GENETIC CHARACTERISTICS AND SPATIAL DISTRIBUTION OF *DICROCOELIUM* IN THE HIMALAYAN RANGES OF PAKISTAN

By

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DEPARTMENT OF ZOOLOGY
FACULTY OF BIOLOGICAL SCIENCES
QUAID-I-AZAM UNIVERSITY
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in

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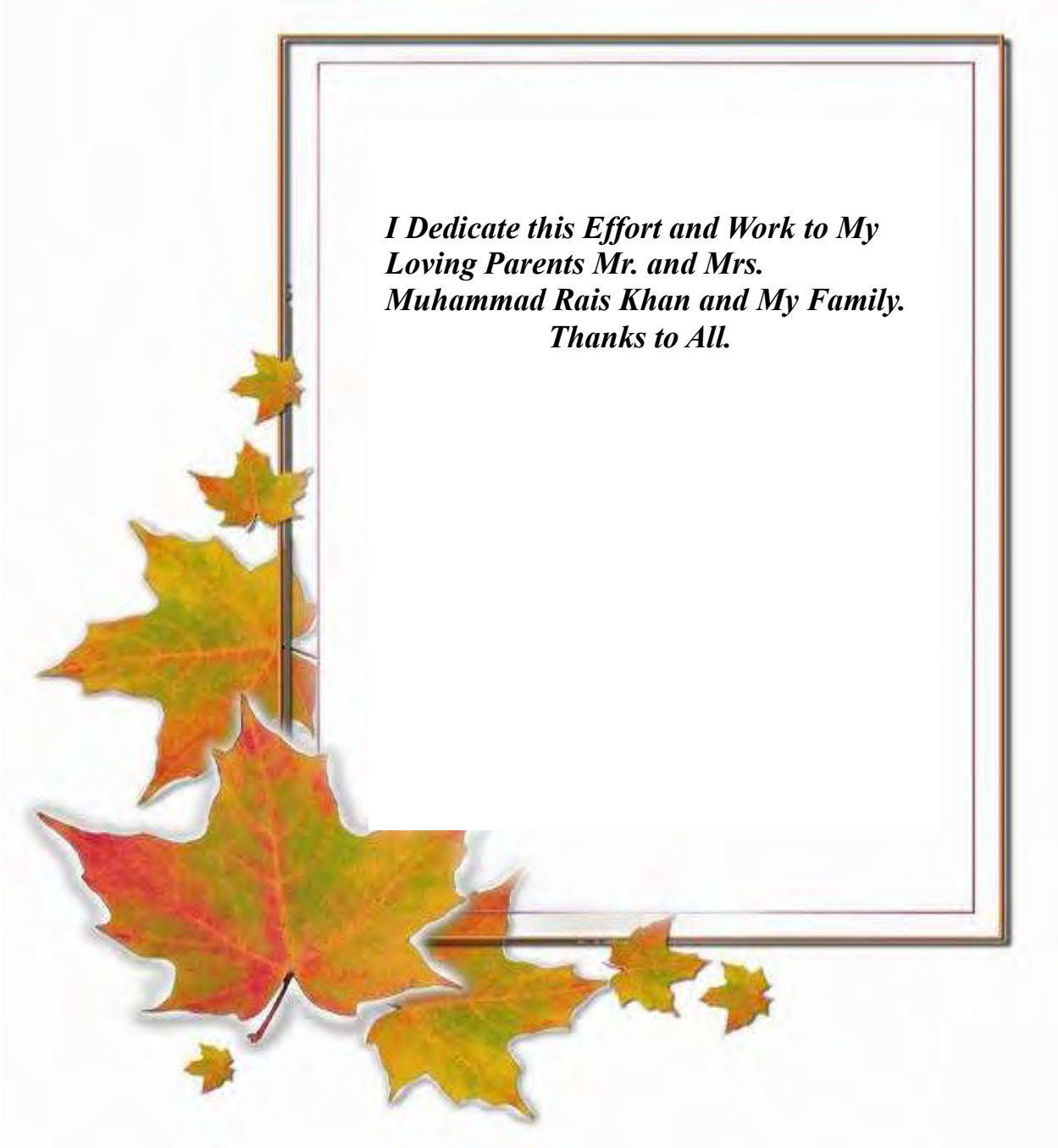

I Dedicate this Effort and Work to My Loving Parents Mr. and Mrs. Muhammad Rais Khan and My Family. Thanks to All.

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LIST OF ABBREVIATIONS

AGPT	Agar Gel Precipitation Test
ATL	Anterior Testes Length
ATW	Anterior Testes Width
BL	Body Length
BW	Body Width
CIEP	Counter Immunoelectrophoresis
COX-1	cytochrome oxidase-1
CPL	Cirrus Pouch Length
CPW	Cirrus Pouch Width
CSV	Comma-Separated Values
CVA	Canonical Variate Analysis
DDT	Dichloro Diphenyl Trichloroethane
DEM	Digital Elevation Model
EL	Egg Length
ELISAs	Enzyme-Linked Immunosorbent Assays
EPG	Egg Per Gram
ES	Excretory/secretory
EW	Egg Width
GDP	Gross Domestic Products
GIS	Geographical Information Systems
GPS	Global Positioning System
ITS-1	Internal Transcribed Spacer Region-1
ITS-2	Internal Transcribed Spacer Region-2
LVL	Left Vitelline Length
LVW	Left Vitelline Width
Max	Maximum

MaxEnt	Maximum Entropy
MCL	Maximum Composite Likelihood
Min	Minimum
ML	Maximum Likelihood
mm	Millimeters
NAD1	NADH dehydrogenase gene
NDVI	Normalized Difference Vegetation Index
NPD	Neglected Parasitic Disease
OL	Ovary Length
OSL	Oral Sucker Length
OSW	Oral Sucker Width
OW	Ovary Width
PCA	Principal Component Analysis
PHT	Passive Hemagglutination Test
PNPP	Para-Nitrophenyl Phosphate
PTL	Posterior Testes Length
rDNA	Ribosomal DNA
RVL	Right Vitelline Length
RVW	Right Vitelline Width
SD	Standard Deviation
SDMs	Species Distribution Models
VSL	Ventral Sucker Length
VSW	Ventral Sucker Width
WHO	World Health Organization

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GENERAL ABSTRACT

Digenean lancet liver flukes of the family Dicrocoeliidae (Trematoda: Digenea) can infect the bile ducts of a variety of wild and domesticated mammals and humans around the globe. Three species of the genus *Dicrocoelium,* namely *Dicrocoelium dendriticum, Dicrocoelium hospes*, and *Dicrocoelium chinensis,* have been described as causes of dicrocoeliasis in domestic and wild ruminants. Among these, *D. dendriticum* is the most common and is distributed throughout Europe, Asia, North and South America, Australia, and North Africa. The main economic impact of dicrocoeliasis in livestock is the rejection of livers from slaughtered animals at meat inspection. However, in severe infections, affected animals may show clinical signs, including poor food intake, ill thrift, poor milk production, alteration in fecal consistency, photosensitization, and anemia. Pakistan is a semi-tropical country with ideal environmental conditions for the survival and reproduction of a wide range of parasitic organisms. Parasitic diseases are of critical importance and cause low productivity in livestock, but they are often overlooked by livestock owners due to the low mortality associated with parasitic infections. In comparison to other parts of Pakistan, the mean daily temperature ranges in the Himalayan ranges create an arid environment with only patchy coniferous tree cover, providing habitats that are mostly hostile to many snail species and potentially creating isolated habitats for *Dicrocoelium* spp. To improve livestock productivity, it is critical to investigate parasite hotspots, identify species, and understand parasite biology. The thesis is composed of various objectives and the first objective was to examine the morphometric variation and histopathological complications caused in the liver of sheep and goats infected with *Dicrocoelium*. For this purpose, one hundred and ninety adult *Dicrocoelium* parasitized sheep and goats from Khyber Pakhtunkhwa and Gilgit Baltistan, Pakistan, were analyzed morphometrically. To evaluate the degree of variation among the population, 26 characters were compared. The principal component analysis showed that the morphological traits of *Dicrocoelium* have no significant variations among the studied population. The histopathological analysis of the liver showed severe infiltration of the inflammatory cells, RBCs congestion, damaged hepatocytes, and sinusoids in the vicinities of the central vein and uterine eggs. The morphologically overlapping traits prevented accurate species identification; therefore, the present study confirmed the species identification of

Dicrocoeliid flukes by using molecular approaches, the molecular markers of rDNA and mitochondrial DNA were used. A phylogenetic comparison of published *D. dendriticum* ribosomal cistron DNA, cytochrome oxidase-1 (COX-1), and NADH dehydrogenase-1 (ND-1) mitochondrial DNA sequences with those from *D. chinensis* was performed to assess inter and intra species variation and reaffirm the use of species-specific single nucleotide polymorphism markers. The results of PCR and sequencing of 34 corresponding fragments of ribosomal DNA, and 14 and 3 corresponding fragments of mitochondrial DNA from the Chitral Valley flukes, reveal 10, 4, and 1 unique haplotypes, respectively. Additionally, these findings confirm the first-time molecular species identity of Pakistani lancet liver flukes as *D. dendriticum* and *D. chinensis.* Moreover, dicrocoeliasis is an important cause of production loss in ruminants due to the cost of liver condemnation at slaughter. Therefore, the objectives of the present study were to determine the prevalence of *Dicrocoelium* species infection and to predict the ecological niches and climatic variables that support dicrocoeliosis in the Himalayan ranges of Pakistan. Thirty-three (33) of 381 liver samples collected at slaughter and two hundred and thirty-eight (238) of 6060 blood samples from sheep and goat herds in the region were positive for *Dicrocoelium*. The results showed that the prevalence of dicrocoeliosis was higher in sheep than in goats and highest in females aged greater than three years. Therefore, an environmental risk map was created to predict active zones of transmission and showed the highest probability values in central parts of the Chitral district in the northwest of Pakistan. Further, the findings depict that the climatic variables of the mean monthly diurnal temperature range (Bio2), annual precipitation (Bio12), and normalized difference vegetation index (NDVI) were significantly ($p<0.05$) associated with the presence of *Dicrocoelium* infection. Similarly, charting antibody dynamics in *Dicrocoelium*-naive animals to determine the time of exposure of infection is needed to improve a strategic control program for liver fluke infection in sheep and goats in Pakistan. The current study also investigated the transmission patterns of *Dicrocoelium* in sheep farms (n=10) and goat (n=10) farms in Khyber Pakhtunkhwa and Gilgit Baltistan, Pakistan, between 2018 and 2019. 15-20 animals from each farm were screened for *Dicrocoelium* infection through ELISA and investigated their time of infection. The results point out that 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 is different in various farms and years. However, the highest infection rate found in sheep farms was 41 in 2018 and 40 in 2019, while in goats the infection rate was 22 in 2018 and 18 in 2019. A minimum infection rate was reported in sheep farms-04, and 05 in 2018 and 2019 respectively. Furthermore, in goats' the lowest infection rate was observed at farm-05 in 2018 and at farm-02, 03, 04, and 05 in 2019. However, the *Dicrocoelium* infection was found most prevalent in sheep and goats in September (n=84) and August (n=63) respectively. In conclusion, this study provides a preliminary illustration of a phylogenetic approach that could be developed to study the ecology, biological diversity, and epidemiology of Dicrocoeliid lancet flukes when they are identified in new settings. The findings of this study demonstrate the most suitable ecological niches and climatic variables influencing the risk of dicrocoeliasis in the Himalayan ranges of Pakistan. The methods and results could be used as a reference to inform the control of dicrocoeliasis in the region.

GENERAL INTRODUCTION

Pakistan is an agricultural country where livestock plays a significant part in the national economy. An excessively large portion of the nation's annual protein requirement is provided by animal products, such as milk, meat, and eggs. Livestock is responsible for nearly 14.0% of Pakistan's GDP and over 61.9% of the country's agricultural value added. Livestock raising is a livelihood for more than 8 million rural families, making up 35-40% of their overall income. In 2021–2022, there were 53.4 million cattle, 43.7 million buffalo, 31.9 million sheep, and 82.5 million goats in the national herd. Gross value addition from livestock increased by 3.26 percent from Rs. 5,269 billion in 2020–21 to Rs. 5,441 billion in 2021–22 (Economic Survey of Pakistan, 2021-2022).

Infectious diseases are a major issue worldwide, and parasites harm tissues, and thereby depleting proteins, reducing productivity and biological efficiency in livestock species. Parasites infest the respiratory, gastrointestinal, liver, and skin systems. There are several approaches to estimating the financial cost of an endemic disease, but these estimates must consider the disease severity as well as the length of time it spends in a herd or flock during its life. Small ruminants in underdeveloped areas are forced to endure the sobering compulsion of clinical and sub-clinical helminth infestation, which lowers their capacity for production and reproduction by reducing voluntary feed intake and/or feed conversion efficiency (Zeryehun, 2012; Ayaz *et al.,* 2013; Kanyari *et al.,* 2017). Poor nutrient use slows growth, and in severe infestations, it can even result in anemia and death (Sykes, 1994; Terefe *et al.,* 2012; Hassan *et al.,* 2011). In addition to these risks, helminth infestations weaken an animal's immune system and increase its risk of contracting other pathogenic diseases, which can cause significant financial losses (Garedaghi *et al.,* 2011). Tropical countries have a much more serious problem with helminth transmission because of the extremely favorable environmental conditions (Mohanta *et al.,* 2007; Tesfaheywet, 2012), the host animal's poor nutrition (Mbuh *et al.,* 2008), and the lack of sanitation in rural areas (Badran *et al.,* 2012). As a result, helminth infections continue to be a major obstacle to small ruminant production in the

tropics (Bersissa *et al.,* 2011), with reports of helminth infestation in as many as 95% of small ruminants living there (Opara *et al.,* 2005; Mbuh *et al.,* 2008; Dechassa *et al.,* 2012). However, because the illness is chronic, the vast majority of helminth-infested animals do not show any clinical symptoms. The three major types of helminths are nematodes (roundworms), cestodes (tapeworms), and trematodes (flatworms) (flukes). Helminth infestation has been studied from a variety of perspectives by a number of researchers in Pakistan (Raza *et al.,* 2007, 2009, 2012; Ijaz *et al.,* 2009; Khan *et al.,* 2010; Farooq *et al.,* 2012). Although many different parasitic genera and species can cause helminth infections in grazing ruminants, the gastrointestinal nematodes, liver fluke, and the bovine lungworm are the most economically significant (Charlier *et al.,* 2014). In Pakistan, studies revealed widespread occurrence of helminths in our domestic animals, and infection rates as high as over 90% are reported (Rhman *et al.,* 2009). These diseases are a major hindrance to the worldwide production of ruminants. In order to increase livestock production to fulfill the needs of a growing and nutritionally more demanding global population, effective management and control of helminth infections are essential (Vercruysse *et al.,* 2018). However, the continued evolution and proliferation of helminth populations resistant to anthelmintics pose a danger to current control (Sutherland and Leathwick, 2011; Sangster *et al.,* 2018).

1.1. Dicrocoeliasis

Dicrocoeliasis, a zoonotic disease that affects human and animals' bile ducts and gallbladders, is caused by one of the three species of the genus *Dicrocoelium* (Khan *et al.,* 2021). *Dicrocoelium dendriticum* has been reported in Europe, Asia, northern Africa, and North America; *D. hospes* is endemic in Sub-Saharan Africa and West Africa; and *D. chinensis* is found in Eastern Asia and Europe (Manga-Gonzalez *et al.,* 2001). *Dicrocoelium* is a common zoonotic helminth, especially in areas with intensive animal husbandry (Sandoval *et al.,* 2013; Beck *et al.,* 2015). *Dicrocoelium* also referred to as the little liver fluke or lancet liver fluke, is a crucial species in the medical, veterinary sciences, and economic industries (Manga-González *et al.,* 2001; Paranjpe *et al.,* 2020). Numerous distinct hosts, primarily domestic and wild ruminants, have been found to harbor this trematode. *Dicrocoelium* has a complicated life cycle that includes three

hosts: terrestrial snails as the first intermediate host, formicid ants as the second, and ruminants as the definitive host (Meshgi *et al.,* 2019). Although dicrocoeliasis in humans is extremely rare compared to domestic ruminants, it is still regarded as a neglected parasitic disease (NPD) (Otranto and Traversa, 2003; Chougar et *al.,* 2019). Cirrhosis, acute urticaria, biliary blockage, cholangitis, and flatulence are all potential outcomes of this disease (Jeandron *et al.,* 2011; Cengiz *et al.,* 2010). The taxonomic position of *Dicrocoelium* is as under:

Kingdom Animalia
Subkingdom Eumetazoa
Phylum Platyhelminthes
Subphylum Neodermata
Class Trematoda
Subclass Digenea
Order Plagiorchiida
Infraorder Plagiorchioidea
Family Dicrocoeliidae
Genus *Dicrocoelium* (La Rue, 1957).

1.2. Morphology

Genus *Dicrocoelium* contains different species which cause dicrocoeliasis, which is a parasitic infection (Dujardin,1845). *Dicrocoelium* spp. has a lancet-shaped body and possesses both an oral and a ventral sucker. The size of the body ranges from 5mm to 10 mm in length, and the width between 2 and 3 mm. The body is semi-transparent and pied. The uterus is black in color, and white vitellaria seems to be with the common eye. The shape of the egg is oval, Dark brown in color operculum is present; typically, egg is small (38-45 um 22-30 um). Two prominent dark points are present which are called “eye spots” and possess a miracidium (Euzebby, 1971). The position of the testis is quite different in species. The position of the test is due to the length of the vitellaria and the sucker, which are ventral in position to the cirrus suck (Schuster, 2002).

Dicrocoelium immature form resembles *Fasciola hepatica*, and for a long time, both trematodes were thought to be the same parasite. In 1803 Rudolphi classified a trematode and, and the first time, described *Dicrocoelium dendriticum* in detail. Buchholz in 1970 isolated this parasite from humans in Weimar and named this parasite *Fasciola lanceolate*. After a few years, this parasite was classified as a *Distoma hepaticum* by Rudolphi. The synonym's nature is complex because of the different generic and specific denominations given to this parasite (Mapes, 1951; Schuster, 1987).

1.3. *Dicrocoelium dendriticum*

Dicrocoelium dendriticum is found in many countries in Europe, Asia, North America, and North Africa (Otranto *et al.,* 2003). *D. dendriticum* intermediate hosts are a wide range of land snails (Pulmonata, Stylommatophora), and numerous species of Formicid ant (Manga-Gonzalez *et al.,* 2001). This parasite affects all ruminants, including ovine, bovine, cervid, elks, and camelids (Malek, 1980b; Soulsby, 1982; Manga-González *et al.,* 1991; Campo *et al.,* 2000; Otranto and Traversa 2002, 2003; Manga-González and González-Lanza 2005; Senlik *et al.,* 2006; Goater and Colwell 2007; Otranto *et al.,* 2007; Colwell and Goater 2010; Manga-González *et al.,* 2010; Sargison *et al.,* 2012; Beck *et al.,* 2015; Lambacher *et al.,* 2016; Mitchell *et al.,* 2017). This parasite is also found in the European brown hare (*Lepus europaeus*) (Diakou *et al.,* 2014) and in rabbits grazing on Machair pastures (Mitchell *et al., 2017*). Humans are also infected by this trematode's parasite (Mohamed and Mummery, 1990; Cengiz *et al.,* 2010; Gualdieri *et al.,* 2011; Jeandron *et al.,* 2011; Pepe *et al.,* 2015). This parasite shows a close resemblance to the immature form of *Fasciola hepatica* because both are trematodes and are found in the liver of ruminants (Mapes, 1951; Schuster, 1987).

1.4. *Dicrocoelium chinensis*

Dicrocoelium chinensis are mostly reported in ruminants from East Asia (Sudarikov and Ryjikov, 1951; Tang *et al.,* 1983, 1985; Taira *et al.,* 2006), and from different countries of Europe in sika deer, probably may exist in Asian countries (Hinaidy, 1983; Poglayen *et al.,* 1996; Otranto *et al.,* 2007). There are molecular differences in adult species among *D. dendriticum* and *D. chinensis*. The intermediate

host of *Dicrocoelium chinensis* is *Stylommatophora* molluscs, genus *Bradybaena* (Bradybaenidae), *Xeropicta* (Helicidae), and *Ganesella* (Pleurodontidae) (Tang *et al.,* 1983; Gu *et al.,* 1990). The second intermediate host of *D. chinensis* is ant, which belongs to the genera *Formica* and *Camponotus* (Tang *et al.,*1983; Gu *et al.,*1990). Phylogenetic relationships among the different populations of China and Japan were studied based on mitochondrial "nad1 gene" sequences (Hayashi *et al.,* 2017). These authors identified the parasites considering the testis orientation and the nucleotide sequences of the ribosomal ITS-2. The molecular variance analysis shows the highest percentage ratio between the countries. *D. chinensis* populations in China and Japan were different genetically. The author strengthens the hypothesis that the introduction of *D. chinensis* in Japan was due to the migration of the infected wild ruminants, in the Pleistocene glaciations, due to which this population becomes differentiated from that of the Chinese population.

1.5. *Dicrocoelium hospes*

D. dendriticum was similar to that of adult Dicrocoeliidae of the Soviet Union and Czechoslovakia on the basis of morphological variability (Bourgat *et al.,* 1976; Kajubiri and Hohorst, 1977; Lucius and Frank, 1978; Malek 1980a, b; Lucius, 1981; Fashuyi and Adeoye, 1986), some authors considered *D. hospes* as a valid species on the basis of molecular characterization of the 28S region, and ITS-2 of this species was done in ruminants (buffaloes, goats, sheep, and cattle) in Sahara of South Africa, and also Savanna, liver fluke is widely distributed (Maurelli *et al.,* 2007; Lucius, 1981). The genus *Limicolaria,* of Stylommatophora molluscs acts as an intermediate host in its biological cycle (Bourgat *et al.,* 1976; Lucius 1981). The genus *Camponotus* (Formicinae), *Crematogaster* (Myrmicinae) and *Dorylus* (Dorylinae) family also act as intermediate host (Bourgat *et al.,* 1976; Roming *et al.,* 1980; Lucius, 1981).

1.6. Life cycle

Land molluscs and ants are the first and second intermediate hosts, respectively, for the completion of the extremely complicated life cycle of the *Dicrocoelium* species; for more than one century details studies were done to know and elucidate the complete life cycle of *D. dendriticum* (Mapes, 1951). Snails were thought to act as an intermediate host for many years, but the assumption of some thought snails to be the definitive host (Mattes, 1936; Nehaus, 1936). *Formica* species is the second intermediate host in the life cycle of *Dicrocoelium* (Krull and Mapes 1952). Liver and bile ducts are the residing places for the adult of *Dicrocoelium* in a definitive host (mainly ruminants, lagomorphs, and equids), where they lay eggs which are embryonated in nature, and these are embryonated eggs pass through the intestine and removed through feces. The hatching of the embryonated eggs and liberation of miracidium get entrance into the walls of the intestine of molluscs and settle down in the hepatopancreas.

The miracidium transforms into a mother sporocyst in the hepatopancreas. Since the sporocyst lacks a wall, it takes on the shape of the spaces between the lobules of the hepatopancreas. Daughter sporocysts develop from the mother sporocyst. Then, they develop into stage cercaria. After maturing, the cercaria migrates into the molluscs' respiratory cavities, where they become covered in slime; that’s why they are called "Slimeballs.” Different species of ants, the second intermediate host, consume these slime balls. The cercaria loses its tail after passing through the ant's tunnel and is occasionally (two, three, or one) referred to as the "brain worm." These brain worms made their home in the ant's ganglion in the sub-esophageal region. In the abdomen, other cercaria changed into metacercaria. Change the ant's behavior, and the ant is attached to the grass, where the final host consumes it—the metacercaria, which has reached maturity in the ant's abdomen, excyst in the intestine. The young fluke enters the liver through the bile duct, matures there, and then begins to lay eggs, which are then removed from the host by defecation to begin the life cycle all over again.

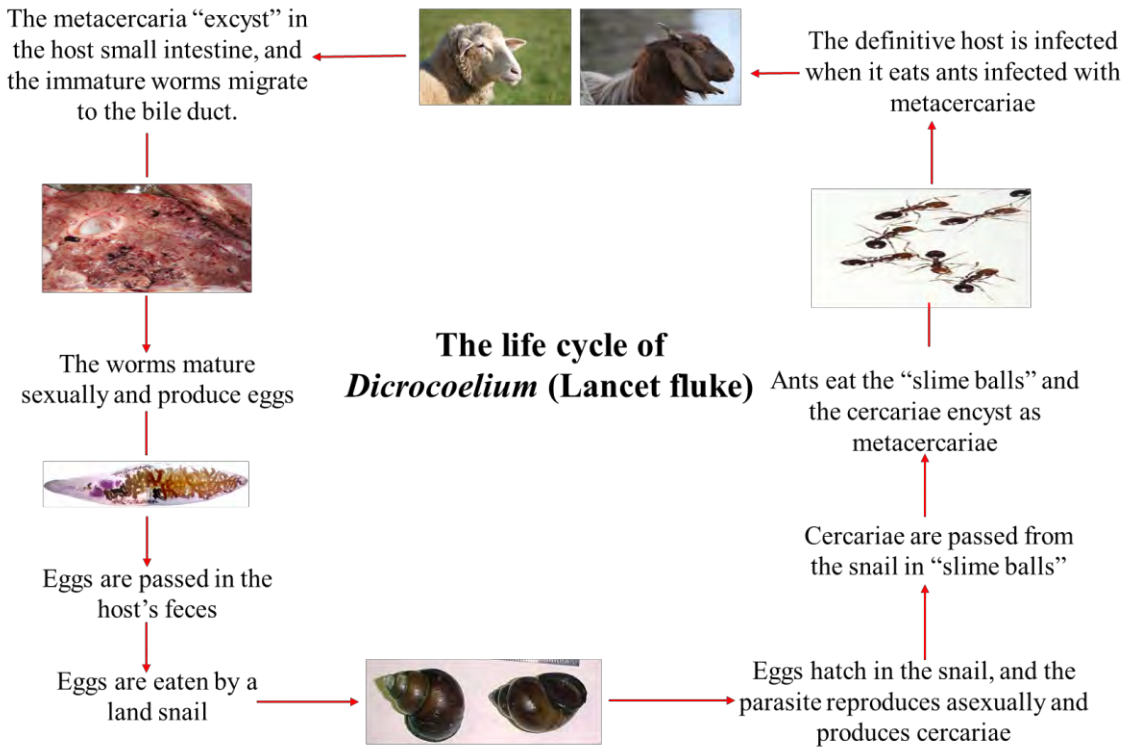


Figure no.1: Life cycle of *Dicrocoelium dendriticum.*

1.7. Environmental factors

Dicrocoelium eggs are widely distributed, and pastures have been contaminated by both domestic and wild animals. (Boray, 1985). Eggs are passed through defecation and exhibit high resistance to temperature changes, even in winter and in postures for up to 20 months. There is no correlation between the infectivity of the eggs and age, according to a study that found no loss in infectivity over a 15-month study period, proving that in the field, egg survival is not age-dependent but rather a seasonal phenomenon (Alunda and Rojo Vazquez, 1983).

Molluscan species, more than hundreds, are the first intermediate hosts for *Dicrocoelium* (Manga-Gonzalez *et al.,* 2001). Development of the larval stages of the parasite in the intermediate host depends on the species, nutritional status of the molluscs, infective dose, ambient temperature, and relative humidity (Manga-Gonzalez *et al.,* 2001). The infection rate was precisely determined in Germany during the grazing

seasons using the *Helicella obvia* snail model. *H. obvia's* population structure underwent changes from April through June when the small snail was present. The large-sized snails predominated in the month of spring of the current year, while the average- or medium-sized snails were present from July to September (Schuster, 1993).

The comparison ratio between the infection rate and snail age reveals that the epidemiology of dicrocoeliasis varies depending on the snail's size. Compared to medium-sized shell-diameter snails, the young snail was less involved in epidemiology, especially in the spring of the second year (Schuster, 1993). The largest snail is thought to be more vulnerable to *D. dendriticum* due to its high metabolic rate and balanced nutritional value for developing sporocysts (Alunda and Rojo-Vazquez, 1983). The infection rate drops in the summer for the second consecutive year because infected snails are more likely to die from sporocyst development, which also disrupts the hepatopancreas. Then it results in a reduction in the activity that could be developed and used to estimate life expectancy (Schuster, 1992).

Then it brings impairment of potential activity for the development and briefing of life expectancy (Schuster, 1992). Temperature, rainfall, and atmospheric carbon dioxide concentration are the main factors that influence growth rates, species distributions, and species interactions in ants (Boukal *et al.,* 2019; Stireman and Singer, 2018; Nelson *et al.,* 2019). Among the different species, and even in the same species of the genus metacercaria, the species of *D. dendriticum* varies per ant. The variation depends upon the season per year, it is higher in summer (Paraschivescu *et al., 1976*), and it also depends upon the affinity of the ant species for slimeballs and the vegetation types and ant species present on vegetation. (Paraschivescu, 1978). In *tetania* “plant topping” by ant is the cause of “brain worm,” a condition in which metacercaria becomes encysted in the ants suboesophageal ganglion, while some other metacercaria lives and becomes mature in the gaster cavity of ants. The behavior of the and which are infected is also dependent on the humid temperature variation. The net result will be that availability of metacercaria for pasture animals will be circadian rhythms. When there is a decrease of solar radiation and temperature at the terminal period of the afternoon then the *tetania* of

the infected ants normally occurs but disappears in the morning when the sun shines, and a temperature increase occurs. This leads ants to alter their behavior and the result will be the intake of the parasites by the definitive host.

1.8. Pathogenesis

When compared to other flukes, *D. dendriticum* hepatic lesions frequently go undetected. In fact, it can be challenging to create experimental infections to study the pathogenesis of *D. dendriticum*, and in the field, infections are typically complicated infections caused by a variety of more pathogenic helminths that obscure the symptoms and lesions that are merely caused by *Dicrocoelium*. In fact, juvenile flukes migrate directly up the biliary duct system of the liver without penetrating the gut wall, liver capsule, or liver parenchyma as in the case of fasciolosis, which may be the cause of the lack of obvious lesions and symptoms (Theodoridis *et al.,* 1991). According to other studies, in cases of extremely severe infections, the main hepatic ducts become thicker, their mucosa enlarges, glands proliferate, connective and muscle tissue increases, there is cellular infiltration, and small bile ducts multiply (Wolff et al., 1984; Camara et al., 1996). The small liver fluke's buccal stilets may also irritate the bile duct surfaces, leading to proliferation and changes in the septal bile ducts at the lobular hepatic edges. There are reports of fibrosis and cirrhosis of the parenchyma in long-term infections (Wolff *et al.,* 1984). The worm burden of infected animals and lesion score were found to be directly correlated. The liver was examined under a microscope along with the lesion and classified as normal, indurated, or scarred. The liver was also severely thickened by fibrosis and a heavy worm burden, as well as posterior liver ducts (Jithendran and Bhat, 1996).

The enlarged state of the liver, thickened ducts, white spots on the surface, scarring, cirrhosis, and cholangitis all contribute to its impairment (Jithendran and Bhat, 1996). Experimentally infected hamsters showed pathological changes such as bile duct proliferation, lymphocyte, eosinophil, and macrophage infiltrations into the entry tract, surface area enlargement, interlobular septa, collagen filtration into the portal tract, and liver distortion (Sanchez-Campos *et al.,* 2000). Oxidative modification and persistent inflammation. Aspartate transaminase and alanine transmission activities increased,

reactive oxygen levels decreased, oxidative liver damage was observed, and superoxide dismutase activity was observed in the mitochondria and gel region of the cytoplasm.

Other studies were conducted in addition to necropsy findings to assess the loss of a vital body component (plasma protein) brought on by dicrocoeliasis. These studies were conducted using radioisotopic methods, which can assess the pathophysiological pattern in animals naturally infected with *D. dendriticum* (Dargie and Allonby, 1975). After injecting sheep with 125-labeled albumins, 51Fe-citrate (Red cell iron incorporation rates), and 51Cr-labeled red cells, no statistically significant loss in the red cell, survival times, or plasma albumin was found (Theodoridis *et al.,* 1991). It was concluded that increased *D. dendriticum* numbers up to 4000 do not cause significant loss of blood or plasma protein in sheep, as reported in the case of fascioliasis and haemonchosis (Dargie and Allonby, 1975).

1.9. Global Prevalence

The most common flukes found in sheep in Europe, Asia, and Africa are the liver flukes *Fasciola* species, *D. dendriticum,* and other rumen flukes. The prevalence of *D. dendriticum* in Europe varies among countries like Italy 6.7-86.2% (Sandoz andrate *et al.,* 2003), Greece 0.2-70% (Kantzoura *et al.,* 2012), Egypt 5% (Haridy *et al.,* 2006), Turkey 3.85-23.55% (Gargili *et al.,* 1999, Kara *et al.,* 2009), Ghana 19.54% (Francis addy *et al.,* 2021) Iran 4.3% (Ali khanjari *et al.,* 2014), Nigeria 39% (Florence Oyibo *et al.,* 2018), Saudi Arabia 10.6% (Murshed *et al.,* 2022), 21.1% in Germany (Uta Alstedt et al., 2022), in China 0.7% (Zhu Dan *et al.,* 2013), in India 18.9% (Godara *et al.,* 2014), and in Pakistan 1.51% (Afzal *et al.,* 1981).

1.10. Diagnosis

Dicrocoeliasis is difficult to clinically detect due to the sub-clinical manifestation. Diagnosis is possible through the egg by coprological examination or by recovering the adult from the liver by necropsy. The coprological examination can be carried out using sedimentation, flotation, and the McMaster technique (Campo *et al.,* 2000; Otranto and Traversa 2002; Manga- González and González-Lanza, 2005). *Dicrocoelium* egg counts per gram (EPG) of feces from infected animals can be determined using these

coprological methods. However, anti-*Dicrocoelium* antibodies have been detected through the use of serological techniques, such as enzyme-linked immunosorbent assays (ELISAs) (Baldelli *et al.,* 1981; Bode and Geyer, 1981; Jithendran and Bhat, 1996; Wedrychowicz *et al.,*1997; González- Lanza *et al.,*2000; Otranto and Traversa, 2002; Sánchez-Andrade *et al.,* 2003) However, this indirect test is not specific because it cannot differentiate between active and past infection (Fagbemi and Obarisiagbon, 1991). The antigens often used to detect antibodies are complex combinations of various protein molecules that can trigger a wide variety of immunological responses and lead to cross-reactivity issues. However, somatic antigen appears to elicit a stronger antibody response in cattle, albeit one with limited specificity.

Other diagnostic methods for *Dicrocoelium* infection in naturally infected sheep investigated were the agar gel precipitation test (AGPT), counter immunoelectrophoresis (CIEP), and passive haemagglutination test (PHT). AGPT had a specificity of 93.3%, CIEP 84.0%, and PHT 93.3%. For the Sero epidemiological survey of dicrocoeliasis in sheep and goats, CIEP proved to be the most sensitive, specific, and fast test (Jithendran *et al.,* 1996). In order to detect *Dicrocoelium* eggs in animal feces, a method was devised to hatch *Dicrocoelium* eggs, collect the miracidium DNA, and created a PCR detection tool (Sandoval *et al.,*2013). The identification of the *Dicrocoelium* larval stages in mollusks and ants, the parasite's first and second intermediate hosts, has also been studied using morphological (Manga-González *et al.,* 2001) and molecular techniques (PCR). Research on adult parasites has been conducted in a number of areas, including isoenzymatic characterization (Campo *et al.,* 1998), genetic variability (Sandoval *et al.,* 1999; Morozova *et al.,* 2002), molecular identification via partial sequencing of 18S rDNA and ITS-2 of rDNA (Otranto *et al.,* 2007), the 28S and ITS-2 (Maurelli *et al.,* 2007). It has been established that *D. dendriticum* and *D. chinensis* are distinct species (Maurelli *et al.,* 2007) as well as that *D. dendriticum* and *D. hospes* are distinct species (Otranto et al., 2007). The ITS-2 rDNA was a suitable marker for determining evolutionary relationships among *Dicrocoelium* species (Biant *et al.,* 2015). Additionally, *D. dendriticum* haplotypes in sheep, goats, and cattle in Iran have been identified using the ITS-2 and the NADH dehydrogenase gene (nad1) (Gorjipoor *et al.,* 2015).

1.11. Treatment, Control, and Prevention

It is challenging to apply preventative and control measures to dicrocoeliasis caused by *Dicrocoelium* due to the complexity of the biological life cycle. The most effective method of preventing the spread of *Dicrocoelium* in ruminants in the absence of a vaccine is to treat them with an effective anthelminthic while also taking into account the epidemiological models for dicrocoeliasis in that area (Manga-González *et al.,* 2001, 2010 Manga-González and González-Lanza, 2005). Some anthelmintics used against *Dicrocoelium* in ovine include albendazole, fenbendazole, luxabendazole, thiophanate, netobimin, and diamphenethide; however, none of these are effective against juvenile and immature stages of *Dicrocoelium* (Stratan, 1986). Restrictive husbandry techniques, such as refraining from grazing in the morning or evening when the herbage is most likely to contain ants in tetany, may be used to control dicrocoeliosis. The use of molluscicides and insecticides to control intermediate hosts is prohibited due to cost and environmental issues (Otranto and Traversa, 2003).

1.12. Current Investigation

Geographically the conditions in the northern areas of Pakistan are conducive to parasitic life because of its diverse climatic conditions. In Pakistan, parasitic outbreaks caused by helminths in domestic animals are frequent, which seriously harms the local animal husbandry industry and, in turn, the nation's economy. Although China, India, and Iran, which are neighbors, have reported a high number of cases of *Dicrocoelium,* there is limited information on the disease in Pakistan. Pakistan shares a border with these countries, and animal migration and transportation are taking place, which increases the chances of disease transmission.

Dicrocoeliasis spread is greatly influenced by the climate. The prevalence of the disease has been linked to a number of environmental factors, including temperature, precipitation, and evapotranspiration rate. The prevalence and geographic distribution of *Dicrocoelium* infection are correlated with these environmental factors, and species distribution models (SDMs) have the potential to identify the spatial pattern of disease and ecological niches supporting infection challenge. SDMs are based on the interaction

of a species' capacity for adaptation and important climatic predictors such as altitude, temperature, rainfall, and humidity (Soberon and Nakamura, 2009; Elith, 2011; Bosso, 2018; Smeraldo, 2018). Geographical Information Systems (GIS) and Maximum Entropy (MaxEnt) are the most commonly used SDMs in fluke parasite research. These models are used to depict the geographical distribution and spatial pattern of fasciolosis and schistosomiasis, as well as the risk factors associated with ecological niches and climatic conditions (McCann *et al.,* 2010; Fox *et al.,* 2011; Chen *et al.,* 2015; Bennema *et al.,* 2017; Meshgi *et al.,* 2019a). The geographic distribution of dicrocoeliasis has only been the subject of a few studies, and as far as we are aware e none has been reported in Asia.

The phenotypic traits of adult worms, such as body width and length, are traditionally used to identify flukes by their species (Valero *et al.,* 2018). However, morphological differences may be skewed due to the existence of intermediate forms, according to a number of studies (Shafiei *et al.,* 2014). Furthermore, Dosay *et al.,* (2005) demonstrated that intraspecific variation was greater than the interspecific variation between different liver fluke species and specimens from various hosts. Molecular methods for amplifying fragments of nuclear ribosomal genes and their internal transcribed spacers or mitochondrial loci DNA have been developed for Dicrocoeliid parasites (Maurelli *et al.,* 2007; Otranto *et al.,* 2007; Martinez-Ibeas *et al.,* 2011; Liu *et al.,* 2014; Bian *et al.,* 2015; Gorjipoor *et al.,* 2015). These methods, which can be used to demonstrate genetic variability and phylogeny, have been used in epidemiological studies of various trematode parasite species affecting livestock (Chaudhry *et al.,* 2016; Ali *et al.,* 2018; Sargison *et al.,* 2019; Rehman *et al.,* 2020).

The diagnosis of diseases in humans and animals with various aetiologies, such as infectious, neoplastic, parasitic, deficient diseases, and intoxications, is assisted by histopathological investigation. Most of the time, parasite illnesses remain misdiagnosed. A superficial diagnosis frequently results in protracted or even unsuccessful treatment. Examining organs or tissues histopathological enables a detailed and precise diagnosis. For differential diagnosis and frequently verifies the existence of specific parasite invasions, detection of histological alterations during investigating histopathological

changes in parasitic infection for parasite differentiation and presence of the parasitic disease (Madej *et al.,* 2008; McGavin *et al.,* 2001; McGavin and Zachary, 2006). Similarly, serology may be a useful tool for detecting exposure as well as existing infections, which are not detected by other diagnostic techniques. Since conventional methods for diagnosing helminths have very low sensitivity and specific antibodies typically decrease six months after treatment, which aids in detecting current and recent infections, antibody serology plays a particularly crucial role in the diagnosis of helminth infection (WHO, 2020; Buonfrate *et al.,* 2015).

Pakistan is a semi-tropical country with ideal environmental conditions for the survival and reproduction of a wide range of parasitic organisms. Parasitic diseases are of critical importance and cause low productivity in livestock, but they are often overlooked by livestock owners due to the low mortality associated with parasitic infections. In comparison to other parts of Pakistan, the mean daily temperature ranges in the studied areas create an arid environment with only patchy coniferous tree cover, providing habitats that are mostly hostile to many snail species and potentially creating isolated habitats for *Dicrocoelium* spp. flukes. To improve livestock productivity, it is critical to investigate parasite hotspots, identify species, and understand parasite biology to implement control measures.

Keeping in view the significance of dicrocoeliasis, the current study was designed with the following objectives.

- To determine the degree of morphometric variability among the different populations of *Dicrocoelium* in the Himalayan ranges of Pakistan and to investigate the pathological complications caused by the *Dicrocoelium* species.
- Molecular characterization and phylogenetic relationship of *Dicrocoelium* spp. Infecting sheep and goats in the Himalayan ranges of Pakistan
- To investigate the prevalence and spatial distribution of dicrocoeliasis in the region and to describe the ecological niches that are favorable for the completion of the *Dicrocoelium* life cycle.
- To estimate the time of infection and characterize the transmission pattern of *Dicrocoelium* in the sheep and goats of the Himalayan ranges of Pakistan.

PHENOTYPING AND HISTOPATHOLOGICAL COMPLICATION IN THE LIVER OF SHEEP AND GOATS INFECTED WITH *DICROCOELIUM* SPECIES

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 aim of the present study was to examine the morphometric variation and histopathological complications in the liver of sheep and goats infected with *Dicrocoelium*. Ten (10) specimens of the lancet fluke from each population were used for comparative analysis by using the available keys. The principal component analysis (PCA) was used for the variability in *Dicrocoelium* species and in population. Liver samples were collected from sheep to investigate the histopathology caused by an infection in the studied areas. One hundred and ninety adult *Dicrocoelium* parasitizing sheep and goats from Khyber Pakhtunkhwa and Gilgit Baltistan, Pakistan, were morphometrically analyzed. To evaluate the degree of variation among the population, 26 characters were compared. These results showed that the morphological traits of *Dicrocoelium* are a little variable among this study's population. The histopathological analysis of the liver showed that animals were heavily infected; the infection of *Dicrocoelium* causes intensive liver destruction and that the presence of adult *Dicrocoelium* spp. in the bile ducts is related to biliary hyperplasia. Severe infiltration of the inflammatory cells, RBCs congestion, damaged hepatocytes, and sinusoids was observed in the infected areas. Congestion in the blood vessels, inflammation of the inflammatory cell, damaged hepatocytes, and sinusoids in the vicinities of the central vein and uterine eggs were observed. Infiltration of inflammatory cells, especially macrophage and lymphocytes, necrosis around the central vein, congestion of blood vessels, and pigmentation were seen in the infected tissues. The accumulation of the lymphocytes causes necrosis which is associated with chronic inflammation.

Conclusion: In conclusion, Molecular analysis on a large scale is necessary to accurately identify whether the studied population constitutes one or several species of *Dicrocoelium*.

Also, it is concluded that *Dicrocoelium* causes severe inflammation and necrosis of liver tissues.

Keywords: *Dicrocoelium*, Morphometry, inflammation, necrosis

INTRODUCTION

Dicrocoelium is a trematode parasite typically found in ruminants. The parasite occasionally results in economic losses due to liver condemnation and causes mild symptoms up until a high infection level (Otranto *et al.,* 2002). *Dicrocoelium* has three host snails, ants, and ruminants where it can complete its life cycle (Dawes, 1968; Krull and Mapes, 1952a; Krull and Mapes, 1952). Lancet flukes lay their eggs inside the definitive host, which they then excrete in feces. The first intermediate host snail, such as *Cochlicopa lubrica,* consumes the miracidium-containing eggs, which then transform into the mother sporocyst. A large number of the infectious cercariae released from the mother sporocyst which grow into metacercaria. When they are consumed by ants of the genus *Formica* (Linnaeus, 1758). The infectious metacercaria then becomes encysted in the ant's abdomen, where it is fed by grazing animals. Metacercaria invades the specific mammalian host as it enters the host body.

Dicrocoelium significantly reduces livestock productivity, by affecting female conception rates, animal development rates, liver condemnation, and milk supply, and increases the cost of anthelmintic treatment (Arbabi *et al.,* 2018). Specific clinical signs of infestation are typically absent, even in severe infections. The primary liver injuries on a macroscopic level are fibrosis, enlargement, and inflammation of the bile ducts (Jithendran and Bhat, 1996). Large lesions caused by liver damage can only be found after a post-mortem examination (Otranto *et al.,* 2003; Rojo *et al.,* 2012), and they are directly correlated with the parasite load (Jithendran and Bhat,1996) and chronic bile duct inflammation (Colwell and Goater, 2010). Histopathological changes in experimentally infected lambs were characterized by a wide spectrum of lesions, including periductal fibrosis, ductal response, and leukocyte infiltration (Ferreras *et al.,* 2007). Very few reports examined the immunopathological characteristics in animals infected with *D. dendriticum,* while numerous studies assessed the phenotypic expression of inflammatory cells in animals infected with *Fasciola hepatica* (Meeusen *et al.,* 1995; Chauvin and Boulard, 1996; Ferreras *et al.,* 2007).

Dicrocoelium dendriticum is most frequently reported throughout Europe, North Asia (China), Japan and Indo- The Malayan region, North Africa, South America, and in a few areas of North America and Australia (Otranto and Traversa, 2003). The rarest species, *D. hospes,* and *D. chinensis,* have been reported from Africa and China (Loss, 1907; Malek, 1980; Tang and Tang, 1978). Species-based identification of lancet flukes is difficult, especially through morphology, and limited reports are available on lancet flukes phenotyping from Pakistan and rest of the world.

Therefore, the present work aimed to determine the degree of morphometric variability among the different populations of *Dicrocoelium* in Khyber Pakhtunkhwa and Gilgit Baltistan province, Pakistan and investigate the pathological injuries caused by the infection in these provinces.

MATERIAL AND METHODS

2.2.1. Study regions

The study area comprises the Gilgit Baltistan and Khyber Pakhtunkhwa provinces of Pakistan (Figure 2.2.1). Gilgit Baltistan has a border with China through the Khunjerab pass, which occupies an area of over 72,971 km southwest of the Karakoram range. The weather conditions include average rainfall of 120 to 240 mm. One district of Gilgit Baltistan was included in the study; (i) Gilgit district annually. Additional irrigation is obtained from the rivers, abundant with melting snow water from higher altitudes. The Khyber Pakhtunkhwa has a border with Afghanistan to the west and north and spreads over an area of over 74,521 km included in the study; (ii) Chitral district to the north of the Indus River, which originates close to the. Three districts of Khyber Pakhtunkhwa were the holy mountain of Kailash in western Tibet. The average elevation is 1,500 m, and the daily mean temperature ranges from 4.1°C to 15.6°C, creating an arid environment with only patchy coniferous tree cover, and providing habitats that are hostile to many snail species; (iii) Swat district surrounded by Chitral and Dir districts. The area is predominantly rural, and most residents live in villages. The average elevation is 980 m, resulting in a considerably cool and wet climate with lush forests, verdant alpine meadows, and snow-capped mountains. The climate of the Swat district is warm and humid, with short and moderate summers, temperature rarely rises above 37°C. The annual rainfall averages around 33 inches, with about 17 inches during June-September; (iv) Dir district borders Afghanistan on the north and the Swat district to the east. The climate is cold, with average rainfall is 700 mm and the temperature varies from 6°C to 38°C.

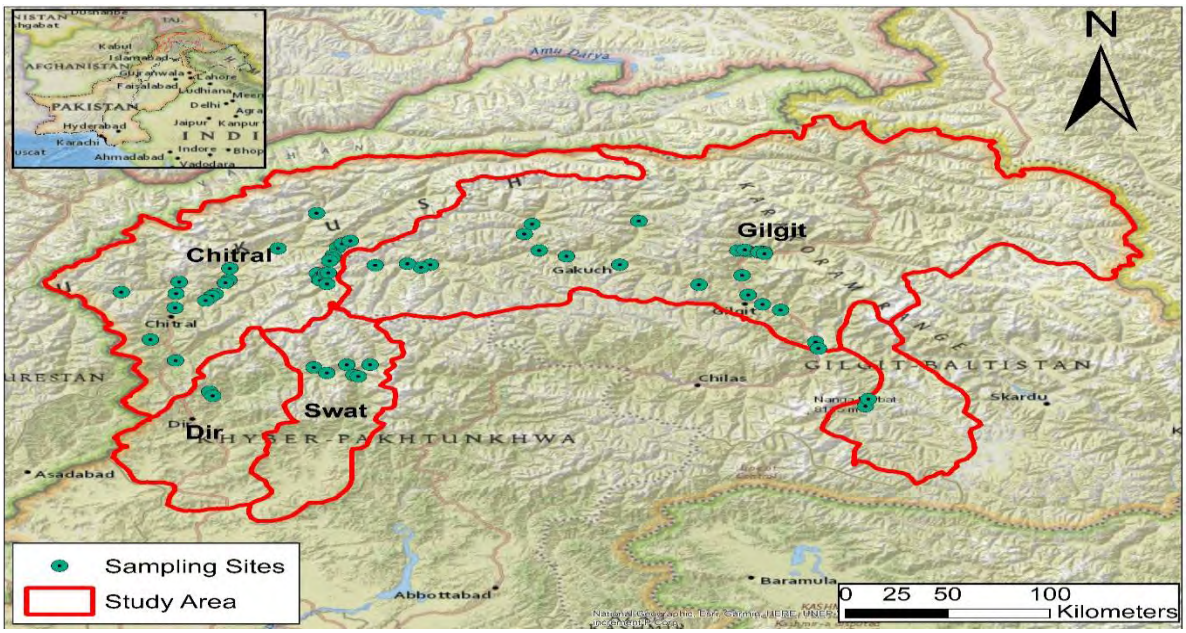


Figure 2.2.1. Map of the study area and sampling sites, Fluke collection from various regions

A total of 169 sheep and 45 goats from the Chitral region were evaluated; the sheep included 68 from Booni, 26 from Torkhow, 33 from Mastuj, 17 from the Laspoor valley, 7 from Mori Payeen, 4 from Drosh, 8 from Brun, and 6 from Garam Chashma. The 45 goats were 19 from Chinar, 14 from Unshit, 7 from Gasht, and 5 from Rondur. Four infected goats and 17 infected sheep that had been slaughtered in various locations in the Chitral district of Khyber Pakhtunkhwa province, Pakistan were found to have adult flukes (25 to 300 per animal) in their livers.

Gilgit Baltistan region provided a total of 64 sheep and 62 goats for evaluation; the sheep included 24 from Dalomal, 14 from Yasin Valley, 11 from Raushan, 7 from Danyor, and 8 from Gorikot, while the 62 goats included 18 from Phander, 11 from Damalgan, and 33 from Chalt Nagar. Seven infected sheep and three infected goats, slaughtered at various locations throughout the region of Gilgit Baltistan, had adult flukes (25 to 300 per animal) found in their livers.

In the Swat district, a total of 15 goats and 26 sheep were inspected; the sheep came from Gabral (n=11), Utrar (n=6), and Boyun (n=9), while the goats came from the Kalam

region. Only 2 infected sheep slaughtered in each of the various locations had adult flukes (ranging in number from 100 to 300 per animal) in their livers. The flukes were cleaned with phosphate-buffered saline (PBS) to get rid of any adhering material before being preserved in 70% ethanol for morphometric and molecular analysis.

2.2.2. Morphological examination of flukes

One hundred and ninety adult flukes were selected from the livers of three infected sheep from Booni (Pop-1, Pop-2, Pop-3), one from Torkhow (Pop-4), one from Mastuj (Pop-5), one from Laspoor (Pop-6), one from Brun (Pop-7), one from Dalomal (Pop-8), one from Yasin Valley (Pop-9), two from Raushan (Pop-10, Pop-11), one from Gabral (Pop-12), one from Boyun (Pop-13), and one goat from Chinar (Pop-14), one goat from Gasht (Pop-15) and one goat from Chalt Nagar (Pop-16). The selected flukes were stained for morphometric analysis.

2.2.3. Staining of lancet flukes

Before fixation, the specimen is fixed between two slides and then fixed in a formalin-acetic alcohol solution for 10-12 hours; likely in figure 2.2.2; the fixed specimen is removed from the slides and put into ethanol. Then they were stained and mounted by following these steps; the specimens were brought down to the water through descending series of ethanol.

100% ethanol ➡ 90% ethanol ➡ 80%ethanol ➡ 70% ethanol ➡ 50% ethanol, then transferred this specimen to a hematoxylin stain (Appendix # 1) until the specimen became purple. The staining period of lancet flukes from 5-10 minutes; after proper staining, the specimen was removed from hematoxylin and transferred to a watch glass containing tap water and left until it became dark blue. A differentiation step followed by discarding the water and applying acid alcohol in the mentioned ratio/volume.

(9.9 vol. of 70% ethanol ± 0.1 vol. of concentrated HCl), the specimens were left for 5 minutes.

70% ethanol ➡ 80% ethanol ➡ 90% ethanol ➡ 100% ethanol. The hydrated specimens were cleared for 3-5 minutes in xylene and finally, the cleared specimens were mounted in Canada Balsam (Luna, 1968).

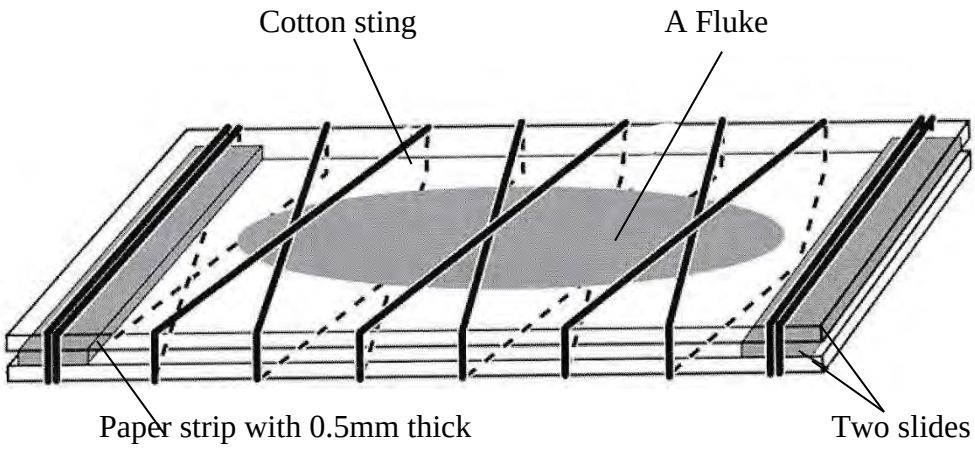


Figure 2.2.3 Fixing of fluke specimen between two slides

2.2.4. Morphological characteristics of adult flukes

The twenty-seven standardized bio morphometric characters were taken for each lancet fluke as described by Falcon-Ordaz *et al., (*2019). Body length (BL), maximal body width (BW), oral sucker length (OSL), oral sucker width (OSW), cirrus pouch length (CPL), cirrus pouch width (CPW), ventral sucker length (VSL), ventral sucker width (VSW), anterior testes length (ATL), anterior testes width (ATW), posterior testes length (PTL), posterior testes width (PTW), right vitelline length (RVL), right vitelline width (RVW), left vitelline length (LVL), left vitelline width (LVW), ovary length (OL), ovary width (OW), distance from the front of the anterior testes to the front of the body, distance from the posterior part of the testes to the front of the body, distance from the right vitelline gland to front of the body, distance from the right vitelline to the tail of the of the body, distance from the left vitelline to the front of the body, distance from the left vitelline to the tail of the body, and distance from the ovary to the front of the body were taken Eggs were isolated from the uterus of adult lancet flukes and egg length (EL) and egg width (EW) were measured. All the measurements were taken in micrometers (µm), while body length and body width were measured in millimeter (mm).

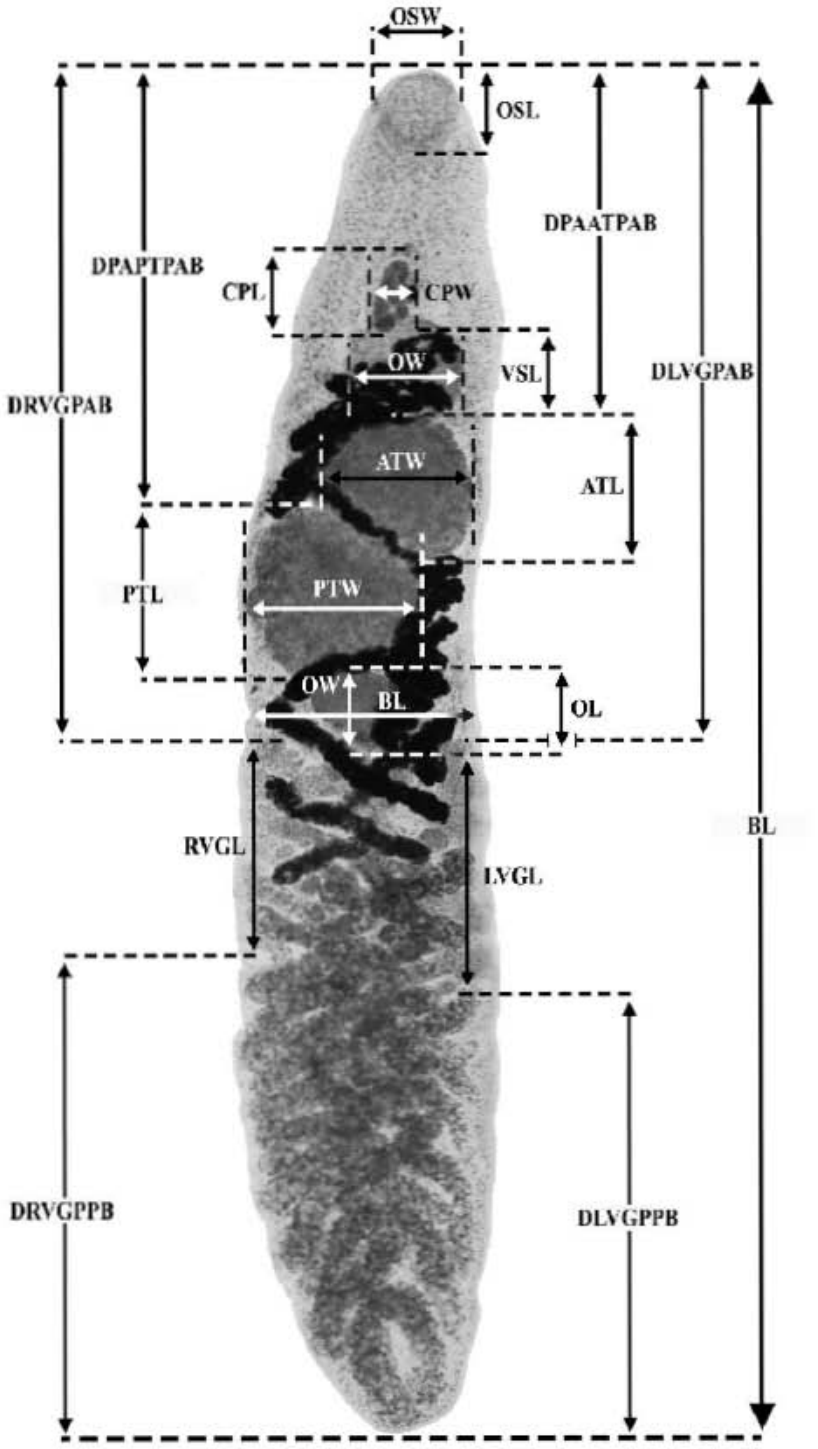


Figure 2.2.4. *Dicrocoelium*: worm showing morphometric features used as variables.

