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A COMPARISON OF RAPID DIAGNOSTIC TEST AND MODIFIED ZIEHL-NEELSEN METHOD IN THE DIAGNOSIS OF CRYPTOSPORIDIOSIS.

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ABSTRACT

Background: *Cryptosporidium parvum* as a leading cause of persistent diarrhoea in developing countries causes a more severe illness in patients infected with human immunodeficiency virus (HIV). The intermittent shedding of *Cryptosporidium parvum* oocysts in stool, even in patients with massive diarrhoea, makes diagnosis difficult. This study evaluated the validity of Crypto-Strip test in comparison to the modified Ziehl-Neelsen for the diagnosis of *Cryptosporidium* infection using polymerase chain reaction (PCR) as gold standard.

Methods: This was a hospital based cross-sectional study of 100 HIV positive patients with diarrhoea at a tertiary health facility in Jos, Nigeria. We collected 15mls of stool sample and relevant information from patients who gave informed consent. The oocytes of *Cryptosporidium parvum* were identified in the stool samples using modified Ziehl-Neelsen stain and Rapid diagnostic test (Crypto-Strip). We also ran all samples using polymerase chain reaction (PCR). Statistical analysis was with statistical package for Social Sciences (SPSS) version 21.

Result: Out of the 100 stool samples analysed, 13 (13%) were positive for cryptosporidiosis by PCR. Crypto-Strips recorded 12 (12%) positive with one (1) being false negative as compared to PCR. Conversely, eight (8%) of the 100 samples collected were positive for *Cryptosporidium* oocysts using modified Ziehl Neelsen (mZN) stain. Out of the eight positives by mZN stain, two were false positive as both tested negative by the PCR. The Crypto-Strip test kit had a sensitivity of 92.3% and a specificity of 100.0% and also positive and negative predictive values of 100.0% and 98.9%, respectively. Whereas, the modified Ziehl Neelsen stain had a sensitivity of 46.2% and a specificity of 97.7% with a positive and negative predictive value of 75.0% and 92.4%, respectively.

Conclusion: In this study, we have shown that Crypto-Strip test was a better diagnostic method for the diagnosis of cryptosporidiosis than mZN stain and should be recommended for use where PCR is not available.

Keywords: Cryptosporidiosis, HIV/AIDS, Polymerase Chain Reaction, Crypto-Strips, modified Ziehl-Neelsen.

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Introduction

Cryptosporidiosis is one of the life-threatening clinical conditions affecting HIV/AIDS patients.¹ The immune deficit in HIV/AIDS patients make them susceptible to cryptosporidiosis with more severe and persistent diarrhoea.² The severity of the disease depends on the CD4 cells count of the patients with high morbidity and mortality in those with CD4 cell count of less than 200 cells/m³.³ Due to the lack of effective drugs to treat cryptosporidiosis, avoiding exposure to the parasite and maintaining immune competence are important ways to prevent infection in these patients.⁴

Although, cryptosporidiosis is most commonly diagnosed by identifying oocysts in the stool specimens of infected persons, diagnostic difficulties arise from the fact that shedding of the *Cryptosporidium parvum* oocysts is intermittent even in patients with massive diarrhoea.⁵ The number of oocysts present in the stool sample may not be adequate for detection and studies have shown that oocysts were better recovered from watery stools than formed stool samples.⁶ Several laboratory techniques have been employed for the detection of *Cryptosporidium parvum* oocysts in stool samples. Among these techniques, conventional modified Ziehl-Neelsen (mZN) and fluorescein-tagged monoclonal antibody immunofluorescence staining techniques are the most commonly used diagnostic modalities for intestinal cryptosporidiosis.⁷ However, polymerase chain reaction (PCR) based methods have been shown to be more sensitive than the conventional micro-

microscopic and immunological methods for detection of *Cryptosporidium parvum* oocysts in human faeces.⁸ Balatbat and his colleagues reported that PCR assay could identify as few as 500 oocysts per gram of stool, which represents a 100-fold increase in sensitivity compared to that of immunofluorescence method.⁹ Thus, PCR assay would contribute to the identification of patients who are asymptomatic but harbour infection at a threshold below that detectable by the current diagnostic tests. Such earlier detection by PCR or a comparable method such as rapid diagnostic strip tests would also help break the chain of transmission.

