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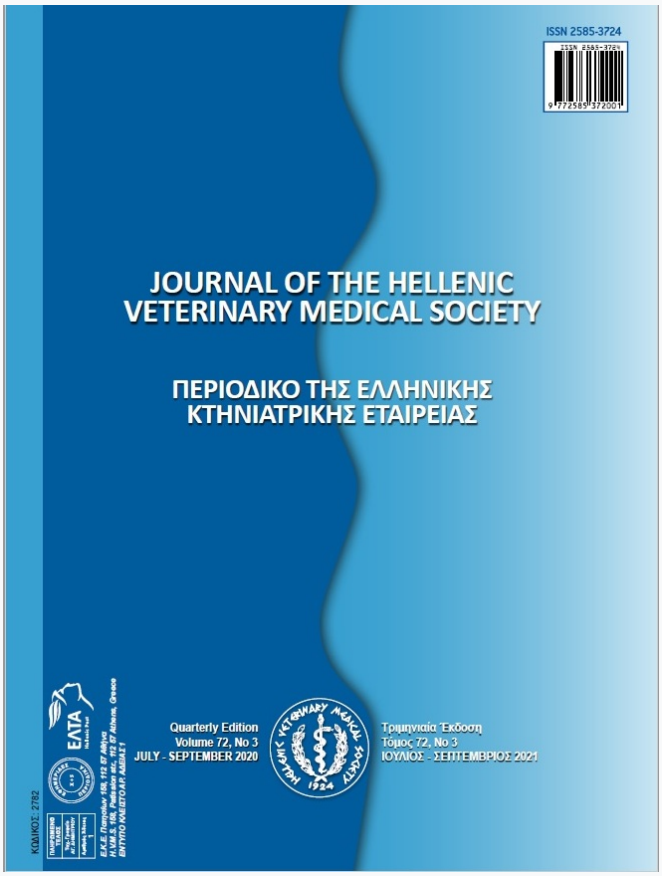

Serological and immuno-histopathological detection of Paramphistomum epiclitum infection in large ruminant population in Punjab, Pakistan

I KHAN, K AFSHAN, R ULLAH, M KOMAL, MA KHAN, S FIRASAT

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Serological and immuno-histopathological detection of *Paramphistomum epiclitum* infection in large ruminant population in Punjab, Pakistan

I. Khan, K. Afshan*, R. Ullah, M. Komal, M. A. Khan, S. Firasat

Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, Pakistan

ABSTRACT: Recent molecular identification of *Paramphistomum epiclitum* in Pakistan raises concerns about its epidemiology and pathologies in infected tissues of ruminants. The present study aimed to find the seroepidemiological and histopathological record of *Paramphistomum epiclitum* from cattle and buffaloes. Indirect ELISA on animal sera and histology of infected rumen with hematological and biochemical analyses were performed. The overall prevalence of *P. epiclitum* was noted as 15.3% in the abattoir survey and 37.6% in the serological examination. The sensitivity and specificity of the diagnostic test were 100% and 83.3% respectively. The paramphistomosis was significantly (p= 0.001) higher during August (6.4%) followed by September (5.4%), whereas the lowest prevalence was recorded during April (0.4%). The hematological and biochemical variations showed significant increase in total leukocyte count (p= 0.002), alanine aminotransferase (p= 0.05), glucose (p= 0.01) and cholesterol (p = 0.024) levels. However, significant decrease in the level of erythrocyte (p= 0.019), hemoglobin (p= 0.001), mean corpuscular hemoglobin concentration (p= 0.05), mean corpuscular volume (p= 0.038) and platelets count (p= 0.048) was observed. The histopathology of rumen tissue showed haemorrhages, atrophy of ruminal papillae, sloughed mucosa, cellular vacuolation, and infiltration of lymphocytes inflammatory cells. The present study provides the prevalence and histopathological record of *P. epiclitum* in Pakistan for the first time in order to take control measures in the country.

Keywords: Indirect ELISA; histopathology; prevalence; *P. epiclitum*; Pakistan

Corresponding Author:
K. Afshan, Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, Pakistan
E-mail address: kafshan@qau.edu.pk

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INTRODUCTION

Paramphistomum digenean trematodes belonged to Paramphistomatidae family are the most prominent parasites, infecting rumen, and reticulum of livestock (Mogdy *et al.,* 2009). *Paramphistomum epiclitum* is one of the most important gastrointestinal trematodes that cause infection in ruminants with a diverse range of geographical distribution (Singh *et al.,* 2012). In the life cycle of paramphistomes, mammals and snails are the definitive and intermediate hosts respectively. Passive ingestion of metacercariae by mammals causes infection. Immature development of the fluke occurs in the small intestine while later development up to the adult stage occurs in the rumen. Paramphistomosis caused by these flatworms is responsible for heavy economic losses to several thousand crore rupees annually to the livestock industry (Ozdal *et al.,* 2010). The highest numbers accounted for in tropical and sub-tropical regions of the world (Ozdal *et al.,* 2010).

