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EXPERIMENTAL CRYPTOSPORIDIOSIS IN MICE, CALVES AND CHICKEN

By

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POHJOLA, SUVI and LARS-AXEL LINDBERG: *Experimental cryptosporidiosis in mice, calves and chicken.* Acta vet. scand. 1986, 27, 80—90. — Experimental infections attributed solely to Cryptosporidium were carried out in newborn SPF mice, calves and chicken in order to study the prepatency, patency and incubation periods, describe the clinical symptoms and find and describe any correlations between the association of Cryptosporidium with the intestinal mucosa and presence of pathological lesions. The paper also gives the clinical and paarsitological parameters of Cryptosporidium infection of calves from a field survey and compares them to the results of experimental study.

parasitological parameters; histopathological lesions.

The minute coccidian protozoan Cryptosporidium, once considered rare and of doubtful clinical significance, is a world-wide newcomer among enteropathogens both in human and veterinary parasitology (reviewed by *Tzipori* 1983, *Anon.* 1984). In veterinary medicine Cryptosporidium has been found most frequently in the alimentary tract of young calves (*Pohlenz et al.* 1978, *Tzipori* 1983, *Stein et al.* 1983, *Henriksen & Krogh* 1985).

The life cycle of the monoxenous Cryptosporidium is rapid and occurs in the brush border of enterocytes (*Tzipori* 1983). Since the infection has an impact on the gastrointestinal tract it may lead to maldigestion, malabsorption and malnutrition; abnormal fecal fat, D-xylose test, and low serum coratene levels have been described in human patients (*Stemmerman* 1980, *Tzipori* 1980, *Sloper* 1982), although the earlier reports at first failed to document malabsorption in connection with Cryptosporidium infection (*Nime* 1976, *Meisel* 1976). In young calves

Cryptosporidium infection has been associated with the cachexia syndrome (*Hage-Noordam et al.* 1982), and the emaciation syndrome (*Krogh & Henriksen* 1985), reflecting the chronic implications of the acute disease.

A 5-month survey among neonatal calves carried out in Finland in 1982, revealed oocysts in 36 out of 68 (53 %) calves under the age of 20 days (*Pohjola et al.* 1986). Since differences between herds in animal husbandry methods and management, the occurrence of other enteropathogens in mixed infections, and the original level of exposure to Cryptosporidium oocysts have certainly influenced the clinical and parasitological parameters, we carried out experimental infections attributed solely to Cryptosporidium in mice, calves and chickens, in order 1) to study the prepatency, patency and incubation periods, 2) to describe the clinical symptoms, 3) to transmit the infection between calves and mice, and calves and chickens, 4) to find and describe any correlations between the association of Cryptosporidium with the intestinal mucosa and the presence of pathological lesions. The present paper also gives the clinical and parasitological parameters of the previous field survey and compares them to the results of the experimental study.

MATERIALS AND METHODS

Animals

M i c e: 120, 1—4 days old offspring of Han-NMRI specific-pathogen-free (SPF) mice (Orion Diagnostica, Espoo, Finland) were used. The litters were kept in plastic cages with their dams, 1 litter per cage. The dams were provided with pelleted food and water ad lib.

C a l v e s: 6 colostrum fed, 2—7 days old Ayrshire calves were purchased from different frams for the experimental study. They were penned individually and fed nonmedicated commercial milk substitute. 5 calves were inoculated with Cryptosporidium oocysts and 1 served as an uninfected negative control. After the sampling was ceased the calves were penned for an additional week before slaughtering was performed to obtain tissue samples.

The 68 calves of the field survey originated from 8 housed herds, which were selected on the ground of a previous history of diarrhea (*Pohjola et al.* 1986).

Chickens: 30 one-day-old broiler chickens of Pilch (Saarioinen Oy, Sahalahti, Finland) were housed in flocks of 10 birds and fed commercial food and water ad lib.

Source and preparation of inocula, inoculation

Two types of inocula were used in the cross-transmission experiments.

Inoculum I, fecal material obtained from a 9-day-old calf, naturally infected with Cryptosporidium, was kindly provided by Dr. *Sv. Aa. Henriksen* (State Veterinary Serum Laboratory, Copenhagen, Denmark). The inoculum was purified by treatment in ethanol (final concentration of 60 %) for 2 h and washed twice in 0.9 % saline by centrifugation at 500 g for 10 min. The absence of bacterial contamination was checked by culturing the seriment on 5 % bovine blood agar and the morphology of the oocysts was examined from the smeared sediment stained with the modified Ziehl-Neelsen technique (*Henriksen & Pohlenz* 1981). 0.05 ml of the fecal sediment (20 % v/v in 0.9 % NaCl) was administered intraesophageally with a stomach tube of 25 gauge to 100 1—4 day old mice, each weighing about 2 g. 20 mice served as uninfected controls. The final oocyst count of inoculum I was not evaluated. One ml inoculum I was also administered into the crop of 20 one-day-old chickens, 10 chickens served as uninfected controls.

