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Investigation of Cryptosporidium Species Antigen by ELISA Method in Stool Specimens Obtained from patients with Diarrhoea in Kosti Teaching Hospital, White Nile State, Sudan

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Abstract:

Cryptosporidium is an important parasitic protozoan causing diarrhoea in developing and developed countries. It causes severe life-threatening diarrhoea especially in immunosuppressed individuals. The diagnosis of the small Cryptosporidium oocysts in stool samples by conventional microscopy is labour intensive, time consuming and relies on stool concentration with subsequent staining and microscopy. The aim of this study is to evaluate the clinical utility and usefulness of capture enzyme-linked immunosorbent assay (ELISA-IDEXX) method in detecting Cryptosporidium oocysts in stool samples from patients admitted with diarrhoea in Kosti Teaching Hospital, White Nile State. In addition, specific antigen by ELISA method in stool was investigated in order to find out whether or not it contributes to the diagnosis of Cryptosporidium species. Stool specimens were collected from 279 patients whose ages ranged from 4 to 85 and examined immediately or stored frozen fresh at -65 C° or in 10% formalin. All specimens were examined for Cryptosporidium species antigen by ELISA and oocysts via gold standard modified Acid Fast staining method. 21 of the specimens were positive by modified Acid Fast staining and by ELISA, 10 addition samples were positive by ELISA

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only, while 248 were negative by both methods; 65 of these negative stool samples containing 97 parasites of 10 different species. ELISA false negative was 8 and 1 false positive were observed. ELISA sensitivity was 93%, specificity was 99% and positive predictive value was 99%. Storage of specimens preserved in 10% formalin or frozen fresh at -65 C° for up to 18 months did not appear to affect the results. There was no cross-reactivity with G. lamblia (14 negative specimens) or with the 9 other species present in the ELISA-negative stool samples. The results of ELISA indicate that it is simple, rapid, accurate, easy-to-read, reliable, sensitive and specific and standardize immunoassay test for routine diagnosis and may be useful for large-scale epidemiological studies of Cryptosporidiosis.

Key words: ELISA for Cryptosporidium antigen, Cold Acid fast staining, Kosti, Sudan.

INTRODUCTION:

Cryptosporidium is a coccidian protozoan parasite that has gained much attention in the last 20 years as a clinically important human pathogen. The discovery of Cryptosporidium is usually associated with E.E. Tyzzer, who, in 1907, described a cell-associated organism in the gastric mucosa of mice (Keusch, *et- al* 1995). For several decades, Cryptosporidium was thought to be a rare, opportunistic animal pathogen, but the first case of human cryptosporidiosis in 1976 involved a 3-year-old girl from rural Tennessee who suffered severe gastroenteritis for two weeks. Electron microscopic examination of the intestinal mucosa led to the discovery that *Cryptosporidium parvum* was the infectious species in humans. In the early 1980s, the strong association between cases of cryptosporidiosis and immunodeficient individuals (such as those with AIDS) brought Cryptosporidium to the forefront as a ubiquitous human pathogen. Presently, the increasing

population of immunocompromised persons and the various outbreaks of cryptosporidiosis through infection by water-borne Cryptosporidium oocysts (often in drinking water) have placed an even greater emphasis on this pathogen. Little is known about the pathogenesis of the parasite, and no safe and effective treatment has been successfully developed to combat cryptosporidiosis (Flanigan and Soave, 1993). Unlike other intestinal pathogens, Cryptosporidium can infect several different hosts, can survive most environments for long periods of time due to its "hardy cyst" (Keusch, *et- al* 1995), and inhabits all climates and locales (Flanigan and Soave, 1993). With the increasing number of individuals with AIDS, cancer patients, and malnourished children suffering from diarrhoeal illness, need for the easy, cheap and quick method for diagnosis is required to reduce the morbidity. Although ELISA has been widely used as a diagnostic tool, availability of this facility is still poor in peripheral set-up (Barua, *et- al* 2013).

None invasive diagnostic technique was first reported in 1978 for calves (Pohlenz*, et- al* 1978) and in 1980 for human (Tzipori, *et- al* 1980), when oocysts were detected in faecal smears stained with Giemsa's stain. Subsequently, numerous techniques to concentrate stool specimens and to stain oocysts have been applied for detection of Cryptosporidium species. There are little consensuses on which methods are most satisfactory.

The present study was conducted to detect the presence of Cryptosporidium oocysts in the stool samples from patients with diarrhoea. Modified Cold Acid Fast (MCAF) staining in direct and concentrated stool examination and ELISA techniques were employed in this study. In addition, specific antigen in stool was investigated by ELISA method in order to find out whether or not it contributes to the diagnosis of Cryptosporidium species.

