Original Article

PREVALENCE AND CORRELATION OF CRYPTOSPORIDIOSIS WITH CD4 COUNT IN HIV/AIDS PATIENTS ATTENDING HIV TREATMENT CLINIC AT THE AHMADU BELLO UNIVERSITY TEACHING HOSPITAL, ZARIA.

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Abstract

Introduction: Cryptosporidiosis is a protozoal infection caused by *Cryptosporidium species* which manifest with chronic diarrhoea among HIV/AIDS patients resulting in high morbidity and mortality among this group. The aim of this study was to correlate the occurrence with CD4 cell count among HIV seropositive patients presenting with diarrhoea at the Ahmadu Bello University Teaching Hospital Zaria. **Methodology:** This was a cross-sectional comparative study among 183 HIV seropositive patients attending the HIV treatment clinic of Ahmadu Bello University Teaching Hospital, Zaria and 183 seronegative controls. Stool samples were collected from each participant and processed using formol ether concentration method and the supernatant from the concentration was used for ELISA (Prospect Oxoid UK) to detect *Cryptosporidium* antigen. The blood samples from participants at the general out-patient department were subjected for HIV screening using Determine while the blood samples from participants at the HIV treatment clinic were analysed using Partec CyFlow® CD4 easy kit for CD4 cell quantification. **Results:** A total of 366 diarrhoeal stool samples were collected from HIV seropositive and HIV seronegative patients, 9 (4.9%) were positive for *Cryptosporidium antigen* by ELISA. In HIV seronegative patients, only 1 (0.55%) *Cryptosporidium* oocyst was positive. Five (55.6%) of the HIV seropositive patients with cryptosporidiosis had CD4+ count less than 200cells/ml and 4 (44.4%) had a CD4+ count between 200-349, there was a significant association between the CD4 cell count and occurrence of Cryptosporidiosis. (OR = 5.55, p = 0.007). **Conclusion:** Cryptosporidiosis was found to be prevalent among HIV patients with low CD4 cell counts

Keywords: Cryptosporidiosis, HIV/AIDS, CD4 cell count, Ahmadu Bello University Teaching Hospital (ABUTH), Zari

INTRODUCTION

Cryptosporidiosis is a protozoal infection caused by the *Cryptosporidium species* which are significant causes of enteritis among HIV/AIDS patients. Although, the parasite was first identified in 1907 by Tyzzer[1], it became of interest after 75years during the emergence of HIV/AIDS. The infection is acquired by ingestion of oocyst leading to gastrointestinal symptoms. In immunocompetent patients, the symptoms are usually mild and self-limiting whereas in immunosuppressed patients the symptoms vary from mild to debilitating diarrhoea depending on the CD4 cell count. Patients who have a $CD4^+$ count of 200-500cells/µl or more usually have self-limiting diarrhoea as compared to those with counts of <200cells/ µl, consequently, $CD4^+$ plays an important role in immunity in cryptosporidiosis.[4] $CD4^+$ T cells are a major component of the human intestinal lamina propria and are among the first T-cell populations to decrease after HIV infection.

HIV infection increases the severity of protozoal infections and as the disease progresses there is reduction in the body's immune cells called the

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CD4+ cells subsequently predisposing such group of people to life threatening opportunistic infections like *Cryptosporidium* infections.[2] Because of the associated co-infections there's increased morbidity and mortality. Similarly, the recovery of an AIDS patient from cryptosporidiosis in response to highly active antiretroviral treatment has been associated with increasing numbers of $CD4^+$ T cells in the intestinal mucosa.[3]

Cryptosporidiosis is said to develop in 10-15% of patients with HIV/AIDS in America and Europe, and 30-50% of patients in developing countries.[4] The greatest burden of cryptosporidiosis is in children and HIV patients. It has been estimated in India among children <2 years, that cryptosporidiosis leads to 3.9–7.1 million diarrheal episodes, 66.4–249.0 thousand hospitalizations, and 5.8–14.6 thousand deaths each year.[5] In HIV seropositive patients, it is a major cause of chronic diarrhoea which subsequently leads to malnutrition and death in severe cases.[6] The aim of this study is to determine the prevalence of *Cryptosporidia* ova in diarrheal stools of HIV patients and to determine the association between $CD4^+$ count level and occurrence of *Cryptosporidium species* among this cohort.

