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# Morphologic and molecular investigation of schistosomes from the mesenteric vein of slaughtered cattle

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***Abstract:*** *In cattle, schistosomosis, caused by different species of helminthes under the genus Schistosoma (Trematoda: Schistosomatidae), is a chronic and wasting disease, contributing considerable economic losses through impairment of production. Accurate diagnosis of Schistosoma spp.by traditional and molecular methods is the key to its management.A totalof 64 cattle mesentery were collected from different slaughter houses of Mymensingh district, Bangladesh. This study has shown that out of 64 cattle examined, 40 were infected with schistosomes species. Tentatively, we identified different species of Schistosoma on the basis of morphologic and morphometric analysis.Up to 1-400 adult Schistosoma indicum or Schistosoma spindale were detected from a positive sample but could not confirm the co-infection state.Histopathological examination revealed cross-section of schistosomes in lymphatics and veins of mesentery. To identify species-specific schistosomes this study developed a new approach where two specific primers (SI16sRNA and SPMit) were designed and used to amplify the genomic DNA of S. indicum and S. spindale on selected eight sample by polymerase chain reaction (PCR). The SPMit primer amplified a fragment of 330bp which was positive for all eight samples (100%) of schistosomes whereas SI16sRNA primer amplified a 606 bp fragment which was positive for three samples (37.5%). Three samples showed mixed infection with both species of S. indicum and S. spindale.In conclusion, Cattle of Mymensingh district were infected/co-infected with schistosomes and can be a cause of ill health, require further observation.*

***Keywords:*** *Mesentery, Molecular Investigation, PCR, Schistosoma indicum, Schistosoma spindale.*

## I. Introduction

The role of livestock sub-sector is vital for the economic development of agro-based Bangladesh. The contribution of livestock to National Gross Domestic Product (GDP) of Bangladesh is 2.5 %. There are about 23.2 million cattle in Bangladesh which play an important role in the national economy [1]. About 44%of the animal protein comes from livestock sources. Moreover, 4.3% of the total export is from the export of leather and leather goods. Furthermore, 20% of the population is directly and 50% of the population is partially dependent on livestock sector [2]. Most cattle population in Bangladesh comes from primitive and low productive breeds. Most animals are reared in house under the shade of old traditional husbandry practices with inadequate and imbalance ration [3]. As a result the general nutritional status of most of the cattle is in subnormal level, which greatly increases susceptibility to parasitic diseases. Among the parasitic diseases, schistosomosis is an important parasitic disease of man and animals [4]. Schistosomosis is one of the most prevalent infectious diseases, endemic in more than 70 countries, mainly within the developing world [5]. About 530 million heads of cattle live in areas endemic for bovine schistosomosis in Africa and Asia while at least 165 million cattle are infected with schistosomes worldwide [6]. Schistosomosis is now well recognized as the fifth major helminthosis of domestic animals in the Indian sub-continent [7].Schistosomes are members of the genus *Schistosoma* belonging to the family schistosomatidae. Adult schistosomes are dioecious and obligate blood flukes of vertebrates. In Asia, cattle are infected with *S. spindale, S.indicum, S.nasale* and *S. japonicum* [8]. *Indoplanorbis exustus* appears to be the only natural intermediate host for *S. indicum, S. spindale* and *S. nasale* in the Indian subcontinent [9].

The prevalence of *S. indicum* and *S. spindale* infection reported was 62.1% among 224 tested cattle of Bangladesh [10]. From another study 11.6% intestinal schistosomosis was found from 1,511 cattle [11]. This disease is generally chronic in nature and signs in majority of animals are insufficient to distinguish the illness from other debilitating infections [8]. Three types of clinical syndromes are seen in animal infection with schistosomosis, an acute intestinal, a chronic hepatic and nasal granulomatous syndrome [12, 13]. The species which commonly occur in ruminants in Bangladesh are *S. indicum, S. spindale* and *S. nasale* [14].