Gut homogenates were collected from the SPF mice, homogenized with a tissue grinder and prepared in the previous manner for inoculum II for the experimental calves. An oocyst count of about 3×10^4 in 5 ml of the inoculum was mixed with 100 ml of warmed milk and given intraruminally with the stomach tube to 5 calves.

Clinical observations and sampling

The experimental animals were observed for a period of 2 weeks. Signs of illness, intake of food and the rectal temperatures of the calves were recorded; 4 mice and 2 chickens were necropsied, and fecal specimens were collected from calves at 12 h intervals beginning at 60 h post-inoculationem.

Since in previous studies we had found that some oocysts may have poor affinity for the Giemsa stain (*Jokipii et al.* 1983) or were not acid-fast at all (*Pohjola et al.* 1986), 3

experimental calves were sampled at 6 h intervals between the 74th and 98th h post-infectionem.

Histology

Tissue samples were taken from all infected mice and 3 calves as quickly as possible after decapitation or slaughter of the animals. The mice were sampled from the terminal part of the ileum, the calves from the following areas: initial and terminal jejunum, terminal ileum, caecum and colon. The samples were fixed in neutral 10 % formalin, dehydrated in ethanol and embedded in paraffin. Sections cut at 5 μm were stained with hematoxylin-eosin (HE) or Giemsa (mice), or with HE (calves).

The remaining part of the intestine from each mouse was homogenized in saline, as was the terminal part of the large intestine from the chickens.

Detection of Cryptosporidium

Gut homogenates from mice and chicken and fecal specimens from calves were smeared on microscope slides and stained with the modified Ziehl-Neelsen technique (*Henriksen & Pohlenz* 1981). The stained smears were examined under oil-immersion at a magnification of 100×. The grade of murine cryptosporidiosis was scored by comparing the means of oocyst numbers counted for 20 fields on each microscope slide.

RESULTS

Cryptosporidiosis in mice

Cryptosporidium oocysts were first detected at 84 h. Patterns of oocysts production are presented in Fig. 1. The number of oocysts reached its first culmination point at 132 h; 20—30 % of the oocysts at this point stained moderately, were thin-walled and showed ruptures in the walls. At 144 h the oocyst number fell to on the level recorded at 108 h. At 180 h it reached a second culmination point, and almost no ruptured oocysts were detected. After that the production of oocysts gradually ceased; the level at 252 h was the same as at 84 h, and at 312 h no more oocysts were detected. No signs of clinical illness were detected during the study period either in the newborn inoculated and control or in adult mice were detected during the study period.

