Morphological and molecular identification of *Cyclospora* species in sheep and goat at Tamil Nadu, India

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Abstract A total of 281 fresh fecal samples (65 sheep and 216 goats) were examined for the presence of *Cyclospora* species in Tamil Nadu from October 2017 to April 2018. All the faecal samples were examined by direct smear method, saturated sucrose floatation technique and modified Ziehl–Neelsen staining method and it was confirmed—at genus level with PCR technique. Overall prevalence of *Cyclospora* infection in—small ruminant was 2.14%. The prevalence of *Cyclospora* species in sheep and goat was 3.08 and 1.85% respectively. *Cyclospora* oocyst was observed mostly during winter season (Dec–Feb) with 7.14% followed by summer (March and April) and Northeast monsoon (October and November) with 1.67 and 1.66% respectively. The oocyst detected from faecal sample through conventional faecal examination was confirmed by PCR.

Keywords *Cyclospora* · Sheep · Goat · Faecal examination · PCR · Tamil Nadu

Introduction

Cyclosporidiosis is a food borne and waterborne tissue invading protozoan parasitic disease which causes gastroenteritis in both immunocompromised and immunocompetent host (Herwaldt 2000; Ortega and Sanchez 2010). *Cyclospora cayentanensis* is reported in poultry, dog, goat and human beings (Garcia-Lopez et al. 1996; Yai et al. 1997; Eberhard et al. 1999; Nimri 2003; Chu et al. 2004). So far, *Cyclospora* infections have been reported only in England, Eastern Europe, Africa, Southeast Asia, North, Central, and South America, the Caribbean, Australia and Nepal (Soave 1996; Chu et al. 2004; Ortega and Sanchez 2010). There is no report on occurrence of *Cyclospora* infection either in livestock or human being in India. Hence, this study was undertaken to know the status of *Cyclospora* infections in Tamil Nadu, India. This paper reports on occurrence, morphological and molecular identification of *Cyclospora* species in sheep and goat at Tamil Nadu, India (Fig. 1a–c).

Materials and methods

A total of 281 fresh fecal samples (65 sheep and 216 goats) were collected from rectum at Large Animal Clinics (LAC), Medicine, Department of Clinics, Madras Veterinary College, Chennai (102), private sheep and goat farms located in Mottur-Siruvaakkam village, Kancheepuram district (62), sheep and goat unit, Instructional Livestock Farm Complex (ILFC), Madhavaram (67) and sheep and goat unit, Post-graduate Research Institute of Animal Sciences (PGRIAS), Kancheepuram district (50) of Tamil Nadu from October 2017 to April 2018. The animal comes to LAC medicine unit belongs to the private owners who were maintained their animals in an extensive system in an unorganized manner.

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Fig. 1 **a** *Cyclospora* spp. unsporulated oocyst (bottom) and sporulated (Top) 100x. **b** *Cyclospora* spp. unsporulated oocyst 400x. **c** *Cyclospora* spp. acid fast oocyst by Ziehl–Neelsen stain 100x. **d** Agarose gel electrophoresis of the PCR (Top) and nested-PCR

products (bottom) obtained from *Cyclospora* spp. amplified using genus specific 18S rRNA primer. M-DNA marker (100 bp), 1, 2, 4 and 5 negative; 3, 6 and 9 positive

The animals at private farms, Kancheepuram district were maintained in a semi-intensive system with wooden reepered slatted floor system in an organized manner. The animals at ILFC, Chennai and PGRIAS at Kancheepuram districts were maintained under semi-intensive system in an organized manner. All the faecal samples were examined by direct smear method, saturated sucrose floatation technique and modified Ziehl–Neelsen staining method (Levine et al. 1988; Scott et al. 1994).

Isolation of *Cyclospora* oocysts

The *Cyclospora* oocysts from faecal samples were isolated by methods of Mochizuki et al. (2006) and Haug et al. (2007) with necessary modifications. Briefly, the faecal pellets of approximately 10 g were homogenized using mortar and pestle with 60 mL of distilled water and the suspension was filtered through a fine metal sieve to remove large faecal debris. A 15 mL aliquot of the filtrate

was transferred into test tubes and centrifuged @ 1000 rpm for 5 min. The supernatant was discarded and the test tube was filled with saturated salt solution (Specific gravity 1.20) instead of saturated sugar solution as given by Mochizuki et al. (2006) and was allowed to stand for 20 min. Subsequently, the top 0.5 mL of the faecal suspension was transferred into 2 mL tube containing 1.5 mL double distilled water and centrifuged @ 2000 rpm for 5 min. The supernatant was discarded and the step was repeated 3–4 times to completely remove the faecal debris. A portion of the sediment was used for studying the morphology of oocysts for identification. The remaining portion of was stored at -20°C for DNA extraction.

DNA extraction

The genomic DNA from the *Cyclospora* oocysts was extracted by following the stool mini kit protocol using QIAamp stool Mini Kit (Qiagen, USA). The extracted DNA samples were stored at -20°C until they were analyzed.

