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Molecular Characterisation of *Cryptosporidium parvum* among Cattle and Cattle Handlers from Tripura (India) and the Associated Risk Factors

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ABSTRACT

Background: *Cryptosporidium parvum* is major protozoan parasite of both animals and humans with zoonotic significance. There is a paucity of information on its occurrence and associated risk factors in North Eastern states, India. The present study aimed at the molecular characterization of Cr*yptosporidium* from cattle and cattle handlers in West district of Tripura and risk associates analysis.

Methods: Faecal samples were randomly collected from cattle and cattle handlers (100 each), of West district, Tripura. The samples were subjected to sheather's sucrose flotation, modified Zeihl Neelsen (mZN) staining and PCR assay targeting the *18S SSUr-RNA* gene for detection of *Cryptosporidium*. The polemerised chain reaction- Restriction length polymorphism (PCR-RFLP) was done to detect *Cryptosporidium* spp. and *C. parvum* genotypes. Association of the epidemiological variables in relation to host and environment were studied for comparative analysis.

Result: The incidence of *Cryptosporidium* was 16 per cent and 7 per cent in cattle and cattle handlers, respectively. The molecular characterisation based on PCR-RFLP analysis revealed 14 *C. parvum* and two *C. andersoni* and three *C. parvum* in cattle handlers. All the 14 *C. parvum* from cattle and one *C. parvum* from cattle handlers belonged to bovine genotype (genotype II) and other two *C. parvum* from cattle handlers belonged to the human genotype (genotype I). Cryptosporidiosis in both cattle and cattle handlers were strongly associated with younger age group and diarrhoeic faecal consistency with significantly ($p<0.01$) higher occurrence. However, significantly ($p<0.05$) higher predominance of monsoon season in occurrence of the disease was observed in cattle and sex predominance was not observed in both cattle and cattle handlers.

Key words: Cattle, Cattle handlers, *Cryptosporidium parvum*, Genotype I, Genotype II, PCR-RFLP.

INTRODUCTION

Cryptosporidium is a common enteric protozoan parasite which is one of the leading causes of gastrointestinal illness in vertebrate animals and human worldwide. The *Cryptosporidium* oocysts are comparatively resistant and ubiquitous in the environment and are mostly transmitted by faeco-oral route. The ocysts are ingested along with contaminated food or water and infect the gastrointestinal tract of the susceptible host (Dankwa *et al*., 2021). *Cryptosporidium parvum* is a zoonotic pathogen and a leading cause of neonatal diarrhoea in young animals, mostly in episodes of outbreak. It often results in stunted growth in humans below five years of age (Bhat *et al*., 2013, Feng and Xiao, 2017; Zhang *et al*., 2020). The prevalence of zoonotic parasites is likely to be underestimated in India owing to the lack of proper surveillance and the shortage of information about the existence of asymptomatic animal carriers. Due to lack of effective vaccine, treatment or intervention strategies against *Cryptosporidium*, control of cryptosporidiosis mainly focuses on its prevention (Jex *et al*., 2011). Detection of the source of infection and mode of transmission is important in identifying a new endemic area for the control of cryptosporidiosis.

Although, cryptosporidiosis in bovines has been found to be highly prevalent in other parts of India (Paul *et al*., 2008) there is paucity of information from North Eastern region (NER). Studies on cryptosporidiosis in zoonotic perspective may be of great public health significance in NER considering the facts that human and animals live in close proximity, improper farming system that lacks in hygienic practice, sharing common water sources for animals and human and high numbers of HIV patients.

Cattle rearing in Tripura are mostly practiced by small and marginal farmers and landless agricultural laborers as

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a part of integrated farming system (Debbarma *et al.,* 2021). Hence, there could be frequent contact of the animals and nearby water bodies. Infective ocysts are passed in the faeces of these animals and the water may contribute significantly to the contamination of the environment for further transmission to other animals and humans. Despite the immense public health and veterinary importance of this parasite, there is limited data on the occurrence and associated risk factors of cryptosporidiosis in the state. Detection of the *C. parvum* among cattle and related human population and their genotyping may help to determine whether calves serve as a major reservoir for *C. parvum* infection in humans. The present study aimed to identify *C. parvum* by PCR based detection of *18S (SSU) r-RNA gene, its genotypes and demographic associates from dairy* cattle and cattle handlers in Tripura, India.

