AN EXPERIMENTAL *CRYPTOSPORIDIUM* INFECTION IN MICE AND GOAT KIDS

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ABSTRACT

Cryptosporidium parvum, a zoonotic protozoan parasite of mammals, occurs throughout the world. Following infection, it multiplies within the microvilli of the enterocytes and produces pathological changes associated with clinical signs in susceptible hosts. Studies on experimental *Cryptosporidium* infections in various species reveal that the infectivity varies with the host species and the strain of the parasite. This paper describes the infectivity, pattern of oocyst shedding, and the morphological changes in the intestine following an experimental *Cryptosporidium* infection in goat kids.

Cryptosporidium oocysts, isolated from adult asymptomatic goats, were identified as *C. parvum* by Polymerase Chain Reaction. Two, 4-day-old goat kids were infected orally with *C. parvum* oocysts (10^5 oocysts in 10 ml phosphate buffered saline/kid) and an age-matched goat kid given an equal volume of phosphate buffered saline (PBS) by the same route served as a control. In addition, eight 1-week-old mice were infected orally (10^3 oocysts/mouse) for comparative purposes. *Cryptosporidium* oocysts were detected in the feces of one infected kid on 3 days post inoculation (dpi) whereas in the other 6 dpi. The faecal oocyst counts gradually increased and the peak counts in both kids were 2×10^6 g^{-1} (on 12 dpi) and 3.2×10^6 g^{-1} (on 14 dpi). The increase in faecal oocyst output coincided with diarrhoea in an infected kid from 10 to 17 dpi. Although, the oocyst excretion declined gradually after the peak, both infected kids excreted oocysts until euthanized on 20 and 22 dpi. Light and scanning electron microscopic investigations of the ileum revealed the developmental stages of the parasite within the brush border of the enterocytes, infiltration of neutrophils and mononuclear cells into the lamina propria, and atrophy, stunting and fusion of villi. All experimental mice excreted oocysts from 3 dpi, and 4 infected mice continued to excrete oocysts until 42 dpi. Thus, the experimental infection in goat kids resembled the natural disease in terms of oocyst excretion, clinical signs, and intestinal pathology. The ability of oocysts excreted by asymptomatic goats to infect goat kids and mice is likely to have a major impact on the epidemiology of cryptosporidiosis in livestock and man.

INTRODUCTION

Cryptosporidium parvum is a minute zoonotic protozoan, which infects the microvillous membrane of enterocytes in a wide variety of animals and humans. The disease is characterized by watery diarrhoea, dehydration and weight loss. Cryptosporidiosis is self-limiting in immunocompetent hosts but it takes a protracted course with severe clinical consequences in young mammals and immunocompromised hosts (O'Donoghue, 1995). Experimental cryptosporidiosis has been studied in many animal models to understand the

pathogenesis of the disease. Although, experimental studies in livestock revealed some similarities in the pathology and the clinical features of the natural diseases, distinct differences among the host species have also been described. For example, in lambs, there is an extensive involvement of the colon in addition to the small intestinal lesions (Mtambo *et al.*, 1996 and Tzipori *et al.*, 1981), while diarrhoea is not a consistent clinical sign in infected piglets (Sanford, 1987). In addition, infected murine hosts often fail to develop clinical signs (Reese *et al.*, 1982 and Mtambo *et al.*, 1996).

Despite increasing recognition of *Cryptosporidium* as a significant pathogen in goats, many features of the disease are poorly understood. Experimental infections in goats have been confined to studies using *Cryptosporidium* isolates derived from an AIDS patient (Current *et al.*, 1983) and bovines (Koudela and Jiri, 1997). Evidence from studies in other livestock species indicates that there is a marked variation in the infectivity, shedding pattern, and virulence of *Cryptosporidium* isolates obtained from different sources. The objectives of the present study were to investigate the infectivity, oocyst shedding pattern, clinical features, and morphological changes (in light and scanning electron microscopy) in the intestine of goat kids infected experimentally with *C. parvum* oocysts isolated from naturally infected asymptomatic adult goats. In addition, these oocysts were inoculated to mice for purposes of comparison.

MATERIALS AND METHODS

Purification of *Cryptosporidium* oocysts

Faecal samples were collected from the rectum of goats from the dry zone, and suspended in PBS. The suspension was sieved sequentially through filters with a final mesh size of 25 μ and the filtrate was concentrated in Sheather's sucrose solution. *Cryptosporidium* oocysts were purified on discontinuous sucrose gradients (Arrowood and Sterling, 1987) and stored at 4°C in PBS.

