Prevalence and molecular epidemiology of *Cryptosporidium parvum* in dairy calves in Punjab (India)

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Abstract *Cryptosporidium parvum* is an important zoonotic protozoan parasite that infects the gastrointestinal tract of vertebrate animals and man. The current study was contemplated for molecular detection of *Cryptosporidium* species prevalent in dairy calves in Punjab, India. A total of 302 faecal samples were screened by modified Ziehl-Neelsen staining technique for the detection of *Cryptosporidium* oocysts. Molecular characterisation was done using PCR followed by sequence analysis of the representative isolates. An overall prevalence of 26.15 % was obtained with the highest prevalence obtained in 0–30 day old calves in both diarrhoeic and non-diarrhoeic animals. PCR analysis revealed the expected bands at 1,325 and 835 bp from all the isolates for primary and secondary/nested PCR respectively. Ten representative samples were sequenced in both directions. Phylogenetic analysis revealed the presence of *C. parvum* in all the samples. The high rate of calves infected with *C. parvum* can act as a great source of zoonotic cryptosporidiosis which indicates a potential risk of zoonotic transmission from animal to human beings in Punjab (India).

Keywords Prevalence · Dairy calves · *Cryptosporidium parvum* · Punjab · India

Introduction

Cryptosporidiosis due to *C. parvum* is an important zoonotic disease caused by a small apicomplexon protozoan

parasites belonging to genus *Cryptosporidium*. Domestic cattle have been considered the major reservoir of *Cryptosporidium* for human infections (Xiao and Fayer 2008). Calves below one month of age are found to be most susceptible to the infection than the other age group (Singh et al. 2006) and a major contributor of zoonotic *C. parvum* (Fayer et al. 2006; Xiao 2010). However with the attainment of immunological maturity the infection subsides in older animals though they remain as a source of infection to other susceptible individuals.

The disease is found to be self-limiting, however in immune-deficient individuals, it may lead to life-threatening chronic diarrhoea. It poses serious public health issues where AIDS is found to be endemic.

Infected humans and animals can seed enormous amounts of the transmission stages of these parasites. The micro-organism is ubiquitous in environment including a number of animal species (Navin and Junarek 1984). So there is every possibility of zoonotic transmission of infection from animal to human beings, especially under poor hygienic conditions.

Due to the lack of distinct morphological features of *Cryptosporidium* oocysts, it cause great inconvenience to clearly differentiate microscopically different species rather genotype (Fall et al. 2003). However, only in the last few years molecular biology based methods have been developed to detect and differentiate *Cryptosporidium* at the species/genotype and subtype levels (Xiao and Ryan 2004; Caccio 2005). Therefore molecular analysis has been widely used to characterise the genetic structure of *Cryptosporidium* spp. and assessment of their zoonotic significance (Xiao 2010). *C. parvum* can be divided into two genetically distinct subpopulations based on the molecular characterization of oocyst: genotype I (anthroponotic genotype) which is associated exclusively with human

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infection and is recently proposed as a new species (*C. hominis*) and genotype II (zoonotic or cattle genotype) which is associated with both human and animal infections (Morgan et al. 2000). *C. parvum* the only prevalent zoonotic species in cattle, is responsible for about 85 per cent of the *Cryptosporidium* infections in pre-weaned calves (Santin et al. 2004; Fayer et al. 2006).

Since its first report in India by Nooruddin and Sarma (1987), the prevalence study on bovine cryptosporidiosis have been conducted microscopically in stained faecal smears of both diarrhoeic and normal cattle in different parts of the country (Dubey et al. 1992; Das et al. 2011; Jeyaba and Ray 2005; Roy et al. 2006; Singh et al. 2006; Bhat et al. 2012; Singh et al. 2010). But limited studies are devoted to ascertain the zoonotic potential of the parasites. A preliminary study using conventional techniques revealed that *Cryptosporidium* spp. appears to be common in dairy calves and an important contributor of calf diarrhoea in Punjab (Singh et al. 2006). The present study was contemplated because of the growing importance of *Cryptosporidium* infection in farm animals together with its immense zoonotic importance as well as inadequate data.

