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Sequence phylogenetic analysis and associative genetic diversity of *Sarcocystis hirsuta* based on 18S rRNA gene

Vikrant Sudan^{1*} , Rahul Kumar², Daya Shanker¹ and Amit Singh¹

Abstract

Background: *Sarcocystis hirsuta*, a tissue cyst-forming coccidian parasite of cattle, is worldwide in distribution. In spite of its global presence, limited literature is available on its characterization studies. No literature is available from India on molecular aspects of *S. hirsuta*. The present study was designed to characterize the isolates of *S. hirsuta* on the 18S gene locus. A total of five isolates of *S. hirsuta* were characterized. PCR products were cloned, sequenced, and compared with other sequences across the world. A phylogenetic tree was constructed based on the maximum parsimony (MP) method with the tree-bisection-regrafting (TBR) algorithm.

Results: An appreciable genetic variability was noticed between various *S. hirsuta* isolates at the 18S gene locus. Sequences generated from the present study (MN121567–MN121571) represented two haplotypes with 99.74–100.00% nucleotide homology within themselves. Alongside, a nucleotide homology of 97.82–99.92% was observed between Indian isolates and isolates across the globe. The two haplotypes were markedly distinct from each other with 3 nucleotide substitutions within themselves. Overall, Indian isolates of *S. hirsuta* were close to those from China and Vietnam than to those from New Zealand, Brazil, and Germany.

Conclusion: The present communication describes the first report of phylogenetic characterization of *S. hirsuta* from India. The findings are very much important in delineating the evolutionary phylogenetics of *S. hirsuta*.

Keywords: 18S, *Sarcocystis hirsuta*, Phylogenetic analysis

1 Background

There are four species of *Sarcocystis* reported from the cattle viz. *S. cruzi*, *S. hirsuta*, *S. hominis*, and *S. rommeli* [1]. Among them, *S. hirsuta* is the only species that forms macroscopic sarcocysts and is reported worldwide [1]. So, as far as the Indian scenario is concerned, multiple reports about the existence of *S. hirsuta* in cattle are available in the literature [2–5].

Differentiation and characterization of various *Sarcocystis* spp. are usually done targeting the 18S rRNA gene [6]. Very limited work has been done in India on the

genetic characterization of various *Sarcocystis* spp. [7–9]. However, globally, the work done on the characterization of *S. hirsuta* [10–13] suggests the existence of genetic variability within its isolates. There is not even a single report about the phylogenetic characterization of *S. hirsuta* from India. So, the present work was undertaken with the objective to genetically characterize Indian isolates of *S. hirsuta* based on the 18S rRNA gene. Alongside, the phylogenetic analysis of the various sequences of *S. hirsuta* that are available worldwide was also done. Lastly, the nucleotide homology of the Indian isolates, with each other and with other isolates of *S. hirsuta* across the world, was computed.

* Correspondence: viks.sudan@gmail.com

¹Department of Parasitology, U. P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan (DUVASU), Mathura 281001, India

Full list of author information is available at the end of the article

2 Methods

2.1 Tissue collection and ethical compliance

Esophageal tissue was collected from the cattle brought for post mortem examination to the College of Veterinary Science and Animal Husbandry, DUVASU, Mathura. The tissue samples were collected in compliance with the ethical standards and guidelines of the Institutional Animal Ethics Committee (IAEC), and permission was granted via order no IAEC/17/24. The samples were collected from dead animals irrespective of their sex and age. Tissue samples were then transported to the Department of Parasitology, DUVASU, on ice and washed thoroughly under running water. After removing the superficial fascia and fat and rinsing them with normal saline, they were immediately processed for the isolation of cysts. Tissue samples were examined for the presence of sarcocysts. The samples were collected as a part of a separate study [5] wherein molecular prevalence and RFLP were standardized for *S. hirsuta*. Out of 25 animals examined in the previous study [5], eight animals were found to be positive for *S. hirsuta* with a prevalence rate of 32%. The examination was done by teasing out of superficial sarcocysts alongside the muscle squash method with the addition of methylene blue [14]. The cysts were identified (Fig. 1) based on their morphological features described elsewhere [1]. Thereafter, the individual sarcocysts (one per animal) were stored at -20°C till DNA isolation.

