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Abstract: Due to increasing numbers of patients with Cryptosporidium, it is important for the physician and clinical laboratory to be aware of the appropriate diagnostic techniques necessary for recovery and identification of organism. Cryptosporidium is found in gastrointestinal tract, the examination of stool specimens is noninvasive procedure and will provide better opportunities for recovery of organism. We compared two procedures for the demonstration of the organism in stool specimens. Of 456 stool specimens tested by both techniques, Sheather sucrose flotation (SSF) identified 89 (19.5%) as positive for Cryptosporidium sp. oocysts. Formalin-ethyl acetate sedimentation (FEA) plus modified cold Kinyoun acid-fast stain (MCK) of sediment identified 93 (20.3%) as positive for Cryptosporidium sp. oocysts. Of 456 samples, 182 were positive for oocysts by both techniques and 274 samples were negative by both techniques. A total of 182 (39.9%) were positive by one technique or other; 107 (23.4%) were positive by both techniques. 37 specimens were positive by SSF but negative by FEA plus MCK, and 38 specimens were positive by FEA plus MCK but negative by SSF. The discrepancies between the two techniques occurred in stool specimens that contained rare to few oocysts. We concluded that FEA plus MCK of sediment was as effective in the concentration and identification of Cryptosporidium sp. oocysts as SSF. F/EA plus MCK may be advantageous as single concentration method for general parasitology when Cryptosporidium sp. is to be identified.

Key Words: Cryptosporidium; Sheathers flotation; Formalin-ethyl acetate.

Introduction

Cryptosporidium, an intracellular protozoan has been reported as an important cause of diarrhea in animals and humans worldwide and the potential for significant morbidity and mortality (Meisel et al., 1976). Until recently, human infections by the coccidian parasite Cryptosporidium sp. have been reported infrequently. The first two documented cases of cryptosporidiosis were reported in 1976 (Meisel et al., 1976; Nime et al., 1976). Case studies of this disease link its occurrence with exposure to infected animals (Current et al., 1983; Reese et al., 1982). The diagnosis of cryptosporidiosis was based originally on finding the organism in histological sections of intestinal tissue obtained by biopsy. Unstained and iodine-stained wet mounts of directly prepared or concentrated fecal material or permanent staining techniques (e.g. trichrome or iron hematoxylin) failed to demonstrate the organism. Cryptosporidium sp. oocysts are small and may easily be confused with yeasts. In 1980 oocysts were found in human stool specimens (Tzipori, 1980). In 1981 the acid-fast nature of the organism was demonstrated (Heniksen and Pohlez 1981). Since this time, new and varied concentration and staining techniques (Bronsdon, 1984) for demonstrating the organism in stools have been reported; without tissue biopsy techniques and allowing routine diagnosis by examination of freshly passed or preserved feces.

Little data are available as to the most effective routine techniques for the recovery and identification of Cryptosporidium sp. We compared two concentration techniques currently recommended for use in the diagnosis of Cryptosporidium sp. infections: (i) The Formalin-ethyl acetate (FEA) (Garcia and Shimizer 1981) concentration procedure combined with a modified
cold Kinyoun acid-fast stain (MCK) of the sediment and (ii) Sheather sucrose flotation (SSF) (Sheather 1923), a specialized procedure adapted from veterinary science to clinical microbiology laboratories (Reese et al.1982) and currently recommended for the diagnosis of cryptosporidiosis (Ma and Soave 1983).

Materials and Methods

Specimen collection. 456 stool samples were chosen to evaluate the two methods. The fresh stool specimens were placed into 10% buffered Formalin preservative. The specimens in Formalin were divided into two parts. One part was processed by FEA sedimentation, and the other part was processed by SSF. The two concentration procedures and examination of slides were performed independent. The fresh specimens were permanently stained and examined under oil immersion magnification for the presence of other parasites.

FEA and MCK. The FEA concentration procedure was performed, 4 to 5 ml of the Formalin-treated stool specimen was washed in 10% Formalin-saline, and the sediment collected by centrifugation at 650 x g for 5 min, was suspended in 8 ml of Formalin-saline-3 ml of ethyl acetate. This mixture was vortexed for 3 min and centrifuged at 500 x g for 5 min, resulting in four layers: a layer of ethyl acetate, a plug of debris, a layer of Formalin-saline, and sediment. The plug was rimmed with an applicator stick, and the top three layers were decanted. One portion of the sediment was placed on a microscope slide and dried for the acid-fast stain. The remainder of the sediment was examined at 100 and 450x for eggs and cysts in saline and iodine wet mounts. The smear was stained by MCK following the protocol for MCK as described previously (6). Each MCK-stained slide was examined under oil immersion power for 15 min.

