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Histopathology and antibody responses describe the seasonal pattern of
dicrocoeliosis in small ruminants in the Himalayan ranges of Pakistan

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Abstract

In some parts of the world, *Dicrocoelium* spp. lancet flukes cause significant production loss in pastoral livestock, and accurate diagnosis of infection is important. The aims of the present study were to describe the histopathology and to investigate the transmission patterns of *Dicrocoelium* amongst ten sheep and goat farms in Khyber Pakhtunkhwa and Gilgit Baltistan, Pakistan. The liver histology and indirect enzyme-linked immunosorbent assay (ELISA) analyses followed standard procedures. The liver histopathology showed intensive tissue destruction and biliary hyperplasia associated with presence of adult flukes, severe inflammatory cell infiltration, congestion of red blood cells, damaged hepatocytes, and sinusoids in the infected areas. The time of onset of infection was investigated by ELISA detection of antibodies in sheep (n=164) and goats (n=152). Colostral transfer of *Dicrocoelium* antibodies from seropositive mothers was detected in sheep and goats up to 16 weeks of age. In both sheep and goats, the estimated time of infection differed between farms and years. Infection was seen in both sheep flocks and goat herds, with high variation between flocks and herds, and the highest infection rate in lambs. *Dicrocoelium* infection was most prevalent in sheep and goats in September (n=84) and August (n=63) respectively.

Conclusions: *Dicrocoelium* causes severe inflammation and necrosis of liver tissues in sheep and goats. Colostral transfer of antibodies can be detected up to about ten weeks of age. Higher infection rates are observed during August and September in sheep than in goats, putatively due to effects of different grazing and browsing behaviors on the ingestion of ants.

Keywords: *Dicrocoelium* spp., anti-*Dicrocoelium* antibodies, liver histopathology, ELISA
Introduction

*Dicrocoelium* spp. are trematode parasites typically found in ruminants. Infection in small ruminants occasionally results in economic losses due to liver condemnation, but clinical signs are only considered to be significant with heavy burdens (Otranto *et al.*, 2002). Three hosts, namely snails, ants, and ruminants are required for completion of the *Dicrocoelium* spp. life cycle (Dawes, 1968; Krull and Mapes, 1952a; Krull and Mapes, 1952). Eggs are shed in the bile ducts of the definitive host, then excreted in faeces. The first intermediate land snail host *ingests* the miracidia contained in the eggs, which then transform into mother sporocysts. Following asexual replication, large numbers of cercariae are released from the mother sporocysts into the slime trails of the snails. These are ingested by ants and develop to metacercariae in the gaster. A single cercaria migrates to the ant’s head and associates with the suboesophageal ganglion, changing the behaviour of the ants, whereby they climb onto and cling to flowers before being eaten by grazing animals. Larvae released from the metacercaria migrate along ghte intestine and common bile duct to the biliary tree of the liver, where they develop to monoecious adults, feed on blood, and shed eggs.