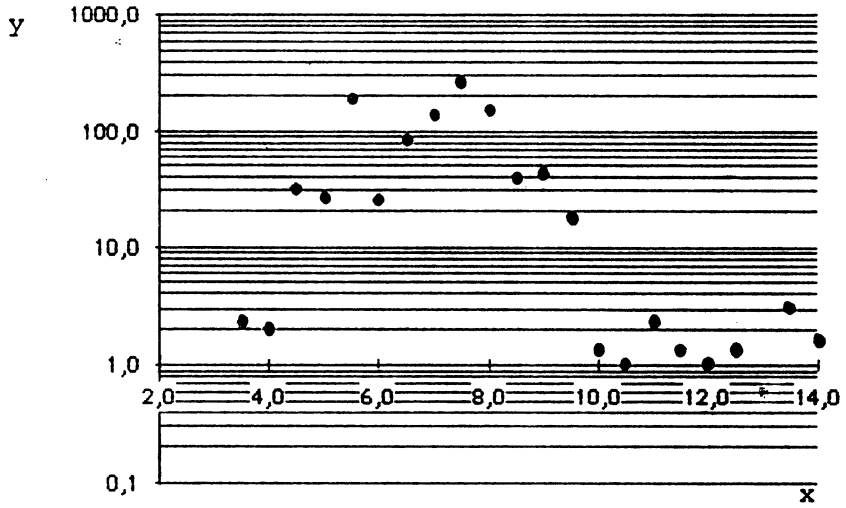


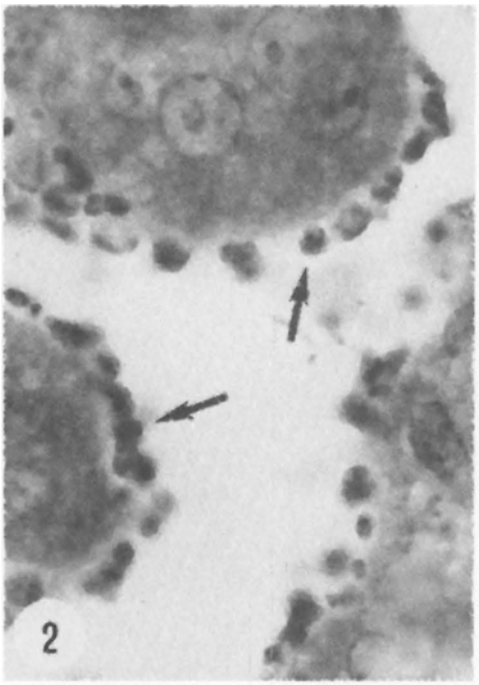
Figure 1. Patterns of oocyst shedding in experimentally infected mice.
(x: days after inoculation
y: means of oocyst numbers of 20 fields (× 1000).

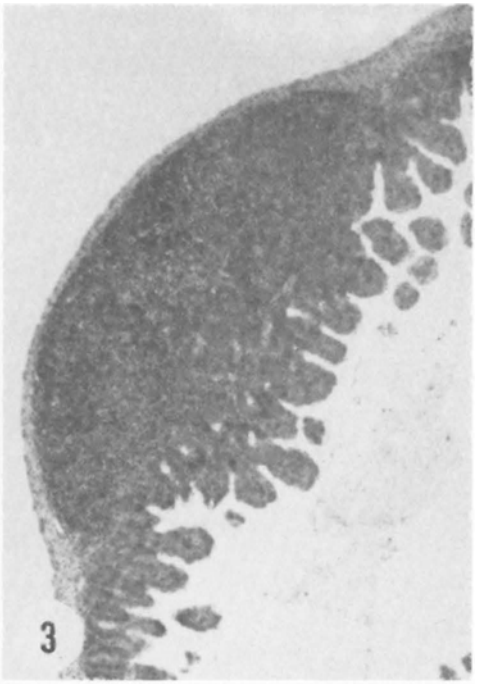
Oocysts from the experimentally infected mice were morphologically indistinguishable from those from the naturally infected calves: 3—6 µm, round, red bodies on a green background. There were some vacuoles and dots inside the oocysts. The thickness of the oocyst walls varied.

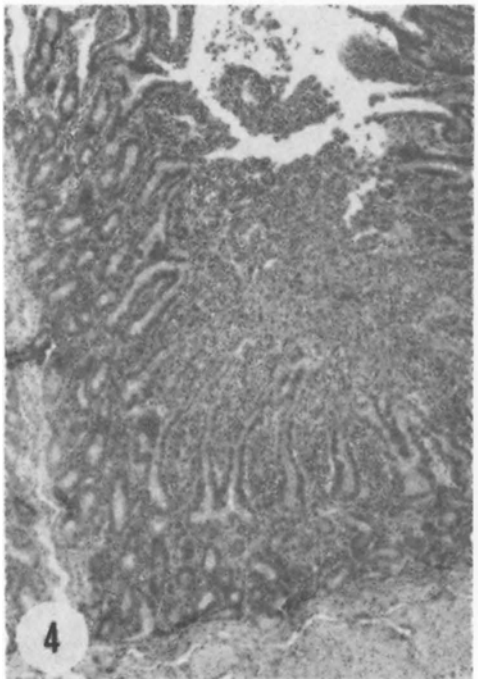
In Giemsa stained sections from inoculated mice cryptosporidia were seen along the striated border of epithelial cells in the terminal ileum (Fig. 2). Areas with numerous inflammatory cells were also seen in the epithelium and lamina propria of the villi (Fig. 3). There were no marked structural changes in the mucosa compared to that of the control mice.

