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Original article

Fascioliasis may promote tuberculous infectivity in small ruminants

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ABSTRACT

Fascioliasis and bovine tuberculosis (TB) are global impediments to livestock development. We investigated the co-infectivity of fascioliasis and TB in small ruminants at slaughter. A total of 84 goats and 16 sheep were investigated from different slaughter houses in Mymensingh city, Bangladesh from June 2019 to February 2020. Grossly, acute fascioliasis was characterized by hemorrhagic tracts in the liver and chronic fascioliasis with biliary cirrhosis and pipe-stem liver. Grossly, seven goats and two sheep were associated with the acute and sixty goats and seven sheep were associated with the chronic phase of fascioliasis. Five goats' livers showed both the acute and chronic phases of fascioliasis. In TB, granulomas with central core of caseous necrosis were seen in the lymph nodes (21), livers (10) and lungs (01) of goats or in the lymph nodes (03) and liver (01) of sheep. Histopathologically, biliary cirrhosis was seen in fascioliasis and granuloma, caseous necrosis and calcification in TB. In co-infection revealed granuloma (TB) with acid-fast bacilli and widespread biliary cirrhosis in the livers of goats (14) and sheep (02). The fragments of the 16S rRNA gene (372 bp, *M. tuberculosis* complex) and MPB83 gene (600 bp, *M. bovis*) were detected in the lymph nodes, livers and lungs using polymerase chain reaction. This study showed the existence of co-infectivity of *Fasciola* and *M. bovis* in goats and sheep in Bangladesh. Chronic fascioliasis may be associated with establishing tuberculous infection in small ruminants. Therefore, extremely zoonotic bovine TB control programs require active management of fascioliasis.

1. Introduction

Parasitic and infectious diseases are the major hurdles to livestock production and development in Bangladesh. The livestock sector is now becoming a more challenging sector because of the presence of co-infection or multiple infections. Co-infection is the simultaneous infection of a host by numerous pathogens, which is more common than a single infection in natural populations (Cox, 2001). Co-infection can aggravate hosts' health and disease conditions as the multiple pathogens interact within the host (Griffiths et al., 2011) and cause difficulties in achieving a diagnosis. This situation is crucial in disease diagnosis, control, eradication and treatment of affected individuals. The co-infection relationship between *Fasciola* spp. and *Mycobacterium bovis* has been extensively studied in cattle (Byrne et al., 2019; Claridge et al., 2012), as globally, both pathogens are common in animals. However, the interaction of these pathogens in small ruminants has not yet been fully elucidated in Bangladesh and remains to be investigated.

Fascioliasis, caused by the infection of the liver fluke *Fasciola hepatica* and *F. gigantica,* is a global problem in ruminants farming (Byrne et al., 2016). *F. gigantica* is the most economically important and widely prevalent liver fluke in ruminants in Asia and Africa, including Bangladesh (Hamond and Sewell, 1990; Rahman et al., 2017). It causes a significant economic loss due to anthelmintic costs, reduction in milk and meat production, condemnation of the liver, loss of draught power and decreased fertility and productivity (Diaw et al., 1988). Fascioliasis is very common in animals in Bangladesh due to its tropical climate (Amin and Samad, 1988). Small ruminants reared destined for slaughter showed higher infectivity due to *Fasciola* (Ezatpour et al., 2015). On the other hand, tuberculosis (TB) is a chronic granulomatous disease of

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domestic and wild animals and humans with a high impact on veterinary and human health (Ayele et al., 2004). TB is a significant zoonotic disease caused by acid-fast bacteria belonging to the *M. tuberculosis* (MTB) complex. *M. bovis* is the universal pathogen among *Mycobacteria* that affects many vertebrates, including humans. Although cattle, goats and pigs are most susceptible, sheep and horses show high natural resistance (Radostits et al., 2000; Thoen et al., 2006). The disease is highly contagious and can be transmitted by inhalation and in association with close contact between infected and susceptible animals. To a lesser degree, the environment makes it very difficult to control.

Although fascioliasis and TB are widespread diseases in animals, they have always been considered separately despite showing a concurrent infection in nature only apparent through abattoir findings. However, co-infection of TB and helminths, particularly *F. gigantica,* is a serious issue in these disease-prone areas (Byrne et al., 2019). *Fasciola* infected animals have significant elevation of immunoglobulin E levels, eosinophilia and Th2 immune responses (Ezenwa et al., 2010), which reduces *M. bovis*-specific Th1 responses (Flynn et al., 2010), resulting in a delay in bacterial clearance from *Fasciola* infected animals (Ezenwa et al., 2010). According to published research, fascioliasis suppresses the host's Th1 immunity, which is a common form of immunological response to TB infection in cattle (Flynn et al., 2007; Flynn and Mulcahy, 2008). Fasciola infections reduce interferon-gamma (IFN-) levels in cattle and inhibit Th1 responses to mycobacterial infection (Brady et al., 1999; Ezenwa et al., 2010; Kelly et al., 2018). The absence of this form of Th1 immune response in a susceptible host can result in an increased risk of TB in cattle (Flynn and Mulcahy, 2008). There is reported increasing evidence of co-infectivity of Fasciola spp. with *M. bovis* in cattle in different parts of the world. Co-infection of *F. gigantica* with bovine TB infection has been reported in Africa (Kelly et al., 2018). Co-infection of *F. hepatica* and *M. bovis* has been reported in an enzootic area of Hidalgo state, Mexico (García-López et al., 2018). Munyeme et al. (2012) reported the interaction between bovine TB and fascioliasis infections in cattle of the Kafue basin ecosystem in Zambia.

However, literature about the co-existence of fascioliasis and TB in small ruminants is scanty. The study aimed to investigate the co-infectivity of fascioliasis and TB in small ruminants at slaughter and explore the association between *F. gigantica* and *M. bovis* in small ruminants, mainly sheep and goats, in Bangladesh and to gain insight into developing protocols for proper detection and management.

2. Materials and methods

2.1. Ethical approval

The research was approved by the committee of ethical standards, Bangladesh Agricultural University Research System (BAURES), with reference no. BAURES//ESRC/VET/05 at dated 11.05.2019.

2.2. Sample collection and gross pathological investigation

A total of 84 goats and 16 sheep at slaughter of both sexes and different age groups were investigated from five different slaughter houses in Mymensingh city, Bangladesh, from June 2019 to February 2020. At slaughter, the liver, lungs and mesenteric lymph nodes were examined for gross lesions suggestive of infectivity due to fascioliasis and TB. In brief, the bile duct, liver, and gall bladders were longitudinally incised with a sharp knife to identify the presence of liver fluke or to see the internal gross pathological changes like pipe-stem liver with fluke migratory tracts. The liver fluke was collected from the gall bladder and bile duct using blunt forceps and identified as the liver fluke species per standard procedure (Mpisana et al., 2022; Ross, 1965; Soulsby, 1986). Immediately after gross observation, the liver, lungs and mesenteric lymph nodes were collected for histopathological and molecular study. Small parts of the liver, lungs and lymph nodes were preserved in 10 % neutral buffered formalin (NBF) and carried out the standard histopathological procedure (Luna, 1968). Small parts of the liver, lungs and lymph nodes were snap-frozen, and preserved at − 20 ℃ for performing polymerase chain reaction (PCR).

