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Comparison between Modified Acid Fast Staining and Antigen Detection Assay as Diagnostic Techniques for *Cryptosporidium parvum*

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Abstract: Cryptosporidiosis, is a common gastro-intestinal illness in both animals and man worldwide and is an important cause of morbidity and mortality in immunosuppressed individuals but self-limiting in immunocompetent hosts. This study aimed to evaluate and compare the use of Direct smear, Formalin-Ethyl Acetate Sedimentation Concentration (MIF), Modified Ziehl-Neelsen staining technique (ZN) and ELISA antigen detection using monoclonal antibody (MAb) for the detection of *Cryptosporidium* in diarrhoea patients. This study was conducted on a total of 92 patients. The study shows that among the diarrheic patients 44 (61.1%), 48 (66.7%) and 52 (72.2%) were positive with *Cryptosporidium* using direct smear, MIF and ZN respectively. The percent positivity among the diarrheic patients using MAb was 98.1 %. The specificity using MAb as a diagnostic tool was 95 %. By comparing our results using MAb with those obtained with the three different microscopic techniques tool for detection of *C. parvum* MAb was the most promising tool for detection of *Cryptosporidium* in diarrhoea patients.

Key words: Cryptosporidiosis · Immunosuppressed · Staining technique · Monoclonal antibody

INTRODUCTION

Cryptosporidiosis is a parasitic disease caused by *Cryptosporidium*, a protozoan in the phylum Apicomplexa. It is an intestinal parasite and is typically an acute short-term infection. It is spread through the fecal-oral route, often through contaminated water; the main symptom is self-limiting diarrhea in people with intact immune systems. Despite not being identified until 1976, it is one of the most common waterborne diseases found worldwide. The parasite is transmitted by environmentally hardy microbial cysts (oocysts) that once ingested exist in the small intestine and result in an infection of intestinal epithelial tissue [1]. Immunocompromised patients, very young or old individuals can develop a more severe form of cryptosporidiosis [2]. Patients with AIDS experience variety of clinical manifestations: approximately 4% of them show no symptoms, 29% have a transient infection, 60% suffer from chronic diarrhea and 8% have a severe, cholera-like infection. With transient infections, diarrhea ends within 2 months and *Cryptosporidium* is no longer found in the faeces. Chronic diarrhea that lasts for 2 months or more might result in losing up to 25 liters water per day [3] since those patients can excrete a bulk 10 times a day. In addition, they experience severe malabsorption and weight loss and many of them couldn't completely eliminate the *Cryptosporidium* from their bodies[4]. Diagnosis of infection generally requires the observation of the infective stage of oocysts, which are usually 4–6µm in size. Ziehl-Neelsen staining technique is used to detect the tiny size oocysts because the wet mount prepared samples make it difficult to differentiate between the oocysts and the debris found in the samples [5]. It becomes more difficult to detect the oocysts in asymptomatic patients or patients with minimal symptoms even by using sugar flotation concentration and modified acid-fast stains techniques [6]. Immunoassays are used widely for detecting the antibodies by fluorescent

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antibody (DFA) techniques and antigen/antibody by immunofluorescence and Enzyme-Linked Immuno-Sorbent Assay (ELISA) [7]. With the ability to interact with matter at the nanoscale, the development of nanotechnology architecture and materials could potentially extend sub-cellular and molecular detection beyond the limits of conventional diagnostic modalities [8]. Biomedical nanotechnology is providing revolutionary opportunities for the rapid and simple diagnosis of many infectious diseases [9]. Given the low sensitivity of microscopy and the varying sensitivity and specificity of ELISA antigen detection, our study aimed to evaluate and compare the use of a staining technique (ZN) and ELISA antigen detection using monoclonal antibody for the detection of *Cryptosporidium* in diarrhoea patients.

