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Comparative molecular epidemiological studies on bovine and human cryptosporidiosis

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ABSTRACT

Cryptosporidium is an intracellular coccidian protozoan parasite, once thought to be rare and host specific now is known to be ubiquitous in nature and infect a wide range of animals, birds, reptiles and human. The present study was carried out both on bovine and human. A total of 500 faecal samples from two different cattle farms and local *Khattal,* and 334 stool samples of children were collected from a hospital of Kolkata, West Bengal. Both the bovine and human samples were classified into three groups, viz. disease status (diarrhoeic and non-diarrhoeic), age and season. The overall 18% and 2.99% cryptosporidiosis was found in cattle and human samples, respectively. The prevalence of *Cryptosporidium* in diarrhoeic and non-diarrhoeic cattle was 22.57% and 9.10%. In human, 4.62% and 1.24% cases were found to be positive for cryptosporidiosis in diarrhoeic and non-diarrhoeic samples, respectively. Age specific distribution of *Cryptosporidium* both in diarrhoeic and non-diarrhoeic cattle suggested that infection was highest (15.75%) in calves (upto 1 year) and lowest (5.83%) in heifer (>1–3 yrs). The prevalence was intermediate (13.95%) in lactating animals (>3 years). In human, prevalence was highest (4.12%) in upto 1 year age group but lowest (1.04%) in >4–5 years of age. Seasonal prevalence revealed that infection was highest during rainy season in both bovine and human. During winter lowest prevalence was found in cattle but no positive case was observed in human. PCR-RFLP analysis and nucleotide sequencing of isolated species revealed that *Cryptosporidium parvum* and *Cryptosporidium hominis* were the species mainly responsible for diarrhoea in cattle and human, respectively. Four *Cryptosporidium parvum* positive cases were also detected from human diarrhoeic samples which had similar nucleotide sequences to that of *Cryptosporidium parvum* isolated from bovine samples. These findings suggest that the zoonotic transmission of the diseases could be possible. PCR-RFLP can be used as a potential and reliable tool for species identification of *Cryptosporidium* in both bovine and human.

Key words: Bovine, *Cryptosporidium*, Diarrhoea, DNA, Human, PCR-RFLP, Sequence, SSU rRNA

Cryptosporidium, a coccidian, intracellular protozoan parasite of the phylum Apicomplexa once thought to be rare and host specific is now known to be ubiquitous and has multiple hosts (Navin and Juranek 1984, Fayer and Ungar 1986). It infects the gastrointestinal tract of both human and livestock and considered the third major cause of diarrhoea worldwide (Spano *et al.* 2000). In pediatric and elderly population, especially in day-care centres and nursing homes, person-to-person transmission probably plays a major role in the spread of this parasite (Neill *et al.* 1996). A variety of domestic and wild animals including calves, pigs, and wild rodents etc. appear to be important zoonotic reservoirs for *Cryptosporidium parvum* to man (Chalmers *et al.* 1997). Pets also play an important role in the transmission of cryptosporidiosis to human (Abe *et al.* 2002). Among the entire animal hosts, calves are the most important from public health point of view, as they may serve as a source of human infection (Current and Garcia 1991, O'Donoghue 1995). In human, the infection in immunocompetent adult is generally acute and self-limiting, whereas immunocompromised individuals can develop chronic and potentially life-threatening diarrhoea particularly in patients suffering from AIDS (Carraway *et al.* 1996). Similar type of life threatening infection has been reported in neonatal animals suffering from malnutrition, hypovitaminosis and immunodeficiency syndromes (Wilson *et al.* 1983, Fukushima and Helman 1984). *Cryptosporidium* has the potential to infect many

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species of animals including man from a point source outbreak (Juranek 1995, Keusch *et al.* 1995). The prevalence of cryptosporidiosis is reported to be generally higher in summer and lower in winter (Chai *et al.* 2001).

The major problems in understanding the transmission of *Cryptosporidium* infection is the lack of species specific morphological features (Fall *et al.* 2003) and the inability to grow the organisms in large numbers from contaminated sources. More precisely, not much information is available on the prevalence of *Cryptosporidium* and its association with causation of diarrhoea in bovine and children in India. Therefore, a lot of information about the clinical, epidemiological features and species variation of existing *Cryptosporidium* are needed to be explored. It is important to know whether the *Cryptosporidium* species isolates from bovine and human origin are same or different. These information will be highly useful for chemoprophylaxis or designing of vaccine. The present study was aimed to assess the prevalence of cryptosporidiosis in bovine and children and identify the species of *Cryptosporidium* using molecular biological techniques.

