Prevalence of cryptosporidiosis in symptomatic immunocompetent children and comparative evaluation of its diagnosis by Ziehl–Neelsen staining and antigen detection techniques

Rumpa Saha, Bhoomika Saxena, Sungtila T. Jamir, Shwetank Shekhar

Departments of Microbiology, University College of Medical Sciences and Guru Teg Bahadur Hospital, Delhi University, ¹MBBS Student, University College of Medical Sciences and Guru Teg Bahadur Hospital, Delhi University, Delhi, India

Abstract

Background: Cryptosporidia is a major pathogen causing diarrhoea and with increasing morbidity and mortality. As persistent diarrhoea from intestinal cryptosporidiosis leads to increased susceptibility to recurrent diarrheal episodes further leading to chronic nutritional and cognitive sequelae or even death, diagnosis is important. Most of the studies done on Cryptosporidium worldwide have focused on immunocompromised patients which have led to a paucity of data on its prevalence among immunocompetent people.

Aims and Objectives: Keeping these facts in mind the present study was aimed to estimate prevalence of cryptosporidiosis in immunocompetent children, and do a comparative evaluation of its detection by microscopy with antigen detection methods.

Material and Methods: 80 immunocompetent children (40 OPD children presenting with diarrhea and 40 children hospitalized for diarrhea) upto age of 5 years were studies and their stool samples were compared by microscopy by mZN with copro-antigen detection methods (using rapid ICT and ELISA) for the diagnosis of Cryptosporidiosis.

Results: A Cryptosporidium prevalence rate of 22.5% was detected in the immunocompetent children upto 5 years of age. Microscopy remained the preferred method of diagnosis for Cryptosporidium being a more sensitive test and considering it’s low cost in resource poor settings. Moderate agreement between mZN and ELISA in Cohen’s kappa test shows that either of the tests can be used for diagnosis of Cryptosporidium from fecal sample. ELISA is time-saving method but ELISA and rapid antigen tests should not be used as the sole method of diagnosis. Keeping in view the ICT kit used in this study is species specific, and the species identification was not carried out in the present study, hence genus specific kits may be useful for diagnosis in such settings.

Conclusion: Microscopy remains the preferred method of diagnosis for Cryptosporidium having good sensitivity and specificity and considering it’s low cost in resource poor settings. ELISA is time-saving method but ELISA and rapid antigen tests should not be used as the sole method of diagnosis.

Keywords: Cryptosporidium, diagnosis, immunocompetent children

Address for correspondence: Dr. Rumpa Saha, 3rd Floor, Department of Microbiology, University College of Medical Sciences and Guru Teg Bahadur Hospital, Delhi University, Delhi - 110 095, India.
E-mail: rumpachatterjee@yahoo.co.in
DOA: 08-03-2019, DOP: 22-05-2019

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

How to cite this article: Saha R, Saxena B, Jamir ST, Shekhar S. Prevalence of cryptosporidiosis in symptomatic immunocompetent children and comparative evaluation of its diagnosis by Ziehl–Neelsen staining and antigen detection techniques. Trop Parasitol 2019;9:18-22.

© 2019 Tropical Parasitology | Published by Wolters Kluwer - Medknow
INTRODUCTION

In India, diarrhea is a leading cause of death among children under 5 years, and Cryptosporidium is one such major pathogen causing diarrhea with increasing morbidity and mortality. Most of the human infections are caused mainly by two species of cryptosporidia, i.e., Cryptosporidium hominis and Cryptosporidium parvum. It has been documented that greater shedding of oocysts for longer duration leading to more severe diarrhea is more common with C. hominis. Although initially reported in immunocompromised individuals, it has also been found in immunocompetent persons.

A high prevalence (27.4%) of Cryptosporidium infection has been reported in immunocompetent children under the age of 5 years in India. Several other studies have shown that the prevalence of Cryptosporidium is high in developing countries.

In immunocompetent hosts, C. parvum has been known to cause acute, self-limiting, watery diarrhea lasting 5–10 days. However, in the immunocompromised and malnourished individuals, diarrhea is usually prolonged. In malnourished children, persistent diarrhea leads to increased susceptibility to recurrent diarrheal episodes, which can lead to chronic nutritional and cognitive sequelae or even death. It has been demonstrated in young Peruvian children that even asymptomatic C. parvum infection may lead to stunting, malnutrition, and lack of catch-up growth. Hence, prompt identification of this agent is important to diagnose and study the etiology of childhood diarrhea.

