

Incidence of *Cryptosporidium andersoni* in diarrheal patients from southern Assam, India: a molecular approach

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Abstract The distribution and public health significance of *Cryptosporidium* species and genotypes in humans and bovine differ across geographical areas. *Cryptosporidium* species causes a disease known as cryptosporidiosis in humans and animals. To characterize the prevalence of cryptosporidiosis in humans in southern Assam, India, stool samples ($n = 1119$) of diarrhea patients were collected from different hospitals and from the community during the period January 2014 to July 2016. Fecal smears were examined microscopically for *Cryptosporidium* species using modified acid fast staining and were screened to ascertain the presence of *Cryptosporidium* antigen by enzyme-linked immunosorbent assay (ELISA). The genomic DNA of positive fecal samples were analyzed by nested polymerase chain reaction (PCR), which were subsequently genotyped by PCR-restriction fragment length polymorphism (RFLP), based on small subunit (SSU) 18S rRNA. It was found that the prevalence of *Cryptosporidium* spp. was high during the monsoon season. The average infection rate of *Cryptosporidium* spp. was found to be 2.4% (27/1119) microscopically. When subjected to nested PCR using amplification of the 18S rRNA gene, *Cryptosporidium* was found to be 8.57% (98/1119). Based on the 18S rRNA gene, two *Cryptosporidium* spp., namely *Cryptosporidium andersoni* (6.97%: 78/1119) and *Cryptosporidium parvum* (1.7%: 20/1119), were identified.

Cryptosporidium andersoni infections were found to be of either zoonotic or anthroponotic origin. The prevalence was statistically significant ($p = 0.03$, $R^2 = 0.042$) considering age, gender, and cast.

Introduction

Cryptosporidium species is the unicellular, microscopic, spore-forming, obligate intracellular organism of the apicomplexa family and is living like a parasite in the microvillus border of the gastrointestinal tract of a wide range of vertebrates, including man [1]. It is a common zoonotic enteric pathogen responsible for diarrheal diseases in humans and a variety of animals around the world [2]. *Cryptosporidium* has emerged as a significant human pathogen, particularly in children, elderly people, and immune-compromised patients, where an untreated infection might be life-threatening [3]. The parasite spreads through the fecal-oral route, through contaminated water or food, and often through contact with animals [3, 4]. However, previous studies suggested that cryptosporidiosis is a zoonotic as well as anthroponotic infection caused by different species, including *C. meleagridis*, *C. felis*, *C. canis*, *C. cuniculus*, *C. ubiquitum*, *C. viatorum*, *C. suis*, *C. muris*, and *C. andersoni* [5–9].

The detection of oocysts of *Cryptosporidium* in stool using the Ziehl–Neelsen modified acid fast stain is generally known as Kinyoun, and it is the most commonly used conventional technique in detecting the *Cryptosporidium* species [10, 11]. So far, 30 *Cryptosporidium* species and over 70 subtypes of *Cryptosporidium* have been recognized in a variety of vertebrate hosts [7, 12]. There is a need to develop fast and high-throughput molecular techniques for the detection and identification of *Cryptosporidium* species and their subtypes infecting humans. Molecular techniques like restriction fragment length polymorphism (RFLP) analysis based upon the gene

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of the small subunit rRNA (SSU rRNA) has been applied for the detection of *Cryptosporidium* species [13–15].

In India, inconceivable discrepancy persists in the characteristic ethnicity, culture, food habit, educational background, and standard of living within the nation [16]. The 2001 survey explains that 72.2% of the inhabitants lived in villages, and over 70% of the countryside inhabitants own domestic animals. Thus, domestic animals play an essential role in the socio-economic life of India. Since *Cryptosporidium* is recognized as one of the organisms which transmit by both anthroponotically and zoonotically, therefore, parasitic zoonoses affect human and animal health directly, and, consequently, affect livestock production [17]. The earliest traces of bovine cryptosporidiosis in India dates back to the late 1980s [18] and, subsequently, the incidence of *C. andersoni* has been reported from cattle [19].

The aim of the present study is to examine *Cryptosporidium* infection in patients with diarrhea, using conventional techniques such as microscopy, serological techniques like enzyme-linked immunosorbent assay (ELISA), and molecular tools such as polymerase chain reaction (PCR) and PCR-RFLP. In our study, samples were collected from various hospitals as well as from local communities of southern Assam, India. We then further examined if any feasible relationship exists between epidemiological data and

clinical manifestations of the predominant *Cryptosporidium* species collected from the different study areas.

Materials and methods

Study design

A cross-sectional study was conducted to determine the prevalence of *Cryptosporidium* species among the patients from January 2014 to July 2016. The study was carried out in southern Assam, India (Cachar, Karimganj, and Hailakandi districts of the Barak Valley) at the level of community and district hospitals (Fig. 1). We used a systematic approach for the determination of the prevalence rate of *Cryptosporidium* species in southern Assam, India (Fig. 2).