2.3. Hematoxylin and eosin (H & E) staining

NBF-fixed tissues were dehydrated, embedded in paraffin, and sectioned at 3–4 μm in thickness. The deparaffinized sections were stained with H & E staining according to Luna, 1968. The stained tissue sections were mounted using DPX, air-dried and examined under a light microscope (ZEISS Primo Star). The images were captured using an electronic device microphotographic method (Cell Bioscience, Alphaimager HP, California, USA).

2.4. Acid-fast staining

Acid-fast staining of tissue sections was performed to identify the acid-fast bacteria in the liver, lungs and lymph nodes (Luna, 1968). NBF-fixed tissue sections were deparaffinized and hydrated in distilled water and stained with carbol fuchsin solution for 30 min. To remove excess stain, the sections were washed with tap water and decolorized in 1 % acid alcohol solution. After decolorization, the sections were counterstained with methylene blue (until the sections turned pale pink). Rinsing was done with water to remove excess color. Finally, the sections were dehydrated and mounted using DPX, air-dried and examined at high (100x) power microscopic fields. The acid-fast bacilli appeared to be red under light microscopic investigation.

2.5. PCR detection of TB

Genomic DNA from the liver, lungs and mesenteric lymph nodes (10–20 mg tissue/organ) was extracted using a commercially available DNA extraction kit (Wizard Genomic purification kit, Promega, USA) according to the manusfacturer's instructions. The purity and concentration of extracted DNA were determined at 260 nm/ 280 nm by a NanodropTMspectrorophotometer (IAEA, Scibersdoff, Vienna). The extracted DNA appeared pure, with an A260/A280 ratio of 1.7–1.9. The concentration of all DNA samples tested in spectrophotometry was reasonably high and was found to contain 350 ng DNA/μl volume. The extracted DNA was then stored at − 20 ℃ for further study.

PCR primers (Table 1) were synthesized from AIT Biotech, Singapore. PCR was carried out at a 25 μl reaction volume using a Master mix solution (One Taq R Quick-Load R 2x Master Mix with standard buffer, New England Biolabs, USA) consisting of 2x PCR master mix, 20 pmol primer in each, 150–200 ng of DNA template and nuclease-free water to make a 25 μl reaction volume. An oil-free thermal cycler (Proplex gradient PCR, USA) was used to amplify fragments of genomic DNA targeted in a PCR setting.

The thermal profile of 35 cycles of PCR reaction consists of an initial denaturation at 94 ℃ for 3 min followed by denaturation at 94 ℃ for 30 *sec*, annealing at 62 ℃ for 2 min (MTB complex), 56 ℃ for 1 min (*M. bovis*), 57 ℃ for 1.5 min (*M. tuberculosis*), extension at 68 ℃ for 5 min and final elongation at 68 ℃ for 7 min. The reaction was held at 4 ℃ and the PCR amplicons were electrophoresed in a 1.5 % agarose gel containing ethidium bromide (0.5 μg/ml) (WSE-1710Submerge-Mini2322100, China) for visualization. The DNA molecular weight marker type 100 bp DNA ladder

Table 1
Oligonucleotide primers used in PCR detection of specific causes of tuberculosis in slaughtered goats and sheep.

Genes targeted	Primers name	Primer sequence (5́-3́)	Organism/ Amplicon size	References
16S rRNA	TB 1-F TB 1-R	gaacaatccggagttgacaa agcacgctgtcaatcatgta	*M. tuberculosis* complex/ 372 bp	Wilton and Cousins, 1992
H37RvHP	H37RvHPF H37RvHPR	gaactcaccgtcggtggtga ccttgctcgatctctgcgtc	*M. tuberculosis*/ 667 bp	Hossain et al., 2016
MPB83	MPB83F MPB83R	cagggatccaccatgttcttagcgggttg tggcgaattcttactgtgccggggg	*M. bovis*/ 600 bp	Jiang et al., 2006

(lnvitrogen, USA) was used and images were captured on a transilluminator (Alpha imager, USA).

2.6. Statistical analysis

To estimate the occurrences of infection, data was entered into (Microsoft, Los Angeles, CA, USA) for descriptive analysis. The Pearson chi-square test for independence relatedness was performed by using SPSS 22 (IBM corporation, USA) to determine the significant differences in the occurrences of fascioliasis, M. bovis infection and co-infection in goats and sheep. A value of $P \leq 0.05$ was considered significant at a 95 % confidence interval.

3. Results

3.1. Gross pathological changes

Grossly, variable changes were seen in goats and sheep's liver, lungs and lymph nodes. In livers, hemorrhagic tracts were seen in 07 infected goats (8.33 %) and two sheep (12.5 %) and were associated with the acute phase of fascioliasis (Fig. 1A). Migratory tracts appeared small to larger and were distributed throughout the affected liver, most commonly in the central and left lobes. The color of the acutely infected liver progressively changed from brown-red to light brown and a red zone of hyperemia surrounded the larger tract. On the other hand, cirrhosis around the bile duct of the liver, forming a pipe-stem appearance, was seen in chronic phases of fascioliasis in 60 goats (71.43 %) and 07 sheep (43.75 %) (Fig. 1B). Fluke migratory tracts and cirrhosis were noted and large numbers of liver flukes were found escaping upon sectioning of the bile ducts (Fig. 1B-D). Moderately dilated and thickened bile ducts were seen protruding from the visceral surface of the central and left lobes. The dilated bile ducts were progressively narrowing from the hilus towards the periphery. There was engorgement of the ducts with brownish mucous exudates. On sectioning of the bile ducts, a gritty sound was felt, indicating a state of calcification. Five livers of goats (5.95 %) showed both the acute and chronic phases of fascioliasis characterized by both hemorrhagic tunnels and pipe-stem liver, respectively.

