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Comparison of modified Ziehl Neelsen staining technique with antigen detection using ELISA in the diagnosis of Cryptosporidiosis at a tertiary hospital in North-Western Nigeria

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Cryptosporidiosis is a diarrhoeal disease caused by *Cryptosporidium* species. It has become a more recognised pathogen especially in immunocompromised patients. It is an important opportunistic infection responsible for significant morbidity and mortality in Human Immunodeficiency Virus (HIV) and AIDS patients. The main aim of this study is to compare microscopy using modified Ziehl-Neelsen (MZN) with ELISA in the detection of *Cryptosporidium* in stool. The study was conducted at the HIV treatment clinic in Ahmadu Bello University Teaching hospital. It was a prospective and cross-sectional type. A total of 183 stool samples were collected and processed for the detection of the *Cryptosporidium* oocyst using modified Zeihl Neelsen stain and the antigen using ELISA. Kappa test was used which showed strong agreement between the two methods in the detection of *Cryptosporidium* in stool. Out of the 183 stool samples analysed, MZN stain identified 7 (3.8%) while ELISA identified 9 (4.9%) of the Cryptosporidia. The sensitivity, specificity, negative predictive value and positive predictive value of ELISA as compared to MZN for the detection of *Cryptosporidium* were 66.9, 99.4, 98.3 and 85.7%, respectively. This study showed that both MZN and ELISA can be used in the detection of *Cryptosporidium* in stool samples, even though ELISA had a higher specificity than MZN.

Key words: Cryptosporidium, ELIZA, modified Ziehl Neelsen, HIV/AIDS.

INTRODUCTION

Cryptosporidiosis is an infection caused by *Cryptosporidium* species which are intestinal pathogens (Saha et al., 2019a). It serves as a common cause of diarrhoea in humans and animals (Elsawey et al., 2020).

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Cryptosporodium associated diarrhoea has become well known as a result of its severe manifestation in acquired immunodeficiency disease syndrome (AIDS) patients (Agholi et al., 2013). It is an important opportunistic infection and is responsible for significant morbidity and mortality in Human Immunodeficiency Virus (HIV) and AIDS patients (Sarkar et al., 2014).

The clinical spectrum seen in HIV infected individuals ranges from asymptomatic infections to severe diseases that are characterized by profuse watery diarrhoea, which may be accompanied by vomiting and abdominal pain.(Nkenfou et., 2013; Petrova et al., 2017).

Effective methods to diagnose cryptosporidiosis especially among immunocompromised have been of great challenge in most laboratories in developing countries like Nigeria. Without a proper diagnostic tool, the burden of the infection and its effect on growth, malnutrition and mortality remains underappreciated. Over the years microscopy using modified Ziehl Neelsen (MZN), has been the main diagnostic method in detecting the oocyst in diarrhoeal stool (Ojuromi et al., 2012). Because of their tiny size (about 4-6 μm), detecting the parasite in diarrhoeal stool using MZN has been a problem (Ojuromi et al., 2012; Kumarya and Gwarzo, 2013). This is because the oocysts are usually confused with other faecal materials, pollen and yeast cells. Another disadvantage of this technique is that large number of oocysts (50,000-500,000) per gram of stool need to be present for detection (Ahmed and Karanis, 2018). Where low oocyst numbers are expected in samples, concentration methods are used to increase the sensitivity of detection (Radfar et al., 2013). Due to its limitations, other more effective methods such as antigen detection using enzyme linked immunosorbent assay (ELISA) which can detects low level of oocysts in a gram of stool, have been developed to give more reliable results (Ghoshal et al., 2018). This method of diagnosing cryptosporidiosis is becoming increasingly used because of their increase in sensitivity and specificity (Gawad et al., 2018).

Studies on cryptosporidiosis remain scanty in Northern Nigeria, especially amongst immunocompromised patients. The greatest burden of cryptosporidiosis is in children and HIV patients (Costa et al., 2018; Karshima and Karshima, 2021). 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 (Sarkar et al., 2014). In HIV seropositive patients, it is a major cause of chronic diarrhoea which subsequently leads to malnutrition and death in severe cases (Iqbal et al., 2012). Therefore, prompt and reliable methods of diagnosis are essential to help curb the severity in such groups of patients.

The main aim of this study is to compare microscopy using Modified Ziehl-Neelsen (MZN) with ELISA in the detection of *Cryptosporidium* in stool.

MATERIALS AND METHODS

The study was a cross sectional and prospective type. A total of 183 HIV seropositive patients attending the HIV treatment clinic were recruited to participate in the study.

Collection and processing of samples

Stool samples were collected in a clean wide mouthed screw capped plastic containers, which were transported to the Medical Microbiology laboratory of ABUTH, Zaria for processing. The samples were preserved and concentrated using the formalin-ether concentration method (Allam et al., 2021). 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 and faecal particles collected on the sieve were discarded. Three millilitres of ether were added to the filtrate and solution was vortexed for 15 s. This was to allow adequate mixing of ether to the filtrate and centrifuged immediately at low speed (500 g) for 1 min. 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 layer of the formol water was carefully transferred to another clean tube using a Pasteur pipette. One millilitre of this was transferred into a clean plain bottle for ELISA analysis, and the remaining was then centrifuged at 1000 g for 10 min. The supernatant was discarded while the sediment was used for making the smear (Tahvildar-biderouni and Salehi, 2014).

