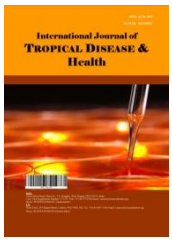

International Journal of TROPICAL DISEASE & Health

42(14): 17-25, 2021; Article no.IJTDH.74514
ISSN: 2278–1005, NLM ID: 101632866

Prevalence of Cryptosporidiosis Detected by Enzyme Immunoassay Coproantigen among People Living with HIV/AIDS Attending Selected Hospitals in Maiduguri, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJTDH/2021/v42i1430513
Editor(s):
(1) Prof. Romulo Dias Novaes, Federal University of Alfenas, Brazil.
Reviewers:
(1) Gamal Ali Abu-Sheishaa, Al Azhar University, Egypt.
(2) Safa Tawfeeq Whaeeb, Albayan University, Iraq.
(3) Madhuri M Suryawanshi, Shree Bhausaheb Hire Govt. Medical College, India.
Complete Peer review History: https://www.sdiarticle4.com/review-history/74514

Original Research Article

Received 15 July 2021
Accepted 23 September 2021
Published 09 October 2021

ABSTRACT

Background: Cryptosporidium species are intestinal parasites that infect both humans and animals; it causes cryptosporidiosis which usually resulted to diarrhea especially among those with impaired immunity. It was observed that enzyme linked immunosorbent assay (ELISA) be ascertain was never been used as one of the techniques in the detection of *Cryptosporidium parvum* antigen in Maiduguri, Borno state.
Materials and Methods: Four hundred stool and blood samples were collected in four selected hospitals in the study area. Stool samples were analyzed by Enzyme link immunosorbent assay to detect fecal Cryptosporidium antigen, while the blood samples were analyzed with Partec sysmex ® flow cytometric machine for CD4 T-lymphocyte counts.

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Results: Demographically, female are 275 while male 125 in number. The result of the stool samples have shown that, of the four hundred samples, seventy nine (79) patients were positive for *Cryptosporidium* species with an infection rate of 19.8%. According to gender, females have the highest infection rate of 14.25% while males 5.5%.based on age, patients between the ages of 20-39 have the highest prevalence of 9.5% followed by 40-59 with 7.5% and lastly 0-19 and >60 old are1.25 and 1.5% respectively. Traders (businessmen and women) demonstrated the highest infection rate of 8.0% followed by housewives 6.25%, civil servants 4.25% and lastly student and farmer with 1.0 and 0.25% respectively. In terms of CD4-T-lymphocyte count, those with CD4 <200cells/µl has an occurrence rate of 8.25% followed by 201-499 with 7.0% and >500 with 4.5%. on the bases of clinical details, the results have shown that patients with diarrhea has a prevalence of 13.0% while those without diarrhea has 6.75%, however, according to the hospitals the patients attended, the result has that, USUMH has 7.0%.Followed by UMTH 6.5%, SSHM 3.75%, and MSMH 2.5% respectively.

Conclusion: It was concluded that those with cd4 count bellow 200 are at risk of contacting the parasites and hence is one of the causes of diarrhea among HIV patients. It is recommended that proper hygiene practice should be encouraged.

Keywords: Cryptosporidium; CD4 T cell; diarrhea; Maiduguri.

1. INTRODUCTION

Though Cryptosporidium was first designated in the laboratory mouse by Tyzzer in 1907 [1], the medical and veterinary significance of this protozoan was not fully appreciated for another 70 years. The interest in Cryptosporidium worsened tremendously over the last two decades, as reflected in the number of publications, which improved from 80 in 1983 to 2850 currently recorded in MEDLINE [1].

The early history of Cryptosporidium is extensively documented in some review articles and volume chapters published recently [2-4]. Taxonomically, *C. parvum* belongs to Phylum Apicomplexa which have an apical complex, Class Sporozoasida which reproduce by asexual and sexual cycles, with oocyst development, Subclass Coccidiasina with a life cycle involving merogony, gametogeny and sporogeny, Order Eucoccidiida in which schizogony occurs, Suborder Eimeriina in which independent micro and macrogamy develop, Family Cryptosporiidae contain four naked sporozoites within oocysts but with no sporocyst [5].