Cryptosporidiosis in calves

After a mean incubation period of 3.6 days (range 3—4 d, S.D. 0.4) the inoculated calves developed signs of illness, characterized by depression, anorexia and profuse yellowish diarrhea, which lasted 6—9 days (mean 7.4 d, S.D. 1.5). The rectal temperatures of all the calves ranged within normal limits (37.9°C—39.1°C) throughout the observation period. Oocysts were first detected after a mean of 4 days (range 3—5 d, S.D. 0.8) and shedding continued for 3 to 8.5 days (mean 6.7 d, S.D. 2.2). Shedding of oocysts







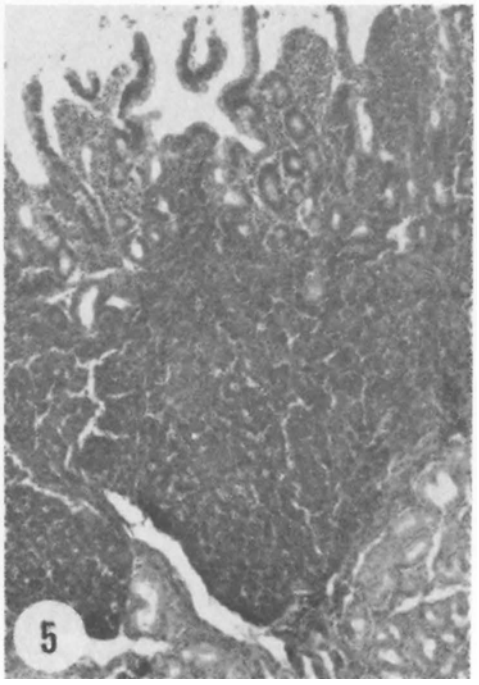


Figure 2. Cryptosporidia (arrows) along the striated border of epithelial cells in the terminal ileum of an experimentally infected mouse. x 1600.
Figure 3. An area with numerous inflammatory cells in the lamina propria in the terminal ileum of an experimentally infected mouse. x 70.
Figure 4. Shortened and fused villi in the jejunum of an infected calf. x 70
Figure 5. An area with inflammatory cells in the epithelium and lamina propria in the jejunum of an infected calf. x 70.

was intermittent in 4 calves. The mean number of days of subclinical shedding observed in all infected calves was 5 days (range 4—7 d, S.D. 1—2). The optimal day for the detection of oocysts was postinfection day 7, when all the infected calves were shedding oocysts. Neither signs of gastrointestinal illness nor shedding of oocysts were detected in the control calf.

In the field survey, 12 out of 36 (33 %) Cryptosporidium positive calves were shedding oocysts for the first time at 5 days of age and 10 (28 %) at 7 days of age. The shortest obvious prepatent period of 3 days was recorded in 4 (11 %) calves and shedding rarely began after 10 days of age (28 %). The patent period in most calves continued for about 1 week; the optimal day for diagnosis was postinfection day 10, when 21 out of 36 (58 %) calves were shedding oocysts. Manifest enteritis was associated with shedding of oocysts in 82 % (9/11) of the calves during their first week of life. In 6 of those calves Cryptosporidium was found alone and in 3 calves it was found rotavirus. The diarrheic feces were described as watery and yellowish, sometimes containing flakes of undigested milk. Subclinical shedding of oocysts was detected in 25 (69 %) calves.

In the present study, with more frequent sampling, oocysts were first recorded in 2 calves at 86 h postinfection, but most of them were not acid-fast. Fecal samples taken at 92 and 98 h postinfection revealed numerous acid-fast oocysts.

In infected calves, mucosal changes were detected mainly in the terminal jejunum and ileum. Almost no changes were seen in the caecum or colon. The villi of affected areas were shorter than in control animals, and often fused together (Fig. 4). The epithelium was low and irregular. Infiltration of inflammatory cells in the epithelium and lamina propria was frequently seen (Fig. 5).

Cryptosporidiosis in chicken

None of the chickens experienced any signs of clinical illness during the study, and Cryptosporidium oocysts could not be demonstrated from their gut contents.

DISCUSSION

In the present cross-transmission experiment, Cryptosporidium infection was transmitted by oral inoculation of

oocysts between calves and mice, but not between calves and chickens. Oocysts demonstratfd from naturally infected calves as compared to oocysts harvested from experimentally infected mice and calves were morphologically indistinguishable, they were equal in shape and size and had similar staining properties, thus strengthening the earlier observation of an organism lacking host-specificity (*Tzipori et al.* 1980, *Current et al.* 1983). However, the failure of the transmission attempt between calves and chickens demonstrated that the lack of host-specificity may occur within each vertebrate class, as suggested by *Levine* in 1984. There has been also suspicion of the occurrence of different strains of Cryptosporidium, e.g. bovine isolates from Scotland have resulted in enteric infections of chickens, whereas Australian isolates have not (*Tzipori et al. 1983).*

The passage of Cryptosporidium through experimental mice seemed not to alter their pathogenicity for calves, since the original calf isolate resulted only in subclinical infections in mice, whereas heavy shedding of oocysts and clinical symptoms was observed in experimental calves. Our results are in agreement with experimental work carried out with laboratory mice, in whom only subclinical and transient infections were successful (*Sherwood et al.* 1982). Contrary to ordinary mice, nude mice (nu/nu, BALB/c) affected with cryptosporidia have shown infection characterized by diarrhea and occasional deaths (*Heine et al.* 1984). The pathogenicity of Cryptosporidium for calves has been demonstrated in experimental monoinfection (*Heine et al.* 1984) as well as in natural infections attributed solely to the organism (*Tzipori et al.* 1980, *Anderson & Bulgin* 1981).

