

**A COMPARATIVE STUDY OF VARIOUS DIAGNOSTIC TECHNIQUES FOR
CRYPTOSPORIDIOSIS IN STOOL SPECIMENS OBTAINED FROM HIV
SEROPOSITIVE INDIVIDUALS IN KHARTOUM AND KOSTI CITIES, SUDAN****Hafiz Yahya Mohammed^{1*}, Abdalmoneim M. Magboul¹, Mohammed A. Suliman¹, Hanan B. Eltahir² and
Ibrahim M. Hassan¹**¹Department of Parasitology and Medical Entomology, Faculty of Medical Laboratory Sciences, El Imam El Mahdi University, Kosti city, Sudan.²Department of Biochemistry, Faculty of Medicine, El Imam El Mahdi University, Kosti city, Sudan.***Corresponding Author: Hafiz Yahya Mohammed**

Department of Parasitology and Medical Entomology, Faculty of Medical Laboratory Sciences, El Imam El Mahdi University, Kosti city, Sudan.

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ABSTRACT

Severe life-threatening diarrheal disease is a common complication of infection with HIV. *Cryptosporidium* has gained importance as an AIDS indicator disease and a cause of intractable diarrhoea in immunosuppressed individuals. With the increasing number of individuals with HIV, cancer patients and malnourished children suffering from diarrheal illness, need for the easy, cheap and quick method for diagnosis is required to reduce the morbidity. There for, this study was conducted to detect *Cryptosporidium* oocysts in the stool samples of HIV-infected patients using Kinyoun Cold Acid Fast (KCAF) staining in concentrated stool and to compare these results with that obtained by ELISA technique. Stool specimens were collected from HIV positive patients with (n= 90) and without (n=90) diarrhea along with their HIV negative counterparts (n=200) and examined using Kinyoun Cold Acid Fast (KCAF) staining method to identify *Cryptosporidium* oocysts. ELISA using *Cryptosporidium* microplate assays for detection of *Cryptosporidium* antigen was also conducted on all stool specimens. The overall prevalence of *Cryptosporidium* was found to be 42/380 (11.1%) and 57/380 (15%) by KCAF staining and ELISA respectively. Detection of *Cryptosporidium* in HIV positive subjects with diarrhea by KCAF staining was 18 (20%) and by ELISA the detection rate went up to 28 (31.1%). All detailed results were statistically compared taking KCAF staining as gold standard which revealed ELISA method to have sensitivity of 83.88% and specificity of 96.55%. Microscopic examinations for ova, cysts and larvae of other parasites were also done using saline and Lugol's iodine preparations. Keeping in mind the present scenario of HIV infection in Sudan and more so in Khartoum and Kosti, it is recommended to include detection of *Cryptosporidium* oocysts in routine parasitological examination of stool specimens.

KEYWORDS: Cryptosporidiosis, ELISA for *Cryptosporidium* antigen, Kinyoun Cold Acid Fast (KCAF) staining, direct stool examination, Kosti, Sudan.**INTRODUCTION**

HIV infected persons like other immunosuppressed individuals, develop serious opportunistic infections. However, all organisms don't represent an equal threat.^[1] Almost 80% of patients with AIDS die from infections other than human immunodeficiency virus HIV.^[2] The gastrointestinal tract is the major surface where contact between man and environment takes place. At sometimes during the clinical course of HIV, diarrhea occurs in almost ninety percent of patients in developing countries^[3] and is the presenting symptom of approximately a third of patients with HIV.^[4] Although HIV-associated parasites are highly prevalence in the Sudan, very few information is available about their accurate diagnosis. Evidenced by deficient data, even the little obtained is unpublished or concentrated on few

specific diseases. *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.^[5] 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.^[6] 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.^[6] Unlike other intestinal pathogens, *Cryptosporidium* can infect several different hosts and can survive most environments for long periods of time due to its "hardy cyst"^[5] and inhabits all climates and locales.^[6] ELISA has been widely used as a diagnostic tool; availability of this facility is still poor in peripheral set-up.^[7]

None invasive diagnostic technique was first reported in 1978 for calves^[8] and in 1980 for human,^[9] when oocysts were detected in faecal smears stained with Giemsa stain. Subsequently, numerous techniques to concentrate stool specimens and to stain oocysts have been applied for detection of *Cryptosporidium* species. There are little consensus on which methods are most satisfactory.

The present study was conducted to detect *Cryptosporidium* oocysts in the stool samples of HIV-infected patients using Kinyoun Cold Acid Fast (KCAF) staining in concentrated stool and to compare these results with that obtained by ELISA techniques.

