ, caecum, proximal colon, spiral colon and ileo-cecal junction at this time. On day 4 PI, the organisms were found in five piglets in Duo., seven in Jej. 100, five in Jej. 200, five in 120PICJ, four in 15PICJ and five in 5PICJ. No organisms were found in the caecum, proximal colon, spiral colon and ileo-cecal junction. On day 6 PI, the organisms were seen in six animals in Duo., eight in Jej. 100, five in Jej. 200, five in 120PICJ, seven in 15PICJ, six in 5PICJ, two in caecum, two in proximal colon, one in spiral colon and five in ileo-cecal junction. On day 8 PI, only four piglets were available for necropsy. Cryptosporidium sp. was present in three of them in Duo., one in Jej. 100, one in Jej. 200, one in 120PICJ, four in 15PICJ, four in 5PICJ, one in caecum, two in proximal colon, two in spiral colon and four in ileo-cecal junction. Change in villous length and Cryptosporidium sp. on villous surface are also illustrated in Figure 3. Generally, there was high correlation of

Figure 3. Distribution of Cryptosporidium sp. in different sites of intestinal sections on the mucosal surface and change in villous length on days 2, 4, 6 and 8 post-inoculation

U: untreated

T: treated with neomycin

A3-D10: identification of experimental piglets

*: villous atrophy

Duo.: duodenum, Jej. 100, Jej. 200 = 100 cm, 200 cm distal to duodenal site

5-, 15-, 120PICJ = 5 cm, 15 cm, 120 cm proximal to ileo-cecal junction

+: <5 organisms present at villous surface (average)

+~+: 5-10 organisms

++: 10-15 organisms

++~++: variable 10-15 or 15-20 organisms

+++: 15-20 organisms

+++~+++: variable 15-20 or >20 organisms

+++: >20 organisms

Site	Duo.		Jej.100		Jej.200		120PICJ		15PICJ		5PICJ		Ileo-cecal junction		Caecum		proximal colon		spiral colon		
Treatment	U	T	U	T	U	T	U	T	U	T	U	T	U	T	U	T	U	T	U	T	
DAY 2PI	++++ +++ ++ +																				
Piglet No.	AABB 3434	CCDD 3434	AABB 3434	CCDD 3434	AABB 3434	CCDD 3434	AABB 3434	CCDD 3434	AABB 3434	CCDD 3434	AABB 3434	CCDD 3434	AABB 3434	CCDD 3434	AABB 3434	CCDD 3434	AABB 3434	CCDD 3434	AABB 3434	CCDD 3434	
DAY 4PI	++++ +++ ++ +																				
Piglet No.	AABB 5656	CCDD 5656	AABB 5656	CCDD 5656	AABB 5656	CCDD 5656	AABB 5656	CCDD 5656	AABB 5656	CCDD 5656	AABB 5656	CCDD 5656	AABB 5656	CCDD 5656	AABB 5656	CCDD 5656	AABB 5656	CCDD 5656	AABB 5656	CCDD 5656	
DAY 6PI	++++ +++ ++ +																				
Piglet No.	AABB 7878	CCDD 7878	AABB 7878	CCDD 7878	AABB 7878	CCDD 7878	AABB 7878	CCDD 7878	AABB 7878	CCDD 7878	AABB 7878	CCDD 7878	AABB 7878	CCDD 7878	AABB 7878	CCDD 7878	AABB 7878	CCDD 7878	AABB 7878	CCDD 7878	
DAY 8PI	++++ +++ ++ +																				
Piglet No.	B 9	C 9	DD 910	B 9	C 9	DD 910	B 9	C 9	DD 910	B 9	C 9	DD 910	B 9	C 9	DD 910	B 9	C 9	DD 910	B 9	C 9	DD 910

villous atrophy and dense concentration of Cryptosporidium sp. organisms on the villous surface.

On day 0 PI, neither Cryptosporidium sp. organisms nor villous atrophy were found in the small or large intestine. Eosinophils and focal neutrophil accumulations were common findings in the lamina propria of the small intestine.

On day 2 PI, only a few Cryptosporidium sp. organisms were attached to the villous surface in Duo. without any change in villous architecture. More organisms were detected in the mid-small intestine (Figure 4), but the lining epithelium was not altered. At this stage, eosinophils and focal collections of neutrophils in the small intestine and focal collections of neutrophils in the large intestine were common cellular infiltrates.

On day 4 PI, there were numerous Cryptosporidium sp. organisms associated with moderate villous atrophy in Duo. (Figure 5). The lining epithelium was irregular, part of which became cuboidal. In Jej. 100 (Figure 6), villous atrophy and fusion, microulceration, epithelial cell disorganization and neutrophil infiltration were prominent. The parasites were densely packed on the surface. In 5PICJ, more organisms were found to adhere to the surface, but the mucosal inflammatory response was mild.

On day 6 PI, the lesions in Duo. were similar to those described in Duo. on day 4 PI except more advanced villous atrophy and an increased amount of plasma cells and lymphocytes were present. In Jej. 200,

