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Prevalence and first molecular identification of *Sarcocystis* species in cattle and water buffaloes in India

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Abstract

The importance of *Sarcocystis hominis* in causing zoonotic infections is well known. Recently, *S. hominis* like cysts have been reported from water buffalo in China. Previous studies indicate prevalence of *Sarcocystis* species in bovine populations in India but molecular evidence is required for proper species differentiation. We examined two hundred and ninety six cardiac tissue samples of Indian water buffaloes and cattle from northern and western parts of the country. Tissues were examined for *Sarcocystis* using intact cyst isolation method, pepsin acid digestion method and *Sarcocystis* 18S rRNA PCR. The combination of primers was used for 18S rRNA PCR amplification followed by sequencing. Twenty five representative samples were sent for sequencing and 19 readable sequences were obtained for phylogenetic analysis. Overall, the *Sarcocystis* cysts/zoites were recorded in 44% (95% CI 38–49%), 58% (95% CI 53–64%) and 68% (95% CI 63–73%) from both cattle and buffalo samples using intact cyst isolation, pepsin-HCl digestion method and conventional PCR, respectively. The results indicate that pepsin-HCl digestion method and conventional PCR are more sensitive than intact cyst isolation for detection of *Sarcocystis* species in tissue samples. The prevalence of *Sarcocystis* species was high in buffalo as compared to cattle intermediate hosts. Phylogenetic analysis indicated that more than one *Sarcocystis* species are circulating in cattle and water buffaloes in India. The results further indicate that experimental transmission studies are required to re-confirm the identities and host ranges of the *Sarcocystis* species in cattle and water buffaloes in India.

Keywords

Cattle, India, molecular identification, prevalence, *Sarcocystis* species, water buffaloes

Introduction

The genus *Sarcocystis* has more than 200 named species that infect mammals, birds, marsupials, and poikilothermic animals (Dubey and Lindsay 2006). Cattle act as intermediate host for three *Sarcocystis* species viz. *Sarcocystis cruzi*, *S. hirsuta* and *S. hominis* which use canids, felids and primates, respectively as definitive hosts. Additionally, a new species *S. sinensis* has also been recognized from cattle in China (Yang *et al*. 2001). Water buffaloes are intermediate hosts for four *Sarcocystis* species viz *S. fusiformis*, *S. buffalonis*, *S. levinei* and *S. dubeyi*. Among these, *S. fusiformis* and *S. buffalonis* form macroscopic cysts with cats as the definitive host, whereas *S. levinei* and *S. dubeyi* form microscopic sarcocysts. Dogs are known to be the definitive hosts for *S. levinei*, however the definitive host (s) for *S. dubeyi* has not yet been identified (Hilali *et al*. 2011).

The definitive host becomes infected after consuming infected meat containing sarcocysts (Gajadhar *et al*. 2015). Although infection usually remains asymptomatic in intermediate hosts, heavy infections could lead to condemnation of carcasses (Olsen 1974). Condemnation of beef due to sarcocysts is a serious economic problem (Dubey *et al*. 1990; Imes and Migaki 1967). In view of the public health and food safety importance of the *S. hominis* (Singh *et al*. 2010) and lack of molecular evidence for proper identification of *Sarcocystis* spp. in India, the current study was carried out to estimate prevalence and molecular identification of *Sarcocystis* species in cattle and water buffaloes in India.

Material and Methods

Place of work

The current study was carried out in School of Public Health & Zoonoses, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana (India).

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Sample collection

Two hundred and ninety six cardiac tissue (152 buffalo and 144 cattle) samples of Indian water buffaloes and cattle were collected from northern and western parts of the country. The buffalo tissue samples were collected from a slaughter house in Uttar Pradesh state of North India. Due to ban on cow slaughter in most of the North Indian states, the cattle tissue samples were collected from a slaughter house in Mumbai, Maharashtra state of Western India. Each sample weighed approximately 50–100 g and was transferred in self-sealed plastic bags to the laboratory within 4–6 hours of collection. The samples were stored at 4ºC until further use. The additional 5–10 g of each sample was stored in 70% ethanol for molecular studies. The 5–10 g of each sample was also stored in 10% formalin for histological studies.