Consent and ethical consideration

All stool samples were collected with the consent of each patient/subject, in the form of questionnaires. All study participants had given written consent before enrollment into the study. Parents/guardians provided consent on behalf of all

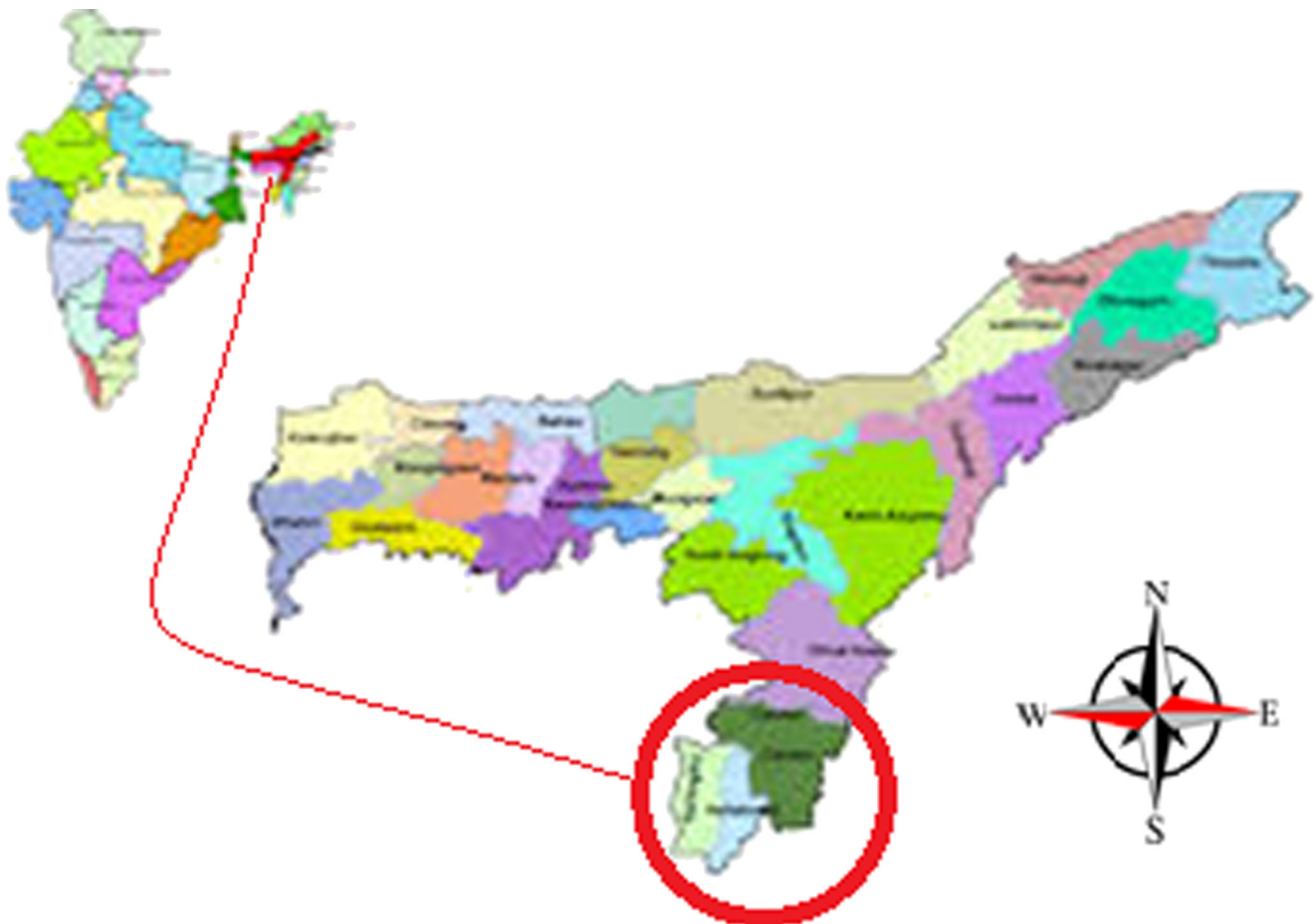
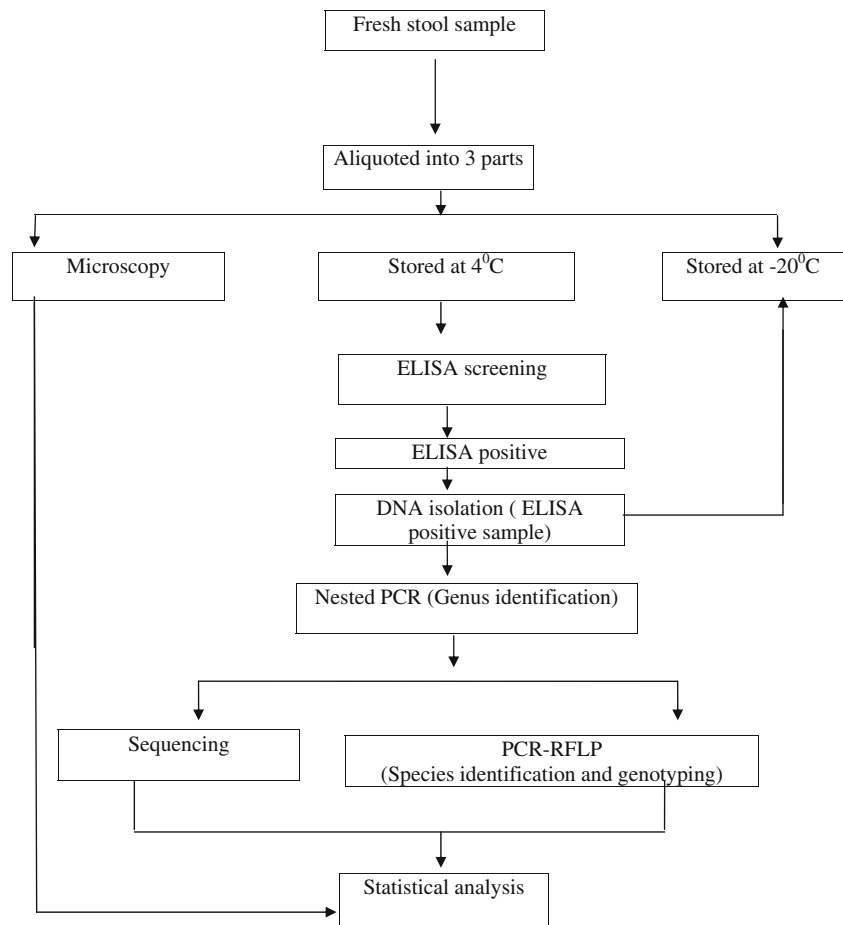