2.2 DNA isolation, primer selection, and cloning of 18S gene of *S. hirsuta*

DNA was isolated from teased out individual sarcocyst using the commercially available DNA isolation kit (Quiagen) following the manufacturer's protocol. Primers for the 18S genes of *Sarcocystis* spp. were custom synthesized from Imperial Life Sciences Pvt. Ltd., Gurugram, India, using sequences described elsewhere

[11]. The PCR reaction was set up into 50 μl volume containing 25 μl of Green PCR Master Mix (0.05/ μl Taq DNA polymerase in reaction buffer, 4 mM MgCl_2 , 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP, and 0.4 mM dTTP), 3 μl of each primer (10pmol/ μl of each primer), and 5 μl of the extracted DNA template. The total volume of the PCR mix was made up to 50 μl using nuclease-free water. The amplified products were visualized in ethidium bromide incorporated 1.25% agarose gel. The PCR products were then purified using gel purification and DNA clean up kit (Fermentas, Germany) following the manufacturer's protocol. Thereafter, the purified products were cloned into CloneJET PCR Cloning Vector (Fermentas, Germany) following the manufacturer's protocol. The recombinant clones were harvested for plasmid isolation. The purified plasmids were sent for outsourced DNA sequencing to Invitrogen Bio Sciences Pvt. Ltd., Gurugram, India using pJET1.2 universal primers (specific for given cloning vector).

2.3 Sequence analysis and construction of phylogenetic tree

Respective accession numbers were assigned to the sequences, generated from the present study, upon their submission into the NCBI portal. A multiple sequence alignment, with Clustal W programme [15] within the MEGA 6 software, was made using a gap opening penalty of 10 and gap extension penalty of 0.1 and 0.2 for the pairwise and multiple alignments, respectively. The sequences were truncated at both ends, so that they started and ended at the homologous nucleotide positions. A phylogenetic tree was constructed employing the maximum parsimony (MP) method with the tree-bisection-regrafting (TBR) algorithm on the available sequences of *S. hirsuta*. Thereafter, nucleotide homology between Indian sequences of *S. hirsuta* and sequences across the world was also calculated. Phylogeny was

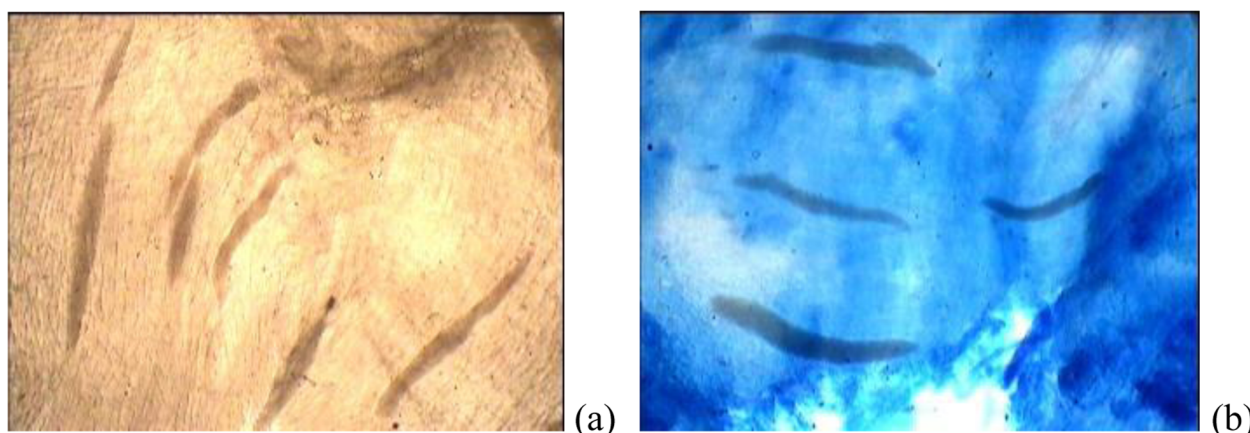


Fig. 1 Muscle squash tissue showing *S. hirsuta* in the esophagus of cattle. **a** Unstained. **b** Methylene blue-stained

tested using 1000 bootstrap replications. A sequence of *Eimeria tenella* (U67121) was used to root the phylogenetic tree. Besides, a sequence (KU247911) of closely related species (*S. buffalonis*) was also kept in the tree.

3 Results

On the basis of morphological features, the sarcocysts were identified as those of *S. hirsuta*. The cysts were comparatively larger in size (up to 8 mm in length and 1 mm width) with a thick wall (Fig. 1). Electron microscopy or histopathology could not be performed owing to the limited funds and lack of facility in the parent department.

3.1 Phylogenetic characterization and nucleotide homology of 18S gene of *S. hirsuta*

A total of five isolates of *S. hirsuta* were characterized for the 18S gene. The accession numbers obtained from NCBI were MN121567–MN121571. These five sequences were aligned with other available sequences of *S. hirsuta*, and a phylogenetic tree was constructed using the MEGA 6 software. Likewise, nucleotide homology of

these isolates with other isolates of *S. hirsuta* was also calculated.

