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Genotyping of *Cryptosporidium* species reveals prevalence of zoonotic *C. parvum* subtype in bovine calves of north India

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ABSTRACT

Faecal samples of diarrhoeic bovine calves (below 2 months of age) collected from government and privately owned dairy farms of north Indian states (Uttar Pradesh, Uttarakhand and Bihar) were stained by modified Zeihl-Neelsen stain and examined microscopically for the presence or absence of *Cryptosporidium* oocysts. Partial 18S rRNA of 25 *Cryptosporidium* positive samples were amplified by nested PCR and subsequently digested by *Ssp*I, *Vsp*I and *Mbo*II restriction enzymes to determine the *Cryptosporidium* species. Based on the PCR-RFLP patterns of 18S rRNA, all the 25 samples were identified as positive for *C. parvum.* For further confirmation, partial 18S rRNA gene of 4 representative samples was cloned and sequenced. The sequence and phylogenetic analysis of PCR-positive specimens also confirmed the presence of *C. parvum*. For subtyping of *C. parvum*, GP60 gene was amplified and sequenced. The sequence analysis of the glycoprotein (GP60) gene revealed that all the 4 isolates belonged to IIdA15G1R1 zoonotic subtype of *C. parvum*. These findings further confirmed that cattle and buffalo calves can be a potent source of cryptosporidial infection for humans and animals.

Key words: Bovine, *Cryptosporidium parvum*, Genotyping, Subtyping

Cryptosporidiosis is characterized by acute gastrointestinal disturbances, mucoid or *haemorrhagic* watery diarrhoea, fever, lethargy, anorexia and loss of condition (Navin and Juranek 1984), leading to significant economic losses in farm animals (Xiao *et al*. 1999). *Cryptosporidium* also infects humans and may cause life threatening disease, especially in immunodeficient patients (Current *et al.* 1983). It was also found associated with numerous water-borne outbreaks of diarrhoea in human beings (Ponka *et al.* 2008). Cryptosporidiosis in animal is seen as a threatening source of infection for humans because of the shedding of huge numbers of resistant oocysts, thus contaminating the surface water. All these findings have given cryptosporidiosis the recognition as an important zoonotic disease. This research encouraged on *Cryptosporidium*. Studies revealed that the relationship between human and animal forms of the disease is far more complex (Widmer 1998). Cryptosporidiosis drew the attention of researchers because it may become harmful and difficult to control because disease in many farm animals. Clinical prevalence of cryptosporidiosis and the associated mortality in animals and humans in India was reviewed by Chhabra and Pathak (2012).

Cattle are infected by *Cryptosporidium parvum*, *C. andersoni* (previously known as *C. muris*), *C. bovis* (previously known bovine genotype B) and *C. ryanae* (previously known as *Cryptosporidium* dear-like genotype) (Fayer *et al.* 2006). Existence of an age-related occurrence of *Cryptosporidium* spp. was observed in dairy cattle*, C. parvum* is mostly found in preweaned calves, *C. bovis* and *C. ryanae* in weaned calves and *C. andersoni* in yearlings and adult cattle (Fayer *et al.* 2006, 2007, Santin *et al.* 2008). These 4 species vary significantly in their pathogenicity; *C. parvum* being the most pathogenic (Fayer *et al.* 2006). It is important to study the prevalence of various species and subspecies of *Cryptosporidium* prevalent in different agro-climatic regions, to understand epidemiological pattern of the disease and for designing effective control strategies. As, it is not possible to differentiate different species and subspecies of *Cryptosporidium* by conventional diagnostic procedures, hence molecular tools are used to characterize the genetic structure and speciation of *Cryptosporidium* and

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for assessment of their zoonotic significance (Xiao 2010). The development of a restriction fragment length polymorphism of 18S rRNA gene fragment of ~ 830bp using restriction enzymes (*Ssp*I, *Vsp*I and *Mbo*II) to differentiate different species of *Cryptosporidium* facilitated the identification of species found in cattle and buffaloes (Feng *et al.* 2007). Also, the GP60 gene showed a high degree of sequence polymorphism among isolates of *Cryptosporidium* species and several subtypes were identified, of which the *C. parvum* IIa and IId were found to be the most common zoonotic species (Xiao *et al.* 2007).

