Title: Molecular characterization and assessment of zoonotic transmission of Cryptosporidium from dairy cattle in West Bengal, India.

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Note: Nucleotide sequence data reported in this paper are available in the GenBank™ database under the following accession numbers: GQ345004-GQ345008.
Abstract

Few studies in the past have examined the genetic diversity and zoonotic potential of Cryptosporidium in dairy cattle in India. To assess the importance of these animals as a source of human Cryptosporidium infections, fecal samples from 180 calves, heifers and adults and 51 farm workers on two dairy farms in West Bengal, India were genotyped by PCR-RFLP analysis of the 18S rRNA gene of Cryptosporidium followed by DNA sequencing of the PCR products. Phylogenetic analysis was carried out on the DNA sequences obtained in the study and those available in GenBank. The overall prevalence of Cryptosporidium in cattle was 11.7% though the infection was more prevalent in younger calves than in adult cattle. The occurrence of C. parvum, C. bovis, C. ryanae and C. andersoni in cattle followed an age-related pattern. A C. suis-like genotype was also detected in a calf. Farm workers were infected with C. hominis, C. parvum and a novel C. bovis genotype. These findings clearly suggest that there is a potential risk of zoonotic transmission of Cryptosporidium infections between cattle and humans on dairy farms in India.

Keywords: Cryptosporidium, dairy cattle, zoonoses, India, Genotyping, Phylogenetic Analysis.
1. Introduction

*Cryptosporidium* species are the most common protozoa causing diarrheal diseases in humans with a significant morbidity and mortality in both the developing and developed world. In addition to humans, they infect a wide variety of domesticated and wild animals including cattle. *Cryptosporidium* from cattle are potential zoonotic pathogens, and contact with animals, manure or contaminated water is believed to lead to infections in humans (Olson *et al.*, 2004).

Cryptosporidiosis causes significant neonatal morbidity in cattle, resulting in weight loss and delayed growth, which leads to large economic losses (McDonald, 2000). In immunocompetent humans, *Cryptosporidium* parasites cause acute infections of the digestive system, but in immunocompromised patients they cause a chronic, life-threatening disease (Xiao *et al.*, 1999a).

*C. hominis* and *C. parvum* are the most common *Cryptosporidium* species found in humans, the others being *C. meleagris*, *C. felis*, and *C. canis* (Xiao *et al.*, 2004). *Cryptosporidium parvum*, *C. bovis*, *C. ryanae* and *C. andersoni* are the major species identified in cattle (Lindsay *et al.*, 2000; Xiao *et al.*, 2002; Santin *et al.*, 2004, 2008; Fayer *et al.*, 2005, 2006, 2008). Over the past two decades, cattle have been identified as being an important reservoir host for *Cryptosporidium* species transmitted from animals to humans. Contact with infected calves has been implicated as the cause of several small cryptosporidiosis outbreaks in veterinary students, animal researchers, and children attending agricultural camps and fairs (Preiser *et al.*, 2003; Smith *et al.*, 2004; Kiang *et al.*, 2006).

Since the first report of the presence of *Cryptosporidium* in Indian calves (Nooruddin and Sarma, 1987), a number of studies based on differential staining and morphology of the oocysts have been made (Dubey *et al.*, 1992; Khubnani *et al.*, 1997; Kumar *et al.*, 2004; Jeyabal and Ray, 2005; Singh *et al.*, 2006). One major problem in understanding the transmission of *Cryptosporidium* infection is the lack of morphologic features that clearly differentiate one *Cryptosporidium* spp. from many others (Fall *et al.*, 2003). Hence, one cannot be sure which...
Cryptosporidium spp. is involved when one examines oocysts in clinical specimens under a microscope. Recently, PCR based molecular epidemiological studies of Cryptosporidium in Indian cattle have been reported (Das et al., 2004; Roy et al., 2006; Feng et al., 2007; Paul et al., 2008, 2009) but none concerning the zoonotic potential of the parasite.