Intact cyst isolation method

Small pieces of suspected tissues (approx. 2–3 cms) and about 5 to 10 g were cut and chopped in normal saline solution (0.85%) with the help of needles and forceps for 2 min in a petri dish and then examined under a stereo microscope (Juyal *et al.* 1989). The cysts were transferred to glass-slides, covered with cover slips and first examined under low (X40) and then under higher magnification (X100).

Pepsin- HCl digestion method

The tissue samples (30–50 g of each sample) previously used for squeezing were digested in peptic digestive solution at room temperature for 2–4 hours by continuous stirring (Jacobs *et al*. 1960). The digestive material was passed through a strainer to remove undigested fat and connective tissue. The strained material was then centrifuged for 10 minutes at 2,000 rpm. The supernatant was decanted and the sediment was re-suspended in one ml of normal saline and observed under microscope. An aliquot of sample was observed under a compound microscope at X40 magnification.

DNA extraction and conventional PCR

DNA was extracted from *Sarcocystis* cysts recovered from 129 samples using an intact cyst isolation and bradyzoites recovered from an additional 44 samples found negative by the intact cyst isolation. For remaining samples, DNA was extracted from tissue samples previously used for squeezing and found negative both in intact cyst isolation and pepsin acid digestion method. The DNA was extracted using Qiagen, DNeasy mammalian genomic DNA extraction kit as per manufacturer's instructions. The eluted DNA was stored at –20° C until further use. The amplification of different fragment sizes was done using 18S rRNA gene primer combinations (Table I) so as to generate the maximum length of 18S rRNA gene of *Sarcocystis* species. The PCR was carried out as per Rosenthal (2010) with slight modifications. The GoTaq green master mix (PROMEGA) was used containing Reaction Buffer (pH 8.5), 400 µM dNTP each, and 3.0 mM $MgCl_2$. The buffer also contained yellow and blue loading dyes. For PCR, the reaction mixture consisted 25µl of GoTaq green master mix, 2µl of 10 pM of each primer, 5 µl of DNA template and 18 µl nuclease free water to make the final volume 50 µl. The thermal cycling conditions were as follows: 95°C for 2 min; 40 cycles of 94° C for 40 sec, 56° C for 50 sec, and 72° C for 60 sec and a final extension at 72° C for 6 min (Rosenthal 2010). The incubation was carried out in Master cycler Pro (Eppendorf, T-Gradient, Hamburg, Germany) thermal cycler. The PCR amplified products were analyzed on 1.5% agarose gel stained with ethidium bromide (0.5 µg/ml) at 70 V (60–90 minutes).

Phylogenetic analysis

The amplified PCR products were purified using commercially available QIAquick PCR Purification kit (QIAGEN) as per manufacturer's instructions. The eluted DNA was stored at –20°C till further use. The sequencing was performed in both the directions at the School of Biotechnology, Punjab Agricultural University (Ludhiana). Purified PCR products were directly sequenced using the BigDye terminator cycle sequencing kits (PE Biosystems, Foster City, California) with an ABI Prism 3730 genetic analyzer automatic sequencer using the same primers as that for PCR.

Table I. Primer sequences used for amplification of 18S rRNA gene of *Sarcocystis* species by using conventional PCR

PCRC	Primer and primer Sequence (5' – 3')	Location in 18S rRNA gene	Size of the product (bp)	References
1	1L–Forward (CCATGCATGTCTAAGTATAAGC)	446–469	1200 bp	Yang *et al.* (2001)
	1H–Reverse (TATCCCCATCACGATGCATAC)	1650–1670		
2	2L–Forward (GGATAAACCGTGGTAATTCTATG)	156–178	915 bp	Rosenthal (2010)
	3H–Reverse (GGCAAATGCTTTCGCAGTAG)	1040–1070		

