Evaluation of various staining techniques for routine microscopy of stool samples for the detection of *Cryptosporidium parvum*

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Abstract

A total of 500 stool samples were subjected to examination with the help of direct microscopy methods. Of these, 39 (7.8%) specimens were positive for *Cryptosporidium parvum*. Among direct microscopy methods: direct wet mount, negative staining and modified Ziehl-Neelsen staining showed a positivity of 22 (4.4%), 14 (2.8%) and 39 (7.8%) respectively. Positivity of *C. parvum* was 1.19 times higher in males as compared to females. The results indicate that the modified Ziehl-Neelsen staining technique to detect *Cryptosporidium parvum* in the stool specimens has significantly higher accuracy (p<0.001) and reliability as compared to the other two techniques.

Keywords: Direct microscopy, *Cryptosporidium parvum*, Ziehl-Neelsen staining, stool specimens.

Introduction

The most common clinical feature of cryptosporidiosis in immunocompetent as well as immunocompromised persons is diarrhea (Current and Garcia, 1991). *Cryptosporidium parvum* is associated with massive diarrhea outbreaks worldwide, generally caused by exposure to drinking or recreational water or direct contact with infected persons through the oral-faecal route (Fayer, 2004; Goncalves et al., 2006). Diarrhea is exceedingly common and exerts an enormous toll in terms of mortality, morbidity, loss of work productivity, consumption of medical resources and social inconvenience. In man, the incidence of *Cryptosporidium* is more in children. Stool is usually watery, greenish in colour containing mucus and vomiting is commonly associated. Other symptoms may be colic by abdominal pain, anorexia, nausea and abdominal distension. Frequencies of passing stool usually 3-6 times in immunocompromised patients but may be up to 20 times a day (Current and Garcia, 1991). Incidence in India is reported to vary from 1.3% to 13% (Nath et al., 1999).

There are studies from India, which entail a number of diagnostic methods without any uniform pattern. The detection of *Cryptosporidium* in India is limited to major research laboratories and does not feature in protocols for routine investigations in most of the clinical laboratories. The complex lifecycle of *Cryptosporidium* species, its small size, and subtle staining characteristics have contributed to the problem for identification of this parasite in routine stool preparations (Bird and Smith, 1980; Current, 1983; Fayer and Unger, 1986). The purpose of this study was to evaluate various direct microscopy methods, taking into account several attributes of diagnostic testing.

The attributes of each of the tests evaluated were diagnostic yield, cost of performing the test, ease of handling, and ability to process large numbers of specimens for screening purposes by batching. A laboratory diagnosis of cryptosporidiosis may have direct benefits to the patient and physician by providing a clinical diagnosis and limiting extensive diagnostic evaluations. It may also reduce the use of empirical therapy for gastroenteritis, which could be ineffective and potentially harmful.

Materials and methods

Test specimen: The study was carried out in a tertiary care hospital over a period of 24 months. During this period, 500 stool samples were received in the department of Microbiology for routine investigation. The study included the patients of all age groups attending the OPD and having the history of diarrhoea admitted in the hospital.

Identification of *Cryptosporidium parvum*: After doing the macroscopic examination, direct microscopic examination was done. All the specimens were subjected to wet saline and iodine preparation (Garcia and Bruckner, 1997; Chatterjee, 2009). Two smears were prepared directly from the specimen and stained with two different stains, modified Ziehl-Neelsen staining (MZN) by using 5% sulphuric acid as a decolourizer and negative staining with carbol fuchsin (NS) respectively (Garcia and Bruckner, 1997; Chatterjee, 2009). MZN smears and NS smears were observed under oil immersion and high power of bright-field microscope respectively.
Results and discussion

Five hundred stool samples were subjected to parasitological examination. Of these, 39 (7.8%) specimens were positive for Cryptosporidium parvum (Fig. 1). Among direct microscopy methods—direct wet mount, NS and MZN showed a positivity of 22 (4.4%), 14 (2.8%) and 39 (7.8%) respectively (Table 1). Incidence of C. parvum was 1.19 times higher in males as compared to females (Table 2). Almost two-third positive cases were aged up to 30 years. Maximum incidence of positive cases (16 i.e. 41%) was during months of June, July and August.

There is difficulty in the identification of Cryptosporidium parvum in stool and in view of this, Centre for Disease Control and Prevention (CDC) in its guidelines has also recommended testing of multiple stool specimens before reporting a test to be negative. Considering high incidence of false negativity, a number of attempts had been made to modify, alter and upgrade the existing laboratory procedures for identification of C. parvum in human faecal specimen. In this study, detection of C. parvum oocysts was done with the help of direct microscopy methods. All the assessments were done in three replicates, one each for wet mount, modified Ziehl-Neelsen stain and negative stain. In this study, overall prevalence of C. parvum in stool specimen tested was 7.8%. Cryptosporidium parvum is an important cause of gastroenteritis especially in children worldwide, with prevalence rates varying from 1 to 4% in the developed world and 6 to 17% in the developing world (Baxby and Hart, 1986; Salon et al., 1990; Enriquez et al., 1997). Among direct microscopy methods—direct wet mount showed a positivity of 22/39 (56.41%), direct staining examination by negative staining showed a positivity of 14/39 (35.89%) while direct staining examination by MZN showed a positivity of 39/39 (100%). Although some workers have reported relatively better efficacy of negative staining method but under electron microscopy (Baxby et al., 1984; Casemore et al., 1985). Negative staining under light microscopy is recommended for capsular structures, as they appear clear in dark background (Black, 2004). Some authors have advocated the use of negative staining for identification and isolation of C. parvum species. They have also illustrated that the quality of negative staining is time dependent and if the samples are not viewed within a specified time then the spores might become less visible (Khurana, 2012). Moreover, the technique requires higher level of technical and optical skill while using the microscope and owing to this the results might vary among persons with different experience and microscopic skills. Among specimen processed, majority were from males (57.6%). Only 212 (42.4%) samples comprised of females. This is in contrast with the observations made by Chang et al. (2006) who observed that functional gastrointestinal disorders were more common in females as compared to males (Halder et al., 2007).