2.2.5. Histopathological examination of infected liver

Histology of infected sheep and goat's liver tissues were carried out to check the histopathological complication of liver infected with *Dicrocoelium*. The infected livers were collected from slaughtered animals; the subsequent processes are as follows (Ullah *et al.,* 2019).

2.2.5.1. Fixation of infected livers

The *Dicrocoelium* infected livers were fixed in PBS formalin (10 %) for 24-48 hours.

2.2.5.2. Dehydration of tissues

After fixation, the water was removed from the fixed tissues by passing through different grades of alcohol, all the steps were carried out at room temperature, the step is as follows.

70 % Ethanol ……120 min

80 % Ethanol ……120 min

90 % Ethanol ……120 min

100 % Ethanol ……...3 times

(Transfer after two hours each time during the last step).

2.2.5.3. Tissues embedding

The dehydrated tissues were then placed in xylene and passed through the following steps of xylene and fixed in paraffin as follows.

Xylene I ---------- 60 min

Xylene I ---------- 60 min

Paraffin I (58 ^{o}C) ----------120 min

The embedded tissues were transferred to paper boats containing melted wax. Bubbles were completely removed from the vax and left to solidify. Before mounting on wooden blocks for section cutting, a knife or scalpel was used for trimming the blocks of paraffin wax.

2.2.5.4. Preparing albumin slides

In the first step, albumen was prepared by adding two egg whites in 1200 ml of deionized water. The solution was stirred on a magnetic stirrer for 5 minutes. 4 ml concentrated ammonium hydroxide was added and mixed well. The albumin was stored in the dark, in a screw-top glass bottle at 40 ^{o}C. The albumen was filtered through a low-grade filter (coffee filter).

2.2.5.5. Coating slides

Coating of slides was done by placing each slide on a slide warmer at a low temperature. A very thin layer of egg albumin was applied on each slide by using a small brush, and the same process was repeated three times at least. After this, the slides were dried on the slide warmer, albumin coated slides were kept at room temperature in the original packaging until use.

2.2.5.6. Microtomy of the tissues

The tissues embedded blocks were placed on a microtome and 2-3 μm thin sections of paraffin-embedded tissues were cut by microtome in which wooden blocks were placed (Shandon, Finesse 325, UK). The long ribbons of wax having tissues were stretched, following fixation on previously prepared albumenized glass slides. These slides were kept on the slides warmer at 60 ^{o}C. To stretch it fully the glass slides were placed in an incubator overnight. The slides were deparaffinized in xylene and the sections were rehydrated in descending grades of alcohol and stained in different grades of chemicals (Appendix No.1). After proper staining the slide was treated with Canada balsam and placed in the incubator for a short period of time.

2.2.5.7. Microscopy and Microphotography

Prepared slides were observed under a light microscope (Leica LB Germany). 2-3μm sections were observed at 10X and 40 X magnification. Hepatocytes, central vein, inflammatory cells, cirrhosis, hyperplasia, etc., were observed under the microscope. A Leica LB microscope (Germany) paired with Canon digital camera (Japan) was used for the microphotography of the sections.

2.2.6. Statistical analysis

Geometrical morphometrics (Rohlf and Marcus, 1993) is a technique for measuring morphological variation that gives an estimate of size by combining various growth axes into a single variable known as the "centroid size" (Bookstein, 1989). Shape is defined as the relative positions of the landmarks after taking into account their sizes, positions, and orientations, with the goal of incorporating the estimate of size into a single variable that reflects variation in as many directions as there are landmarks being taken into account. With the aid of these data, important biological and epidemiological aspects can be quantified more accurately, and the associated software to carry out complex analysis is readily available (Dujardin, 2008). Modern statistical morphometric techniques can be used to test the null hypothesis that conspecific populations are merely the allometric extension of one another if a common allometric trend can be found (Rohlf and Marcus, 1993; Dujardin and Le Pont, 2004). Multivariate analyses were used to identify phenotypic variations in lancet fluke samples, with size-free canonical discriminant analysis being applied to the covariance of log-transformed observations. Canonical discriminant analysis was also performed using PAD V. 98. (Dujardin, 2010). These studies are employed to reduce the contribution of each characteristic to the first pooled within-group principal component (a multivariate size estimator), thereby removing the contribution of ontogenetic changes within the group (Dos Reis *et al.,* 1990). A large number of variables in a dataset are reduced to a manageable number of dimensions using principal component analysis (Dujardin and Le Pont, 2004). The resulting "allometry-free" or size-free variables were subjected to a canonical variate analysis (CVA), and Mahalanobis distances were calculated (Mahalanobis, 1936). Multiple methods of phenotypic analysis of lancet flukes were carried out using the CLIC program (Dujardin, 2002). $P<0.05$ indicated that the results were statistically significant.

RESULTS

2.3.1 Comparative biometric analysis amongst *Dicrocoelium* species

Table 2.3.1 provides comparative characteristics (measurements in mm and µm) with their extreme values, mean and standard deviation. *D. dendriticum* has a translucent, dorsoventrally flattened body that is between 1.6 and 8 mm long and 0.48 and 1.84 mm wide, whereas *D. chinensis* is between 5.68 and 8 mm long and 1.2 and 1.28 mm wide (Figure 2.3.1). The oral sucker of *D. dendriticum* and *D. chinensis* is subterminal and measures 80 - 400 µm and 168 - 408 µm, respectively. The slightly larger ventral sucker (80 - 408 µm and 160 - 560 µm) is in the anterior quarter of the body. Just behind the intestinal bifurcation is where the genital pore is located. The ovary is located beneath the posterior testis and measures 40 – 320 µm by 40 - 480 µm in *D. dendriticum*, and 164 - 416 µm by176 - 412 µm in *D. chinensis*. The anterior end of the slightly lobed testes is close to the posterior margin of the ventral sucker, and they are arranged in the body in an oblique manner (Figure 2.3.2). The anterior testis of *D. dendriticum* is approximately 88-640 µm long and 92 - 736 µm wide, while the posterior testis is 88-728 x 120-896 µm. *D. chinensis* anterior testis measures about 408-736 x 328 - 880 µm and the posterior testis is 332-732 x 488-808 µm. At the level of the ventral sucker, the vasa efferentia unite to form a short vas deferens that enters the cirrus pouch and forms a seminal vesicle. For both *Dicrocoelium* species, the operculate eggs have a diameter of 12 x 8 µm (Figure 2.3.3). However, variations and overlapping ranges of morphometric measurements were detected for various traits between both species.

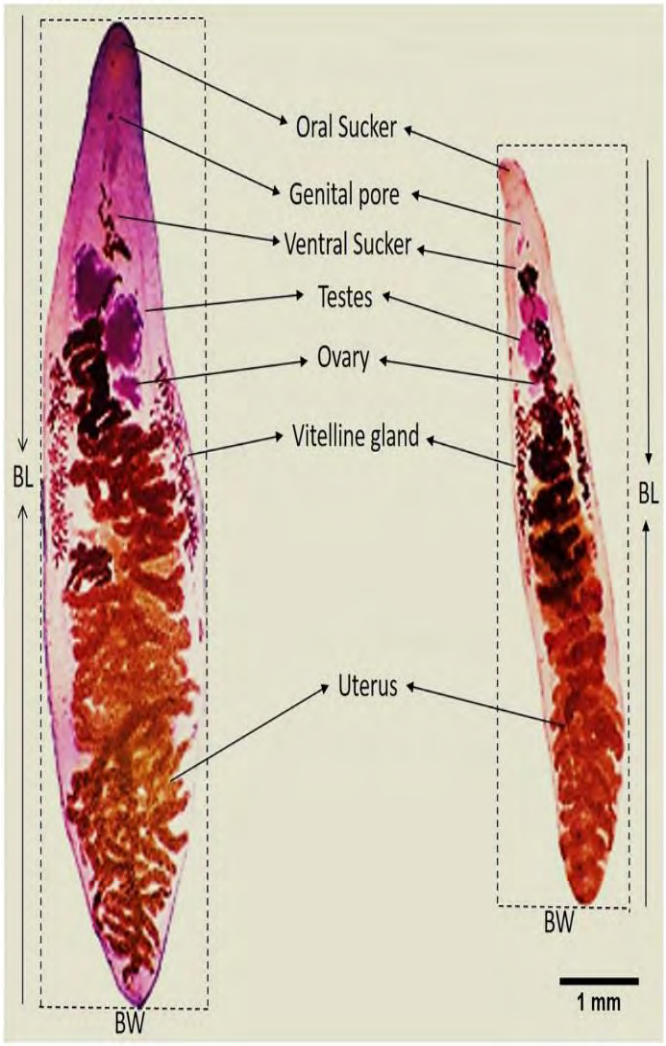


Figure 2.3.1. Light micrograph of hematoxylin-stained flukes from the present study. *Dicrocoelium dendriticum* (left) and *Dicrocoelium chinensis* (right). The bodies are pointed at both ends and semi-transparent, with a pair of lobate testes behind the ventral sucker. The ovary is small, and the uterus has both ascending and descending limbs and white vitellaria. The morphometric measurements and orientation of the testes of 160 flukes were similar to those shown on the left, and the equivalent features of 15 flukes were similar to those shown on the left.

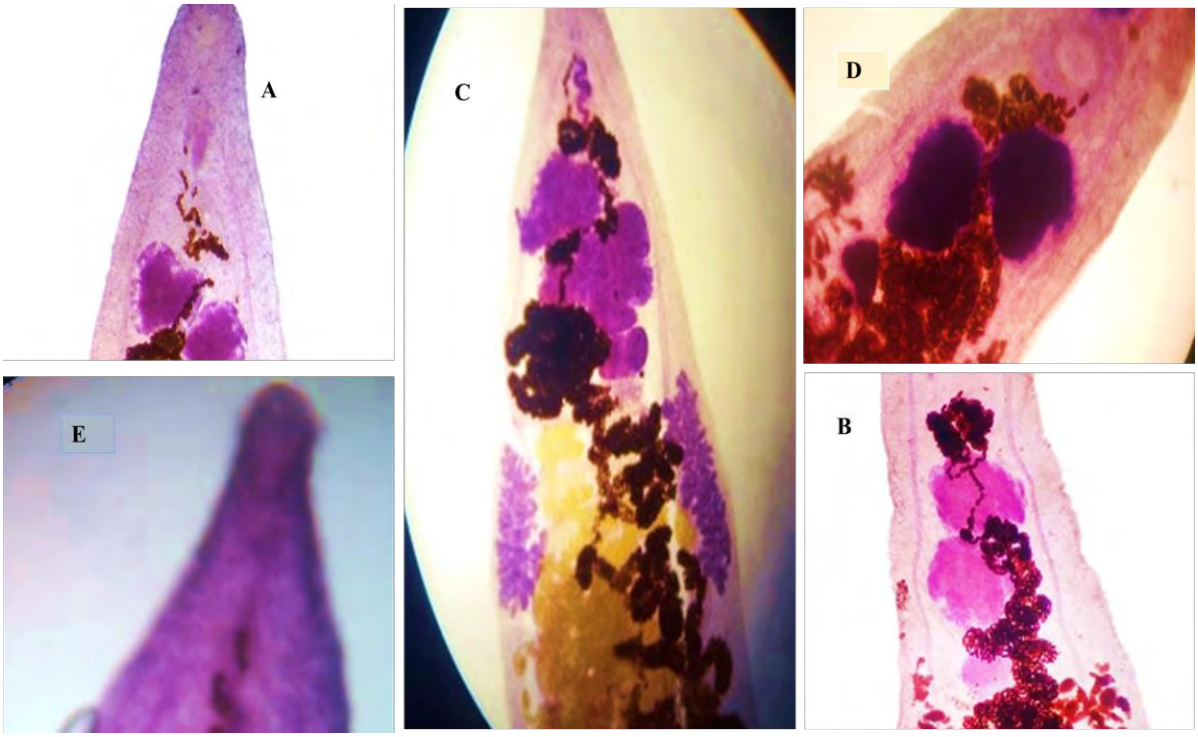


Figure 2.3.2. (A), (B), (C), (D), and (E): hematoxylin-stained flukes, variation in testis shape, ovary, and vitelline glands of *Dicrocoelium* spp.

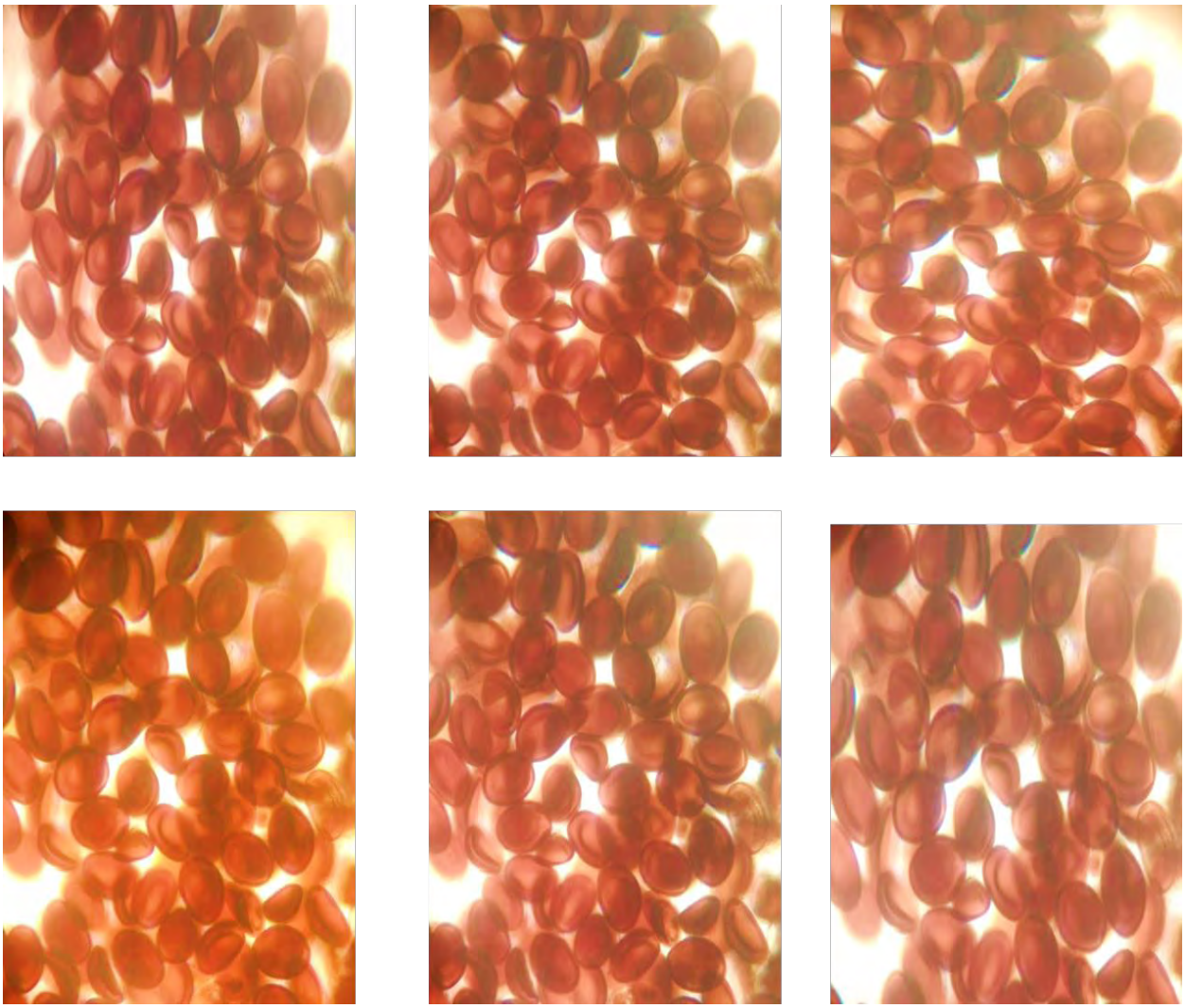


Figure 2.3.3. Microscopic images of the uterine eggs of *Dicrocoelium* collected from northern area of Pakistan.

Table 2.3.1. Comparative morphometric values (minimum values - maximum values, mean ± standard deviation) of *Dicrocoelium dendriticum* and *Dicrocoelium chinensis* from studied populations.

Species	*D. dendriticum* (n=160)		*D. chinesis* (n=15)	
Measurement Parameters	**Mean ± St.D**	**Min - Max**	**Mean ± St.D**	**Min - Max**
Body length (BL) (mm)	3.75 ± 1.55	1.6 – 8	6.97 ± 0.62	5.68 - 8
Maximal body width (BW) (mm)	0.94 ± 0.33	0.48 - 1.84	1.21 ± 0.02	1.2 - 1.28
Oral sucker length (OSL)	175.34 ± 75.53	80 – 400	320.53 ± 70.3	168 - 408
Oral sucker width (OSW)	168.26 ± 70.31	80 – 328	268.53 ± 61.6	168 - 400
Ventral sucker length (VSL)	194.68 ± 80.08	80 – 408	332.53 ± 96.51	160 - 560
Ventral sucker width (VSW)	191.48 ± 76.84	80 – 404	322.4 ± 88.54	160 - 488
Cirrus pouch length (CPL)	137.6 ± 62.43	80 – 328	280.53 ± 84.76	240 - 560
Cirrus pouch width (CPW)	70.83 ± 31.09	40 – 252	116 ± 37.68	80 - 160
Anterior testes length (ATL)	265.18 ± 115.26	88 – 640	535.73 ± 99.49	408 - 736
Anterior testes width (ATW)	299.73 ± 140.59	92 – 736	648.27 ± 130.06	328 - 880
Posterior testes length (PTL)	290.23 ± 141.93	88 – 728	568.8 ± 116.38	332 - 732
Posterior testes width (PTW)	310.83 ± 148.92	120 – 896	606.13 ± 142.37	488 - 808
Right vitelline length (RVL)	877.73 ± 415.72	404 - 2172	1615.73 ± 338.97	1120 - 2320
Right vitelline width (RVW)	160.53 ± 76.99	40 – 408	301.6 ± 37.85	244 - 336
Left vitelline length (LVL)	862.85 ± 397.47	400 - 2120	1544 ± 359.06	1200 - 2240
Left vitelline width (LVW)	166.15 ± 76.55	40 – 400	380 ± 240.79	244 - 1208
Ovary length (OL)	117.38 ± 65.63	40 – 320	293.6 ± 84.85	164 - 416
Ovary width (OW)	167.45 ± 99.6	40 – 480	260 ± 75.31	176 - 412
Egg length (EL)	12 ± 0	12 – 12	12 ± 0	12 - 12
Egg width (EW)	8 ± 0	8 – 8	8 ± 0	8 - 8
Distance from front of the anterior testes to front of the body	757.73 ± 335.63	304 - 1880	1354.67 ± 447.44	720 - 2080
Distance from posterior part of the testes to front of the body	941.3 ± 387.65	320 - 2400	1569.33 ± 416.81	960 - 2160
Distance from the right vitelline gland to the front of the body	1327.5 ± 528.48	648 - 3040	2422.67 ± 406.44	1924 - 3048
Distance from the right vitelline the tail of the body	1500.83 ± 702.05	560 - 3612	2837.07 ± 619.99	1760 - 3520

Distance from the right vitelline to the tail of the body	1347.55 ± 516.78	648 - 2880	2509.33 ± 429.13	2000 - 3360
Distance from the left vitelline to the front of the body	1580.7 ± 746.21	568 - 3696	2947.47 ± 631.3	1768 - 3612

Min: minimum, Max: maximum; SD: Standard deviation; mm: millimeters;

2.3.1.1 Multivariate analysis amongst species

Multivariate analysis was used to measure the changes in the size of *D. dendriticum* and *D. chinensis*. First, a principal component analysis was performed, and the 12 non-redundant measurements were analyzed altogether (BL, BW, OSL, OSW, VSL, VSW, ATL, ATW, PTL, PTW, OL, OW). The two species appear to overlap in the factor map, i.e., *D. dendriticum* and *D. chinensis.* The contribution of the two principal components was PCI: 74% and PCII: 10%. The results showed that the sizes of *D. dendriticum* and *D. chinensis* are very close to each other (Figure 2.3.4). The centroid size variation (Figure 2.3.5) of *Dicrocoelium* species showed that *D. dendriticum* had larger than *D. chinensis*. Size-free conical discriminant analyses of *Dicrocoelium* adults from Pakistan were carried out. The scatter plot of conical factors (CFI and II) showed that the standard population of *D. dendriticum* and *D. chinensis* are very close. The large (close to 1) value of Wilks' lambda = 0.617, $P \leq 0.05$ showed that the two populations are not significantly discriminated and overlapping of size was observed (Figure 2.3.6).

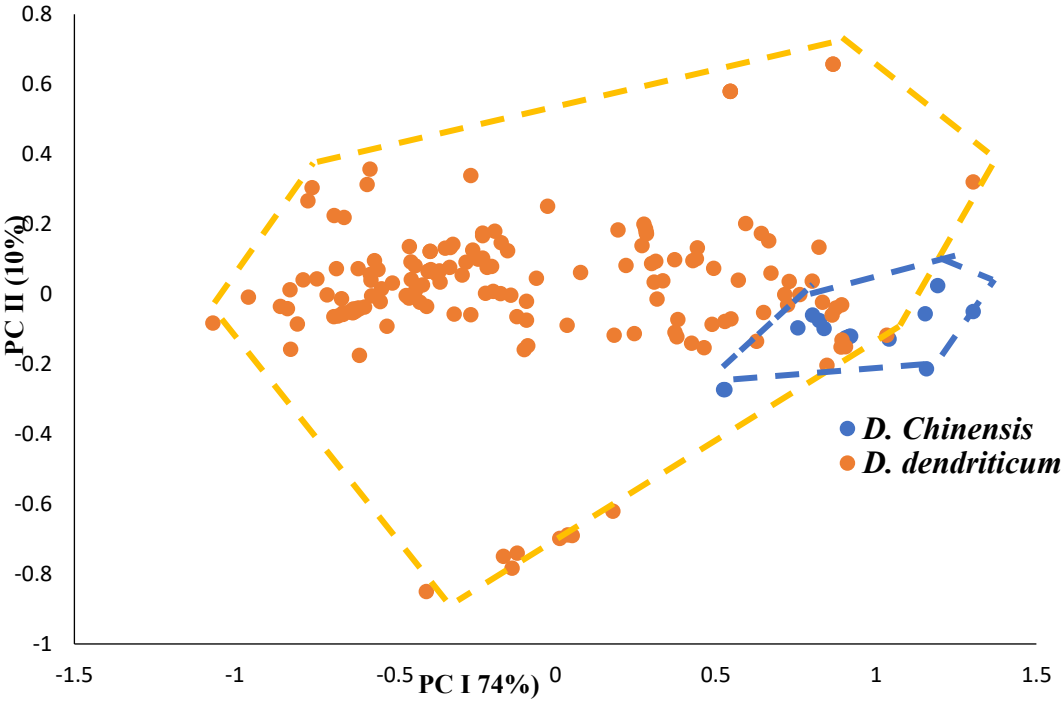


Figure 2.3.4. Principal component analysis factor map of *D. dendriticum* and *D. chinensis*.

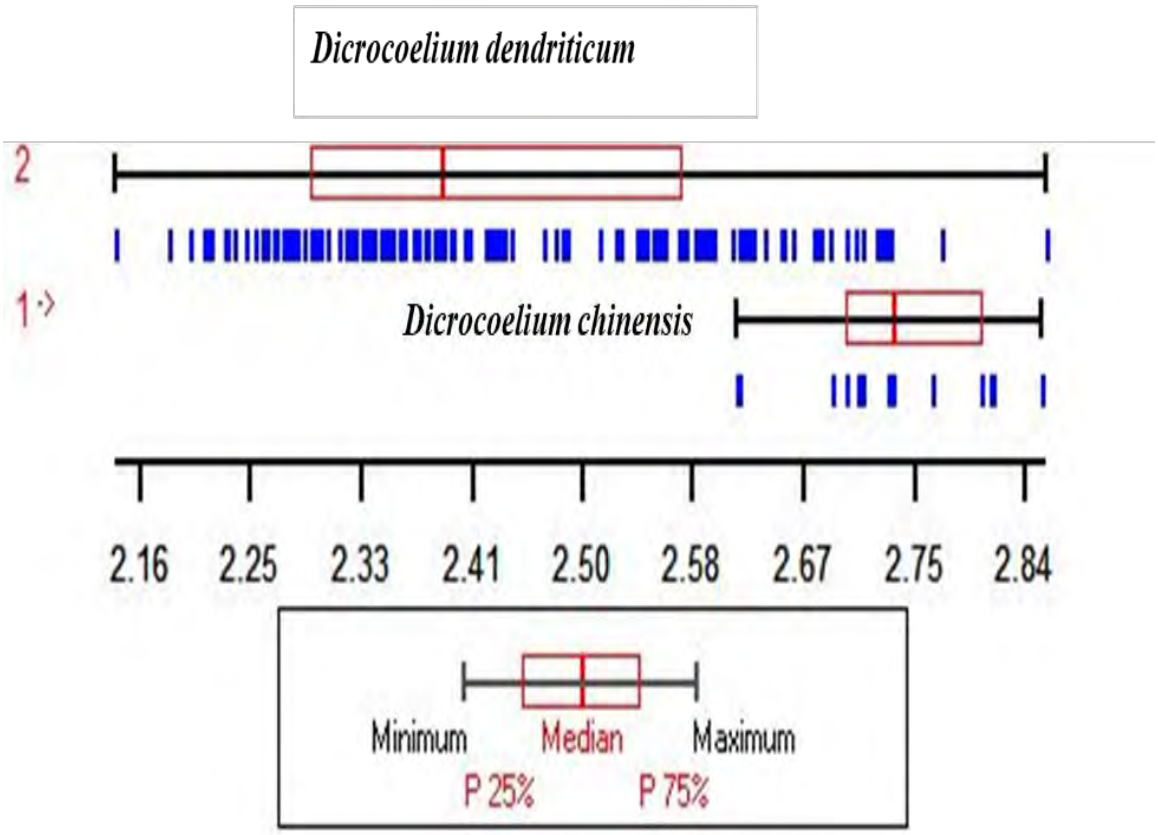


Figure 2.3.5. Centroid size variations among *Dicrocoelium* species are presented as quantile plots. Vertical lines under the quantiles are the number of organisms examined. Each box characterizes the median as a line across the middle and quartiles (25 percentiles) as its ends. Units are in millimeters (mm).

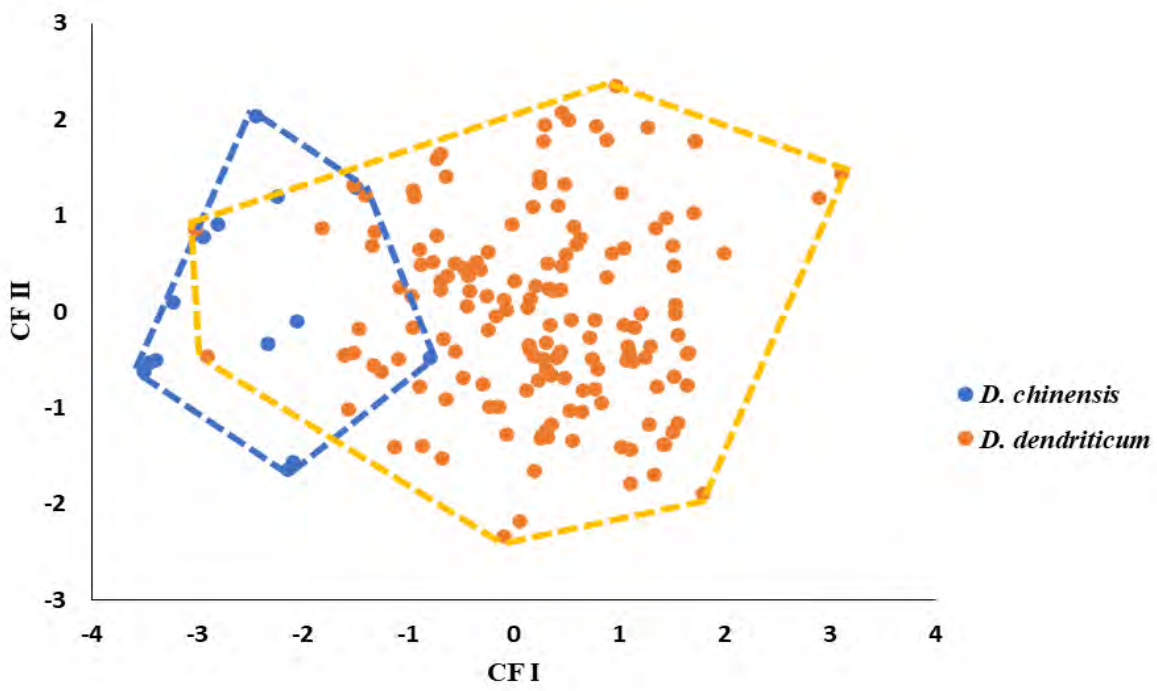


Figure 2.3.6. Discriminant analysis, showing conical factors (CFI and CFII) map of *D. dendriticum* and *D. chinensis*.

2.3.2. Comparative biometric and multivariate analysis of *Dicrocoelium* amongst geographical area

Table 2.3.2 provides comparative characteristics (measurements in mm and µm) with their extreme values, mean and standard deviation. *Dicrocoelium* from Gilgit measured 1.8-6.8 mm in length and 0.48-1.6 mm in width, whereas *Dicrocoelium* from Chitral and Swat measured 1.6-8.0, 2.28-4.4 mm in length and 0.56 to 1.84 and 0.64 to 0.92 mm in width, respectively. In all study districts, the ventral and oral suckers of *Dicrocoelium* were about the same size. However, variations and overlapping ranges of morphometric measurements were detected for various traits.

In the factor map, the size of *Dicrocoelium* species from Gilgit and Chitral is nearly in the same range. The factors (PCI and II) map showed that Chitral and Gilgit populations are clearly discriminated. The Swat population showed overlapping traits, with contribution PCI was 70%, and PCII was 12% (Figure 2.3.7). The centroid size variations (Figure 2.3.8) showed that *Dicrocoelium* from Chitral were larger than Gilgit and Swat populations. The discriminant analysis of the 12 non-redundant measurements was analyzed all together (BL, BW, OSL, OSW, VSL, VSW, ATL, ATW, PTL, PTW, OL, OW). The conical factors (CFI and II) map of the *Dicrocoelium* population from Chitral, Gilgit, and Swat displayed overlapping traits. The large (close to 1) value of Wilks' lambda = 0.66, $P \leq 0.05$ showed that three populations were not significantly discriminated and overlapping of size was observed between them (Figure 2.3.9).

Table. No.2.3.2: Area wise comparative morphometric values (minimum values - maximum values, mean ± standard deviation) of *Dicrocoelium* species from studied populations.

Worms Collected Areas	Gilgit		Swat		Chitral	
Measurement Parameters	Mean ± St.D	Min - Max	Mean ± St.D	Min – Max	Mean ± St.D	Min - Max
Body length (BL) (mm)	2.86 ± 0.97	1.8 - 6.8	3.19 ± 0.52	2.28 - 4.4	4.37 ± 1.69	1.6 -8
Maximal body width (BW)(mm)	0.78 ± 0.26	0.48 - 1.6	0.78 ± 0.08	0.64 - 0.92	1.07 ± 0.34	0.56 -1.84
Oral sucker length (OSL)	145.52 ± 78.92	80 - 400	144 ± 27.22	80 – 200	198.87 ± 72.98	80 -328
Oral sucker width (OSW)	138 ± 69.61	84 - 328	142.4 ± 27.17	80 – 200	190.82 ± 69.48	80 -328
Ventral sucker length (VSL)	173.2 ± 79.89	80 - 400	148 ± 29.31	80 – 200	216.98 ± 80.66	88 -408
Ventral sucker width (VSW)	174.08 ± 81.15	80 - 404	146 ± 26.83	80 – 200	211.24 ± 75.76	88 -404
Cirrus pouch length (CPL)	128 ± 65.52	80 - 328	105.6 ± 34.49	80 – 200	150.04 ± 62.51	80 -328
Cirrus pouch width (CPW)	65.52 ± 19.97	40 - 88	54.8 ± 18.03	40 - 80	77.33 ± 36.35	40 -252
Anterior testes length (ATL)	235.52 ± 107.3	96 - 640	204.6 ± 50.72	160 – 320	295.12 ± 120.92	88 -576
Anterior testes width (ATW)	261.04 ± 134.79	92 - 736	211 ± 62.41	120 – 336	340.93 ± 141.96	120 -652
Posterior testes length (PTL)	250.8 ± 124.02	96 - 728	209 ± 54.66	160 – 336	330.18 ± 151.37	88 -724
Posterior testes width (PTW)	266.8 ± 117.94	160 - 572	220 ± 60.9	160 – 320	355.47 ± 161.9	120 -896
Right vitelline length (RVL)	707.44 ± 326.11	404 - 2000	730 ± 172.6	480 – 1080	1005.16 ± 454.12	408 -2172
Right vitelline width (RVW)	119.04 ± 48	40 - 248	137.2 ± 52.64	80 - 240	188.76 ± 82.68	40 -408
Left vitelline length (LVL)	669.52 ± 328.18	400 - 2080	732 ± 155.25	480 - 1080	999.34 ± 417.26	408 -2120
Left vitelline width (LVW)	133.92 ± 61.9	40 - 328	140.8 ± 51.55	80 - 240	189.7 ± 80.64	80 -400
Ovary length (OL)	124.8 ± 87.9	40 - 320	83.2 ± 16.7	56 - 128	120.85 ± 55.45	40 -280
Ovary width (OW)	163.84 ± 141.56	80 - 480	121.2 ± 28.93	80 - 200	179.73 ± 76.62	40 -332
Egg length (EL)	12 ± 0	12 - 12	12 ± 0	12 - 12	12 ± 0	12 -12
Egg width (EW)	8 ± 0	8 - 8	8 ± 0	8 - 8	8 ± 0	8 -8
Distance from front of anterior testes to front of the body	583.12 ± 240.84	304 - 1568	646 ± 78.16	520 - 840	879.56 ± 364.78	360 -1880

Distance from posterior part of the testes to the front of the body	751.84 ± 293.33	408 - 2000	886 ± 135.66	560 - 1080	1058.84 ± 427.01	320 -2400
Distance from the right vitelline gland to the front of the body	1070.24 ± 401.67	728 - 2800	1208 ± 184.72	800 - 1640	1496.98 ± 577.41	648 -3040
Distance from the right vitelline the tail of the body	1057.68 ± 331.04	648 - 2240	1350 ± 343.82	800 - 1920	1780.53 ± 774.85	560 -3612
Distance from the right vitelline to the tail of the body	1096.48 ± 389.96	656 - 2732	1210 ± 177.88	800 - 1600	1517.6 ± 563.67	648 -2880
Distance from the left vitelline to the front of the body	1076 ± 327.84	640 - 2016	1420 ± 303.94	800 - 2000	1896.8 ± 816.05	568 -3696

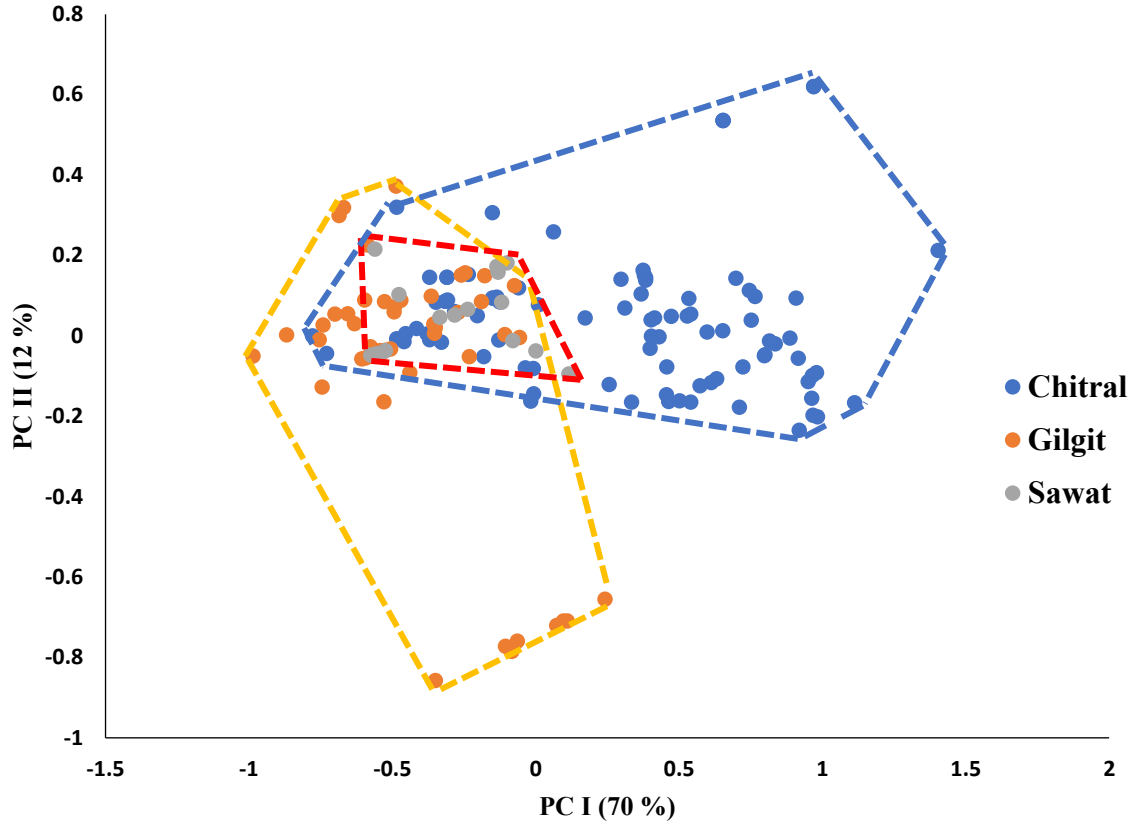


Figure 2.3.7. Principal component analysis factor map *Dicrocoelium* species-based study districts.

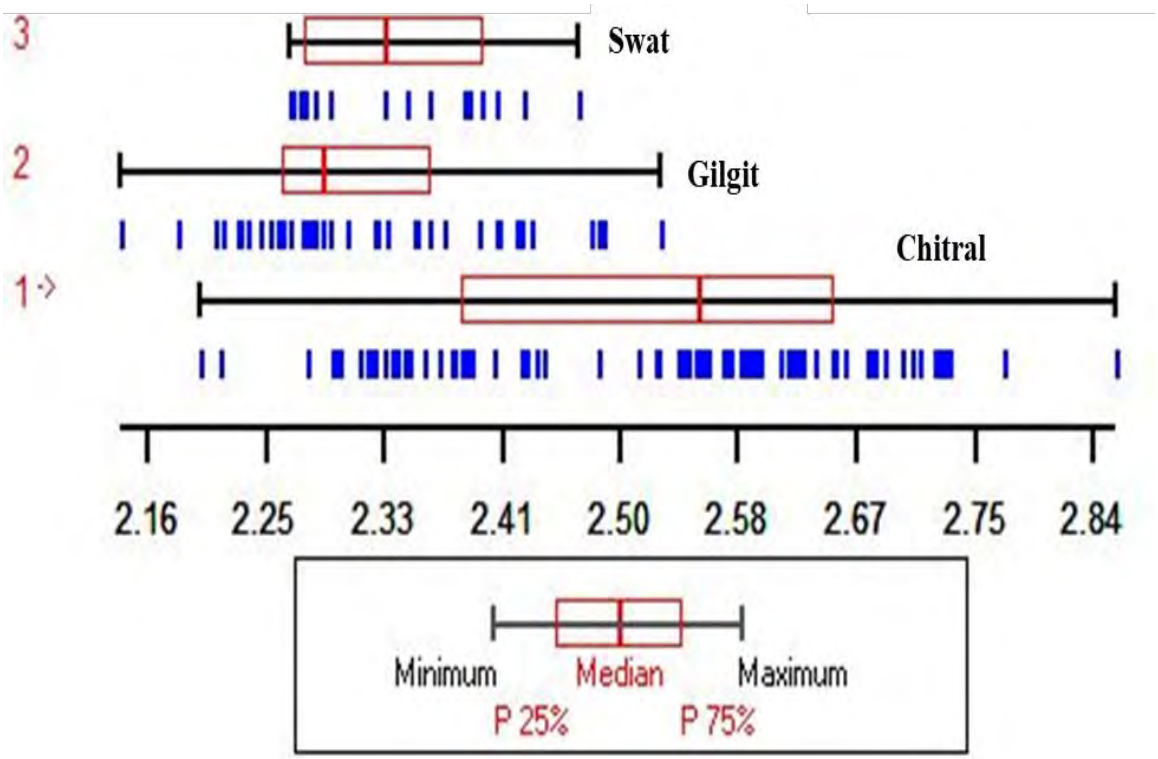


Figure 2.3.8. Centroid size variations among *Dicrocoelium* species from different study districts are presented as quantile plots. Vertical lines under the quantiles are the number of organisms examined. Each box characterizes the median as a line across the middle and quartiles (25 percentiles) as its ends. Units are in millimeters (mm).

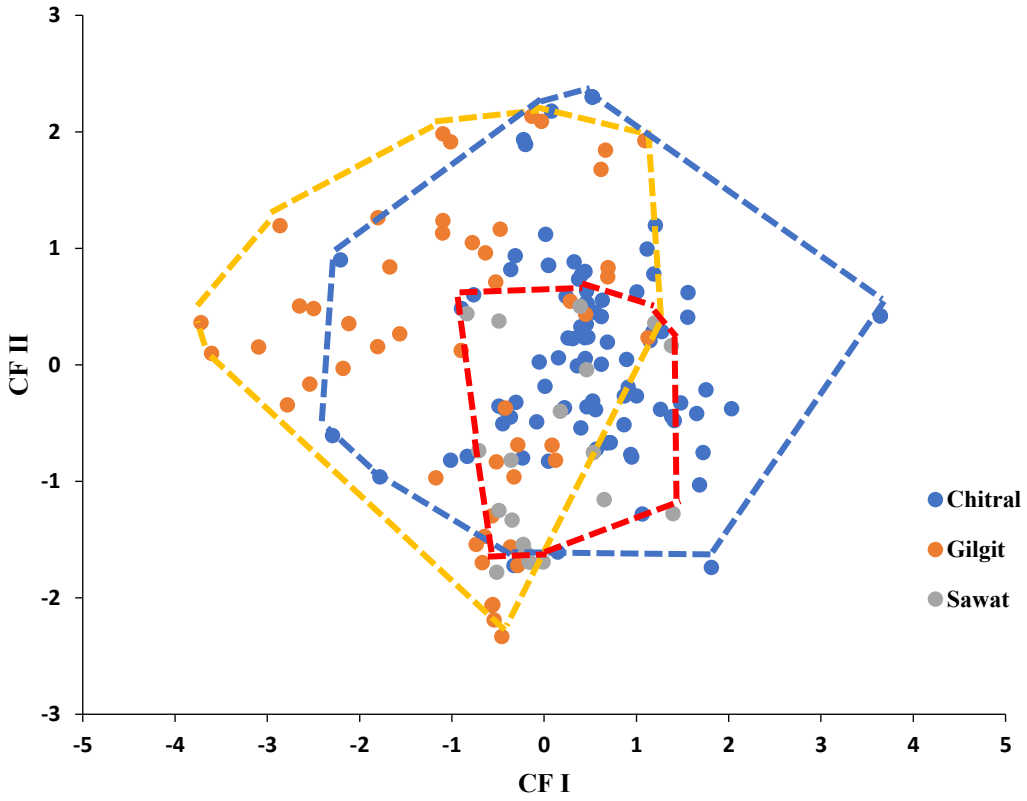


Figure 2.3.9. Discriminant analysis, showing conical factors (CFI and CFII) maps of *Dicrocoelium species* based on different study districts.

2.3.3. Comparative biometric and multivariate analysis of *Dicrocoelium* amongst Host

Dicrocoelium measurements ranged from 2.28 to 8 mm in length and 0.56 to 1.84 mm in width in goats, and from 1.6 to 7.2 mm in length and 0.48 to 1.6 mm in width in sheep (Table 2.3.3). In both hosts, oral and ventral suckers were similar in size. *Dicrocoelium* ovary measurements in goats ranged from 40 to 256 µm, while sheep measured 40 to 480 µm. In goats, the anterior testis of the *Dicrocoelium* measured roughly 160-568 x 160-644 µm, while the posterior testis measured 160-652 x 160-652 µm. The dimensions of the sheep's anterior and posterior testicles were 88–640 x 92–736 µm. and 88–728 x 120–896 µm, respectively. The diameter of the operculate eggs was 12 x 8 µm. (Fig,2.3.3). However, differences and overlaps in morphometric measurements for different traits between the two host species were found.

Factor map of *Dicrocoelium* in both hosts i.e., sheep and goats overlapped. The contribution of the principal component was PCI: 70% and PCII: 12% (Figure 2.3.103). Variation in the centroid size was larger in sheep than goats (Figure 2.3.11). The canonical factor map between hosts did not discriminate traits and showed close similarity. The large (close to 1) value of Wilks' lambda = 0.829, $P \leq 0.05$ showed that both groups are not significantly discriminated and overlapping of size was observed (Figure 2.3.12).

Table 2.3.3. Host wise comparative morphometric values (minimum values - maximum values, mean ± standard deviation) of *Dicrocoelium* species from studied populations

Host	Goat		Sheep	
Measurement Parameters	**Mean ± St.D**	**Min - Max**	**Mean ± St.D**	**Min - Max**
Body length (BL) (mm)	3.87 ± 1.57	2.28 - 8	3.71 ± 1.55	1.6 - 7.2
maximal body width (BW) (mm)	0.93 ± 0.37	0.56 - 1.84	0.95 ± 0.32	0.48 - 1.6
Oral sucker length (OSL)	174.85 ± 68.1	120 - 328	175.5 ± 78.12	80 - 400
Oral sucker width (OSW)	169.05 ± 64.24	120 - 324	168 ± 72.48	80 - 328
Ventral sucker length (VSL)	179.5 ± 71.96	120 - 328	199.73 ± 82.27	80 - 408
Ventral sucker width (VSW)	177.3 ± 68.29	120 - 336	196.2 ± 79.19	80 - 404
Cirrus pouch length (CPL)	122.8 ± 62.56	80 - 328	142.53 ± 61.86	80 - 328
Cirrus pouch width (CPW)	66.8 ± 45.48	40 - 252	72.17 ± 24.62	40 - 252
Anterior testes length (ATL)	256.9 ± 118.1	160 - 568	267.94 ± 114.67	88 - 640
Anterior testes width (ATW)	288 ± 145.02	160 - 644	303.63 ± 139.49	92 - 736
Posterior testes length (PTL)	276.2 ± 134.39	160 - 652	294.9 ± 144.59	88 - 728
Posterior testes width (PTW)	283.5 ± 143.55	160 - 652	319.93 ± 150.15	120 - 896
Right vitelline length (RVL)	887.4 ± 482.16	480 - 2160	874.5 ± 393.3	404 - 2172
Right vitelline width (RVW)	166.5 ± 81.37	80 - 332	158.53 ± 75.73	40 - 408
Left vitelline length (LVL)	879 ± 418.3	480 - 2120	857.47 ± 391.95	400 - 2080
Left vitelline width (LVW)	169.7 ± 79.87	80 - 336	164.97 ± 75.72	40 - 400
Ovary length (OL)	107.1 ± 47.86	40 - 256	120.8 ± 70.4	40 - 320
Ovary width (OW)	151.5 ± 64.58	80 - 328	172.77 ± 108.51	40 - 480
Egg length (EL)	12 ± 0	12 - 12	12 ± 0	12 - 12
Egg width (EW)	8 ± 0	8 - 8	8 ± 0	8 - 8
Distance from the front of the anterior testes to front of the body	746.3 ± 353.26	480 - 1880	761.53 ± 330.99	304 - 1680
Distance from the posterior part of the testes to front of the body	954.1 ± 449.65	560 - 2400	937.03 ± 366.67	320 - 2000
Distance from the right vitelline gland to front of the body	1327.8 ± 584.72	800 - 3040	1327.4 ± 511	648 - 2800
Distance from the right vitellineto the tail of the of the body	1568 ± 768.56	800 - 3200	1478.43 ± 680.39	560 - 3612

Distance from the right vitelline to the tail of the of the body	1350.4 ± 529.99	800 - 2880	1346.6 ± 514.55	648 - 2732
Distance from the left vitelline to the front of the body	1666.3 ± 779.7	800 - 3520	1552.17 ± 735.85	568 - 3696

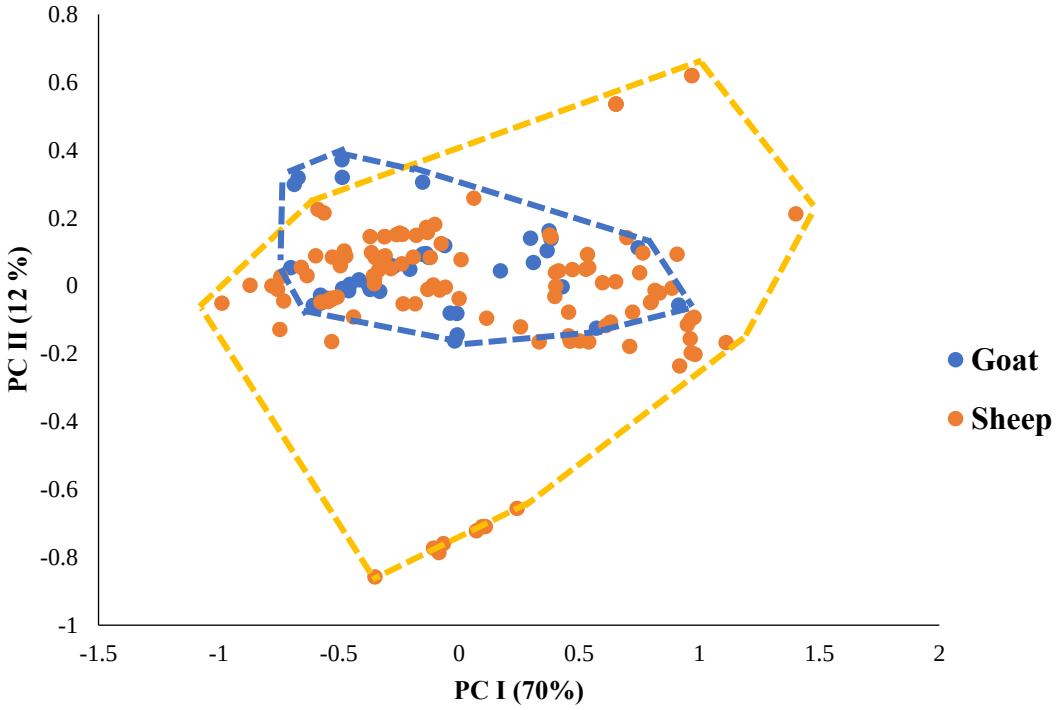


Figure 2.3.10. Principal component analysis factor map of *Dicrocoelium species* with respect to host (sheep and goats).

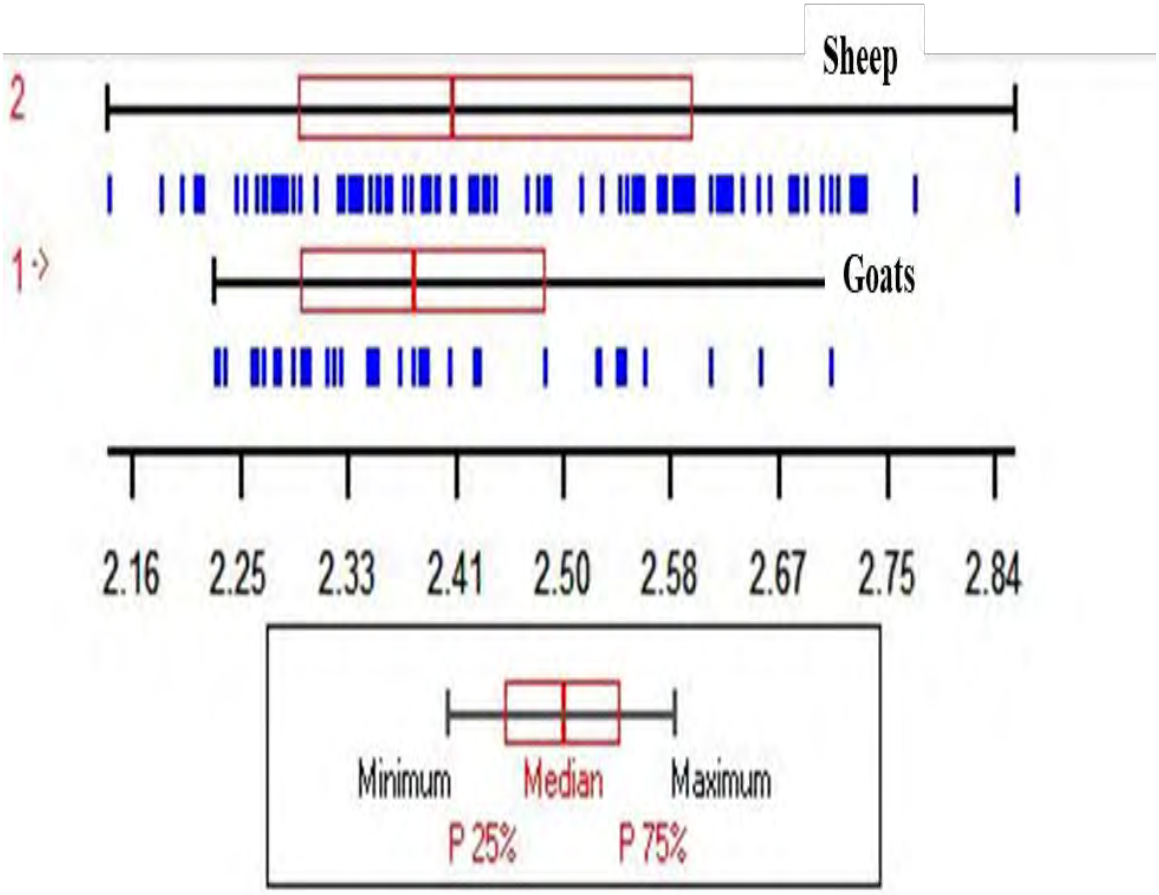


Figure 2.3.11. Centroid size variations among *Dicrocoelium* species from sheep and goats are presented as quantile plots. Vertical lines under the quantiles are the number of organisms examined. Each box characterizes the median as a line across the middle and quartiles (25 percentiles) as its ends. Units are in millimeters (mm).

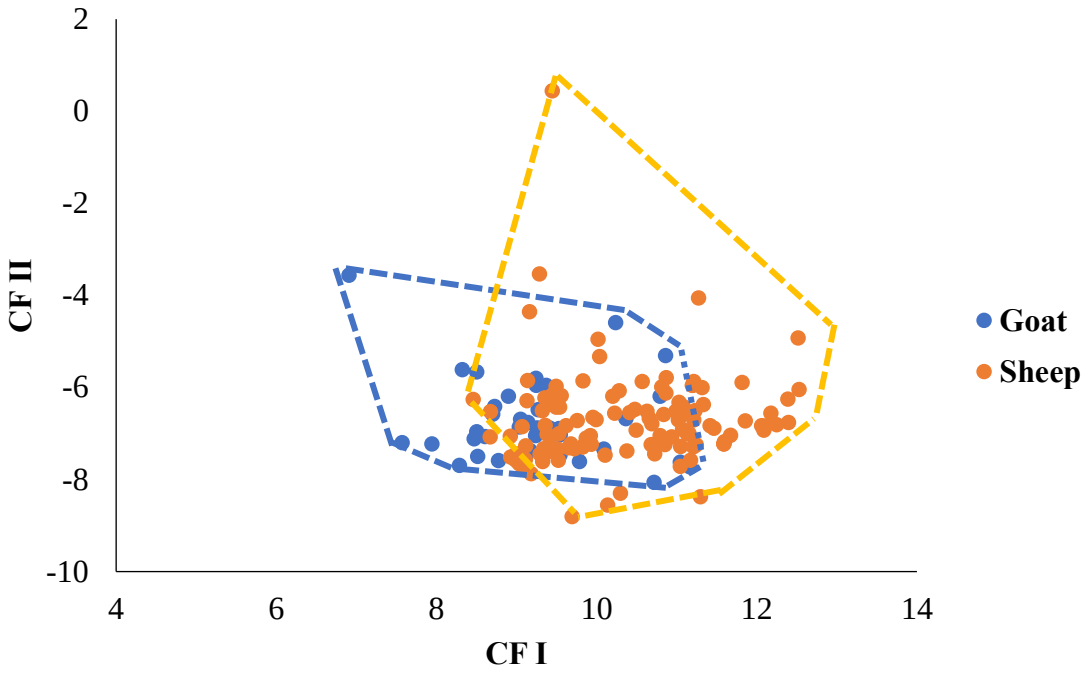


Figure 2.3.12. Discriminant analysis, showing conical factors (CFI and CFII) maps of *Dicrocoelium* species from sheep and goats.

Mahalanobis distances between each pair of groups were calculated for each discriminant analysis performed (Table 2.3.4ABC). The 12 non-redundant measurements were analyzed all together (BL, BW, OSL, OSW, VSL, VSW, ATL, ATW, PTL, PTW, OL, OW). *D. dendriticum* and *D. chinensis* present larger distances between them than among other groups i.e., geographical areas (Table 2.3.4B) and host (2.3.4C).

