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Original Article

Cryptosporidiosis among HIV/AIDS Patients with Diarrhoea and Associated Risk Factors in Jos, North-Central Nigeria

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ABSTRACT

Cryptosporidiosis is an opportunistic, parasitic infection caused by *Cryptosporidium parvum.* It is transmitted via faecal-oral route and causes life-threatening, cholera-like diarrhoea in immunocompromised individuals such as HIV/AIDS patients. This study aimed to determine the prevalence of *cryptosporidiosis* and associated risk factors among HIV/AIDS patients with diarrhoea. This was a cross-sectional study of 100 HIV/AIDS patients with diarrhoea in a tertiary health institution in Jos, North-central Nigeria between April and November 2019. Samples were collected from each patient after signing a consent form and filling a well-structured questionnaire. The oocytes of *Cryptosporidium parvum* were identi?ed in the stool samples using modified Ziehl-Neelsen stain and polymerase chain reaction (PCR). The results obtained were computed using SPSS version 21. The mean age (standard deviation) of the study participants was 37.0 (± SD9.6), with a minimum age of 20years and a maximum age of 63 years. The study comprises of 53(53.0%) males and 47(47.0%) females. Fifty-four (54) of the participants were on antiretroviral (ARV) drugs while 46 were ARV drug naïve. The prevalence of cryptosporidiosis among the study population was 13.0%. Cryptosporidiosis was found in 10(21.7%) of the 46 ARV drugs naïve participants and in 3(5.6%) of the 54 participants on antiretroviral therapy. This was statistically significant at $p = 0.016$. There was also a significant relationship ($p = 0.012$) between the prevalence of cryptosporidiosis and the level of $CD4^+$ T-lymphocytes count of the study participants. The infection was more among participants with $CD4^+$ T-lymphocytes count less than 200 cells/µl. This stress the need for good personal hygiene, sanitation and compliance to antiretroviral treatment among HIV/AIDS patients to reduce the risk of opportunistic infections such as cryptosporidiosis.

Keywords: *Cryptosporidium parvum, Cryptosporidiosis,* HIV/AIDS, $CD4^+$ T-lymphocytes

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INTRODUCTION

Cryptosporidium is an intracellular, microscopic protozoan within the phylum Apicomplexa. It is ubiquitous in its geographic distribution and range of vertebrate hosts including humans.[1] *Cryptosporidium parvum* causes many of human infections, although other species such as *Cryptosporidium hominis*, *Cryptosporidium muris*, *Cryptosporidium felis* and *Cryptosporidium meleagridis* have been reported to cause infection in some individuals especially immunocompromised patients.[2,3]

Cryptosporidium parvum is transmitted through faecal-oral route and can be spread from person to person, and from animals to humans via contaminated food and water. It has been reported as the most common cause of waterborne disease in the United Kingdom due to contaminated drinking water and swimming pools.[4] Although cryptosporidiosis is not the commonest cause of waterborne outbreak in the United States, it was responsible for one of the largest waterborne outbreaks ever described.[5] This link between *Cryptosporidium* infection and drinking water has led authorities in both the United Kingdom and United State to issue advice to immunocompromised patients to boil water before drinking.[6]

Infection in immunocompetent individuals often results in asymptomatic or mild self-limited disease, while in immunocompromised individuals such as HIV/AIDS patients, infection may result in chronic or life-threatening cholera-like diarrhoea.[7] Infected individuals usually present commonly with persistent diarrhoea that may lead to chronic malabsorption of fluids, nutrients, vitamins, and electrolytes with resultant wasting.[8] This warrants a prompt diagnosis and an early institution of therapy to reduce the morbidity associated with the disease. In Jos University Teaching Hospital (JUTH), the prevalence of cryptosporidiosis has been largely reported among under-fives and to the best of researchers' knowledge, no study has been conducted among adult HIV positive patients.[9] This study aimed at determining the prevalence of cryptosporidiosis and associated risk factors among HIV/AIDS patients with diarrhoea in a tertiary health institution Jos, North-central Nigeria.