MATERIALS AND METHODS

The study was a cross-sectional, comparative study carried out at Ahmadu Bello University Teaching Hospital (ABUTH), Zaria. The centre serves as a referral centre from most regions in the Northwest and neighbouring states like Katsina, Sokoto, Kano, Zamfara, and beyond. The study was carried out among consecutive, consenting adult male and female subjects seeking care at the HIV treatment clinic and HIV seronegative subjects attending the General Out Patient clinic (GOPC), ABUTH Zaria. Subjects meet the inclusion were recruited consecutively and samples from patients were collected until sample size was achieved. Based on a previous prevalence of 4%[7] and using a comparative cross-sectional sample size calculator on Epi info 7, a total sample size 366 (183 HIV seropositive and 183 seronegative) patients was obtained.

Ethical approval was obtained from the Ahmadu Bello University Teaching Hospital, Zaria ethical committee before the commencement of the study. Also, a written informed consent was sought from all participants in the study. Confidentiality of participants was ensured during and after the study.

Sample Collection and Storage

For each recruited participant, a blood and stool sample each were collected. Four millilitres of blood were collected in EDTA bottle while stool samples were collected in a clean wide mouthed screw capped plastic container, which were transported to the Medical Microbiology laboratory of ABUTH, Zaria for processing.

Blood Sample Processing

The blood collected from GOPC participants were initially subjected for HIV screening; using Determine® rapid test kits according to manufacturer instructions, with only seronegative subjects being further included in the study. Briefly, the test pads were exposed and about 50µl of plasma was picked using a precision pipette and placed on the test pads. Results from the test were read after 10-15minutes as positive (pink/red line was seen at the control region and another one at the test region) or negative (pink/red line was seen only at the control region.[8] The blood samples from participants from the HIV treatment clinic were analysed using the flow cytometer for CD4 quantification.

Stool Sample Processing

The samples were preserved and concentrated using the formalin-ether concentration method.[9] In this method, 2 ml of watery stool was collected in a tube and then 10 ml of 10% formalin added. This was mixed well then filtered using a coffee strainer into another tube with disposal of faecal particles collected on the strainer. Three (3) millilitres of ether were added to the filtrate and the solution was vortexed for 15 seconds to allow adequate mixing of ether to the filtrate and subsequently centrifuged immediately at low speed (500g) for 1 minute. After centrifuging, four layers were formed: topmost layer represented the ether, then faecal debris layer followed by formol water layer and lastly the sediment layer. The formol water later was then carefully transferred to another clean tube using a Pasteur pipette for ELISA analysis. [10]

Antigen Detection

The processed stool sample (formol water layer) was used for this procedure using ProspecT™ (Oxoid, Basingstoke, UK). This is a rapid ELISA kit for detection of Cryptosporidium antigen in the stool. Test was carried out according to the manufacturer's instructions.[11] First, required number of the micro wells were identified, including two additional wells for positive and negative controls. Four drops of the negative and four drops of the positive control were respectively added in wells A1 and B1. Also, 200ul of the supernatant from stool sample was added to subsequent wells. The plate was covered using a microplate cover and incubated for 60 minutes at room temperature. The contents of the wells were then discarded into a waste container, washed 3 times using a diluted wash buffer (1 part of wash buffer concentrate to 9 parts distilled water). After the last wash, the plate was tapped on a paper towel to remove excess wash buffer. Four drops (200ul) of enzyme conjugate were added to each well and incubated for 30 minutes at room temperature. Contents of the wells were discarded into a waste container, washed using diluted wash buffer for 5 times and excess wash buffer removed as above. Four drops of colour substrate were added into each well and incubated for 10 minutes at room

temperature. Finally, a drop (50ul) of stop solution was added to each well and results read within 10 minutes at room temperature.[11] Positive wells were seen as pale to deep yellow while negative wells remained colourless.[11]