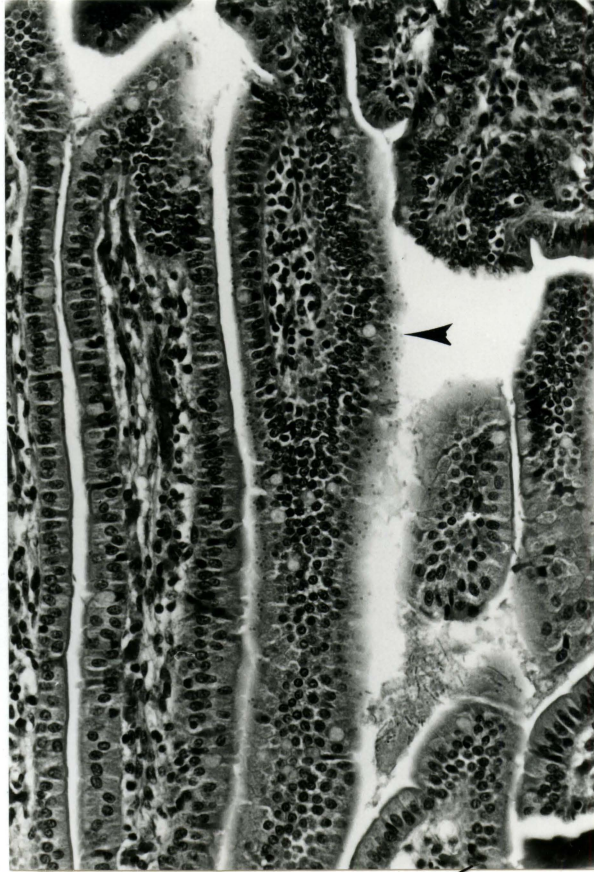


Figure 4. Jej. 200, day 2 PI, untreated group

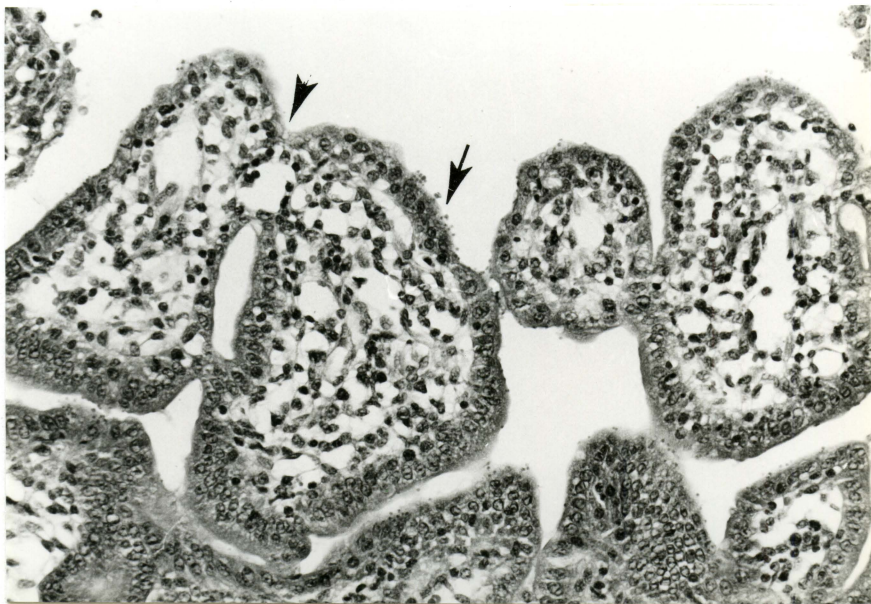
H&E stain: Many Cryptosporidium sp. organisms (arrowhead) on the surface of villous epithelial cells. No morphological alterations present in the villi (110x)

Figure 5. Duo., day 4 PI, treated group

H&E stain: Numerous Cryptosporidium sp. organisms (arrowhead) attached to the atrophied villi with the formation of synechia, elongated hyperplastic crypts in the deeper mucosa (33x)

Figure 6. Jej. 100, day 4 PI, untreated group

H&E stain: Fusion occurring in the atrophied villi (arrowhead), flat to cuboidal lining epithelial cells carrying Cryptosporidium sp. organisms (long arrowhead) on their surface (180x)



(Figure 7), less Cryptosporidium sp. organisms were detected. The villi were not atrophied and eosinophils were seen as a common cellular infiltrate. Numerous organisms associated with severe shortening of villi and mucosal inflammatory response as described earlier were observed in 5PICJ (Figure 8) and 15PICJ. Very few organisms were found in the caecum (Figure 9), proximal colon and spiral colon. Comparing mucosal changes between the uninoculated piglets and the inoculated piglets, the latter had some degree of disorganization of lining epithelial cells in which the nuclei were not uniform in size. At the ileo-cecal junction (Figure 10), numerous organisms lined the villi. Flattened epithelium on villous surface and atrophied and fused villi with increased infiltration of neutrophils were often seen.

On day 8 PI, blunt villi with the organisms adherent to the surface were still present in Duo. Moderate crypt hyperplasia with an increase of plasma cells and lymphocytes were observed in the deeper mucosal layer. In Jej. 200 (Figure 11), fewer organisms lined the compact villi, with varying degrees of cellular response. Moderate villous shortening and adherence of numerous parasites were seen in 5- and 15PICJ. Very few organisms were detected in the large intestine. The mucosal epithelium was unaltered, however, macrophages in the lamina propria appeared to be increased. The mucosal changes at the ileo-cecal junction were similar to those described on day 6 PI.

Pyloro-duodenal junction of the stomach was found to be affected by Cryptosporidium sp. in three piglets (A8, C7 and C9), but more or-

Figure 7. Jej. 200, day 6 PI, untreated group

H&E stain: Crypt hyperplasia with regenerated villi.
A few Cryptosporidium sp. organisms are present on the
villous surface (130x)

Figure 8. 5PICJ, day 6 PI, untreated group

H&E stain: Villous atrophy and crypt hyperplasia.
Cryptosporidium sp. organisms (arrowhead) on the
epithelial surface of dome (a), enteroabsorptive
cells (b) and crypt cells (c) (188x)

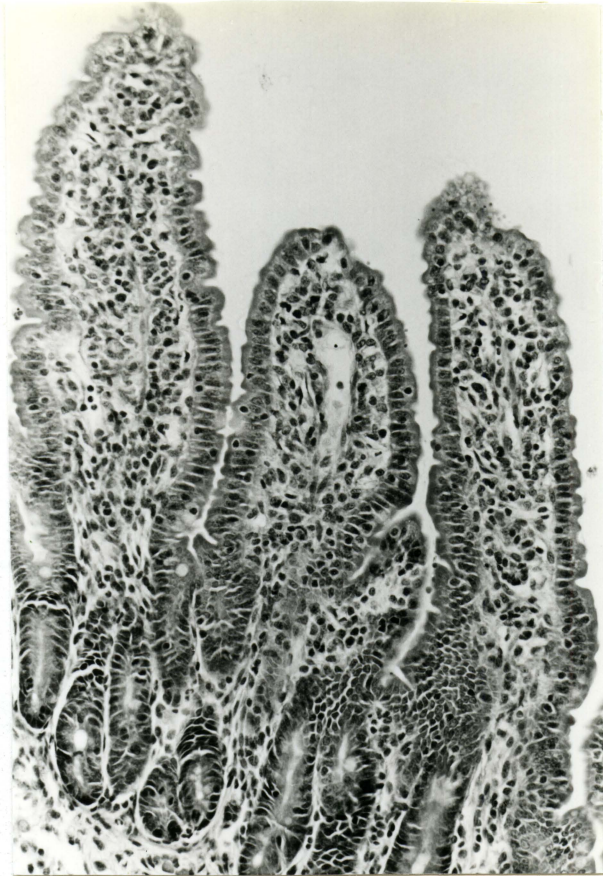
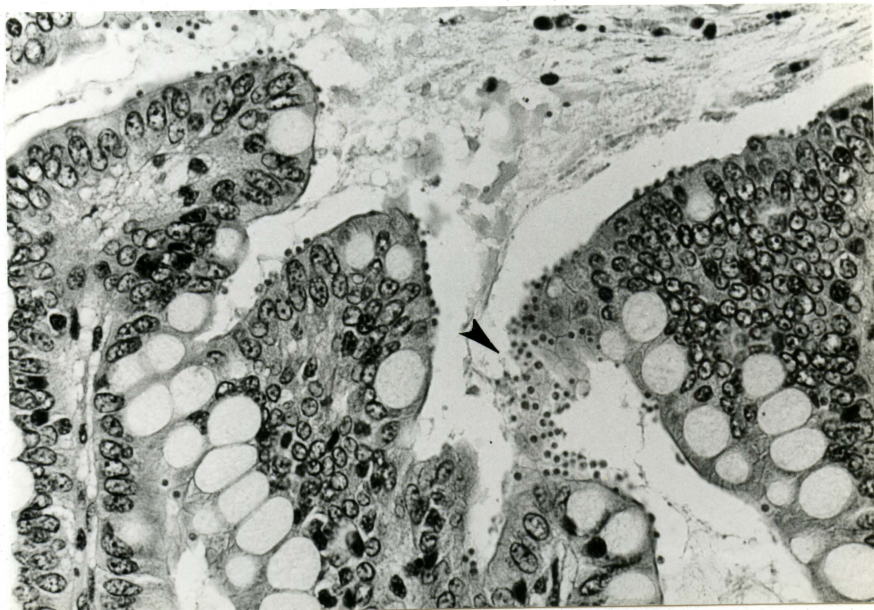
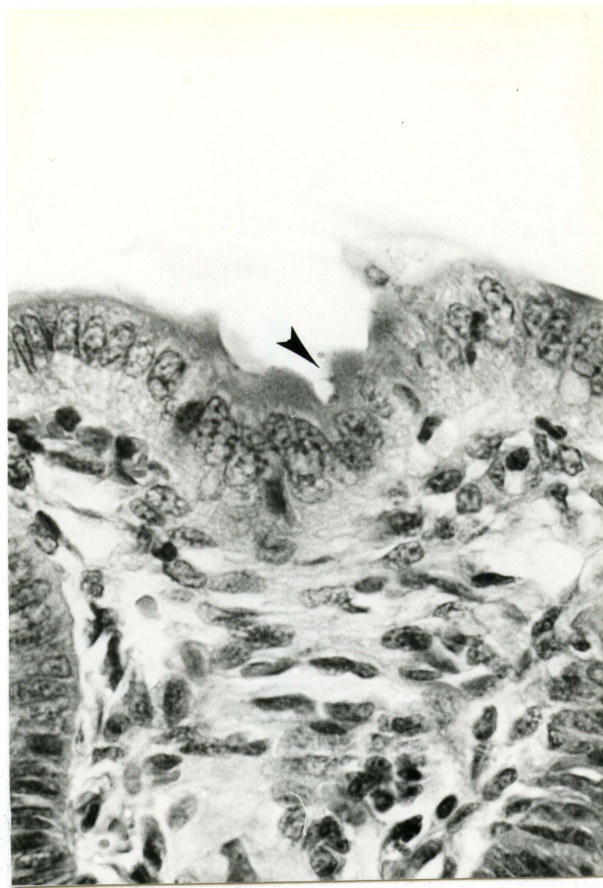