Table 2.3.4. Values of Mahalanobis distance between species (*D. dendriticum* and *D. chinensis*), geographically and host wise from studied population.

A	**Species**	***D. dendriticum***	***D. chinensis***	
	D. dendriticum	0.00		
	D. chinensis	2.80	0.00	
B	**Geographical Area**	**Chitral**	**Gilgit**	**Swat**
	Chitral	0.00		
	Gilgit	1.38	0.00	
	Swat	1.01	1.03	0.00
C	**Host Type**	**Goat**	**Sheep**	
	Goat	0.00		
	Sheep	1.04	0.00	

2.3.4. Gross Liver Pathology

Large numbers of flukes were detected in the bile ducts of each liver. The infected livers were cirrhotic and scarred, and the bile ducts were markedly distended, with thickened and fibrosed walls.

2.3.5. Histopathological Analysis

Histopathological examination of the infected sheep liver revealed a clear cross-sectional part of the oral sucker within the bile duct, severe infiltration of inflammatory cells, and RBC congestion (figure 2.3.13). The worm was found to be causing epithelium erosion, damage sinusoids and hepatocytes. Inflammation of the inflammatory cells and congestion of the liver's blood vessels at the portal areas were noted. Hepatocytes and inflammatory cells also clogged the central vein, and sinusoids nearby the central vein were also affected (Figure 2.3.14). The damaged hepatocytes, inflammatory cells and sinusoids are shown in figure 2.3.15. An adult lancet fluke was found in the bile duct, causing hyperplasia of the duct. Proliferative cholangitis is characterized by a severe proliferation of bile duct epithelial cells and a mild proliferation of goblet cells. Desquamative-necrotic cholangitis was characterized by epithelial cell degeneration and desquamation into the lumen. Bile duct epithelium necrosis was seen to exist (Figure 2.3.16). Additionally, uterine eggs were discovered; these eggs' shallow operculum, thick shell, small shoulders, and presence of miracidia served as distinguishing features; the sinusoid and hepatocytes in these regions were also completely damaged (Figure 2.3.17). Furthermore, these areas showed severe inflammation (Figures 2.3.18, 2.3.19). There was also a significant amount of periportal infiltration of inflammatory cells, particularly macrophages and lymphocytes, coagulative necrosis around the central vein, congestion of the blood vessels, and pigmentation (Figures 2.3.20, 2.3.21). Connective tissue was also seen to spread in either wide or narrow streaks between the hepatocytes of neighboring lobuli (Figure 2.3.22). The region was infiltrated by lymphocytes, macrophages, and a few eosinophils. In the portal region, there was much swollen connective tissue made up of fibroblasts and collagen (Figure 2.3.23). Kupffer cells can be round, oval, or triangular in shape and are widely distributed in constrained sinusoid regions. The bile duct lumen was filled with fluid and debris because of pericholangitis, and it was also obstructed

by lymphocytes, macrophages, and eosinophils. Livers of the control group showed no histological changes (Figures 2.3.24-2.3.26).

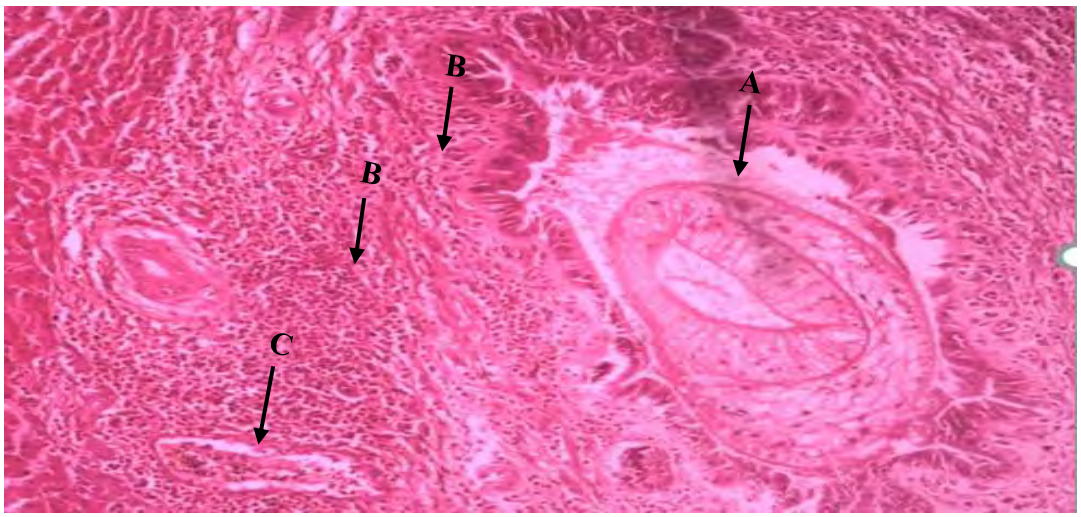


Figure 2.3.13: Histological section of sheep liver infected with *Dicrocoelium*, (A): cross-sectional section of parasite sucker in the lining epithelial cells of a septal bile duct, (B): showed RBCs congestion, (C): showed severe infiltration of the Inflammatory cells. Swelling of bile duct mucosal glands due to *Dicrocoelium* infection. Hematoxylin and eosin (HE). 100X

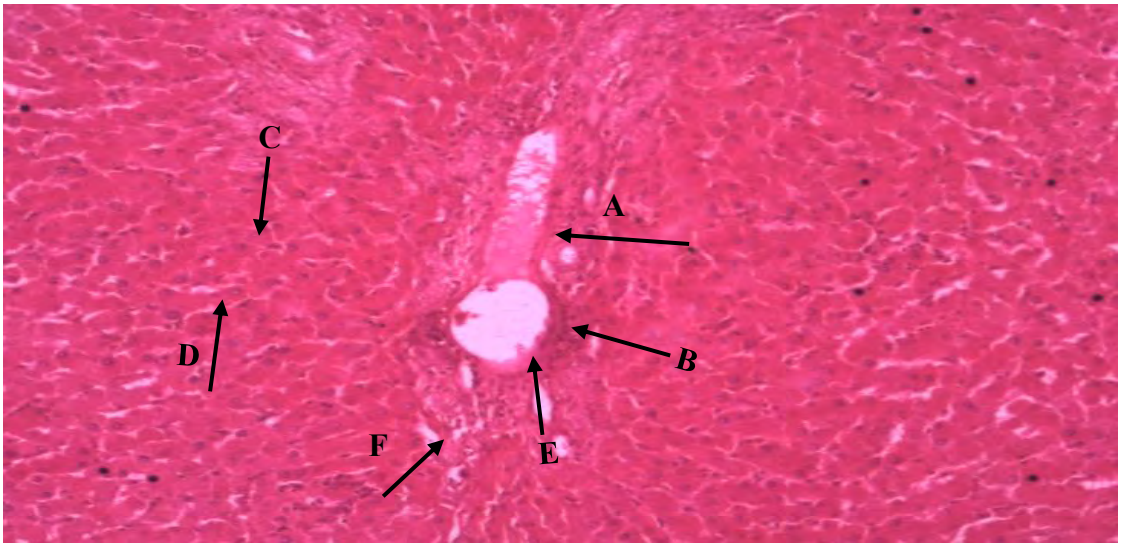


Figure 2.3.14 Histological section of sheep liver infected with *Dicrocoelium*. (A): showed congestion in liver blood vessels at the portal area (B): with inflammation of inflammatory cells (C): showed normal hepatocytes in the vicinities area while the nearest are the damaged hepatocytes (D): showed normal sinusoids (E): Central vein and (F): near the central vein are the affected sinusoids and hepatocytes. Hematoxylin and eosin (HE). 100X

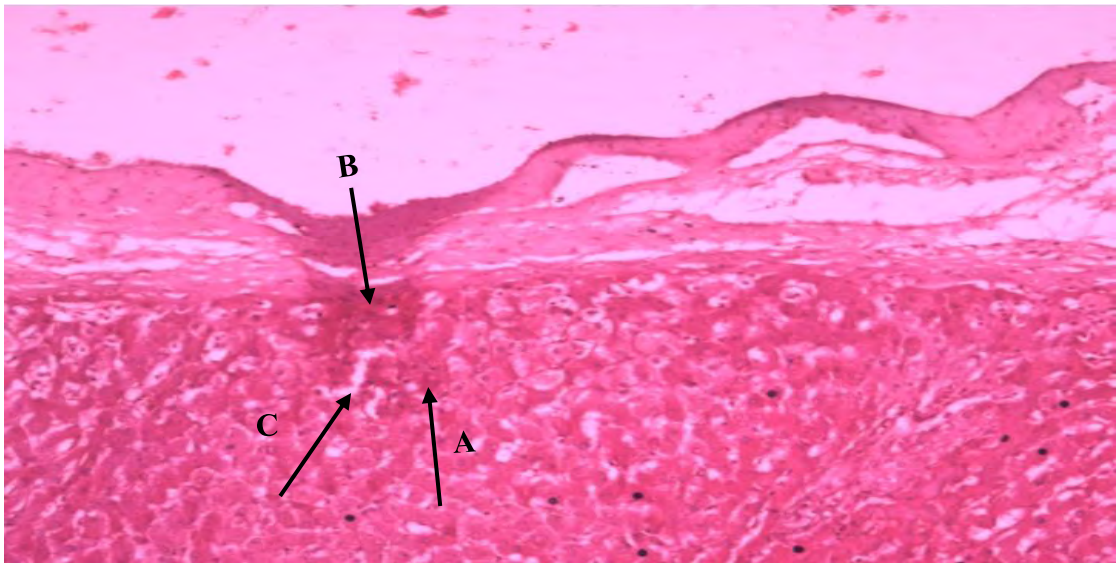


Figure 2.3.15 Histological section of sheep liver infected with *Dicrocoelium*, showed the absence of normal liver histology (A): damaged hepatocytes (B): Severe inflammation of the inflammatory cells (C): damaged sinusoids. Dark patches are also observed over the section. Hematoxylin and eosin (HE). 100X

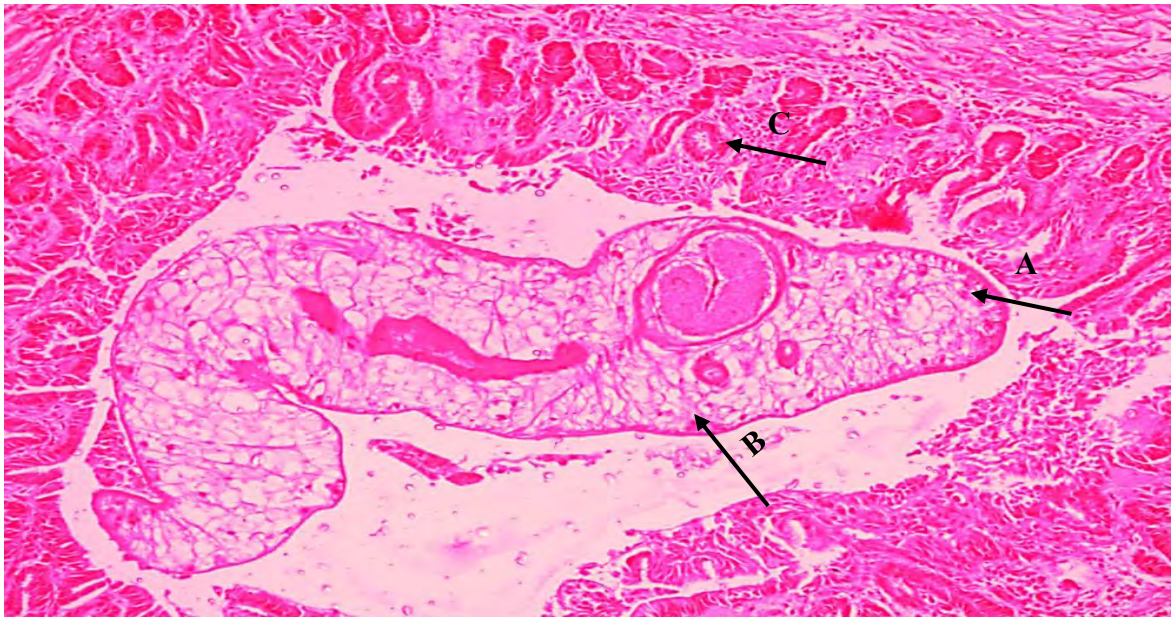


Figure 2.3.16 Histological section of sheep liver infected with *Dicrocoelium*, (A) showed the presence of adult *Dicrocoelium* worms in the bile duct (arrow), causing hyperplasia of the bile duct. (B) Histological appearance of a septal bile duct with severe epithelial papillary hyperplasia, degeneration, and desquamation of epithelial cells into the lumen. (C) Fibrosis and leukocyte infiltration around the biliary ducts in a severely infected liver. Hematoxylin and eosin (HE). 100X

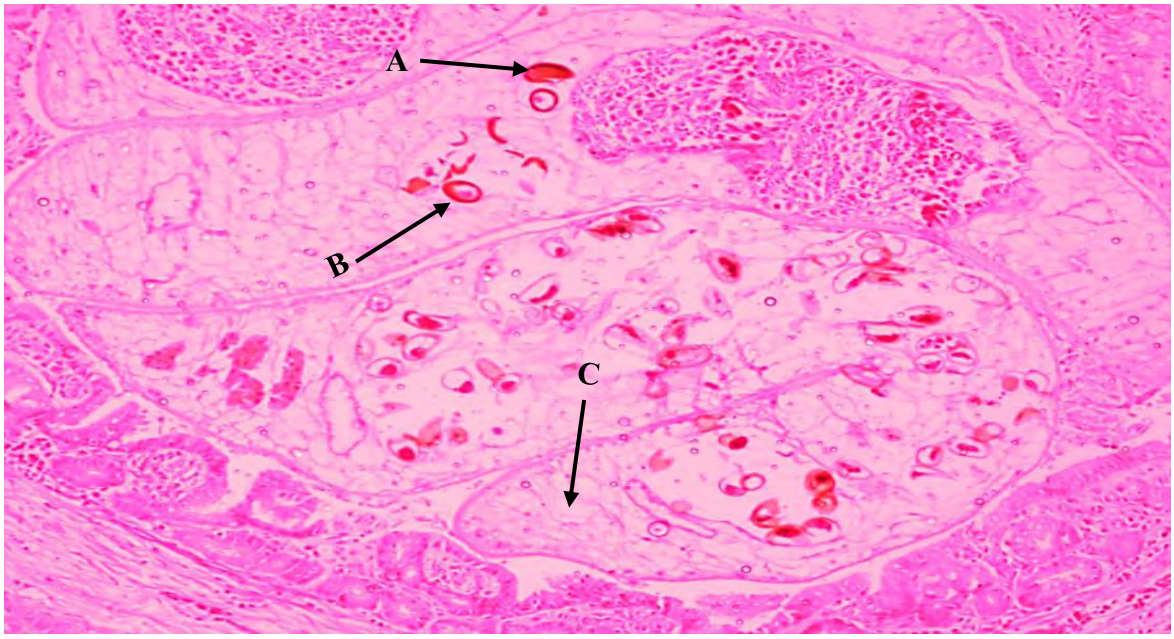


Figure 2.3.17 The histological section of an infected liver, revealing of hyperplasia and inflammatory cells. A and B: *Dicrocoelium* eggs inside the worm uterus are present in the bile duct of the sheep liver. C: The cross-sectional part of the *Dicrocoelium* specimen. Hematoxylin and eosin (HE). 100X

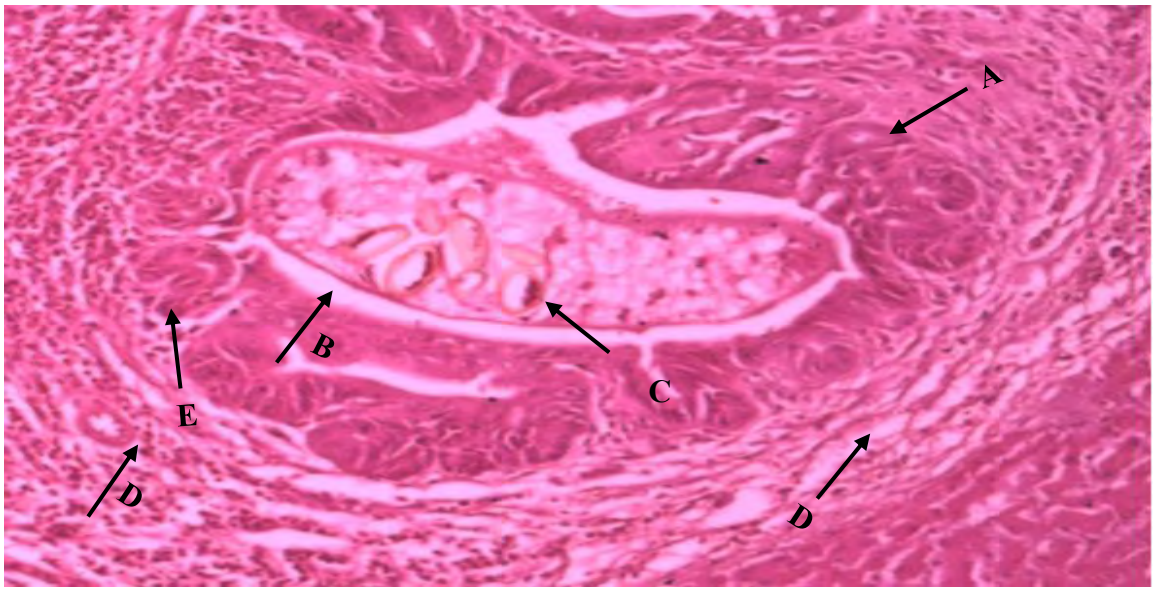


Figure 2.3.18 (A): Cross-sectional of *Dicrocoelium* inside the central vein (B): Central vein occupied by the parasite. (C): Eggs with miracidium (D): Rupture area (Sinusoid, hepatocytes) by the worm. (E): Inflammatory cells. Hematoxylin and eosin (HE). 100X

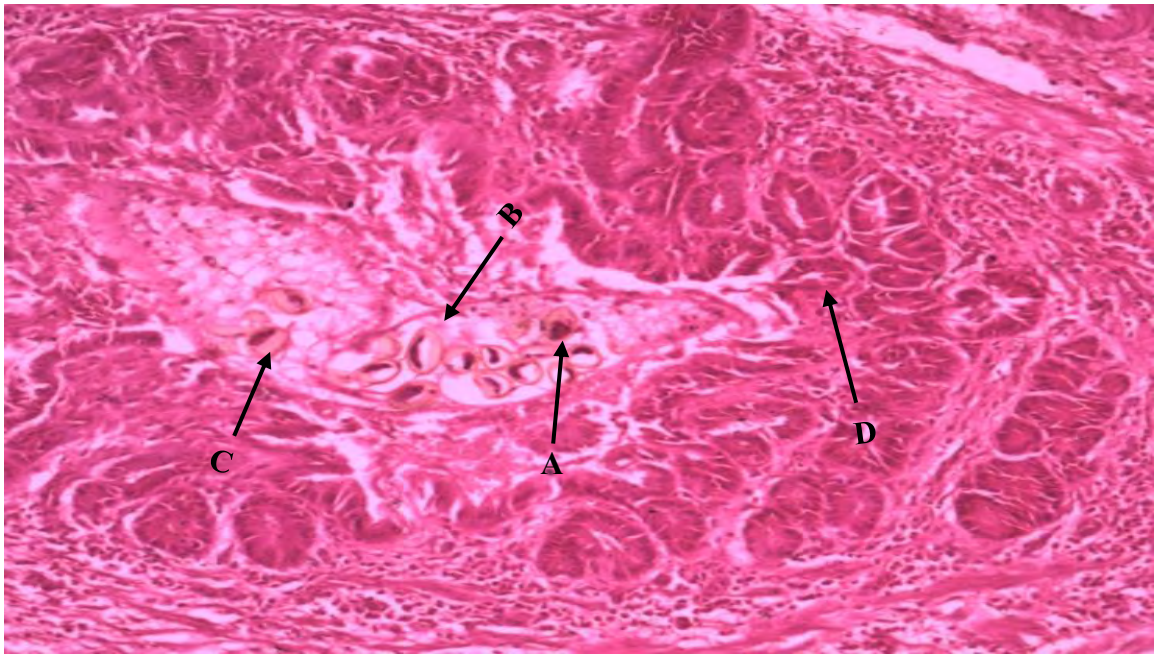


Figure 2.3.19 Section of liver of affected sheep with *Dicrocoelium* revealing the presence of biliary hyperplasia, inflammatory cells and the parasitic section containing Several defining characteristics including (A): Shows egg containing miracidia. (B): Eggs thick shell. (C): Show operculated egg. (D): Shows cross-sectional parts of *Dicrocoelium*. Hematoxylin and eosin (HE). 100X

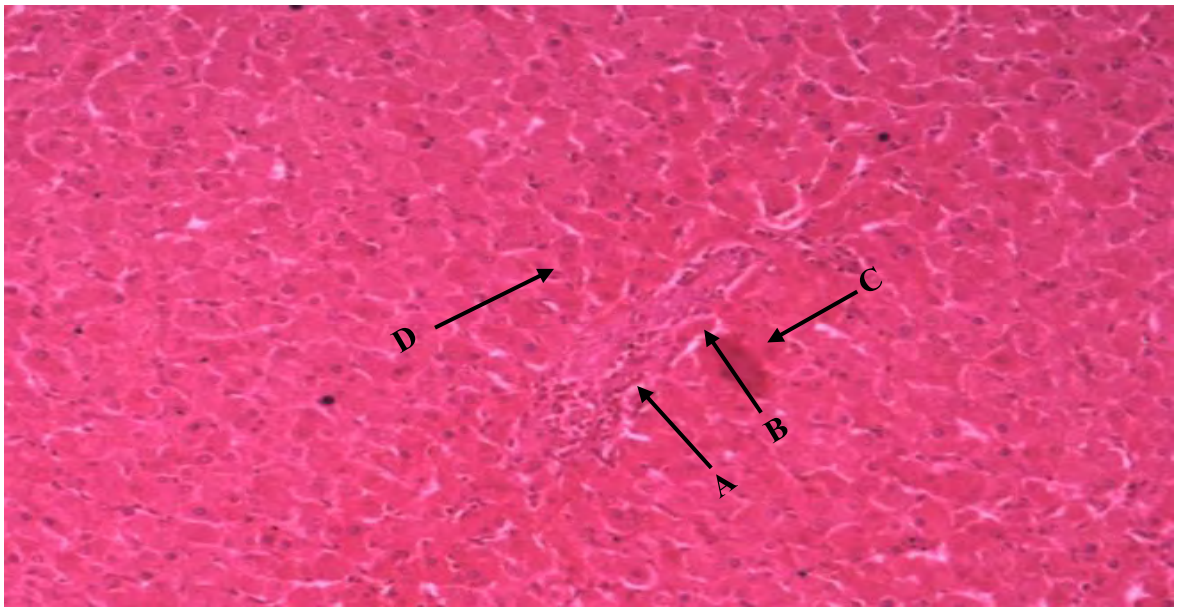


Figure 2.3.20 Histological section of sheep liver infected with *Dicrocoelium.* (A): Showed severely congested blood vessels in the central vein. (B): Infiltration of inflammatory cells. (C): Represents the pigmentation. (D): Showed inflammatory regions. Hematoxylin and eosin (HE). 100X

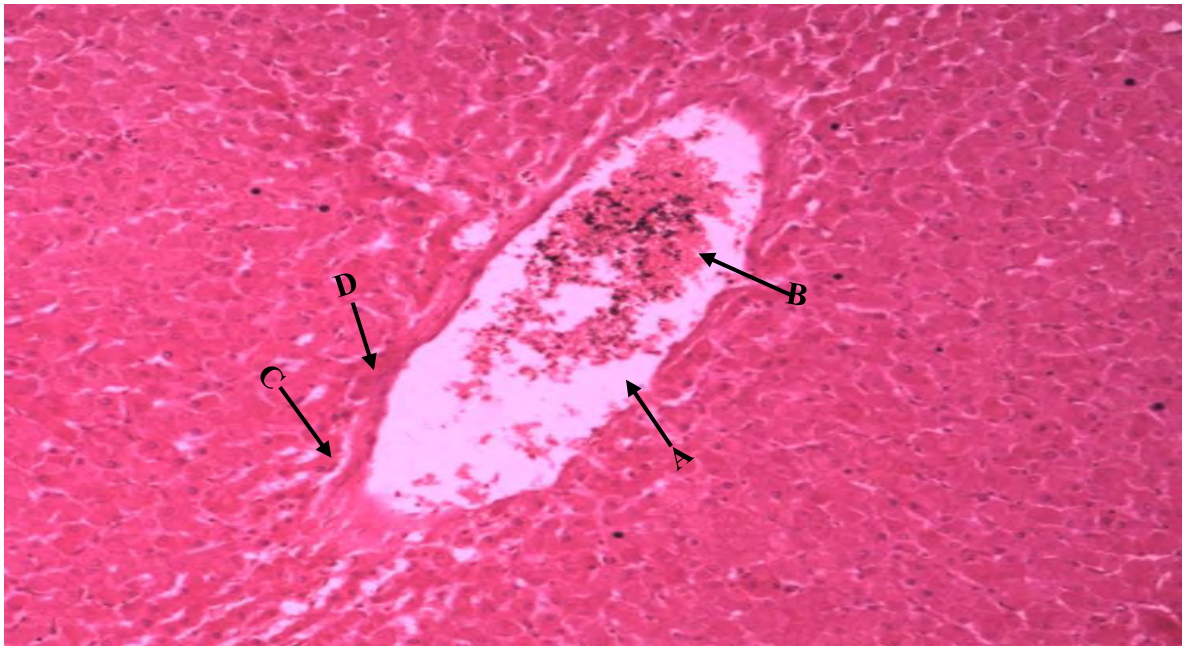


Figure 2.3.21 (A): Shows the central vein. (B): Ingestion inside the central vein. (C): The damaged sinusoids. (D): Histological section of sheep liver infected with *Dicrocoelium* showed inflammation of the inflammatory cells. Hematoxylin and eosin (HE). 100X

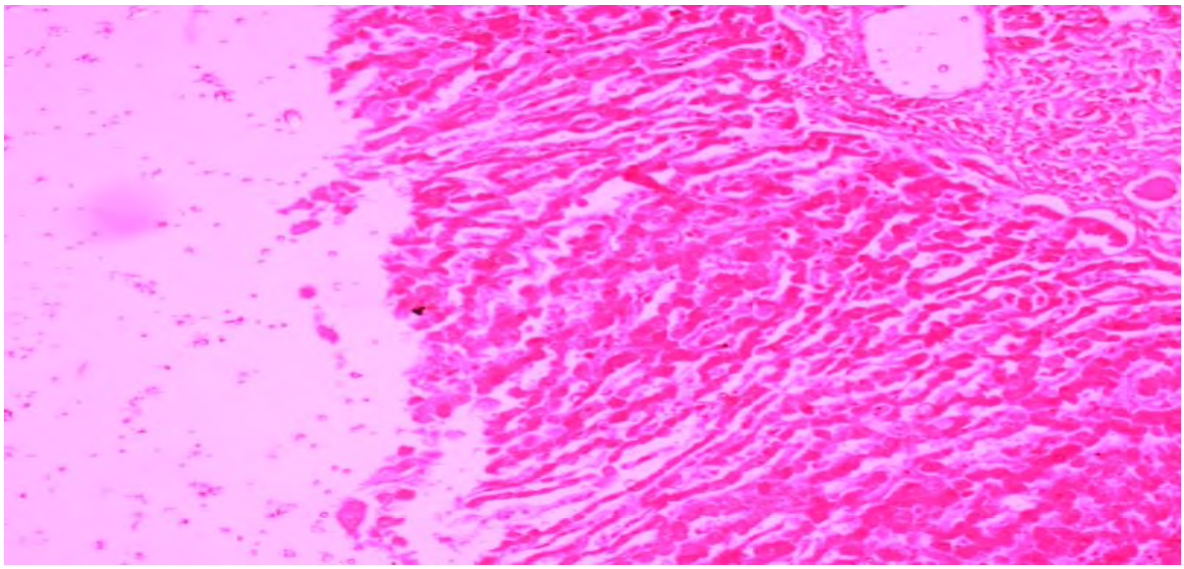


Figure 2.3.22 Histological section of sheep liver infected with *Dicrocoelium*, showed the absence of normal liver histology. Narrow and wide streaks of connective tissues, infiltration of lymphocytes, macrophages and eosinophils. Hematoxylin and eosin (HE). 100X

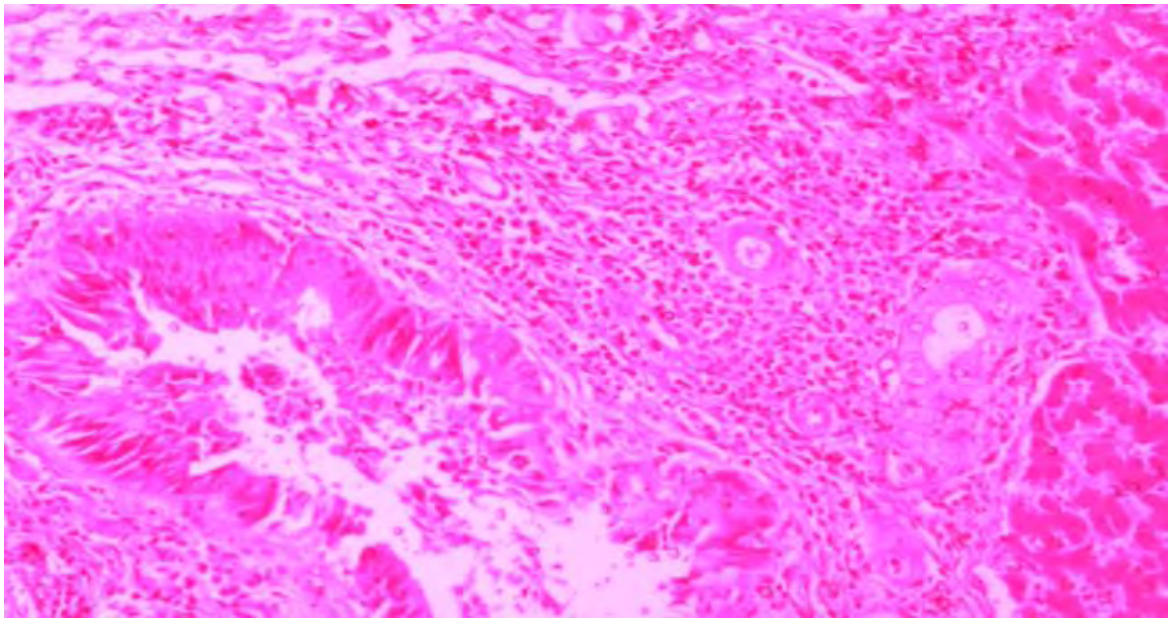


Figure 2.3.23 Histological section of sheep liver infected with *Dicrocoelium*, showed the absence of normal liver histology. The presence of a greater amount of edematous connective tissue in the portal area and septal tapes, connective tissue infiltrated with lymphocytes and macrophages. Hematoxylin and eosin (HE). 100X

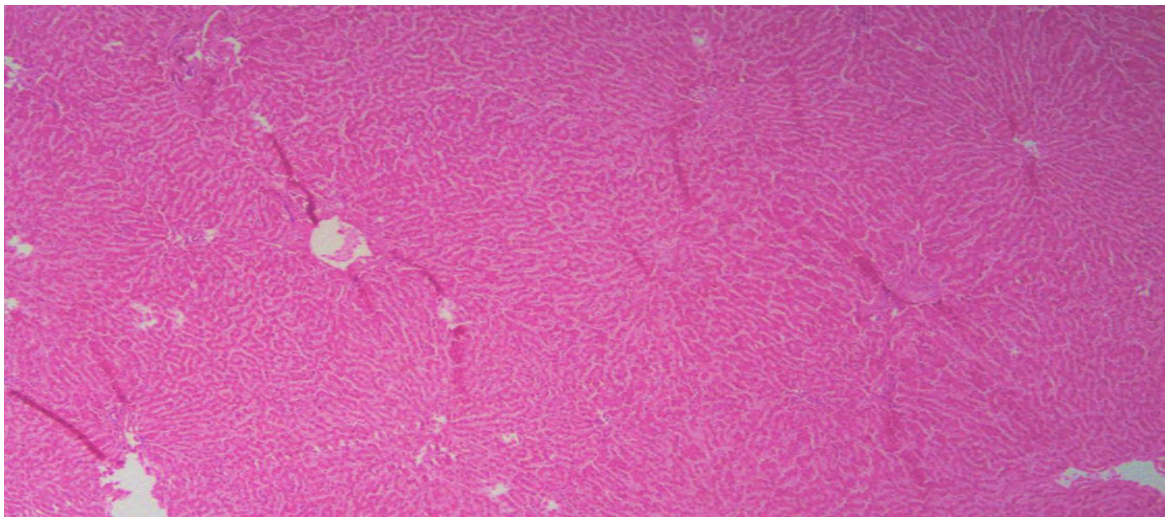


Figure 2.3.24 Histological section of sheep of normal liver, showing healthy integrity of hepatic tissue construction. Hematoxylin and eosin (HE). 100X

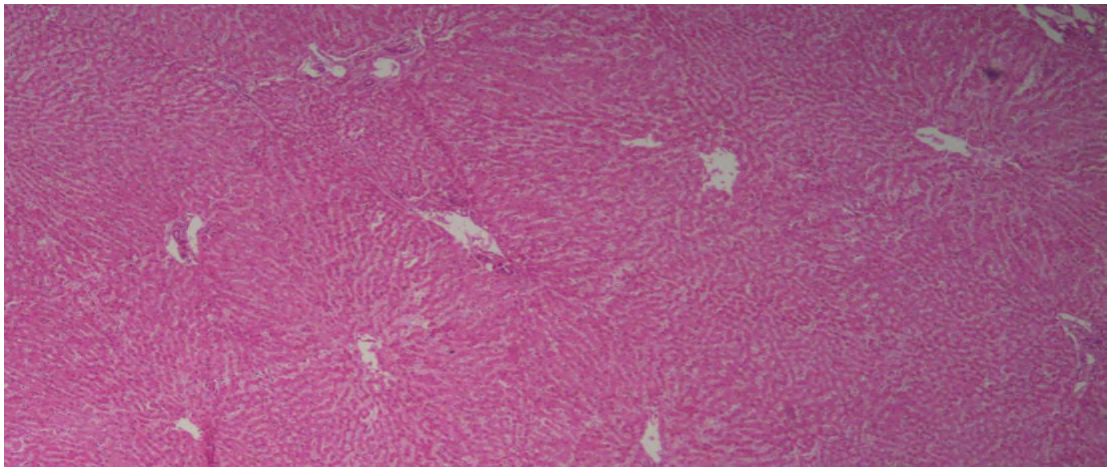


Figure 2.3.25 Histological section of sheep of normal liver, showed normal liver histology. Hematoxylin and eosin (HE). 100X

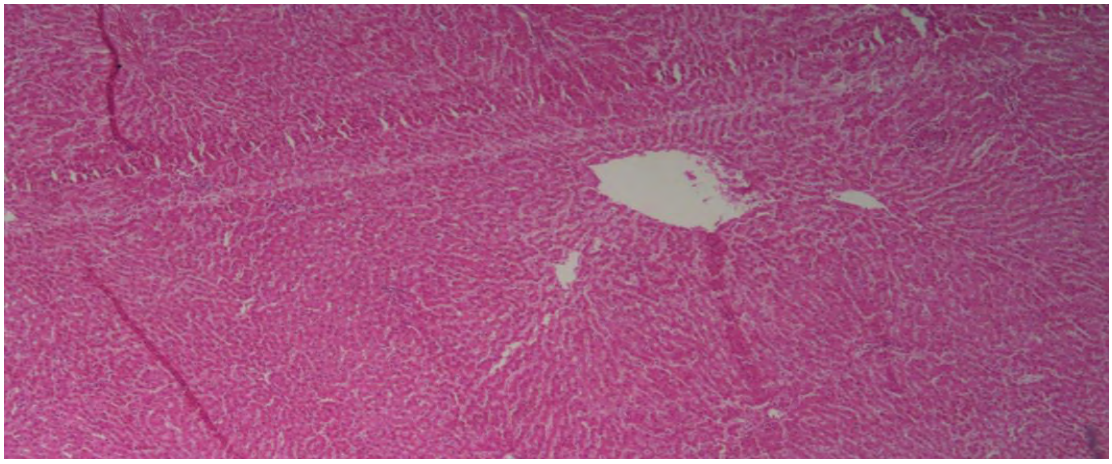


Figure 2.3.26 Histological section of sheep of normal liver, showed normal liver histology. Hematoxylin and eosin (HE). 100X

DISCUSSION

The economics of livestock production is marginal in the studied region, hence better understanding of any potential production-limiting disease, such as dicrocoeliasis is important. The environment of study area provides suitable habitats for the mud snail intermediate hosts of *Dicrocoelium* species. Previous studies have shown the value of morphological and molecular-based methods for the accurate species differentiation of *D. dendriticum* and *D. chinensis* (Otranto *et al.,* 2007; Bian *et al.,* 2015; Gorjipoor *et al.,* 2015). The present work for the first time conducted a phenotypic study of lancet flukes infecting sheep and goats in Chitral, Gilgit and Swat, Pakistan. The specimens were morphologically identified in the Dicrocoeliidae genus based on morphological keys. The testes' orientation, overall size, and level of maximum body width as described by Otranto *et al.* (2007), were consistent with the morphological identity of both *D. dendriticum* and *D. chinensis*. The morphological methods confirmed the species identity of *D. dendriticum* lancet flukes collected from abattoirs, local markets in the Chitral valley, Swat, and Gilgit Baltistan of Pakistan; although *D. dendriticum* has been reported in neighboring Himalayan India, (Bian *et al.,* 2015; Hayashi *et al.,* 2017), China (Shah and Rehman, 2001; Jithendran *et al.,* 1996) and Iran (Khanjari *et al.,* 2014; Meshgi *et al.,* 2019). Based on morphological characteristics of adult flukes, including the size, shape, length, and width of several internal body organs, the lancet fluke found in this investigation is identical to the previously identified *D. dendriticum* (Rudolphi 1802; Murshed *et al.,* 2022; Kuchai *et al.,* 2011; Kruchynenko *et al.,* 2020). *D. dendriticum* had a body length of 1.6 to 8 mm and a body width of 0.48 to 1.84 mm, which was consistent with earlier reports that the body length of *D. dendriticum* obtained from sheep was 7.2 mm and the body width was 2.5 mm (Kuchai *et al.,* 2011). Similar to this, studies have shown that *D. dendriticum* ranges in size from 6.10 to 8.07 mm and 1.52 to 1.94 mm, while *D. chinensis* ranges in size from 7.33 to 9.20 mm and 1.90 to 3.10 mm (Otranto *et al.,* 2007; Kruchynenko *et al.,* 2020; Murshed *et al.,* 2022). Variation between hosts was seen in *D. dendriticum* (Otranto *et al.,* 2007).

One of the most popular multivariate statistical techniques for examining biological patterns and models based on large collections of correlated variables is principal component analysis (PCA). PCA is a method for reducing the number of variables in multivariate data sets by as much as possible. It does this by using orthogonal transformation to transform multiple variables into a set of orthogonal, uncorrelated axes, also known as principal components (Hotelling, 1993; James and McCulloch, 1990; Legendre and Legendre, 1998; Harper, 1998; Robertson et al., 2001; Gotelli and Ellison, 2004; Hammer and Harper, 2006; Janzekovic and Novak, 2012). Numerous biological and ecological studies have utilized this strategy extensively. Similarly, by projecting a multivariate data set down to one dimension and maximizing separation between groups that have been previously separated, discriminant analysis (DA) was used to test hypotheses of morphologic similarities or differences using pairwise comparisons between two groups (Hammer and Harper, 2006; Sokal and Rohlf, 1995). In the current study, the principal component analysis along with discriminant analysis of species based on size measurements showed closed morphological resemblance between *D. dendriticum* and *D. chinensis*. Both species showed similarities in most of the values. However, Falcon-Ordaz *et al.,* (2019), reported morphometric variation within and between the *Dicrocoelium rileyi* population. Other trematodes studies also supported population variation (Valero *et al., 2018*; Afshan *et al.,* 2014). The observed morphological and morphometric variances may be attributed to a number of factors, including the type of fixation and preparation of the specimens under investigation. This variable can cause organisms to shorten or lengthen, which in turn affects morphological trait measurements (Poulin, 2009, Barger and Wellenstein, 2015). Parasite species' microevolutionary processes are affected and regulated by several factors, some of which may have contributed to the emergence of variety (Paterson and Gray, 1997). Parasite populations vary in size and distribution based on several parameters, including intermediate host type, life cycle complexity, parasite prevalence, definitive host specificity, and host immune system. Distance between parasite populations increases the likelihood of genetic divergence, which increases the likelihood of phenotypic variance (Poulin, 1998, 2007; Criscione *et al.,* 2005; Alemán-Muoz *et al.,* 2013). (Criscione *et al.,* 2005; Chibwana and Nkwengulila, 2010). Digenea's body proportions, as well as the shape and position of its internal organs, are the main diagnostic criteria for identifying the species. In present study host and geographical areas did not show significant variation in body size. However,

differences in infection load and host identity are both potential causes of phenotypic variance. There is a chance that species-specific variation could emerge because of these phenotypic effects, leading to incorrect identification (Hildebrand *et al.,* 2015; Nolan and Cribb, 2005; Stunkard, 1957).

In the present study, the histological findings agreed with the previous studies (Sanchez-Campos *et al.,* 1999; Manga-González *et al.,* 2004), indicating that dicrocoeliasis had caused hepatic damage. The pathological changes observed in dicrocoeliasis 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 that results in the pathological alterations seen in dicrocoeliasis (Manga-González *et al.,* 2004). Another study also supported these findings that pathological changes observed in dicrocoeliasis are caused by direct mechanical stimulation, probably due to the suckers of the adult flukes, in combination with fibrosis-promoting substances released by leukocytes and toxic metabolites released by the adult flukes, inducing inflammatory reaction (Samadieh *et al.,* 2017).

The microscopic examination of the infected liver 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. Leukocytic infiltration and periductal fibrosis were also observed, consistent with previous studies (Manga-Gonzalez *et al.,* 2004; Samadieh *et al.,* 2017). Moreover, our findings are consistent with the finding of other similar studies (Changizi *et al.,* 1998; Samadieh *et al.,* 2017; Sato *et al.,* 2019 and Nelwan *et al.,* 2019; Pour *et al.,* 2020; Piegari *et al.,* 2021). Pour *et al.* (2020) recorded the presence of adult worms in the bile ducts caused swelling, enlargement, damage to the epithelial tissue, villous atrophy, hyperplasia of bile duct mucosal glands, tissue nodularity and bile ducts showed accumulation of neutrophils and eosinophils. Piegari *et al.* (2021), in a study on sheep naturally infected by *D. dendriticum* reported various degrees of fibrosis, bile duct hyperplasia, severe leukocyte infiltration, and sinusoids. Similarly, Murshed *et al.* (2022) recorded various degrees of fibrosis, hyperplasia, infiltration, dilation of bile ducts and portal areas, leukocyte infiltration, and lymphoid aggregation.

CONCLUSION

The results of this study demonstrated that the *Dicrocoelium* species found in the samples taken from sheep and goats in the north of Pakistan were *D. dendriticum* and *D. chinensis.* Regarding the economic and health significance of dicrocoeliasis, it is suggested that the prevalence and incidence of this disease be studied more thoroughly. Furthermore, research is necessary to correctly identify and distinguish the parasite isolates based on the sequencing of relevant genes.

MOLECULAR CONFIRMATION OF *DICROCOELIUM DENDRITICUM* IN THE HIMALAYAN RANGES OF PAKISTAN

ABSTRACT

Lancet liver flukes of the genus *Dicrocoelium* (Trematoda: Digenea) are recognized parasites of domestic and wild herbivores. The present study aimed to confirm the species identity of Dicrocoeliid flukes collected from the Chitral valley in the Himalayan ranges of Pakistan. The morphology of 48 flukes belonging to eight host populations was examined, but overlapping traits prevented accurate species designation. PCR and sequencing of fragments of ribosomal cistron DNA, and cytochrome oxidase-1 (COX-1) and NADH dehydrogenase-1 (ND-1) mitochondrial DNA from 34, 14, and 3 flukes revealed 10, 4, and 1 unique haplotypes, respectively. Single nucleotide polymorphisms in these haplotypes were used to differentiate between *D. chinensis* and *D. dendriticum* and confirm the molecular species identity of each of the lancet flukes as *D. dendriticum*. Phylogenetic comparison of the *D. dendriticum* rDNA, COX-1, and ND-1 sequences with those from *D. chinensis*, *Fasciola hepatica*, and *Fasciola gigantica* species was performed to assess within and between species variation and validate the use of species-specific markers for *D. dendriticum*. Genetic variations between *D. dendriticum* populations derived from different locations in the Himalayan ranges of Pakistan illustrate the potential impact of animal movements on gene flow. This work provides a proof of concept for the validation of species-specific *D. dendriticum* markers and is the first molecular confirmation of this parasite species from the Himalayan ranges of Pakistan. This work provides a preliminary illustration of a phylogenetic approach that could be developed to study the ecology, biological diversity, and epidemiology of Dicrocoeliid lancet flukes when they are identified in new settings.

Keywords: Dicrocoeliid, lancet flukes, *Dicrocoelium dendriticum,* Morphological traits Ribosomal and mitochondrial markers

INTRODUCTION

Digenean lancet liver flukes of the family Dicrocoeliidae (Trematoda: Digenea) can infect the bile ducts of a variety of wild and domesticated mammals and humans around the globe. Three species of the genus *Dicrocoelium,* namely *Dicrocoelium dendriticum, Dicrocoelium hospes*, and *Dicrocoelium chinensis,* have been described as causes of dicrocoeliasis in domestic and wild ruminants (Otranto and Traversa, 2003). Among these, *D. dendriticum* is the most common and is distributed throughout Europe, Asia, North and South America, Australia, and North Africa (Arias *et al.,* 2011). The main economic impact of dicrocoeliasis in livestock to the rejection of livers from slaughtered animals at meat inspection (Rojo-Vazquez *et al.,* 2012). However, in severe infections, affected animals may show clinical signs, including poor food intake, ill thrift, poor milk production, alteration in fecal consistency, photosensitization, and anemia (Manga-Gonzalez *et al.,* 2004; Sargison *et al.,* 2012).

The life history of *Dicrocoelium spp*. is indirect and may take at least six months to complete. Monoecious sexually reproducing and self-fertilizing adults are found in the bile ducts. Eggs containing fully developed miracidia are shed in feces and must be eaten by land snails before hatching. Miracidia penetrate the gut wall of the snails and undergo asexual replication and development into cercariae, which then escape from the snails in their slime trails and are eaten by ants. One cercaria migrates to the head of the ant and associates with the sub-oesophageal ganglion, while about 50 cercariae encyst in the gaster as metacercariae (Martín-Vega *et al.,* 2018). The larval stage that develops in the ant's head alters its behavior, making it cling to herbage and increasing the probability of its being eaten by a definitive herbivorous host. Unlike *Fasciola* spp., larval flukes migrate to the liver via the biliary tree and develop into adults in the bile ducts (Manga-Gonzalez *et al.,* 2001). Several species of land snails and ants are known to be intermediate hosts within the same geographical location (Mitchell *et al.,* 2017); nevertheless, the geographical distribution of *Dicrocoelium* spp. is constrained by the precise conditions required for the completion of the parasite's life history.

Molecular methods amplifying fragments of nuclear ribosomal genes and their internal transcribed spacers, or mitochondrial loci DNA (Maurelli *et al.,* 2007; Otranto *et al.,* 2007; Martinez-Ibeas *et al.,* 2011; Bian *et al.,* 2015; Liu *et al.,* 2014; Gorjipoor *et al.,* 2015) have been developed for Dicrocoeliid parasites; but as with all molecular diagnostic tools, these depend on accurate morphological speciation of the reference materials. These methods are adaptable to demonstrate genetic variability and phylogeny and have been applied to epidemiological studies of various trematode parasite species affecting ruminant livestock (Chaudhry *et al.,* 2016; Ali *et al.,* 2018; Sargison *et al.,* 2019; Rehman *et al.,* 2020). However, in the study of Dicrocoeliid genera, the value of phylogenetic analyses to detect intraspecific variation is limited by the availability of comparative sequence data.

In this study, we describe the molecular identification of *Dicrocoelium* spp. Infecting sheep in the Himalayan ranges of Pakistan. The fragments of ribosomal DNA, cytochrome oxidase-1 (COX-1), and NADH dehydrogenase-1 (ND-1) mtDNA were amplified to confirm the species identity. We aimed to use these data to describe any possible phylogenetic relationships within and between Pakistani *D. dendriticum* and the limited number of other *Dicrocoelium* spp. for which matching sequence data are publicly available.

MATERIALS AND METHODS

3.2.1 Adult flukes Collection

A convenience sampling method was used to examine the livers of 144 sheep slaughtered at four abattoirs in the Chitral valley of the Khyber Pakhtunkhwa province of Pakistan. These comprised 68 from Booni, 26 from Torkhow, 33 from Mastuj, and 17 from the Laspoor valley. Overall, 639 typical adult Dicrocoelid flukes (25 to 50 per liver) were recovered from the livers of 16 infected sheep. The isolates used for molecular analysis are shown in Table 3.2.1.

Table. 3.2.1: *Dicrocoelium* isolates based on the host and geographical origin

Isolate	Region	Host	Isolate	Region	Host
1	Booni	Sheep	18	Mastuj	Sheep
2	Booni	Sheep	19	Mastuj	Sheep
3	Booni	Sheep	20	Mastuj	Sheep
4	Booni	Sheep	21	Mastuj	Sheep
5	Booni	Sheep	22	Mastuj	Sheep
6	Booni	Sheep	23	Torkhow	Sheep
7	Booni	Sheep	24	Torkhow	Sheep
8	Booni	Sheep	25	Torkhow	Sheep
9	Booni	Sheep	26	Torkhow	Sheep
10	Booni	Sheep	27	Torkhow	Sheep
11	Booni	Sheep	28	Torkhow	Sheep
12	Booni	Sheep	29	Laspoor	Sheep
13	Booni	Sheep	30	Laspoor	Sheep
14	Booni	Sheep	31	Laspoor	Sheep
15	Booni	Sheep	32	Laspoor	Sheep
16	Mastuj	Sheep	33	Laspoor	Sheep
17	Mastuj	Sheep	34	Laspoor	Sheep

3.2.2. Genomic DNA Isolation from Worm Tissues

Genomic DNA was successfully extracted from 34 individual adult flukes (3 to 6 flukes per animal), adult flukes were selected from each of the livers of infected sheep from Booni (Pop-1, Pop-2, Pop-3, Pop-4), Laspoor (Pop-5), Mastuj (Pop-6, Pop-7) and from Thorkhow (Pop-8) for molecular analysis. The phenol-chloroform method (Sambrook *et al.,* 1989), a conventional technique for genomic DNA extraction, and PCR lysate were used to extract high-quality DNA from lancet flukes.

3.2.3. Extraction of Genomic DNA through the Phenol-Chloroform method

DNA extraction was performed according to Sambrook *et al.,* (1989) with some modifications. Firstly, the lancet fluke was cut and ground into small pieces by adding 600 µl of solution A [10 mM Tris, pH 7.5, 0.32 M sucrose, 5 mM $MgCl_2$, and triton X-100 (1% V/V)]. Mix it by inverting the tubes 4-5 times and place them at room temperature for 35 minutes. The tubes were centrifuged at 13000 rpm for two minutes, the supernatant was discarded and 500 µl of solution A was added again to dissolve the pellet. Centrifugation was carried again at 13000 rpm for 2 minutes and the supernatant was discarded. 400 µl solution B (10 mM Tris, pH 7.5, 400 mM NaCl, and 2 mM EDTA), 20 % SDS, and 5 µl of Proteinase K were added to the DNA pellet incubated overnight at 37 ºC. Nucleic acids were extracted by adding 500 µl equal volume of a freshly prepared mixture of chloroform- isoamyl alcohol (24:1) and phenol and shaking for one minute. The aqueous phase was transferred into the new eppendorf by centrifugation [10 minutes at 13000 revolutions per minute (rpm)]. 500 µl chloroform-isoamyl alcohol (24:1) was added and centrifuged at 13000 rpm for 10 minutes. The upper layer was transferred by adding 60 µl sodium acetate (3M, pH 4.5-6) and 500 µl ice-cold isopropanol. After centrifugation at 13000 rpm for 10 minutes, the genomic DNA goes into a pellet. 200 µl 70% ethanol was added into the pellet and centrifuged at 13000 rpm for 7 minutes; ethanol was discarded, and the tubes were dried at room temperature for 10-15 minutes. In the final step, 0.2 mM TE buffer (80-150 µl) was added and kept overnight at 37 ºC.

The extracted DNA was checked for shearing and concentration by 1% agarose gel electrophoresis in 1X TBE buffer and visualized by 0.5 µg/ml ethidium bromide staining. A molecular weight marker ranging from 100 to 3000 bp (Solis BioDyne) was chosen to evaluate DNA migration. 3 µl sample of DNA and 3 µl loading dye (Bromophenol blue and sucrose). Electrophoretic migration of DNA was done at 120 V, 120 current, and run for 30 minutes. The Ethidium bromide-stained agarose gel was visualized under UV light.

3.2.4. Genomic DNA extraction through direct PCR lysis buffer

To avoid contamination with eggs, a small tissue portion of around 2 mg was taken from the head of each worm for DNA extraction. Each piece was rinsed twice in a petri dish with distilled water (dH2O) for 5 minutes each before being lysed in a 25 ul worm-lysis solution made by mixing 50 ul Proteinase K (10 mg/ml, New England BioLabs) and 50 l 1 M Dichloro Diphenyl Trichloroethane (DDT) in 1 ml of Direct PCR Lysis Reagent (Viagen). The lysates were then incubated at 60 °C for 2 hours, followed by 15 minutes at 85 °C. Lysates were kept at -80 °C until needed. The Ethical Review Committee approved the abattoir-based study.

3.2.5. PCR Amplification of the Internally Transcribed Spacer Regions (ITS)

A 1,152 bp fragment of the nuclear ribosomal DNA (rDNA) cistron, comprising the ITS-1, 5.8 S, ITS-2, and 28 S (Figure 3.2.1) flanking region, was amplified by using two sets of universal primers (Table 3.2.2) previously reported (Gorjipoor *et al.,* 2015). The PCR was carried out in a total volume of 25 µl PCR reaction mixtures consisting of 2 µl of PCR buffer (1X) (Thermo Fisher Scientific, USA), 2 µl $MgCl_2$ (25 mM), 2 µl of 2.5 mM dNTPs, 0.7 µl of primer mix (10 pmol/µl final concentration of each primer), 2 µl of gDNA and 3 µl of Taq DNA polymerase (5U/µl) (Thermo Fisher Scientific, USA), and 16 µl ddH_2O. The PCR was performed in a My Cycler ™ Thermal Cycler (Bio-Rad). The thermocycling conditions were as follows: after an initial denaturation at 96ºC for 10 minutes, samples were subjected to 35 cycles of amplification (denaturation at 96ºC for 1 minute, primer annealing at 60.9 ºC (BD1-F-rDNA/ BD1-R-rDNA), or 61.4 ºC F-rDNA/ Dd-R-rDNA) for 1 minute and 72 ºC for 1 min, with a final extension of 72 ºC for 5 minutes. PCR products were checked for size and purity through 1% agarose gel electrophoresis in 1X TBE buffer and visualized by 0.5 µg/ml

Ethidium bromide staining. A molecular weight marker ranging from 100 to 3000 bp (Solis BioDyne) was chosen to evaluate DNA migration. Samples were prepared with 3 µl of PCR amplified product and 3 µl loading dye (Bromophenol blue and sucrose). Electrophoretic migration of DNA was done at 120 V for 35 minutes, and the Ethidium bromide-treated agarose gel was visualized under UV light.

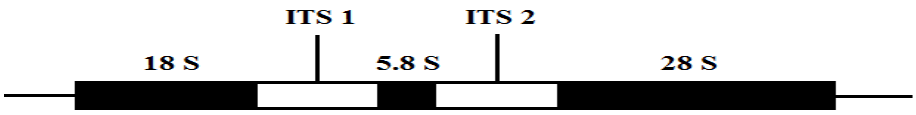


Figure 3.2.1: Ribosomal DNA region contains 18 S, ITS-1, 5.8 S, ITS-2, and 28 S

3.2.6. PCR Amplification of Cytochrome C Oxidase Subunit-1 (COX-1) Gene

A 1536 bp fragment of mitochondrial cytochrome c oxidase subunit 1(cox*1*) gene was amplified using newly developed forward and reverse primers, designed with reference sequences downloaded from NCBI using the 'Primers 3' online tool (Table 3.2.2). The PCR was carried out in a total volume of 25 µl PCR reaction mixtures consisting of 2 µl of PCR buffer (1X) (Thermo Fisher Scientific, USA), 2 µl $MgCl_2$ (25 mM), 2 µl of 2.5 mM dNTPs, 0.7 µl of primer mix (10 pmol/µl final concentration of each primer), 2 µl of gDNA and 3 µl of Taq DNA polymerase (5U/µl) (Thermo Fisher Scientific, USA), and 16 µl ddH_2O. The PCR was performed in a My Cycler ^{TM} Thermal Cycler (Bio-Rad), and the thermocycling conditions were as follows: after an initial denaturation at 96ºC for 10 minutes, samples were subjected to 35 cycles of amplification (denaturation at 96 ºC for 1 minute, primer annealing at 55 ºC (D-F-cox11/D-R-cox11), 53 ºC (D. cox1-2-F/ D. cox1-2-R, D.cox1-3-F/D.cox1-3-R) for 1 minute and 72 ºC for 1 min, followed by a 10 minutes final elongation at 72ºC. PCR products were checked for size and purity through 1% agarose gel electrophoresis in 1X TBE buffer and visualized by 0.5 µg/ml ethidium bromide staining. A molecular weight marker ranging from 100 to 3000 bp (Solis BioDyne) was chosen to evaluate DNA migration. Samples were prepared with 3 µl of PCR amplified product and 3 µl loading dye (Bromophenol blue and sucrose). Electrophoretic migration of DNA was done at 120 V for 35 minutes, and the ethidium bromide staining treated agarose gel was visualized under UV light.

