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Genotyping of *Cryptosporidium* spp. isolated from young domestic ruminants in some targeted areas of India

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ABSTRACT

Faecal samples (363) from kids, lambs, calves and buffalo calves of below 3 months of age were collected from various parts of India and screened microscopically for *Cryptosporidium* oocysts using modified Ziehl–Neelsen method of staining. Microscopically positive samples (20) were genotyped by PCR amplification of the partial 18S rRNA region and subsequent digestion by *Ssp*I, *Vsp*I and *Mbo*II restriction enzymes. Based on the PCR-RFLP patterns of 18S rRNA, all the 20 samples were found positive for *C. parvum.* All the positive samples were also used for amplification of partial actin gene of *Cryptosporidium* spp. For further confirmation of the species of *Cryptosporidium,* amplified 818 bp partial actin gene of 3 representative isolates was cloned and sequenced. The sequence and phylogenetic analysis of PCR-positive samples confirmed the presence of *C. parvum*. Thus, actin gene can also be used for specific molecular diagnosis of *Cryptosporidium* spp., in addition to 18S rRNA. These findings also indicated that young domestic ruminants can be a potent source of cryptosporidial infection for humans and animals in India.

Key words: 18S rRNA, Actin gene, *Cryptosporidium parvum*, Genotyping, India

Currently 26 species of *Cryptosporidium,* the ubiquitous, obligatory intracellular but extracytoplasmic enteric protozoa that infect over 170 vertebrate species (Maurya *et al*. 2013b), are known (Chalmers and Katzer 2013). Cryptosporidiosis in domestic ruminants is basically a disease of young animals below 2 to 3 months of age as adult animals, although harbour the parasite, do not exhibit any symptom. The recent studies used 18S rRNA (SSU rRNA) based genotyping tools, which are genus specific and highly sensitive because of multiple copy number of the target (Xiao 2009). However, genus-specific confirmative genotyping tools based on other genes are also needed because genetic diversity in 18S rRNA gene among *Cryptosporidium* parasites is restricted to a few polymorphic regions of the gene, indicating that 18S rRNA gene is not selectively neutral, and that homoplasy is a possibility because of restriction of heterogeneity to small regions. It can be argued that conclusions made on the basis of genetic differences and relatedness at 18S rRNA locus may not reflect phylogenetic relationship at whole genome level. Differences in branch orders and fine grouping indicated that there is a need for analysis at additional genetic loci (Sulaiman *et al*. 2002). For this reason in the present study, in addition to18S rRNA region another gene i.e., actin, which codes for a ubiquitous and highly conserved microfilament protein, was also used. Fixed differences in the sequence of this gene among *Cryptosporidium* species is useful in genotyping of *Cryptosporidium* species. In light of this knowledge, present study was undertaken to genotype the *Cryptosporidium* spp. isolated from domestic ruminants below 3 months of age.

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MATERIALS AND METHODS

Sample collection and microscopic examination: Faecal samples of young domestic ruminants (363 from 204 cattle, 70 buffalo, 57 goat and 32 sheep) below 3 months of age were collected from various livestock farms and small holdings in the following districts: Bareilly, Jhansi, Lucknow (Uttar Pradesh), Dehradun, Nainital, Udham Singh Nagar, (Uttarakhand), Bengaluru, Chickballapur, Mysore (Karnataka), Kolkata (West Bengal), Bhojpur (Bihar), Jammu (Jammu & Kashmir), Gurgaon (Haryana) and Kollam (Kerala) of India. Each faecal sample was collected directly from the rectum separately in a clean, sterile plastic bag or vial. After collection, the samples were brought to the laboratory without using any fixatives. Samples were screened for *Cryptosporidium* oocysts using modified Ziehl-Neelsen staining (Henricksen and Pohlenz 1981) under compound microscope. Positive samples were stored in 2.5% potassium dichromate solution at 4°C until

DNA extraction was accomplished.

DNA extraction: DNA was extracted from 20 faecal samples positive for *Cryptosporidium* oocysts (12 cattle, 6 buffalo, 2 goat) by using faecal stool DNA extraction kit as per the manufacturer's protocol, with the addition of 15 freeze-thaw (freezing in liquid nitrogen and immediate thawing at 90°C) cycles prior to resuspension in lysis buffer to rupture the oocysts. DNA was stored at –20°C before being used in polymerase chain reaction (PCR).