MATERIALS AND METHODS

Collection of faecal samples and case history: The bovine diarrhoeic and nondiarrhoeic faecal samples were collected both from calves and adult cattle in 100 ml sterile plastic containers. The epidemiology and clinical history of each case were also recorded. A total of 500 bovine samples were collected from the Haringhata cattle farm (212), Dairy Farm. Narendrapur Ramakrishna Mission (212) and local *Khattal* (76); out of these, the diarrhoeic cases (total 259) were 111, 109 and 39, respectively, from these three locations; the rest of the cases were nondiarrhoeic. The diarrhoeic and nondiarrhoeic samples collected, were from three age groups, viz. calves (up to 1 year), heifer (>1–3 years), lactating animals (>3 years). The samples were collected in summer, rainy and winter seasons. The different breakup of samples is given in the respective result table (Tables 2, 3). A total of 334 stool samples, comprising 173 diarrhoeic and 161 nondiarrhoeic cases, were collected in sterile collection vials from human patients of different age groups (up to 1, 1–2, 2–3, 3–4, 4–5 year) during three different seasons (summer, rainy and winter) from B.C. Roy Memorial Children Hospital, Kolkata.

Staining of Cryptosporidium *oocyst:* Staining of faecal samples was done by standard modified acid fast staining protocol of Garcia *et al.* (1983). The stained slides were observed under oil immersion lens.

Isolation and quality, purity and concentration checking of DNA: DNA was isolated from faecal samples both using laboratory made solutions and commercial kit as per the manufacturer's protocol. The quality of isolated genomic DNA was checked by electrophoresis in 0.8% (w/v) agarose gel in 0.5x TBE through ethidium bromide staining and visualization under UV light. The purity was analyzed in spectrophotometer by taking optical densities (OD) at 260 and 280 nm wavelengths. The samples having OD_{260}/OD_{280} ratio between 1.7 and 1.9 were used for subsequent study. The concentration of DNA was calculated using standard formula.

Polymerase chain reaction: The nested polymerase chain reaction was carried out based on SSU rRNA gene of *Cryptosporidium* species. The primers for the primary and secondary (nested) PCR are given in Table 1. The primary PCR was carried out in a 25 µl volume containing ~50–100 ng genomic DNA, 40 ng of each of primer (AL 1687 and AL 3417), 1×s PCR buffer, 1.5 µM $MgCl_2$, 200 µM dNTPs each and 0.25 U *Taq* DNA Polymerase. The thermal cycling conditions were; initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec and extension at 72°C for 1 min. The final extension step was carried out at 72°C for 5 min. The products of primary PCR were analyzed by electrophoresis in ethidium bromide stained 1.5% (w/v) agarose gel in 0.5x TBE buffer and visualized under UV light. The secondary PCR was performed in a 25 µl volume containing 2.5 µl primary PCR products as template and all other components as mentioned for primary PCR, except the primers. The forward and reverse primers used for nested PCR were AL 3032 and AL 1598. The PCR cycling conditions were almost like that of primary PCR given above with annealing at 58°C for 45 sec. The products of secondary PCR were also resolved in agarose gel as mentioned above.

Restriction fragment length polymorphism (RFLP) analysis: The RFLP analysis was carried out using *Ssp*I and *Vsp*I restriction enzymes. Each of the nested PCR products was separately digested by these 2 restriction enzymes in a 25 µl reaction volume. Each digestion reaction contained 5 µl nested PCR product, 0.5 µl (5 U) specific restriction enzyme, 2.5 µl specific buffer (react 6 buffer for *Ssp*I and react 2 buffer for *Vsp*I) and sterile water. Both the reactions were incubated in a water bath at 37°C for 3 h. The digested

Table 1. Primers used for the primary and secondary PCR

Primer Name	Type	Sequence (5' to 3')	Function
AL 1687	Primary PCR	TTCTAGAGCTAATACATGCG	Forward primer
AL 3417		CCCATTTCCTTCGAAACAGGA	Reverse primer
AL 3032	Secondary PCR	GAAGGGTTGTATTTATTAGATAAAG	Forward primer
AL 1598		AAGGAGTAAGGAACAACCTCCA	Reverse primer

Table 2. Prevalence of *Cryptosporidium* species in two farms and local *Khattal*

Place	Diarrhoeic samples	% +ve *Cryptosporidium*	Non-diarrhoeic samples	+ve% *Cryptosporidium*	Per cent affected (mean±SE)
Harringhata cattle farm	111	28.83	10122	11.88	14.90±3.5[a]
Dairy Farm (R.K.Mission)	109	27.52	103	10.68	13.90±3.9[a]
Local *Khattal*	39	12.82	37	5.41	7.18±2.8[b]
Overall	259	22.57±0.42[a]	241	9.1±0.13[b]	

The mean comparison between the overall *Cryptosporidium* positive diarrhoeic and nondiarrhoeic cases are significant at P≤0.01 and that among the farms is significant at P≤0.05.

Table 3. Age specific distribution of *Cryptosporidium* infection in diarrhoeic cattle

Place	Up to 1 year (calves)		1–3 years (heifer)		3 years (lactating animal)	
	Diarrhoea	Affected (%)	Diarrhoea	Affected (%)	Diarrhoea	Affected (%)
Haringhata cattle farm	82	28.05	12	16.67	15	20.00
Dairy Farm (R.K.Mission)	82	32.93	15	13.33	14	21.43
Local *Khattal*	10	10.00	0	0	29	13.79
Overall	174	22.73±0.79	27	14.96±0.05	58	18.28±0.10

products were then electrophoresed in a 2% (w/v) agarose gel in 0.5× TBE buffer. The gel was visualized under UV light through ethidium bromide staining.