In Iran, dicrocoeliosis affects agricultural productivity, due to reduced reproduction, growth and milk production, liver condemnation, and the cost of anthelmintic treatment (Arbabi *et al.*, 2018). Specific clinical signs of infestation are typically absent, even in severe infections. The primary macroscopic liver pathologies are fibrosis, enlargement, and inflammation of the bile ducts (Jithendran and Bhat., 1996). The severity of the bile duct inflammation is correlated with the parasite load (Otranto *et al.*, 2003; Colwell and Goater, 2010, Rojo *et al.*, 2012). Histopathological changes in experimentally infected lambs were characterised by periductal fibrosis, ductal response, and leukocyte infiltration (Ferreras *et al.*, 2007). However, while numerous studies assessed the phenotypic expression of inflammatory cells in animals infected with the *Fasciola* spp. liver flukes (Meeusen *et al.*, 1995; Chauvin and Boulard., 1996; Ferreras *et al.*, 2007); none have examined the immunopathology in animals naturally infected with *Dicrocoelium* spp.
The diagnosis of dicrocoeliosis is frequently made after characteristic eggs are found in the feces of infected animals (Ferre et al., 1994), but these tests are frequently negative in small ruminants with fewer than 100 flukes (Ambrosi, 1991). The absence of clinical signs in animals with such low levels of infection presents diagnostic challenges, and led to the development of antigen detection methods, such as the ELISA. The ovine immune response to Dicrocoelium spp. has not been extensively studied. Gonzalez-Lanza et al. (2000) demonstrated that antibodies to D. dendriticum are first detectable in experimentally infected sheep by indirect enzyme-linked immunosorbent assay (ELISA) from 30 days after infection, during the liver-migration phase of the immature flukes. About 60 days after infection, the maximum antibody levels were reached, and they persisted at high levels at least until day 180 after infection. The average prepatent period after infection was 59 days according to earlier research (Campo et al., 2000). According to Sanchez-Andrade et al., (2003), a significant portion of sheep tested positive for D. dendriticum by the ELISA, but tested negative by egg detection. As is the case with fasciolosis (Paz et al., 1998; Sanchez-Andrade et al., 2003, 2001), a positive ELISA test result can also show past exposure to the parasite without an active infection.

A thorough understanding of the epidemiology and seasonal transmission patterns of Dicrocoelium is needed to improve the strategic control of liver fluke infection in sheep and goats in Pakistan. In this study, we charted antibody dynamics to determine the time of first exposure to Dicrocoelium spp., similar to previous studied in F. hepatica naive animals (Novobilský et al., 2014). To the best of our knowledge, this is the first study designed to investigate pathology and describe the transmission pattern of Dicrocoelium in sheep and goats in Asia.

**Materials and Methods**

**Study areas**

The study was carried out in 2018 and 2019 on farms where dicrocoeliosis was known to be present. A total of five sheep flocks (01, 02, 03, 04, and 05) and five goat herds (06, 07, 08, 09, and 10) were chosen in the Khyber Pakhtunkhwa and Gilgit Baltistan regions of Pakistan (Table 1). Herds and flocks were selected based on field
and abattoir reports of condemned livers. Unique codes were assigned to each sheep and goat, and proformas were filled out with information about the flock or herd, farm location, date of birth of each lamb and kid, breed, and month of sample collection.

**Ethical approval:**
The study was conducted by following the guideline approved (No. #BEC-FBS-QAU2017) by the Ethical Committee of Quaid-i-Azam University Islamabad, Pakistan. The sheep and goats used in the study were slaughtered solely to fulfill the meat demand of the local population.

**Histopathological examination of infected liver**

Histology of infected sheep (n=25) liver tissues was carried out to describe the histopathology of *Dicrocoelium* spp. infected livers. The infected livers were collected from slaughtered animals at local butcher shops in Chitral and Gilgit Baltistan. The histology was performed as described by Ullah *et al.*, (2019). Briefly, after 48 hours of fixation in formalin (10 %), the infected livers were dehydrated with alcohol and cleared with xylene. 2-3 μm paraffin sections were cut and stained with hematoxylin and eosin to assess the standard histology of the infected tissues. Prepared slides were observed and microphotographed under a light microscope (Leica LB Germany) paired with Canon digital camera (Japan) at 10X and 40X magnification.

**Collection of serum samples and ELISA**

Blood samples were taken from lambs and goat kids born between 2018 and 2019. The same 10-15 animals from each farm were subjected to blood sampling up to three times each during the two years. All blood samples were centrifuged at 3000 g for 10 min, and the separated sera were then stored at -20 °C until needed.

