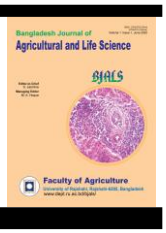
Original Article

Bangladesh Journal of Agriculture and Life Science
Faculty of Agriculture, University of Rajshahi
Rajshahi-6205, Bangladesh
Vol. 01(1): 1-10, 2020
www.dept.ru.ac.bd/bjals/



Epidemiology and pathogenesis of Fasciola-infected goat liver lesions collected from abattoirs in Rajshahi Metropolitan area of Bangladesh

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ARTICLE INFO

Article History
Received: January 20, 2020
Accepted: March 5, 2020
Online: October 25, 2020

Keywords
Epidemiology
Fibrosis
Fasciola
Goat liver
Pathogenesis

ABSTRACT

Liver fluke infection is an important cause of morbidity and mortality in small ruminants worldwide. The mechanism behind the fluke-induced liver lesions is poorly unveiled. We investigated Fasciola-infected liver lesions in goats grossly and histopathologically by routine and special staining methods. A total of 12 (11.76%) livers out of 102 showed gross changes including enlargement with the tense capsule, hemorrhage, or multi-focal depression with whitish discoloration and fluke within the bile ducts. The prevalence of Fasciola infections were 10.84 % in Black Bengal and 15.79% in Jamnapari goats. Female goats (13.20%) were more affected than the male goats (10.20%). Age-wise prevalence were 16.67%, 11.90%, 3.70%, 14.29%, 50.00% with the age of 1-2 years, 2-3 years, 3-4 years, > 4 years and aged goats, respectively. Goats were more infected in the rainy season (21.21%) in contrast to 8.82% in the winter and 5.71% in the summer seasons. Histopathologically, control livers showed normal architecture with scant fibrous connective tissue and a few inflammatory cells in the portal area. In Fasciola-infected livers, deposition of fibrous connective tissue increased with an increase in the infiltration of macrophages and eosinophils indicating their pivotal roles in fibrosis. An interesting finding was the deposition amyloid in the blood vessel in a Fasciola-infected liver section.

Introduction

Fascioliasis, also known as liver fluke infection, is an important cause of morbidity and reduced productivity in small ruminants worldwide. It is regarded as one of the most common single helminth infection of small ruminants in Asia and Africa (Hamond and Sewell, 1990). Fascioliasis has been considered as one of the major constraints of the production of goats in Bangladesh and it affects the liver of all ages (Talukder et al. 2010). *Fasciola gigantica* is the prevalent species in Bangladesh that causes anorexia, loss of body weight, occasional diarrhea, anemia, and even abortion (Soulsby, 1982). It causes serious damage to the liver including bile duct obstruction, pipe stem liver, biliary fibrosis, cirrhosis, and cancer (Machicado et al. 2016). Migration of immature flukes through the liver

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parenchyma causes hemorrhage and necrosis of hepatocytes (Soulsby, 1982). The prevalence of fascioliasis in goats has increased dramatically in the last few years. The geo-climatic conditions of Bangladesh are highly favorable for the growth and multiplication of parasites. The epidemiology of fascioliasis has a spatial element due to the free-living stages of *Fasciola giantica* as well as the intermediate snail host *Lymnaea auricularia* var. *rufescens* (Chowdhury et al. 1994; Islam et al. 2016) and the influence of climatic and environmental conditions (Charlier et al. 2011). However, the prevalence and the factors affecting the prevalence are largely unknown in the Barind tract areas of Bangladesh.

views, the current study was undertaken to shed some light on the factors affecting the prevalence of fascioliasis in goats of the Barind tract and to characterize fasciola-infected liver lesions histopathologically focusing on the mechanism of disease progression.