MATERIALS AND METHODS

The present study was undertaken in the Department of Veterinary Public Health and Epidemiology, College of Veterinary Sciences and AH, Aizawl, Mizoram during 2019-20. A total of 100 faecal samples each from both the cattle and cattle handlers were screened from West district of Tripura. The calendar year was distributed into three seasons, summer (March-May), monsoon (June-September) and winter (October-February). The demographic associates, namely age, sex and faecal consistency in cattle and cattle handlers that determines the occurrence of *Cryptosporidium* were studied. The distribution of *Cryptosporidium* in different age groups of cattle (0-6 months and >6 months) and human (0-5, 6-10 and >10 years) was recorded. The consistency of faeces was recorded as diarrhoeic and non diarrhoeic. The faecal samples were collected per rectum in clean, leak-proof containers and examined for the presence of *Cryptosporidium* oocyst by using mZN staining after concentrating the samples by sheather's sucrose floatation technique (Casemore *et al.,* 1985).

For molecular confirmation of the protozoan parasite, the faecal samples which were positive on light microscopy were subjected to PCR based analysis of *18S (SSU) r-RNA* gene by using published primers (CRP-DIAG F1:5′-AACCTGGTTGATCCTGCCAGTAGTC-3′ and CRP-DIAG R1: 5′TGATCCTTCTGCAGGTTCACCTACG-5). Genomic DNA was extracted from the faecal samples by using conventional technique (Sambrook *et al.,* 2001). The *18S (SSU) rRNA* gene confirmed amplicons were subjected to nested PCR and subsequently PCR based restriction fragment length polymorphism (RFLP) analysis to detect *Cryptosporidium* species and genotypes of *C. parvum*. In nested PCR, two sets of primers were used in two successive reactions which increased the specificity of DNA amplification. In primary PCR, the *18S (SSU) r-RNA* gene was amplified by using the first set of oligonucleotide primers (F: 5′-TTCTAGAGCTAATACATGCG-3′and R: 5′- CCCTAAT CCTTCGAAACAGGA-3′) and the primary PCR products were amplified in secondary PCR by using the second set of oligonucleotide primers (F: 5′-GGAAGGGTTGTATTTA TTAGATAAAG-3′ and R: 5′-AAGGAGTAAGGAACAA CCTCCA-3′) (Xiao *et al.,* 1999). The 25 µl PCR mixture was composed of 12.50 µl PCR master mix, 1µl forward and reverse primer (10 pmol/ µl), each, 1µl DNA template and 9.50 µl nuclease free water. The thermal cycling conditions are mentioned in Table 1. Gel electrophoresis of amplified DNA was done in 1.5 per cent agarose gel for 2 hours at 80V using Tris Acetate EDTA(1X TAE) running buffer (Sambrook *et al.,* 2001).

The RFLP analysis was done using 10 µl purified nested PCR products. The nested PCR products were separately subjected to restriction endo-nuclease enzyme digestion with *SspI* and *VspI*enzymes for detection of *Cryptosporidium* species and its genotypes, respectively. The 20 µl reaction mixture consisted of 10 µl DNA amplicon, 2 µl RE buffer (10X), 1 µl enzyme (10 IU/µl) and nuclease free water up to 20µl and digested at 37°C for 4 hours in humid condition. The digested product was fractionated in 3 per cent agarose gel and visualized by ethidium bromide staining.

Data were analyzed by SPSS 17.0. Qualitative data were compared by chi-square test or Fisher exact test, as applicable.

RESULTS AND DISCUSSION

Detection and molecular characterization of *Cryptosporidium*

For detection of *Cryptosporidium* in faecal samples of cattle and cattle handlers, sheather's sucrose flotation, mZN staining and PCR-RFLP tests were performed. In Sheather's sucrose floatation, the oocysts appeared as round or oval, refractile bodies with a thin cytoplasmic membrane. However, in mZN staining, the oocysts appeared as spherical to ellipsoidal shaped pink to red stained bodies containing four sporozoites against a pale green background. All the faecal samples which were positive on microscopy were also