The results were recorded as the average number of oocysts observed per oil immersion field.

Sheathers Sugar Flotation. Approximately 2 ml of the Formalin-treated stool suspension was strained through two layers of gauze into a conical tube. Eight ml of Sheather sugar solution was added, and the suspension was mixed thoroughly by inversion. The tube was capped and centrifuged at 500 x g for 15 min. After centrifugation, material at the surface of the suspension was removed with a loop, placed on a slide and covered with a cover slip. Each slide was examined for approximately 5 min for pink, oocysts by microscopy. Results were recorded as the average number of oocysts observed per high-power dry field.

Data analysis: Statistical analysis was done by the chi-square test. Probability level of p < 0.05 was considered significant.

RESULTS

A total of 456 stool specimens were examined by the two procedures and the prevalence of Cryptosporidium infection is shown in Table 1. Cryptosporidium sp. oocysts were identified in 182 (39.9%) specimens. Using SSF, we identified oocysts in 89 (19.5%) specimens. When FEA sedimentation plus MCK was employed only 93 (20.3%) specimens were positive. There was no significant difference (p > 0.05) in number of positive samples between SSF and FEA. The calculated sensitivities of FEA and SSF were 51% (93/182) and 49% (89/182) respectively under the assumption that there were no false negatives. A total of 274 specimens were negative for Cryptosporidium sp. oocysts by both techniques. A total of 37 specimens that were positive by SSF but negative by FEA plus MCK contained less than one oocyst per high-power field. In 38 specimens oocysts were recovered by only FEA plus MCK.

Table 1. Comparison of positivity among two different fecal examination techniques

<table>
<thead>
<tr>
<th>Method</th>
<th>No of specimens</th>
<th>No of positive</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FES</td>
<td>456</td>
<td>93</td>
<td>20.3%</td>
</tr>
<tr>
<td>SSF</td>
<td>456</td>
<td>89</td>
<td>19.5%</td>
</tr>
<tr>
<td>Total</td>
<td>456</td>
<td>182</td>
<td>39.9%</td>
</tr>
</tbody>
</table>

FES: formalin–ether sedimentation technique, SSF: Sheathers Sucrose flotation technique.

Table 2. Distribution of number of oocysts detected by the two techniques

<table>
<thead>
<tr>
<th>No. of oocysts found</th>
<th>Number of oocysts detected by (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FEA</td>
</tr>
<tr>
<td>1</td>
<td>38(41)</td>
</tr>
<tr>
<td>2-10</td>
<td>19(20)</td>
</tr>
<tr>
<td>11-50</td>
<td>16(17)</td>
</tr>
<tr>
<td>51-100</td>
<td>11(12)</td>
</tr>
<tr>
<td>&gt;100</td>
<td>9(10)</td>
</tr>
<tr>
<td>Total</td>
<td>93(100)</td>
</tr>
</tbody>
</table>
DISCUSSION

The FEA concentration procedure which is generally used for recovery of parasites was compared with the more classical method (SSF) for the detection of Cryptosporidium sp. There was no significant difference between these two methods in rates of recovery of Cryptosporidium sp. oocysts. All disparate results were associated with the presence of very few oocysts. The use of both techniques resulted in a 39.9% positivity rate, as compared with rates of approximately 20% for either technique alone. In view of the large number of specimens included in this study, this difference in positivity rates may appear to demand the recommendation that both methods be used for the optimal detection of Cryptosporidium sp. However, an examination of multiple specimens by either technique may provide a similar increase in sensitivity. As the FEA concentration procedure is already performed routinely in most clinical microbiology laboratories, preparation of the acid-fast smear for detecting Cryptosporidium sp. in the same sediment may reduce cost and technical time. The acid-fast smear provides a permanent record of the results, but the preparation and reading of the smear require some technical expertise. SSF concentration is easy to perform, and the pink, refractile oocysts are easily recognized under high-power magnification, but the wet mounts should be examined within 15 min after preparation or the oocysts may collapse.

We examined each MCK-stained slide for 15 min under oil immersion magnification to cover approximately 100 fields. This amount was roughly equal to the area covered when we examined each SSF wet mount for 5 min under high-power dry magnification. We chose these two parameters to standardize our procedures and make our data available for comparison. Although there are several reports on the prevalence of Cryptosporidium in human patients, very few studies used SSF technique.

We concluded that FEA plus MCK of the sediment was as effective in the concentration and identification of Cryptosporidium sp. oocysts as SSF. FEA plus MCK may be advantageous as single concentration method for general parasitology when Cryptosporidium sp. is to be identified.

References