Genotyping of Cryptosporidium *spp. by PCR-RFLP pattern of 18S rRNA*: The 18S rRNA nested PCR protocol was adopted for detection of *Cryptosporidium* species as described previously (Xiao *et al.* 1999, 2001) with minor modifications. In the first step, partial 18S rRNA gene of *Cryptosporidium* was amplified in a 25 µl reaction mixture containing 20 pmol of each primer (CR-DIAG1 Forward: 5' TTC TAG AGC TAA TAC ATG CG 3' and CR-DIAG1 Reverse: 5' CAT TTC CTT CGA AAC AGG A 3'), 2.5 µl of 10X *Taq* polymerase buffer, 2mM $MgCl_2$, 0.5mM of each dNTP, 1U of *Taq* polymerase and 50ng of genomic DNA. Polymerase chain reaction was performed in an automated programmed manner using PTC-200 Peltier thermal cycler with initial denaturation at 94°C for 5 min, followed by 35 cycles each of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 1 min. This was followed by final extension for 10 min at 72°C.

For the nested PCR, 1µl of the primary PCR product was used as a template and 20 pmol of primers (CR-DIAG2 Forward: 5' GGA AGG GTT GTA TTT ATT AGA TAA AG 3' and CR-DIAG2 Reverse: 5' AAG GAG TAA GGA ACA ACC TCC A 3') were used in the 50 µl reaction mixture. The PCR reaction and cycling condition were identical to the conditions used for primary PCR, except that the annealing temperature was 60°C.

Restriction fragment length polymorphism (RFLP) pattern analysis of amplified 18S rRNA nested PCR products was conducted by using the restriction enzymes *Ssp*I, *Vsp*I and *Mbo*II (Xiao *et al.* 2001; Feng *et al.* 2007, Khan *et al.* 2010). 10 µl of the purified nested PCR product (approx. 834 bp) was separately subjected to restriction enzyme mediated digestion using 10 IU each of *Ssp*I, *Vsp*I and *Mbo*II in 20 µl reaction mixture for 3 h at 37°C. The digested product was electrophoresed in submarine horizontal electrophoresis apparatus using 2% agarose gel and viewed and photographed using gel documentation system to study the specific RE digestion pattern for identification of the species (genotype) of *Cryptosporidium*.

Genotyping of Cryptosporidium *spp. by PCR based amplification and sequencing of actin gene*: All the faecal DNA samples positive for *Cryptosporidium* spp. at the 18S rRNA locus were again used as templates for amplification of partial actin gene of *Cryptosporidium* spp., as described previously (Ng *et al.* 2006) with minor modifications. In the first step, partial actin gene of *Cryptosporidium* was amplified in a 25 µl reaction mixture containing 20 pmol of each primer (CR-ACTIN1 Forward: 5' ATG CCV GGW RTW ATG GTD GGT ATG 3' and CR-ACTIN1 Reverse: 5' GGD GCA ACR ACY TTR ATC TTC 3'), 2.5 µl of 10X *Taq* polymerase buffer, 3mM $MgCl_2$, 0.5mM of each dNTP, 1U of *Taq* polymerase and 50ng of genomic DNA. Polymerase chain reaction was performed in an automated programmed manner with a preliminary cycle of denaturation at 94°C for 2 min., annealing at 58°C for 1 min. and extension at 72°C for 2 min followed by 50 cycles each of denaturation at 94°C for 30 sec, annealing at 58°C for 20 sec, and extension at 72°C for 40 sec. This was followed by final extension for 7 min at 72°C.

For the nested PCR, 1µl of the primary PCR product was used as a template and 20 pmol of primers (CR-ACTIN2 Forward: 5' GAY GAR GCH CAR TCV AAR AGR GGT AT 3' and CR-ACTIN2 Reverse: 5' TTD ATY TTC ATD GTH GAH GGW GC 3') were used in the 50 µl reaction mixture. The PCR reaction and cycling condition were identical to the conditions used for primary PCR.

