MORPHOLOGICAL, HISTOPATHOLOGICAL AND IMMUNOFLUORESCENCE CHARACTERIZATION OF CRYPTOSPORIDIUM PARVUM NATURAL INFECTION IN GOAT KIDS FROM ROMANIA

BEJAN A, BOLFA P, MAGDAS C, POP R, MIRCEAN VIOРИCA, CĂTOI C, TAULESCU M and COZMA V

University of Agricultural Sciences and Veterinary Medicine, Faculty of Veterinary Medicine, Cluj-Napoca, Romania

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Few studies were conducted to investigate the pathogenesis and to characterize the histopathological alterations in the intestinal mucosa of kids with cryptosporidiosis. In our study, 58 kids were presented for necropsy, and the faeces of another 83 goat kids were studied. Animals were aged between 1 day and 3 weeks. Infection was confirmed by oocyst identification in the faeces (Ziehl-Neelsen stain modified by Henriksen). Gross examination was followed by examination of the tissue using histopathological procedures (Hematoxylin-Eosin stain), scanning electron microscopy (SEM) and immunofluorescence. Pathological changes occurred mainly in the ileum, but also jejunum and caecum of the infected animals, at the apical surface of intestinal epithelial cells. Oocysts appeared as round or oval formations, with a diameter ≤4.5 μm, sometimes covering the atrophic and denuded free border of intestinal villi. Most histological and morphological characteristics of infection with C. parvum are common on the whole to other Cryptosporidium spp. Massive dentification of cryptosporidiosis in kids indicates the possible role of these animals as disease reservoirs for other animals and for humans. This is the first study to characterize the Cryptosporidium infection by confocal technique and SEM in Romania.

Key words: Cryptosporidium, immunofluorescence, kids, oocysts, SEM

INTRODUCTION

Cryptosporidium spp. are coccidia with an increased capacity to replicate and disseminate. Some Cryptosporidium species infect farm animals and the economic impact of cryptosporidiosis is dependent on the affected species.

Cryptosporidiosis is a disease diagnosed in humans and animals, with a favourable evolution in immunocompetent individual organisms and severe evolution in immunodeficient organisms. It is characterized clinically mainly by gastrointestinal disorders and less often by respiratory, hepatic and pancreas
disorders. Cryptosporidiosis occurs in all farming systems and produces large economic losses, especially when the etiologic agent, is involved in triggering neonatal diarrhea.

Caprine cryptosporidiosis was first reported in 1981, in a two weeks old kid with diarrhea from Australia (Mason et al., 1981). In goats, infection with Cryptosporidium spp. is considered extremely important economically, because of the animal losses it produces, but also in terms of human health, due to its zoonotic potential. In kids, natural infection with C. parvum led to a difference of minus 2 kg body weight in four weeks compared to uninfected kids of the same age (de Graaf et al., 1999).

Economic losses associated with cryptosporidiosis in small ruminants are reflected not only in mortality, but also in growth delay, and cost of medicines and veterinary assistance. In the absence of other enteropathogen agents, mortality is higher in kids than in calves, and may reach 100% (de Graaf et al., 1999). Anorexia is very prominent from the beginning both in kids and in lambs.

Molecular data on caprine cryptosporidiosis are very limited. Cryptosporidium parvum, Cryptosporidium hominis and goat genotype, represent the only species and genotypes identified so far in goats. Since human infections are mostly caused by Cryptosporidium parvum and Cryptosporidium hominis, both species reported in goats, caprine cryptosporidiosis should be considered as a potential zoonosis.

MATERIAL AND METHODS

Biological material

In February 2009, we identified an outbreak of cryptosporidiosis, with watery diarrhoea in goat kids aged between 1 day and 3 weeks, and in a goat farm from Cluj County, Romania. A total of 141 animals were included in the present study. The faeces of 58 dead kids and of another 83 living kids were examined for the identification of etiological agents by coproparasitological techniques.

