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

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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. meleagridis*, *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´-CCCATTTCCTTCGAAACAGGA-3´). The primary PCR mixture (50 µl) consisted of 1× buffer containing 6 mM $MgCl_2$, 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´-GGAAGGGTTGTATTTATTAGATAAAG-3´) and the reverse primer Cr18SR2 (5´-AAGGAGTAAGGAACAACCTCCA-3´). The PCR reaction mixture consisted of 1× buffer containing 3 mM $MgCl_2$, 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 *Ssp*I, *Vsp*I or *Mbo*II (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 *Ssp*I/*Mbo*II (New England Biolabs) and 2 µl of corresponding 10× buffer. Similarly restriction analysis by *Vsp*I involved digestion of 10 µl secondary PCR products using 6 units of *Vsp*I (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.

185 Phylogenetic analysis of *Cryptosporidium* species resulted in formation of two clades with
186 good statistical reliability. One clade contained *C. muris*, *C. serpentis*, *C. galli* and *C. andersoni*
187 while the other clade contained *C. baileyi*, *C. felis*, *C. canis*, *C. meleagridis*, *C. suis*, *C. suis*-like
188 genotype, *C. bovis*, *C. bovis* human genotype, *C. ryanae*, *Cryptosporidium* deer genotype, *C.*
189 *hominis*, *C. wrairi* and *C. parvum* (Fig. 2). As expected, *C. suis*-like genotype clustered together
190 with *C. suis* while *C. bovis* human genotype (isolated from a farm worker) related most closely to
191 *C. bovis*. Similarly, *C. ryanae* clustered together with the *Cryptosporidium* deer genotype.
192
193 *3.2. Nucleotide sequence accession numbers*
194 Representatives for species/ genotypes of *Cryptosporidium* identified in this study have
195 been submitted to GenBank under the accession numbers: GQ345004 to GQ345008.
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198 **4. Discussion**

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

212 Interestingly, cattle in this study were found to be infected with a number of species and
213 genotypes in addition to *C. parvum*: *C. bovis*, *C. ryanae*, *C. andersoni* and a *C. suis*-like genotype.
214 Although *C. bovis* has been detected from Indian cattle recently (Feng *et al.*, 2007), this study
215 provides the first report for the detection of *C. ryanae* and the *C. suis*-like genotype in cattle of
216 India. The lack of finding of *C. bovis* and *C. ryanae* in previous epidemiological studies in India is
217 probably partially due to the use of older genotyping tools (Das *et al.*, 2004; Roy *et al.*, 2006; Paul
218 *et al.*, 2008, 2009). Since there are only minor differences in 18S rRNA based RFLP patterns
219 among *C. parvum*, *C. bovis* and *C. ryanae*, their reliable differentiation generally requires either
220 DNA sequencing of the secondary PCR products (Santin *et al.*, 2004, 2008; Fayer *et al.*, 2006,
221 2007; Feng *et al.*, 2007) or the use of an additional restriction enzyme *Mbo*II in conjunction with
222 *Ssp*I and *Vsp*I (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

248 zoonotic genotypes of *Cryptosporidium* could pose a significant public health risk directly to cattle
249 handlers or indirectly as an important reservoir for human waterborne outbreaks of
250 cryptosporidiosis.
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References

Das, G., Sarkar, S., Panja, P., Das, P., 2004. PCR based detection of *Cryptosporidium parvum* in cattle. J. Vet. Parasitol. 18, 43-44.

Dubey, J.P., Fayer, R., Rao, J.R., 1992. Cryptosporidial oocysts in faeces of water buffalo and zebu calves in India. J. Vet. Parasitol. 6, 55-56.

Fall, A., Thompson, R.C., Hobbs, R.P., Morgan-Ryan, U., 2003. Morphology is not a reliable tool for delineating species within *Cryptosporidium*. J. Parasitol. 89, 399-402.

Fayer, R., Santin, M., Trout, J.M., 2007. Prevalence of *Cryptosporidium* species and genotypes in mature dairy cattle on farms in eastern United States compared with younger cattle from the same locations. Vet. Parasitol. 145, 260-266.

Fayer, R., Santin, M., Trout, J.M., 2008. *Cryptosporidium ryanae* n. sp. (Apicomplexa: Cryptosporidiidae) in cattle (*Bos taurus*). Vet. Parasitol. 156, 191-198.

Fayer, R., Santin, M., Trout, J.M., Greiner, E., 2006. Prevalence of species and genotypes of *Cryptosporidium* found in 1-2-year-old dairy cattle in the eastern United States. Vet. Parasitol. 135, 105-112.