For further species level confirmation, 818 bp nested PCR products (1 each from Izatnagar cattle, Izatnagar buffalo and Izatnagar kid) were purified using Gel/PCR DNA fragment extraction kit following the manufacturer's protocol and were cloned into pTZ57R/T cloning vector using standard protocol (Sambrook *et al.* 2001). Stab cultures of positive recombinant clones harboring the desired 18S rRNA gene were custom sequenced in an automated DNA sequencer at DNA sequencing facility, Delhi University, South Campus, New Delhi. The sequence information received was analysed using DNA Star, MEGA 4 software and Basic local alignment search tool.

RESULTS AND DISCUSSION

Genotyping of Cryptosporidium *isolates using 18S rRNA*: Nested PCR amplification of 18S rRNA of *Cryptosporidium* spp. yielded a product of approximately 834 bp (Fig. 1) in all the 20 samples of domestic ruminants below 3 months of age which further confirmed the presence of *Cryptosporidium* spp. in all of them. For genotyping of *Cryptosporidium* species, a restriction fragment length polymorphism (RFLP) pattern analysis of nested PCR products was conducted by using the restriction enzymes *Ssp*I, *Vsp*I and *Mbo*II. Three fragments of 450 bp, 267 bp and 108 bp, 2 fragment of 628 bp and 115 bp, and 2 fragments of 771 bp and 76 bp were observed on digestion by *Ssp*I, *Vsp*I and *Mbo*II, respectively (Fig. 2). These typical RFLP patterns indicated the presence of *C. parvum* in all the isolates screened.

PCR-RFLP analysis of the nested PCR products of 18S rRNA gene or other targets serve as a potential technique for describing the speciation of the *Cryptosporidium* isolates at the molecular level. Genotyping of *Cryptosporidium* spp. based on the PCR-RFLP pattern was first described by Xiao *et al*. (1999). In India Roy *et al.* (2006), Paul *et al.* (2008), Khan *et al.* (2010), Venu *et al.* (2012), Maurya *et al.* (2013a) identified *C. parvum* as the most prevalent causative agent of cryptosporidial diarrhoea among bovine calves by PCR-RFLP analysis of nested PCR product of 18S rRNA gene. Khan *et al.* (2010) and Venu *et al.* (2012) also identified *C.*

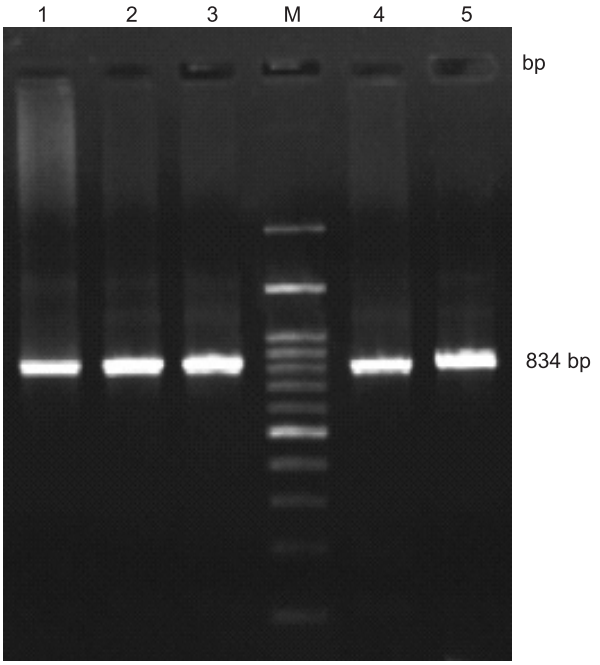

Fig.1. Nested PCR amplification of 18S rRNA of *Cryptosporidium* spp. (Lane M: 100 bp plus DNA ladder, Lane 1–3: Bovine isolates, Lane 4–5: Caprine isolates).