Table 1. Age-wise prevalence of liver fluke infection in goats

Group	Age of goats	No. of livers examined	No. of livers infected	Prevalence (%)
1	< 2 year	24	4	16.67
2	2-3 year	42	5	11.90
3	3-4 year	27	1	3.70
4	> 4 year	9	2	22.22
	Total	102	12	11.76

Materials and Methods

The liver samples from goats were collected from three abattoirs (Shaheb Bazar, Kazla and Binodpur Bazar) under the Rajshahi Metropolitan area. The health condition of the goats, age, sex, breed, and date of sample collections was recorded in a prescribed form. The goats with broad chest, small ears, small to medium horns and short body were categorized as Black Bengal and goats with highly convex nose, long pendulous ears and long legs were grouped as Jamnapari. A total of randomly selected 102 goats were examined for liver lesions from August 2016 to July 2017. The age of the goats was determined by the physical examination of teeth and matching with a dental chart (Vatta et al. 2007). The livers of the goats were examined immediately after slaughter and gross abnormalities were recorded. For histopathologic examination representative tissue samples from control (4 livers) and Fasciola-infected livers (12 livers) were placed immediately

Table 2. Sex-wise prevalence of liver fluke infection in goats

Sex	Breed	Number of livers examined	Number of livers infected	Prevalence (%)
Male	Black Bengal	41	4	10.20
	Jamnapari	8	1	
Female	Black Bengal	42	5	13.21
	Jamnapari	11	2	
	Total	102	12	11.76

Fibrosis is an intractable disease characterized by the deposition of an excessive amount of extracellular matrices (ECMs), mainly collagens. It is the net balance between the production and degradation of collagen produced by the complex interaction of inflammatory cells and cytokines resulting in portal hypertension and acute liver failure (Wynn and Ramalingam, 2012). The mechanism of fibrosis has been well investigated in laboratory rodents, however, the pathogenesis behind the fasciola-induced liver fibrosis in goat is largely unknown (Golbar et al. 2016; Golbar et al. 2017; Wijesundera et al. 2014). Considering these

into 10% neutral buffered formalin and brought to the Veterinary Pathology Laboratory at the Rajshahi University Department of Veterinary and Animal Sciences. The tissues were trimmed and placed in 10% neutral buffered formalin for overnight and processed following standard procedure, the detailed methodology has been described elsewhere (Bondoc et al. 2016). Briefly, the tissues were dehydrated through ascending grades of alcohol, cleared in xylene and embedded in paraffin. The tissue sections, cut at 5 µm in thickness by using a rotary microtome (Mosbi, China), were stained with hematoxylin and eosin (Bancroft and Gamble, 2007). For Congo red staining, after deparaffinization, the sections were incubated in Congo red solution for 20 minutes (Bancroft and Gamble, 2007). The deparaffinized sections were also incubated in Direct red 80 solution for 30 minutes for the staining (Bancroft and Gamble, 2007). All the stained sections were dehydrated through ascending grades of alcohol, cleared in xylene and mounted with cover glass using Canada Balsam ™ (Sigma-Aldrich, Germany). The sections were examined under a light microscope at low and high magnification. Photographs from the selected sections were grabbed by using a photographic microscope system (Camera model: LC-20, Labomed, Inc., USA fitted with microscope model: MBL-2100, Krüss, Germany).

Results

Among the 102 goat livers examined, 12 were infected with *Fasciola gigantica*, the overall prevalence was 11.76%.

Age-wise prevalence of liver fluke infection in goats

The goats examined were divided into 4 age-groups based on age as determined by the dental chart. Group 1, goats below two years of old were placed in this group and examined 24 goats of which 16.67% livers (4 goats) were infected with fluke (Table 1). Group 2 consisted of goats of 2-3 years old and examined the highest number of goats (42 goats) and the prevalence of liver infection with this parasite in this group was 11.90% (5 livers). Goats of 3-4 years old belonged to group 3 and consisted of 27 goats of which 3.70% livers (1 liver) showed infection with the fluke. Finally, goats with age above 4 years were categorized in group 4 and examined 9 goats of which 22.22% liver (2 livers) showed infection with the parasite. Goats with chronic infection were weak with poor health conditions and suffered from malnutrition.

Table 3. Season-wise prevalence of liver fluke infections in goats

Breeds	Summer (April-June)			Rainy (June-September)			Winter (November-February)		
	Total goats Examined	Total goats infected	Prevalence (%)	Total goats Examined	Total goats infected	Prevalence (%)	Total goats Examined	Total goats infected	Prevalence (%)
Black Bengal	26	1	3.85	29	6	20.69	28	2	7.14
Jamnapari	9	1	11.11	4	1	25.00	6	1	16.67
Total	35	2	5.71	33	7	21.21	34	3	8.82

Breed-wise prevalence of liver fluke infection in goats

Two types of goat breeds prevailing in the Barind tract area were encountered during the investigation of liver fluke infections and they were Black Bengal and Jamnapari. Among the 102 goats investigated 83 were Black Bengal of which 10.84% goats (9 goats) were infected with liver flukes (supplementary Table 1).