Figure 9. Caecum, day 6 PI, treated group

H&E stain: A few Cryptosporidium sp. organisms (arrowhead) on epithelial surface only. Mild mononuclear sub-epithelial infiltration and disarrangement of lining cells (400x)

Figure 10. Ileo-cecal junction, day 6 PI, treated group

H&E stain: Numerous Cryptosporidium sp. organisms (arrowhead) attached to the mucosal surface (400x)



ganisms were detected in the duodenal site than the pyloric site (Figure 12). Mucosal areas intimately associated with the organisms were mildly irregular, with lymphocytes and plasma cell infiltrates in the lamina propria.

The presence of Cryptosporidium sp. in crypts of the intestinal mucosa in any of the sites investigated was not common until day 6 and 8 PI. In all intestinal sections in which domes were cut, the parasite was found adherent to dome epithelial cells from day 4 PI on. There were no significant differences of lesions and numbers of the organisms between dome epithelium and villous epithelium in various stages of infection.

4.3. Morphometry and statistical analyses

As described earlier, there were only four piglets (one untreated, B9, and three treated, C9, D9, and D10) left to be killed on day 8 PI. Thus, the data from these animals were not included into the statistical analyses. To obtain equally balanced data, the analytic evaluation was confined to the measurement from small intestinal localizations of the piglets killed on days 0, 2, 4 and 6 PI.

There were five morphometric parameters taken in this experiment: numbers of Cryptosporidium sp. on the villous surface, mitotic index, vl, cd and vd/cd ratio. The range and mean value of the above parameters from the group treated with neomycin and untreated group are listed in Table 2. On the basis of statistical analyses, none of the above five parameters were significantly different between the treated and un-

Figure 11. Jej. 200, day 8 PI, untreated group

H&E stain: Crypt hyperplasia with regenerated villi.
Very few organisms detected (90x)

Figure 12. Pyloro-duodenal junction, day 6 PI, treated group

H&E stain: A few Cryptosporidium sp. organisms present
on the gastric mucosal surface. Mononuclear cells
infiltrate in the subepithelial region (860x)

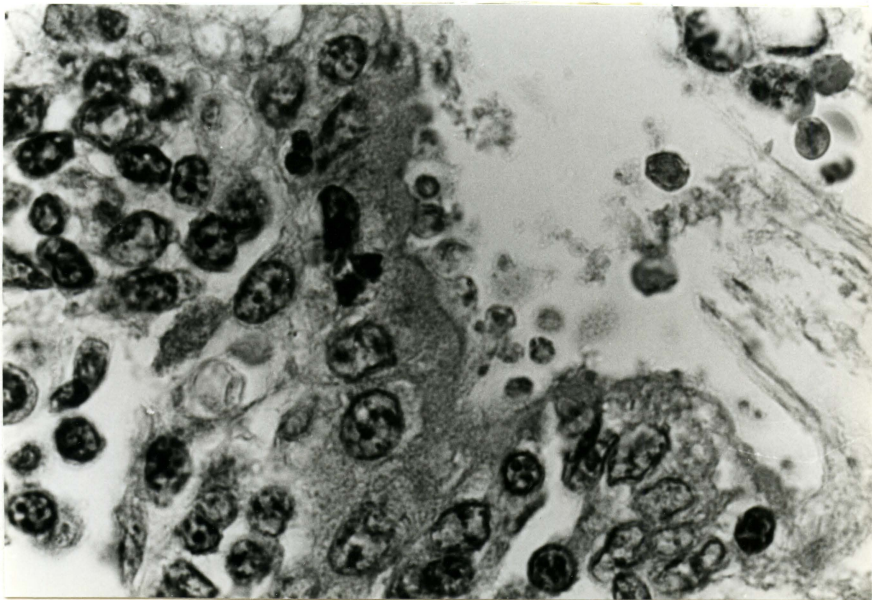
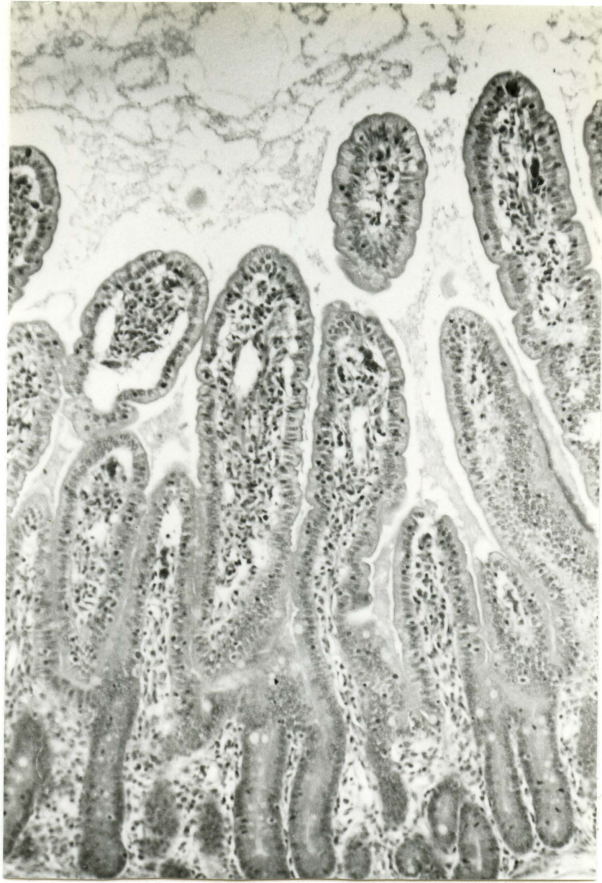