Speciation by PCR

Cryptosporidium oocysts were ruptured by 5 sequential cycles of rapid freezing in liquid nitrogen and rapid thawing in a 60°C water bath. The lysate was de-proteinated by Proteinase-K, and the DNA was extracted by phenol-chloroform method. The DNA was suspended in sterile-water and stored at -20°C until used. To speciate the above isolate, polymerase chain reaction (PCR) was performed using *C. parvum* specific primers 5'CTCTTAATCCAATCATTACAAC3' and 5'GGATCAAATGGAAGACCAACC3' as described by Carraway *et al.* (1997). Briefly, the DNA was amplified with *Taq* DNA polymerase and the PCR conditions were 35 cycles of 94°C for 50 seconds, 52°C for 1 min and 72°C for 50 seconds with a 5 min extension at 72°C. *C. parvum* genomic DNA (obtained from Dr. G. Widmer, Tufts Veterinary School, USA) served as a positive control in PCR. The amplicons were electrophoretically separated in 1.5 % agarose gels, stained with ethidium bromide, and visualized under ultraviolet light.

Preparation of the inoculum

The inoculum was prepared by diluting the oocysts in PBS containing penicillin (60 μg/ml) and streptomycin (100 μg/ml) (Miller *et al.*, 1990). The number of oocysts in the suspension was determined by counting the oocysts in smears prepared from 10 μl aliquots, following staining with the modified Ziehl Neelsen (MZN) method (Casemore, 1991).

Oocyst inocula contained no observable contaminants as determined by bright field and phase contrast microscopy.

Inoculation of mice and goat kids

Sixteen one-week-old mice were divided into two equal groups. The mice in Group 1 were given 10^3 *C. parvum* oocysts suspended in 2 ml of PBS, orally. The mice in the Group 2 were given equal volume of PBS via the same route. Faecal oocyst excretion was monitored daily for 42 days. Four mice were necropsied on day 8-post infection and the entire gastro-intestinal tracts were fixed in 10% buffered formalin for histological examinations.

Three, 4-day-old *Cryptosporidium* negative indigenous goat kids (Kid 1, 2 & 3) were used in the experiment. The kids were separated from their dams within 24 hours after birth and bottle-fed daily with 400-500 ml of heat-treated cow's milk. Clean water was provided *ad libitum*. Two kids (Kid 1 & 2) were each dosed orally with 10^5 oocysts of *C. parvum* in 10 ml of PBS. The control kid (Kid 3) was inoculated with an equal volume of PBS by the same route. The infected kids and the control kid were housed separately in a disinfected experimental animal unit and observed daily for clinical evidence of diarrhoea. Faecal samples were collected daily from the rectum and concentrated by sucrose floatation technique. Ten µl of concentrated faecal suspensions were uniformly spread onto glass slides, stained with MZN, and examined for *Cryptosporidium* oocysts. The number of oocysts per gram of faeces was counted as described previously (Noordeen *et al.*, 2000). The infected Kid 1 & Kid 2 were euthanized and necropsied on 20 and 22 dpi, respectively. Impression smears obtained from the mucosa of the abomasum, duodenum, jejunum, ileum, caecum and the colon were examined for *Cryptosporidium* by staining with MZN. Tissue sections from the aforementioned sites were fixed in 10% buffered formalin for light microscopy and in 2.5% glutaraldehyde solution for scanning electron microscopy.

RESULTS

Speciation of *Cryptosporidium*

Species of the *Cryptosporidium* isolates were determined by PCR. Fig. 1 shows the PCR amplicons on 1.5 % agarose gel. DNA from the *Cryptosporidium* isolates and positive control DNA yielded a 417 bp amplification product. No amplicons were detected from negative controls. This confirms the identity of the isolate as *Cryptosporidium parvum.*

Pattern of oocyst shedding in infected kids

Cryptosporidium oocysts were detected in the faeces of Kids 1 and 2, three days and six days post inoculation (dpi), respectively. The number of oocysts per gram of faeces ranged from 1.3×10^4 to 2.0×10^6, with the highest number on 12 dpi.

Kid 2 started to shed *Cryptosporidium* oocysts from 6 dpi. The oocyst count ranged from 2×10^4 to 3.2×10^6 oocysts g^{-1} faeces during the course of the infection. The oocyst excretion reached a peak on day 14 with a count of 3.2×10^6. The oocyst count gradually declined from 17 dpi and the faeces was positive for *Cryptosporidium* oocysts until 22 dpi, when it was necropsied following euthanasia.

