Pakistan J. Zool., vol. 49(2), pp 979-987, 2017. DOI: http://dx.doi.org/10.17582/journal.pjz/2017.49.3.979.987

Phenotyping of Amphistomes, and Pathological, Hematological and Bile Biochemical Response to *Gigantocotyle explanatum* Infection in Buffaloes

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ABSTRACT

The present study aims to determine the morphological features of amphistome adults and eggs combined with examining pathological, hematological and bile biochemical response in buffaloes to *Gigantocotyle explanatum* infection. Of the 558 slaughtered buffaloes from central Punjab, Pakistan, 18.99% were infected, and morphometric analyses were made of 115 mature amphistomes and gravid eggs with traditional microscopic methods on the basis of standardized measurements. Amphistomes were flattened and stained in Borax carmine, some were sectioned in the median sagittal plane and histological slides of the worms were prepared for detailed morphological studies. Bile tissues, aspirates and blood samples of the same animals were analysed. The infection was found prevalent throughout the year but during post monsoon season it was relatively high. Morphological identification of *G. expalanatum* was carried out on the basis of size and shape of worm. The hematological and bile biochemical changes were found in two groups. Pathologically significant changes were observed in the infected tissue. The parasites had formed pocked in the bile duct mucosa, glandular hyperplasia, thickened blood vessels. The results demonstrate that morphological markers, pathological, hematological and bile biochemical studies are effective tools that could be used as complementary in diagnosis of amphistome infections in ruminants.

Article Information
Received 26 July 2016
Revised 22 October 2016
Accepted 26 November 2016
Available online 09 May 2017

Authors' Contributions
MQ and KA conceived and designed the experiments; SIM performed the experiments; KA analyzed the data and KA, SIM and MQ wrote the paper.

Key words
Amphistomes, Abattoirs, Gigantocotyle explanatum, Morphometry, Buffaloes, Biochemical analysis.

INTRODUCTION

Helminth infections are prevalent throughout the world, affecting both humans as well as livestock animals and cause huge economic losses (Morphew *et al*., 2011). Amphistomes that have been identified in ruminants encompass more than 70 species. Most of these species are pathogens that cause serious morbidity in ruminants. *Gigantocotyle explanatum* is one of neglected amphistome parasite infecting the bile duct of water buffalo (Ichikawa *et al*., 2013). The fresh water snail *Gyrulus convexiculus* serve as the intermediate host (Patzelt, 1993). The prevalence of infection is very high in Indian subcontinent; about 60% buffaloes sacrificed at the local abattoirs had *G. explanatum* infection (GoI, 2011-2012). The exact estimates of economic loss in the world due to *G. explanatum* are not available.

Adult amphistomes remained attach to the epithelium of the bile duct of domestic ruminants where they inflict sever damage (Fukui, 1929; Mazahery *et al*., 1994; Singh, 1958). The pathologies caused by *G. expalanatum* are proliferation of connective tissues, hemorrhage, hypertrophy and hyperplasia in the bile duct, thereby affecting the health and productivity of the livestock animals (Swarup *et al*., 1987). Amphistomes produce "granulomatous nodules" at attachment sites and infiltrated by numerous inflammatory cells (Haque *et al*., 2011). The severity of hepatocellular injury, degree of cholestasis and synthesizing capacity of the liver can be detected by using serum biochemical tests including serum enzymes (Lee *et al*., 2005; Hodzic *et al*., 2013). It is, therefore, very important to make an accurate identification of amphistomes species for timely diagnosis of infection, as well as conducting epidemiological surveys to obtain a better picture of the infection dynamics of this species.

Pakistan is one of the Asian countries where livestock provides sources of income for 60% of rural population (GoP, 2012). In the Punjab province *G. expalanatum* infection rate was reported to be 44.44% in Gujarat, 33.33% Pindi Bhatian and Chiniot, 23.25% Sargodha, 19.35% Jhelum, 18.60% Mandibahudin and 17.39% in Faisalabad (Iqbal *et al*., 2014). The *Gigantocotyle explanatum* has economic importance in ruminants but presently no reliable methods are available to identify and differentiate

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this parasite from other amphistomes. Although diagnosis of amphistome infection relies on detection of parasite eggs during faecal examination, it is impossible to identify which species is present from morphological analysis of the eggs alone. Therefore, reliable morphological marker for adult species identification is urgently required.