Features of the life cycle of Cryptosporidium of mice and calves resembled each other. The shortest prepatent period in both species took an average of 3 to 4 days, after a patency lasting on average between 7 to 10 days, the host was free of infection. Self-limiting infections have been recorded with similar parasitological parameters in both natural and experimental infections in these and other species of animals as well as humans (*Sherwood et al.* 1982, *Current et al.* -983, *Heine et al.* 1984).

Two types of oocysts, thin-walled and thick-walled, as seen in the present study, have also been recorded in vivo in mice as also in vitro using chicken embryos. Rupture of the thin-walled oocysts have been suggested as representing the pattern of endogenous autoinfection (*Current & Reese* 1982, *Current & Long*

1983), it also may explain the intermittent shedding of oocysts observed in experimentally infected calves as also in calves from the field.

Asymptomatic shedding of oocysts seemed to be frequent among calves from the field; diarrhea was most frequently associated with Cryptosporidium infection during the first week of life. The significance of asymptomatic shedding of Cryptosporidium oocysts with respect to the zoonotic transmission of infection is still obscure, as the number of oocysts excreted has not yet been evaluated in asymptomatic versus clinical infection and the infectious dose of oocysts for man and animals is not known. So far, diarrheic calves shedding oocysts have been reported to be a source of human cryptosporidiosis (*Anderson et al.* 1982, *Reese et al.* 1982, *Current et al.* 1983, *Rahaman et al.* 1984, *Pohjola et al.* in print).

The difference in the optimal day of diagnosis, postinfection day 10 in the field as compared with postinfection day 7 in experimental conditions, suggests smaller and later initial infectious challenge in the field, since in experimental work the prepatency has been delayed by applying contact transmission of infection instead of direct ionculation of oocysts (*Tzipori et al.* 1983). The symptoms of Cryptosporidium infection in calves resembled earlier descriptions (*Sanford et al.* 1982, *Tzipori et al.* 1983), and were not different from other enteric infections. It cannot be ignored that during the first week of life Cryptosporidium alone was a sufficient cause of diarrhea.

The degree of infection, mucosal injury and severity of disease have been reported to correlate with each other (*Tzipori* 1983) — a phenomen which was also seen in this study. Cryptosporidium in mice resulted only in transient subclinical infections with no marked histopathological changes, whereas in calves the infection may result in asymptomatic or symptomatic enteric infection with more prominent changes in intestinal architecture. In calves the predominant histopathological changes were found in the posterior half of the small intestine, in the caudal jejunum and the ileum, whereas no changes occurred in caecum and colon. The morphological changes, such as villus blunting and fusion, are nonspecific except that endogenous stages of the parasite were found in the tips of the villi, but have been thought to explain the functional lesions and to reduce the absorptive surface (*Pohlenz et al.* 1984). Low levels of

microvillar disacchridases (sucrase, lactase and/or maltase) have been reported in infected animals (*Tzipori et al.* 1982 a, *Tzipori* 1982 b), reflected as malabsorption and/or maldigestion and diarrhea (*Pohlenz et al.* 1984, *Heine et al.* 1984). Mucosal destruction by parasite metabolites or toxins has also been suggested (*Tzipori* 1983). The morphological changes observed in the present study might have been more prominent if the calves had been slaughtered while still shedding oocysts. Since there is so far no treatment for Cryptosporidium, every effort should be made to reduce the fecal contamination of premises where newborn and young calves are being reared.

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SAMMANDRAG

Experimentell cryptosporidios hos möss, kalvar och kycklingar.

Experimentella infektioner tillskrivna enbart Cryptosporidium genomfördes på nyfödda SPF möss, kalvar och kycklingar för att studera prepatens, patens och inkubationsperioder, beskriva kliniska symptom och finna och beskriva samband mellan förekomst av Cryptosporidium vid tarmväggens mukosa och patologiska förändringar i tarmen. Artikeln ger också de kliniska och parasitologiska parametrarna för Cryptosporidium infektion hos kalvar i en fältstudie, och jämför dem med de experimentella resultaten.

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