Fig. 1 Study site of the districts of southern Assam, India

Fig. 2 Systematic approach which attempted to derive the prevalence rate of the parasite *Cryptosporidium* spp. in southern Assam, India



infant participants. The study protocol was reviewed and approved by the Institutional Ethical Committee (ref. no. GCC/9440) of Gurucharan College, Silchar, Assam, before the commencement of the study and Silchar Medical College and Hospital, Silchar, Assam.

Collection of stool samples

A total of 1119 stool samples were obtained randomly from various hospitals and the locality of Cachar, Karimganj, and Hailakandi of southern Assam, India. The stool samples were collected in fresh, broad-mouthed, twist-cap disposable plastic containers. Within 3 h of collection, each stool sample was divided into three parts, each having equal quantity; one part was directly used for microscopic scrutiny; the second part was stored at 4°C in formal ether, which was later used for the serological analysis (ELISA); and the third part was stored at -20°C for PCR analysis.

Microscopy

This analysis was carried out according to standard techniques described earlier [20]. Slides were observed at 40× and then confirmed in oil immersion at 100× magnification under a phase contrast microscope (CX31, Japan).

ELISA

The *Cryptosporidium* antigen in the sample was distinguished by microwell ELISA. The ELISA was carried out with an existing kit according to the manufacturer's instructions (*Cryptosporidium* 2nd Generation, Fecal, Diagnostic Automation, Inc., cat #8301–3).

Extraction of DNA

The DNA was isolated from the frozen stool samples using a Nucleopore™ stool DNA kit (Genetix Biotech Asia Pvt. Ltd., New Delhi, India), according to the procedure given by the provider, with minor modifications. In addition to seven freeze-thawing cycles, the samples were vortexed for 30 min in lysis buffer FL containing thrashing beads (Genetix Biotech Asia Pvt. Ltd., New Delhi, India). The samples were then processed according to the manufacturer's instructions. The eluted DNA was quantified spectrophotometrically (BioPhotometer plus) and stored at -20°C for further use.

Nested PCR

The PCR amplification of the 18S rRNA gene was accomplished from individual samples using the oligonucleotide primers for

species- and strain-specific identification of *Cryptosporidium* species. The sequences unique to all *Cryptosporidium* species were detected from the multiple alignments (CLUSTALW) and used as primers in a nested PCR procedure. For the major PCR step, a PCR product that was about 1325 bp long was amplified by using primers 5'-TTCTAGAGCTAATACATGCG-3' and 5'-CCCTAATCCTTCGAAACAGGA-3' [21] (Table 1). PCR amplifications were carried out in a volume of 20 μ L with 100 ng of sample DNA, 1 μ M of each primer, 1 \times PCR buffer, 2 mM MgCl₂, 1 \times BSA, 0.2 Mm dNTPs, and 1U of Taq DNA Polymerase (Thermo Scientific, USA) in the MJ Mini™ (Bio-Rad Laboratories, Hercules, CA, USA) thermal cycler. A total of 35 cycles, each consisting of 94°C for 45 s, 55°C for 45 s, and 72°C for 90 s, were performed; an initial hot start at 94°C for 3 min and a final extension step at 72°C for 7 min were also incorporated. For the secondary PCR step, a PCR product that was 819 to 825 bp long (depending upon the species) was amplified by using 2 μ L of the primary PCR product and primers F-5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and R-5'-AAGGAGTAAGGAACAACCTCCA-3' [21]. The PCR mixture and the standard PCR conditions were kept the same as the conditions used for the primary PCR step, except that 3 mM MgCl₂ was used for the PCR mixture and the annealing temperature was 57.8°C. The amplification of specific PCR product was checked by gel electrophoresis using 1% agarose.

