



PCR-RFLP at 18S rRNA gene for identification of *Sarcocystis* species and their prevalence in cattle of Andhra Pradesh

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Sarcocystosis, caused by *Sarcocystis* spp. is an economically important zoonotic disease involving carnivores and humans as definitive hosts, and domestic animals as intermediate hosts. An individual host may harbour more than one species of *Sarcocystis*. In cattle, *Sarcocystis cruzi*, *S. hirsuta* and *S. hominis* are the common species but occasionally is infected by *Sarcocystis* spp. of buffaloes (Yang *et al.* 2002, Gjerde *et al.* 2016). Parasitic cysts (50%) found in muscles of cattle belong to *S. hominis* (WHO 1981), which is zoonotic (Fayer 2004) and regarded as one of the important opportunistic agents in acquired immune deficiency syndrome patients (Velasquez *et al.* 2008).

The prevalence of sarcocysts in slaughtered cattle in India was reported based on conventional techniques (Dafedar *et al.* 2011, Mounika *et al.* 2017). However, reports on molecular characterization of *Sarcocystis* species in India are still limited. Molecular studies based on variable region of 18S rRNA are routinely employed as an economical and accurate approach to differentiate *Sarcocystis* spp. in bovines (Jehle *et al.* 2009) and it gives the similar results as sequence analysis based labeling. Keeping in view the zoonotic importance of *S. hominis*, the present work was aimed at species specific differentiation of *Sarcocystis* cysts in slaughtered cattle in Andhra Pradesh using 18S rRNA gene as a marker.

Tissue samples were collected from different predilection sites of 150 slaughtered cattle that were brought to various local slaughter houses in Chittoor district, Andhra Pradesh, India. On macroscopic and microscopic examination of these samples, sarcocysts were identified in 137 cattle (Mounika *et al.* 2018). Among the 137 cattle, macroscopic sarcocysts were noticed exclusively in the oesophagus of 9 cattle. Bradyzoites were isolated by pepsin digestion method

from macroscopic sarcocysts and tissue samples collected from predilection sites of all the 137 cattle and were preserved at –20°C for DNA isolation. Genomic DNA was extracted from each of the bradyzoite positive isolates using the phenol chloroform protocol (Sambrook *et al.* 1989).

PCR amplification was performed using the primers (Li *et al.* 2002) specific for 18S rRNA gene with 60°C annealing temperature (1 min) and final elongation at 72°C (10 min). A negative (ultra pure water in substitution of DNA) and positive (DNA of macroscopic cyst) control was run along with the samples at every PCR setup. Each PCR amplicon was visualized on 2% agarose gel electrophoresis and the sizes were verified by comparison with DNA ladder. Restriction enzyme fingerprinting was performed subsequently on each amplicon (5 µl) with *Bse*LI enzyme (10 units) in an incubator at 55°C for 5 h. The digested products were analyzed on a 2% agarose gel to identify the *Sarcocystis* species.

18S rRNA gene was amplified in all positive samples with an expected amplicon size of 900 bp. Oligonucleotide primers did not yield PCR product with negative control. On digestion with *Bse*LI, three different patterns were visualized on electrophoresis, one with 513 bp and 343 bp (*S. cruzi*), other with 525 bp, 241 bp and 141 bp (*S. hirsuta*), and another with 532 bp and 335 bp (*S. fusiformis*). Out of 9 macroscopic cysts, 6 were differentiated into *S. hirsuta* and 3 were differentiated into *S. fusiformis*. RFLP with *Bse*LI can differentiate *Sarcocystis cruzi*, *S. hirsuta*, *S. hominis*, *S. sinensis*, *S. buffalonis* and *S. fusiformis* (Jehle *et al.* 2009). The number of consistent nucleotide differences between *S. cruzi* and *S. fusiformis* was low which could be due to the selection of 18S rRNA gene as target that was also observed by Gjerde *et al.* (2016) while characterization of *Sarcocystis* spp. in buffaloes and opined that the *cox*I gene was superior to the 18S and 28S rRNA genes to unequivocally differentiate the species within each species pair.