Fayer, R., Santin, M., Xiao, L., 2005. *Cryptosporidium bovis* n. sp. (Apicomplexa: Cryptosporidiidae) in cattle (*Bos taurus*). J. Parasitol. 91, 624-629.

Feng, Y., Ortega, Y., He, G., Das, P., Xu, M., Zhang, X., Fayer, R., Gatei, W., Cama, V., Xiao, L., 2007. Wide geographic distribution of *Cryptosporidium parvum* and deer like genotype in bovines. Vet. Parasitol. 144, 1-9.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41, 95-98.

Higgins, D., Thompson, J., Gibson, T., Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through

294 sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids Res. 22,
295 4673-4680.
296 Huetink, R.E., van der Giessen, J.W.B., Noordhuizen, J.P.T.M., Ploeger, H.W., 2001.
297 Epidemiology of *Cryptosporidium* spp. and *Giardia duodenalis* on a dairy farm. Vet. Parasitol.
298 102, 53-67.
299 Jeyabal, L., Ray, D.D., 2005. Cryptosporidial infection in cattle and buffaloes. J. Vet. Parasitol. 19,
300 165-166.
301 Khubnani, H., Sivarajan, K., Khubnani, A.H., 1997. Study of cryptosporidiosis in a rural area of
302 Maharashtra. Indian J. Pathol. Microbiol. 40, 33-36.
303 Kiang, K.M., Scheftel, J.M., Leano, F.T., Taylor, C.M., Belle-Isle, P.A., Cebelinski, E.A., Danila,
304 R., Smith, K.E., 2006. Recurrent outbreaks of cryptosporidiosis associated with calves among
305 students at an educational farm programme, Minnesota, 2003. Epidemiol. Infect. 134, 878-886.
306 Kumar, D., Sreekrishnan, R., Das, S.S., 2004. Cryptosporidiosis in man and animals in
307 Pondicherry. Ind. J. Anim. Sci. 74, 261-263.
308 Langkjaer, R.B., Vigre, H., Enemark, H.L., Maddox-Hyttel, C., 2007. Molecular and phylogenetic
309 characterization of *Cryptosporidium* and *Giardia* from pigs and cattle in Denmark. Parasitology
310 134, 339-350.
311 Lindsay, D.S., Upton, S.J., Owens, D.S., Morgan, U.M., Mead, J.R., Blagburn, B.L., 2000.
312 *Cryptosporidium andersoni* n. sp. (Apicomplexa: Cryptosporiidae) from cattle, *Bos taurus*. J.
313 Eukaryot. Microbiol. 47, 91-95.
314 McDonald, V., 2000. Host cell-mediated responses to infection with *Cryptosporidium*. Parasite
315 Immunol. 22, 597-604.
316 Mendonca, C., Almeida, A., Castro, A., de Lurdes Delgado, M., Soares, S., da Costa, J.M., Canada,
317 N., 2007. Molecular characterization of *Cryptosporidium* and *Giardia* isolates from cattle from
318 Portugal. Vet. Parasitol. 147, 47-50.

319 Nooruddin, M., Sarma, D.K., 1987. Role of *Cryptosporidium* in calf diarrhea, Annual report.
320 Livestock Advisor. 12, 49.
321 Olson, M.E., O'Handley, R.M., Ralston, B.J., McAllister, T.A., Thompson, R.C.A., 2004. Update
322 on *Cryptosporidium* and *Giardia* infections in cattle. Trends Parasitol. 20, 185-191.
323 Paul, S., Chandra, D., Ray, D.D., Tewari, A.K., Rao, J.R., Banerjee, P.S., Baidya, S., Raina, O.K.,
324 2008. Prevalence and molecular characterization of bovine *Cryptosporidium* isolates in India. Vet.
325 Parasitol. 153, 143-146.
326 Paul, S., Chandra, D., Tewari, A.K., Banerjee, P.S., Ray, D.D., Raina, O.K., Rao, J.R., 2009.
327 Prevalence of *Cryptosporidium andersoni*: A molecular epidemiological survey among cattle in
328 India. Vet. Parasitol. 161, 31-35.
329 Preiser, G., Preiser, L., Madeo, L., 2003. An outbreak of cryptosporidiosis among veterinary
330 science students who work with calves. J. Am. Coll. Health 51, 213-215.
331 Roy, S.S., Sarkar, S., Batabyal, S., Pramanik, A.K., Das, P., 2006. Observation on the
332 epidemiology of bovine Cryptosporidiosis in India. Vet. Parasitol. 141, 330-333.
333 Santin, M., Trout, J.M., Fayer, R., 2008. A longitudinal study of cryptosporidiosis in dairy cattle
334 from birth to 2 years of age. Vet. Parasitol. 155, 15-23.
335 Santin, M., Trout, J.M., Xiao, L., Zhou, L., Greiner, E., Fayer, R., 2004. Prevalence and age-related
336 variation of *Cryptosporidium* species and genotypes in dairy calves. Vet. Parasitol. 122, 103-117.
337 Singh, B.B., Sharma, R., Kumar, H., Banga, H.S., Aulakh, R.S., Gill, J.P.S., Sharma, J.K., 2006.
338 Prevalence of *Cryptosporidium parvum* infection in Punjab and its association with diarrhoea in
339 neonatal dairy calves. Vet. Parasitol. 140, 162-165.
340 Smith, K.E., Stenzel, S.A., Bender, J.B., Wagstrom, E., Soderlund, D., Leano, F.T., Taylor, C.M.,
341 Belle-Isle, P.A., Danila, R., 2004. Outbreaks of enteric infections caused by multiple pathogens
342 associated with calves at a farm day camp. Pediatr. Infect. Dis. J. 23, 1098-1104.