As is evident from the current situation, further studies on amphistomes epidemiology and transmission to animals are required to obtain the baseline data, on which appropriate control measures could be established. This study represents a step further in this endeavour, by analysing the morphometric characteristics of amphistomes adults infecting the buffalo, one of the main livestock species present. We estimated the monthly prevalence and also compared the *G. expalanatum* associated hematological alterations in the buffalo population sampled in this study.

MATERIALS AND METHODS

Collection and examination of amphistomes

The study area comprises irrigated land with a well-established water canal system of the Indus river basin, commonly categorized as Central Punjab in Pakistan. The existence of rivers and canals provides excellent breeding grounds for the development and survival of freshwater snails serving as potential intermediate hosts for a variety of digenetic trematode parasites.

In 2010-2011, the post-mortem examinations of 553 buffaloes were conducted immediately after slaughtering to ascertain the presence of adult amphistomes. The adult worms found in bile ducts were collected with the help of rubber-coated forceps in order to avoid any structural damage. Only adult amphistomes having gravid uteri (n= 115) were included in the study. The eggs included in the study are collected from uterus of Adult worm. Amphistome specimens providing the largest worm variability in their size, maturity and gravid uteri were used for characterization. Amphistome specimens were fixed in Bouin's solution between two slides with a little pressure, depending on the thickness of the worm. The worms were stained with Grenacher's borax carmine and subsequently differentiated, dehydrated and mounted in Canada balsam.

Morphometrics

An accurate morphometric study has been conducted to establish phenotypic markers for adult amphistomes found in naturally infected buffaloes. All standardized measurements were made with a microscope and images captured by a digital camera (Nikon DS-Fi-1). Micrometry was performed using ocular micrometer and stage micrometer (0.01 mm) at 40x objective of microscope.

For adult Amphistomes and their eggs, the following measurements were taken for whole mount and sagittal section (Fig. 1):

Lineal biometric characters (mm)

Body length (BL), body width (BW), acetabulum diameter minimum (AD min), acetabulum diameter maximum (AD max), pharynx length (PL), pharynx width (PW), pharynx one half width (P1/2 W), oesophagus length (Oes L), intestinal caeca length (InL), anterior testis length (T_1L), anterior testis width (T_1W), posterior testis length (T_2L), posterior testis width (T_2W), ovary length (Ov L), ovary width (Ov W), mehli's gland length (MGL), mehli's gland width (MGL), egg length (EL) and egg width (EW).

Ratios

Body length over body width (BL/BW), and egg length over egg width (EL/EW).

Pathological examination and tissue sampling

The bile tissues from slaughtered animals were appropriately examined for the presence of *G. expalanatum* and its gross pathology. Diameter and wall thickness of both normal and infected bile ducts was measured from three different sides. The primary examination involves visualization and palpation of the organs; secondary examination involves more incision of bile duct. The gross pathological changes of bile duct as well as the distribution of the lesion were thoroughly examined and recorded. Then parts of the affected organ were sampled into 10% neutral buffered formalin. For histological lesion characterization, the fixed tissue samples were trimmed to 5 mm and processed. The tissues were sectioned at 5µm and stained with haematoxylin and eosin (Okaiyeto et al., 2012), and examined.

Hematological and bile biochemical analysis of blood samples

Then blood sample was taken during ante mortem from jugular vein using sterile EDTA coated 5ml vacutainer tubes, labelled according to the neck tag of animals and taken to laboratory. At the laboratory, blood samples were rendered to hematological analysis included packed cell volume (PCV), total erythrocyte count (TEC), hemoglobin (Hb) concentration and red cell indices, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total leukocyte count (TLC) and ratio of TEC/TLC (Linne and Ringsurd, 1999). Bile present in the gall bladder was aspirated with a needle and syringe. The concentration of Alanine transaminase (ALT), total serum protein and aspartate aminotransferase (AST) were calculated in the bile juice collected from both infected and control group.