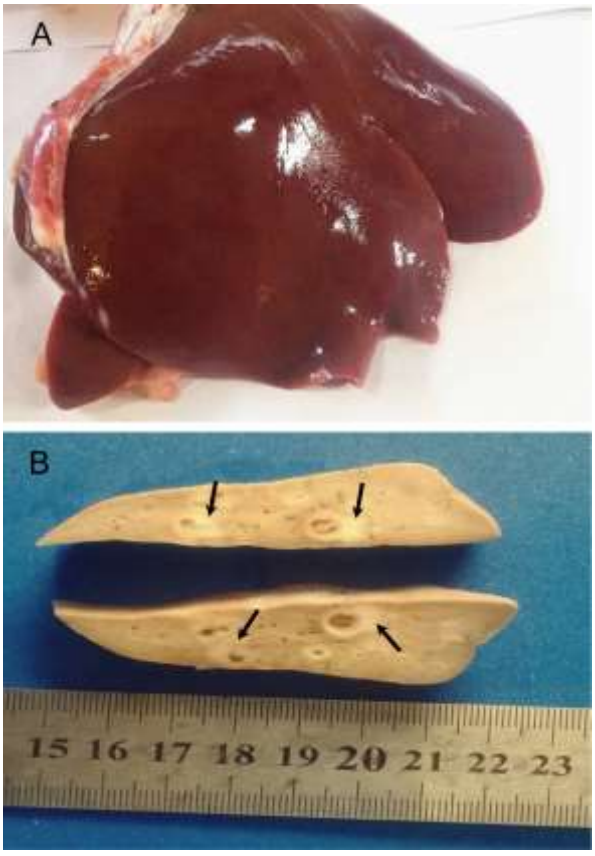

Fig. 1. Gross images of goat liver. Control liver showing no abnormality (A). Liver from chronic fascioliasis showing the whitish fibrotic area (arrows) around the bile ducts (B).

By contrast, out of 19 Jamnapari goats examined 15.79% (3 goats) were infected with the flukes. This result showed a higher infection rate in Jamnapari goats compared to Black Bengal goats.

Sex-wise prevalence of liver fluke infection in goats

Sex was an important determinant for liver fluke infection in goats. Of the 102 goats examined, male goat was 49 (41 Black Bengal and 8 Jamnapari) and female goat was 53 (42 Black Bengal and 11 Jamnapari) and the prevalence were 10.20% (5/49) and 13.21% (7/53), respectively (Table 2). This finding indicates that liver fluke infection is higher in female goats than in male goats in both the breeds.

Season-wise prevalence of liver fluke infection in goats

The prevalence of liver fluke infection was different in different seasons. Among the 102 goats examined, overall, goats were more affected in the rainy season (21.21%, 7/33) compared to in the winter (8.82%, 3/34) and in the summer (5.71%, 2/35) seasons (Table 3). The liver fluke infection in Black Bengal goats concerning seasons was also the highest in the rainy season (20.69%, 6/29) in contrast to that in the winter (7.14%, 2/28) and in the summer (3.85%, 1/26) seasons. Similarly, the infections were also the highest in the rainy season (25.00%, 1/4) than in the winter (16.67%, 1/6) and in the summer (11.11%, 1/9) seasons in Jamnapari goats. The results also showed that Jamnapari goats were more affected than the Black Bengal goats in the summer (11.11% vs. 3.85%), rainy (25.00% vs. 20.69%) and winter (16.67% vs. 7.14%) seasons.

Gross Necropsy findings

The livers from healthy goats devoid of any abnormality were wedge-shaped and reddish-brown and served as controls (Fig. 1A). The surfaces of the *Fasciola gigantica* infected livers were pale to a large extent than the color of control livers. Gross pathological changes due to acute liver fluke infection included slight swelling of the liver with rounded edges, thickened capsule and soft in consistency with hemorrhagic spots on the surfaces. In some chronic infection cases, flukes were seen in the intrahepatic bile ducts with thickening in the bile duct wall (Fig. 1B). Pre-mature and mature flukes were lodged in the intra-hepatic bile ducts and protruded from the cut surface. The affected bile ducts were distended. The affected livers were reduced in size with irregular and granular surfaces in chronic form. The color of the livers was changed and turned into pale and the capsules were thick, rough and were closely adhered to the parenchyma. Whitish color fibrous connective tissues covered the

parietal surface of the liver. The liver was hard to cut due to the presence of fibrous connective tissue.