MATERIALS AND METHODS

This was a cross-sectional study of 100 HIV/AIDS patients with diarrhoea attending a tertiary health institution in Jos, north-central Nigeria. Stool samples were collected in sterile universal specimen containers after informed consent and assurance of confidentiality. About 2mls of blood sample in EDTA bottles were collected from each patient to determine their $CD4^+$ T-lymphocytes count. A well-structured questionnaire was also used to collect demographic information. The stool samples were transported to the laboratory immediately for analysis. The samples were divided into two portions; one portion was stored at -20^0C until used for Polymerase Chain Reaction (PCR). The other portion was immediately used for modified Ziehl-Neelsen to identify the oocytes of *Cryptosporidium*.

Modified Ziehl-Neelsen (mZN)

A smear was made directly from the fresh stool specimen and allowed to air dry. The smear was fixed with methanol for 2-3 minutes to prevent washing off during staining. The fixed smear was stained with strong carbol-fuchsin for 15 minutes and rinsed thoroughly with tap water to wash off the excess carbol-fuchsin. It was then decolourized with 1% acid alcohol for 15-20 seconds.[10] The smear was rinsed thoroughly with tap water and allowed to drain. It was counterstained with 0.4% malachite green or methylene blue for 30-60 seconds. The smear was rinsed thoroughly with tap water and air-dried. After air-drying, the smear was examined microscopically for *Cryptosporidium parvum* oocysts using x100 oil immersion objective. The oocysts appeared pink-red against the light green or blue background.[11]

DNA Extraction from Faecal Specimen

Deoxyribonucleic acid (DNA) extraction from faecal samples was carried out using Quick-DNA™ Faecal/Soil Microbe Miniprep Kit (Zymo Research Corporation, South Africa). The procedure was carried out following manufacturer's instructions. Approximately 200mg of faecal sample was added to a ZR Bashing Bead™ lysis tube (0.1 & 0.5 mm) and 750µl Bashing Bead™ Buffer was added to the tube. The mixture was secured in a bead beater fitted with a 2ml tube holder assembly and process at maximum speed for 5 minutes.[12] After the bashing, the ZR Bashing Bead™ lysis tube was centrifuged in a microcentrifuge at 14,000g for 1 minute to pellet stool particles. About 400µl of the supernatant was pipetted out and transferred to a Zymo-Spin™ III-F filter in a collection tube. This was centrifuged at 8,000g for 1 minute. About 1,200µl of genomic lysis buffer was added to the filtrate in a collection tube and mixed well. Approximately 800µl of the mixture was transferred to a Zymo-Spin™ IICR column in a collection tube and centrifuge at 10,000g for 1 minute. The flow-through from the collection tube was discarded and this last step was repeated. About 200µl of DNA pre-wash buffer was added to the Zymo-Spin™ IICR column in a new collection tube and centrifuge at 10,000g for 1 minute.[13] Approximately 500µl of g-DNA wash buffer was added to the Zymo-Spin™ IICR column and centrifuge at 10,000g for 1 minute. The Zymo-Spin™ IICR column was transferred to a clean 1.5 ml microcentrifuge tube and 100µl of DNA elution buffer was added directly to the column matrix and centrifuged at 10,000g for 30 seconds to elute the DNA. The Zymo-Spin™ III-HRC filter was placed in a clean collection tube and 600µl prep solution added to it and centrifuged at 8,000g for 3 minutes. The eluted DNA was transferred to a prepared Zymo-Spin™ III-HRC filter in a clean 1.5ml microcentrifuge tube and centrifuged at exactly 16,000g for 3minutes. The filtered DNA was now suitable for PCR and other downstream applications.[14] The eluted DNA was stored at -20°C until it was used for PCR.

18S rRNA Gene Amplification

Cryptosporidium oocysts were identified using a small-subunit (18S) ribosomal RNA (rRNA) gene-based on primary and nested PCR. The primary PCR was performed using the primer set, CPr I (5'-AAA CCC CTT TAC AAG TAT CAA TTG GA-3') -forward and CPr II (5'-TTC CTA TGT CTG GAC CTG GTG AGT T-3') -reversed. The resultant amplicons from the primary PCR were used for the nested PCR using the primer set CPr III (5'-TGC TTA AAG CAG GCA TAT GCC TTG AA-3') -forward and CPr IV (5'-AAC CTC CAA TCT CTA GTT GGC ATA GT-3') -reversed.