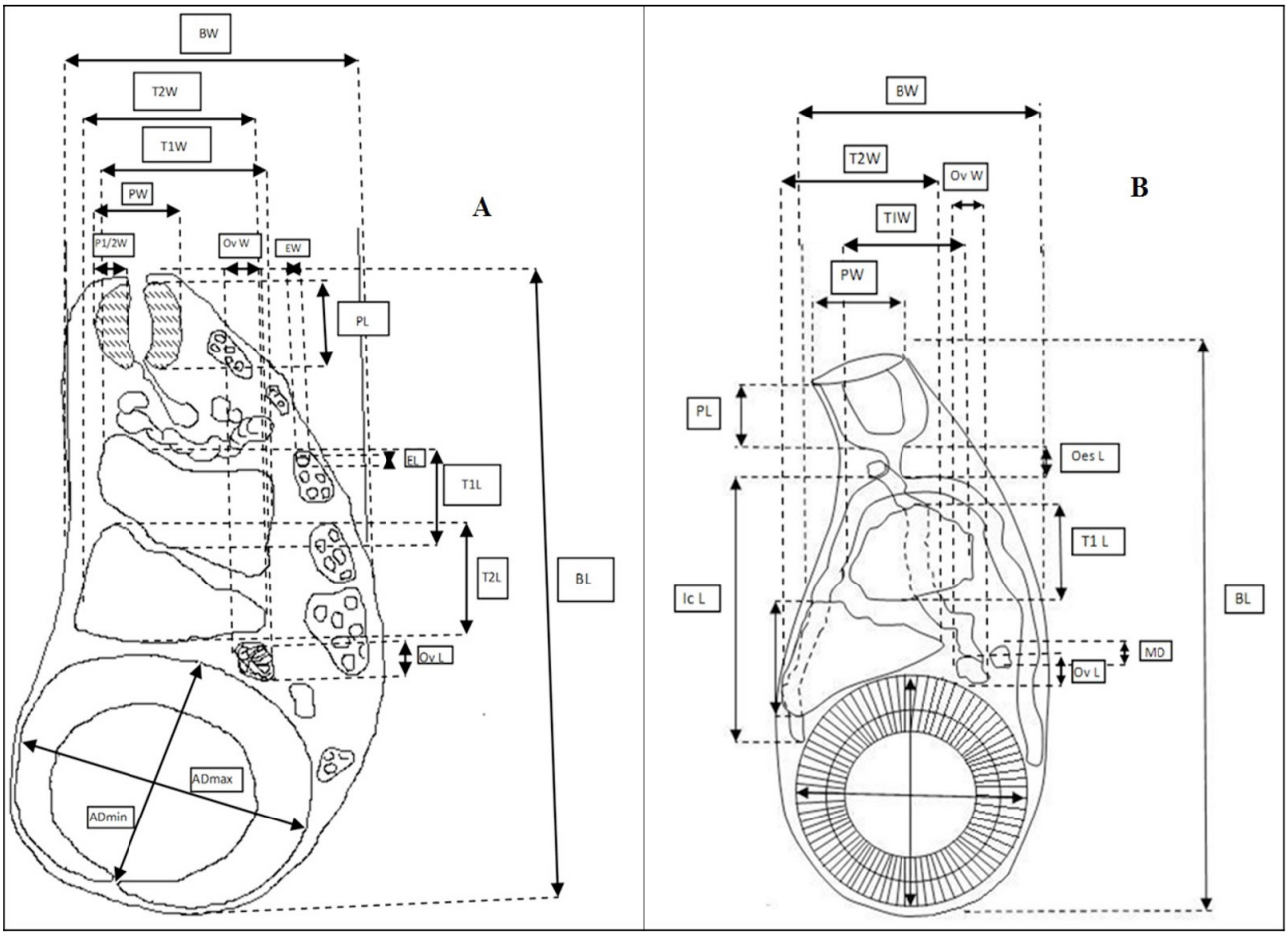

Fig. 1. Standardized measurements of **(A)** Whole mount **(B)** Sagittal section of adult Amphistomes. The key to abbreviations for all measurements is shown in Table I.

Data management and analysis

The data expressed as mean ± standard deviation and level of significance $p \leq 0.05$ was considered as statistically significant at 95% CI. The hematological and bile biochemical parameters of infected groups were compared with those of the controls/non-infected by using t-test. Data was analysed by using SPSS statistical software version 17.

RESULTS

Prevalences

The results of the gross pathology showed that 18.99% (n=558) of buffaloes were found to be infected with *G. expalanatum*. The result also depicted the infection was prevalent throughout the studied year (Fig. 2).

Morphological identification

The comparative morphometric measurements of adult amphistomes species from Pakistan are shown in the Table I with their mean and standard deviation. A total of 115 adult worms and egg were measured. Morphological

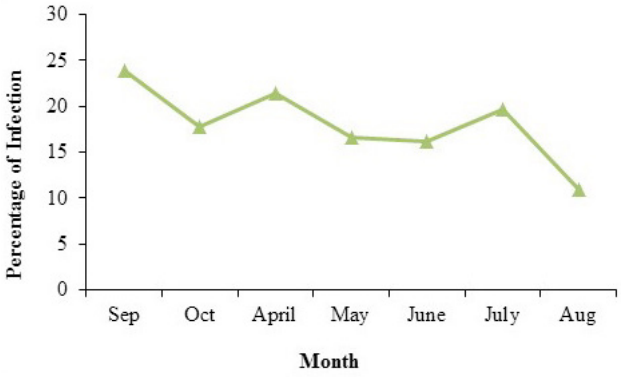

Fig. 2. Prevalence of amphistomes in buffaloes of central Punjab in different months of the year 2010-2011.

