Bangl. J. Vet. Med. (2014). 12 (2): 185-190 ISSN: 1729-7893 (Print), 2308-0922 (Online)

PREVALENCE OF CRYPTOSPORIDIOSIS IN CROSSBRED CALVES IN TWO SELECTED AREAS OF BANGLADESH

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ABSTRACT

A cross-sectional study was conducted to determine the prevalence of bovine cryptosporidiosis using 110 fecal samples of crossbred diarrhoeic calves from two different areas (Muktagacha, Mymensingh and Shajadpur, Sirajgonj) in Bangladesh during April 2012 to September 2014. The fecal samples were screened by rapid detection kit and confirmed by Modified Ziehl- Neelsen staining, and polymerase chain reaction (PCR). The positive samples along with standard positive control yielded 1325bp band on PCR. The overall prevalence of cryptosporidiosis in crossbred calves was 28.18% (31/110) by rapid detection kit. The higher prevalence of cryptosporidiosis was found in the calves from Shajadpur (29.76%) than the calves from Muktagacha (23.08%).The prevalence of cryptosporidiosis was significantly ($p<0.001$) higher in calves between 1-2 months (70%) age group than less than one month age group (24.49%). Cryptosporidiosis was not observed in calves over two months age. The prevalence of cryptosporidiosis was higher in males (34.75%) than females (24.64%) although not significant statistically. It is evident that the prevalence of cryptosporidiosis in bovine in these areas is under diagnosed and the clinical status of infection is potentially high.

Key words: Cryptosporidiosis, Prevalence, Calves, Oocysts,

INTRODUCTION:

Cryptosporidiosis is an emerging zoonotic disease of global importance caused by the apicomplexan protozoan parasite which is one of the most common causes of diarrhea in humans and livestock worldwide. Oocysts of cryptosporidium are usually transmitted by the feco-oral route, through direct host-to-host contact, and indirect contamination of food or water (Sayers *et al*., 1996). The zoonotic transmission has been confirmed by epidemiological studies involving pets, farm animals and by accidental infection of veterinary workers (Ahmed., 1984; Webster, 1993; Casemore *et al*., 1997; Saini *et al*., 2000; Nydam *et al.*, 2005; Collick *et al*., 2006). Ruminants are reported to be the major source of *Cryptosporidium* (*C.*) *parvum* transmission to humans (Xiao *et al*., 2004a, b; Caccio, 2005). *Cryptosporidium spp.* infection is well known as a major cause of morbidity and mortality particularly in immune compromised hosts and young animals (Graff *et al*., 1999). It causes self-limited watery diarrhoea in immunocompetent subjects but has far more devastating effects on immunocompromised patients and in some cases can be life threatening due to dehydration caused by chronic diarrhea (Alves *et al*., 2001; Mohandas *et al*., 2002; Caccio *et al.*, 2005; Chen *et al.*, 2005).In livestock the disease may lead to economic loss due to mortality, retarded growth of the animals, cost of drugs, veterinary assistance and increased staff labor (De Graaf *et al*., 1999). It has been reported as an important cause of calf mortality (Moon *et al*., 1982; Tzipori *et al.*, 1982; Hoffman and Sandoval, 1989). Cryptosporidium oocysts may remain viable in water for over 140 days and are very resistant to the most common disinfectants making them difficult to destroy by conventional chlorination treatment (Ahmed, 1984).

Cryptosporidiosis is considered as the third major cause of diarrheal disease worldwide (Janoff and Reller, 1987; Casemore *et al*., 1997; Fayer *et al*., 1997, 2000; Morgan *et al*., 1999; Spano and Crisanti, 2000). Cryptosporidiosis in cattle has been reported from different parts of the world with prevalence ranging from 24.5% to 45.5% (Kumar *et al*., 2005). The calf mortality in Bangladesh up to 12 months of age was reported from 9% under rural (Debnath *et al*., 1990) to 13.4% under a farm (Debnath *et al*., 1995) conditions. Reports on entero-pathogens associated with calf diarrhea are very limited from Bangladesh (Samad *et al*., 1977, 2001). There is no published report on the prevalence of cryptosporidiosis in crossbred calves in Bangladesh. This study describes the prevalence of cryptosporidiosis in crossbred calves under large and small holder dairy farms in some selected areas of Bangladesh

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MATERIALS AND METHODS

Study areas, period and population

Shahjadpur Upazilla of Sirajgonj District and Muktagacha Upazilla of Mymensingh District in Bangladesh, the most important dairy zone of Bangladesh were selected as study area. Five hundred (500) farms having at least two cross-bred dairy cattle were selected conveniently. Calves from day old to 1 year of age were included in this study. Active monitoring and surveillance system was used to collect sample from the selected farms over a period of 30 months from April 2012 to September 2014.

