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Molecular detection and phylogenetic analysis of *Schistosoma indicum* in slaughtered cattle from Chennai city, India

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Abstract

Bovine visceral schistosomosis is a neglected tropical blood fluke disease causing economic losses in cattle industry. One hundred and eighty mesentery samples of cattle were collected from Perambur slaughter house, Chennai, Tamil Nadu, during a period of October 2019 - March 2020 for screening visceral schistosomosis. *Schistosoma indicum* was identified in eleven mesenteries based on gross and microscopic characters and worm number varied from 1-114 per mesentery. Morphological identification was further confirmed by PCR targeting the 16S rRNA gene (606 bp). The amplified product was also subjected to sequencing and phylogenetic analysis, which revealed three different clades with the present isolate belonging to clade II. The percentages of nucleic acid identity for the sequence of Indian *S. indicum* isolate obtained in this study revealed 100 % identity to *S. indicum* of Bangladesh. The present study revealed the occurrence of *S.indicum* in cattle and phylogenetic analysis of gene sequence was also carried out from Tamil Nadu for the first time. Broader studies are needed to understand the epizootiology of this important disease affecting cattle in India.

Keywords: *Schistosoma indicum*, Cattle, Tamil Nadu, Molecular identification, Phylogenetic analysis.

Introduction

Bovine visceral schistosomosis (BVS) is a snail borne trematode infection which is considered as neglected tropical disease. BVS is well recognized as the fifth major helminthosis of domestic animals in the Indian subcontinent (Sumanth *et al.,* 2004). This infection is caused mainly by *Schistosoma indicum* and *S. spindale* in India (Agrawal and Southgate, 2000). Hence, the species need to be differentiated based on morphology of adult worm tegument and number of testes in male as well as the shape of ova in female (Roy and Tandon, 1992; Agrawal, 2012a). *S. indicum* is an obligate parasite of blood vascular system residing in the portal and mesenteric veins of ruminants. The blood fluke infection causes chronic wasting illness and is characterized with haemorrhagic diarrhoea, emaciation, anaemia which overlaps with other existing debilitating diseases (De Bont and Vercruyesse, 1998). It also causes reduced milk yield, severe mortality with outbreaks leading to high death rates in cattle (Agrawal, 2012a). Diagnostic methods include direct parasitological examination of ova and miracidium from faeces/rectal pinch which is time consuming and limited in sensitivity since, *S. indicum* ova in cattle are found mostly in the mucous membrane of intestine causing cellular lesions

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(Krishnamurthi, 1956). In erstwhile Madras province *S. indicum* infection was uncommon and the cases were reported from Kurnool, Chittor and Nellore districts, currently in the state of Andhra Pradesh of India (Alwar, 1950). Mitochondrial markers, particularly the species barcoding gene cytochrome c oxidase subunit I (*COX* I), 16S and 12S ribosomal subunit RNA gene are being used as target genes for species identification and phylogenetic analysis (Jones *et al.,* 2020). The current study was carried out to know the occurrence of BVS due to *S. indicum* in Chennai, Tamil Nadu, India, which was not recorded since decades and to verify the *S. indicum* 16S rRNA gene based molecular confirmation and phylogenetic studies.

Materials and Methods

Fluke collection:

A total of 180 mesentery samples of cattle were randomly collected during a period from October 2019 to March 2020 from Perambur slaughter house, Chennai, Tamil Nadu, India (Latitude 13.1038° North, 80.2612° East) in order to detect visceral schistosomosis. The tissue samples soaked in normal saline for two hours to collect the blood flukes present, if any. The veins of the mesentery were punctured by holding it against sunlight for recovery of the adult blood flukes (Fig.1a). The collected flukes were ndividually counted to ascertain the intensity of infection and subjected to microscopic examination.

Morphological identification:

The adult flukes were examined for the structural characteristics such as size, tegument, sucker position, gynaecophoric canal, number of testes, and morphology of ova under an inverted microscope at 40X magnification to confirm their identity (Singh, 1958; Srivastava and Dutt, 1962; Agrawal, 2012a).