MATERIAL AND METHOD

This prospective study was conducted over a period of two years, from July 2015 to - July 2017. Stool samples were collected from Kosti Teaching Hospital, White Nile State and Bashair care center, Bashair hospital, Khartoum state where HIV/AIDS patients with different age admitted for follow-up. All samples were carried to the laboratory of Microbiology and Parasitology, Faculty of Medicine, University of Khartoum for diagnosis. The confirmed cases of the studied groups were subjected to standardize questionnaire interview. Informed consent was obtained before inclusion in the study which was reviewed and approved by the Ethical Committee of Faculty of Medical Laboratory Sciences, University of Gezira and health administration in these states.

380 stool samples were collected in wide-mouth stool containers. Each container was labeled clearly with patient's number and name, and immediately transferred to the laboratory and examined using direct stool examination and concentration techniques, (KCAF) and ELISA techniques. Study group included all HIV seropositive individuals; they were further subdivided into two groups on the basis of presence of recurrent attacks of diarrhea along with passage of blood and mucus, abdominal pain and weight loss. The other group included those individuals having no gastrointestinal symptoms. The control group consisted of HIV seronegative individuals with or without diarrheal manifestations. A total of 180 randomly selected

seropositive individuals (study group); along with 200 HIV seronegative individuals (control group) were enrolled for this study as per the above criteria.

Microscopic examinations of stool included direct normal saline and Lugol's iodine wet mounts and concentration techniques were conducted in all stool samples. Diarrheal stool specimens usually contain enough oocysts to be readily identified^[10,11]. Also the ova, larva, trophozoites and cysts of other intestinal parasites were detected.

Aliquot of each sample was examined using Kinyoun Cold Acid Fast (KCAF) stain, briefly the stool smears were firstly air dried and fixed in absolute alcohol then stained with (KCAF) staining and examined under 40X and 100X objectives for detection of *Cryptosporidium* oocysts.^[12]

Another aliquot of all stool samples (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 380 subjects included in the study were divided into 4 groups based on HIV seropositivity or negativity and presence or absence of diarrhea (table 1). In this study, the overall prevalence of *Cryptosporidium* was found to be 11.1% (42/380) and 15% (57/380) using (KCAF) staining and ELISA techniques respectively. (KCAF) staining method detected *Cryptosporidium* oocysts in stool of 31/180 (17.2%) subjects of HIV Seropositive groups and 11/200 (5.5%) HIV Seronegative group of which 18/90 (20%) HIV infected subjects with diarrhea. In contrast only 8/100 (8%) of HIV seronegative individuals with diarrhea had *Cryptosporidium* oocysts in their stool samples (table 2).

ELISA for *Cryptosporidium* antigen showed positive results in 22.87% (41/180) HIV Seropositive subjects and 8% (16/200) of HIV Seronegative subjects (table 3). In the present study population, 152/380 (40%) of subjects had parasitic infection, out of which 66/152 (43.4%) were found in HIV positive subjects with diarrhea. Helminthes formed the major parasites detected 38/152 (25%). Protozoan parasites accounted for 34.7% (132/380), *Cryptosporidium* species was the major species in this category 42/112 (37.5%). The cyst or spore forming protozoan parasites encountered by any of the methods employed includes *Cryptosporidium* 11.1% (42/380), *E. histolytica* 9.2% (35/380), *G. Lamblia* 9.2%

(35/380) and *Isospora* 0.01% (2/380). Special attention was however given to *Cryptosporidium*. The results showed that *E. histolytica* and *G. Lambli*a cysts were detected in 12.2 % (22/180), 10% (18/180) of HIV Seropositive and 6.5% (13/200), 8.5% (17/200) of HIV seronegative individuals respectively (table 4).

25 of 30 samples positive for *Cryptosporidium* by KCAF staining were also positive by ELISA. Additionally 10 samples were found positive by ELISA (table 5).

Table 1: Details of subjects included in the study.

Study group	Description	Number studied
Group I	HIV positive with diarrhea	90
Group II	HIV positive without diarrhea	90
Group III	HIV negative with diarrhea	100
Group IV	HIV negative without diarrhea	100
Total		380

Table 2: Detection of *Cryptosporidium* in stool sample by Kinyoun acid fast staining method.

Result	Group I	Group II	Group III	Group IV
<i>Cryptosporidium</i> positive	18 (20%)	13 (14.4%)	8 (8%)	3 (3%)
<i>Cryptosporidium</i> negative	72 (80%)	77 (85.6%)	92 (92%)	97 (97%)
Total	90	90	100	100

Chi-square: $p < 0.01$

Interpretation: highly significant

Table 3: Detection of *Cryptosporidium* antigen in stool samples using ELISA test.