Light microscopy using Kinyoun’s-modified acid-fast staining is the most commonly used method for its diagnosis. Nowadays, the application of Cryptosporidium antigen detection techniques on stool samples is becoming increasingly popular. Copro-antigen techniques, include rapid antigen immunochromatographic tests (ICTs) and enzyme-linked immunosorbent assay (ELISA), act as easy diagnostic tools.

Most of the studies published on Cryptosporidium worldwide have focused on immunocompromised patients which have led to a paucity of data on its prevalence among immunocompetent people. Keeping these facts in mind, the present study was focused to estimate this parasite burden in immunocompetent children under the age of 5 years presenting with diarrhea and to evaluate modified Ziehl–Neelsen (mZN) staining with copro-antigen detection methods for its diagnosis.

METHODOLOGY

This cross-sectional study conducted during May–July 2018 was done in the department of microbiology of our tertiary care hospital. The study population included children <5 years of age presenting with acute or persistent diarrhea at the pediatric outpatient department (OPD) and those requiring admission to the hospital for diarrhea. Diarrhea was defined as ≥3 loose/liquid stools in a 24-h period or when bowel movements occurred more frequently than the child’s normal amount. Symptoms lasting <2 weeks were considered acute diarrhea, while 14–29 days of symptoms was considered persistent diarrhea. Children with known immunodeficiency, those with known allergy to lactose, gluten, or any other food and those receiving antiparasitic medication for the current episode of diarrhea within the past 7 days, were excluded from this study. All diarrhea patients who (i) had severe dehydration, (ii) were not accepting orally, (iii) had repeated vomiting, (iv) were not passing urine, (v) were drowsy, (vi) had any other coinfection like pneumonia, and (vii) had persistent diarrhea were admitted to the hospital.

Eighty stool samples were collected from 40 OPD children presenting with diarrhea and 40 children hospitalized for diarrhea.

Each sample was divided into three parts as follows:

1. The first part was concentrated by formol-ether concentration technique and smear prepared from deposit. Smears were stained by mZN staining after fixation in absolute alcohol for 10 min. Cryptosporidium was identified by its characteristic size (4.5–6 μm), round to slight ovoid shape, and staining variably pink with unstained sporulated crescentic forms seen within the oocyst.

2. The second part was subjected to rapid antigen detection by EpiTuub® Fecal C. parvum antigen rapid ICT kit as per the manufacturer’s instruction (Epitope Diagnostics, Inc., San Diego, USA).

3. The third part was preserved in 10% formalin and was subjected to ELISA following manufacturer’s instruction (DRG Diagnostics, Germany).

All the samples were discarded as per the BMW guidelines following completion of the work.

All the data collected were entered in MS-Excel and SPSS v20.0 were used for data analysis (IBM, Armonk, NY, USA). All qualitative assessment is expressed as percentage. Comparison of abilities of the various methods used for diagnosis and detection of cryptosporidiosis was done by
Chi-square test. \( P < 0.05 \) was considered as statistically significant. As there was no positivity with ICT test, the diagnostic accuracy (inter-rater reliability) of mZN and ELISA was assessed by Cohen’s kappa test.

RESULTS

In the present study, 80 stool samples were collected. Forty samples were collected from children hospitalized for diarrhea. Forty samples were from children attending an outpatient clinic with a history of diarrhea.

Of the total 80 samples, 18 were found positive for Cryptosporidium by any one technique(s), indicating 22.5% prevalence in immunocompetent children <5 years.

By microscopy (mZN), total positive samples were 16 (20%) (mZNs positivity in OPD: hospitalized = 6 [15%]:10 [25%]), ICT total positive samples were nil, and ELISA total positive samples were 8 (10%) (ELISA positivity in OPD: hospitalized = 3 [7.5%]: 5 [12.5%]). Eight samples were positive by microscopy alone, whereas two samples were positive by only ELISA. Six samples (7.5%) were positive by both microscopy and ELISA.

Sample positivity by various techniques in both hospitalized and OPD children are shown in Figure 1.

Of the total 18 positive samples, 7/40 (17.5%) were from the OPD patients and 11/40 (27.5%) were from the hospitalized patients who were positive for Cryptosporidium.

Keeping ELISA as the gold standard, the sensitivity, specificity, positive predictive value (PPV), and negative predictive value were 75%, 86.11%, 37.5%, and 96.87%, respectively, for mZN and 0%, 100%, 0%, and 90%, respectively, for ICT [Table 1].

As there was no positivity with ICT test, the diagnostic accuracy (inter-rater reliability) of mZN was assessed by Cohen’s Kappa test, and the results were interpreted as per Table 2.\(^{[17]}\) The interrater reliability (kappa value) by comparing ELISA with mZN was calculated to be 0.423, thus suggesting a moderate agreement between two tests.

