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ORIGINAL ARTICLE

Comparison of various staining techniques in the diagnosis of *Coccidian parasitosis* in HIV infection

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Diarrhoea due to oppourtunistic coccidian parasites is a common clinical presentation in HIV infection. Its management differs from that of diarrhoea due to other protozoa, improvement of immune status being the mainstay while specific drug treatment is available for other aetiologies, hence, the need for its accurate identification when present. This can be achieved via various diagnostic techniques, commonly microscopy in this environment, hence the need to compare the efficacy of the commonly used stains in our locality. The objective of the study is to identify the most effective of the commonly used stains in identifying these parasites. Diarrhea stool samples from 250 adult HIV positives and an equal number of age and sex matched HIV negative controls were screened, staining with trichrome, auramine and modified Ziehl Neelsen stain. A positivity rate of 55% was reported. Modified Ziehl Neelsen, when compared with trichrome staining had 81% sensitivity, 77.3% specificity, positive predictive value of 70.4%, negative predictive value of 85.9% and when compared to auramine staining, had 80% sensitivity, 76.7% specificity, positive predictive value of 69.9%, negative predictive value of 85.2% in test subjects. There was a significant moderate level of agreement between the staining methods though trichrome showed a stronger agreement than auramine when compared with Modified ZN in test ($\varkappa$ value 0.569 and 0.553 respectively), and a significant, fair level of agreement between the methods with Auramine showing a stronger agreement than trichrome when both were compared with Modified ZN ($\varkappa$ value 0.399 and 0.332 respectively) in controls. Auramine and trichrome techniques are preferred for screening and diagnosis based on findings. Of these two techniques, auramine is preferred.

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INTRODUCTION

HIV infection is endemic in sub-sahara Africa with as much as 25.8 million of the people living with HIV residing in sub-Saharan Africa, accounting for 70% of the global total (USAIDS, 2015). Of all people living with HIV globally, 9% of them live in Nigeria with about 3.5 million people living with HIV infection and an average of 250,000 new infections in the year 2015 (UNAIDS, 2016). In Nigeria, HIV scourge is a menace affecting more of the reproductive age group with a reported prevalence rate of 3.2% among adults aged 15-49 (UNAIDS, 2016). This, in addition to the effect of diseases like malaria, malnutrition has had its toll on the economy of the country by reducing the workforce. One of the common clinical presentations and the commonest cause of mortality in the HIV infected is diarrhoea disease (Dikman *et al.*, 2015). Diarrhoea disease is due to many aetiology, ranging from bacterial cause to parasitic causes. Commonly associated with diarrhoea in the HIV infected is the opportunistic coccidian parasites (Deorukhkar *et al.*, 2011).

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The coccidia are a group of obligate tissue parasites within the subphylum sporozoa. Of importance in gastrointestinal infection is the cryptosporidium, isospora and the cyclospora species (Winn and Koneman, 2006). Their life cycle requires an external intermediate host, usually an animal, in which sporogenesis and oocyst formation take place. They

cause enterocolitis in a variety of domestic animals and humans become infected either through direct contact with infected animals or from ingestion of feacally contaminated food or water. With the advent of HIV/AIDS epidemic, infection by this group of parasite has evolved as a new emerging infectious disease (Buckley and Fox, 1989; Winn and Koneman, 2006). The parasite sheds millions of oocysts in the stool and hence, the diagnosis of cryptosporidiosis is generally made by assessment of stool samples (Sterling and Adam, 2004).

Management options for diarrhoea in HIV vary depending on the aetiology. Even amongst parasitic diarrhoea causes, management options vary. For other intestinal parasitic infection, antiparasitic agents like metronidazole, tinidazole, paromomycin are useful (Chacon-Cruz and Mitchell, 2003). For coccidian parasitic infection however, the management differs, improvement of immune status being the mainstay of management as there is no specific drug treatment but rather, recovery and clearance of the infection depends on improved immune status (Chacon-Cruz and Mitchell, 2003).

