MOLECULAR CHARACTERISATION OF SNAIL BORNE TREMATODES IN DAIRY CATTLE OF KERALA

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Abstract: The most common and economically important snail borne trematodes parasitizing bovines in Kerala are schistosomes and amphistomes. They adversely affect the production and productivity of dairy cattle. There has been no attempts to characterize these species by molecular tools and hence a study was designed to analyse the 18S ribosomal RNA genes of *Schistosoma spindale, S.indicum, Gastrothylax crumenifer and Fischoederius elongatus* isolated from naturally infected cattle. A gradient PCR protocol with novel primers, designed based on sequences of schistosomes available in Genbank yielded a specific 432bp amplicon for all the four species. Bidirectional nucleotide sequences of the purified amplicons were aligned and the sequences were analysed by BLASTn for sequence similarity. Phylogenetic analysis were performed with Mega 6.0 to identify the evolutionary relationship of these Indian isolates of trematodes. The present study provided an insight into the high sequence similarity of 18S rRNA gene of schistosomes and pouched amphistomes and possible differentiation of the species based on restriction enzyme digestion.

Keywords: Schistosomes, pouched amphistomes, 18S rRNA, Phylogeny, Kerala.

Introduction: The most common and economically important snail borne trematodes parasitizing bovines in Kerala are schistosomes and amphistomes. They adversely affect the production and productivity of dairy cattle. Schistosomosis has been recognised as one of the major parasitic diseases of livestock and human beings. It is speculated that at least 165 million cattle are infected with schistosomes worldwide, while another 530 million live in endemic areas [1]. The major species affecting the ruminant population of India are *S.spindale, S.indicum and S.nasalis*. The economic importance of bovine schistosomosis is mainly attributed to morbidity, mortality, liver condemnation, reduced productivity and poor subsequent reproductive performance [2].Besides pathology in animals, it has been long known that cercariae of *S. spindale* are a common cause of dermatitis in human beings in Asia, especially in India and that the fresh water snail, *Indoplanorbis exustus* is a major source of infection. Although this is not a life-threatening zoonosis, *S. spindale* assumes public health significance owing to its high prevalence in definitive hosts. Several species of amphistomes reside in the rumen causing disturbances in digestion. Moreover, the immature amphistomes causes inflammation of small intestine leading to diarrhea and loss in production. The presence of vector snails in water logged areas of the state has led to a high prevalence of these infection in Kerala. However, there has been no attempts to characterize these species by molecular tools. Ribosomal DNA forms a tandem array of repeat sequences and each repeat includes 18S, 5.8S and 28S subunits separated by spacers [3]. The 18S and 28S ribosomal DNA sequences have been identified as good genetic markers for identification and phylogenetic

studies. Hence a study was designed to analyse the 18S ribosomal RNA genes of *Schistosoma spindale, S.indicum, Gastrothylax crumenifer* and *Fischoederius elongatus* isolated from naturally infected cattle.

Materials and Methods: *Collection of Samples*: Adult schistosome worms were recovered from the mesentery of cattle (n=50) slaughtered in local abattoir of Thrissur district following a previously described method [4]. Adult amphistomes were also collected from the rumen of slaughtered bovines (n=50). These trematodes were washed several times in phosphate buffered saline (PBS, pH 7.4), identified morphologically [5] and stored at -20^{o}C until DNA extraction. The adult schistosomes were identified as *S. spindale* and *S. indicum*. The amphistomes recovered from the rumen were *G. crumenifer* and *F. elongatus*.

Polymerase Chain Reaction: Total DNA was extracted from parasites using QIAGEN DNeasy Blood and Tissue Kit (Germany). The sequences of forward and reverse primers designed corresponding to 18S r RNA gene of schistosomes [6] are as follows:
Forward -5'- ACTTCCAGCTCCAAAAGCGTA-3'
Reverse – 5'-CGATCCCAGAATTTCACCTC-3'

A gradient PCR was done to standardize the protocol with DNA extracted from adult *S. spindale*. These primers were also tested with DNA extracted from the most commonly occurring amphistome species in Kerala *viz*., *F. elongatus* and *G. crumenifer*. The PCR was performed in a 25 µl reaction volume containing 2.5 µl of buffer (10X) without $MgCl_2$, 200 µM each of dNTP, 25 pmol each of forward and reverse primer, 1.5 mM of $MgCl_2$, 1U of Taq DNA polymerase and 5.0 µl of template DNA. All the reagents were procured from Sigma Aldrich (USA). A gradient thermal cycling program (MJ Mini, Biorad, USA) was set with initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation (94°C, 1 min), annealing (55°C to 67°C, 30s) and extension (72°C, 1 min). A final extension was adopted at 72^{o}C for 7 min. The amplicons were electrophoresed in 2 per cent agarose gel and sizes were resolved using 100 bp ladder (Thermoscientific, USA). The amplicons were column purified and sequenced using Sangers dideoxy chain termination method (Sci Genom PvtLtd, Cochin).

