TRADITION AND MODERNITY IN VETERINARY MEDICINE, 2025, vol. 10, No 1(18): 70–77

PATHOMORPHOLOGICAL STUDIES IN CLINICAL CASES OF CRYPTOSPORIDIUM AND EIMERIA ENTERITIS IN LAMBS

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(Submitted: 10 January 2025; Accepted: 19 May 2025; Published: 25 June 2025)

ABSTRACT

Gastroenteritis in newborn and adolescent lambs and goat kids is a complex multifactorial disease. Its manifestation is related to the immune status of the animals on the farm, the feeding conditions, as well as the presence of infectious agents (bacterial, viral and parasitic) in the environment. The main goal of the conducted field and laboratory studies was to identify the main protozoan agents causing neonatal diarrhea in newborn and adolescent lambs in sheep farms on the territory of Bulgaria. As well as analyzing the pathoanatomical and histopathological changes in the different segments of the intestinal tract. The study included 1100 newborn and adolescent lambs from 7 private dairy sheep farms aged from 24 h postnatal to day 30. For rapid antigen diagnostics were used *Rainbow calf scour 5 BIO K 306 Detection of Rota, Corona, E. coli F5, Crypto and Clostridium perf. in bovine stool* (*BIOX Diagnostics, Belgium*), as well as coprological studies. Pathoanatomical and histopathological examinations were performed on tissue samples obtained from 56 lamb carcasses that died after developing gastrointestinal infections. Based on the obtained results, the main enteropathogens causing enteritis in lambs were defined as: *Cryptosporidium parvum*, *Eimeria spp*., rotaviruses, coronaviruses and *Escherichia coli*.

Key words: cryptosporidiosis, eimeriosis, lambs, pathomorphology, enteritis.

Introduction

Neonatal diarrhea observed in goat kids and lambs is one of the most common diseases causing high morbidity and mortality in this category of animals. The values of these indicators (morbidity and mortality) depend on the conditions of cultivation and management of the farm, varying from 25% to 50%. Delayed growth and development of newborn goat kids and lambs suffering from gastroenteritis increase treatment costs, and high morbidity and mortality rates lead to large economic losses for farmers (Kalkanov *et al.,* 2021).

Authors such as (Sambeth *et al.,* 1993) define enteritis in lambs and goat kids as a complex multifactorial disease. Its appearance is related to the immune status of farm animals, feeding conditions, as well as the presence of infectious agents (bacterial, viral and parasitic) in the environment. Gastrointestinal infections caused by apicomplexan coccidia belonging to the genera *Eimeria* and *Cryptosporidium* are common in young lambs and goat kids. Coccidiosis and cryptosporidiosis are the most common protozoan diseases in small ruminants during the first weeks after birth (Fayer & Ungar, 1986). And according to Norton *et al.,* (1986) have been implicated as the main etiological agent causing neonatal diarrhea in lambs and goat kids.

According to (Nalan *et al.,* 2009), the most common protozoan intestinal infections in lambs in the first month after birth are: Coccidiosis, Giardiasis and Cryptosporidiosis. Highly polluted environment and overpopulation of animals, mixing of animals of different ages, as well as high humidity predispose to the occurrence of these diseases (Taylor, 2017).

In Turkey, screening studies were carried out on a sheep farm and it was found that more than one protozoan agent was present in 98 out of a total of 132 lambs with gastroenteritis examined. The obtained data indicate that in 74.24% of the cases a mixed invasion between *Eimeria spp*. and *Cryptosporidium spp.* was found. (Nalan *et al.,* 2009).

Epidemiological results obtained from outbreaks of diarrhea in ruminants on randomly selected farms around the world support the proposition that protozoan infections were widespread in lambs with diarrhea. Which necessitates consideration of *Eimeria, Giardia*, and *Cryptosporidium spp*. as the main protozoan agents causing intestinal problems in small ruminants (Causapé *et al.*, 2002; De Graaf *et al.*, 1999).