Paramphistomosis causes fatal diarrhea, weakness, dehydration, enteritis, low milk yield, sub-maxillary edema of the clinically ill hosts, and deaths (Bianchin *et al.,* 2007). The immature flukes penetrate the duodenal mucosa causes severe damage and increased mortality rate (Millar *et al.,* 2012). In the small intestine, the juvenile *Paramphistomum* causes erosions, petechiae, necrotic areas, mucus, and eosinophilic infiltrate. Due to intestinal discomfort loss of appetites occurs and some animals showed anorexia. Other symptoms are the loss of albumen, in severe cases, hypoalbuminemia occurs with oedema in several body parts. The clinical signs are mostly related to juvenile helminths; however, after two weeks of post-infection, the adults also cause problems. Mature paramphistomes are associated with ruminal papillae atrophy and ulceration at the point of attachment (Fuertes *et al.,* 2015). Heavy adult infestation rate is characterized by anemia, leucopenia, diarrhea, loss of appetite, loss of body weight, and malabsorption (Rolfe and Boray, 1993).

Immature stages of flukes are incapable to lay eggs and cause severe pathogenicity (Magdy *et al.,* 2009), while adult release eggs with faeces and does not harm the animals severely (Kelly and Henderson, 1973). Early detection methods are required for its treatment and control before irreversible damage occurs (Wang *et al.,* 2006). Immunodetection with the standard parasitological technique could play a dependable means for monitoring the infection (Anuracpreeda *et al.,* 2008).

In Pakistan prevalence of paramphistomes were 75.07% and 50.7% in buffaloes and cattle, respectively (Cheema *et al.,* 1997). These highest incidences may occur due to the canal system extensions in the country, where buffaloes and cattle were exposed to infective larvae and metacercariae of helminths (Misra *et al.,* 1997). Recently, we reported the genome and morphology of this parasite for the first time in Pakistan (Khan *et al.,* 2020). To the best of our knowledge, no study has previously been conducted on the seroepidemiology and histopathology of *Paramphistomum epiclitum* in the country. Therefore, the present study was aimed to find the epidemiological and histopathological records of *P. epiclitum* in Pakistan to reduce the impact of disease in livestock.

MATERIALS AND METHODS

Study area

This study was carried out from September 2017 to February 2019, which comprised of local abattoir of Pothwar region in north-eastern Pakistan, forming the northern part of Punjab Province (Figure. 1). This area has a semi-arid and sub-humid climate with four different seasons namely hot (April-Jun), cold (December-March), monsoon (July-September), and post-monsoon (October-November).

Collection of adult worms

Adult worms were collected from the rumen of slaughtered cattle and buffaloes. A total of 215 slaughtered animals were thoroughly examined for the collection of adult *P. epiclitum*. The worms were identified with morphological and molecular analysis (part of other reported study by Khan *et al.* (2020). The detail of animals including host type, sex, age, breed, body condition and month of infection were recorded. Worms were collected from rumen with the help of forceps to avoid any type of damage to the parasite. Collected parasites were washed several times with 0.01M phosphate buffer saline (PBS- pH-7.2) and immediately transferred to the laboratory.