There has been considerable interest in recent years in the potential for zoonotic transmission of Cryptosporidium spp. with respect to cattle and other livestock. Till now, very little has been known about the genetic diversity of Cryptosporidium spp. in Indian dairy cattle. Also, there is lack of information regarding the zoonotic potential of these parasites for human beings working at dairy farms in a developing country like India. The environment at dairy farms in India is such that close contact of humans with animals occurs regularly, putting farm workers, cattle handlers and veterinarians at risk of contracting zoonotic diseases. Consequently, this study has been formulated to provide valuable information on both aspects viz. the genetic diversity and the zoonotic potential of Cryptosporidium spp. from Indian dairy cattle.
2. Materials and Methods

2.1. Collection of fecal samples

Bovine fecal samples used in this study were collected from 180 dairy cattle including 40 pre-weaned calves (0-2 months old), 72 post-weaned calves (3-12 months old) and 68 heifers and adults (> 12 months) from two dairy farms: the Harringhata Cattle Farm, Nadia and Ramakrishna Mission Dairy Farm, Narendrapur, West Bengal, India from October 2008 to August 2009. Feces were collected directly from the rectum of each animal with a gloved hand and transferred into sterile wide mouthed, labeled plastic containers and immediately placed into an insulated container packed with ice or cold packs. In addition to these, stool samples were also collected from 51 farm workers of these two farms who were in direct or indirect contact with these animals but showed no visible clinical signs of cryptosporidiosis. Specimens were transported to the Division of Parasitology, National Institute of Cholera and Enteric Diseases, Kolkata, India as early as possible and processed within 1-3 days of collection. Three aliquots of each sample were frozen without preservative in 1.5 ml cryovials at -80 °C for ELISA and PCR studies.

2.2. Parasite Detection

Microscopic examination was performed on all samples within 48 hours after collection. Modified Kinyoun’s Acid fast staining was performed according to the Centers for Disease Control and Prevention (CDC) method (http://www.dpd.cdc.gov/dpdx/HTML/DiagnosticProcedures.htm). For microscopic screening, parasite oocysts present in fecal samples were first concentrated using a FPC® Fecal Parasite Concentrator (Evergreen Scientific, Los Angeles, CA, USA). Antigen capture Enzyme Linked Immunosorbent Assay (ELISA) was also performed with all the frozen samples for detection of Cryptosporidium spp. using a commercially available kit CRYPTOSPORIDIUM II (TECHLAB, Blacksburg, VA, USA). The monoclonal antibody based ELISA test was used as instructed by the manufacturer.
2.3. DNA extraction

Genomic DNA was extracted from frozen samples from individuals that were positive by microscopy and ELISA using the QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer’s instructions except that the stool lysis temperature was increased to 80 °C. The eluted DNA was quantified spectrophotometrically and stored at -20 °C for further use.

2.4. PCR analysis

The 18S rRNA nested PCR was performed for detection of Cryptosporidium species as described previously (Xiao et al., 1999a, 2001). In the primary PCR, a 1325 bp PCR product was amplified using the forward primer Cr18SF1 (5´-TTCTAGAGCTAATACATGCG-3´) and reverse primer Cr18SR1 (5´-CCCATTTCCTTCGAACAGGA-3´). The primary PCR mixture (50 µl) consisted of 1× buffer containing 6 mM MgCl₂, 200 µM of each dNTP, 10 pmol of each primer, 2.5 units of Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany), 1-3 µl of DNA and non-acetylated bovine serum albumin (BSA; New England Biolabs, Beverly, MA, USA) to a final concentration of 0.1 µg/µl. The templates were subjected to an initial denaturation at 94 for 3 min followed by 35 amplification cycles (94 °C for 45 s, 55 °C for 45 s, 72 °C for 60 s) followed by a final extension of 7 min at 72 °C. In the secondary PCR, a ~830 bp PCR product was amplified using the forward primer Cr18SF2 (5´-GGAAGGGTTGTATTATAAAG-3´) and the reverse primer Cr18SR2 (5´-AAGGATAGGAACACCTCCA-3´). The PCR reaction mixture consisted of 1× buffer containing 3 mM MgCl₂, 200 µM of each dNTP, 10 pmol of each primer, 2.5 units of Taq DNA polymerase (Roche Diagnostics), and 1 µl of primary PCR product in a final volume of 50 µl. Cycling conditions for the secondary PCR were the same as described for the primary PCR. All PCR products were analysed by 1.5% agarose gel electrophoresis and visualised after ethidium bromide staining.
2.5. Restriction fragment length polymorphism (PCR) analysis