Eimeria infection in newborn lambs and goat kids occurs after ingestion of oocysts that are released by adult animals. The sporozoites then penetrate the epithelial cells lining the villi of the small intestine. After their penetration, several stages of merogony follow, the first of which produces giant meronts that release large numbers of merozoites that re-penetrate the mucosal epithelial cells and produce a second generation of small meronts (Taylor, 2002). *Cryptosporidium spp*. are small intracellular parasites found in many species of mammals, birds, reptiles, and fish (Fayer & Ungar 1986). *C. parvum* infects a wide range of mammals and is highly prevalent in ruminants, particularly calves, lambs and goat kids. The life cycle and infection of *C. parvum* are similar to those of coccidia, but unlike *Eimeria spp*., their oocysts are sporulated (Taylor *et al.*, 2006).

The clinical signs observed in eimeriosis and cryptosporidiosis were: apathy and depression, anorexia accompanied by abdominal pain, abdominal discomfort, dehydration and diarrhea. The color of the feces in affected animals usually varies from white to yellowish with blood and the consistency can be from soft pasty to watery with an unpleasant odor. Diarrhea usually lasts 3–5 days, while in severe cases, it can last up to 15 days (Tzipori *et al.*, 1981; Johnson *et al.*, 1999).

Macrolesions on the mucosa of the small intestine resembling punctate white formations have been described in eimeria, representing the giant meronts themselves. The macroscopic changes described in *Eimeria spp.*, and *Cryptosporidium spp*. were expressed on the mucosa of the small and large intestines, and numerous developmental forms of gamonts and oocysts were observed histologically (Ruiz *et al.*, 2012).

The main objectives of the present study are to identified the main protozoan agents involved in the neonatal diarrhea complex in newborn and growing lambs raised on sheep farms in Bulgaria. Also to establish the characteristic macroscopic and microscopic changes in the digestive system caused by them with the aim of their application in the pathomorphological diagnosis of gastrointestinal diseases in lambs.

Materials and methods

A total of 1100 newborn and growing lambs from 7 private dairy sheep farms from Southern Bulgaria were covered in the study. The animals were of the Asaf, Lakon, Marishka vakla and Pleven black-headed breeds. The lambs were aged from the 24th hour after birth to the 30th day, unvaccinated and treated against infectious and protozoal diseases. Gastrointestinal diseases were observed in newborn and adolescent lambs in all farms. In all 7 farms, clinical and epidemiological studies of newborn and adolescent lambs were carried out.

Rapid 5 valent antigen tests were used, *Rainbow calf scour 5 BIO K 306 Detection of Rota, Corona, E. coli F5, Cryptosporidium parvum and Clostridium perfingens type A. in stool (BIOX Diagnostics, Belgium*), detecting the presence of antigens of the indicated pathogens in fecal content.

Coprological samples were obtained manually from the ampula recti of each animal with proven coproantigens for *Cryptosporidium parvum* by the rapid antigen tests and stored at 4 ºC until analyzed by parasitological examinations by the methods of Henriksen (Zill-Nielsen) and Fühleborn.

According to the standard autopsy protocol, 56 carcasses of lambs that died after suffering from gastrointestinal diseases were autopsied. Tissue samples for histopathological examination measuring 1 cm x 1 cm were obtained from the affected sections of the proximal and distal parts of the gastrointestinal tract: abomasum, duodenum, jejunum with mesenteric lymph nodes, ileum, cecum, colon and rectum – 2.5 cm long. Materials for histopathological examination were fixed in 10% neutral buffered formalin for 48–72 hours and embedded in paraffin. Sections with a thickness of 4 μm were prepared from the obtained paraffin blocks using a "Leica" RM 2235 microtome and conventionally stained with hematoxylin-eosin (H/E).

Results

Clinical and epidemiological studies carried out in a total of 7 sheep farms investigated proved the wide spread of gastrointestinal infections in newborn and adolescent lambs. Lambs aged between 1 and 30 days showed smudges in the perianal area and pelvic limbs with diarrheic slimy faeces having a yellowish color and copious amounts of blood, dehydration, pale visible mucous membranes, weight loss and recumbency, internal body temperature was normal.