It is on this premise that the presence of coccidian parasite need to be accurately identified with certainty in any HIV infected individual for a missed diagnosis affects the management and hence the prognosis in such an individual. Various techniques can be used in the identification and diagnosis of this infection. This include staining techniques like giemsa, modified acid fast ziehl neelsen, trichrome, auramine phenol, modified cold Kinyoun, etc. The most commonly used staining method is the acid fast modified ziehl neelsen stain (Chalmers *et al.*, 2011).

Other diagnostic method includes molecular diagnostic techniques using the Polymerase Chain Reaction to amplify 18S rRNA gene in the organism, the immunological techniques such as enzyme immunoassays and serologic diagnosis (Ghaffari and Kalantari, 2014). Studies have shown the molecular diagnostic method to be more sensitive and specific than other methods (Ghaffari and Kalantari, 2014) but it is also the most expensive and widely not available in this part of the world. The immunoassay like ELISA are more sensitive than microscopy methods (Elgun and Koltas, 2011). The microscopy techniques are however more widely used in this environment, hence the need to compare the commonly used staining techniques in our locality. The study therefore sick to identify coccidian parasites in stool of HIV infected presenting with diarrhea using various staining techniques and to identify the most effective of the techniques in identifying these parasites.

MATERIALS AND METHODS

Study population

A total of 250 patients presenting to the adult ARV clinic of the University of Ilorin Teaching Hospital with diarrhea were included in the study while the HIV positive without diarrhea were excluded from the study. Sex and age matched HIV negative patients presenting with diarrhea were recruited from the ARV clinic and the General Outpatient Clinic of the hospital as controls.

Data collection

Spot stool sample were collected from each study subject at presentation, and the stool concentrated using the sedimentation method. The sediment is smeared on three glass slides and each slide stained with one of the staining procedure to be compared: the modified ziehl nelson method, the auramine and the trichrome staining method.
The auramine stained slides were examined using florescent microscope, the trichrome and modified ziehl neelsen using the light microscope. Ova of parasite appears as acid fast oocyst on the trichrome and modified ziehl neelsen (Figure 3,4) and appear as green fluorescing oval structures of auramine stain (Figure 5).

Statistical analysis

Data was entered into Microsoft excel spread sheet and exported to SPSS version 20.0 for analysis. Data was presented in numbers (count), percentages and charts. Chi square test (with Fisher's exact *p* value) was used to compare findings from the three techniques. Cohen's kappa coefficient was used to test agreement between each of the methods.

RESULTS

All the three staining technique were found to identify coccidian parasites in the stool sample of patients. Sample is said to be truly positive for coccidian parasite if the parasites is identified by at least two of the three staining techniques. An overall positivity rate of 55% was recorded. Using the modified ziehl neelsen staining technique, the parasite was detected in 111 of the samples stained, 100(90.0%) of which were samples from test subjects. With the auramine flourochrome stain, the parasite was detected in 123 samples, 115(93.5%) of which were from HIV positive test subjects. The trichrome staining technique detected parasite in 121 samples, 115(95.0%) of which were from the test subjects (Table 1).

Of the 100 test samples that were positive for the acid fast oocysts of coccidian parasite using the modified ziehl neelsen technique, 80(80.0%) of them were also positive with the auramine fluorochrome stain and 81(81.0%) were also positive with the trichrome stain. Of the 150 test samples that were negative with the modified ziehl neelsen staining technique, 115(76.7%) were also negative with auramine stain but 35(23.3%) of them were positive with the auramine stain. Likewise, of this 150 that were negative with modified ziehl neelsen stain, 116 (77.3%) were also negative with the trichrome stain and 34(22.7%) of them were positive with the trichrome stain (Table 2).