Table 1: PCR conditions applied for detection of *Cryptosporidium.*

Stages	*SSU rRNA* gene	Nested PCR assay		No of cycles
		Primary PCR	Secondary PCR	
Initial denaturation	94°C for 5 minutes	94°C for 5 minutes	94°C for 3 minutes	One
Denaturation	94°C for 45 seconds	94°C for 1 minute	94°C for 45 seconds	35
Annealing	53.6°C for 45 seconds	49.5°C for 1 minute	55°C for 45 seconds	
Extension	72°C for 1 minute	72°C for 1 minute	72°C for 1 minute	
Final extension	72°C for 10 minutes	72°C for 10 minutes	72°C for 7 minutes	One

positive for *18S (SSU) r-RNA* gene with an amplicon size of 1745 bp (Fig 1). These gene amplicons were subjected to nested PCR and subsequently PCR-RFLP analysis to detect *Cryptosporidium* species and genotypes of *C. parvum* considering its zoonotic significance. Nested PCR assay revealed the 1325 bp band in primary PCR and 825 bp bands in the secondary PCR (Fig 2 and 3). The nested PCR product of 825 bp size holds the key information for species differentiation of *Cryptosporidium*.

The incidence of *Cryptosporidium* was recorded as 16 per cent and 7 per cent in cattle and cattle handlers, respectively from West district of Tripura. Brar *et al.* (2017) reported 25 per cent and 33 per cent incidence of cryptosporidiosis in bovine calves by mZN staining and commercial ELISA, respectively. Thakre *et al.* (2017) also reported higher incidence (41.59%) of *Cryptosporidium* in bovine faeces.

The PCR-RFLP analysis using restriction enzyme *SspI* revealed 14 bovine *Cryptosporidium* as *C. parvum* and two as *C. andersoni* while out of the seven human *Cryptosporidium*, three were *C. parvum*. The PCR-RFLP analysis using restriction enzyme *SspI* revealed distinct band patterns for *C andersoni* (448 bp and 370 bp) (Fig 4) and *C. parvum* (108 bp, 267 bp and 449 bp) (Fig 5). Similar band pattern in PCR-RFLP analysis for *C. parvum* and *C. andersoni* in faeces from cattle was earlier reported by Xiao *et al.* (1999) and Feng *et al.* (2007).

Cryptosporidium parvum was previously reported as the most prevalent *Cryptosporidium* species in India (Khan *et al*., 2010). However, simultaneous detection of *C. andersoni* and *C. parvum* in cattle indicates the possibility of cross contamination and easy transmission of this parasite (Zhao *et al*., 2014). Rekha *et al.* (2016) reported 5.71 per cent *Cryptosporidium* in bovine with occurrence of *C. parvum* in calves and *C. andersoni* in adult animals.

Further, RFLP analysis using restriction enzyme *VspI* revealed that all the 14 *C. parvum* of cattle origin and one *C. parvum* of human origin belonged to bovine genotype (genotype II) and other two human *C. parvum* belonged to the human genotype (genotype I). In RFLP analysis by using *VspI* enzyme, two distinct bands of 628 bp and 104 bp (Fig 6) represented bovine genotype (genotype II) and two distinct bands of size 556 bp and 104 bp (Fig 7) represented human genotype (genotype I) of *C. parvum.* Similar band patterns of *C. parvum* genotype I and genotype II had been reported by Xiao *et al.* (1999). However, Feng *et al.* (2007) reported the possibility of some missing bands in agarose gel electrophoresis subsequent to RFLP analysis of *C. parvum* due to small band size.

Demographic and environmental associates of Cryptosporidiosis

Demographic, geographic, seasonal and socioeconomic status has been contributing to infection sources and transmission routes in the distribution of *Cryptosporidium* spp. in animals and humans. Risk factor association analysis of cryptosporidiosis has revealed that *Cryptosporidium*

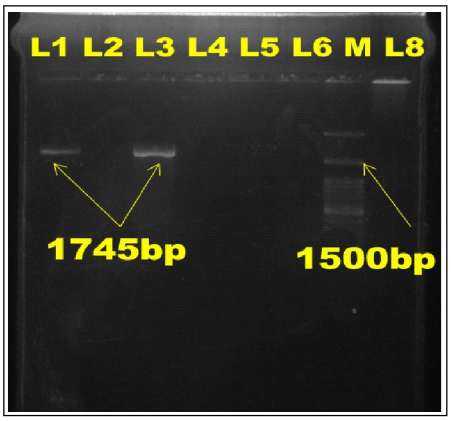

Fig 1: PCR amplification of*18S (SSU) rRNA g*ene of *Cryptosporidium;*
Lane M: 100 bp ladder; Lane 1: Positive control); Lane 2: Negative control; Lane 3: *Cryptosporidium* (1745 bp); (Lane 4, 5, 6 and 8: Blank.