Table 3 shows the age group distribution of children affected by Cryptosporidium. Children <1 year of age were most affected in the present study. Females were overall twice more affected with the disease (M:F = 1:2).

DISCUSSION

In the present study, 18 samples were positive for Cryptosporidium by either one of the diagnostic techniques giving a prevalence rate of 22.5% in the immunocompetent children <5 years, which is comparable with studies from India (3%–30%).\(^{[18,19]}\) A study from the same institution 6 years back also documented a similar prevalence (27.4%) in under 5 years immunocompetent children.\(^{[9]}\) The present study showed that children hospitalized for diarrhea had higher positivity of Cryptosporidium as compared to the OPD patients with diarrhea indicating greater severity in those who required hospitalization. This finding is comparable to a Brazilian study which reports more severe diarrhea with C. hominis.\(^{[6]}\) Although children <1 year were most affected in the present study, most studies from India

---

**Table 1: Comparison of modified Ziehl-Neelsen and immunochromatographic test with enzyme-linked immunosorbent assay**

| Detection technique | ELISA (Gold standard) | Total |
|---------------------|-----------------------|-------|
|                      | Positive | Negative |       |
| mZN                 | Positive | 6         | 10    | 16   |
|                     | Negative | 2         | 62    | 64   |
|                     | Total    | 8         | 72    | 80   |
| ICT                 | Positive | 0         | 0     | 0    |
|                     | Negative | 8         | 72    | 80   |
|                     | Total    | 8         | 72    | 80   |

ELISA: Enzyme-linked immunosorbent assay, mZN: Modified Ziehl-Neelsen, ICT: Immunochromatographic test

**Table 2: Interpretation of Cohen’s kappa test**

| \( \kappa \) | Interpretation         |
|--------------|------------------------|
| <0           | Less than chance agreement |
| 0.01-0.20    | Slight agreement     |
| 0.21-0.40    | Fair agreement      |
| 0.41-0.60    | Moderate agreement   |
| 0.61-0.80    | Substantial agreement|
| 0.81-0.99    | Almost perfect agreement |

**Table 3: Cryptosporidium positivity in different age groups**

| Age group (years) | Total positives (%) |
|-------------------|---------------------|
| 0-1               | 8 (10)              |
| >1-2              | 5 (6.25)            |
| >2-3              | 3 (3.75)            |
| >3-4              | 2 (2.5)             |
| >4-5              | 0                   |

---

![Figure 1: Cryptosporidium positivity by different techniques](image-url)
and outside India have shown the highest prevalence in children under 5 years.\[22\]

About 20% positivity was seen by microscopy, whereas 10% were positive by ELISA indicating higher positivity by microscopy in comparison to ELISA. Analogous results have been documented from the USA and Egypt.[21,22] Low positivity in ELISA may be because the monoclonal antibodies used by different commercially available copro-antigen detection ELISA kits may recognize different sets of surface epitopes and thus may not react with antigens of different Cryptosporidium species resulting in false negativity in ELISA. Two samples which were positive only by ELISA, but negative microscopically, can be false positive due to clearance of oocyst from the gut, but persistence of antigen in patients who have been recently treated.[19] Low sensitivity of Cryptosporidium ELISA test has been reported from Atlanta, and hence the results should be confirmed with mZN staining, especially in low-prevalence areas.[23] Advantages of ELISA over mZN staining are that more number of samples can be tested at the same time. Moreover, microscopy skills and concentration of the fecal samples are also not required for antigen detection.

In our study, ICT (Epitope Diagnostics, Inc. San Diego, USA) gave no positive results. As the kit used in the present study detects only C. parvum antigen, so this could be one of the possible reasons that the diarrheal infections in the current study may have been caused by other species of Cryptosporidium.[18] Ideally, a genus specific would be more appropriate for routine diagnosis, especially in areas where species prevalence is unknown. A recent community-based study from Vellore found C. hominis in children to be the most common Cryptosporidium species.[24] Another reason for false negativity can be a low parasite load in the stool. A parasite load of >175 oocysts/10 μl fecal sample is necessary for ICT positivity as reported by Johnston et al.[23] The same authors suggest that the sensitivity of ICT varies between 67% and 91%, with a higher specificity of up to 99% comparable to the present study. Researchers from various studies who have evaluated the following ICT kits have detected C. parvum-specific antigen in ImmunoCard STAT (Meridian Bioscience Inc., USA); RIDA®QUICK Cryptosporidium (R Biopharm, Germany); and CoproStrip Cryptosporidium (Savyon Diagnostics Ltd., Israel) with a 67%–84% sensitivity and 98%–100% specificity. However, Dia-Pro CA-RT cryptosporidium (Dia-pro diagnostics, Italy) and Crypto-Strip (Coris BioConcept Inc., Belgium) have used genus-specific antigen detection kits having similar sensitivity (61%–86%) and specificity (99%–100%).[22,25] The specificity of the ICT kit used in the present study although comparable with the above, a larger sample size would be needed for appropriate evaluation of this kit. To the best of our knowledge from the available literature, Epitope Diagnostics, Inc., San Diego, USA, EpiTuub® Fecal C. parvum antigen detection kit has not been used so far by any documented studies. Hence, the use of this kit should be further evaluated by documented studies.