Histopathologic findings

Histopathologically, control liver tissues showed cords of hepatocytes in the lobules with scant fibrous connective tissue and a few inflammatory cells in the portal triad (Fig. 2A). The portal triad contained a hepatic artery, portal vein, and a few bile ducts. In some acute Fasciola-infected liver sections, small foci consisting of mainly eosinophil aggregates were found. There were proliferations of spindle-shaped myofibroblasts and deposition of fibrous connective tissues in the portal triad along with the infiltration of inflammatory cells consisting mainly of macrophages and eosinophils. Depending on the extent of fibrous connective tissue deposition and the number of inflammatory infiltrates, portal fibrosis was categorized into grade-1, grade-2, and grade-3.Grade-1 fibrosis was characterized by the deposition of a small amount of fibrous connective tissues with infiltration of a small number of eosinophils and macrophages (Fig. 2B). In grade-2 fibrosis, there was deposition of moderate amount of fibrous connective tissue and infiltration of huge eosinophils and macrophages (Fig. 2C). Extensive deposition of fibrous connective tissue along with the infiltration of few to moderate number of inflammatory cells was categorized as grade-3 fibrosis (Fig. 2D). Cross-sections of immature and

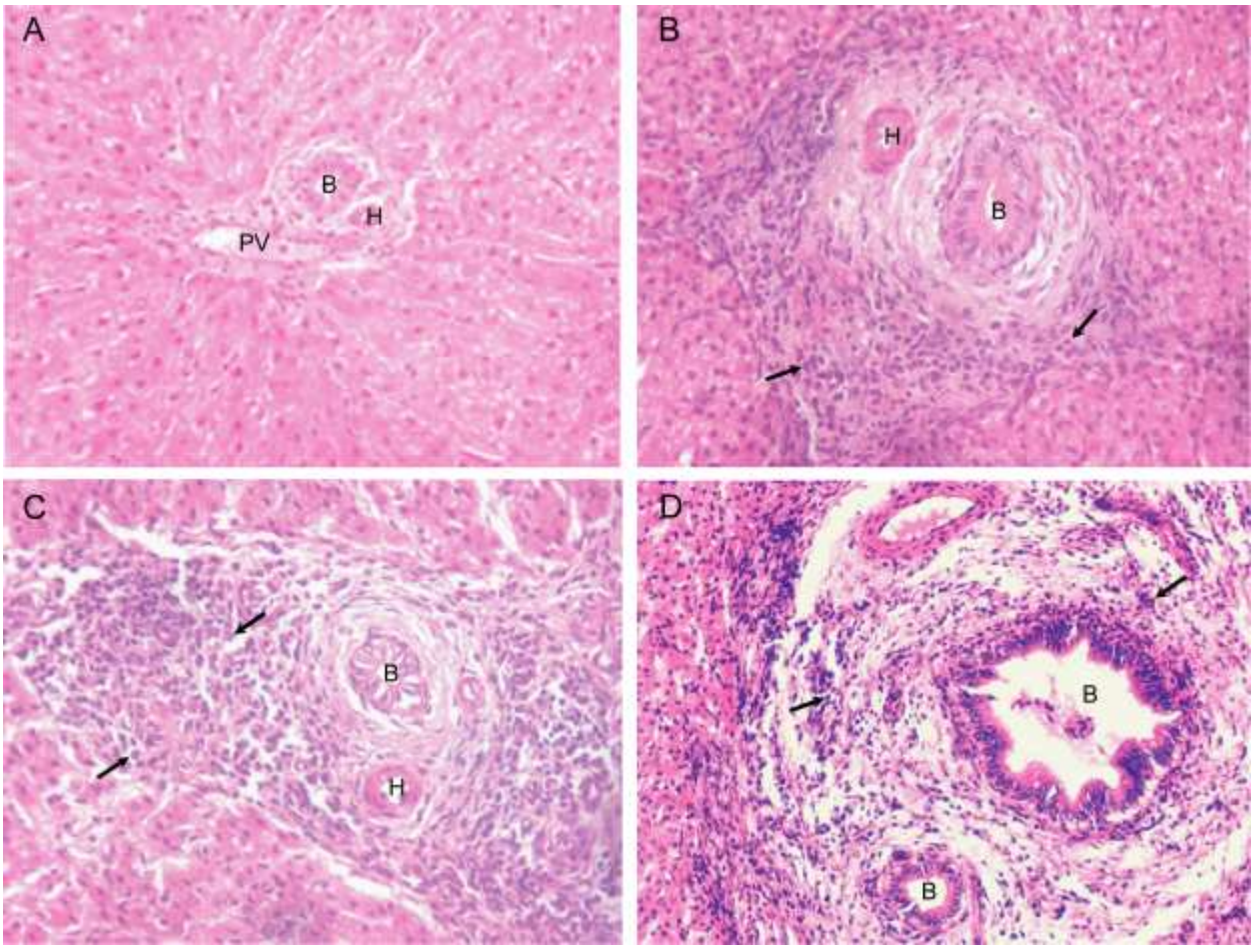

Fig. 2. Microscopic images of goat liver sections. Section from control liver showing hepatic lobules and hepatocytes arranged in cord with scant fibrous connective tissue and a few inflammatory cells in the portal triad (A). Fasciola-infected liver sections showing mild fibrosis associated with scant fibrous connective tissue (FCT) deposition and infiltration of a few inflammatory cells consisting chiefly macrophages and eosinophils (B), moderate fibrosis with a moderate amount of FCT and huge infiltration of inflammatory cells (C) and extensive fibrosis with deposition large amount of FCT and infiltration of a small number of inflammatory cells (D) in the portal area. Hematoxylin and eosin stain.

