ORIGINAL ARTICLE

Evaluation of laboratory diagnostic methods for cryptosporidiosis among HIV-seropositive patients in Kano, Nigeria

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ABSTRACT

Background: The laboratory diagnosis of *Cryptosporidium parum* infection involves the demonstration of the infective oocysts in stool specimen. The conventional method of modified Ziehl-Neelsen (MZN) is very laborious, and stool debris can be mistaken for the parasite oocytes.

Objective: This research was set to evaluate the diagnostic efficacy of microscopic Modified Ziehl-Neelsen (MZN) stain, Florescence Auramine-O-phenol (AOP) stain and ELISA method.

Methodology: This is a prospective descriptive cross – sectional study of 182 consecutive adults and children of both genders, aged 1-65 years, who were HIV-seropositive and receiving treatment at Prof Sadiq Wali Treatment Centre, Aminu Kano Teaching Hospital, Kano State, Nigeria.

Result: The overall seropositivity of *C. parvum* among HIV-seropositive clients receiving treatment at the Sadiq Wali Centre, using all the three techniques, was 29.7% (range 26.9% - 31.9%). Furthermore, the sensitivity of the techniques ranged 57.1% - 93.1% (MZN to ELISA) with ELISA techniques showing the highest sensitivity at 93.1% and specificity of the three techniques ranged 51.6% - 96.1%. The highest specificity at 96.1% was recorded with the Auramine-O-phenol fluorescence stain while 93.2% and 51.6% specificity were recorded with the MZN and ELISA techniques, respectively.

Conclusion: The ELISA technique proved more suitable for the determination of the presence of cryptosporidium oocysts in stool. All stool specimens should be examined for opportunistic parasites, especially in immunosuppressed patients.

INTRODUCTION

Cryptosporidiosis is an opportunistic infection caused by *Cryptosporidium* species. These intestinal protozoa, which may be associated with immune-incompetent individuals, also contribute to enteric disease in calves, lambs, foals and piglets. They are normally passed in the faeces of infected persons and animals in the form of cysts.[1] The conventional differential staining via modified Ziehl- Neelsen (MZN) stain cannot differentiate the species.[1,2,3]

Cryptosporidiosis is an important problem in HIV-infected patients and the severity of the infection could result in severe dehydration, electrolyte imbalances, malnutrition, wasting and eventual death, since curative treatment is very disappointing.[4]

Diagnosis of the infection generally requires the observation of the infective stage of oocysts in infective stool, which is usually done via microscopy, antigen detection and polymerase chain reaction (PCR). Compared to antigen detection, the sensitivity of stool examination by acid-fast staining remains poor and requires, at least, an oocyst concentration of over 500,000 per mL in formed stools.[3,5]

The global burden of this disease may be high; more so that the current available detection systems are inadequate and there is no acceptable laboratory investigation adapted for worldwide surveillance. Chronic diarrhoea is a common presentation among HIV-infected Africans even as *C. parvum* is the most common microbial cause of diarrhoea in the HIV- infected population, worldwide. Furthermore, in Nigeria, laboratory investigations for the detection of the parasite are not routinely done for HIV/AIDS patients, except when indicated by the clinician.[6,7,8,9,10]

Conventional microscopic examination which are relatively available with less sophistication in terms of use, are being replaced with techniques which rely on molecular recognition of specific pathogenic species. Techniques such as PCR have the advantages of improved sensitivity, specificity and positive predictive value. However, these methods have limited applicability at point-of-care or resource poor healthcare system due to their costs and high technical expertise involved. The PCR was used to a great extent in the detection of *Cryptosporidium* in clinical samples.[8,11,12,13]

In two studies on rural and urban population to determine the prevalence of intestinal parasites in normal population, 29.7% and 19.4% prevalence rates, respectively were recorded for high *C. parvum*. Currently, paucity of data in northern Nigeria regarding the impact of cryptosporidiosis among HIV-seropositives, and the importance of sero-diagnosis in HIV-seropositive patients, had contributed to poor understanding of its infections and thus, made preventive and control programmes very difficult.[14]

