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Cryptosporidium Infection in Asymptomatic Dairy Calves in Ludhiana District of Punjab, India

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***Cryptosporidium*, an apicomplexan protozoon, is a well-recognized cause of diarrhoea in humans and animals throughout the world. A total of 157 faecal samples were collected from asymptomatic cattle and buffalo calves of five commercial dairy farms in and around Ludhiana, Punjab to investigate the carrier status of *Cryptosporidium* spp infection. Overall 26.75% faecal samples were found to be positive for 834bp amplicon specific for *Cryptosporidium* spp. after application of nested PCR using genus specific primers directed against small subunit ribosomal RNA gene. Percentage positivity declined with increase in the age with highest (53.84%) and lowest (5.56%) prevalence values recorded in 0-30 days and 4-5 months age group, respectively. A high degree of negative correlation (r=-0.92) was recorded between percentage positivity and age groups and difference between the age related prevalence values was statistically significant (p≤0.01). In terms of seasonal variation, diarrhoeic calves showed the prevalence of the cryptosporidiosis as maximum in summer (29.09%), followed by monsoon (28.07%) and winter (22.22%) and the variation was statistically significant (p≤0.05). And regarding sex wise distribution, female calves showed relatively higher prevalence (22.95%) than male calves (29.17%). In conclusion, *Cryptosporidium* spp infection in clinically asymptomatic calves indicated a carrier status of cattle and buffalo, which may act as reservoir of infection and transmit it to neonatal calves.**

Key words: *Cryptosporidium* spp., Nested PCR, Prevalence, asymptomatic.

Cryptosporidium, a zoonotic protozoan, is a well-recognized cause of diarrhoea in immunocompetent and immunocompromised humans and animals throughout the world (Fayer *et al.,* 2000). It is an apicomplexan protozoon with a life cycle involving both asexual and sexual reproduction, which it completes within an individual host (monoxenous). Transmission is via an environmentally robust oocyst stage and occurs through faeco-oral route (O' Donoghue, 1995). *C. parvum* is the major zoonotic species and causes neonatal diarrhoea in livestock, with consequent economic loss (Smith, 2008). The manifestation of infection in individual hosts can range from asymptomatic to profuse diarrhea; in immunocompromised individuals, the disease can be fatal (Navin and Juranek, 1984; Singh *et al.,* 2006). *Cryptosporidium* is increasingly gaining attention as a human and animal pathogen mainly due to its dominant involvement in worldwide waterborne outbreaks (Karanis*et al* 2007).

Microscopic methods are commonly used for its diagnosis and faecal smears are examined for *Cryptosporidium* oocysts after modified Zeihl-Neelsen Staining. The immunological approaches like direct immunofluorescence and detection of *Cryptosporidium* antigens by enzyme linked immunosorbent assay and immunochromatography for the detection of

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Cryptosporidium oocysts have proven useful (OIE, 2008). Polymerase chain reaction (PCR) has an advantage over these techniques in that it offers both lower limits of detection and the potential to differentiate species. Cryptosporidiosis has been reported from different states in India like Uttar Pradesh (Dubey *et al.,* 1992; Jeyabal and Ray, 2005), West Bengal (Prasad *et al.,* 1989; Chattopadhyay *et al.,* 2000; Das *et al.,* 2004), Andhra Pradesh (Shobhamani *et al.,* 2005), Pondicherry (Kumar *et al.,* 2004), Punjab (Singh *et al.,* 2006) but the carrier status of cryptosporidial infections in dairy animals from India in general and in Punjab state in particular is not fully investigated. It is difficult to ascertain in great detail the prevalence of cryptosporidial infections with conventional parasitological methods due to their inherent limitations because majority of the cases go undiagnosed due to small size of oocysts. Therefore, the present study was performed to investigate the carrier status of *Cryptosporidium* spp. infection in assymptomatic dairy cattle and buffalo calves in Ludhiana district of Punjab, India.

MATERIALS AND METHODS

Study area

Ludhiana is located in the central plain zone of Punjab state, with latitudes and longitudes of 30.55° N and 75.54° E and has an area of 3767 Km^2. Dairy farms situated in and around Ludhiana were selected for collection of faecal samples from clinically normal cattle and buffalo calves showing no symptoms of diarrhea and dehydration.

Sample collection

A total of 157 faecal samples were collected from normal buffalo and crossbred cattle calves, below 5 months of age, directly from the rectum without any clinical symptom from July 2009 to June 2010. The data related to risk factors like age, sex and breed was collected from each animal through a questionnaire. The samples, after collection, were quickly transported to the Postgraduate Laboratory, Department of Veterinary Parasitology and were stored at 4°C in 2.5 % potassium dichromate solution till further use. For DNA extraction some quantity of the collected faecal samples was stored at -20°C.