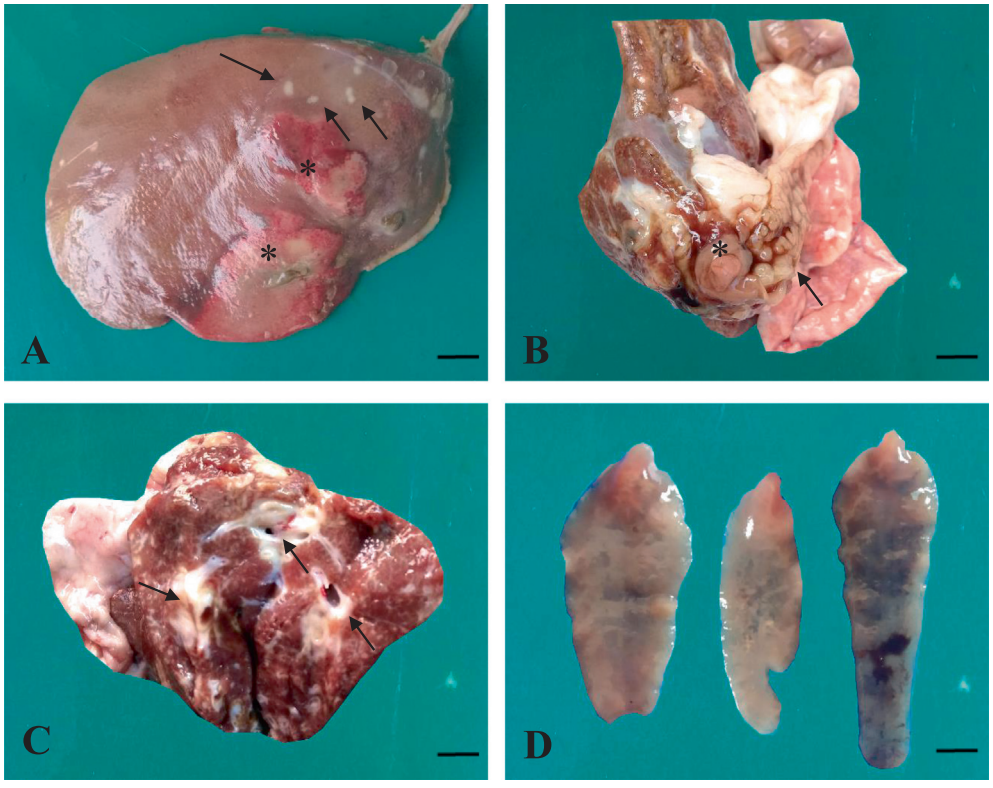

Fig. 1. Gross lesions of the livers of Fasciola infected goats. A: small size multiple white necrotic foci (arrows) and hemorrhages (asterisks) were seen on the parietal surface of the liver in acute fascioliasis. B: pipe-stem liver (arrow) and fluke was found to escacpe following sectioning of the bile ducts of the liver (asterisk) in chronically *Fasciola* infected goat. C: cirrhosis with migratory tracts of flukes were seen following sectioning of the liver (arrows). D: flattened *F. gigantica* flukes collected from gall bladder and bile ducts. Bar = 3 cm.

Granuloma with thick fibrous connective tissue encapsulation was seen in the sheep and goat liver, resembling lesions of tuberculous infection. Extensive cavitary lesions characteristic of tuberculous nodules were seen in the livers of 10 goats (11.9 %) and one sheep (6.25 %) (Fig. 2A). Among them, five goats and a sheep liver showed tuberculous nodules and pipe-stem liver. The liver of three goats showed tuberculous nodules but lacked lesions of pipe-stem appearance. Seven goat livers showed an irregular outer surface with yellowish discoloration and icteric. Nodular development in the bile ducts and obstructive jaundice were seen in the livers of two goats. Thick fibrous connective tissue encapsulation around the tuberculous nodules and a pipe-stem liver suggest co-infectivity due to *Fasciola* and TB (Fig. 3).

About 08 to 12 % of the mesenteric lymph nodes of the slaughtered goats (n = 21) and sheep (n = 03) examined showed overall enlargement. Grossly, the affected lymph nodes showed general enlargement of 25 % lymph nodes, apparently twofold larger than the normal ones, and the remaining 75 % affected lymph nodes showed a moderate to slight increase in size. Following sectioning of the enlarged lymph nodes, multiple white or pale yellowish colored small to medium-sized granulomas with central caseous necrosis were seen (Fig. 2B). There was mineralization in the caseous necrotic center (Fig. 2B), as detected by feeling the herd sound upon sectioning.

Lungs were mildly affected in slaughtered goats and sheep. Grossly visible tuberculous granulomas lesions were seen in the lungs of a goat (1.19 %). Three to four white or pale yellowish colored small to medium-sized granulomas with central caseous necrosis and calcification were seen (Fig. 2C). The nodules were slightly raised over the middle lobe of the right lung.

3.2. Histopathological changes

Histopathology of *Fasciola* and TB infected goats and sheep livers showed variable changes depending upon the extent and severity of infection with *Fasciola,* TB and combined infection due to *Fasciola* and TB. The microscopic changes in goats (n = 07) and sheep (n = 02) livers revealed hemorrhages (Fig. 4A-B), necrosis and the presence of migratory tracts of flukes in case of acute fascioliasis. The migrational tracts in the liver were mainly composed of eosinophilic debris of disintegrated hepatocytes infiltrated by numerous eosinophils and some neutrophils, lymphocytes and macrophages (Fig. 4B). In smaller tracts, the tendency to hemorrhage was low compared to widespread hemorrhages in larger tracts. There was pronounced coagulative necrosis of hepatic tissues surrounding the larger tracts. The incomplete rim of degenerated hepatocytes surrounding the larger tracts appeared more acidophilic compared to other areas distant from hemorrhagic tracts. Healing migratory tracts were seen in 04 goats of large necrotic areas. Macrophages were abundant in the areas of larger tracts. Proliferating fibroblasts noticed the reabsorbed areas of tracts. The proliferation of bile ductules was extensive along with the healing tracts and nearby portal triads. The epithelial cells in the bile ductules were hyperplastic and did not appear uniform. Arteritis and phlebitis were frequently seen (04 out of 07 livers of goats and 01 out of 02 livers of sheep) in the migratory areas. Microthrombi were seen in the blood vessels of adjoining migratory tracts.

In case of chronic fascioliasis, goat liver sections (n = 60) and sheep (n = 07) revealed widespread portal cirrhosis characterized by fibrous connective tissue proliferation around the portal areas of the liver accompanied by infiltration of mononuclear cells. Extensive fibrosis and calcification of the bile ducts were seen as characteristics of the pipe-stem liver /clay pipe liver. There was loss of the lobular pattern, proliferation of bile ducts and bile duct epithelium, and fibrosis in portal areas. The broad zone of compound glandular mucosa of the main bile ducts consisting of numerous irregularly dispersed tubuli and acini were severely necrosed. The walls of the ducts became infiltrated with eosinophils, lymphocytes, and macrophages and ultimately became thickened due to fibrous connective tissue proliferation. Calcified plaques were seen in the cicatrized areas of the bile duct. The intense proliferation and various regressive changes, including complete erosion caused by the spiny cuticles of liver flukes, were seen in the bile duct epithelium. In non-infected healthy areas of the bile duct, the epithelial cells were not traumatized by flukes, were tall, columnar and clearly defined with pale cytoplasm and several nucleoli in the basal nucleus. There was pressure necrosis of hepatocytes around the thickened and distended bile duct and the necrosed hepatocytes stained moderate to pink while staining the sections with H & E intensely. Also, biliary cirrhosis (Fig. 4C), portal triaditis, fibrous cholangitis and hepatitis associated with cholestasis were seen in chronic fascioliasis in goats. The livers of five goats showed both hemorrhagic tunnels and cirrhosis in the livers and the lesions consisted of both the acute and chronic phases of fascioliasis.