Table I.- Morphometrics with Mean values ±SD of n=115 adult amphistomes in buffaloes from Pakistan, lineal biometric characters in mm, and ratios without units.

Adult Measurements (mm)	Whole Mount	Sagittal Section
Body length (BL)	14.16 ± 2.09	7.65 ± 1.25
Body width (BW)	6.69 ± 0.92	3.47 ± 0.63
Acetabulum diameter min (AD min)	5.59 ± 0.62	2.47 ± 0.64
Acetabulum diameter max (AD max)	5.99 ± 0.68	3.24 ± 0.57
Pharynx length (PL)	1.09 ± 0.12	0.86 ± 0.11
Pharynx width (PW)	1.04 ± 0.13	0.67 ± 0.13
Pharynx one half width (P1/2 W)	-	0.30 ± 0.1
Oesophagus length (Oes L)	0.96 ± 0.29	-
Intestinal caeca length (InL)	8.98 ± 1.15	-
Anterior testis length (T_1L)	2.25 ± 0.58	1.06 ± 0.29
Anterior testis width (T_1W)	3.98 ± 0.80	1.80 ± 0.45
Posterior testis length (T_2L)	2.24 ± 0.45	1.04 ± 0.32
Posterior testis width (T_2W)	4.31 ± 1.00	1.82 ± 0.56
Ovary length (Ov L)	0.07 ± 0.37	0.42 ± 0.15
Ovary width (Ov W)	0.30 ± 0.06	0.36 ± 0.14
Mehli's gland length (MGL)	0.26 ± 0.06	-
Mehli's gland width (MGL)	0.20 ± 0.04	-
Egg length (EL)	0.13 ± 0.00	0.08 ± 0.01
Egg width (EW)	0.08 ± 0.00	0.05 ± 0.01
Ratio between BL/BW	2.13 ± 0.29	2.25 ± 0.46
Ratio between EL/EW	1.55 ± 0.01	1.61 ± 0.35

identification of *G. expalanatum* was carried out on the basis of size and shape of worm and position of elliptical or round ventral sucker (acetabulum). In the present study, most of the species were identify as *G. expalanatum*, and found mainly in the bile duct, where we distinguished notable light pink or grey in colour with large muscular and strongly developed acetabulum than to rest of the body. The body of *G. expalanatum* was ovoid or conical and were firmly attached with the walls of bile duct. The whole mount of worm measures about 14.165± 2.096 mm in length and 6.693± 0.923 mm in width, while sagittal section (Fig. 3) of worm about 7.652±1.25 mm in length and 3.476±0.63 mm in width. The papillae were absent, vitelline follicles were present extending from pharynx to the acetabulum along the lateral sides of the body. The digestive system comprised of pharynx, oesophagus and intestinal caeca. The oesophageal bulbs were absent and shortly divided in to two intestinal caeca. The intestinal caeca were almost straight and run longitudinally along the length of the body reaching the acetabular region. The male reproductive organs included two testes seminal vesicle and a genital pore; testes were lobate and somewhat diagonally placed to each other. The ovary was post testicular and lead to uterus, which was somewhat convoluted and filled with eggs and lead to the genital pore. The eggs were oval, filled with yolk and surrounded by thick shell.

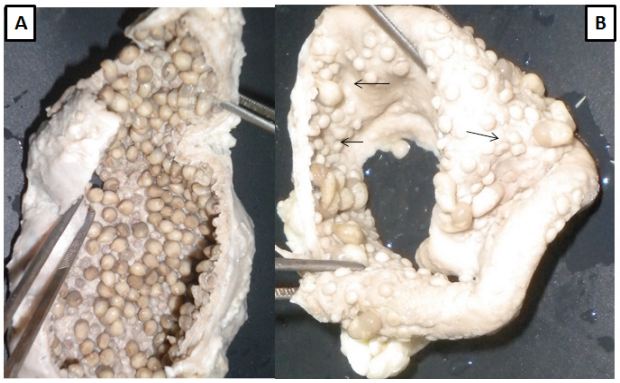

Fig. 3. A bile duct contains numerous amphistomes **(A)** and dome-like protuberances" after removal of the amphistomes **(B)** indicated with arrows.