Secondary PCR products were purified using the High Pure PCR product purification kit (Roche Diagnostics). For RFLP analysis the PCR products were digested with SspI, VspI or MboII (Xiao et al., 1999a, 2001; Feng et al., 2007). Briefly, 10 µl of purified secondary PCR products of the 18S rRNA gene were digested in a final volume of 20 µl with 5 units of SspI/MboII (New England Biolabs) and 2 µl of corresponding 10× buffer. Similarly restriction analysis by VspI involved digestion of 10 µl secondary PCR products using 6 units of VspI (Promega, Madison, WI, USA) and 2 µl of 10× buffer in a final volume of 20 µl. All restriction digestions were carried out at 37 °C for 2 hours. Restriction products were fractionated on a 2% agarose gel and visualised after ethidium bromide staining.

2.6. DNA Sequencing and Phylogenetic Analysis

To confirm the PCR-RFLP results, all purified secondary PCR products that were positive for Cryptosporidium spp. were directly sequenced in both directions using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA) with forward and reverse primers. Sequences obtained were analyzed and assembled using CLUSTAL W software (Higgins et al., 1994). The obtained nucleotide sequences were used to search the GenBank nucleotide sequence database for sequence similarities using BLAST software (NCBI, Bethesda, MD, USA). Multiple alignments of these sequences were made using the BioEdit program (Hall, 1999).

For comparative phylogenetic analysis, reference sequences retrieved from the GenBank were aligned with the representative sequences of each species or genotype of Cryptosporidium obtained in this study and a neighbor-joining tree was constructed using TREECON for Windows version 1.3b (Van de Peer and De Wachter, 1994). Distance estimations were carried out using the Jukes and Cantor correction. The branch reliability of the neighbor-joining tree was assessed by the bootstrap method with 1000 replications. The nucleotide sequence of Eimeria tenella (GenBank
accession no. AF026388) was used as an outgroup to root the neighbor-joining tree since the construction of an unrooted tree showed it to be the most divergent member under analysis.
3. Results

The overall prevalence of *Cryptosporidium* in cattle was 11.7% (Table 1). 8 out of 40 pre-weaned calves (20%), 10 out of 72 post-weaned calves (13.9%) and 3 out of 68 heifers and adults (4.4%) were positive for *Cryptosporidium*. Among farm workers, *Cryptosporidium* was present in 11.8% of the screened samples. Successful PCR amplification of the *Cryptosporidium* 18S rRNA gene was accomplished for all the samples positive by microscopy and ELISA.

3.1. Genotyping and Phylogenetic Analysis of Cryptosporidium

The occurrence of *Cryptosporidium* spp. in cattle followed an age-related pattern: the zoonotic *C. parvum* was found only in pre-weaned calves; *C. bovis* and *C. ryanae* found mostly in post-weaned calves whereas *C. andersoni* was found mostly in heifers and adults (Table 1). A *C. suis*-like genotype (GenBank accession no. GQ345008) was also detected in a post-weaned calf.

Restriction digestion by *Ssp*I, *Vsp*I and *Mbo*II generated two (453 and 365 bp), three (630, 104 and 103 bp) and two (774 and 64 bp) bands respectively (Fig. 1). This restriction profile was similar to that of *C. suis* (Xiao et al., 1999b). However, DNA sequencing results showed a mismatch of 1 base pair (A to T) at 481 position and a single gap after 478 position when aligned with the reference sequence for *C. suis* (GenBank accession no. AF115377).