The purpose of this study was to document the prevalence of *Cryptosporidium* spp. infections in bovine calves from dairy herds in different parts of India using molecular tools based on the genus-specific amplification of a gene of a small ribosomal subunit followed by PCR-RFLP analysis and to assess distribution and diversity of potentially zoonotic subtypes of *C. parvum* by sequence analyses of a gene encoding a 60 kDa glycoprotein.

MATERIALS AND METHODS

Sample collection and microscopic examination: Faecal samples (225) were collected from diarrhoeic bovine and bubaline calves below 2 months of age (142 cattle, 83 buffalo) from 2 Government and 16 privately owned dairy farms located in Uttar Pradesh, Uttarakhand and Bihar. Each faecal sample was collected directly from the rectum separately in a clean, sterile plastic bag or vial. The samples were brought to the laboratory without using any fixatives. Samples were screened for *Cryptosporidium* oocysts using modified Ziehl-Neelsen stain (Casemore *et al*. 1985) under compound microscope. Positive samples were stored in 2.5% potassium dichromate solution at 4°C prior to DNA extraction.

DNA extraction: DNA was extracted from 25 *Cryptosporidium* positive faecal samples (representing different farms of different states) by using faecal stool DNA extraction kit as per the manufacturer's protocol, with the addition of 8 freeze-thaw (freezing in liquid nitrogen and immediate thawing at 90°C) cycles prior to resuspension in lysis solution to rupture *Cryptosporidium* oocysts. DNA was stored at –20°C before being used in polymerase chain reaction (PCR).

PCR amplification of 18S rRNA: The 18S rRNA nested PCR protocol was adopted for detection of *Cryptosporidium* species (Xiao *et al.* 1999, 2001) with minor modifications. In the first step, partial 18S rRNA of *Cryptosporidium* was amplified in a 25 µl reaction mixture containing 20 pmol of each primer (CRP-DIAG1 Forward: 5' TTC TAG AGC TAA TAC ATG CG 3' and CRP-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 gene Amp PCR system 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 (CRP-DIAG2 Forward: 5' GGA AGG GTT GTA TTT ATT AGA TAA AG 3' and CRP-DIAG2 Reverse: 5' AAG GAG TAA GGA ACA ACC TCC A 3') were used in 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 at 60°C.

Differentiation of Cryptosporidium *spp. by RFLP pattern:* For genotyping of *Cryptosporidium* sp., 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 (Xiao *et al.* 2001, Feng *et al.* 2007, Khan *et al.* 2010); 10 µl of the purified nested PCR product (approximately 834 bp) was separately subjected to restriction enzyme mediated digestion using 10 IU each of *Ssp*1, *Vsp*1 and *Mbo* II in 20 µl reaction mixture for 4 h at 37°C. The digested products were electrophoresed in submarine horizontal electrophoresis apparatus using 2% agarose gel containing ethidium bromide and viewed and photographed using gel documentation system for identification of the species of *Cryptosporidium*.

Cloning, sequencing and phylogenetic analysis of 18S rRNA gene: For further confirmation of the species of *Cryptosporidium,* 4 nested PCR products identified as *C. parvum* by PCR-RFLP (1 each from cattle and buffalo from Uttar Pradesh, a cattle from Uttarakhand and a buffalo from Bihar) were purified using Gel/PCR DNA fragment extraction kit following the manufacturer's protocol and were cloned into pTZ57R/T cloning vector. Stab cultures of positive recombinant clones harboring the desired 18S rRNA gene were sequenced in an automated DNA sequencer at DNA sequencing facility, Delhi University, South Campus, New Delhi. The sequences obtained in the present study were analysed by Basic local alignment search tool and aligned with published sequences of different *Cryptosporidium* species viz. *C. parvum* (Accession no. AJ853994, EF611871 and AF164102), *C. bovis* (Accession no. GU831567), *C. andersoni* (Accession no. GU831568), *C. ryanae* (Accession no. EU410344), *C. cuniculus* (Accession no. HQ397716), *C. hominis* (Accession no. GU319779), *C. xiaoi* (Accession no. GU174540), *C. ubiquitum* (Accession no. JN642225), *C. suis* (Accession no. GU254176), *C. serpentis* (Accession no. EU553575), *C. muris* (Accession no. EU553592), *C. meleagridis* (Accession no. JQ217141), *C. galli* (Accession no. GU816046), *C. baileyi* (Accession no. JQ217142), *C. wrairi* (Accession no. AF115378), *C. canis* (Accession no. AF112576) and *C. felis* (Accession no. JQ312664) using DNA Star, Laser gene software by Clustal W method. A phylogenetic tree was constructed using MEGA4 (Tamura