Hematological / Bile biochemical analysis

The haematological and bile biochemical analysis values of the animals studied are summarized in Table II. A decrease in the mean hemoglobin (Hg) value was observed in infected animals. The level of the packed cell volume (PCV) did not significantly vary. Similarly, there was statistically non-significant ($p>0.05$) difference observed in the values of mean corpuscular haemoglobin (MCH). However, there was increase in the values of mean corpuscular volume (MCV), and a decrease in the values of mean corpuscular haemoglobin concentration (MCHC). The statistically non-significant ($p>0.05$) increase in the value of total erythrocyte count (TEC) and total leukocyte count (TLC) was observed in infected animals.

The bile enzyme alanine transferase level was found higher in infected animals as compared to the control group but the difference was statistically non-significant ($p>0.05$). A statistically significant ($p>0.05$) high aspartate aminotransferase (AST) level was measured in infected buffaloes as compared to non- infected/control group. The level of total serum proteins among control and infected buffaloes were found same (Table II).

Pathological lesions

The common bile and the hepatic duct were

distended and lumen of the bile duct was embedded with numerous small parasites (Fig. 4). There were numerous nodular structures on the inner and outer surface of the bile duct due to attachment of parasites by means of their acetabulum (Fig. 5A, B). Haemorrhagic spots were present on the surface of liver and bile duct. On the inner surface of the bile duct dilations were prominent due to parasite attachment. In severe cases the lumen of bile duct was blocked with parasites. The average diameter of the infected bile duct was greater than duodenum. There was increase in wall thickness due to hyperplasia.

Microscopically, the most common finding was the proliferation of the mucosal cells with the hyperplasia in the mucosal epithelium (Fig. 5C). The mucosal glands were enlarged and their shapes were distorted. The sub-mucosal region was also showing marked proliferation of glands. There were villi like appearance of the mucosa due to glandular degeneration (Fig. 5D, K). The upper columnar epithelium lining was distorted (Fig. 5E). The degeneration of the glands was most obvious at the site of fluke attachment (Fig. 5D). The mucosa packed inside the acetabulum was found different as compared to the rest of the glandular mucosa. The glands were distended but in the cases of severe infection glands were totally distorted and were gradually replaced by the inflammatory cells (Fig. 5I). In chronic cases only a few glands could be seen. The inflammatory cells included lymphocytes, which

Table II.- Hematological profiles and bile biochemical analysis in non-infected and G*igantocotyle explanatum* infected buffaloes as detected by different methods.

Parameters	Control	
	Non-Infected Mean± SD	**Infected Mean± SD**
Erythrocyte indices		
Hb (g / dl)	11.4 ± 1.06[a]	10.71 ± 2.55[a]
PCV (%)	29.28 ± 6.4[a]	33.57 ± 6.26[a]
MCV (FL)	36.43 ± 6.36[a]	48.78 ± 24.8[a]
MCH (pg)	15.1 ± 3.01[a]	14.51 ± 7.26[a]
MCHC (g / dl)	40.91± 12.7[a]	29.85 ± 3.94[b]
TEC X 10^6 µL	7.84 ± 1.01[a]	8.16 ± 3.20[a]
TLC / mm^3	5.35 ± 1.45[a]	7.21 ± 2.34[a]
TEC / TLC	1.45 ± 0.37[a]	1.24 ± 0.49[a]
Serum enzyme level		
ALT	5.8 ± 0.0[a]	6.86 ± 1.39[a]
TSP	0.79 ± 0.0[a]	0.79 ± 0.0[a]
AST	90.52 ± 137.2[a]	238.2 ± 319.2[b]

Means followed by different superscripts (a, b) within the same row are significantly different at ($p< 0.05$). Two means followed with the same letter implies that they are not significantly different ($p>0.05$). ALT, alanine transaminase; AST, aspartate aminotransferase; Hb, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PCV, packed cell volume; TEC, total erythrocyte count; TLC, total leukocyte count; TSP, total serum protein.

