COMPARATIVE MOLECULAR & LABORATORY DIAGNOSTIC TECHNIQUES OF *CRYPTOSPORIDIUM PARVUM* IN DIARRHOIEC HIV POSITIVE INDIVIDUALS IN LAGOS

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ABSTRACT

The swift resurgence of Cryptosporiosis as an opportunist parasitic agent in asymptomatic immunocompromised patients makes it absolutely newly emerging pathogens although self-limiting in immunocompetent individuals. This study evaluates the comparative efficacy of molecular-based Polymerase Chain Reaction (PCR) over both Florescence microscopy (FM) and Ziehl-Nielsen (ZN) laboratory diagnostic techniques of Cryptosporidiosis in diarrheic HIV positive individuals.

120 volunteered individuals comprising of thirty six (30%) males and eighty four (70%) females participated in this study. Appropriately homogenized fractional stool samples from diarrheic, non-diarrheic HIV positive patients and HIV negative (control) participants collected into separately marked specimen bottles were subjected to PCR analysis and compared to the modified ZN and FM laboratory diagnosis of *Cryptosporidium parvum*. PCR amplification of purified DNA was done using primers' sequences for *C. parvum* tagged DNA Primers (5'

TTCTAGAGCTAATACATGCG3' and 5' CCCTAATCCTTCGAAACAGGA3') targeting the region of the 18s rRNA gene to give an expected amplicon of 1325bp.

98 individuals were HIV positive while 22 were HIV negative (control). Sixty-six (55%) of the 98 presented with mucoid and watery diarrhea while 32(26.7%) were non-diarrheic. Further macroscopic analysis of diarrheic samples revealed 60(91.93%) mucoid and six (9.07%) watery consistencies. All watery stool samples revealed sensitivities to 3(4.5%) PCR; 2(3.0%) modified ZN and 2(3.0%) FM. There was no statistical significance in the efficacy of PCR over the microscopy techniques ($p > 0.05$).

Cryptosporidiosis, nevertheless predominantly presents with non-specific symptoms in the immunocompromised individuals. Being understudied and under-diagnosed due to poor laboratory facilities & experty; therefore, a molecular–based diagnostic technique is most ideal option relative to other complimentary laboratory staining methods in screening for the protozoa in diarrhoiec individuals.

Keywords: Cryptosporiosis, PCR, HIV, diarrhoiec, stool, staining.

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INTRODUCTION

Infectious diseases appear to be receding from the developed countries, while the threat of microbial agents to health remains a source of global concern, particularly in developing countries of Africa. Of all the infectious agents reported across the globe, *Cryptosporidium*

parvum, an important enteric & zoonotic parasitic pathogen, aetiological agent of diarrheal illness in humans and animals (Casemore & Jackson 1984; Casemore *et al* 1987; Casemore *et al* 1997; CDC, 1997; Hunter & Nichols 2002), is now recognized as the most common opportunistic pathogens in HIV infected patients (Mwachari 1998; Gupta. 2008).

However, it is likely that *Cryptosporidium spp* is under-diagnosed because clinicians often fail to consider the diagnosis in patients with diarrheal disease (particularly immunocompetent adults and children). As a result clinicians do not request stool analysis for *Cryptosporidium*, a test not normally included in routine stool analysis (Clark, 1999). And in some cases, its resurgence makes it absolutely becoming newly emerging pathogens in immunocompromised patients (Samie 2006). But such infection is usually self-limiting in immunocompetent individuals (CDC, 1997).

These protozoa complete their life cycles in a single host but their oocysts are usually transmitted by indirect zoonosis through contaminated water or food, products of animal origin, such as milk and meat, while direct zoonosis is by contact with oocysts in animal faeces, as in the case of animal handlers, farmers and their families, farm or petting zoo visitors and contaminated water (Hunter and Thompson 2005). And due to their small size (4-5 µm in diameter) and resistance to chlorine and other disinfectants, water treatment plants cannot usually guarantee total removal of *C. parvum* oocyst.

Different variables such as aetiological and ecological or environmental factors--- expansion of human habitat into the niche of virulent organism(s) and or its vector; new strain of an existing microbe, drug-resistance---give rise to new infections hence, increased host susceptibility which is a major contributing factor to emergence of *cryptosporidium parvum* infections. Although clinically persistent infections could be life threatening (Mwachari,1998), immunocompetent

persons incubates the parasites over a period of about six to 12 days with characteristically chronic, non bloody, unremitting, profuse, watery diarrhoea but less commonly with abdominal discomfort, anorexia, fever, nausea, mal-absorption and weight loss. However, it is usually self-limited with asymptomatic carriers (CDC, 1997).

The diarrhoea usually takes the form of a secretory process but such infections hardly cause disruption of bowel epithelium. In addition to small intestine, the parasites may be found in the colon, rectum, and oesophagus (CDC, 1997). It may also spread into the biliary tract causing diseases in other individuals. *Cryptosporidium parvum* may account for 10-20% of diarrheoic cases in HIV-infected patients living in developed countries and about 50% in the developing countries (Navin *et al*., 1999).

Consequently, the surveillance for cryptosporidiosis is recommended as an initial step in the disease control since it represents a classic 'emerging infection', previously unknown in such severity. This has become important owing to its potential for fatal outcomes in HIV individuals. Since treatments are still in the developmental phase, current control efforts are largely directed at prevention methods. The concurrent rising population of immune-compromised patients and outbreaks of cryptosporidiosis via drinking water-borne cryptosporidium oocysts have enhanced the biological significance of the pathogenic protozoan which is often drug-resistant (*CDC,1997*).