As none of our samples were positive for C. parvum antigen by ICT, the possibility that C. hominis can be the pathogenic Cryptosporidium in our patients cannot be ruled out. This, however, needs confirmation using molecular methods. A multicentric study from India (Delhi, Trichy, and Vellore) found C. hominis in children to be the most common Cryptosporidium species.[24] As coinfections with other diarrheal agents were not assessed in the present study, hence Cryptosporidium cannot be labeled as the sole etiological agent of diarrhea in the present study.

Moderate agreement between mZN and ELISA in Cohen’s kappa test may be due to the small sample size of the size. The present study had good sensitivity and specificity of mZN and suggested that microscopy by mZN can be used for the diagnosis of Cryptosporidium from the fecal sample. Further, it detects all species although cannot differentiate between the species and hence will detect all Cryptosporidium oocysts. Studies from abroad have reported higher sensitivity of microscopy as compared to ELISA[22,23] and studies from Chandigarh and the UK report higher specificity for mZN.[26,27] As predictive values of a disease vary with the prevalence of the disease, the low PPV of mZN in the present study can be due to the low prevalence of the disease in our study population.

CONCLUSION

A Cryptosporidium prevalence rate of 22.5% was detected in the immunocompetent children up to 5 years of age. Microscopy remains the preferred method of diagnosis for Cryptosporidium having good sensitivity and specificity and considering its low cost in resource-poor settings. ELISA is a time-saving method, but ELISA and rapid antigen tests should not be used as the sole method of diagnosis.

Acknowledgment

The authors acknowledge the help of Mr. Room Ram Prami (technical assistant, Microbiology Laboratory, University College of Medical Sciences and Guru Teg Bahadur Hospital) for his technical assistance in the laboratory.
Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

REFERENCES

1. Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, et al. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (The Global Enteric Multicenter Study; GEMS): A prospective, case-control study. Lancet 2013;382:209-22.

2. Mor SM, Tsiangor S. Cryptosporidiosis in children in Sub-Saharan Africa: A lingering challenge. Clin Infect Dis 2008;47:915-21.

3. Molbak K, Hoijang N, Gottschau A, Sá JC, Ingholt L, da Silva AP, et al. Cryptosporidiosis in infancy and childhood mortality in Guinea Bissau, West Africa. BMJ 1993;307:417-20.

4. Mohandas, Sehgal R, Sud A, Malla N. Prevalence of intestinal parasitic pathogens in HIV-seropositive individuals in Northern India. Jpn J Infect Dis 2002;55:83-4.

5. Lanjewar DN, Rodrigues C, Saple DG, Hira SK, DuPont HL. Cryptosporidium, Isospora and Strongyloides in AIDS. Natl Med J India 1996;9:17-9.

6. Bushen OY, Kohli A, Pinkerton RC, Dupnik K, Newman RD, Sears CL, et al. Heavy cryptosporidial infections in children in Northeast Brazil: Comparison of Cryptosporidium hominis and Cryptosporidium parvum. Trans R Soc Trop Med Hyg 2007;101:378-84.

7. Houpt ER, Bushen OY, Sam NE, Kohli A, Asgharpour A, Ng CT, et al. Short report: Asymptomatic Cryptosporidium hominis infection among human immunodeficiency virus-infected patients in Tanzania. Am J Trop Med Hyg 2005;73:520-2.

8. Xiao L, 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.

9. Bera P, Das S, Saha R, Ramachandran VG, Shah D. Cryptosporidium infection in children with diarrhea: A hospital-based study. Indian Pediatr 2014;51:906-8.

10. Checkley W, White AC Jr., Jaganath D, Arrowood MJ, Chalmers RM, Chen XM, et al. A review of the global burden, novel diagnostics, therapeutics, and vaccine targets for Cryptosporidium. Lancet Infect Dis 2015;15:85-94.

