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

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Abstract
Schistosoma spindale is an obligate parasite of blood vascular system causing Bovine visceral schistosomiasis. It is economically important chronic disease characterized by hemorrhagic diarrhoea, weight loss, weakness, anemia and stunted growth in cattle and often goes under diagnosed. The present study was conducted to know the presence of *Schistosoma spindale* infection in Chennai, Tamil Nadu by morphology and molecular confirmation by PCR One hundred and eighty mesentery samples of cattle were collected from Perambur slaughter house, Chennai, Tamil Nadu, India during a period of October 2019 - March 2020 for screening visceral schistosomiasis.. Adult flukes were collected from 57 cattle by screening mesentery samples ranging from 1-808 / mesentery. Morphological identification was further confirmed by PCR by targeting the 16S rRNA gene. Upon gel documentation amplicon of size 330 bp specific for *Schistosoma spindale* was observed and confirmed by sequencing studies. Phylogenetic analysis revealed existence of commom ancestor from *S. spindale* of Thailand and Sri Lankan isolate.

Keywords: *Schistosoma spindale*, Cattle, Tamil Nadu, molecular identification, phylogenetic analysis

Introduction
Bovine Visceral schistosomiasis (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) [13]. This infection is caused mainly by *Schistosoma spindale and Schistosoma indicum* in India (Agrawal, 2000) [1]. 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 (Agrawal, 2012) [2]. *S. spindale* 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, anemia which overlaps with other existing debilitating diseases (De Bont and Vercruyesse, 1998) [4]. It also causes reduced milk yield, severe mortality with outbreaks leading to high death rates in cattle (Agrawal, 2012) [2]. Diagnostic methods include direct parasitological examination of ova and miracidium from faeces/rectal pinch which is time consuming and limited in sensitivity Mitochondrial markers particularly the species barcoding gene cytochrome c oxidase subunit I (*COX* I), 16S and 12S ribosomal subunit RNA gene have been currently used as target genes for species identification and phylogenetic analysis (Jones *et al.* 2020) [8]. The current study was carried out to know the occurrence of BVS due to *S. spindale* in Chennai, Tamil Nadu, India, which was not recorded since decades and to verify the *S. spindale* 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 check Bovine visceral schistosomiasis. The mesentery was soaked in normal saline to collect the blood flukes present if any for two hours. The veins of the mesentery were punctured by holding it against sunlight for recovery of the adult blood flukes (Fig.1). The collected flukes were subjected to microscopic examination followed by molecular confirmation.

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 (Agrawal, 2012) [2].

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. spindale* was amplified by polymerase chain reaction using species specific oligonucleotide primers (SPMitF - CTTGGAGTCGGGTTGTTTGAG, SPMitR - CAGACCCTCACACCAACAGTG) previously used by Lakshmanan *et al.* (2014) [10] and Hossain *et al*. (2015) [6]. 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 III), 10pmol of each primer (2µl each) and 3.5 µl of nuclease free water. The following amplification protocol was employed in a thermal cycler (BioRad, USA): 95 °C for 5 minutes, followed by 35 cycles each of 95 °C for 1 minute (denaturation), 54 °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 (1.5%) and sized by comparison with GeneDirex ® 100 bp DNA ladder as molecular marker. Gels were photographed using Gel Doc 2000 (BioRad, 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. spindale* 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 (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). 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 *et al.*, 2013; Onile *et al.*, 2014) [5, 11]. Published 16S rRNA gene sequences of different *Schistosoma* spp. from different geographical regions available in GenBank database were used for the phylogenetic analysis.