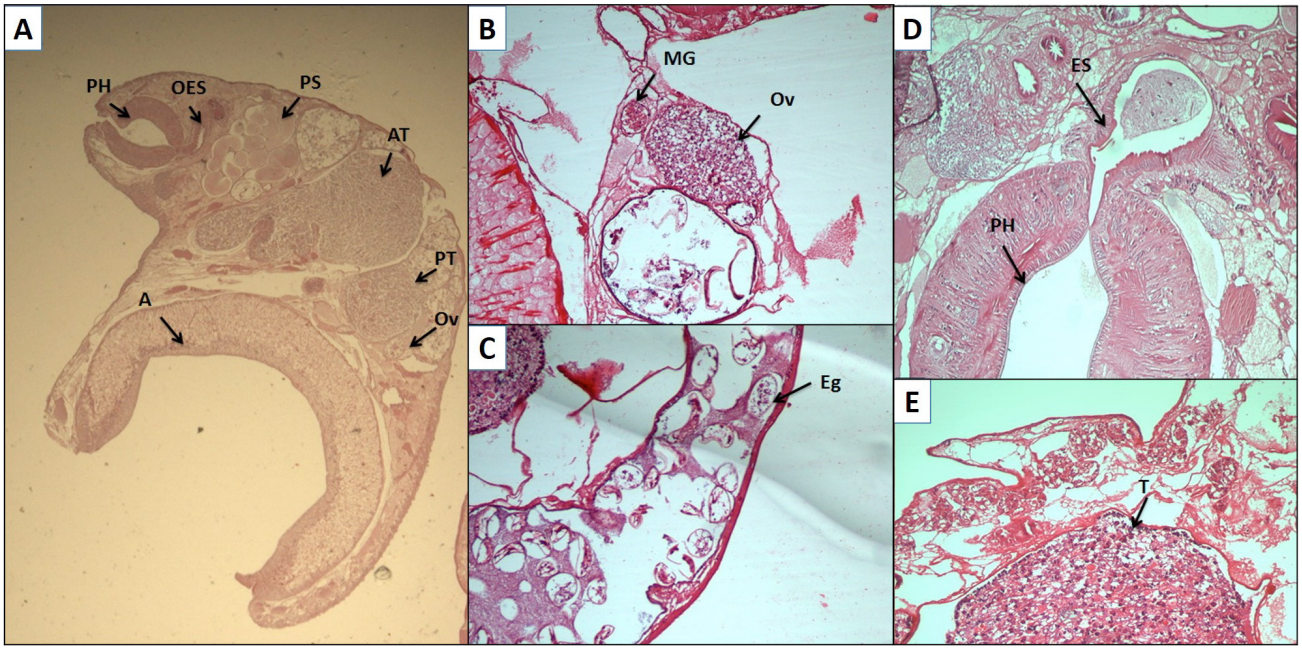

Fig. 4. **A,** Representative sagittal sections of *Gigantocotyle explanatum* indicates PH, pharynx; Es, esophagus; PS, pars seminalis; AT, anterior testis; PT, posterior testis; Ov, ovary and A, acetabulum. **B,** an enlarged image of ovary and Mehli's gland; **C,** egg; **D,** pharynx, esophagus; **E,** testis.