In developing countries, most laboratories rely on conventional microscopy of concentrated stool specimen making identification more difficult.¹⁰ Simple and rapid point-of-care tests for the detection of *Cryptosporidium* infection have been developed over the years; however, there is limited information on the diagnostic validity of these rapid diagnostic tests (RDT) in most health institutions. Therefore, this study aimed at evaluating the validity of Crypto-Strip test in comparison to the modified Ziehl-Neelsen for the detection of *Cryptosporidium* in stool samples using PCR as gold standard.

Methodology and Materials:

This was a cross-sectional study of 100 HIV/AIDS patients with diarrhoea attending a tertiary health institution in Jos, north-central Nigeria. The sample size was determined using the formula provided by Araoye, 2003,¹¹ at 95% confidence

interval and a 5% expected error of margin. A local prevalence of 6.0% obtained from a previous study was used.¹² By calculating and adding 10% attrition, sample size of 100 was arrived at. The participants were recruited using total sampling technique over seven (7) months because of limited number of HIV patients presenting with diarrhoea. Stool samples were collected in sterile universal specimen containers after an informed consent and assurance on confidentiality. The stool samples were transported to the laboratory immediately for analysis. The samples were divided into two portions; one portion was stored at -20°C until used for PCR whilst the other portion was immediately used for Crypto-Strip test (Coris Bioconcept, Belgium) and modified Ziehl-Neelsen stain to identify the oocysts of *Cryptosporidium*.

Modified Ziehl-Neelsen (mZN):

A smear was made directly from the fresh stool specimen and allowed to air dry. After a fixation period of 2-3 minutes in methanol to prevent washing off during staining, the mZN staining described by Henriksen and Pohlenz, 1981 was carried out.¹³ The stained smear was examined microscopically for *Cryptosporidium parvum* oocysts using x100 oil immersion objective. In a positive sample, the oocysts typically appeared pink red against the light green or blue background.¹³

Crypto-Strip Test Method:

This test is an immunochromatographic strip test and is based on the homogeneous membrane system technology with colloidal gold particles. Monoclonal antibodies directed against *Cryptosporidium parvum* oocyst's membrane antigens as indicated by the colour change on the strip.¹⁴ This was performed according to manufacturer's instructions. Positive result was indicated by two (2) red lines, one at control line and one at test line while negative result was indicated by only one red line at control line.¹⁴

Crypto-Strip

Figure 1: Crypto-Strip Test Procedure. Double red lines (control and test line) seen on a strip indicates a positive result.¹⁵

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), following the manufacturer's recommendations with little modification. 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 3 minutes. The filtered DNA was now suitable for PCR and other downstream applications.¹⁶ 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 (17). The primary PCR was performed using the primer set (Inqaba Biotech, West Africa Ltd), 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 (Inqaba Biotech, West Africa Ltd), 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 MgCl₂, a 0.5µM concentration of each primer sets, 1.5U of DNA Taq polymerase, and a 100µM concentration of each deoxynucleoside triphosphate (dNTP).¹⁸ 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.¹⁸ 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 was 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.¹⁷ and amplicons of 300bp were considered positive (Figure 2).

Ethical Consideration:

This research 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 respondents before enrolment in the study.

Data Analysis:

The data entry and analysis were performed using Statistical Package for Social Sciences (SPSS) version 21 (IBM SPSS Inc, USA) and the validity of mZN and Crypto-strip was calculated using PCR as a reference method.

Results:

The validity of Ziehl Neelsen stain and Crypto-Strip test were compared with positive results from PCR considered as true positive. Out of the 100 stool samples analysed, 13 (13.0%) were positive for cryptosporidiosis using polymerase Chain Reaction (PCR). Crypto-Strip correctly identified twelve (12) positive with one (1) false negative as compared to PCR result (Table 1). Eight (8) of the 100 samples collected were positive using Modified Ziehl Neelsen (mZN). Out of the 8 positives by mZN, two (2) were false positive as both were negative by the PCR (Table 2). The Crypto-Strip test kit had a sensitivity of 92.3% and a specificity of 100.0% while, the modified Ziehl Neelsen staining had a sensitivity of 46.2% and a specificity of 97.7%. The positive and negative predictive values for Crypto-strip test kit and mZN were 100.0% and 98.9%; and 75.0% and 92.4% respectively. These results indicated that the Crypto-Strip test demonstrated a better comparative performance for the diagnosis of cryptosporidiosis in stool than mZN (Table 3).

