Indian Journal of Medical Microbiology, (2007) 25 (2):133-6

Original Article

MOLECULAR CHARACTERISATION OF *CRYPTOSPORIDIUM*: AN EMERGING PARASITE

*** K Nagamani, PRR Pavuluri, M Gyaneshwari, K Prasanthi, MIS Rao, NK Saxena**

Abstract

Purpose: The aim of the present study was to determine the prevalence of *Crytposporidium* in local population and to understand its epidemiology by molecular methods. **Methods:** Faecal samples from 681 children and 804 adults, admitted to tertiary care hospitals in twin cities of Hyderabad and Secunderabad with complaints of diarrhoea; and six calves with diarrhoea, were screened for *Cryptosporidium* oocysts by microscopy and enzyme linked immunosorbent assay (ELISA). Polymerase chain reaction restriction fragment length polymorphism (PCR RFLP) based identification of *Cryptosporidium* species in positive specimens was done to elucidate epidemiology of *Cryptosporidium.* **Results**: *Cryptosporidium* was found in 52 (7.6%) children and 7(0.9%) adults and 1(16.6%) calf with diarrhoea. The prevalence of *Cryptosporidium* in children below five years of age was 8.2% and 14.3% in children in the age group of six months to one year. Of the 42 samples genotyped 29 (69%) were *C. hominis* and 8 (19%) were *C. parvum* and 5 (11.9%) were mixed infection with the two species. **Conclusions**: Children in the age group of six months to one year were found to be the most vulnerable. The occurrence of *C. parvum*, in nearly one third of cases in the present series indicates that the zoonotic transmission is of considerable significance in the epidemiology *of Cryptosporidiosis* in the study area.

Key words: *Cryptosporidium, diarrhoea, polymerase chain reaction, restriction fragment length polymorphism*

Cryptosporidium is an intracellular, extracytoplasmic coccidian parasite with a monoxenous life cycle. It invades the microvillous border of gastrointestinal and respiratory epithelium of a wide range of vertebrate animals and is associated with watery diarrhoea in mammals, diarrhoea and respiratory illness in birds and gastroenteritis in reptiles and fish. Towards the end of twentieth century *Cryptosporidium* emerged as an important etiologic agent of epidemic and endemic diarrhoeal disease worldwide affecting mostly children and immunocompromised individuals.[1-4]

Recognition of existence of genetic diversity in *Cryptosporidium* led to the development of molecular techniques for identification of morphologically indistinguishable species. This helped researchers to understand the epidemiology and public health significance of *Cryptosporidium* spp. found in animals and environment.[2,5,6] Of the 13 species named, six have been reported to be responsible for human infection. *C. hominis* is the most common species reported from developing countries suggestive of anthroponotic transmission.[7] In contrast in United Kingdom and Kuwait *C. parvum* was found to be the most prevalent species indicating the role of zoonotic transmission.[8,9] Besides *C. muris, C. felis, C. meleagridis, C. canis* have been infrequently reported in children and immunocompromised persons.[10-12]

In India, several studies have documented the prevalence of *Cryptosporidium* in paediatric diarrhoea (1.3 to 13.1%) from different parts of the country based on microscopic detection of oocysts in faecal specimens.[13-15] However, there are no published reports on species identification of the parasite in paediatric or animal isolates which can be useful in understanding the epidemiology of cryptosporidiosis.

The present study was undertaken to determine the prevalence of *Cryptosporidium* in and around Hyderabad and Secunderabad and also to understand its epidemiology by using molecular methods.

Materials and Methods

The institutional ethical committee approved the study. Faecal samples were collected from 681 children and 804 adults admitted in the tertiary care hospitals in twin cities of Secunderabad and Hyderabad between 2003 and 2006. The inclusion criteria included complaints of diarrhoea and other gastrointestinal symptoms. Relevant demographic, clinical and epidemiological data was recorded regarding the duration of symptoms, age, gender, socioeconomic status, source of water and exposure to animals. In addition, faecal samples were collected from six calves with diarrhoea from a nearby farm.