Amplification Reactions

The reaction mixture of the primary PCR consisted of 10µl template DNA in a total volume of 50µl with final concentrations of 10mM Tris-HCl (pH 8.3), 50mM KCl, and 2.5mM $MgCl_2$, a 0.5µM concentration of each primer sets, 1.5U of DNA Taq polymerase, and a 100µM concentration of each deoxynucleoside triphosphate (dNTP). The reaction mixtures of the primary PCR were thermally cycled at initial denaturation at 94°C for 5min, 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and the final extension was performed at 72°C for 10min. The reaction mixture of the nested PCR was identical, except that 1ul of the first reaction and a 0.5µM concentration of each primer of the nested PCR primer sets were used. For the nested PCR, reaction mixtures were thermally cycled once at 94°C for 5min, 30 times at 94°C for 30s, at 65°C for 30s, and at 72°C for 1 min, and then once at 72°C for 5min.[15] The final PCR products were analysed by electrophoresis on 1.5% agarose gel.

Agarose Gel Electrophoresis

Approximately, 1.5g of agarose gel was weighed and mixed with 100ml of Tris-Acetate EDTA (TAE) buffer in a conical flask and was heated in a microwave for 5 mins at 15°C and allowed to cool. About 1.5µl of ethidium bromide solution was added. The mixture was poured into a gel casting glass and combs were carefully removed

and the cast was then placed into the electrophoretic tank. Loading buffer of 1µl was mixed with 8µl of amplicon in a loading tray and was dispensed into the various wells in the gel. Eight (8µl) of 100bp ladder was placed in the 1st well. The gel was then run at 120 volts for 40 minutes and the result was visualized under a UV transilluminator[13] and amplicons of 300bp were considered positive (Figure 1).

Data Analysis

The data obtained from the study were analyzed using Statistical Package for Social Sciences (SPSS) version 21 (IBM SPSS Inc, USA). Proportions were compared using Ch-square with confidence limit (*p*-value) of < 0.05 considered significant.

RESULTS

Out of the 100 HIV/AIDS patients presenting with diarrhoea, 13(13.0%) were positive for cryptosporidiosis. Antiretroviral drug naive HIV/AIDS patients were responsible for a higher prevalence rate. Cryptosporidiosis was found in 10(21.7%) of 46 ARV drug-naïve HIV/AIDS patients recruited while only 3(5.6%) of 54 HIV/AIDS patients on antiretroviral therapy had cryptosporidiosis. The difference in *Cryptosporidium parvum* infection among the antiretroviral drugnaive HIV patients and HIV positive patients on antiretroviral therapy was statistically significantly ($p = 0.016$) (Table 1).

There was no significant difference in *Cryptosporidium* infections among the age groups ($p = 0.250$) (Table 2). A higher infection rate was observed in patients aged 20-29 years old as 22 were recruited, with 6(27.3%) positive. Age group 30-39 accounted for 3(7.7%) of the infection out of 39 patients recruited within this age group. The least prevalence rate of infection was observed among patients above 60 years. None of those recruited within this age group was positive for cryptosporidiosis in this study.

There was a significant relationship ($p = 0.012$) (Table 3) between prevalence of cryptosporidiosis and the level of CD4 cell count of the study participants. Patients with CD4 cell count of <200 cells/µl had a prevalence of 30.4% (7 out of 23 tested). Forty-seven (47) of the participants had CD4 cell count between 200-499 cells/µl with 5(10.6%) positive for cryptosporidiosis. Based on our findings, the infection was lower in those with CD4 cell count ≥500 cells/µl as 30 were screened with only 1(3.3%) positive.

Table 4 summarized the rate of *Cryptosporidium parvum* infection to some selected socio-demographic factors. Based on sex, 53 of the subjects were males with 8(15.1%) diagnosed to have cryptosporidiosis while 5(10.6%) of the female subjects had cryptosporidiosis. Though the infection was more among the male subjects, the difference was not statistically significant ($p = 0.508$).