Antigen tests

From the conducted antigenic tests, co-infections between viral, protozoan and bacterial agents (*Cryptosporidium parvum*, rotaviruses, coronaviruses and *Escherichia coli*) were proven as etiological agents in 40% of gastroenteritis cases. In 45% of cases, *Cryptosporidium parvum* invasions were registered, and in the remaining 15% coli infections **(**Fig. 1/Tabl. 1).

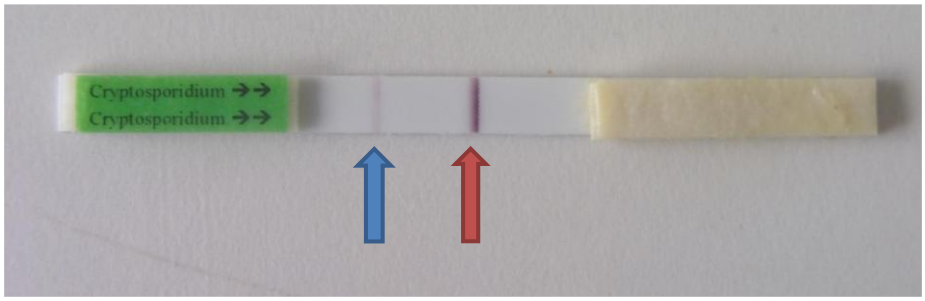

Figure 1: Positive result for the presence of *Cryptosporidium parvum* antigens in fecal samples from diarrheic lambs (blue arrow – control line red arrow – positive result).

Table 1: Results from rapid 5 valent antigen tests Rainbow calf scour 5 BIO K 306 Detection of Rota, Corona, *E. coli F5*, *Cryptosporidium parvum* and *Clostridium perfingens type A* in stool.

Detecting of antigens of the pathogens in fecal content	Co-infections between Cryptosporidium parvum, rotaviruses, coronaviruses and Escherichia coli	Cryptosporidium parvum	Escherichia coli
Result	**40%**	**45%**	**15%**

Coprological tests

The results established by coproantigen tests proving the presence of cryptosporidial enteritis in 45% of the lambs with gastrointestinal diseases were verified by stained faecal smears. Multiple

sporulated oocysts of *Cryptosporidium parvum* were observed in fecal smears stained by the Henriksen method (Emmonya Biotech, Chelopech) – a modified Ziehl-Neelsen stain for acid-fast organisms (Fig. 2). Fühleborn flotation studies were performed in parallel with Henriksen-stained fecal smears, which revealed multiple Eimeria oocysts in 18% of 45% with cryptosporidiosis, demonstrating the presence of 18% mixed invasion between the two protozoan agents (Fig. 3). The results obtained from the conducted coprological studies prove 18% mixed invasion between *Cryptosporidium spp.* and *Eimeria spp.* in the studied cases of neonatal diarrhea in lambs.

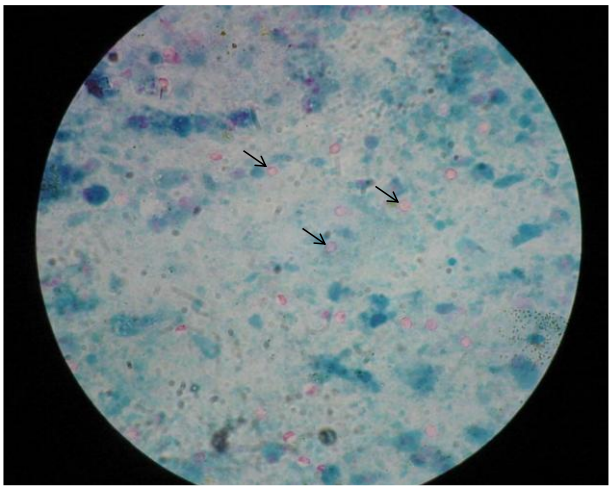

Figure 2: Multiple sporulated oocysts (arrows) of *Cryptosporidium spp*, Ziehl-Neelsen stained faecal smear.