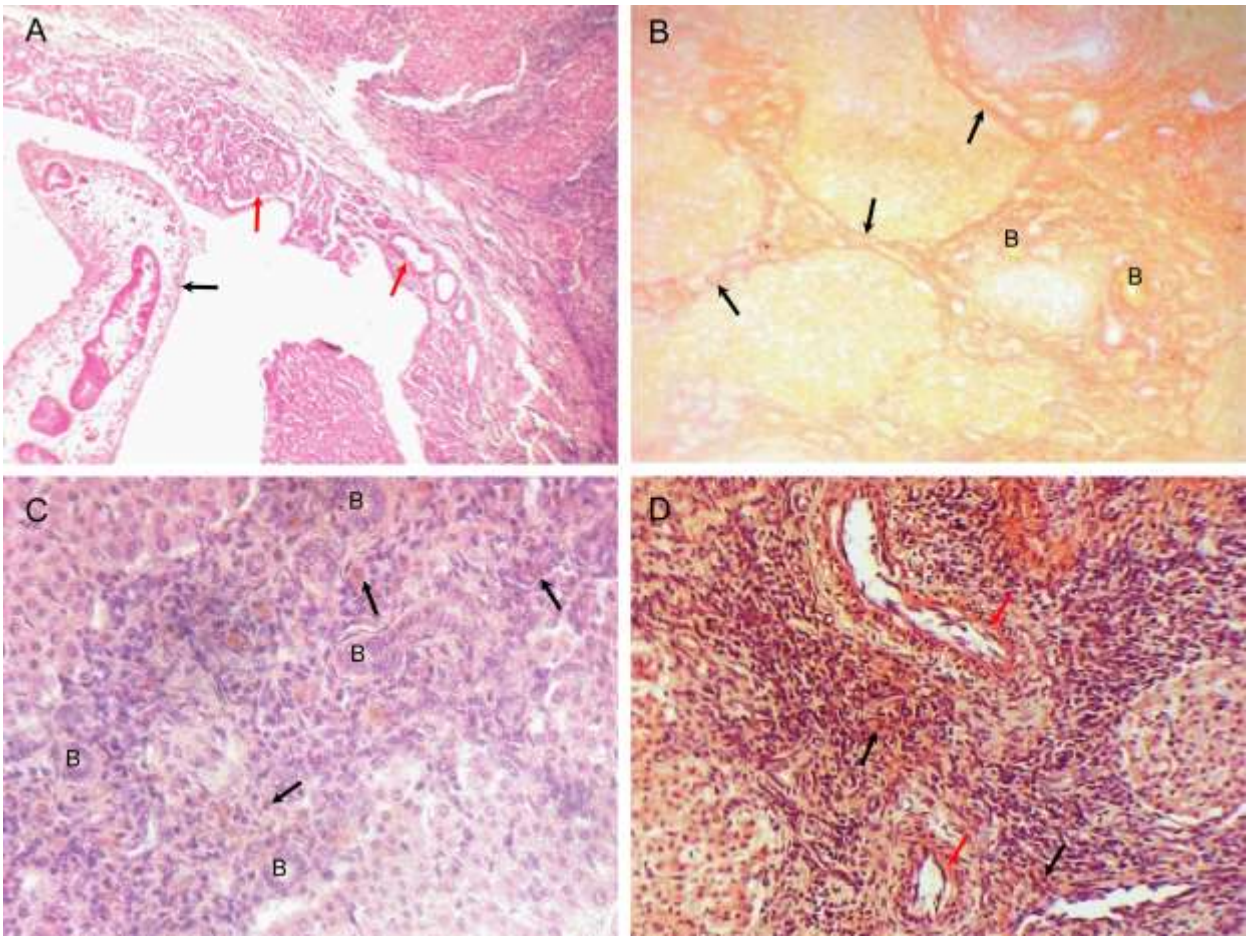

Fig. 3. Fasciola-infected liver sections in goat. Cross-section of liver fluke (black arrow) within the bile duct and bile duct hyperplasia with infiltration of inflammatory cells (red arrows) in a liver section (A). Deposition of extensive fibrous connective tissue in the portal area as well as bridging fibrosis (arrows) among portal areas (B). Section showing infiltration of macrophages and eosinophils (arrows) in the portal area (C). Deposition of amyloid in the blood vessels (red arrows), infiltration of macrophages and eosinophils (black arrows) in the portal area (C). B, bile ducts. Hematoxylin and eosin (A), Direct red 80 (B) and Congo red (C-D) stains.

adult flukes were seen within the bile duct of the chronically infected liver sections along with hyperplasia of bile ductular epithelia, proliferation of fibrous connective tissue around the bile ducts and infiltration of inflammatory cells in the portal connective tissue and periportal parenchyma (Fig. 3A). Besides, extensive portal fibrosis, portal to portal bridging fibrosis was evident in grade-3 fibrosis (Fig. 3B). The macrophages were mononuclear with abundant cytoplasm with hematoxylin and eosin stain. The nuclei of eosinophils were lobulated and cytoplasm stained orange-red with Congo red (Fig. 3C). An interesting finding in this study was the deposition of orange-red amyloid stained with Congo red in some blood vessels of a fluke infected liver section (Fig. 3D). It was worthy to note that the number of inflammatory cells decreased with the advancement of fibrosis.

Discussion

Fascioliasis is an endemic disease of goats in Bangladesh. Liver lesions associated with liver fluke infection in goats have not been adequately investigated in the Barind tract areas. Therefore, this study was carried out to shed some light on the epidemiology and pathogenesis of Fasciola-infected liver lesions collected from goats of the Barind tract. Because goats slaughtered at the abattoirs of the Rajshahi metropolitan area came from the Upazilas of Rajshahi and nearby districts, the samples represented the liver lesions due to natural infection with Fasciola in goats of the