Schistosomosis is considered as one of the most neglected tropical disease, based on the limited resources invested in diagnosis, treatment and control and its strong association with poverty (NTD). In Bangladesh there are no preventive measures to control the parasitic diseases. In developed countries the

principles of control of parasitic disease are based on pasture and barn management and protective treatment [15]. In fact, regular deworming is the only way to lower the prevalence of schistosomosis but it was evident that efficacy of some anthelmintic is variable against *Schistosoma* spp. The study and development of new diagnostic techniques for schistosomosis are still necessary in view of the difficulties to evaluate infection patterns accurately and to control the disease [16].

Molecular techniques based on genomes are very useful for the epidemiological diagnosis as well as for research on genetic variation of the parasitic organism [17].Molecular analysis can provide information that can help to define an organism and their ranking according to the number of close relatives and their phylogenetic position. During the last few years, several authors have tried to set new diagnostic tools with higher specificity and sensitivity, some based on the detection of *Schistosoma* DNA by the polymerase chain reaction (PCR) technique, specifically, an *S. mansoni* sequence containing 121-base pair tandem repeats [18] was used to design specific primers which have been further applied for the amplification of the corresponding sequence in human patients' stool and serum [19, 20, 21, 22, 23]. Some other *Schistosoma* sequences have been described and used to design PCR reactions [24, 25, 26].

Considering the economic importance and pathological effects of schistosomosis in cattle, the present study was aimed at morphological investigation of ova and adult parasites of *Schistosoma* spp. along with pathologic investigation and designing of PCR protocols for the specific detection of *Schistosoma* spp. in cattle.

## II. Materials And Methods

A total of 64 mesentery with its intestinal part were collected from cattle of different slaughter houses of Mymensingh Sadar. The samples were collected during the period of December, 2013 to May, 2014. Samples collected early in the morning and dispatched to the Parasitology Laboratory, Department of Parasitology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh.

### 2.1 Processing, preservation and examination of samples

The mesentery was separated from its attachments and cut into small pieces and immersed in large glass jars containing normal saline and left undisturbed for 4-5 hours to recover schistosomes. Tissues were squeezed and removed. The saline was therefore, searched for blood flukes. Collected parasites were tentatively identified preparing permanent slide examining under light microscope following the keys and previous description [27, 28, 29].The intensity of infection was considered to be mild (1-20 worms per sample), moderate (21-100 worms per sample) and heavy (>100 worms) [7].

Side by side, parasites were preserved at -20℃ by keeping them in a properly labelled vial containing normal saline. Finally samples were transported to the Molecular Biology Laboratory, Department of Pathology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh for PCR amplification and characterization.

### 2.2 Histopathological investigation of mesentery and intestine

Schistosomes affected mesenteries and intestines were collected and fixed in 10% buffered neutral formalin. Formalin fixed tissue samples were processed and stained with hematoxyline and eosine as per method described previously [30].

### 2.3 Molecular detection of *Schistosoma* spp.

#### 2.3.1 DNA extraction

For DNA extraction from schistosomes traditional DNA extraction method was followed. Briefly, stored *Schistosoma* spp. was chopped by using fine head scissors. About 20-30 *Schistosoma* spp. was chopped in a 1.5ml Eppendorf tubes containing 600µl cell lysis buffer and vortexed to wet the tissue. The mixture was incubated at 56℃ for 2 hours to lyse the tissue cells, and then cooling it at room temperature. The solution was centrifuged at 5000g for five minute and the supernatant was collected. After that equal volume of PCI was added and vortexed vigorously at high speed for 20 seconds to mix the sample with the PCI and centrifuged at 5000g for one minute. The clear supernatant (top layer) containing the DNA was collected in a micro centrifuge tube. Then $1/10^{th}$ volume of 5N NaCl was added and mixed. After that 2.5 times ice cool absolute ethanol was added and kept at -20℃ for 10 minute. The solution was centrifuged at 14000g for 15 minute, the supernatant was discarded and desalted twice with 80% ethanol. The mixture was then centrifuged at 14000g for 15 minute at room temperature and carefully aspirated the 80% ethanol**.** The DNA pellet was very loose at this point and care was taken to avoid aspirating the pellet into the pipette tips. The pellet was allowed to dry in air for 15 minute, 25 µl of nuclease free water was added and concentration of DNA was measured using spectrophotometry (A260 and A280) and agarose gel electrophoresis. The DNA suspension was labelled and stored at -20℃ until PCR was carried out. Four set of primers (Table 1) were used to identify the species of *Schistosoma* using PCR.