3.2.7. PCR Amplification of Mitochondrial NADH Dehydrogenase Subunit 1 (ND-1) Gene

A 659 bp fragment of the mitochondrial NADH dehydrogenase subunit1(ND-1) gene was by using two sets of universal primers (Table 3.2.2) previously reported (Hayashi *et al.,* 2016). The PCR was carried out in a total volume of 25 µl PCR reaction mixtures consisting of 2 µl of PCR buffer (1X) (Thermo Fisher Scientific, USA), 2 µl $MgCl_2$ (25 mM), 2 µl of 2.5 mM dNTPs, 0.7 µl of primer mix (10 pmol/µl final concentration of each primer), 2 µl of gDNA and 3 µl of Taq DNA polymerase (5U/µl) (Thermo Fisher Scientific, USA), and 16 µl ddH_2O. The PCR was performed in a My Cycler ^{TM} Thermal Cycler (Bio-Rad), and the thermocycling conditions were as follows: after an initial denaturation at 96ºC for 10 minutes, samples were subjected to 35 cycles of amplification (denaturation at 96ºC for 1 minute, primer annealing at 55 ºC for 1 minute and 72 ºC for 1 min, followed by a 10-minute final elongation at 72ºC. PCR products were checked for size and purity through 1% agarose gel electrophoresis in 1X TBE buffer and visualized by 0.5 µg/ml ethidium bromide staining. A molecular weight marker ranging from 100 to 3000 bp (Solis BioDyne) was chosen to evaluate DNA migration. Samples were prepared with 3 µl of PCR amplified product and 3 µl loading dye (Bromophenol blue and sucrose). Electrophoretic migration of DNA was done at 120 V for 35 minutes, and the ethidium bromide staining treated agarose gel was visualized under UV light.

Table. 3.2.2: Primer sequences for the amplification of *Dicrocoelium* spp. ITS rDNA, mt-ND-1, and mt- COX-1 mtDNA fragments.

Primer Name	Sequences (5′ - 3′)	
BD1-F-rDNA	GTCGTAACAAGGTTTCCGTA	
BD2-R-rDNA	TATGCTTAAATTCAGCGGGT	(Gorjipoor *et al.,* 2015)
Dd-F-rDNA	ATATTGCGGCCATGGGTTAG	
Dd-R-rDNA	ACAAACAACCCGACTCCAAG	(Gorjipoor *et al.,* 2015)
D-F-cox1-1	GTTTGTGGGGTGGTCTTACT	
D-R-cox1-1	AAGCACTAGCAAAATGACGA	
D.cox1-2	TGAGGTCTTGGATCGTGTTA	
D. cox1-2	AAACCACCAACTCACCAAAC	
D.cox1-3	GTTTATGGGATCCGGTT	
D. cox1-3	CAAAAGCACCATTCTCATCA	
D-F-nad1	GGAGTGTGGTGTTTTGGTTT	
D-R-nad1	AACAACGAACTAACCCAAGC	(Hayashi *et al.,* 2016)

3.2.8. Purification of the Amplified PCR Products

The amplified PCR product of ITS-1, 5.8 S, ITS-2, 28 S, ND-1, and COX-1 genes was cleaned using a WizPrep ™ Gel/PCR Purification Mini kit 202 (Seongnam 13209, South Korea). The PCR products were transferred to 1.5ml Eppendorf tubes, and 5 volumes of GP buffer were added to 1 volume of the PCR amplified products and mixed well. The mixture of the products was transferred to a spin column, and the filtrate was discarded after centrifugation at 13000 rpm for 1 minute. Now 700 µl of ethanol was added wash buffer to the spin column, and again, the filtrate was discarded after centrifugation at 13000 rpm for 30 seconds, the centrifugation step was repeated, and the spin column was transferred to a new 1.5ml new tube. After this step, the product was eluted by applying 50 µl of elution buffer in the center of the column, and the columns were placed for 1 minute at room temperature and centrifuged for 1 minute at 13000 rpm. Finally, the purified products of PCR were checked for size and purity through 1% agarose gel electrophoresis in 1X TBE buffer and visualized by 0.5 µg/ml ethidium bromide staining. A molecular weight marker ranging from 100 to 3000 bp (Solis BioDyne) was chosen to evaluate DNA migration. Samples were prepared with 3 µl of PCR amplified product and 3 µl loading dye (Bromophenol blue and sucrose).

Electrophoretic migration of DNA was done at 120 V for 35 minutes, and the ethidium bromide-treated agarose gel was visualized under UV light.

3.2.9. Sequencing of the Purified Products

The purified PCR products of ITS-1, 5.8 S, ITS-2, 28 S, ND-1, and COX-1 genes of *Dicrocoelium* were used to sequence both strands using an Applied Biosystems 3730Xl genetic analyzer. The same amplification primers were used to sequence these regions. Both strands of rDNA, COX-1 and ND-1 sequences from each fluke were assembled, aligned, and edited to remove primers from both ends using Geneious Pro 5.4 software (Drummond, 2012). The sequences were then aligned with previously published NCBI GenBank rDNA, COX-1, and ND-1 sequences of *D. dendriticum*, *D. chinensis, F. hepatica,* and *F. gigantica*. All sequences in the alignment were trimmed based on the length of the shortest sequence available that still contained all the informative sites. Sequences showing 100% base pair similarity were grouped into single haplotypes using the CD-HIT Suite software (Huang *et al.,* 2010).

3.2.10. Molecular phylogeny of the rDNA, COX-1, and ND-1 data sets

The generated haplotypes were imported into MEGA 7 (Tamura *et al.,* 2013) and used to determine the appropriate model of nucleotide substitution. Molecular phylogeny was reconstructed from the rDNA, COX-1, and ND-1 sequence data by the Maximum Likelihood (ML) method. The evolutionary history was inferred by using the ML method based on the Kimura 2-parameter model for rDNA and the Hasegawa-Kishino-Yano model for the COX-1 and ND-1 loci. The bootstrap consensus tree inferred from 1,000 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test was shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log-likelihood values. All positions containing gaps and missing data were eliminated. There were 698 bp, 187 bp, and 217 bp in the final datasets of rDNA, COX-1, and ND-1, respectively. A split tree was created in the SplitTrees4 software by using

the UPGMA method in the HKY85 model of substitution. The appropriate model of nucleotide substitutions for UPGMA analysis was selected using the jModeltest 12.2.0 program.

3.2.11. Published accession numbers used in the phylogenetic trees

The published sequences of *Dicrocoelium* deposited in the GenBank (GenBank accession numbers) were used in the analysis of the phylogenetic tree (Table 3.2.3).

Table. 3.2.3: Reference sequences obtained from GenBank for comparison.

No	COX-1		ND-1		ITS	
	Acc. Numbers	Country	Acc. Numbers	Country	Acc. Numbers	Country
1	LC333992.1	Japan	KF318787.1	China	KF734787.1	China
2	LC333991.1	Japan	LC159517.1	Japan	KF734786.1	China
3	JX509886.1	China	LC159520.1	Japan	KF734791.1	China
4	KF318786.1	China	LC159519.1	Japan	KF734795.1	China
5	LC333989.1	Japan	LC159518.1	Japan	KF734778.1	China
6	LC333993.1	Japan	LC159525.1	Japan	KF734794.1	China
7	LC333994.1	Japan	LC159524.1	Japan	KF734783.1	China
8	JX509885.1	China	LC159523.1	Japan	KF734782.1	China
9	JX509887.1	China	JX050117.1	Iran	KF734781.1	China
10	JX509890.1	China	JX050124.1	Iran	KF734785.1	China
11	LC333990.1	Japan	JX050130.1	Iran	KF734792.1	China
12	KX827441.1	Iran	JX050133.1	Iran	KF734793.1	China
13	KX781719.1	Iran	JX050115.1	Iran	KF734780.1	China
14	LC333985.1	Japan	LC159521.1	Japan	KF734779.1	China
15	KX827430.1	Iran	MG889416.1	Iran	KF734788.1	China
16	KX827436.1	Iran	MG889420.1	Iran	KF734784.1	China
18	KX827434.1	Iran	JX050116.1	Iran	KF734773.1	China
19	KX827439.1	Iran	KC164155.1	Iran	KF734774.1	China
20	KX827438.1	Iran	JX050126.1	Iran	KC774524.1	China
21	KX827432.1	Iran	MG889409.1	Iran	KC774511.1	China
22	KX827435.1	Iran	KC164152.1	China	KC774514.1	China
23	KX827431.1	Iran	LC159522.1	China	KC774515.1	China
24	KX827429.1	Iran	JX050121.1	Iran	KC774516.1	China
25	KX827428.1	Iran	JX050110.1	Iran	KC774517.1	China
26	KX827427.1	Iran	MG149594.1	Iran	KC774508.1	China
27	KX781718.1	Iran	MG889402.1	Iran	KC774510.1	China
28	KX781720.1	Iran	MG889412.1	Iran	KC774502.1	China
29	KC164176.1	China	MG889407.1	Iran	KC774501.1	China

30	KC164174.1	China	MG889405.1	Iran	KC774522.1	China
31	KX781722.1	Iran	MG889406.1	Iran	KC774500.1	China
32	KX827440.1	Iran	MG889399.1	Iran		
33	KX827437.1	Iran	MG889400.1	Iran		
34	KX827444.1	Iran	MG889403.1	Iran		
35	KX781721.1	Iran	MG889404.1	Iran		
36	KX827433.1	Iran	MG889401.1	Iran		
37	KX827442.1	Iran	KC164169.1	China		
38	KX827443.1	Iran	JX050127.1	Iran		
39	KC164177.1	China	MG889419.1	Iran		
40	KX827441.1	Iran	KC164158.1	China		
41	KX781719.1	Iran	MG889421.1	China		
42	LC333985.1	Japan	MG889413.1	China		
43	KX781722.1	Iran	MG889410.1	China		
44	KX781721.1	Iran	MG889411.1	China		
45	KX827444.1	Iran	MG889414.1	China		
46	KX827442.1	Iran	MG889415.1	China		
47	KX827443.1	Iran	MG889418.1	China		
48	KX827437.1	Iran	JX509884.1	China		
49	KX827433.1	Iran	JX509864.1	China		
50	KX827440.1	Iran	LC333983.1	Japan		
51	KC164174.1	China	JX509881.1	China		
52	KX827431.1	Iran	JX509871.1	China		
53	KX827429.1	Iran	KF318786.1	China		
54	KX827430.1	Iran	LC333982.1	Japan		
55	KX827434.1	Iran	JX509866.1	China		
56	KX827436.1	Iran	LC333981.1	Japan		
57	KX827432.1	Iran	JX509862.1	China		
58	KX827435.1	Iran	LC160342.1	Japan		
59	KX827428.1	Iran				
60	KX827439.1	Iran				
61	KX781720.1	Iran				
62	KX781718.1	Iran				
63	KX827438.1	Iran				
64	KX827427.1	Iran				
65	KC164176.1	China				

RESULTS

3.3.1. Nuclear and Mitochondrial DNA Extraction

From each lancet fluke sample, DNA was successfully extracted. A smear was visible in almost all the lanes in the agarose gel electrophoresis (Figure 3.3.1), indicating that the DNA concentration was too high. It was diluted to a working concentration of roughly 20–50 ng/ul for all samples.

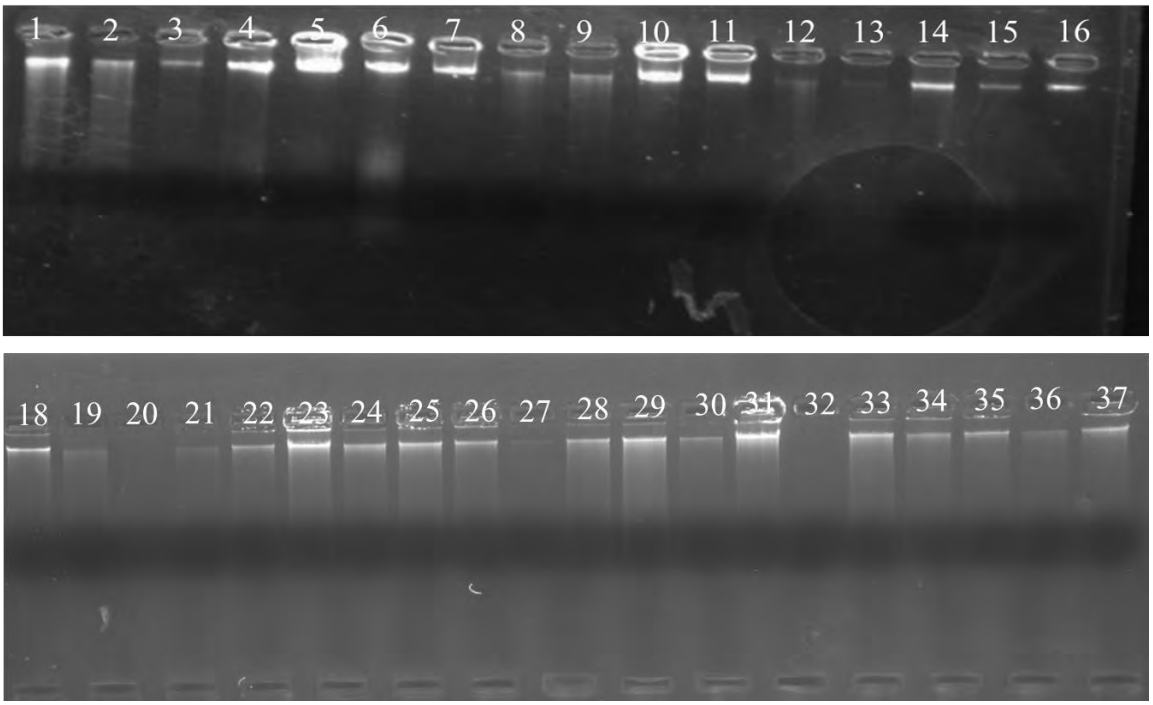


Figure 3.3.1. Agarose gel electrophoresis of DNA extraction from lancet flukes' isolates

3.3.2. Molecular confirmation of *Dicrocoelium* species identity

The extracted DNA from all lancet flukes' isolates was submitted to PCR for amplification with specific primers to amplify a region of the rDNA 18S (partial), ITS1, 5.8S, ITS2, and a partial region of 28S. A monomorphic DNA segment with approximately 1152bp was visible for *Dicrocoelium* isolates of each host (Figure 3.3.2).

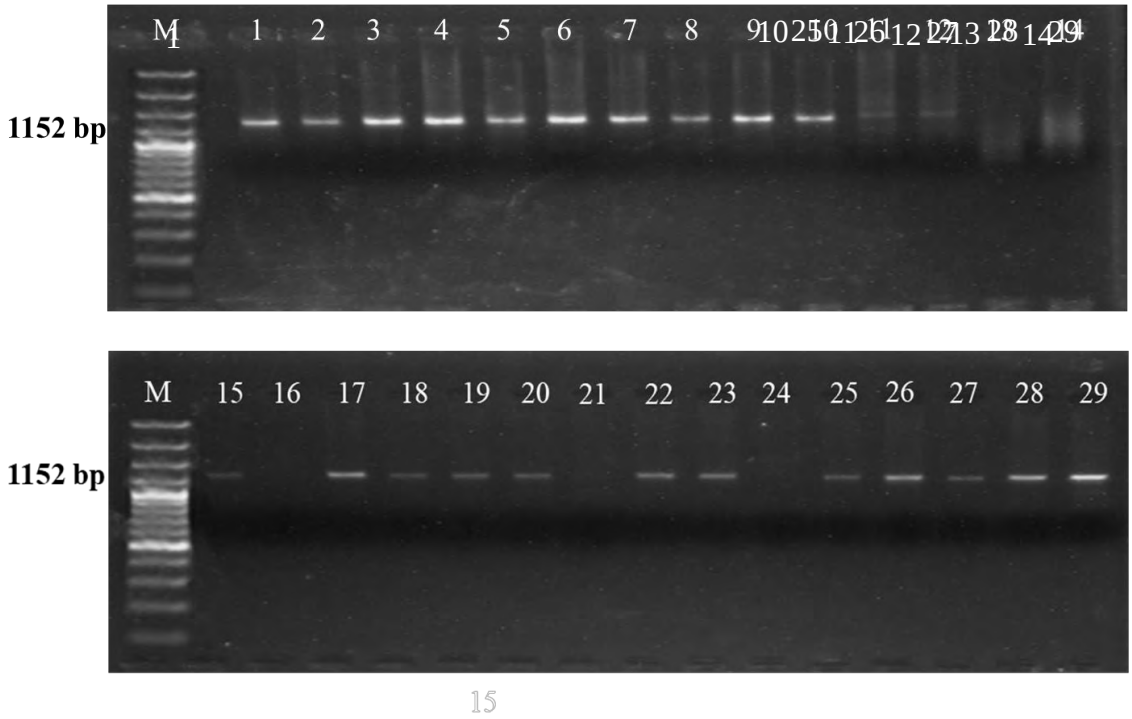


Figure 3.3.2 Agarose gel electrophoresis of the rDNA regions (18S, ITS1, 5.8S, ITS2, and 28S) PCR products from the isolates. M-100 bp molecular weight marker.

3.3.3. Ribosomal DNA haplotypes

The rDNA sequences of each of the 34 flukes from which genomic DNA was successfully extracted were aligned with 12 sequences of *D. dendriticum* and 18 sequences of *D. chinensis* (Annexure 3.1) available on the Mendeley database and trimmed to 698 bp length. This included 4 informative sites to describe intraspecific variation within *D. dendriticum* and 6 sites to describe intraspecific variation within *D. chinensis*. The alignment confirmed 21 species-specific fixed SNPs to differentiate between *D. dendriticum* and *D. chinensis* (Table 3.3.1); this allowed the molecular species identity of each of the 34 flukes to be confirmed as *D. dendriticum*. The 12 *D. dendriticum* and 18 *D. chinensis* sequences from the public database were examined, along with the 34 *D. dendriticum* rDNA sequences from the present study. Sequences showing 100% base pair similarity were grouped into single haplotypes generating 19 unique *D. dendriticum* and 4 unique *D. chinensis* haplotypes (Annexure 3.1). An ML tree was constructed from the 23 rDNA haplotypes to examine the evolutionary relationship between *D. dendriticum* and *D. chinensis*. The phylogenetic tree shows that *D. dendriticum* and *D. chinensis* form discrete species-specific clades (Figure 3.3.3).

Ten haplotypes generated from the 34 rDNA sequences from the Chitral Valley were clustered in the *D. dendriticum* clade. Comparison of these haplotypes with those originating from the Shaanxi province of China showed some common origins and close relationships (Table 3.3.2), but there were insufficient published sequence data to meaningfully describe the emergence and spread of the Pakistani *D. dendriticum*. The rDNA haplotypes generated from the eight Pakistani *D. dendriticum* populations are shown in a split tree (Figure 3.3.8), albeit there is too little data to draw any conclusions.

Table. 3.3.1 Sequence variation in a 698 bp fragment of rDNA, differentiating between *D. dendriticum* and *D. chinensis*. The rDNA data are based on 12 sequences identified as *D. dendriticum* and 18 sequences identified as *D. chinensis* on NCBI Gene Bank. Informative sites to describe intraspecific variation are shown in bold.

rDNA nucleotide position	*D.dendriticum*	*D.chinensis*
18	**T/A**	**T**
56	C	T
58	G	A
60	T	-
82	**T/C**	**T**
119	G	A
134	G	T
221	**C**	**C/A**
222	**T**	**T/A**
228	**A**	**A/G**
240	**A**	**A/G**
358	T	C
367	G	A
370	C	T
405	G	A
423	**C/T**	**T**
424	C	T
469	T	C
489	**T/C**	T
535	T	C
541	A	G
550	T	C
571	G	A
630	T	A
632	G	T
654	T	A
655	C	T
671	**C**	**C/A**
680	G	A
681	T	G
689	**G**	**A/G**

Table 3.3.2. *D. dendriticum rDNA* haplotypes showing the sequences representing unique alleles and the country of origin. The materials and methods section describes the accession number of all the sequences.

rDNA haplotypes	Total number of sequences	Countries
*D.dendriticum*16	12	Pakistan
*D.dendriticum*17	1	Pakistan
*D.dendriticum*18	2	Pakistan, China
*D.dendriticum*19	1	Pakistan
*D.dendriticum*20	1	Pakistan
*D.dendriticum*21	1	Pakistan
*D.dendriticum*22	1	Pakistan
*D.dendriticum*23	6	Pakistan, China
*D.dendriticum*24	1	China
*D.dendriticum*25	1	China
*D.dendriticum*26	1	China
*D.dendriticum*27	1	China
*D.dendriticum*28	1	China
*D.dendriticum*29	1	China
*D.dendriticum*30	1	China
*D.dendriticum*31	1	China
*D.dendriticum*32	1	China
*D.dendriticum*33	9	Pakistan
*D.dendriticum*34	3	Pakistan

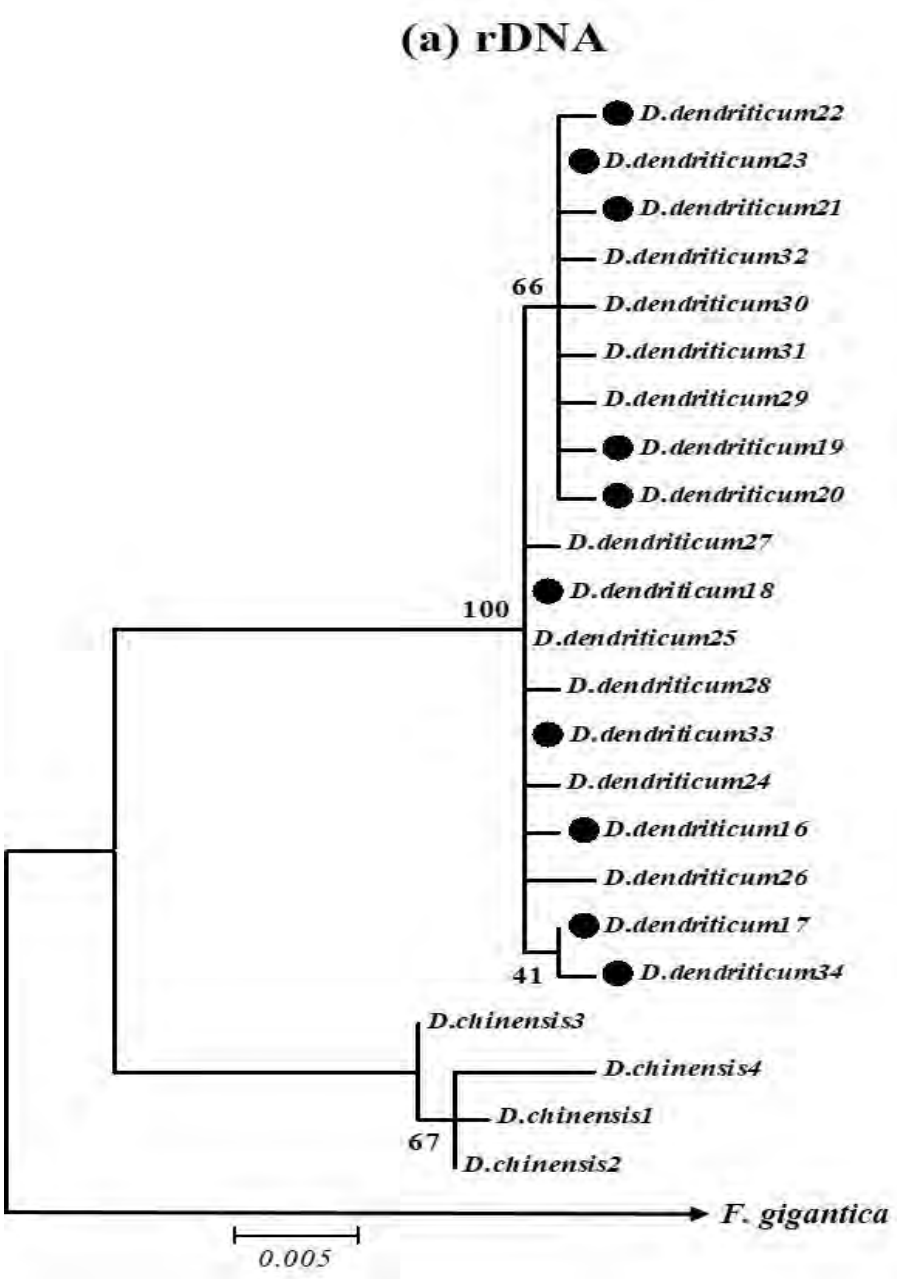


Figure 3.3.3. Maximum-likelihood trees were obtained from the rDNA sequences of *D. dendriticum* and *D. chinensis*. Thirty-eight unique rDNA haplotypes. The haplotype of each species is identified with the name of the species, and black circles indicate *D. dendriticum* haplotypes originating from the Chitral valley of Pakistan.

3.3.4. PCR Amplification of the Cytochrome C Oxidase Subunit 1(COX-1) Gene

DNA from all the lancet flukes was submitted to PCR amplification with specific primers to amplify the COX-1 region. A monomorphic DNA band with approximately 500 bp was visible for *Dicrocoelium* isolates of each host (Figures 3.3.4).

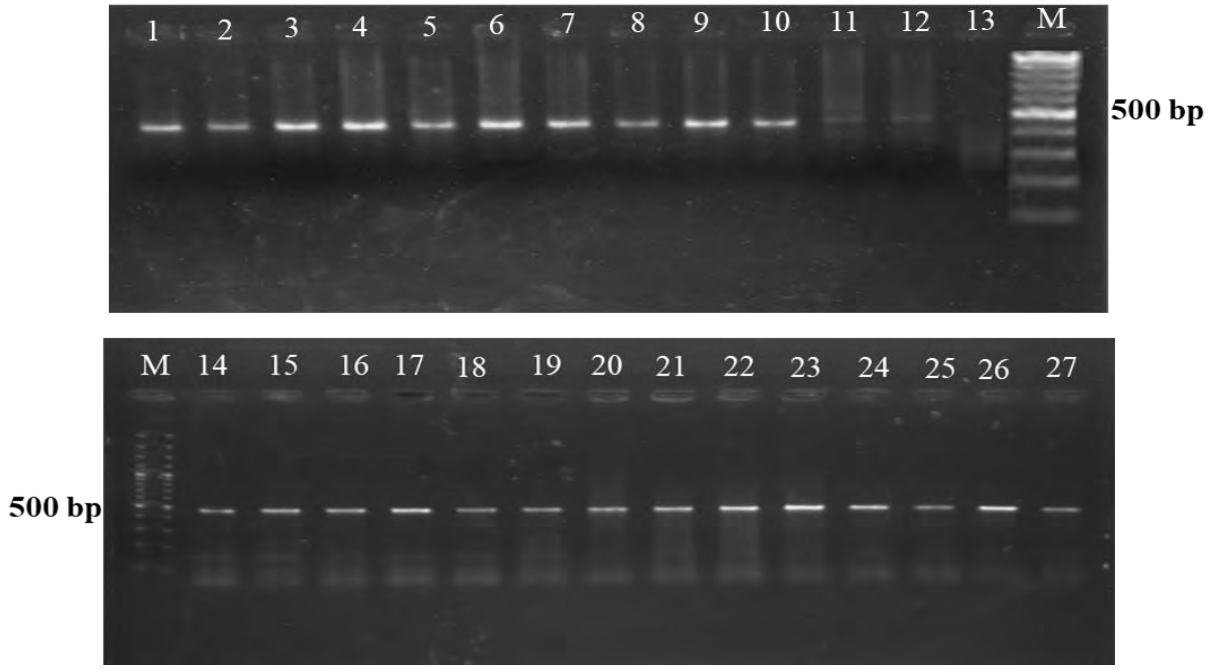


Figure 3.3.4. Agarose gel electrophoresis of the mtDNA regions (COX-1) PCR products from the isolates. M-100 bp molecular weight marker.

3.3.5. Mitochondrial COX-1 haplotypes

Unfortunately, COX-1 sequences of sufficient quality were generated from only 14 of the 34 flukes from which genomic DNA was successfully extracted. These were aligned with 56 *D. dendriticum* sequences and 11 *D. chinensis* sequences available on the public NCBI Mendeley database (Annexure 3.2) and trimmed to 187 bp length. This included 7 informative sites of intraspecific variation within *D. dendriticum* and 3 sites of intraspecific variation within D. chinensis. The alignment confirmed 12 species-specific fixed SNPs to differentiate between *D. dendriticum* and *D. chinensis* (Table 3.3.3) and allowed the molecular species identity of the 14 flukes to be re-affirmed as *D. dendriticum*. The 56 COX-1 *D. dendriticum* and 11 *D. chinensis* sequences from the public database were examined, along with the 14 *D. dendriticum* mtDNA sequences from the present study. Sequences showing 100% base pair similarity were grouped

into single haplotypes generating 8 unique *D. dendriticum* and 3 unique *D. chinensis* haplotypes (Annexure 3.2). An ML tree was constructed from the 11 unique COX-1 haplotypes to examine the evolutionary relationship between *D. dendriticum* and the other liver flukes. The phylogenetic tree re-affirms that *D. dendriticum* and *D. chinensis* form discrete species-specific clades (Figure 3.3.5) Four haplotypes generated from the 14 COX-1 sequences from the Chitral valley were clustered in the *D. dendriticum* clade. Comparison of these haplotypes with those originating from the Shaanxi province of China, Shiraz province of Iran, and Japan showed some common origins and close relationships (Table 3.3.4), but there were insufficient published sequence data to meaningfully describe the emergence and spread of the Pakistani *D. dendriticum*.

Table 3.3.3. The COX-1 data are based on 56 sequences identified as *D. dendriticum* and 11 sequences identified as *D. chinensis* on NCBI Gene Bank. Informative sites to describe intraspecific variation are shown in bold.

COX-1 nucleotide position	*D.dendriticum*	*D.chinensis*
9	T	A
12	T	C
15	T	C
24	G	T
30	G	T
54	**T/C**	**T**
57	**T/A**	**T**
63	**T**	**T/C**
66	**G/A**	**G**
75	**T**	**A/T**
78	**C/T**	**T**
84	**A**	G
111	G	A
114	C	T
129	T	A
132	**A**	G
135	T	A
138	**T/C**	**T**
141	T	A
143	**T/G**	**T**
177	**C/T**	**T**
183	**T**	**C/T**

Table 3.3.4. *D. dendriticum* mtCOX-1 haplotypes showing the number of sequences representing unique alleles and the country of origin. The materials and method section describes the accession number of all the sequences.

mt-COX-1 haplotypes	Total number of sequences	Countries
*D.dendriticum*1	4	Iran
*D.dendriticum*2	2	Japan
*D.dendriticum*3	2	Iran
*D.dendriticum*4	33	Iran, China, Pakistan
*D.dendriticum*5	2	Iran
*D.dendriticum*6	19	Iran, China, Pakistan
*D.dendriticum*7	7	Pakistan
*D.dendriticum*8	1	Pakistan

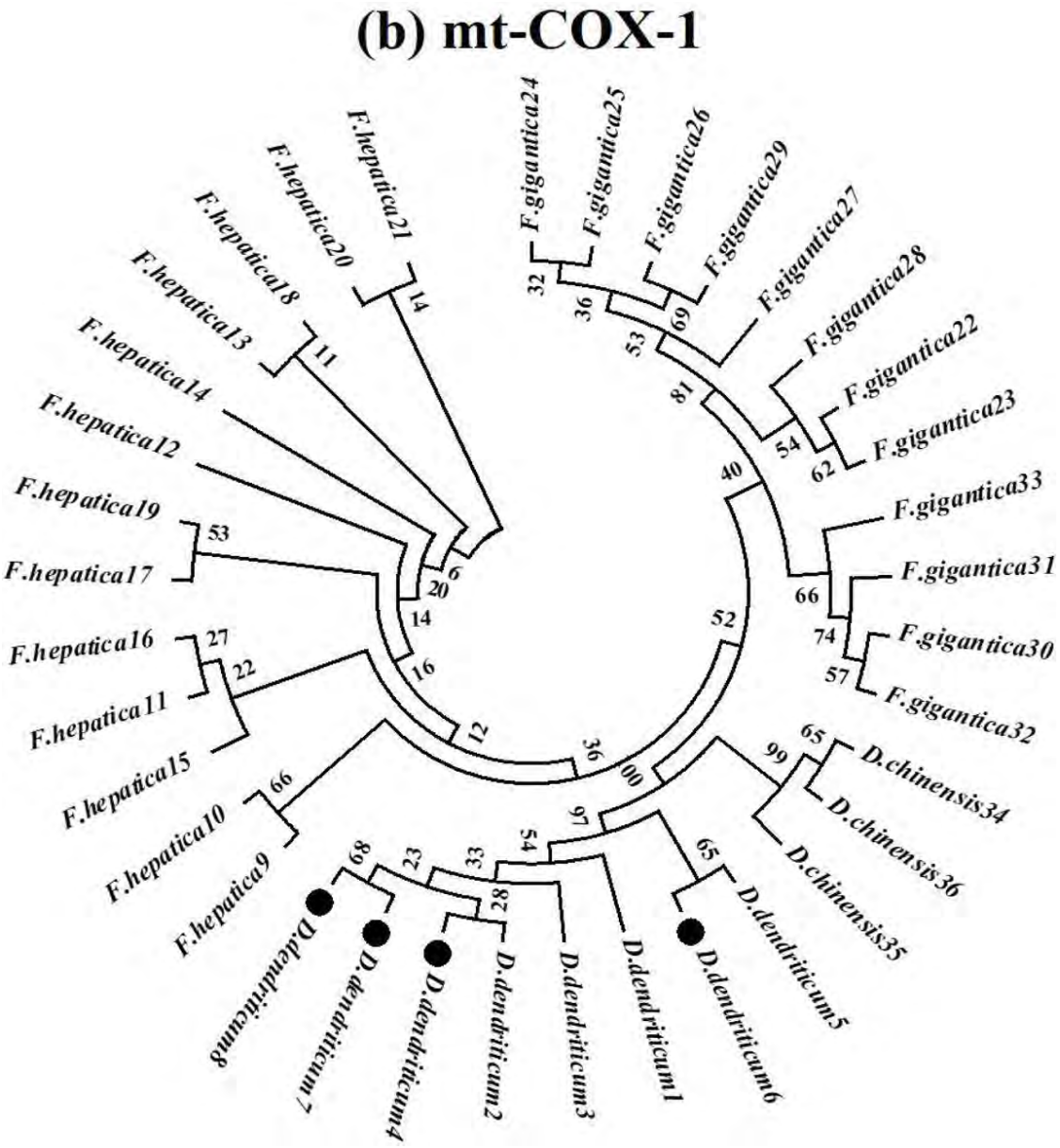
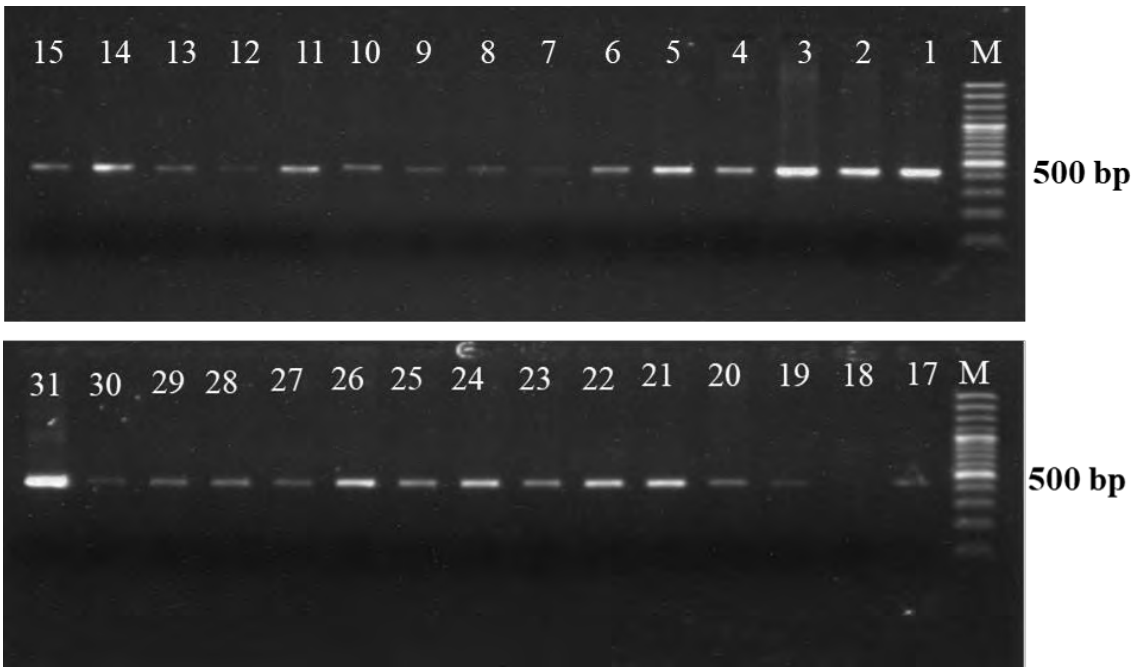


Figure 3.3.5: Maximum-likelihood trees were obtained from the COX-1 sequences of *D. dendriticum, D. chinensis, F.hepatica, and F. gigantica.* Thirty-six unique COX-1mtDNA haplotypes. The haplotype of each species is identified with the name of the species, and black circles indicate *D. dendriticum* haplotypes originating from the Chitral valley of Pakistan.

3.3.6. PCR Amplification of the Mitochondrial NADH Dehydrogenase Subunit 1 (NAD-1) Gene

DNA from all the lancet flukes was submitted to PCR amplification with specific primers to amplify the mitochondrial ND-1 region. A monomorphic DNA band with approximately 500 bp was visible for *Dicrocoelium* isolates of each host (Figure 3.3.6).



Figures 3.3.6: Agarose gel electrophoresis of the mtDNA regions (ND-1) PCR products from the isolates. M-100 bp molecular weight marker.

3.3.7. Mitochondrial ND-1 haplotypes

ND-1 sequences were generated from only 3 flukes from the present study. These were aligned with 46 sequences of *D. dendriticum* from NCBI GenBank and 11 sequences of *D. chinensis* available on the public database (Annexure 3.3) and trimmed to 217 bp length. This included 17 informative sites of intraspecific variation within *D. dendriticum* and 4 sites of intraspecific variation within *D. chinensis*. The alignment confirmed 19 species-specific fixed SNPs to differentiate between *D. dendriticum* and *D. chinensis* (Table 3.3.5), allowing the molecular species identity of the 3 flukes to be confirmed as *D. dendriticum*. The 46 *D. dendriticum* sequences from NCBI GenBank and 3 *D. dendriticum* ND-1 sequences from the present study were examined, along with 11 D. chinensis, 100 *F. hepatica*, and 31 *F. gigantica* sequences from the public

database. Sequences showing 100% base pair similarity were grouped into single haplotypes generating 14, 4, 11, and 23 unique *D. dendriticum*, D. chinensis, *F. hepatica*, and *F.gigantica* haplotypes, respectively (Annexure 3.3). An ML tree was constructed from the 52 ND-1 haplotypes to examine the evolutionary relationship between *D. dendriticum* and the other liver flukes. The phylogenetic tree indicates four species-specific clades (Figure 3.3.7). One haplotype generated from the 3 ND-1 sequences from the Chitral valley was in the *D. dendriticum* clade (Figure 3.3.8). Haplotype *D. dendriticum* 14 represented sequences from Pop-5, Pop-6, and Pop-8, which originated from the Laspoor, Mastuj, and Torkhow regions, while the closely related haplotypes *D. dendriticum* 2 and 3 represented sequences from China (Table 3.3.6).

Table 3.3.5: Sequence variation in a 217 bp fragment of ND-1 mtDNA, differentiating between *D. dendriticum* and *D. chinensis*. The ND-1 data are based on 46 sequences identified as *D. dendriticum* and 11 sequences identified as *D. chinensis* on NCBI GeneBank. Informative sites to describe intraspecific variation are shown in bold.

ND-1 Nucleotide position	*D.dendriticum*	*D.chinensis*
3	**T/A**	**T**
4	**A/G**	**A**
6	T	C
9	**G/A**	**G**
36	**A/G**	**A**
37	A	T
39	T	A
45	T	C
50	**G**	**G/C**
51	T	C
57	T	G
66	G	T
78	G	A
90	G	A
96	G	T
99	T	A
103	**G**	**G/A**
111	A	T
114	**G/A**	**G**
118	**G/A**	**T**
126	**T/C**	**T**
139	**G/A**	**G**
142	**C/A/G**	**A**
150	**A/G**	**G**
156	**A/G**	**G**
160	A	G
165	**C/T**	**T**
167	**C/T**	**C**
174	A	G
177	**C/T**	**G**
178	G	T
180	**G/A**	**G**
187	A	T
189	A	G
199	A	G

200	**T**	**T/C**
204	**C/T**	**T**
208	**T**	**T/C**
216	**A/G**	**G**

Table 3.3. 6. *D. dendriticum mt*-ND-1 haplotypes showing the number of sequences representing unique alleles and the country of origin. The accession number of all the sequences are described in the materials and method section.

mt-ND-1 haplotypes	Total number of sequences	Countries
*D.dendriticum*1	27	Japan, China, Iran
*D.dendriticum*2	1	Japan
*D.dendriticum*3	1	Japan
*D.dendriticum*4	1	Japan
*D.dendriticum*5	1	Japan
*D.dendriticum*6	1	Japan
*D.dendriticum*7	1	Japan
*D.dendriticum*8	1	Japan
*D.dendriticum*9	6	Japan, China, Iran
*D.dendriticum*10	1	Iran
*D.dendriticum*11	1	Iran
*D.dendriticum*12	3	Japan
*D.dendriticum*13	1	Japan
*D.dendriticum*14	3	Pakistan

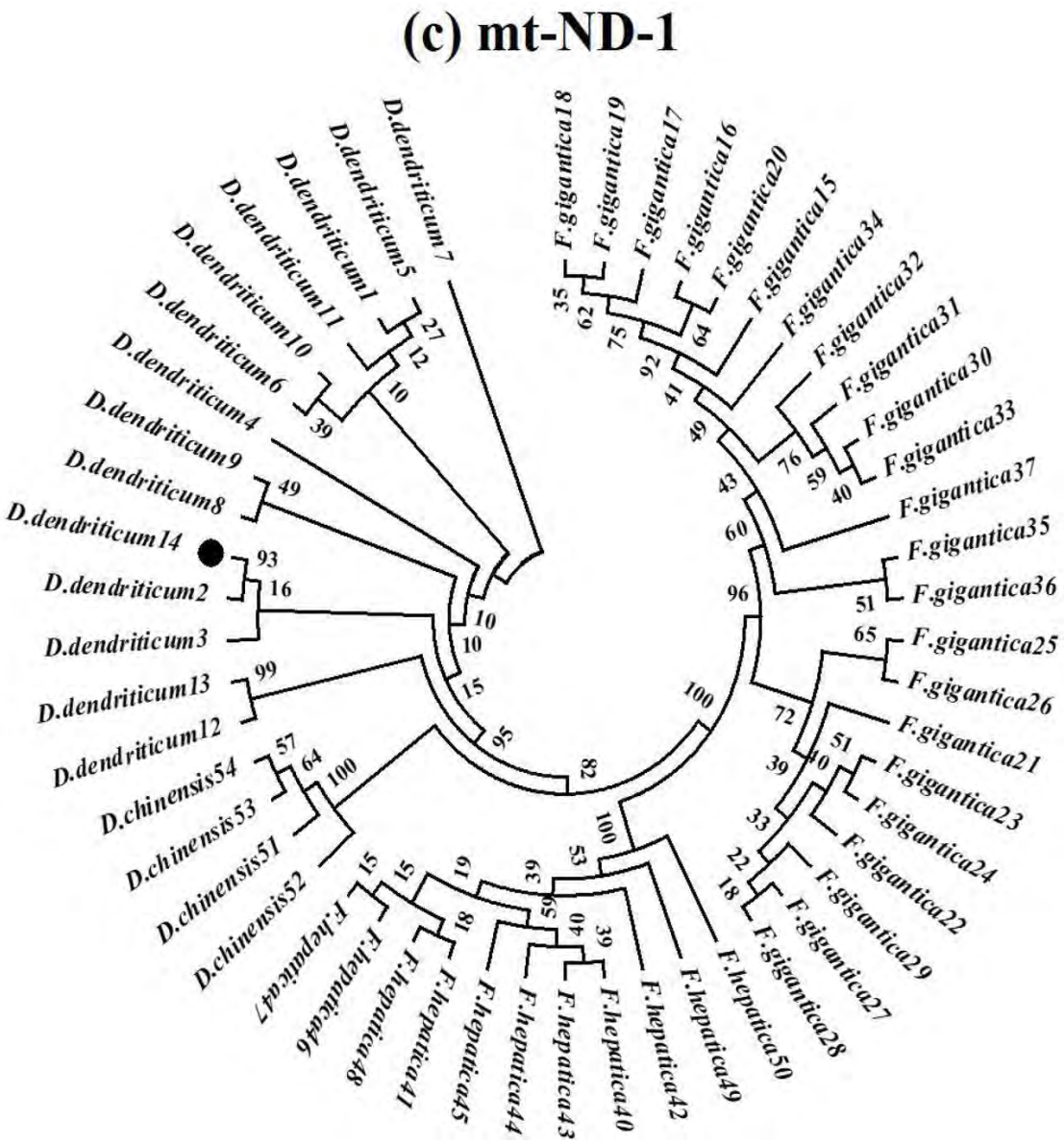


Figure 3.3.7. Maximum-likelihood trees were obtained from the ND-1 mtDNA sequences of *D. dendriticum, D. chinensis, F.hepatica, and F. gigantica.* Fifty-four unique ND-1mtDNA haplotypes. The haplotype of each species is identified with the name of the species, and black circles indicate *D. dendriticum* haplotypes originating from the Chitral valley of Pakistan.

The rDNA haplotype *D. dendriticum 18* represented two sequences from Pop-1 and Pop-7, which originated from the Booni and Mastuj regions, and sequences reported from the Shaanxi province of China. The rDNA haplotype *D. dendriticum 16* represented 12 sequences from Pop-2, Pop-3, Pop-4, Pop-6, and Pop-8, which originated from the Booni, Mastuj, and Torkhow regions, while the closely related rDNA haplotypes *D. dendriticum 24, 25, 26, 27* and *28* represented sequences from the Shaanxi province of China. The rDNA haplotype *D. dendriticum 23* represented 4 sequences from Pop-1 and Pop-5, which originated from the Booni and Laspoor regions, and sequences reported from the Shaanxi province of China. The rDNA haplotypes *D. dendriticum 19, 20,* and *21* each represented single sequences from Pop-1 and Pop-2, originating from the Booni region, while the closely related rDNA haplotypes *D. dendriticum 29, 31,* and *32* represented sequences from the Shaanxi province of China. The rDNA haplotype *D. dendriticum 33* represented 9 sequences from Pop-1, Pop-2, Pop-3, and Pop-5, originating from the Booni and Laspoor regions. The rDNA haplotype *D. dendriticum 34* represented 3 sequences from Pop-1 and Pop-6, originating from the Booni and Mastuj regions. The rDNA haplotypes *D. dendriticum 17* and *22* represented single sequences from Pop-1 and Pop-6, originating from the Booni and Mastuj regions, while the closely related rDNA haplotypes *D. dendriticum 25 and 30* represented sequences from the Shaanxi province of China. The mtDNA haplotype *D. dendriticum 4* represented 33 sequences from Pop-2, Pop-3, Pop-5, Pop-6, and Pop-7, originating from the Booni, Mastuj, and Laspoor regions and sequences reported from the Shaanxi province of China and Shiraz province of Iran. This haplotype was closely related to *D. dendriticum 2* reported from Japan. The mtDNA haplotype *D. dendriticum 7* represented sequences from Pop-1, Pop-4, Pop-5, Pop-6, and Pop-8, originating from the Booni, Mastuj, Laspoor, and Torkhow regions. The mtDNA haplotype *D. dendriticum 8* represented one sequence from Pop-3, which originated from the Booni regions. The mtDNA haplotype *D. dendriticum 6* represented 17 sequences from Pop-1, Pop-5, and Pop-6, originating from the Booni, Mastuj, and Laspoor regions and sequences reported from the Shaanxi province of China and Shiraz province of Iran. Haplotype *D. dendriticum 14* represented sequences from Pop-5, Pop-6, and Pop-8, which originated from the Laspoor, Mastuj, and Torkhow regions, while the closely related haplotypes *D. dendriticum 2* and *3* represented sequences from China. An ML tree was constructed from the 23 rDNA haplotypes to examine the evolutionary relationship between *D. dendriticum* and D. chinensis. The phylogenetic

tree shows that *D. dendriticum* and *D. chinensis* form discrete species-specific clades (Figure 3.3.3). Ten haplotypes generated from the 34 rDNA sequences from the Chitral valley were clustered in the *D. dendriticum* clade. Comparison of these haplotypes with those originating from the Shaanxi province of China showed some common origins and close relationships (Table 3.3.2), but there were insufficient published sequence data to meaningfully describe the emergence and spread of the Pakistani *D. dendriticum*. The rDNA haplotypes generated from the eight Pakistani *D. dendriticum* populations are shown in a split tree (Figure 3.3.8), albeit there are too little data to draw any conclusions.

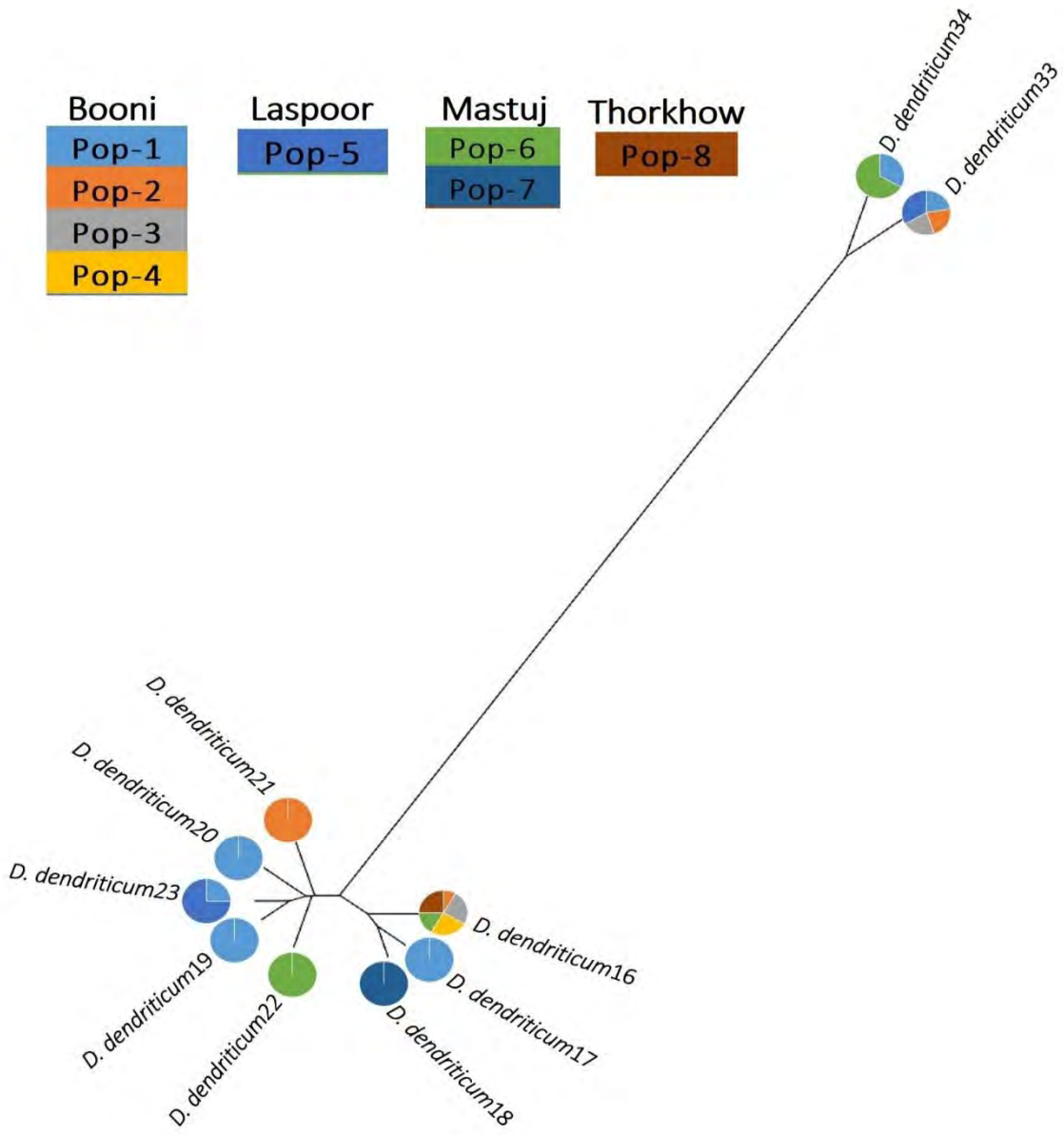


Figure 3.3.8. Split tree of 10 rDNA haplotypes generated from eight *D. dendriticum* populations. The tree was constructed with the UPGMA method in the HKY85 model of substitution in the Split Trees4 software. The pie chart circle represents the haplotype distribution from each of the eight populations. The color of each haplotype's circle represents the percentage of sequence reads generated per population

DISCUSSION

The economics of livestock production is marginal in the Himalayan ranges of Pakistan; hence better understanding of any potential production-limiting disease, such as dicrocoeliasis, is important. The prevalence of fasciolosis is also high in the region, where suitable habitats for the mud snail intermediate hosts of *Fasciola* spp. are created all year round in margins of monsoon rainfall-filled ponds, where animals are taken to drink. While various flukicidal anthelmintic drugs are available for use in the control of fasciolosis, few have high efficacy against all stages of *D. dendriticum (*Sargison *et al.,* 2012). Control of dicrocoeliasis in livestock, therefore, depends on evasive grazing management and strategic use of anthelmintic drugs. However, while an obvious management control measure for fasciolosis is to provide livestock with clean piped drinking water, the current understanding of where and when *D. dendriticum* metacercarial challenge arises is inadequate to inform management for the control of dicrocoeliasis. Accurate diagnostic tests are, therefore, required to improve our understanding of the epidemiology of dicrocoeliasis in specific regions where livestock are kept.

Dicrocoeliid liver flukes have previously been reported in Himalayan India (Jithendran and Bhat, 1996), China (Jithendran and Bhat, 1996; Hayashi *et al.,* 2017), and Iran (Khanjari *et al.,* 2014; Meshgi *et al.,* 2019); but there are only anecdotal and unconfirmed reports from Pakistan (Shah and Rehman, 2001). The molecular methods used in the present study confirm for the first time the species identity of *D. dendriticum* lancet flukes collected from abattoirs in the Chitral valley. Knowledge of the parasite species infecting livestock in any geographical region is of particular importance when considering effective and sustainable control strategies. Our confirmation of *D. dendriticum* in Pakistan highlights the need for a better understanding of aspects of the parasite's biology, such as the identification of the species of snail and ant that may act as competent intermediate hosts.

Previous studies have shown the value of morphological and molecular-based methods for the accurate species differentiation between *D. dendriticum* and *D. chinensis* (Otranto *et al.,* 2007; Liu *et al.,* 2014; Bian *et al.,* 2015; Gorjipoor *et al.,*

2015). The specimens collected from the Chitral valley were identified as the Dicrocoeliid genus, but overlapping morphological traits prevented their accurate species designation.

Our analyses of ribosomal cistron, mitochondrial COX-1, and ND-1 loci sequences of *D. dendriticum* and *D. chinensis* from the public database showed consistent inter-specific variations. Twenty-one discriminatory ribosomal cistron SNPs, 12 discriminatory COX-1 SNPs, and 19 discriminatory ND-1 SNPs were identified, allowing practical differentiation of the Dicrocoelid family as previously described (Liu *et al.,* 2014). *D. hospes* was not included in our analyses because directly comparable sequence data are not publicly available. The ML tree that was generated shows separate clades of *D. dendriticum* and D. chinensis; hence in the case of co-infections, molecular differentiation between each of the species is possible. The Chitral valley lancet flukes were all *D. dendriticum*, irrespective of their morphological identity. Morphological traits may be influenced by the stages of maturation of the flukes at the time of collection; the intensity of infection; factors linked to the host species; normal biological variation; or errors introduced during processing. Furthermore, the published morphological keys used for *D. chinensis* are based on parasites recovered from sika deer, whereas those for *D. dendriticum* are based on parasites recovered from sheep, albeit with no significant intraspecific variation reported from cattle-derived parasites (Otranto *et al.,* 2007). Consideration of these factors highlights the complementary value of morphological and molecular methods in fluke species identification.

Our analyses of ribosomal cistron and mitochondrial COX-1 loci sequences of *D. dendriticum* and *D. chinensis* from the public database showed consistent intra-specific variations. Ten haplotypes in the ribosomal cistron fragment and four in the mitochondrial COX-1 sequences from the Chitral valley of Pakistan were then used to analyze gene flow. Mitochondrial ND-1 haplotypes had to be excluded from the analysis of gene flow because insufficient DNA sequences were generated. Unfortunately, comparable sequence data for European and North American *D. dendriticum* populations were unavailable in the public database for analysis. There were unique and common haplotypes in each *D. dendriticum* population from the Chitral valley, some of which were also present in populations from China, Iran, and

Japan. There are insufficient data on which to base firm conclusions; hence further studies based on larger population sizes and using, for example next, generation methods as described for Calicophoron daubneyi rumen flukes and F. gigantica liver flukes (Sargison *et al.,* 2019; Rehman *et al.,* 2020) are needed to describe gene flow and the role of animal movement in the spread of *D. dendriticum*.

CONCLUSION

Our findings support the potential for the development of population genetics tools to improve understanding of the molecular evolutionary biology and phylogenetics of *D. dendriticum*. This is needed to study changing epidemiology of the parasite, potentially arising as a consequence of changing management and climatic conditions, as previously described using a panel of microsatellites and a COX-1 mtDNA sequence marker (Paridon *et al.,* 2017).

SPATIAL DISTRIBUTION OF *DICROCOELIUM* IN THE HIMALAYAN RANGES: POTENTIAL IMPACTS OF ECOLOGICAL NICHES AND CLIMATIC VARIABLES

ABSTRACT

Dicrocoeliosis can be an important cause of production loss in ruminants due to the cost of liver condemnation at slaughter. The aim of the present study was to determine the prevalence of *Dicrocoelium* infection and to predict the ecological niches and climatic variables that support dicrocoeliasis in the Himalayan ranges of Pakistan. *Dicrocoelium* was detected in 33 of 381 liver samples and 238 of 6060 blood samples taken from sheep and goat herds in the area. The prevalence of dicrocoeliasis was higher in sheep than in goats and highest in females aged greater than three years. An environmental risk map was created to predict active zones of transmission and showed the highest probability values in central parts of the Chitral district in the northwest of Pakistan. Climatic variables of the mean monthly diurnal temperature range (Bio2), annual precipitation (Bio12), and normalized difference vegetation index (NDVI) were found to be significantly ($p<0.05$) associated with the presence of *Dicrocoelium* infection. Together, the findings of this study demonstrate the most suitable ecological niches and climatic variables influencing the risk of dicrocoeliasis in the Himalayan ranges of Pakistan. The methods and results could be used as a reference to inform the control of dicrocoeliasis in the region.