For RFLP analysis, the secondary PCR product was digested in a 25- μ L reaction mixture containing 5 U of SspI (Thermo Scientific, USA) (for species diagnosis) or 5 U of VspI (Thermo Scientific, USA) (for genotyping of *C. parvum*) (Table 2), and 2.5 μ L of 10 \times restriction buffer at 37°C for 2.5 h, under conditions recommended by the supplier. The digested products were fractionated on a 2.0% agarose gel and visualized by ethidium bromide in a transilluminator [21, 22].

DNA sequence analysis

Positive secondary PCR products were subjected to sequencing from both directions using the secondary primers by the ABI 3500 genetic analyzer (Applied Biosystems Inc., Foster City, CA, USA) from the Genome Service of the Department of Biotechnology, Assam University, Silchar. Sequences obtained were analyzed and assembled using Sequencher software (Gene Codes Corp.). The obtained nucleotide sequences were used to search the GenBank nucleotide sequence database for sequence similarities using BLAST software (NCBI, Bethesda, MD, USA). Multiple alignments of these sequences were made by using the BioEdit program. The accuracy of the sequencing data was confirmed manually by looking at the overlapping regions. The sequences were deposited in GenBank (accession numbers KJ188438 and KJ719487).

Statistical analysis

All of the statistical analyses were performed using SPSS software v.21 (IBM Software Company, Armonk, NY, USA). The two-factor factorial analysis of variance technique was used to adjudge the effects of gender, caste, and age, along with their possible interactions. The parameters were further compared with Duncan's test at the 1% level of significance.

Results

We tested a total of 1119 fecal samples: 694 were from males and 425 were from females. The mean age of males and females was 21.34 \pm 20.11 and 24.77 \pm 21.36 years, respectively.

Table 1 Prevalence of *Cryptosporidium andersoni* and *Cryptosporidium parvum* on the basis of age and gender

Parameter	No. of screened samples (n)	No. of positive samples (%)	<i>Cryptosporidium andersoni</i> (%)	<i>Cryptosporidium parvum</i> (%)
Cases	*1119	98 (8.75)	78 (6.97)	20 (1.7)
**Age category				
<5	215	34 (15.81)	32 (14.88)	02 (0.93)
5–12	301	21 (6.9)	15 (4.98)	06 (1.99)
13–19	205	11 (5.3)	00 (0.0)	11 (5.3)
20–29	70	03 (4.2)	02 (2.8)	01 (1.4)
30–39	60	02 (3.3)	02 (3.3)	00 (0.0)
40–49	48	04 (8.3)	04 (8.3)	00 (0.0)
>50	220	23 (10.45)	23 (10.45)	00 (0.0)
Gender				
Male	694	69 (9.94)	57 (8.21)	12 (1.7)
Female	425	29 (6.82)	21 (4.9)	08 (1.8)

*All specimens were collected from January 2014 to December 2015 and *Cryptosporidium* species were detected by the 18S rRNA gene analysis

**Age category shows a significant difference compared with *C. parvum* [F(1, 1115) = 4.572, p = 0.03] and there was no significant difference compared with *C. andersoni* [F(1, 1115) = 0.044, p = 0.8]

Table 2 Regional prevalence of *Cryptosporidium* spp. infection in humans, southern Assam, India

*Region	No. of screened samples (n)	No. of positive samples (%)	<i>Cryptosporidium andersoni</i> (%)	<i>Cryptosporidium parvum</i> (%)
Cachar	472	51 (10.80)	50 (10.59)	1 (0.21)
Karimganj	390	32 (8.20)	7 (1.7)	15 (3.8)
Hailakandi	257	25 (9.7)	21 (8.1)	4 (1.5)

*Regional variation shows a significant difference compared with *C. andersoni* [F(1, 1116) = 4.128, $p = 0.04$] and there was no significant difference compared with *C. parvum* [F(1, 1115) = 3.601, $p = 0.05$]

Smears were prepared from fecal material and stained with a modified acid fast technique. The oocysts appeared as pink and round and the size varied from 3 to 5 μm in a blue background of methylene blue stain. The ELISA method (Luminoskan Ascent™ Microplate Luminometer) detected 8.7% (98/1119) of positive samples. The absorbance range of 0.15–2.44 OD indicated that the presence of *Cryptosporidium* antigen was extremely stable. The nested PCR showed specific amplification of 825 bp of the 18S rRNA gene in 98 samples.

Based on the present study, the overall prevalence of *C. andersoni* was found to be 6.97%, followed by *C. parvum* (1.7%). A two-way analysis of variance (ANOVA) implicated that the correlation between the dependent variables like gender, age, and the prevalence of the parasite had no significant effect ($p = 0.33$, $R^2 = 0.015$) (Table 1). An inverse relationship between the age of the host and infection with *Cryptosporidium* species was recorded, with maximum prevalence (13.4%) in infants aged <5 years, followed by the age groups 40–49 years (8.3%), 5–12 years (7.6%), >50 years (6.03%), 13–19 years (5.3%), and 20–29 years (4.2%), with the lowest prevalence (3.3%) in adults aged 30–39 years (Table 1). A two-way ANOVA was also conducted to explore the impact of age on the prevalence of *C. parvum* and *C. andersoni*. The results indicated that there was a significant mean effect for age on the prevalence of *C. parvum* [F(1, 1115) = 4.572, $p = 0.03$] and there was no statistically significant mean effect for age on the prevalence of *C. andersoni* [F(1, 1115) = 0.044, $p = 0.8$] (Table 1).