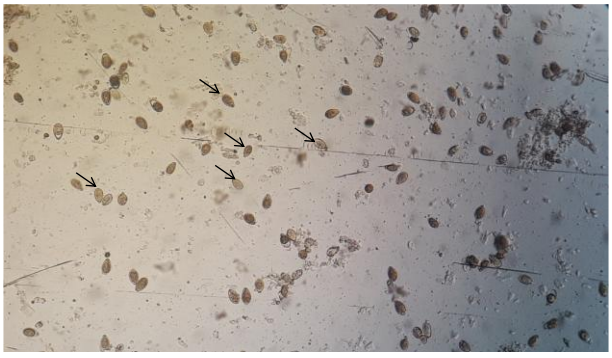

Figure 3: Multiple oocysts (arrows) of *Eimeria spp*, Fühleborn method.

Pathoanatomical studies

A total of 56 lamb carcasses, all of which died from neonatal diarrhea, were pathologically examined. Macroscopic changes on the carcass were enophthalmos, pale conjunctival mucous membranes, and profuse staining of the perianal region, base of the tail, and pelvic limbs with pale yellow mixed with blood diarrheal feces. At a section of the abdominal cavity, macroscopic lesions were expressed on the organs of the gastrointestinal tract. They were expressed with signs of catarrhal-hemorrhagic enterocolitis in the cases of eimeriosis and cryptosporidiosis. In the remaining 40% of complicated cases of co-infections between viruses and bacteria, changes were observed in addition to the intestines and on the abomasum: its mucosa was highly edematous, intensely red and dotted

with numerous erosions the size of millet. The contents were mucilaginous with a grey-brown color and mixed with milky coagulum. The mesenteric lymph nodes were pale, enlarged in size and bean-shaped, their core was pink on section. The serosa of the small intestine was strongly folded and thickened, and whitish nodule-like formations were visualized in places. Injection of the serosa vessels was noted (Fig. 4). The large intestines were ballooned, and in places the intestinal wall was severely thinned with virtualization of the contents. On sectioning of the gastrointestinal tract, the intestinal mucosa was pale pink and shiny. In 18% of cases of mixed invasion between coccidiosis and cryptosporidiosis, numerous whitish papillomatous raised nodular formations are observed on the mucosa of the ileum. The intestinal contents are dark red to brown in color with a watery consistency, mixed with blood coagulum (Fig. 5). In 27% of cases of cryptosporidial enteritis, whitish papillomatous nodular formations were absent on the ileal mucosa, in the remaining intestinal segments the intestinal wall was thinned, and the contents were pale yellow mixed with blood.

In 15% the cases of Escherichia coli infection, in addition to intestinal changes and pneumonic foci of a lobular nature in the lung, the bronchial and mediastinal lymph nodes reacted. At section of the heart, petechial hemorrhages on the epicardium and hematomas on the bicuspid valves were found. There were no changes in other organs and systems.

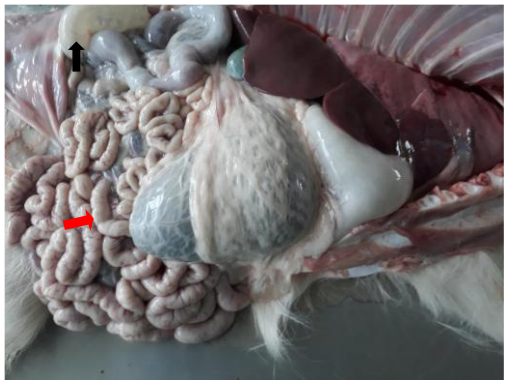

Figure 4: Strongly folded and thickened wall of the small intestine, injection of vessels on the surface of the intestinal serosa (red arrow). Presence of gas in the colon (black arrow). Lamb with cryptosporidium and eimeria enteritis.