et al. 2007) by Neighbor-Joining method (Saitou and Nei 1987) and the evolutionary distances were calculated with Kimura 2-parameter model (Kimura 1980). The nucleotide sequence of *Eimeria tenella* (Accession no. AF026388) was used as an outgroup to root the neighbor-joining tree.

Subtyping of C. parvum*:* Nested PCR amplification of the GP60 gene was performed for subtyping of *C. parvum* isolates (1 each from cattle and buffalo from Uttar Pradesh, a cattle from Uttarakhand and a buffalo from Bihar) as per Brook *et al.* (2009) with minor modifications. In the first step, GP60 gene of *C. parvum* was amplified in a 25 µl reaction mixture containing 20 pmol of each primer (GP601F- 5' ATA GTC TCC GCT GTA TTC 3', GP601R- 5' GAG ATA TAT CTT GGT GCG 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 with initial denaturation at 94°C for 5 min, followed by 35 cycles each of denaturation at 94°C for 1 min, annealing at 50°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 (GP602F- 5' TCC GCT GTA TTC TCA GCC 3', GP602R- 5' CGA ACC ACA TTA CAA ATG AAG 3') were used in the 25µl reaction mixture. The PCR reaction and cycling condition were identical to the conditions used for primary PCR, except that the annealing temperature was 54°C.

The purified nested products of Gp60 gene were custom sequenced at DNA sequencing facility, Delhi University, South Campus, New Delhi. The sequence information for Gp60 gene was analysed by using DNA Star, Laser gene software and Basic local alignment search tool. Nomenclature system of *C. parvum* subtypes based on sequence polymorphism of Gp60 gene as described by Sulaiman *et al.* (2005) was used for identification of subtypes of *C. parvum*.

RESULTS AND DISCUSSION

Microscopic examination of diarrhoeic faecal samples revealed that 20.0% bovine neonates (18.31% cattle and 22.89% buffalo) were positive for *Cryptosporidium* oocysts, with 28.24% prevalence in Uttar Pradesh, 25.0% in Bihar and 14.00% in Uttarakhand. Out of the 45 positive faecal samples, 25 (15 from cattle calves and 10 from buffalo calves) samples representing different farms of different states were randomly selected for genotyping of *Cryptosporidium* species.

Genotyping of Cryptosporidium *isolates:* Nested PCR amplification of 18S rRNA of *Cryptosporidium* sp. yielded a product of approximately 830 bp in all the 25 microscopically positive faecal samples (15 from cattle calves and 10 from buffalo calves), which further confirmed the presence of *Cryptosporidium* spp. in all of them. Digestion of nested products with the enzyme *Vsp*I yielded 2 bands at 628 bp and 115 bp, while *Ssp*I yielded 3 bands at 450 bp, 267 bp, 108 bp in all the products. The restriction enzyme *Mbo*II digested all the nested products, yielding 2 bands at 771 bp and 76 bp (Fig. 1). These typical RFLP patterns indicated the presence of *C. parvum* in all the isolates screened.