Faecal sample collection and examination

A total of 110 faecal samples from diarrhoeic calves of research area (Muktagacha and Shajadpur) were collected and examined for the presence of Cryptosporidium. Fecal samples were collected directly from the rectum of the animals or from the faecal mass immediately after defecation in stool pot with detail history of age group and sex and were immediately capped, labeled accordingly which included sample identification and site of collection. The collected samples were placed on ice in an insulated container in order to maintain low temperature of the samples. Feces were transported to the Laboratory of Department of Medicine, Bangladesh Agriculture University, Mymensingh and processed within 1–3 days of collection. The samples were examined in the field and in the Laboratory soon after collection or after preservation by freezing at -20^{0}C. In this study, stool samples collected from diarrheic calves were tested by rapid detection kit (Rainbow Calf scour 5, BioX Diagnostics, Belgium) to detect Cryptosporidium and other enteropathogens from diarrheal fecal samples as per manufacturer's instruction(Fig.1). A total of 4 representative samples from 31 positive for cryptosporidiosis by BioK-306 were mixed together and a thin smear was prepared and stained using a modified Ziehl-Neelsen method for further confirmation of *Cryptosporidium* spp (Fig-2). Samples were treated with carbol-fuchsin solution for 3 minutes recommended by Lennette *et al*., 1985. The discoloration procedure was realized with discoloration solution (95% ethyl alcohol-50ml and 95% Acetone-50ml) for 15-20 seconds used instead of the ethyl alcohol-sulfuric acid 5% recommended by Henriksen and Polhenz (1981). These modifications promoted a better washing out of the excess of carbol fuchsin therefore increasing the dye efficiency. In such conditions, the visualization of protozoan oocysts on the slides examined became easier. Smears were washed with running water and counterstained with solution of 0.4% malachite green or methylen blue at 1% for 1 minute. After the final wash with water, slides were dried at room temperature and then examined using X40 and X100 magnification under microscope. The positive samples were stored at -20˚C for DNA extraction.

DNA Extraction

DNA was extracted from concentrated mixture of 4 positive samples. *Cryptosporidium* oocysts were purified using a NaCl flotation procedure. Purified oocysts were washed three times in DDW/PBS in 50ml tube. After wash, the sediment was re-suspended with 45ml saturated salt solution. Five milliliter DDW was layered above the re-suspended samples. Samples were then centrifuged at 2300rpm for 30 minutes without break. *Cryptosporidium* oocysts were deposited in the upper layer and were collected and transferred to another tube by pipette. The oocysts were then subjected to 5-8 freeze-thaw cycles and DNA extraction was carried out using a Promega DNA extraction kit according to the manufacturer's instructions. The DNA extracted from the concentrated oocyst was used for polymerase chain reaction (PCR). The amplified products obtained from PCR assay were visualized after running through agarose gel electrophoresis.

Cryptosporidium detection by PCR

Cryptosporidium oocysts were confirmed by polymerase chain reaction (PCR). Primary PCR was performed by primers SSU-F2: (5 TTCTAGAGCTAATACATGCG 3) and SSU-R2: (5'-CCCATTTCCTTCGAAACAGGA 3) (Xiao *et al*.,1999; Limor *et al*., 2002; Park *et al*., 2006; Schindler *et al*., 2005). The primary PCR mixtures contained 5µl of template, 2X PCR Master mix (Promega, USA)-12.5µl, 1µl of each primer (10 pmol/µl) and DDW-5.5µl in a 25µl reaction volume. Thermocycling parameters were 3 minutes at 94°C hot start (initial heat activation step), followed by 35 cycles of 45 seconds at 94°C, 45 seconds at 55°C and 1 minute at 72°C, with a final extension of 7 minutes at 72°C (Xiao *et al*., 1999). The PCR product was loaded on 1.5% agarose gel, electrophoresis was done for 1 hour. The gel was stained with ethidium bromide and the products (1325bp) were visualized under a UV transilluminator.

Statistical analysis

The association of cryptosporidiosis with other variable like area, age and sex were assessed by Chi-square test. The Chi-square test and 95% confidence interval of prevalence were performed in R 3.1.0 (The R foundation for Statistical Computing).