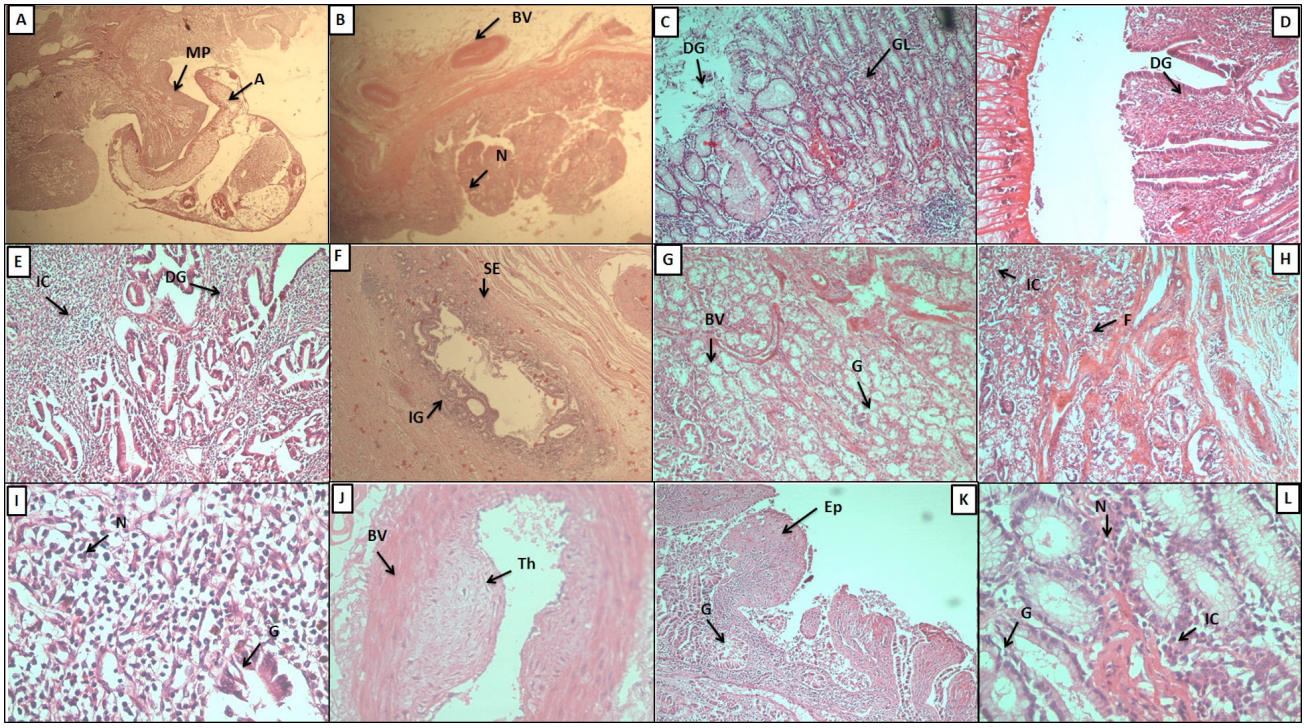

Fig. 5. Histopathological study of buffalo bile duct. **A:** Cross section of the infected bile duct; A, acetabula; MP, mucosal plug. **B:** mucosal plugs formed due to parasite attachment; BV, blood vessel; N, nodule. **C:** the glandular mucosa showing extensive glandular hyperplasia; DG, degenerated gland; GC, glandular cells. **D:** the glandular degeneration and villi like appearance at the site of fluke attachment. **E:** massive glandular degeneration and inflammation; IC, inflammatory cells. **F:** glandular infiltration in serosal layer; IG, inflammed glands; SE, serosa. **G:** sub mucosal layer showing increased glandular hyperplasia; BV, blood vessel; G, gland. **H:** serosal layer of infected bile duct showing fibrosis; IC, inflammatory cells; F, fibrosis. **I:** inflammatory cells; N, nucleus; G: gland. **J:** thick walled blood vessel showing hyperplasia and thrombus formation. **K:** villi like appearance of hyperplastic mucosa and degeneration of upper epithelial layer; EP, epithelial layers; GL: gland. **L:** increased nuclear size in infected tissue; N, nucleus; IC, intestinal caeca.

were most prominent. Also eosinophils could be seen in the lamina propria that gradually replaced the glandular tissue. In the infected tissue, the glandular cells were different in their sizes and shapes. Also the nuclear sizes were increased due to inflammation (Fig. 5L). The infiltration of the glandular tissue was also seen in the serosal and the muscular layer (Fig. 5F). The glands were present along with the inflammatory tissue in the muscular layer and serosa (Fig. 5G, H). The blood vessels were also affected from the infection. Obliteration and thrombus formation was a common finding. The blood vessels were thickened due to hyperplasia (Fig. 5J). The size of blood vessel was larger as compared to the normal tissue. There were hemorrhagic spots on the cut surface of the bile duct.

DISCUSSION

Morphological, hematological and histological identification is important features used to differentiate amphistomes. Several earlier studies have investigated morphological and histological variation in amphistomes species. The described parasites belong to the phylum Platyhelminthes, class Trematoda and subclass Digenea (Miller and Harly, 2010). The domesticated buffaloes are frequently infested with snail borne parasitic diseases like fasciolosis and amphistomosis due to their inherent affinity for water logging areas (Swarup *et al*., 1987). The present study gives the information on the prevalence of *G. explanatum* in different months of the study. It is observed that the prevalence of the parasite was remarkably high during all the months of the year. The parasite was affecting the haematological as well as the biochemical profile of the animal. The histopathological changes due to the parasite are: causing a serious damage to the host affecting its overall efficiency in terms of milk yield, meat and growth. This leads to a great economical loss to the dairy industry as a larger part of our national economy lies on the livestock.