Sample analysis using Modified Zeihl Neelsen staining technique

The smears were allowed to air dry and then fixed using 95% ethanol. After fixing, the slides were stained with concentrated carbol fuchsin, after which it was heated from below until vapour was seen. This stain was left for 5 min before it was rinsed with water. This was followed by decolourization using 1% sulfuric acid for 1 min and then washed. Lastly 1% methylene blue was used to counterstain for 1 min, and finally washed. The smeared slide was then air dried and viewed under ✕100 objectives. Under oil immersion, *Cryptosporidium* oocyst appeared as oval pinkish red on a blue background.

Sample analysis using ELISA

The supernatant from the previously preserved stool sample 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.[18]

First, required number of the micro wells was identified, including two additional wells for positive and negative controls. Four drops of the negative and four drops of the positive control were added in wells A1 and B1, also 200 μl of the supernatant from stool sample was added to subsequent wells. The plate was covered using a microplate cover and incubated for 60 min 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 (200 μl) of enzyme conjugate were added to each well and incubated for 30 min at room temperature. Contents of the wells were discarded into a waste container, washed using diluted wash buffer for 5 times and still excess wash buffer removed as earlier done. Four drops of colour substrate were

Table 1. Age and sex distribution of of cases of *Cryptosporidium* in stool

Variables	HIV Seropositive	
	Frequency	Percentage
Age group		
16-25	22	12.0
26-35	92	50.3
36-45	46	25.2
46-55	16	8.7
55-65	7	3.8
Total	183	100.0
Sex		
Male	51	27.9
Female	132	72.1
Total	183	100.0

Source: Author

added into each well and incubated for 10 min at room temperature. Finally, a drop (50 µl) of stop solution was added to each well and results read within 10 min at room temperature (Thermofisher, 2017).

Washing of wells consisted of filling each well to overflowing and decanting contents. After the last fill, contents were discarded into a container, and the plate turned upside down on a paper towel. Results were read both visually and using an ELISA reader. Visually, positive result was seen as pale to deep yellow while negative wells will remain colourless. Wells were read at 450 nm. Absorbance of 0.15 OD and above was read as positive while absorbance reading at less than 0.15 OD was recorded as negative.

RESULTS

A total of 183 diarrheal stool samples were collected from March to December 2017 from HIV seropositive patients attending the HIV treatment clinic. Ninety-two (50.3%) participants were in the age group 26-35 years while the 36-45 years group had 46 (25.2%). Only 7 (3.8%) of the participants were from the age group 56-65 years. Out of the 183 participants, 132 (72.1%) were females and 51 (27.9%) were males (Table 1).

+Modified Ziehl-Neelsen stain identified 7 (3.8%) *Cryptosporidia* in stool of the HIV seropositive while ELISA identified 9 (4.9%) of these cases (Figure 1). However, using a Kappa test there is strong agreement between the two methods in the detection of *Cryptosporidium* in the stool.

The sensitivity, specificity, negative predictive value and positive predictive value of ELISA as compared to MZN for the detection of *Cryptosporidium* were 66.9, 99.4, 98.3 and 85.7%, respectively.

DISCUSSION

In this study, all samples were analysed using MZN and ELISA. Out of the 183 stool samples obtained, MZN and ELISA detected 7 (3.8%) and 9 (4.9%) cases, respectively of *Cryptosporidium* in HIV seropositive cases in this study (Table 2). There was no statistically significant difference between MZN and ELISA (p<0.05) in the detection of *Cryptosporidium* in stool. However, test of agreement between MZN and ELISA in the detection of *Cryptosporidium* showed a significant degree of agreement between the two tests (k= 0.854, p< 0.0005). These results indicate that both tests can be equally used in the detection of *Cryptosporidium* in stool, although this study suggests that ELISA may have a slightly higher rate of detection.

The sensitivity and specificity results showed that ELISA has a significantly higher sensitivity than MZN in the detection of *Cryptosporidium* in stool; although the specificity of MZN is, still high (Table 3). These findings are buttressed by several studies in various parts of the world that revealed the higher sensitivity and specificity of ELISA as compared to MZN (Cengiz et al., 2016; Aly Shalash et al., 2016; Omoruyi et al., 2014). However, one study by Mittal et al. (2014) revealed that MZN staining is a more sensitive method than ELISA for detection of *Cryptosporidium* in stool samples but that the specificity of ELISA was more than MZN (Mittal et al., 2014; Saha et al., 2019b). Another study in India showed that MZN and ELISA have comparative sensitivity and specificity. They also concluded that ELISA may be used as a reliable substitute for microscopy in setups where the case load is higher or expertise in special staining techniques is unavailable and that the cost of the ELISA kits can only be justified if the sample load is sufficiently high or if immunocompromised patients form a significant patient population (Ghoshal *et al.*, 2018; O'Leary et al., 2021).

