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Original paper

Associative genetic diversity among *Sarcocystis cruzi* isolates from Northern India based on 18S ribosomal gene

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ABSTRACT. *Sarcocystis cruzi* is perhaps the most common *Sarcocystis* spp. affecting cattle throughout the world. Despite its wide presence across Indian subcontinent, very limited studies are reported from India describing the phylogenetics of the parasite. The present communication describes the phylogenetic characterization of various isolates of *S. cruzi*. The isolates were characterized at 18S gene locus. An appreciable among of genetic variability was noticed between various *S. cruzi* isolates. Sequences generated from the present study (MN121572–MN121576) represented two haplotypes with 99.0–100.0% nucleotide homology within themselves. Alongside, nucleotide homology of 98.36–100.0% was observed between Indian isolates and isolates across the globe. The two haplotypes were markedly distinct from each other with 10 nucleotide substitutions within themselves. Moreover, there was a deletion of G nucleotide at position 545 in one of the sequences (MN121575). In general, the Indian isolates were seen closer to isolates from China than to the isolates from Iran, Argentina, Japan, Australia and Netherlands. Results clearly depicted the presence of multiple lineages of *S. cruzi* in Indian cattle. The findings are very much important in delineating the evolutionary phylogenetics of *S. cruzi* from India and abroad.

Keywords: phylogenetic analysis, *Sarcocystis cruzi*, 18S rRNA

Introduction

Four valid species of *Sarcocystis* have been reported from the cattle *viz., S. cruzi*, *S. hirsuta*, *S. hominis* and *S. rommeli* [1]. Among them, *S. cruzi* is often regarded as the most pathogenic and widely prevalent *Sarcocystis* spp. affecting cattle throughout the world [1,2]. It can cause fever, anorexia, anaemia, weight loss, muscle twitching, prostration, abortions, hyper salivation, neurological signs and sometimes, death, depending upon the isolate involved and number of sporocysts ingested [1,3]. The parasite is, sometimes, also associated with fatal acute eosinophilic myocarditis [3] characterized by severe, disseminated eosinophilic granulomatous necrotizing myocarditis with myocardial fibrosis. The parasite has been reported worldwide with very high prevalence rates [2,4]. As far as Indian scenario is concerned, there are multiple reports about prevalence of *S. cruzi* throughout India [4–7]. Religious sediments often limits the wide epidemiological data covering the exact prevalence of *S. cruzi* across India, yet many abattoir [4–6] and post mortem [7] studies do suggest a high prevalence rate of the parasite across India.

Molecular analysis based on variable region of 18S rRNA is routinely used to differentiate *Sarcocystis* spp. [8,9]. Molecular characterization studies on *Sarcocystis* species in India are very much limited [9–11]. However, globally work done on molecular characterization work done on *S. cruzi* [12–14] suggests genetic variability amongst its isolates. Baring a single report [10] authors did not find any appreciable literature on phylogenetic analysis of *S. cruzi* from India. So the present study was designed to genetically characterize Indian isolates of *S. cruzi* based on 18S ribosomal (rRNA) gene.

Materials and Methods

Sample collection

Heart tissue samples were collected from the cattle brought for post mortem examination to the College of Veterinary Science and Animal Husbandry, DUVASU, Mathura as a part of separate study [7]. 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 superficial fascia and fat and rinsing them with normal saline, they were immediately processed for isolation of cysts. Tissue samples were examined microscopically for the presence of sarcocysts. Examination was done by muscle squash method with addition of methylene blue [15]. The cysts were identified (Fig. 1) based on their morphological features described elsewhere [1]. A total of 24 animals were screened in that study out of which 15 animals (62.50%) were found to be infected with sarcocysts of *S. cruzi.* Individual sarcocyst was teased out (one per animal) of cardiac tissue and stored at –20°C till DNA isolation.