Sera were examined by indirect ELISA using *Dicrocoelium* excretory-secretory antigen (ES Ag), as described by Khan *et al.* (2023). Briefly, 100 μl of coating buffer NaHCO3/Na2CO3 (Merck) was added to each well of the microtiter plate, and each eluted antigen was mixed with it in an equal proportion (1:1). The microtiter plate was then incubated overnight at 4 °C. The plates were blocked with 0.05% BSA for 2 hours at room temperature after being washed with PBS containing 0.05% Tween 20 (Merck). Each well received 100 μl of the diluted sera and was then incubated for two
hours at 37 °C and subject to three rounds of washing. Following washing, 100 µl/well goat anti-bovine IgG secondary antibodies (1: 10,000) conjugated with alkaline phosphatase (Invitrogen™ Cat. nos. WP20006, WP20007) were added and incubated for 1 h. The plates were then washed before 100 µl of the substrate para-nitrophenyl phosphate (PNPP) (Thermo Scientific™ Cat. No. 37621) was added and incubated for 20 min at room temperature. An automated microplate reader was used to record the optical density (OD) value at 405 nm after the reaction was finally stopped by the addition of 50 l of 3 N NaOH solution. The ELISA results were expressed as a percentage of the mean optical density (OD) of the positive control as % of positivity = (mean OD of the tested sample /mean OD of the positive control) × 100. The cut-off value was calculated as the mean of the negative control sera absorbance values plus 3 standard deviations.

**Statistical Analyses**

GraphPad Prism version 5.02 (GraphPad Software, USA) was used to construct scatter plots of OD values, and descriptive statistics was used to calculate the cut-off values.

**Results**

**Screening of antibodies**

The specificity of the E/S antigen was tested against *Dicrocoelium* spp. positive control sera, and the calculated cut-off values for lambs and goats ELISA were 0.4314 and 0.411, respectively (supplementary Table 1). In sheep flocks 03, 04, and 05 in 2018 and on farms 02 and 03 in 2019, fifteen lambs from the first sampling period in June and July tested seropositive. The same lambs all tested negative in the second sampling period in July and August (Fig. 1 and 2). In goat herd 07 in 2018 and in goat herds 06 and 09 in 2019, three goat kids from the June blood collection tested seropositive. The same goat kids tested negative in the second sampling period in August (Fig. 3 and Fig. 4). In both 2018 and 2019, lambs in flocks 01, 03, and 04 that had tested negative during the first sampling period seroconverted by the second sampling period in August and remained positive in the third sampling period in September. Seroconversion occurred in lambs between the second and third sampling period, in flock 02 in 2018 and 2019, and in flock 05 in 2019. However, in 2018 and 2019, most lambs in flock 01 were already seropositive in June, and their ELISA OD values increased further during the following sampling months.
In comparison to antibody dynamics in lambs, no positive goat kids were detected at the first sampling period in June and July. Goat kids had seroconverted by the second sampling period in August in herds 06, 08, 09, and 10 in 2018, and in herds 08 and 10 in 2019, and remained positive at the third sampling period in September (Fig. 3 and 4). For a detailed presentation of the serological data for individual animals, see supplementary Tables 2-11.

Gross Liver Pathology and Histology

Large numbers of flukes were found in the bile ducts of each of the infected livers. The bile ducts had noticeably enlarged, thickened, and fibrosed walls, and the infected livers were cirrhotic and scarred. Histopathological examination (Fig.5) revealed cross-sectional parts of the oral suckers within the bile ducts, severe infiltration of inflammatory cells, and RBC congestion. The presence of flukes was associated with epithelium erosion, damaged sinusoids and hepatocytes. Inflammation and congestion of the liver's blood vessels at the portal areas were noted. Hepatocytes and inflammatory cells blocked the central vein, and sinusoids nearby were also affected. An adult lancet flukes found in the bile ducts, associated with biliary hyperplasia. Dicrocoelium spp. eggs were identified in the bile ducts, characterized by a shallow operculum, thick shell, small shoulders, and presence of miracidia; the sinusoids and hepatocytes in these regions were completely damaged. There was significant periportal infiltration of inflammatory cells, particularly macrophages and lymphocytes, coagulative necrosis around the central vein, congestion of the blood vessels, and pigmentation. Connective tissue was seen to spread in both wide and narrow streaks between the hepatocytes of neighboring lobuli.

