Prevalence of Cryptosporidiosis in Neonatal Buffalo Calves in Ludhiana District of Punjab, India

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ABSTRACT

In neonatal buffaloes calves Cryptosporidium spp. infection was investigated to determine its importance as etiological agent of diarrhoea syndrome. A total of 162 faecal samples were collected from neonatal buffalo calves of below 5 months age and examined for the Cryptosporidium spp. infection. Overall prevalence of the disease was observed as 38.3%. A gradual decline in the prevalence values was seen with increase in the age, highest in 0-30 days age group i.e., 65.71% and lowest in 4-5 months age group i.e., 5.88%. This trend of decline in prevalence values was observed in both diarrhoeic as well as non-diarrhoeic calves. A high degree of association was seen between Cryptosporidium infection and diarrhoea with infected cases at relatively higher risk to diarrhoea than non-infected. The highest prevalence (40.55%) of infection was recorded during the monsoon season followed by pre monsoon season (39.35%) and lowest prevalence (34.04%) was recorded in the post monsoon season. Female calves showed higher prevalence (40.35%) than the male calves (33.3%).

Key words: Cryptosporidium spp., diarrhoea, mZN staining, nested PCR, prevalence

INTRODUCTION

Cryptosporidium, an intracellular (but extra-cytoplasmic) apicomplexan protozoan (Tyzzer, 1907), infects the gastrointestinal tract of a wide range of vertebrates including humans, domestic and wild animals and also birds (Fayer et al., 2000). Cryptosporidiosis is commonly associated with enteritis and is usually characterized by acute, watery, or steatorrheic diarrhea and colic (Chen et al., 2002; Kosek et al., 2001), although asymptomatic infection can occur (Checkley et al., 1997; Skerrett and Holland, 2001). Bovine cryptosporidiosis is a common disease affecting newborn calves (Del Coco et al., 2009). With the attainment of immunological maturity infection subsides in the older cattle. Affected animals upon recovery become carriers and hence act as source of infection to the susceptible individuals. Cryptosporidium parvum is incriminated for causing intestinal cryptosporidiosis in newborn calves (Nolan et al., 2009). In India, human cases of cryptosporidiosis with diarrhoea first appeared in the mid-1980’s (Mathan et al., 1985; Das et al., 1987; Malla et al., 1987) while the first report of calf diarrhea associated with Cryptosporidium came around the same time (Nooruddin and Sarma, 1987). Cryptosporidium sparked great Public health interest after the large human water borne outbreaks in Milwaukee in 1993 and rapidly was recognized as on of the most serious water borne pathogens to date. The diagnosis of
Cryptosporidiosis relies on the identification of oocysts in faecal samples. As such there are only very few known reports on the prevalence and epidemiology of cryptosporidiosis in India. *Cryptosporidium* is increasingly gaining attention as a human and an animal pathogen mainly due to its dominant involvement in worldwide waterborne outbreaks (Karanis *et al.*, 2007).

Environmentally resistant oocysts are transmitted by faeco-oral route but zoonotic infection and individual-to-individual transmission is also known (O’Donoghue, 1995). Diagnosis is established microscopically, with the modified Ziehl-Neelsen (mZN) or Auromine phenol methods using unconcentrated or concentrated faecal smears. The immunological approaches like direct immunofluorescence and detection of *Cryptosporidium* antigens by enzyme linked immunosorbent assay and immunochromatography for the detection of *Cryptosporidium* oocysts have proven useful but inherit limitations (OIE, 2008).