Genomic DNA extraction, PCR and sequencing:

Genomic DNA was isolated from the adult blood flukes using DNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany). The concentration of the genomic DNA extracted was estimated using Biospectrophotometer (Eppendorf, USA). The 16S rRNA mitochondrial gene specific for *S. indicum* was amplified by polymerase chain reaction using species specific oligonucleotide primers (SI16sRNAF-GAGTTTGTAAATGGAGGCTGAG, SI16sRNAR-CCTTATTCAGCCTCTACACCG) previously used by Attwood *et al.* (2007) and Hossain *et al*. (2015). PCR amplification was performed in a total volume of 25 µL, including 110 ng of genomic DNA (5 µL), 12.5 µL of *Taq* DNA polymerase Red-Dye Master mix (Ampliqon), 10 pmol of each primer (2µL each) and 3.5 µL of nuclease free water. The following amplification protocol was employed in a thermal cycler (Bio-Rad, USA): 95°C for 5 minutes, followed by 35 cycles each of 95°C for 1 minute (denaturation), 52°C for 1 minute (annealing) 72°C for 1 minute (extension), followed by 72°C for 10 minutes (final extension). Negative controls (no DNA template) were included in the PCR reactions which were run in the same thermal cycler. Amplicons were resolved in ethidium bromide-stained agarose gel (1.2%) and sized by comparison with GeneDirex® 100 bp DNA ladder as molecular marker. Gels were photographed using Gel Doc 2000 (Bio-Rad, Hercules, CA, USA). PCR product was gel purified and sequenced using the Sanger's method. Nucleotide sequence analysis was done by Nucleotide BLAST (BLASTn) algorithm. The *S. indicum* nucleotide sequence databases available from GenBank were retrieved from National Centre for Biotechnological Information (NCBI) and used for nucleotide BLAST algorithm similarity search and phylogenetic analysis (Table 1, http://www.ncbi.nlm.nih.gov).The nucleotide sequence was assembled and analyzed using Seqman and MegAlign programs of Lasergene package (version 7.1.0, DNA Star Inc. Madison, WI, Table 1). Nucleotide sequence alignment was performed by ClustalW method with MegAlignTM program (DNA Star Inc). Phylogenetic analysis of 16s rRNA gene was performed using maximum likelihood method of Tamura-Nei model analysis with 1000 bootstrap replication in the MEGA software version 7.0 (Hall, 2013; Onile *et al.,* 2014). Published 16S rRNA gene sequences of different *Schistosoma* spp. from different geographical regions available in GenBank database were used for the phylogenetic analysis.

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Table 1: Reference sequence used in this study for phylogenetic analysis

S. No	Accession Number	Organism/Species	Country
1.	MZ433263 (From this study)	*Schistosoma indicum*	India
2.	KR423833	*Schistosoma cf. indicum W528*	Nepal
3.	EF534290	*Schistosoma spindale*	Thailand
4.	EF534288	*Schistosoma spindale*	Bangladesh
5.	KR423842	*Schistosoma spindale*	Nepal
6.	KR423836	*Schistosoma cf. indicum W557*	Nepal
7.	KR423843	*Schistosoma nasale*	Nepal
8.	EF534284	*Schistosoma indicum*	Bangladesh
9.	EU567131	*Schistosoma haematobium*	East Africa
10.	MK253577	*Schistosoma haematobium*	Tanzania
11.	L03651	*Schistosoma bovis*	Nigeria
12.	AP017708	*Schistosoma curassoni*	Japan
13.	L03653	*Schistosoma bovis*	Nigeria
14.	MN637820	*Schistosoma spindale*	Bangladesh
15.	KF850451	*Schistosoma indicum*	India
16.	AY446259	*Schistosoma mansoni*	Ghana
17.	AY446247	*Schistosoma mansoni*	Egypt