Two haplotypes were noticed. While sequence MN121567–68 represented one haplotype, sequences MN121569–71 represented the second haplotype. All the five Indian isolates formed a separate portion of a large clade on the phylogenetic tree and were seen close to a particular isolate (AF176938) from China. On broader aspects, the Indian isolates were globally seen closer to isolates from Vietnam and Brazil. These Indian sequences showed a marked distance from sequences of *S. hirsuta* originating from New Zealand and Germany (Fig. 2). The two Indian haplotypes differ by 3 nucleotide substitutions between them viz. C→G at nucleotide position 355, T→A at nucleotide position 494, and A→T at nucleotide position 598. The two haplotypes shared 99.74–100.00% nucleotide homology between themselves; however, the nucleotide homology levels of 97.82–99.92% were noticed with other isolates of *S. hirsuta* across the globe. The sequence of *S. buffalonis* was seen distinctly separate from the sequences of *S. hirsuta*.

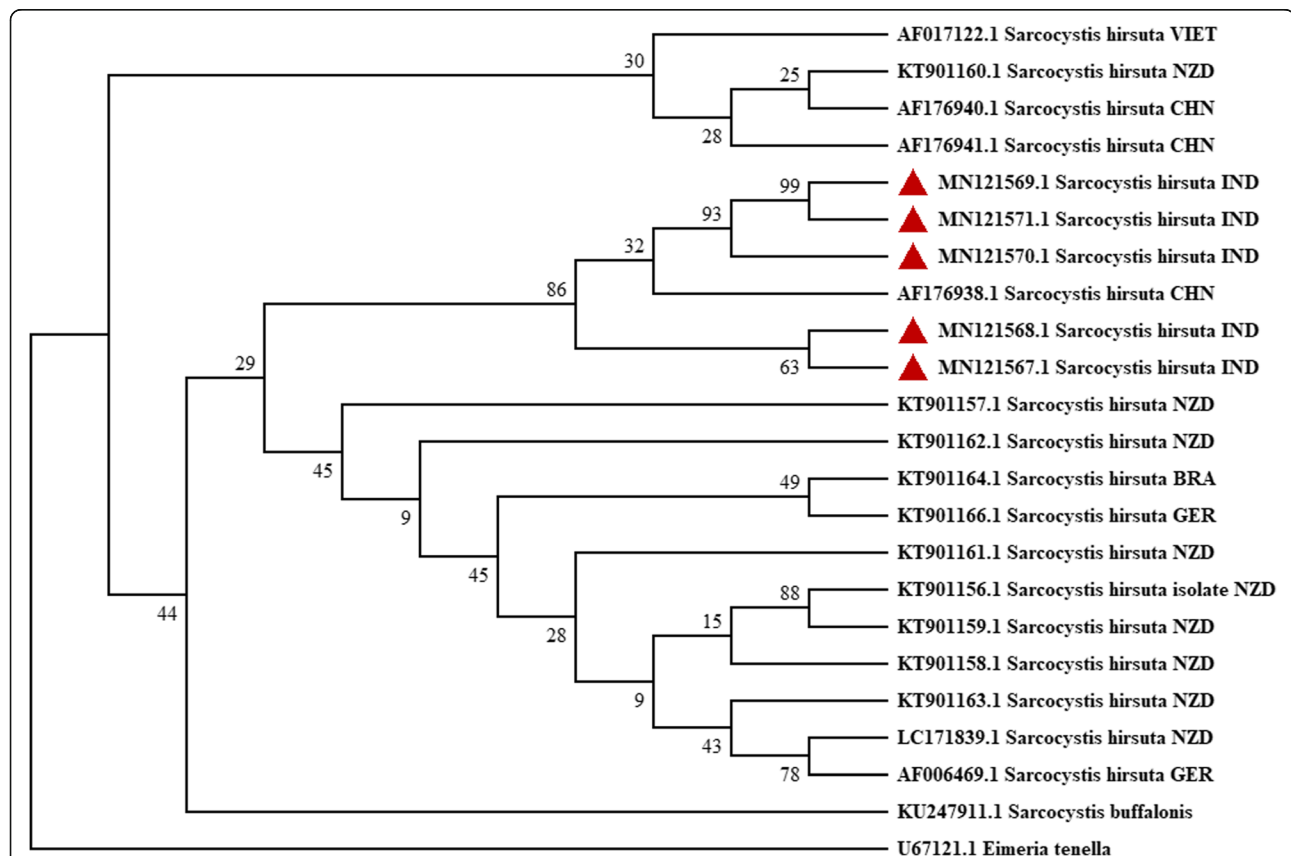


Fig. 2 Phylogenetic relationship of *S. hirsuta* isolates across the world based on 18S rRNA gene. All accession numbers correspond to different *S. hirsuta* isolates followed by their country of origin. The sequences generated in the present study are marked as a red triangle. CHN, China; GER, Germany; IND, India; NZD, New Zealand; VIET, Vietnam