Elisa Reader

Wells were read at 450nm. Absorbance values of 0.15 and above OD were read as positive while absorbance reading at less than 0.15 OD was recorded as negative.

Flow Cytometry

The process was analysed using the Partec CyFlow® CD4 easy kit with code 05-8401.[12] About 20µl of the whole blood was added to the Partec test tube. Then 20µl of the CD4 mAb PE Z (PE conjugated monoclonal antibody to human CD4 cells) was added to the whole blood and mixed. The mixture was incubated for 15 minutes at room temperature. About 800µl of no lyse buffer was added to above. Subsequently the mixture was subjected to the Partec device for analysis.[12] Counting results were displayed automatically as CD4 T cells per µl of whole blood using the "gating" method to separate the two types of cells. The count of the CD4 T cells was automatically displayed by the Partec Cyflow counter and recorded. [12]

Data Analysis

Data collected was entered into a personal computer and analysed using the software - Statistical Package for Social Sciences (SPSS™) version 20. Data is presented using tables and percentages. A confidence interval of 95% was used in this study with significance levels set at $P < 0.05$. Regression analysis was carried out to investigate the relationship between CD4 count and cryptosporidial diarrhoea.

RESULT

A total of 366 diarrheal stool samples were collected, of which 183 were from HIV seropositive patients attending the HIV treatment clinic and 183 from HIV seronegative attending the GOPC at ABUTH. The ages of the subjects ranged between 16 to 65 years.

Ninety-two (50.3%) and 95 (51.8%) participants were in the age group 26-35 years of the HIV seropositive and seronegative patients respectively, while the 36-45 years group had 46 (25.2%) and 40 (21.8%) participants in the HIV seropositive and seronegative subjects respectively. Seven (3.8%) and 4 (2.2%) of the participants were from the age group 56-65 years in HIV seropositive and seronegative subjects respectively.

Out of the 183 participants in both the HIV seropositive and seronegative participants, 132 (72.1%) and 115 (62.8%) were females respectively. One hundred and fifty-three (83.6%) participants were married in HIV seropositive patients and 144 (78.7%) seronegative patients.

Table 1. Demographic characteristics of HIV seropositive and seronegative patients.

Variables	HIV Seropositive		HIV Seronegative	
	Frequency	Percentage (%)	Frequency	Percentage (%)
Age group				
16-25	22	12.0	32	17.5
26-35	92	50.3	95	51.9
36-45	46	25.2	40	21.8
46-55	16	8.7	12	6.6
55-65	7	3.8	4	2.2
Total	183	100.0	183	100.0
Sex				
Male	51	27.9	68	37.2
Female	132	72.1	115	62.8
Total	183	100.0	183	100.0
Marital status				
Single	15	8.2	21	11.5
Married	153	83.6	144	78.7
Divorced	10	5.5	5	2.7
Widowed	5	2.7	13	7.1
Total	183	100.0	183	100.0
Educational status				
Primary	10	5.5	37	20.2
Secondary	118	64.4	74	40.4
Tertiary	51	27.9	48	26.2
Informal	4	2.2	24	13.2
Total	183	100.0	183	100.0
Occupation				
Civil servant	59	32.2	48	26.2
Self employed	70	38.3	82	44.8
Unemployed	36	19.7	20	10.9
Retired	5	2.7	12	6.6
Others	13	7.1	21	11.5
Total	183	100.0	183	100.0

Table 2. Age and sex distribution of cases of *Cryptosporidium* in stool

	HIV Seropositive		HIV Seronegative	
	Freq	(%)	Freq	(%)
Age Group (Years)				
16-25	1	11.1	0	0.0
26-35	3	33.4	0	0.0
36-45	2	22.2	1	100.0
46-55	2	22.2	0	0.0
56-65	1	11.1	0	0.0
Total	9	100.0	1	100.0
Sex				
Male	2	22.2	1	100.0
Female	7	77.8	0	0.0
Total	9	100.0	1	100.0

Table 3. Relationship between CD4 count and occurrence of *Cryptosporidium*.

