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Molecular characterization and histopathological studies on *Fasciola gigantica* in Mithun (*Bos frontalis*)

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ABSTRACT

Mitochondrial DNA sequence of the sub-unit 1 of cytochrome c oxidase (Cox1) and ribosomal DNA sequence of the first internal transcribed spacer (ITS-1) of *Fasciola* collected from mithun and cattle from Arunachal Pradesh, India were characterized at molecular level in order to identify the species of parasite. Based on sequence and phylogenetic analysis, the identity of the parasite was confirmed as *Fasciola gigantica* in mithun as well as from cattle. In order to know histopathological alteration in *Fasciola* infection, a histopathological study was performed on eight liver specimens, out of 110 animals studied during the period from 2010-2016. Histopathological examination of *F.gigantica* infected liver showed extensive fibrous connective tissue proliferation with necrosis of hepatocytes and infiltration of polymorphonuclear cell. There was evidence of migratory tracts of parasites with losing of normal lobular hepatic architecture. Bile duct proliferation was followed by congestion of portal vein with perivascular cuffing with surrounding degeneration of hepatocytes. The hepatocytes showed pyknosis with hyperplasia of bile duct.

Key words: *Fasciola gigantica*, Mithun, Molecular identification, Pathological alteration.

INTRODUCTION

Mithun (*Bos frontalis*), a magnificent bovine species, is confined to North Eastern hilly region of India particularly in the states of Arunachal Pradesh, Nagaland, Manipur and Mizoram. Besides, this animal is also found in Chittagong district of Bangladesh, Myanmar, Malaysia and Yunan province of China. Like other bovines, mithuns are susceptible to various diseases including parasitic diseases causing mortality and high morbidity (Rajkhowa *et al*. 2005, Chamuah *et al.* 2009, Chamuah *et al*. 2013a, Chamuah *et al*. 2013b and Chamuah *et al.* 2015). Further endoparasitic infection produces ill effects, immuno- suppression and predisposes the animals for various potential pathogens (Kaur *et al.* 2019; Moudgil *et al.* 2018). Establishing a data base on the species infecting particular host and to predict the pathogenesis of the disease by performing epidemiological studies is of utmost importance and needs attention (Bal *et al.* 2014; Kaur *et al.* 2016). Fasciolosis, one of the most important helminthic diseases, is associated with decreased productivity in terms of meat and milk and huge economic losses due to the condemnation of infected liver and death of the animals. Though the prevalence of *F. gigantica* infection in mithun is reported (Tandon *et al.* 2005, Rajkhowa *et al*. 2005), the information on genetic characterization of the *Fasciola* in mithun is scarce.

Molecular methods have assisted in the identification and genetic characterization of *Fasciola* species in different geographical regions. The high level of diversity within the mitochondrial genes allows the differentiation of parasite populations. The distribution of *Fasciola* spp. seems to follow a confined geographical pattern with a predominant distribution with *F. hepatica* and *F. gigantica* in temperate and tropical regions of Asia and Africa, respectively (Terasaki *et al.* 1998; Ashrafi *et al.* 2006). Molecular characterization along with identification of helminth parasites in mithun was also achieved by Chamuah *et al*. (2016a). Nevertheless, it is quite interesting to note their co-existence in some geographical areas (Itagaki *et al*. 2005). The parasites with hybrid / introgressed genotypes between *F. hepatica* and *F. gigantica* are also reported which represent the emergence of probably a new species so as to adapt to the changing climatic conditions. This has led to a curiosity to find the species of parasite prevalent in mithun population of North Eastern states of India.

Molecular phylogeny using highly polymorphic mitochondrial genes such as cytochrome oxidase 1 (Cox1) elucidates the origin and source of infection besides the identification of species of the parasite. Also, ribosomal DNA markers like internal transcribed spacer regions (ITS) are available in high copy numbers and contain variable regions flanked by more conserved regions. Therefore, the present study was designed for accurate identification of the parasite based on the mitochondrial marker (Cox1) and ribosomal

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marker (ITS-1) along with studying the pathological alteration of *Fasciola* infected mithun from Arunachal Pradesh of India.