Results and Discussion

The adult flukes harvested from the mesenteric veins were grossly milky white in color with the length of 8 to 16 mm in male and 20 to 23 mm in female adult worms. When examined under the inverted microscope revealed the presence of atuberculated body surface in male, presence of oral and ventral sucker in anterior end, male and female flukes found in copulation (Fig.2), spindle shaped ova with terminal spine in uterus (Fig.3). Based on these characters the flukes were morphologically identified as *Schistosoma spindale*. Among 180 cattle mesenteries, 82 were of female and 98 of male out of which 57 showed presence of *S. spindale* with 31.67 percent infection and worm number varied from 1-808 in number per mesentery. Co-infection with *S. indicum* was also noticed in 4 mesenteries with the ratio of 1:3 of *S. indicum* and *S. spindale* respectively. Abattoir surveys revealed that 30-68 per cent of bovines in South Indian states such as Karnataka, Tamil Nadu, Kerala and Telangana are infected with visceral schistosomiasis caused by *S.spindale* (Sumanth *et al*., 2004; Jeyathilakan *et al*., 2008; Lakshmanan *et al*., 2011 and Sudhakar *et al*., 2016) [15, 7, 9, 12].

The phylogenetic analysis of 16SrRNA mitochondrial gene of *Schistosoma* spp. forms different clades (Fig.5). The present study isolate *S. spindale* belongs to clade I which comprises of *S. spindale* from Bangladesh (LC224106), Kerela isolate, India (KF425713), Nepal (KR423862) and Sri Lanka (AY157257). This *S. spindale* isolate might have been have existed a common ancestor from *S. spindale* of Thailand and Sri Lankan isolate. The results of the PCR assay in the present study were in concordance with the work carried out by Lakshmanan *et al.*, 2014 [10], Hossain *et al.*, 2015 [6] and Bulbul *et al.*, 2019 [3] in which the DNA were isolated and characterized molecularly by targeting species specific mitochondrial gene in Kerala, Bangladesh and Assam respectively.

Fig 1: Gross adult worms in the mesenteric veins of cattle held against sunlight shows the predilection site of parasite

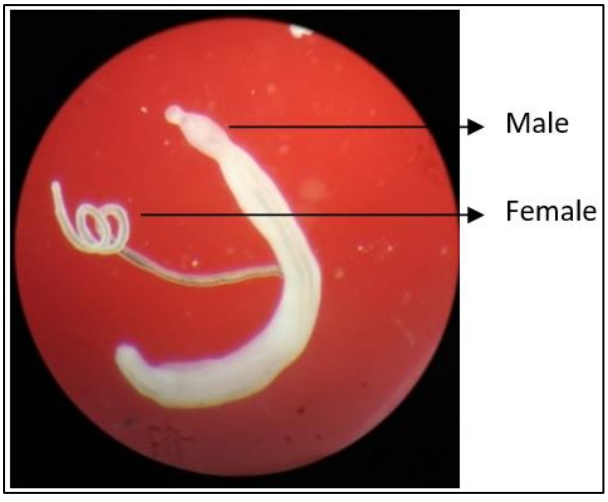

Fig 2: In copulo adult *Schistosoma spindale* male and female worm showing tuberculated tegument (5X)

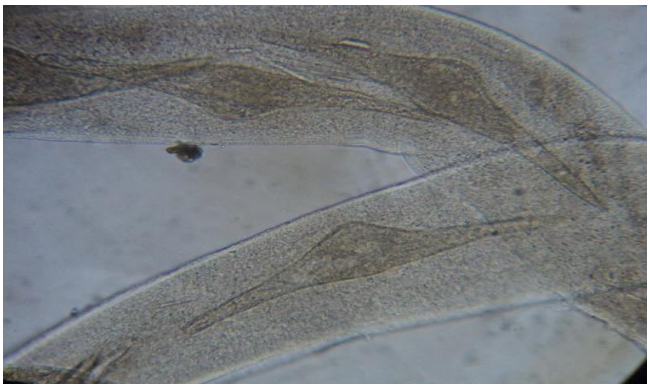

Fig 3: Spindle shaped ova with terminal spine *in utero* in *S. spindale* females (40X)