MATERIALS AND METHODS:

This study was conducted over a period of one year, from February 2016 to February 2017 in Kosti Teaching Hospital, White Nile State, Sudan and department of Microbiology and Parasitology, faculty of Medicine University of Khartoum. The studied patients were subjected to standardize questionnaire interview. 279 stool samples were collected in wide mouth containers. Each stool container was labelled clearly with patient's number and name, and immediately transferred to the laboratory and examined using direct stool examination and concentration techniques, modified Acid Fast staining and ELISA techniques. Study group (n=179) included all individuals on the basis of presence of recurrent attacks of diarrhoea. The control group (n=100) included those individuals having no gastrointestinal symptoms or without diarrhoeal manifestations.

Microscopic examination of stool included direct normal saline and Lugol's iodine wet mounts and concentration technique. Diarrhoeal stool specimens usually contain enough oocysts to be readily identified (Garcia, *et- al* 1993, Weber and Philip, 1993).

Slides were Air dried and fixed in absolute alcohol then stained with (MCAF) staining and examined under 40× and 100× objectives and through direct observation in saline(0.85% NaCl solution), for the detection of ova, larva, trophozoite and cysts of intestinal parasites (Morello, *et- al* 2006).

An aliquot each of all stool samples (fresh, frozen or preserved) were subjected to ELISA test using Cryptosporidium microplate assay (IDEXX). This ELISA is used for qualitative detection of Cryptosporidium specific antigen in aqueous extracts of faecal specimens. The protocol was followed as per the manufacturer's instructions.

STATISTICAL ANALYSIS:

All tests performed for evidence of Cryptosporidium were compared and evaluated statistically using chi-square test as the criteria for significance of test values.

RESULTS:

A total of 279 subjects included in study, were divided into 2 groups based on presence or absence of diarrhoea (table: 1). In the parasitological examination of stool sample, protozoan parasites accounted for 24.01% (67/279), whereas helminths were observed in 16.48% (46/279) of all subjects studied. In the entire study population, the spore forming protozoan parasites encountered by any of the methods employed include Cryptosporidium (21/279; 7.52%), *E. histolytica* (20/279; 7.16%), *G. Lamblia* (21/279; 7.52%) and Isospora (2/279; 0.71%). Special attention was however given to Cryptosporidium (table: 2).

Using (MCAF) staining method, Cryptosporidium oocysts were detected in 18 subjects with diarrhoea (18/179; 10.05%). Only 3 none diarrhoeal subjects (3/100; 3%) showed the presence of Cryptosporidium oocysts in their stools. This observed difference in isolation of Cryptosporidium oocysts in the two groups with or without diarrhoea using (MCAF) staining method, is highly significant ($p<0.01$) (table: 3).

Results of direct and concentration (D&C) methods on all stool samples show that *G. Lamblia* and *E. histolytica* cysts were detected in 10.05.% (18/179), 8.37% (15/179) of patients with diarrhoea and 6% (6/100), 5% (5/100) of individuals without diarrhoea.

ELISA for coprodiagnosis of Cryptosporidium parvum antigen showed a positive results in 29/179 (16.20%) of patients with diarrhoea and negative results 150/179 (84.80%) of individuals without diarrhoea. The difference in

Cryptosporidium antigen isolation using ELISA test in stool samples of the two study groups is statistically highly significant $p<0.01$ (table: 4). Storage of specimens preserved, fresh or frozen at-65 C^o for up to 18 months did not appear to affect the results. There was no cross-reactivity with the 10 other parasite species present in the ELISA-negative stool samples.

Taking (KCAF) staining method as gold standard for the diagnosis of Cryptosporidium, the results of ELISA test for Cryptosporidium antigen detection when correlated with results of gold standard the p value (<0.01) suggests that the ELISA test result is significantly useful for predicting positive and negative results with reference to MCAF staining as the gold standard (table: 5). Comparison of parameters for the two tests performed in this study reveals (table: 6) that ELISA test is most sensitive single test for detecting the infestation, assuming MCAF staining as gold standard.

Table 1: Details of subjects included in the study

Study group	Description of subjects	Number studied
Group I	with diarrhoea	179
Group II	without diarrhoea	100
Total		279

Table 2: Parasites detected in faecal samples of all two study group individuals

Parasites detected	Group I N= 179	Group II N= 100
Protozoa		
Cryptosporidium spp.	18 (10.05)	3 (3)
Isospora spp.	2 (1.67)	-
E. histolytica	15 (8.37)	5 (5)
G. lamblia	18 (10.05)	6 (6)
Nemato-helminth		
Ascaris lumbricoides	2 (1.67)	-
Strongyloides stercoralis	4 (2.34)	1(1)
Entrobius vermicularis	5 (2.79)	1(1)
Platy-helminth		

Taenia spp.	4 (2.34)	1(1)
Hymenolepis nana	17 (9.49)	2(2)
Schistosoma mansoni	6 (4.46)	3(3)
Total number isolated	75 (41.89)	22(22)

Table 3: Detection of Cryptosporidium in stool sample by Kinyoun acid fast staining method

Result	Group I	Group II
Cryptosporidium positive	18(10.05)	3(3)
Cryptosporidium negative	161(89.95)	9 (97)
Total	179	100