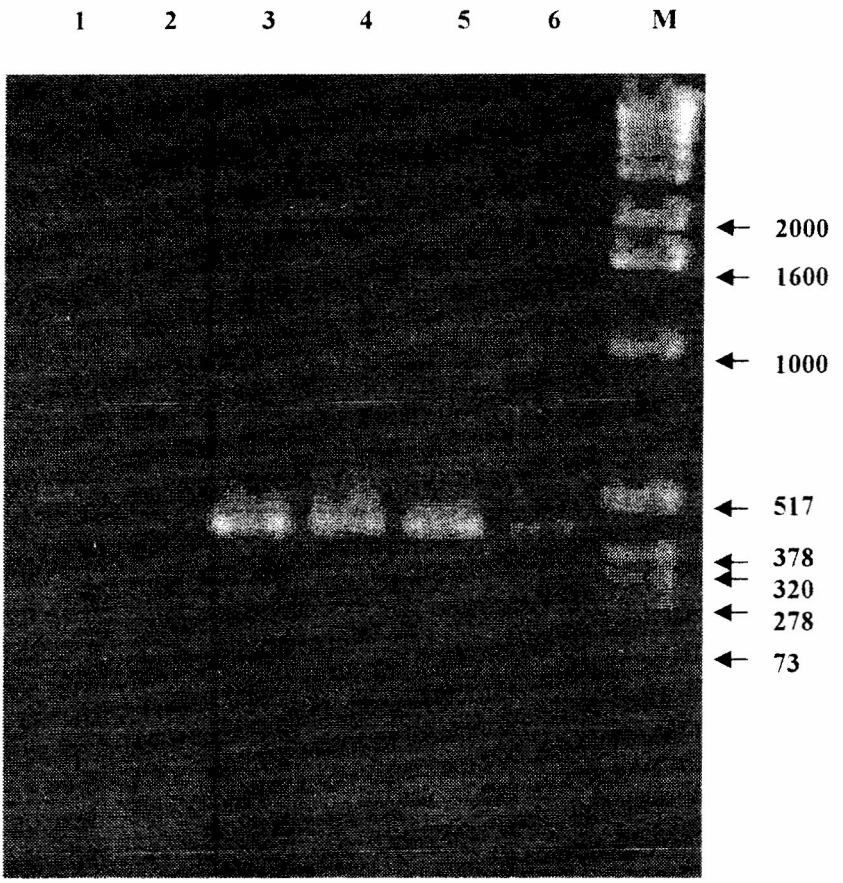

Fig 1. Ethidium bromide-stained 1.5 % agarose-gel showing PCR amplified products of *Cryptosporidium* isolates. Lane 1 = Reaction control (no DNA); lane 2 = Negative control (*Escherichia coli* DNA); lanes 3 and 4 = *Cryptosporidium* isolates; lane 5 and 6 = positive controls (*C. parvum* genomic DNA); and lane M = molecular marker. Molecular weights are indicated on the right in base pairs (bp).

Clinical signs and morphological changes

Kid 1 did not show any signs of diarrhoea but Kid 2 had severe watery diarrhoea between 10 and 17 dpi during which period the oocyst excretion was at its maximum. Among the impression smears taken from the gastrointestinal tract, *Cryptosporidium* oocysts were detected only in the smears obtained from ileum of both infected kids. Histological sections of the ileum of both infected kids revealed endogenous stages of the *C. parvum* on the brush border of the enterocytes, infiltration of neutrophils and mononuclear cells into the lamina propria. Further, the organisms were demonstrated on the villous surface by scanning electron microscopy. Light (Fig. 2b) and scanning electron microscopic (Fig. 3b) investigations of the ileum from infected kids also revealed morphological changes of the intestines, such as, atrophy, stunting, fusion and denudation of villi. Infected mice began to excrete *Cryptosporidium* oocysts from 3 dpi with some of the infected mice continuing to excrete oocysts until 42 dpi. Histologically, the ileum of infected mice contained the endogenous stages of *Cryptosporidium* on the brush border (data not shown). *Cryptosporidium* oocysts were not detected in the faeces of control goat kid and control mice, and no pathological changes were observed in the ileum (Fig. 2a).

Fig. 2a. Histological section of the ileum of the control kid (kid 3) *x* 400

Fig. 2b. Histological section of the ileum of the infected kid (kid 1) depicting stunting and fusion of villi *x* 400