Reports from southern Nigeria by Nwokediuko revealed rarity of *C. parvum*, as apparently, no case of *C. parvum* was reported among the investigated HIV-infected patients in South-East Nigeria.[15]

The use of ELISA methods has proved useful in patients with low ova concentrate. This is a recent method using fixed antigens in micro well. Among HIV patients with diarrhoea, *Cryptosporidium* was found in many children with chronic diarrhoea in developing countries. However, infection was not more frequent in HIV patients during the Milwaukee outbreak. The prevalence of cryptosporidiosis also varies. In Nigeria, 2.9% was reported in the South-South.[16,17]

OBJECTIVE

The aim was to evaluate three laboratory diagnostic methods for cryptosporidiosis among HIV seropositive patients presenting at the Prof Sadiq Wali HIV Treatment Centre in Aminu Kano Teaching Hospital (AKTH), Kano State, Nigeria.

METHODOLOGY

Study Area

The study was carried out at the Prof Sadiq Wali Centre in Aminu Kano Teaching

Hospital (AKTH) HIV Treatment Centre in Kano. The AKTH is a 600-bedd tertiary health institution serving Kano State and majority of the States in the North-West Zone of Nigeria. Kano State has a population of 12,178,712 people, with a population density of 403 per square kilometre, based on 2006 National population census.[18]

Study Population
Consecutive adult and paediatric patients, male and female, aged 1-65years, who were HIV-seropositive and receiving anti-retroviral therapy at the centre were enrolled.

Study Design
This was a prospective descriptive cross–sectional study which spanned over four months (October 2010 - January 2011).

Study Sample Size
The minimum sample size was calculated using the formula by Araoye.[19]Therefore, 182 subjects participated in the study.

LABORATORY PROCEDURE: **Specimen Collection, Transportation and Processing**
Stool specimens were collected in universal transparent containers with screw cap lid. Blood specimens were also collected in EDTA bottles. Samples were transported to the laboratory using suitable transport bags (with biohazard label).

Macroscopy: Stool samples were each examined for colour and consistency. Evidence of blood in the stool will be noted and was centrifuged.

Microscopy: For each specimen, a drop of stool was placed on the slide and covered with a cover slip and viewed under high powerfield(x400 magnification) with iodine background, for the presence of ova and parasites. Another faecal smear was, also, made with a drop of stool and allowed to dry in the air, and stained with acid-fast technique, for evidence of *Cryptosporidium* cyst which will appear pink or reddish, and oval in shape.

Formalin Ether Concentration Technique for stool examination: The modified formol-ether concentration method by use of sedimentation technique as described by Freeman.[20]

Microscopic Examination (Modified Ziehl-Neelsen): Stool samples were collected and smeared on clean glass slides, followed by fixation with 95%absolute methanol and stained using the modified Ziehl-Neelsen stain and air dried. Afterwards, the smear was further stained with cold carbol fuchsin and allowed to stand for 10 minutes after which it was washed off with clean tap water. The smear was decolorized with 3% hydrochloric acid (HCl) in95% ethanol, rinsed off and counter-stained with 0.25% weight per volume malachite green. The smear was, again, washed off with clean tap water and air-dried. The slide was then observed microscopically for oocysts. A confirmed positive specimen was used as quality control slide and for comparative purposes.[21]

Fluorescence Staining Method (Auramine-O-phenol, Fluorescent Stain, AOPmethod)
This simple two-step method combines fluorescent staining (using auramine) with negative staining by strong carbol fuchsin to mask background material. A confirmed positive specimen was used as quality control slide and for comparative purposes.[21,22]

Determination of Cryptosporidium Antigen via ELISA: The kit produced by DIAGNOSTIC AUTOMATION, INC. CALABASAS, USA. This was performed according to the manufacturers' instructions.

Data Analysis: Data were analysed using SPSS Version 16.0 software. The *Chi square* test and *Fischer Exact* test were used to perform and establish any statistical significance. Probability values of <0.05 were considered as statistically significant. Sensitivity, specificity, positive and negative predictive values of various techniques were determined following the methods of Galen and Gambino.[24,25]

Ethical Considerations: The study was approved by the Ethical Committee of Aminu Kano University Teaching Hospital and each participant signed the Informed Consent Form.