Likewise, of the 11 control sample that was positive for the acid fast oocyst of coccidian parasites using the modified ziehl neelsen stain, 4 was also positive using auramine flourochrome stain and 3 using trichrome stain but 7 were negative for the parasite with auramine and 8 were negative with trichrome

Table 1: Frequency of parasite identification per stain

	Coccidian Parasite				
Staining technique	**Subject, n(%)**	**Control, n(%)**	**Total, n(%)**	**X^2**	**p-Value**
Modified Zeel Neelsen					
Present	100(90.0%)	11(10.0%)	111(100.0%)		
Absent	150(38.6%)	239(61.4%)	389(100.0%)	92.76	0.001
Auramine Test					
Present	115(93.5%)	8(6.5%)	123(100.0%)		
Absent	135(35.8%)	242(64.2%)	377(100.0%)	124.64	0.001
Trichrome Test					
Present	115(95.0%)	6(5.0%)	121(100.0%)		
Absent	135(35.6%)	244(64.4%)	379(100.0%)	133.49	0.001

Data presented as number and percentage. X^2 – chi square value. P-value < 0.05 was statistical significant

Table 2: Test of agreement between Modified Zeel Neelson and Staining technique (Test group)

	Modified Zeel Neelson				
Staining Technique	Positive, n(%)	Negative, n(%)	Total, n(%)	**k**	**p-value**
Auramine Test					
Positive	80 (80.0%)	20 (20.0%)	100(100.0%)	0.553	<0.001*
Negative	35 (23.3%)	115 (76.7%)	150(100.0%)		
Trichrome Test					
Positive	81(81.0%)	19(19.0%)	100(100.0%)	0.569	<0.001*
Negative	34(22.7%)	116(77.3%)	150(100.0%)		

κ: Kappa; *: p value <0.05 (i.e. statistically significant); Sensitivity: 80.0%; Specificity: 76.7%; Positive predictive value: 69.6%; Negative predictive value: 85.2%

stain. Of the 239 control samples that were negative for the parasite with the modified ziehl neelsen stain, 235 were also negative with auramine, 236 with trichrome (table 3).

When the conventional modified Ziehl Neelsen staining method was compared with trichrome staining in identifying parasite in test subjects, it was found to be 81% sensitivity but 77.3% specificity with a positive predictive value of 70.4% and negative predictive value of 85.9%. When compared to auramine staining in identifying parasite in test subjects, it was found to be 80% sensitivity and 76.7% specificity with a positive predictive value of 69.9% and negative predictive value of 85.2% (tables 2 and 3). There is a significant moderate level of agreement between the various test methods. Trichrome however showed a stronger agreement than Auramine when both were compared with Modified ZN in detecting acid fast oocysts in test subjects ($\varkappa$ value 0.569 and 0.553 respectively). In control subjects without disease, there is a significant fair level of agreement between the various test methods. Auramine however showed a stronger agreement than Trichrome when both were compared with Modified ZN ($\varkappa$ value 0.399 and 0.332 respectively).

The presence of intestinal parasitosis diagnosed by each of the staining methods was correlated with the CD4 count (Figure 1) and the viral load (Figure2) of test subjects using the spearman's correlation. The presence of coccidian parasitosis diagnosed by each of the staining methods revealed a statistically significant, direct relationship with the viral load (Table 4). A weak positive monotonic correlation was noted with modified ziehl neelsen staining technique (R=0.31, n=250, p<0.05) while moderate positive monotonic correlation was noted with both trichrome (R=0.48,n=250,p<0.05) and auramine staining techniques (R=0.44, n=250, p<0.05). The presence of coccidian parasitosis diagnosed by each of the staining methods correlated with the CD4 count revealed a statistically significant, indirect relationship (Table 5). A weak negative monotonic correlation was seen with each of the method, the strength of which is more with trichrome (R= -0.35,n=250,p<0.05) followed by auramine (R= -

Table 3: Test of agreement between Modified Zeel Neelsen and staining technique (Control group)