Fig. 3a Scanning electron micrograph of the ileum of control kid (kid 3) *x* 120

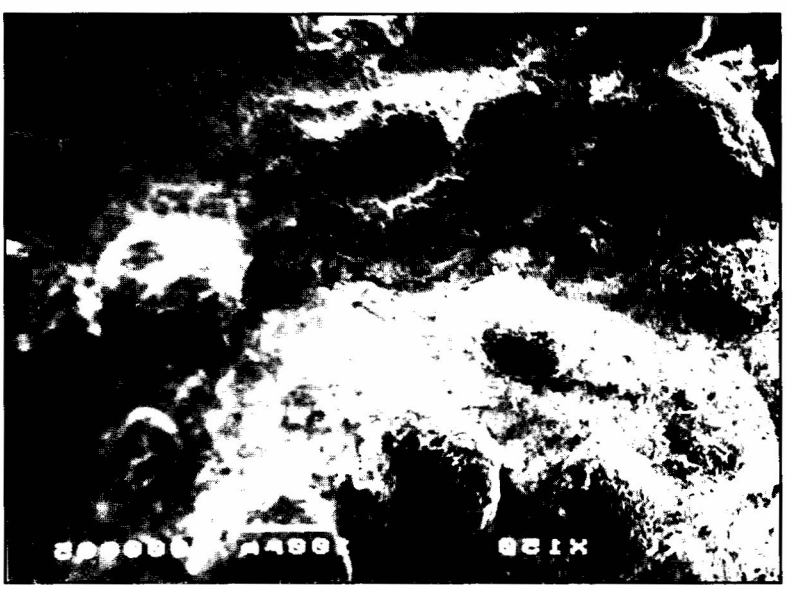

Fig. 3b. Scanning electrom micrograph showing the villous alterations in the ileum of an infected goat kid (kid 2) *x* 120

DISCUSSION

Experimental cryptosporidiosis in goats has been documented with isolates of the organism recovered from clinically affected calves and immunocompromised humans (Current *et al.*, 1983; Koudela and Jiri, 1997). The present study appears to be the first one that explains the development of the disease in goats using caprine isolates of *C. parvum*.

As in other ruminants, natural *Cryptosporidium* infection in goats primarily affects the neonates of this species and causes intestinal pathology that results in self-limiting watery diarrhoea with high mortality (Graff *et al.*, 1999). In the adults, the infection is sub-clinical or chronic and is manifested by a progressive weight loss; a large number of infected adults are carriers (Smith and Sherman, 1994). The lack of clinical manifestations in adult infected animals may be due to age-related immunity or the variation in the infectivity and virulence of different isolates of *C. parvum*. Indeed, studies in cattle have demonstrated a variation in the virulence of *Cryptosporidium* oocyst where an isolate from a diarrhoeic calf failed to cause the disease and mortality when administered to experimental calves (Fayer *et al.*, 1985). In the present study, the oocysts used in the experimental infection were isolated from asymptomatic, adult goats. The fact that these oocysts were able to infect both goat kids and mice, and produce the clinical signs of natural cryptosporidiosis in one kid, indicates that the asymptomatic carriers of the infection do have a major role in the epidemiology of the disease.

The incubation period of cryptosporidiosis in goats is short and has been noted to vary from 4 to 7 days (Graff *et al.*, 1999). In the experimental infection described in this study, oocyst excretion was found to commence between 3 and 6 days after infection and reach a maximum to coincide with the onset of diarrhoea in Kid 2, thereby reflecting the natural disease to a great extent (Thamsborg *et al.*, 1990; Vieira *et al.*, 1997).

In the present study, there were very minimal gross changes in the gut of the infected goats and this observation is in close agreement with other reports of experimental and natural cryptosporidiosis in goats as well as in other ruminants (Tzipori, 1983). The location of lesions in the intestines was guided by the impression smears, which demonstrated the presence of oocyst only in the ileum in both kids. Experimental studies in goats reported by Koudela and Jiri (1997) however have demonstrated lesions in the posterior jejunum in addition to the ileum, while in the natural disease, the entire intestine and the initial part of the large intestine were also found to be involved (Vieira *et al.*, 1997). The variation in the location of lesion in the natural and experimental disease is difficult to explain as there is a dearth of information on the virulence of different isolates of *Cryptosporidium* and the role of the caprine host in the pathogenesis and the clinical course of the disease.

Histological changes observed in the ileum in the present study included villous alterations characterized by atrophy, blunting, and fusion as well as infiltration of neutrophils and mononuclear cells into the lamina propria. These morphological changes bear remarkable similarities to those reported in experimental *Cryptosporidium* infections in neonates of other ruminant species (Moon and Bemrick, 1981; Sanford and Josephsen, 1982; Pearson *et al.*, 1982). Scanning electron microscopy further illustrated histological changes associated with atrophy, blunting and fusion of villi (Fig. 2 and 3).

All experimentally infected mice were found to shed *Cryptosporidium* oocysts in their faeces from 3 dpi indicating the establishment of the infection. The relatively long excretion period of oocysts in mice, as observed in this study, may have important implications in the spread of the infection to livestock, and indeed to man through contaminated water and food.

In conclusion, the experimental infection in goat kids mimics the natural disease in terms of oocyst excretion, clinical signs, and intestinal pathology. Diarrhoea was the salient clinical feature and it coincided with the highest oocyst excretion. The infection was localized in the ileum where light and scanning electron microscopy revealed morphological changes in the villi. The ability of oocyst from asymptomatic goats to produce infection in young goat kids and mice is likely to have major implications on the epidemiology of the disease in susceptible livestock and man.

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