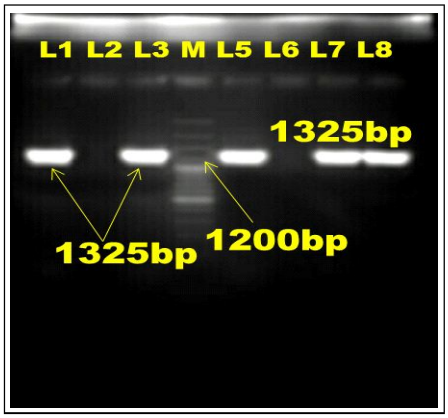

Fig 2: Primary PCR amplification of *18S (SSU) rRNA* sample in Nested PCR; Lane M: 100 bp ladder, Lane 1: Positive control; Lane 2: Negative control; Lane 3, 5, 7 and 8: *Cryptosporidium* (1325bp); Lane 6: Blank.

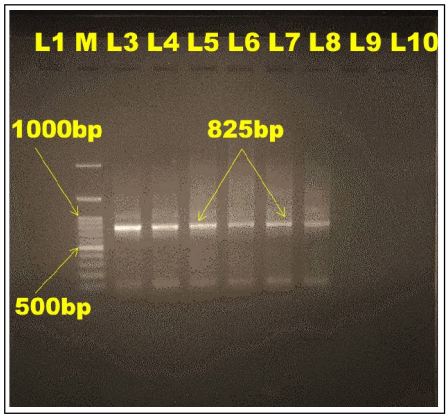

Fig 3: Secondary PCR amplification of *18S (SSU) rRNA* sample in Nested PCR
Lane M: 100 bp ladder; Lane 1: Negative control, Lane 3: Positive control; Lane 4, 5, 6, 7 and 8: *Cryptosporidium* (825 bp); Lane 9, 10: Blank.

infection is strongly associated with age and faecal consistency of infected cattle and cattle handlers.

A significantly ($p<0.01$) higher incidence of *Cryptosporidium* was recorded in young calves of below 6 months (32.35%) than above 6 months (7.57%) (Table 2). Age related variations in the distribution of *Cryptosporidium* had been observed in earlier studies and pre-weaned calves were the major sources of *C. parvum*. Decreased rate of infection along with increased age might be ascribed to strengthened immunological competence of the host with increased age and thereby suppressing the infection to a latent stage and thus the adult animals might act as asymptomatic carriers and act as a source of infection for young animals (Xiao and Feng, 2008; Das *et al.,* 2015). It has been indicated that bovine cryptosporidiosis is a disease of neonates and the higher susceptibility of calves to *Cryptosporidium* has been recorded from different states of India, namely Assam (28.41%) (Das *et al.,* 2015), Kashmir (29.37%) (Sheikh *et al*., 2007), Pondicherry (25.00%) (Kumar *et al.*, 2004), Uttar Pradesh (35.50%) (Jeyabal and Ray, 2005) and Punjab (33%) (Brar *et al*., 2017). The susceptibility of bovine calves to *C. parvum* significantly ($p<0.05$) decreased with increasing age, below one month (67.26%), 1-3 months (37.11%), 4-8 months (30%) and 9-12 months (17.65%) (Thakre *et al*., 2017).

In cattle handlers, 17.65 per cent and 5.19 per cent incidence of *Cryptosporidium* was recorded in 0-5 years and >10 years age group, respectively (Table 2). Age specific distribution of human cryptosporidiosis showed the highest prevalence in 0 to 24 months age group with the consistent peak occurrence in 0 to 12 months age groups (Pal *et al.*, 2010; Das *et al.,* 2011). The majority of the children in this study were the members of the cattle farmer's family who live in close contact with animals and belonged to low socio economic status with poor personal hygiene. Thus, direct animal to human and person to person transmission probably played an important role in the epidemiology of *Cryptosporidium* in children. In addition, most of the infected children and adults probably shared the common water sources with the animals like pond, stream and other natural water bodies.