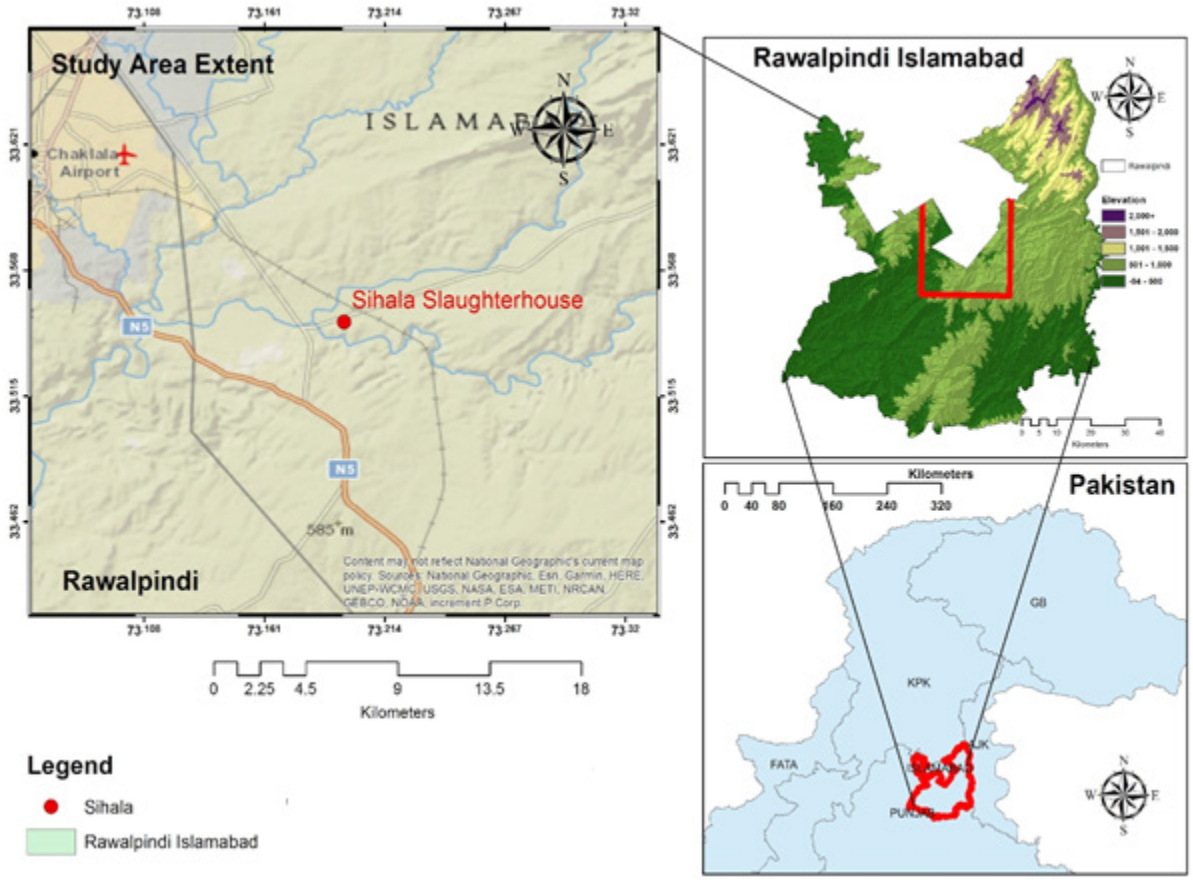

Figure 1: Study area indicating location of slaughterhouse in Rawalpindi district of Punjab Province, Pakistan.

Preparation of somatic antigen

For the extraction of somatic antigen, the parasite (whole worm) was homogenized via tissue grinder in tissue lysis buffer (50 mM HEPES- Free Acid (Roth), 150 mM Sodium Chloride (Sigma), 0.02% Sodium Azide (Sigma, 0.1% SDS (Reidel de hein), 1% Triton X-100, 0.1 mg/ml PMSF. According to the weight of the parasite, approximately 10 µL/mg of lysis buffer was added. Then the homogenate was centrifuged in an Eppendorf tube at 10000 rpm for 10 min at 4 ºC. The supernatant was taken, and the pellet was discarded. Bradford's method (Bradford, 1979) was applied to determine protein concentration and was stored at - 20 ºC until used.

Blood sample collection

A total of 500 blood samples were randomly collected from farm animals to find out the seroepidemiology of paramphistomosis in cattle and buffaloes. The sera were separated and stored at - 20 ºC until used. For establishment of in-house ELISA, control sera were collected from confirmed positive animal with *P. epiclitum* (n= 27), *Fasciola hepatica* (n= 10), *Gigantocotyle explanatum* (n= 10) and from negative control (n= 30) obtained from 2-week-old kids born to herd having history of stall feeding.

Indirect ELISA

ELISA was performed on a polystyrene microtiter plate having 96 wells with previously determined all incubation time by checkerboard titration and followed the method described by Faghemi *et al.* (1997) and Ferre *et al.(*1997). Briefly, the antigen was diluted in coating buffer in (1:1), coated (200 µl) in each well of a microtiter plate, and kept at 4 ºC overnight. The next day, the plate was washed 3 times with 0.01 M PBS Tween-20. The plate was filled with (200 µL) blocking solution (10 ml PBS+ T-20 (0.02% + 0.2g BSA (2%) and incubated at room temperature for 2 hours. Then the plate was washed 3 times with 0.01M PBS, Tween-20. Each well of microtiter plate was treated with 100 µL of diluted primary antibodies (infected bovine serum) and incubated for 2 hours at room temperature. Again, the washing was done 3

times followed by the addition of 100 µL secondary antibodies (Goat Anti-Bovine IgG (H+L) Antibodies) diluted 1:5000 to each well, incubated for 120 minutes at room temperature. Then the plate was washed 3 times with 0.01M PBS Tween-20 and to each well, 100 µL of PNP (para-Nitrophenyle Phosphate) substrate was added and incubated at room temperature for 15-30 minutes in dark. The reaction was stopped by adding 50 µL stopping solution (2M H_2SO_4) to each well. Results were analyzed by ELISA reader at OD value 405 nm.