The results were matched using NCBI BLAST software (Altschul *et al.* 1990). The sequences were aligned using ClustalW method with pairwise and multiple alignment without using negative matrix in Mega6 (Tamura *et al.* 2013). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.* 2004) and are in the units of the number of base substitutions per site. The sequences were compared with previously published sequences of seven *Sarcocystis* species (GenBank AF017120, AF017121, JX679467, KJ917940, JQ713823, AF017121, AF006469, U03071, AF176945, KT901120, AF176930, KT901129, KT901152 and KF954728) recorded from cattle and water buffaloes. *Toxoplasma gondii* (GenBank L37415), *Besnoitia besnoiti* (GenBank DQ227419) and *Eimeria bovis* (GenBank AB769589) were used as out-groups. A dendogram using a neighbor joining algorithm (Saitou and Nee 1987) was constructed to depict the genetic relationship and phylogeny of isolates. The reliability and robustness of phylogenetic tree was tested by bootstrap analysis with 500 replications (Felsenstein 1985).

Histopathological examination

The samples for which readable sequences were available were histopathologically examined. The formalin fixed tissues were processed by an acetone benzene method (Luna 1968). The paraffin blocks were prepared and sections of 4–5 µm thickness were obtained on glass slides with rotator microtome. These paraffin sections were stained with haematoxylin and eosin stain (H & E) for routine histopathology.

Statistical analysis

The lower and upper limits of the 95% Confidence Interval for the disease prevalence were estimated as per the method of Newcombe (1998) and Wilson (1927).

Results

Intact cyst isolation method

The sarcocysts were detected in 70 (46%, 95% Confidence Interval [CI] 38–54%) buffalo and 59 (41%, 95% CI 33–49%) cattle samples with an overall prevalence of 44% (95% CI 38–49%). Numerous elongated complete cysts were obtained. The mature cysts when crushed over the glass-slide revealed banana shaped cystozoites. Only micro sarcocysts were encountered from the tissues.

Pepsin-HCl digestion method

The pepsin-HCl digestion method revealed an overall prevalence of 58% (95% CI 53–64%). The prevalence was found to