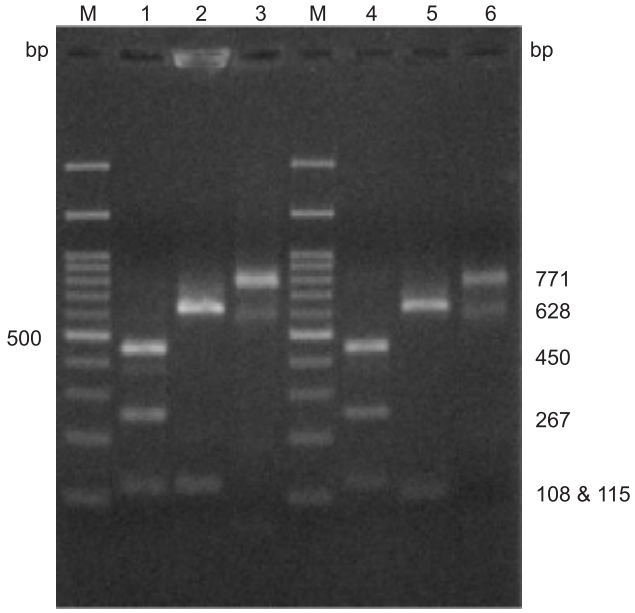

Fig. 2. PCR-RFLP profile of 18S rRNA gene of bovine (lane 1–3) and caprine (lane 4–6) isolates of *C. parvum.* (Lane M: 100 bp plus DNA ladder; Lane 1&4: *Ssp*I digestion- 450 bp, 267 bp, 108 bp; lane 2&5: *Vsp*I digestion - 628 bp, 115 bp; lane 3&6: *Mbo*II digestion - 771 bp, 76 bp).

bovis, C. ryanae and *C. andersoni* in bovines by using this technique.

Genotyping of Cryptosporidium *spp. using actin gene*: All the faecal DNA samples positive for *Cryptosporidium* spp. at the 18S rRNA locus were again used for amplification of actin gene of *Cryptosporidium* spp. in a nested PCR format. Nested PCR amplification of actin gene

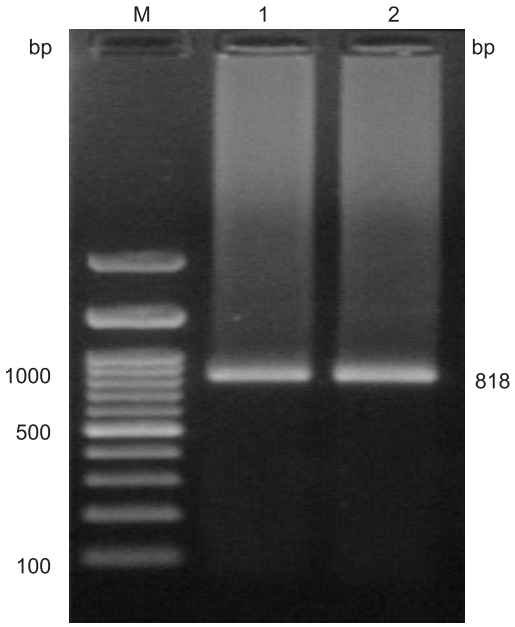

Fig. 3. Nested PCR amplification of actin gene of *Cryptosporidium* spp. (Lane M: 100 bp plus DNA ladder; lane 1: bovine isolate; lane 2: caprine isolate).

of *Cryptosporidium* spp. yielded a product of approximately 818 bp (Fig. 3) further confirming the presence of *Cryptosporidium* spp. in all of them.

The molecular characterization of *Cryptosporidium* spp. using nested PCR of actin gene was first described by Sulaiman *et al.* (2002) who stressed the importance of multilocus analyses in phylogenetic studies, as all genetic loci are not free of the influence of selection and the rate of gene evolution may not be the same for all members tested. The group amplified a product of approximately 1,095 bp by primary PCR and approximately 1,066 bp by secondary PCR. Ng *et al.* (2006) standardized a nested PCR protocol for amplification of actin gene locus of *Cryptosporidium* spp. which amplified a product of 830 bp in primary PCR and 818 bp in secondary PCR which was adopted in this study.

For further identification of the species of *Cryptosporidium,* 818 bp partial actin gene nested PCR products of 3 isolates of *C. parvum* (1 each from Izatnagar cattle, Izatnagar buffalo and Izatnagar kid), already identified by PCR-RFLP of amplified 18S rRNA were cloned and sequenced. Sequence analysis of these actin gene nested PCR products further confirmed that they belonged to *C. parvum*. The partial actin gene sequences obtained in the present study are available in the GenBank with accession numbers as KC469977, KC469978 and KC469979. Phylogenetic analysis revealed that the actin gene sequences of *C. parvum* isolates, identified in the present study clustered together with already published sequences of *C. parvum* from different parts of the world, which further confirmed presence of *C. parvum* in the isolates identified in the present study (Fig. 4).