Keywords: Dicrocoeliosis, Himalayas range, Ecological niches, Climatic variables, Sheep, Goats

INTRODUCTION

Dicrocoeliosis is an important parasitic disease caused by three species of the genus *Dicrocoelium*, namely *Dicrocoelium dendriticum*, *Dicrocoelium hospes* and *Dicrocoelium chinensis* (Otranto and Traversa, 2003). Among these, *D. dendriticum* is the most common and is distributed throughout Europe, Asia, North and South America, Australia, and North Africa. The other species have limited distribution and are present in Asia, Africa and some parts of Europe (Arias *et al.,* 2011). *Dicrocoelium* can infect the bile ducts of a variety of wild and domesticated mammals. Dicrocoeliosis causes overt economic loss due to the condemnation of livers with cholangitis from slaughtered animals at meat inspection (Jahed Khaniki *et al.,* 2013). Clinical signs of poor food intake, ill thrift, poor milk production, alteration in fecal consistency, photosensitization, and anemia have been described in animals with high burdens (Manga-Gonzalez *et al.,* 2001; Sargison *et al.,* 2012), and subclinical infection might cause reduced growth, although this is seldom measured.

Dicrocoelium has an exceptional life cycle that can take at least six months to complete. Within the same geographical location, several species of land snails and ants can be involved as first and second intermediate hosts, respectively (Mitchell *et al.,* 2017). Adult flukes are found in the bile ducts of their definitive herbivorous hosts. Eggs containing fully developed miracidia are shed in feces. They must be ingested by the snails before hatching and undergoing asexual replication and development into cercariae, which are shed by the snails and then eaten by ants. One cercaria migrates into the head of the ant and associates with the sub-oesophageal ganglion, while up to about 50 encyst in the gaster as metacercariae (Martín-Vega *et al.,* 2018). The larval stage that develops in the ant's head alters its behavior, making it cling to herbage and increasing the probability of its being eaten by a definitive host. Following encystment of the metacercariae, larval flukes migrate to the liver via the biliary tree and develop into adults (Manga-Gonzalez *et al.,* 2001).

Several studies have described the prevalence of *Dicrocoelium* in endemic regions; between 4.8 and 11% in Iran (Ezatpour *et al.,* 2015; Meshgi *et al.,* 2019b), between 5 and 30% in Canada (Dempsey *et al.,* 2019; van Paridon *et al.,* 2017), 0.7% in China (Zhu *et al.,* 2013) and 22% in Japan (Waki *et al.,* 2021). Due to its unique life cycle involving two intermediate hots, *Dicrocoelium* is highly affected by climatic factors. Temperature and humidity influence the survival of eggs containing miracidia and the development of snail and ant intermediate hosts in their respective environmental niches (Dempsey *et al.,* 2019; Meshgi *et al.,* 2019b). A seasonal pattern of the probability of infection has been shown in Canadian livestock, with the highest rate in mid-summer followed by an autumn decline (Dempsey *et al.,* 2019).

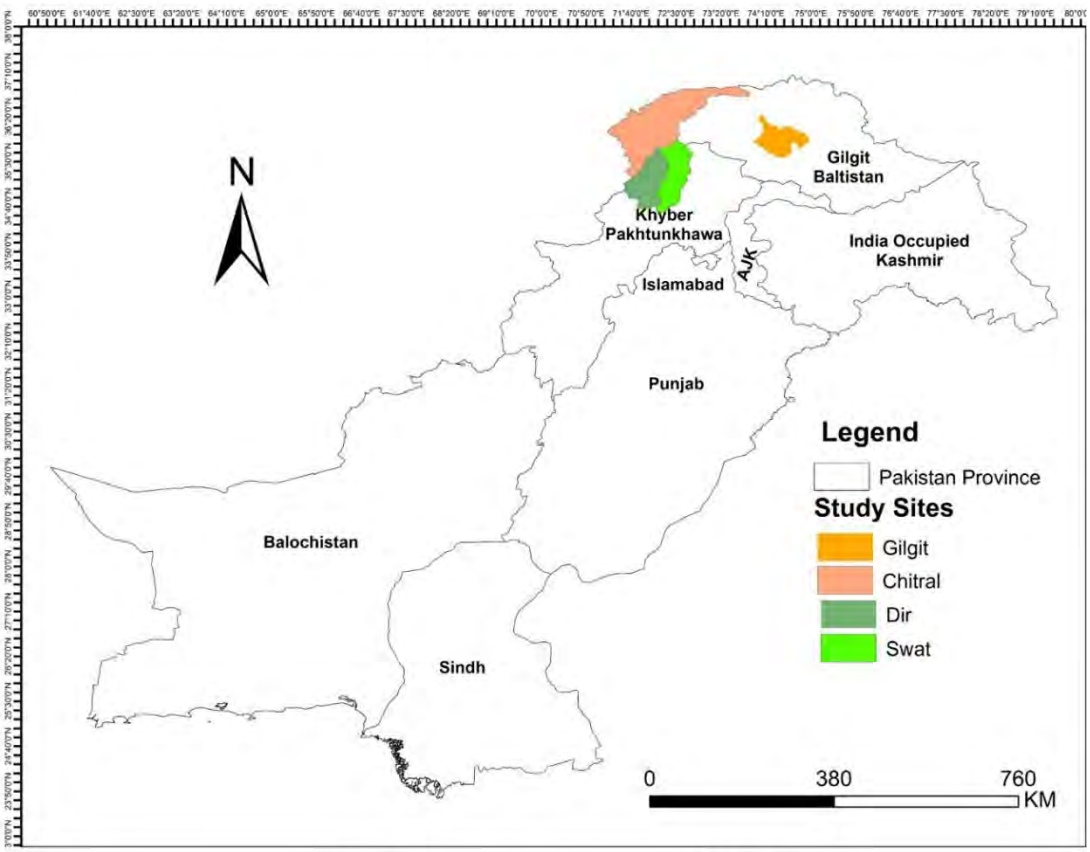
Due to the association between these environmental factors and the prevalence and geographical distribution of *Dicrocoelium* infection, species distribution models (SDMs) have the potential to determine the spatial pattern of disease and ecological niches supporting infection challenge. SDMs are based on the interaction between species adaptability and key predicting climatic factors informed by humidity, rainfall, temperature, and altitude (Bosso, 2018; Elith, 2011; Smeraldo, 2018; Soberón and Nakamura, 2009). Geographical Information Systems (GIS) and Maximum Entropy (MaxEnt) are the most widely used SDMs in the study of fluke parasites. These models have been used to show the geographical distribution and spatial pattern of fasciolosis or schistosomiasis and their risk factors associated with the ecological niches and climatic conditions (Bennema *et al.,* 2017; Chen *et al.,* 2015; Fox *et al.,* 2011; McCann *et al.,* 2010; Meshgi *et al.,* 2019a).

Dicrocoelium was first identified in the Himalayan ranges of Pakistan by Khan *et al.,* (2021). There have been few studies that provide information on the spatial distribution of dicrocoeliasis, and none in Asia. The present study was, therefore, undertaken to determine the prevalence and spatial distribution of dicrocoeliasis in the region and to describe the ecological niches that are favorable for the completion of the *Dicrocoelium* life cycle.

MATERIALS AND METHODS

4.2.1. Study Area

The study area is comprised of the Gilgit Baltistan and Khyber Pakhtunkhwa provinces of Pakistan (Figure 4.2.1). Gilgit Baltistan has a border with China through the Khunjerab pass, which occupies an area of over 72,971 km2. One district of Gilgit Baltistan was included in the study: (i) Gilgit district in the southwest of Karakoram range. The weather conditions include an average rainfall of 120 to 240 mm annually. Additional irrigation is obtained from the rivers, which are abundant with melting snow water from higher altitudes. The Khyber Pakhtunkhwa has a border with Afghanistan to the west and north and spreads over an area of over 74,521 km2. Three districts of Khyber Pakhtunkhwa were included in the study; (ii) Chitral district to the north of the Indus River, which originates close to the holy mountain of Kailash in western Tibet. Page 4/19 The average elevation is 1,500 m and the daily mean temperature ranges from 4.1°C to 15.6°C, creating an arid environment with only patchy coniferous tree cover, and providing habitats that are hostile to many snail species; (iii) Swat district surrounded by Chitral and Dir districts. The area is predominantly rural, and most residents live in villages. The average elevation is 980 m, resulting in a considerably cool and wet climate with lush forests, verdant alpine meadows, and snow-capped mountains. The climate of the Swat district is warm and humid with short and moderate summer, temperature rarely rises above 37°C. The annual rainfall averages around 33 inches with about 17 inches during June-September; (iv) Dir district borders to Afghanistan on the north and the Swat district to the east. The climate is cold, with average rainfall is 700 mm and the temperature varies from 6°C to 38°C.



Province	Gilgit Baltistan		Khyber Pakhtunkhwa	
District	Gilgit	Chitral	Swat	Dir
Coordinates	35.35°N 75.9°E	35.7699°N 71.7741° E	35°12′N 72°29′E	35°12′N 71°52′E
Elevation (m)	1500	1494	980	1420
Average precipitation (mm)	107.8	418	1011.5	1468.8
Average temperature (Min- Max) °C	6.2-19.4	11.4 -21.4	10-15	7.8-22.6
Liver samples examined	126	214	41	0
Blood samples examined	3020	2140	670	230

Figure 4.2.1 Areas of study in Chitral, Gilgit, Swat and Dir, Pakistan.

4.2.2. Study Design and Sample Collection

The study was carried out from July 2018 to September 2019. A random sampling was conducted and a total of 381 animals [Gilgit (n = 126), Chitral (n = 214), Swat (n = 41)] were examined for flukes' recovery, animals belonged to 56 sheep flocks and 24 goat herds. 10.04% of the sheep and 5.74% of the goat's livers had flukes. Adult Dicrocoelid flukes (50-100, 100-300 flukes per liver) were recovered from the liver of 26 sheep and 7 goats. The flukes were washed with phosphate-buffered saline (PBS) to remove adherent debris followed by Dicrocoelid morphological identification. A total of 6060 blood samples [Gilgit (n = 3020), Chitral (n = 2140), Swat (n=670) and Dir (n = 230)] were collected from 112 sheep and 48 goat herds. The blood samples were taken from the jugular vein of the animal herds and stored at 4°C for 4-6 hours before sera were separated. The number of blood samples to be collected was determined using

the formula: $n = Z^2P(1-P)/d^2$ (Daniel and Cross, 1999), where n was the sample size, Z was the desired confidence interval (95%), P was a conservative estimate of the proportion of infected animals in the population (0.5) and d was precision of estimation or range in which the true population proportion is estimated to be (5%). The samples were transferred to Department of Zoology, Quaid-I-Azam University, Islamabad, Pakistan, for further processing.

4.2.3. Herds Information, Screened through ELISA for Dicrocoeliosis in Gilgit

The regions with a significant concentration of cases were included in this study, a total of 28 counties were selected from Gilgit, and total of 72 herds from the mentioned counties were selected, out of which 48 herds were from sheep and 24 herds from goats, all the herds were tested for *Dicrocoelium* infection. The 72 herds consist of 5710 animals, out of which 3020 animals were screened through ELISA for Dicrocoeliosis presence, counties, and herds information were shown in Table 4.2.1.

4.2.4. Herds Information, Screened through ELISA for Dicrocoeliosis in Chitral

A total of 29 counties were selected from the Chitral region, and total of 54 herds from the selected counties were selected, out of which 36 herds were from sheep and 18 herds from goats, all the herds were tested for *Dicrocoelium* infection. The 72 herds consist of 3190 animals, out of which 2070 animals were screened through ELISA for Dicrocoeliosis presence, counties, and herds information were shown in Table 4.2.2.

4.2.5. Herds Information, Screened through ELISA for Dicrocoeliosis in Swat and Dir

A total of 08 counties were selected from Swat (6) and Dir (2), and total of 34 herds from these counties (30 from Swat and 4 from Dir) were selected, out of which 28 herds from sheep (which 24 herds from Swat and 4 sheep herds from Dir) 6 herds from goats (all six herds from Swat regions), all the herds were tested for *Dicrocoelium* infection. The 34 herds consist of 1266 animals, out of which 70 animals were screened through ELISA for Dicrocoeliosis presence, information regarding counties and herds was shown in Table 4.2.3.

4.2.6. Herds Information, Screened for Adult Flukes in the Study Area

A total of 381 slaughtered animals were examined for the presence of adult flukes (*Dicrocoelium*). Out of a total, 259 sheep animals and 122 goats were included in this study. From Gilgit 36 herds (126 animals) were examined, From Chitral 27 herds (214 animals) were examined and from Swat region 17 herds (41 animals) were examined, County-wise herds examined sheep and goats and the number of totals animals' details were present in below Table 4.2.4.

Table 4.2.1. Herds information, screened through ELISA for Dicrocoeliosis in Gilgit

Gilgit	Longitudes	Latitude	Herds (N)	Sheep Herds (N)	Goat Herds (N)	Animals (N)
Phander	72.92577582	36.16830481	4		4	150
Dalomal	72.88445966	36.15165566	3		3	120
Khonan Deh	72.82447566	36.17374448	3		3	110
Barsat	72.68235822	36.16476267	2	2		150
Yasin Valley	73.33959436	36.3602194	2	2		130
Damalgan	73.40479532	36.25741282	2	2		140
Sandhi	73.37310738	36.42244227	2	2		145
Raushan	73.52507938	36.22116713	2	2		150
Gahkuch	73.75971508	36.16805181	2	2		125
Barjangle	73.84378483	36.44135909	2		2	200
Singul	74.10879847	36.04304903	2		2	160
Rahim Abad	74.29851057	36.10135473	4	4		180
Chilmish Das	74.32483288	35.97944698	4	4		170
Danyor	74.38810427	35.91952078	4	4		180
Oshikhandas	74.46766955	35.88427908	2	2		130
Jaglot	74.62235228	35.68355741	3	3		290
Chalt Nagar	74.3230176	36.25213215	3	3		270
Chaprot	74.27928954	36.25747796	3	3		200
Hussain Abad	74.39550816	36.24808754	4	4		200
Rabat	74.31028973	36.26074172	3	3		110
Khizar Abad	74.37048165	36.24989659	3		3	280
Sikandar Abad	74.37292439	36.24490334	3		3	300
Jafar Abad	74.39680721	36.23677301	2		2	190
Harcho	74.79649251	35.45187006	2		2	230
Bunji	74.63417457	35.64362222	2	2		400
Doian	74.70315045	35.54201132	2	2		400

Gorikot	74.84184321	35.28217529	1	1		400
Bolan	74.85432454	35.32722239	1	1		200
	Total		**72**	**48**	**24**	**5710**

Table 4.2.2. Herds information, screened through ELISA for Dicrocoeliosis in Chitral

Chitral	Longitudes	Latitude	Herds (N)	Sheep Herds (N)	Goat Herds (N)	Animals (N)
Booni	72.25408342	36.27065815	4	4		252
Mastuj	72.51646335	36.27580273	4	4		192
Chinar	72.53666957	36.29905292	2	2		160
Chuinj	72.57234087	36.3177618	2	2		180
Unshit	72.49777774	36.22792404	2		2	80
Shaidas	72.492333	36.20836874	2		2	86
Gasht	72.48216148	36.19227539	2		2	114
Phargram	72.42720205	36.11420605	2		2	80
Phort	72.45249212	36.09822992	1		1	50
Lasht	72.45401066	36.09195636	1		1	45
Brock	72.45406227	36.08664618	2		2	65
Huzun	72.43538897	36.07516096	1	1		130
Balim	72.44061593	36.07008078	1	1		140
Raman	72.46423496	36.11582726	2	2		169
Harchin	72.4762235	36.11772126	2	2		157
Sor Laspor	72.47027663	36.04724606	2		2	130
Mori	71.97788528	35.98527822	1	1		70
Mori Payeen	71.96235341	35.9717946	1	1		60
Kaghozi	71.93570885	35.94411601	1		1	134
Singoor	71.80078909	35.89817402	1		1	120
Rondur	71.80499303	35.98730362	2	2		90
Riri Qwir	72.04230382	36.14513295	2	2		84

Barenis	72.03799451	36.072131	3	3		80
Pret	72.02233612	36.0547482	2	2		60
Kiyar	71.81919176	36.06065706	1	1		30
Drosh	71.80337168	35.5687402	2	2		90
Brun	71.69266145	35.69949912	2	2		82
Garam- Chashma	71.56388325	35.99694023	2	2		96
Torkhow	72.42492036	36.49056069	2		2	176
	Total		**54**	**36**	**18**	**3202**

Table 4.2.3 Herds information, screened through ELISA for Dicrocoeliosis in Swat and Dir

Swat	Longitudes	Latitude	Herds (N)	Sheep Herds (N)	Goat Herds (N)	Animals (N)
Bankhwar	72.5574839	35.54150413	7	7		220
Gabral	72.41215702	35.52524005	7	7		180
Utrar	72.46900083	35.49101312	6	6		260
Kalam	72.58858522	35.47791333	4	4		156
Boyun	72.60772352	35.46922477	3		3	130
Matiltan	72.65968608	35.54221495	3		3	90
	Total		**30**	**24**	**6**	**1036**
Dir						
Katair Dogdara	71.95316986	35.37313758	2	2		160
Maina Doag	71.96703689	35.34783052	2	2		110
	Total		**4**	**4**	**Zero**	**270**
	Total		**160**	**112**	**48**	**10218**

Table 4.2.4 Herds information, screened for adult flukes in the study area.

Municipality	Longitudes	Latitudes	No of Herds	Sheep Herds (N)	Goat Herds (N)	Animal (N)
Phander	72.9257758	36.16830481	5		5	18
Dalomal	72.8844597	36.15165566	3	3		24
Yasin Valley	73.3395944	36.3602194	3	3		14
Damalgan	73.4047953	36.25741282	2		2	11
Raushan	73.5250794	36.22116713	7	7		11
Danyor	74.3881043	35.91952078	8	8		7
Chalt Nagar	74.3230176	36.25213215	5		5	33
Gorikot	74.8418432	35.28217529	3	3		8
Booni	72.2540834	36.27065815	2	2		68
Mastuj	72.5164634	36.27580273	2	2		33
Chinar	72.5366696	36.29905292	2		2	19
Unshit	72.4977777	36.22792404	3		3	14
Gasht	72.4821615	36.19227539	3		3	7
Sor Laspor	72.4702766	36.04724606	1	1		17
Mori Payeen	71.9623534	35.9717946	3	3		7
Rondur	71.804993	35.98730362	1		1	5
Drosh	71.8033717	35.5687402	2	2		4
Brun	71.6926615	35.69949912	3	3		8
Garam Chashma	71.5638833	35.99694023	3	3		6
Torkhow	72.4249204	36.49056069	2	2		26
Gabral	72.412157	35.52524005	7	7		11
Utrar	72.4690008	35.49101312	5	5		6
Kalam	72.5885852	35.47791333	3		3	15
Boyun	72.6077235	35.46922477	2	2		9
	Total		**80**			**381**

4.2.7. Liver Sample Processing for Antigen Extraction

The liver samples were inspected for Dicrocoelid flukes to determine the infection rate among sheep and goats. Excretory/secretory (ES) and somatic antigens were extracted from Dicrocoelid flukes recovered from 33 positive liver samples as described by González-Lanza *et al.,* (2000) with some modifications. Briefly, flukes were incubated in RPMI 1640 medium (Biosera, Boussens, France) supplemented with 200 mM N-acetyl-L-alanyl-L-glutamine (Sigma), 0.3 g/l sodium bicarbonate 7.5% (Sigma) and 40 mg/l gentamycin at 37°C for 48 h. After removal of the flukes, the medium was collected and centrifuged at 10,000 g for 15 min at 4°C. To obtain a somatic extract, flukes were homogenized in tissue lysis buffer and added according to the weight of tissue in a ratio of 1000 µl buffer/100 mg of tissue. The homogenate was then transferred to pre-chilled eppendorf tubes and centrifuged at 10,000 rpm at 4°C for 10 minutes. The supernatant was filtered through 0.22 µm pore size filter units and Protease Inhibitor Cocktail (P8340; Sigma) was added. Protein concentration was determined by the Bradford method (Bradford, 1967). Samples were aliquoted and stored at -80°C until further processing.

4.2.8. Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed on 96 wells of microtiter plates as previously determined all incubation time by checkerboard titration method (Anuracpreeda *et al.,* 2016). Briefly, each eluted antigen was mixed with coating buffer NaHCO3/Na2CO3 (Merck) in equal proportion (1:1) and 100 µl was added to each well of the microtiter plate and incubated overnight at 4°C. The plates were washed three times with PBS containing 0.05% Tween 20 (Merck) and blocked with 0.05% BSA for 2 hours at room temperature. 100 µl of the diluted sera from infected and control animals was added to each well and incubated for 2 hr at 37°C and washed three times with PBS containing 0.05% Tween 20. After washing, 100 µl/well goat anti-bovine IgG secondary antibodies (1: 10,000), conjugated with alkaline phosphatase (InvitrogenTM Cat. nos. WP20006, WP20007) were added and incubated for 1 hr at room temperature. After washing the plates, 100 µl of the substrate para-nitrophenyl phosphate (PNPP) (Thermo Scientific TM Cat. No. 37621) was added and incubated at room temperature for 20 min. Finally, the reaction was stopped by the addition of 50 µl of 3N NaOH solution, and the optical density (OD)

value was recorded at 405 nm using an automated microplate reader. The sensitivity of the test was measured at 88%, and the specificity was 95%, respectively (Table 4.2.5). The sensitivity of the assay was determined using the formula: Sensitivity = [a / (a+c)]×100; where 'a' is the number of animals positive by ELISA and liver analysis (true positive), while 'c' is the number of animals positive by liver analysis but negative by ELISA (false negative). Similarly, Specificity = [d / (b+d)] ×100; where 'd' is the number of animals negative by ELISA and liver analysis (true negative), while 'b' is the number of animals negative by liver analysis but positive by ELISA (false positive). The cut-off was calculated by the mean optical density (OD) of the negative reference serum, plus three times standard deviations (0.14+3*0.08=0.38). The cut-off value was set at 0.38, and sera with OD value higher or equal to 0.38 were considered positive.

4.2.9. Species distribution models (SDMs)

Nineteen bioclimatic variables were obtained from the WorldClim (https://www.worldclim.org) global climate database (Fick and Hijmans, 2017) with the finest available resolution of approximately 1 km2 (Table 4.2.6). These layers were readable in ASCII format using ArcGIS 10.2 (ESRI, Redlands, CA, USA). The spatial patterns of *Dicrocoelium* infection were measured with MaxEnt-based modeling with MaxEnt version 3.4.4 (Phillips *et al.,* 2004; 2006). Maxent is freely downloadable at http://www.cs.princeton.edu/~schapire/maxent/. Field visits were conducted to obtain the geographic coordinates of *Dicrocoelium*-infected animals, and Global Positioning System (GPS) location was used to obtain the precise coordinates of infected animal flocks and herds. If a flock or herd had multiple infected animals, only one point was recorded to avoid the spatial clusters of localities.

The occurrence data of *Dicrocoelium* based on liver and blood samples were filtered to reduce bias and to improve the performance of the ecological niche modeling. The SDM toolbox in ArcGIS 10.2 software (ESRI, Redlands, CA, USA) was used to reduce the occurrence locations of each infected animal to a single point within 5 km. By eliminating duplicate occurrence points within the same pixel, *Dicrocoelium* presence points were reduced to 63 points from 160 presence points; 80% were used for the training and 20% for testing the model. 10045 points were used to determine the MaxEnt distribution (background Page 6/19 points and presence points). The model

was run with the logistic output format where predicted values range from 0 (impossible) to 1 (optimal).

The performance of predicting the ecological niches of *Dicrocoelium* infection was evaluated using threshold-independent receiver operating characteristic (ROC) assessment, where the area under the ROC (AUC) was obtained for plotting the model's sensitivity and specificity in MaxEnt. The geographical distribution of *Dicrocoelium* infection was mapped using a geographic information system (GIS). The presence points were marked on a world geodetic system (WGS84) reference coordinate system using high-resolution Google Earth and GIS coordinates. The parasite data were saved in an excel sheet and comma-separated values (CSV) files were used for the analysis. Compilation of geographic data and mapping was done by converting the excel data to the GIS format through Arc-Map (ESRI, Redlands, CA, USA).

To remove the autocorrelation among the 19 bioclimatic variables, Pearson's correlation was used at ($r2 \geq |0.8|$) through the SDM Tools function in ArcGIS 10.2 (Universal tool; Explore climate data; Remove highly correlated variable). Five bioclimatic variables [Bio2 = Mean Diurnal Range (Mean of monthly (max temp - min temp), Bio4 = Temperature Seasonality (standard deviation ×100), Bio6 = Min Temperature of Coldest Month, Bio12 = Annual Precipitation and Bio15 = Precipitation Seasonality (Coefficient of Variation)] were used for the analysis. Additional variables with the same resolution as the bioclimatic variables were included in the evaluation; these were normalized difference vegetation index (NDVI) extracted from moderate resolution imaging spectroradiometer (MODIS) images, calculated from the visible and near-infrared light reflected by vegetation (NDVI data are available in Raster data images, each of which has several blocks which have specific values for different vegetation; and can be processed in a MaxEnt readable format using specific conversion tools), forest cover, elevation, derived from the digital elevation model (DEM) in ArcGIS 10.2, and distance to buildings or settlements. The environmental variables used in the MaxEnt model are summarized in table 4.2.5. The environmental variables associated with dicrocoeliasis were generated using a jackknife test in MaxEnt version 3.4.4 (Phillips *et al.,* 2004; 2006).

4.2.10. Statistical analysis

The relatedness of *Dicrocoelium* prevalence, based on blood and liver samples examination, with associated environmental and climatic risk factors, was calculated by using the chi-square test of independence in a statistical package for the social sciences (SPSS) version 20 (Armonk, NY: IBM Corp). The level of significance was set at $P \leq 0.05$.

Table 4.2.5 Diagnostic efficacy of ELISA established for ES/ and somatic antigens.

Test	ELISA Test			Sensitivity	Specificity	KAPPA
Liver examination	Positive	Negative	Total	95% CI	95% CI	
Positive	29	4	33	87.9% (71.8-96.6)	94.6% (81.8-99.3)	Kappa= 0.823
Negative	2	33	35			SE of kappa = 0.069
			68			95% confidence interval: 0.688 to 0.958

Table 4.2.6. List of environmental variables used in the MaxEnt model.

Environmental variables	Interpretation	Source
bio1	Annual mean temperature	http://www.worldclim.org
bio2	Mean diurnal range (mean of monthly [max temp—min temp])	http://www.worldclim.org
bio3	Isothermality (Bio2/Bio7) (*100)	http://www.worldclim.org
bio4	Temperature Seasonality (standard deviation*100)	http://www.worldclim.org
bio5	Max Temperature of Warmest Month	http://www.worldclim.org
bio6	Min Temperature of Coldest Month	http://www.worldclim.org
bio7	Temperature Annual Range (Bio5-Bio6)	http://www.worldclim.org
bio8	Mean Temperature of Wettest Quarter	http://www.worldclim.org
bio9	Mean Temperature of Driest Quarter	http://www.worldclim.org
bio10	Mean Temperature of Warmest Quarter	http://www.worldclim.org
bio11	Mean Temperature of Coldest Quarter	http://www.worldclim.org
bio12	Annual precipitation	http://www.worldclim.org
bio13	Precipitation of Wettest Month	http://www.worldclim.org
bio14	Precipitation of driest month	http://www.worldclim.org
bio15	Precipitation Seasonality (Coefficient of Variation)	http://www.worldclim.org
bio16	Precipitation of Wettest Quarter	http://www.worldclim.org
bio17	Precipitation of Driest Quarter	http://www.worldclim.org
bio18	Precipitation of Warmest Quarter	http://www.worldclim.org
bio19	Precipitation of Coldest Quarter	http://www.worldclim.org
NDVI	Normalized difference vegetation index	NASA: http://modis-land.gsfc.nasa.gov/vi.html
Land Cover		
Elevation	elevation of the areas	Derived in ArcGIS 10.2 from DEM
forest cover	Type of forest	
distance to buildings/settlements	The density of settlements (m)	Calculated in Arc GIS 10.2

RESULTS

4.3.1. Prevalence of *Dicrocoelium*

Overall, Dicrocoelid flukes were identified in 33 of 381 (8.66%) liver samples, and 238 of 6060 (3.93%) blood samples were positive for both *Dicrocoelium* IgG antibodies (Table 4.3.1). *Dicrocoelium* was isolated from the liver samples of 20 of 56 sheep flocks and 13 of 24 goat herds, and blood samples showed the presence of *Dicrocoelium* IgG antibodies in 108 of 112 sheep flocks and 44 of 48 goat herds, respectively (Table 4.3.2 and 4.3.3). The highest month-wise proportion of *Dicrocoelium* was in September 2018 (14.8% and 6.31%) and August 2018 (15.22% and 5.92%) in liver and blood samples analysis, respectively. However, the significantly different ($p=0.0001$) prevalence was recorded in different months for blood sample analysis, while difference was not significant ($p=0.239$) for liver samples. The seasonal percentage of *Dicrocoelium* positive liver samples was higher during the summer and autumn (10.88% and 10%, respectively) than during the winter and spring (5.22% and 6.96%, respectively); and a similar trend was seen in the blood samples, but neither of these seasonal differences was significant ($p>0.05$). The percentage of *Dicrocoelium* positive blood samples was significantly higher ($p =0.0001$) in females (4.93%) than in male hosts (1.47%), and a similar, but non-significant trend was seen in the liver samples. The percentage of *Dicrocoelium* positive blood samples was significantly higher ($p =0.05$) in animals aged more than 3 years (4.5%) than in animals aged less than 1- year-old (3.26%), or 1 to 2 years old (3.33%). Similar, but non-significant trends were seen in the liver samples. The percentage of *Dicrocoelium* positive blood samples was significantly higher ($p =0.0001$) in goats (7.39%) than in sheep (3.29%); while the percentage of *Dicrocoelium* positive liver samples was significantly higher ($p =0.0001$) in sheep (10.04%) than in goats (5.74%). These data are shown in Table 4.3.1.

4.3.2. Geographical Distribution of *Dicrocoelium*

The prevalence of *Dicrocoelium* was highest in the Chitral district (7.1% and 9.81% positive blood and liver samples, respectively); followed by the Gilgit district (2.58% and 7.94% positive blood and liver samples, respectively); and lowest in Swat (1.19% and 4.88% positive blood and liver samples, respectively) and Dir (no positive samples, albeit the numbers of animals sampled in these districts, were small). Within each region, the prevalence of *Dicrocoelium* positive samples varied between different valleys from 0.5% (Doian valley in Gilgit) to 17.5% (Pret valley in Chitral) of blood samples and 3.85% (Torkhow valley in Chitral) to 18.18% (Raushan valley in Gilgit) of liver samples, as shown in Table 4.3.4). *Dicrocoelium* positive samples were identified in each valley in the Chitral and Swat districts. No *Dicrocoelium* positive samples were detected in the Barjangle, Singul and Bolan valleys in the Gilgit district; or in the Katair Dogdara and Maina Doag valleys of Dir district.

Table 4.3.1: Overall Prevalence of *Dicrocoelium* based on month, season, sex, age, and host during the study period 2018–2019.

Variables		Blood samples			Liver analysis		
		Animals	*Positive n (%)*	*P-Value*	*Animals*	*Positive n (%)*	*P-Value*
Month	Jul-18	500	24 (4.8)	$\chi 2 = 35.85$	63	7 (11.11)	$\chi 2 = 13.89$
	Aug-18	540	32 (5.92)	p=0.0001**	46	7 (15.22)	p=0.239NS
	Sep-18	570	36 (6.31)		54	8 (14.8)	
	Oct-18	520	24 (4.61)		36	1 (2.8)	
	Nov-18	440	16 (3.64)		34	0 (00)	
	Mar-19	500	8 (1.6)		33	4 (12.12)	
	Apr-19	520	22 (4.23)		24	2 (8.33)	
	May-19	520	20 (3.85)		24	0 (00)	
	Jun-19	540	12 (2.22)		16	1 (6.25)	
	Jul-19	520	8 (1.54)		13	1 (7.7)	
	Aug-19	450	20 (4.44)		23	1 (4.35)	
	Sep-19	440	16 (3.64)		15	1 (6.7)	
Season	Spring (March-April)	1020	30 (2.94)	$\chi 2 = 7.294$	29	2 (6.96)	$\chi 2 = 2.96$
	Summer (May-Aug)	3070	116 (3.78)	p=0.063NS	147	16 (10.88)	p=0.398NS
	Autumn (Sept-Oct)	1530	76 (4.97)		90	9 (10)	
	Winter (Nov-Feb)	440	16 (3.64)		115	6 (5.22)	
Sex	Female	4296	212 (4.93)	$\chi 2 = 39.69$	222	22 (9.91)	$\chi 2 = 1.05$
	Male	1764	26 (1.47)	p=0.0001**	159	11 (6.92)	p=0.306NS
Age	<1 year	920	30 (3.26)	$\chi 2 = 5.718$	43	3 (6.98)	$\chi 2 = 4.73$

	>1 year	1984	66 (3.33)	p=0.05*	152	8 (5.26)	p=0.094NS
	In any age	3156	142 (4.5)		186	22 (11.83)	
Host	Sheep	5113	168 (3.29)	$\chi 2 = 5536.3$	259	26 (10.04)	$\chi 2 = 349.7$
	Goat	947	70 (7.39)	p=0.0001**	122	07 (5.74)	p=0.0001**
Total		**6060**	**238 (3.93)**		**381**	**33 (8.66)**	

Table 4.3.2: Presence of *Dicrocoelium* in sheep and goat herds according to liver samples analysis during the study period 2018–2019.

Host	Breed	Liver samples				
		Total number of flocks/herds examined	***Dicrocoelium*-positive flocks/herds in Chitral**	***Dicrocoelium*-positive flocks/herds in Gilgit**	***Dicrocoelium*-positive flocks/herds in Swat**	***Dicrocoelium*-positive flocks/herds in Dir**
Sheep	Kelli	5	2	-	-	-
	Ramghani	9	5	-	-	-
	Balkhi	23	3	7	2	-
	Waziri	10	-	1	7	0
	Katchli	9	-	5	-	-
	Total	**56**	**10**	**13**	**9**	**0**
Goat	Khurasani	13	7	5	-	0
	Cross Beetal	4	1	0	0	0
	Waziri	7	0	5	-	0
	Total	**24**	**8**	**10**	**0**	**0**

Table 4.3.3: **Presence of *Dicrocoelium* in sheep and goat herds according to blood samples analysis during the study period 2018–2019.**

Host	Breed	blood samples				
		Total number of flocks/herds examined	***Dicrocoelium*-positive flocks/herds in Chitral**	***Dicrocoelium*-positive flocks/herds in Gilgit**	***Dicrocoelium*-positive flocks/herds in Swat**	***Dicrocoelium*-positive flocks/herds in Dir**
Sheep	Kelli	10	10	-	-	-
	Ramghani	18	18	-	-	-
	Balkhi	46	8	28	10	-
	Waziri	20	-	2	14	0
	Katchli	18	-	18	-	-
	Total	**112**	**36**	**48**	**24**	**0**
Goat	Khurasani	26	14	6	-	2
	Cross Beetal	8	4	2	0	2
	Waziri	14	-	12	-	2
	Total	**48**	**18**	**20**	**0**	**6**

Table 4.3.4. Prevalence of *Dicrocoelium* determined in liver and blood samples.

Locations		*Blood samples*			*Liver samples*		
District	*Valley*	*Number of animals*	*Number positive (%)*	*P-Value*	*Number of animals*	*Number positive (%)*	*P-Value*
Gilgit							
	Phander	100	7 (7)	$\chi^2 =$ 213.39	18	1 (5.56)	χ^2 19.064
	Dalomal	70	4 (5.71)	p=0.0001**	24	4 (16.67)	p=0.697NS
	Khonan Deh	70	5 (7.14)				
	Barsat	100	3 (3)				
	Yasin Valley	80	2 (2.5)		14	1 (7.14)	
	Damalgan	80	1 (1.25)		11	0	
	Sandhi	100	9 (9)				
	Raushan	95	7 (7.37)		11	2 (18.18)	
	Gahkuch	85	6 (7.06)				
	Barjangle	120	0				
	Singul	100	0				
	Rahim Abad	110	5 (4.55)				
	Chilmish Das	110	4 (3.64)				
	Danyor	100	4 (4)		7	0	
	Oshikhandas	80	3 (3.75)				
	Jaglot	150	2 (1.33)				
	Chalt Nagar	140	2 (1.43)		33	2 (6.06)	
	Chaprot	120	2 (1.67)				
	Hussain Abad	60	1 (1.67)				

Table 4.3.4 Continuation

Locations		*Blood samples*			*Liver samples*		
District	***Valley***	***Number of animals***	***Number positive (%)***	***P-Value***	***Number of animals***	***Number positive (%)***	***P-Value***
	Rabat	50	1 (2)				
	Khizar Abad	140	2 (1.43)				
	Sikandar Abad	150	2 (1.33)				
	Jafar Abad	120	1 (0.83)				
	Harcho	90	1 (1.11)				
	Bunji	150	2 (1.33)				
	Doian	200	1 (0.5)				
	Gorikot	180	1 (0.56)		8	0	
	Bolan	70	0				
	Total	*3020*	*78 (2.58)*		*126*	*10 (7.94)*	
	Mean ±SEM	*10.86 ±6.82*	*2.79 ±0.44 (3.25 ±0.48)*		*15.75 ±3.15*	*1.25 ±0.49 (10.72 ±2.18)*	
Chitral							
	Booni	200	22 (11)		68	12 (17.65)	
	Mastuj	140	18 (12.86)		33	2 (6.06)	
	Chinar	110	3 (2.73)		19	2 (10.53)	
	Chuinj	110	3 (2.73)				
	Unshit	60	3 (5)		14	1 (7.14)	
	Shaidas	60	3 (5)				
	Gasht	80	4 (5)		7	1 (14.29)	
	Phargram	70	4 (5.71)				

Table 4.3.4 Continuation

Locations		*Blood samples*			*Liver samples*		
District	*Valley*	*Number of animals*	*Number positive (%)*	*P-Value*	*Number of animals*	*Number positive (%)*	*P-Value*
	Phort	40	4 (10)				
	Lasht	40	3 (7.5)				
	Brock	50	5 (10)				
	Huzun	75	8 (10.67)				
	Balim	75	6 (8)				
	Raman	80	5 (6.25)				
	Harchin	80	6 (7.5)				
	Sor Laspor	100	4 (4)		17	1 (5.88)	
	Mori	33	2 (6.06)				
	Mori Payeen	27	1 (3.7)		7	0	
	Kaghozi	80	4 (5)				
	Singoor	80	4 (5)				
	Rondur	34	4 (11.76)		5	0	
	Riri Qwir	26	2 (7.69)				
	Barenis	50	7 (14)				
	Pret	40	7 (17.5)				
	Kiyar	30	2 (6.67)				
	Drosh	88	3 (3.41)		4	0	

Table 4.3.4 Continuation

Locations		*Blood samples*			*Liver samples*		
District	***Valley***	***Number of animals***	***Number positive (%)***	***P-Value***	***Number of animals***	***Number positive (%)***	***P-Value***
	Brun	82	4 (4.88)		8	1 (12.5)	
	Garam Chashma	60	3 (5)		6	0	
	Torkhow	140	8 (5.71)		26	1 (3.85)	
	Total	*2140*	*152 (7.1)*		*214*	*21 (9.81)*	
	Mean ±SEM	*73.79 ±7.3*	*5.24 ±0.83 (7.25 ±0.67)*		*17.83 ±5.26*	*1.75 ±0.95 (9.74 ±1.39)*	
Swat							
	Bankhwar	130	2 (1.54)				
	Gabral	110	2 (1.82)		11	1 (9.09)	
	Utrar	130	1 (0.77)		6	0	
	Kalam	90	1 (1.11)		15	0	
	Boyun	130	1 (0.77)		9	1 (11.11)	
	Matiltan	80	1 (1.25)				
	Total	*670*	*8 (1.19)*		*41*	*2 (4.88)*	
	Mean ±SEM	*111.67 ±9.1*	*1.33 ±0.21 (1.21 ±0.17)*		*10.25 ±1.89*	*0.5 ±0.29 (10.1 ±0.71)*	
Dir							
	Katair Dogdara	120	0				
	Maina Doag	110	0				
	Total	*230*	*0*		*0*	*0*	
	Mean ±SEM	*115 ±5*	*0*		*0*	*0*	
Overall		***6060***	***238 (3.93)***		***381***	***33 (8.66)***	

4.3.3. Prevalence of *Dicrocoelium* in Chitral

A total of 2140 animal blood samples were examined and 152 (7.1%) were found positive for *Dicrocoelium*, while from 214 liver samples 21(9.81%) were positive for *Dicrocoelium* (Table 4.3.5). The highest month-wise proportion of *Dicrocoelium* was in September 2018 (10.58%) in blood samples analysis and August 2018 (18.2%) in liver samples analysis, respectively. The seasonal proportion of *Dicrocoelium* was recorded in both liver and blood samples, and the prevalence was highest during summer (13.8% to 7.01%) followed by autumn (9.6% to 8.4%), respectively. The proportion of *Dicrocoelium* was highest in females (10.9% in liver samples and 8.61% in blood samples. Overall, *Dicrocoelium* was recorded higher in sheep (10.05%, 7.9%) than goats (8.88%, 5.75%) for liver and blood samples, respectively (Table 4.3.5).

4.3.4. Prevalence of *Dicrocoelium* in Gilgit Baltistan

A total of 3020 animal blood samples were examined and 78 (2.58%) were found positive for *Dicrocoelium*, while 126 livers examined 10(7.93%) were positive for *Dicrocoelium* (Table 4.3.6). The highest month-wise proportion of *Dicrocoelium* was in September 2018 (12.5% and 5.92%) and October 2018 (9.1% and 4.8%) for liver and blood samples analysis, respectively. The seasonal proportion of *Dicrocoelium* was recorded in both liver and blood samples, and the prevalence was highest during summer (8.75% to 2.22%) followed by autumn (11.11% to 4.55%), respectively. The proportion of *Dicrocoelium* was highest in females (10.13% for liver and 3.43% blood samples). Overall, *Dicrocoelium* was recorded higher in sheep (10.93%, 2.72%) than goats (4.84%, 2.3%) in liver and blood samples, respectively (Table 4.3.6).

4.3.5. Prevalence of *Dicrocoelium* in Dir and Swat

A total of 900 animal blood samples were examined and 08 (0.98%) were found positive for *Dicrocoelium*, while 41 livers examined 2 (4.87%) were positive for *Dicrocoelium* (Table 4.3.7). The highest month-wise proportion of *Dicrocoelium* was in August 2019 (0.00% and 2.5%) and September 2018 (11.11% and 1.53%) in liver and blood samples analysis, respectively. The seasonal proportion of *Dicrocoelium* was recorded in liver and blood samples, and the prevalence was highest during autumn (9.1% to 1.3%) followed by winter (6.25% to 2.5%), respectively. The proportion of

Dicrocoelium was highest in females and overall, *Dicrocoelium* was recorded higher in sheep (7.7%, 0.99%) than goats (0.00%, 0.95%) in liver and blood samples, respectively (Table 4.3.7).

Table 4.3.5. Prevalence of *Dicrocoelium* based on month, season, sex, age, and host in Chitral district, Khyber Pakhtunkhwa

Variables		Blood samples		Liver samples	
		Animals	*Positive n (%)*	*Animals*	*Positive n (%)*
Month	July-2018	200	14 (7)	37	6 (16.21)
	August-2018	200	20 (10)	22	4 (18.2)
	September-2018	170	18 (10.58)	29	5 (17.24)
	October-2018	160	10 (6.25)	23	0 (00)
	November-2018	140	6 (4.28)	25	0 (00)
	March-2019	210	8 (3.81)	8	2 (25)
	April-2019	220	22 (10)	9	1 (11.11)
	May-2019	200	16 (8)	13	0 (00)
	June-2019	220	12 (5.45)	13	1 (7.7)
	July-2019	200	6 (3)	7	0 (00)
	August-2019	120	12 (10)	15	1 (.66)
	September-2019	100	8 (8)	13	1 (7.7)
Seasons	**Spring (March-April)**	430	30 (6.97)	20	1 (5)
	Summer (May-Aug)	1140	80 (7.01)	87	12 (13.8)
	Autumn (Sept-Oct)	430	36 (8.4)	52	5 (9.6)
	Winter (Nov-Feb)	140	6 (4.35)	55	3 (5.45)
Sex	Female	1650	142 (8.61)	110	12 (10.9)
	Male	490	10 (2.04)	104	9 (8.65)
Age	<1 year	338	22 (6.51)	15	1 (6.66)
	>1 year	648	38 (5.86)	83	3 (3.61)
	In any age	1154	92 (7.97)	116	17 (14.65)
Host	Sheep	1340	106 (7.9)	169	17 (10.05)
	Goat	800	46 (5.75)	45	4 (8.88)
Total		**2140**	**152 (7.1)**	**214**	**21 (9.81)**

Table 4.3.6. Prevalence of *Dicrocoelium* based on month, season, sex, age, and host in Gilgit Baltistan

Parameters		Blood samples		Liver samples	
		Animals	*Positive n (%)*	*Animals*	*Positive n (%)*
Month	July-2018	240	10 (4.26)	20	1 (5)
	August-2018	260	12 (4.61)	17	3 (17.65)
	September-2018	270	16 (5.92)	16	2 (12.5)
	October-2018	250	12 (4.8)	11	1 (9.1)
	November-2018	220	8 (3.64)	5	0 (00)
	March-2019	240	0 (00)	18	1 (5.55)
	April-2019	240	0 (00)	11	1 (9.1)
	May-2019	260	4 (1.54)	10	0 (00)
	June-2019	260	0 (00)	3	0 (00)
	July-2019	260	2 (0.77)	6	1 (6.66)
	August-2019	250	6 (2.4)	7	0 (00)
	September-2019	270	8 (2.96)	2	0 (00)
Seasons	**Spring (March-April)**	480	0 (00)	9	1 (11.11)
	Summer (May-Aug)	1530	34 (2.22)	46	4 (8.75)
	Autumn (Sept-Oct)	790	36 (4.55)	27	3 (11.11)
	Winter (Nov-Feb)	220	8 (3.64)	44	2 (4.54)
Sex	Female	1962	66 (3.43)	79	8 (10.13)
	Male	1058	12 (1.13)	47	2 (4.25)
Age	<1 year	490	8 (1.63)	28	2 (7.14)
	>1 year	1040	24 (2.31)	57	5 (8.77)
	In any age	1490	46 (3.17)	41	3 (7.32)
Host	Sheep	2060	56 (2.72)	64	7 (10.93)
	Goat	960	22 (2.3)	62	3 (4.84)
Total		**3020**	**78 (2.58)**	**126**	**10 (7.93)**

Table 4.3.7. Prevalence of *Dicrocoelium* based on month, season, sex, age, and host in Dir, Swat districts of Khyber Pakhtunkhwa

Parameters		Blood samples		Liver samples	
		Animals	*Positive n (%)*	*Animals*	*Positive n (%)*
Month	July-2018	60	0 (00)	6	0 (00)
	August-2018	80	0 (00)	7	0 (00)
	September-2018	130	2 (1.53)	9	1 (11.11)
	October-2018	110	2 (1.82)	2	0 (00)
	November-2018	80	2 (2.5)	4	0 (00)
	March-2019	50	0 (00)	7	1 (14.3)
	April-2019	60	0 (00)	4	0 (00)
	May-2019	60	0 (00)	1	0 (00)
	June-2019	60	0 (00)	0	0 (00)
	July-2019	60	0 (00)	0	0 (00)
	August-2019	80	2 (2.5)	1	0 (00)
	September-2019	70	0 (00)	0	0 (00)
Seasons	**Spring (March-April)**	110	0 (00)	0	0 (00)
	Summer (May-Aug)	400	2 (0.5)	14	0 (00)
	Autumn (Sept-Oct)	310	4 (1.3)	11	1 (9.1)
	Winter (Nov-Feb)	80	2 (2.5)	16	1 (6.25)
Sex	Female	684	4 (0.64)	33	2 (6.1)
	Male	216	4 (1.85)	8	0 (00)
Age	<1 year	92	0 (00)	0	0 (00)
	>1 year	296	4 (1.35)	12	0 (00)
	In any age	512	4 (0.81)	29	2 (6.89)
Host	Sheep	690	6 (0.99)	26	2 (7.7)
	Goat	210	2 (0.95)	15	0 (00)
Total		**900**	**8 (0.98)**	**41**	**2 (4.87)**

4.3.6. Prevalence of *Dicrocoelium* infection among sheep and goats herds and other animals

A total of 80 herds, (56 herds of sheep and 24 herds of goats) were included in the liver sample examination. The total number of animals examined other than herds were 381 (259 sheep and 122 goats). 50 (62.5%) herds were found to be positive, of which 32 (57.14%) were sheep herds and 18 (75%) were goats' herds. A total of 33 animals were found positive (26 sheep and 7 goats). A total of 160 herds were tested for the presence of *Dicrocoelium* infection. Out of a total of 152 (95%), herds were found seropositive, 108 (96.43%) sheep herds, and 44 (91.77%) goats' herds tested positive. A total of 238 animals other than herds were serologically positive, and the number of positive sheep and goats were 168 and 70 respectively (Table 4.3.8).

Table 4.3.8 Prevalence of *Dicrocoelium* infection among sheep and goats herds and other animals.

	Liver samples		Blood samples	
Animals	**N**	**n (%)**	**N**	**n (%)**
Sheep Herds	56	32 (57.14)	112	108 (96.43)
Goat Herds	24	18 (75)	48	44 (91.77)
Total	80	50 (62.5)	160	152 (95%)
Sheep	259	26 (10.04)	7178	168 (2.34)
Goat	122	07 (5.74)	3040	70 (2.33)
Total	381	33 (8.66)	10218	238 (2.34)

4.3.7 Predictive prevalence of *Dicrocoelium* based on liver samples

Figure 4.3.1, display the potential geographic distribution of *Dicrocoelium* infection predicted by the Maxent program. The highest level of infection in most of our study areas was recorded in Phander, Yasin valley, Unshit, Gasht, Sor Laspor, Brun, Garam Chashma, Torkhow, Boyun, Raushan, Chalt Nagar, Mastuj, Chinar, in Dalomal and Booni region highest infection rate was observed in the animals. In figure 4.3.1, the red color predicts the highest prevalent areas and the greenish areas are less prevalent or infection-free areas. The highest number of infections was predicted in Chitral and from north to east, and in central areas of Gilgit and the southern parts of Gilgit. A low number of cases were predicted in eastern parts of Swat, while the probability of *Dicrocoelium* infection in the south of Swat and Dir region was near zero.

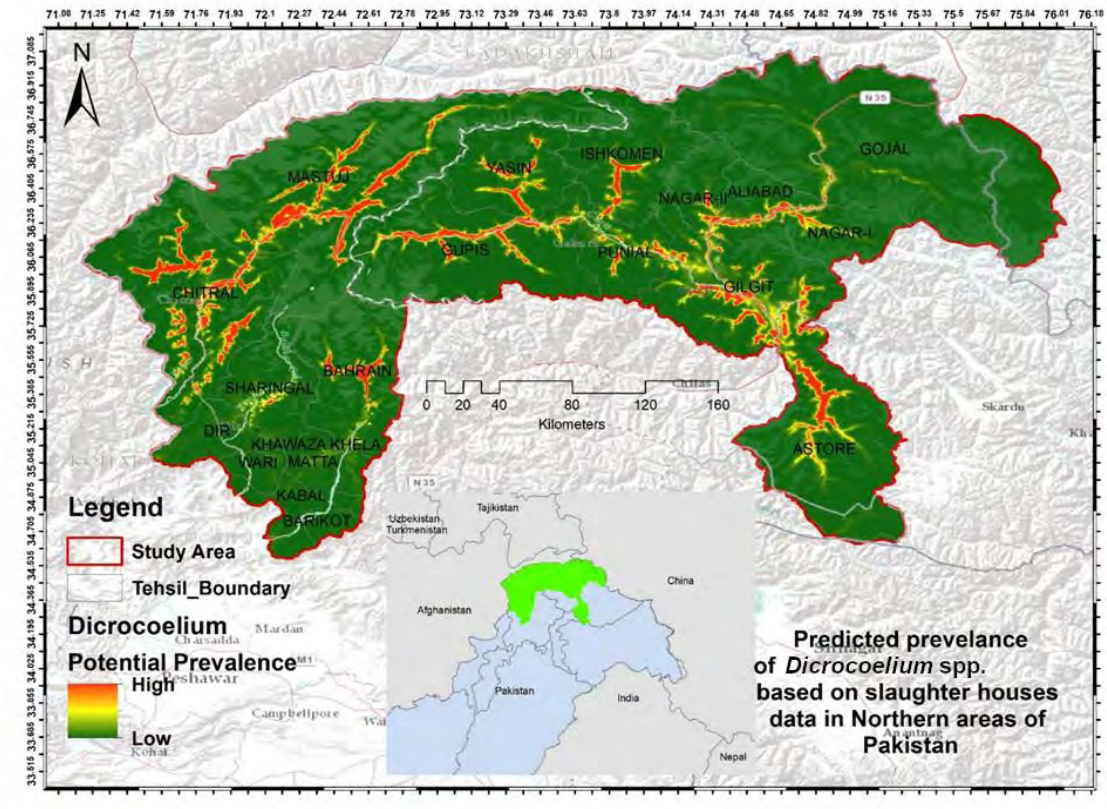


Figure 4.3.1: Predictive prevalence of *Dicrocoelium* based on a slaughterhouse in liver samples analysis from northern areas of Pakistan.

4.3.8. Predictive prevalence of *Dicrocoelium* based on blood samples

Figure 4.3.2, display the potential geographic distribution of *Dicrocoelium* infection predicted by the Maxent program. The prediction was based on the presence points of seropositive animals by ELISA test, these points are in Gilgit, Chitral, and Swat. The highest level of probability for the geographic distribution of *Dicrocoelium* infections covers the central parts of Chitral (Booni, Mastuj, Chinar, Chuinj, Unshit, Shaidas, Gasht, Phargram, Phort, Last, Brock, Huzun, Balim, Raman, Harchin, Sor Laspor, Mori, Mori Payeen, Kaghozi, Sindoor, Rondur, Riri Qwir, Barents, Pret, Kiyar, Drosh, Brun, Garam Chashma, Torkhow) extends towards the east to Gilgit and spreads towards its southern region. The program predicted *Dicrocoelium* infection in Bahrain and Sharingal, and infection decreased towards the south of Chitral.

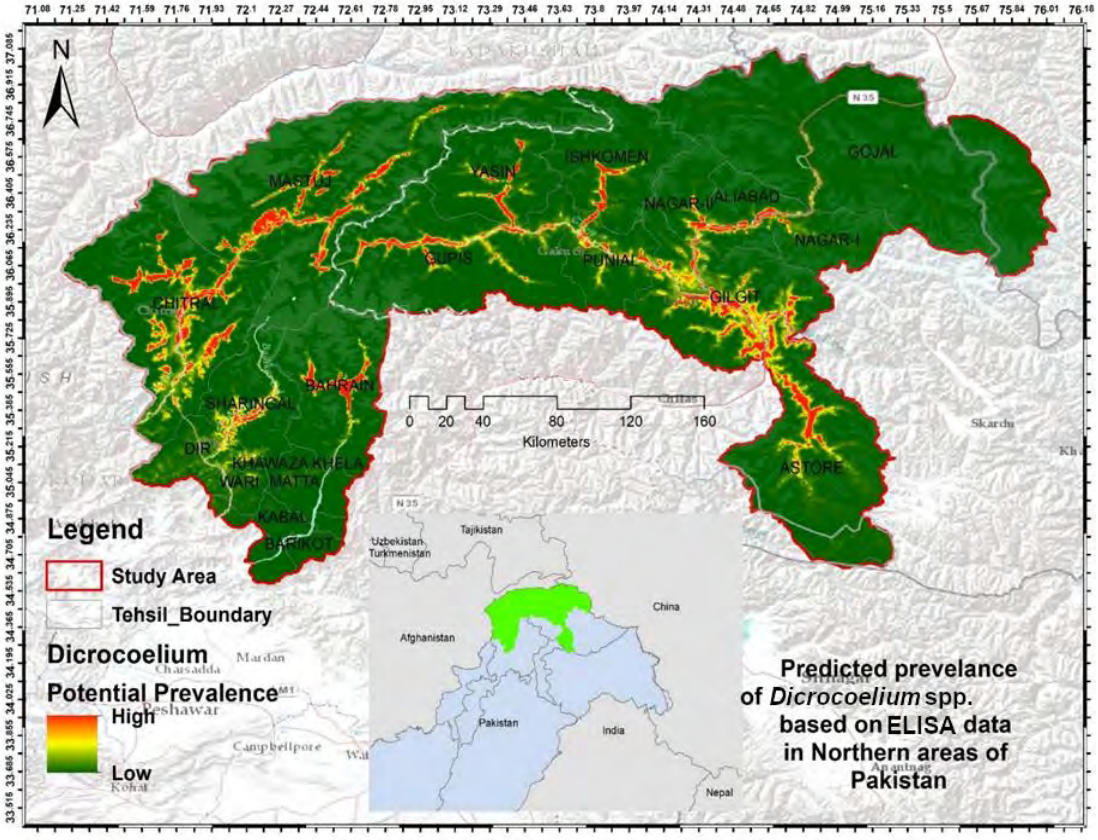


Figure 4.3.2: Predictive prevalence of *Dicrocoelium* based on ELISA in blood samples analysis from northern areas of Pakistan.

4.3.9. Spatial patterns of *Dicrocoelium* infection

The map based on the *Dicrocoelium* occurrence of positive samples predicted the most likely ecological niches to support *Dicrocoelium* infection to be in the central parts of Chitral, extending towards the upper and lower Chitral districts (Figure 4.3.3). Although *Dicrocoelium* infection was identified from parts of Gilgit, and areas of Swat and Dir bordering Chitral, MaxEnt modeling predicted lover risk of *Dicrocoelium* occurrence in these overall study regions. The MaxEnt model predicted that the two climatic variables of the mean diurnal temperature range (Bio2) and temperature seasonality (Bio4) contributed most to the occurrence of dicrocoeliasis in the Gilgit and lower and upper parts of the Chitral (Figure 4.3.3 a, b). However, annual precipitation (Bio 12) and distance to built-up areas were predicted to contribute most to the occurrence of dicrocoeliasis in upper Dir and Sawat districts (Figure 4.3.3 c, d); while summer NDVI values predicted *Dicrocoelium* active zones in the upper Dir and lower Chitral districts (Figure 4.3.3 e).