The genotyping of *Cryptosporidium* spp. based on the PCR-RFLP pattern was first described by Xiao *et al.* (2000). In India Roy *et al.* (2006) and Paul *et al.* (2008) identified *C. parvum* to be the main causative agent of cryptosporidial diarrhoea among bovine calves by PCR-RFLP analysis of nested PCR product of 18S rRNA gene with *Vsp*I and *Ssp*I restriction enzymes. Also, Paul *et al.* (2009) and Xiao *et al.* (2004) used the RFLP pattern of 845bp nested fragment of 18S rRNA to identify and characterize *C. andersoni* using *Vsp*I and *Ssp*I restriction enzymes. But, since there are minor differences in 18S rRNA based RFLP patterns among *C. parvum*, *C. ryanae* and *C. bovis*, their reliable differentiation generally requires DNA sequencing of nested PCR products when only these 2 restriction enzymes (*Vsp*I and *Ssp*I) are used. However, the use of an additional restriction enzyme *Mbo*II in conjugation with *Vsp*I and *Ssp*I can overcome this problem (Feng *et al.* 2007). For these reasons, the PCR-RFLP studies using 3 restriction enzymes namely, *Vsp*I, *Ssp*I and *Mbo*II for identifying the species of *Cryptosporidium* in positive faecal samples were carried out in the present study. Similar PCR-RFLP studies using 3 restriction enzymes were carried out by Khan *et al.* (2010) to assess the importance of cattle as a source of *Cryptosporidium* infections to human beings. They were able to identify *C. parvum*, *C. bovis*, *C.*

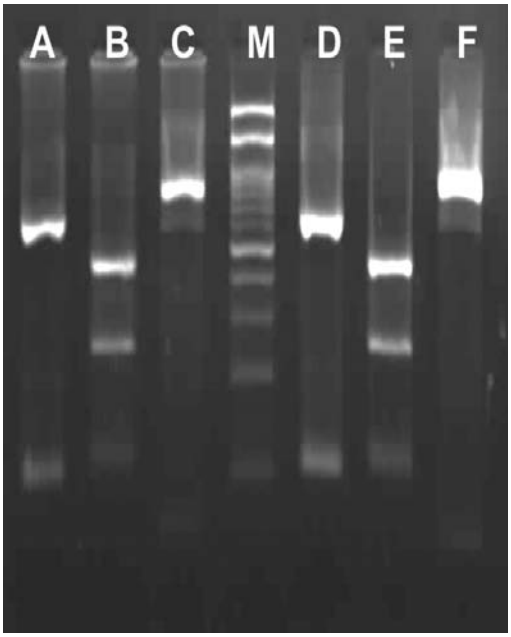

Fig. 1. PCR-RFLP profile of 18S rRNA gene of cattle isolates of *Cryptosporidium*. (Lane A, D: *Vsp*I digestion, lane B, E: *Ssp*I digestion, lane C, F: *Mbo*II digestion, lane M: 100bp DNA ladder).

ryanae, *C. andersoni* and *C. suis* like genotype in dairy cattle from West Bengal, India. They also demonstrated that the farm workers were infected with *C. hominis*, *C. parvum* and a novel *C. bovis* genotype, suggesting the potential risk of zoonotic infections being transmitted from infected calves to the dairy farm workers in India.

Sequence and phylogenetic analysis: For further confirmation of the species of *Cryptosporidium,* 4 nested PCR products identified as *C. parvum* by PCR-RFLP (1 each from cattle and buffalo from Uttar Pradesh, a cattle from Uttarakhand and a buffalo from Bihar) were cloned and sequenced. Sequence analysis of these nested PCR products further confirmed that they belonged to *C. parvum*. Phylogenetic analysis revealed that the 18S rRNA 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 this study (Fig. 2). The partial 18S rRNA gene sequences obtained in this study are available in the GenBank with accession numbers as JN836324, JN967646, JN967647 and JN967648.

Phylogenetic relationship of the sequences were studied with the reference published sequence information available in GeneBank, as a final step for the confirmation of the species or genotype identified by PCR-RFLP by Fayer and Xiao (2008), who utilized the molecular taxonomy studies to identify multiple species of *Cryptosporidium*. Karanis *et al.* (2010) used phylogenetic analysis of the PCR amplified sequences of 18S rRNA gene to confirm 33 isolates of *Cryptosporidium* as that of *C. parvum*. Thus, sequencing of the PCR amplified fragments of *Cryptosporidium* isolates followed by their alignment with the reported reference sequences of various species of the same organism is a sort of proof reading to confirm the genotypic results obtained from PCR-RFLP.