DNA isolation, primer selection and cloning of 18S gene of S. cruzi

DNA was isolated from teased out individual sarcocyst using the commercially available DNA isolation kit (Quaigen) following the manufacturer's protocol. Primers for 18S genes of *Sarcocystis* spp. were custom synthesized from Imperial Life Sciences Pvt. Ltd., Gurugram, India using sequences described elsewhere [16]. The PCR reaction were set up into 50 µl volume containing 25 µl of Green PCR Master Mix (0.05/µlTaq 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 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).

Sequence analysis and construction of phylogenetic tree

The sequences, hence obtained, were submitted into NCBI and corresponding accession numbers were obtained. A multiple sequence alignment was generated with the Clustal W programme within MEGA 6 software [17], using a gap opening penalty of 10 and gap extension penalty of 0.1 and 0.2 for the pair wise and multiple alignments, respectively. The sequences were truncated at both ends, so that nearly all sequences started and ended at the

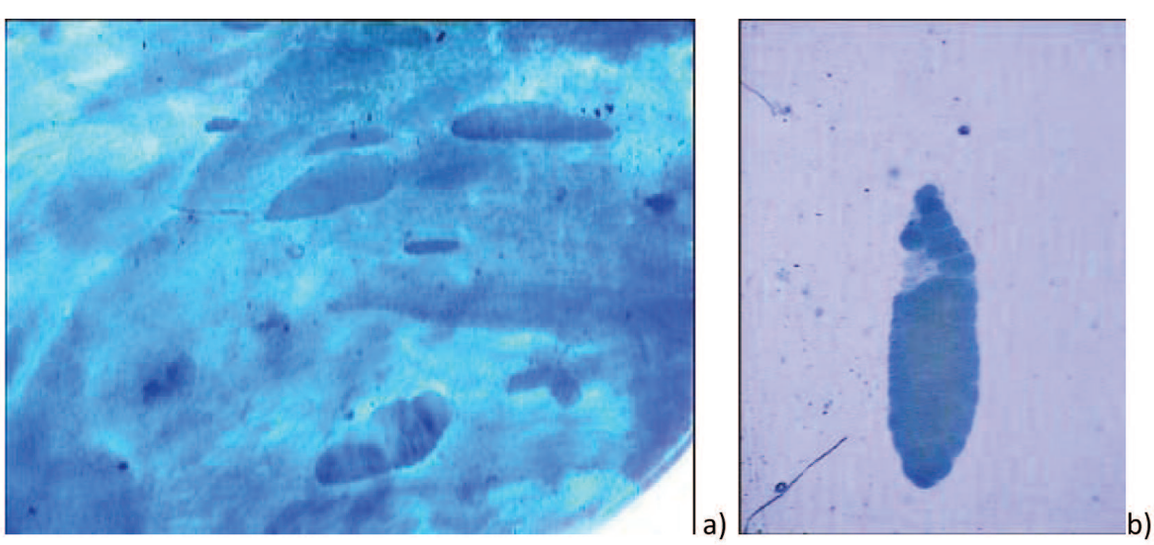

Figure 1. *Sarcocystis cruzi* from heart tissue of cattle upon methylene blue staining. (a) muscle squash method, (b) teased out individual sarcocyst

Table 1. Table depicting nucleotide substitutions alongside the position of substitution and the nucleotide involved in studied sequences of *S. cruzi*

Nucleotide position	25	53	114	204	445	603	793	903	953	960
Sequences										
MN121572–MN121573	T	C	G	T	A	G	A	G	T	A
MN121574–MN121576	C	T	A	A	T	A	G	A	A	C