**2.3.2 PCR**

PCR was performed in a total of 25 µl reaction volume consisting of 2x PCR master mix (Promega® Inc, USA), 20pmol of primer in each and 150-300ng of DNA template. A total of 35 cycles of PCR amplification reactions were carried out. The cycling parameter for the PCR identification of two genes in a thermal cycler (Master Cycler Gradient, Eppendorf, Germany) was an initial denaturation at 95℃ for 5minute. The PCR identification of SI16sRNA gene (Table 1) in a thermal cycler included the denaturation at 95℃ for 1 minute, annealing at 52℃ for 1 minute and extension at 72℃ for 1 minute. The PCR amplification for the identification of SPMit (Table 1) was the denaturation at 95℃ for 1 minute, annealing at 54℃ for 1 minute and extension at 72℃ for 1 minute. The final extension for PCR was set at 72℃ for 10 minute and the reactions were held at 4℃.Finally,the PCR reactions were terminated by adding 3µl 50mM EDTA and PCR products were analyzed by electrophoresis in 1.5% agarose gel, stained with ethidium bromide and examined under UV light using an image documentation system (Cell Biosciences, Alphalmager HP, USA).

**Table 1:** Primers and their sequences used to identify species of *Schsitosoma* with the disease processes

| Name of primer | Primer sequences(5´-3´)designed | Amplicon size | Targeted gene/ accession number | Species of *Schistosoma* | Reference (Gene bank accession No.) |
|---|---|---|---|---|---|
| SI16sRNAF | gagtttgtaaatggaggctgag | 606bp | 16sRNA/ EF534284.1 | *S. indicum* | Attwood et al., 2007 |
| SI16sRNAR | ccttattcagcctctacaccg | | | | |
| SPMitF | cttggagtcgggttgtttgag | 330bp | Mit/ KF425713.1 | *S. spindale* | Bindu et al., 2013 |
| SPMitR | cagaccctcacaccaacagtg | | | | |

## III. Results And Discussion

In this study, *S. indicum* and *S. spindale*, were tentatively identified and differentiated by their characteristic egg morphology under microscopes. Eggs having oval shape with lateral spine were identified as *S. indicum* female whereas spindle shaped eggs with terminal spine were identified as *S. spindale* female (Fig.1A and 1B). Copulated adult *S. indicum* and *S. spindale* were also identified by preparation of permanent slide (Fig.1C). Length of adult and eggs of female *S. spindale* and their spine were longer than *S. indicum* (Fig.1D). The most common test for the detection of schistosomosis diagnosis is still the microscopic examination of feces or visceral organs. However, microscopy has limited sensitivity, especially when parasitaemia is low and immature stages (schistosomulae) of parasites. Although microscopic examination can detect species of *Schistosoma* spp., accurate identification is difficult until eggs were passed through feces. About 1-400 adult *S. indicum* or *S. spindale* were detected (Fig.1E) from each of the 40 positive samples (out of 64 samples) but could not confirm the co-infection state. For more accuracy, histopathological and molecular investigation was carried out.