Sequencing: The PCR products showing different RFLP patterns for both the restriction enzymes were sequenced. The sequencing was performed in both forward and reverse direction by an automated genetic analyzer following the dideoxy chain-termination method (Sanger *et al*. 1977).

Sequence analysis: The obtained sequences were used for Basic Local Alignment Search Tool (BLAST) analysis using National Centre for Biotechnology Information's (NCBI) online facility (www.ncbi.nlm.nih.gov) for searching the sequences with significant homology. The sequences were also analyzed in *ClustalW* subprogramme of *MegAlign* programme of LASERGENE software for generating multiple sequence alignment reports, sequence similarity/divergence and phylogenetic trees among them.

Statistical analysis: The percentage data was angularly transformation and analyzed using appropriate statistical techniques as described by Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

Clinical features (diarrhoeic animals and children): Among the 259 diarrhoeic cases in bovine, 26.59% had severe, 24.78% moderate and 49.13% mild diarrhoea. There were incidences of 5–10 episodes of diarrhoea/day. The degree of dehydration, temperature, pulse rate and respiratory rate varied according to the degree of severity of diarrhoea. The respiratory rate and heart rate were higher in early stages. The signs of trachycardia and bradycardia were also observed.

Out of 173 diarrhoeic human cases, 24.01% had severe, 28.09% moderate and 47.90% mild diarrhoea. There were 5–12 episodes of incidence per day. Like the bovine cases, the other symptoms varied according to the degree of severity of the cases. There was varying degree of anorexia, emaciation and weakness.

Prevalence of Cryptosporidium *by microscopy:* There were overall 18% *Cryptosporidium* positive cases found from the 500 bovine diarrhoeic and nondiarrhoeic samples screened. The overall mean prevalence of *Cryptosporidium* in diarrhoeic and nondiarrhoeic cattle were 22.57±0.42 and 9.1±0.13%, respectively. The disease status (diarrhoeic and nondiarrhoeic) of cattle had significant (P≤0.01) effect on the mean per cent affected. The difference in prevalence between diarrhoeic and nondiarrhoeic cattle was also significant (P≤0.01). The highest proportion of cases of both diarrhoeic and nondiarrhoeic cattle from which *Cryptosporidium* was detected, were those from Haringhata cattle farm. However, the local diarrhoeic and nondiarrhoeic cattle samples collected from *Khattals* had least presence of *Cryptosporidium* oocysts (Table 2).

From 334 human samples studied, *Cryptosporidium* was detected in 2.67±0.28% cases. Among the diarrhoeic and nondiarrhoeic samples, *Cryptosporidium* was detected in 4.62 and 1.24% cases, respectively. Pal *et al.* (1989) and Das *et al.* (1993) also found similar results from the cases of the same hospital. This finding also corroborates with earlier observations from other developing countries (Tzipori *et al.* 1983, Shahid *et al.* 1985, Fayer and Unger 1986). But Mathan *et al.* (1985) recorded as high as 9.8% *Cryptosporidium* prevalence from nondiarrhoeic persons in South India.

Clinical features in Cryptosporidium positive cattle and humans: Out of 22.57±0.42 % *Cryptosporidium* positive samples from the diarrhoeic cattle, dehydration was seen in 71.42%, weakness and debility in 57.14%, pasty faeces in

38.77%, watery diarrhoea in 34.70%, semisolid faeces in 26.53% and anorexia in 18.36% cases. Fayer and Ungar (1986) found that calves often had dehydrated, rough hair coat and were debilitated. Thomas *et al.* (1984) recorded acute cryptosporidiosis, characterized by watery diarrhoea, anorexia and weight loss. Naciri *et al.* (1993) observed that experimental *C. parvum* infection caused a severe clinical disease with profuse watery diarrhoea, high oocyst shedding and mortality (3 out of 5) in the untreated group.

Out of 4.62% *Cryptosporidium* positive samples from the diarrhoeic human cases, dehydration was seen in 70%, watery diarrhoea in 100%, fever in 20% and vomiting in 30% cases. These findings corroborate the earlier observations made by Pal *et al.* (1989).

Source (farm) specific distribution of Cryptosporidium *in cattle:* The farms had significant (P≤0.05) effect on the occurrence of cryptosporidiosis. The source wise mean prevalence was 14.90±3.50, 13.90±3.90 and 7.18±2.80 percent, respectively, in samples from Haringhata cattle farm, Dairy farm (Ramakrishna Mission) and local *Khattal* (Table 2). Duncan's multiple range test showed that the prevalence rates between the 2 farms did not differ significantly, whereas it differed significantly (P≤0.05) from that of local *Khattal.* This might be due to the biasness in sample collection from local *Khattal,* as *Khattal* owner mainly maintain lactating animals which are naturally resistant to cryptosporidiosis. Dubey *et al*. (1990) recorded 45.5% *Cryptosporidium* prevalence in USA 24.5% in UK 26% in formerly USSR., 40% in Germany and 27% in Hungary. Garcia *et al.* (1993) and Kaminjolo *et al.* (1993) observed 27.87% and 19.6% *Cryptosporidium* cases in cattle. Garcia *et al.* (1993) found 31.40% and 21.62% diarrhoeic and non-diarrhoeic *Cryptosporidium* positive cases in Brazil. However, information on prevalence of *Cryptosporidium* spp. in Indian cattle irrespective of diarrhoea or non-diarrhoea is very scanty so no comparison could be possible with the findings of the present study.