Hematological and Biochemical Analysis

The blood (2 ml) was directly taken from the external jugular vein of cattle and buffaloes through a syringe in EDTA coated vacutainers and stored at 4 ºC till analyzed through an auto hematology analyzer (URIT - 2900Vet Plus, China). The complete blood profile i.e. red blood cells count (RBCs), total leucocytes count (TLCs), platelets count (PLT), hemoglobin concentration (HGB), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV) and hematocrit (HCT) level was measured. For biochemical analysis, sera were analyzed for liver function tests, total cholesterol, and triglycerides concentrations through a semi-auto-chemistry analyzer. The activities of alanine aminotransferase (ALT) and glucose were measured at 37 ºC and the results were presented in respective units.

Histological Studies

A portion of rumen tissues from infected animals was separated and preserved in 10% (w/v) formaldehyde at room temperature for histological study. For histopathology, preserved rumen tissues were fixed overnight in 10% buffered formalin, dehydrated in different alcoholic grades and then placed in xylene with two changes and transferred to molten paraffin wax preheated to 59 ºC and kept overnight. Second wax infiltration was done, and tissues were finally embedded in paraffin wax. After removing bubbles from wax, solidify, trimmed and mounted on wooden blocks for section cutting. Albumin coated slides were prepared and stained with haematoxylin and eosin (H&E) following standard histopathological procedures (Stevens, 1996). Sections were photographed on a compound photomicroscope (Leica, Germany).

Statistical Analysis

The data on the prevalence of *P. epiclitum* infected cattle and buffaloes with respect to age, sex, breed, and months were calculated by using the chi-square test. The hematological and biochemical data were analysed by t-test and presented with mean ± S.E values. The level of significance was set at $p < 0.05$. The O.D value of the serum sample was observed at 405 nm and compared with the cut-off value. The cut-off was calculated by the mean optical density (OD) of the negative reference serum, plus three times standard deviations (0.11+3*0.01=0.14). The cut-off value was set at 0.14, and sera with OD value higher or equal to 0.14 consider as positive. The sensitivity (a / a + b) and specificity (d / c + d) were calculated, where (a) is the number of cases in the diseased group that test positive and negative (*b*); and the number of cases in the non-diseased group that test positive (*c*) and negative (*d*).

RESULTS

Prevalence of *P. epiclitum*

The sensitivity and specificity of the diagnostic test for *P. epiclitum* were 100% and 83.3%, respectively. The kappa value calculated for the test was 0.81 and revealed that the strength of agreement was 'perfect' (Table 1). The value of R^2 calculated from the linear regression equation was 0.96 and the protein concentration of somatic antigens was determined (Figure. 2). The OD value of control sera used in the validation of ELISA is given in Figure 3.

The overall prevalence of *P. epiclitum* was noted as 15.3% in the abattoir survey and 37.6% in serological examination (Table 2). In the abattoir survey, the highest rate of infection was found 12.6% in females, while in the seroprevalence method male had a higher infection (24.6%), while statistically non-significant ($p > 0.05$). Higher prevalence of infection was noted 6% in 5-7 year of the old age group in the abattoir survey, and the serologically highest rate of infection was 13.6% in 8-10 years age group, while statistically not significant ($p > 0.05$). The animals with semi-extensive grazing habit showed a 7% infection rate in the abattoir survey, while ELISA was 15.6% in animals with intensive grazing habit. However, the difference was not significantly ($p > 0.05$) associated with the infection. The abattoir survey revealed a significantly ($p = 0.001$) higher infection rate of 11.2% in animals with poor body condition as compared to healthy animals. Similarly, a higher seroprevalence rate was noted 22.2% in poor body condition animals, while not significantly ($p > 0.05$) different. Among

breeds paramphistomosis based on abattoir survey was 5.6% in Kundhi fallowed by Nili Ravi 4.7%, Azi Kheli 2.8% breed of buffaloes, while 1.4% in Sahiwal breed of cattle. The seroprevalence of *P. epiclitum* was 11.2% in Kundhi and 7.2% in Sahiwal breed of buffalo and cattle respectively. However, the breed did not show significant ($p > 0.05$) association with disease. The infection was found higher in buffaloes (11.6%, 25.8%) as compared to cattle (3.7%, 11.8%) with abattoir and serological survey, respectively; however, the difference was not significant ($p > 0.05$). The animals having contact with pond water showed a high prevalence (6%, 12.6%), but the association was not significant ($p > 0.05$). The paramphistomosis was significantly ($p = 0.001$) higher during August (6.4%) followed by September (5.4%), whereas the lowest prevalence was recorded during April at 0.4%.

Table 1: Validation parameters of indirect ELISA developed for identification of specific antibodies against *Paramphistomum epiclitum.* Isolation of adult flukes from rumen of slaughtered animals was considered as gold standard.