Suspected parts of infected mesentery and intestines were subjected to routine histopathological examination. The results of histopathological examination revealed *Schistosoma* egg granulomas in the cortex and medulla of the mesenteric lymph nodes (Fig.2). Abundant inflammatory cells in infiltrates consisted of eosinophils and hypertrophy of the tunica media of veins in the mesenteric lymph nodes was seen. The intestinal wall was thickened and mesenteric lymph nodes were edematous and atrophied. Hyperplastic portal and mesenteric lymph nodes with the formation of granuloma was seen in affected tissues which conforms with the lesions observed previously in liver, intestine and mesenteric lymph nodes of cattle elicited by *Schistosoma* spp. [31, 32]. This study disclosed that lesions associated with schistosomes were not restricted to a single organ rather many other organs. This was not unusual but most of the parasite during histopathological examination was seen in lymphatics of mesentery and serosa of intestine. It needs to study further whether the *Schistosoma* changes its living behaviour in lymphatics rather than mesenteric veins.

PCR was designed and carried out with the DNA extracted from the *S. indicum* and *S. spindale* with specific primers targeting genomic fragment of SI16sRNA and SPMit genes. Result of PCR amplification showed that, all of thetested samples (100 %) were positive to *S. spindale* infection and 37.5%cases were positive to *S. indicum* infection (Fig.3 and 4). About 37.5% sample showed mixed infection with both species of *S. indicum* and *S. spindale*. During the last decade, diagnostic methods for schistosomosis based on the specific detection of antigens or antibodies have been developed, but the sensitivity and specificity of these tests remains a problem [33]. Thus, a considerable number of schistosomosis cases was misdiagnosed. In addition, false positives results mainly due to cross-reactivity of the currently used serological tests were also relatively unreliable [34]. Antibody tests are usually negative during acute symptomatic schistosomosis. Assays that detect circulating antigens seem very promising in the early phase of infection and still lack of sensitivity in the diagnosis of light infections [21], consequently, there was a need for the development of new, more sensitive and specific diagnostic tools with potential application in the routine schistosomosis diagnosis. Here, this study

designed a new sensitive PCR-based approach for the amplification of the fragment of 16sRNA gene of *S. indicum* and mitochondrial gene of *S. spindale*. To date, this is the first specific primer based PCR tool described for the specific detection of *S. indicum* and *S. spindale* using genomic DNA of the parasites. The results of this study can be used as a baseline of relationship for future molecular analysis of *S. indicum* and *S. spindale* in cattle of Bangladesh. The present study analyzed selected cases. It needs to sequence the amplified gene following PCR with the collected sample to identify genomic lineage and understand molecular epidemiology of the parasite in Bangladesh. Moreover, it is essential to test large number of cases from different geoclimatic areas of Bangladesh to know the distribution pattern of *Schistosoma* spp. with their molecular epidemiology.