Age specific distribution of Cryptosporidium *both in diarrhoeic and non-diarrhoeic cattle and human:* Age had significant (P≤0.01) effect on mean per cent prevalence in cattle. Among the 500 bovine samples *Cryptosporidium* was detected in 15.75±4.00, 5.83±2.81 and 13.95±3.22% cases in calves, heifer and lactating animals, respectively. Duncan's multiple range test showed that the difference in prevalence was nonsignificant between calves and lactating animals, whereas prevalence in heifer significantly (P≤0.05) differ from that of calves and lactating animals. Out of 22.57±0.42% *Cryptosporidium* positive cases from diarrhoeic samples, the highest prevalence was in calves (upto 1 year) whereas it was least in heifers (>1–3 years) (Table 3). Out of overall 9.10±0.13% *Cryptosporidium* positive nondiarrhoeic samples, the highest prevalence was found in lactating animals, while heifer showed least prevalence (Table 4). The results supported the findings of Mtambo *et al.* (1997) who reported higher infection in calves of less than 3 months of age. Olson *et al.* (1997) observed the prevalence of *C. parvum* in 59% cases and it was predominant in 2-to 4-week-old calves. Quilez *et al.* (1996) recorded 44.4% prevalence in 3–4 day old calves and 76.7% during 6–15 days of age. The insignificant difference in prevalence between calves and lactating animals may be due to the fact that during the study period 26.78% lactating animals were considered within 1 week after parturition and these animals showed very high prevalence. These higher percentages of *Cryptosporidium* in parturient animals may have totally increased the prevalence of *Cryptosporidium* among the lactating animals. The higher infection just after parturition may be ascribed to the lack of immunity due to calving related stress of the animals. Occurrence of *Cryptosporidium* in clinically asymptomatic animals indicated that the particular age group of animals might be reservoir for the parasite. This result was in concordance with the findings of Malgorzata *et al.* (1998).

In children, the mean prevalence of cryptosporidiosis in up to 1, 1–2, 2–3, 3–4, 4–5 year age groups were 4.12±1.94, 3.20±1.44, 0.98±0.98, 0.88±0.88 and 1.04±1.04 percent, respectively. Out of 4.6% *Cryptosporidium* positive diarrhoeic cases, 8.33% and 5.71% cases were from up to 1 year and 1–2 year age group, respectively, whereas rest of the age groups had 2.94% positive cases (Table 5). From the *Cryptosporidium* positive non-diarrhoeic samples, 3.13% and 2.94% cases were recorded upto 1 year and 1–2 year age groups, respectively (Table 5). The higher prevalence upto 1 year and 1–2 year than the other age groups of human well substantiates the observation made by Pal *et al.* (1989) and Das *et al.* (1993).

Seasonal distribution of Cryptosporidium *both in diarrhoeic and non-diarrhoeic cattle and human:* The season

Table 4. Age specific distribution of *Cryptosporidium* infection in non-diarrhoeic cattle

Place	Up to 1 year (calves)		1–3 years (heifer)		3 years (lactating animal)	
	Non-diarrhoea	Affected (%)	Non-diarrhoea	Affected (%)	Non-diarrhoea	Affected (%)
Harringhata cattle farm	80	11.25	10	0	13	15.38
Dairy Farm (R.K.Mission)	80	13.75	9	0	12	8.33
Local *Khattal*	8	0.03	0	0	29	6.90
Overall	168	5.96±1.32	19	0.03±0.0	54	9.93±0.18

Table 5. Age specific distribution of cryptosporidiosis in children

Age group (year)	Diarrhoeic samples	Percent affected	Non-diarrhoeic samples	Percent affected	Mean ±SE
Up to 1	36	8.33	32	3.13	4.12±1.94[a]
1-2	35	5.71	34	2.94	3.2±1.44[a]
2-3	34	2.94	32	0.00	0.98±0.98[b]
3-4	34	2.94	32	0.00	0.88±0.88[b]
4-5	34	2.94	31	0.00	1.04±1.04[b]

Means within a column having different superscripts differ significantly (P≤0.05).

Table 6. Seasonal distribution of cryptosporidiosis in diarrhoeic and non-diarrhoeic children

Season	Diarrhoeic samples	Percent affected	Non-diarrhoeic samples	Percent affected	Mean±SE
Summer	58	5.17	54	0.00	1.45±1.01[b]
Rainy	58	8.62	54	3.7	4.63±1.11[a]
Winter	57	0.00	53	0.00	
Overall	173	4.62	161	1.24	

Seasonal means having different superscripts differ significantly (P≤0.01).

had significant (P≤0.05) effect on prevalence of *Cryptosporidium* in cattle. The season wise overall mean prevalence were 13.97±2.52, 21.05±4.67 and 2.76±1.27% during summer, rainy and winter, respectively. Mean comparison showed that summer and rainy season had significantly (P≤0.05) higher prevalence than winter. This was in agreement with the findings of other workers who observed that during the warm and humid months (August-September) the infection was highest, whereas in dry and cool months (December-January) the prevalence was low (Prasad *et al.* 1989, Chai *et al.* 2001).