N.B. Figures in parentheses indicate percentage
Chi-quare :p <0.01
Interpretation: Highly significant

Table 4: Detection of Cryptosporidium antigen in stool samples using ELISA test

Result	Group I	Group II
Cryptosporidium positive	29(16.20)	2(2)
Cryptosporidium negative	150 (84.80)	98(98)
Total	179	100

N.B. Figures in parentheses indicate percentage
Chi-quare :p <0.01
Interpretation: Highly significant

Table 5: Comparison of antigen detection by ELISA with acid fast staining method

Gold standard → test↓	Positive by Kinyoun cold acid fast staining	Negative by Kinyoun cold acid fast staining	Total
ELISA positive for Cryptosporidium antigen	25	10	35
ELISA negative for Cryptosporidium antigen	5	239	244
Total	30	249	279

Sensitivity =83.33%
Specificity =96.13%
Positive predictive value =71.42%
negative predictive value =98.24
Chi-quare: p <0.01
Interpretation: Highly significant

DISCUSSIONS:

Cryptosporidium species is an important parasitic protozoan causing diarrhoea in developing and developed countries (Elgun and Koltas, 2001). The agent causes severe life-threatening diarrhoea especially in immunocompromised hosts (Gellin and Soave, 1992, Elgun and Koltas, 2001). A number of workers have studied the possible association between this coccidian parasite and AIDS patients (Kasper and Buzoni- Gatel, 1998, Hunter and Nichols, 2002, Matos, *et- al* 2004, Ajjampur, *et- al* 2008, Gupta, *et- al* 2008).

In the present study, the overall prevalence of Cryptosporidium was found to be 11.11% (31/279), the prevalence however was 2% in those individuals having no gastrointestinal symptoms or without diarrhoeal manifestations but it was still higher in subjects suffering from diarrhoea (16.20%). The possible role of Cryptosporidium causing diarrhoea in these patients may be attributable in some, to the enterocyte or neural dysfunction related to infections. Alternatively, there may be quantitative differences in parasite burden between patients with and without diarrhoea (Batman, *et- al* 1991, Bartlett, *et- al* 1992). In this study however, the oocysts detected were not quantified.

In the present study population, 34.76% subjects had parasitic infection, out of which 96.98% were found in subjects with diarrhoea. Helminth formed the major parasites detected (16.48%), Cryptosporidium species was the major protozoan in this category (70.42%). A study conducted in patients with abdominal disturbances in Mayo area; Khartoum; Sudan, showed the overall prevalence of helminthic infection to be 32.4% using concentration methods (Arabi, 2002). A lower isolation of helminth in the present study although concentration techniques were used, could be attributed to the area to which study was conducted. The area being urban, a

better sanitary environment and a moderate personal hygiene could have contributed to a lower prevalence of parasite infection. Secondly, the population coming to the hospital is not a true representation of general population as a whole. Among the protozoan, higher isolation rate of Cryptosporidium species could be due to special emphasis given to this parasite, or probably it was a true higher incidence, although further studies are required in this field to ascertain the prevalence of other coccidian parasites in the community.

Using MCAF staining procedure 10.05% of subjects with diarrhoea in the study were identified as being infected with Cryptosporidium oocysts. In contrast only 3% of individuals without diarrhoea had Cryptosporidium oocysts in their stool samples. Comparative studies rank MCAF staining method highest in terms of the reagent cost, hands-on time required, yield, ease of handling and ability to process large number of specimens, although there was some difficulty of interpretation at times (Mac Pherson and Mc Queen, 1993, Kehl, *et- al* 1995). Keeping these points in mind and also that MCAF staining technique is the method of choice for the detection Cryptosporidium most of the parasitological laboratories (Henricksen and Pohlenz, 1981), we selected MCAF staining method for identification of Cryptosporidium oocysts in stool samples of our subjects as well as the "gold standard" for our study when comparing the other methods.

All stool samples were subjected to ELISA test using Prospect Cryptosporidium microplate assay (IDXX) for detection of Cryptosporidium antigen. We documented the presence of Cryptosporidium antigen in 16.20% of diarrhoeal persons studied. 25 of 30 samples positive for Cryptosporidium by MCAF staining were also positive by ELISA. Additionally 10 samples were found positive by ELISA. The sensitivity and specificity were observed to be 83.33% and 96.13% respectively. These figures correlate closely with two other studies

documented (Ungar, 1990, Newman, *et- al* 1993). Possible reasons for microscopy negative, ELISA positive result of a specimen may be attributed to the fact that a fewer number of oocysts have to be present for their detection by microscopy. ELISA on other hand detect even disintegrating organisms and their products (Ungar, 1990, Current and Garcia, 1991, Nichols, 2000).

CONCLUSION:

ELISA was found to be simple, rapid, accurate, easy-to-read, reliable, sensitive and specific and standardize immunoassay test for routine diagnosis and may be useful for large-scale epidemiological studies of Cryptosporidiosis.

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