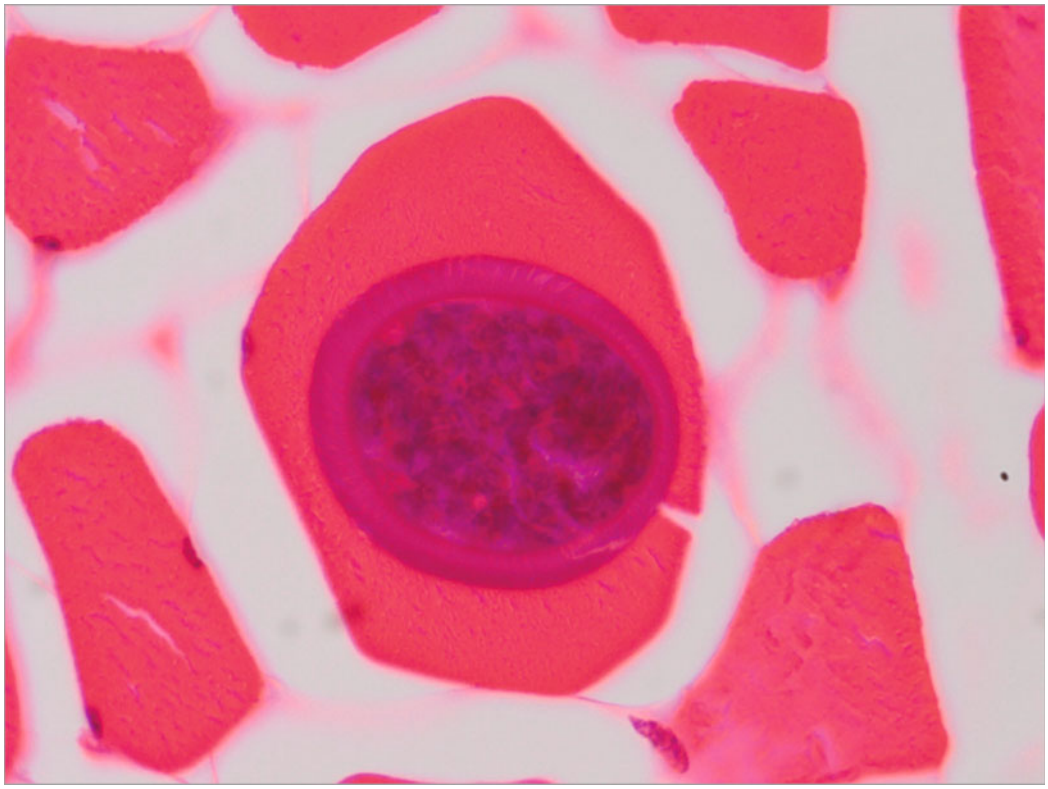

Fig. 1. Thick walled sarcocysts in tissue samples of naturally infected water buffalo (X 100, H and E stain)

be 66% (95% CI 58–73%) and 50.0% (95% CI 42–58%) in buffalo and cattle populations, respectively.

Conventional PCR

The prevalence of *Sarcocystis* in buffalo and cattle was found to be 79% (95% CI 71–84%) and 57% (95% CI 49–65%), respectively with an overall prevalence of 68% (95% CI 63–73%) in both the species.

Phylogenetic analysis

Nineteen readable sequences of approximately 900 to 1500 bp were obtained after sequencing of the samples in both the directions. Representative sequences were submitted to the NCBI, GenBank and accession numbers (KT306826-KT306829, KM588350) were obtained. In the present study all the sequenced samples were of *Sarcocystis* species. Nine buffalo nucleotide sequences showed homology (95–100%) with *S. cruzi and S. cruzi like* taxon with those in GENBANK (Accession number AB682779, AF176935, JX679467 and KJ917940). One of the samples (23A- GenBank accession number KT306826) from buffalo revealed sequence homology of 94% with *S. sinensis*, 93 % *with S. bovini and* 93 % *with S. bovifeliS.* (GENBANK accession number KT901111, KT901141, and KT901120 respectively). This sample (23a) formed a different branch in the phylogenetic tree (Fig. 1). This sample could be *S. dubeyii* as thick walled cysts were found when histologically examined but no similar nucleotide sequence was found registered in NCBI, GenBank.

In cattle, 8 nucleotide sequences showed homology (96–99%) with *S. cruzi* (GENBANK accession numbers KJ917940, AB682779, JX 679467.1, AF017120, AF176935). One cattle isolate (15C) showed homology of 92% with S. *sinesnis* (JQ 713823), 92% with *S. bovinii* (KT901147), 92% with *S. bovifelis* (KT901120), and 90% with *S. hominis (*KF954731. This isolate (15c) formed a different branch in the phylogenetic tree (Fig. 1). This species could be identical with *S. sinensis*, the taxonomic status of which is uncertain and has been debated (Dubey *et al*. 2014).

Histopathology

The thin walled sarcocysts were recorded in the tissue sections of 9 buffaloes. The thick walled cysts (Fig. 2) were recorded in an isolate phylogenetically different from others. The results were consistent with the phylogenetic analysis and indicated that *Sarcocysts levinei* (*S.cruzi* like taxon) and *S. dubeyii* are prevalent in India.

In cattle only thin walled cysts were seen in the tissues sections (Fig. 3). Thin walled cysts were of *S. cruzi* and were consistent with the sequencing result of the samples which had homology with *S. cruzi*. However, thin walled cysts were also recorded in a sample phylogenetically not similar with *S. cruzi*. No major changes were seen in the histology of the