Methods

For staining of oocysts Ziehl-Neelsen modified by Henriksen stain was used (Henricksen and Pohlenz, 1981).

Necropsy examination of the 58 kids was followed by sampling intestinal segments, mainly from the distal ileum, jejunum and proximal colon.

For histopathological examination, samples from different portions of the jejunum and ileum were fixed in 10% formalin for 24 hours, embedded in paraffin. Sections were cut at 6 micrometers with a microtome (Leica RM 2125 RT model) deparaffinised, hydrated, dehydrated and stained by Hematoxilin-Eosin method. Images were acquired and examined using an Olympus image acquisition and processing system (Olympus BX51 microscope and Olympus cell B software).

Samples of the intestine from three goat kids were prepared for scanning electron microscopy (SEM). The specimens were prefixed in 2.7% glutaraldehyde in phosphate buffer 0.1M, pH 7.4, for 90 minutes at 4°C, thereon washed in three consecutive phosphate buffer baths 60 minutes each, followed by a fourth bath,
overnight at 4°C. Postfixation was with osmic acid (OsO₄) in phosphate buffer 0.15M, pH 7.4 for 90 minutes at 4°C. Samples were then dehydrated at critical point, in acetone which was then replaced with liquid CO₂ under pressure. Samples were covered with a gold pellicle under vacuum condition, introduced into the microscope and examined.

The protocol followed for immunofluorescence staining started from paraffin embedded sections. For deparaffinization/rehydration sections were incubated in three washes of xylene for 5 minutes each, followed by two washes of 10 minutes each in 100% ethanol, two washes of 10 minutes each in 95% ethanol then rinsed twice in dH₂O for five minutes each. For antigen unmasking, slides were placed in 10 mM sodium citrate buffer (pH 6.0), then boiled for 10 minutes, cooled and rinsed in dH₂O three times for 5 minutes each, then finally rinsed for 5 minutes in PBS. The specimens were blocked with blocking buffer for 60 minutes, incubated with primary monoclonal antibodies for Cryptosporidium parvum oocysts 1:1000 (VMRD cat. No. 16.87.16) overnight at 4°C. The next day, specimens were rinsed three times in PBS for 5 minutes each, then incubated in 1:1000 diluted secondary antibody goat polyclonal to mouse IgG - (FITC labelled) for 2 hours at room temperature, in dark. Slides were then rinsed in PBS, and nuclei were counterstained with DRAQ5 (Cell Signaling, Cat No. 4084) for 10 minutes, and rinsed again twice in PBS. Slides were mounted and examined using a confocal microscope (Zeiss LSM 710) equipped with Zen software for image processing.

RESULTS

Coproelimination of Cryptosporidium spp. oocysts were detected in 115 from a total of 141 goat kids on the farm (81.56%). All kids positive for Cryptosporidium infection, eliminated through their faeces more than 100 oocysts/100 microscopical fields (Fig. 1). In one dead kid, examination of faeces revealed 3400 oocysts/100 fields, meaning that infection was massive.
Coproelimination of Cryptosporidium oocysts in kids from the investigated farm is detailed in Table 1.

Table 1. Prevalence of cryptosporidiosis among goat kids aged up to 3 weeks

<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>%</th>
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<tbody>
<tr>
<td>Positive</td>
<td>115</td>
<td>81.6</td>
</tr>
<tr>
<td>Negative</td>
<td>26</td>
<td>18.4</td>
</tr>
<tr>
<td>Total</td>
<td>141</td>
<td>100</td>
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Figure 2. Section in the distal jejunum – the presence of the parasite at the apical poll of the enterocytes; superficial chorionic congestion; abundant inflammatory infiltrate with plasma cells, lymphocytes, macrophages and sparse neutrophils.

Figure 3. Section from proximal caecum portion; intestinal mucosa membrane with various parasitic stages on the surface; areas of enterocytes undergoing necrosis and degeneration (arrow).

Figure 4. Section in the distal ileum – epithelial displasia, vesicle enterocyte, microvilli disappearance in the area where the oocysts are present on the mucous surface. Numerous plasma cells infiltrating the lamina propria.