MATERIALS AND METHODS

This cross-sectional study was carried out from January 2014 to January 2015 at the outpatient clinics of different Hospitals belonged to Cairo University (Kasr-EL-Ini. Abu Reesh Hospital. A total of 119 patients were included in the initial enrollment in the present study, but only 92 patients continued in the study. Those outpatients attended the clinics for upper respiratory tract infections as well were included in the study. Children with chronic illness or severe conditions were excluded. According to the results of stool examination, the patients were divided into three groups; one of them contains patients (52) infected with *Cryptosporidium*, another group of patients (20) infected with different protozoa (*Entamoeba histolytica*, *Escherichia coli* and *Blastocystis*) and the third group included non-infected persons (20).

Written informed consents were obtained from all patients' caregivers.Three consecutive fresh stool samples were collected from all patients into clean cups and examined by direct microscopy of both unstained and iodine-stained smears for the presence Crypto oocysts or other protozoa.

Microscopic Examination of *Cryptosporidium* Oocysts

Direct Smear: Direct microscopic examination of faecal smears prepared from fresh or concentrated samples is still widely used to detect protozoan cysts in faeces. A certain amount of each stool sample was taken and emulsified in normal saline and smeared on a clean glass slide then examined by 10X and 40X.

Formalin-Ethyl Acetate Sedimentation Concentration: Approximately a 2 cc. of stool were collected, 5 ml were strained, 0.85% saline or 10% formalin was added through the debris on the gauze to bring the volume 15 ml. Then, the samples were centrifuged at 500 × g for 10 minutes, supernatant was decanted, a 10 ml of formalin (10%) was added to the sediment and mixed thoroughly. Finally, 4 ml of ethyl acetate was added and the sample was recentrifuged at 500 × g for 10 minutes.The plug of debris and the top layers of supernatant were removed. Several drops of formalin (10%)were added to suspend the concentrated specimen and proceed with the applicable method.

Modified Ziehl-Neelsen Method

Preparation of Reagents and Staining: Carbolfuchsin (1%) was prepared from 10 g of basic fuchsin (Hi-Media) dissolved in 100 ml of methanol (Qualigens) and 50 ml of melted phenol (Qualigens) in a flask maintained at 60°C in a water bath. This solution was made up to 1,000 ml with distilled water and filtered after proper mixing. Sulfuric acid (25%) was prepared from 250 ml of concentrated sulfuric acid (Qualigens) slowly added to 750 ml of distilled water. Methylene blue (0.1%) was prepared from 1 g of methylene blue (Hi-Media) dissolved in 1,000 ml of distilled water. Gabbett's Methylene blue was prepared as described by Vasantha kumara *et al.* [14] and Gokhale *et al*. [15]. For staining, the glass slides were kept in a staining rack with the smear side facing upwards and flooded with Carbolfuchsin (1%) solution. After 5 minutes the slides were washed gently with running water, excess water was drained off and 25% Sulphuric acid was poured onto the slides and allowed to stand. Two to three minutes later, the slides were washed in running water and excess was drained off. Methylene blue solution (0.1%) was poured on the slides and allowed to stand. After 1 minute the slides were rinsed in running water, air dried and examined using oil immersion objective.

Cryptosporidium Specific Antigen Detection by Commercial MAb Based Sandwich ELISA Technique:

Cryptosporidium-specific antigens were detected in stool samples using monoclonal antibody test (MOCI Ltd, West Sussex, UK) in accordance with manufacturer's instructions. ELISA microtiter plates were coated with fluorescent monoclonal antibody specific to crypto-antigen, washed trice with 300µl Supper Block® blocking buffer (Antimicrobial Agent, Rockford, IL, USA) and dried. The supernatant of fecal suspension

was added to the wells. Two hundred microliter of horseradishperoxidase (HRP)-labelled mouse IgG monoclonal anti-CSA conjugate was added to each well of the plate, covered and incubated for 60 minutes at 20 °C with shaking. The plates were washed five times with buffer to remove unbound antibody conjugate. A colorless single-component enzyme substrate (tetra methyl benzidine; TMB) was added, the plates incubated for 10 min at 20 °C and observed for a color change. A stop solution was added and the optical density (OD) was read on an ELISA plate reader (Model 680, Bio-Rad Laboratories, Hercules, CA, USA) at an absorbance of 450 nm. Cryptosporidial antigen was used as a positive control. The cut-off value for a positive reaction was calculated to be double the optical density value of the negative control. OD values >0.05were considered positive following the manufacturer's guidelines.