Figure 5: Multiple whitish papillomatous raised nodules on the mucosa of the ileum (arrows), dark red intestinal contents with a watery consistency mixed with blood clots. Lamb with cryptosporidium and eimeria enteritis.

Histopathological examinations

The observed microlesional changes from the performed histological examinations of various segments of the intestinal tract (abomasum, duodenum, jejunum with mesenteric lymph nodes, ileum, cecum, colon and rectum), as well as in the mesenteric lymph nodes. They demonstrated intensively expressed hyperemia of the blood vessels with hemorrhages located in the mucosa and submucosa in the small and large intestines. Submucosal and intermuscular edema was also noted, and the glands were hypersecreting. Histopathological changes in the villi presented disintegration of the epithelium, degenerative-necrobiotic processes with desquamation of the cells in the intestinal lumen. In coccidiosis and cryptosporidiosis, in contrast to other enteropathogens (viruses and bacteria), villi atrophy was observed in the ileum and colon. Multiple forms of development in 18% of cases of *Cryptosporidium spp.* and *Eimeria spp.* were observed on and in the intestinal epithelial cells of all lamb carcasses examined with cryptosporidium and eimeria enteritis (Fig. 6). In the

intestinal lumen there were numerous oocysts mixed with catarrhal-hemorrhagic exudate. In 27% of cases, there were numerous only developmental forms of *Cryptosporidium spp.*, but not of *Eimeria spp.* The propria in protozoal enteritis (18% *Cryptosporidium spp. + Eimeria spp;* 27% *Cryptosporidium spp.*) was infiltrated with numerous lymphocytes, macrophages and single eosinophils. Neutrophils and lymphocytes were present in cases with other enteropathogens such as viruses and bacteria. Intense hyperemia was observed in the medullary zone of the mesenteric lymph nodes. Intense hyperemia was observed in the medullary zone of the mesenteric lymph nodes.

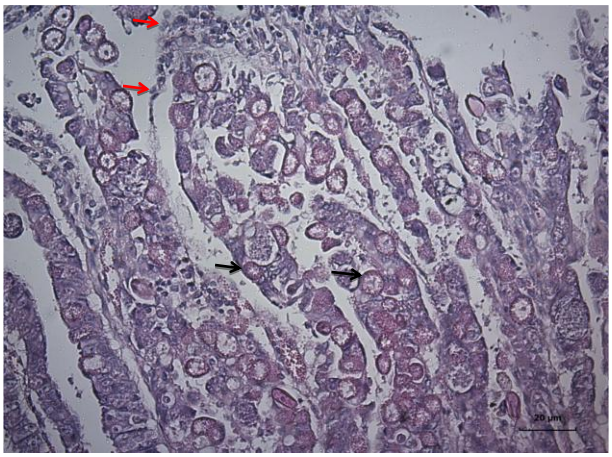

Figure 6: Desquamation accompanied by degenerative-necrobiotic changes in epithelial cells of the intestinal villi. Multiple developmental forms of *Cryptosporidium spp.* (red arrows) and *Eimeria spp.* (black arrows). Lamb with cryptosporidium and eimeria enteritis. H/E, bar=20 µm.

Discussion

Interpreting the data from the results of the conducted: epidemiological, clinical, etiological, patho-anatomical and pathomorphological studies among the population of newborn and adolescent lambs in a total of 7 private sheep farms under investigation proved to be widely distributed enteropathogens: *Cryptosporidium spp*., *Eimeria spp*., rotaviruses, coronaviruses and *Escherichia coli*, in lambs at an early age.

The results of the conducted clinical, epidemiological and etiological studies on newborn and adolescent lambs with neonatal diarrhea in the 5 sheep farms studied support the thesis of authors such as Sambeth *et al.*, (1993) and Taylor, (2017) that gastrointestinal infections in lambs are a multifactorial disease. Depending on the hygiene and environmental conditions, the immune status of the herd on the farm, the feeding and rearing conditions of the newborn animals (lambs), the observance of zoohygiene requirements, the management of the farm, as well as the presence in the environment of various enteropathogens (bacteria, viruses and protozoa). Similar results were obtained in our previous research conducted in goat farms and sheep farms in the country (Kalkanov *et al.*, 2021).