Conventional screening of all the faecal specimens by microscopy after concentration by formalin ethyl acetate sedimentation method was carried out for detection of various

*Corresponding author (email: <nagamaniy2k03@rediffmail.com>) Departments of Microbiology (NK, KP, NKS) and Paediatrics (MISR), Gandhi Medical College, Secunderabad - 500 020; Department of Biotechnology (PRRP), College of Veterinary Sciences, Hyderabad - 500 030; and Department of Microbiology (GM), Shadan Institute of Medical Sciences, Hyderabad - 500 008, Andhra Pradesh, India

Received : 23-08-06
Accepted : 06-12-06

This PDF is available for free download from a site hosted by Medknow Publications (www.medknow.com).

ova and cysts. Modified Ziehl-Neelsen (ZN) stain using 5% H_2SO_4 was used to stain smears. They were examined by bright field microscopy by screening 200 oil immersion fields for oocysts of *Cryptosporidium*.[16]

All the samples were also tested for *Cryptosporidum* antigen by ELISA using Ridascreen (R.Biopharma, Darmstadt, Germany) as per manufacturers' instructions**.** DNA extraction was done from the samples positive for *Cryptosporidium* as described by Xiao and the DNA thus obtained was purified by using QIA amp DNA stool kit to remove inhibitors and was stored at -20°C. Further genomic analysis was carried out by a two-step nested PCR protocol for amplification followed by RFLP analysis.[6]

A two-step nested PCR protocol was used to amplify the SSU rRNA gene using primers, which amplified sequences unique to all species of *Cryptosporidium* genus.

A PCR product of 1.3 kbp was amplified using forward. (5'TTCTAGAGCTAATACATGCG-3) and reverse (5' CCATTTCCTTCGAAACAGGA - 3') primers. Each PCR mixture (100 µL volume) contained 10 µL of 10x Taq Buffer, dNTP (10 mM each), primers 20 pM each, MgCl 1.5 mM, bovine serum albumin 0.01.mg, 2.5 U Taq polymerase and 2 µL of DNA template.

A total of 35 cycles each consisting of 94°C for 45 seconds, 55°C for 45 seconds and 72°C for 60 seconds were performed with initial hot start at 94°C for three minutes and a final extension step at 72°C for seven minutes. For the secondary nested PCR step, a PCR product that was 836-849 bp long (depending on species) was amplified by using 2 µL of a primary PCR product and primers (5'GGAAGGGTTGTATTTATTAGATAAAG3') and (5'CTCATAAGGTGCTGAAGGAGTA -3). The PCR mixture and cycling conditions were identical to the conditions used for primary PCR except that the annealing temperature was at 58°C.

Restriction fragment length polymorphism

For restriction fragment length analysis 20 µL of nested PCR product was digested in a 50 µL reaction mixture containing 10 units of Sspl or Vsp1 (Fermentas, Life science) for genotyping and 5 µL of appropriate restriction buffer at 37°C overnight. The digested products were fractionated on a 2% agarose gel containing ethidium bromide at a concentration of 0.5 µg/mL.

Results

Out of 1485 faecal samples examined, 59 (4.0%) samples were positive for *Cryptosporidium* (51 by microscopy and ELISA, 8 by ELISA). *Cryptosporidium* was found in 52 out of 681 (7.6%) children and seven out of 804 (0.9%) of adults with diarrhoea. Highest prevalence was in the age group of six months - one year (14.3%). The prevalence progressively decreased with increasing age (Table) and was very low (0.9%) in the adult population. The association between age and *Cryptosporidium* infection was found to be highly significant on statistical analysis ($P<0.01$)**.** The youngest child to be infected in our study was one month old. Of the six bovine samples examined, only one (16.7%) was positive for *Cryptosporidium*.

All the microscopy positive samples appeared dark green to olive green in colour, watery or semi formed with foul odour. From four children with cryptosporidiosis, stool samples could be repeated every day. It was observed that as these samples became negative on microscopy the character of stool changed from watery green colour to formed yellow colour. There were no leukocytes or erythrocytes on microscopy.

Cryptosporidium cases occurred through out the year, but highest prevalence was seen during the monsoon in the months of August and September (Fig. 1). The duration of illness ranged from 3 to 30 days, the median duration was 4 days. There was no significant difference in infection rate among male (31) and female (28) cases. All cases were from families of low socioeconomic status. Tap water supplied by the municipal corporation was the only source of water. There was no correlation between the species isolated and contact with animals. No history of contact with animals could be elicited in the 13 cases in which *C. parvum* was detected. In four cases with history of contact with cattle, *C. hominis* was isolated.