RESULTS

Frequency and Percentage Distribution of Age and Sex Within Studied Patients: This study was conducted on a total of 92 patients. Male patients were 54 (58.7 %) and 38 female patients (41.3%) (Table1). The frequency distribution of the patients in the different age groups is demonstrated in Table (2). The age ranged from 10 – 58 years. The mean age in male patients was 42.5 year and the mean age in female patients was 33.7 year. Most of cases suffered from diarrhea (n=72, 78.3 %). The rest of patients have no symptoms (n=20, 21.7 %).

Microscopic Examination of *Cryptosporidium* Oocysts

Direct Smear: In bright-field microscopy using differential interference contrast (DIC), oocysts appear as small round structures (4 to 6 µm) similar to yeasts. They do not autofluoresce. Among the diarrheic patients, 44 were positive with *Cryptosporidium*, 8 positive with *Entamoeba histolytica*, 5 positive with *Escherichia coli* and 3 with *Blastocystis* (Table 3).

Formalin-Ethyl Acetate Sedimentation Concentration: In bright-field microscopy using differential interference contrast (DIC), oocysts appear as small round structures (4 to 8 µm) similar to yeasts. They do not autofluoresce. Among the diarrheic patients, 48 were positive with *Cryptosporidium*, 8 positive with *Entamoeba histolytica*, 6 positive with *Escherichia coli* and 6 with *Blastocystis* (Table 4).

Table 1: Sex distribution among studied patients.

	Male		Female		Total
Patients	No	%	No	%	No
Sex	54	58.7 %	38	41.3 %	92

No significant difference between number of male and female patients

Table 2: Age distribution among studied patients

Age group (Years)	Males	Females	Total	Frequency (%)
10-15	4	2	6	6.5 %
15-20	13	8	21	22.8 %
21-30	18	10	28	30.4 %
31-40	8	8	16	17.4 %
41-50	8	7	15	16.4 %
51-58	3	3	6	6.5 %
Total	54	38	92	100%

Table 3: Microscopic Examination of *Cryptosporidium* Oocysts by Direct Smear

Patients	No. of positive by Direct Smear	% positive samples/ diarrheic patients (72)
Cryptosporidium	44***	61.1 %***
Entamoeba histolytica	8	11.1 %
Escherichia coli	5	6.9 %
Blastocystis	3	4.1 %
Healthy control	0	0 %

*** P < 0.001 high significant difference relative to healthy control

Table 4: Microscopic Examination of *Cryptosporidium* Oocysts by Formalin-Ethyl Acetate

Patients	No. of positive by Formalin-Ethyl Acetate	% positive samples/ diarrheic patients (72)
Cryptosporidium	48***	66.7 %***
Entamoeba histolytica	8	11.1 %
Escherichia coli	6	8.3 %
Blastocystis	6	8.3 %
Healthy control	0	0 %

*** P < 0.001 high significant difference relative to healthy control

Modified Ziehl-Neelsen Method: Bright-field microscopy using oil immersion (x100) differential interference contrast (DIC), oocysts appeared as small round structures. Oocysts (4 to 8 µm) had distinct oocyst walls and stained from light pink to bright red. However, staining may be variable (Fig. 1). Among the diarrheic patients, 52 were positive with *Cryptosporidium*, 8 positive with *Entamoeba histolytica*, 6 positive with *Escherichia coli* and 6 with *Blastocystis* (Table 5).