Table 2. Range and mean value of five morphometric parameters (villous length, crypt depth, ratio of villous/crypt depth, mitotic index and numbers of Cryptosporidium sp. organisms) on villous surface from group treated with neomycin (treated) and untreated group (untreated)^a

Morphometric parameter	Postinoculation (days)	Villous length (µm)		Crypt depth (µm)	
		Treated	Untreated	Treated	Untreated
		0	Range	237.5-1228.75	352.5-1112.5
	Mean value	722.5	610	106	121
2	Range	205-1207.5	261.5-1003.75	96-287	95-202
	Mean value	542.5	590	135	121
4	Range	167.5-812.5	131.25-1111.25	87-172	110-221
	Mean value	450	545	126	146
6	Range	41.25-871.25	120-1068.75	93-271	110-228
	Mean value	412.5	472.5	150	160

^aGrading scale "0-4" in this table represent "0-++++" used in the measurement of histologic sections.

Ratio of villous length per crypt depth		Mitotic index		<u>Cryptosporidium</u> sp. on the villous surface	
Treated	Untreated	Treated	Untreated	Treated	Untreated
0.81-16.17	2.20-10.70	2-13	1-10	0-0	0-0
7.33	5.42	6.25	5.21	0	0
1.17-8.75	2.51-9.65	2-13	1-10	0-4	0-4
4.43	5.05	6.67	4.08	0.48	0.48
1.09-7.21	0.59-10.01	3-14	1-11	0-4	0-4
3.73	4.10	7.83	4.33	1.21	1.58
0.21-6.86	0.70-9.72	4-23	2-18	0-4	0-4
3.07	3.03	11.17	11.13	1.79	2.42

treated groups ($p > 0.05$). Regardless of the treatment, the number of organisms on the villous surface and mitotic indices increased significantly ($p < 0.05$) with the days post-inoculation (Figure 13 and Figure 14). Additionally, the mitotic index (Figure 14) increased approximately two times on day 6 PI compared with day 0 PI.

Vl, cd and vl/cd ratio measured from wet tissues sectioned by vibratome were not significantly different between the treated and untreated groups ($p > 0.05$). Without regard to the treatment, mean values of villous lengths from the treated and untreated groups decreased significantly ($p < 0.05$) with the days postinoculation (Figure 15). But, mean values of crypt depths of the two groups increased significantly ($p < 0.05$) with the days postinoculation (Figure 16). Similarly, significant increase ($p < 0.05$) was seen in the mean values of vl/cd ratio from the groups with the days postinoculation (Figure 17). By LSD analysis, mitotic index (Figure 14), vl (Figure 15), cd (Figure 16) and vl/cd ratio (Figure 17) also show that none of the treatment comparisons at the same day postinoculation are significantly different.

To compare the vl/cd ratio in different sites of the small intestine in all experimental piglets, three segments: anterior (Duo.), mid (Jej. 100, Jej. 200 and 120PICJ) and posterior (15- and 5PICJ) were evaluated. Vl/cd ratio in three of them (Figure 18, Figure 19, and Figure 20) was decreased on subsequent days postinoculation. The changes from days 0 to 6 PI are approximately from 3:1 (2.91:1, true value)

Figure 13. Mean numbers of Cryptosporidium sp. organisms on villous surface of the small intestine on days 0, 2, 4 and 6 after inoculation with 10^6 oocysts in group treated with neomycin and untreated group

Least significant difference (LSD) ($\alpha = 0.05$) = 21.63 for treatment comparison at the same day postinoculation

Arrowhead = day of inoculation

Figure 14. Mean mitotic index (numbers of mitoses per twenty crypts) of the small intestine on days 0, 2, 4 and 6 after inoculation with 10^6 oocysts in group treated with neomycin and untreated group

Least significant difference (LSD) ($\alpha = 0.05$) = 63.42 for treatment comparison at the same day postinoculation

Arrowhead = day of inoculation

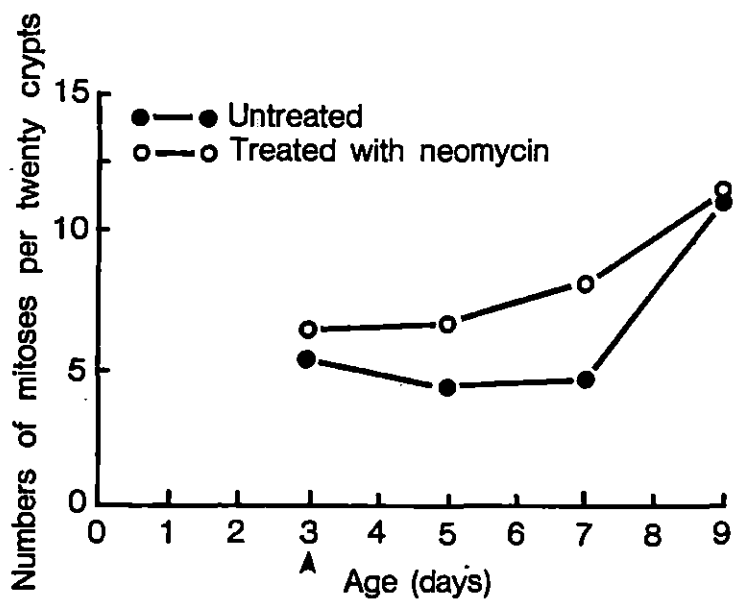
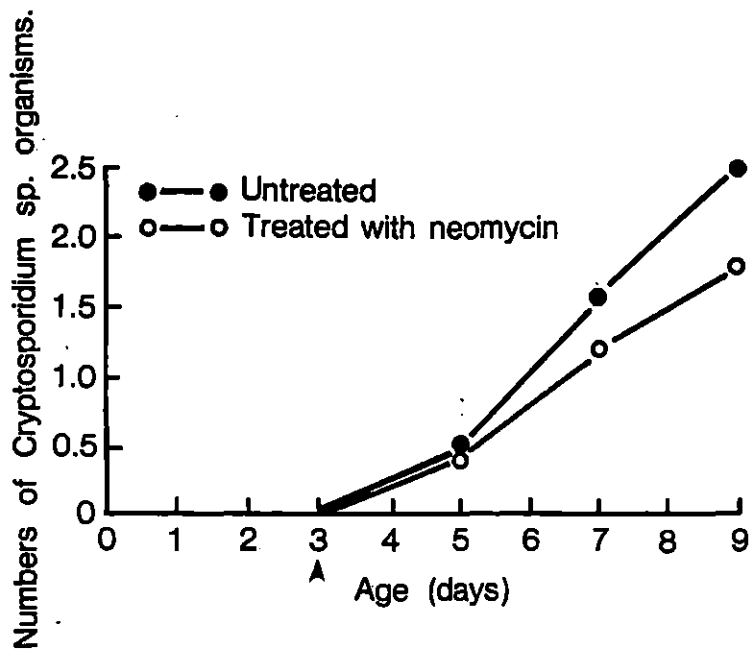