Table: Age wise distribution of patients with *Cryptosporidium* infection

Age	Total no. of patients with diarrhoea	*Cryptosporidium* positive cases No. (%)
0-6 months	264	16 (6.1)
6 months - 1 year	105	15 (14.3)
1-2years	93	8 (8.6)
2-5 years	117	8 (6.8)
5-12 years	102	5 (4.9)
>12 years	804	7 (0.9)

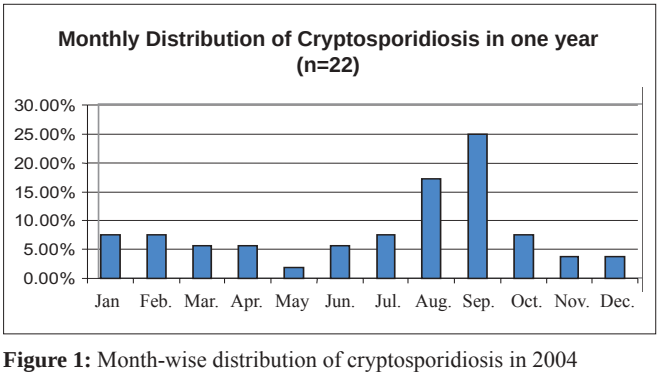

Figure 1: Month-wise distribution of cryptosporidiosis in 2004

This PDF is available for free download from a site hosted by Medknow Publications (www.medknow.com).

Forty-two samples, 39 from children and three from adults were genotyped. *C. parvum* and *C. hominis* generated three visible bands of 447, 270 and 101 bp on Ssp1 digestion. The two species were differentiated by the vsp1 digestion pattern. *C. parvum* produced two visible bands of 627 and 115 bp. *C. hominis* generated two visible bands at 556 and 115 bp due to presence of one additional vsp1 restriction site (Fig. 2).

RFLP analysis of the nested PCR products showed that 29 (69.0%) were *C. hominis*, eight (19.0%) were *C. parvum*. Mixed infection with *C. parvum* and *C. hominis* was found in five (11.90%). Occurrence of mixed infection in these cases was confirmed by overnight digestion of nested PCR product using double the concentration of the enzymes. Only *Cryptosporidium* found in the bovines was identified as *C. parvum*.

Discussion

In the developing world, the association of *Cryptosporidium* with acute and persistent diarrhoea in children is striking. Several cross sectional studies in children with diarrhoea suggest that cryptosporidiosis is endemic in developing world with prevalence of up to 26% in Mexican and 16.5% in Brazilian children with diarrhoea.[4] Prevalence in African countries, Central and South American countries, Asian countries is greater than in Europe and North America.[16] In our study the prevalence in children under five years was 8.12 and 7.63% in children under 12 years. In the neighbouring countries of Pakistan and Indonesia the parasite has been found in 10.3 and 8.2% of children with diarrhoea respectively.[17,18] In India, most of the earlier studies in paediatric diarrhoea were based on microscopic examination of faecal samples. The parasite was detected in 1.4% of cases of paediatric diarrhoea in North, 5.5% from East, 5.6% from West and 13.1% from South Indian children.[13-15]

Cryptosporidium has been reported in persons from three days to 95 years of age. But data suggest that young children are more susceptible to infection.[2] In our study, children in the age group of six months to one year were found to be the most vulnerable. This corresponds to the weaning period when the children are exposed to contaminated environment, food and water. Similar observations were made in Indonesia, Pakistan and Ghana where children less than 2 years old were found to be the most susceptible.[17,18] In contrast, higher age group (4.5 years) was found to be the most susceptible in Kuwait.[9]

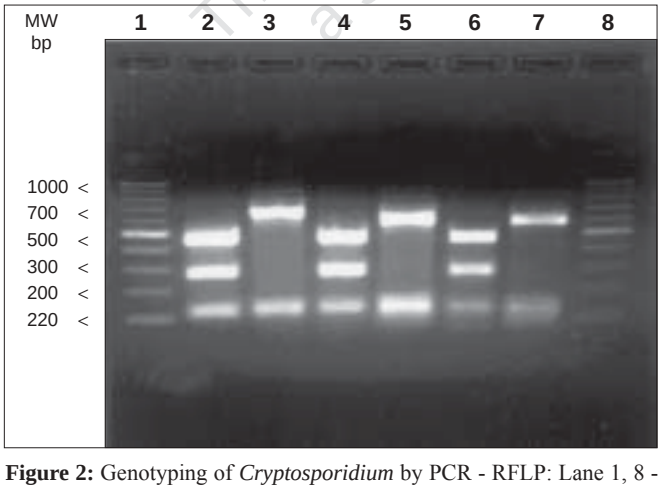

Figure 2: Genotyping of *Cryptosporidium* by PCR - RFLP: Lane 1, 8 - 100bp marker; Lane 2, 3 - *C. parvum* (lane 2: SSP1, lane-3: VSP1); Lane 4, 5, 6, 7 - *C. hominis* (lane- 4, 6: SSP1, lane- 5,7: VSP1)