Parameters	Results	Kappa correlation index
Number of tested sera	500	
Number of truly positive sera	27	Kappa = 0.811 almost perfect agreement
Number of truly negative sera	30	SE of kappa = 0.072
Number of false-positive	6	95% confidence interval:
Number of false negatives	0	From 0.669 to 0.952
Cut-off	0.14	
Sensitivity (%)	100	
Specificity (%)	83.3	
*Accuracy (%)	89.6	
*Positive Predictive Value (%)	52.01	
*Negative Predictive Value (%)	100	
*Disease prevalence (%)	15.3	

*These values are dependent on disease prevalence.
Positive predictive value = Sensitivity x Prevalence / Sensitivity x Prevalence + (1- Specificity) x (1- Prevalence)
Negative predictive value = Specificity x (1 − Prevalence)/ (1-Sensitivity) x Prevalence + Specificity x (1- Prevalence)
Accuracy = Sensitivity × Prevalence + Specificity × (1 − Prevalence)+

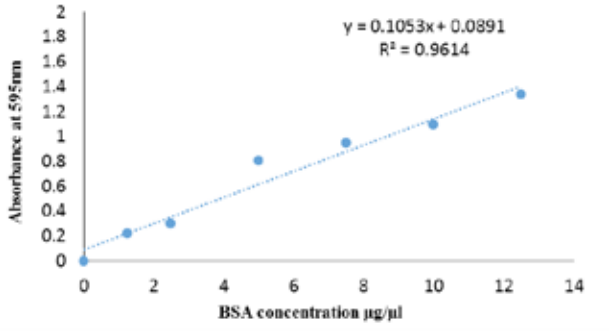

Figure 2: Standard protein curve to determine the protein concentration of antigen used in establishment of indirect ELISA.

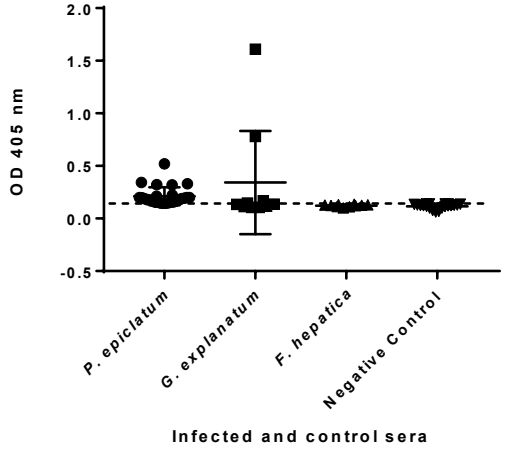

Figure 3: The optical density values (OD) of positive and control sera for identification of specific antibodies against *Paramphistomum epiclitum.* The cut-off value was set at 0.14.

Table 2: Prevalence of *Paramphistomum epiclitum* based on postmortem examination and indirect ELISA in Pakistan.

	Abattoir Survey				Seroprevalence				
Variable	**No. examined**	**Positive n (%)**	**χ^2**	**p-value**	**No. examined**	**Positive n (%)**	**OD Mean ± SD (Min-Max)**	**χ^2**	**p-value**
Gender									
Male	60	6(2.8)	1.83	0.176	315	123(24.6)	0.24±0.14 (0.14-0.72)	0.76	0.383
Female	155	27(12.6)			185	65(13.0)	0.23±0.13 (0.14-0.80)		
Age									
2-4 year	33	3(1.4)	2.93	0.403	84	36(7.2)	0.23±0.13 (0.14-0.58)	1.44	0.696
5-7 year	67	13(6.0)			115	44(8.8)	0.23±0.14 (0.14-0.80)		
8-10 year	80	10(4.7)			187	68(13.6)	0.22±0.13(0.14-0.72)		
11-12 year	35	7(3.3)			114	40(8.0)	0.24±0.15 (0.14-0.60)		
Grazing Habitat									
Intensive	70	11(5.1)	5.54	0.063	202	78(15.6)	0.22±0.12 (0.14-0.58)	0.34	0.846
Extensive	22	7(3.3)			162	58(11.6)	0.26±0.17 (0.14-0.80)		
Semi-Extensive	123	15(7.0)			136	52(10.4)	0.21±0.11 (0.14-0.58)		
Body Condition									
Poor	65	24(11.2)	33.38	0.001**	291	111(22.2)	0.21±0.11 (0.14-0.59)	0.09	0.767
Good	150	9(4.2)			209	77(15.4)	0.25±0.16 (0.14-0.80)		
Breed									
Kundhi	70	12(5.6)	5.37	0.251	153	56(11.2)	0.23±0.12 (0.14-0.58)	3.66	0.453
Azi Kheli	66	6(2.8)			112	47(9.4)			
Nili Ravi	40	10(4.7)			69	30(6.0)			
Sahiwal	20	3(1.4)			104	36(7.2)			
Dhani	19	2(0.9)			62	19(3.8)			
Host Type									
Buffalo	148	25(11.6)	0.78	0.351	331	129(25.8)			
Cattle	67	8(3.7)			169	59(11.8)			
Water Bodies									
Canal	40	6(2.8)	3.92	0.270	132				
Reservoir	55	5(2.3)							
Pond	58	13(6.0)							
All Type	62	9(4.2)							
Month	na	na	na		na				
April						2(0.4)	0.17±0.16 (0.16-0.18)	74.47	0.001**
May						16(3.2)	0.18±0.06 (0.14-0.31)		
Jun						12(2.4)	0.21±0.13 (0.14-0.59)		
July						26(5.2)	0.24±0.16 (0.14-0.72)		
August						32(6.4)	0.30±0.18 (0.14-0.80)		
September						27(5.4)	0.22±11 (0.14-0.52)		
October						5(1.0)	0.20±0.06 (0.14-0.20)		
November						25(5.0)	0.19±0.08 (0.14-0.47)		
December						22(4.4)	0.27±0.15 (0.14-0.65)		
January						21(4.2)	0.19±0.10 (0.14-0.51)		