Cryptosporidium infects the intestine of young calves, humans and other animals resulting in acute enteritis and diarrhoea and the intensity of shedding oocyst has been higher in calves with diarrhoea (Khan *et al.,* 2010; Das *et al.,* 2015). There was a positive association between *Cryptosporidium* infection and diarrhoeic stools in both cattle and human. The occurrence of *Cryptosporidium* infection was significantly ($p<0.01$) higher in diarrhoeic cattle (31.25%) than non-diarrhoeic (8.82%) cattle. However, only diarrhoeic cattle handlers (29.16%) were positive for *Cryptosporidium* (Table 2). Similarly, higher incidence of *Cryptosporidium* has been reported in diarrhoeic cattle (24.20%, 50.00%, 32.90%, 81.00%, 24.20% and 59.54%) than non-diarrhoeic cattle (16. 60%, 25. 68%, 7.40%, 18.99%, 16.60% and 29.41%) from Karnataka, Punjab,

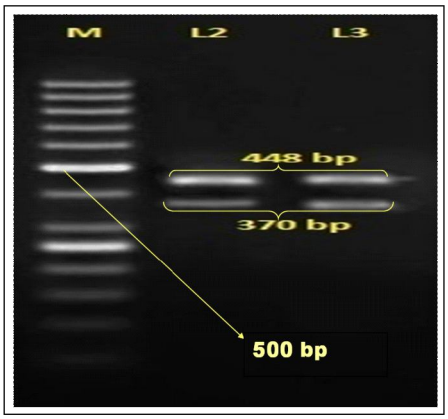

Fig 4: The PCR-RFLP analysis using restriction enzyme *SspI* representing *C. andersoni*; Lane M: 50 bp ladder; Lane 2: Positive control; Lane 3: *C. andersoni* (448 bp, 370 bp).

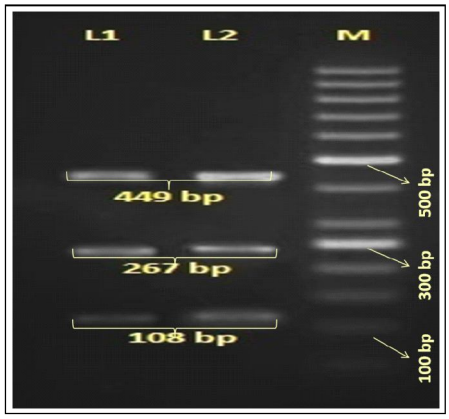

Fig 5: The PCR-RFLP analysis using restriction enzyme *SspI* representing *C. parvum*; Lane 1: positive control; Lane 2: *C. parvum* (449 bp, 267 bp, 108 bp); Lane M: 50 bp ladder.

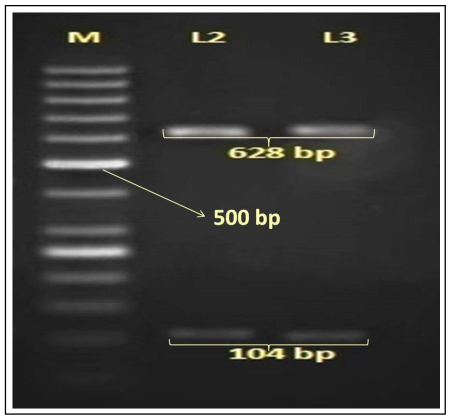

Fig 6: The PCR-RFLP analysis using restriction enzyme *VspI* representing bovine genotype (genotype II); Lane M: 50 bp ladder; Lane 2: Positive control; Lane 3: *C. parvum* genotype II (628 bp, 104 bp).

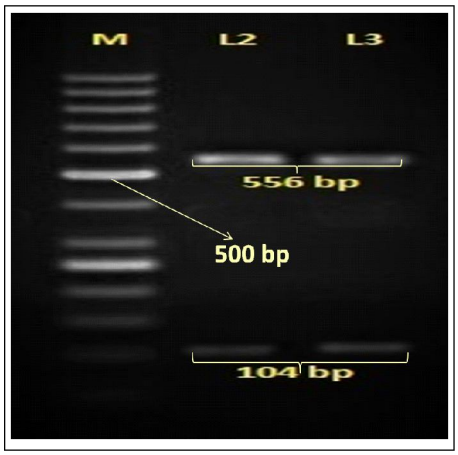

Fig 7: PCR-RFLP analysis using restriction enzyme *VspI* representing human genotype (genotype I); Lane M: 50 bp ladder; Lane 2: Positive control; Lane 3: *C. parvum* genotype I (556 bp, 104 bp).

West Bengal, Assam, Bangaluru and Gujarat irrespective of organized and un-organized farming system (Mallinath *et al.*, 2009; Singh *et al.*, 2006; Das *et al.*, 2011; Das *et al.*, 2015; Rekha *et al.*, 2016; Thakre *et al*., 2017), respectively. Similarly, 13.80 per cent diarrhoeic and 3.70 per cent non-diarrhoeic human stool samples were found to be positive for *Cryptosporidium* (Das *et al.,* 2011).

