Scholars Journal of Applied Medical Sciences (SJAMS) **ISSN 2320-6691 (Online)**
Sch. J. App. Med. Sci., 2017; 5(9D):3805-3810 **ISSN 2347-954X (Print)**

(An International Publisher for Academic and Scientific Resources)
www.saspublisher.com

Evaluation of Different Diagnostic Techniques for Detection of *Cryptosporidium spp*. in Faecal Specimens

Farhat Tahira[1], Amit Kumar Singh[2*], Malay Banerjee[1], Taiyaba[3], Haris M. Khan[4], Indu Shukla[4]
1Department of Microbiology, Career Institute of Medical Sciences and Hospital, Lucknow
2Department of Microbiology, Mayo Institute of Medical Sciences, Barabanki
3Department of Microbiology, GCRG Institute of Medical Sciences and Hospital, Lucknow
4Department of Microbiology, Jawaharlal Nehru Medical College and Hospital, AMU, Aligarh

***Corresponding author**
Amit Kumar Singh

Article History
Received: 10.09.2017
Accepted: 16.09.2017
Published: 30.09.2017

DOI:
10.21276/sjams.2017.5.9.58

Abstract: *Cryptosporidium spp*. is a 4-6 µm coccidian parasite causing mild to fulminant diarrhoea especially in immunocompromised persons. It remains largely under-diagnosed by using current routine diagnostic techniques in microbiology laboratories. The aim of our study was to compare four different diagnostic techniques for the detection of *Cryptosporidium spp*. in faecal specimens in cases of both acute and chronic diarrhoea. The present study was carried out in the Department of Microbiology for the detection of *Cryptosporidium spp.* in stool samples collected from 177 children suffering from diarrhoea. All the stool samples were examined microscopically after concentration by the formol-ether sedimentation technique. Sediments were examined by iodine-stained wet mount preparations and were stained with two staining techniques – Modified Ziehl Neelsen (Z-N) and Safranine methylene blue staining techniques. Samples were further subjected to Enzyme Linked Immunosorbent Assay (ELISA). Chi-square and z tests were used to compare differences between the groups. A p value of ≤ 0.05 was considered significant. ELISA gave the highest rate of positivity (12.99%) followed by Safranine methylene blue staining and Z-N staining in which the yield was 11.9%. The wet mount preparation technique yielded the lowest number of positive samples (9%). We considered cryptosporidiosis to be a definite diagnosis if the organisms were found in any two of the four techniques. The overall prevalence of *Cryptosporidium spp*. was found to be 11.9%. Both the conventional staining methods and ELISA had similarities in sensitivity and sensitivity patterns. The conventional staining methods were found to be more cost-effective in comparison to ELISA but were found to be time-consuming, labour-intensive and required greater skill and experience.
Keywords: evaluation, diagnostic methods, cryptosporidium, children, diarrhoea .

INTRODUCTION

*Cryptosporidium spp.*have emerged as an important cause of diarrhoea among humans and animals [1]. It is a 4- to 6- µm coccidian parasite infecting the intestinal tract of humans. It causes mild to fulminant diarrhoea in patients, especially in immunocompromised persons. Children and elderly are at risk for more severe infections [2, 3].

Cryptosporidiosis is prevalent worldwide. Its prevalence varies widely in different parts of the world. A review of 78 reports showed *Cryptosporidium spp*. infection in 2.1% and 6.1% of immunocompetent persons with diarrhoea in industrialized and developing countries respectively [4].

For the routine diagnosis of cryptosporidiosis, either fresh or preserved specimens are examined using the routine stool formalin-ethyl acetate sedimentation or Sheathers sugar floatation technique and microscopic examination of smears by the modified acid-fast, safranine-methylene blue or auramine rhodamine stains . The use of immunoassays has proven to be very helpful in providing a very sensitive method of detecting organisms in stool specimens [2, 5]. Assays based on immunofluorescence methods are more sensitive than conventional staining methods [6, 7]. PCR technology is more sensitive and easier to interpret but requires more 'hands-on' time and expertise, as well as being more expensive. Unlike the ELISA and PCR techniques, which may not distinguish between active and resolved infections, microscopy has the advantage of indicating active infections [8]. We conducted a

study to compare four different diagnostic techniques for the detection of *Cryptosporidium spp*. in faecal specimens in cases of both acute and chronic diarrhoea taking into account several attributes of diagnostic testing like ease of use, ease of interpretation, performance and cost.