Histopathologically, the liver showed granulomas (including microgranulomas) in 14 goats (16.67 %) and two sheep (12.5 %). Microscopically, the granulomas consist of three layers (i.e, the outer zone composed of a large number of lymphocytes and mononuclear macrophages in the external layer; langhans giant cells and epitheloid cells in the intermediate layer; and the central core of the caseous necrosis and mineralization) (Fig. 5A). In some cases, the granulomas lacked characteristic three layers; the granulomas were infiltrated with lymphocytes and monocytes (Fig. 5B-C). Lesions consistent with both tuberculosis (tuberculous nodules) and fascioliasis (widespread biliary cirrhosis) were seen in the livers of 12 goats and two sheep. Two goats' liver showed tuberculous nodules; these livers lacked adult *Fasciola* in the bile ducts, but there was biliary cirrhosis and pipe-stem liver. Among them, fibrous cholangitis, hepatitis, cholestasis, biliary and portal cirrhosis with microgranulomas were seen in four goats and in a sheep

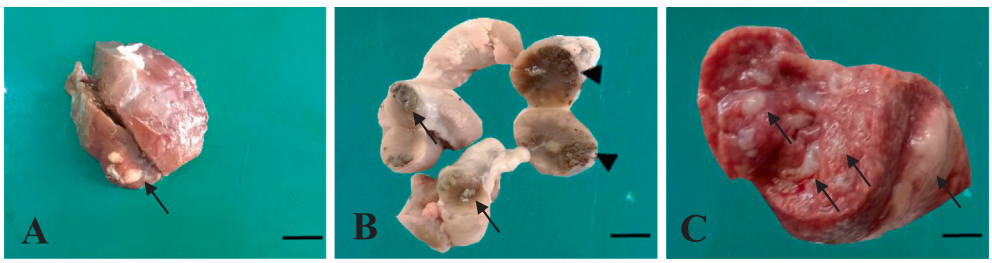

Fig. 2. Gross lesions of the liver, mesenteric lymph nodes and lung of tuberculosis infected goats. A: whitish multiple small sized caseous nodule (arrow) was seen on the parietal surface of the liver. B: calcification (arrowheads) and caseation (arrows) were seen following cross section of the mesenteric lymph node. C: whitish to yellowish small sized multiple nodule was seen in the lung (arrows). Bar = 3 cm.

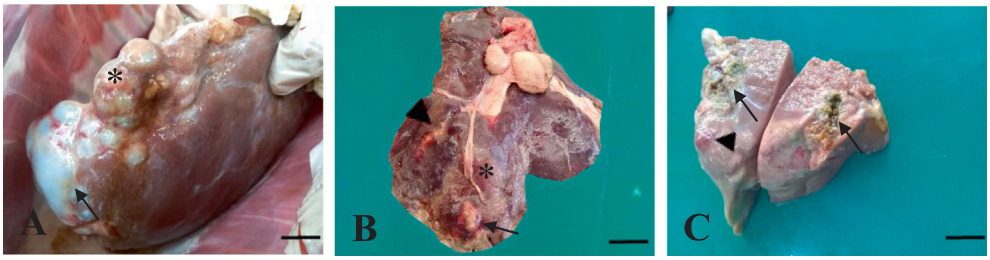

Fig. 3. Gross liver lesions of co-infectivity in tuberculosis and *Fasciola* infected goats (A-B) and sheep (C). A: caseous nodule (asterisk) and cirrhosis (arrow) were seen in goat liver. B: caseous nodule (arrow), icterus (asterisk) and pipe-stem liver (arrowhead) were seen in goat liver. C: caseous nodule (arrowhead) and calcified bile ducts (arrows) were seen in sheep liver. Bar = 3 cm.

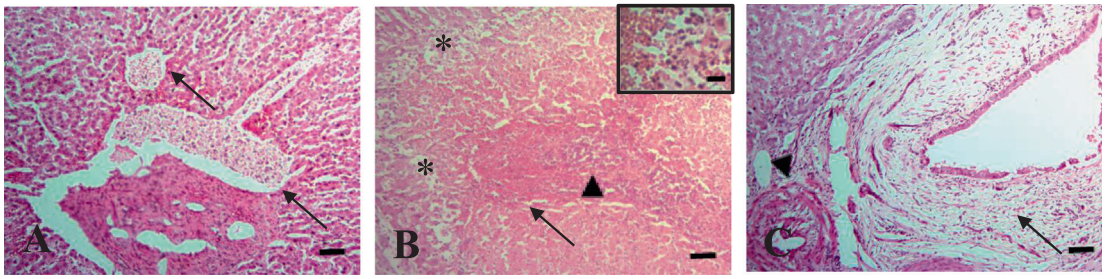

Fig. 4. Histopathology of the liver of *Fasciola* infected goats (A, C) and sheep (B). A: hemorrhages (arrows) were seen in the goat liver in acute fascioliasis. B: hemorrhages (arrow), necrosis (asterisks) of hepatocytes and focal infiltration of inflammatory cells (arrowhead), mainly neutrophils and mononuclear cells (inset: in higher magnification) were seen in sheep liver in acute fascioliasis. C: biliary cirrhosis characterized by proliferation of fibrous connective tissues (arrow) and hyperplasia of bile duct (arrowhead) were seen in chronically *Fasciola* infected goat liver. H & E stain. Bar (A-C = 100 μm; B (inset) = 50 μm).