343 Thompson, H.P., Dooley, J.S., Kenny, J., McCoy, M., Lowery, C.J., Moore, J.E., Xiao, L., 2007.
344 Genotypes and subtypes of *Cryptosporidium* spp. in neonatal calves in Northern Ireland. Parasitol.
345 Res. 100, 619-624.
346 Van de Peer, Y., De Wachter, R., 1994. TREECON for Windows: a software package for the
347 construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput.
348 Appl. Biosci. 10, 569-570.
349 Xiao, L., Bern, C., Limor, J., Sulaiman, I., Roberts, J., Checkley, W., Cabrera, L., Gilman, R.H.,
350 Lal, A.A., 2001. Identification of 5 types of *Cryptosporidium* parasites in children in Lima, Peru. J.
351 Infect. Dis. 183, 492-497.
352 Xiao, L., Escalante, L., Yang, C., Sulaiman, I., Escalante, A.A., Montali, R.J., Fayer, R., Lal, A.A.,
353 1999a. Phylogenetic analysis of *Cryptosporidium* parasites based on the small-subunit rRNA gene
354 locus. Appl. Environ. Microbiol. 65, 1578-1583.
355 Xiao, L., Fayer, R., Ryan, U., Upton, S.J., 2004. *Cryptosporidium* taxonomy: recent advances and
356 implications for public health. Clin. Microbiol. Rev. 17, 72-97.
357 Xiao, L., Herd, R.P., 1994. Infection patterns of *Cryptosporidium* and *Giardia* in calves. Vet.
358 Parasitol. 55, 257-262.
359 Xiao, L., Morgan, U.M., Limor, J., Escalante, A., Arrowood, M., Shulaw, W., Thompson, R.C.,
360 Fayer, R., Lal, A.A., 1999b. Genetic diversity within *Cryptosporidium parvum* and related
361 *Cryptosporidium* species. Appl. Environ. Microbiol. 65, 3386-3391.
362 Xiao, L., Sulaiman, I.M., Ryan, U.M., Zhou, L., Atwill, E.R., Tischler, M.L., Zhang, X., Fayer, R.,
363 Lal, A.A., 2002. Host adaptation and host-parasite co-evolution in *Cryptosporidium*: implications
364 for taxonomy and public health. Int. J. Parasitol. 32, 1773-1785.
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367 **Tables**

368 Table 1.

369 Detection of *Cryptosporidium* species and genotypes by PCR-RFLP and DNA sequencing of the

370 18S rRNA gene in fecal specimens collected from different age groups of dairy cattle and dairy

371 farm workers.

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Source	**Sample size**	***Cryptosporidium***					
		C. parvum	*C. hominis*	*C. bovis*	*C. ryanae*	*C. andersoni*	*C. suis*-like
Pre-weaned calves	40	6	0	1	1	0	0
Post-weaned calves	72	0	0	6	2	1	1
Heifers and Adult Cattle	68	0	0	1	0	2	0
Farm Workers	51	2	3	1	0	0	0

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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). Lane 1: *C. parvum*, lane 2: *C. bovis*, lane 3: *C. ryanae*, lane 4: 100 bp plus marker, lane 5: *C. andersoni*, lane 6: *C. hominis*, lane 7: *C. suis*-like genotype and lane 8: *C. bovis* (human source).