Barind tract. Fasciola-infection affects the liver functions and results in decreased performances including milk, meat and skin production as well as affects their quality (Skapetas and Bampidis, 2016). As Fasciola infection is affected by geo-climatic conditions, availability of intermediate host, temperature, moisture, immune and nutritional status, and management systems of the goats, this study investigated the prevalence of Fascioliasis for one year to represent the infectivity concerning seasonal variations prevailing in the area. In this study, a total of 12 goat livers were found infected with liver fluke out of the 102 goats examined which resulted in an overall prevalence of 11.76%. The overall prevalence of the parasitic infections in our study is similar to the findings of Islam et al. (2016) who recorded 10.10% prevalence of Fascioliasis in goats Sylhet region of Bangladesh. However, the prevalence of Fascioliasis of our study is lower than the prevalence (20.75%) recorded from the slaughterhouse survey conducted by other authors in the Sylhet district (Hossain et al. 2011). It is important to note that the geo-climatic conditions of the Barind tract vary greatly from those of Sylhet areas. Additionally, susceptibility to infection with Fasciola may be due to the breed of the goats. Exotic breeds, a few Jamnapari goats as used in the current study, may have increased susceptibility to liver fluke infection. Therefore, the overall low prevalence found in the present study compared to prevalence recorded by Hossain et al. (2011) may be due to extremely hot weather and decreased rainfall in the Barind tract and increased susceptibility of Jamnapari goats in contrast to Black Bengal goats.