Figure 5. Section in the distal ileum – disappearance of microvilli in the area where Cryptosporidium oocysts are located on the mucous membrane surface; inflammatory infiltrate with plasma cells and neutrophils in lamina propria.
A complete coproparasitological, bacteriological and virological exam was performed from the faeces of all subjects. Other pathogens that were detected, were not at a significant level in order to determine a clinical infection (unpublished results).

The 83 kids that survived revealed clinical changes in 63 animals (75.9%) with watery diarrhoea in 57 kids (68.68%), kyphosis in 8 goatlings (9.64%) and dehydration in 37 kids (44.58%). Mortality rate in the investigated farm was 41.13%.

Minor changes were detected in the investigated organs or tissues, mainly in the gastrointestinal tract. These changes were represented by a congestion of the terminal segment of the small intestine and the beginning of the large intestine, which was distended, with a yellowish and watery content (from the distal ileum to the proximal colon).

The lesions we found during histopathological examination of samples from the ileum and colon which included alterations of the microvillus border of the intestinal epithelium characterized by atrophy, denudation and fusion along with

![Figure 6](image1.png)  
**Figure 6.** SEM of ileal mucosa of a goat kid naturally infected with Cryptosporidium parvum showing extensive denudation of villous surface.

![Figure 7](image2.png)  
**Figure 7.** Atrophy, stunting and fusion of villi in the distal jejunal segment.

![Figure 8](image3.png)  
**Figure 8.** Large numbers of C. parvum oocysts on the epithelial cells of the ileum.

![Figure 9](image4.png)  
**Figure 9.** Shape, structure and the attachment organelle of C. parvum oocysts at the ileal mucosa surface.
crypt hyperplasia. Thus, the normal citoarchitecture of enterocytes was conserved on most of the affected mucosa. *Cryptosporidium spp.* infection, represented by different parasitic stages, was limited to the apical pole of the enterocytes, with intracellular extracytoplasmatic localization (Figure 2 and Figure 3). Some infected enterocytes presented vacuolar dystrophy, while in some areas the cells were flattened or cuboidal (Figure 4). These areas alternate with areas of normal enterocytes, as reported by Vitovec and Koudela (1992). Congestion of the affected segments accompanied by a moderate degree of infiltration with lymphocytes, plasma cells, only few neutrophils and eosinophils was found in the lamina propria (Figure 5). On some areas, the enterocytes were undergoing necrosis and degeneration (Figure 3).

Large scale enlargement of ileal epithelium from kids with cryptosporidiosis revealed ultrastructural changes similar to those previously described in goat kids from Tanzania (Matovel et al., 1984), and identical with those described in calves (Heine and Boch, 1981), piglets (Vitovec and Koudela, 1992) and in newly born mice (Vitovec and Koudela, 1988). The intestinal segment (where the parasites were localised) showed extensive denudation of the villous surface, atrophy, stunting and fusion of villi in the jejunal segment (Figure 6 and Figure 7). Under SEM, numerous round-to-spherical organisms consistent with *Cryptosporidium parvum* were observed on the surface of the intestinal mucosal epithelium, mainly

![Image](image-url)

**Figure 10.** Confocal image from the ileum of infected kids: *C. parvum* oocysts are present at the free border of enterocytes (arrows).

**Figure 11.** Presence of *C. parvum* positive parasitic stages in the intestinal lumen of the distal jejunum.

**Figure 12.** Ileum goat kids: presence of *C. parvum* oocysts at the surface of the intestinal mucosa.
with ileal localization (Figure 8 and Figure 9). Occasionally, hatching organisms of *C. parvum* and the remaining shells were observed. In some images, we observed the craters that remained after parasites have ruptured host cells at the attachment site (Figure 8).