Discussion

The economics of livestock production is marginal in the studied region, hence better understanding of any potential production-limiting disease, such as dicrocoeliosis is important. The severity of the hepatic damage due to dicrocoeliosis that was seen in the present histopathological study was consistent with previous reports (Sanchez-Campos
et al., 1999; Manga-González et al., 2004). Microscopic examination of the infected livers in current study showed different degrees of hyperplasia, desquamation, necrosis of the mucosal epithelium and a superficial erosive effect of the parasite sucker on the lining of epithelial cells. The pathological changes observed in dicrocoeliosis are caused by direct mechanical stimulation, probably from the suckers of the adult flukes, along with fibrosis-promoting factors released by leukocytes and toxic metabolites released by the adult flukes, inducing an inflammatory reaction (Manga-González et al., 2004; Samadieh et al., 2017). Leukocytic infiltration and periductal fibrosis were also observed, consistent with previous studies (Changizi et al., 1998; Manga-Gonzalez et al., 2004; Samadieh et al., 2017; Sato et al., 2019 and Nelwan et al., 2019; Pour et al., 2020; Piegari et al., 2021).

Experimental studies provide limited data on the immune response to D. dendriticum (Piergili Fioretti et al., 1980; Wedrychowicz et al., 1997; Gonzalez-Lanza et al., 2000). The current study of naturally-infected sheep flocks and goat herds demonstrates that tracking antibody dynamics may be an effective way to establish when grazing animals are first exposed to Dicrocoelium spp. infection. The indirect ELISA can detect antibodies against D. dendriticum infected lambs from 30 days after infection, being at least one month before coprological examination to identify eggs shows a positive result (Gonzalez-Lanza et al., 2000; Manga-Gonzalez and Gonzalez-Lanza 2005; Broglia et al., 2009). Lambs infected with a low and high dose showed the same timing and amplitude of response (Broglia et al., 2009), and the IgG levels remained high in lambs for a period of 150 days following infection (Gonzalez-Lanza et al., 2000; Manga-Gonzalez and Gonzalez-Lanza 2005; Broglia et al., 2009). In the present study of naturally infected animals, some lambs were seropositive at the first sampling period before first grazing and became seronegative one month later. These results imply passive transfer of colostral antibodies. Novobilsky et al. (2014) reported similar findings in cases of fasciolosis, and demonstrated the mechanism of antibody transmission from mother to infant via colostrum intake. According to Mezo et al., (2010), Fasciola hepatica colostral antibodies are transferred to dairy calves, and can be detected up to 12 weeks after birth.

Varying increases in Dicrocoelium spp. antibody ELISA OD values were seen in lambs and following the start of their grazing period. Beck et al., (2014)
demonstrated that the high variation in *D. dendriticum* abundance in naive calves was due to accidental ingestion of infected ants that contain variable numbers of metacercariae. The current finding could be explained by both post-infection and colostral antibody transfer as the source of seropositivity. According to several studies (Cornelissen *et al.*, 2001; Novobilsk'y *et al.*, 2014; Phiri *et al.*, 2006), the typical dynamics of antibodies during early *F. hepatica* infection in ruminants are characterised by antibody responses first appearing from about 4 weeks post-infection and then gradually increasing until about 12 weeks post-infection. Several mechanisms have been proposed to explain peak antibody abundance patterns with host age (Anderson and Gordon, 1982; Duerr *et al.*, 2003).

The antibody levels most lambs in flock 01 that were already seropositive at the first sampling period due to passive colostral transfer, further rose during the subsequent sampling periods. The high prevalence of dicrocoeliosis in this flock could be assessed by the widespread nature of ecological niches that can support the continuity of the *Dicrocoelium* life cycle in the northwest of Pakistan (Khan *et al.*, 2023). The suitability of environmental factors for the development and growth of intermediate snails and ant hosts, as well as grazing patterns that allow exposure to metacercaria-infected ants, will vary over the course of the year. Calcium-rich, alkaline soils and diverse vegetation help to provide overlapping niches suitable for each of the intermediate and definitive hosts (Manga-Gonzalez *et al.*, 2001).