RESULTS AND DISCUSSION

A total of 110 fecal samples from diarrhoeic calves were examined where 31 samples were positive for *Cryptosporidium spp*. by rapid detection kit (Biok-306). *Cryptosporidium spp* oocysts were observed in stained smear (Fig. 2). The positive samples along with positive control yielded 1325bp (Fig. 3) band on visualization which was supported by earlier report (Hassanain *et al*., 2011). The overall prevalence of cryptosporidiosis in calves below six months of age was 28.18% (Table 1).

Prevalence of Cryptosporidiosis was higher in Shajadpur (29.76%) in comparison to that in Muktagacha (23.08%). However, this difference was not significant statistically (Table 1). Based on the results of our study, it is evident that bovine cryptosporidiosis is endemic and locally widespread in Bangladesh. Some studies have shown that *Cryptosporidium* oocysts are able to survive for extended periods in faeces and environment, and very low dose of viable oocysts can cause an infection (Chako *et al*.,2010).The apparent variability of prevalence between geographical localities may reflect differences in the levels of calf management practices employed at farm level, housing-related factors (i.e., single housed calves, cleanness of the calf sleeping places), calf-related factors at a time of sampling (diarrhoeic versus nondiarrhoeic), nature of the study (cross-sectional versus prospective longitudinal studies), and fecal screening technique used (EL-Shazly *et al*., 2002; Kaushik *et al*.,2008).The prevalence of cryptosporidiosis was significantly ($p<0.001$) higher in calves between 1-2 months of age (70%) in comparison to those of one month of age (24.49%). This finding is also supported by other authors (Swai and Schoonman, 2010; Maldonado-Camargo *et al*., 1998; Gow and Waldner, 2006; Paul *et al*., 2008). Several authors reported higher prevalence among calves less than 6 months of age (Ongerth and Etibbs 1989; Shovamoni 2005; Jayabal and Ray 2005; Roy *et al.* 2006; Mehdizami 2007). The prevalence of cryptosporidiosis did not vary significantly according to sex of calves as also reported by others (Rehman *et al.,* 1985; Shovamani, 2005). However, Nouri and Toroghi (1991) recorded a higher infection in male diarrheic calves than in female calves.

Table 1. Prevalence of cryptosporidiosis in crossbred calves

Variable		Tested	Positive	Prevalence (%)	95% Confidence Interval
Area	Muktagacha	26	6	23.08	8.97-43.65
	Shajadpur	84	25	29.76	20.27-40.73
Age	Upto1 month	98	24	24.49	16.36-34.21
	More than 1 to 2 months	10	7	70.0	34.75-93.32**
	More than 2 months	3	0	0	0-70.76*`
Gender	Male	41	14	34.15	20.08-50.59
	Female	69	17	24.64	15.05-36.49
Overall		110	31	28.18	

*97.5% confidence interval ** Significant at $p<0.001$

Figure 1. Rainbow Calf Scour 5 (Bio K306

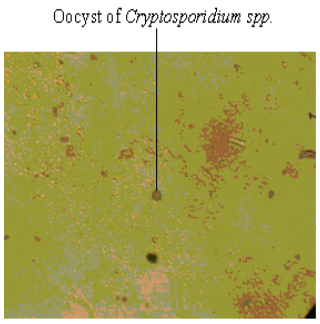

Figure 2. *Cryptosporidium spp* Oocyst

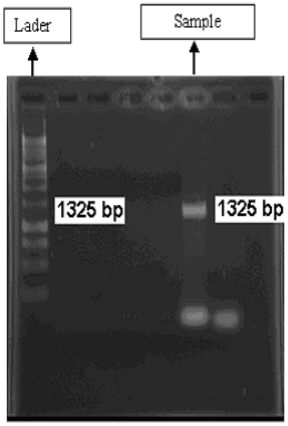

Figure 3. PCR result of *Cryptosporidium spp*

CONCLUSIONS

Results of this study indicate that the prevalence of cryptosporidiosis in crossbred calves in these areas is under diagnosed and the clinical status of infection is potentially high. The prevalence of the *Cryptosporidium* species/genotypes appeared to be age related. Because calves less than 3 months of age are the predominant population infected with *C. parvum* (zoonotic species), any effort designed to control this infection must be directed primarily at this age group.

A further prospective study, capturing seasonal variations to elucidate the magnitude of the disease (mortalities and reduced production), is desirable. Moreover, studies to understand the dynamics of transmission cycles and the genetic diversity of *Cryptosporidium* spp. on the farms should be undertaken.

ACKNOWLEDGEMENTS

The authors thank Krishi Gobeshona Foundation, BARC, Farmgate, Dhaka for funding of this research.

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