MATERIALS AND METHODS

Collection of samples

A total of 110 mithuns, sacrificed during different tribal rituals and festivals in Itanagar, Arunachal Pradesh between 2010-2016 were examined for fasciolosis. The adult flukes were detected from the liver of eight animals (7.27%).

Gross identification of parasite

Fasciola flukes were washed with phosphate buffer saline (pH 7.2) and transported in same buffer to the laboratory. The grossly visible parasites collected from livers were identified based on the morphological characteristics (Soulsby, 1986).

Histopathology

At the time of collection of the parasite, gross lesions of liver were recorded. The infected tissue samples were fixed in 10% formalin followed by dehydration in ascending grade of alcohol. The routine histopathological studies were carried out by haematoxylin and eosin staining method as described by Luna (1968) during the period of 2018.

Molecular identification based on Cox1 and ITS-1

Genomic DNA was extracted from each fluke using commercial genomic DNA extraction kit according to the manufacturer's instructions (DNeasy® Blood and Tissue Kit, Qiagen, Germany). The DNA concentration was measured in Nanodrop spectrophotometer and stored at -20°C until use. For PCR amplification of ITS-1 and Cox I regions, 100 ng of DNA was used as a template in a reaction volume of 25 µl. DNA fragments of each target region were amplified using gene specific primers (Table 1). The PCR conditions consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles with denaturation at 94°C for 60 s, annealing at 60°C for 45 s and primer extension at 72°C for 60 s with a final extension of 72°C for 10 min. The resulting amplicons were checked by agarose gel electrophoresis. The 100 bp DNA ladder was used to estimate the sizes of the amplicons. The PCR products were purified using Gel extraction kit (QIAquick® Gel Extraction Kit, Qiagen) and custom sequenced at University of Delhi, New Delhi.

Phylogenetic analysis

The sequence was analyzed by various bioinformatics tools like GeneTool, DNA STAR and MEGA 6.0 software for identification of the parasite up to the species level. The retrieved sequences were aligned with the existing sequences of isolates from different countries available in National Centre for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST). The phylogenetic tree was constructed using neighbor-joining method. All characters were given equal weightage and the alignment gaps were treated as missing data. Bootstrap analysis was conducted using 1000 replicates.

RESULTS AND DISCUSSION

The adult flukes were detected in the liver of eight (7.27%) animals. Morphologically, adult parasites were large, leaf shaped, elongated, the cephalic cone prominent without shoulders, characteristics branching of intestinal caeca and uterus confirmed the identity of parasites as an *F. gigantica*.

Genotypic characterization based on cytochrome oxidase 1 (Cox1) and ITS1 markers

The PCR targeting Cox1 gene of *F. gigantica* (mithun isolate) yielded a product of 447 bp (Fig 1). The sequence results were used to identify the species and genotype of the parasite. The Cox 1 gene showed 100% similarity with other isolates of Thailand and China (KF687896; AJ628033 and AJ628022). Likewise, ITS-1 sequence analysis also showed 100% identity with Indian isolate of *F. gigantica* (KX198631), Tehran isolate and Egypt isolate (FJ756395; LCO776127. Sequence analysis of cattle isolate also shared same sequence with *F.gigantica*.