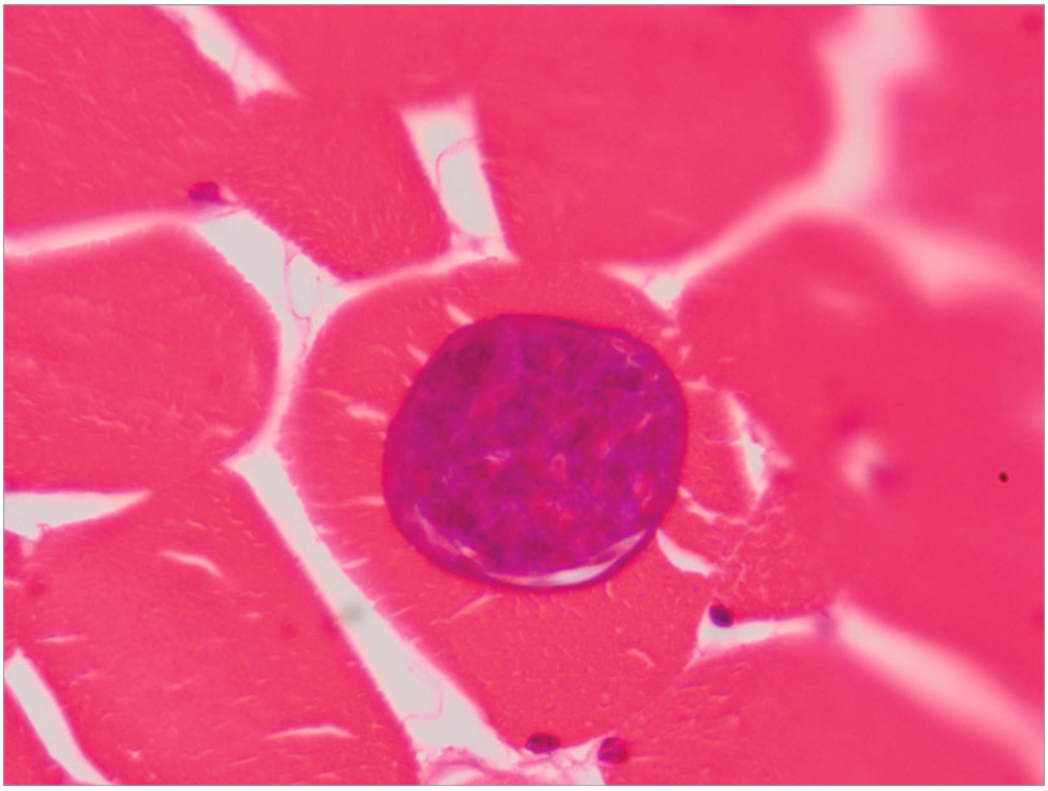

Fig. 2. Thin walled sarcocysts in tissue samples of naturally infected cattle (X100, H and E stain)