11. Sarkar R, Tate JE, Ajijumpur SS, Kattula D, John J, Ward HD, et al. Burden of diarrhoea in children. PLoS Negl Trop Dis 2014;8:e3042.

12. Hunter PR, Nichols G. Epidemiology and clinical features of Cryptosporidium infection in immunocompromised patients. Clin Microbiol Rev 2002;15:145-54.

13. Khalil IA, Troger C, Rao PC, Blacker BF, Brown A, Brewer TG, et al. Morbidity, mortality, and long-term consequences associated with diarrhoea from Cryptosporidium infection in children younger than 5 years: A meta-analyses study. Lancet Glob Health 2018;6:e758-68.

14. Checkley W, Epstein LD, Gilman RH, Black RE, Cabrera L, Sterling CR, et al. Effects of Cryptosporidium parvum infection in Peruvian children: Growth faltering and subsequent catch-up growth. Am J Epidemiol 1998;148:497-506.

15. Tahvildar-Biderouni F, Sakhi N. Detection of Cryptosporidium infection by modified Ziehl-Neelsen and PCR methods in children with diarrheal samples in pediatric hospitals in Tehran. Gastroenterol Hepatol Bed Bench 2014;7:125-30.

16. Government of India. Ministry of Environment, Forest and Climate Change. New Delhi: Government of India; 2016. Available from: http://www.mpecb.gov.in/biomedical/pdf/BMW_Rules_2016. pdf. [Last accessed on 2018 Oct 04].

17. McHugh ML. Interrater reliability: The kappa statistic. Biochem Med (Zagreb) 2012;22:276-82.

18. Ghoshal U, Dey A, Ranjan P, Khanduja S, Agarwal V, Ghoshal UC, et al. Identification of opportunistic enteric parasites among immunocompetent patients with diarrhoea from Northern India and genetic characterisation of Cryptosporidium and Microsporidia. Indian J Med Microbiol 2016;34:60-6.

19. Ghoshal U, Jain V, Dey A, Ranjan P. Evaluation of enzyme linked immunosorbent assay for stool antigen detection for the diagnosis of cryptosporidiosis among HIV negative immunocompromised patients in a tertiary care hospital of Northern India. J Infect Public Health 2018;11:115-9.

20. Murugesan M, Ganesan SK, Ajijumpur SS. Cryptosporidiosis in children in the Indian subcontinent. Trop Parasitol 2017;7:18-28.

21. Newman RD, Jaeger KL, Wubah T, Lima AA, Guerriart RL, Sears CL. Evaluation of an antigen capture enzyme-linked immunosorbent assay for detection of Cryptosporidium oocysts. J Clin Microbiol 1993;31:2080-4.

22. Mohamed AM, Ahmed MA, Zagool DA, Ahmed SA. Molecular evaluation of conventional microscopic method versus fecal antigen capture ELISA and rapid immunochromatographic assay for diagnosis of Cryptosporidium infection. Infect Dis Clin Pract 2015;23:26-31.

23. Johnston SP, Ballard MM, Beach MJ, Causer L, Wilkins PP. Evaluation of three commercial assays for detection of Giardia and Cryptosporidium organisms in fecal specimens. J Clin Microbiol 2003;41:623-6.

24. Ajijumpur SS, Sankaran P, Kang G. Cryptosporidium species in HIV-infected individuals in India: An overview. Natl Med J India 2008;21:178-84.

25. Chalmers RM, Campbell BM, Crouch N, Charlett A, Davies AP. Comparison of diagnostic sensitivity and specificity of seven Cryptosporidium assays used in the UK. J Med Microbiol 2011;60:1598-604.

26. Destura RV, Cena RB, Galarion MJ, Pangilinan CM, Arevalo GM, Alba RO, et al. Advancing Cryptosporidium diagnostics from bench to bedside. Curr Trop Med Rep 2015;2:150-60.

27. Khurana S, Sharma P, Sharma A, Malla N. Evaluation of Ziehl-Neelsen staining, auramine phenol staining, antigen detection enzyme linked immunosorbent assay and polymerase chain reaction, for the diagnosis of intestinal cryptosporidiosis. Trop Parasitol 2012;2:20-3.

28. Kaushik K, Khurana S, Wanchu A, Malla N. Evaluation of staining techniques, antigen detection and nested PCR for the diagnosis of cryptosporidiosis in HIV seropositive and seronegative patients. Acta Trop 2008;107:1-7.