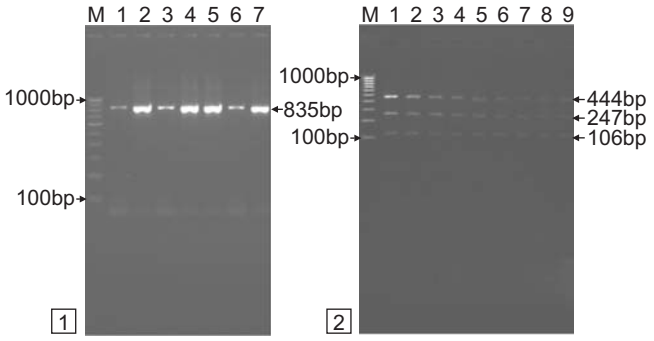
In children, 1.45±1.01 and 4.63±1.11% *Cryptosporidium* positive cases were found during summer and rainy season. However, none of the winter samples were positive for *Cryptosporidium*. Out of the diarrhoeic cases, 5.17 and 8.62% were positive for *Cryptosporidium* in summer and rainy season. Among the non-diarrhoeic cases, 3.7% cases showed cryptosporidiosis during rainy season (Table 6). Comparison of seasonal mean prevalence showed that rainy season had significantly (P≤0.01) higher prevalence than summer. These findings are similar with the previous results of other workers who observed that during the monsoon and post-monsoon months, prevalence was higher (Pal *et al.* 1989).

Molecular biological studies

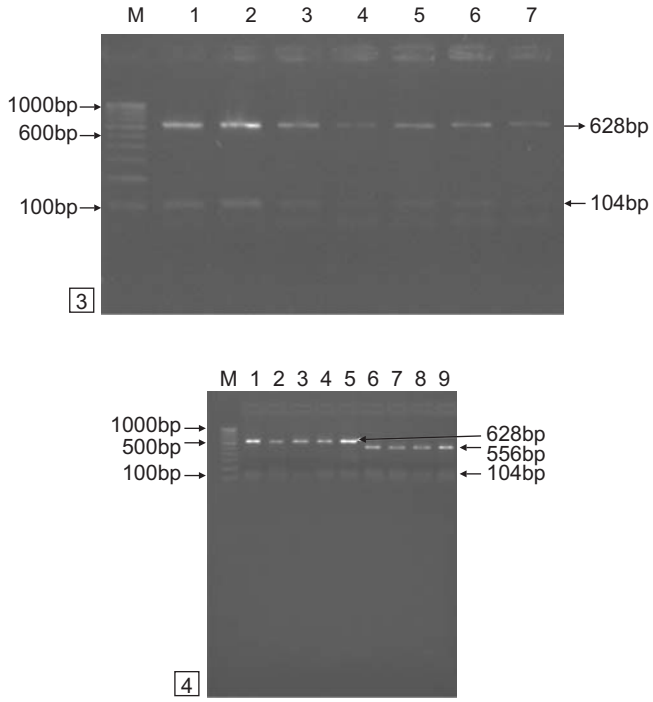
Isolation and quality of genomic DNA: DNA was isolated from 180–190 mg of faecal sample. The DNA samples of standard quality, purity and requisite concentration were used in the study.

PCR based diagnosis of Cryptosporidium*:* The primary PCR was done with a pair of forward and reverse primers specific for SSU rRNA gene of *Cryptosporidium.* It yielded a product of 1321 bp size. The nested PCR was carried out with a second set of primers and it produced a product of 835 bp length (Fig. 1). It is important to mention that in some samples the primary PCR did not give any amplification to produce band of 1321 bp but all the *Cryptosporidium* positive samples showed band of 835 bp bands from secondary PCR. Two step PCR was used because the basic idea was to first amplify the product and nested PCR has enhanced the sensitivity and specificity of the test to the extent of 35 folds (Xiao *et al.* 1999).

Cryptosporidium *species identification by RFLP analysis:* The *Ssp*I restriction enzyme digestion of the nested PCR products both from cattle and human samples revealed 3 distinct bands of 444, 247 and 106 bp in all the samples. These banding patterns clearly indicated that the samples were *Cryptosporidium parvum* or *Cryptosporidium hominis* (Fig. 2). This result was in complete agreement with that of Spano *et al.* (1997), Sulaiman *et al.* (1998) and Xiao *et al.* (1999, 2000 and 2001). It is clear from the findings that PCR-RFLP can be a potential and reliable technique for detection of *Cryptosporidium* spp. On the other hand, *Vsp*I digestion of nested PCR products of all bovine samples revealed two distinct bands of 628 and 104 bp. This result suggested that the identified species was *Cryptosporidium parvum* (Fig. 3). Thus, it indicated that *Cryptosporidium parvum* was mainly responsible for the Cryptosporidiosis in cattle. When the nested PCR products from human samples were digested with *Vsp*I, most of the samples produced 2 distinct bands of 556 and 104 bp. This banding pattern confirmed that the species was *Cryptosporidium hominis*, which was mainly responsible for cryptosporidiosis in human (Xiao *et al.* 1999). However, the nested PCR products of 4 human samples after digestion