	Modified Zeel Neelson			k	p-value
Staining Technique	**Positive, n(%)**	**Negative, n(%)**	**Total, n(%)**		
Auramine Test					
Positive	4 (36.4%)	7 (63.6%)	11 (100.0%)	0.399	<0.001*
Negative	4 (1.7%)	235 (98.3%)	239 (100.0%)		
Trichrome Test					
Positive	3 (27.3%)	8 (72.7%)	11 (100.0%)	0.332	<0.001*
Negative	3 (1.3%)	236 (98.7%)	239 (100.0%)		

$\varkappa$: Kappa; *: p value <0.05 (i.e. statistically significant); Sensitivity: 36.4%; Specificity: 98.3%; Positive predictive value: 50.0%; Negative predictive value: 97.1%

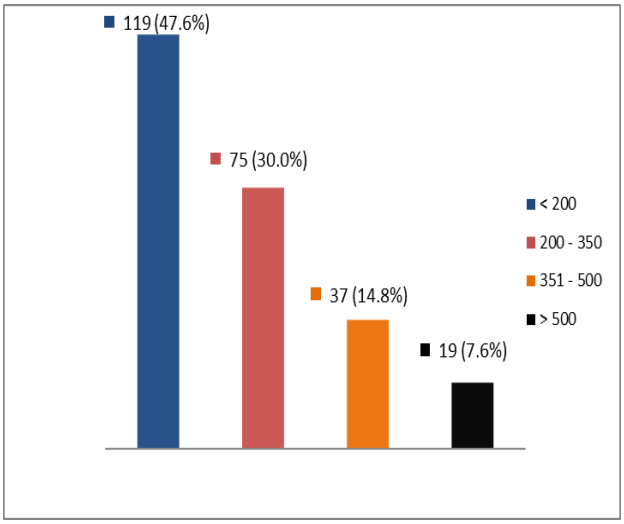

Figure 1: CD4+ count range in test subjects

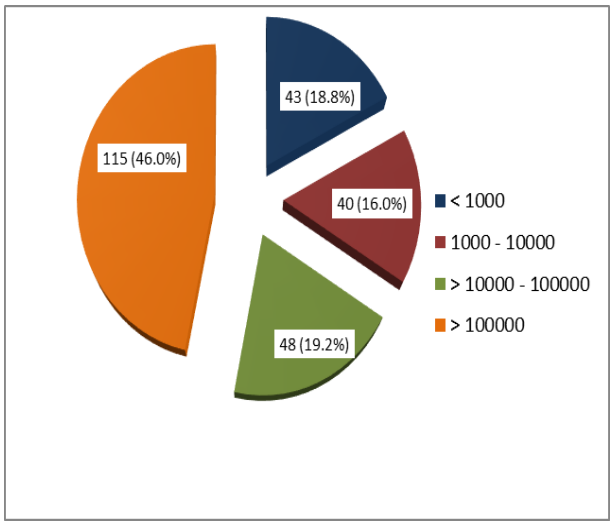

Figure 2: HIV viral load range in test subjects

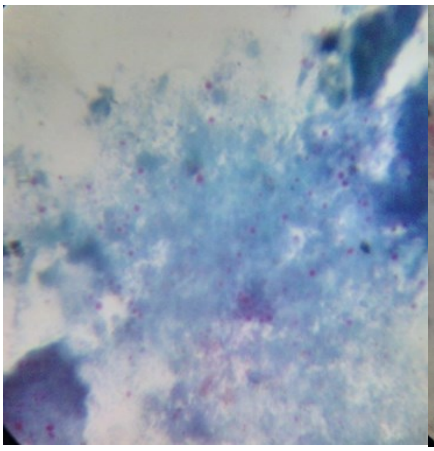

Figure 3: Micrograph of Acid Fast Oocysts On Modified Zeihl Neelsen Stain

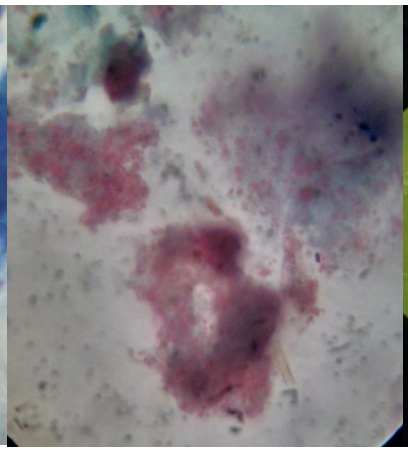

Figure 4: Micrograph of Acid Fast Oocysts On Trichrome Stain

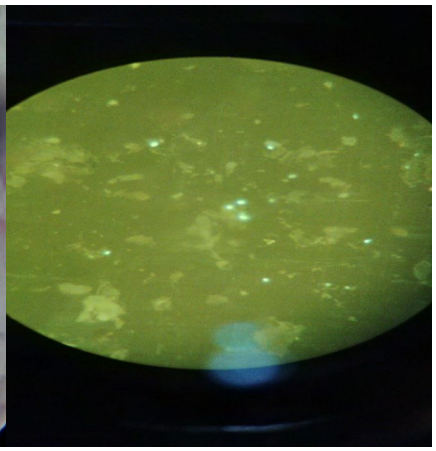

Figure 5: Micrograph of fluo-rescing Acid Fast Oocysts when stained with auramine

Table 4: Relationship between parasitosis per stain and viral load

Tests	r	P value	interpretation
Modified ZN	0.306	0.001*	Weak, positive, monotonic correlation
Auramine	0.440	0.001*	Moderate positive monotonic correlation
Trichrome	0.481	0.001*	Moderate positive monotonic correlation

r: Spearman coefficient of correlation; *: P value < 0.05 (i.e. statistically significant); 0.00-0.19: very weak; 0.2-0.39: weak; 0.4-0.59: moderate; 0.6-0.79: strong;0.8-1.0: very strong

Table 5: Relationship between parasitosis per stain and CD4+

Tests	r	p-value	Interpretation
Modified ZN	- 0.269	0.001*	Weal negative monotonic correlation
Auramine	- 0.326	0.001*	Weak negative monotonic correlation
Trichrome	- 0.351	0.001*	Weak negative monotonic correlation

r: Spearman coefficient of correlation; *: P value < 0.05 (i.e. statistically significant); 0.00-0.19: very weak; 0.2-0.39: weak; 0.4-0.59: moderate; 0.6-0.79: strong; 0.8-1.0: very strong