Amplification by PCR

Cyclospora genus specific primers viz, F1E (5'-TACC-CAATGAAAACAGTTT-3') and R2B (5'-CAGGA-GAAGCCAAGGGAGG-3') as an external primers to amplify the 636 bp partial 18S rRNA gene in 20 μL reaction volumes containing 10 μL EmeraldAmp GT PCR Master Mix (Takara, Japan), 2 μL DNA template, 0.5 μL (10 pmol/ μL) forward primer and 0.5 μL (10 pmol/ μL) reverse primer and 7 μL nuclease free water (NFW). Further internal primer viz., CC719 (5'-GTAGCCTTCCGCGCTTCG-3') to amplify the 298 bp partial 18S rRNA gene in 20 μL reaction volumes containing 10 μL EmeraldAmp GT PCR Master Mix (Takara, Japan), 0.5 μL primarily amplified PCR product as template, 0.5 μL (10 pmol/ μL) forward primer and 0.5 μL (10 pmol/ μL) reverse primer and 8.5 μL nuclease free water (NFW). DNA was amplified

with an initial denaturation step of 15 min at 95°C , 35 cycles at 94°C for 30 s, annealing temperature of 53°C for 30 s and 72°C for 1.5 min and a final elongation step for 10 min at 72°C for the primary amplification. Cycle condition with an initial denaturation step of 15 min at 95°C , 25 cycles at 94°C for 15 s, annealing and extension at 66°C for 15 s for the nested PCR amplification (Orlandi et al. 2004).

Results and discussion

Out of 281 faecal samples examined from small ruminants, 2.14% (6/281) of animals showed positive for *Cyclospora* species. The prevalence of *Cyclospora* species in sheep and goat was 3.08 (2/65) and 1.85% (4/216) respectively (Table 1). Nimri (2003) observed *Cyclospora* oocysts in goat, chicken and human in Jordan. *Cyclospora* species was detected in all the methods namely, direct smear method, saturated sucrose floatation technique and modified Ziehl–Neelsen staining method. *Cyclospora* oocyst was identified based on the shape, size and acid fast staining (Nimri 2003). The size of the oocyst was 10 micron (Fig 1c).

Among the area wise prevalence, *Cyclospora* oocyst was observed only at LAC, Madras Veterinary College (3.92%) and private farm at Kancheepuram district, Tamil Nadu (1.61%). The animals from organized farm namely ILFC, Madhavaram, Chennai and PGRIAS, Kancheepuram district were free from *Cyclospora* oocyst. The result showed that the animals reared under free ranging in an unorganized and semi-intensive system of management in organized private farms only had *Cyclospora* oocyst infection.

In the present study, *Cyclospora* oocysts were observed in sheep and goats mostly during winter season (Dec–Feb) with 7.14% followed by summer (March and April) and Northeast monsoon (October and November) with 1.67 and 1.66% respectively. In contrast Nimri, (2003) reported 75% of *Cyclospora* cases during spring season (April and May)

Table 1 Prevalence of *Cyclospora* species in sheep and goat

Place	Prevalence (%)		Total prevalence (%)
	Sheep	Goat	
Large Animal Clinics (LAC), Medicine, Department of Clinics, Madras Veterinary College, Tamil Nadu	16.67 (2/12)	33.33 (3/90)	3.92 (4/102)
Private sheep and goat farms located in Mottur-Siruvaakkam village, Kancheepuram district of Tamil Nadu	0.00 (0/18)	2.27 (1/44)	1.61 (1/62)
Sheep and goat unit, Instructional Livestock Farm Complex (ILFC), Madhavaram, Tamil Nadu	0.00 (0/10)	0.00 (0/57)	0.00 (0/67)
Sheep and goat unit, Postgraduate Research Institute of Animal Sciences (PGRIAS), TANUVAS, Tamil Nadu	0.00 (0/25)	0.00 (0/25)	0.00 (0/50)
Total	3.08 (2/65)	1.85 (4/216)	2.14 (6/281)

Table 2 Prevalence of *Cyclospora* spp. in dung samples by conventional microscopic method/PCR

Method	No. of samples screened	No. of samples positive	Positive (%)
Direct smear	281	0.0	0.0
Sucrose floatation	281	6.0	2.14
Modified Ziehl–Neelsen	281	6.0	2.14
PCR	50	6.0	12.0

and Ortega and Sanchez (2010) reported overall prevalence of *Cyclospora* infection in HIV patients (2.3%) during warmer month.

In this study, polymerase chain reaction was carried out by using DNA isolated from faecal oocyst. The 636 bp product size specific to *Cyclospora* and *Eimeria* species were amplified in primary PCR and 298 bp product size specific to *Cyclospora* species visualized under gel documentation system. The oocysts detected from faecal sample through conventional faecal examination was confirmed by PCR and showed 12.00% (6/50) positive amplification for *Cyclospora* (Table 2). Similarly, *Cyclospora* was confirmed by many workers with PCR technique in various countries (Eberhard et al. 1999; Relman et al. 1996; Chu et al. 2004; Li et al. 2015).

Conclusion

From this study it was concluded that *Cyclospora* species was recorded in sheep and goat in Tamil Nadu, India as the first report.

Author's contribution KB Collection and processing of samples and literature collection. KN Assistance in literature collection, typing and preparation of manuscript. CS Assistance in literature collection, typing and preparation of manuscript. SV Assisted in manuscript preparation. VSR Assisted in manuscript preparation.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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