The amplification of ITS-1 yielded a product of 490 bp in all the specimens (Fig 2). The sequence analysis of ITS-1 region of *Fasciola* from mithun with that of *F. gigantica* (EF 198867) from India revealed an identity of 98.4% with

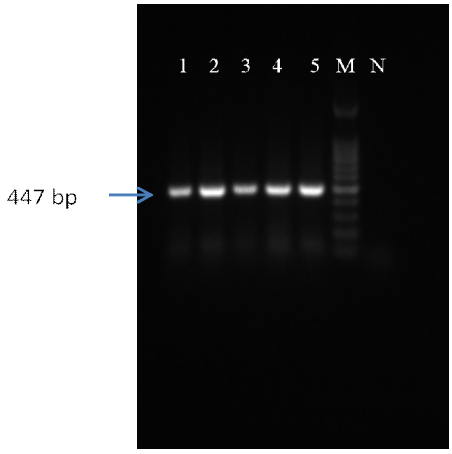

Fig 1: PCR amplification of CO1 region of *Fasciola* sample; 1 to 5=*Fasciola* sample,M=Marker, N=Negative control.

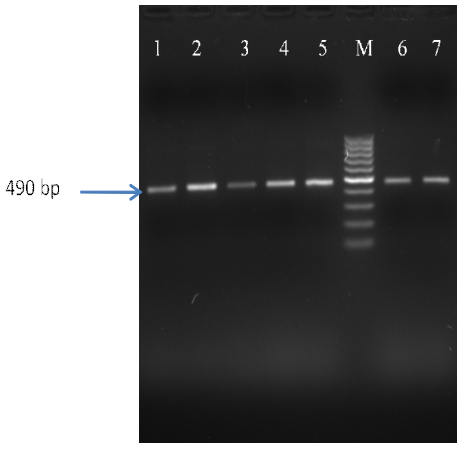

Fig 2: PCR amplification of ITS-1 region of *Fasciola* sample no.1 to 7, M=Marker.

six mismatches in positions 24 (C to T), 114 (A to T), 208 (C to T), 286 (T to A), 306 (C to T) and 371 (G to C). The identity between *F. hepatica* and *Fasciola* from mithun ranged from 93.8%-96.8%. This clearly indicates that the liver flukes collected from mithun are maternally linked to *F. gigantica*. The phylogenetic analysis also revealed clustering of *Fasciola* from mithun with *F. gigantica* (Fig 3).

The Phylogenetic trees were constructed by comparing ITS1 sequence of *Fasciola* collected from mithun with those of other fasciolid species deposited in the GenBank (Fig 4). Neighbor-joining algorithms were used to construct phylogenetic tree and the phylogenetic tree showed that *F. hepatica* and *F. gigantica* were separated in two clusters and *Fasciola* from mithun clustered with *F. gigantica* isolated from different parts of the country.

Gross pathology

Grossly, the infected liver was pale, swollen, firm in consistency with the small irregular whitish area. On histopathological examination, varying degree of changes was observed depending on the intensity of infection. Multiple adult *Fasciola* parasites were seen embedded in the liver parenchyma. There was extensive necrosis of hepatocytes, hemorrhage along with fibrous tissue proliferation around the parasites with infiltration of inflammatory cell (Fig 5). There was also evidence of migratory tracts of the parasites with eosinophilic debris.

Necrosed hepatocytes surrounding such tracks appeared more dark-stained compared to adjacent areas which showed fibrous tissue proliferation and cellular infiltration (Fig 6). In some of the sections, parasitic eggs were also noted. Mild to extensive biliary proliferation and cirrhosis with eosinophilic and macrophagic infiltrations in the large portal tracts were evident (Fig 7). In the case of chronic infection, hyperplasia of bile duct with massive infiltration of inflammatory cells and fibrosis were observed (Fig 8). The walls were thickened and fibrotic with a rugged inner surface. The lobular hepatic architecture of the organ is lost due to heavy infection in most of the cases. Cases with mild infection, congestion of portal area with perivascular cuffing with surrounding degeneration in the cytoplasm and pyknosis of the nuclei of hepatocytes was noticed adjacent to the portal area (Fig 9). The adjoining portal areas and the hepatic sinusoids were abundantly infiltrated by numerous eosinophils and some lymphocytes and macrophages.