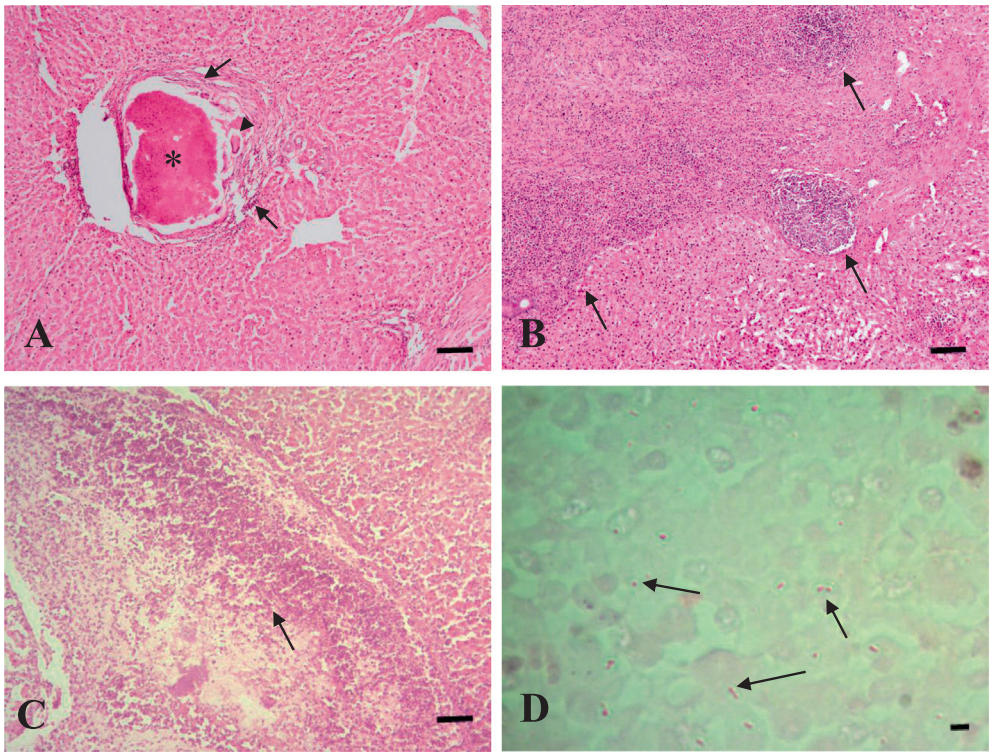

Fig. 5. Histopathology of the liver of tuberculosis infected goats (A-B) and sheep (C-D). A: caseous necrosis (asterisk) surrounded by fibrous connective tissues and accompanied by mononuclear cells (arrows) and langhans type giant cell (arrowhead) were seen in the goat liver. B: multifocal granuloma was seen in goat liver (arrows) C: granuloma (arrow) was seen in sheep liver. D: few acid-fast bacilli (red color) were seen in the macrophages of the granuloma (arrows). H & E stain (A-C); acid-fast stain (D). Bar (A-C = 100 μm; D = 50 μm).

(Fig. 7A and D), but the granulomas lacked characteristic three layers; the microgranulomas were infiltrated with lymphocytes and monocytes (Fig. 7A). Widespread necrosis of hepatocytes, cavitation and calcification, were seen in four goats and a sheep. In addition, widespread cirrhosis, hemorrhages and granuloma were seen in the livers of six goats (Fig. 7C). The bile duct wall was also thick-

ened and hyperplastic, forming a gland-like structure due to advanced stages of fascioliasis. Acid-fast staining of the liver section identified acid-fast bacteria in the macrophages of the granuloma in 14 goats (16.67 %) and two sheep (12.5 %) (Fig. 5D and 7B).

Enlarged mesenteric lymph nodes demonstrated granulomatous nodules in 21 goats (25 %) and 03 sheep (18.75 %). Encapsulation, caseous necrosis and calcification were seen in the granulomas (Fig. 6). In addition, acid-fast bacteria were seen in the cytoplasm of RE cells in 15 goats (17.86 %) and two sheep (12.5 %) as detected by acid-fast staining. Lung tissue sections stained with H & E and acid-fast staining showed granulomas in two goats (2.38 %). None of the sheep's lungs showed granulomas during the histopathological investigation.

3.3. PCR detection of TB

PCR targeting to the 16S rRNA gene of MTB complex amplified 372 bp fragments (Fig. 8) specific for infectivity due to MTB complex in the lymph nodes (n = 21), liver (n = 17) and lungs (n = 02) of goats. Sheep samples tested with MTB-specific PCR showed amplification of 372 bp fragments (Fig. 8) in the lymph nodes (n = 03), liver (n = 02) and lungs (n = 01). To identify further the specific cause of TB, PCR was performed based on the MPB83 and the H37RvHP genes to detect the infectivity due to *M. bovis* and *M. tuberculosis*, respectively. MPB83 gene of *M. bovis* specific PCR was carried out and a 600 bp amplicon (Fig. 9A-B) was generated in the lymph nodes, liver and lungs of 12, 10 and 02 goats,

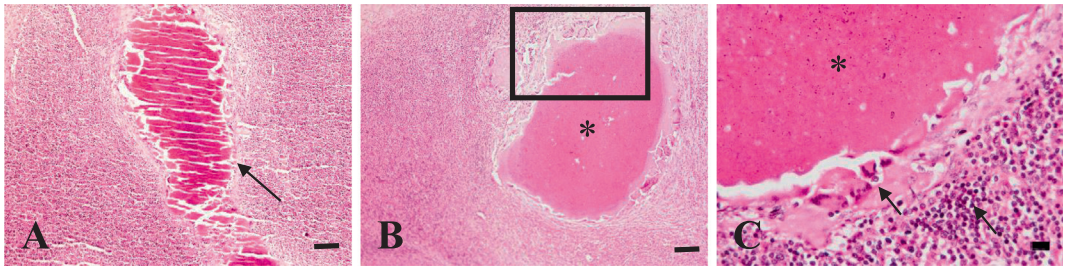

Fig. 6. Histopathology of the mesenteric lymph nodes of tuberculosis infected goats. A: calcification (arrow) surrounded by fibrous connective tissues were seen. B-C: caseous necrosis (asterisk) surrounded by fibrous connective tissues accompanied by infiltrates with mononuclear cells and langhans types giant cells were seen (arrows). H & E stain. Bar = 100 μm.

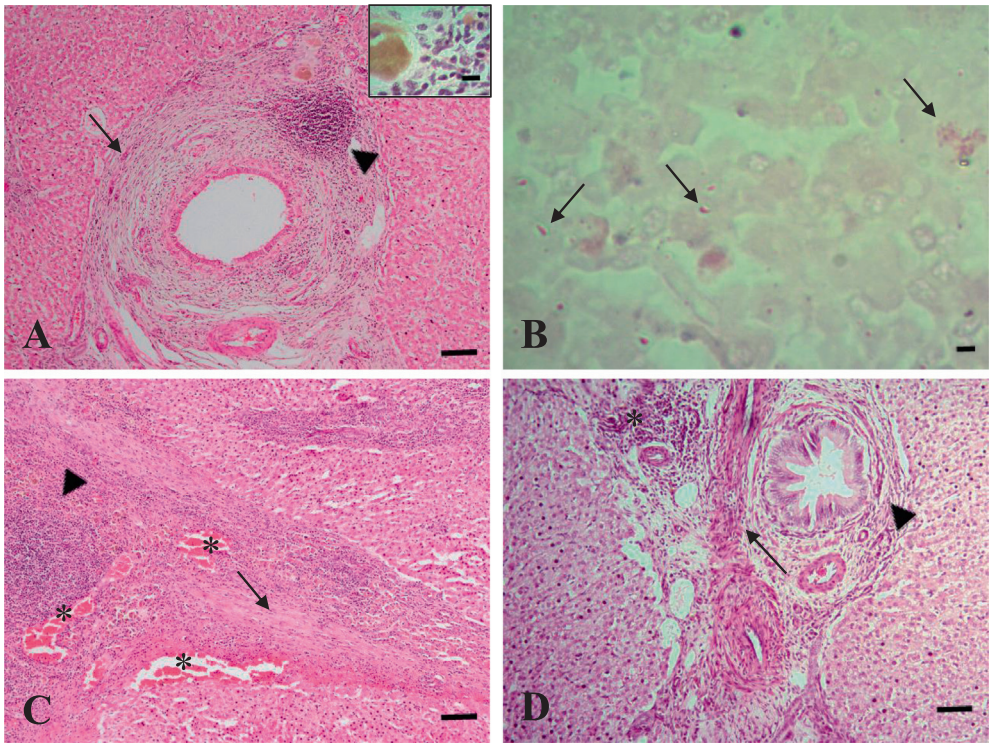

Fig. 7. Histopathology of the liver of co-infectivity in *Fasciola* and tuberculosis infected goats (A-C) and sheep (D). A: biliary cirrhosis characterized by proliferation of fibrous connective tissues (arrow), granuloma (arrowhead) characterized by infiltrates with macrophages and lymphocytes and retention of bile pigments in the canaliculi were seen (inset: in higher magnification) in goat liver. B: few acid-fast bacilli (red color) were seen in the macrophages of the granuloma (arrows) in goat liver. C: widespread cirrhosis characterized by proliferation of fibrous connective tissues (arrow) with granuloma (arrowhead) and hemorrhages (asterisks) were seen. D: portal cirrhosis (arrow), portal granuloma (asterisk) and hyperplasia of bile duct (arrowhead) were seen in sheep liver. H & E stain (A, C-D); acid-fast stain (B). Bar (A, C-D = 100 μm; A (inset) and B = 50 μm).