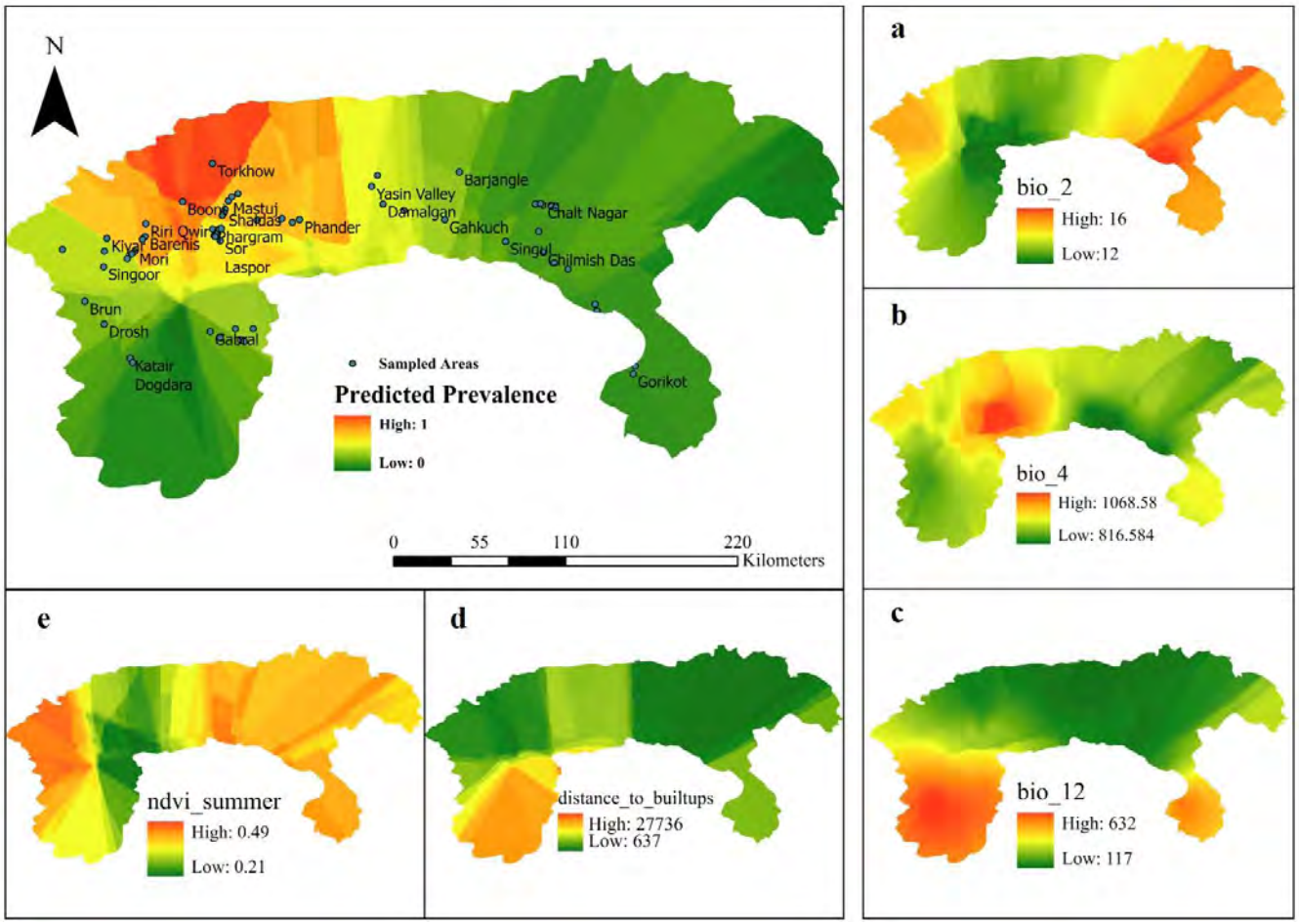


Figure 4.3.3: Predicted spatial pattern based on blood and liver sample results of ecological niches predicted to support *Dicrocoelium* infection. Red shading indicates the most suitable niches for Dicrocoelid flukes, and green shading predicts the least suitable conditions. The MaxEnt model predictions for the contributions of variables to the occurrence of dicrocoeliasis are shown in a) (mean monthly diurnal temperature range), b) (temperature seasonality), c) (annual precipitation), d) (distance from built-up areas), and e) (normalized difference vegetation index).

4.3.10. Contribution of Ecological Niches and Climatic Variables on *Dicrocoelium* Infection

4.3.10.1 Factors determining habitat suitability

Variables with higher contributions in the Maxent model were normalized difference vegetation index (55.8), annual precipitation (12.4), mean diurnal range (mean of monthly max temp-min temp) (9.3), and distance from the population (8.7). The contribution of other variables included in the model was low (Table 4.3.9). The results of the jackknife analysis performed on five climatic and 4 geographical variables are shown in figure 4.3.4. The Jackknife test of variable importance showed that the environmental variable with the highest gain, when used in isolation, is Bio2, which therefore appears to have the most useful information by itself. The environmental variable that decreases the gain the most when it was omitted was the NDVI summer, which therefore appears to have the most information that is not present in the other variables. The values shown are averages over replicate runs (Figure 4.3.4).

Table 4.3.9 Estimates of relative contributions of the environmental variables to the Maxent model

Variable	Interpretation	Percent contribution	Permutation importance
NDVI-S	Normalized difference vegetation index in summer	55.8	38.4
bio12	Annual precipitation	12.4	10.7
bio2	Mean diurnal range (mean of monthly [max temp-min temp])	9.3	6
dist.- 2-built up	Distance from population	8.7	0.3
bio4	Temperature seasonality (standard deviation *100)	5.6	7.1
bio15	Precipitation seasonality (Coefficient of variation)	3.6	6.9
forest cover	Type of forest	2	2.1
elevation	Elevation of the area	1.3	3.1
bio6	Mean temperature of the coldest month	0.7	22.8
NDVI-fall	Normalized difference vegetation index in fall	0.6	2.6

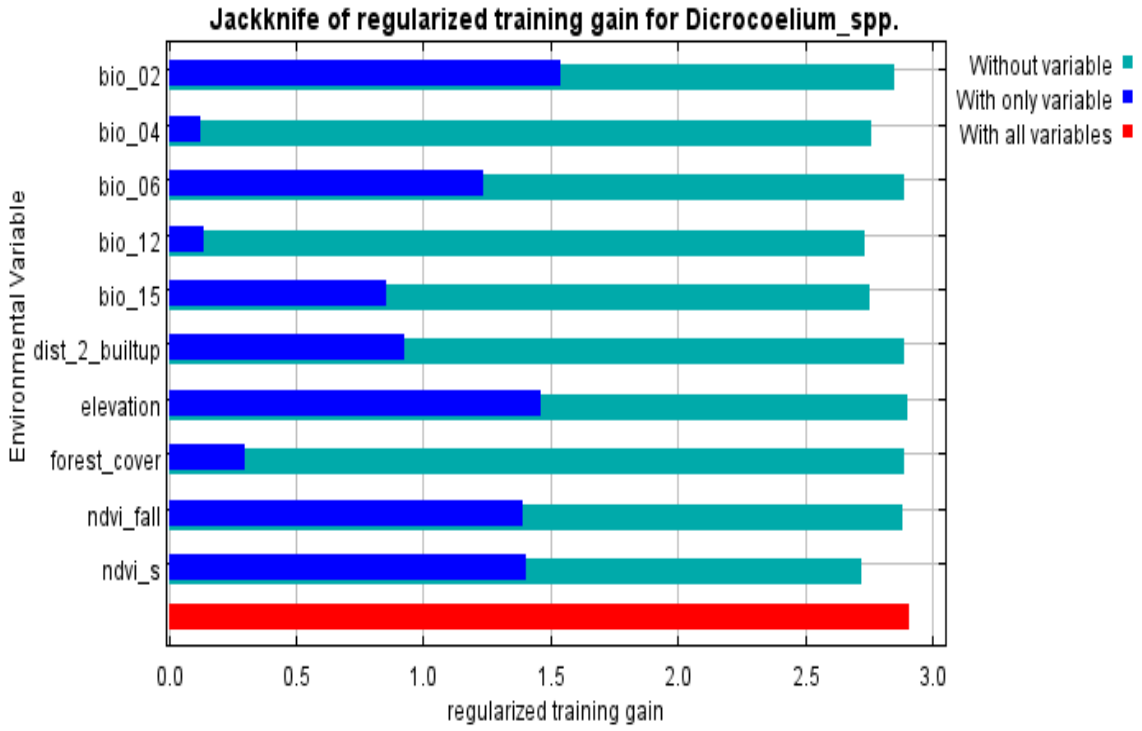


Figure 4.3.4: Jackknife test of regularised training gain of variables examined in the *Dicrocoelium* habitat suitability model. Blue bars represent the gain when the environmental variable is used in isolation; green bars represent the gain when the environmental variable is omitted; the red bar represents the gain when using all the environmental variables.

4.3.10.2 Model evaluation and threshold selection

The AUC values for the training and test data were 0.987 and 0.985, respectively, suggesting an excellent predictive power for the model (Figure 4.3.5, a, b). The ROC curve (Figure 4.3.5 b) for the data was also calculated by MaxEnt, again, averaged over the replicate runs. Here, specificity is defined using the predicted area rather than true commission. The average test AUC for the replicate runs was 0.985.

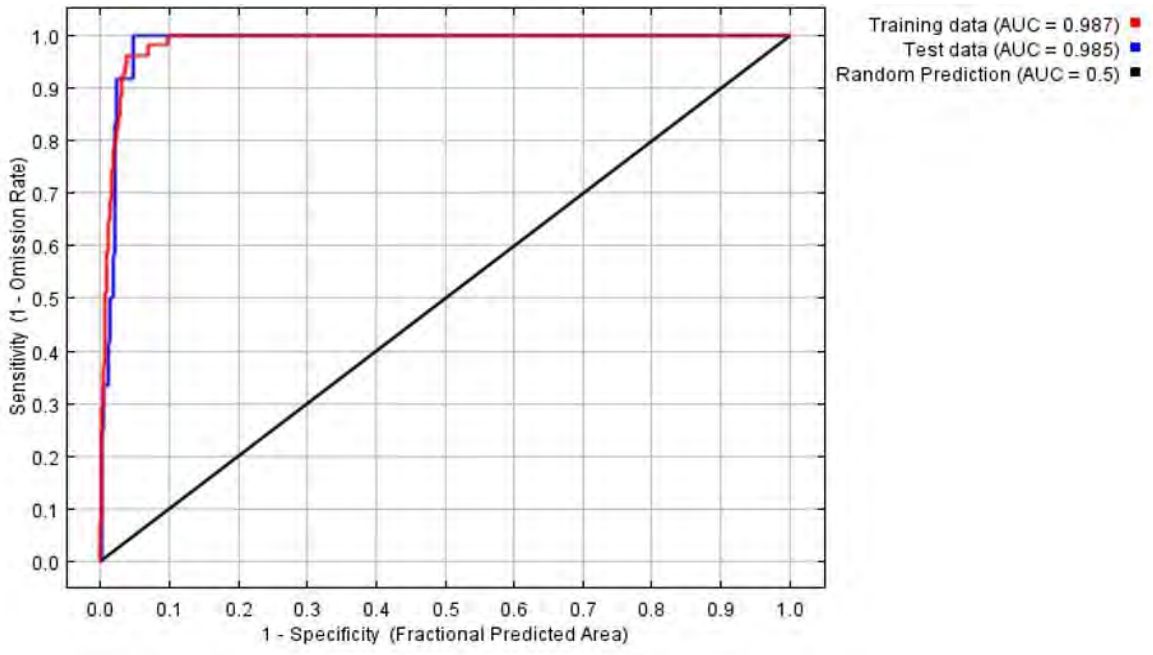


Figure 4.3.5 a: ROC curve calculated by MaxEnt plotting average sensitivity against 1 - specificity for prediction of *Dicrocoelium*.

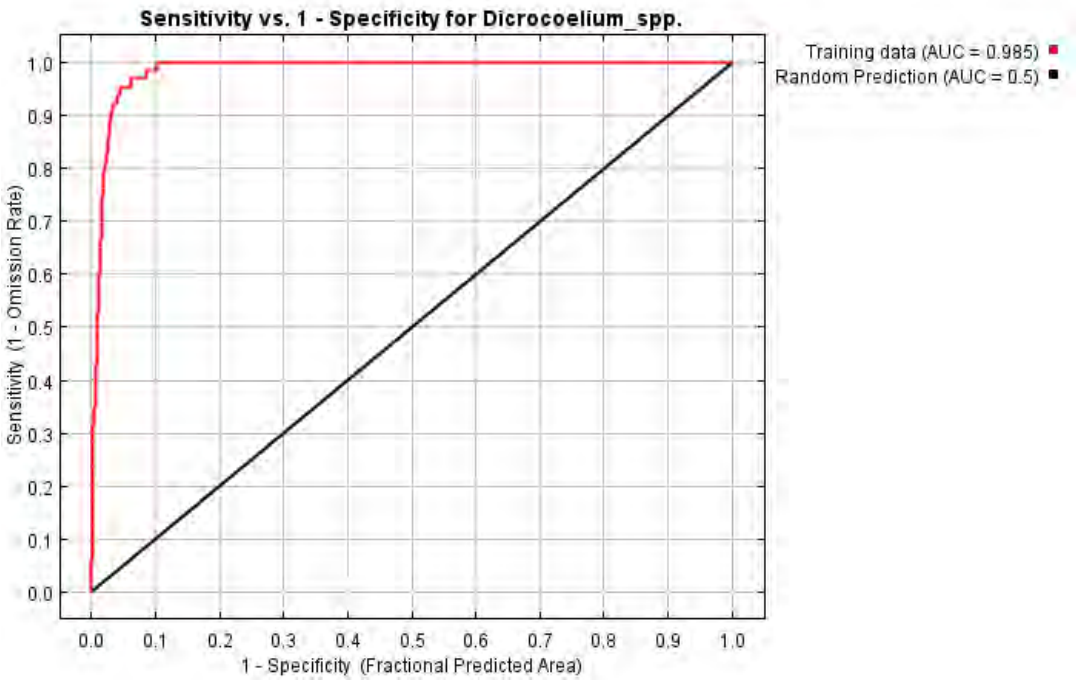


Figure 4.3.5 b: Model evaluation, The ROC curve calculated by MaxEnt as averaged sensitivity versus 1-specificity for *Dicrocoelium.*

4.3.10.3 Description of the Model curves

The six most influential variables observed in the present study are shown in Figure 4.3.6. The result shows that the occurrence of *Dicrocoelium* infection was directly related to the mean of the monthly diurnal temperature range (Bio2), temperature seasonality (Bio4), mean temperature of the coldest month (Bio6), distance from population built-up areas and summer NDVI. An inverse relationship was observed between annual precipitation (Bio12) and the identification of *Dicrocoelium* infection.

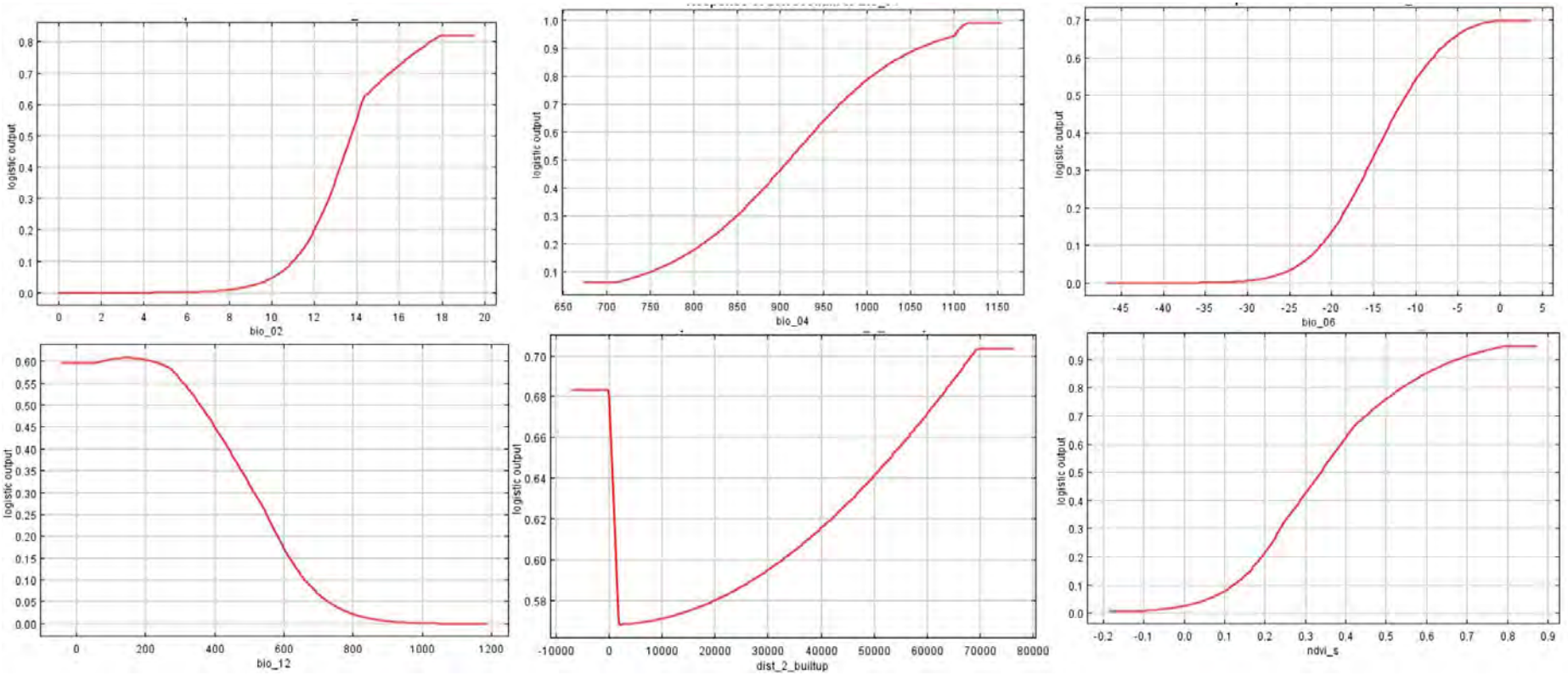


Figure 4.3.6: The response curves for suitable variables were obtained by the logistic output format for mean diurnal temperature range (bio2), seasonal temperature variation (bio4), mean temperature of the coldest month (bio6), annual precipitation (bio12), distance to build-up areas, and summer normalized digital vegetation index.

DISCUSSION

In the present study, 381 liver samples and 6060 blood samples provide a valuable resource which can be used to describe aspects of the epidemiology of dicrocoeliosis in the Himalayan ranges of Pakistan. The estimated prevalence of dicrocoeliosis in sheep and goats in the Gilgit and Chitral districts was higher than reported incomparable Asian studies conducted in India (Godara *et al.,* 2014), Iran (Najjari *et al.,* 2020), and Iraq (Manuchar *et al.,* 2021). While direct comparisons are biased by differences in study design, the relatively high prevalence confirms the widespread nature of ecological niches that can support the continuity of the Dicrocoelium life cycle in the northwest of Pakistan. Characteristics including calcium-rich, alkaline soils and diverse vegetation help to provide overlapping niches that are suited to each of the intermediate and definitive hosts (Manga-Gonzalez *et al.,* 2001). The prevalence of *Dicrocoelium* was highest during the summer and autumn, as previously described in Algerian cattle (Chougar *et al.,* 2019), but the seasonal differences were not significant, and potentially may have been confounded by factors such as the age, species and breed of the animals and sampling location. The suitability of environmental factors for the development and growth of intermediate snails and ant hosts and grazing patterns enabling exposure to metacercaria-infected ants (Manga-Gonzalez *et al.,* 2001), will vary throughout the year. However, in the absence of effective anthelmintic treatments for dicrocoeliosis (Sargison *et al.,* 2012), animals accumulate infections acquired during different periods throughout their lives; consequently, a cross-sectional study involving animals more than 1-year-old cannot identify seasonal infection risks. Extreme cold weather conditions in the Himalayan ranges of Pakistan preclude grazing of animals on open pastures during the winter months and imply that the greatest risk of infection is during the spring and summer when conditions are also favorable for intermediate host development (Cabeza *et al.,* 2011). The estimated prevalence of *Dicrocoelium* was higher in female hosts and highest in animals aged more than 3 years. Previous reports have shown higher prevalence's in female hosts (Cabeza *et al.,* 2011; Bihaqi *et al.,* 2017), and suggested a relationship between periparturient susceptibility due to pregnancy and lactation stress (Shubber *et al.,* 1981). However, female animals are more

likely to be retained for breeding, hence live for longer and have more opportunities to become infected with *Dicrocoelium*. The specie prevalence could be explained by the possibility of higher susceptibility of sheep than goats. Higher prevalence and worm burden in sheep could be the result of more sensitive species, but goats have contact "infection" with *Dicrocoelium*, but this does not go advance. This could explain the higher prevalence of antibodies, but not found in adults. The different results further highlight challenges of sample size and diagnosis of adults could be less sensitive, with a high number of false negatives in goats than in sheep. It has been suggested that browsing goats are less likely to be infected than grazing sheep (Bihaqi *et al.,* 2017), albeit *Dicrocoelium*-infected ants may migrate high enough onto herbage to be ingested by browsing animals. However, the ecological information on ants and land snails involved as intermediate hosts in these areas is still unknown.

The highest occurrence of *Dicrocoelium* infection was recorded in the Chitral district, consistent with its high-altitude pastureland fed by melting of glacier water and high seasonal rainfall providing the most suitable conditions for completion of the parasite's life history. A similar situation has been described in Spain, where *Dicrocoelium* infection is most frequent in areas with high altitudes, lower winter temperatures and high rainfall (Diaz *et al.,* 2017). The occurrence of *Dicrocoelium* infection in the Gilgit, Swat and Dir districts was moderate to low associated with lower rainfall and more humid environments. Prediction of the environmental suitability and geographical distribution of ecological niches, climatic and anthropomorphic factors that are suited to the completion of the *Dicrocoelium* life cycle is needed to inform strategic disease control. SDMs have been used to predict the special distribution of *Dicrocoelium* infection in Iran (Meshgi *et al.,* 2019), and Spain (Diaz *et al.,* 2017). The ROC test showed a high validity of the SDM in predicting favorable ecological niches for these parasites in the Himalayan ranges of Pakistan. The MaxEnt model revealed that the most influential climatic variables associated with a positive effect on the risk of dicrocoeliosis were the mean of the monthly diurnal temperature range (Bio2), temperature seasonality (Bio4) and the mean temperature of the coldest month (Bio6); while an inverse relationship was observed for annual

precipitation (Bio12). The results suggest that these factors play a key role in the development, survival and transmission of Dicrocoelid flukes and their intermediate hosts. The results also found a high correlation between distance from population built-up areas and summer NDVI and the presence of *Dicrocoelium* infection, explained by the observation that forest areas with permanent pastures, good water availability and suitable soil type provide suitable habitats for land snails and ant intermediate hosts, and opportunities for final host infection (Ekstam *et al.,* 2011).

Overall, this study shows a high estimated prevalence of dicrocoeliosis in the Himalayan ranges of Pakistan. The ecological niche model helps to describe factors that increase the risk of infection, providing information that might help in the development of targeted evasive management strategies and in predicting the potential spread of *Dicrocoelium* to other suitable habitats in the region.

CONCLUSION

In the present study, the diagnosis of dicrocoeliosis was based both on the identification of Dicrocoelid flukes in the livers of slaughtered animals and on positive blood sample results using a bespoke combination of ES and somatic antigen ELISAs. The random sampling methods that were used to collect the diagnostic samples helped describe the spatial distribution of *Dicrocoelium* infection and provided a crude estimation of the parasite's prevalence. However, the fold difference in overall prevalence estimates obtained from the liver (~ 9%) and blood (~ 4%) sample results highlight important difficulties in the accurate determination of the prevalence of fluke parasites; namely the adequacy of the sample size, precise knowledge of the sensitivities and specificities of the diagnostic tests used, and the representativeness of the study populations. In the current study, the blood sample size was adequate, but the number of liver samples was too low to allow for precise analysis; the true sensitivities and specificities of the diagnostic tests were unknown; and the live and slaughtered animal populations may have different in their origins, grazing management, and are known to differ in demographic characteristics such as sex, age, species and breed. The number of samples that could be collected and processed was constrained by the remoteness and poor supporting infrastructure of the study region.

Nevertheless, the 381 liver samples and 6060 blood samples provide a valuable resource which can be used to describe aspects of the epidemiology of dicrocoeliosis in the Himalayan ranges of Pakistan. In the absence of a gold standard, the accurate determination of the sensitivities and specificities of diagnostic tests for the study of fluke parasite epidemiology is challenging (Mazeri *et al.,* 216), and requires different samples to be collected from the same animals in a manner which was not feasible in the current study.

TRANSMISSION PATTERN OF *DICROCOELIUM* TO SHEEP AND GOATS IN PAKISTAN

ABSTRACT

Lancet flukes cause a lot of damage to the livestock industry annually. So, the rapid diagnosis of infection is very important. The main aim of the present study was to investigate the transmission patterns of *Dicrocoelium* in sheep farm (n=10) and goat (n=10) farms in Khyber Pakhtunkhwa and Gilgit Baltistan, Pakistan, between 2018 and 2019. The preparation of the blood samples for ELISA analysis followed standard procedures. 15-20 animals from each farm were screened for *Dicrocoelium* infection through ELISA and investigated their time of infection. These animals were screened to investigate the colostral transfer of *Dicrocoelium* antibodies from infected mothers to young ones and for how much duration these antibodies were detected. The time of infection of *Dicrocoelium* was investigated in different months 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 is different in various farms and years. The highest infection rate was investigated in sheep farms (n=41) in 2018 and 40 lambs in 2019, in goats the infection rate in 2018 was 22 and 18 goats were infected in 2019. A limited infection rate was investigated in sheep farm-04, and 05 in 2018 and 2019 respectively. In goats' the lowest infection was in farm-05 in 2018 and farm-02, 03, 04, and 05 in 2019. The *Dicrocoelium* infection was found most prevalent in sheep and goats in September (n=84) and August (n=63) respectively.

Conclusion: In conclusion, colostral transfer of antibodies was detected up to 9-11 weeks of age. The highest infection was observed in sheep because of the grazing behavior and the infection was highest in September and August.

Keywords: *Dicrocoelium*, antibodies, colostral

INTRODUCTION

Dicrocoelium dendriticum is one of the dominant specie of lancet flukes found in the livers of infected animals. The diagnosis of this parasitosis is frequently made after eggs are found in the feces of infected animals (Ferre *et al.,* 1994), despite the fact that tests are frequently negative in sheep with fewer than 100 flukes (Ambrosi, 1991). The lack of symptoms of low infection makes dicrocoeliasis diagnosis in small ruminants challenging. As a result, routine tests utilizing antigen detection techniques, such as the ELISA technique, were developed. The ovine immune response to *D. dendriticum* has not been extensively studied. Gonza'lez-Lanza *et al.,* (2000) demonstrated that antibodies to *D. dendriticum* are first detectable by indirect-ELISA 30 days after infection, during the liver-migration phase of the immature flukes, in experimentally infected sheep. 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 test but tested negative for the production of eggs. This suggests that *D. dendriticum* was migrating in these sheep. As is the case with fasciolosis (Paz *et al.,* 1998; Sa'nchez-Andrade *et al.,* 2000, 2001), a positive ELISA test result can also show past exposure to the parasite without an active infection.

Similar, to charting antibody dynamics in *F. hepatica*-naive animals (Novobilsk'y *et al.,* 2014), we studied antibody dynamics to determine the time of exposure of *D. dendriticum.* A thorough understanding of the epidemiology and seasonal transmission patterns of *Dicrocoelium* is needed to improve a strategic control program for liver fluke infection in sheep and goats in Pakistan. To the best of our knowledge, this is the first study designed to determine the time of infection and describe the *D. dendriticum* transmission pattern in sheep and goats.

MATERIALS AND METHODS

5.2.1. Study areas

The study was as carried out in 2018 and 2019 on farms where *Dicrocoelium* was most prevalent. In order to study the *Dicrocoelium* transmission pattern in sheep and goats, five sheep herds (01, 02, 03, 04, and 05) and five goat herds (01, 02, 03, 04, and 05) were chosen annually in various regions of Khyber Pakhtunkhwa and Gilgit Baltistan. These herds were chosen based on reports from abattoirs on condemned livers and field reports. A summary of all farms, including geographical location is shown in Table 5.2.1. Unique codes were assigned to each sheep and goat, and proformas were filled out with information about the herd, farm location, date of birth of each lamb and kid, breed, and month of sample collection.

Table No. 5.2.1: Sampling sites and number of samples collected from lambs and goat kids

Areas	Longitudes	Latitudes	No of lambs samples		No of goat's samples	
			2018	2019	2018	2019
Booni	72.25408342	36.27065815	17	20	18	15
Mastuj	72.51646335	36.27580273	16	15	15	15
Sor Laspor	72.47027663	36.04724606	15	17	16	15
Chalt Nagar	74.3230176	36.25213215	18	15	14	14
Torkhow	72.42492036	36.49056069	15	16	14	16

5.2.2. Collection of serum samples and ELISA

Blood samples were taken from lambs and goat kids born between 2018 and 2019 to determine the approximate time the animals became infected while grazing. The same 10-15 herd members 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 collected sera were then kept at 20 °C until needed.

Sera were examined by indirect enzyme-linked immunosorbent assay (ELISA) using excretory-secretory antigen of *Dicrocoelium* (ES Ag). Detailed methodology is

already discussed in Chapter 4. In brief, sera were diluted 1:50 in buffer (0.05% Tween 20, 5% non-fat milk in phosphate-buffered saline, pH = 7.2), and each serum sample was tested in duplicate. The same positive and negative reference samples were included in all assays. The ELISA results were expressed as a percentage of the mean optical density ratio (ODR) of the positive control as % of positivity = (mean OD of the tested sample (n = 2)/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.

5.2.3. Statistical Analysis

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

RESULTS

The specificity of the E/S antigen was tested against *Dicrocoelium* and the calculated cut-off values for lambs and goats ELISA were 0.4314 and 0.411, respectively (Table 5.3.1). The specificity of the ES antigen was tested against positive sera from *Fasciola hepatica, Haemoncus contortus,* and *Peraphistomom cervi*, and no cross-reaction was observed.

Table No. 5.3.1: OD values of control samples of goat and sheep

Goat No.	OD values	Sheep No.	OD values
Goat No.1	0.285	Sheep No.1	0.288
Goat No.2	0.278	Sheep No.2	0.286
Goat No.3	0.271	Sheep No.3	0.278
Goat No.4	0.056	Sheep No.4	0.281
Goat No.5	0.115	Sheep No.5	0.272
Goat No.6	0.133	Sheep No.6	0.096
Goat No.7	0.044	Sheep No.7	0.128
Goat No.8	0.029	Sheep No.8	0.135
Goat No.9	0.123	Sheep No.9	0.048
Goat No.10	0.156	Sheep No.10	0.128
Goat No.11	0.161	Sheep No.11	0.162
Goat No.12	0.086	Sheep No.12	0.153
Goat No.13	0.118	Sheep No.13	0.121
Goat No.14	0.135	Sheep No.14	0.148
Goat No.15	0.314	Sheep No.15	0.132
Goat No.16	0.138	Sheep No.16	0.291
Goat No.17	0.101	Sheep No.17	0.316
-	-	Sheep No.18	0.118
-	-	Sheep No.19	0.098
-	-	Sheep No.20	0.111
Mean	**0.149**		**0.1795**
Standard deviation	**0.087**		**0.084**
Cut off value	**0.41**		**0.431**

On farms 03, 04, and 05 in 2018 and on farms 02 and 03 in 2019, total of fifteen (15) lambs from the June and July (first blood collection) blood collection tested seropositive, but the same lambs later tested negative in the second blood collection in July and August respectively (Figure 5.3.1 and Figure 5.3.2). Similar to this, on farm 02 in 2018 and on farm 01 and 04 in 2019 only three (3) goat kids from the June blood collection tested seropositive, but the same goat kids later tested negative in the following blood collection in August (Figure 5.3.3. and Figure 5.3.4). On the other hand, other lambs seroconverted in August and remained positive in September in 2018 and 2019 (at farms 01, 03, and 04). Lamb seroconversion was noted on farm 02 in 2018 and 2019 between the months of August and October, and at farm 05 in 2019 between the months of September and November. However, in 2018 and 2019, most lambs on farms 01 were already seropositive in June, and their antibody levels increased further during the following sampling months.

Similar to lambs, goat kids seroconverted in August at farms 01, 03, 04, and 05 in 2018, and 03 and 05 in 2019 remain positive in the next sampling month of September (Figures 5.3.3 and 5.3.4). In comparison to antibody dynamics in lambs, no positive goat kids were detected in the first blood collection in June 2018 (at farms 03, 04 and 05) and 2019 (at farm O5). For a detailed presentation of the serological data for individual animals, see appendix Tables.

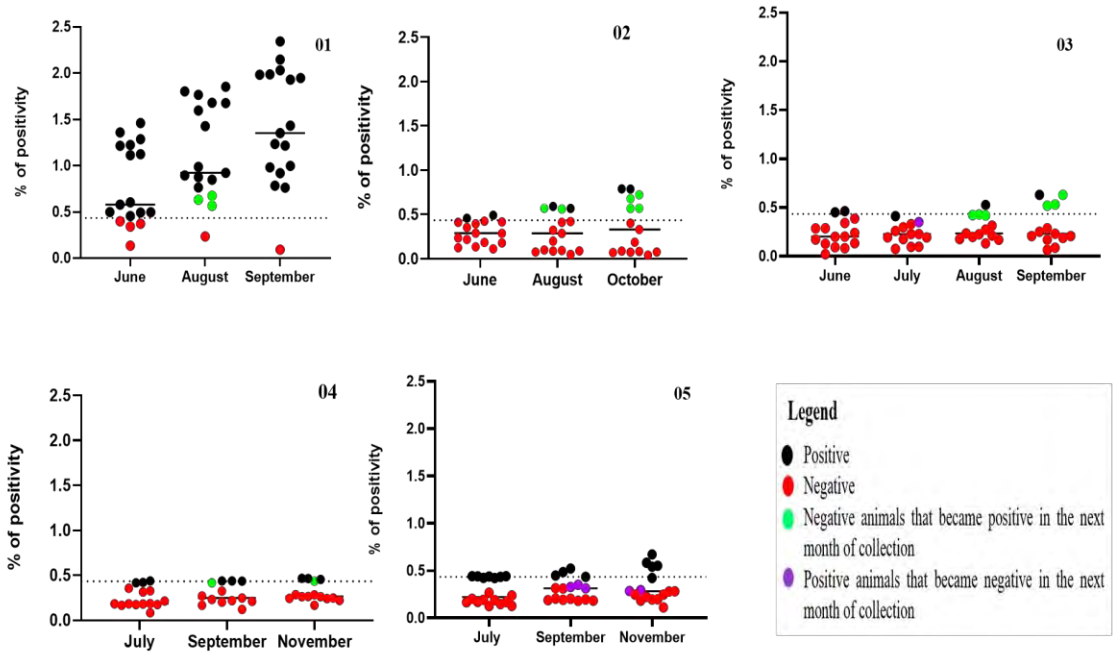


Figure 5.3.1: Dynamics of *Dicrocoelium* antibodies in lambs on sheep farms 01, 02 03, 04, and 05 during 2018. The dashed line is the cut-off limit (cut-off = 0.431% of positivity).

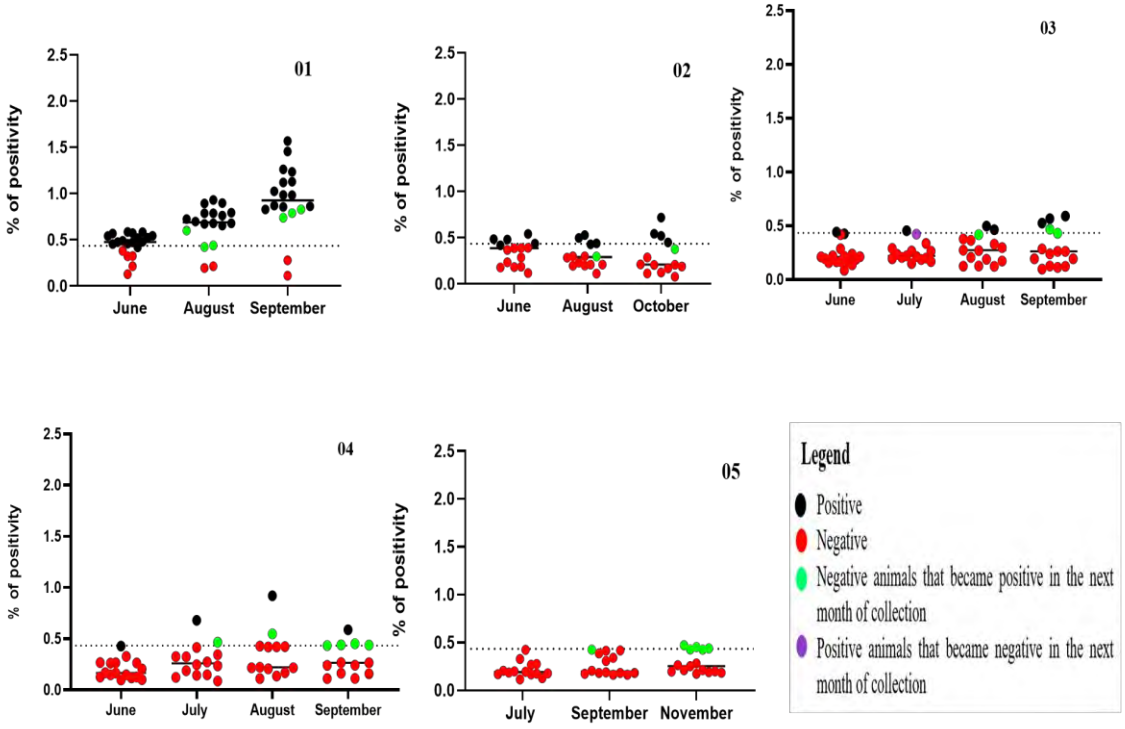


Figure 5.3.2: Dynamics of *Dicrocoelium* antibodies in lambs on sheep farms 01, 02 03, 04, and 05 during 2019. The dashed line is the cut-off limit (cut-off = 0.433% of positivity).

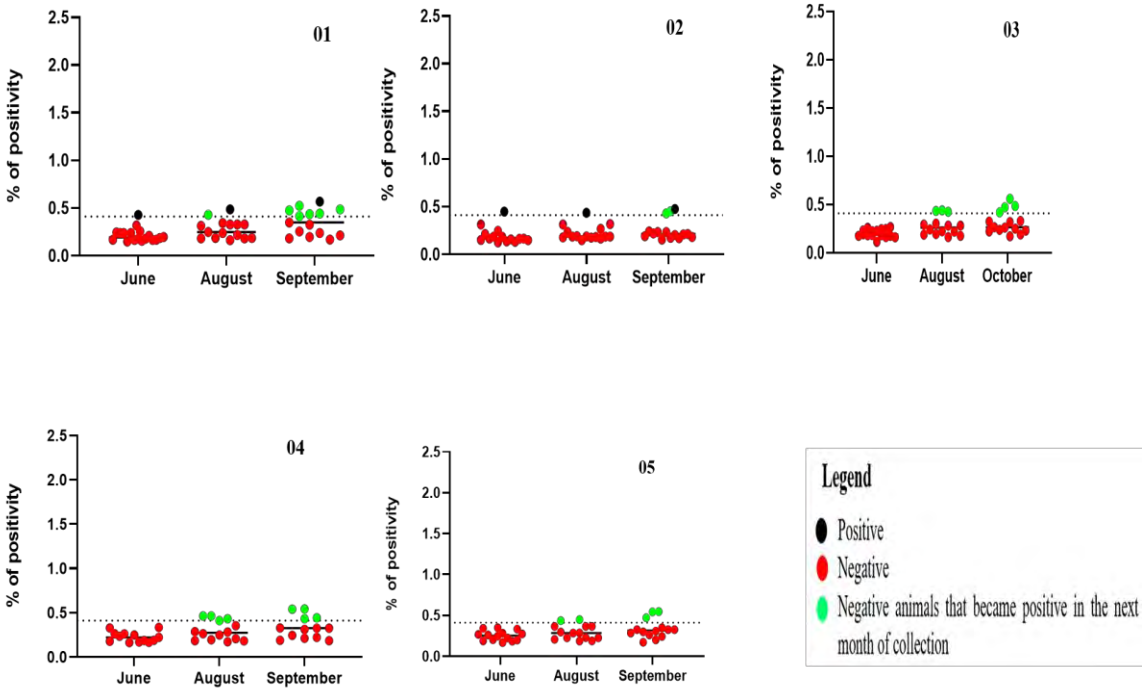


Figure 5.3.3: Dynamics of *Dicrocoelium* antibodies in kids on goat farms 01, 02 03, 04, and 05 during 2018. The dashed line is the cut-off limit (cut-off = 0.411% of positivity).

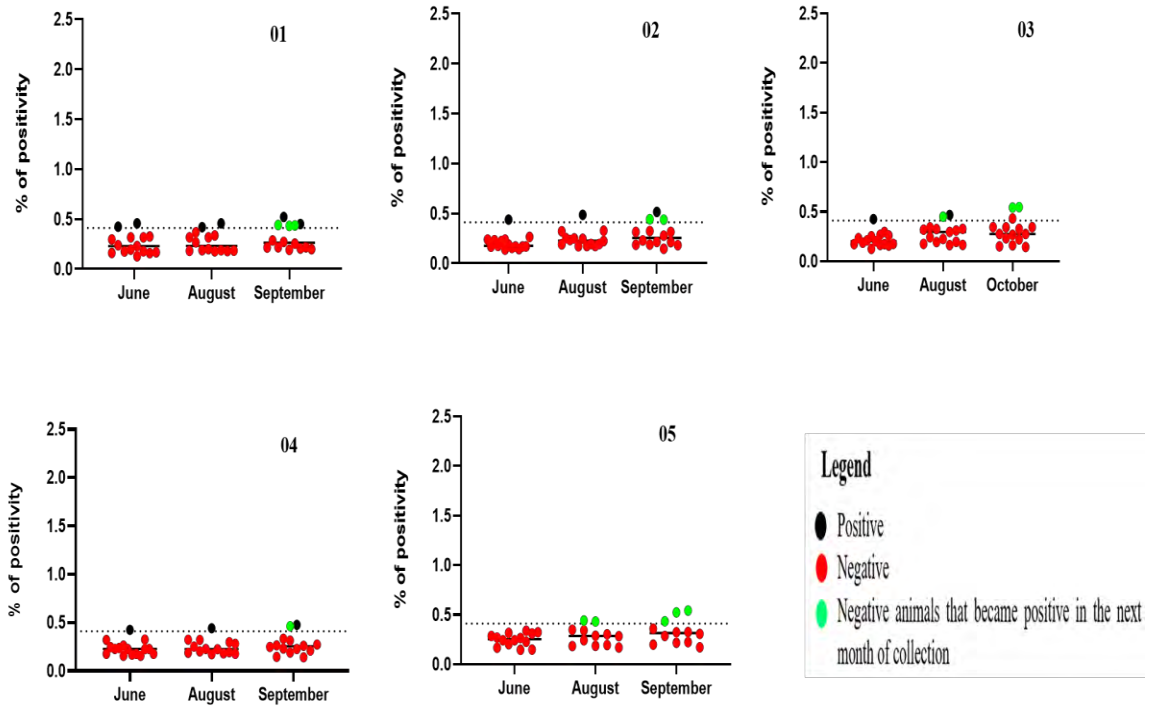


Figure 5.3.4-: Dynamics of *Dicrocoelium* antibodies in kids on goat farms 01, 02 03, 04, and 05 during 2019. The dashed line is the cut-off limit (cut-off = 0.411% of positivity).

DISCUSSION

Based on the fact that ELISA can detect antibodies against *D. dendriticum*-infected lambs between 30 days after infection, thus a month before the coprological examination returned a positive results (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 throughout the entire study period of 150 days following infection (Gonzalez-Lanza *et al.,* 2000; Manga-Gonzalez and Gonzalez-Lanza 2005; Broglia *et al.,* 2009). Despite the limited data on the immune response to *D. dendriticum* obtained from experimental studies (Piergili Fioretti *et al.,* 1980; Wedrychowicz *et al.,* 1997; Gonzalez-Lanza *et al.,* 2000), the current study demonstrates that tracking antibody dynamics may be an effective way to pinpoint when grazing animals were first exposed to *D. dendriticum* infection.

Seropositive lambs after birth to first grazing at farms 03 (Laspo-0366, Laspo-0380), 04 (Thork-0572), and 05 (Naga-0719a, Naga-0720a, Naga-0762a, Naga-0763a) in 2018 and lambs at farm 02 in 2019 (Masj-1305a, Masj-1306a, Masj-1329) and 03 (Laspo-1529, Laspo-1565) were seronegative one month after blood collection. Similar to this, goat kids at farm 02 (Masj-0617) in 2018 and at farm 01 (Boon-1403), 04 (Thork-1921) in 2019, that were seropositive changed to negative during the subsequent blood collection month. Novobilsky *et al.,* (2014) also reported these seroconversions in fasciolosis seropositive animals, and they demonstrated the mechanism of antibody transmission from mother to infant via colostrum intake. during colostrum. According to Mezo *et al.,* (2010), *Fasciola hepatica* colostral antibodies are transferred to dairy calves, and it has been suggested that maternal antibodies can be found up to 12 weeks after birth.

An increase in antibodies level was seen with the start of grazing that occurs 2 months later at farm 02 lambs (Masj-127, Masj-0179, Masj-0142, Masj-0195a), 03 (Laspo-0369, Laspo-0328a, Laspo-0373, Laspo-0390), 04 (Thork-0507a, Thork-0556, Thork-0577, Thork-0516), 05 (Naga-0773, Naga-0705, Naga-0756a, Naga-0757a) in 2018, and at farm 02 lambs (Masj-1302, Masj-1451, Masj-1444, Masj-1388), 03 (Laspo-1591,

Laspo-1561), 04 (Thork-1882, Thork-1833, Thork-1856, Thork-1871, Thork-1851) and at farm 05 lambs (Naga-0931, Naga-0923a, Naga-0924a, Naga-0913a, Naga-0914a) in 2019. Similarly, goat kids displayed high antibody titers at farms 01 (Boon-0498, Boon-0449, Boon-0411, Boon-0472, Boon-0460, Boon-0475; Boon-1411, Boon-1415, Boon-1420, Boon-1446, Boon-1467), 02 (Masj-0647, Masj-0660a, Masj-0612; Masj-1603, Masj-1602, Masj-1689), 03 (Lasp-0502, Lasp-0561, Lasp-0577, Lasp-0552; Lasp-1753, Lasp-1776, Lasp-1712), 04 (Thork-1018a, Thork-1058, Thork-1028a, Thork-1022; Thork-1941, Thork-1965) and at farm 05(Naga-1210, Naga-1230, Naga-1273; Naga-2048, Naga-2059, Naga-2044) in 2018 and 2019, respectively. 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 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 response first appearing between 2 and 4 weeks post-infection and then gradually increasing until 10-12 weeks. Several mechanisms have been proposed to explain peaked abundance patterns with host age (Anderson and Gordon, 1982; Duerr *et al.,* 2003).

However, the majority of lambs on farms 01 in 2018 and 2019 were already seropositive by June, and their antibody levels further rose during the subsequent sampling months. The high prevalence of *Dicrocoelium* infection at 01 lamb farm could be assessed in relation to the ecological features of the study area, specifically taking into account the biotopes of the intermediate hosts, as discussed by Broglia *et al.,* (2009) to assesdicrocoeliasisis seroprevalence in ovine flocks from Italy.

According to the present study, the majority of lambs and goat kids seropositive at farms in 2018 and 2019 had metacercariae at the very beginning of August. During the ant's active season in late summer (July-August) the infected snails could shed cercariae. In terms of the dynamics of disease transmission, this is a critical time for dicrocoeliasis in

the flocks of nomadic goats that graze on 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.

CONCLUSION

The study concludes that the time of year and the types of grazing areas have a significant role in predisposing sheep and goats to dicrocoeliasis. The study may be useful in creating an epidemiological map of dicrocoeliosis and will be very helpful in developing effective control strategies to stop the disease and maintain the maximum growth and productivity of sheep and goats.

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APPENDICES

Chapter # 1:

Appendix no1.

Preparation of hematoxylin stain

Hematoxylin stains contain the following chemicals.

- Hematoxylin ---------- 2g
- Ammonium alum ---------- 3g
- Sodium iodate ---------- 0.24 g
- Acetic acid ---------- 10 ml
- Absolute ethanol ---------- 100 ml
- Distilled water ---------- 100 ml
- Glycerol ---------- 100 ml

Two-gram hematoxylin was dissolved in 100 ml of ethanol. To prepare the Ammonium alum solution, 3-gram alum was dissolved in distilled water and then heated to 100 ºC. solution of ammonium alum was added to an inhomogeneous mixture of hematoxylin, then add sodium iodate and glycerol properly, and in the last add acetic acid and properly stir the solution.

To remove the paraffin, slides were passed in xylene:

- Xylene I -------- 3 min
- Xylene II -------- 3 min

Hydration. Sections were rehydrated in descending grades of alcohol

- 100% alcohol I --------3 min
- 100% alcohol II -------- 3 min
- 90% alcohol -------- 3 min
- 70% alcohol -------- 3 min
- Washed with water -------- 3 min.

Now the slides were stained in different grades as follows:

- Hematoxylin -------- 8 min

- Washing -------- 2 min
- Acidified alcohol -------- 1 min
- Washing -------- 2 min
- Bluing solution (1mL NH_4OH ± 300mL water) -------- 2 min
- Washed with water -------- 2 min

Dehydration

- 90% alcohol --------- 10 dips
- Eosin ---2 min
- Washed with water -------- 2 min
- 90% alcohol -------- 5 min
- Absolute alcohol I -------- 5 min
- Absolute alcohol II -------- 5 min
- Absolute alcohol III -------- 5 min
- Xylene I -------- 5 min
- Xylene II -------- 5 min

After staining, a small amount of Canada balsam was placed on the slides and concealed with xylene-dipped coverslips, and carefully positioned on the slides before placing them in an incubator for one night.

Chapter # 3:

Appendix no1.

Reagents

Reagents for antigen preparation

Washing solution

- Hank's solution 40.0 ml
- Penicillin/streptomycin (10.000 IU/ml) 20.0 ml
- Distilled H2O 360.0 ml

Culture medium

- Dulbecco modified Eagle's medium 100 ml
- Penicillin/streptomycin (10.000 IU/ml) 0.5 ml
- HEPES buffer solution 1.0 ml
- L-Glutamin 1.0 ml

Reagent for ELISA test

Carbonate buffer pH 9.6

- Na2CO3 1.12g
- NaHCO3 2.92 g
- NaN3 0.20 g
- dist. H2O ad 1000 ml

Phosphate buffered saline/Twen20 (PBS-T) pH 7.3-7.4

- NaCI 16.0 g
- KCI 0.4 g
- Na2HPO4 x 12 H2O 5.8 g
- KH2PO4 0.4 g
- Tween 20 1.0 ml
- dist. H2O 2000 ml.

ABTS solution

ABTS (Sigma): 2, 2'azino-bis (3-ethylbenzthiazoline 6 sulfonic acid)

- 2 tablets solved in 20.0 ml dist. H2O.

Table No.1: Dynamics of *Dicrocoelium* antibodies in lambs on sheep farms 01 during 2018 and 2019. The cut-off limit (cut-off = 0.431% and 0.433% of positivity respectively

2018 Sheep Farm No.1						2019 Sheep Farm No.1					
			% of positivity						% of positivity		
Sheep code	**Breed**	**Birth date**	**June**	**Aug**	**Sept**	**Sheep code**	**Breed**	**Birth date**	**June**	**Aug**	**Sept**
Boon-0097	Balkhi	13 March	0.578	0.893	1.234	Boon-1177	Balkhi	15 March	0.474	0.672	0.828
Boon-0078	Ramghani	15 March	0.604	0.987	1.352	Boon-1146	Balkhi	22 March	0.213	0.194	0.111
Boon-0089	Balkhi	19 March	0.398	0.565	0.783	Boon-1129	Balkhi	27 March	0.128	0.213	0.278
Boon-0047	Kelli	20 March	1.124	1.596	1.983	Boon-1103	Balkhi	28 March	0.417	0.598	0.789
Boon-0063	Ramghani	24 March	1.215	1.765	2.147	Boon-1151	Balkhi	28 March	0.453	0.678	1.129
Boon-0051	Ramghani	24 March	0.497	0.875	1.432	Boon-1133 a	Balkhi	29 March	0.458	0.761	0.869
Boon-0096	Balkhi	26 March	0.456	0.763	0.997	Boon-1134 a	Balkhi	29 March	0.521	0.786	1.119
Boon-0057	Balkhi	26 March	1.358	1.802	2.342	Boon-1112	Balkhi	31 March	0.541	0.723	1.234
Boon-0048	Balkhi	28 March	0.498	0.923	1.217	Boon-1153	Balkhi	3 April	0.471	0.679	0.984
Boon-0052	Ramghani	28 March	0.492	0.847	0.981	Boon-1169	Ramghani	4 April	0.321	0.421	0.862
Boon-0093	Balkhi	2 April	1.112	1.426	1.947	Boon-1123	Ramghani	5 April	0.381	s	s
Boon-0080	Ramghani	3 April	1.224	1.675	1.929	Boon-1106	Balkhi	5 April	0.518	m	0.828
Boon-0086	Ramghani	7 April	1.461	1.853	2.031	Boon-1119	Ramghani	9 April	0.478	0.653	0.856
Boon-0081	Ramghani	11 April	1.286	1.679	1.986	Boon-1130	Balkhi	21 April	0.572	0.792	0.982
Boon-0071	Kelli	11 April	0.341	0.675	0.918	Boon-1172	Balkhi	23 April	0.543	0.697	s
Boon-0060	Balkhi	13 April	0.134	0.234	0.092	Boon-1139	Balkhi	24 April	0.582	0.893	1.456
Boon-0044	Kelli	18 April	0.371	0.632	0.761	Boon-1113	Balkhi	27 April	0.324	0.438	0.739
						Boon-1137	Balkhi	27 April	0.489	0.787	1.023
						Boon-1109 a	Balkhi	29 April	0.567	0.931	1.567
						Boon-1110 a	Balkhi	29 April	0.584	0.897	1.261

Table No.2: Dynamics of *Dicrocoelium* antibodies in lambs on sheep farms 02 during 2018 and 2019. The cut-off limit (cut-off = 0.431% and 0.433% of positivity respectively).

2018 Sheep Farm No.2			% of positivity			2019 Sheep Farm No.2			% of positivity		
Sheep code	**Breed**	**Birth date**	**June**	**Aug**	**Oct**	**Sheep code**	**Breed**	**Birth date**	**June**	**Aug**	**Oct**
Masj-0153	Balkhi	24 March	0.134	0.093	0.087	Masj-1302	Balkhi	26 March	0.389	0.438	0.543
masj-0103 b	Balkhi	27 March	0.218	0.286	0.331	Masj-1409	Balkhi	29 March	0.234	0.197	0.114
Masj-0104 b	Balkhi	27 March	0.123	0.098	0.076	Masj-1388	Kelli	29 March	0.187	0.428	0.449
Masj-0113	Balkhi	02 April	0.287	0.198	0.187	Masj-1451	Kelli	30 April	0.391	0.496	0.519
Masj-0163	Balkhi	04 April	0.234	0.321	0.402	Masj-1481	Balkhi	05 April	0.367	0.285	0.189
Masj-0121	Balkhi	09 April	0.395	0.411	0.569	Masj-1444	Balkhi	13 April	0.437	0.529	0.718
Masj-127	Balkhi	11 April	0.412	0.591	0.786	Masj-1305 a	Waziri	14 April	0.481	0.231	0.123
Masj-0167	Balkhi	12 April	0.184	0.088	0.083	Masj-1306 a	Waziri	14 April	0.541	0.198	0.167
Masj-0157	Balkhi	15 April	0.354	0.419	0.568	Masj-1329	Waziri	27 April	0.484	0.296	0.205
Masj-0179	Balkhi	17 April	0.458	0.569	0.721	Masj-1358	Balkhi	01 May	0.183	0.209	0.287
Masj-0137	Balkhi	17 April	0.289	0.087	0.074	Masj-1391	Balkhi	01 May	0.287	0.209	0.208
Masj-0111	Balkhi	17 April	0.416	s	s	Masj-1467	Balkhi	01 May	0.387	s	s
Masj-0142	Balkhi	21 April	0.423	0.561	0.679	Masj-1349	Balkhi	03 May	0.418	0.298	0.212
Masj-0117	Balkhi	24 April	0.111	0.048	0.041	Masj-1373	Balkhi	05 May	0.117	0.111	0.078
Masj-0195 a	Balkhi	28 April	0.178	0.075	0.069	Masj-1341	Waziri	07 May	0.178	0.298	0.376
Masj-0195 a	Balkhi	28 April	0.491	0.568	0.789						

Table No.3: Dynamics of *Dicrocoelium* antibodies in lambs on sheep farms 03 during 2018 and 2019. The cut-off limit (cut-off = 0.431% and 0.433% of positivity respectively).

2018 Sheep Farm No.3			% of positivity				2019 Sheep Farm No.3			% of positivity			
Sheep code	**Breed**	**Birth date**	**June**	**July**	**Aug**	**Sept**	**Sheep code**	**Breed**	**Birth date**	**June**	**July**	**Aug**	**Sept**
Laspo-0343	Ramghani	17 March	0.171	0.194	0.169	0.231	Laspo-1572	Ramghani	09 April	0.189	0.291	0.208	0.241
Laspo-0369	Ramghani	17 March	0.387	0.412	0.527	0.631	Laspo-1521 a	Ramghani	12 April	0.238	0.265	0.365	0.432
Laspo-0309	Ramghani	21 March	0.201	d	d	d	Laspo-1522 a	Ramghani	12 April	0.212	0.169	0.126	0.098
Laspo-0380	Ramghani	22 March	0.464	0.331	0.274	0.252	Laspo-1611	Ramghani	17 April	0.287	0.222	0.189	0.128
Laspo-0327 a	Ramghani	29 March	0.134	0.194	0.132	0.065	Laspo-1565	Ramghani	20 April	0.426	0.337	0.298	0.264
Laspo-0328 a	Ramghani	29 March	0.021	0.076	0.419	0.631	Laspo-1591	Ramghani	23 April	0.418	0.456	0.498	0.591
Laspo-0366	Balkhi	01 April	0.451	0.351	0.235	0.207	Laspo-1548	Ramghani	23 April	0.16	0.188	0.174	0.124
Laspo-0373	Balkhi	01 April	0.093	0.098	0.429	0.519	Laspo-1509	Ramghani	24 April	0.217	0.195	0.127	0.263
Laspo-0390	Balkhi	05 April	0.129	0.298	0.422	0.532	Laspo-1632	Ramghani	24 April	0.169	0.201	0.379	0.469
Laspo-0317 a	Ramghani	05 April	0.287	s	s	s	Laspo-1537	Ramghani	28 April	0.196	0.208	0.418	0.526
Laspo-0318 a	Ramghani	05 April	0.343	0.228	0.213	0.189	Laspo-1501	Ramghani	29 April	0.088	s	s	s
Laspo-0323	Balkhi	11 April	0.286	0.261	0.315	0.288	Laspo-1516	Ramghani	29 April	0.214	0.236	0.331	0.286
Laspo-0346	Balkhi	19 April	0.082	0.096	0.176	0.088	Laspo-1561	Ramghani	29 April	0.168	0.424	0.465	0.568
Laspo-0373	Balkhi	26 April	0.203	0.176	0.226	0.208	Laspo-1583	Waziri	01 May	0.139	0.216	0.271	0.195
Laspo-0319	Balkhi	26 April	0.24	0.228	0.198	0.169	Laspo-1578	Waziri	01 May	0.168	0.15	0.124	0.114
							Laspo-1529	Ramghani	03 May	0.443	d	d	d
							Laspo-1541	Ramghani	05 May	0.221	0.267	0.275	0.194

Table No.4: Dynamics of *Dicrocoelium* antibodies in lambs on sheep farms 04 during 2018 and 2019. The cut-off limit (cut-off = 0.431% and 0.433% of positivity respectively).