Similar to the results obtained following SEM examination, immunofluorescence staining of *C. parvum* oocysts, revealed the presence of the parasitic stages mainly in the ileal intestinal segment (Figure 10 and Figure 12), but also in the distal jejunum (Figure 11) and the proximal colon. The parasite was localized at the apical surface of the infected enterocytes. The typical spherical shape of oocysts, with characteristic green fluorescence delineating the oocyst wall using a fluorescein isothiocyanate-labeled monoclonal antibody was observed at the confocal microscope examination (Figure 13).

**DISCUSSION**

Cryptosporidiosis is frequently diagnosed in newly born farm animals, with morbidity up to 100% until the end of the birth season. The disease has been detected in all ages of goats, but prevalence is higher in kids (Mišić *et al.*, 2006). The prevalence of infection in the kids from the investigated farm was of 81.6%. This percentage, confirms the fact that in kids, the highest oocyst counts are detected in the first months of life, as they acquire the infection as neonates (Noorden *et al.*, 2001; Mišić *et al.*, 2006).

Goat kids, similar to other ruminants such as bovine and ovine, are highly sensitive to *Cryptosporidium spp.* infection, but their resistance increases with aging (Molina *et al.*, 1994). As most human infections are caused by *C. hominis* and *C. parvum*, both reported in goats, caprine cryptosporidiosis should be considered to be a potential zoonosis (Fayer and Xiao, 2008).

The pathological changes at the intestinal level are translated in transient watery diarrhoea and increased mortality (Graff *et al.*, 1999). In adult animals the infection evolves chronically, characterized by weight loss, or in most of the cases the animals are asymptomatic carriers (Smith and Sherman, 1994). Lack of clinical signs in adult animals could be explained by acquired immunity or
variance of the infecting capacity and of the parasite virulence, as demonstrated by bovine studies (Fayer et al., 1985). The fact that oocysts infected goat kids, producing clinical signs of disease, indicates that asymptomatic carriers play a key-role in the pathogenesis and epidemiology. Experimental studies in caprines reported by Koudela and Vitovec (1997), revealed the presence of lesions both in the distal jejenum and in the ileum.

The severity of the inflammatory cell reaction in the lamina propria, as observed by histopathological examination, is more intense in infections caused by Cryptosporidium parvum compared to other Cryptosporidium spp. (Deng et al., 2004b; Enemark et al., 2002; Fayer et al., 1997; Sacco et al., 1998; Wyatt et al., 1997). The degree of injury and the parasitic burden, in our study, varied from area to area, and was localized in the distal jejunum, ileum and the proximal colon. Inflammatory infiltrate in the lamina propria of infected goatlings, ranged from minimal in some areas to substantial in other zones, including abundant plasma cells, lymphocytes and macrophages, and also some polymorphonuclear leukocytes. Areas of denudation were also observed, with a lack of microvilli. Molecular details as to how Cryptosporidium mediates intestinal epithelial cell apoptosis and secretion of proinflammatory cytokines are just beginning to be clarified and suggest that infection with C. parvum alters the host biochemical pathways by affecting gene expression (Deng et al., 2004a).

On some intestinal segments, vesiculated enterocytes appeared (Figure 4), containing different parasitic stages. In some severe forms of evolution, the tall columnar intestinal epithelium was replaced by distorted, disorganized cells undergoing necrosis and degeneration (Figure 3). The relatively mild host reaction in some cases, might relate to the long term colonic infection, as other authors suggested (Masuno et al., 2006).

Morphological changes described in this study, are similar to those reported in experimental infections with Cryptosporidium spp. in newly-born from other ruminant species (Moon and Bemrick, 1981; Sanford and Josephsen, 1982; Pearson et al., 1982), and with intestinal lesions of other newborn animals infected with Cryptosporidium parvum (Heine and Boch, 1981; Vitovec and Koudela, 1988; Vitovec and Koudela, 1992). The major difference between natural and experimental infection with C. parvum in mammals consists in the intestinal localization. In our study, different parasitic stages were detected in the small intestine and in the colon. No signs of cryptosporidiosis, or associated lesions were found in other tissues. The same localization was described by other authors (Heine and Boch, 1981; Vitovec and Koudela, 1992), but Noordeen et al. (2002), described the parasite to be localized only at the level of the ileum.