MATERIAL AND METHODS:

The present study was carried out in the Department of Microbiology for the detection of Cryptosporidium sp. on stool samples from children attending the out-patient and in-patient sections of Department of Paediatrics at J. N. Medical College, AMU, Aligarh over a period of one and a half years. A total of 177 children up to the age of 12 years suffering from diarrhoea were selected. Patients who had received anti-parasitic drugs within 3-4 weeks were not included in the study. Informed consent was obtained from each study participant/ parents/ guardians.

A portion of each specimen was immediately frozen at -20˚C until further processing for Cryptosporidium antigen detection by Enzyme Linked Immunosorbent Assay (ELISA). All the stool samples were examined microscopically after concentration by the formol-ether technique [9].

Wet mount preparations

A drop of Lugol's iodine solution was placed on the slide. Using an applicator stick, a small amount of fresh specimen was mixed with the drop. The preparation was covered with a coverslip. Iodine wet mount helped to study nuclear details of protozoan cysts and glycogen inclusions [9]. Lugol's 1% iodine was also used to differentiate *Cryptosporidium spp.* oocysts from yeast cells: the former do not accept the iodine stain, so they appear transparent; yeast cells accept the stain and appear deep yellow [10].

Staining Methods

All the smears were stained by the Modified Ziehl Neelsen technique and the Safranine-methylene blue staining technique. Modified Ziehl Neelsen Staining was performed according to the procedure described by Henriksen and Pohlenz [11]. The Safranine-Methylene blue staining method was performed according to the protocol given by Baxby and Blundell [12].

ELISA

The Cryptosporidium Microwell ELISA was performed using the kit manufactured by IVD Research Inc. Carlsbad, CA, US. as per the manufacturer's instructions. The results were read visually or at 450/620-650 nm.

Statistical Analysis

Chi-square and z tests were used to compare differences between the groups. A p value of ≤ 0.05 was considered significant.

RESULTS

Table-1: Detection of Cryptosporidium by Individual Diagnostic Methods (n=177)

Tests	Positive cases (%)
1. Wet mount preparation	16 (9)
2. Modified Z-N staining	21 (11.9)
3. Safranine methylene blue staining	21 (11.9)
4. ELISA	23 (12.99)

Figures in parentheses indicate percentage

- Out of 23, 20 of the samples were also microscopy-positive. 3 samples which were detected by ELISA could not be confirmed by staining techniques. However, 1 sample which was microscopy-positive was negative by ELISA.
- Till date, there is no approved 'gold standard' for the detection of Cryptosporidium in human stool specimens. Therefore, we considered cryptosporidiosis to be a definite diagnosis if the organisms were found in any two of the four techniques.
- There were 21 samples for which confirmed identification was made by any two of the tests.
- The overall prevalence of Cryptosporidium was found to be 11.9%.

Table-2: Detection of Cryptosporidium by Combinations of Two Methods (n=177)

a. Wet mount + Modified Z-N staining	16 (9)
b. Wet mount + Safranine methylene blue staining	16 (9)
c. Wet mount + ELISA	16 (9)
d. Modified Z-N staining + ELISA	20 (11.3)
e. Modified Z-N staining + Safranine methylene blue staining	21 (11.9)
f. Safranine methylene blue staining + ELISA	20 (11.3)

Figures in parentheses indicate percentage

Table-3: Comparison of Four Methods in Diagnosis of Cryptosporidium (n=177)