The Polymerase Chain Reaction (PCR) has provided the basis for the development of a new generation of diagnostics. Absence of an effective drug or vaccine against cryptosporidiosis has made control of the disease cumbersome. This has restricted the path of control in dairy farms to the utilisation of effective managerial practices, hygiene and sanitation of dairy premises along with diagnostic tools. Though, apicomplexan protozoan parasite has been detected in water buffaloes in India (Dubey *et al.*, 1992; Singh *et al.*, 2006) as well as in other countries e.g., Italy (Canestri-Trotti *et al.*, 1984; Condoleo *et al.*, 2007), Spain (Gomez-Couso *et al.*, 2005), Egypt (Iskander *et al.*, 1987) and Brazil (Araujo *et al.*, 1996). However, data on the distribution of this protozoan in water buffaloes are quite fragmentary. The purpose of present study was to determine the distribution of *Cryptosporidium* in neonatal buffalo calves of commercial dairy farms and assess the impact of risk factors associated with it.

**MATERIALS AND METHODS**

**Animal source of faecal sample collection:** Different dairy farms located in the periurban surroundings of district Ludhiana, Punjab were selected. The area is located 254 m above mean sea level and lies between north latitude 30°-34' and 31°-01' and east longitude 75°-18' and 76°-20' with a total geographical area of 3767 km². A total of 162 faecal samples (4-5 g) were collected from neonatal buffalo calves of both the sexes below 5 months of age directly from the rectum using a disposable latex glove. Samples were collected without prior information of disease status of the farm. Samples were stored in 2.5% potassium dichromate solution and kept at refrigeration temperature. For DNA extraction and subsequent PCR analysis samples were kept at -20°C.

**Examination of faecal smears:** In direct faecal smear examination, a thin and transparent faecal smear was made by with the help of ear bud or applicator stick and air dried. The air dried smear was fixed in methanol for 3 minutes, air dried, stained by modified Ziehl-Neelsen staining method and examined at 40 and 100x objective by following the procedure recommended by OIE (2008).

In concentration methods, faecal smears were made after suspension of faeces in floatation medium (zinc sulphate solution, sp. gr. 1.18 and sugar solution, sp. gr. 1.18), dried and fixed in methanol followed by modified Ziehl-Neelsen staining.

**Prevalence:** The prevalence study was based on the observation of *Cryptosporidium* oocysts (gold standard) in the faecal samples and confirmed by application of nested PCR. The study was conducted from June, 2009 to May, 2010 in different seasons of the year (pre-monsoon, monsoon
and post-monsoon) to record the occurrence of the disease and influence of possible risk factors associated with it like age, sex and season in which faecal sample was collected and to determine association of the cryptosporidiosis and diarrhoea.

**DNA extraction:** Faecal samples detected positive for *Cryptosporidium* oocysts by modified Ziehl-Neelsen staining after floatation were taken for DNA extraction and subsequent PCR analysis for confirmation. 180-220 mg of frozen faecal sample was taken in 2 mL micro centrifuge tube and DNA was extracted by using QIAGENS mini stool QIAamp DNA extraction kit. Elution was done in 100 μL AE buffer, to increase the quantity of extracted DNA. Elute was preserved at -20°C until further use. Concentration of the extracted DNA from samples was measured in Nanodrop instrument. Purity of the extracted DNA was estimated by observing the ratio of A₂₆₀ and A₂₈₀. Samples with the ratio of A₂₆₀ and A₂₈₀ falling in the range of 1.8 to 1.9 were used for PCR.

**18S rRNA gene amplification:** A nested PCR method (reference standard) was followed to amplify small subunit (18S) ribosomal RNA gene. Primers specific for the 18S rRNA gene, previously described by Xiao et al. (1999) with minor modifications by Paul et al. (2008), were used. For primary PCR forward primer 5’-TTCTAGAGCTAAATACATGCCG-3’ and reverse primer 5’-CGTTTACCTCGGAAACAGGA-3’ was used and for secondary/nested PCR forward primer 5’-GGAGGAGTATTTATTTATTAAGAAG-3’ and reverse primer 5’-AAGGAAGAACAAACCTCAG-3’ was used. The 25 μL primary PCR mixture prepared was composed of 2.5 μL of 10X Taq polymerase buffer, 2.0 μL MgCl₂ (25 mM), 0.5 μL of each dNTP mixture (10 mM), forward primer, reverse primer and Taq polymerase, 4.0 μL Template (genomic DNA) and double distilled water was added to make final volume 25 μL. The thermocyclic parameters kept were: initial denaturation at 94°C for 5 minutes; 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and elongation at 72°C for 1 min; final elongation at 72°C for 10 min. For secondary/nested PCR similar quantities of the PCR mixture constituents except 1.5 μL MgCl₂ (25 mM) and 3 μL of template was used. Identical thermocyclic parameters were kept in secondary/nested PCR except annealing was done at 57°C. Nested PCR products were visualized after electrophoresis in 1% agarose gel containing ethidium bromide (0.5 μg mL⁻¹) and recorded by UV transilluminator.

