Molecular approaches in species identification of Sarcocysts in Indian buffalo meat

C. Ramakrishna*, S. Vaithiyanathan, M. Muthukumar, L.R. Chatlod, P. Lavanya and V.V. Kulkarni

ICAR-National Research Centre on Meat, Hyderabad-500 092, Telangana, India.
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ABSTRACT

DNA was extracted from sarcocysts (visible on naked eye examination) collected from 168 buffaloes belonging to Mumbai (60), Hyderabad (54) and Kolkata (54) cities of India. They were subjected to PCR assay using 18S rRNA gene primer. All the PCR amplicons of about 900 bp were subjected to restriction enzyme digestion with four different restriction enzymes (BslI, DraI, FokI and RsaI). PCR amplicons showed two different patterns (Pattern A and Pattern B) on RFLP. Twenty one PCR products from Pattern A and one PCR product from Pattern B were subjected to DNA sequencing. *S. fusiformis* and *S. taeniata* were identified from Pattern A and *S. buffalonis* was identified from Pattern B on sequencing.

Key words: Buffalo, Identification, Meat, PCR, RFLP, Sarcocysts.

INTRODUCTION

Sarcocystosis is a protozoan disease of several domestic and wild animals having obligatory two host life cycle. Carnivores act as definite hosts and herbivores and omnivores act as intermediate hosts. During the life cycle, the fodder contaminated by sporocysts released by carnivores is eaten by herbivores. The sporozoites are released from sporocysts in the intestine of herbivores and reach different parts of body to form sarcocysts. Sarcocysts appear as opaque white cucumber seed shaped cysts with different sizes lodging in muscles all over body. The main predilection is oesophagus (Dubey et al., 1989).

The presence of Sarcocystis species in water buffalo was first observed by Railliet in 1897. He briefly described the large “balbiands” and named the parasite *Balbiana fusiformis* (Later it was renamed *Sarcocystis fusiformis*). This is the largest among the Sarcocystis species found in water buffalo, which use cats as definitive hosts. A second Sarcocystis species in buffalo, *Sarcocystis levinei*, is smaller and uses canines as its definitive host (Dissanaike and Kan, 1978). Since the 1980s, several other Sarcocystis species in water buffalo have been reported. For example, *S. cruzi* uses dogs as its definitive hosts (Dubey et al., 1989). The specimens type of at least four species of Sarcocysts have been documented and described in the water buffalo, including two macroscopic species *S. fusiformis*, commonly occurring in south-east Asia and *S. buffalonis* and two microscopic species *S. levinei* and *S. dubeyi* (Huong, 1999).

Presence of sarcocysts in buffalo meat creates economic loss particularly during export of buffalo meat due to 1) aesthetic value and 2) zoonotic significance. *S. fusiformis* with cat as definitive host and *S. levinei* with dog as definitive host are not zoonotic. However, *S. hominis* with human being as definitive host is zoonotic. It is difficult to identify the species of Sarcocyst based on morphological examination. Few workers identified species of Sarcocysts based on ultrastructural studies (Fayer, 2004). The authentic identification of species of Sarcocysts is only possible with molecular methods. In India, several reports are available on prevalence studies (Juyal and Bhatia, 1989). Identification of species of Sarcocysts plays a major role in finding the zoonotic species (*S. hominis*) particularly in export / import of buffalo meat. Reports on identification of species of Sarcocysts in India is scanty. Thus, the present study is carried out to find out the species of Sarcocysts in India in buffalo meat through PCR-RFLP and DNA sequencing analysis.

MATERIALS AND METHODS

A total of 2,304 buffaloes slaughtered at major cities (Hyderabad, Kolkata, Mumbai and New Delhi) of India were examined for presence of Sarcocysts in meat portion of oesophagus, cervical and masseter region. Out of 550 buffaloes positive for Sarcocysts, randomly, Sarcocysts were collected from 168 buffaloes belonging to Mumbai (60), Hyderabad (54) and Kolkata (54) cities of India. DNA was extracted from these 168 Sarcocysts (visible on naked eye examination) using Dneasy® blood and tissue kit method supplied by Qiagen India (P) Ltd. The amplification of the extracted DNA was carried out in 50µl of reaction mixtures using forward primer (5’-3’ GGATAACCTGGTAATT CTATG) and reverse primer (5’-3’ GGCAAAATGCTTTCC CAGTAG) with expected amplicon size of 900bp. PCR amplicons of the 18S rRNA gene were subjected to restriction enzymes.