CD4+	Number of cases (n=9)	Occurrence of Cryptosporidium (%)	Total (%) (n=183)
>500	0	0.0	0.0
350-499	0	0.0	0.0
200-349	4	44.4	2.2
<200	5	55.6	2.7
Total	9	-	4.9

Table 4. Association between CD4 count and occurrence of *Cryptosporidium*.

	Cryptosporidium Present	*Cryptosporidium* Absent	Total
<200 cells/µl	5 (13.5%)	32 (86.5%)	37
>200 cells/µl	4 (2.7%)	142 (97.3%)	146
Total	9	174	183

OR= 5.55 (CI= 1.41-21.82); p=0.007

Cryptosporidia were identified in diarrhoeal stools of 9 (4.9%) of the HIV seropositive cases, and only 1 (0.5%) of the HIV seronegative participants. The age distribution for *Cryptosporidium* detection cut across all age groups: 3 (33.4%) in the age group 26 to 35 years, and 2 (22.2%) in both 36-45 years and 46-55 years age groups. Only an ovum (11.1%) each was detected in age groups < 26 years and > 55 years, as shown in Table 4.2.

Seven (77.8%) of the parasites detected in HIV seropositive subjects were picked in females and the remaining 2 (22.2%) were detected in males. The only *Cryptosporidium* detected in HIV seronegative patients was found in a male subject.

Five (55.6%) of the nine cases were detected in patients with CD4+ count less than 200cells/ml, while the remaining 4 (44.4%) occurred in patients with CD4+ counts between 200-349 cells/ml. There was a positive correlation between lower CD4 counts and the occurrence of *Cryptosporidium*, with an odds ratio (OR) = 5.55 (CI=1.41-21.82) and p= 0.007

DISCUSSION

Cryptosporidiosis is one of the major opportunistic infections seen in HIV seropositive individuals especially in those with CD4 count of <200cells/ml. In this study, a prevalence of 4.9% and 0.5% was obtained among HIV seropositive and HIV seronegative patients respectively using ELISA. The prevalence observed in this study is slightly higher than that of a study conducted in Kano that reported a 4.0% prevalence.[7] It is however markedly lower when compared to studies conducted in Ilorin, Borno, Adamawa, and Edo states which reported prevalence rates of 37.7%,[13] 24.1%[14], 20.8%[15] and 39.0%[16] respectively. Additionally, a study in Lagos revealed a prevalence rate of 18.7%.[17] These marked differences in prevalence rates may be partly explained by the fact that these studies employed only MZN as the method of detection of *Cryptosporidium* and so there is the possibility of occurrence of false positives due to the similarity of staining characteristics of *Cryptosporidium* with other intestinal parasites such as oocysts of *Cyclospora* and *Microsporidium* and the spores of yeasts. Another study in Imo State, Nigeria, found a prevalence of 32.5% which is markedly higher than the finding of 4.9% but the Imo study was a large population-based study among 1960 patients across three zones in the state.[18] The markedly higher sample size and the fact that MZN was the detection method employed in the study may all have contributed to this marked difference.