Figure 15. Mean villous length of the small intestine on days 0, 2, 4 and 6 after inoculation with 10^6 oocysts in group treated with neomycin and untreated group

Least significant difference (LSD) ($\alpha = 0.05$) = 3037.27
for treatment comparison at the same day postinoculation

Arrowhead = day of inoculation

Figure 16. Mean crypt depth of the small intestine on days 0, 2, 4 and 6 after inoculation with 10^6 oocysts in group treated with neomycin and untreated group

Least significant difference (LSD) ($\alpha = 0.05$) = 432.75
for treatment comparison at the same day postinoculation

Arrowhead = day of inoculation

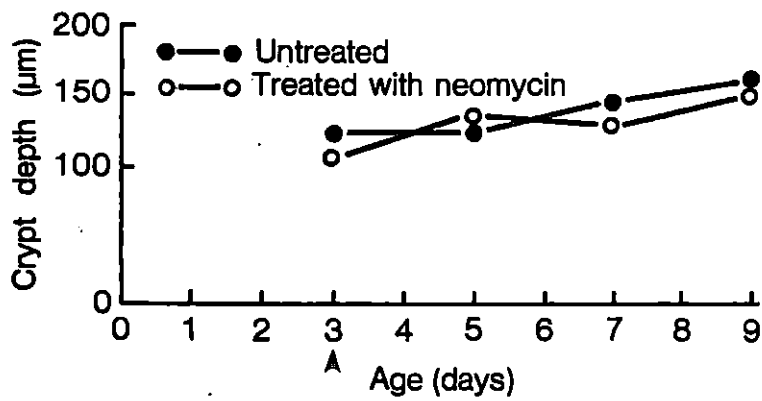
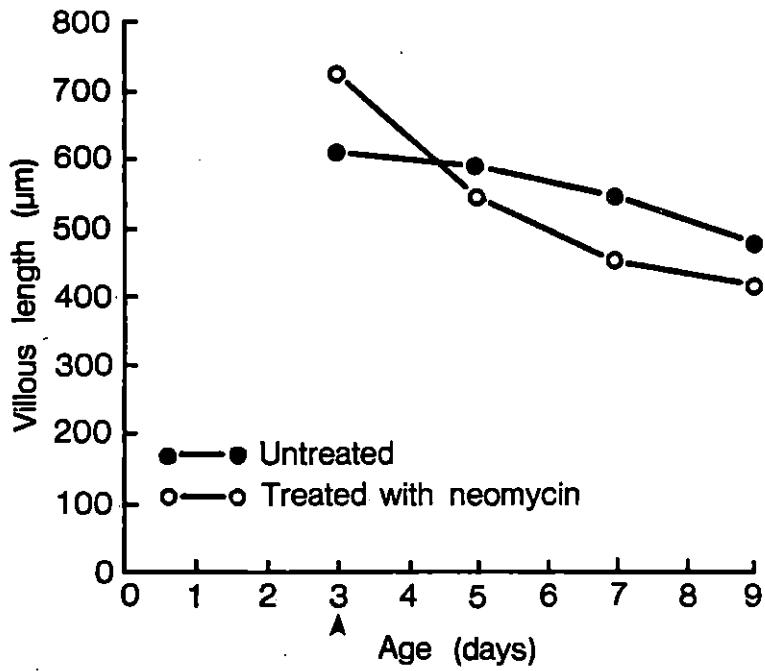


Figure 17. Mean ratio of villous length/crypt depth (vl/cd) of the small intestine on days 0, 2, 4 and 6 after inoculation with 10^6 oocysts in group treated with neomycin and untreated group

Least significant difference (LSD) ($\alpha = 0.05$) = 33.36 for treatment comparison at the same day postinoculation

Arrowhead = day of inoculation

Figure 18. Mean ratio of villous length/crypt depth of the duodenum (Duo.)