In the previous studies carried by Cheema *et al*. (1997) the parasites were found ovoid or conical in shape, 7-13 mm in length and 4-5 mm in largest diameter and light pink or grayish in color. Singh (1958) described the morphological characters and life history of *G. explanatum*. Size ranged from 14.54-16.62 mm in length and 5.77-6.69 mm in width. Shape was conical due to comparatively large size of acetabulum. Pharynx was 0.6–0.76 mm in length. Papillae were absent. Acetabulum was elliptical or oval opening ventrally. Eggs were light greenish yellow with thin colorless shells and full of yolk cells. The results of the current study were found very much near to the findings of Nasmark (1937) described length as 8.48 mm and width as 4.24 mm. The acetabulum was 4.0 mm in diameter and pharynx was found 1.10 mm in length.

The possible cause of anemia in the present study may be assigned to the parasitic infection, which feed on host nutrition thus resulting in the depletion of nutrients and improper digestion. Furthermore, due to obstruction of bile duct by these parasites, the flow of bile is not possible and the outcome is a problem in digestion. This over all affects the animal efficiency including the hematological parameters. The non-significant change in the values of TEC and PCV may be due to haemoconcentration resulting from diarrhea. Mudaliar (1945) found decrease in haemoglobin and packed cell volume of goat infected with *C. cotylophorum*. Horak and Clark (1963) reported an increase in hemoglobin percentage; packed cell volume and erythrocyte count in sheep artificially infected with *P. microbothrium*. Chhabra *et al*. (1972) reported a significant decrease of hemoglobin concentration without significant variation in total leucocytic count in a cross bred calf naturally infected with paramphistomes.

In current study the possible reason for high level of ALT and AST in the bile juice in the case of infected animals is the increase production of these enzymes by the cells due to inflammatory reactions and release in to the bile. Both enzymes are considered as a measure of tissue necrosis. Tanritanir *et al*. (2009) recorded that pathological changes in tissues and organ are associated with differences in actions of liver enzymes like AST, ALT, GGT, ALP, LDH and GLDH. The hepatocellular damage and hepatocyte proliferation elevates the plasma concentration of ALT and AST (Hodzic *et al*., 2013; Salem and Hassan, 2011). Rumosa *et al*. (2012) reported non-significant differences in serum level of both AST and ALT enzymes among goats suffering with liver infections which are partially consistent with current results.

Fripp (1967) and Senft (1968) observed that the trematode parasites that live in the biliary system of the animal get their nutrients from the bile or the blood. This gives one reason for our results. Many workers have reported that changes in the composition of bile exposed to the action of pathologically altered gallbladders tended to diminish the effectiveness of the bile as a solvent for cholesterol (Drury *et al*., 1924; Ravdin *et al*., 1932; Andrews *et al*., 1932).

The present study is in agreement to previous results that recorded lesions due to chronic infection by *G. explanatum* (Eduardo and Manual, 1979; Ghosh *et al*., 1982; Swarup *et al*., 1987; Hafeez and Rao, 1989; Khan and Anjum, 1994). Ghosh *et al*. (1982) found neoplasia in the infected tissue. The neoplastic changes were leading to the mucosal adenoma and in rare cases adenocarcinoma was also observed. Metastatic infiltration of the malignant cells in the muscular and serosal layer was also observed. The cells had hyperchromatic nuclei and heavy fibrosis was observed. Although in the present study no neoplastic changes could be observed, the infiltration of the serosal and muscular layer by the glandular tissue and fibrosis was observed.

Cheema *et al*. (1997) reported *G. explanatum* in the common bile duct, cystic and hepatic duct. There was increase in the length, wall thickness and diameter of the infected duct. The amphistomes were attached on the inner side of the bile duct drawing mucosal plugs inside their acetabulum. Mucosal hyperplasia was present due to marked proliferation the mucosa formed villi like structures in the lumen of the bile duct. The same results were observed in the present study. The current findings are in agreement with Khatoon *et al*. (2003) reported bile duct hyperplasia, disintegration of the bile duct epithelium and inflammatory cell reaction.

The present study gives information about the morphological parameter of the *G. explanatum* in order to identify it and compare it on the specie level with the identifications given by parasitologists of the other countries. We also conclude that the buffaloes from central Punjab studied are highly infected by *G. explanatum* and react differently to the infection as showed by the histopathological, hematological and bile biochemical observations. These analyses could be used as complementary in diagnosis of ruminant amphistomes infection. Furthermore, it is important to emphasize the risk of dissemination of these parasites due to presence of its snail vectors, experimental studies with case-control should be done to establish better association between liver, hepatic duct and bile pathological changes and serum biochemical alteration.

ACKNOWLEDGMENTS

The authors acknowledge the help rendered by the Institute of Health, Park Road Islamabad, Pakistan for

providing histology facilities.

Conflict of interest statement

We declare that we have no conflict of interest.

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