4 Discussion

A peculiar feature in sequence phylogenetic analysis of various *Sarcocystis* spp. is the existence of genetic variation within the isolates of the concerned species [8, 9, 16]. Most of these variations are attributed to intra-isolate heterogeneity due to the presence of certain indels [8, 9, 13, 16]. It is hard to assign various sequence variants to a particular *Sarcocystis* spp. until it is fully ascertained that they all have been originated from a single sarcocyst [17].

There are limited reports on the characterization studies pertaining to *S. hirsuta* throughout the globe and subsequently, limited information is available about the associative genetic diversity within its isolates [10–13]. Though there are many reports about the presence of *S. hirsuta* from India [7–9] including a few reports on PCR-RFLP-based differentiation of *S. hirsuta* from *S. cruzi* [8, 9], yet the authors could not find a single literature regarding the genetic characterization of *S. hirsuta* from the Indian context. So, the present study appears to be the pioneer documentation regarding the genetic characterization and subsequent, sequence phylogenetic analysis of *S. hirsuta* isolates from India. Alongside, the animals used for the collection of sarcocysts in the present study were mostly adult farm animals. The area where the animals are kept had an open access to dogs and cats alongside, major chances of contamination of their feeds and fodder with feces of these animals, accounting for a high prevalence of infections like sarcocystosis and hydatidosis [5, 14].

A total of 21 sequences of *S. hirsuta* were compared on the phylogenetic tree. This included five sequences from India (generated in the present study), and subsequently, two haplotypes were noticed among the studied Indian isolates. The Indian isolates differed within themselves owing to three nucleotide substitutions within themselves. They showed marked closeness with particular Chinese isolate (AF176938) than with other *S. hirsuta* isolates from China and across the world. On a broader term, Indian isolates were seen closer to isolates from China, Germany, Brazil, and Argentina. The Indian isolates of *S. hirsuta* were seen markedly distinct from isolates from New Zealand and Vietnam. This clearly suggests the presence of an appreciable amount of genetic variability within *S. hirsuta* isolates across the globe.

The Indian isolates shared 99.74–100.00% nucleotide homology within themselves alongside 97.82–99.92% nucleotide homology with other isolates of *S. hirsuta* across the globe. Deletion of few indels was reported in selective *S. hirsuta* isolates, upon full sequence analysis of the 18S gene [11], accounting for nucleotide homology levels of 98.3.0–100.0% within various *S. hirsuta* isolates. Gjerde et al. [13] stressed upon the use of multiple marker genes (28S, ITS, and cox 1), besides the 18S

rRNA gene, for the clear-cut differentiation of *S. buffalo-nis* in buffaloes and *S. hirsuta* in cattle. Both these species were considered to be monophyletic sister groups based on the detailed evaluation of these four marker genes [13].

5 Conclusion

In conclusion, two haplotypes of *S. hirsuta*, sharing 99.74–100.00% nucleotide homology, were reported in the present study. They differ by three nucleotide substitutions within themselves. In general, the Indian isolates were found to be close to a selective isolate from China suggesting possible common ancestry. Overall, Indian isolates of *S. hirsuta* were close to those from China and Vietnam than to those from New Zealand, Brazil, and Germany. More well-planned studies, using multiple marker genes and using isolates all across the country, are required for the delineation of the evolutionary phylogenetics of *S. hirsuta* from India.

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Authors' contributions

All authors have read and approved the final manuscript. VS performed the study and prepared the manuscript. DS helped in the identification of the parasite. RK collected the tissue samples and the parasite. AS helped in the manuscript writing and isolation of cysts.

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Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Declarations

Ethics approval and consent to participate

The tissue samples were collected in compliance with the ethical standards and guidelines of the Institutional Animal Ethics Committee (IAEC), and permission was granted via. order no IAEC/17/24.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹Department of Parasitology, U. P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan (DUVASU), Mathura 281001, India. ²Department of Pathology, U. P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan (DUVASU), Mathura 281001, India.

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