**Statistical analysis:** Data was analysed by Chi-square test using SPSS 18.0 software.

**RESULTS**

**Observation of Cryptosporidium spp. oocysts:** *Cryptosporidium* spp. oocysts stained apple red on a pale green background (Fig. 1). The degree and proportion of staining varied with individual oocysts with internal structures taking up the stain to varying degrees. Smears stained after floatation show oocysts smaller in size than the normal due to shrinking in hypertonic solutions. Colour of the background was dependant on exposure time of carbol-fuchsin, malachite green and decolouriser.

**Prevalence:** Out of 162 faecal samples collected, 75 samples were diarrhoeic and 87 were non-diarrhoeic in consistency. Forty nine percent faecal samples were detected positive among diarrhoeic while 28.73% were found positive among non-diarrhoeic samples for *Cryptosporidium* spp. oocysts. Thirty five faecal samples were collected from buffalo calves in the age group of 0-30 days and this age group recorded the highest occurrence of infection i.e., 65.71% with 59.56% positive cases in diarrhoeic and 58.33% in non-diarrhoeic samples. The rate of infection peaked in
Fig. 1: Bright pink coloured oocysts of *Cryptosporidium* sp., mZN staining under 100x objective

### Table 1: Age related prevalence of cryptosporidiosis in neonatal dairy buffalo calves

<table>
<thead>
<tr>
<th>Age</th>
<th>Diarrhoeic</th>
<th></th>
<th>Non-diarrhoeic</th>
<th></th>
<th>Total</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of animals examined</td>
<td>No. of positive cases</td>
<td>% positive</td>
<td>No. of animals examined</td>
<td>No. of positive cases</td>
<td>% positive</td>
</tr>
<tr>
<td>0-30 days</td>
<td>23</td>
<td>16</td>
<td>69.56</td>
<td>12</td>
<td>7</td>
<td>58.33</td>
</tr>
<tr>
<td>1-2 months</td>
<td>19</td>
<td>11</td>
<td>57.89</td>
<td>18</td>
<td>7</td>
<td>38.89</td>
</tr>
<tr>
<td>2-3 months</td>
<td>16</td>
<td>7</td>
<td>43.75</td>
<td>22</td>
<td>6</td>
<td>27.27</td>
</tr>
<tr>
<td>3-4 months</td>
<td>9</td>
<td>2</td>
<td>22.22</td>
<td>26</td>
<td>5</td>
<td>19.23</td>
</tr>
<tr>
<td>4-5 months</td>
<td>8</td>
<td>1</td>
<td>12.50</td>
<td>9</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Total</td>
<td>75</td>
<td>37</td>
<td>49.30</td>
<td>87</td>
<td>25</td>
<td>28.73</td>
</tr>
</tbody>
</table>

\[\chi^2 (p<0.05) = 1.15\]  

\[\chi^2 (p<0.05) = 1.08\]