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

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.

Source	**Sample size**	***Cryptosporidium***					
		C. parvum	*C. hominis*	*C. bovis*	*C. ryanae*	*C. andersoni*	*C. suis*-like
Pre-weaned calves	40	6	0	1	1	0	0
Post-weaned calves	72	0	0	6	2	1	1
Heifers and Adult Cattle	68	0	0	1	0	2	0
Dairy Farm Workers	51	2	3	1	0	0	0

Figure

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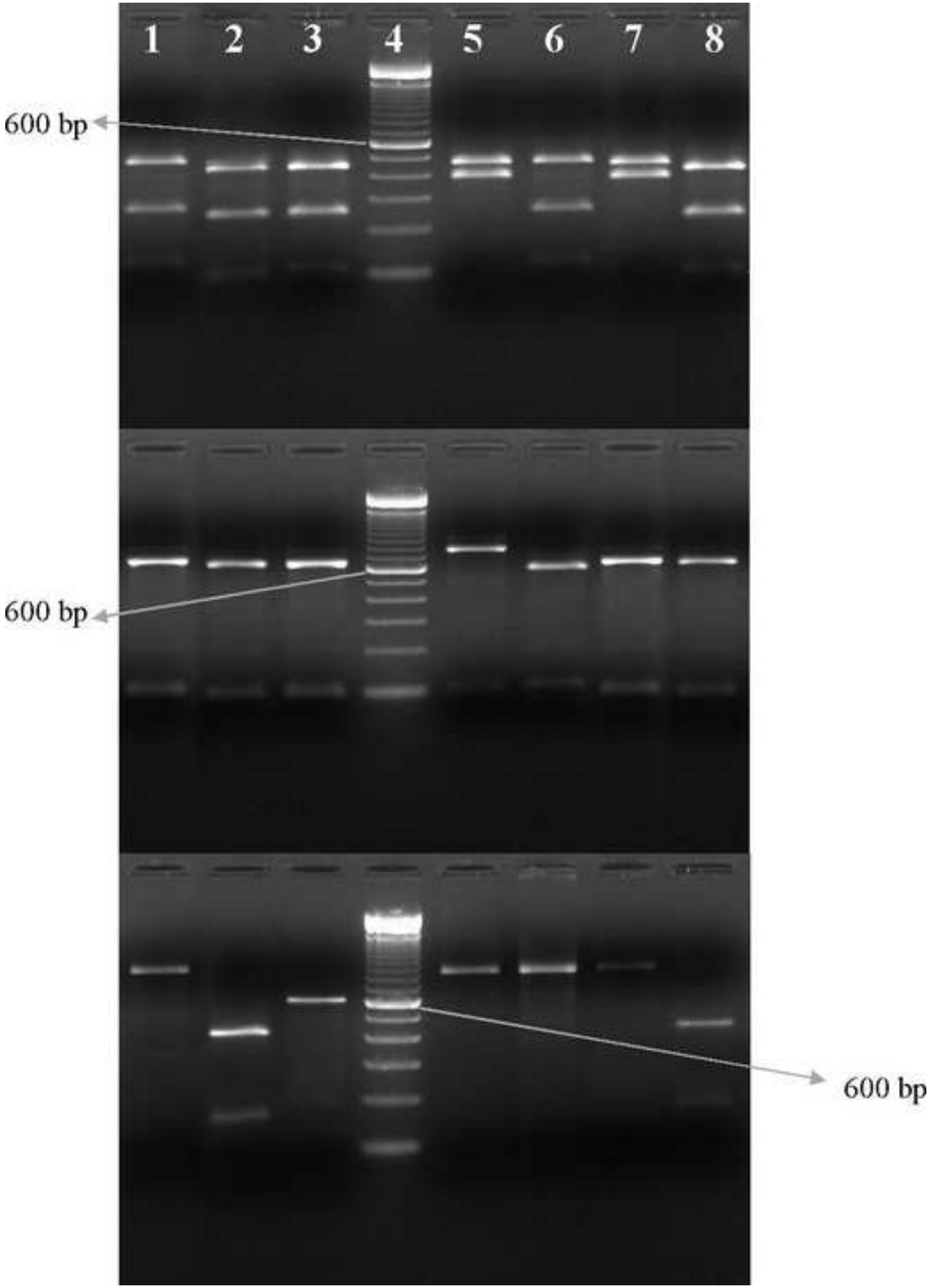

Figure

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