**Significant (p <0.05); na data not calculated

Table 3: Hematological and biochemical analysis of *Paramphistomum epiclitum* infected cattle and buffaloes along with control groups.

Parameters	Infected (n=28) Mean ± SEM	Control (n= 10) Mean ± SEM	p-values
Hematological Analysis			
TLC/mm3	9.35 ± 0.67	7.20 ± 1.96	0.002**
TEC × 106µL	5.41 ± 0.26	6.62 ± 0.34	0.019**
HCT%	30.10 ± 1.62	33.45± 1.32	0.087^{NS}
HGB (g/ dl)	9.42 ± 0.60	11.48 ± 0.46	0.001**
MCH (pg)	15.65 ± 0.93	17.82 ± 0.55	0.05*
MCHC (g / dl)	31.00 ± 0.95	33.60 ± 0.43	0.21^{NS}
MCV (FL)	49.56 ± 1.91	53.33 ± 1.50	0.038*
PLT ×10^9/L	129.00 ± 11.98	149 ± 9.70	0.048*
Biochemical analysis			
ALT (U/I)	50.50 ± 3.06	41.12 ± 3.05	0.005**
Sugar (mg/dl)	66.75 ± 2.73	62.37 ± 2.41	0.01*
Cholesterol (mg/dl)	118.50 ± 7.6	103.87 ± 5.01	0.024*
Triglycerides (mg/dl)	126.12 ± 3.09	126.12 ± 3.09	0.42 ^{NS}

^{NS} non-significant (p >0.05), *,** Significant (p <0.05; p <0.01)

Hematological and Biochemical Analysis

Hematological parameters showed significant increase in number of TLC (p = 0.002) and decrease in the level of TEC (p = 0.019), HGB (p = 0.001), MCH (p = 0.05), MCV (p = 0.038) and PLT (p = 0.048) in infected animals than controls. Significantly increased level of ALT (p = 0.05), glucose (p = 0.01) and cholesterol (p = 0.024) was observed in infected as compared to non-infected animals (Table 3).

Histopathology of Rumen

The infected rumen tissue with *P. epiclitum* fluke microscopically showed haemorrhages and atrophy at attachment site to the ruminal papillae (Figure. 4), necrosis of epithelial papilla, and thickening of the wall and ulceration. The most prominent histopathological changes were sloughed mucosa, cellular vacuolation, submucosal edema, and disruption of the stratified epithelium with dilated lymphatics in the submucosa. Focal hyperplasia of the ruminal epithelium in different regions was also prominent and infiltration of lymphocytes inflammatory cells were observed around them. The pathogenicity was associated with the number of parasites sucking the ruminal mucosa.

DISCUSSION

Paramphistomosis is usually diagnosed by examination of parasite eggs in host feaces, but immature paramphistomes that cause pathogenicity are incapable of laying eggs. ELISA is an immunodiagnostic tool used for the detection of early infection in the host (Ahmad *et al.,* 2014). To develop a reliable and specific diagnostic tool many investigations have been performed to detect a related trematode infection in the host body (Raina *et al.,* 2006). In the present study antigen prepared from somatic extract were subjected to ELISA test for early diagnosis of paramphistomiasis. Similarly, reported that the antigen of *P. cervi* has been used to detect antibodies of paramphistomosis (Alabay, 1981; Keller, 1983). Diaz *et al.* (2006) conducted a study utilizing ELISA to analyze IgG antibody response in cattle against *Calicophoron daubneyi.*

The present study recorded the seroprevalence and abattoir-based prevalence of *P. epiclitum* in cattle and buffalo to find out the accurate status of the disease in the country. So far the reported prevalence of other species of *Paramphistomum* in Punjab was 22.29% (Javed *et al.,* 2006), and 11% in buffalo, and 7% in cattle (Raza *et al.,* 2007). In the current study, the overall prevalence of *P. epiclitum* was observed 15.3% on slaughterhouse-based investigation and 37.6% with serological technique. The present result showed a higher prevalence than other reported studies of which 11.25% in Sudan (Alkareem *et al.,* 2012), 7% in Spain (Sanchís *et al.,* 2013) and 1.99% to 3.4% in India (Shabih and Juyal, 2006). However, a higher prevalence as compared to present work was reported 53.1% in Bangladesh (Paul *et al.,* 2011) and 45.8% in Ethiopia (Yeneneh *et al.,* 2012). These differences in results could be explained with sample size, geographical region, and practicing of communal traditional grazing and examination techniques.