### Table 2: Seasonal prevalence of cryptosporidiosis in neonatal dairy buffalo calves

<table>
<thead>
<tr>
<th>Season</th>
<th>Diarrhoeic</th>
<th></th>
<th>Non-diarrhoeic</th>
<th></th>
<th>Total</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of animals examined</td>
<td>No. of positive cases</td>
<td>% positive</td>
<td>No. of animals examined</td>
<td>No. of positive cases</td>
<td>% positive</td>
</tr>
<tr>
<td>Pre monsoon</td>
<td>31</td>
<td>15</td>
<td>48.39</td>
<td>27</td>
<td>8</td>
<td>29.63</td>
</tr>
<tr>
<td>Monsoon</td>
<td>23</td>
<td>12</td>
<td>52.17</td>
<td>34</td>
<td>11</td>
<td>32.35</td>
</tr>
<tr>
<td>Post monsoon</td>
<td>21</td>
<td>10</td>
<td>47.61</td>
<td>26</td>
<td>6</td>
<td>23.07</td>
</tr>
</tbody>
</table>

\[\chi^2 (p<0.05) = 3.10\]  

\[\chi^2 (p<0.05) = 6.95\]

this age group and declined in the subsequent age groups. 1-2 month age group revealed 48.65%, 2-3 month age group showed 34.21%, 3-4 month age group showed 20.00% prevalence of the disease. The minimum occurrence of *Cryptosporidium* spp. infection was observed in 4-5 months age group and was recorded as 5.88%. This trend of decline in prevalence was recorded in both diarrhoeic as well as non-diarrhoeic calves (Table 1). Maximum infection was recorded during the monsoon season (40.35%) followed by summer season (39.65%). The lowest occurrence of 34.04% was recorded during the winter season (Table 2). The occurrence of the *Cryptosporidium* spp. infection was recorded to be higher among females (40.35%) than the males (33.3%) (Table 3).
Table 3: Sex related prevalence of cryptosporidiosis in neonatal dairy buffalo calves

<table>
<thead>
<tr>
<th>Sex</th>
<th>No. of animals examined</th>
<th>No. of positive cases</th>
<th>% positive</th>
<th>No. of animals examined</th>
<th>No. of positive cases</th>
<th>% positive</th>
<th>Total positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>15</td>
<td>7</td>
<td>46.67</td>
<td>33</td>
<td>9</td>
<td>27.27</td>
<td>33.30</td>
</tr>
<tr>
<td>Female</td>
<td>60</td>
<td>30</td>
<td>50.0</td>
<td>54</td>
<td>16</td>
<td>29.63</td>
<td>40.95</td>
</tr>
</tbody>
</table>

$\chi^2$ (p<0.05) 0.056 0.037 0.735

Fig. 2: Lane a, b and c showing 834 bp PCR amplified product, I represents 100 bp DNA ladder (fermentas)

**PCR amplification of the *Cryptosporidium* DNA:** The amplified products obtained from nested PCR assay were visualized after running through agar gel electrophoresis. The positive samples along with standard positive yielded 834 bp band on visualization (Fig. 2).

**DISCUSSION**

The study was purposefully done to assess the effect of age, season and sex on the occurrence of the *Cryptosporidium* spp. infection along with the effect of the infection on faecal consistency. From the results, it was observed that the infection was highest in the 0-1 month age group in comparison to other groups and rate of infection decreased with increase in age. High degree of negative correlation (r = -0.95) between the percentage distribution of positive cases and age was seen with reference to age groups of diarrhoeic and non diarrhoeic calves. Susceptibility of different age groups of cattle and buffalo dairy calves to the *Cryptosporidium* spp. infection has been reported from abroad (Quilez et al., 1996; Mtambo et al., 1997; Olson et al., 1997; Sturdee et al., 2003; Santin et al., 2004) and in India also (Saha et al., 2006; Singh et al., 2006; Paul et al., 2008). Higher incidence of the infection among cattle calves of 2-4 weeks of age was recorded by Olson et al. (1997). Quilez et al. (1996) reported higher prevalence of the *Cryptosporidium* infection.
in 6-15 days cattle calves. Results of the present study were in conformity with the findings of Paul et al. (2008) who reported the age related susceptibility of calves to Cryptosporidium infection and recorded the highest prevalence of infection (45.1%) in calves in the age group of 0-15 days. Results obtained in the present study are in accordance with the findings of Singh et al. (2006) who reported 79.41% prevalence of Cryptosporidium parvum infection among 0-30 days old dairy calves of cattle and buffalo in Punjab. Similar findings were reported by Saha et al. (2006) who reported the decline in the rate of infection with increase in the age of calves and incidence of infection peaked in 0-1 month age group. Results obtained by Kumar et al. (2004) and Shobhamani et al. (2006) in their respective studies in cattle calves also support the findings of present study.