The stool consistency was observed to have a significant relationship with cryptosporidiosis. Patients who presented with loose/watery stool were more infected as 10(24.4%) of 41 participants with loose/watery stools were positive. Those with bloody diarrhoea (dysentery) were the least infected with 1(4.0%) positive out of the 25 examined. About 34 of the patients recruited had diarrhoea with mucus and 2(5.9%) of them were positive for cryptosporidiosis. This difference in infection base on stool consistency was statistically significant at $p = 0.018$, (Table 4). Another factor that was considered among the study population was the use of antibiotics. Eighty-one (81) of the patients were on antibiotics such as flagyl, cotrimoxazole, and ciprofloxacin. About 14.8% (12 out of 81) had cryptosporidiosis while only 1(5.3) of the 19 patients that were not on antibiotics were positive. This was not statistically significant and may suggest that these antibiotics are not effective against *Cryptosporidium parvum*.

Sources of water for drinking and domestic use was also analysed and was not found to be statistically significant among the study population. Out of 42 participants that use borehole as their source of drinking water, 4(9.5%) had cryptosporidiosis. Thirty-one (31) use tap water out of which 6(19.4%) were positive. The remaining 27 of the

participants use well as their source of water out of which 11.1% were positive for *cryptosporidiosis* (p = 0.440) (Table 4).

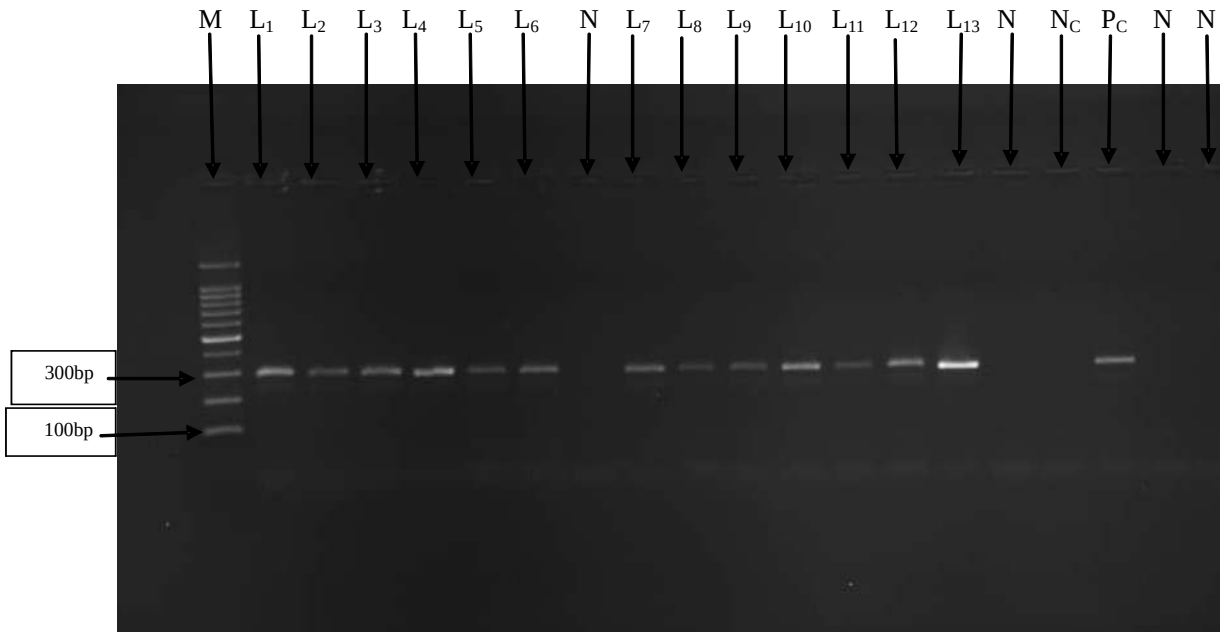

Figure 1: The gel electrophoresis of the PCR product after amplification of 18S rRNA gene of *C. parvum*.

Key:

***M** = ladder which was 100 base pairs (100bp); **$L_1 - L_{13}$** = Positive results with bands at 300bp; **N** = Negative results with no bands; **Nc** = Negative control showing no band; **Pc** = Positive control showing band at 300bp © – 1085/TT)*

Table 1: Prevalence of *Cryptosporidium parvum* infection among antiretroviral drug naive HIV/AIDS patients and HIV/AIDS patients on antiretroviral therapy.