Subtyping of C. parvum *isolates:* Nested PCR amplification of GP60 gene of *C. parvum* yielded a product of approximately 340 bp. The purified 340 bp products were sequenced and analysed, which revealed the presence of *C. parvum* sub-genotype IId. The subtypes were named according to a system suggested by Sulaiman *et al.* (2005). Sequence analysis of all the 4 isolates examined in the present study revealed that each isolate had 15 copies of the TCA repeat and 1 copy of the TCG repeat, with ACATCA sequence present immediately after the trinucleotide repeats (TCA or TCG). Therefore, all these 4 isolates were designated as IIdA15G1R1 genotypes. The GP60 gene sequences of *C. parvum* IIdA15G1R1 generated in the present study are available in GenBank under accession numbers JN967649 to JN967652.

The sub-genotype IId of *C. parvum* reported in the present study is of immense zoonotic potential as it has been reported from humans in Asia (Iran, Kuwait), Europe (Spain, Netherlands, Portugal, Hungary, Germany, Belgium, Switzerland and Serbia) and Australia (Sulaiman *et al.* 2005, Alves *et al.* 2006, Geurden *et al.* 2007, Plutzer and Karanis 2007, Broglia *et al.* 2008, Quilez *et al.* 2008). The same workers working on the genotyping and subtyping of the *C. parvum* isolates from human beings and cattle, discovered the prevalence of the IId sub-genotypes among the bovine calf populations in Australia; Kuwait in Asia; Spain, Portugal, Serbia, the Netherlands, Hungary, Switzerland, Belgium and Germany in Europe. However, to the best of our knowledge this is the first report of *C. parvum* IIdA15G1R1 isolates of animal origin from north India. The only previous report of sub-genotyping of *C. parvum* isolates of calves from India revealed prevalence of zoonotic sub-types IIaA15G2R1, IIaA13G2R2, IIaA14G2R1a and IIaA14G2R1b (Feng *et al.* 2007). However, the sub-genotype IId of *C. parvum* has once been reported from a child at St. Stephens Hospital, New Delhi, India (Ajjampur *et al.* 2010). The prevalence of zoonotic sub-genotype IId of *C. parvum* in bovine calves in the present study and the finding of the same genotype in a child from St. Stephens Hospital, New Delhi re-emphasizes the importance of *C. parvum* as emerging zoonoses in India.

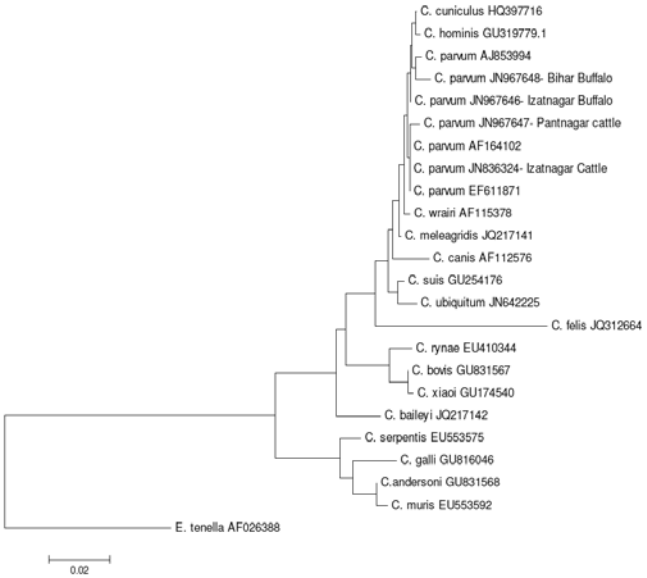

Fig. 2. Phylogenetic relationship between *C. parvum* obtained in this study and other previously published *Cryptosporidium* spp. inferred by Neighbor-Joining analysis of 18S SSU rRNA gene sequence, based on genetic distances calculated by Kimura 2-parameter method.

The information generated in this study will be helpful in assessing the zoonotic potential of *Cryptosporidium* species and the source of human infection. It will also be helpful in monitoring the transmission dynamics of cryptosporidiosis in different geo-climatic areas and in determining the host specificity of the parasite. Further studies on this aspect will provide a valuable basis for the predictive epidemiology of cryptosporidial infection in India.

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