Dairy calves shedded 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. These results of the present study (Table 2) are in congruent with Paul et al. (2008) who recorded the prevalence of cryptosporidiosis in the monsoon months as 37.3% which was higher than the dry pre-monsoon (25.6%) and cooler post-monsoon months (19.6%). The findings of present study also corroborates the results obtained in the earlier studies done in India by Prasad et al. (1989) and Saha et al. (2006) who observed the infection highest in warm and humid months in bovine calves.

As per the earlier studies, the relationship between occurrence of the Cryptosporidium infection and sex of the individual is vague and ambiguous. Results of Maliath et al. (2009) are in congruent with the findings of present study. Higher incidence of the infection was recorded in male calves than in female calves by Paul et al. (2008) but the difference was not significantly apart. Rehman et al. (1985) and Bollam (2005) observed Cryptosporidium infection among the dairy calves to be unrelated with the sex. In contrast Nouri and Toroghli (1991) reported higher rate of infection in male diarrhoeic calves than female calves. As most dairy complexes used to cull the male calves soon after birth therefore, the number of faecal samples collected from the male calves was comparatively lesser than the female calves. This may be the reason for the significant difference of Cryptosporidium spp. infection between male and female calves in the present study (Table 3).

The dairy buffalo calves which revealed infection with the Cryptosporidium spp. were found to be relatively at higher risk (1.906 times) to diarrhoea than non-infected calves. Also, the diarrhoeic calves provide a better chance for oocyst shedding and propagation as compared to the non-diarrhoeic calves. Results revealed that the prevalence of cryptosporidiosis was significantly higher (p≤0.05) in the diarrhoeic than the non-diarrhoeic individuals indicating the importance of Cryptosporidium in the causation of diarrhoea. There was gradual decline in the occurrence of the infection with increase in the age in both diarrhoeic as well as non-diarrhoeic dairy 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 both diarrhoeic (86.36%) and non-diarrhoeic (66.6%) among neonatal calves in Punjab, India. Paul et al. (2008) reported the incidence of infection greater among the diarrhoeic (32.33%) than the non-diarrhoeic (22.64%). Saha et al. (2006) recorded Cryptosporidium infection in 26.79 and 8.13% in first year and 27.49 and 8.59% in second year out of 50.21% diarrhoeic and 49.79% non-diarrhoeic samples. Small sample size in the study of Singh et al. (2006) may be the cause of higher prevalence in the diarrhoeic calves than the present study. Percentage occurrence of the cryptosporidiosis both in diarrhoeic and non-diarrhoeic dairy calves observed in the present study corroborates the findings of Gracia and Lima (1993), Kaminjolo et al. (1993), Saha et al. (2006) and Paul et al. (2008). The

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shedding of Cryptosporidium oocysts by clinically asymptomatic calves i.e., non-diarrhoetic 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. The present study is supported by Singh et al. (2006) who recorded 35.34% Cryptosporidium infection in buffalo in Punjab, India. In conclusion, Cryptosporidium is prevalent in neonatal buffalo calves, especially in young age groups and it is suggested that veterinarians should consider this protozoan for differential diagnosis while investigating the etiology of diarrhea in young calves.

REFERENCES