Antiretroviral	No. Positive (%)	No. Negative (%)	Total
Yes	3(5.6)	51(94.4)	54
No	10(21.7)	36(78.3)	46
Total	**13(13.0)**	**87(87.0)**	**100**

p = 0.016; χ^2 = 5.752; df = 2

Table 2: Prevalence of *Cryptosporidium parvum* infections in relation to age among HIV/AIDS patients in a tertiary health institution in Jos North-central Nigeria.

Age Group (years)	No. Positive (%)	No. Negative (%)	Total
20-29	6(27.3)	16(72.7)	22
30-39	3(7.7)	36(92.3)	39
40-49	3(10.7)	25(89.3)	28
50-59	1(11.1)	8(88.9)	9
≥60	0(0.0)	2(100.0)	2
Total	**13(13.0)**	**87(87.0)**	**100**

$p = 0.250$; $\chi^2 = 5.391$; df = 4

Table 3: Prevalence of *Cryptosporidium parvum* in relation to CD4 cell count in HIV/AIDS patients in a tertiary health institution in Jos, North-central Nigeria.

CD4 Cell Count (cells/µl)	No. Positive (%)	No. Negative (%)	Total
?200	7(30.4)	16(69.6)	23
200-499	5(10.6)	42(89.4)	47
≥500	1(3.3)	29(96.7)	30
Total	**13(13.0)**	**87(87.0)**	**100**

$p = 0.012$; $\chi^2 = 8.892$; df = 2

Table 4: *Cryptosporidium parvum* infection among HIV/AIDS patients in a tertiary health institution in Jos, North-central Nigeria.

Variables	No. Tested	No. Positive (%)	χ^2	P-value
Sex				
Male	53	8(15.1)	0.437	0.508
Female	47	5(10.6)		
Sources of water				
Well	27	3(11.1)	1.641	0.440
Borehole	42	4(9.5)		
Tap water	31	6(19.4)		
Stool consistency				
Loose/watery	41	10(24.4)	8.017	0.018
Bloody stool	25	1(4.0)		
Mucoid	34	2(5.9)		
Antibiotics used				
Yes	81	12(14.8)		
No	19	1(5.3)	1.242	0.265
Marital status				
Single	15	3(20.0)	2.149	0.341
Married	71	7(9.9)		
Widower/widow	14	3(21.4)		

DISCUSSION

The prevalence of cryptosporidiosis among HIV/AIDS patients attending a tertiary health institution in Jos, North-central Nigeria was found to be 13.0% in this study. This prevalence was higher than the 3.8% previously reported in under-5 malnourished children with diarrhoea in the same institution.[9] Also in South-south Nigeria, Erhabor and his colleagues[16] in 2011 reported a lower prevalence of 2.9% among 105 HIV positive patients, while 6.3% was reported among HIV infected patients on antiretroviral drugs in Southeastern Nigeria.[17] A higher prevalence rate of 15.1% was reported in another study by Nwabuisi in Kwara State , Northcentral Nigeria among children within the ages 0-14 years old with diarrhoea.[18] In Southwestern and North-eastern Nigeria, two separate studies also reported a higher prevalence of 52.7% and 42.9% respectively.[19,20] The variations in prevalence rates across Nigeria could also be explained by the differences in methodologies employed for the study. This research was conducted using polymerase chain reaction (PCR) which has been demonstrated to be more sensitive than conventional microscopy mostly used in the previous studies.

The reported high prevalence rate in Northeastern Nigeria may not be unconnected with the ongoing fight against insurgency in the area. The war has led to the displacement and relocation of people to IDP camps and crowded communities. The resultant overcrowding, inadequate basic amenities and poor standard of living could increase the spread of infectious diseases including cryptosporidiosis. It has been established that exposure to contaminated water, food, and animals are significant risk factors to water-borne diseases such as cryptosporidiosis[21] and these factors are commonly associated with war-torn areas.

In other African countries, a lower prevalence of 2.2% was reported in South-west Uganda[22] and 11.9% in Kenya among 151 samples studied.[23] While, a higher prevalence of 18.0% have been reported in Limpopo Province of South Africa.[24] This variation in prevalence rate across African countries could be attributed to the methodology used by different researchers, immune status of the study population, lack of portable drinking water, poor personal hygiene and poor sanitary facilities which are key factors in the dissemination of cryptosporidiosis. It was also observed that some of these researches were conducted in both symptomatic and asymptomatic patients which could explain the low prevalence rate compared to our study that was conducted among symptomatic HIV patients only.