Data pooled from both groups

Arrowhead = day of inoculation

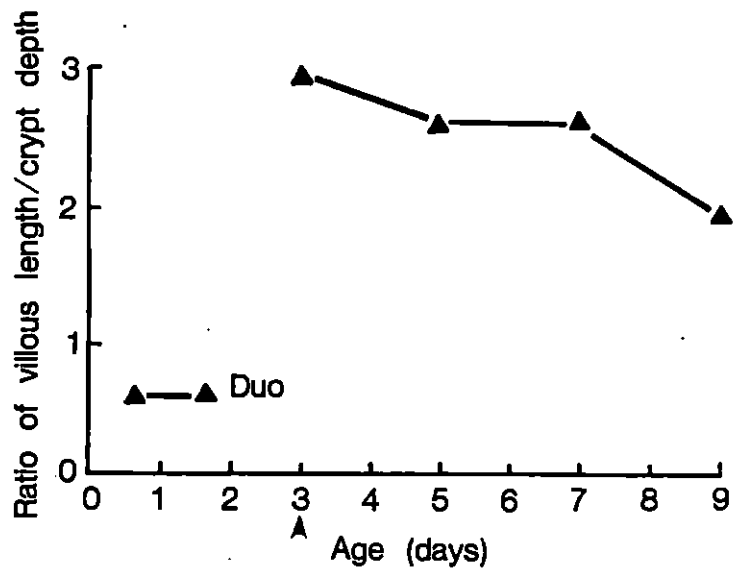
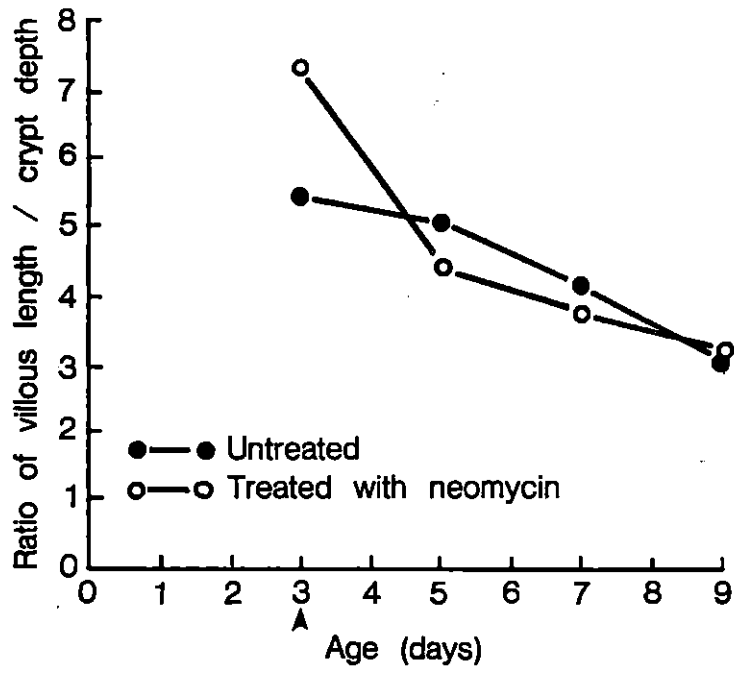
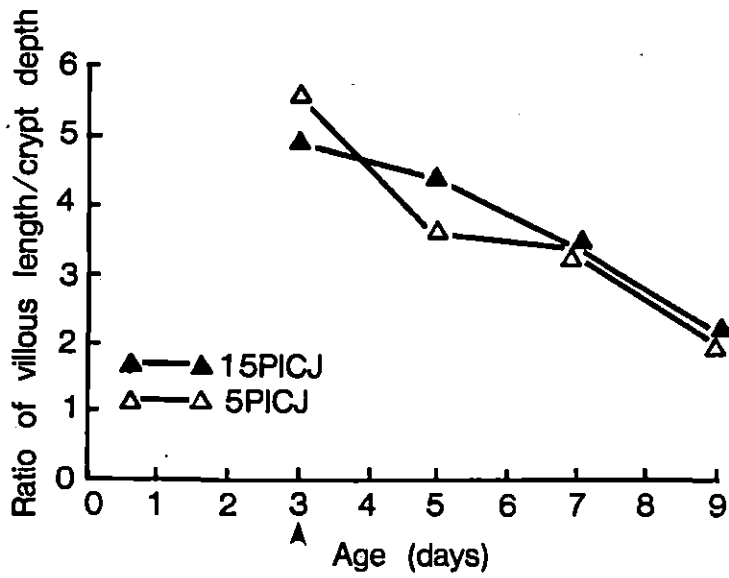
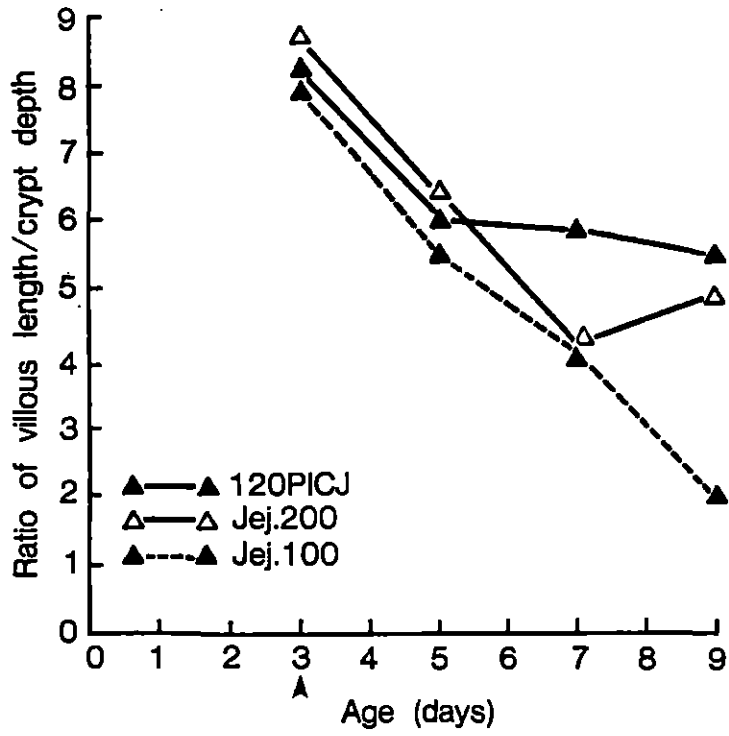


Figure 19. Mean ratio of villous length/crypt depth of 100 cm,
200 cm distal to duodenal site (Jej. 100 and Jej. 200)
and 120 cm proximal to ileo-cecal junction (120PICJ)
Data pooled from both groups
Arrowhead = day of inoculation

Figure 20. Mean ratio of villous length/crypt depth of 5 cm and
15 cm proximal to ileo-cecal junction (5PICJ and
15PICJ)
Data pooled from both groups
Arrowhead = day of inoculation



to 2:1 (1.89:1, true value) in the anterior small intestine, 8:1 (7.91:1 in Jej. 100, 8.67:1 in Jej. 200 and 8.28:1 in 120PICJ, true value) to 2:1 (2.02:1 in Jej. 100, true value) or 5:1 (4.88:1 in Jej. 200 and 5.44:1 in 120PICJ, true value) in the mid-small intestine, and 5:1 (5.53:1 in 5PICJ and 4.93:1 in 15PICJ, true value) to 2:1 (2.00:1 in 5PICJ and 2.09:1 in 15PICJ, true value) in the posterior small intestine.

5. DISCUSSION

Four litters of conventionally born piglets raised with the sow were orally inoculated with 10^6 Cryptosporidium sp. oocysts. The inoculum was derived from an earlier obtained isolate taken from a calf infected with Cryptosporidium sp. In all experimental piglets, except one killed on day 2 PI, Cryptosporidium sp. was detected in the gastrointestinal tract. This high morbidity shows that piglets raised under normal conditions and fed with colostrum are still susceptible to infection with Cryptosporidium sp. The amount of 10^6 oocysts contained in the inoculum per piglet is estimated to be less than probably contained in one ml of fecal material taken from an infected calf under field conditions. The successful inoculation confirms previously reported results (63,103) which demonstrated that porcine cryptosporidium infection is possible with an inoculum taken from a different species -- in this experiment bovine origin -- without being passed through an intermediate host. Although outbreaks of piglet diarrhea associated with Cryptosporidium sp. have not been described, this experiment demonstrates that Cryptosporidium sp. should be considered as a potential cause of diarrhea in newborn piglets in the daily diagnostic work. Diarrhea as described in other experimental infections with Cryptosporidium sp. (63,65,103,105) was seen in 35% of animals killed on days 4, 6 and 8 PI, but not all animals developed diarrhea. It is not unlikely that some piglets are more resistant to this protozoal infection than others.

As shown in this experiment where stomach, six sites of the small intestine and three sites of the large intestine were investigated (see Figure 3), there are individual differences in the amount of Cryptosporidium sp. present at a given section and in the severity of lesions associated with the presence of this parasite. Other workers (103,105) observed vomiting and inappetence. The latter was not recorded in this experiment and vomiting was not seen in individual animals. There was, however, vomited stomach contents found on the floor of the pens of three of the four litters, indicating that some piglets did vomit after inoculation. The cause of vomiting is unknown. The rather intense duodenal infection in most of the animals on day 4 PI and the infection of the pyloric region in three animals suggest that the organism can cause vomiting. It is unknown whether Cryptosporidium sp. influences intestinal motility. Duodenal villous atrophy as seen in rotavirus infection (55) can cause vomiting in piglets, but viral infections were not demonstrated in these litters of piglets. It is possible that Cryptosporidium sp. can cause vomiting in some affected piglets.

As shown by others in this experiment, oocysts were first found on day 4 PI (65,103) in fecal samples from four animals and later (days 6 and 8 PI) in those from most animals. In two piglets in which no Cryptosporidium sp. oocysts were detected in the fecal samples by the methods used here, the organism was found in several histologic sections. It is possible that these animals had a very mild infection in which not many oocysts were produced and that methods used to detect the para-

site in fecal smears are not sensitive enough to find low numbers of these coccidia.