The duration of illness ranged from two days to 30 days. Median duration of diarrhoea was four days, which is similar to the report from a cohort study in Brazil in which the median number of days per diarrhoeal episode was three days.[4]

In tropical countries, prevalence of cryptosporidiosis is highest in rainy season. This could be due to contamination in surface waters in catchments areas following rains. Moreover oocysts of *Cryptosporidium* are sensitive to heat and resistant to chlorine.[2] Viable oocysts are likely to be present in more numbers in water during rainy season. Similarly in the present study cryptosporidiosis was found to be a seasonal disease. Highest number of cases occurred in the months of August and September when the rainfall is highest. Similar observations were made in Brazil and Indonesia.[4,18] In Kuwait where rainfall is scanty highest prevalence was found in cool months of November to April.[9]

Development of molecular tools to identify morphologically indistinguishable species has played a key role in defining the relationship between parasite species, potential hosts and pathways of transmission. Even though the prevalence of cryptosporidiosis in tropical countries is high, limited studies have been conducted to characterize *Cryptosporidium* species from humans at molecular level. Characterization of *Cryptosporidium* isolates from Brazil, Malawi, Kenya, Vietnam and Peru have shown *C. hominis* to be the most prevalent indicating the predominance of anthroponotic transmission.[7,10] There are no published reports on *Cryptosporidium* species identification from children in India. However, in South Indian HIV infected patients *C. hominis* was found to be the predominant species.[11] In this study also *C. hominis* was the most common species suggestive of anthroponotic transmission. In contrast, in Kuwait and England, zoonotic species *C. parvum* was found to be the most common species reported in 94% and 84% cases respectively indicating the importance of zoonotic transmission in these countries.[9,8]

Other zoonotic species like *C. meleagridis, C. felis* and *C. muris* were also reported from children from Peru which were not found in our study.[10] However, *C. parvum* was found in 30.9% cases either singly (19.0%) or as mixed infection with *C. hominis* (11.9%) in this study. There was no correlation of species identified with animal contact and municipal tap water was the only source of water. Besides *C. parvum* was found in 16.6% of calves tested. In a study from rural area in Maharashtra five out of 13 calves tested were found to be shedding oocysts of *C. parvum*.[19] In Australia, 48.1% of calves

This PDF is available for free download from a site hosted by Medknow Publications (www.medknow.com).

were found to be infected with *Cryptosporidium* spp. when the infection appeared within one - three weeks after birth.[20] Genotyping of *Cryptosporidium* isolates from human cases from Kuwait and UK revealed zoonotic sources of infection involving livestock to be major route of transmission in humans.[8,9]

Cattle rearing are a common occupation in urban and suburban areas of Andhra Pradesh. The occurrence of *C. parvum* in nearly one third of cases of cryptosporidiosis indicates zoonotic transmission to be of considerable significance in the epidemiology of *Cryptosporidium* in this State. In tropical countries, waterborne transmission is considered a major route in epidemiology of cryptosporidiosis. Similarly, water may be the major route of transmission of *C. hominis* and *C. parvum* genotypes which is evident from existence of significant proportion of mixed infections with *C. parvum* and *C. hominis* and the high prevalence during rainy season. Besides, there is no correlation of species identified and animal contact and municipal tap water was the only source of water. This shows the need for better protection of water catchments from livestock and improved drinking water treatment.

Further studies on characterization and subtyping of human, animal and environmental isolates are required to evaluate the public health significance of *C. parvum* infection in livestock, the exact sources and routes of transmission and population dynamics of *Cryptosporidium*. Control measures based on such knowledge shall be useful in reducing morbidity and mortality due to cryptosporidiosis in children.

Acknowledgement

This study was supported by a grant from Department of Biotechnology, Government of India, New Delhi (BT/PR/2716/MED). We thank LiHua Xiao of the CDC, Atlanta, USA, for supplying positive control slides and his expert guidance in this study.