Tests	Sensitivity (%)	Specificity (%)	Predictive Value (%)	
			Positive (PPV)	Negative (NPV)
Wet mount preparation	76.2	100	100	96.9
Modified Z-N staining	100	100	100	100
Safranine methylene blue staining	100	100	100	100
ELISA	95.2	98.1	87	99.4

Table-5: Detection of Cryptosporidium by Different Combinations of the Methods (n=177)

Test	Number
2. Any of the 4 tests positive	24 (13.56)
3. Any 3 tests positive a. Wet mount + Modified Z-N staining + Safranine methylene blue staining	16 (9.04)
b. Wet mount + Modified Z-N staining + ELISA	16 (9.04)
c. Wet mount + Safranine methylene blue staining + ELISA	16 (9.04)
d. Safranine methylene blue staining + Modified Z-N staining + ELISA	20 (11. 3)
4. Any 2 tests positive a. Wet mount + Modified Z-N staining	16 (9.04)
b. Wet mount + Safranine methylene blue staining	16 (9.04)
c. Wet mount + ELISA	16 (9.04)
d. Modified Z-N staining + ELISA	20 (11.3)
e. Modified Z-N staining + Safranine methylene blue staining	21 (11.9)
f. Safranine methylene blue staining + ELISA	20 (11.3)
5. Only ELISA positive	3 (1.7)
6. All the 4 tests positive	16 (9)

Figures in parentheses indicate percentage

Table 13 shows the comparison of the four methods used in diagnosis of Cryptosporidium. When wet mount was used in combination of any 2 or 3 of the remaining tests, the isolation rates were 16 (9%). However when wet mount was excluded, the isolation rates increased to 20 (11.3%) by the combination of the other 3 tests. When any 2 methods were taken, the combinations of Safranine methylene blue staining with ELISA and modified Z-N staining with ELISA both yielded 20 (11.3%) positive samples. But the combination of modified Z-N staining with safranine methylene blue staining yielded 21 (11.9%) positive samples. For the purpose of our study we have considered 21 (11.9%) samples to be confirmed positive.

DISCUSSION

The study aimed to compare the different methods used for the diagnosis of Cryptosporidium. Various tests were employed to detect Cryptosporidium oocysts in the stool samples of 177 children. The aim of this work was to compare the utility of ELISA techniques with conventional microscopy.

Examination with Lugol's iodine, the sediment of formalin-ether concentration, yielded only 16 of a total of 177 samples (9%). The sensitivity and specificity of the test came out to be 76.2% and 100%. Martina Sanchez *et al*. have also reported a low yield of iodine wet mount preparation (40%) in their study [13]. Mahgoub *et al*. reported a yield of only 17.3% when direct wet mount with iodine was performed [10]. Since the Cryptosporidium oocyst is very small in size, it can easily be mistaken in stool debris for artefacts. Also, it is easy to confuse with other oocysts, such as those of Cyclospora spp., and cells, especially yeast cells, which resemble Cryptosporidium oocysts in size and morphology [14].

The results were then compared with Modified Ziehl-Neelsen and Safranine methylene blue staining techniques which confirmed the results of wet mount preparation. In addition, both detected 5 more samples which were negative by wet mount examination. Hence, both the staining methods gave a total yield of 21 (11.99%) cases. The sensitivity and specificity of both the tests were found to be 100%. Interestingly, both showed an excellent diagnostic correlation. Sethi *et al*.

have also shown an excellent correlation between the two staining techniques [15]. However, Bogaerts *et al*. have revealed in their study that safranine methylene blue combined the same specificity as the Ziehl-Neelsen method with greater sensitivity. This was explained by the occurrence on the Ziehl-stained smears of empty 'ghosts' having the typical size and shape of the oocysts [16]. In contrast to this, a few authors have reported that it is difficult to find oocysts by safranine methylene blue staining method when the oocysts are few [17, 18].