There was no close correlation between shedding of oocysts and diarrhea (see Table 1) and several animals did shed parasites without evidencing disease. This observation is not consistent with other reports (64,65,103,105) and remains unexplained. It is, however, necessary to point out that clinically healthy piglets have to be considered silent carriers of the organism.

Cryptosporidium sp. was not found in tissues other than the gastrointestinal tract. This finding confirms previous reports that the gastrointestinal tract is the main site in oral infection with this parasite (63,65,103,105). Respiratory cryptosporidiosis in pigs occurring following oral infection with Cryptosporidium sp. was described by Schloemer (82) and was mentioned by Tzipori (95), but this did not occur in this experiment and has not been reported by other workers (63,65,103,105). Conjunctival and tracheal infection as reported by Heine et al. (38) was not seen in these piglets. It is speculated that respiratory infection results from aerogenic exposure. Inhalation of vomitus or inoculum containing Cryptosporidium sp. organisms is possible. It is not believed that the infection spreads hematogenous-ly in contrast to Ma et al. (59) who considered the possibility of hematogenous spread due to Cryptosporidium sp. oocysts in pulmonary macrophages.

Histopathologically, hyperkeratosis with a mild inflammatory re-

sponse was a common finding in the esophageal mucosa of the control piglets and more severe lesions were seen after inoculation. This is interpreted to be most likely due to the application of the inoculation by gavage.

The distribution of Cryptosporidium sp. on the villous surface was investigated in six different segments of the small intestine (see Materials and Methods). The mid-small intestine (Jej. 100, Jej. 200 and 120PICJ) was infected more heavily by parasite than other sites of the intestine on day 2 PI. Snodgrass et al. (89) reported that Cryptosporidium sp. was first detected in the mid-gut and ileum at 12 to 24 hours postinoculation (in experimentally infected germ-free lambs). Tzipori et al. (103) reported in experimental porcine cryptosporidiosis that piglets killed shortly after the onset of clinical signs had mild histologic lesions restricted to the mid-gut. These results indicate that mid-jejunum is probably the first target site in the gastrointestinal tract after ingestion of Cryptosporidium sp. organisms.

On day 4 PI, Cryptosporidium sp. organisms were found throughout the small intestine indicating that the parasite spread anteriorly to the duodenum and posteriorly to the ileum. The parasite was not detected in any site of the large intestine, though two of these piglets shed the organisms in the feces at this time. The cause remains unexplained.

On day 6 PI, numerous Cryptosporidium sp. organisms were predominantly distributed in both anterior and posterior small intestine. The mid-small intestine, however, was less intensely infected. Close correlation between numbers of the organisms and villous atrophy was also noted (see Figure 3) suggesting a correlation between the number of Cryptosporidium sp. organisms attached to the villous surface and severe villous atrophy. There were, however, a few exceptions, e.g., Jej. 100 and Jej. 200 in piglet B8 and Duo. and Jej. 100 in piglet A8, where the parasite was present in high numbers on the villous surface, but villous length was not decreased. It is possible that this parasite just completed attachment to the villus and villous atrophy had not developed. Histologically, a few organisms associated with increased mitotic figures and crypt depth, but without presence of villous atrophy were noted in the mid-small intestine. This distribution pattern of Cryptosporidium sp. organisms was not reported in other porcine cryptosporidial infections (63,65,103,105).

On day 8 PI, there were two piglets (B9 and D10) with a similar distribution pattern as seen on day 6 PI. Mid-small intestine of these piglets at this time again had fewer organisms on the villous surface with an increase of both crypt depth and mitotic figures as seen on day 6 PI.

Many reports (63,65,105) indicated that the ileum is a commonly affected site in porcine cryptosporidiosis. This was seen in this ex-

periment starting from day 4 PI and more intense on days 6 and 8 PI. In all ileal sections where Cryptosporidium sp. was found on entero-absorptive cells of villi, the organisms were also found adhering to dome epithelium. Wolf and Bye (109) have defined membraneous epithelial (M) cells as being "specialized epithelial cells overlying the gut-associated and bronchial-associated lymphoid tissues (GALT and BALT) that transport antigens from the lumen to the extracellular space, allowing access to lymphocytes, macrophages and plasma cells." The authors also mentioned that reovirus types 1 and 3, mycobacteria, chlamydia, Vibrio cholerae, horseradish peroxidase, some ferritins and agglutinins have been transported by M cells. It is suggested that antigen derived from Cryptosporidium sp. on dome epithelium can induce an immunologic response to the organism.

The ileo-cecal junction was affected by numerous Cryptosporidium sp. organisms from day 6 PI, at the same time, where the ileum was more intensely parasitized. Since this site of the intestine contains gut-associated lymphoid tissue, it is possible that the parasite can induce a mucosal immune response similar as in their association to the dome epithelium at this site.

In three piglets, Cryptosporidium sp. was found at the pyloro-duodenal junction. This observation and the finding that the duodenum appears to be regularly affected is unusual and has not been reported earlier except in one experiment, in which Schloemer (82) described

this parasite in the stomach. As discussed earlier, the parasites at the pyloro-duodenal junction and in the duodenum may cause functional disorders resulting in vomiting.

Cecum and two sites of colon were found to be affected on day 6 PI in two out of eight animals only and in two animals out of four on day 8 PI. This result is in agreement with the report from Tzipori et al. (103) but in disagreement with the reports from Moon et al. (63,65) and Tzipori et al. (105) who described a colitis in experimental porcine cryptosporidiosis. Typhlitis and colitis in experimentally-infected gnotobiotic calves were recently reported by Pohlenz et al. (76). It remains unexplained why this discrepancy exists. In this experiment, 10^6 oocysts were used as inoculum which is considered to be a very small amount of infectious organism. A detailed dosage concerning the inoculum used in other porcine experiments (63,65,103) was not given, except in one experiment (105); thus, a comparison is difficult.

The most striking histologic lesions in the small intestine observed in this experiment were villous fusion, villous atrophy and flat to cuboidal epithelium on villous surface associated with crypt hyperplasia and an increase of mitotic figures in crypts. These findings have been described by others in experimental porcine cryptosporidiosis (63, 65,103,105) and have been reported as typical lesions in the intestinal mucosa in other species (39,96).

Though there were no matched controls in this experiment and piglets killed on day 3 of life before the inoculation were considered to

serve as controls, we feel that the villous atrophy observed in the small intestine on day 4, 6 and 8 PI is a result of the parasitic infection. Especially since on day 2 PI, villous atrophy was not detected and was not present in the less severely affected mid-small intestine on day 6 PI. Mean villous length of entire small intestine in all experimental piglets (Figure 15) killed on day 6 PI was decreased 1.5 times compared with those on day 0 PI. These data also support that villous atrophy develops during the cryptosporidial infection.