2018 Sheep Farm No.4 % of positivity						2019 Sheep Farm No.4 % of positivity						
Sheep code	**Breed**	**Birth date**	**July**	**Sept**	**Nov**	**Sheep code**	**Breed**	**Birth date**	**June**	**July**	**Aug**	**Sept**
Thork-0507 a	Waziri	28 March	0.328	0.418	0.436	Thork-1882	Balkhi	07 April	0.428	0.468	0.548	0.588
Thork-0508 a	Waziri	28 March	0.186	0.219	0.266	Thork-1839	Balkhi	07 April	0.126	0.148	0.136	0.114
Thork-0550	Waziri	29 March	0.084	0.168	0.242	Thork-1856	Balkhi	09 April	0.165	0.326	0.422	0.434
Thork-0556	Waziri	11 April	0.422	0.438	0.455	Thork-1820	Balkhi	09 April	0.098	0.124	0.168	0.158
Thork-0577	Waziri	11 April	0.358	0.436	0.468	Thork-1841	Balkhi	11 April	0.102	0.68	0.92	0.112
Thork-0584	Waziri	12 April	0.176	0.122	0.168	Thork-1869	Balkhi	12 April	0.119	0.088	0.112	0.164
Thork-0516	Waziri	27 April	0.418	0.438	0.465	Thork-1871	Balkhi	12 April	0.268	0.345	0.424	0.452
Thork-0553	Waziri	29 April	0.176	0.232	0.284	Thork-1889	Balkhi	14 April	0.169	0.143	0.207	0.266
Thork-0533	Balkhi	01 May	0.316	0.272	0.248	Thork-1803	Balkhi	16 April	0.126	0.248	0.218	0.242
Thork-0519	Balkhi	03 May	0.184	s	s	Thork-1809	Balkhi	23 April	0.206	0.274	0.218	0.24
Thork-0580	Balkhi	04 May	0.168	0.204	0.244	Thork-1829	Balkhi	20 April	0.264	s	s	s
Thork-0587	Waziri	12 May	0.216	m	0.286	Thork-1847	Balkhi	03 May	0.266	s	s	s
Thork-0572	Waziri	14 May	0.438	0.326	0.264	Thork-1851	Balkhi	07 May	0.148	0.236	0.42	0.438
Thork-0559	Waziri	19 May	0.184	0.249	0.222	Thork-1833	Balkhi	08 May	0.328	0.416	0.428	0.44
Thork-0538	Waziri	25 May	0.182	0.212	0.264	Thork-1837	Balkhi	08 May	0.148	0.188	0.222	0.267
						Thork-1887	Balkhi	14 May	0.262	0.324	s	s

Table No.5: Dynamics of *Dicrocoelium* antibodies in lambs on sheep farms 05 during 2018 and 2019. The cut-off limit (cut-off = 0.431% and 0.433% of positivity respectively).

2018 Sheep Farm No.5 % of positivity						2019 Sheep Farm No.5 % of positivity					
Sheep code	**Breed**	**Birth date**	**July**	**Sept**	**Nov**	**Sheep code**	**Breed**	**Birth date**	**July**	**Sept**	**Nov**
Naga-0773	Waziri	13 April	0.128	0.434	0.552	Naga-0923 a	Waziri	17 April	0.194	0.386	0.428
Naga-0721	Waziri	13 April	0.146	0.202	0.196	Naga-0924 a	Waziri	17 April	0.206	0.414	0.438
Naga-0705	Waziri	18 April	0.423	0.448	0.542	Naga- 0939	Balkhi	19 April	0.128	0.164	0.196
Naga-0712	Waziri	21 April	0.192	0.196	0.286	Naga- 0908 a	Waziri	19 April	0.186	0.188	0.282
Naga-0725	Waziri	29 April	0.169	0.188	0.247	Naga- 0909 a	Waziri	23 April	0.175	0.183	0.199
Naga-0756 a	Waziri	05 May	0.424	0.486	0.583	Naga-0931	Balkhi	23 April	0.328	0.427	0.453
Naga-0557 a	Waziri	05 May	0.438	0.522	0.671	Naga-0905	Balkhi	23 April	0.169	0.183	0.212
Naga-0762 a	Waziri	09 May	0.439	0.312	0.28	Naga-0913 a	Waziri	23 April	0.275	0.341	0.425
Naga-0763 a	Waziri	09 May	0.442	0.316	0.294	Naga-0914 a	Waziri	23 April	0.268	0.418	0.472
Naga-0779 a	Waziri	09 May	0.188	0.204	0.282	Naga-0942	Balkhi	11 May	0.116	m	0.173
Naga-0780 a	Waziri	09 May	0.166	0.182	0.246	Naga-0916	Waziri	12 May	0.167	0.173	0.185
Naga-0719 a	Waziri	13 May	0.434	0.327	0.222	Naga-0961	Balkhi	19 May	0.179	163	0.191
Naga-0720 a	Waziri	13 May	0.441	0.314	0.198	Naga-0955	Balkhi	19 May	0.423	0.307	0.263
Naga-0731 a	Waziri	18 May	0.268	0.351	0.423	Naga-0973 a	Waziri	26 May	m	0.183	0.212
Naga-0732 a	Waziri	18 May	0.168	s	s	Naga-0974 a	Waziri	26 May	0.198	0.205	0.254
Naga-0755 a	Waziri	18 May	0.126	0.184	0.112						
Naga-0756 a	Waziri	18 May	0.202	0.192	0.184						
Naga-0783	Waziri	20 May	0.238	d	d						

Table No.1: Dynamics of *Dicrocoelium* antibodies in kids on goat farms 01 during 2018 and 2019. The limit (cut-off = 0.431% and 0.433% of positivity respectively).

2018 Goat Farm No.1			% of positivity			2019 Goat Farm No.1			% of positivity		
Goat code	**Breed**	**Birth date**	**June**	**Aug**	**Sept**	**Goat code**	**Breed**	**Birth date**	**June**	**Aug**	**Sept**
Boon- 0411	Khurrassani	11 March	0.239	0.328	0.442	Boon-1400	Waziri	11 March	0.128	0.189	0.271
Boon-0498	Khurrassani	11 March	0.167	0.431	0.567	Boon-1403	Waziri	12 March	0.458	0.318	0.287
Boon-0443	Khurrassani	17 March	0.158	0.187	0.183	Boon-1404	Waziri	13 March	0.179	0.188	0.206
Boon-0433	Waziri	18 March	0.179	0.182	0.196	Boon-1411	Waziri	14 March	0.297	0.419	0.451
Boon-0449	Khurrassani	24 March	0.428	0.487	0.523	Boon-1415	Waziri	15 March	0.423	0.458	0.521
Boon-0472	Waziri	24 March	0.209	0.328	0.486	Boon-1419	Waziri	16 March	0.239	0.267	0.218
Boon-0493	Waziri	28 March	0.187	0.164	0.169	Boon-1420	Khurassani	17 March	0.317	0.368	0.436
Boon-0459	Waziri	01 April	0.245	s	s	Boon-1422	Waziri	18 March	0.167	0.181	0.193
Boon-0463	Khurrassani	03 April	0.168	0.183	0.214	Boon-1437	Waziri	27-Mar	0.175	0.184	0.198
Boon-0460	Khurrassani	03April	0.197	0.249	0.476	Boon-1445	Waziri	28-Mar	0.162	0.179	0.215
Boon-0475	Khurrassani	09 April	0.238	0.342	0.437	Boon-1446	Khurassani	2-Apr	0.231	0.317	0.432
Boon-0469	Khurrassani	15 April	0.318	d	d	Boon-1454	Khurassani	4-Apr	0.159	0.183	0.217
Boon-0417	Khurrassani	16 April	0.178	0.239	0.326	Boon-1461	Khurassani	11-Apr	0.326	s	s
Boon-0456	Khurrassani	19 April	0.165	0.187	0.238	Boon-1463	Khurassani	17-Apr	0.194	0.199	0.256
Boon-0441 a	Waziri	23 April	0.149	0.313	0.416	Boon-1467	Waziri	17-Apr	0.318	0.337	0.441
Boon-0442 a	Waziri	23 April	0.165	0.218	0.256						
Boon-0445 a	Khurrassani	29 April	0.239	s	s						
Boon-0446 a	Khurrassani	29 April	0.258	0.329	0.348						

Table No.2: Dynamics of *Dicrocoelium* antibodies in kids on goat farms 02 during 2018 and 2019. The limit (cut-off = 0.431% and 0.433% of positivity respectively).

2018 Goat Farm No.2			% of positivity			2019 Goat Farm No.2			% of positivity		
Goat code	**Breed**	**Birth date**	**June**	**Aug**	**Sept**	**Goat code**	**Breed**	**Birth date**	**June**	**Aug**	**Sept**
Masj-0619	Cross beetal	18 March	0.137	0.165	0.183	Masj-1602	Cross beetal	2 Aril	0.238	0.322	0.439
Masj-0660 a	Waziri	22 March	0.133	0.237	0.453	Masj-1603	Cross beetal	8-Apr	0.438	0.487	0.516
Masj-0661 a	Waziri	22 March	0.127	0.187	0.213	Masj-1607	Cross beetal	11-Apr	0.169	0.173	0.182
Masj-06673	Waziri	25 March	0.214	0.189	0.173	Masj-1608	Cross beetal	13-Apr	0.138	0.171	0.147
Masj-0647	Waziri	27 March	0.314	0.436	0.476	Masj-1611	Cross beetal	16-Apr	0.168	0.195	0.216
Masj-0632 a	Waziri	27 March	0.166	0.187	0.235	Masj-1613	Cross beetal	18-Apr	0.143	0.247	0.188
Masj-0633 a	Waziri	27 March	0.184	0.191	0.156	Masj-1645	Cross beetal	28-Apr	0.175	0.191	0.213
Masj-0656	Cross beetal	28 March	0.148	0.183	0.19	Masj-1661	Cross beetal	28-Apr	0.239	0.252	0.315
Masj-0612	Waziri	01 April	0.246	0.313	0.431	Masj-1665	Cross beetal	1-May	0.169	0.173	0.231
Masj-0617	Waziri	01 April	0.448	0.317	0.169	Masj-1674	Cross beetal	3-May	0.223	s	s
Masj-0661	Waziri	10 April	0.166	0.187	0.209	Masj-1687	Cross beetal	5-May	0.232	0.194	0.324
Masj-0666	Cross beetal	12 April	0.187	0.175	0.234	Masj-1689	Cross beetal	6-May	0.265	0.328	0.442
Masj-0614	Waziri	24 April	0.157	0.149	0.219	Masj-1694	Cross beetal	9-May	0.189	0.223	0.316
Masj-0676 a	Waziri	27 April	0.163	0.267	0.241	Masj-1698	Cross beetal	13-May	0.156	0.234	0.278
Masj-0677 a	Waziri	27 April	0.152	0.178	0.203	Masj-1692	Cross beetal	12-May	0.169	0.254	0.187

Table No.3: Dynamics of *Dicrocoelium* antibodies in kids on goat farms 03 during 2018 and 2019. The limit (cut-off = 0.431% and 0.433% of positivity respectively).

2018 Goat Farm No.3 % of positivity						2019 Goat Farm No.3 % of positivity					
Goat code	**Breed**	**Birth date**	**June**	**Aug**	**Oct**	**Goat code**	**Breed**	**Birth date**	**June**	**Aug**	**Oct**
Lasp-0502	Cross beetal	16 March	0.231	0.428	0.562	Lasp-1707	Cross beetal	16 March	0.176	0.243	0.276
Lasp-0540	Cross beetal	16 March	0.163	0.178	0.232	Lasp-1712	Cross beetal	16 March	0.254	0.328	0.432
Lasp-0561	Cross beetal	22 March	0.248	0.437	0.473	Lasp-1718	Cross beetal	22 March	0.235	0.342	0.328
Lasp-0577	Cross beetal	22 March	0.165	0.442	0.486	Lasp-1723	Cross beetal	22 March	0.174	0.177	0.162
Lasp-0508	Cross beetal	22 March	0.184	m	0.241	Lasp-1731	Cross beetal	22 March	0.262	0.296	0.268
Lasp-0531	Cross beetal	27 March	0.179	0.191	0.224	Lasp-1738	Cross beetal	27 March	0.189	0.195	0.222
Lasp-0549	Cross beetal	27 March	0.184	0.227	0.322	Lasp-1753	Cross beetal	27 March	0.426	0.468	0.543
Lasp-0552	Cross beetal	28 March	0.226	0.294	0.422	Lasp-1768	Cross beetal	28 March	0.174	0.196	0.232
Lasp-0568	Cross beetal	30 March	0.178	0.196	0.193	Lasp-1776	Waziri	30 March	0.212	0.452	0.547
Lasp-0516 a	Cross beetal	30 March	0.194	0.221	0.254	Lasp-1781	Waziri	30 March	0.159	0.163	0.152
Lasp-0517 a	Cross beetal	30 March	0.234	0.284	0.325	Lasp-1788	Cross beetal	30 March	0.268	0.326	0.342
Lasp-0545	Cross beetal	27 March	0.262	s	s	Lasp-1791	Cross beetal	27 March	0.165	0.227	0.276
Lasp-0538	Cross beetal	28 March	0.116	0.165	0.173	Lasp-1793	Cross beetal	28 March	0.126	0.165	0.144
Lasp-0521	Cross beetal	04 April	0.246	0.306	0.332	Lasp-1797	Cross beetal	04 April	0.204	0.32	0.344
Lasp-0506	Cross beetal	06 April	0.269	0.285	0.263	Lasp-1798	Cross beetal	06 April	0.296	0.311	0.344
Lasp-0587	Cross beetal	06 April	0.187	0.244	0.268						

Table No.4: Dynamics of *Dicrocoelium* antibodies in kids on goat farms 04 during 2018 and 2019. The limit (cut-off = 0.431% and 0.433% of positivity respectively).

2018 Goat Farm No.4 % of positivity						2019 Goat Farm No.4 % of positivity					
Goat code	**Breed**	**Birth date**	**June**	**Aug**	**Sept**	**Goat code**	**Breed**	**Birth date**	**June**	**Aug**	**Sept**
Thork- 1001	Khurassani	06 March	0.248	0.286	0.312	Thork- 1903	Waziri	16 March	0.169	0.195	0.232
Thork-1017 a	Khurassani	12 March	0.222	0.283	0.327	Thork- 1904	Waziri	16 March	0.227	0.254	0.262
Thork- 1018 a	Khurassani	12 March	0.189	0.433	0.442	Thork- 1909	Waziri	22 March	0.158	0.173	0.142
Thork- 1042	Waziri	25 March	0.264	0.249	0.325	Thork- 1916	Waziri	22 March	0.228	0.298	0.335
Thork-1058	Khurassani	25 March	0.334	0.412	0.432	Thork- 1921	Waziri	22 March	0.426	0.324	0.229
Thork-1063	Khurassani	27 March	0.186	0.194	0.188	Thork- 1926	Waziri	27 March	0.178	0.188	0.146
Thork-1006	Khurassani	28 March	0.165	0.172	0.185	Thork- 1928	Waziri	27 March	0.238	0.283	0.316
Thork-1027 a	Khurassani	28 March	0.234	0.354	0.328	Thork- 1932	Waziri	28 March	0.321	s	s
Thork-1028 a	Khurassani	28 March	0.262	0.463	0.542	Thork- 1935	Khurassani	30 March	0.326	0.226	0.266
Thork-1036	Khurassani	03 April	0.169	0.182	0.211	Thork- 1941	Khurassani	30 March	0.228	0.443	0.478
Thork-1022	Khurassani	03 April	0.328	0.464	0.54	Thork- 1944	Waziri	30 March	0.264	m	m
Thork-1054	Khurassani	05 April	m	0.264	s	Thork- 1946	Waziri	27 March	0.178	0.204	0.248
Thork-1068	Khurassani	05 April	0.178	0.208	0.244	Thork- 1952	Waziri	28 March	0.228	0.179	0.212
Thork-1072	Khurassani	14 April	0.164	0.184	0.222	Thork- 1956	Waziri	04 April	0.157	0.182	0.191
						Thork- 1961	Waziri	06 April	0.179	0.228	0.274
						Thork- 1965	Khurassani	06 April	0.246	0.321	0.464

Table No.5: Dynamics of *Dicrocoelium* antibodies in kids on goat farms 05 during 2018 and 2019. The limit (cut-off = 0.431% and 0.433% of positivity respectively).

2018 Goat Farm No.5			% of positivity			2019 Goat Farm No.5			% of positivity		
Goat code	**Breed**	**Birth date**	**June**	**Aug**	**Sept**	**Goat code**	**Breed**	**Birth date**	**June**	**Aug**	**Sept**
Naga-1219	Waziri	24 March	0.273	0.228	0.242	Naga-2002	Waziri	06 March	0.148	0.184	0.223
Naga-1242	Waziri	27 March	0.246	0.226	0.325	Naga-2007	Waziri	12 March	0.228	0.194	0.218
Naga-1208	Waziri	27 March	0.342	0.366	0.298	Naga-2011	Waziri	12 March	0.242	0.342	0.286
Naga-1224	Cross beetal	27 March	0.189	0.226	0.284	Naga-2014	Waziri	25 March	0.324	s	s
Naga-1262	Waziri	29 March	0.283	0.366	0.348	Naga-2019	Waziri	25 March	0.168	0.187	0.199
Naga-1273	Waziri	29 March	0.184	0.366	0.472	Naga-2021	Waziri	27 March	0.243	0.288	0.326
Naga-1210	Waziri	30March	0.349	0.436	0.542	Naga-2027	Waziri	28 March	0.269	0.242	0.324
Naga-1230	Cross beetal	08 April	0.332	0.449	0.546	Naga-2029	Waziri	28 March	0.146	0.169	0.172
Naga-1238 a	Waziri	08 April	0.262	0.186	0.172	Naga-2033	Waziri	28 March	0.265	0.304	0.359
Naga-1239 a	Waziri	08 April	0.268	0.296	0.308	Naga-2039	Waziri	03 April	0.338	s	s
Naga-1216 a	Waziri	11 April	0.198	0.208	0.262	Naga-2044	Waziri	03 April	0.311	0.349	0.435
Naga-1217 a	Waziri	11 April	0.204	0.284	0.326	Naga-2048	Waziri	05 April	0.286	0.432	0.542
Naga-1280	Waziri	11 April	0.167	0.188	0.202	Naga-2055	Waziri	05 April	0.204	0.284	0.308
Naga-1245	Cross beetal	14 April	0.224	0.286	0.326	Naga-2059	Waziri	14 April	0.317	0.443	0.524

ANNEXURE

Annexure #3.1

D. dendriticum and *D. chinensis* rDNA haplotypes

D.dendriticum16

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*D.dendriticum*26
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*D.dendriticum*27
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*D.dendriticum*29
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*D.dendriticum*31
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*D.dendriticum*33
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*D.dendriticum*34
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*D.chinensis*1 (reversed)
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*D.chinensis*2 (reversed)
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*D.chinensis*3 (reversed)
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*D.chinensis*4 (reversed)
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Annexure 3.2

D. dendriticum and *D. chinensis* COX1 haplotypes

1. *D. dendriticum*
ggatggtgagtcatgtgtgtatgtgtgtgaccaataatgattcgttgtttggttatatgggattggtgtttgcgatggtggctattgtgtgccttggt
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2. *D. dendriticum*
ggatggtgagtcatgtgtgtatgtgtgtgactaataatgattcgttgtttggttacatgggattggtgtttgcgatggtggctattgtgtgccttggt
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3. *D. dendriticum*
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4. *D. dendriticum*
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5. *D. dendriticum*
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6. *D. dendriticum*
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7. *D. dendriticum*
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8. *D. dendriticum*
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9. *D. chinensis*
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10. *D. chinensis*
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11. *D. chinensis*
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Annexure. 3.3

***D. dendriticum_ D. chinensis _ F. gigantica_F. hepatica* ND1 haplotypes**

*D.dendriticum*1
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*D.dendriticum*2
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*D.dendriticum*3
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cccgtgtgttatatattttggttgattggcgtgttgtgtgaat

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*D.dendriticum*11
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Molecular confirmation of *Dicrocoelium dendriticum* in the Himalayan ranges of Pakistan

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ABSTRACT

Lancet liver flukes of the genus *Dicrocoelium* (Trematoda: Digenea) are recognised parasites of domestic and wild herbivores. The aim of the present study was to confirm the species identity of Dicrocoeliid flukes collected from the Chitral valley in the Himalayan ranges of Pakistan. The morphology of 48 flukes belonging to eight host populations was examined; but overlapping traits prevented accurate species designation. Phylogenetic comparison of published *D. dendriticum* ribosomal cistron DNA, and cytochrome oxidase-1 (COX-1) mitochondrial DNA sequences with those from *D. chinensis* was performed to assess within and between species variation and re-affirm the use of species-specific single nucleotide polymorphism markers. PCR and sequencing of 34 corresponding fragments of ribosomal DNA and 14 corresponding fragments of mitochondrial DNA from the Chitral valley flukes, revealed 10 and 4 unique haplotypes, respectively. These confirmed for the first time the molecular species identity of Pakistani lancet liver flukes as *D. dendriticum*. This work provides a preliminary illustration of a phylogenetic approach that could be developed to study the ecology, biological diversity, and epidemiology of Dicrocoeliid lancet flukes when they are identified in new settings.

1. Introduction

Dicrocoeliid liver flukes can infect the bile ducts of a variety of wild and domesticated mammals and humans around the globe. Three species of the genus *Dicrocoelium*, namely *Dicrocoelium dendriticum*, *Dicrocoelium hospes* and *Dicrocoelium chinensis* have been described as causes of dicrocoeliosis in domestic and wild ruminants [1]. Among these, *D. dendriticum* is the most common and is distributed throughout Europe, Asia, North and South America, Australia, and North Africa [2]. The main economic impact of dicrocoeliosis in livestock is due to the rejection of livers with cholangitis from slaughtered animals at meat inspection [3]. However, in severe infections, affected animals may show clinical signs including poor food intake, ill thrift, poor milk production, alteration in faecal consistency, photosensitisation and anaemia [4,5].

The life history of *Dicrocoelium* spp. is indirect and may take at least six months to complete. Monoecious and both sexually reproducing and self-fertilising adults are found in the bile ducts. Eggs containing fully-developed miracidia are shed in faeces and must be eaten by land snails before hatching. Miracidia undergo asexual replication and development into cercariae, which then escape from the snails in their slime trails, and are eaten by ants. One cercaria migrates to the head of the ant and associates with the sub-oesophageal ganglion; while up to about 50 cercariae encyst in the gaster as metacercariae [6]. The larval stage that develops in the ant's head alters its behaviour, making it cling to herbage and increasing the probability of its being eaten by a herbivorous definitive host. Larval flukes migrate to the liver via the biliary tree and develop to adults in the bile ducts [4]. Several species of land snails and ants are known to be intermediate hosts within the same geographical location [7]; nevertheless the geographical distribution of *Dicrocoelium* spp. is constrained by the precise conditions required for completion of the parasite's life history.

Molecular methods amplifying fragments of nuclear ribosomal genes and their internal transcribed spacers, or mitochondrial loci DNA [8–13] have been developed for Dicrocoeliid parasites; but as with all molecular diagnostic tools, these depend on accurate morphological speciation of

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Table 1
Primer sequences for the amplification of *Dicrocoelium* spp. ITS-2 rDNA and mt-COX-1 mtDNA fragments.

Primer name	Sequences (5′-3′)	
BD1-F-rDNA	GTCGTAACAAGGTTTCCGTA	
BD2-R-rDNA	TATGCTTAAATTCAGCGGGT	[13]
Dd-F-rDNA	ATATTGCGGCCATGGGTTAG	
Dd-R-rDNA	ACAAACAACCCGACTCCAAG	[13]
D-F-cox11	TGAGGTCTTGGATCGTGTTA	
D-R-cox11	AAACCACCAACTCACCAAAC	

the reference materials. These methods are adaptable to demonstrate genetic variability and phylogeny, and have been applied to epidemiological studies of various trematode parasite species affecting ruminant livestock [14–17]. However, in the study of Dicrocoeliid genera, the value of phylogenetic studies to detect intraspecific variation is limited by the availability of comparable sequence data.

The Chitral valley has economic and strategic importance as a route of human and animal movements to and from south-east Asia through what is known as the China-Pakistan economic corridor. The average elevation is 1500 m and the daily mean temperature ranges from 4.1 °C to 15.6 °C, creating an arid environment with only patchy coniferous tree cover, and providing habitats that are mostly hostile to many snail species. Moving north from Peshawar over the Lowari Pass into Chitral valley the snail fauna changes completely [18], potentially creating isolated habitats for *Dicrocoelium* spp. flukes in this region as compared to other parts of Pakistan. The economics of livestock production is marginal in this region, hence any improved understanding of any potential production limiting disease, such as dicrocoeliosis is important.

In this study, we describe variability in morphological features of Dicrocoeliid parasites recovered from slaughtered sheep in the Chitral valley, along with corresponding genomic sequence data for fragments of ribosomal DNA and cytochrome oxidase-1 (COX-1) mtDNA. Our aim was to use these data to describe any possible phylogenetic relationships within and between Pakistani *D. dendriticum* and the limited number of other *Dicrocoelium* spp. for which matching sequence data are publicly available.

2. Materials and methods

2.1. Fluke collection

A convenience sampling method was used to examine the livers of a total of 144 sheep slaughtered at four abattoirs in the Chitral valley of the Kyber Pakhtunkhwa province of Pakistan. These comprised of 68 from Booni, 26 from Torkhow, 33 from Mastuj and 17 from the Laspoor valley. Overall, 639 typical adult Dicrocoelid flukes (25 to 50 flukes per liver) were recovered from the livers of 16 infected sheep. The flukes were washed with phosphate-buffered saline (PBS) to remove adherent debris and fixed in 70% ethanol for subsequent morphometric and molecular characterisation.

2.2. Morphological examination

Six adult flukes were selected from each of the livers of four of the 12 infected sheep from Booni (Pop-1, Pop-2, Pop-3, Pop-4), the one infected sheep from Laspoor (Pop-5), the two infected sheep from Mastuj (Pop-6, Pop-7) and the one infected sheep from Thorkhow (Pop-8) and stained for morphometric analysis. The flukes were fixed between two glass slides in formalin-acetic acid alcohol solution, stained with hematoxylin (Sigma-Aldrich) and mounted in Canada balsam. Standardised measurements were taken and orientation of the testes was noted as previously described [9,19].

2.3. PCR amplification and sequence analysis of ribosomal and mitochondrial DNA

Genomic DNA was successfully extracted from 34 individual adult flukes (3 to 6 flukes per animal) from the livers of the same eight sheep (Pop-1 to Pop-8), using a standard phenol-chloroform method [20]. 1152 bp of the rDNA cistron, comprising of fragments of the ITS1, 5.8S, ITS2 and 28S flanking region, were amplified by using two sets of universal primers (Table 1) as previously reported [13]. A 215 bp fragment of cytochrome *c* oxidase subunit-1 (COX-1) mtDNA was amplified using newly developed forward and reverse primers, designed with reference sequences downloaded from NCBI using the 'Primers 3' online tool (Table 1). The 25 μl PCR reaction mixtures consisted of 2 μl of PCR buffer (1×) (Thermo Fisher Scientific, USA), 2 μl $MgCl_2$ (25 mM), 2 μl of 2.5 mM dNTPs, 0.7 μl of primer mix (10 pmol/μl final concentration of each primer), 2 μl of gDNA, and 0.3 μl of Taq DNA polymerase (5 U/μl) (Thermo Fisher Scientific, USA) and 16 μl ddH_20. The thermocycling conditions were 96 °C for 10 min followed by 35 cycles of 96 °C for 1 min, 60.9 °C (BD1-F-rDNA/ BD1-R-rDNA), or 61.4 °C (Dd-F-rDNA/ Dd-R-rDNA), or 55 °C (D-F-cox11/ D-R-cox11) for 1 min and 72 °C for 1 min, with a final extension of 72 °C for 5 min.

PCR products were cleaned using a WizPrepTM Gel/PCR Purification Mini kit (Seongnam 13,209; South Korea) and submitted for sequencing of both strands on an Applied Biosystems 3730Xl genetic analyser (Eurofins Genomics LLC), using the same amplification primers. Both strands of rDNA, and COX-1 sequences from each fluke were assembled, aligned and edited to remove primers from both ends using Geneious Pro 5.4 software [21]. The sequences were then aligned with previously published NCBI GenBank rDNA and COX-1 sequences of *D. dendriticum* and *D. chinensis*. All sequences in the alignment were trimmed based on the length of the shortest sequence available that still contained all the informative sites. Sequences showing 100% base pair similarity were grouped into single haplotypes using the CD-HIT Suite software [22].

2.4. Molecular phylogeny of the rDNA and COX-1 data sets

The generated haplotypes were imported into MEGA 7 [23] and used to determine the appropriate model of nucleotide substitution. Molecular phylogeny was reconstructed from the rDNA and COX-1 sequence data by the Maximum Likelihood (ML) method. The evolutionary history was inferred by using the ML method based on the Kimura 2-parameter model for rDNA and Hasegawa-Kishino-Yano model for the COX-1 locus. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test was shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log-likelihood values. All positions containing gaps and missing data were eliminated. There were totals of 698 bp, and 187 bp in the final datasets of rDNA and COX-1, respectively. A split tree was created in the SplitTrees4 software by using the UPGMA method in the HKY85 model of substitution. The appropriate model of nucleotide substitutions for UPGMA analysis was selected by using the jModeltest 12.2.0 program.

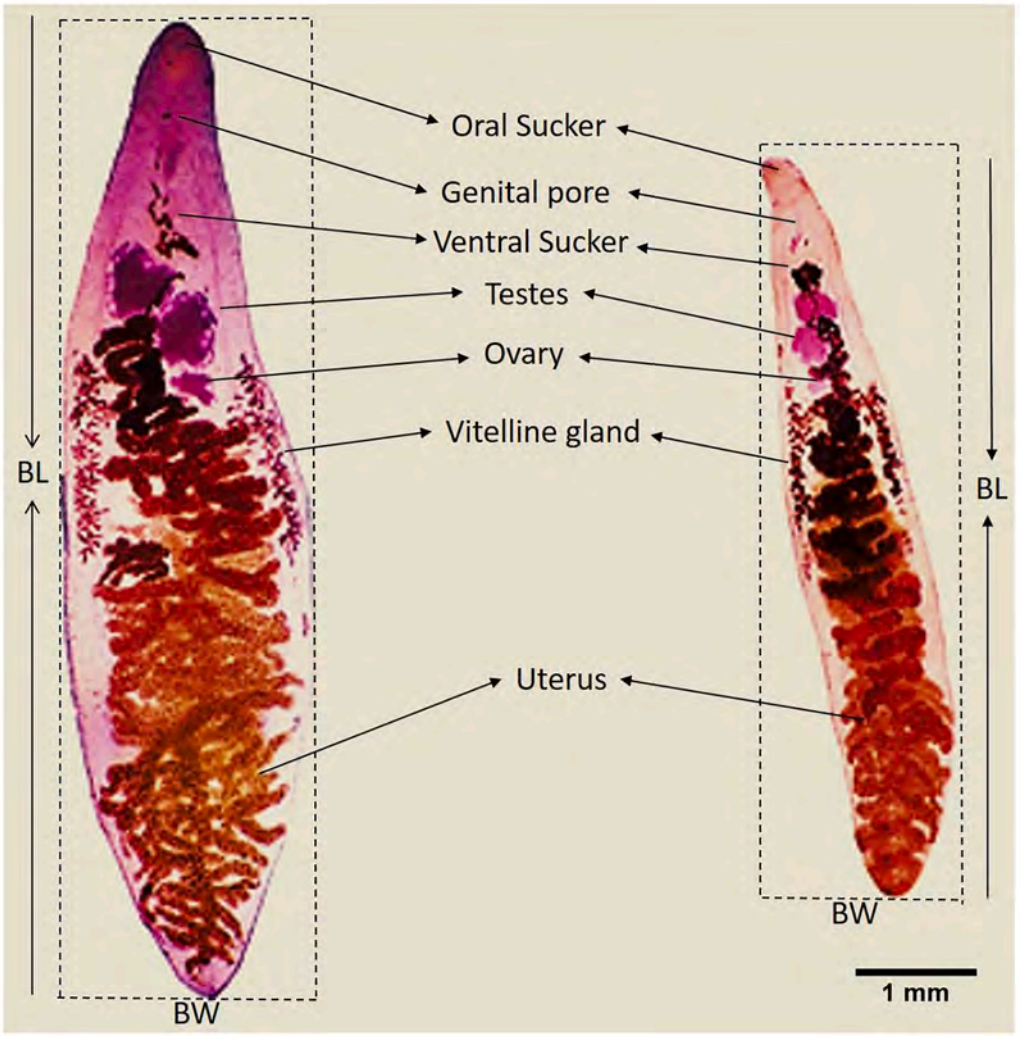

Fig. 1. Light micrograph of hematoxylin-stained flukes from the present study. The bodies are pointed at both ends and semi-transparent, with a pair of lobate testes behind the ventral sucker. The ovary is small, and the uterus has both ascending and descending limbs and white vitellaria. The morphometric measurements and orientation of the testes of 23 flukes were similar to those shown on the left, and the equivalent features of 25 flukes were similar to those shown on the left.

Table 2
Sequence variation in a 698 bp fragment of rDNA and a 187 bp fragment of COX-1 mtDNA, differentiating between *D. dendriticum* and *D. chinensis*. The rDNA data are based on 12 sequences identified as *D. dendriticum* and 18 sequences identified as *D. chinensis* on NCBI GeneBank. The COX-1 data are based on 56 sequences identified as *D. dendriticum* and 11 sequences identified as *D. chinensis* on NCBI GeneBank. Informative sites to describe intraspecific variation are shown in bold.

rDNA nucleotide position	*D. dendriticum*	*D. chinensis*	COX-1 nucleotide position	*D. dendriticum*	*D. chinensis*
18	**T/A**	**T**	9	T	A
56	C	T	12	T	C
58	G	A	15	T	C
60	T	–	24	G	T
82	**T/C**	**T**	30	G	T
119	G	A	54	**T/C**	**T**
134	G	T	57	**T/A**	**T**
221	**C**	**C/A**	63	**T**	**T/C**
222	**T**	**T/A**	66	**G/A**	**G**
228	**A**	**A/G**	75	**T**	**A/T**
240	**A**	**A/G**	78	**C/T**	**T**
358	T	C	84	A	G
367	G	A	111	G	A
370	C	T	114	C	T
405	G	A	129	T	A
423	**C/T**	**T**	132	A	G
424	C	T	135	T	A
469	T	C	138	**T/C**	**T**
489	**T/C**	**T**	141	T	A
535	T	C	143	**T/G**	**T**
541	A	G	177	**C/T**	**T**
550	T	C	183	**T**	**C/T**
571	G	A			
630	T	A			
632	G	T			
654	T	A			
655	C	T			
671	**C**	**C/A**			
680	G	A			
681	T	G			
689	**G**	**A/G**			

3. Results

3.1. Fluke morphometric characteristics

Forty-eight hematoxylin-stained liver flukes were examined. The flukes were all lanceolate, with flattened bodies, and were 5.65 to 8.7 mm in length and 1.3 to 2.2 mm in width. The overlapping ranges of morphometric measurements and orientation of the testes of the examined flukes did not allow for accurate species differentiation between *D. dendriticum* and *D. chinensis* (Fig. 1).

3.2. Molecular confirmation of Dicrocoelium species identity

3.2.1. Ribosomal DNA haplotypes

The rDNA sequences of each of 34 flukes from which genomic DNA was successfully extracted were aligned with 12 sequences of *D. dendriticum* and 18 sequences of *D. chinensis* (Supplementary Data S1) available on the Mendeley database and trimmed to 698 bp length. This included 4 informative sites to describe intraspecific variation within *D. dendriticum* and 6 sites to describe intraspecific variation within *D. chinensis*. The alignment confirmed 21 species-specific fixed SNPs to differentiate between *D. dendriticum* and *D. chinensis* (Table 2); this allowed the molecular species identity of each of the 34 flukes to be confirmed as *D. dendriticum*.

The 12 *D. dendriticum* and 18 *D. chinensis* sequences from the public database were examined along with the 34 *D. dendriticum* rDNA sequences from the present study. Sequences showing 100% base pair similarity were grouped into single haplotypes generating 19 unique *D. dendriticum* and 4 unique *D. chinensis* haplotypes (Supplementary Data S1). A ML tree was constructed from the 23 rDNA haplotypes to examine the evolutionary relationship between *D. dendriticum* and *D. chinensis*. The phylogenetic tree shows that *D. dendriticum* and *D. chinensis* form discrete species-specific clades (Fig. 2a). Ten haplotypes generated from the 34 rDNA sequences from the Chitral valley were clustered in the *D. dendriticum* clade. Comparison of these haplotypes with those originating from the Shaanxi province of China showed some common origins and close relationships (Table 3), but there were insufficient published sequence data to meaningfully describe the emergence and spread of the Pakistani *D. dendriticum*. The rDNA haplotypes generated from the eight Pakistani *D. dendriticum* populations are shown in a split tree (Fig. 3); albeit there are too few data to draw any conclusions.

3.2.2. Mitochondrial COX-1 haplotypes

Unfortunately, COX-1 sequences of sufficient quality were generated from only 14 of the 34 flukes from which genomic DNA was successfully extracted. These were aligned with 56 *D. dendriticum* sequences and 11 *D. chinensis* sequences available on the public NCBI Mendeley database (Supplementary Data S2) and trimmed to 187 bp length. This included 7 informative sites of intraspecific variation within *D. dendriticum* and 3 sites of intraspecific variation within *D. chinensis*. The alignment confirmed 12 species-specific fixed SNPs to differentiate between *D. dendriticum* and *D. chinensis* (Table 2), and allowed the molecular species identity of the 14 flukes to be re-affirmed as *D. dendriticum*.

The 56 COX-1 *D. dendriticum* and 11 *D. chinensis* sequences from the public database were examined along with the 14 *D. dendriticum* mtDNA

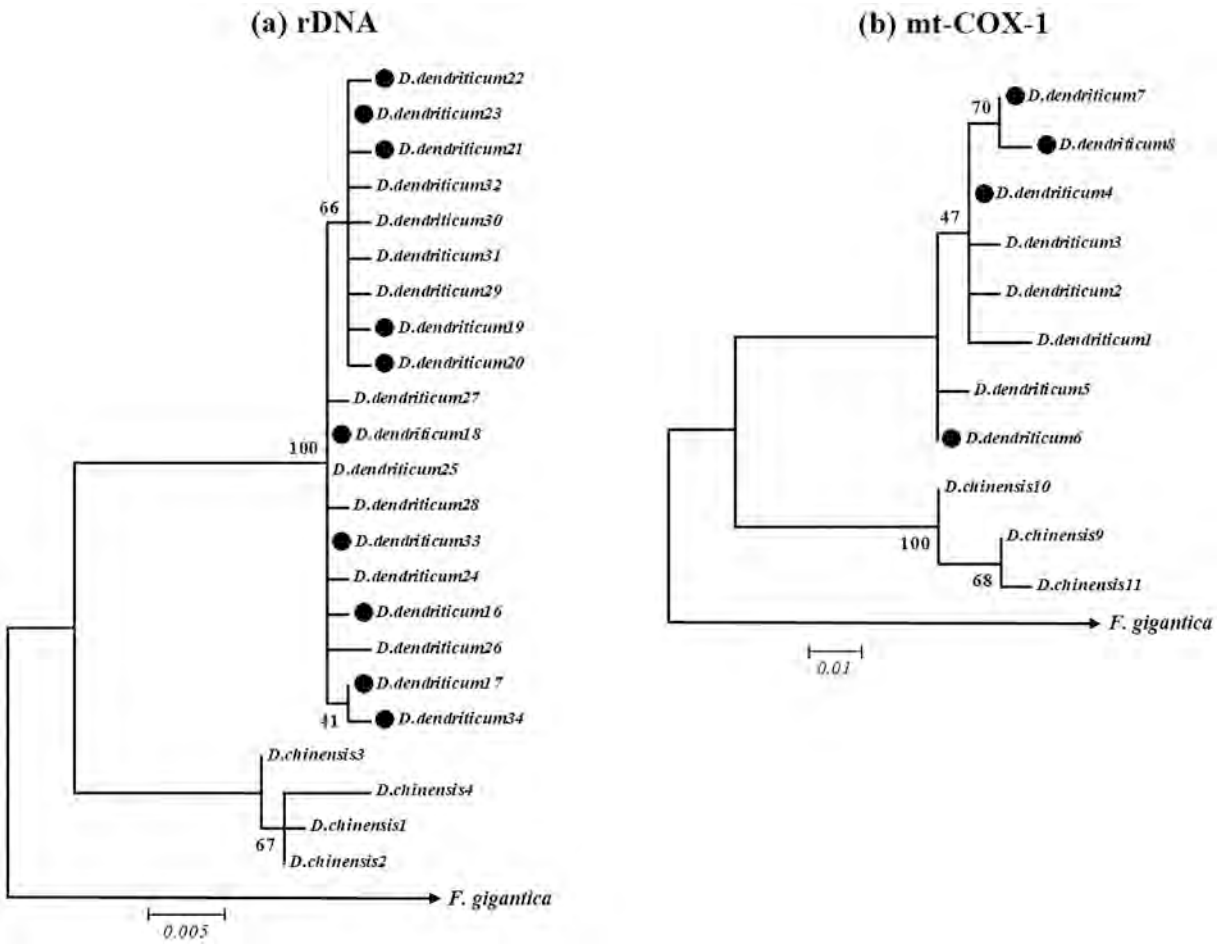

Fig. 2. Maximum-likelihood trees were obtained from the rDNA and COX-1 mtDNA sequences of *D. dendriticum* and *D. chinensis*. (a) Thirty-eight unique rDNA haplotypes. (b) Thirty-six unique COX-1 mtDNA haplotypes. The haplotype of each species is identified with the name of the species and black circles indicate *D. dendriticum* haplotypes originating from the Chitral valley of Pakistan.

sequences from the present study. Sequences showing 100% base pair similarity were grouped into single haplotypes generating 8 unique *D. dendriticum* and 3 unique *D. chinensis* haplotypes (Supplementary Data S2). A ML tree was constructed from the 11 unique COX-1 haplotypes to examine the evolutionary relationship between *D. dendriticum* and the other liver flukes. The phylogenetic tree re-affirms that *D. dendriticum* and *D. chinensis* form discrete species-specific clades (Fig. 2b). Four haplotypes generated from the 14 COX-1 sequences from the Chitral valley were clustered in the *D. dendriticum* clade. Comparison of these haplotypes with those originating from the Shaanxi province of China, Shiraz province of Iran and Japan showed some common origins and close relationships (Table 3), but there were insufficient published sequence data to meaningfully describe the emergence and spread of the Pakistani *D. dendriticum*.

4. Discussion

Dicrocoeliid liver flukes have previously been reported in Himalayan India, [24], China [25,26] and Iran [27,28]; but there are only anecdotal and unconfirmed reports from Pakistan [29]. The molecular methods used in the present study confirm for the first time the species identity of *D. dendriticum* lancet flukes collected from abattoirs in the Chitral valley. Knowledge of the parasite species infecting livestock in any geographical region is of particular importance when considering effective and sustainable control strategies. Our confirmation of *D. dendriticum* in Pakistan highlights the need for better understanding of aspects of the parasite's biology, such as the identification of the species of snail and ant that may act as competent intermediate hosts.

Previous studies have shown the value of morphological and molecular-based methods for the accurate species differentiation between *D. dendriticum* and *D. chinensis* [9,11–13]. The specimens collected from the Chitral valley were identified as Dicrocoeliid genus, but overlapping morphological traits prevented their accurate species designation.

Our analyses of ribosomal cistron and mitochondrial COX-1 loci sequences of *D. dendriticum* and *D. chinensis* from the public database showed consistent inter-specific variations. Twenty-one discriminatory ribosomal cistron SNPs and 12 discriminatory COX-1 SNPs were identified, allowing practical differentiation of the Dicrocoeliid family as previously described [11]. *D. hospes* was not included in our analyses,

Table 3
D. dendriticum rDNA and COX-1 haplotypes showing the number of sequences representing unique alleles and the country of origin. The accession numbers of all of the sequences are described in Supplementary Data S1 and S2 files.

rDNA haplotypes	Total number of sequences	Countries	mt-COX-1 haplotypes	Total number of sequences	Countries
*D. dendriticum*16	12	Pakistan	*D. dendriticum*1	4	Iran
*D. dendriticum*17	1	Pakistan	*D. dendriticum*2	2	Japan
*D. dendriticum*18	2	Pakistan, China	*D. dendriticum*3	2	Iran
*D. dendriticum*19	1	Pakistan	*D. dendriticum*4	33	Iran, China, Pakistan
*D. dendriticum*20	1	Pakistan	*D. dendriticum*5	2	Iran
*D. dendriticum*21	1	Pakistan	*D. dendriticum*6	19	Iran, China, Pakistan
*D. dendriticum*22	1	Pakistan	*D. dendriticum*7	7	Pakistan
*D. dendriticum*23	6	Pakistan, China	*D. dendriticum*8	1	Pakistan
*D. dendriticum*24	1	China			
*D. dendriticum*25	1	China			
*D. dendriticum*26	1	China			
*D. dendriticum*27	1	China			
*D. dendriticum*28	1	China			
*D. dendriticum*29	1	China			
*D. dendriticum*30	1	China			
*D. dendriticum*31	1	China			
*D. dendriticum*32	1	China			
*D. dendriticum*33	9	Pakistan			
*D. dendriticum*34	3	Pakistan			

The rDNA haplotype *D. dendriticum 18* represented two sequences from Pop-1 and Pop-7, which originated from the Booni and Mastuj regions and sequences reported from the Shaanxi province of China. The rDNA haplotype *D. dendriticum 16* represented 12 sequences from Pop-2, Pop-3, Pop-4, Pop-6 and Pop-8, which originated from the Booni, Mastuj and Torkhow regions, while the closely related rDNA haplotypes *D. dendriticum 24, 25, 26, 27* and *28* represented sequences from the Shaanxi province of China. The rDNA haplotype *D. dendriticum 23* represented 4 sequences from Pop-1 and Pop-5 which originated from the Booni and Laspoor regions, and sequences reported from the Shaanxi province of China. The rDNA haplotypes *D. dendriticum 19, 20* and *21* each represented single sequences from Pop-1 and Pop-2 which originated from the Booni region, while the closely related rDNA haplotypes *D. dendriticum 29, 31* and *32* represented sequences from the Shaanxi province of China. The rDNA haplotype *D. dendriticum 33* represented 9 sequences from Pop-1, Pop-2, Pop-3 and Pop-5 which originated from the Booni and Laspoor regions. The rDNA haplotype *D. dendriticum 34* represented 3 sequences from Pop-1 and Pop-6 which originated from the Booni, and Mastuj regions. The rDNA haplotypes *D. dendriticum 17* and *22* represented single sequences from Pop-1 and Pop-6 which originated from the Booni and Mastuj regions, while the closely related rDNA haplotypes *D. dendriticum 25 and 30* represented sequences from the Shaanxi province of China.
The mtDNA haplotype *D. dendriticum 4* represented 32 sequences from Pop-2, Pop-3, Pop-5, Pop-6 and Pop-7, which originated from the Booni, Mastuj and Laspoor regions and sequences reported from the Shaanxi province of China and Shiraz province of Iran. This haplotype was closely related to *D. dendriticum 2* reported from Japan. The mtDNA haplotype *D. dendriticum 7* represented sequences from Pop-1, Pop-4, Pop-5, Pop-6 and Pop-8, which originated from the Booni, Mastuj, Laspoor and Torkhow regions. The mtDNA haplotype *D. dendriticum 8* represented one sequence from Pop-3, which originated from the Booni regions. The mtDNA haplotype *D. dendriticum 6* represented 17 sequences from Pop-1, Pop-5 and Pop-6 which originated from the Booni, Mastuj and Laspoor regions and sequences reported from the Shaanxi province of China and Shiraz province of Iran.

because directly comparable sequence data are not publicly available. The ML tree that was generated shows separate clades of *D. dendriticum* and *D. chinensis*, hence in the case of co-infections, molecular differentiation between each of the species is possible. The Chitral valley lancet flukes were all *D. dendriticum*, irrespective of their morphological identity. Morphological traits may be influenced by: the stages of maturation of the flukes at the time of collection; the intensity of infection; factors linked to the host species; normal biological variation; or errors introduced during processing. Furthermore, the published morphological keys used for *D. chinensis* are based on parasites recovered from sika deer, whereas those for *D. dendriticum* are based on parasites recovered from sheep, albeit with no significant intraspecific variation reported from cattle derived parasites [9]. Consideration of these factors highlights the complementary value of morphological and molecular and methods in fluke species identification.

Our analyses of ribosomal cistron and mitochondrial COX-1 loci sequences of *D. dendriticum* and *D. chinensis* from the public database showed consistent intra-specific variations. Ten haplotypes in the ribosomal cistron fragment and four in the mitochondrial COX-1 sequences from the Chitral valley of Pakistan were then used to analyse gene flow. Unfortunately, comparable sequence data for European and north American *D. dendriticum* populations were unavailable in the public database for analysis. There were both unique and common haplotypes in each *D. dendriticum* population from the Chitral valley, some of which were also present in populations form China, Iran and Japan. There are insufficient data on which to base firm conclusions; hence further studies based on larger population sizes, and using for example next generation methods as described for *Calicophoron daubneyi* rumen flukes and *F. gigantica* liver flukes [16,17] are needed to describe gene flow and the role of animal movement in the spread of *D. dendriticum*.

Our findings support the potential for the development of population genetics tools to improve understanding of the molecular evolutionary biology and phylogenetics of *D. dendriticum*. This is needed to study changing epidemiology of the parasite, potentially arising as a consequence of changing management and climatic conditions, as previously described using a panel of microsatellites and a COX-1 mtDNA sequence marker [30].

Declaration of Competing Interest

The authors declare no conflicts of interest related to this work.

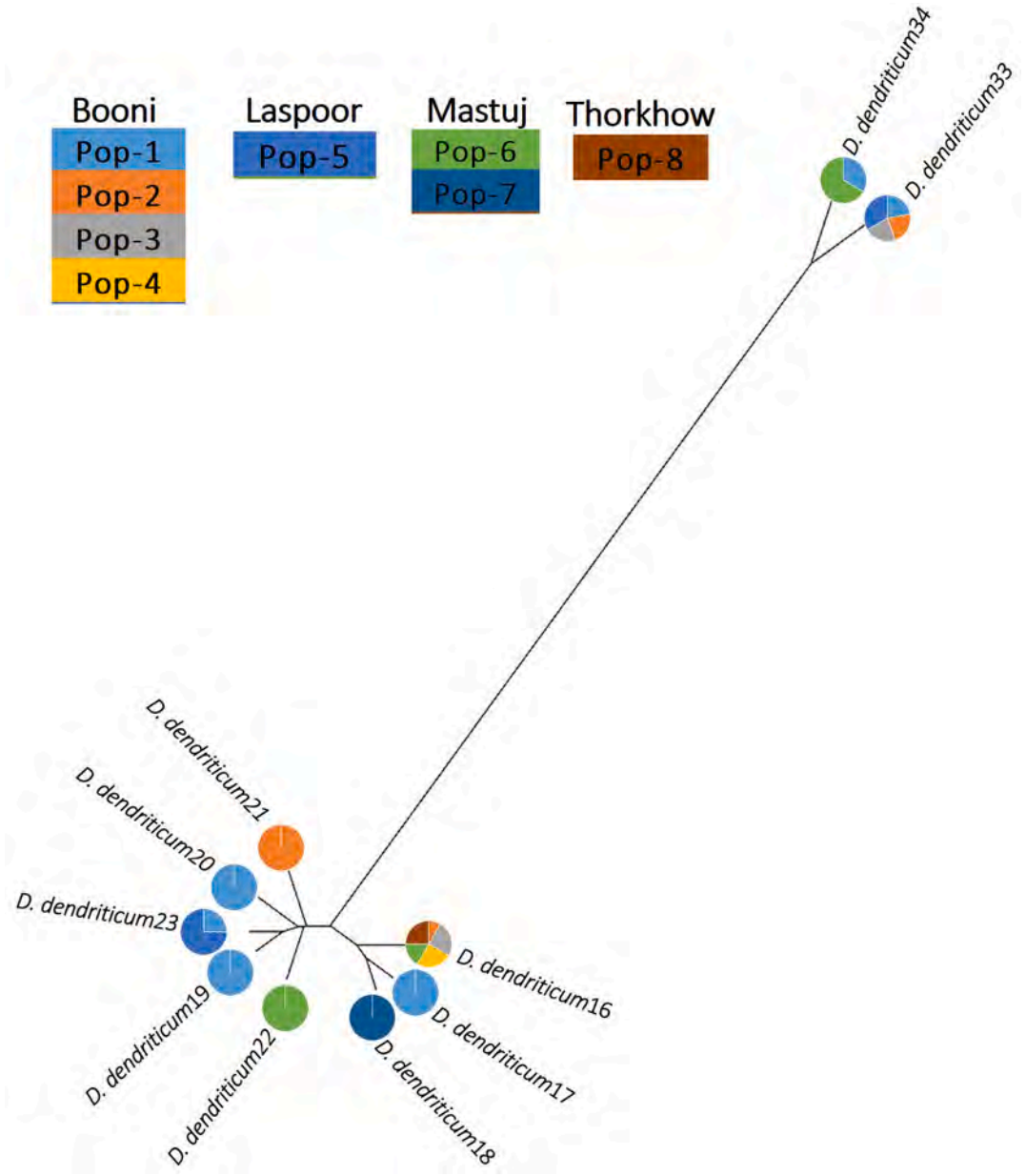

Fig. 3. Split tree of 10 rDNA haplotypes generated from eight Pakistani *D. dendriticum* populations. The tree was constructed with the UPGMA method in the HKY85 model of substitution in the SplitsTrees4 software. The pie chart circle represent the haplotype distribution among each of the eight populations. The colour of each haplotype circle represents the percentage of sequence reads generated per population.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2020.102276.

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ORIGINAL PAPER

Spatial Distribution of *Dicrocoelium* in the Himalayan Ranges: Potential Impacts of Ecological Niches and Climatic Variables

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Abstract

Purpose Dicrocoeliosis can be an important cause of production loss in ruminants due to the cost of liver condemnation at slaughter. The aim of the present study was to determine the prevalence of *Dicrocoelium* infection and to predict the ecological niches and climatic variables that support dicrocoeliosis in the Himalayan ranges of Pakistan.

Methods and Results *Dicrocoelium* was detected in 33 of 381 liver samples and 238 of 6060 blood samples taken from sheep and goat herds in the area. The prevalence of dicrocoeliosis was higher in sheep than in goats and highest in females aged more than 3 years. An environmental risk map was created to predict active zones of transmission and showed the highest probability values in central parts of the Chitral district in the northwest of Pakistan. Climatic variables of the mean monthly diurnal temperature range (Bio2), annual precipitation (Bio12), and normalised difference vegetation index (NDVI) were found to be significantly ($p < 0.05$) associated with the presence of *Dicrocoelium* infection.

Conclusion Together, the findings of this study demonstrate the most suitable ecological niches and climatic variables influencing the risk of dicrocoeliosis in the Himalayan ranges of Pakistan. The methods and results could be used as a reference to inform the control of dicrocoeliosis in the region.

Keywords Dicrocoeliosis · Himalayas range · Ecological niches · Climatic variables · Sheep · Goats

Introduction

Dicrocoeliosis is an important parasitic disease caused by three species of the genus *Dicrocoelium*, namely *Dicrocoelium dendriticum*, *Dicrocoelium hospes* and *Dicrocoelium chinensis* [1]. Among these, *D. dendriticum* is the most common and is distributed throughout Europe, Asia, North and South America, Australia, and North Africa. The other species have limited distribution and are present in Asia, Africa and some parts of Europe [2]. *Dicrocoelium* can infect the bile ducts of a variety of wild and domesticated mammals. Dicrocoeliosis causes overt economic loss due to the condemnation of livers with cholangitis from slaughtered animals at meat inspection [3]. Clinical signs of poor food intake, ill thrift, poor milk production, alteration in faecal consistency, photosensitisation and anaemia have been described in animals with high burdens [4, 5], and subclinical infection might cause reduced growth, although this is seldom measured.

Dicrocoelium has an exceptional life cycle that can take at least 6 months to complete. Within the same geographical location, several species of land snails and ants can be involved as first and second intermediate hosts, respectively [6]. Adult flukes are found in the bile ducts of their definitive herbivorous hosts. Eggs containing fully developed miracidia are shed in faeces and must be ingested by the snails before hatching and undergoing asexual replication and development into cercariae, which are shed by the snails and then eaten by ants. One cercaria migrates into the head of the ant and associates with the suboesophageal ganglion, while up to about 50 encyst in the gaster as metacercariae

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[7]. The larval stage that develops in the ant's head alters its behaviour, making it cling to herbage and increasing the probability of its being eaten by a definitive host. Following the encystment of the metacercariae, larval flukes migrate to the liver via the biliary tree and develop into adults [4].

Several studies have described the prevalence of *Dicrocoelium* in endemic regions; 4.8 and 11% in Iran [8, 9], between 5 and 30% in Canada [10, 11], 0.7% in China [12] and 22% in Japan [13]. Due to its unique life cycle involving two intermediate hots, *Dicrocoelium* is highly affected by climatic factors. Temperature and humidity influence the survival of eggs containing miracidia and the development of snail and ant intermediate hosts in their respective environmental niches [9, 10]. A seasonal pattern of the probability of infection has been shown in Canadian livestock, with the highest rate in mid-summer followed by an autumn decline (Dempsey, Burg [10].