Age-wise prevalence of liver fluke infection in goats

Parasitic infection is eliminated by the so-called self-cure phenomenon (Islam et al. 2016). Under this phenomenon, the infection reduces with the increase of age due to the development of the immune system (Hossain et al. 2011; Keyyu et al. 2003; Tasawar et al. 2007). A host may recover from parasitic infection with increasing age and may become resistant day by day (Keyyu et al. 2003; Tasawar et al. 2007; Winkler 1982). In our study, goats < 2 years showed higher prevalence (16.67%) compared to 2-3 years (11.90%) and 3-4 years (3.70%) age groups, the decrease of infections might be due to increase of immunity with the advancement of the age of goats. By contrast, higher prevalence in young goats may be due to less immune protection and a gradual decrease in maternal antibody (Islam et al. 2016). On the other hand, the highest prevalence (22.22%) was recorded in goats >4 years, this finding might be due to the investigation of only a few goats within the age group. Decrease of immune function due to malnutrition, inclement weather and stress may favor the higher incidence of liver fluke infection in the goats of this age group.

Sex-wise prevalence of liver fluke infection in goats

The prevalence of Fascioliasis was different due to the difference in sex. Female goats (13.21%) were more affected than the male goats (10.20%) in the current study. Female goats are generally kept for breeding purposes. The stress of milk yield or kidding may reduce body immunity which may result in increased and chronic infection in female goats (Hossain et al. 2011; Islam et al. 2016). Islam et al. (2016) also showed 1.85 times more susceptibility in females to *Fasciola gigantica* infection than males in Black Bengal goats. Research from Islam and Ripa (2015) also showed a higher incidence of Fascioliasis in female goats. Additionally, Jamnapari female goats were 1.53 times more susceptible than Black Bengal female goats and Jamnapari male goats were 1.28 times more susceptible than Black Bengal males in the current study. Blood et al. (2006) documented that female goats which are usually weak and malnourished, consequently are more susceptible to infections.

Breed-wise prevalence of liver fluke infection in goats

The breed is an important risk factor of diseases in animals. Jamnapari goats were more susceptible to Fascioliasis compared to Balck Bengal goats in the current study. Reports from several other authors also recorded higher susceptibility of diseases including parasitic infection in exotic animals than in native animals (Brotherstone et al. 2010; Wambura et al. 1998). Although Black Bengal goats are widely distributed throughout the country, the Jamnapari goats are available only in the Rajshahi areas.

Although the Jamnapari goats showed higher susceptibility to infection with Fasciola than the Black Bengal goats in the present study, data were unavailable to compare the findings.

Season-wise prevalence of liver fluke infection in goats

The liver fluke infection depends on ecology, geographical and agro-climatic conditions associated with the survival of intermediate host essential for the development of the pre-infection stages of the parasite (Islam et al. 2014). Rainfall is frequently associated with the reproduction of intermediate hosts (snails) and with their longer survival due to moist conditions (Ahmed et al. 2007). The infection is also associated with open grazing in the submerged area, especially after heavy rainfall (Hossain et al. 2011). The environmental condition of Rajshahi is suitable for the development of larval stages of *Fasciola gigantica* and to complete their life cycle. The heavy rainfall and open grazing system might be the cause of a higher incidence of *Fasciola gigantica* infection in the rainy season than other seasons in the current study (Islam et al. 2014; Jithendran and Bhat, 1999; Tamloorkar et al. 2002). Infection with Fasciola depends on the availability of an intermediate host. The liver fluke of the present study was identified as *Fasciola gigantica* based on the morphology of the parasite. Unavailability of snail (intermediate host) essential for the completion of the life cycle of other Fasciolas excluded the presence of other Fasciola species.