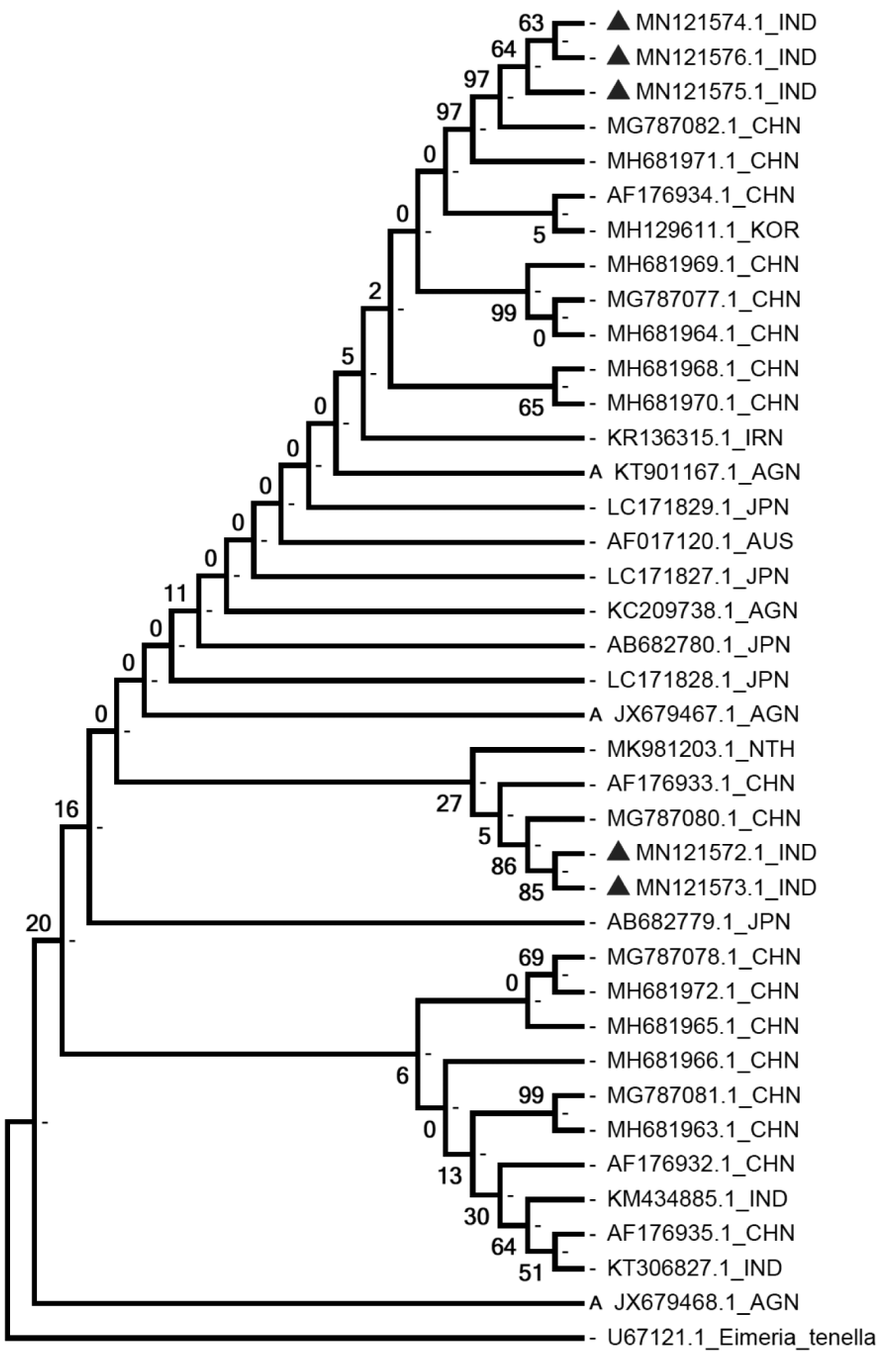

Figure 2. Phylogenetic relationship of *S. cruzi* isolates across the world based on 18S gene. All accession numbers corresponds to different *S. cruzi* isolates followed by their country of origin. The sequences generated in the present study are marked as red triangle. (AGN: Argentina; CHN: China; IND: India; JPN: Japan; KOR: Korea; NTH: Netherlands)