Among 137 sarcocysts isolates, 128 belonged to *S. cruzi* (only microscopic cysts), 6 to *S. hirsuta* (both macro and microscopic cysts) and 3 to *S. fusiformis* (only macroscopic cyst). The rate of infection of *S. cruzi*, *S. hirsuta* and *S. fusiformis* in cattle in the study area was 93.43, 4.38 and

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2.19% respectively and differed significantly ($P < 0.01$). The high prevalence of *S. cruzi* in cattle in the present study could be due to contamination of cattle feed and water with faeces of dogs as a result of stray dogs in the proximity of cattle. Likewise molecular approaches carried out in different parts of world also indicated high prevalence of *S. cruzi* in cattle (Badawy *et al.* 2012, Hornok *et al.* 2015). Co-infection with more than one *Sarcocystis* spp. in cattle is frequent (Dubey *et al.* 1989, Domenis *et al.* 2011) though were not detected in the present study. This might be due to very low prevalence of *S. hirsuta* and *S. fusiformis* in the study area.

Earlier, *S. fusiformis* was thought to parasitize both cattle and buffalo (Huong *et al.* 1997). Attempted transmission of *Sarcocystis* species of rodents and other mammals indicated that *Sarcocystis* spp. are generally host specific (Dubey *et al.* 1989). Combining the morphology and sequence data, Yang *et al.* (2002) reported that *S. hirsuta* and *S. hominis* found in water buffalo were not new species but are in fact *S. hirsuta* and *S. hominis* as found in cattle, supporting the idea that *Sarcocystis* spp. are not restricted to one or other host group which was later evidenced by Jehle *et al.* (2009) and Xiang *et al.* (2011) based on PCR-RFLP analysis of 18 sRNA gene. Accordingly, *S. fusiformis* might have used cattle as an intermediate host in the studied area. Occurrence of macroscopic cysts of *S. fusiformis* in cattle indicated that *Sarcocystis* species of cattle and water buffaloes are not strictly intermediate host specific but might occasionally infect water buffaloes and cattle, respectively, where both hosts occur and natural cross transmission through dogs are possible as also reported by Gjerde *et al.* (2016).

Sarcocystis hominis, the only species of *Sarcocystis* in cattle that possesses zoonotic importance was not identified in the study area. The prevalence of *S. hominis* in cattle varied between 6.2 to 97.4% in Europe, Asia and South America (Vangeel *et al.* 2007, Domenis *et al.* 2011). The prevalence rate of *S. hominis* had been over calculated in past studies depending either on mere morphology of cyst wall or on DNA based techniques that were incapable to differentiate *S. hominis* and *S. bovis* (Gjerde *et al.* 2016). Perusal of literature did not reveal any report on prevalence of *S. hominis* in India. The absence of *S. hominis* in this region could be inferable to taboo on beef eating on religious grounds by majority of the people and traditional cooking practices by beef-eaters followed in Indian subcontinent. Though this result outweighs the zoonotic concerns, further studies on large number of samples using mitochondrial *cox1* gene to unequivocally differentiate various *Sarcocystis* spp. are warranted to re-confirm the *Sarcocystis* spp. in cattle in Andhra Pradesh.

SUMMARY

In the present study, the species specific determination of *Sarcocystis* cysts isolated from 137 naturally infected and slaughtered cattle from different regions of Chittoor district, Andhra Pradesh, India was achieved by PCR-RFLP.

Diagnosis of sarcocysts in cattle was based on macroscopic examination and pepsin digestion method. Genomic DNA was extracted individually from bradyzoites of all 137 sarcocystosis positive cattle and all the isolates were characterized at 18S rRNA. Digestion of PCR amplicons (900 bp) with restriction endonuclease revealed 3 different electromorphs which were referred to *Sarcocystis cruzi* (513 and 343 bp), *S. hirsuta* (525, 241 and 141 bp) and *S. fusiformis* (532 and 335 bp). *Sarcocystis cruzi* (93.43%) was significantly more prevalent in comparison with the *S. hirsuta* (4.38%) and *S. fusiformis* (2.19%). Infection of cattle with *S. hominis* was not observed in the study area. Occurrence of *S. fusiformis* in cattle supports that *Sarcocystis* species of buffaloes are not strictly intermediate host specific however can infect cattle.

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