The prevalence rate of *Cryptosporidium* species was compared between the different districts of Assam; the rate was highest in the district of Cachar (10.80%), followed by Hailakandi (9.7%) and Karimganj (8.20%) districts. The two-way ANOVA indicated that there was a significant mean effect for different districts on the prevalence of *C. andersoni*

[F(1, 1116) = 4.128, $p = 0.04$] and there was no significant mean effect for different districts on the prevalence of *C. parvum* [F(1, 1115) = 3.601, $p = 0.05$] (Table 2). The prevalence of the disease was highest during the monsoon season (14.48%), followed by pre-monsoon (7.36%) and post-monsoon months (4.1%). The two-way ANOVA indicated that there was no statistically significant difference for season in the prevalence of *C. parvum* [F(1, 1115) = 0.333, $p = 0.56$] and *C. andersoni* [F(1, 1115) = 1.403, $p = 0.14$] (Table 3).

The samples collected from the community included members of three different communities, i.e., Hindus, Muslims and Christians, with a mean participant age of 24.77 ± 21.36 . The prevalence of the *Cryptosporidium* species was maximum in the Muslim population (12.79%), followed by the Christians (12.24%) and the Hindus (7.2%). The prevalence of *C. andersoni* found in the Hindu population (7.14%) was highest, followed by the Christian (10.20%) and Muslim (5.8%) individuals. Similarly, the prevalence of *C. parvum* was highest in the Muslim population (6.9%) followed by the Christian (2.04%) and the Hindu (0.12%) populations. The two-way ANOVA results indicated that there was a significant mean effect for different communities in the prevalence of *C. parvum* [F(1, 1115) = 5.903, $p = 0.01$] and there was no statistically significant mean effect for different communities in the prevalence of *C. andersoni* [F(1, 1115) = 0.000, $p = 0.9$] (Table 4).

In this study, the patients were divided into five groups in different districts (Group 1: 0–12 years; Group 2: 12–24 years; Group 3: 24–36 years; Group 4: 36–48 years; Group 5: 48–60 years). The prevalence of *C. andersoni* in Cachar district was highest in Group 2 (1.5%), followed by Group 1 (1.34%), Group 3 (0.80%), Group 4 (0.44%), and Group 5 (0.35%). The prevalence of *C. parvum* was only found in Group 4 (0.08%). The two-way ANOVA implicated that there was a

Table 3 Seasonal prevalence of *Cryptosporidium* spp. infection in humans

*Season	No. of screened samples (n)	No. of positive samples (%)	<i>Cryptosporidium andersoni</i> (%)	<i>Cryptosporidium parvum</i> (%)
Pre-monsoon	475	35 (7.36)	26 (5.47)	9 (1.8)
Monsoon	350	51 (14.57)	49 (14.0)	2 (0.57)
Post-monsoon	292	12 (4.1)	03 (1.0)	8 (2.7)

*Seasonal variation shows that there was no significant difference compared with *C. parvum* [F(1, 1115) = 0.333, $p = 0.56$] and *C. andersoni* [F(1, 1115) = 1.403, $p = 0.14$]

Table 4 Community-wise prevalence of *Cryptosporidium* spp. infection in humans, southern Assam, India

*Caste	Total no. of samples (n)	No. of positive samples (%)	<i>Cryptosporidium andersoni</i> (%)	<i>Cryptosporidium parvum</i> (%)
Hindu	812	59 (7.2)	58 (7.14)	1 (0.12)
Muslim	258	33 (12.79)	15 (5.8)	18 (6.9)
Christian	49	6 (12.24)	5 (10.20)	1 (2.04)

*Community-wise variation shows that there was a significant difference compared with *C. parvum* [F(1, 1115) = 5.903, $p = 0.01$] and there was no significant difference compared with *C. andersoni* [F(1, 1115) = 0.000, $p = 0.9$]

significant mean effect for Cachar district in the prevalence of *C. andersoni* [F(1, 1109) = 12.801, $p = 0.000$] and there was no statistically significant mean effect for Cachar district in the prevalence of *C. parvum* [F(1, 1109) = 0.480, $p = 0.48$] (Table 5). The prevalence of *C. andersoni* in Karimganj district was highest in Group 1 (0.26%), followed by Group 2 (0.17%), Group 3 (0.08%), Group 5 (0.08%), and Group 4 (0.0%). Similarly, the prevalence of *C. parvum* was found to be highest in Group 1 (0.71%), followed by Group 2 (0.44), Group 3 (0.17), Group 4 (0.0), and Group 5 (0.0). The two-way ANOVA results indicated that there was a significant mean effect for Karimganj district in the prevalence of *C. parvum* [F(1, 1116) = 18.175, $p = 0.000$] and there was no statistically significant mean effect for Karimganj district in the prevalence of *C. andersoni* [F(1, 1116) = 2.727, $p = 0.09$] (Table 5). The prevalence of *C. andersoni* in Hailakandi district was highest in Group 1 (1.16%), followed by Group 2 (0.35%), Group 3 (0.26%), Group 4 (0.08%), and Group 5 (0.0%). Similarly, the prevalence of *C. parvum* was found to be highest in Group 1 (0.26%), followed by Group 4 (0.08), Group 2 (0.0), Group 3 (0.0), and Group 5 (0.0). The two-way ANOVA results indicated that there was a significant mean effect for Hailakandi district in the prevalence of *C. andersoni* [F(1, 1116) = 23.813, $p = 0.000$] and there was no statistically