Examination of faecal smears

The faecal samples (1–2 g) were suspended in floatation medium (zinc sulphate solution, sp. gr. 1.18 and sugar solution, sp. gr. 1.18) for 20 min. After this, the meniscus was gently removed with a disposable pipette and dispensed gently on to a microscope slide to prepare faecal smears. Faecal smear was air dried, fixed in methanol for 3 minutes, air dried and stained by modified Ziehl-Neelsen (mZN) staining method as recommended by OIE. (2008). In brief, after fixation, smears were stained with 1 % cold Carbol-Fuchsin solution for 15 min and rinsed thoroughly in tap water. Then decolorization was done in 1 % acid methanol for 15 s and again the smears were rinsed with tap water and then, the smears were counterstained with 0.4 % Malachite Green for 30s. The smears were finally washed in tap water, air-dried and were examined microscopically.

Genomic DNA isolation

For conducting the PCR, whole-genomic DNA was isolated from faecal sample using QIAamp® DNA mini stool kit (QIAGEN, GmbH, Germany) following the manufacturer's recommendations with minor modifications. In brief, approximately 200 mg of the faecal sample was mixed with 1.4 ml ASL buffer in 2.0 ml microcentrifuge tube. The homogenous suspension was heated in water bath at 80°C for 5 minutes and then centrifuged for 1 min at 14000 rpm to pellet stool particles. Supernatant (1.2 ml) was pipetted out in new 2 ml centrifuge tube, one inhibit EX tablet was added and then vortex. After one minute incubation, the sample was centrifuged at 14000 rpm for 3 minutes to pellet out inhibitors bound to inhibitEX. Supernatant (200µl) was added to new 1.5 ml centrifuge tube containing 30 µl of proteinase K and after vortexing, 200 µl of AL buffer was added. This lysate was incubated at 70°C for 10 min, 200 µl of ethanol was added, and the mixture was applied to QIAamp Mini spin column and centrifuged at 8000 rpm for 1 min. Thereafter, 2 washings were given with wash buffers and DNA was eluted in 150 µl of elution buffer and stored at -20°C till use. Concentration of the extracted DNA from samples was measured in Nanodrop instrument. Genomic DNA of *Cryptosporidium* spp was isolated from faeces sample positive and revealing large number of oocysts in faecal smear examination and was utilized as positive control.

Genomic DNA was also isolated from the faeces of infection-free, neonatal bovine calf and used as a negative control.

18S rRNA gene amplification

The PCR (primary as well as nested) was optimized to identify the small subunit (18S) ribosomal RNA gene as described by Paul *et al*. (2009). The sequences of the primers were as follows:

For primary PCR

CRP-DIAG1 forward	5¹-TTCTAGAGCTAATACA TGCG-3¹
CRP-DIAG1 reverse5 ¹	-CATTTCCTTCGAAAC AGGA-3 ¹
For nested PCR	
CRP-DIAG2 forward 5	-GGAAGGGTTGTATTTA TTAGATAAAG-3 ¹
CRP-DIAG2 reverse 5	-AAGGAGTAAGGAACAA CCTCCA-3 ¹

Two rounds of PCR in a final volume of 25 µl were carried out in a PCR thermal cycler (Eppendorf, Germany). In the primary PCR assay, the master solution consisted of 2.5 µl of 10X

PCR buffer (Bangalore Genei), 0.5 µl of 10 mMdNTP mix (Bangalore Genei), 2.0 µl of 25 mM $MgCl_2$ (Bangalore Genei), 0.5 µl Taq DNA polymerase (Bangalore Genei), 0.5 µl each (20 pmol) of the external forward (CRP-DIAG1 forward) and external reverse (CRP-DIAG1 reverse) primers and 4.0 µl of template DNA isolated from faecal samples. The volume was made up to 25 µl with nuclease-free water. The cycling conditions were as: Initial denaturation at 94°C for 5 min, 34 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min, and the final extension was performed at 72°C for 10 min. For nested PCR similar quantities of the PCR mixture constituents except 1.5 µl $MgCl_2$ (25 mM) and 3 µl of template was used. Identical thermocyclic parameters were kept in nested PCR except annealing was done at 57°C. The PCR product was checked for amplification by electrophoresis on a 1.5% agarose gel and visualized using gel documentation system (Syngene, UK).

Statistical analysis

Statistical analysis was performed on data by SPSS 13.0 software by applying Chi-Square test and statistical differences ($p \leq 0.01$ and $p \leq 0.05$) between various groups were calculated.