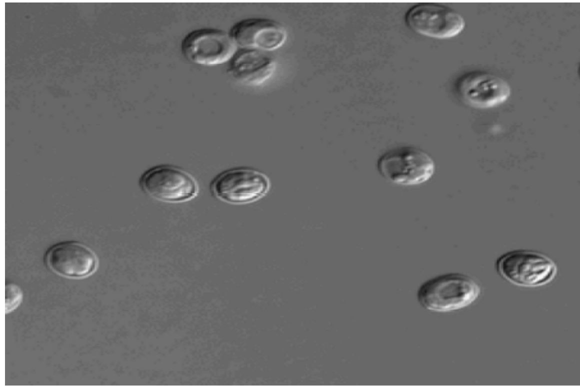

Fig. 1: Microscopic Examination of *Cryptosporidium* Oocysts Direct smear

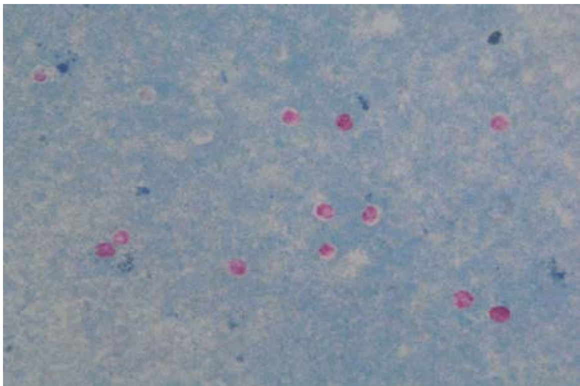

Fig. 2: Microscopic Examination of *Cryptosporidium* Oocysts by Modified Ziehl-Neelsen Method

Table 5: Microscopic Examination of *Cryptosporidium* Oocysts by Modified Ziehl-Neelsen Method

Patients	No. of positive by Modified Ziehl-Neelsen	% positive samples/ diarrheic patients (72)
Cryptosporidium	52***	72.2 %***
Entamoeba histolytica	8	11.1 %
Escherichia coli	6	8.3 %
Blastocystis	6	8.3 %
Healthy control	0	0 %

*** P < 0.001 high significant difference relative to healthy control

Table 6: Detection of *Cryptosporidium* Specific Antigen by commercial MAb based sandwich ELISA

Patients	No. of positive by commercial MAb	% positive samples
Cryptosporidium	51***	98.1 %***
Entamoeba histolytica	0	0 %
Escherichia coli	0	0 %
Blastocystis	1	5 %
Healthy control	0	0 %

*** P < 0.001 high significant difference relative to healthy control

Table 7: Comparison between the different methods used in detection of *Cryptosporidium* infection

Patients	% positive samples/ diarrheic patients (72)
Direct Smear	61.1 %
Formalin-Ethyl Acetate	66.7 %
commercial ELISA	98.1 %***$
Modified Ziehl-Neelsen	72.2 %##

*** P < 0.001 high significant difference between commercial ELISA and both direct and formalin-ethyl acetate.

P < 0.01, moderate significant difference between Modified Ziehl-Neelsen and both direct and formalin-ethyl acetate.

$ P <0.05 light significant difference between commercial ELISA and Modified Ziehl-Neelsen.

Detection of *Cryptosporidium* Specific Antigen by Commercial MAb Based Sandwich ELISA: The concentration of *Cryptosporidium* Specific Antigen in stool samples of different studied groups were measured by the commercial –monoclonal antibody based sandwich ELISA. Fifty-one of 52 *Cryptosporidium* infected samples showed positive results and the sensitivity of the assay was 98.1 %. All the 20 negative controls were below the cut off value while 1 out of 20 of other parasites groups was at the border line of the cut off value giving 95 % specificity. The positive case belonged to the group of patient infected with *Blastocystis* (Table 6 and Fig. 2).