Result	Group I	Group II	Group III	Group IV
<i>Cryptosporidium</i> positive	28 (31.1%)	13 (14.4%)	16 (16%)	0
<i>Cryptosporidium</i> negative	62 (69.9%)	77 (85.6%)	86 (86%)	100 (100%)
Total	90	90	100	100

Chi-square: $p < 0.001$

Interpretation: highly significant.

Table 4: Parasites detected in faecal samples of all four study group individuals.

Parasites detected	Group I N= 90	Group II N= 90	Group III N= 100	Group IV N= 100
Protozoa				
<i>Cryptosporidium spp.</i>	18 (20%)	13 (14.4%)	8 (8%)	3 (3%)
<i>Isospora spp.</i>	0	0	2 (2%)	0
<i>E. histolytica</i>	18 (20%)	4 (4.4%)	8 (8%)	5 (5%)
<i>G. lambli</i> a	16 (17.7%)	2 (2.2%)	11 (11%)	6 (6%)
Nematehelminth				
<i>Ascaris lumbricoides</i>	5 (5.6%)	2 (2.2%)	3 (3%)	2 (2%)
<i>Strongyloides stercoralis</i>	2 (2.2%)	0	2 (2%)	0
<i>Entrobious vermicularis</i>	1 (1.1%)	0	3 (3%)	1 (1%)
Platyhelminth				
<i>Taenia spp.</i>	0	0	2 (2%)	1 (1%)
<i>Hymenolepis nana</i>	6 (6.7%)	1 (1.1%)	7 (7%)	0
Total number isolated	66 (73.3%)	22 (24.4%)	46 (46%)	18 (18%)

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 <i>Cryptosporidium</i> antigen	25	10	35
ELISA negative for <i>Cryptosporidium</i> antigen	5	280	285
Total	30	290	320

Sensitivity 83.33%

Specificity 96.55%

Positive predictive value 71.42%

Negative predictive value 98.24%

chi-square: $p < 0.001$

Interpretation: highly significant

DISCUSSION

Cryptosporidium species is an important parasitic protozoan causing diarrhea in developing and developed countries.^[13] The agent causes severe life-threatening diarrhea especially in immunocompromised hosts.^[13,14] A number of workers have studied the possible association between this coccidian parasite and AIDS patients.^[2,15,16,17,18]

The prevalence of *Cryptosporidium* was higher in HIV positive subjects suffering from diarrhea (31.1%). The possible role of *Cryptosporidium* causing diarrhea in HIV Seropositive patients may be attributable in some, to the enterocyte or neural dysfunction related to HIV infection.^[19,20] Alternatively, there may be quantitative differences in parasite burden between patients with and without diarrhea. In this study, the oocysts detected were not quantified.

In our study, KCAF staining procedure can detect *Cryptosporidium* oocysts in stool samples of HIV Seropositive and HIV seronegative individuals with diarrhea. This observed highly significant difference ($P < 0.01$) in isolation of *Cryptosporidium* oocysts in various groups of HIV Seropositive and HIV seronegative individuals with or without diarrhea. Comparative studies rank KCAF 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.^[21,22] Keeping these points in mind and also that KCAF staining technique is the method of choice for the detecting *Cryptosporidium* for most of the parasitological laboratories,^[23] we selected KCAF staining method for identification of *Cryptosporidium* oocysts in stool samples of our subjects as well as the "gold standard" for our study when comparing with 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 41/180 of HIV Seropositive persons studied. The sensitivity and specificity were observed to be 83.33% and 96.55% respectively. These figures correlate closely with two other studies documented.^[24,25] Possible reasons for microscopy negative and ELISA positive results 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 detects even disintegrating organisms and their products.^[25,26,27] The difference in *Cryptosporidium* antigen isolation using ELISA test in stool samples of various study groups is statistically highly significant $P < 0.01$.

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 KCAF staining as the gold standard. Comparison of parameters for the two tests performed in this study reveals that ELISA test is most sensitive single test for detecting the infestation, assuming KCAF staining as gold standard.

As the other intestinal parasitic infections were concerned, helminthes formed the major parasites detected in HIV positive subjects with diarrhea. A study conducted in normal healthy population in Goa; India, showed the overall prevalence of helminthic infection to be 41% using concentration methods.^[28] A lower isolation of helminthes in the present study (25%) although concentration techniques were used, could be attributed to the area in 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.

CONCLUSION

The study concluded that ELISA test is most sensitive single test for detecting the *Cryptosporidium* antigen, assuming KCAF staining as gold standard.

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