The lack of microvilli, at the surface of enterocytes, most probably impairs digestion and absorption, resulting in or contributing to diarrhea. Previous reports regarding the pathogenesis of natural or experimental intestinal cryptosporidiosis in young animals were incomplete, due to the lack of pathogenesis studies and detailed characterization of the disease in this age category (Mancassola et al., 1995). The model of clinical infections with Cryptosporidium parvum described in the newborn of most ruminant species represents currently the only available source for the production of a large number
of oocysts (O’Donoghue, 1995). Newborn animals present the advantage of being less expensive and could serve as a model for pathogenesis studies, to determine the resistance mechanisms and to evaluate the therapeutic and prophylactic potential (Mancassola et al., 1995).

The intensity of the fluorescence observed under confocal examination of C. parvum oocysts varies. The parasite was not always uniformly coloured. Some oocysts were observed containing the characteristic crescentic form of sporozoites. The shape and size of the oocysts, corresponded to C. parvum, with a diameter of ≤4.5 μm.

CONCLUSION

Most histological and ultrastructural features of C. parvum infection and its mechanisms of attachment to intestinal epithelial cells are similar on the whole to those in other Cryptosporidium spp. Infection was generally confined to the laminal border of the enterocytes, where the parasite was attached to the cell surface. The degree of injury and the parasitic burden varied from area to area, and was localized in the distal jejunum, ileum and the proximal colon. Our findings demonstrate the presence of Cryptosporidium infection in goat kids from Cluj County, Romania and indicate the possible role of these animals as disease reservoirs. This is the first report in which the confocal technique and SEM were used to characterize the Cryptosporidium infection from Romania.

__Adress for correspondence:__
Dr. Bolfa Pompei Florin, DVM, PhD
Pathology Department
Faculty of Veterinary Medicine, USAMV
Calea Manastur 3-5
Cluj-Napoca, 400372, Romania
E-mail: pompeibolfa@gmail.com

REFERENCES


MORFOLOŠKA, HISTOPATOLOŠKA I IMUNOFLOURESCENTNA KARAKTERIZACIJA CRYPTOPODIDIUM PARVUM KOD PRIRODNO INFICIRANE JAGNJADI U RUMUNIJI

BEJAN A, BOLFA P, MAGDAS C, POP R, MIRCEAN VIORICA, CĂTOI C, TAULESCU M i COZMA V

SADRŽAJ

Do sada je sprovedeno samo nekoliko studija radi istraživanja patogeneze i karakterizacije histopatoloških promena na intestinalnoj mukozi mladunčadi sa kriptosporidiozom. U našem ispitivanju je izvršena obdukcija 58 jарица, a ispitan je feces kod ukupno 83 jedinke. Ispitivane životinje su bile u uzrastu od jednog dana do tri nedelje. Infekcija je potvrđena prisustvom oocista u stolici (Ziehl-Neelsen bojenje modifikovano po Henriksenu). Nakon obdukcije, isečci tkiva su ispitivani svetlosnom mikroskopijom (bojenje hematoksilin-eozin, skening elektronskom mikroskopijom (SEM) i imunofluorescentnom metodom. Patološke promene su uočene uglavnom na ileumu, ali i na jejunumu i spleom crevu inficiranih životinja, na apikalnoj površini intestinalnih epitelnih celija. Oociste su bile ovalnog ili okruglog oblika, prečnika ≤4,5 µm i ponekad su prekrivale atrofičnu i ogoljenu granicu crevnih resica. Većina histoloških i patomorfoloških promena, nastalih infekcijom sa C. parvum, je bila ista kao i kod infekcija sa ostalim parazitima Crypto- sporidium spp. Masovno prisustvo kriptospridija kod jaradi ukazuje da su ove životinje mogući rezervoar zaraze za ostale životinje i ljude.