Figs 1–2. **1.** Lane 1–7 showing the band of nested PCR amplified products of 835 bp of different *Cryptosporidium* isolates using primers AL-3032 and AL-1598; M-represents 100bp DNA ladder. **2.** SspI restriction digestion pattern of nested PCR products of *Cryptosporidium* isolates from bovine and human; lane 1–9 showing bands of 444, 247 and 106 bp; M-represents 100 bp DNA ladder.



Figs 3–4. *VspI* restriction digestion pattern of nested PCR products of *Cryptosporidium* isolates from bovine; lane 1-7 showing bands of 628 and 104 bp (*C. parvum*); M-represents 100 bp DNA ladder. **4.** *VspI* restriction digestion pattern of nested PCR products of *Cryptosporidium* isolates from human; lane 1-5 showing bands of 628 and 104 bp (*C. parvum*) and, lane 6-9 showing bands of 556 and 104 bp (*C. hominis*); M-represents 100bp DNA ladder.

with *VspI* showed bands of 628 and 104 bp similar to the bovine samples, suggesting the presence of *Cryptosporidium parvum* induced infection in human (Fig. 4). *Cryptosporidium parvum* isolated from human suggested that the zoonotic transmission of this disease could be possible. These results were supported by the similar findings of Xiao *et al.* (1999).

Sequencing and sequence analysis: Sequencing of the secondary PCR products of the isolated species of *Cryptosporidium* was carried out for further confirmation of the presence of concerned species in bovine and human population.

Sequence variation: There was no difference in the nucleotide sequences of *Cryptosporidium parvum* isolated from bovine and human origin. All *Cryptosporidium hominis* isolates from human origin were having similar type of nucleotide sequence. There were few nucleotide variations (Fig. 5) found in the sequences of *Cryptosporidium parvum* and *Cryptosporidium hominis* isolates when compared with their respective sequences available in the NCBI public database.

Sequence similarity: The nucleotide sequences of all the *C. parvum* isolates of both bovine and human origin showed 100% similarity among them (Fig. 6). But all these isolates were 93.0% similar to the GenBank sequence AF159111.1. These sequences were 93.1% and 92.8% similar to *C. meleagridis* (EU814439.1) and *C. wriari* (AF115378.1) sequences, respectively. Again, all the *Cryptosporidium hominis* isolates were identical to each other. When compared to a reference sequence of *C. hominis* (FJ707311.1) each of these isolates were found to be 99.2% similar. They showed 98.2% and 98% similarity to the sequences of *C. meleagridis* (EU814439.1) and *C. wriari* (AF115378.1), respectively. The *C. parvum* sequences were 92.8% identical to the *C. hominis* sequences.

Phylogenetic analysis: Phylogenetic analysis based on the nucleotide sequences revealed that all the *Cryptosporidium parvum* isolates form a single group irrespective of their source (Fig. 7). On the other hand, both the *C. hominis* isolates showed distinct divergence from the point of origin and form a separate group which is closely located to the reference sequence of *Cryptosporidium parvum* (AF159111.1)

The results obtained from our study were in consonance with those reported by earlier researchers (Carraway *et al.* 1996, Vasquez *et al.* 1996, Peng *et al.* 1997, Spano *et al.* 1997, Sulaiman *et al.* 1998, Xiao *et al.* 1999).

The finding of identical sequences of *C. parvum* isolates from both bovine and human origin revealed that the same was transmitted to human from cattle either through direct contact with infected animals or through contaminated drinking water. The most of the children considered in this study were from the urban slums of Kolkata or villages in and around Kolkata. They reside in overcrowded rooms and belong to the low socio-economic strata with poor hygienic condition and most of the families have either cattle or goat as one of their important livelihood. The case control study revealed that the four *Cryptosporidium parvum* of human origin came from different villages in and around Kolkata and the concerned families also have lactating cattle and calves. Faecal sample was also collected from all cattle and calves of these families and all the cases were positive