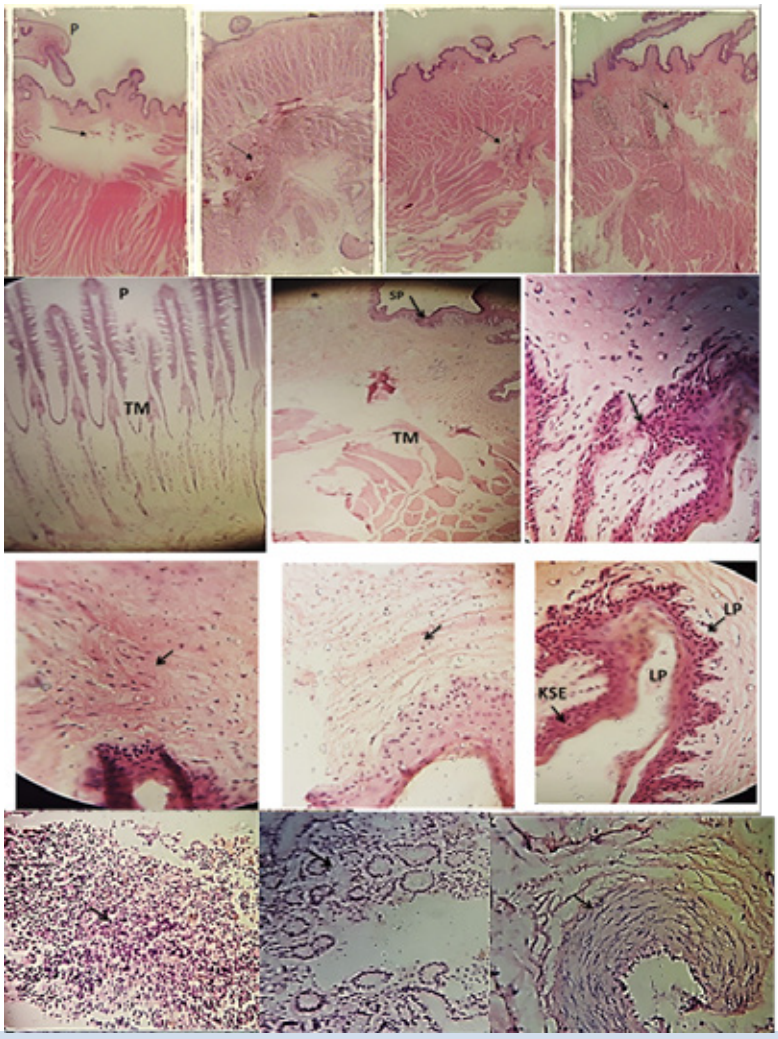

Figure 4: Histopathology of rumen tissues of buffaloes (H&E; Scale Bar = 100 μm), showing fluke(P) attached to rumen papilla, keratinized stratifies squamous epithelium (KSE) divisible into basal dark and light superficial layer, Lamina propria (LP), Long papilla (LP), short papilla (SP) and tunica muscularis (TM). Necrosis of squamous epithelial cells at the tip of the papilla and submucosal neutrophil and edema fluid accumulations and an infiltrate of a few lymphoid inflammatory cells into the epithelial layer are present. Rumen papilla of buffaloes showing infiltrates of lymphoid inflammatory cells form a dense aggregate, cellular vacuolation.

In the present abattoir survey, the prevalence of *P. epiclitum* was higher in females, in agreement with Alim *et al*. (2012). This may be due to changes in the physiological condition of female animals at the time of pregnancy, lactation, low resistance, or temporarily near parturition the loss of acquired immunity. Our findings with seroprevalence of *P. epiclitum* showed higher antibody titer in males, in agreement with Khedri *et al.* (2015). The reason is due to the social practice of females, which keep under better feeding and good management condition as compared to the male which are let loose in posture for freely grazing (Iqbal *et al.,* 2013).