RESULTS
This study was carried out among 182 subjects, with the three diagnostic methods comparedfor their capacity to detect

Cryptosporidium in HIV-seropositive patients.There were 74 males (40.7%) and 108 females (59.3%), a male:female ratio of 1:1.5. The mean age of the subjects was 26 ± 14.7years. Twenty-one out of the 74 samples from male patients were positive with all the three techniques, while 37 samples out of the 108 female samples were positive in at least one technique tested. The rest, 124samples, were negative with all three techniques. This distribution was not statistically significant (*p* =0.403; *see Table 2*).

From the 182 subjects, the overall sero-positivity for *C. parvum* using all the three techniques was 29.7% (range 26.9% - 31.9%). Of the age groups 10, 8 and 8 tested positive from the 0 – 10year age group giving a prevalence rate of 40.0%, 32.0% and 32.0% respectively; 17 samples out of 58 samples in the 21-30year age group, representing 29.3%, were positive with all the three techniques; 6 (40%) sample out of the 15 samples tested within the 41-50year age group were positive (*p= 0.001, see Table 1*).

In comparing the three methods used in the identification of the *C. parvum* parasite among the HIV-seropositive patients, the percentage detection rates were 26.9% (MZN), 30.2% (AOP) and 31.9 (ELISA). A lower isolation rate was observed using the Ziehl-Neelsen method, with 49 specimens reported positive with a prevalence rate of 26.9%. This was statistically significant (*p =0.001, see Table 3*).

Furthermore, the sensitivity of the techniques ranged from 57.1% to 93.1% (MZN to ELISA) with ELISA techniques showing the highest sensitivity at 93.1% in HIV-seropositive patients and the lowest sensitivity at 57.1% with modified Ziehl-Neelsen and specificity of the three techniques ranged 51.6%-96.1%. The highest specificity at 96.1% was recorded with the Auramine-O-Phenol fluorescent stain while 93.2% and 51.6% specificity recorded with the MZN and ELISA, respectively. The positive predictive values of

the kits/methods in HIV-seropositive patients were 75.7%, 88.4%, and 47.4% for MZN, fluorescence technique and ELISA methods, respectively; while, the negative predictive values were 85.5%, 87.8% and 94.1% for MZN, Auramine O phenol stains and ELISA. This was not statistically significant (*p =0.011*, see *Table 4*).

Table 1.
Age distribution of *Cryptosporidium parvum* and the diagnostic techniques among HIV-seropositive patients

Age group (Years)	Total (%)	Percent C. parvum Positivity		
		ELISA (%)	MZN (%)	AOP(%)
0-10	25	13.7	10(40.0)	8(32.0)	8(32.0)
11-20	39	21.4	11(28.2)	9(23.1)	10(25.6)
21-50	58	31.9	17(29.3)	17(29.3)	17(29.3)
31-40	29	15.9	12(41.3)	5(17.2)	5(17.2)
41-50	15	8.2	6(40.0)	6(40.0)	6(40)
51-60	12	6.6	1(8.3)	2(16.7)	6(50)
61-70	4	2.2	1(2.5)	2(50)	3(75)
Total	**182**	**100**	**58(31.9)**	**49(26.9)**	**55(30.2)**

P =0.001   df=6 MZN= Modified Ziehl-Neelsen
ELISA= Enzyme Linked Immunoabsorbant Assay
AOP =Auramin- O-Phenol Fluorescence Stain

Table 2.
Gender Distribution of *Cryptosporidium parvum* among HIV seropositive patients

Gender	Total (%)	Percentage C. parvum Positivity		
		ELISA(%)	MZN(%)	AOP(%)
Male	74(40.7)	21(28.3)	21(28.3)	21(28.3)
Female	108(59.3)	37(34.2)	28(28.9)	34(31.4)
Total	**182(100)**	**58(31.9)**	**49(26.9)**	**55(30.2)**

p= 0.403   𝒳²= .699   df= 1

Table 3.   Efficacy of laboratory diagnostic method of *C. parvum* among HIV-seropositives