Flukes belonging to the genus *Fasciola* cause substantial economic losses to the livestock industry and significant public health problems (Chhabra and Singla 2009; Lin *et al.,* 2011). There are increasing reports about the

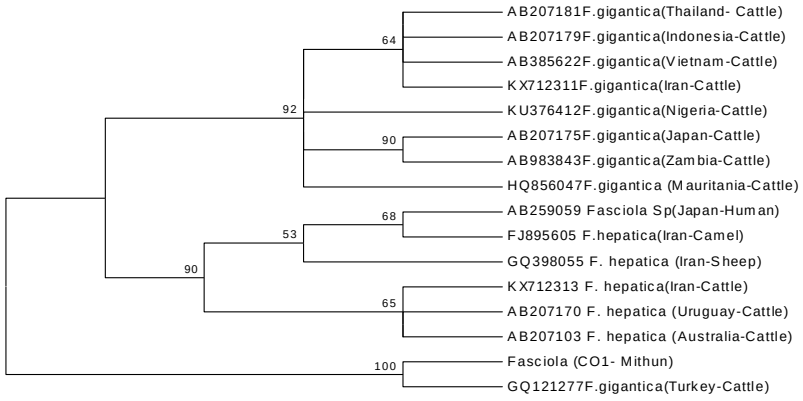

Fig 3: Phylogenetic tree of *Fasciola* from mithun inferred from the nucleotide sequences of partial CO1 and the neighbor-joining method using MEGA 6 software.

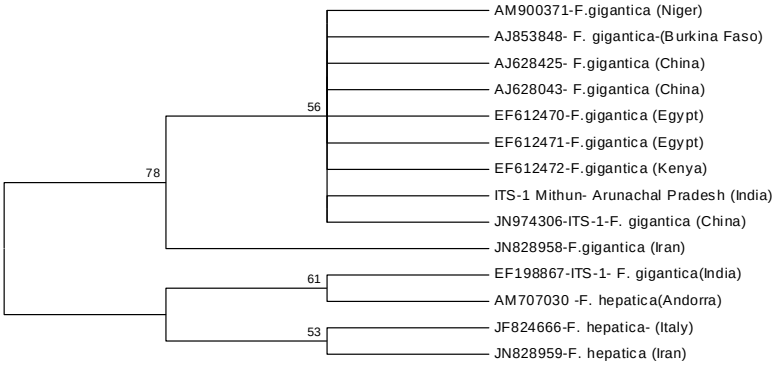

Fig 4: Phylogenetic tree of *Fasciola* from mithun inferred from the nucleotide sequences of ITS-1 and the neighbor-joining method using MEGA 6 software.

existence of pure and mixed forms of *Fasciola* particularly in Asian countries like Japan (Terasaki *et al.* 1998, Itagaki *et al*. 2005), Iran (Ashrafi *et al.* 2006) Vietnam (Le *et al.* 2008, Itagaki *et al*. 2009) and China (Huang *et al.* 2004, Lin *et al.* 2007, Peng *et al*. 2009). In India, fasciolosis is very common in cattle, buffalo and sheep with *F. gigantica* being the predominant species while *F. hepatica* has been reported from high altitude (Himalayan region) of the country (Sharma *et al.* 1989). The prevalence of fasciolosis in both free ranging and semi intensive system of mithun rearing was also recorded by Chamuah *et al.* (2014). Interestingly, in an earlier study by Varma (1953), existence of flukes with morphology neither typical of *F. gigantica* nor *F. hepatica* was reported in India which was assigned to a new species as *F. indica*. But further confirmation on the existence of *F. indica* in India is lacking. However, the existence of both pure *F. gigantica* and *Fasciola* with mixed genotype prevalent in the domestic ruminants in India was confirmed based on ITS-2 sequence (Raina *et al*. 2015).