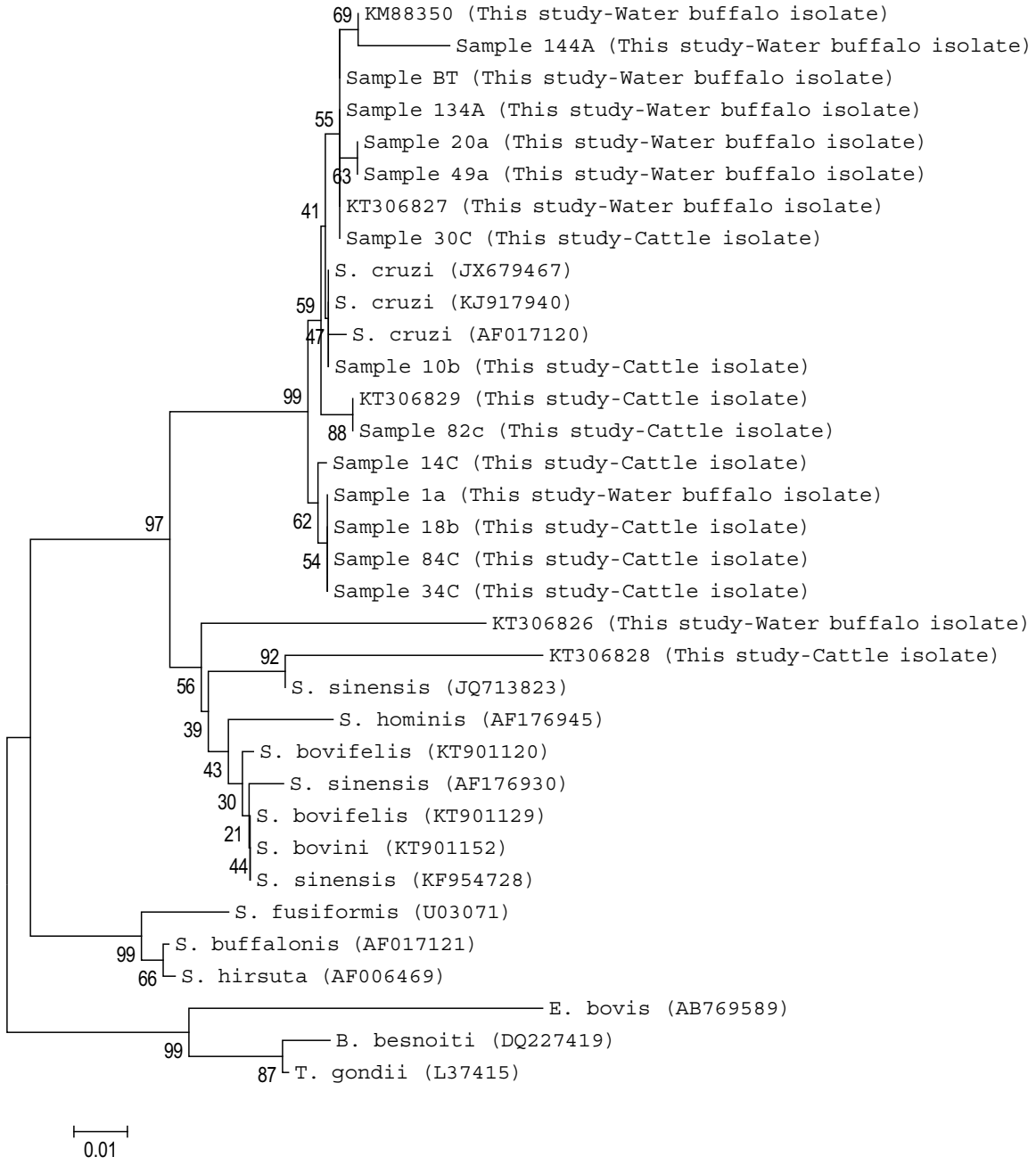

Fig. 3. Phenogram construction of the 18S rRNA gene of *Sarcocystis* isolates from cattle and water buffaloes sourced in this study together with GenBank reference strains. Numbers shown at different nodes represent percent confidence limits obtained in the bootstrap analysis. The optimal tree with the sum of branch length = 0.42892411 is shown

cells. However hypertrophy of the cell was seen where sarcocysts were present.

Discussion

The prevalence of *Sarcocystis* was low in cattle as compared to buffalo populations. Previous studies also indicate similar trends (Mohanty *et al*. 1995a; Venu and Hafeez 2000). We recorded only microscopic cysts. Similarly, Mehlhorn *et al*. (1976), Dubey (1982a, b) and Latif (1999) recorded the presence of only microscopic sarcocysts. The overall prevalence of *Sarcocystis* infested meat using PCR was 68% as compared to 58% by using pepsin-HCl digestion method. The results indicate that pepsin HCl digestion method and conventional PCR are more sensitive than intact cyst isolation for detection of *Sarcocystis* species in tissue samples. The prevalence was high in buffalo as compared to cattle intermediate hosts. Other

studies also report molecular identification to be more sensitive as compared to routine techniques (Domenis *et al*. 2011). Phylogenetic analysis indicated that more than one *Sarcocystis* species are circulating in cattle and water buffaloes in India. The results further indicate that experimental transmission studies are required to re-confirm the identities and host ranges of the *Sarcocystis* species in cattle and water buffaloes in India. Bovine sarcocystosis could be an important public health risk and transmission studies must be carried out to evaluate the role of human/ non-human primates (as definitive host) in the life cycle of this parasite in India.

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