Results: Gradient PCR with annealing temperatures varying from 55°C to 67°C using SS 18 primers, amplified a 432 bp product at all temperatures, when template DNA of *S. spindale*, *S.indicum, F. elongatus* and *G. crumenifer* were used (Fig 1).

The partial sequence of 18S rRNA of *S.spindale, S.indicum, G.crumenifer* and *F.elongatus* obtained in this study were submitted to Genbank and assigned with accession numbers . Nucleotide blast analysis of sequence data of *S. spindale* DNA amplified with SS 18 primers revealed 100 per cent similarity with 18S ribosomal RNA gene of Sri Lanka isolate of *S. spindale* (Z 11979), 99 per cent with that of *S. margrebowiei*(AY 157233), *S.mattheei*(AY 157237), *S. haematobium* (Z 11976), Bangladesh isolate of *S. indicum* (AY 157231), *S. bovis* (AY 318828) and *S. mansoni* (U 65657), and 98 per cent with that of Sri Lanka isolate of *S. nasale* (AY 157232). The DNA sequence from morphologically identified *F. elongatus* and *G. crumenifer* amplified using SS 18 primers revealed no identity with sequences of same species, as complete sequence data of these parasites were not available in published databases. Nevertheless, *G. crumenifer* sequence revealed 99 per cent similarity with 18S rRNA sequences of several Schistosoma sp. (Z 11979, AY 157237, AY 157236, AY 157234, AY 157231, AY 318828) and 94 per cent identity with *Paramphistomidae* sp. (FJ 550131), a closely related amphistome. Sequences of *F. elongatus* amplified with SS 18 primers revealed 97 per cent similarity with 18S r RNA gene sequences of *Schistosoma* sp. (Z 11979, AY 157237, AY 157236, AY 157231 and Z 11976) and 87 per cent identity with 18S rRNA gene sequence of *Paramphistomidae* (FJ 550131). BLAST analysis also showed that 18S rRNA gene sequence of *Paramphistomidae* share 90 per cent identity with that of *S.spindale* (Z11979).

Partial 18S rRNA sequence of *S. spindale, S. indicum, F. elongatus* and *G. crumenifer* analysed using the online software, NEB cutter, revealed several restriction enzyme (RE) recognition sites within the sequences. The analysis of *S. spindale* sequence revealed that *TspR1* could leave the sequence into 98 bp and 334 bp fragments, while digestion with *BtslMutI* could generate 91 bp and 341 bp fragments. These enzymes had no recognition sites within the sequence of *S. indicum*. The sequence of *F. elongatus* had unique recognition sites for *DraIII* which cuts the sequence at 124 bp and *BamI* which cut at 71 bp position. Other recognition sites for this sequence included those for *NspI* and *SphI,* which could produce 210 bp and 222 bp products, as well as for *EcilI* which could yield 171 bp and 261 bp products, upon RE digestion. The recognition sites for these enzymes were lacking in the 18S rRNA sequences of *S. spindale*, *S. indicum* and *G. crumenifer*.

Phylogenetic analysis of schistosomes was performed with the sequences obtained in the present study along with the 18S rRNA sequence of *S. haematobium* (Z 11976), *S. mattheei* (AY 157237), *S. mansoni* (U 65657), *S. bovis* (AY 318828), *S. incognitum* (JQ 408706), *S. spindale* Sri Lanka (Z 11979), *S. nasale*, Sri Lanka (AY 157232), *S. indicum*, Bangladesh (AY 157231), *Bivitellobilharzia nairi* (AY 829261) which were downloaded from online database (www.nchi.nlm.nih.gov). The evolutionary history was inferred using neighbour joining method constructed in Mega 6.0. The bootstrap consensus tree was obtained from 1000 replicates (Fig 2) The evolutionary divergence between sequences were analysed using maximum composite likelihood model. The numbers of base substitutions per site, between the sequences, are depicted in Table 1. Standard error estimates are shown above the diagonal. The sequence *S. spindale*, Kerala showed closest relationship with that of *S. incognitum*, and both of these clustered as a single group. *Schistosoma indicum* shared close relationship with Sri Lankan isolate of *S. spindale*

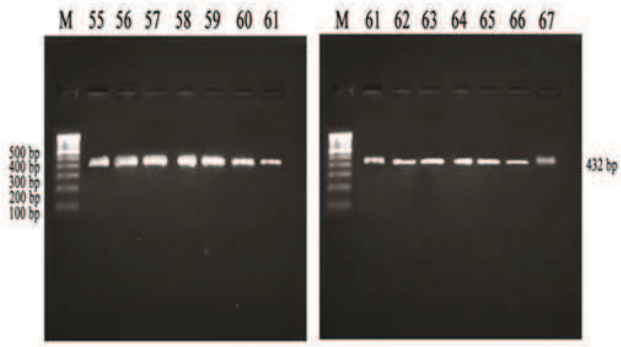

Fig.1: PCR Amplicons of *S.Spindale* with SS 18 Primers
Lane Titles Denote Annealing Temperature;
M-100 bp DNA Ladder