The mitotic index in this experiment was defined as the number of mitotic figures per twenty straight cut crypts. Comparing trends (see Figure 14) between the group treated with neomycin and the untreated group, it is evident that the mean mitotic index of the untreated group in all days postinoculation is lower than that in the treated group. These differences are not significant and it is unknown whether neomycin influences the mitotic rate. There is a marked increase of mitotic figures on day 6 PI in both groups, which is most likely due to the infection. Increased mitotic rate in crypts infected with Cryptosporidium sp. was reported in pigs (50), lambs (7) and monkeys (108), but these findings were from histologic observation without morphometric data. The increased mitotic figures as described above and an increase of crypt depth during the infection (see Fig. 16) indicate that crypt hyperplasia does occur due to Cryptosporidium sp. infection. On day 6 PI, there were remarkably less Cryptosporidium sp. organisms in the mid-

small intestine compared with those in the anterior and posterior small intestine. However, an increase of crypt depth and mitotic figures found in the mid-gut suggests that crypt hyperplasia occurred after a few days of infection (on day 6 PI in this experiment). Crypt hyperplasia indicates an increased cellular turnover by which surface cells are replaced. With this the organisms may leave with exfoliated villous epithelium and probably regenerated villous epithelium may resist the reinfection with this parasite. This observation is supported from findings on day 8 PI, where the amount of Cryptosporidium sp. on the villous surface decreased and villous atrophy in the mid-small intestine was not found in four piglets.

The ratio of villous length/crypt depth (vl/cd ratio) in this experiment was defined as mean twenty villous lengths divided by mean ten crypt depths and used to evaluate the correlation of villi and crypt in the entire small intestine. Vl/cd ratio had a significant decrease with the days postinoculation. The reasonable explanation of decreased vl/cd ratio is directly related to decreased villous length and increased crypt depth as described earlier; decreased vl/cd ratio in the ileum with Cryptosporidium sp. was previously described in rabbits (44). Weinstein et al. (107) reported vllunted villi, long crypts and 2:1 of vl/cd ratio in duodenal biopsies from human cryptosporidiosis. In addition, 4:1 of vl/cd ratio in jejunal biopsies was mentioned in other cases of human cryptosporidiosis. From these reports and our data of decreased vl/cd ratio in the cryptosporidial infection has to be con-

sidered as a consistent finding.

In this experiment, no severe inflammatory cell response was observed. Neutrophils and eosinophils infiltrating the lamina propria of the small intestine were common during the infection. But in some of the controls killed on day 0 PI, eosinophils and neutrophils were also found. There was an increase of plasma cells and lymphocytes starting on day 4 PI and more prominent on days 6 and 8 PI, particularly in the anterior small intestine. It is not unlikely that the appearance of plasma cells and lymphocytes in the mucosa may suggest a local mucosal immune response due to this parasitic infection.

A vibratome technique was employed to prepare wet sections to measure villous length and crypt depth and then determine vl/cd ratio. As described in the Materials and Methods, the criteria of villi selected were entire villous length with an intact villous architecture from the base of villi to the tip. Crypts were chosen for measurement only when visible from the lamina muscularis to the crypt opening. A series of at least three sections were cut and only those villi and crypts were measured, which fulfilled the criteria described above to avoid mismeasurement. The vibratome was used to standardize the measurement working at consistent sections of 60-80 μm . Using wet mounted formalin fixed material appears advantageous for measurements, because the tissue is not altered by paraffin embedding and staining procedures.

Two piglets from litter C and three piglets from litter D had diarrhea before inoculation. No viral agents were found in fecal materials

of these piglets. Sow C refused to feed for two days after parturition. It is possible that these piglets from sow C did not receive enough milk and took urine from the floor, which may have induced diarrhea. Since piglets from litters C and D only developed diarrhea before inoculation it is possible that neomycin induced diarrhea in these treated groups. Histologic changes and morphometric parameters were not significantly different between the group treated with neomycin (litters C and D) and untreated group (litters A and B). There were also no significant differences in the treatment comparisons from days 0 and 6 PI.

It has been shown in human pathology that high dosage of neomycin induced a malabsorption syndrome (47,48) and an intestinal mucosal alteration compatible with tropical sprue (35). These biological effects may be produced by as little as 3 gm of the drug per day per person but are more intense with a dose of 12 gm per day per person. Thus, it may be possible that infection after pretreatment with neomycin would promote the infection. This has not been observed in this experiment. It is not known whether the dosage used did not reach the level high enough to produce this effect, which may be different in piglets.

Cryptosporidium sp. organisms were detected in histologic sections in all piglets from litters C and D killed on days 2, 4, 6 and 8 PI. Though these animals had received neomycin for three days before inoculation and for two days after inoculation, the inoculation resulted in infection. These results show that neomycin used in this experiment

neither prevented nor treated cryptosporidial infection, and did not appear to promote the infection.

6. SUMMARY

Four litters (A-D) of conventionally born piglets were reared with the sow and fed with colostrum ad libitum. Nineteen of these piglets from litter C(1-9) and litter D(1-10) were orally treated with 140 mg neomycin per piglet per day for five days from day 1 of life. Seventeen of these piglets from litter A(1-8) and litter B(1-9) remained untreated. Two piglets of each litter (A-D) were killed on day 3 of life (day 0 postinoculation, PI), and considered as controls. The remaining piglets were orally inoculated with 10^6 Cryptosporidium sp. by gavage at day 3 of life.

More than half of the piglets developed diarrhea after inoculation, a few vomited. Piglets shed oocysts from day 4 PI. None of the experimental piglets had inappetence. Some piglets had no diarrhea but oocysts were present in the feces.

On day 2 PI, more Cryptosporidium sp. organisms were found in the mid-small intestine and less in the duodenum and the ileum. On day 4 PI, the organisms were spread throughout the small intestine. On day 6 PI, numerous organisms were distributed on both anterior and posterior small intestine and less organisms were seen in the mid-small intestine. A similar distribution of Cryptosporidium sp. was observed on day 8 PI. Ileo-cecal junction was severely affected on days 6 and 8 PI. Very few organisms with mild lesions appeared in the caecum and colon on days 6 and 8 PI. There were no significant differences of lesions and the organism distribution between dome epi-

thelium and villous epithelium. Cryptosporidium sp. was detected in the stomach (pylorus) in three piglets. The organisms were not present in any tissue outside the gastrointestinal tract in any experimental piglet.

Histopathologically, villous atrophy, crypt hyperplasia, fusion of villi, cuboidal to flattened lining epithelial cells and microulceration with neutrophils, eosinophils and mononuclear cell infiltrations were common lesions in the small intestine.

Five morphometric parameters [villous length (vl), crypt depth (cd), ratio of villous length/crypt depth (vl/cd ratio), mitotic index and the number of Cryptosporidium sp. on villous surface in the small intestine] were used to evaluate the mucosal response and compare treated and untreated groups. To investigate vl and cd, a vibratome technique was first employed and preliminary finding indicated that it is practical and time-saving. From the statistical analyses on days 0, 2, 4 and 6 PI, there were a significant increase ($p < 0.05$) in cd, mitotic index and number of organisms on villous surface, and a significant decrease ($p < 0.05$) in vl and vl/cd ratio with days post-inoculation. However, no significant differences ($p > 0.05$) of these parameters were present between treated and untreated groups. Treatment comparisons at the same day postinoculation also did not reveal significant differences between the two groups. On the basis of clinical signs, histologic lesions and morphometric parameters, neomycin used in this experiment did not predispose or impede cryptosporidial infection.

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