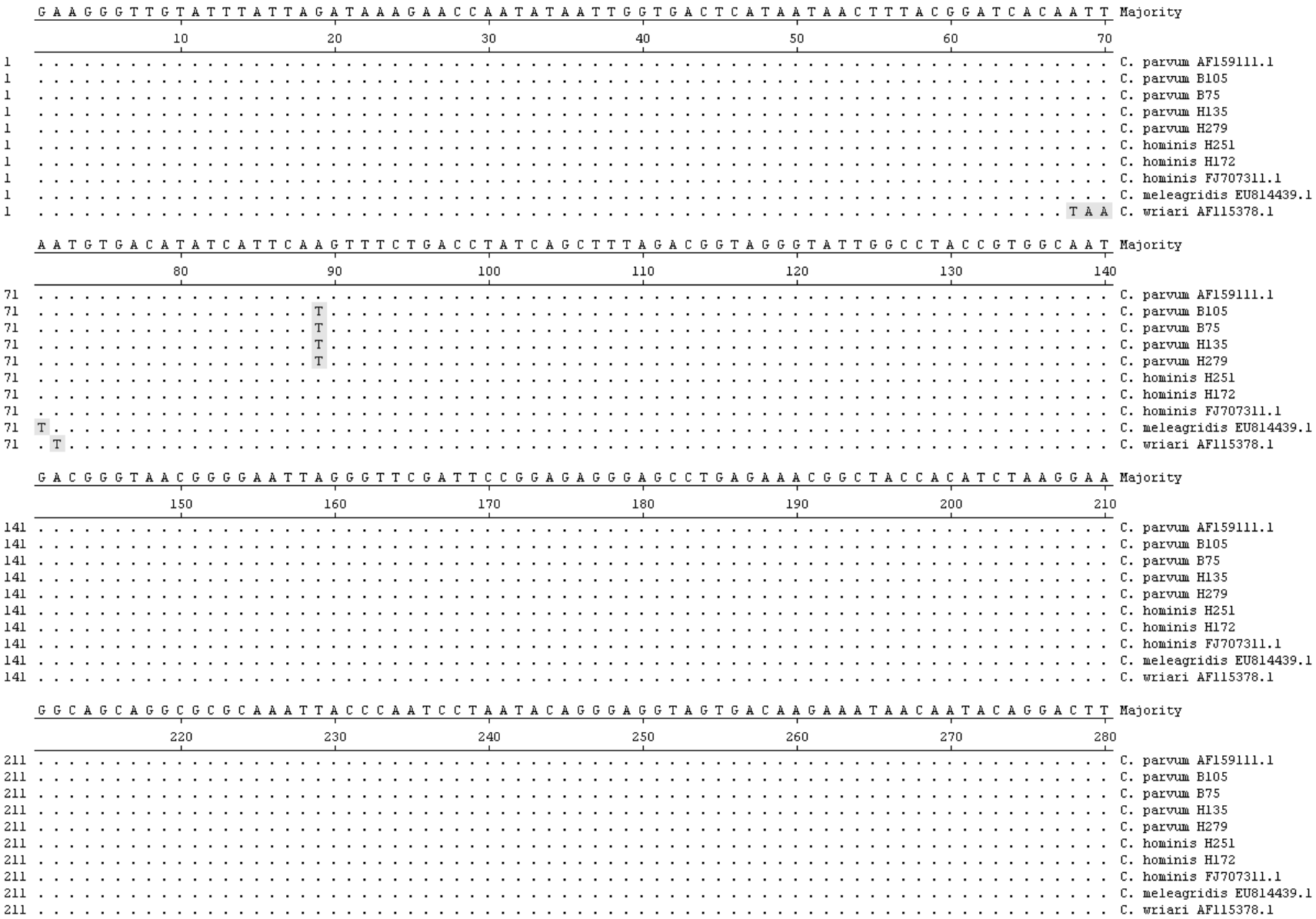


Fig. 5. Nucleotide sequence alignment report of SSU rRNA gene among different species of *Cryptosporidium* of bovine and human origin

T T T G G T T T T G T A A T T G G A A T G A G T T A A G T A T A A A A C C C C T T T A C A A G T A T C A A T T G G A G G G C A A G T C T G G G T
281 . . . . . C. parvum AF159111.1
281 . . . . . C. parvum B105
281 . . . . . C. parvum B75
281 . . . . . C. parvum H135
281 . . . . . C. parvum H279
281 . . . . . C. hominis H251
281 . . . . . C. hominis H172
281 . . . . . C. hominis FJ707311.1
281 . . . . . C. meleagridis EU814439.1
281 . . . . . C. wriari AF115378.1
Majority
G C C A G C A G C C G C G G T A A T T C C A G C T C C A A T A G C G T A T A A A G T T G T T G C A G T T A A A A A G C T C G T A G T T
351 . . . . . C. parvum AF159111.1
351 . . . . . C. parvum B105
351 . . . . . C. parvum B75
351 . . . . . C. parvum H135
351 . . . . . C. parvum H279
351 . . . . . C. hominis H251
351 . . . . . C. hominis H172
351 . . . . . C. hominis FJ707311.1
351 . . . . . C. meleagridis EU814439.1
351 . . . . . C. wriari AF115378.1
Majority
G G A T T T C T G T T A A T A A A T T T A T A A A A A T A T T T T G A A T A T T T A T A A T A A A T T C A T A A C A T A A T T C A T A T T A A
421 . . . . . C. parvum AF159111.1
421 . . . . . T . . . . . C . . . . . C . . . . . C. parvum B105
421 . . . . . T . . . . . C . . . . . C . . . . . C. parvum B75
421 . . . . . T . . . . . C . . . . . C . . . . . C. parvum H135
421 . . . . . T . . . . . C . . . . . C . . . . . C. parvum H279
421 . . . . . C. hominis H251
421 . . . . . C. hominis H172
421 . . . . . T . . . . . C. hominis FJ707311.1
421 . . . . . T . . . . . - . . . . . T . . . . . C. meleagridis EU814439.1
421 . . . . . T . . . . . - A . . . . . C. wriari AF115378.1
Majority
C T A T A T T T T T T - - - A G T A T A T G A A A A A T T T T A C T T T G A G A A A A A T T A G A G T G G C T T A A A A G C A G G G C A T A T G C C T T
491 . . . T . . . - - - . . . . . G . . . . . C. parvum AF159111.1
491 . . . . . A . . . - - - . . . . . G . . . . . C. parvum B105
491 . . . . . A . . . - - - . . . . . G . . . . . C. parvum B75
491 . . . . . A . . . - - - . . . . . G . . . . . C. parvum H135
491 . . . . . A . . . - - - . . . . . G . . . . . C. parvum H279
491 . . . . . T . . . T T T . . . . . G . . . . . T . . . . . C. hominis H251
491 . . . . . T . . . T T T . . . . . G . . . . . T . . . . . C. hominis H172
491 . . . . . T . . . T T T . . . . . G . . . . . T . . . . . C. hominis FJ707311.1
490 . . . . A . . . - - - . . . . . C. meleagridis EU814439.1
490 . . . . . A . . . T - . . . . . C. wriari AF115378.1