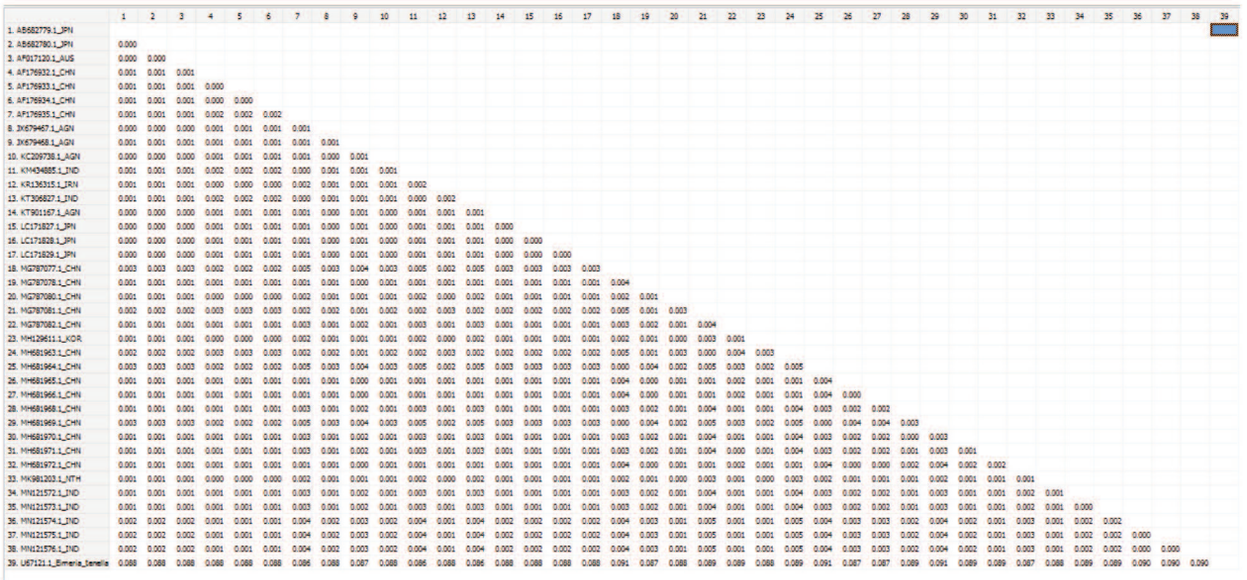

Figure 3. Nucleotide homology table of various isolates of *S. cruzi* across the globe. All accession numbers corresponds to different *S. cruzi* isolates followed by their country of origin. The sequences generated in the present study are marked as red triangle. (AGN: Argentina; CHN: China; IND: India; JPN: Japan; KOR: Korea; NTH: Netherlands)

homologous nucleotide position. Phylogenetic tree was constructed using present sequences alongside other available sequences of *S. cruzi* employing maximum parsimony (MP) method with the tree-bisection-regrafting (TBR) algorithm. Thereafter, nucleotide homology between Indian sequences of *S. cruzi* and sequences across the world was also calculated. Phylogeny was tested with bootstrap method using 1000 bootstrap replications. GenBank sequence U67121 of *E. tenella* of chickens was used as out-group species to root the tree and sequence AF176935 was taken as the standard *S. cruzi* sequence for comparing all the sequences.

Ethical statement

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 number IAEC/17/24.

Results

The sarcocysts were identified as those of *S. cruzi* based on the morphological features described elsewhere [1]. The microscopic cysts were thin walled (Fig. 1). A total of five sarcocysts (one per animal) were analyzed.

Phylogenetic analysis and nucleotide homology of 18S gene sequences of the present Indian isolates with other isolates of S. cruzi across the world

A total of five clones of *S. cruzi* were characterized for 18S ribosomal gene. The nucleotide sequences obtained were submitted in NCBI and corresponding accession numbers (MN121572-MN121576) were obtained. These sequences were aligned with other sequences of *S. cruzi* available in NCBI and phylogenetic tree was drawn using Mega 6 software. Likewise, nucleotide homology of present isolates with other isolates of *S. cruzi* was also calculated.