0.33,n=250,p<0.05) then modified ziehl neelsen (R= -0.27,n=250,p<0.05).

DISCUSSION

The staining techniques compared in this study were modified ziehl neelsen (MZN), auramine and trichrome stains. All the three identified satisfactorily acid fast oocysts in stool of both HIV positive test and HIV negative control. The technique that required shorter time to stain and screen stained slide was the auramine fluorochrome stain. The widely used technique in this environment is the modified ziehl neelsen stain hence the need to compare the other two methods with it. This is because most centres do not have the needed fluorescent microscope required to view auramine stained slide and the trichrome stain is not readily available and expensive in the study environment. The process of trichrome staining technique is also cumbersome hence not routinely used in most clinical laboratories. The reagents used in the modified ziehl neelsen staining is however readily available, affordable and cheaper compared to the reagents used in the other sta ining techniques being reviewed.

This finding is similar to the report of Pakavadee, et al in Thailand who compared four methods for staining *Cryptosporidium* and *Isospora* in stool specimens (Siriprasert *et al.*, 2011). The auramine-phenol and modified ziehl neelsen were among the stains compared and they reported that all four methods were found to give satisfactory results. In this study however, MZN was used as a standard against the other two and according to the statistics, the auramine and trichrome staining were found to be more sensitive and specific than the modified ziehl neelsen**.** We can however say that Trichrome was more sensitive than Auramine. This could be due to the false positive rates associated with MZN because where yeast and other spherical objects staining red can be difficult to discriminate from acid fast oocysts in stool (Khurana *et al.*, 2012).