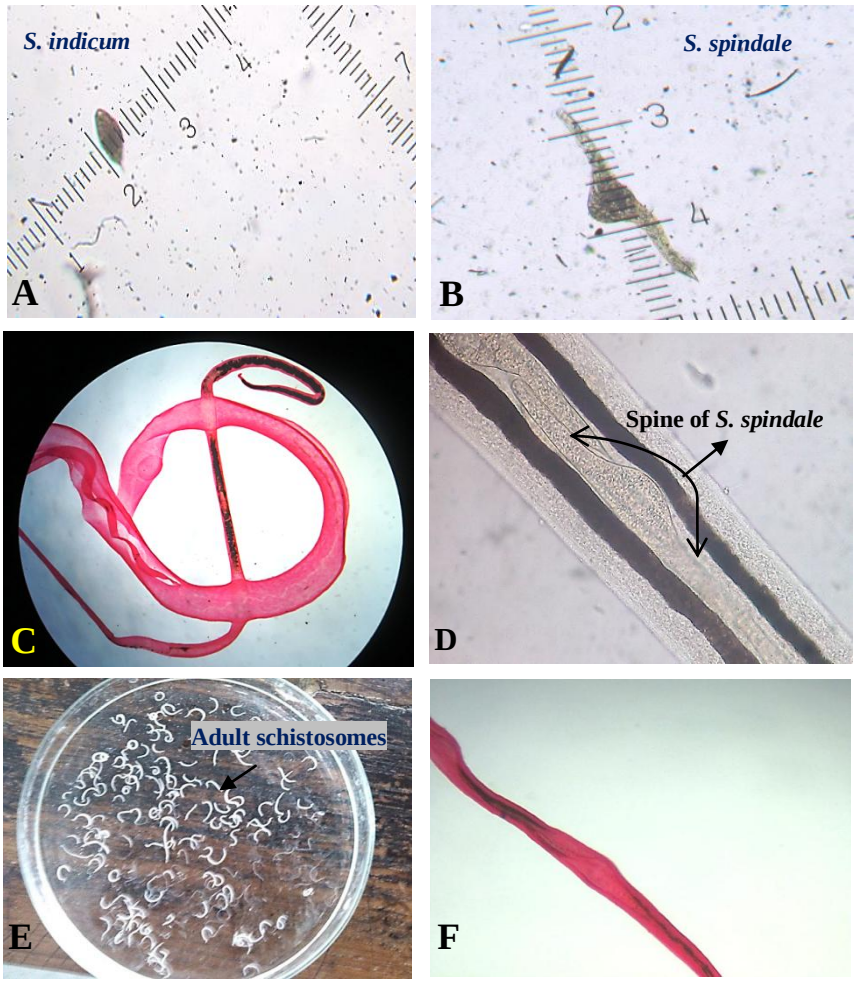


**Fig.1Morphological features of bovine schistosomes and their eggs. (A)** Measuring size of egg of S. indicum **(B)** Measuring size of egg of *S. spindale* (10X)**(C)** Copulated male and female *Schistosoma* sp. **(D)**Egg of *S. spindale* in female genital tract (40X). **(E)** Isolated adult schistosomes (>400) collected from mesentery of a bullock. (4X) and **(F)** Permanent slide indicates female schistosomes posterior part (10X).