significant mean effect for Karimganj district in the prevalence of *C. parvum* [F(1, 1116) = 3.324, $p = 0.06$] (Table 5).

All of the 98 ELISA-positive samples showed amplification when nested PCR was carried out. The species identification was performed by nested PCR using the 18S rRNA gene, which yielded 1325-bp fragment in primary PCR and a 825-bp fragment in secondary PCR (Table 6) [21]. To identify the species and genotype of *Cryptosporidium* isolates present in the sample, the nested PCR products were digested with SspI and VspI endonuclease enzymes. *Cryptosporidium* species and genotypes were diagnosed by comparing band patterns with those published previously [21, 22]. The secondary PCR-RFLP analysis using SspI digestion of nested PCR products yielded three bands of about 448 bp, 247 bp, and 106 bp, for which the banding pattern of 247 bp suggested that *C. parvum* (human genotype) was present, and the two bands of about 448 bp and 106 bp in different areas suggested that *C. parvum* in the bovine genotype originated from humans and cattle, respectively. *Cryptosporidium andersoni* generated two visible bands of 448 bp and 397 bp in three areas, which indicates the bovine and human genotypes, respectively (Table 7). In the PCR-RFLP analysis of the nested PCR products of *Cryptosporidium*-positive samples, the VspI digestion showed bands of 556 bp and 104 bp, which indicates the

Table 5 Distribution of *Cryptosporidium* spp. infection in different districts of southern Assam on the basis of age group

Age group	*Cachar		**Karimganj		***Hailakandi	
	<i>C. parvum</i>	<i>C. andersoni</i>	<i>C. parvum</i>	<i>C. andersoni</i>	<i>C. parvum</i>	<i>C. andersoni</i>
0–12	0	15	8	3	3	13
12–24	0	17	5	2	0	4
24–36	0	9	2	1	0	3
36–48	1	5	0	0	1	1
48–60	0	4	0	1	0	0

*The results indicated that there was a significant mean effect for Cachar district in the prevalence of *C. andersoni* [F(1, 1109) = 12.801, $p = 0.000$] and there was no statistically significant mean effect for Cachar district in the prevalence of *C. parvum* [F(1, 1109) = 0.480, $p = 0.48$]

**The results indicated that there was a significant mean effect for Karimganj district in the prevalence of *C. parvum* [F(1, 1116) = 18.175, $p = 0.000$] and there was no statistically significant mean effect for Karimganj district in the prevalence of *C. andersoni* [F(1, 1116) = 2.727, $p = 0.09$]

***The results indicated that there was a significant mean effect for Hailakandi district in the prevalence of *C. andersoni* [F(1, 1116) = 23.813, $p = 0.000$] and there was no statistically significant mean effect for Karimganj district in the prevalence of *C. parvum* [F(1, 1116) = 3.324, $p = 0.06$]

Table 6 List of primers used in this study

SL no.	Primer and sequence	Location in genome	Amplicon size	Annealing temperature	References
1.	<i>Cryptosporidium</i> species 5'TTC TAG AGC TAA TAC ATG CG 3' 5'CCC TAA TCC TTC GAA ACA GGA 3' 5'GGA AGG GTT GTA TTT ATT AGA TAA AG 3' 5'AAG GAG TAA GGA ACA ACC TCCA 3'	18S rRNA	1325 825	55 °C	Xiao et al. [21] and Feng et al. [22]

presence of *C. parvum* human genotype, and 628 bp and 104 bp for most samples, which also indicates the presence of *C. parvum* bovine genotype. In another case, the samples of VspI digestion showed bands of 730 bp and 115-bp, confirming the identification of *C. andersoni*. Some variation has been shown in the bands, as one is very dark and another is light. The dark band indicates the *C. andersoni* (bovine genotype), while the light band indicates the *C. andersoni* (human genotype) (Table 7).

The RFLP analysis yielded typical restriction patterns for *C. andersoni* in 78 cases and *C. parvum* in 20 cases. The prevalence of *C. andersoni* and *C. parvum* infection stratified by three districts, viz., Cachar, Karimganj, and Hailakandi, is shown in Fig. 3. Restriction analysis of secondary PCR products with SspI and VspI revealed the presence of *C. andersoni* and *C. parvum*, and further confirmed the presence of human and bovine genotypes (eight samples were of the *C. parvum* human genotype and 12 samples were of the bovine genotype) in the districts of Cachar, Karimganj, and Hailakandi. The presence of *C. andersoni* was found predominantly in the samples of these regions under study and the *C. parvum* human genotype was found only in a single isolate of Cachar district. In addition, of the total *C. andersoni*-positive samples, 31 samples were of the bovine genotype and 19 were of the human genotype. *Cryptosporidium andersoni* was the major species found in Cachar in comparison to the other districts.