Furthermore, MZN may detect some false positives as indicated by the positive predictive value of 85.7% and above. One of the major advantages of ELISA is in its
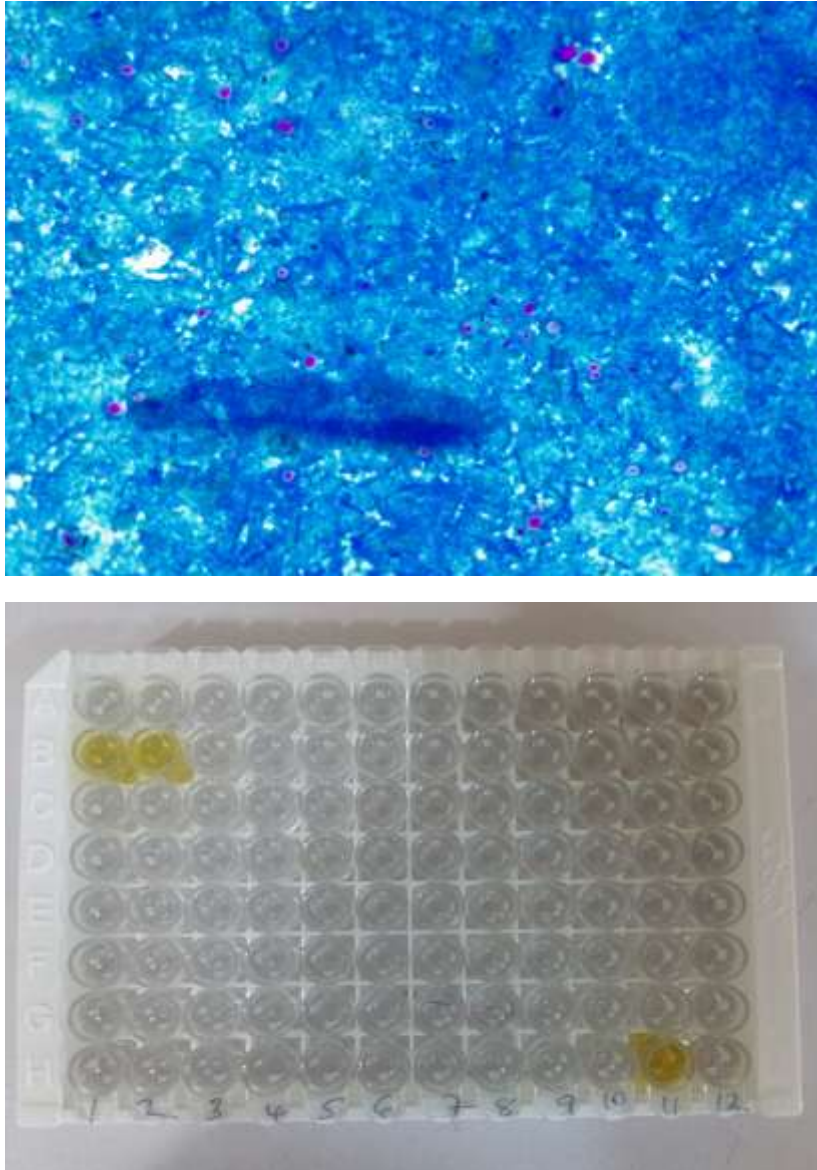
Figure 1. Modified Ziehl Neelsen stain (MZN).
Source: Author

Table 2. Comparison of MZN and ELISA

Cryptosporidium identification	Modified Ziehl Neelsen stain	Percentage	ELISA	Percentage
Positive	7	3.8	9	4.9
Negative	176	96.2	174	95.1
Total	183	100	183	100

K= .854, p < .0005
Modified Ziehl Neelsen stain (MZN)
Enzyme immunosorbent assay (ELISA)
Source: Author

detection of Cryptosporidium antigens during asexual multiplication which cannot be picked on MZN (Ghoshal et al., 2018). In this study, 2 cases of *Cryptosporidium* were detected in stools which were reported as negative by MZN, further buttressing the relative superiority of ELISA over MZN. Despite being a bit costly, ELISA

Table 3. Sensitivity and Specificity of MZN

ELISA	Percentage
Sensitivity	66.9
Specificity	99.4
Positive predictive value	85.7
Negative predictive value	98.3

Source: Author

should be considered for diagnosis in situation where there is a low concentration of parasite in the faecal samples and to overcome the lack of experienced technicians who can carry out MZN (Hassan et al., 2021).

Conclusion

This study showed that both MZN and ELISA can be used in the detection of *Cryptosporidium* in stool samples as both tests showed strong agreement with each other. However, ELISA can be used as a confirmatory test for the diagnosis of cryptosporidiosis.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests

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