There was no significant (p>0.05) sex predominance in the occurrence of *Cryptosporidium* in cattle and figured as 12.00 per cent and 17.33 per cent in male and female, respectively (Table 2). However, the lower incidence of cryptosporidiosis in male animals might be attributed to the smaller number of male calves screened since most of them were culled after birth and the female calves were in more contact with the cows. Thakre *et al.* (2017) and Bhat *et al.* (2019) also reported that both sexes of ruminants are equally susceptible to cryptosporidiosis. Similarly, there was no sex wise variation in the incidence of *Cryptosporidium* in cattle handlers, 5.45 per cent in male and 8.89 per cent

Table 2: Occurrence of *Cryptosporidium* in cattle and cattle handlers and its associated risk factors.

Parameter	Cattle		Cattle handlers	
	Sample tested	Sample positive	Sample tested	Sample positive
Cryptosporidium	100	16 (16.00%)	100	7 (7.00%)
Species				
C. andersoni	100	4 (4.00%)	100	-
C. parvum		12 (12.00%)		3 (3.00%)
Other species		-		4 (4.00%)
Total	100	16 (16.00%)	100	7 (7.00%)
***C. parvum* genotypes**				
Genotype I	-	-	3	2 (66.66%)
Genotype II	12	12 (100.00%)	3	1 (33.33%)
Age				
0-6 months	34	11 (32.35%)	-	-
≥6months	69	5 (7.57%)	-	-
Total	100	16 (16.00%)**		
0-5 years	-	-	17	3 (17.65%)
5-10 years	-	-	6	0 (0.00%)
>10 years	-	-	77	4 (5.19%)
Total	-	-	100	7 (7.00%) NS
Faecal consistency				
Diarrhoeic	32	10 (31.25%)	24	7 (29.16%)
Non diarrhoeic	68	6 (8.82%)	76	0 (0.00%)
Total	100	16 (16.00%)**	100	7 (100%)
Sex				
Male	25	3 (12.00%)	55	3 (5.45%)
Female	75	13 (17.33%)	45	4 (8.89%)
Total	100	16 (16.00%) NS	100	7 (7.00%) NS
Season				
Summer	28	4 (14.29%) ab	36	2 (5.56%)
Monsoon	41	11 (26.63%) b	32	5 (15.63%)
Winter	31	1 (3.23%) a	32	0 (0.00%)
Total	100	16 (16.00%)*	100	7 (7.00%)

Non significant: NS; Significant at p<0.05*; Significant at p<0.01**.

in female. The involvement of both man and woman in the small traditional farming activities in NER probably attributes to the less gender wise difference in the occurrence of *Cryptosporidium* in human.

The seasonal variation of *Cryptosporidium* infection in cattle and cattle handlers revealed highest incidence in monsoon (26.83% and 15.63%) followed by summer (14.29% and 5.56%), respectively. However, the incidence was significantly ($p<0.05$) lower in cattle (3.23%) and not detectable in cattle handlers during winter season (Table 2). Similar findings were also recorded by Das *et al.* (2015) with highest occurrence in monsoon (27.88%) followed by pre-monsoon (20.14%) and post-monsoon (8.38%). The highest prevalence of cryptosporidiosis in cattle (45.15%) and human (6.30%) during rainy season was also recorded by Das *et al*. (2011) and Thakre *et al*. (2017), respectively. In India, environmental ecology has major effect on transmission of cryptosporidiosis and high incidence of cryptosporidiosis during monsoon has been reported in earlier studies in both cattle and human (Bhat *et al.* 2014; Kali, 2014). High temperature and humidity along with frequent rains in monsoon season enabled the faster transmission of the oocysts. Further, due to the longer viability of oocysts in water and its resistance towards chlorine, sporadic cases as well as waterborne outbreaks of *Cryptosporidium* are common in monsoon season.

CONCLUSION

The prevalence of *Cryptosporidium* infection among cattle and cattle handlers of Tripura indicated a potential public health risk. Detection of *C. parvum* bovine genotype (genotype II) in human living in close contact with animals indicated bovine as a probable source of human infection. The risk factors associated with *C. parvum* infection has shown significant incidence in young animals and human along with diarrhoeic episodes.

Conflict of interest: None.

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