The occurrence of *P. epiclitum* was found higher in old aged animals, which corroborates the observation of Iqbal *et al.* (2013). An increase in worm burden is the reason due to, exposure to grassland, high grazing, and absence of helminthic drugs (Iqbal *et al.,* 2013). This may also be explained by environmental factors, faulty management, and increased disease incidence (Javed *et al.,* 2006). The present result showed a higher infection rate in animals with semi-extensive (7%) and intensive (15.6%) grazing management, which is not in agreement with the study by Bilal *et al.* (2009). However, studies reported the chances to ingest the eggs of parasites by free-grazing animals are more due to their increased exposure to contaminated pastures (Bilal *et al.,* 2009).

The body condition of animals was recorded poor with high *P. epiclitum* infection in the current investi-

gation, consistent with Bashier (2014). This could be explained by the fact that ruminants have high protein loss and lower resistance to fluke (Bashier, 2014).). The present result of breed and host type did not significantly associate with *P. epiclitum* infection. Tehmina *et al.* (2016) recorded different results and found a significant association of breed with *Paramphistomum cervi* infection. The present result showed the highest prevalence of paramphistomosis in buffaloes as compared to cattle. Similarly, Raza *et al.* (2009) observed high incidences of infection in buffalo as compared to cattle. The reason may be due to moistening areas and water-loving habit of buffaloes, whereas intermediate host (snail) is more than grassland for transmission of the disease (Raza *et al.*, 2009). In the current investigation, no significant interaction was found between the rate of infection and water bodies. Although, it is suggested that among water bodies risk of infection is variable, generally shallow-water provides a favorable habitat.

In the current study, the month of August (6.4%) and September (5.4%) showed a higher prevalence of *P. epiclitum* agrees with other researchers. Ozdal et al. (4) recorded *Paramphistomum* spp. infection in cattle and sheep during the September (14.1%) to November (8.33%). Hassan *et al.* (2005) in domesticated ruminant documented the highest infection 8.06% in July to October and least in November to December (0.49%). Tariq *et al.* (2008) recorded the maximum intensity of infection in sheep during the late summer and drier autumn season. A study in Mexico recorded infection in cattle in early winter as animals mainly pick infection in rainy and windy summer and appeared in early autumn (Rangel-Ruiz *et al.,* 2003). Similar findings were recorded by Rolfe *et al.* (1991) in eastern Australia. Soulsby (1968) described the paramphistomosis outbreak usually occur in early winter or drier months. The results highlighted that moisture and temperature are the two main factors, affecting the hatching of parasites ova, snail's population, and accessibility of encysted cercaria which ultimately increase the incidence of paramphistomosis in animals (Soulsby, 1968).

The cattle and buffaloes infected with *P. epiclitum* had low TEC, HGB, MCH, MCV, and PLT, suggesting anemia. The increased TLC showed an inflammatory response caused by the fluke, results showed agreement with other researchers. Chauhan *et al.* (1972) recorded a significant reduction in the mean hemoglobin and total erythrocyte count in infected animals. Singh *et al.* (1984) reported decrease in TEC ($P < 0.05$), Hb ($P < 0.01$), and PCV ($P < 0.01$), while an increase in eosinophilic count ($P > 0.01$) as compared to the healthy animals was observed. Chhabra *et al*. (1972) conducted a study on amphistomes infected cross-bred calf and reported a reduction in hemoglobin. The possible cause of anemia may be due to worms, which feed on the host resulting in the depletion of nutrients and improper digestion.

The current pathology of rumen tissues showed an increase in the level of liver enzymes (ALT), glucose, and cholesterol. The possible cause of higher ALT results attributed to liver damage; could be necrosis of the liver due to toxemia from the damaged rumen mucosa (Garry, 2002). The increase in glucose level may be due to stress leading to adrenocorticosteroid release, which has the glycogenolytic effect, causing hyperglycemia. The Lipoproteins are synthesized in the liver and hepatic dysfunction may results in a disturbance in serum cholesterol levels.

The current histopathology results showed necrosis of epithelial papilla and increased infiltration of lymphocytes inflammatory cells, consistent with other researches. Rolfe *et al.* (1991) reported rumen with increased infiltration of eosinophils in the mucosa. Love & Hutchinson (2003) found adult flukes do not cause pathogenicity, although heavy fluke infestation is associated with chronic ulcerative rumenitis with atrophy of ruminal papillae. Singh *et al.* (1984) reported severe damage in the duodenal tissue caused by immature flukes, whereas the adult form inflicted mild tissue damage in the rumen.

CONCLUSION

In conclusion, this study provides the prevalence rate of *P. epiclitum* in Pakistan for the first time. The infected rumens were microscopically examined and showed damage of epithelial papilla and infiltration of lymphocytes inflammatory cells, resulting in hematological and biochemical disturbance associated with liver dysfunctions. In order to take control measures and determine precisely the prevalence of *P. epiclitum* in Pakistan, further seroepidemiological studies are required to determine the infection caused by immature flukes.

CONFLICT OF INTERESTS

The author(s) declare no potential conflicts of interest.

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