When ELISA was performed on 177 samples, 23 (12.99%) yielded positive results. Out of 23, 20 of the samples were also microscopy-positive. 3 samples which were detected by ELISA could not be confirmed by staining techniques. However, 1 sample which was microscopy-positive was negative by ELISA. If we had considered all the samples detected by any one of the 4 methods as confirmed positive, the yield would have been 24 out of 177 (13.56%). Taking the combination of any 3 tests as positive, the yield would have been 20 (11.3%). Since no 'gold standard' for the detection of Cryptosporidium oocysts in human stool specimens has yet been established, we considered cryptosporidiosis to be a definite diagnosis if the organisms were found in any two of the four techniques employed [19]. There were 21 samples for which confirmed identification was made by any two of the tests; for the remaining 3 samples, confirmed identification could not be made. Hence, 21 (11.86%) samples were considered positive in our study.

The ELISA for Cryptosporidium antigen detection in faecal specimens described here offers a diagnostic alternative to direct microscopy. The sensitivity, specificity and positive predictive value of the assay were 95.2%, 98.1% and 87% respectively. In the 3 ELISA- positive and microscopy- negative cases, ELISA may have detected cryptosporidiosis more efficiently than other means of diagnosis and may be important in identifying persons not actively excreting oocysts at the time of specimen collection [20]. Another possibility may be that the above mentioned cases may be recovering from cryptosporidiosis and may represent the continued presence of cryptosporidial antigens in the stool without the presence of whole oocysts [21]. Samples positive only on ELISA may therefore not be false-positives. Nevertheless, for the sake of this study, these results were considered discordant.

The failure of the ELISA to detect infection in 1 specimen which was microscopy - positive may suggest that these specimens contained antigen that was inaccessible or not recognized by the detecting polyclonal antibodies. In an infected individual, different antigens may be present and detectable at different life cycle stages of the parasite. Another possibility, particularly in Indian patients may be that Cryptosporidium isolates are antigenically distinct from the isolates from the United States used to produce antisera. Particularly, in our set-up where there are frequent power-cuts, the specimen might have undergone repeated freezing and thawing and may have lost recognizable antigen. Alternatively, the inability of the ELISA to detect Cryptosporidium antigen from this patient may simply mean that these samples contained an amount of free antigen below the sensitivity of this assay [22].

Several reports with other versions of an ELISA for detecting *Cryptosporidium spp*. in stool specimens are available. Baveja undertook a study in a Delhi hospital to compare the efficacy of ELISA in comparison to Modified Ziehl-Neelsen staining and found it to be 100% sensitive and 99.1% specific [23]. Rosenblatt and Sloan reported that a commercially available EIA was 93% sensitive and 99% specific as compared with Ziehl-Neelsen staining and the IFA [24]. Dagan *et al*. evaluated the diagnostic utility of a commercially available EIA and reported both the sensitivity and specificity to be 98% [25]. Parisi and Tierno compared the results of EIA with those of Modified Ziehl-Neelsen staining and found the sensitivity to be 100% while specificity was 98.5% [26]. Garcia and Shimizu evaluated two ELISA kits for Cryptosporidium and found that the sensitivity ranged from 98% to 99% and specificities were 100% [7].

In the present study, no significant difference was seen in the sensitivities of the staining techniques and ELISA. Previous studies have also found similar results for the two methods [27, 28, 21, 24].

The conventionally stained smears are difficult to interpret requiring examination at 100x oil magnification to identify the organisms. They are also prone to error if staff is inexperienced; difficulties in distinguishing Cryptosporidium oocysts from non-cryptosporidial bodies have been reported [29]. The EIAs can be easily read visually, or with a spectrophotometer.

In addition, the ELISA kit is far less time-consuming than microscopy if more than several samples are to be examined. Because there is currently no treatment for Cryptosporidium infection and quick diagnosis is therefore not essential, samples batched and run by ELISA may represent significant time savings for a busy parasitology laboratory [21].

Nina *et al*. have reported that the antigenic differences between isolates of *C. parvum* are not marked [30]. The presence of these common antigens provides some assurance that immunoassays are efficient in detecting antigens of *C. parvum* from a

variety of different patients. This has also been proved in our study. The fact that high sensitivity and specificity were obtained from specimens tested in our country, while the kit was prepared and pretested in the United States, is reassuring with regard to the potential universal application of this test.