The prevalence rates in a large-scale survey of faecal *Cryptosporidium* oocyst excretion in developed countries in Europe and North America ranges from 1-3%.[25] It also shows that cryptosporidiosis in these developed countries is more common in children, especially those younger than 2 years old.[21,26,27] The low prevalence in Europe and other developed countries is probably justified by the fact that these countries have improved access to potable drinking water, good drainage system and proper waste disposal mechanism, thus limiting the spread of cryptosporidiosis. This study revealed that the prevalence of cryptosporidiosis was not statistically significant for age. The infection was higher among the age group 20-29 years with a prevalence of 27.3%. These findings were similar to a study conducted in Bushenyi, Uganda where a higher prevalence was obtained among the group 25-34 years,[28] but contrary to the report of Nakibirango and co-workers[22] who reported that cryptosporidiosis was more common among the age group 31-40 years which was corroborated by Masarat and his colleagues[29] who reported similar findings in HIV positive immigrants in Kashmir, India.

There was a significant relationship between cryptosporidiosis and CD4 cell count. The infection was higher in patients with CD4 T lymphocytes of <200 cells/µl. The positive correlation between CD4 cells count and *Cryptosporidium parvum* infection may be because HIV infection depletes CD4 T-lymphocytes with progressive impairment of cell-mediated immunity

leading to increased susceptibility to opportunistic infections such as cryptosporidiosis,[30] and once the infection is established, it is difficult to treat leading to severe complications.[31] This has been corroborated by several other studies that cryptosporidiosis is more common in patients with CD4 T lymphocytes of <200 cells/µl.[32,33,34]There was a varied association between social demographic factors and *Cryptosporidium parvum* infections. It was observed that cryptosporidiosis was significantly associated with stool consistency. The infection was more in participants with loose/watery stool than in those with mucoid and bloody diarrhoea. These findings were similar to the report of a study conducted in Kano, North-western Nigeria.[34] This also gives credence to the fact that *Cryptosporidium parvum* infection presents with non-bloody, non-mucoid diarrhoea and does not cause invasion of the bowel.[35]

This study also revealed that cryptosporidiosis was more in male than in female participants. This difference was not statistically significant, indicating that gender was not a risk factor for cryptosporidiosis. This was in agreement with the report of Adesiji[19] in Osun State, Nigeria but contrary to some other documented research findings that reported a higher prevalence of cryptosporidiosis among females.[36,37,38] The reason for this difference is not clear but may probably be because males constitute majority of the population in this study and are more adventurous than females which may predispose them to more infection.Other factors such as marital status and the role of antibiotics did not show any significant correlation to cryptosporidiosis. This has been corroborated by other studies.[39,40] Significant number of the patients were on routine antibiotics such as flagyl, cotrimoxazole, and ciprofloxacin and still reported high cases of cryptosporidiosis. This may suggest that these antibiotics were not effective for the treatment of cryptosporidiosis and should not be used in established case of infection. The drug recommended for the treatment of cryptosporidiosis by the United State Food and Drug Administration is nitazoxanide[40] and none of these patients was on this drug.

CONCLUSION

Cryptosporidiosis infection was found to be more among HIV/AIDS patients drug naïve than HIV positive patients on antiretroviral drugs. Patients with CD4 cell count of <200cells/µl were more infected than patients with higher CD4 cell counts. This lends credence to the fact that the infection is more in patients with immunosuppression. People living with HIV/AIDS should be encouraged on adequate use of antiretrovirals, good personal hygiene and sanitation to protect against opportunistic infections such as cryptosporidiosis.

Ethical Consideration

This study was approved by the research ethical committee of Jos University Teaching Hospital with reference number JUTH/DCS/ADM/127/XIX/6584. Written informed consents were also signed by all participants before enrolment in the study.

Consent for Publication

All the authors reviewed and gave their approval for this article to be submitted for publication.

Competing of Interest

There are no conflicts of interests among the authors.

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