Like other enteric coccidia of vertebrates, Cryptosporidium has a monoxenous life cycle that is primarily completed within the gastrointestinal tract of a single host. There are, however, many unique features that distinguish Cryptosporidium from other coccidia, of which the relative lack of host and organ specificity, resistance to antimicrobial agents, ability for autoinfection and the curious location it occupies within the host cell membrane are the most obvious [6]. Between 1980 and 1993, three broad articles of cryptosporidiosis became recognized [7]. The first was the revelation in 1980 that Cryptosporidium was, in fact, a shared, yet serious, primary cause of outbreaks as well as sporadic cases of diarrhea in certain mammals [6].

From 1983 onwards, with the onset of the AIDS epidemic, Cryptosporidium emerged as a life-threatening disease in this subpopulation [8–11]. In 1993, it reached the public domain when it became widely recognized as the most serious and difficult to control, cause of waterborne-related diarrhea [12]. The first glimpse of the seriousness of Cryptosporidium in mammals, mainly in calves, was pro- vided in the late 1970s [13,14]. Until then, Cryptosporidium was frequently identified histologically in infected gut sections or in biopsy specimens [15,16] and was an opportunistic protozoan that caused a few or no symptoms. However, there is little awareness on the issue of cryptosporidiosis in Maiduguri, Borno state hence the need for the study.

2. MATERIALS AND METHODS

2.1 Study Area

The study was conducted in Maiduguri, the capital of Borno state. The city is located in the north-eastern part of Nigeria with an area of 69,436km^2 within latitude 10.30˚N and longitude 12.15˚E in the Sudano-Sahelian savanna zone. The state comprises of 27 local government areas and shares boundries with the republic of Niger to the North, republic of Chad to the

northeast, Cameroun to the east Adamawa state to the southwest and Yobe to the west. With a dense population that is mostly crop farmers, fishermen, herdsmen and traders Udo, (1978).

Based on the national census conducted in 2006, Borno state has a population as estimated to be 4,151,193 million NPC (2006). The climate of the region is dry season between November and April with hottest in April and May with a temperature of about 31-44^0 C. it is cold from November to February with the dusty harmattan period in December and January. The wet season begins between late April to May and lasts for about 5 months with rainfall of about 25inches.

2.2 Inclusion Criteria

a. HIV Seropositive patients.
b. HIV Seropositive in- and out-patients.

2.3 Exclusion Criteria

a. Patient that refused consents to be part of the study,
b. Patients that are not HIV positive

2.4 Study Design

The study was a hospital-based cross –sectional study.

2.5 Sample Collection

A total of 400 Stool samples was collected using a wide mouth sterile universal transparent container with screw cap lid and 400 blood samples were collected in an EDTA bottle from HIV positive patients attending the four selected hospitals, (100) University of Maiduguri Teaching Hospital (UMTH), (100) State Specialist Hospital (SSH), (100) Mamman Shuwa Memorial Hospital (MSMH) and (100) Umaru Shehu Ultra-Modern Hospital (USUMH) in Maiduguri for analysis.

2.6 Detection of cryptosporidium by Enzyme Link Immunosornbent Assay (ELISA) *Principle of Assay*

During the first incubation, Cryptosporidium specific antigen present in the stool specimens were captured by antibodies attached to the microwells. The wells were incubated and washed before anti-Cryptosporidium antibodies conjugated to horseradish peroxidase are added. The enzyme conjugate was “sandwich” any antigen bound to the wells. After washings to remove unbound enzyme, a chromogen was added which develops a blue color in the presence of the enzyme complex. The stop solution ends the reaction and turns the blue color to yellow.

Map 1.

2.7 Procedure

Sample was prepared by dilutions in tubes using 0.7 ml of Dilution Buffer and 0.1 g, about the size of a small pea, of fecal sample using an applicator stick. It was mixed thoroughly before using.1 ml of dilution buffer was added to the dilution tube. The swab was coated with a thin layer of specimen and mix into dilution buffer, expressing as much fluid as possible. It was Mixed thoroughly before using, the wells was break off (number of samples plus 2 for controls) and place in holder. Using a micropipette, 100µl of negative control was added to well 1, Using a micropipette, 100µl of positive control was added to well 2, 100µl of diluted sample was added to each well, Incubate for 60 minutes at room temperature (15-25°C), then wash. After last wash, slap the wells out on a clean absorbent towel to remove excess wash buffer. 2 drops of Enzyme Conjugate was added to each well. Incubate for 30 minutes at room temperature (15-25°C), then wash. After last wash, slap the wells out on a clean absorbent towel to remove excess wash buffer. 2 drops of Chromogen was added to each well. Incubate for 10 minutes at room temperature (15-25°C). 2 drops of Stop Solution was added to each well. Mix wells by gently tapping the side of the strip holder with index finger for approximately 15 seconds. Read reaction within 5 minutes after adding stop solution. The results were read using an ELISA plate reader.