A total of two haplotypes were noticed within the studied *S. cruzi* sequences in which sequences MN121572 and MN121573 represented one haplotype while the sequences MN121574–MN121576 constituted other haplotype. These two haplotypes were seen distinctly on phylogenetic tree forming two opposite sub-positions on a large clade. Both these haplotypes were close to different individual isolates from China depicting vast genetic variability. On a whole, the Indian isolates were globally closer to Chinese isolates than to the Iranian, Argentinean, Japanese, Australian and Netherlands counterparts (Fig. 2). The two haplotypes showed 10 nucleotide substitutions between them. The details of the nucleotide substitutions in these haplotypes are given in table 1. Alongside, a peculiar deletion of G was noticed at nucleotide position 545 in sequence MN121575 in respect to all other sequences. These five sequences constituting the two haplotypes had

99.0–100.0% nucleotide homology between themselves, however, the nucleotide homology levels of 98.36–100.00% was noticed with other isolates of *S. cruzi* across the globe (Fig. 3).

Discussion

Marked genetic variability, among the respective isolates of various *Sarcocystis* spp., is a peculiar feature noticed throughout the globe [11,18–20]. Even though there are many reports on characterization studies related to *S. cruzi* throughout the globe, only a handful of information is available about the associative genetic diversity within the isolates of *S. cruzi* [12–14]. There is only one literature available about genetic characterization of *S. cruzi* from Indian context [10].

A total of 38 *S. cruzi* sequences including 5 (MN121572–MN121576) generated in the present study were compared and two haplotypes were noticed among the studied Indian isolates. The present Indian haplotypes formed two parts of a broader clade in the phylogenetic tree and were seen as markedly distinct from each other owing to 10 nucleotide substitutions within themselves. Moreover, there was a deletion of G nucleotide at position 545 in one of the sequences. Additionally, both these haplotypes showed marked similarity with specific isolates from China. On a broader term, Indian isolates were seen to be closer to few of the Chinese isolates than to the isolates from Iran, Argentina, Japan, Australia and Netherlands. In contrast, the sequences earlier reported from India showed marked resemblance with another set of Chinese isolates and they together formed a separate clade. This clearly suggests presence of different lineages of *S. cruzi* among Indian cattle population.

A substantial amount of intra isolate sequence variation in the 18S rRNA gene has been recorded in several *Sarcocystis* spp. [11–14,18–20]. However, this intra-isolate heterogeneity is mostly attributed to indels which makes it a necessity to clone the PCR products before sequencing as it is hard to assign various sequence variants to a particular *Sarcocystis* species until it is fully ascertain that they all have been originated from a single sarcocyst [19]. Hence, many authors have reported this ambiguity in *Sarcocystis* spp. owing to direct sequencing of their PCR products [21,22]. However, it has already been proven that 18S gene alone can be used to differentiate between *S. cruzi* and other closely related *Sarcocystis* spp. [14].

The studied Indian isolates showed 99.0–100.0% nucleotide homology within themselves alongside, 98.36–100.00% nucleotide homology with other available isolates across the globe. In contrast, Daptardar et al. [10] reported much lesser levels (96.0-99.0%) of nucleotide homology within Indian isolates. This could be very much justified on the above stated facts that fact that they opted for direct sequencing of PCR products rather than cloning alongside, the portion of 18S gene used by them for phylogenetic analysis was different from that used in present study.

In conclusion, a total of two haplotypes were observed sharing 99.0–100.0% and 98.36–100.0% nucleotide homology within themselves and with isolates across the globe, respectively. The two haplotypes showed 10 nucleotide substitutions within themselves alongside a unique deletion of G nucleotide at position 545 in one of the sequences. In general, the Indian isolates were seen closer to isolates from China than to the isolates from Iran, Argentina, Japan, Australia and the Netherlands. Presence of multiple lineages, as evident by nucleotide homology and wider distance on phylogenetic tree, of *S. cruzi* clearly states of vast genetic diversity of the parasite in Indian cattle. More well planned studies, using multiple marker genes, are thereby warranted to actually delineate the evolutionary phylogenetics of *S. cruzi* from India. So far no work has been reported from India on *S. cruzi* using any other marker gene.

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