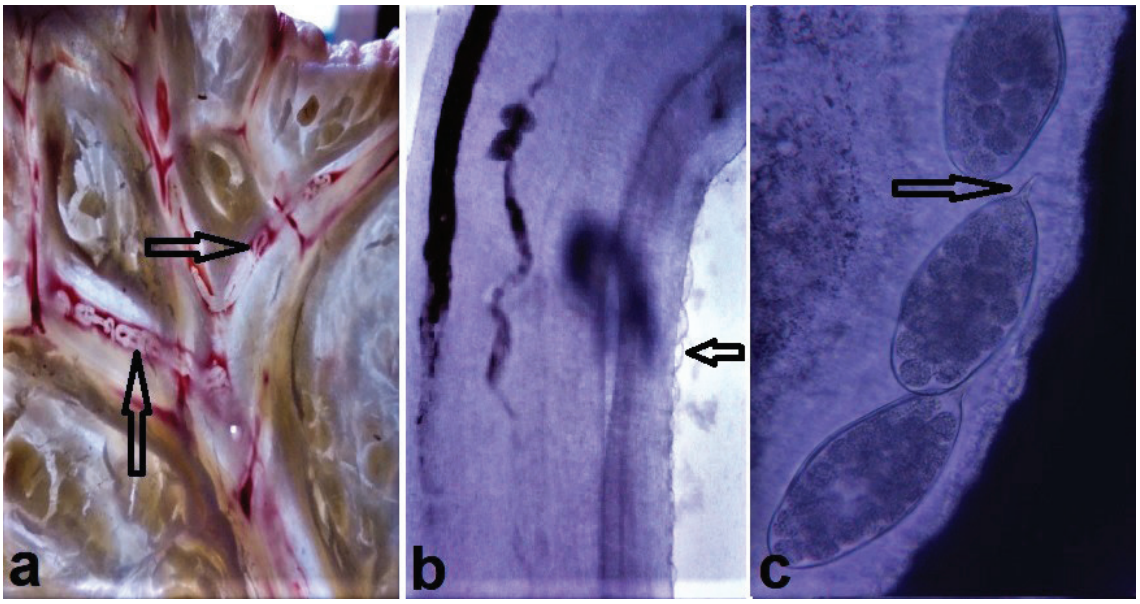

Fig. 1: Gross appearance (a) of *Schistosoma indicum* adult worms (arrows) in the mesenteric veins of cattle held against sunlight shows the parasite predilection site. *In copulo* adult *S. indicum* male and female worms (b) showing tuberculated tegument (arrow, 5X). Oval shaped ova (c) with terminal spine (arrow) in utero in *S. indicum* females (40X).

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Results and Discussion

The adult flukes harvested from the mesenteric veins were grossly milky white in colour with length of 9 to 18 mm in male and 21 to 24 mm in female adult worms. When examined under the inverted microscope they revealed the presence of tuberculated body surface in male, presence of oral and ventral sucker in anterior end, male and female flukes found in copulation (Fig. 1b), oval shaped ova with terminal spine in uterus (Fig. 1c). Based on these characters the flukes were morphologically identified as *Schistosoma indicum*. Among 180 cattle mesenteries, (82 females and 98 males), 11 showed presence of *S. indicum* with 6.11% infection. The worm number varied from 1-114 in number per mesentery. The mean number of the flukes was found to be 25. All the affected cattle were males, nine of them being adult and two were calves. Co-infection with *S. spindale* was also noticed in four mesenteries with three adult male and one female wherein the number of *S. indicum* were 3 to 4 times more than that of *S. spindale.*

Bovine visceral schistosomosis caused by *S. indicum* is a neglected tropical disease in Southeast Asia. It was first identified in horse, donkey and sheep in north India (Montgomery, 1906). It has also been recorded in cattle, buffaloes, goat and camel. The record of this infection in erstwhile Madras province is not uncommon though infrequent (Rao, 1939; Alwar, 1950). Banerjee *et al*. (1972) also reported a clinical case of the infection in crossbred Holstein bull in West Bengal based on coprological examination. In Northern states of India such as Haryana, Himachal Pradesh, Punjab and Rajasthan, the incidence rate of *S. indicum* in cattle was around 2.3% as per the study and it was also noted that the infection of *S. indicum* was more widespread than that of *S. spindale* (Chaudhri and Singh, 2007). Central Indian states also showed this infection in cattle (Giri *et al*., 2018). The prevalence of *S. indicum* infection was also reported from examination of faecal samples of small ruminants in Karnataka with a relatable infection rate (Cherian and D'Souza, 2009). Prevalence of *S. indicum* in cattle was also reported from Kerala with low infection rate (Chirayath, 2007; Divya *et al.,* 2012).

In an earlier abattoir survey conducted at Perambur slaughter house (Jeyathilakan *et al.,* 2008), 114 cattle mesenteries were examined, among which the prevalence of *S. spindale* was about 30.7%. However, *S. indicum* infection was not recorded, even though common intermediate host *Indoplanorbis exustus* is prevalent in Tamil Nadu. The present abattoir study conducted in Chennai revealed the occurrence of *S. indicum* in cattle for the first time. Identification of *Schistosoma* spp. can be done by examination of characteristic morphology of ova upon coprological survey. However, visual appraisal of the affected animal does not aid in diagnosing this condition since the infected animal shows clinical signs overlapping with other debilitating diseases. The 16S rRNA gene sequence analysis has been used for accurate confirmation of *S. indicum*.

An amplicon of 606 bp (Fig. 2) was obtained with DNA of *S. indicum*, which was later sequenced and analysed by BLASTn to confirm the identity . The 16S rRNA nucleotide sequences were aligned by ClustalW method with MegAlignTM program (DNA Star Inc) and sequence distances were performed with other 16s rRNA sequences retrieved from Genbank of NCBI. The 16S rRNA sequences of *S. indicum* in this study revealed 100% similarity with EF534284 of *S. indicum* from Bangladesh. The sequence was submitted to GenBank (accession No.MZ433263).