CONCLUSION

The study reflects that since the sensitivity and specificity of the methods are comparable, the choice of method must be based on other criteria such as ease of use, ease of interpretation and cost, in addition to performance.

The conventional microscopic methods are cumbersome and require initial processing of the specimens prior to staining i.e. concentration of the stool specimens. In contrast, the ELISA is easy to perform, samples are easy to prepare and eliminates some of the skill needed in performing labour intensive concentration and staining procedures. The fact that the test can be performed on unprocessed stool specimens and that result can be read visually or spectrophotometrically renders this test a simple one to perform.

Cost may be a limiting feature of the ELISA kit. The conventional microscopic techniques in comparison to ELISA are quite cheaper especially when used for a small number of cases.

The presently studied ELISA offers a combination of high sensitivity, specificity and simplicity without the need for laborious and skilled work that is involved in the standard diagnostic techniques. It is particularly useful in laboratories not accustomed to diagnosing cryptosporidiosis often, in epidemiologic studies in need of diagnostic standardization, and in situations when batch specimen processing may be crucial.

REFERENCES

1. Clark DP. New insights into human cryptosporidiosis. Clinical Microbiology Reviews. 1999; 12: 554-63.
2. Fayer R. General Biology. In: Fayer R, Xiao L, editors. Cryptosporidium and cryptosporidiosis. 2 ed. New York: *CRC Press*; 2008; p. 1-42.
3. Tumwine JK, Kekitiinwa A, Nabukeera N, Akiyoshi DE, Rich SM, Widmer G, Feng X, Tzipori S. Cryptosporidium parvum in children with diarrhea in Mulago Hospital, Kampala, Uganda. The American Journal of Tropical Medicine and Hygiene. 2003; 68 (6):710–5.
4. Adal KA, Sterling CR, Guerrant RL. *Cryptosporidium* and related species. In: Blaser MJ, Smith PD, Ravdin JI, Greenberg HB and Guerrant RL. Infections of the gastrointestinal tract: New York: Raven Press; 1995; 72:1107-28.
5. 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. Journal of Clinical Microbiology. 2003; 41(2):623–6.
6. Huang DB, White AC. An updated review on Cryptosporidium and Giardia. Gastroenterology Clinics of North America. 2006; 35(2):291–314.
7. Garcia LS, Shimizu RY. Evaluation of nine immunoassay kits (enzyme immunoassay and direct fluorescence) for detection of Giardia lamblia and Cryptosporidium parvum in human fecal specimens. Journal of Clinical Microbiology. 1997; 35(6):1526–9.
8. Morgan UM, Pallant ML, Dwyer BW, Forbes DA, Rich G, Thompson RCA. Comparison of PCR and microscopy for detection of *Cryptosporidium parvum* in human fecal specimens: clinical trial. Journal of Clinical Microbiology. 1998; 36: 995- 8.
9. Garcia LS, Bruckner DA, Brewer TC, Shimizu RY. Techniques for the recovery and identification of *Cryptosporidium* oocysts from stool specimens. Journal of Clinical Microbiology. 1983; 18: 185-90.
10. Mahgoub ES, Almahbashi A, Abdulatif B. Cryptosporidiosis in children in a north Jordanian paediatric hospital. Eastern Mediterranean Health Journal. 2004; 10:494-501.
11. Henriksen SA, Pohlenz JFL. Staining of cryptosporidia by a modified Ziehl-Neelsen technique. Acta Veterinaria Scandinavica. 1981; 22: 594- 6.
12. Baxby D, Blundell N. Sensitive, rapid, simple methods for detecting *Cryptosporidium* in faeces. Lancet. ii. 1983; 1149.
13. Martin-Sanchez AM, Rodriguez-Hernandez J, Canut-Blasco A., Dovigo-Prieto C. The detection of *Cryptosporidium spp*. in the feces of a preschool population: a comparison of 5 staining methods. Revista Clinica Espanola. 1993; 192: 63-6.
14. Fayer R, Speer SA, Dubey JP. The general biology of *Cryptosporidium*. In: Fayer R, ed. *Cryptosporidium* and cryptosporidosis. Boca Raton, Florida, CRC Press. 1997; 1-41.
15. Sethi S, Sehgal R, Malla N, Mahajan RC. Cryptosporidiosis in a tertiary care hospital. The National Medical Journal of India. 1999; 12: 207-9.
16. Bogaerts J, Lepage P, Rouvroy D, Vandepitte J. *Cryptosporidium spp*., a Frequent Cause of Diarrhoea in Central Africa. Journal of Clinical Microbiology. 1984; 20: 874-6.
17. Hart CA, Baxby D, Blundell N. Gastroenteritis due to *Cryptosporidium*: a prospective survey in a children's hospital. The Journal of Infection. 1984. 9; 264-70.