Intra-species variations were also found in *C. parvum* and *C. andersoni*. The most significant observation was found with *C. parvum*, where two genotypes of *C. parvum* differed from each other in the three different areas. The human genotypes were isolated only from humans, whereas the bovine genotypes were isolated from both cattle and humans.

Cryptosporidium andersoni isolates from the human and bovine genotypes were found to be different from that of the *C. parvum* genotypes isolated from the three districts of Barak Valley, southern Assam. It was also evident from the sequencing results that the differences between the two genotypes (bovine and human) of *C. parvum* as well as *C. andersoni* were confirmed by sequence analysis of the 18S rRNA gene locus.

The sequences of *C. andersoni* and *C. parvum* have been deposited in GenBank under accession numbers KJ188438 and KJ719487, respectively. These samples showed 100% similarity with the *C. andersoni* sequence (GenBank accession number KF826294) and 99% similarity with the *C. parvum* sequence (GenBank accession number KF146224) (Table 8).

Discussion

The coexistence of *Cryptosporidium* with severe and constant diarrhea in children is prominent in the developing countries. A number of cross-sectional studies in children with diarrhea revealed that cryptosporidiosis was common in developing countries with a prevalence of up to 26% in Mexican and 16.5% in Brazilian children with diarrhea [23, 24]. The prevalence of *Cryptosporidium* in Asian countries, African countries, and Central and South American countries is greater than in Europe and North America [25]. In our study, the prevalence of *Cryptosporidium* in infants (<5 years old) was 13.4% and it was 7.6% in children (aged 5–12 years). In Pakistan and Indonesia, the parasite has been reported in 10.3 and 8.2% of

Table 7 List of restriction enzymes used in this study

SL no.	Restriction enzyme	Restriction site	Target gene	Restriction fragments (bp)	<i>Cryptosporidium</i> spp.	References
1.	SSPI	5' AAT↓ ATT 3' 3' AAT ↑ATT 3'	18S rRNA	448, 247,106 448,397	<i>Cryptosporidium parvum</i> <i>Cryptosporidium andersoni</i>	Xiao et al. [21] and Feng et al. [22]
2.	VSPI	5' AT↓TAAT 3' 3' TAAT↑TA 3'	18S rRNA	556,104 628,104 730,115 730,115	<i>Cryptosporidium parvum</i> (human genotype) <i>Cryptosporidium parvum</i> (bovine genotype) <i>Cryptosporidium andersoni</i> (bovine genotype) <i>Cryptosporidium andersoni</i> (human genotype)	Xiao et al. [21] and Feng et al. [22]