RESULTS AND DISCUSSION

The purpose of this study was to determine the carrier status of *Cryptosporidium* infection in non-diarrhoeic dairy calves of Punjab. The study was conducted from June, 2009 to May, 2010 and was based upon the appearance of 834 bp, specific for *Cryptosporidium* spp., obtained after agar gel electrophoresis of nested PCR products (Fig 1). Overall 26.75% faecal samples were found to be positive for *Cryptosporidium* spp infection. Occurrence of the infection was found to be related with age with highest (53.84%) and lowest prevalence (5.56%) recorded in 0-30 days and 4-5 months age group, respectively. High degree of negative correlation (r=”0.92) between the percentage distribution of positive cases and age was seen with reference to age groups. There was gradual decline in the occurrence of infection with increase in the age in and this pattern was observed in both cattle and buffalo calves. Results of the present study corroborate the earlier findings of Singh *et al.* (2006) who reported the prevalence of the infection peaked in the young calves between 0 and 30 days in non-diarrhoeic (66.6%) among neonatal calves in Punjab, India. Paul *et al.* (2008) reported 22.64% positivity in non-diarrhoeic bovines and Roy *et al*. (2006) recorded *Cryptosporidium* infection in 8.13% in first year

Table 1. Overall age related prevalence

Age	No. of animals examined	No. of positive cases	% positive
0-30 days	26	14	53.84
1-2 months	29	10	34.48
2-3 months	41	10	24.39
3-4 months	43	7	16.27
4-5 months	18	1	5.56
Total	157	42	26.75
χ^2 ($p \geq 0.01$)			1.72
Season			
Pre Monsoon	55	16	29.09
Monsoon	57	16	28.07
Post Monsoon	45	10	22.22
χ^2($p \geq 0.05$)			0.507
Sex			
Male	61	14	22.95
Female	96	28	29.17
χ^2($p \geq 0.05$)			0.704

and 8.59% in second year out of 49.79% non-diarrhoeic bovine samples. The presence *Cryptosporidium* spp infection in clinically asymptomatic calves i.e. non-diarrhoeic as recorded in the present study and other workers in India (20-25%) indicated a carrier status of cattle and buffalo, which may act as reservoir of infection and transmit it to neonatal calves.

Asymptomatic shedding of *Cryptosporidium* oocysts has been reported in cattle in other parts of the world also by different authors (Scott *et al.,* 1995, Lorenzo-Lorenzo *et al.,* 1993; Castro-Hermida*et al.,* 2002, 2006; Fayer*et al.* 2000; Wade *et al.,* 2000; Ralston *et al*., 2003).

In terms of seasonal variation, prevalence of the *Cryptosporidium* spp infection as maximum in monsoon (28.07%), followed by summer (29.09%) and winter (22.22%) and the variation was statistically significant between winter and other seasons ($p \leq 0.05$). This may be due to the fact that high temperature and humidity along with frequent rains in the monsoon season enabled the faster transmission of the oocysts. The results are in consistent with Prasad *et al.* (1989), Roy *et al.* (2006) and Paul *et al.* (2008) who recorded highest infection rates in warm and humid months. Das *et al.,* (2004) reported higher percentage of infection in winter (45.16%), than summer (27.2%) and rainy season (19.2%). This anomaly has not been recognized, although it could be attribute to management practices like keeping calves indoor with minimal spacing in winter as compared to other seasons. Other factors like precipitation of the area, sunlight, desiccation, hygiene etc. cannot be ruled out.

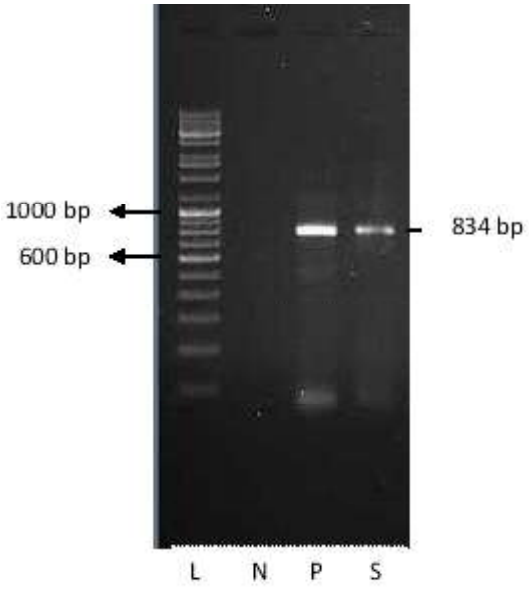

Fig. 1. Lane L is ladder (medium range, Banglore Genei), Lane N, P and S are negative control, positive control and sample, respectively

As regards sex wise distribution, female calves showed relatively higher prevalence (22.95%) than male calves (29.17%). Results of Mallinath *et al.* (2009) and Paul *et al.,* (2008) are in congruent with the findings of present study. Rehaman *et al.* (1985) and Shobhamani (2005) observed no sex preponderance in C*ryptosporidium* infection amongst the calves while higher positivity of infection was reported in male diarrhoeic calves than in female calves by Nouri and Toroghi. (1991). The practice of culling of male calves soon after birth by most of dairy farmers results in collection of comparatively lesser number of faecal samples from the male calves than the female counterparts. This may be the cause for skewed distribution of prevalence percentage towards male calves.

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