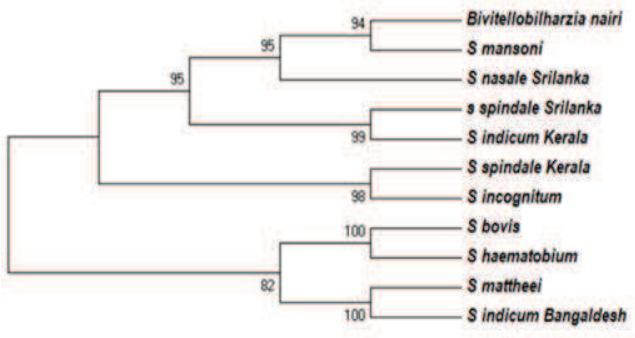

Fig.2: Phylogenetic Tree of *S. Spindale*

Table 1: Estimates of Evolutionary Divergence between Schistosomes Based on 18S rRNA Sequences

	1	2	3	4	5	6	7	8	9	10	11
1. S spindale Kerala		15.444	13.894	18.485	13.885	15.081	18.485	17.184	7.153	15.339	17.187
2. Bivitellobilharzia nairi	14.024		6.786	14.669	6.790	0.307	14.669	22.456	14.444	0.009	22.456
3. s spindale Srilanka	14.316	6.183		16.094	0.003	4.162	16.083	20.386	18.765	6.846	20.386
4. S bovis	18.329	16.311	16.531		16.069	14.331	0.002	15.544	22.179	14.761	15.497
5. S indicum Kerala	14.582	6.100	0.005	16.776		4.165	16.065	20.372	18.757	6.849	20.372
6. S nasale Srilanka	14.555	0.144	4.167	16.664	4.110		14.330	25.381	13.715	0.038	25.381
7. S haematobium	18.329	16.311	16.532	0.002	16.778	16.666		15.545	22.179	14.757	15.509
8. S mattheei	16.282	18.023	14.330	14.063	14.053	18.762	14.065		18.242	22.545	0.003
9. S incognitum	6.018	12.779	17.575	18.214	17.788	12.739	18.214	16.560		14.782	18.225
10. S mansoni	14.077	0.029	6.183	16.310	6.100	0.131	16.311	17.991	13.298		22.545
11. S indicum Bangaldesh	16.283	18.023	14.330	14.050	14.053	18.762	14.051	0.005	16.545	17.991	

Discussion: The complete 18S rRNA gene of *S. spindale* contained sequences suitable for use as species-specific probes besides being highly conserved [7]. Sequence data analysis of *S. japonicum* isolates in mainland of China also suggested that 18S rRNA and 28S rRNA were effective genetic markers and could be useful in identification of schistosomes [8]. No information is available on small subunit ribosomal RNA sequence of pouched amphistome species prevalent in the country. Besides, the nucleotide sequence anlaysis of this gene of Kerala isolates of schistosomes were also not studied. The present investigation provided evidence for the high similarity of 18 and 28 S r RNA sequences of pouched amphistomes and schistosomes isolated from cross bred cattle of the State. Previous report suggests that primers targeting the 18S and 28 S rDNA of schistosomes could not be incorporated as ideal markers in the diagnostic protocols of schistosomosis [6]. Nevertheless,

both these sets of primers could be used to identify the snail vectors harbouring the developmental stages of schistosomes or amphistomes or both. This protocol could thus be effectively translated for accurate monitoring of infected snails in an area, which would, in turn, be useful to direct snail control measures to targeted population alone.

Further investigation into nucleotide sequence similarity with *Fischoederius* sp. and *Gastrothylax* sp. proved unsuccessful, since complete 18S rRNA gene sequence data of these flukes were unavailable in databases. There are no previous published documents regarding restriction enzyme mapping of the sequences of *S. spindale, S. indicum, F. elongatus or G.crumenifer*. But, a PCR RFLP of a 480 bp PCR product containing ITS-2 region using the restriction enzymes, Taq 1 and Sau 3A1 was reported to be helpful for differentiation of *S. bovis* and *S. haematobium* [9].The restriction map analysis revealed that there were subtle differences in nucleotide sequences of 18S rRNA gene between species which could be well detected by RFLP.

Phylogenetic tree based on partial 18S rRNA gene sequence showed that *S. spindale*, Kerala was closest to *S. incognitum* and occurred as sister clade to *S. spindale*, Sri Lanka and *S. indicum*, Kerala and had exhibited good bootstrap support. Evolutionary divergence revealed that *S. spindale*, Kerala was farthest from S. *bovis* and *S. haematobium*. *Schistosoma spindale* clustered strongly with the African species (*S. mansoni* and *S. haematobium*) based on V4 region and D1 domain of ribosomal gene sequences [10]. However, the phylogenetic analysis was performed using short nucleotide sequences obtained in the study and hence the conclusions drawn need not be absolute. The present scenario of bovine schistosomosis and amphistosmosis in Kerala demands much of research towards analyzing the nucleotide sequence of complete ribosomal gene to unravel the genetic diversity of different isolates infecting cattle population throughout the State.

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