References

1. Current WL, Garcia LS. *Cryptosporidiosis*. *Clin Microbiol Rev* 1991;**4:**325-58.
2. Fayer R**.** *Cryptosporidium:* A water borne zoonotic parasite. *Vet Parasitol* 2004**;126:**37-56.
3. Mac Kenzie WR, Hoxie NJ, Procter ME, Gradus MS, Blair KA, Peterson DE, *et al*. A massive outbreak in Milwaukee of *Crytposporidium* infection transmitted through the public water supply. *N Eng J Med* 1994**;331:**161-7.
4. Newman RD, Sears CL, Moore SR, Nataro JP, Wuhib T, Agnew DA, *et al***.** Longitudinal study of *Cryptosporidium* infection in children in northeastern Brazil. *J Infect Dis* 1999;**180:**167-75.
5. Xia L, Fayer R, Ryan U, Upton SJ. *Cryptosporidium* taxonomy: Recent advances and implications for public health. *Clin Microbiol Rev* 2004;**17:**72-97.
6. Xiao L, Morgan UM, Limor J, Escalante A, Arrowood M, Shulaw W, *et al*. Genetic diversity within *Cryptosporidium parvum* and related *Cryptosporidium* species. *Appl Environ Microbiol* 1999;**65:**3386-91.
7. Gatei W, Greensill J, Ashford RW, Cuevas LE, Parry CM, Cunliffe NA, *et al*. Molecular analysis of 18s r RNA gene of *Cryptosporidium* parasites from patients with or without human immunodeficiency virus infections living in Kenya, Malawi, Brazil, The United Kingdom and Vietnam. *J Clin Microbiol* 2003;**41:**1458-62.
8. Goh S, Reacher M, Cassemore DP, Verlande NQ, Chalmers R, Knowles M, *et al*. *Sporadic Cryptosporidiosis*, North Cumbria, England, 1996-2000. *Emerg Infect Dis* 2004;**10**:1007-15**.**
9. Sulaiman IM, Hira PR, Zhou I, Al-Ali FM Shelahi FA, Shweiki HM, Iqbal J, *et al*. Unique endemicity of *Cryptosporidiosis* in children in Kuwait. *J Clin Microbiol* 2005;**43:**2805-9.
10. Xiao L, Bern C, Limor J, Sulaiman I, Roberts J, Checkley W, *et al***.** Identification of 5 types of *Cryptosporidium* parasites in Children in Lima, Peru. *J Infect Dis* 2001;**183:**492-7.
11. Muthusamy D, Rao SS, Ramani S, Monica B, Banerjee I, Abraham OC, *et al***.** *Multilocus* genotyping of *Cryptosporidium* sp. isolates from human immunodeficiency virus-infected individuals in South India. *J Clin Microbiol* 2006;**44:**632-4.
12. Pieniazek NJ, Bornay-Llinares FJ, Slemenda SB, Alexander da Silva J, Moura IN, Arrowood MJ, *et al***.** *New cryptosporidium genotypes* in HIV infected persons. *Emerg Infect Dis* 1999; **5:**444-9.
13. Sehgal R, Rai SK, Sharma P, Malla N, Ganguly NK, Walia BN, *et al***.** Cryptosporidium as a cause of diarrhoea in children. *Indian J Med Microbiol* 1989;**7:**130-3.
14. Saraswathi K, Pandit DV, Deodhar LP, Bichiles LS. Prevalence of *Cryptosporidia* in patients with diarrhoea in Bombay**.** *Indian J Med Res* 1988;**87:**221-4.
15. Mathan MM, Venkatesan S, George R, Mathew M, Mathan VI**.** *Cryptosporidium* and diarrhoea in southern Indian children. *Lancet* 1985;**2**:1172-5.
16. Garcia LS, Bruckner DA, Brewer TC, Shimizu RY. Techniques for the recovery and identification of *Cryptosporidiuum* oocysts from stool specimens. *J Clin Microbiol* 1983;**18:**185-90.
17. Iqbal J, Munir MA, Khan MA**.** *Cryptosporidium* infection in young children with diarrhea in Rawalpindi, Pakistan. *Am J Trop Med Hyg* 1999;**60**:868-70.
18. Katsumata T, Hosea D, Wasito EB, Kohno S, Soeparto P, Ranuh IG**.** *Cryptosporidiosis* in Indonesia: A hospital based study and a community based survey. *Am J Trop Med Hyg* 1998;**59:**628-32.
19. Khubnani H, Sivarajan K, Khubnani AH**.** *Study of Cryptosporidiosis* in a rural area of Maharashatra**.** *Indian J Patho Microbiol* 1997**;40:**33-6.
20. Becher KA, Robertson ID, Fraser DM, Palmer DG, Thompson RC. Molecular epidemiology of *Giardia and Cryptosporidium* infections in dairy calves originating from three sources in Western Australia**.** *Vet Parasitol* 2004;**123:**1-9.

Source of Support: Department of Biotechnology, Government of India, New Delhi (BT/PR/2716/MED).,**Conflict of Interest:** None declared.

This PDF is available for free download from a site hosted by Medknow Publications (www.medknow.com).