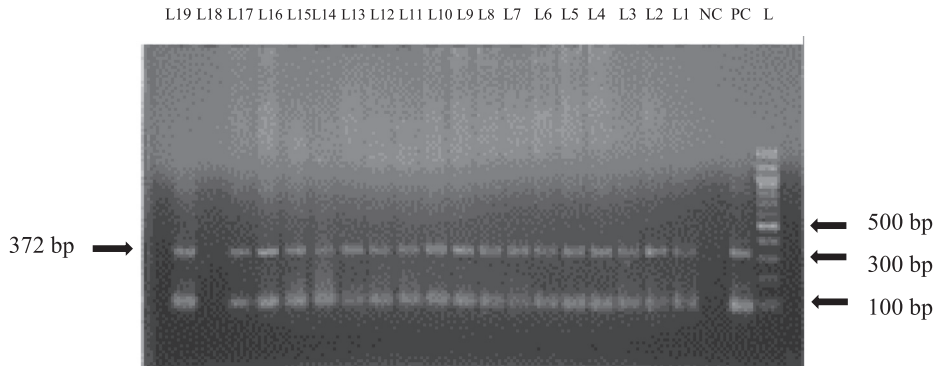

Fig. 8. PCR amplified products of 372 bp fragments of the 16S rRNA gene of *M. tuberculosis* (MTB) complex isolates from goats and sheep. L = DNA marker (100 bp), PC = Positive control, NC = Negative control, Lane 01–10 = representative MTB complex isolates from the mesenteric lymph nodes of goats; lane 11–15 = representative MTB complex isolates from the livers of goats; lane 16–17 = representative MTB complex isolates from the lungs of goats and lane 19 = representative MTB complex isolates from the liver of sheep.

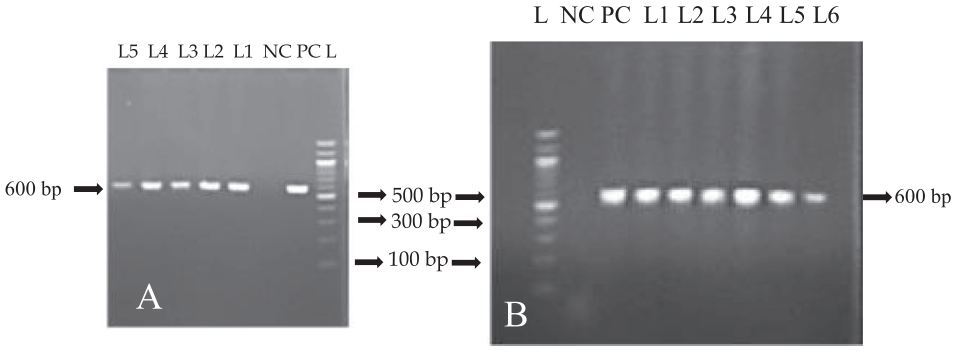

Fig. 9. PCR amplified products of 600 bp fragments of the MPB83 gene of *M. bovis* isolates from goats (A-B) and sheep (B). L = DNA marker (100 bp), PC = Positive control of *M. bovis*, NC = Negative control. A: Lane 01–03 = representative *M. bovis* isolates from the mesenteric lymph nodes of goats and lane 04–05 = representative *M. bovis* isolates from the lungs of goats. B: Lane 01–03 = representative M. bovis isolates from the livers of goats; lane 04–05 = representative *M. bovis* isolates from the livers of sheep and lane 6: representative *M. bovis* isolates from the mesenteric lymph node of sheep.

respectively. MPB83 gene specific amplification of genomic DNA was seen in the lymph nodes and liver of 02 sheep (Fig. 9B). Thus, 12 goats (14.29 %) and 02 sheep (12.5 %) were clinically or subclinically infected with *M. bovis*. However, the H37RvHP gene of *M. tuberculosis* (667 bp) specific amplification was not seen with the genomic DNA extracted from visceral organs and lymph nodes of goats and sheep. Specific mycobacterial infectivity in nine goats was not identified in this study. PCR results showed that TB occurrences in goats and sheep were higher in the mesenteric lymph nodes followed by liver and lungs.

3.4. Overall summary statistics

Out of 84 goats tested, 62 (73.81 %) were infected with *Fasciola* and 12 (14.29 %) with *M. bovis*; 12 (14.29 %) goats were co-infected with both *Fasciola* and *M. bovis* (Fig. 10). On the other hand, out of 16 sheep samples tested, 09 sheep (56.25 %) were infected with *Fasciola;* 02 (12.5 %) with *M. bovis*; and 02 sheep (12.5 %) were co-infected with both *Fasciola* and *M. bovis* (Fig. 10). *Fasciola* infection was significantly higher ($\chi 2 = 5.677$, df = 1, p = 0.017) in goats compared to sheep. The occurrences of *M. bovis* infection and co-infection due to *Fasciola* and *M. bovis* were relatively higher in goats compared to sheep, but not at a significant level ($\chi 2 = 0.0361$, df = 1, p = 0.850). Out of 21 goat samples examined for infectivity due to MTB, 12 goats showed *M. bovis* specific infectivity. This study could not identify specific causes of mycobacterial infectivity in 09 goats. None of the goats and sheep were infected with *M. tuberculosis*.

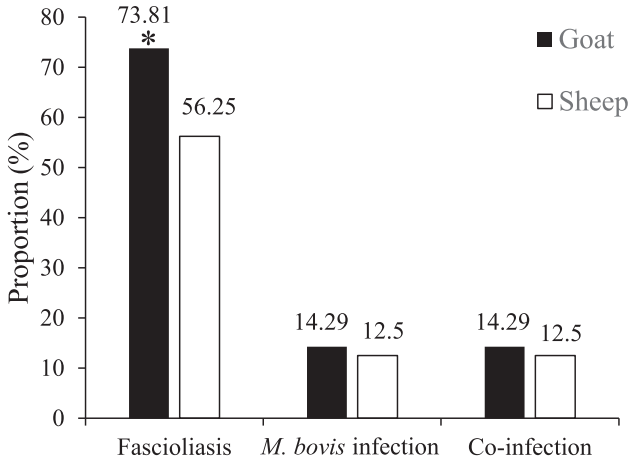

Fig. 10. The percentages of fascioliasis, *M. bovis* infection and co-infection in naturally infected goats and sheep. *p = 0.017 indicates significant differences in fascioliasis between goats and sheep.