*Corresponding author’s e-mail: drcramakrishna@gmail.com*
enzyme digestion with four different restriction enzymes (BslI, DraI, FokI and RsaI). Reaction mix was prepared by mixing 10µl PCR product and 0.4 µl of restriction enzyme, 1.6 µl of respective buffer and 8µl of nuclease free water and incubated overnight in water bath with different temperature conditions as described. Digested product was visualized after electrophoresis in 2.5% agarose gel. The 18S rRNA amplified PCR products were sequenced by outsourcing at M/s Bioserve Biotechnologies (India) Pvt. Ltd., Hyderabad. The nucleotide sequences obtained were blasted in the NCBI to find out the similarity with known sequence in database.

RESULTS AND DISCUSSION

The average yield of DNA was 220.16±19.27 ng/µl, 204.88±27.88 ng/µl and 344.84±44.5 ng/µl at Hyderabad city, Kolkata city and Mumbai city, respectively. All the 168 DNA samples from 3 different cities were amplified as per the targeted gene in the PCR assay. All the amplicons showed the 900 bp bands which were clearly identified by using the 100 bp ladder. The RFLP analysis of all the 167 DNA samples showed similar pattern (Pattern A) and only one DNA sample belonging to Mumbai city showed different pattern (Pattern B) (Fig. 5 and 6). In the present study, 4 different enzymes (BslI, DraI, FokI and RsaI) on RFLP differentiated S. fusiformis and S. taeniata from S. buffalonis but could not differentiate S. fusiformis from S. taeniata. From pattern A, few selective PCR products (Hyderabad city -3; Kolkata – 10, Mumbai – 8) and from pattern B, one PCR product (Mumbai – 1) were sequenced. Out of 21 PCR products sequenced from Pattern A, 18 showed S. fusiformis (Fig. 7) and 3 showed S. taeniata (Fig. 8). Out of 1 PCR product sequenced from Pattern B, showed S. buffalonis (Fig. 9). In the present study, 3 different Sarcocyst species were identified. The zoonotic S. hominis could not be identified.

S. fusiformis: In the present study, S. fusiformis cysts appeared macroscopic, opaque white in color, spine-shaped (Fig. 1 and 2). Similar observations were made by El – Seify et al. 2014. S. fusiformis infecting water buffaloes was studied in different countries and showed high incidences like 83.3% in Turkey (Retzlaff and Weise, 1969), 94% in China (Xiao et al., 1988) and 88% in Vietnam (Huong et al., 1995). In Egypt, reports about S. fusiformis prevalence were variable from high (100% Ghaffar et al., 1978) to low (6.9% El-Dakhly et al., 2011). Oryan et al. (2011) opined that based on the molecular results of the study, it seems that the microscopic forms are most likely the younger stages or a typical developing cysts of S. fusiformis and are not distinct Sarcocystis species.

S. taeniata: It appears that S. taeniata observed in the present study (Fig. 3) may be the first report from buffaloes in India. Gjerde, Bjorn (2014) reported S. taeniata in Canadian moose. Prakas et al. (2016) reported S. taeniata in sika deer.
**Fig 5:** RFLP fragment pattern of PCR products belonging to BslI and Dral restriction enzymes.

(Lane L – 100 kb Ladder; Lane 1 to 8 - different amplified DNA samples; UD – Undigested)

**Sarcocystis fudiformis** 18S ribosomal RNA gene, partial sequence.

> ATTTTCACTTTATACATCTCGGGGTTATAAGTGTTTTATTAGATACTAGAAGAAAGTTCACTAAGCCGATCCTATTATATCTCCTATTGCACCCTTTGCTAGAAGCCATTCGCTACAGA

**Fig 6:** RFLP fragment pattern of PCR products belonging to Mumbai city with FokI and RsaI restriction enzymes.

(Lane L – 100 kb Ladder; Lane 1 to 8 - different amplified DNA samples; UD – Undigested)

**Fig 7:** DNA sequence information of *S. fudiformis*
In the present study, zoonotic *S. hominis* could not be identified in the Sarcocysts (collected on naked eye examination from slaughtered buffaloes) subjected for molecular biology methods (PCR – RFLP and DNA examination from slaughtered buffaloes) subjected for molecular biology methods (PCR – RFLP and DNA examination from slaughtered buffaloes)
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**Sarcocystis buffalonis** 18S ribosomal RNA gene, complete sequence.

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