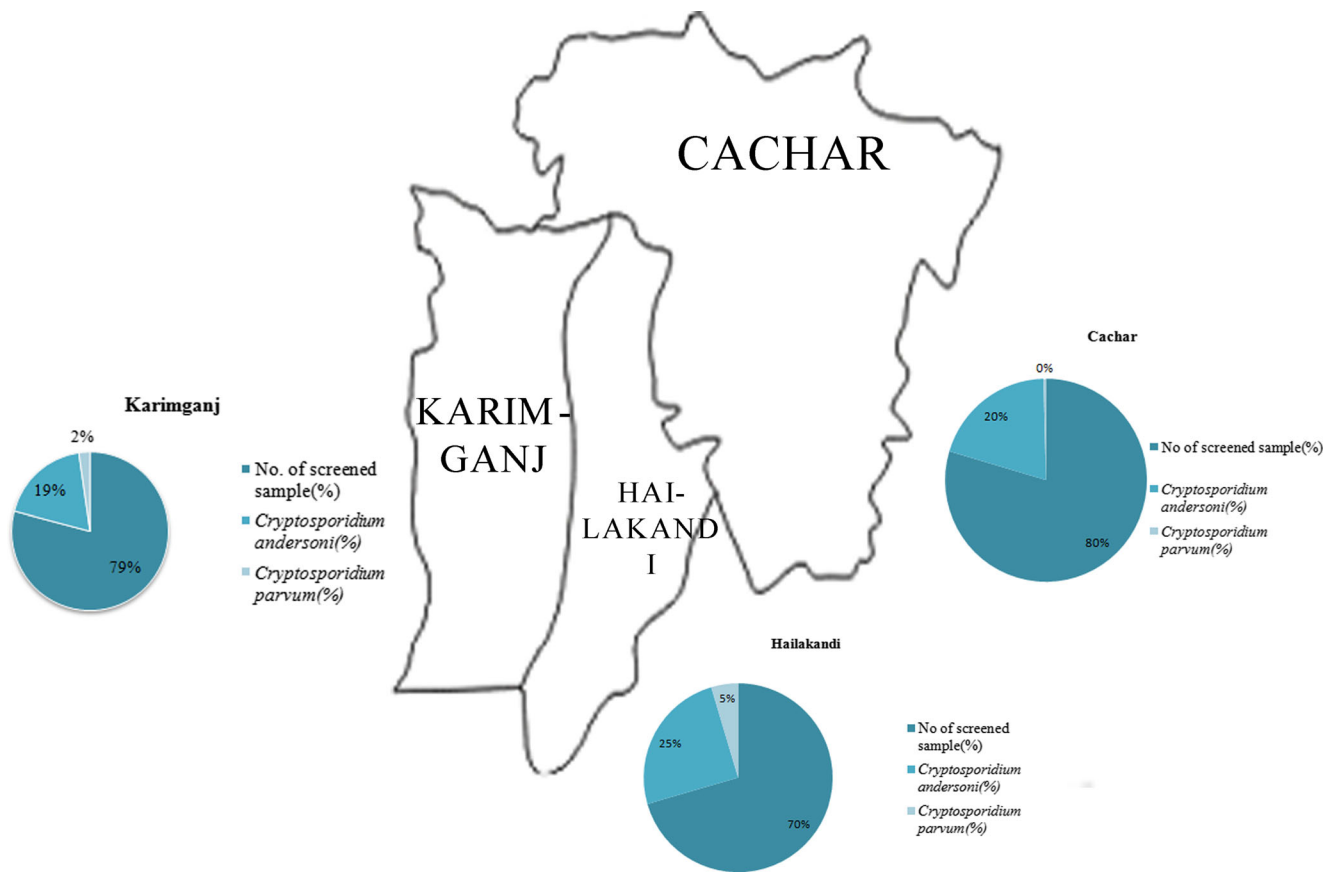


Fig. 3 Prevalence of *C. andersoni* and *C. parvum* infection stratified by three districts, viz., Cachar, Karimganj, and Hailakandi, in southern Assam, India, during January 2014 to December 2015

children with diarrhea, respectively [26, 27]. The parasite was detected in pediatric diarrhea in 1.4% of cases in North India, 5.5% from East India, 5.6% from West India, and 13.1% from South Indian children [28–30]. In a study from Hyderabad, the prevalence was reported to be as low as 2.99% in children and 0.12% in adults [31]. Previous studies also stated that the prevalence of *Cryptosporidium* was 7.9% in Indian adults [32], which is supported by our current study (6.06%). In our study area, the highest prevalence was found to be (14.5%) in the monsoon season, followed by pre-monsoon (7.36%) and post-monsoon (4.1%) months. Water may be the major route of transmission of *C. andersoni*, *C. hominis*, and *C. parvum* genotypes, which are evident to the existence of high prevalences of *C. parvum* and *C. andersoni* during the rainy season [33]. From the present study, the prevalence of

C. andersoni (6.97%) is higher than that of *C. parvum* (1.7%). No differences were observed morphologically between the dimensions of the *C. andersoni* and *C. parvum* oocysts isolated from the humans in our current study and those reported previously [34]. However, our study showed that the rate of *Cryptosporidium* prevalence was 6.06% in elderly patients (>50 years age), but earlier studies reported that the prevalence was 7.9% in Indian adults [32]. We also found an infection rate of 13.4% in infants in our study, which was higher than those from studies (4.3–13.3%) [29, 30, 35]. *Cryptosporidium andersoni* is also a major etiological agent of human cryptosporidiosis in India, especially in infants and the elderly (>50 years age) [32]. However, the results from our study clearly showed that *C. andersoni* has become a novel predominant species (78 positive for *C. andersoni* out of 98 cases)

Table 8 List of sequences submitted to GenBank, along with accession number

SL no.	Species	Accession number	Reference sequence at GenBank	Identities
1	<i>Cryptosporidium andersoni</i>	KJ188438	KF826294	100%
2	<i>Cryptosporidium parvum</i>	KJ19486	KF146224	99%

among the hospital-based and community-level patients with diarrhea in southern Assam, India. Only a few cases of *C. andersoni* infection in humans have been reported in France [36], Malawi [37], and England [38]. As per our knowledge, this might be the first report on the prevalence of *C. andersoni* in southern Assam, India, using molecular techniques. We have attempted to study the prevalence and distribution of two species in the study population and found significant impacts of age and season on the prevalence of these species. Therefore, screening for this pathogen should obviously be done among animals for national or international trade. The findings also clearly suggest the existence of two genotypes, including two of potential animal origin. Further genotyping, subtyping, and phylogenetic analysis with large sample sizes and extensive collection of epidemiological data are needed for a better understanding of cryptosporidiosis transmission in India.

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Compliance with ethical standards

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Conflict of interest No conflict of interest.

Ethics approval The study protocol was reviewed and approved by the Institutional Ethical Committee (ref. no. GCC/9440) of Gurucharan College, Silchar, Assam, before the commencement of the study and Silchar Medical College and Hospital, Silchar, Assam.

Informed consent All stool samples were collected with the consent of each patient/subject, in the form of questionnaires. All study participants had given written consent before enrollment into the study. Parents/guardians provided consent on behalf of all infant participants.

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