Previous studies based on ITS-2 marker revealed *F. gigantica* as the predominant species in cattle in the North Eastern region of India (Prasad *et al*. 2009). In the present study *Fasciola* flukes collected from mithun were genetically characterized based on ITS-1 and Cox1 molecular markers which identified the species in mithun as *F. gigantica*. At the same time, sequence analysis of ITS-1 and Cox1 from cattle and buffalo also revealed the same identity of parasites. In an earlier attempt to genetically characterize *Fasciola* in mithun, ribosomal DNA sequences of the ITS-2 and 28S rDNA were targeted which revealed the identity of the parasite as *F. gigantica* (Chamuah *et al.* 2015). However, the present study was carried out on ITS-1 and Cox1 that

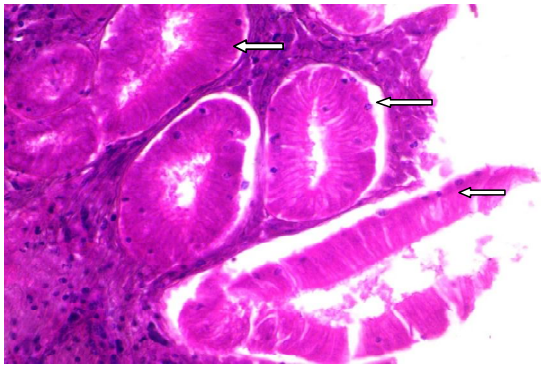

Fig 5: Multiple Adult *Fasciola* parasites (arrows) seen embedded in the liver parenchyma. Necrosis of heparocytes, haemorrhage and fibrous tissue proliferation around the parasite with infiltration of polymorphonuclear cell. X200. H& E.

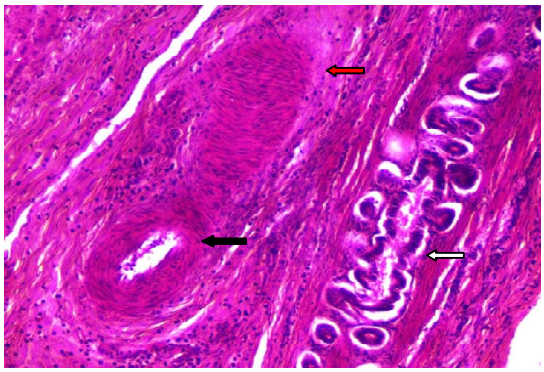

Fig 6: Parasitic tract of the parasite with fibrous proliferation (white arrow) and cirrhosis (red arrow) is observed in the liver parenchyma. Bile duct proliferation (black arrow) with infiltration of inflammatory cells. X100. H&E.

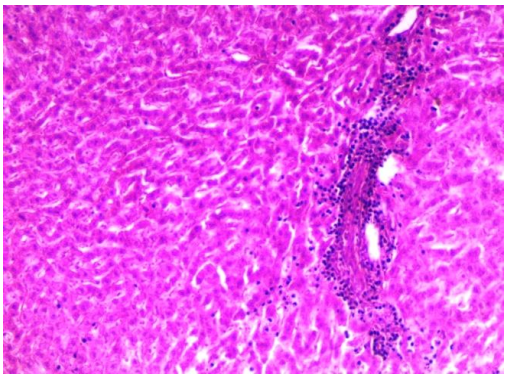

Fig 7: The chronic case of the infection showing cellular infiltrations of the tracts, necrosis of the hepatocytes and vascular walls Degeneration in the cytoplasm of hepatocytes with pyknosis of the nuclei. x200 H&E.

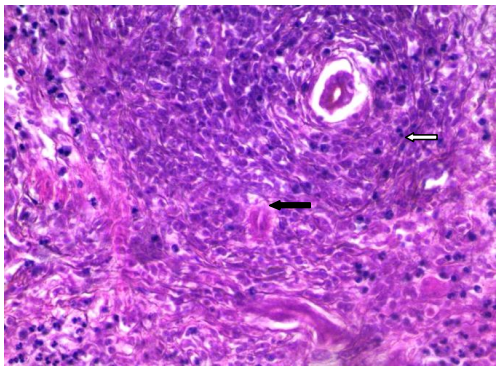

Fig 8: Hyperplasia of bile duct with massive migration of inflammatory cells and fibrosis (white arrow). Parasitic eggs (black arrow) were also observed. X400 H&E.