'.' indicates the residues that match the consensus exactly and residues with grey colour differ from the consensus

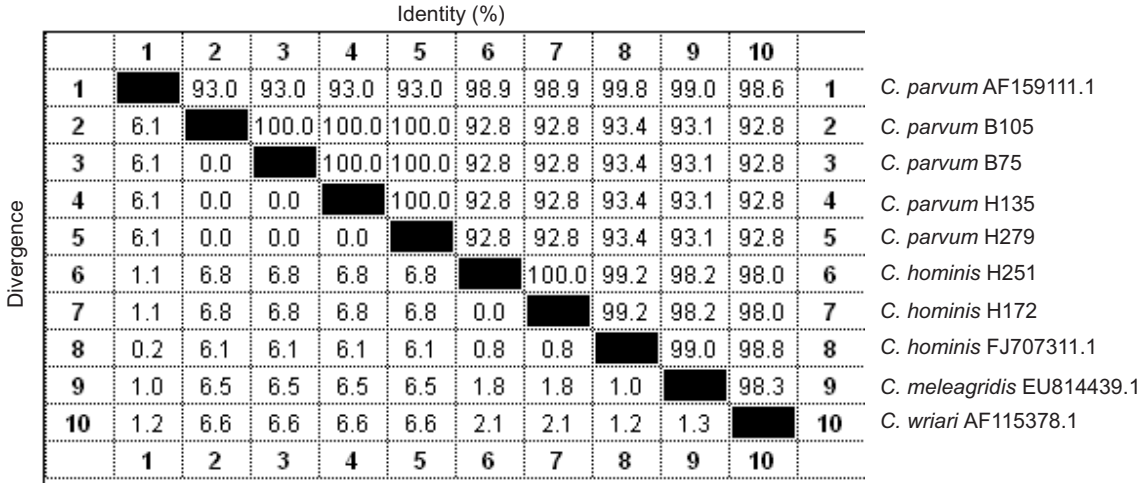


Fig. 6. Per cent similarity/divergence among nucleotide sequences of SSU rRNA gene among different species of *Cryptosporidium* of bovine and human origin.

for *Cryptosporidium parvum.* In all the four cases the common space used for playing by the children during morning and evening is also shared by calves due to overcrowding. So during playing, children unconsciously come in direct contact with the animals, their faecal material or contaminated mud. Due to poor personal hygiene and socio-economic condition they are not habituated to wash

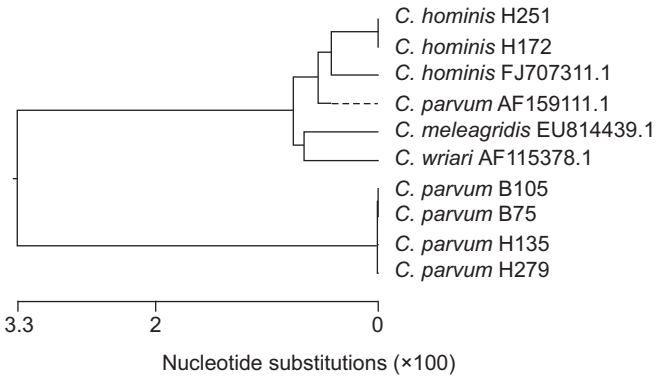


Fig. 7. Phylogenetic analysis based on nucleotide sequences of SSU rRNA gene among different species of *Cryptosporidium* of bovine and human origin.

their hands properly before taking their meals. Therefore, it is quite possible that the children got the infection directly from the cattle. These findings clearly indicate the presence of zoonotic transmission of *Cryptosporidium parvum* from bovine to human. Further studies with large number of samples and extensive epidemiological data are required for a better characterization and transmission pattern of cryptosporidiosis in India. PCR-RFLP can be used as a potential and reliable tool for species identification of *Cryptosporidium* in both bovine and human.

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