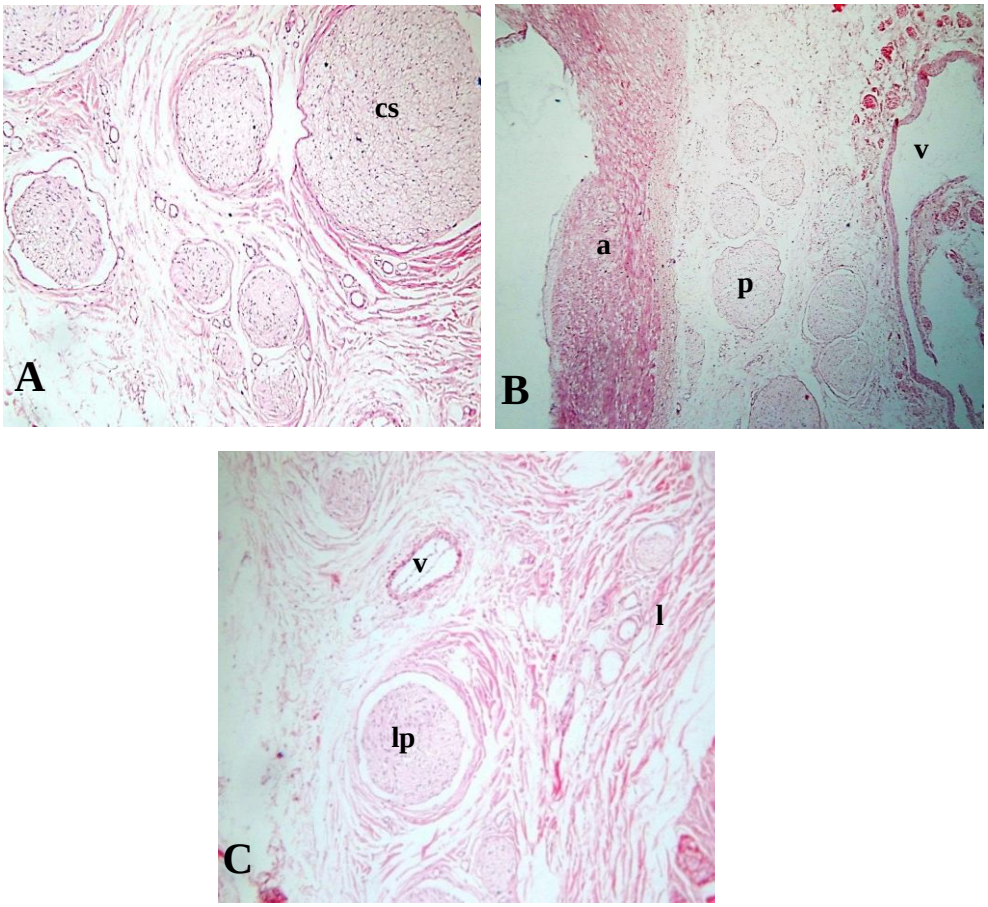


**Fig.2 Histological features of tissues infected *Schistosoma*. (A)** Mesentery of cattle infect with *Schistosoma* and stained with H or E. Cross-section of parasite was seen in the lymphatics of mesentery; cs= cross-section of *Schistosom*a spp. **(B)** Serosa of intestine collected from suspected case of schistosomosis. Section of parasite was seen in the lymphatics of intestine (40x); v= vein, p= section of parasite and a= artery. **(C)** Dilatation of the lymphatics containing parasites; v= vein, l= lymphatics and lp= lymphatic containing parasite.

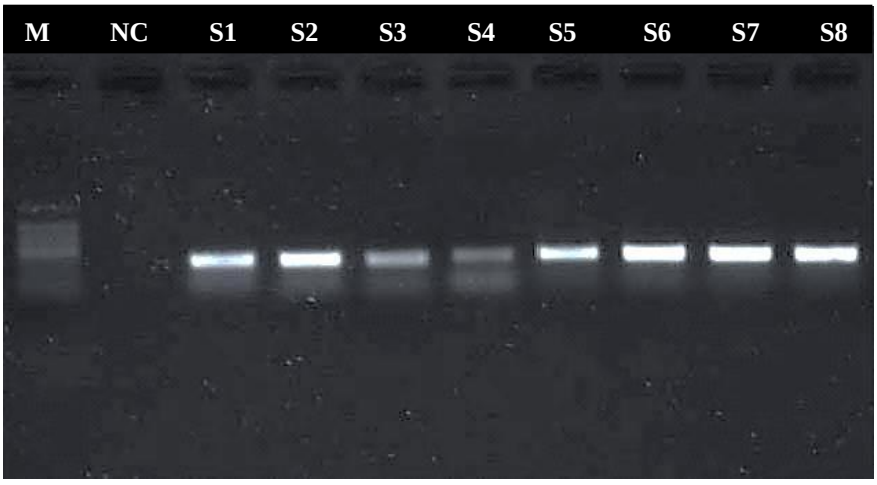


**Fig.3 Confirmatory identification of *S. spindale* by PCR**. Lane 1-8 represents the positive amplicons of *S. spindale* with SPMit primers; M, molecular weight marker (100 bp DNA ladder) and NC, negative control.


**Fig.4 Confirmatory identification of *S. indicum* by PCR.** Lane 4, 6 and 8 represents the positive amplicons of *S. indicum* with SI16sRNA primers; M, molecular weight marker (100 bp DNA ladder) and NC, negative control.

## IV. Conclusions

In conclusion, specific PCR based identification provide powerful resolution and are helpful tool for the investigation of species of *S. indicum* and *S. spindale* in cattle. This technique provide a method for the rapid differentiation of different species of Schistosoma that may in combination with biochemistry and molecular biology, help us to understand and take effective control measures against schistosomosis in endemic regions.

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