Fig. 1. Oocysts of C. parvum in modified Ziehl-Neelsen stain preparation under oil immersion (X100).

Table 1. Microscopic examination of stool samples for C. parvum.

<table>
<thead>
<tr>
<th>Direct microscopy (n=500)</th>
<th>Positive cases</th>
<th>Total positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet mount</td>
<td>22</td>
<td>4.4%</td>
</tr>
<tr>
<td>Negative staining</td>
<td>14</td>
<td>2.8%</td>
</tr>
<tr>
<td>Modified ZN staining</td>
<td>39</td>
<td>7.8%</td>
</tr>
</tbody>
</table>

Table 2. Gender wise distribution of cases of C. parvum.

<table>
<thead>
<tr>
<th>Sex</th>
<th>No. of suspects (n=500)</th>
<th>Positive No. of cases (n=39)</th>
<th>% (out of total in corresponding gender)</th>
<th>% (out of total positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>288</td>
<td>24</td>
<td>8.3%</td>
<td>61.5%</td>
</tr>
<tr>
<td>F</td>
<td>212</td>
<td>15</td>
<td>7.0%</td>
<td>38.5%</td>
</tr>
</tbody>
</table>

The contrast in our study could be because of the difference in hospital healthcare seeking behaviour of Indian population wherein females are generally tended to seek hospital healthcare only for life-threatening problems and for minor ailments, they generally seek home remedies (Bentley and Parekh, 1998; Sen, 2012). Table 2 shows the proportion of males was higher as compared to that of females both for positivity (8.3% against 7%) as well as for proportion of positives (61.5% against 38.5%). In Cryptosporidiosis surveillance, the proportion of females was found to be 1.15 times higher in females as compared to males (Yoder et al., 2012). However, Pereira et al. (2002) have also shown that the risk of infection among male patients was 2.2 times higher as compared to that in females among children hospitalized for diarrhoea in Brazil. Kimani et al. (2012) have also observed the proportion of males to be 3.35 times higher as compared to that of females in a study conducted at Kenya. Higher risk to males in developing countries could be attributed to higher prevalence of manual jobs, unhygienic conditions of work and eating environment and gender wise differentiation of occupations, which involve higher proportion of males as compared to females thus enhancing the risk of males as compared to females.

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As for as the age distribution was concerned among specimen tested positive, the proportion of those aged <10 years and aged between 21-30 years was maximum (23.1%). Almost two-third (64.1%) of positive cases was from among those aged up to 30 years. Hlavsa et al. (2005) in their nationwide surveillance study on Cryptosporidiosis reported that greater number of cases was observed in age group 1-9 and 30-39 years compared with other age groups. Prevalence of *C. parvum* has been reported to be more than 10 times that of adult cases in paediatric cases with diarrhoea (Natividad et al., 2008). The higher rate of prevalence among paediatric age group might be attributed to the lack of maintenance of hygiene while younger age group is generally more active in life and spends a lot of their time outdoors and is more exposed to various risk factors such as unhygienic food, inability to maintain hygiene and tendency to experiment with a variety of food stuff which may be unhygienic in nature. A high seasonal variability was observed in number of samples obtained during different months. It has been reported maximum during the rainy season because of the availability of favourable conditions for growth of microbes and possibility of opportunistic infections (Prieto et al., 2009).

On comparing the different diagnostic methods for their relative accuracy (accuracy to predict, true positive and true negative cases precisely), direct wet mount examination had significantly lower diagnostic accuracy as compared to direct staining examination by MZN. Direct staining examination by MZN showed significantly higher accuracy as compared to direct wet mount (p<0.05) and direct staining by negative staining (p<0.001). In this study, the negative staining method was incorporated because of the reasons that it is quick and simple to perform (as it uses only one reagent). Also negative stain slide is examined under high power, which conventionally improves the screening results. However, direct staining by negative staining had significantly lower accuracy as compared to direct examination by MZN. It was even poorer than the wet mount preparation. Therefore, if the MZN is applied in routine stool examination this is likely to improve the detection of *Cryptosporidium parvum*. This method is simple and does not consume much time. The negative staining seems to have many variables and may not be fruitful and hence not recommended.

**Conclusion**

Despite the comparative efficacy of different techniques and relatively better performance of modified Ziehl-Neelsen staining, it is essential to make a note that identification of *Cryptosporidium parvum* is a daunting task for a microbiologist with varying accuracy. In this study, the criteria for evaluating performance of different methods were taken as efficacy of each method against a positive finding in a given sample.

Modified Ziehl-Neelsen staining showed 100% positivity against the other two techniques and hence can be considered as the best technique for routine stool examination. It improves the positivity considerably and is simple to perform and less time consuming. The negative staining did not find a favour in the study and was poorer than wet mount.

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**References**