DISCUSSION

There is an increasing demand for diagnostic testing for *C. parvum*, with a priority being placed on obtaining diagnostic results in an efficient and timely manner. Staining of fecal smears may help to identify *Cryptosporidium* oocysts and despite being an inexpensive technique, it still requires experienced personnel for the microscopic examination of stained smears capable of differentiating oocysts from similarly stained particles such as spores of fungi or yeast and oocysts, which is time consuming [16,17]. Direct and indirect immunofluorescence assays are more costly than the conventional microscopic techniques, but oocysts are readily identified [18,19]. On the other hand, the PCR is still a more expensive technique than the immunofluorescence, more time consuming and require specialized personnel and expensive equipment [6]. In Egypt, uneven results of prevalence were reported since the late of 20th century, when several Egyptian institutions started surveillance in livestock animals and/or humans targeting *Cryptosporidium spp.* [20-34. Those studies conducted various diagnostic techniques including

microscopy, serology and molecular examinations and sometimes a modification of techniques was developed to use combinations of any of those methods [35]. Recent report on prevalence of *Cryptosporidium* in human has estimated 47% incidences [36]. Earlier, the reported antigen Copro-prevalence of *Cryptosporidium* infection in Egypt was demonstrated 17% in diarrheic stools among 1275 children below 5 years of age [37]. In 2006, El-Mohamady and co-workers found that 15% in diarrheal faecal samples of children less than 5-year old in a district of Fayoum governorate. In our study, we examined the fecal samples of patients with age groups ranged from 10-58 years old. Surprisingly, the highest total frequency of infection (30.7%) was detected in the age group 21-30 and was more frequent in males than females. The data analysis of the three microscopic examination methods depicted that the least positivity (around 60%) to detect the parasite was recorded by the direct smear technique and the greatest positive percentage (~70%) was in favor of the Ziehl-Neelsen method. However, the positivity of detection of *Cryptosporidium* cysts with the Formalin-Ethyl Acetate was almost the midpoint between the other two techniques. It is well-known that there is so much difficulty in distinguishing artefacts from the oocysts in the faecal samples when examined by microscopy [38, 39]. The gradual improvement observed in the positivity of the two latter microscopic examination techniques, in our work, may be due to fixation of the oocysts which maintained their morphological entity [38]. Advantageously, staining the samples with Methylene blue in Ziehl-Neelsen method brought about the best medium for *Cryptosporidium* oocysts to be detected the faeces. Recognition of oocyst morphological features via microscopy, after ZN staining, is the convention in the diagnosis of cryptosporidiosis, however, this techniques is laborious and less sensitive and thus prone to error [10]. *Cryptosporidium* oocysts are quite tiny and consequently can easily be mistaken in stool debris as an artifact. Also, they may be easily be confused with other oocysts, such as those of *Cyclospora* species and yeast cells [2]. Conversely, this method would not differentiate between *Cryptosporidium* species oocysts because they similarly takes up a red to pink color as do other fecal components, which is the shortfall of this technique compared to others. However, it is affordable, hence resource-poor countries still rely on the technique as has been previously reported [11-19]. Unlike FEA and ZN, unfixed fresh fecal samples were used for ELISA for optimum performance and the positivity was the greatest amongst all. On comparing ELISA with the ZN, we may conclude that the first is a highly sensitive and specific technique, which is more timely efficient than the staining method. In spite of being a bit costly, ELISA should be considered for diagnosis as to determine the low concentration of parasite in the fecal samples and overcome the lack of experienced technicians. Since 1990, several studies [36-38] recommended using ELISA for diagnosis of *Cryptosporidium* describing it “a simple, quick, easily standardized test. In our study, the concentration of *Cryptosporidium* Specific Antigen in stool samples of different studied groups were measured by the commercial –monoclonal antibody based sandwich ELISA. Fifty-one of 52 *Cryptosporidium* infected samples showed positive results. The sensitivity and specificity of the assay was 98.1 and 95 % respectively. In conclusion, measurement of Cryptosporidium specific antigen using monoclonal antibody based sandwich ELISA is highly sensitive and specific than other techniques. Yet, in-housing preparation of monoclonal anti-oocyst wall might definitely make ELISA affordable and choice of the diagnostic laboratories all over the country.

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