The current findings suggest that browsing goat kids are less likely to be infected than grazing lambs, which is consistent with Bihaq *et al.* (2017). Most of the seropositive lambs and goat kids in the present study would have ingested ants with metacercaria by the beginning of August. This is therefore a critical time in terms of the dynamics of disease transmission in the flocks and herds of nomadic sheep and goats that graze on Himalayan pastures (Godara *et al.*, 2014). Jithendran and Bhat (1996) previously discovered a higher prevalence of dicrocoeliosis in sheep and goats during the post-rainy and winter seasons. Extreme cold weather conditions in Pakistan's Himalayan ranges hinder grazing of animals on open pastures during the winter months, implying that the greatest risk of infection occurs during the spring and summer months, when conditions are also favorable for intermediate host development (Cabeza-Barrera *et al.*, 2011; Khan *et al.*, 2023).
Conclusion

This study has described the severe histopathology caused by *Dicrocoelium* spp. infection. Indirect ELISAs have shown for the first time the presence of colostral antibodies, and have identified the timing of first infection in lambs and goat kids. The results will be useful in creating an epidemiological map of dicrocoeliosis in the Himalayan ranges, and will aid in the development of effective disease control strategies to ensure optimal growth and productivity of sheep and goats.

References


**Fig. 1:** Dynamics of *Dicrocoelium* antibodies in lambs on sheep flocks 01, 02, 03, 04, and 05 during 2018. The dashed line is the cut-off limit (cut-off = 0.434% of positivity).

**Fig. 2:** Dynamics of *Dicrocoelium* antibodies in lambs on sheep flocks 01, 02, 03, 04, and 05 during 2019. The dashed line is the cut-off limit (cut-off = 0.434% of positivity).

**Fig. 3:** Dynamics of *Dicrocoelium* antibodies in kids on goat herds 06, 07, 08, 09, and 10 during 2018. The dashed line is the cut-off limit (cut-off = 0.411% of positivity).

**Fig. 4:** Dynamics of *Dicrocoelium* antibodies in kids on goat herds 06, 07, 08, 09, and 10 during 2019. The dashed line is the cut-off limit (cut-off = 0.411% of positivity).

**Figure 5:** Histological sections of sheep livers infected with *Dicrocoelium* spp.

1. Shows cross-section of a parasite sucker in the lining epithelial cells of a septal bile duct (A), RBCs congestion (B) severe infiltration of inflammatory cells (C).
2. Shows congestion in blood vessels at the portal area (A), infiltration of inflammatory cells (B), normal hepatocytes in the vicinity of damaged hepatocytes (C), normal sinusoids (D), central vein and (E), near the central vein affected sinusoids and hepatocytes (F).
3. Presence of adult *Dicrocoelium* flukes in the bile duct, causing hyperplasia of bile duct (A), Histological appearance of a septal bile duct with severe epithelial papillary hyperplasia (B), Fibrosis and leukocyte infiltration around the biliary ducts in a severely infected liver (C).
4. Shows hyperplasia and inflammatory cells. *Dicrocoelium* eggs in a bile duct (A and B), cross-sectional part of the *Dicrocoelium* specimen (C).
5. Shows severely congested blood vessels in the central vein (A), Infiltiration of inflammatory cells (B), pigmentation (C), inflammatory regions (D).
6. Infected liver shows the damaged central vein (A), hepatocytes and inflammatory cells clogged the central vein (B), damaged sinusoids (C), infiltration of inflammatory cells (D).
7. Shows biliary hyperplasia, inflammatory cells, and a parasitic section containing several defining characteristics including egg containing miracidia (A), thick egg shell (B), operculated egg (C) cross-sectional parts of *Dicrocoelium* (D).
8. Shows narrow and wide streaks of connective tissues, infiltration of lymphocytes, macrophages, and eosinophils. Hematoxylin and eosin (HE) 100X.