Methods	C. parvum Positive	C. parvum negative	Total
ELISA	58	124	182
Modified Ziehl-Neelsen	49	133	182
O-Phenol	55	127	182
Total	**162**	**384**	**546**

P =0.001   df=2

Table 4. **Sensitivity, specificity, positive predictive value of the methods of** *C. parvum* **among HIV-seropositive patients**

Methods (Percent)	Prevalence (%)	Sensitivity (%)	Specificity Positive (%)	Predictive Value Negative (%)	
MZN	49(26.9)	57.1	93.2	75.7	85.5
AOP	55(30.2)	69.1	96.1	88.4	87.8
ELISA	58(31.9)	93.1	51.6	47.4	94.1

p =0.011   df=2

DISCUSSION

The laboratory diagnosis of cryptosporidiosis in the tropics, and indeed in the developed countries, is very important and is the first step in recognition of the intestinal opportunistic parasite, and should, therefore, be taken seriously.

Cryptosporidium antigen detection by ELISA showed sensitivity as high as 93.1% in HIV-positive subjects, which illustrates a high predictive true positive rate and a few false negatives. However, this technique does not ascertain if a positive result is an active infection or due to a previous exposure. The detection technique by Modified Ziehl-Neelsen (MZN) showed specificity as high as 93.2% in subjects investigated while the third method of Auramine-O-Phenol (AOP) recorded a better and higher specificity (96.1%) than the Modified Ziehl-Neelsen test. Although both techniques involve the use of varying magnifying and modifying microscopic methods, this outcome was similar to studies in Nigeria and around the world.[15,16,17,22]

Modified ZN method had shown a lower sensitivity, hence, is not fit as a screening tool, especially for HIV patients who have no symptoms. The method is available in most clinical and research laboratories but is less sensitive, more cumbersome to process, time consuming, and can confuse small ova (measures 4-6µm) for yeast cells. Despite the problems of inter-observer variability, especially with microscopy, this technique will not differentiate the pathogenic species (*C. parvum*) from other species.

Although, the technique to detect *Cryptosporidium* antigen have been reported with variable sensitivity and specificity ,yet, due to manufacturing instructions on use of kits, the sensitivity recorded in this study was high and not different from other reported studies. Therefore, this technique provides an excellent screening tool and can provide useful data for epidemiological studies. This technique has a low detection limit, and does not give information of on-going active infection and previous infection.[22,23]

We also observed higher isolation rate with the ELISA method (58) than with modified Ziehl-Neelsen method (49). This implies that the latter, though the conventional technique, still has several drawbacks which were eliminated using the ELISA method. This finding was, nonetheless, contrary to the finding by Yemisi and colleagues who got a much higher isolation rate of (52.7%) using the MZN method.[26]

The efficacy of the methods in this study has revealed that there is no one method that is fool proof and, that, the combination of any two of the three methods evaluated will be the better option. This is very important in the sense that no patients with immunosuppression should harbour *C. parvum* and left undetected, and that no diarrhoeal stool which is infested with *C. parvum* should leave the laboratory without detection. Therefore, regular and rapid investigation using clear and unambiguous techniques in the diagnosis of cryptosporidiosis in the laboratory should be encouraged.

CONCLUSION
Cryptosporidiosis is an opportunistic infection caused by *C. parvum*, commonly seen in immunosuppressive states such as

HIV infection. Individually, the ELISA method offers a more reliable tool for the screening of HIV-infected patients for the presence of this infection than either microscopy or fluorescence methods, standing alone. But, where the facilities are available, a combination of microscopy and ELISA methods gives a higher yield of positive detection and should be employed in the diagnosis of cryptosporidiosis.

RECOMMENDATIONS
1. All individuals living with HIV should have a routine examination for *C. parvum* infection.
2. Patients that are HIV-positive should be encouraged to chlorinate their drinking water.

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