4. Discussion

Co-infection of small ruminants has devastating economic consequences and, in most cases, co-infectivity due to peste des petits ruminants virus (PPRV) and goatpox virus (GTPV) is widely reported in Asia and Africa (Akanbi et al., 2020; Zhang et al.,

2021). The clinical manifestation, pathology and immunity of a single infection in small ruminants are incredibly varied from case to case across a population, making it difficult to conclude an accurate diagnosis. The clinical manifestation and detection are a bit more complicated in the presence of multiple infections or co-infections in small ruminants. Immunoregulation of helminths, like 'bystander' immunity to other pathogens, is a well-known phenomenon (Elias et al., 2007). The interactions of co-infecting pathogens are species-specific (both for host and parasite) and depend on context (du Plessis and Walzl, 2014; Rafi et al., 2012). There may be co-infection exacerbation effect of several diseases, including TB and fascioliasis, on the health of sheep and goats, because both of the diseases remain undiagnosed in flocks or grazing fields. Naturally, immunity in small ruminants against TB and *Fasciola* co-infected cases is variable and disease-specific immune response is difficult to detect. However, literatures describe that the host Th1 immunity is a common form of immunological response to TB infection in cattle, which is suppressed by fascioliasis (Flynn et al., 2007; Flynn and Mulcahy, 2008). *Fasciola* infections inhibit Th1 responses to mycobacterial infection and reduce interferon-gamma (IFN-γ) levels in cattle (Brady et al., 1999; Ezenwa et al., 2010; Kelly et al., 2018). The absence of this form of Th1 immune response in a susceptible host can result in an increased risk of TB in cattle (Flynn and Mulcahy, 2008). Nowadays, there is increasing evidence of *F. gigantica* co-infection with *M. bovis* in cattle (Kelly et al., 2018). However, data is lacking regarding the clinical manifestation, pathology, detection and immune response to co-infectivity of small ruminants due to *F. gigantic*a and *M. bovis* infection.

In this study, we confirmed the co-infection of *F. gigantica* with *M. bovis* in goats and sheep for the first time under natural conditions in Bangladesh by examining visceral organs collected at slaughter. It was impossible to determine which disease infected first or dominating infection and the development of subsequent pathological processes. In natural conditions, the order in which animals are exposed to each pathogen and the length of time elapsed between the exposures and the development of clinical pathology is unknown, rather the animals slaughtered were apparently healthy before sacrifice.

Diagnosing *F. gigantica* by visualization of the fluke at necropsy is considered the gold standard with 100 % specificity and sensitivity (Mazeri et al., 2016; Rapsch et al., 2006). In this study, *F. gigantica* was confirmed by observing the characteristic gross pathology in the liver and the presence of the fluke. The level of acute fascioliasis in goats (8.33 %) and sheep (12.5 %) appeared lower compared to chronic infectivity (71.43 % and 43.75 % in goats and sheep, respectively). In most cases, the goats and sheep are allowed to graze freely on the pasture land and consistently pick up the infection, thus, the level of chronic *Fasciola* infectivity is higher. Moreover, Bangladesh's geographical and climatic conditions are favorable for the development and propagation of *F. gigantica*. The prevalence of fascioliasis in Bangladesh vary from 10 to 32 % in goats (Islam et al., 2014, 2008; Rahman et al., 2014; Sammadar et al., 2015) and 8.4 to 31 % in sheep (Islam et al., 2008; Sangma et al., 2013). Here, we also observed that fascioliasis was higher in goats (73.81 %) than in sheep (56.25 %). A total of 17 chronically infected goats (28.33 %) and a sheep (14.29 %) showed pipe-stem liver but lacking adult parasites at necropsy, indicating that the goats and sheep may have been treated with flukicides. However, pipe- stem liver was still a dominant pathology observed at necropsy.

Grossly, *Fasciola* infected livers were mostly fibrotic with the pipe-stem appearance of bile ducts; this alteration may be due to constant irritation of adult flukes in the bile duct, which resulted in swollen bile ducts, the hyperplastic proliferation of duct epithelium and deposition of calcium salts with severe ductular fibrosis (Dharanesha et al., 2015; Talukder et al., 2010). Sometimes, bile ducts were blocked by flukes and the gall bladder and bile ducts were engorged with bile. Histopathologically, in acute fascioliasis, infected livers showed migratory tracts of immature flukes and hemorrhages accompanied by infiltrates of neutrophils and mononuclear cells, both in goats and sheep, which is consistent with acute fascioliasis. Cellular inflammatory reactions are due to the migration of immature liver flukes through the tissue, resulting in hemorrhages and inflammation. In case of chronic fascioliasis, there was biliary and portal cirrhosis due to proliferation of fibrous connective tissue accompanied by infiltration of mononuclear cells, thickening and hyperplastic changes in the lining epithelium of the bile duct. This may be due to the response of macrophages and lymphocytes in the necrotic areas during the later stages of fascioliasis and the merging fibrous tissues into the healing sites (El-Dakhly et al., 2008; Sayed et al., 2008). Fibrous cholangitis was observed in five cases. However, goats (n = 17) and sheep (n = 01) livers lacking adult *Fasciola* parasites in the gall bladder and distended bile ducts showed biliary and portal cirrhosis, thickening of bile ducts, calcification of bile ducts and fibrosis in bile ducts, characteristics of chronic fascioliasis.

Bovine TB is one of the most disreputable problems in the livestock industry worldwide, including in Bangladesh (Al Mamun et al., 2016; Hossain et al., 2016; Nahar et al., 2011; Yasmin et al., 2017). Small ruminants can serve as a silent reservoir of TB and dissemination of infection. Previously, goats and sheep were thought to have a minor role in tuberculous epidemiology as they were considered disease-resistant hosts or spillover hosts (Daniel, 2009). However, recent studies have demonstrated that goats and sheep can also be infected with TB at a higher rate (Gelalcha et al., 2019; Omar et al., 2021; Sanou et al., 2021; Zanardi et al., 2013). The caprine and ovine outbreaks of TB are caused predominantly by M. bovis and M. caprae (Gelalcha et al., 2019; Hiko and Agga, 2010) and few are caused by M. tuberculosis (Cadmus et al., 2009; Tschopp et al., 2011). The present study revealed M. bovis infection in goats and sheep, as they are closely related with regard to the immune response and pathological characteristics of *M. bovis* infection in cattle (Marianelli et al., 2010). Lesions suggestive of TB in goats and sheep revealed encapsulated caseo-calcified changes in the mesenteric lymph nodes. The liver and lungs also dominated caseous inflammatory lesions (Davidson, 1981; Hossain et al., 2016). Tuberculous lesions of small ruminants are mainly similar to the lesions seen in cattle (Daniel et al., 2009; Naima et al., 2011). In this study, granulomatous lesions were predominantly found in affected mesenteric lymph nodes (n = 21), followed by liver (n = 14) and lungs (n = 02) of goats. Microscopic lesions revealed granulomatous inflammation, consisting of central caseous necrosis with or without calcifications, infiltrated with macrophages, epithelioid cells, lymphocytes and langhans types of giant cells surrounded by fibrous connective tissues in the mesenteric lymph nodes, liver and lungs. The lesions were more extensive in the mesenteric lymph nodes than in the liver and lungs. However, Aljameel et al. (2017) reported that the lungs were the most frequently affected organs. Few acid-fast bacilli were seen in the edges of the caseous necrosed area, in epithelioid cells and Langhans types of giant cells (Malone et al., 2003). The mesenteric lymph nodes (n = 03) and liver (n = 02) of sheep were found to contain granulomatous lesions characteristic of TB. To the best of our knowledge, this is for the first time describing the details of the pathological investigation of TB in small ruminants in Bangladesh. As both large and small ruminants are maintained closely in the grazing field, this may increase susceptibility and transmission of TB in small ruminants in Bangladesh. However, it is unclear whether the infectivity of small ruminates due to TB or fascioliasis alone or in combination favors the development of pathology in infected animals. Diagnosing sub-clinically infected or co-