In farm workers, *C. hominis* was the most prevalent species (3 out of 6 *Cryptosporidium* positive isolates) followed by *C. parvum* (2 out of 6 *Cryptosporidium* positive isolates) (Table 1). A novel *C. bovis* genotype (GenBank accession no. GQ345006) was also detected in a worker, showing a mismatch of 1 base pair (G to A) at 491 position with the partial 18S rRNA sequence of *C. bovis* isolated from a calf (GenBank accession no. GQ345005). RFLP analysis by *Ssp*I, *Vsp*I and *Mbo*II produced a banding pattern similar to *C. bovis* (Fig. 1) clearly indicating that the single base substitution did not affect its restriction profile.
Phylogenetic analysis of *Cryptosporidium* species resulted in formation of two clades with good statistical reliability. One clade contained *C. muris*, *C. serpents*, *C. galli* and *C. andersoni* while the other clade contained *C. baileyi*, *C. felis*, *C. canis*, *C. meleagris*, *C. suis*, *C. suis*-like genotype, *C. bovis*, *C. bovis* human genotype, *C. ryanae*, *Cryptosporidium* deer genotype, *C. hominis*, *C. wrairi* and *C. parvum* (Fig. 2). As expected, *C. suis*-like genotype clustered together with *C. suis* while *C. bovis* human genotype (isolated from a farm worker) related most closely to *C. bovis*. Similarly, *C. ryanae* clustered together with the *Cryptosporidium* deer genotype.

3.2. Nucleotide sequence accession numbers

Representatives for species/ genotypes of *Cryptosporidium* identified in this study have been submitted to GenBank under the accession numbers: GQ345004 to GQ345008.
4. Discussion

Overall, Cryptosporidium was detected in 11.7% (21/180) of the bovine fecal samples collected from two dairy farms in the present study. These results corroborate similar findings observed in a previous study on the prevalence of Cryptosporidium in adult cattle and calves in Maharashtra, India (Khubnani et al., 1997). Further, infections by Cryptosporidium spp. were more prevalent in calves than in adult cattle, which agrees with previous reports (Huetink et al., 2001; Olson et al., 2004; Mendonca et al., 2007). Additionally, 6 out of 8 (75%) Cryptosporidium positive pre-weaned calf isolates genotyped in this study through PCR-RFLP and DNA sequencing were identified as C. parvum (Table 1). This finding was expected and is in agreement with abundant literature data that have indicated C. parvum as the most frequently found species in pre-weaned calves (Xiao et al., 2002; Santin et al., 2004; Thompson et al., 2007; Feng et al., 2007). Similarly, an age-related variation was also seen in the occurrence of other Cryptosporidium spp. detected in cattle, thereby supporting observations from similar studies (Santin et al., 2004, 2008; Fayer et al., 2006, 2007; Langkjaer et al., 2007).

Interestingly, cattle in this study were found to be infected with a number of species and genotypes in addition to C. parvum: C. bovis, C. ryanae, C. andersoni and a C. suis-like genotype. Although C. bovis has been detected from Indian cattle recently (Feng et al., 2007), this study provides the first report for the detection of C. ryanae and the C. suis-like genotype in cattle of India. The lack of finding of C. bovis and C. ryanae in previous epidemiological studies in India is probably partially due to the use of older genotyping tools (Das et al., 2004; Roy et al., 2006; Paul et al., 2008, 2009). Since there are only minor differences in 18S rRNA based RFLP patterns among C. parvum, C. bovis and C. ryanae, their reliable differentiation generally requires either DNA sequencing of the secondary PCR products (Santin et al., 2004, 2008; Fayer et al., 2006, 2007; Feng et al., 2007) or the use of an additional restriction enzyme MboII in conjunction with SspI and VspI (Feng et al., 2007).
The partial 18S rRNA sequence of the *C. suis*-like genotype differed from that of *C. suis* (GenBank accession no. AF115377) by just two nucleotides and both related closely to each other under phylogenetic analysis. A *C. suis*-like genotype identical to the one found in this study has been detected previously in three calves in Denmark with no history of contact with pigs (Langkjaer *et al.*, 2007). Similarly, pigs were not present on any of the cattle farms involved in our study. Therefore, the presence of the *C. suis*-like genotype in cattle cannot be explained by proximity between pigs and cattle.