The submission of Annam et al was in support of this finding for they found flouorescent microscopy to be more advantageous than the ziehl neelsen for detection of acid-fast bacilli in a study done to correlate the fluorescent microscopy method to the conventional ziehl neelsen method for the detection of acid-fast bacilli (Annam *et al.*, 2009). They concluded that the fluorescent microscopy has the advantage of speed and ease of screening and reduces observer fatigue. Rigor and Franco though observed a superiority of the MZN stain above the trichrome stain, hence concluded in their study comparing the modified ziehl neelsen and trichrome in fecal screening for *cryptosporidium* and *isospora*, that the modified ziehl neelsen technique is still the most indicated for routine use in clinical analysis laboratories (Rigo and Franco, 2002). Despite their submission, they concurred with the fact that the trichrome, if modified, could be a simple and inexpensive technique appropriate for use routinely in the diagnosis of intestinal coccidia. In another study carried out by Rosileia et al to detect *cryptosporidium* oocysts by auramine and ziehl neelsen staining methods, found that auramine has a greater affinity for the cryptosporidium oocyst wall than fuschin used in ziehl neelsen. They concluded that auramine had more advantages over the ziehl

neelsen method by being quicker, to perform, and read and ideal for population-based studies (Taboada, 1993; QUADROS *et al*., 2006). The stained slides, if protected from light can last for months and can be later stained by the ziehl neelsen technique (Taboada, 1993; QUADROS *et al*., 2006).

The auramine-rhodamine and indirect immunofluorescence techniques required investment in a fluorescence microscope. The Ziehl-Neelsen technique specimens were however easy to handle and to batch. The technique was relatively inexpensive, though was not as sensitive as the other techniques used in a study carried out in San Franscisco (Cryptosporidiosis, 1998).

Sensitivity of a test, a parameter that assess the presence of disease in diseased population hence its relevance in diagnosis, was noted to be high in trichrome and auramine stains. Specificity on the other hand assesses the absence of disease in those without the disease and is ideal for screening. An ideal test to detect the presence of disease (if truly present) should be highly sensitive and highly specific to rule out the presence of disease if it is not there. For the test group with the disease, a test that has high specificity and sensitivity is required and trichrome and auramine tests have comparable sensitivity and specificity with modified ziehl neelsen. The control group without the disease need a test that is highly specific and the trichrome and auramine tests are also noted to have this characteristic. The higher the predictive value, the more likely a positive test result means disease is present and a negative test result means disease is absent. Hence, for an ideal test to screen out presence of oocyst in non-diseased controls, the test must have a high negative predictive value and to detect the presence of disease in diseased test subjects, a high positive predictive value is required. From the analysis in this study, the auramine and trichrome staining technique has high negative predictive value compared to modified ziehl neelsen when used on population without the disease, and a higher positive predictive value when used on a population with the disease. Relationship between HIV load and coccidian parasites was similar to the finding from a study carried out in Zaria, Nigeria where a correlation between the presence of coccidian parasites and HIV infection was reported (Aminuand and Yakubu, 2008). Likewise, Hafix et al in a study carried out in India found out that high parasite load is associated with high plasma viral load and a reduction of the parasite load by instituting antiparasitic therapy significantly lowered the plasma viral load (Hafiz Ahmad, 2016).

CONCLUSION

Irrespective of the staining method used, coccidian parasites were significantly present in HIV infection. Of all these staining methods however, auramine and trichrome techniques are preferred for screening and diagnosis based on the specificity and sensitivity obtained in this study. Of these two techniques, auramine is preferred due to the ease of staining, faster, less observer fatigue when reporting, less skill required when screening and reporting for the fluorescing oocysts are easily identified and the slide can be stained with modified ziehl neelsen staining technique at a later date. It is therefore recommended that, stool samples from all HIV infected patients presenting with diarrhoea are to be screened for coccidian parasites using the fluorochrome auramine.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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