18. Ha F, Xu S. Etiological diagnosis of cryptosporidiosis. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi. 1989; 7: 1-3.
19. Weber R, Bryan RT, Bishop HS, Wahlquist SP, Sullivan JJ, Juranek DD. Threshold of detection of *Cryptosporidium* oocysts in human stool specimens: evidence for low sensitivity of current diagnostic methods. Journal of Clinical Microbiology. 1991; 9:1323-7.
20. Crawford FG, Vermund SH. Human cryptosporidiosis. Critical Reviews in Microbiology. 1988; 16: 113- 59.
21. Newman RD, Jaeger KL, Wuhib T, Lima AA, Guerrant RL, Sears CL. Evaluation of an antigen capture enzyme-linked immunosorbent assay for detection of *Cryptosporidium* oocysts. Journal of Clinical Microbiology. 1993; 31:2080-4.
22. Ungar BLP. Enzyme-linked immunoassay for detection of *Cryptosporidium* antigens in fecal specimens. Journal of Clinical Microbiology. 1990; 28:2491-5.
23. Baveja UK. Acid fast staining versus ELISA for detection of *Cryptosporidium* in stool. Journal of Communicable Diseases. 1998; 30: 241-4.
24. Rosenblatt JE, Sloan LM. Evaluation of an Enzyme-Linked Immunosorbent Assay for Detection of *Cryptosporidium spp*. in Stool Specimens. Journal of Clinical Microbiology. 1993; 31:1468- 71.
25. Dagan R, Fraser D, El-On J, Kassis Imad I, Deckelbaum R, Turner S. Evaluation of an enzyme immunoassay for the detection of *Cryptosporidium spp*. in stool specimens from infants and young children in field studies. The American Journal of Tropical Medicine and Hygiene. 1995; 52:134- 8.
26. Parisi MT, Tierno Jr. PM. Evaluation of new rapid commercial enzyme immunoassay for detection of *Cryptosporidium* oocysts in untreated stool specimens. Journal of Clinical Microbiology. 1995; 33:1963- 5.
27. Rusnak J, Hadfield TL, Rhodes MM, Gaines JK. Detection of *Cryptosporidium* oocysts in human fecal specimens by an indirect immunofluorescence assay with monoclonal antibodies. Journal of Clinical Microbiology. 1989; 27: 1135- 6.
28. Kehl KS, Cicirello H, Havens PL. Comparison of four different methods for detection of *Cryptosporidium* species. Journal of Clinical Microbiology. 1995; 33:416-8.
29. Ma P, Soave R. Three step stool examination for Cryptosporidiosis in 10 homosexual men with protracted watery diarrhoea. The Journal of Infectious Diseases. 1983; 147: 824- 8.
30. Nina JM, McDonald V, Dyson DA, Catchpole J, Uni S, Iseki M, Chiodini PL and McAdam KP. Analysis of oocyst wall and sporozoite antigens from three ***Cryptosporidium*** species. Infection and Immunology. 1992; 60:1509- 13.