The phylogenetic analysis of 16SrRNA mitochondrial gene of *Schistosoma* spp. (*S. mansoni* was used as outgroup) formed three different clades (Fig. 3). The first clade comprised of *S. haematobium, S. curassoni* and *S. bovis*. The *S. indicum* isolate obtained in the present study (MZ433263) belonged to clade II with *S. spindale, S. nasalis* and *S. indicum* from Bangladesh. The third clade comprised of *S. spindale* and *S. indicum* from United Kingdom.

Indian isolate of *S. indicum* showed 100% identity to the Bangladesh isolate of *S. indicum*. The phylogenetic tree revealed that the *S. indicum* of Indian isolate formed the clade with *S. indicum* of Asian continent (Nepal, Bangladesh and Thailand).. The earlier reported isolate of

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S. indicum from India formed a separate clade along with *S. spindale* of UK origin. There are three different clades and the *S. indicum* of Chennai falls inside the clade II of *Schistosoma*. Presence of *S. indicum* in three different clades suggests the sympatric speciation in the *S. indicum* group, possibly *S. indicum* evolving from *S. spindale* due to genetic polymorphism within the population. It also suggests the extent of genetic diversity in the *S. indicum* group. However, *S. indicum* group was regarded to be paraphyletic in nature (Agatsuma *et al.,* 2002; Attwood *et al.,* 2002; Morgan *et al.,* 2003; Webster *et al.,* 2006). *S. indicum*-group includes *S. indicum, S. nasalis* and *S. spindale* (Rollinson and Southgate, 1987). *S. spindale* and *S. indicum* are considered as sympatric species. *Indoplanorbis exustus* is the common intermediate host for *S. spindale, S. indicum* and *S. nasalis*. *COX*1 and 16S rRNA gene based studies indicated the existence of five separate species within the *S. indicum* group*: S. nasalis, S. indicum, S. cf. indicum*, with *S. spindale* split into two distinct species groups which are Sri Lanka/ Malaysia and Bangladesh/Nepal (Jones *et al.,* 2020). Despite their sympatric nature, inter-species mating between *S. spindale* and *S. indicum* and the production of viable hybrid eggs have been recorded under laboratory conditions (Agrawal, 2012b).The present study revealed the occurrence of *S.indicum* in cattle and phylogenetic analysis of gene sequence was also carried out from Tamil Nadu for the first time.

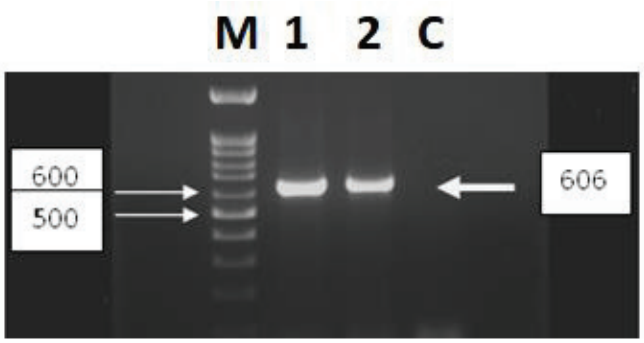

Fig. 2: Agarose gel (1.2 %) showing amplification of 16S rRNA mitochondrial gene specific to *S. indicum* using genomic DNA of adult *S. indicum* isolated from cattle mesentery. L- 100 bp DNA marker, lanes 1 & 2- amplification of 16S rRNA of *S. indicum* of product size 606 bp, C-Negative control.

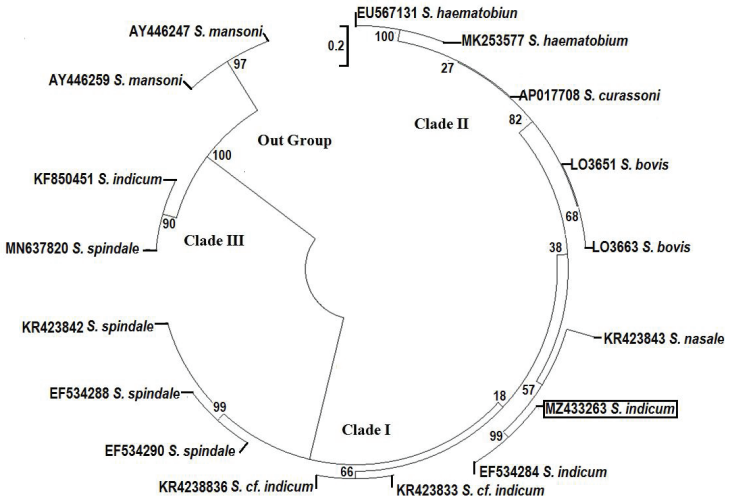

Fig. 3: Molecular phylogenetic analysis of *S. indicum* based on 16S rRNA gene from India in MEGA 7.0. The sequence obtained in this study is represented in a box

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