Elisa Reader Zero reader on air, Read all wells at 450/620-650 nm.

2.8 Reactive

Absorbance reading of 0.08 OD units and above indicates the sample contains Cryptosporidium antigen.

2.9 Non-reactive

Absorbance reading less than 0.08 OD units indicates the sample does not contain detectable levels of Cryptosporidium antigen.

2.10 CD4 T- Lymphocytes Count by Partec Sysmex ® Cyflow Machine

2.10.1 Principles of operation

An aliquot of an EDTA whole blood sample was mixed with the antibody (CD4) conjugated to the flourochrome in a 1:1ratio after a fixed incubation time, the buffer was added, and the sample was ready for analysis on the Cyflow counter flow cytometer. The light source excites the fluorescein dye linked with the stained cell and the emitted light was detected while a precise a volume of blood sample was run through the instrument. The integrated software calculates the concentration of the detected cell population.

2.11 Procedure

The blood sample in the ethylene diamine tetra acetic acid (EDTA) was inverted in a collection tube gently for 8-10times, 20µl of the blood sample was pipetted into a sample bottle, and 20µl of CD4 mAb PE was added directly into the whole blood and mixed. The mixture was incubated for 15minute at 15- 30^0C in the dark, 800µl no lyses buffer and vortex briefly. The menu for cd4 was chosen on the cyflow counter, the sample tube was inserted into the sample port and measurement was taken.

2.12 Data Analysis

Data was analyzed using SPSS 19.0 software. The chi-square test and Fischer exact test was used to perform and establish any statistical difference. Probability values of <0.05 was considered as significant.

3. RESULTS

Of the four hundred samples collected, the results have shown that seventy nine (79) patients were positive for *Cryptosporidium* species giving an infection rate of 19.8%. According to the gender, females have the highest infection rate of 14.3% while males 5.5%.Patients between the ages of 20-39 have the highest prevalence of 9.5% followed by 40-59 with 7.5% and lastly 0-19 and >60 old are1.3% and 1.5% respectively(X^2 = 2.320 *p value* 0.5088). Trader (business men and women) demonstrated the highest infection rate of 8.0% according to occupation followed by housewives 6.25%, civil servants 4.25% and lastly student and farmer with 1.0% and 0.3% respectively. In terms of CD4 T-lymphocyte count, those with <200cells/µl has an occurrence rate of 8.25% followed by 201-499 cells/µl with 7.0% and >500 cells/µl with 4.5%. on the bases of clinical details, the result have shown that patients with diarrhea has a prevalence of 13.0% while those without diarrhea has 6.75%, however, according the hospitals attended, the result has that, USUMH has 7.0%. Followed by UMTH 6.5%, SSHM 3.75%, and MSMH 2.5% respectively.

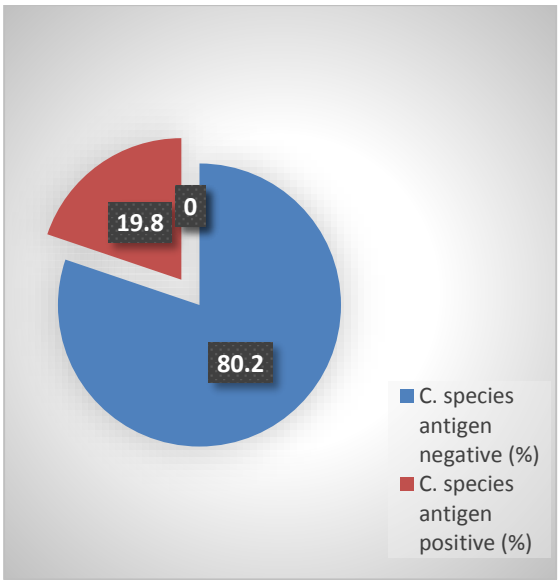


Fig. 1. Prevalence of faecal *cryptosporidium species* among HIV infected persons by (a) ELISA

Table 1. Seroprevalence of molecular detections of cryptosporidiosis Demographic variables of HIV infected persons