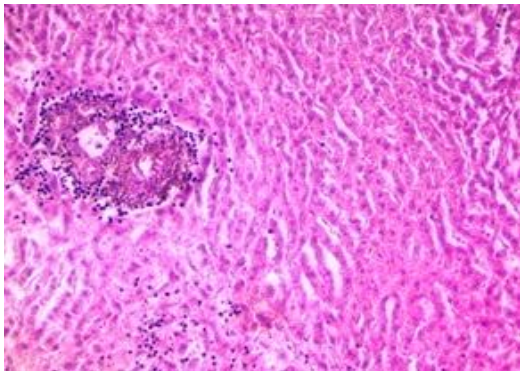

Fig 9: Parasitic tract of the parasite with fibrous proliferation and cirrhosis is observed in the liver parenchyma. Bile duct proliferation with infiltration of inflammatory cells. X100. H&E.

further confirmed the *Fasciola* in mithun as *F. gigantica*. In the present study, we could not come across the parasites with mixed genotypes/ hybrid forms.

Prasad *et al.* (2008) studied the phylogenic relationship of *Fasciola* spp. of Assam, India based on rDNA molecular data. ITS regions were sequenced and compared with other species of trematodes in the family fasciolidae. The phylogenetic tree based on the ITS (1 and 2) sequences revealed a close relationship with isolates of *F. gigantica* from China, Indonesia, Japan, Egypt and Zambia; the isolate from China with significant bootstrap values being the closest. In Thailand identification of *Fasciola* species as *F.gigantica* and their intermediate forms were identified based on mitochondrial enzyme Nad1 and Cox1 (Wannasan *et al*. 2014).

In the present study on histopathology, biliary hyperplasia, fibrous tissue proliferation with perivascular cuffing and loss of normal hepatic architecture was in quite agreement with the earlier finding of Chamuah (2005). The pathological study on helminth parasitic infection was also reviewed in mithun from north eastern hilly region of India (Chamuah *et al.* 2017, Chamuah *et al.* 2016b). In *F. gigantica* infection, fibrosis and calcification were common in condemned livers and in most cases these were severe in the vicinity of bile ducts (Phiri *et al.* 2006). In most of the cases, migratory juvenile flukes digest hepatic tissues causing extensive parenchymal destruction with intensive haemorrhagic lesions and immunological reactions as recorded by Gajewska *et al*. (2005). Severe destruction was noticed due to release of enzyme protease which has direct negative impact on liver parenchyma (Gajewska *et al*. 2005). Lesions associated with the migration of immature flukes through the parenchyma were a prominent feature of infection with *F. gigantica* (Wiedosari *et al.*1991). The size of the hepatic lesions increased during the course of infection and with progressive increase in cellular infiltration, biliary hyperplasia also increased. The mithun studied in the present research work hailed from the area of Papumpare district particularly area of Doimukh and Saglee which shares boundary with low lying area of Lakhimpur district of Assam where *Lymnaea* snail intermediate host is quite abundant that could be the source of infection to mithun. The present findings of pathological alterations in the mithun liver are in quite agreement with findings in cattle and buffalo (Haroun and Hussein, 1975).

CONCLUSION

In the present study, *Fasciola gigantica* was identified based on the mitochondrial DNA sequence of cytochrome c oxidase sub-unit 1(Cox1) and ribosomal DNA sequence of the first internal transcribed spacer (ITS-1) of *Fasciola* collected from mithun and cattle of Arunachal Pradesh, India. In the histopathological examination of affected liver, extensive fibrous connective tissue proliferation along with necrosis of hepatocytes and infiltration of polymorphonuclear cell along with evidence of migratory tracts of parasites with hyperplasia of bile duct was observed.

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CONFLICT OF INTEREST

There is no conflict of interest among the authors.

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