Materials and methods

Collection of samples and identification of *Cryptosporidium* oocysts

A total of 302 faecal samples (both diarrhoeic and non-diarrhoeic) were collected from neonatal bovine calves. Samples were screened by modified Ziehl-Neelsen staining methods (OIE 2008). Sheather's sucrose flotation method was used for the concentration of oocysts. Examination for the presence of oocysts was done by scanning the slide using the 40× objective lens of a bright-field microscope. Presence of oocysts in the smear was confirmed under the oil immersion objective lens. The positive samples were then stored at 4 °C for further molecular analysis.

Isolation of DNA

DNA extraction was carried out using QIAamp Stool Mini Kit (Qiagen) as per protocol. The eluted DNA was quantified spectrophotometrically (Nanodrop, Thermo) and stored at −20 °C till further use.

PCR amplification and sequencing

The small sub-unit (18S) rRNA gene of *Cryptosporidium* was amplified using the oligonucleotides primers CRP-DIAG1 forward (5'-TTCTAGAGCTAATACATGCG-3')

and CRP-DIAG1 reverse (5'-CAT-TTCCTTCGAAACAGGA-3') (Paul et al. 2008). The PCR was set up in 25 µl reaction consisting of 2.5 µl of 10× PCR *Taq* buffer (MBI Fermentas), 0.5 µl of 10 mM dNTP mix (MBI Fermentas), 1.5 µl of 25 mM MgCl₂ (MBI Fermentas), 0.5 µl of 1.0 U of *Taq* DNA polymerase (recombinant) (MBI Fermentas), 20 pmol each of the respective primers and 5 µl of template as DNA source. The final volume was made up to 25 µl by adding nuclease free water. The reaction mixture were subjected to initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, elongation at 72 °C for 1 min and 1 cycles of final elongation at 72 °C for 10 min. For the secondary/nested PCR 1 µl of primary PCR products was used as template and the products were amplified using the primers CRP-DIAG2 forward (5'-GGAAGGGTTGTATTTATTAGATAAAG-3') and CRP-DIAG2 reverse (5'-AAGGAGTAAGGAACAACCTCCA-3') (Paul et al. 2008). The thermo cyclic parameters were kept same as that of primary PCR except the annealing temperature was increased by one degree. The amplified products were visualized by 1.5 % agarose gel electrophoresis using ethidium bromide staining. PCR products were purified using HiPurA™ PCR Product Purification Kit (Himedia) and 10 purified samples were sequence in both directions by Department of Animal Biotechnology, Lala Lajpat Rai University of Veterinary & Animal Sciences, Hisar (India).

Phylogenetic analysis

Sequence chromatograms were analysed using the Bioedit, ClustalW and Mega 5.0 computer software programs. The results were matched using NCBI BLAST software. These were aligned and compared with previously published sequences of *C. Parvum* (Gene Bank accession numbers AB746195.1), *C. bailey* (Gene Bank accession numbers L19068.1), *C. bovis* (Gene Bank accession numbers AB746197.1) and *C. andersoni* (Gene Bank accession numbers FJ463171.1). *Eimeria tenella* (Gene Bank accession number EUO425114.1) was used as an outgroup. Distance-based analyses were conducted and trees were constructed using the Neighbor-Joining algorithm using Mega 5.0 software.

Statistical analysis was evaluated using χ^2 test. Probability (p) of <0.05 was defined as statistically significant.

Results

The overall prevalence of *Cryptosporidium* species was found to be 26.15 per cent using modified ziehl-neelson (mZn) staining technique (Fig. 1). The highest prevalence

Fig. 1 Modified Ziehl–Neelsen stained faecal smear ($\times 100$) showing *Cryptosporidium* oocysts

of 37.25 per cent was recorded in 0–30 days calves followed by 22.97 per cent, 20.27 and 17.30 per cent in 1–2 months age groups, 2–3 months age groups and in 3–4 months of age groups and above respectively (Tables 1, 2). The rate of infection peaked in the age group 0–30 days and declined in the subsequent age groups and difference was found to be statistically significant ($p < 0.05$). The prevalence values decreased with increase in age in both diarrhoeic as well as non-diarrhoeic calves. Higher rate of negative correlation ($r = -0.898$) was observed between the rate of infection with increase in age. Prevalence rate was found to be highest during monsoon (42.85 %) followed by pre-monsoon (30.66 %). PCR