infected cases of TB and fascioliasis of small ruminants are significant concerns for detecting and managing these disorders.

The accurate diagnosis of bovine TB in sub-clinically infected cases is hindered by *Fasciola* infection, as fascioliasis reduces the IFN-γ level, resulting in false-negative results (García-López et al., 2018; Kelly et al., 2018). Moreover, hypersensitive skin tests like the cervical intradermal tuberculin test and comparative intradermal tuberculin are seldom practiced in the small ruminants industry due to the thinness of the skin. Furthermore, lesion detection and bacterial culture also confer poor applicability of TB detection technology in live animals (Howell et al., 2019). The development of technologies for the detection of TB in live small ruminants is a big deal, and detection of lesions or tuberculous organisms at necropsy are acceptable alternatives. Therefore, the level of infectivity, the link between tuberculous infection and risk of catching liver fluke infestation, and alternatively, liver fluke infestation and predisposition to tuberculous infections of farm ruminants remain unreported. A study in Zambia revealed that liver fluke-infected cattle showed five times more chance of catching bovine TB than those without fascioliasis (Munyeme et al., 2012). Similar findings were also reported in cross-bred cattle in an African tropical ecology (Kelly et al., 2018). In both cases, F. gigantica was reported to be the endemic liver fluke, and the dairy cattle in these areas were more exposed to bovine TB, and a link between liver fluke infestation and tuberculous infectivity was proposed. Like Zambia and tropical African countries, fascioliasis is endemic in cattle and small ruminants in Bangladesh, but there is no routine TB testing or surveillance program for small ruminants. Therefore, this study was designed to identify the infectivity of slaughtered goats and sheep with *Fasciola* and TB using necropsy, histopathology and PCR.

The co-infected liver showed caseous nodule, icterus and thick fibrous capsule encapsulation over the parietal and visceral surface in this study. Firm whitish areas within the liver parenchyma regarded as fibrosis were seen and these areas were considered as migratory tracts of flukes. Microscopically, the lesions revealed widespread biliary cirrhosis with thickened and proliferated bile ducts forming a gland-like appearance. In some cases, granulomas, hepatitis associated with cholestasis and granuloma, caseous necrosis encapsulated with fibrous connective tissues, fibrous cholangitis with microgranuloma and the presence of acid-fast bacteria in the macrophages of the granuloma were the predominant lesions observed, which was consistent with the lesions of both TB and fascioliasis (Aljameel et al., 2017; El-Dakhly et al., 2008; Talukder et al., 2010). Lesions suggestive of both TB were further tested by PCR targeting the16S rRNA gene of MTB complex, the MPB83 gene of M. bovis and the H37RvHP gene of M. tuberculosis. We confirmed M. bovis infection in goats and sheep by using PCR, but none of the samples yielded M. tuberculosis specific amplicon. In this study, 21 goats (mesenteric lymph nodes/ liver/ lungs) showed MTB specific amplification in PCR (372 bp) and M. bovis infectivity was confirmed in 12 cases. Nine cases appeared to yield MTB specific amplicon (372 bp) in PCR, but specific species of *Mycobacterium* were not identified; these goats may have been infected with M. caprae or other species of *Mycobacterium.* Previously, different diagnostic techniques such as necropsy and histopathology, acid-fast staining, culture and IFN-γ assay were conducted to detect co-infectivity due to *Fasciola* and TB (Kelly et al., 2018). Here, we performed necropsy and histopathology, acid-fast staining and PCR technology; PCR appeared to be the most sensitive, accessible and accurate method to detect the specific cause of TB in slaughtered tissue samples (Ramos et al., 2015). In contrast to goats, the tuberculous infection or liver fluke infestation was lower in sheep. Sheep have traditionally been considered less susceptible to tuberculous and *Fasciola* infection than goats (Caswell and Williams, 2007). Fascioliasis affects the liver and the liver is the site of protein synthesis, thus, fascioliasis suppresses Th1 and Th2 immune responses to the host (Cwiklinski et al., 2016). This study showed widespread fascioliasis in small ruminants. The affected animals showed hepatic lesions to varying extents. Therefore, a suppressed Th1 immune response due to *Fasciola* infection is proposed and this may favor M. bovis infection in goats and sheep.

5. Conclusion

This is the first time describing the co-infection of goats and sheep due to *F. gigantica* and *M. bovis* in natural conditions in Bangladesh. Fascioliasis affects the liver and consequently the immune system; thus, the infected liver may render susceptibility to tuberculous infectivity in small ruminants. The infectivity of mesenteric lymph nodes appeared much higher than in the liver and lungs, indicating an oral route of infection. This study showed that goats and sheep destined for slaughter were clinically or sub-clinically infected with fascioliasis and *M. bovis. M. bovis* is a highly zoonotic pathogen and small ruminants at slaughter and in the grazing field may silently disseminate *M. bovis* to other susceptible hosts. Since bovine TB control and eradication are the current global issues, therefore, during an active bovine TB control and eradication program, *F. gigantica* infestation should be considered. Goats and sheep are the neglected small ruminants in Bangladesh, and very rarely are the herd health or health of individual goats and sheep monitored at the household level. Thus, infectivity in goats and sheep due to bovine TB and fascioliasis remains unidentified in Bangladesh. Sheep and goats require deworming at regular intervals to control liver fluke infection. Routine TB testing or surveillance programs for small ruminants should be strengthened. As tuberculin tests are seldom performed on small ruminants, it needs to develop technologies to detect TB in small ruminants in order to minimize the spread of TB from slaughtered sheep and goats.

6. Authors' contributions

NS, MP and MAHNAK: planned, designed, generated research funds and developed the manuscript for submission. NS, SS, MM and TTM helped in collecting and processing samples and did DNA extraction. NS: conducted PCR, gel electrophoresis and statistical analysis. NS and MP: conducted histopathology. NS, MP and MAHNAK: Drafted and revised the final manuscript. All authors read and approved the final submission.

Declaration of Competing 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.

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