In the current study, *C. parvum* was detected in both dairy cattle and farm workers. Significantly, DNA sequencing results and phylogenetic studies showed that *C. parvum* isolates from calves and human workers on the dairy farm were genetically identical to each other. Furthermore, detection of *C. bovis* in a farm worker in the current study represents the first report of the detection of this species in human beings. It showed only one nucleotide change in the 18S rRNA target sequence when compared to *C. bovis* isolated from bovines and was also phylogenically related to *C. bovis*, *C. ryanae* and the *Cryptosporidium* deer genotype. Identification of this cattle-specific species in a farm worker in close contact with dairy cattle on the farm suggests that ‘unusual’ species may play a role in human infections but such findings are very rare and therefore the resultant public health significance is also minimal. However, the occurrence of genetically identical *C. parvum* isolates in both dairy cattle and human workers in this study is of potential zoonotic concern. On the other hand, *C. hominis* was not detected in cattle in the present study although it was the most prevalent species found in farm workers thus indicating anthroponotic transmission of this genotype is more common.

In conclusion, results of this study clearly indicate that the epidemiological picture and genetic diversity of *Cryptosporidium* spp. in Indian dairy cattle is quite different from what it was previously thought to be. Additionally, results also provide useful evidence on the zoonotic transmission of this parasite between cattle and farm workers. Even a few calves infected with
zoonotic genotypes of *Cryptosporidium* could pose a significant public health risk directly to cattle handlers or indirectly as an important reservoir for human waterborne outbreaks of cryptosporidiosis.
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References


102, 53-67.


Detection of *Cryptosporidium* species and genotypes by PCR-RFLP and DNA sequencing of the 18S rRNA gene in fecal specimens collected from different age groups of dairy cattle and dairy farm workers.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sample size</th>
<th><em>C. parvum</em></th>
<th><em>C. hominis</em></th>
<th><em>C. bovis</em></th>
<th><em>C. ryanae</em></th>
<th><em>C. andersoni</em></th>
<th><em>C. suis-like</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-weaned calves</td>
<td>40</td>
<td>6</td>
<td>0</td>
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<td>1</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Post-weaned calves</td>
<td>72</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>2</td>
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<td>1</td>
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<tr>
<td>Heifers and Adult Cattle</td>
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<td>0</td>
<td>1</td>
<td>0</td>
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<tr>
<td>Farm Workers</td>
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<td>3</td>
<td>1</td>
<td>0</td>
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</table>
**Figure Captions**

Figure 1. Genotyping of *Cryptosporidium* isolates by RFLP analysis based on digestion of 18S rRNA gene PCR products by *Ssp*I (upper panel), *Vsp*I (middle panel) and *Mbo*II (lower panel).


Figure 2. Phylogenetic relationship among *Cryptosporidium* isolates as inferred by neighbor-joining analysis of the partial 18S rRNA nucleotide sequences. Bootstrap values above 50% out of 1000 replicates are indicated at each node. Accession numbers for sequences obtained from GenBank are given in parentheses. The sequence of *Eimeria tenella* was used as an outgroup.
Table 1.

Detection of different species and genotypes of *Cryptosporidium* by PCR-RFLP and DNA sequencing of the 18S rRNA gene in fecal specimens collected from different age groups of dairy cattle and dairy farm workers.

<table>
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<tr>
<th>Source</th>
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<td></td>
<td></td>
<td><em>C. parvum</em></td>
<td><em>C. hominis</em></td>
<td><em>C. bovis</em></td>
<td><em>C. ryanae</em></td>
<td><em>C. andersoni</em></td>
<td><em>C. suis-like</em></td>
</tr>
<tr>
<td>Pre-weaned calves</td>
<td>40</td>
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<td>0</td>
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<td>Post-weaned calves</td>
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<td>0</td>
<td>6</td>
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<td>1</td>
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<td>Heifers and</td>
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<td>0</td>
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<td>2</td>
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<tr>
<td>Adult Cattle</td>
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<td>Dairy Farm Workers</td>
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