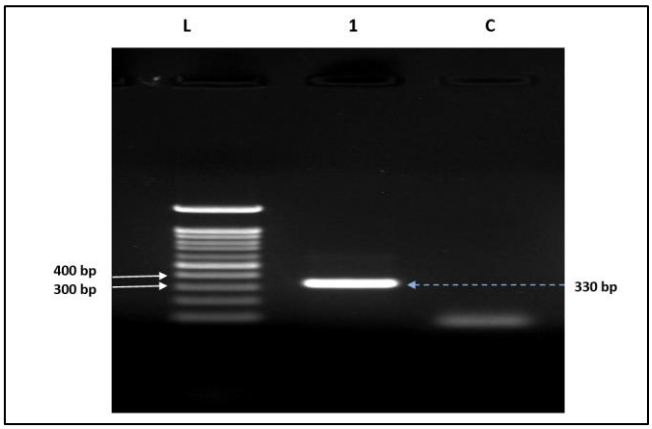

Fig 4: Agarose gel (1.5 %) showing amplification of 16S rRNA mitochondrial gene specific to *S. spindale* using genomic DNA of adult *S. spindale* isolated from cattle mesentery L- 100 bp DNA marker, lanes 1 - amplification of 16S rRNA of *S. spindale* of product size 330 bp, C- control

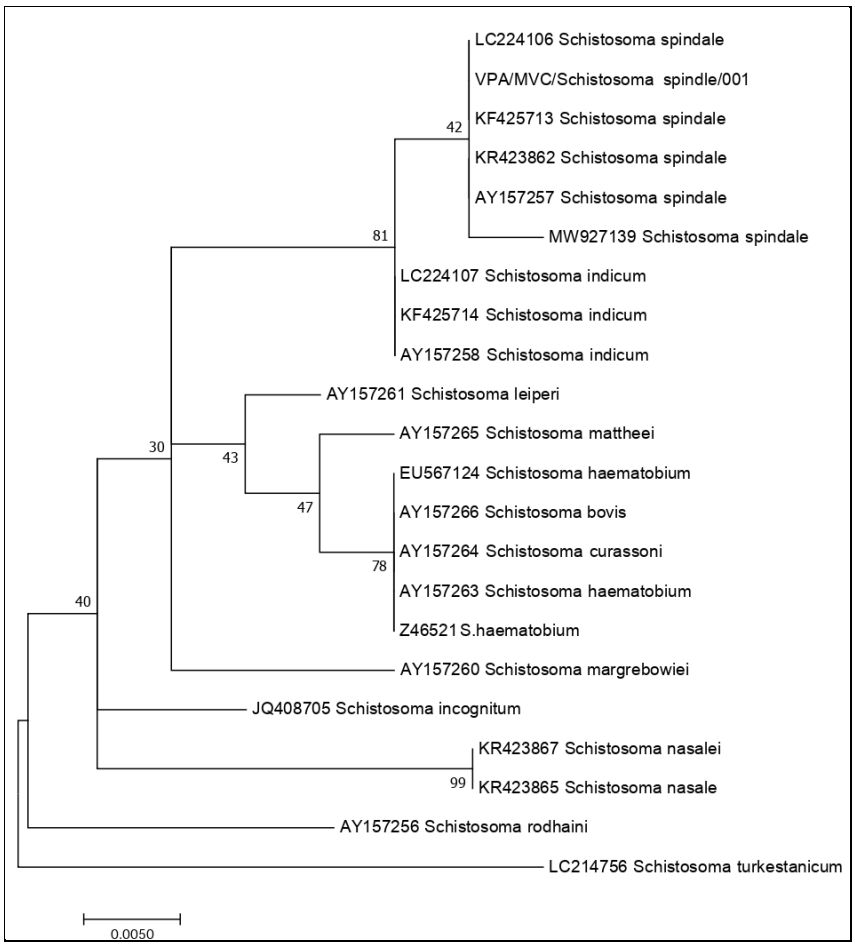

Fig 5: Molecular phylogenetic analysis of *S. spindale* based on 16S rRNA gene from India in MEGA 7.0.

Conclusion

The current study confirms the presence of active bovine visceral schistosomiasis due to *S. spindale* in cattle, Chennai, Tamil Nadu at molecular level using PCR targeting species specific mitochondrial gene and hints existence of common ancestor from *S. spindale* of Thailand and Sri Lanka. Since, the disease condition is oftenly undiagnosed with the low sensitive parasitological tests, molecular detection using the species specific primers helps in early and confirmative diagnosis of the bovine visceral schistosomiasis in cattle.

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