Due to the association between these environmental factors and the prevalence and geographical distribution of *Dicrocoelium* infection, species distribution models (SDMs) have the potential to determine the spatial pattern of disease and ecological niches supporting infection challenge. SDMs are based on the interaction between species adaptability and key predicting climatic factors informed by humidity, rainfall, temperature and altitude [14–17]. Geographical Information Systems (GIS) and Maximum Entropy (MaxEnt) are the most widely used SDMs in the study of fluke parasites. These models have been used to show the geographical distribution and spatial pattern of fascioliosis or schistosomiasis and their risk factors associated with the ecological niches and climatic conditions [18–22]

Dicrocoelium was first identified in the Himalayan ranges of Pakistan by Khan, Afshan [23]. There have been few studies that provide information on the spatial distribution of dicrocoeliosis, and none in Asia. The present study was, therefore, undertaken to determine the prevalence and spatial distribution of dicrocoeliosis in the region and to describe the ecological niches that are favourable for the completion of the *Dicrocoelium* life cycle.

Materials and Methods

Study Areas

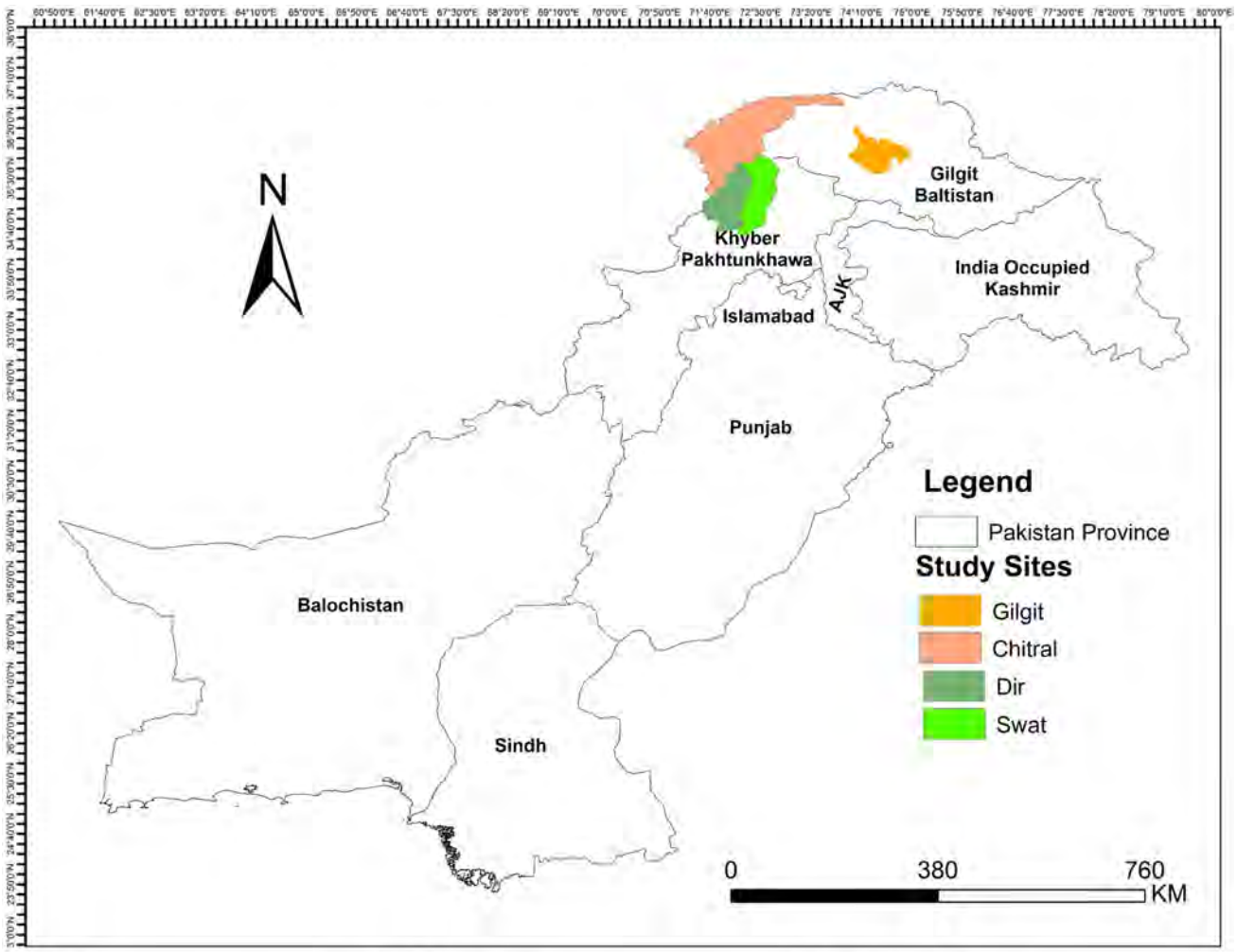
The study area is comprised of the Gilgit Baltistan and Khyber Pakhtunkhwa provinces of Pakistan (Fig. 1). Gilgit Baltistan has a border with China through the Khunjerab pass, which occupies an area of over 72,971 km^2. One district of Gilgit Baltistan was included in the study; (i) Gilgit district in the southwest of Karakoram range. The weather conditions include average rainfall of 120–240 mm annually. Additional irrigation is obtained from the rivers, which are abundant with melting snow water from higher altitudes. The Khyber Pakhtunkhwa has a border with Afghanistan to the west and north and spreads over an area of over 74,521 km^2. Three districts of Khyber Pakhtunkhwa were included in the study; (ii) Chitral district to the north of the Indus river, which originates close to the holy mountain of Kailash in western Tibet. The average elevation is 1500 m and the daily mean temperature ranges from 4.1 °C to 15.6 °C, creating an arid environment with only patchy coniferous tree cover, and providing habitats that are hostile to many snail species; (iii) Swat district surrounded by Chitral and Dir districts. The area is predominantly rural, and most residents live in villages. The average elevation is 980 m, resulting in a considerably cool and wet climate with lush forests, verdant alpine meadows, and snow-capped mountains. The climate of the Swat district is warm and humid with short and moderate summers, temperature rarely rises above 37 °C. The annual rainfall averages around 33 inches with about 17 inches during June–September; (iv) Dir district borders to Afghanistan on the north and the Swat district to the east. The climate is cold, with average rainfall is 700 mm and the temperature varies from 6 °C to 38 °C.

Study Design and Sample Collection

The study was carried out from July 2018 to September 2019. Random sampling was conducted and a total of 381 animals [Gilgit ($n=126$), Chitral ($n=214$), Swat ($n=41$)] were examined for flukes recovery, animals belonging to 56 sheep flocks and 24 goat herds. The flukes were washed with phosphate-buffered saline (PBS) to remove adherent debris followed by Dicrocoelid morphological identification. A total of 6,060 blood samples [Gilgit ($n=3020$), Chitral ($n=2140$), Swat ($n=670$) and Dir ($n=230$)] were collected from 112 sheep and 48 goat herds. The blood samples were taken from the jugular vein of the animal herds and stored at 4 °C for 4–6 h before sera were separated. The number of blood samples to be collected was determined using the formula: $n = Z^2P(1-P)/d^2$ [24], where n was the sample size, Z was the desired confidence interval (95%), P was a conservative estimate of the proportion of infected animals in the population (0.5) and d was precision of estimation or range in which the true population proportion is estimated to be (5%).

Liver Sample Processing for Antigen Extraction

The liver samples were inspected for Dicrocoelid flukes to determine the infection rate among sheep and goats. Excretory/secretory (ES) and somatic antigens were extracted from Dicrocoelid flukes recovered from 33 positive liver samples as described by Gonzalez-Lanza, Manga-Gonzalez [25] with some modifications. Briefly, flukes were incubated



Province	Gilgit Baltistan		Khyber Pakhtunkhwa	
District	Gilgit	Chitral	Swat	Dir
Coordinates	35.35°N 75.9°E	35.7699°N 71.7741° E	35°12′N 72°29′E	35°12′N 71°52′E
Elevation (m)	1500	1494	980	1420
Average precipitation (mm)	107.8	418	1011.5	1468.8
Average temperature (Min- Max) °C	6.2-19.4	11.4 -21.4	10-15	7.8-22.6
Liver samples examined	126	214	41	0
Blood samples examined	3020	2140	670	230

Fig. 1 Locations of the Chitral, Gilgit, Swat and Dir study districts in the Himalayan ranges of Pakistan

in RPMI 1640 medium (Biosera, Boussens, France) supplemented with 200 mM N-acetyl-L-alanil-L-glutamine (Sigma), 0.3 g/l sodium bicarbonate 7.5% (Sigma) and 40 mg/l gentamycin at 37 °C for 48 h. After removal of the flukes, the medium was collected and centrifuged at 10,000 g for 15 min at 4 °C. To obtain a somatic extract, flukes were homogenised in tissue lysis buffer, and added according to the weight of tissue in a ratio of 1000 µl buffer/100 mg of tissue. The homogenate was then transferred to pre-chilled Eppendorf tubes and centrifuged at 10,000 rpm at 4 °C for 10 min. The supernatant was filtered through 0.22 µm pore size filter units and Protease Inhibitor Cocktail (P8340; Sigma) was added. Protein concentration was determined by the Bradford method [26]. Samples were aliquoted and stored at −80 °C until further processing.

Enzyme-linked Immunosorbent Assay

ELISA was performed on 96-well microtiter plates as previously determined all incubation times by checkerboard titration method [27]. Briefly, each eluted antigen was mixed with coating buffer NaHCO3/Na2CO3 (Merck) in equal proportion (1:1) and 100 µl was added to each well of the microtiter plate and incubated overnight at 4 °C. The plates were washed three times with PBS containing 0.05% Tween 20 (Merck) and blocked with 0.05% BSA for 2 h at room

temperature. 100 µl of the diluted sera from infected and control animals was added to each well and incubated for 2 h at 37 °C and washed three times with PBS containing 0.05% Tween 20. After 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 at room temperature. After washing the plates, 100 µl of the substrate para-nitrophenyl phosphate (PNPP) (Thermo Scientific™ Cat. No. 37621) was added and incubated at room temperature for 20 min. Finally, the reaction was stopped by the addition of 50 µl of 3 N NaOH solution, and the optical density (OD) value was recorded at 405 nm using an automated microplate reader. The sensitivity of the test was measured at 88% and the specificity was 95%, respectively (Supplementary Table S1). The sensitivity of the assay was determined using the formula: Sensitivity $= \left[a / (a + c)\right] \times 100$; where '$a$' is the number of animals positive by ELISA and liver analysis (true positive), while 'c' is the number of animals positive by liver analysis but negative by ELISA (false negative). Similarly, Specificity $= \left[d / (b + d)\right] \times 100$; where '$d$' is the number of animals negative by ELISA and liver analysis (true negative), while 'b' is the number of animals negative by liver analysis but positive by ELISA (false positive). The cut-off was calculated by the mean optical density (OD) of the negative reference serum, plus three times standard deviations (0.14 + 3*0.08 = 0.38). The cut-off value was set at 0.38, and sera with OD value higher or equal to 0.38 were considered positive.

Species Distribution Models (SDMs)

Nineteen bioclimatic variables were obtained from the WorldClim (https://www.worldclim.org) global climate database (Fick and Hijmans, 2017) with the finest available resolution of approximately 1 km^2. These layers were readable in ASCII format using ArcGIS 10.2 (ESRI, Redlands, CA, USA). The spatial patterns of *Dicrocoelium* infection were measured with MaxEnt based modelling with MaxEnt version 3.4.4 [28] Maxent is freely downloadable at http://www.cs.princeton.edu/~schapire/maxent/. Field visits were conducted to obtain the geographic coordinates of *Dicrocoelium*-infected animals, and Global Positioning System (GPS) location was used to obtain the precise coordinates of infected animal flocks and herds. If a flock or herd had multiple infected animals, only one point was recorded to avoid the spatial clusters of localities.

The occurrence data of *Dicrocoelium* based on liver and blood samples were filtered to reduce bias and to improve the performance of the ecological niches modelling. The SDM toolbox in ArGIS 10.2 software (ESRI, Redlands, CA, USA) was used to reduce the occurrence locations of each infected animal to a single point within 5 km. By eliminating duplicate occurrence points within the same pixel, *Dicrocoelium* presence points were reduced to 63 points from 160 presence points; 80% were used for the training and 20% for testing the model. 10,045 points were used to determine the MaxEnt distribution (background points and presence points). The model was run with the logistic output format where predicted values range from 0 (impossible) to 1 (optimal).

The performance of predicting the ecological niches of *Dicrocoelium* infection was evaluated using threshold-independent receiver operating characteristic (ROC) assessment, where the area under the ROC curve (AUC) was obtained for plotting the model's sensitivity and specificity in MaxEnt. The geographical distribution of *Dicrocoelium* infection was mapped using a geographic information system (GIS). The presence points were marked on a world geodetic system (WGS84) reference coordinate system using high-resolution Google Earth and GIS coordinates. The parasite data were saved in an excel sheet and comma-separated values (CSV) files were used for the analysis. Compilation of geographic data and mapping was done by converting the excel data to the GIS format through Arc-Map (ESRI, Redlands, CA, USA).

To remove the autocorrelation among the 19 bioclimatic variables, Pearson's correlation was used at ($r^2 \geq |0.8|$) through the SDM Tools function in ArcGIS 10.2 (Universal tool; Explore climate data; Remove highly correlated variable). Five bioclimatic variables [Bio2 = mean diurnal range (mean of monthly (max temp—min temp), Bio4 = temperature seasonality (standard deviation × 100), Bio6 = min temperature of coldest month, Bio12 = annual precipitation and Bio15 = precipitation seasonality (coefficient of variation)] were used for the analysis. Additional variables with the same resolution as the bioclimatic variables were included in the evaluation; these were normalised difference vegetation index (NDVI) extracted from moderate resolution imaging spectroradiometer (MODIS) images, calculated from the visible and near-infrared light reflected by vegetation (NDVI data are available in Raster data images, each of which has several blocks which have specific values for different vegetation; and can be processed in a MaxEnt readable format using specific conversion tools), forest cover, elevation, derived from the digital elevation model (DEM) in ArcGIS 10.2, and distance to buildings or settlements. The environmental variables used in the MaxEnt model are summarised in Supplementary Table S2. The environmental variables associated with dicrocoeliosis were generated using a jacknife test in MaxEnt version 3.4.4 [28].

Statistical Analysis

The relatedness of *Dicrocoelium* prevalence, based on blood and liver samples examination, with associated environmental and climatic risk factors, was calculated using Chi-squared test of independence in a statistical package for

the social sciences (SPSS) version 20 (Armonk, NY: IBM Corp). The level of significance was set at $P \leq 0.05$.

Results

Prevalence of *Dicrocoelium*

Overall, Dicrocoelid flukes were identified in 33 of 381 (8.66%) liver samples, and 238 of 6060 (3.93%) blood samples were positive for both *Dicrocoelium* IgG antibodies. *Dicrocoelium* was isolated from the liver samples of 20 of 56 sheep flocks and 13 of 24 goat herds, and blood samples showed the presence of *Dicrocoelium* IgG antibodies in 108 of 112 sheep flocks and 44 of 48 goat herds, respectively (Table 1). The seasonal percentage of *Dicrocoelium* positive liver samples was higher during the summer and autumn (10.88% and 10%, respectively) than during the winter and spring (5.22% and 6.9%, respectively); and a similar trend was seen in the blood samples, but neither of these seasonal differences was significant ($p > 0.05$). The percentage of *Dicrocoelium* positive blood samples was significantly higher ($p = 0.0001$) in females (4.93%) than in male hosts (1.47%), and a similar, but non-significant trend was seen in the liver samples. The percentage of *Dicrocoelium* positive blood samples was significantly higher ($p = 0.05$) in animals aged more than 3 years (4.5%) than in animals aged less than 1- year-old (3.26%), or 1 to 2 years old (3.33%). Similar, but non-significant trends were seen in the liver samples. The percentage of *Dicrocoelium* positive blood samples was significantly higher ($p = 0.0001$) in goats (7.39%) than in sheep (3.29%); while the percentage of *Dicrocoelium* positive liver samples was significantly higher ($p = 0.0001$) in sheep (10.04%) than in goats (5.74%). These data are shown in Table 2.

Geographical Distribution of *Dicrocoelium*

The prevalence of *Dicrocoelium* was highest in the Chitral district (7.1% and 9.81% positive blood and liver samples, respectively); followed by the Gilgit district (2.58% and 7.94% positive blood and liver samples, respectively); and lowest in Swat (1.19% and 4.88% positive blood and liver samples, respectively) and Dir (no positive samples, albeit the numbers of animals sampled in these districts, were small). Within each region, the prevalence of *Dicrocoelium* positive samples varied between different valleys from 0.5% (Doian valley in Gilgit) to 17.5% (Pret valley in Chitral) of blood samples and 3.85% (Torkhow valley in Chitral) to 18.18% (Raushan valley in Gilgit) of liver samples, as shown in Table 3. *Dicrocoelium* positive samples were identified in each valley in the Chitral and Swat districts. No *Dicrocoelium* positive samples were detected in the Barjangle, Singul and Bolan valleys in the

Table 1 Presence of *Dicrocoelium* in sheep and goat herds during the study period 2018–2019

Host	Breed	Liver samples						Blood samples					
		Total number of flocks/herds examined	*Dicrocoelium* positive flocks/herds in Chitral	*Dicrocoelium* positive flocks/herds in Gilgit	*Dicrocoelium* positive flocks/herds in Swat	*Dicrocoelium* positive flocks/herds in Dir		Total number of flocks/herds examined	*Dicrocoelium* positive flocks/herds in Chitral	*Dicrocoelium* positive flocks/herds in Gilgit	*Dicrocoelium* positive flocks/herds in Swat	*Dicrocoelium* positive flocks/herds in Dir	
Sheep	Kelli	5	2	–	–	–		10	10	–	–	–	
	Ramghani	9	5	–	–	–		18	18	–	–	–	
	Balkhi	23	3	7	2	–		46	8	28	10	–	
	Waziri	10	–	1	7	0		20	–	2	14	0	
	Katchli	9	–	5	–	–		18	–	18	–	–	
Total		56	10	13	9	0		112	36	48	24	0	
Goat	Khurasani	13	7	5	–	0		26	14	6	–	2	
	Cross Beetal	4	1	0	0	0		8	4	2	0	2	
	Waziri	7	0	5	–	0		14	–	12	–	2	
Total		24	8	10	0	0		48	18	20	0	6	

Table 2 Prevalence of *Dicrocoelium* based on month, season, sex, age and host during the study period 2018–2019

Variables	Blood samples			Liver samples		
	Animals	Positive *n* (%)	*P* value	Animals	Positive *n* (%)	*P* value
Season						
Spring (March–April)	1020	30 (2.94)	$\chi 2 = 7.294$	29	2 (6.9)	$\chi 2 = 2.96$
Summer (May–Aug)	3070	116 (3.78)	$p = 0.063^{NS}$	147	16 (10.88)	$p = 0.398^{NS}$
Autumn (Sept–Oct)	1530	76 (4.97)		90	9 (10)	
Winter (Nov–Feb)	440	16 (3.64)		115	6 (5.22)	
Total	6060	238 (3.93)		381	33 (8.66)	
Sex						
Female	4296	212 (4.93)	$\chi 2 = 39.69$	222	22 (9.91)	$\chi 2 = 1.05$
Male	1764	26 (1.47)	$p = 0.0001^{**}$	159	11 (6.92)	$p = 0.306^{NS}$
Total	6060	238 (3.93)		381	33 (8.66)	
Age						
< 1 year	920	30 (3.26)	$\chi 2 = 5.718$	43	3 (6.98)	$\chi 2 = 4.73$
1–2 year	1984	66 (3.33)	$p = 0.05^{*}$	152	8 (5.26)	$p = 0.094^{NS}$
> 3 year	3156	142 (4.5)		186	22 (11.83)	
Total	6060	238 (3.93)		381	33 (8.66)	
Host						
Sheep	5113	168 (3.29)	$\chi 2 = 5536.3$	259	26 (10.04)	$\chi 2 = 349.7$
Goat	947	70 (7.39)	$p = 0.0001^{**}$	122	7 (5.74)	$p = 0.0001^{**}$
Total	6060	238 (3.93)		381	33 (8.66)	

Gilgit district; or in the Katair Dogdara and Maina Doag valleys of Dir district.

Spatial Patterns of *Dicrocoelium* Infection

The map based on the *Dicrocoelium* occurrence of positive samples predicted the most likely ecological niches to support *Dicrocoelium* infection to be in the central parts of Chitral, extending towards the upper and lower Chitral districts (Fig. 2). Although *Dicrocoelium* infection was identified from parts of Gilgit, and areas of Swat and Dir bordering Chitral, MaxEnt modelling predicted lower risk of *Dicrocoelium* occurrence in these overall study regions.

The MaxEnt model predicted that the two climatic variables of the mean diurnal temperature range (Bio2) and temperature seasonality (Bio4) contributed most to the occurrence of dicrocoeliosis in the Gilgit and lower and upper parts of the Chitral (Fig. 2a,b). However, annual precipitation (Bio 12) and distance to built-up areas were predicted to contribute most to the occurrence of dicrocoeliosis in upper Dir and Sawat districts (Fig. 2c,d); while summer NDVI values predicted *Dicrocoelium* active zones in the upper Dir and lower Chitral districts (Fig. 2e).

Contribution of Ecological Niches and Climatic Variables on *Dicrocoelium* Infection

The AUC values for the training and test data were 0.987 and 0.985, respectively, suggesting an excellent predictive power for the model (Supplementary Fig. S1). The results of jackknife analysis performed on five climatic and four geographical variables are shown in Supplementary Figure S2. Cross comparison of these nine variables in MaxEnt revealed that only four, namely annual precipitation (10.4%), mean diurnal range (mean of monthly max temp-min temp) (7.7%), distance from population built-up areas (9.1%) and vegetation index in spring (56.7%), were effective and would have contributed most to the model development. The six most influential variables observed in the present study are shown in Supplementary Fig. S3. The result shows that the occurrence of *Dicrocoelium* infection was directly related to the mean of the monthly diurnal temperature range (Bio2), temperature seasonality (Bio4), mean temperature of the coldest month (Bio6), distance from population built-up areas and summer NDVI. An inverse relationship was observed between annual precipitation (Bio12) and the identification of *Dicrocoelium* infection.

Discussion

In the present study, 381 liver samples and 6060 blood samples provide a valuable resource which can be used to describe aspects of the epidemiology of dicrocoeliosis in the Himalayan ranges of Pakistan. The estimated prevalence of dicroceliosis in sheep and goats in the Gilgit and Chitral districts was higher than reported incomparable Asian studies conducted in India [29], Iran [30], and Iraq [31]. While

Table 3 Prevalence of *Dicrocoelium* in different localities of Gilgit, Chitral, Swat and Dir districts

Locations		Blood samples			Liver samples		
District	Valley	Number of animals	Number positive (%)	*P* value	Number of animals	Number positive (%)	*P* value
Gilgit							
	Phander	100	7 (7)	$\chi 2 = 213.39$	18	1 (5.56)	$\chi 2 = 19.064$
	Dalomal	70	4 (5.71)	$p = 0.0001^{**}$	24	4 (16.67)	$p = 0.697^{NS}$
	Khonan Deh	70	5 (7.14)				
	Barsat	100	3 (3)				
	Yasin Valley	80	2 (2.5)		14	1 (7.14)	
	Damalgan	80	1 (1.25)		11	0	
	Sandhi	100	9 (9)				
	Raushan	95	7 (7.37)		11	2 (18.18)	
	Gahkuch	85	6 (7.06)				
	Barjangle	120	0				
	Singul	100	0				
	Rahim Abad	110	5 (4.55)				
	Chilmish Das	110	4 (3.64)				
	Danyor	100	4 (4)		7	0	
	Oshikhandas	80	3 (3.75)				
	Jaglot	150	2 (1.33)				
	Chalt Nagar	140	2 (1.43)		33	2 (6.06)	
	Chaprot	120	2 (1.67)				
	Hussain Abad	60	1 (1.67)				
	Rabat	50	1 (2)				
	Khizar Abad	140	2 (1.43)				
	Sikandar Abad	150	2 (1.33)				
	Jafar Abad	120	1 (0.83)				
	Harcho	90	1 (1.11)				
	Bunji	150	2 (1.33)				
	Doian	200	1 (0.5)				
	Gorikot	180	1 (0.56)		8	0	
	Bolan	70	0				
	Total	3020	78 (2.58)		126	10 (7.94)	
	Mean ± SEM	10.86 ± 6.82	2.79 ± 0.44 (3.25 ± 0.48)		15.75 ± 3.15	1.25 ± 0.49 (10.72 ± 2.18)	
Chitral							
	Booni	200	22 (11)		68	12 (17.65)	
	Mastuj	140	18 (12.86)		33	2 (6.06)	
	Chinar	110	3 (2.73)		19	2 (10.53)	
	Chuinj	110	3 (2.73)				
	Unshit	60	3 (5)		14	1 (7.14)	
	Shaidas	60	3 (5)				
	Gasht	80	4 (5)		7	1 (14.29)	
	Phargram	70	4 (5.71)				
	Phort	40	4 (10)				
	Lasht	40	3 (7.5)				
	Brock	50	5 (10)				
	Huzun	75	8 (10.67)				
	Balim	75	6 (8)				
	Raman	80	5 (6.25)				
	Harchin	80	6 (7.5)				

Table 3 (continued)

Locations		Blood samples			Liver samples		
District	Valley	Number of animals	Number positive (%)	*P* value	Number of animals	Number positive (%)	*P* value
	Sor Laspor	100	4 (4)		17	1 (5.88)	
	Mori	33	2 (6.06)				
	Mori Payeen	27	1 (3.7)		7	0	
	Kaghozi	80	4 (5)				
	Singoor	80	4 (5)				
	Rondur	34	4 (11.76)		5	0	
	Riri Qwir	26	2 (7.69)				
	Barenis	50	7 (14)				
	Pret	40	7 (17.5)				
	Kiyar	30	2 (6.67)				
	Drosh	88	3 (3.41)		4	0	
	Brun	82	4 (4.88)		8	1 (12.5)	
	Garam Chashma	60	3 (5)		6	0	
	Torkhow	140	8 (5.71)		26	1 (3.85)	
Total	2140	152 (7.1)		214	21 (9.81)		
Mean ± SEM	73.79 ± 7.3	5.24 ± 0.83 (7.25 ± 0.67)		17.83 ± 5.26	1.75 ± 0.95 (9.74 ± 1.39)		
Swat							
	Bankhwar	130	2 (1.54)				
	Gabral	110	2 (1.82)		11	1 (9.09)	
	Utrar	130	1 (0.77)		6	0	
	Kalam	90	1 (1.11)		15	0	
	Boyun	130	1 (0.77)		9	1 (11.11)	
	Matiltan	80	1 (1.25)				
	Total	670	8 (1.19)		41	2 (4.88)	
	Mean ± SEM	111.67 ± 9.1	1.33 ± 0.21 (1.21 ± 0.17)		10.25 ± 1.89	0.5 ± 0.29 (10.1 ± 0.71)	
Dir							
	Katair Dogdara	120	0				
	Maina Doag	110	0				
	Total	230	0		0	0	
	Mean ± SEM	115 ± 5	0		0	0	
Overall		6060	238 (3.93)		381	33 (8.66)	

direct comparisons are biased by differences in study design, the relatively high prevalence confirms the widespread nature of ecological niches that can support the continuity of the *Dicrocoelium* life cycle in the northwest of Pakistan. Characteristics including calcium-rich, alkaline soils and diverse vegetation help to provide overlapping niches that are suited to each of the intermediate and definitive hosts [4]. The prevalence of *Dicrocoelium* was highest during the summer and autumn, as previously described in Algerian cattle (Chougar, Harhoura [32], but the seasonal differences were not significant, and potentially may have been confounded by factors such as the age, species and breed of the animals and sampling location. The suitability of environmental factors for the development and growth of intermediate snails and ant hosts and grazing patterns enabling exposure to metacercaria-infected ants [4] will vary throughout the year. However, in the absence of effective anthelmintic treatments for dicrocoeliosis [5], animals accumulate infections acquired during different periods throughout their lives; consequently, a cross-sectional study involving animals more than 1-year-old cannot identify seasonal infection risks. Extreme cold weather conditions in the Himalayan ranges of Pakistan preclude grazing of animals on open pastures during the winter months and imply that the greatest risk of infection is during the spring and summer when conditions are also favourable for intermediate host development [33].

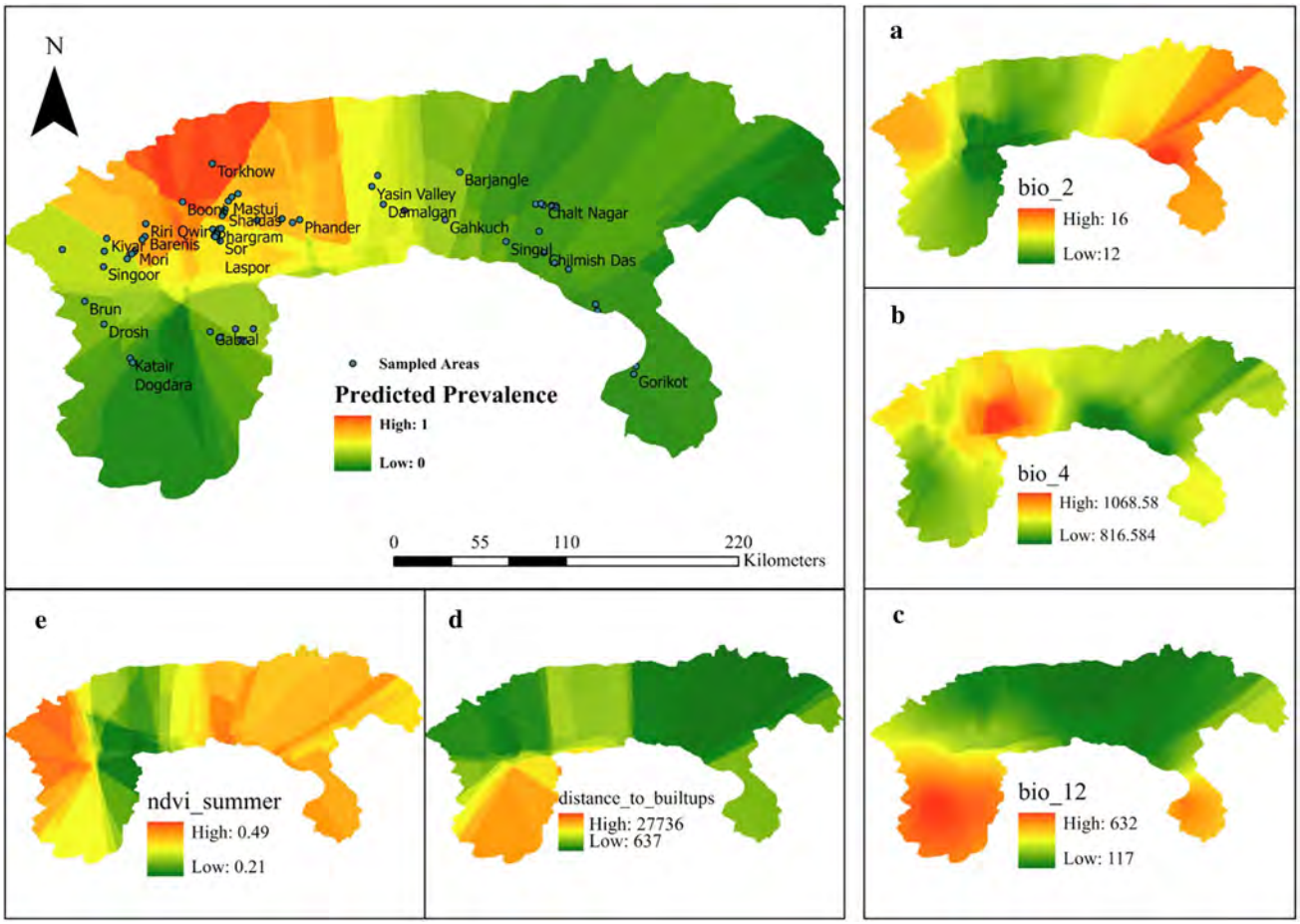


Fig. 2 Predicted spatial pattern based on blood and liver sample results of ecological niches predicted to support *Dicrocoelium* infection from 2018 to 2019. Red shading indicates the most suitable niches for Dicrocoelid flukes, and green shading predicts the least suitable conditions. The MaxEnt model predictions for the contributions of variables to the occurrence of dicrocoeliosis are shown in **a** (mean monthly diurnal temperature range), **b** (temperature seasonality), **c** (annual precipitation), **d** (distance from built-up areas) and **e** (normalised difference vegetation index)

The estimated prevalence of *Dicrocoelium* was higher in female hosts and highest in animals aged more than 3 years. Previous reports have shown higher prevalences in female hosts [32, 34] and suggested a relationship between periparturient susceptibility due to pregnancy and lactation stress [35]. However, female animals are more likely to be retained for breeding, hence live for longer and have more opportunities to become infected with *Dicrocoelium*. The specie prevalence could be explained by the possibility of higher susceptibility of sheep than goats. Higher prevalence and worm burden in sheep could be the result of more sensitive species, but goats have contact "infection" with *Dicrocoelium*, but this does not go advance. This could explain the higher prevalence of antibodies, but not found in adults. The different results further highlight challenges of sample size and diagnosis of adults could be less sensitive, with a high number of false negatives in goats than in sheep. It has been suggested that browsing goats are less likely to be infected than grazing sheep [34], albeit *Dicrocoelium*-infected ants may migrate high enough onto herbage to be ingested by browsing animals. However, the ecological information on ants and land snails involved as intermediate hosts in these areas is still unknown.

The highest occurrence of *Dicrocoelium* infection was recorded in the Chitral district, consistent with its high altitude pastureland fed by melting of glacier water and high seasonal rainfall providing the most suitable conditions for completion of the parasite's life history. A similar situation has been described in Spain, where *Dicrocoelium* infection is most frequent in areas with high altitudes, lower winter temperatures and high rainfall [36]. The occurrence of *Dicrocoelium* infection in the Gilgit, Swat and Dir districts was moderate to low associated with lower rainfall and more humid environments.

Prediction of the environmental suitability and geographical distribution of ecological niches, climatic and

anthropomorphic factors that are suited to the completion of the *Dicrocoelium* life cycle is needed to inform strategic disease control. SDMs have been used to predict the special distribution of *Dicrocoelium* infection in Iran [19] and Spain [36]. The ROC test showed a high validity of the SDM in predicting favourable ecological niches for these parasites in the Himalayan ranges of Pakistan. The MaxEnt model revealed that the most influential climatic variables associated with a positive effect on the risk of dicrocoeliosis were the mean of the monthly diurnal temperature range (Bio2), temperature seasonality (Bio4) and the mean temperature of the coldest month (Bio6); while an inverse relationship was observed for annual precipitation (Bio12). The results suggest that these factors play a key role in the development, survival and transmission of Dicrocoelid flukes and their intermediate hosts. The results also found a high correlation between distance from population built-up areas and summer NDVI and the presence of *Dicrocoelium* infection, explained by the observation that forest areas with permanent pastures, good water availability and suitable soil type provide suitable habitats for land snails and ant intermediate hosts, and opportunities for final host infection [37].

Overall this study shows a high estimated prevalence of dicrocoeliosis in the Himalayan ranges of Pakistan. The ecological niche model helps to describe factors that increase the risk of infection, providing information that might help in the development of targeted evasive management strategies and in predicting the potential spread of *Dicrocoelium* to other suitable habitats in the region.

Conclusion

In the present study, the diagnosis of dicrocoeliosis was based both on the identification of Dicrocoelid flukes in the livers of slaughtered animals and on positive blood sample results using a bespoke combination of ES and somatic antigen ELISAs. The random sampling methods that were used to collect the diagnostic samples helped describe the spatial distribution of *Dicrocoelium* infection and provided a crude estimation of the parasite's prevalence. However, the fold difference in overall prevalence estimates obtained from the liver (~9%) and blood (~4%) sample results highlight important difficulties in the accurate determination of the prevalence of fluke parasites; namely the adequacy of the sample size, precise knowledge of the sensitivities and specificities of the diagnostic tests used, and the representativeness of the study populations. In the current study, the blood sample size was adequate, but the number of liver samples was too low to allow for precise analysis; the true sensitivities and specificities of the diagnostic tests were unknown; and the live and slaughtered animal populations may have differed in their origins, grazing management, and are known to differ in demographic characteristics such as sex, age, species and breed. The number of samples that could be collected and processed was constrained by the remoteness and poor supporting infrastructure of the study region. Nevertheless, the 381 liver samples and 6060 blood samples provide a valuable resource which can be used to describe aspects of the epidemiology of dicrocoeliosis in the Himalayan ranges of Pakistan. In the absence of a gold standard, the accurate determination of the sensitivities and specificities of diagnostic tests for the study of fluke parasite epidemiology is challenging [38], and requires different samples to be collected from the same animals in a manner which was not feasible in the current study.

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Author Contributions Conceptualization: KA, UC; sample collection: MAK, SF, KA and methodology: KA, SF, UC, NDS; formal analysis: KA, UC, MAK, MB; data curation: KA and MAK, UC, NDS; writing—original draft preparation: KA, MAK, UC, MB, NDS; writing—review and editing: U.C. NDS, MB. All authors read and approved the final manuscript.

Availability of Data and Material Maxent software is freely downloadable at http://www.cs.princeton.edu/~schapire/maxent/. The data files used to calculate the model are available on request.

Declarations

Conflicts of Interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics Declarations The collection of flukes was from the animals slaughtered for other purposes at local abattoirs to meet the high protein demand of the population. The study was approved by the Animal Ethics Committee of the Quaid-i-Azam University, Islamabad.

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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 blood vessels, 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. This study concluded *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. The results will aid in the development of effective disease control strategies to ensure optimal growth and productivity of sheep and goats.

1. 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 and Traversa (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, 1952b). Eggs are shed in the bile ducts of the definitive host, then excreted in faeces. The most common snail intermediate hosts include *Helicella itala*, Cochlicella *acuta, Cochlicella aspersum, Coinella lubrica, Vitrina pellucida*, *Lauria cylindracea* and *Oxychilus* spp. (Tarry, 1969). 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 gut. 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 the intestine and common bile duct to the biliary tree of the liver, where they develop to monoecious adults, feed on blood, and shed eggs (Sargison et al., 2012). While it is unknown how long adults live, the lifetime of adults can also be affected by the host's longevity (Roberts and Janovy, 2008).

In Iran, dicrocoeliosis affects agricultural productivity, due to

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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 and Traversa (2003); Colwell and Goater, 2010, Rojo-Vázquez et al., (2012)). Histopathological changes in experimentally infected lambs were characterised by periductal fibrosis, ductal response, and leukocyte infiltration (Ferreras-Estrada 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-Estrada 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 using an indirect enzyme-linked immunosorbent assay (ELISA) 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 with fasciolosis(Paz et al., 1998; Sanchez-Andrade et al., 2003, 2001), a positive ELISA test result can also indicate past parasite exposure without a present infection.

In Pakistan, livestock accounts for almost 14.0 % of Pakistan's GDP and more than 61.9 % of agricultural value added (Pakistan Economic Survey, 2021–2022). Economic losses in the country have been recorded by a number of parasitic infections, including toxoplasmosis (Rafique et al., 2022), theileriosis (Zaman et al., 2022), trypanosomiosis (Yasein et al., 2022), echinococcosis (Mahmood et al., 2022; Alvi et al., 2022), haemonchosis (Qamar et al., 2022; Bibi et al., 2017), fasciolosis (Afshan et al., 2021; Mufti et al., 2015) and dicrocoeliosis (Khan et al., 2021). Many researchers focused on use of nanoparticles, synthetic and biological compounds for the control of parasitic infection (Rehman et al., (2020); Nawaz et al., 2022; Kandeel et al., 2022).

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.

2. Materials and methods

2.1. 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.

Table 1
Sampling sites and number of samples collected from lambs and goat kids.

Areas	Longitudes	Latitudes	No of lamb sample		No of goats sample	
			2018	2019	2018	2019
Booni	72.25408342	36.27065815	17	20	18	15
Mastuj	72.51646335	36.27580273	16	15	15	15
Sor Laspor	72.47027663	36.04724606	15	17	16	15
Chalt Nagar	74.3230176	36.25213215	18	15	14	14
Torkhow	72.42492036	36.49056069	15	16	14	16

2.2. 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.

2.3. Histopathological examination of infected liver

Histology of infected sheep (n = 25) liver tissues was carried out. 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 h 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.

2.4. 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 h 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 ScientificTM 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.

2.5. 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.

3. Results

3.1. 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 (Figs. 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 (Figs. 3 and 4). For a detailed presentation of the serological data for individual animals, see supplementary Tables 2 to 11.

3.2. Gross liver pathology and histology

Large numbers of flukes were found in the bile ducts of twenty-five 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.

4. 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,

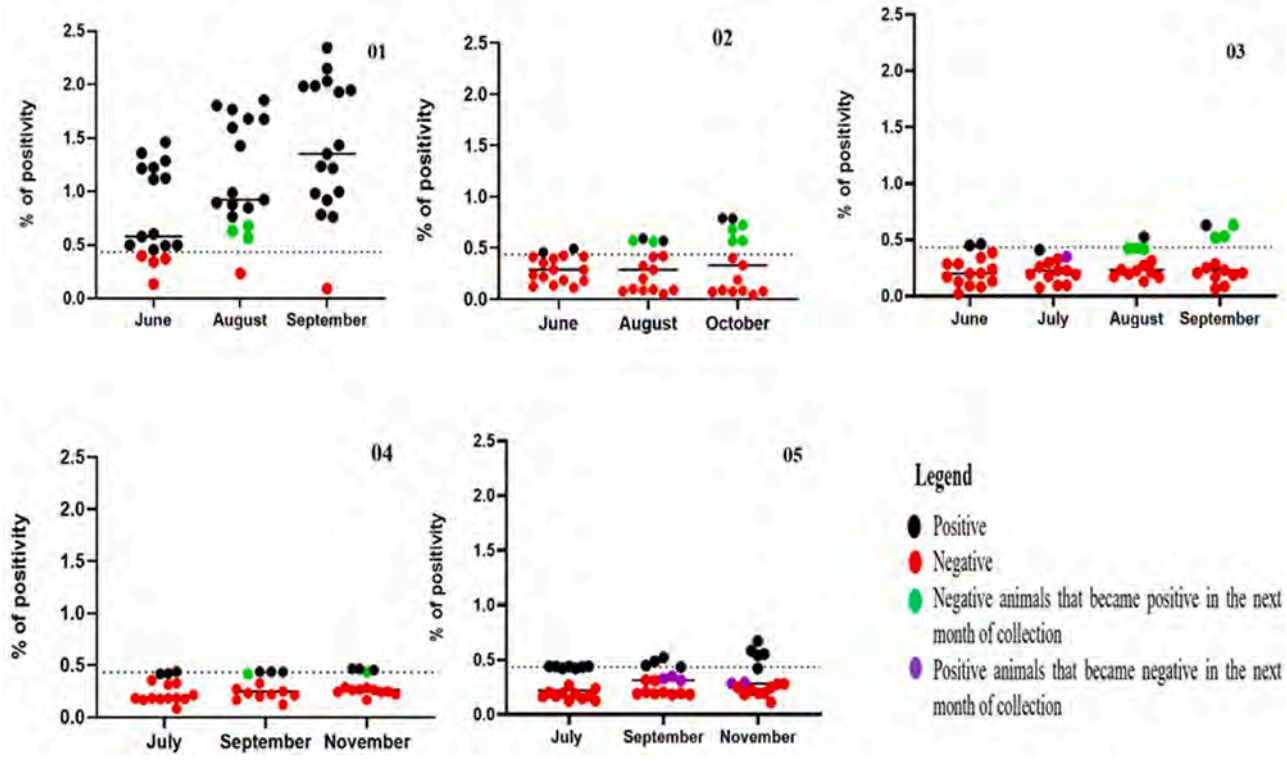

Fig. 1. Dynamics of *Dicrocoelium* antibodies in lambs on sheep flocks 01, 02 03, 04, and 05 during 2018. The dashed line is the cut-off limit (cut-off = 0.434 % of positivity).

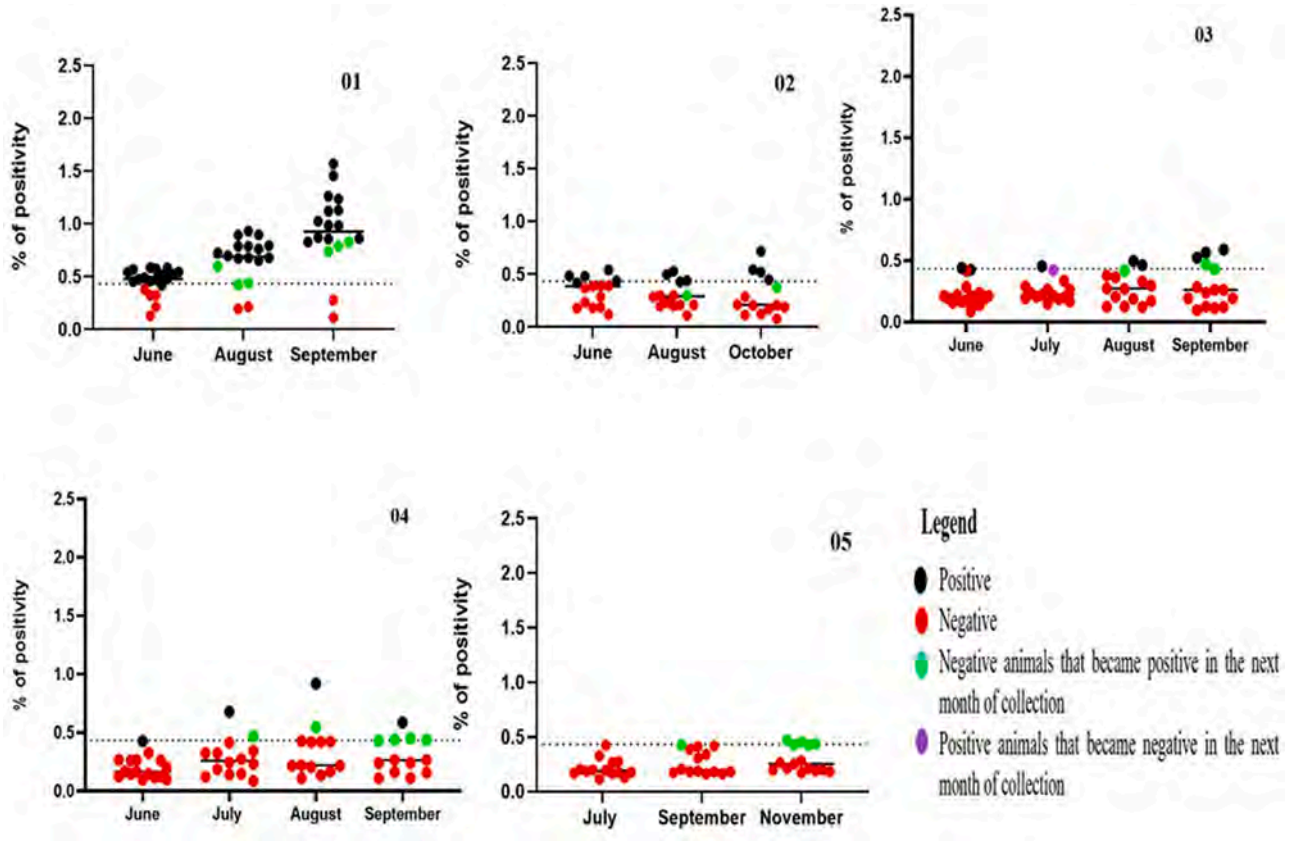

Fig. 2. Dynamics of *Dicrocoelium* antibodies in lambs on sheep flocks 01, 02 03, 04, and 05 during 2019. The dashed line is the cut-off limit (cut-off = 0.434 % of positivity).

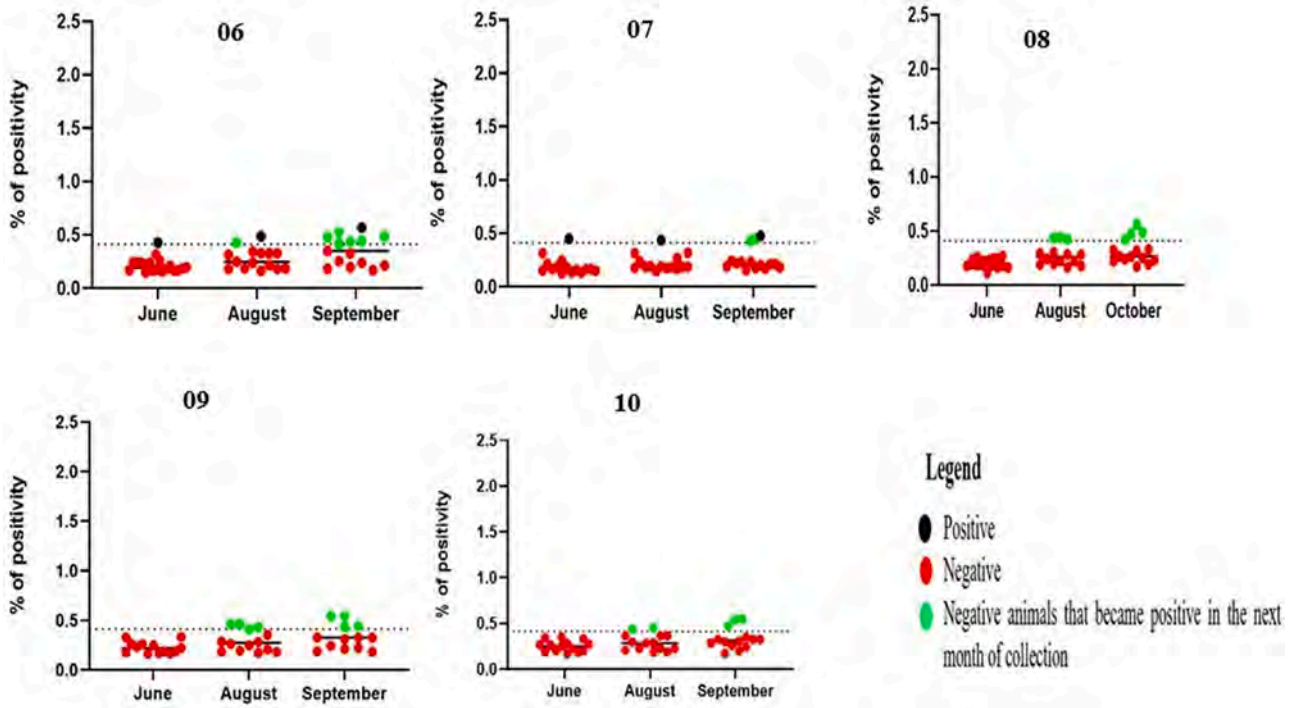

Fig. 3. Dynamics of *Dicrocoelium* antibodies in kids on goat herds 06, 07, 08, 09and 10 during 2018. The dashed line is the cut-off limit (cut-off = 0.411 % of positivity).

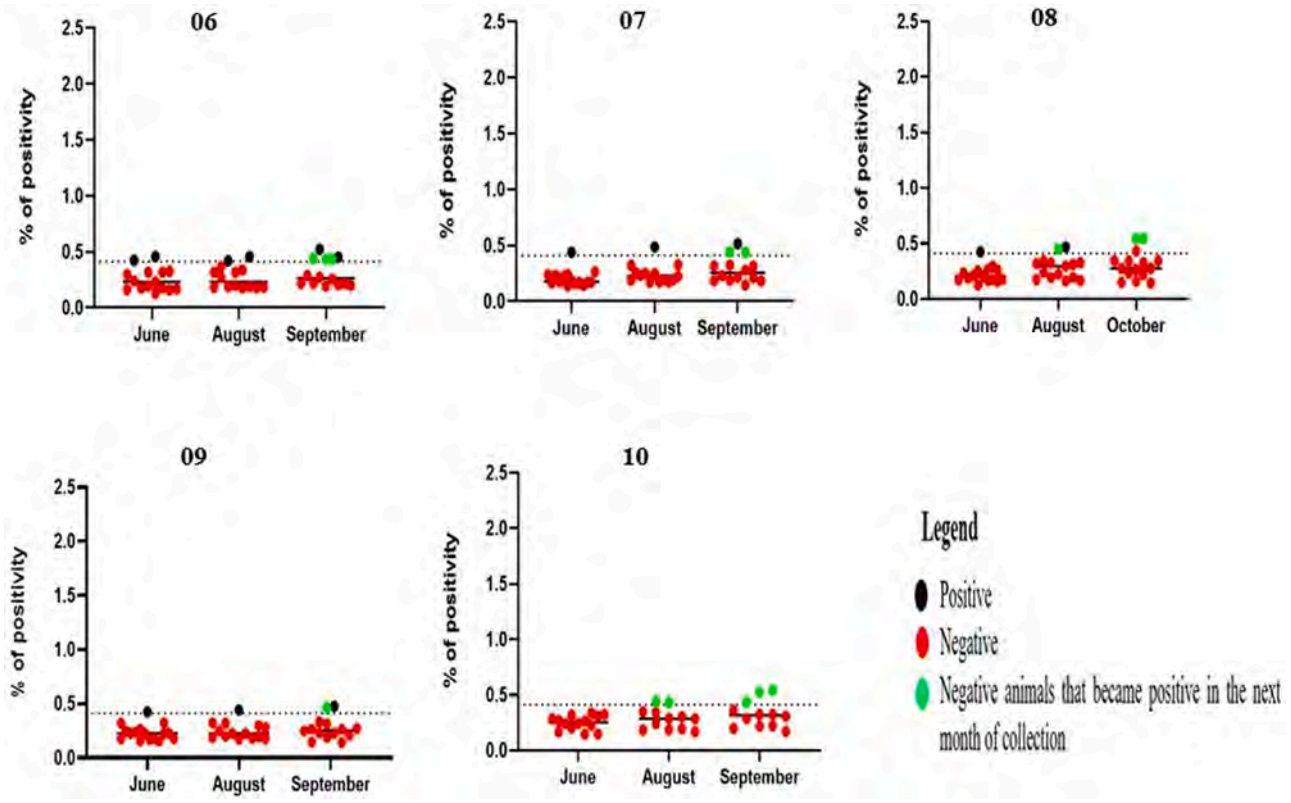

Fig. 4. Dynamics of *Dicrocoelium* antibodies in kids on goat herds 06, 07 08, 09, and 10 during 2019. The dashed line is the cut-off limit (cut-off = 0.411 % of positivity).

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 (Chngizi et al., (1998); Manga-Gonzalez et al., 2004; Samadieh et al., 2017; Sato et al., 2019 and Nelwan (2019); Ebrahim 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 may detect antibodies against *D. dendriticum* infected lambs 30 days after infection, which is at least one month before coprological analysis to identify eggs produces a positive result (Gonzalez-Lanza et al., 2000; Manga-González and González-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-González and González-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. Novobilský 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; Novobilský 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 Bihaqi 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

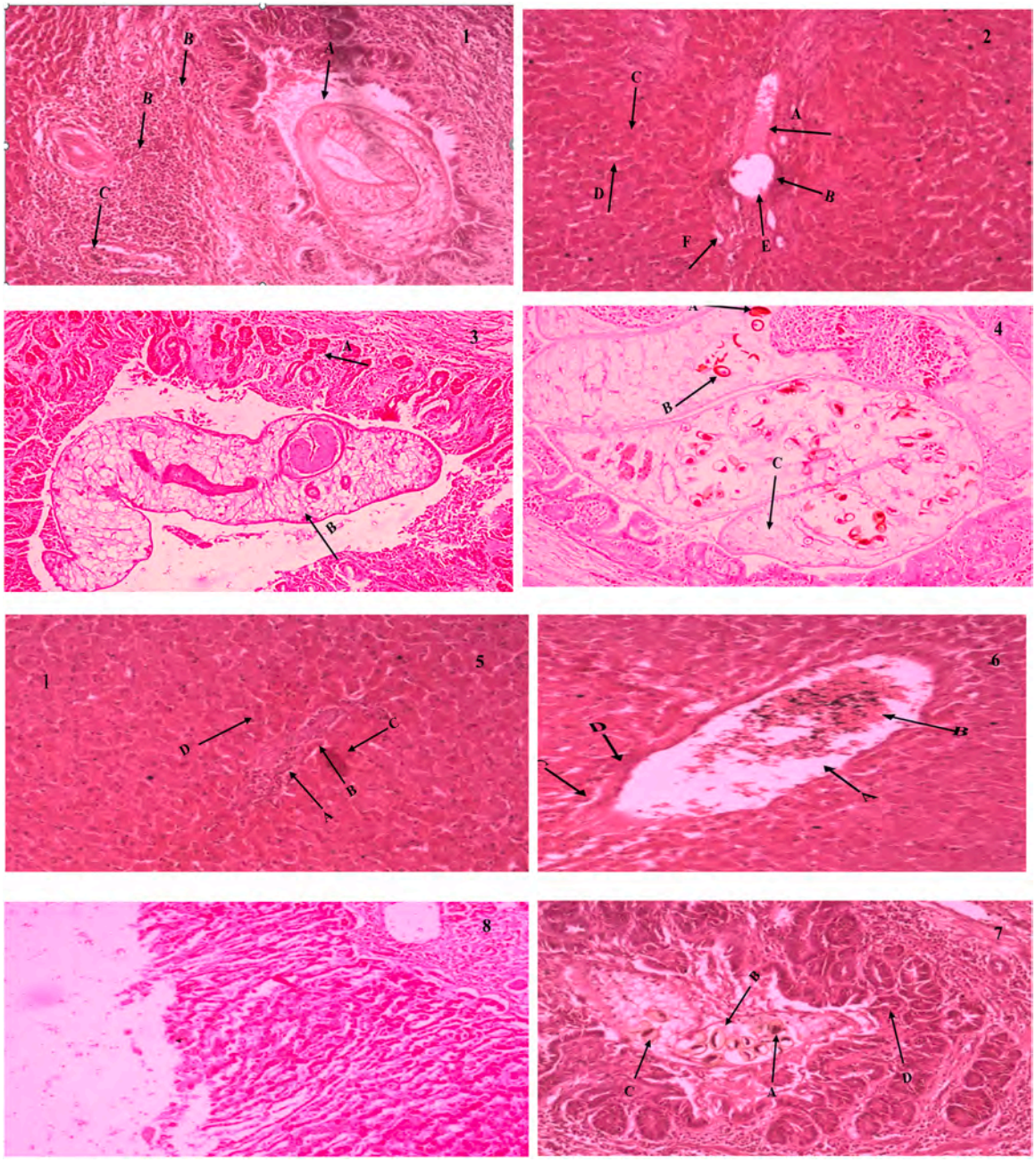

Fig. 5. Histological sections of sheep livers infected with *Dicrocoelium* spp. (1) shows cross-section of a parasite sucker in the lining epithelial cells of a septal bile duct (A), RBCs congestion (B) severe infiltration of inflammatory cells (C). (2) Shows congestion in blood vessels at the portal 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), Infiltration 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.

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).

5. 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.

CRediT authorship contribution statement

Khan Muhammad Asim: Writing – original draft, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Sargison Neil D.:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Firasat Sabika:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Chaudhry Umer:** Writing – review & editing, Visualization, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation. **Afshan Kiran:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare no personal or financial conflicts of interest related to this work.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2023.109975.

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