Gross and microscopic features of liver fluke infection in goats

The gross and microscopic features of the liver vary with the duration (acute or chronic), load and stages (larva, adults) of the parasites causing infection. The acutely infected livers were enlarged characterized by tensed capsule and rounding of edges, soft in consistency and sometimes with associated hemorrhages. By contrast, livers with chronic infection showed multifocal depression with whitish discoloration. Thickening of the bile ducts, so-called pipe stem liver, with lodgment of flukes within the intrahepatic ducts were evident (Jones et al. 1997). These gross findings are in agreement of the findings with other authors (Adama et al. 2011; Affroze et al. 2013; Ahmedullah et al. 2007; Alim et al. 2000; Islam et al. 2016; Masuduzzaman et al. 1999; Okaiyeto et al. 2012; Rahman et al. 2007). Peribiliary fibrosis induced by Fasciola was documented by Elshraway and Mahmoud (2017). Histopathologic findings of scant fibrous connective tissue with a few inflammatory cells in the portal area of control livers served as a basis of comparison with Fasciola-infected livers. In Fasciola-infected livers, the amount of fibrous connective tissue increased with an increase in inflammatory infiltrates consisting mainly of eosinophils and macrophages (Howlader and Huq, 1997). The eosinophils and macrophages accumulated might have mediated the fibrosis reaction by secreting some factors responsible for transdifferentiation of myofibroblast that lead to the deposition of fibrous connective tissue, which was graded as mild, moderate or intense (Golbar et al. 2013). It has been documented that macrophages are the prime source of TGF-β1, a potent fibrogenic factor (Wynn and Barron, 2010). Eosinophils are also known to secrete TGF-β1 (Shen et al. 2008). Infiltration of a large number of macrophages and a moderate number of eosinophils were documented in the current study. It was interesting to note that there were more inflammatory infiltrates in moderately fibrotic areas compared to the mild and intense fibrotic areas. It may be due to that the inflammatory cells mediated the fibrotic reaction and when fibrosis progressed advanced the inflammatory cells disappeared. The eosinophils were stained with Congo red stain showing orange-red cytoplasm with coarse granules and bluish nucleus with 2-3 lobes (Desroches et al. 2009; Joshi and Kaijkar, 2013). The macrophages were differentiated from eosinophils by the non-segmentation of the nucleus. An interesting finding was the deposition of amyloid, orange-red in color, in the blood vessels stainable with Congo red in a Fasciola-infected female Jamnapari goat liver section. Amyloidosis is the deposition of ultra-structurally similar but biochemically distinct protein (Jones et al. 1997). Amyloidosis is not uncommon in domesticated animals but rare in goats. There are a few reports describing amyloid deposition in the liver in goats (Farnsworth and Miller, 1985; Gaffney et al. 2015; Ménsua et al. 2003). Amylodosis in the liver may be suspected in patients with hepatomegaly (Ludlage et al. 2005). However, the relation between Fasciola infection and amyloid deposition remains to be

clarified in further studies. In chronic Fascioliasis, fibrosis of portal area and thickening of the bile ducts were observed. These observations are consistent with chronic fascioliasis of goat, sheep, and pig (Chiezey et al. 2013; Farnsworth and Miller, 1985; Islam et al. 2016; Masuduzzaman et al. 2005; Okaiyeto et al. 2012). An explanation on the pathogenesis of Fasciola infection and portal fibrosis may be that there were huge infiltration of eosinophils and macrophages, which secrete transforming growth factor TGF-β1 resulting in transdifferentiation of portal fibroblasts into myofibroblasts; the myofibroblasts are the prime source of extracellular matrices leading to fibrosis (Golbar et al. 2013). This observation states that eosinophils and macrophages have crucial roles in mediating portal fibrosis.

Conclusion

Barind tract is unique in terms of geo-climatic conditions and the goat population concerning Fascioliasis. Jamnapari goat, in addition to Black Bengal, is only available in the Barind tract area. Works on epidemiology and pathogenesis of fascioliasis in goats of the Barind tract is the first attempt of this kind in this area. This study, for the first time, elucidated the interactions of macrophages and eosinophils on the pathogenesis of Fasciola-induced liver lesions in goats. This study also reports the characterization of amyloidosis in goats for the first time in Bangladesh.

Acknowledgment

The work was supported in part by a grant from Rajshahi University No: A-848-5/52/UGC/Agri-36/2015 (to Hossain M. Golbar).

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How to cite this article: Rehana Parvin, Afsana Akta, Rubina Khatun, Most. Nahida Khatun, Nurularifa Khatun, Shah M. A. Rauf and Hossain M. Golbar (2020). Epidemiology and pathogenesis of Fasciola-infected goat liver lesions collected from abattoirs in Rajshahi City area of Bangladesh. Bangladesh Journal of Agriculture and Life Science 1(1): 1−10.