The lower prevalence obtained in this study could be explained by the fact that adherence to HAART has increased over time since its introduction in the 1990s which has been shown to improve immunity in HIV seropositive patients. This in turn helps in limiting the occurrence of opportunistic infections, including Cryptosporidiosis.[19] Chronic diarrhoea and *Cryptosporidial* infection often resolve with increase in CD4 count; in addition, the use of co-trimoxazole as a prophylaxis against *Pneumocystis jiroveci*, has been shown to have some activity against *Cryptospridium spp*., which could contribute to the low prevalence obtained.

Other studies in Kenya and Rwanda reported prevalence rates of 34.0%[20] and 34.2% respectively but these utilized PCR rather than MZN or ELISA in the primary detection of *Cryptosporidium* in stools of HIV seropositive patients. Studies from Ethiopia have revealed various prevalence rates ranging from 11-25%.[2,21,22] An Indian study that employed ELISA and MZN for the detection of Cryptosporidium in stools of HIV/AIDS patients reported a prevalence of 5.4%, but the sample size of 73 was significantly smaller than what was used in this study and most other studies.[23] Two studies from Malaysia and Turkey revealed prevalence rates of 12.4%[24] and 11.3%[25] respectively; although the methodology used in these studies were MZN and ELISA respectively. The findings reflect the varying detection rates of the parasite depending on the detection method used. Results from a global systematic review and meta-analysis, published in 2018 revealed a 14% pooled prevalence of cryptosporidium in stools of HIVAIDS patients and the authors concluded that HIV-infected people have a high prevalence of *Cryptosporidium* and other opportunistic intestinal protozoa, especially in sub-Saharan Africa.[26]

In this study, females were found to be more infected than the males, this may not be surprising as more females participated in the study. However, bivariate analysis did not show any statistical significant difference between males and females (p=0.70). This result is similar to the one obtained in Kaduna,[27] but different when compared to other studies in Benin city,[28] Enugu[29] and Sokoto.[30]

The prevalence is found to be higher in age groups 26-35 which was 33.4%. This young, active age group are perhaps more likely to engage in practices like patronising food vendors which may increase the likelihood of infection. This result agrees with a report from Michika, Adamawa state,[15] India[31] and China[32].

The findings of this study showed that HIV seropositive patients are significantly more likely to be infected with *Cryptosporidium* than HIV seronegative patients (4.9% versus 0.5% respectively; P=0.0032). The finding is consistent with reports in other parts of Nigeria that showed that HIV seropositive patients are

much more prone to the infection [15,18]. This is not surprising as the relationship between immunodeficiency and the occurrence of opportunistic infections, including cryptosporidiosis, has long been established.

Immunity has been seen to play a vital role especially HIV immunosuppression in the natural history of cryptosporidiosis. It showed significant association between low CD4 counts and the occurrence of the parasite in HIV seropositive patients (p=0.007). Also, the odds ratio (OR) of 5.55 obtained shows that the infection is about 6x more likely to occur in patients with CD4 count <200 cells/ml than in those with a CD4 count of >200 cells/ml. This study showed no parasite detection at CD4 counts >350 cells/ml. This finding is in keeping with observations from a study conducted by Akinbo *et al* on HIV positive patients in Benin City, Nigeria.[33] Similar findings were obtained from studies in Kaduna[34] and Jos.[35] Another study in Iran further buttressed this assertion as the mean CD4 cell count at which oocysts were picked was 215 cells/ml and the highest number of oocysts was picked at CD4 count < 200 cells/ml.[36] Similar observations were made from studies in Ghana,[37] Egypt[38] and India[39,40]. These studies also revealed that the severity and duration of diarrhoea is significantly associated with the level of CD4 cell count with lower CD4 counts being associated with more severe diarrhoea disease. Also, the risk of clinical disease increases with decreasing CD4 count explaining why patients with CD4 cell count less than 100 cells/ml have more life-threatening infection.

CONCLUSION

This study has recognised the role of immunity especially the CD4 cells in playing a vital role in the protection of HIV/AIDS patients in developing cryptosporidiosis and it is still a threat in the management of opportunistic infection.

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