Based on antigenic and parasitological studies, we found that 45% of cases of enteritis in lambs were caused by cryptosporidiosis. In 18% of them, there were mixed infestations between coccidiosis and cryptosporidiosis. Which allows us to the thesis of (Nalan et al., 2009) and (Taylor, 2017) that coccidiosis and cryptosporidiosis are the most common protozoal intestinal infections in lambs and kids in the first month after birth, as in many cases they are attributed to a mixed invasion between these two protozoal agents. A similar opinion is expressed by other researchers in the field

such as Fayer and Ungar, (1986) and Norton *et al.,* (1986). As co-infections with the participation of other enteropathogens such as rota and coronaviruses, *E. coli* and other pathogens are also possible. This opinion is also proven by our results of past studies in the area of neonatal diarrhea in ruminants Kalkanov *et al.,* (2015), Kalkanov *et al.,* (2019), Kalkanov *et al.,* (2021).

The macrolesions described in the small and large intestines in the pathologically examined 56 lamb carcasses, register the location and the characteristic pathological changes in the individual intestinal segments of the diagnosed diseases (*Cryptosporidium parvum*, *Eimeriosis*, rotaviruses, coronaviruses and *Escherichia coli*). The data obtained, with the exception of 18% of cases of mixed invasion between cryptosporidiosis and coccidiosis are similar to those described in the intestinal sections of our other studies on gastrointestinal diseases in newborn and growing calves, heifers and kids (Kalkanov *et al.,* 2015; Kalkanov *et al.,* 2019; Kalkanov *et al.,* 2021).

Microscopic changes in the intestinal villi expressing, degenerative-necrobiotic processes of the epithelial cells, accompanied by atrophy of the villi in the ileum and colon characteristic of Coccidiosis and Cryptosporidiosis. Which were not found in other enteropathogens (viruses and bacteria) and co-infections between them. According to us and authors such as Ruiz *et al.,* (2012) the observed multiple developmental forms of *Cryptosporidium spp.* and *Eimeria spp.* in the intestinal epithelium, in addition to pathomorphological, they also have a diagnostic value, in the case of protozoan enteritis. In our opinion, the observed cellular proliferators represented by eosinophils, lymphocytes and macrophages in the intestinal compartments would complement the histopathological diagnosis. They can also be used for differential diagnosis between protozoal, viral and bacterial enteritis in lambs. The described pathohistological lesions in cryptosporidial enteritis in 45% of cases in lambs to some extent coincide with those described by us in cryptosporidiosis in calves (Kalkanov *et al.,* 2015, Kalkanov *et al.,* 2019), as well as with published scientific reports by western authors working in the field of protozoa enteritis in small and large ruminants (Taylor, 2017). In 18% of cases of mixed invasion, no macroscopic and microscopic similarities are found in the intestinal tract. The recorded histopathological changes in the intestinal tract in 40% of coinfections with other enteropathogens: rotaviruses, coronaviruses and E. coli correlate to some extent with the cases of intestinal infections in lambs and kids on the farms we described (Kalkanov *et al.,* 2021).

Conclusion

In conclusion, the results of the conducted studies prove the involvement of protozoan agents in 45% of cases of neonatal diarrhea in lambs. Represented mainly by Cryptosporidium spp., and in 18% there are mixed invasions between *Cryptosporidium spp.* and *Eimeria spp*. Which make the methods used reliable and applicable in practice. Macro- and microlesional manifestations described in the individual intestinal segments in diagnosed intestinal infections and co-infections. They complement their diagnosis and differential diagnosis between other diseases: Giardiasis, Anaerobic dysentery, Salmonellosis, etc., involved in neonatal diarrhea in lambs in sheep farms on the territory of Bulgaria.

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