Fig. 2 PCR amplification of 18S rRNA gene of bovine *Cryptosporidium* with $\sim 1,325$ bp amplicons. From left to right DNA marker, Lane 1 to 2 DNA samples. Lane N negative control

analysis of all the samples showed expected bands at 1,325 and 835 bp in primary PCR and secondary PCR respectively (Figs. 2, 3). The nucleotide sequence alignment and

Table 1 Prevalence of *Cryptosporidium* spp. infection in different age group in diarrhoeic and non-diarrhoeic dairy calves in Punjab (India)

Age group (months)	Diarrhoeic			Non-diarrhoeic			Total (%) prevalence
	No. of screened samples	No. of positive samples	% positive	No. of screened samples	No. of positive samples	% positive	
0–30 days	40	25	69.44	62	13	20.96	37.25
1–2 months	29	10	34.61	45	7	15.15	22.97
2–3 months	45	7	15.00	29	8	27.58	20.27
3 months and above	21	5	23.80	31	4	12.90	17.30
Total	135	47	34.81	167	32	19.16	26.15

Table 2 Seasonal prevalence of *Cryptosporidium* spp. infection in dairy calves in Punjab (India)

Season	Diarrhoeic		Non-diarrhoeic		Prevalence (%)
	No. of screened samples	No. of positive samples	No. of screened samples	No. of positive samples	
Pre Monsoon	36	12	49	8	23.52
Monsoon	42	27	65	12	37.14
Post Monsoon	57	8	53	15	20.90

Fig. 3 Nested PCR amplification of 18S rRNA gene of bovine *Cryptosporidium* with ~ 835 bp amplicons. From left to right DNA marker, Lane 1 to 4 DNA samples. Lane 5 negative control

BLAST search analysis of the isolates revealed homology between the 825 bp amplified fragments and the sequences obtained in the Gene bank. Phylogenetic analysis revealed the presence of *C. parvum* in all the samples (Fig. 4). None of the samples was found positive for *C. bovis* and *C. andersoni*.

Discussion

Our observations are in confirmatory with O’Handley et al. (1999) who reported that *Cryptosporidium* is often the only pathogen detected in diarrheic calves. The calves observed

to be infected with *Cryptosporidium* spp. were found to be relatively at higher risk (1.81 times) to diarrhoea than non-infected calves. The result of age wise distribution of the parasite demonstrated that the majority of the *Cryptosporidium* cases were from neonates. Similar observations have also been reported by Paul et al. (2008), Kumar et al. (2004) and Shobhamani et al. (2005). In our study a negative correlation ($r = -0.89$) was found between infection rate and age. The results are in accordance with the findings of Singh et al. (2006) who reported 79.41 per cent prevalence of *C. parvum* infection among 0–30 days old dairy calves in Punjab and infection was found to be negatively correlated with age. From our findings it is evident that dairy calves shed *Cryptosporidium* oocysts apparently higher in the warm and humid season than the dry and cold winter. High temperature and humidity along with frequent rains in the monsoon season enabled the transmission of the oocysts faster. Similar observations have also been reported by Paul et al. (2008) who recorded the prevalence of cryptosporidiosis in the monsoon months as 37.3 per cent which was higher than the dry pre-monsoon (25.6 %) and cooler post-monsoon months (19.6 %). Results of the sequence analysis of the nested PCR products revealed that all the isolates were *C. parvum* and can be regarded as the most prevalent species among bovine calves in this part of the country. Paul et al. (2008) have also reported *C. parvum* as the only species of *Cryptosporidium* prevalent in bovine calves in three different geographical regions of India and Roy et al. (2006) confirmed that *C. parvum* is responsible for bovine cryptosporidiosis in India. Infected calves can act as a great source of zoonotic cryptosporidiosis. The high prevalence rate of infection among bovine calves with the zoonotic *C. parvum* indicates a potential risk of transmission from animal to human beings.

Fig. 4 Phylogenetic relationship among *Cryptosporidium* isolates as inferred by neighbor-joining analysis of 18S rRNA nucleotide sequences

Conflict of interest No financial or personal relationships between the authors and other people or organizations have inappropriately influenced (bias) this work.

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