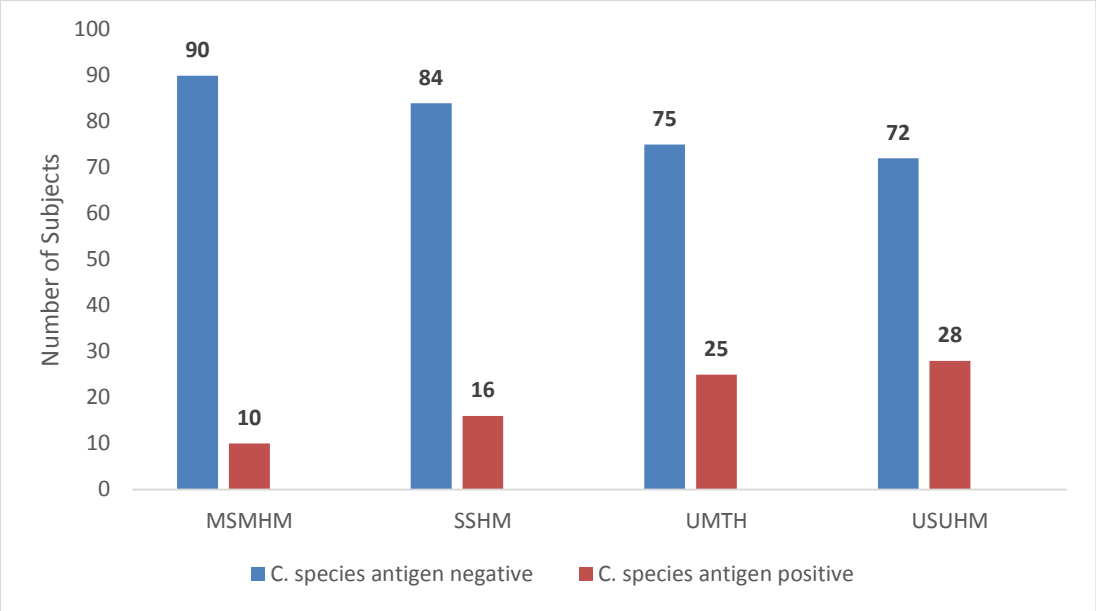
Parameter	No. of Subjects Tested	No. with reactive C. *species* (ELISA) (%)	Chi-squared	*p* value
Age(years)				
0-19	26	5 (1.3)		
20-39	189	39 (9.8)	2.320	
40-59	166	29 (7.3)		0.5088
>60	13	6 (1.5)		
Sex				
Male	125	22 (5.5)		
female	275	57 (14.3)	0.529	0.4670
Occupation				
Housewife	136	25 (6.3)		
Business	148	32 (8.0)		
Civil Servant	77	17 (4.3)		
Student	31	4 (1.0)		
Others	8	1 (0.25)	1.933	0.7481

*= *p<0.05*

Table 2. Seroprevalence of Cryptosporidiosis by Clinical presentation of HIV infected persons

Parameter	No. of Subjects Tested	No. with reactive C. *species* (%)	Chi-squared	*p* value
Diarrhea				
No	274	53 (13.3)		
YES	126	26 (6.5)	0.091	0.7633
CD4 Sage				
>500	100	19 (4.8)		
350- 499	57	9 (2.3)		
200-349	243	51 (12.8)	0.834	0.6589

*= *p<0.05*



Chi-squared= 12.918, DF=3, P = 0.0048

Fig. 2. detection of cryptosporidium antigens according to the hospitals in the study area

Table 3. Impact of HIV and *Cryptosporidium parvum* coinfection on CD4+ T cell counts of Subjects

Laboratory detection methods		
Parameter	**HIV patients with *C. species* antigen positive results (n=79)**	**HIV patients with *C. species* antigen negative results (n=321)**
CD4+ T cell counts (cells/mm^3) Mean± SD	348.7±301.3	326.3±263.8
t value	0.6063	
p value	0.5447	

**= p<0.05*

4. DISCUSION

Cryptosporidium is a well-recognized cause of diarrhea among HIV infected patients worldwide with occurrence of infection ranging from 3% in advanced countries to 50% in developing countries [18]. The results in this study by ELISA method identified all the cryptosporidium species in the fecal samples to be 19.8%.The prevalence rate of the study is less than the studies carried out in kano by [19] with an infection rate of 31.9% and higher than the study in south-south Nigeria with 2.9% [18] and higher in North West Nigeria with 25% respectively [20]. In another studies in Jos, reported by [21] with a high prevalence rate of 83.3% in contrast with the present study, and higher than the report of [22] However, this variation may be due to the fact that the prevalence of *C. parvum* varies curiously among regions of the world as well as among communities depending on the level of uncleanness of piped and drinking water with human and animal excreta. In this study, so many factors have also contributed to the spread of this parasite especially the activities of insurgency in the Northeastern part of Nigeria, Borno state. People were exiled from their rural populations to find refugee camps in the capitals, thereby alter their socioeconomic well-being. They lack basic amenities and hence defecate indiscriminately in an open field close to rivers and streams in the study area where the water serves as the sources of irrigation and drinking for human and animal's consumption.