Thus in the current study, genotype analysis using 18S rRNA and actin genes revealed that *C. parvum* is the only

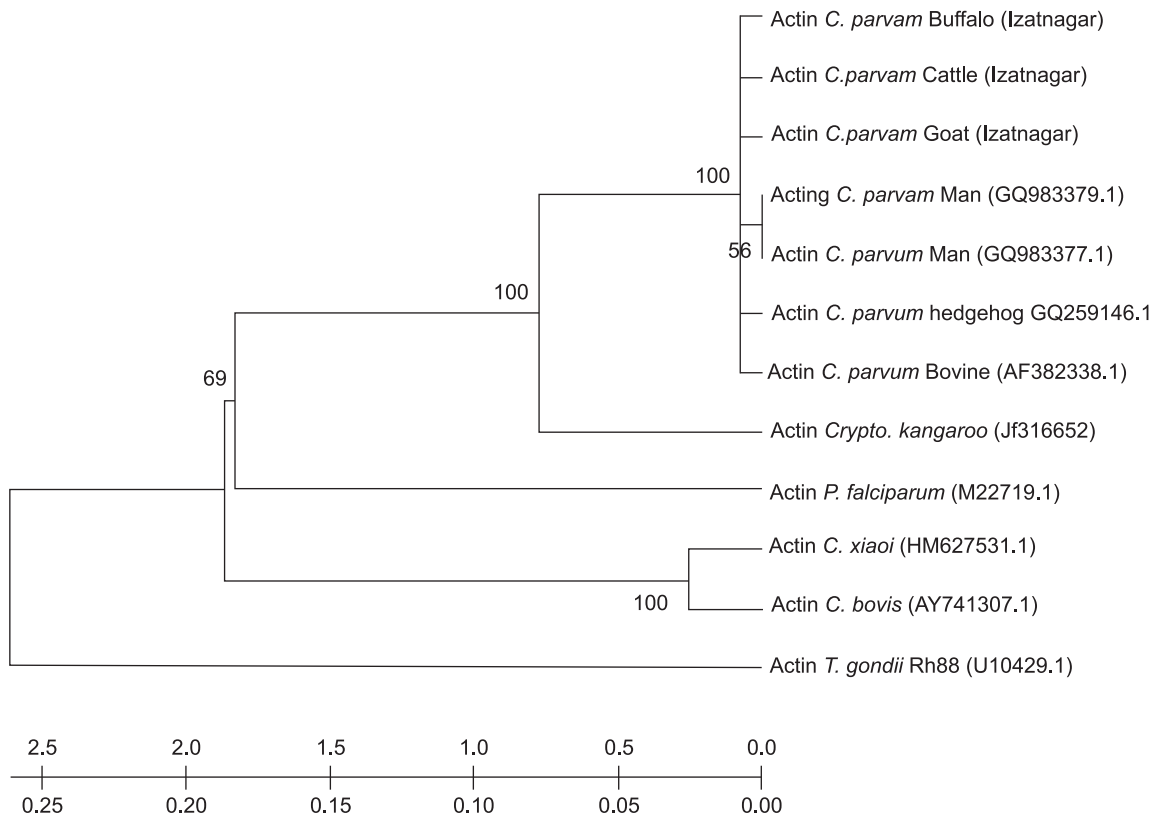

Fig. 4. Phylogenetic tree of actin gene of *Cryptosporidium* spp. isolates.

species prevalent in all the 20 samples of domestic ruminants below 3 months of age. These results are of potential zoonotic concern as cryptosporidiosis due to *C. parvum* is an important direct zoonosis.

In conclusion, actin gene based genotyping can serve as a reliable alternative for accurate identification of *Cryptosporidium* spp. infecting domestic ruminants. The results of this study clearly indicate that the molecular epidemiological picture of *Cryptosporidium* spp. in young domestic ruminants of India is quite alarming. Even few infected animals could act as a source of infection to other young animals in the herd leading to severe economic losses in the farm and also pose a significant public health risk directly to animal handlers or indirectly as an important reservoir for human waterborne outbreaks of cryptosporidiosis.

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