Figure 2: 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)

L₁ – L₁₃ = 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 (C – 1085/TT)

Table 1: Correlation of Crypto-Strip test and Polymerase Chain Reaction.

Crypto -Strip	Polymerase Chain Reaction		Total
	Positive	Negative	
Positive	12	0	12
Negative	1	87	88
Total	13	87	100

Table 2: Correlation of Ziehl-Neelsen Staining method and Polymerase Chain Reaction.

Modified Ziehl-Neelsen	Polymerase Chain Reaction		Total (%)
	Positive	Negative	
Positive	6	2	8(8.0)
Negative	7	85	92(92.0)
Total (%)	13(13.0)	87(87.0)	100

Table 3: Comparison of the validities of Modified Ziehl-Neelsen and Crypto-Strip test.

Validities	Ziehl-Neelsen (%)	Crypto-Strip test (%)
Sensitivity	46.2	92.3
Specificity	97.7	100.0
PPV	75.0	100.0
NPV	92.4	98.9

PPV = Positive predictive value; NPV = Negative predictive value

Discussion:

Cryptosporidiosis is responsible for life-threatening diarrhoea in developing countries, mostly among HIV/AIDS patients. The diagnosis in developing countries have been impeded by lack of advanced diagnostic techniques. Many health centres rely on conventional microscopy for the diagnosis of cryptosporidiosis despite its disadvantages of low sensitivity and specificity.⁵ This necessitated the need for rapid diagnostic tests (RDT) as a point-of-care test for the diagnosis of cryptosporidiosis. Several RDTs are commercially available but their validity needs to be evaluated as their performance can be affected by many factors. This study evaluated the sensitivity and specificity of modified Ziehl Neelsen (mZN) stain compared with that of Crypto-Strip using PCR as gold standard. It was observed that mZN was considerably less sensitive and less specific than Crypto-Strip test. The Crypto-Strip kit which is an immunochromatographic technique used to detect *Cryptosporidium parvum* oocyst's membrane antigen directly in fresh faeces demonstrated greater performance over mZN with a sensitivity of 92.3% and a specificity of 100% compared to 46.2% and 97.7% for mZN, respectively. The Crypto-Strip has the advantage of being rapid and can be performed at the bed side leading to earlier diagnosis of cryptosporidiosis. This was comparable to the report of Olopade and his co-workers that Crypto-strip kit was more sensitive and specific than mZN.¹⁹ The sensitivity and specificity of the Crypto-Strip kit as reported by Olopade and his colleagues was 60% and 94.4% respectively. Though these values are lower than what was reported by our study, the discrepancy could be attributed to technical issues or storage conditions of the strip. In another research conducted in the United Kingdom (UK) in 2011 where the sensitivity and specificity of diagnostic methods were compared, it was reported that immunochromatographic test was more sensitive and specific than mZN stain in the diagnosis of cryptosporidiosis.²⁰ However, in a research conducted by Mohamed and his colleagues,²¹ conventional microscopy was more sensitive than Crypto-Antigen Rapid test but less specific. A possible explanation for this might be due to differences in commercial products and genetic diversity of *Cryptosporidium* with geographical regions. This variation in validity of rapid diagnostic test from different studies also emphasizes the need to carefully evaluate the performance of available commercial immunochromatographic tests before their use as standard tools in clinical diagnosis of cryptosporidiosis.

Conclusion:

Based on the findings of this research, the Crypto-Strip test demonstrated greater sensitivity and specificity than the modified Ziehl-Neelsen and could be employed for routine diagnosis of cryptosporidiosis. In areas where diagnostic options are limited, this Crypto-strip test could be used as point-of-care test for screening large population for cryptosporidiosis in high-risk individuals such as HIV/AIDS patients.

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.

Limitations to the study:

The modified Ziehl-Neelsen method could be affected by observer or instrument error. To overcome this, the procedure was quality controlled using positive and negative control. Also, the stained smears were examined and reported by three independent microscopists.

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