In this study, it has also shown that female has the higher infection rate of 14.3% while male has 5.5%, this was not statistically significant. However, it may be attributed to the fact that polygamous family settings is highly encouraged,

with this, higher number of females are infected with HIV thereby increasing the chances of more females acquiring opportunistic infection such as *C. parvum* parasite. It could also be due to the facts that high number of female attending antiretroviral clinic to collect antiretroviral drugs to their spouses; this is in agreement with studies carried out by [19] with female 25% and male 22.1%. Similar study was carried out in Kano with female having 63.7% as reported by [20]. Also a study was carried out in northeast Nigeria among children by [23] where female has the infection rate of 52.0% to male with 48%.

In this study, it was observed that patients between the age group of 20-39 has the highest prevalence rate of 9.5% this is not in agreement with study conducted by [20] with a higher rate of 34.9% and this may not be in connection with the habit of eating in commercial food areas. In a similar study at kano by [21] that the age group between 20-30 years old has the highest prevalence rate of 29.3% higher than the present study.

According to occupation of the people living with AIDS in the study area, the result has shown that housewives and business individuals predominate, which is statistically no relationship. These findings were consistent with other findings and support the facts that cryptosporidium parvum is more likely identified from low socioeconomic status that engage in trading and artisans while some housewives use to engage in sewing caps for sale and doesn't bother in their hygiene condition of their environment which is the common practice in the study area.

According to diarrhea, it is statistically not significant and has shown a prevalence rate of 13.0% in contrast with those without diarrhea having 6.8% infection rate. The diarrhea could be associated with the immune response to the invasion of the oocysts in the mucosa leaning of the intestine and this is in conformity with several studies across the world.

There was no relationship between the CD4 stages of infection, the parasites and the HIV seropositive patients in the study area; however, the parasites occur most among the CD4 count of less than 200cell/µl which is the advance stage of the infection with prevalence rate of 8.3%. This is contrary with the studies carried out by [21], [24] and [18]. However, there was a positive correlation between CD4count of 600cells/µl and above and infection with C. parvum. HIV destroys the cell mediated immune system which is provided by the CD4 lymphocytic cell, these lymphocytes when significantly destroyed below 200 predisposes the patients to opportunistic infection and invariably more chance of acquisition of C. parvum infection.

Cryptosporidium species have been estimated to infect up to 500 million people annually in developing countries, in Africa about 20-35% people are infected with the parasites and 32.5-40% harbor this parasites in sub-Saharan Africa [24].

This study recorded similar report on low CD4 count among HIV/AIDS patient's as reported by [20], [17] and [15]. The immunodefficient state of HIV/AIDS patient makes them more susceptible to cryptosporidium and once established they are not able to prevent the proliferation or clear the infecting agent, [24].

According to the four selected hospitals in Borno state of the study area, is statistically significant. The infection rate is higher in USUHM with 7.0% compare to UMTH, SSH, and MSMHM. This could be because of the location of the hospital is in the vicinity of Internally Displaced Camps (IDP) and Bulunkutu area where low income earners live and visit the hospital for their routine health care delivery.

5. CONCLUSION

The findings in this study indicate that Cryptosporidium antigens are prevalent in the study population. Patients with age group of 20-39years of age had higher prevalence of cryptosporidiosis. Clinical presentations of diarrhea were found to be associated with cryptosporidiosis. The occurrence based on hospitals has no association with the parasite but has indicated that patients with CD4 <200µl are more vulnerable to contact the parasites. More studies are recommended to determine the molecular contour of the different species and genotypes of Cryptosporidium in circulation in Borno State, Northestern Nigeria.

CONSENT

The participants were given a consent form attached to a questionnaire (17), the specimens were labeled appropriately and registered with the patient's study number, and Samples was

transported to the laboratory. On arrival in the laboratory, safety precautions were observed throughout the period of processing the specimens.

ETHICAL APPROVAL

The study was approved by the Ethical Committee of University of Maiduguri Teaching Hospital, Umaru Shehu ultramodern Hospital, State Specialist Hospital, and Mamman shuwa memorial Hospital Maiduguri, with approval number UMTH/REC/558.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
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