In time past, diagnostic approach to cryptosporidiosis using microscopic screening methods and the non-availability of any known special acid-fast stain for the opportunistic parasites never made its diagnosis relevant despite its prevalence in the study communities (Kaushik, 2008).

Hence, this study was aimed at evaluating the comparative efficacies of molecular diagnostic (Polymerase Chain Reaction) technique of Cryptosporidiosis over the conventional laboratory Florescence microscopy (FM) and Ziehl Neelsen (ZN) staining methods in diarrheic HIV positive individuals attending the study centre. This would establish if there is an alternative and possibly a more accurate, reliable screening method for prompt and accurate detection of the parasitic agents in patients' diarrheic stool samples since PCR is one of the few ways to distinguish accurately between *C. parvum* and non-*C. parvum (*Kaushik *et al* 2008 and Zaidah (2008).

MATERIALS & METHOD

This study was carried out on in- and out-patients attending HIV clinics, General Hospital, Lagos Island, between April 2009 and January, 2010. Ethical approval was obtained from the authorities of the Health Service Commission, Lagos State. And 120 individuals comprising of thirty six (30%) males and eighty four (70%) females whose informed consents were sought and obtained willingly participated in the study. HIV positive patients' diarrhoiec stool samples were separated from non-diarrhoiec stools samples in order to comparatively investigate for cryptosporidiosis using PCR, ZN and FM methods while HIV negative individuals served as the control population.

Stool samples were collected and transported in fixatives-- 5 to 10% buffered Formalin/ Sodium Acetate-Formalin (SAF) and concentrated by the Formalin ethyl acetate method. Macroscopic faecal examinations required the visual observation of the stool samples checking out for stool colour, consistencies (soft, formed, semi-formed or watery), absence or presence of blood and mucus. Microscopic faecal examination carried out comprised of Modified Ziehl–Neelsen

staining Technique (Kinyoun carbol-fuchsin); Formol–Ether Concentration technique and Fluorescence staining Technique (auramine-rhodamine nonspecific fluorescent stain) according to the methods described by (Júnior & Souza 2007; Kaushik, 2008 and Zaidahs, 2008).

And DNA purification and its PCR amplification was carried out as described by Morgan *et al* (1999) .The primers and their sequences used for amplification are *C. parvum* DNA Primers (5' TTCTAGAGCTAATACATGCG3' and 5' CCCTAATCCTTCGAAACAGGA3'). A region of the 18s rRNA gene was targeted using primers 5´ TTCTAGAG CTAATACATGCG 3´ and 5´ CCCTAATCCTTCGAAACAGGA 3´ to give an expected amplicon of 1325bp.The PCR mix contained 5 µl of 5x Go Taq pcr buffer (Promega®). And 200µm (each) dNTPs,100 µm (each) primer, 6mM $mgcl_2$, 2.5U Tac polymerase and 1µl of DNA template in a total 25µl reaction volume.Samples were placed in a PCR thermocycler and set according to methods described by Ekou *et al*, 2013. After initial denaturation at 95^0C for three minutes, the samples were subjected to 35 cycles at 95^0C denaturation for 60 seconds, annealing 42^0C for 45 seconds and extension for 90 seconds at 72^0C followed by seven minutes final extension at 72^0C. After PCR amplification product was resolved on 1% agarose gel electrophoresis at 80 volts for four hours and then visualized after staining with ethidium bromide and photographed using digital camera. Statistical analysis was carried out using Instat® computer software for unpaired t-test with Welsh correction and Fisher's Exact test analysis of data obtained thereof at 95% confidence interval.

RESULTS

One hundred and twenty individuals comprising of 36(30%) males and 84(70%) female, age ranged between 17 and 53 years old were engaged for the study. They were categorized as 98(81.7%) HIV positive and 22(18.3%) HIV negative (control) participants in all (tables 1 & 2).

Of the total enrolled participants, 66 (55%) across genders were diarrhoiec HIV positive subjects of interest to this study. They were subjected to molecular PCR analysis and compared to the modified ZN and FM laboratory diagnostic methods of screening for *Cryptosporidium parvum*. However, the gender distribution of the HIV negative participants were 6(7%) and 16(18%) females. And none of the 22 apparently healthy control subjects was found to be infected with Cryptosporidium by any of the techniques. Hence, there was no significant association in the gender distribution between diarrhoeic HIV +ve and non diarrhoeic HIV –ve (control) population neither was there a significant association in gender distribution between diarrhoeic and non diarrhoeic HIV +ve individuals (tables 3 & 4).

The 66 diarrhoeic HIV positive individuals presented with 60(91.9%) mucoid and 6(9.1%) watery stool subject to macroscopic stool examination. Their gender distribution revealed 16(24.2%) males and 50(75.8%) females. Screening for cryptosporidium oocytes showed no oocyte in all the stool samples examined except in the watery stool samples. And comparatively, in all the watery stool samples examined, PCR sensitivity revealed 3(4.5%) cryptosporidiosis positivity with a 1500 basepairs (bp) at lanes 39, 42 and over 6000bps at lane 32 (plates 1 & 2) respectively; while modified ZN staining technique revealed 2(3.0%) and fluorescence microscopy picked 2(3.0%) cryptosporidiosis.

The analysis of the relative different in the sensitivities between the methods of identification of *C. parvum* oocytes indicated no strong statistical significance in the efficacy of PCR over the microscopy techniques as $P > 0.05$.

TABLE 1: Age / Gender distribution of diarrhoeic HIV positive individuals

Age Range	Males	Females	Total
16-20	1	3	4
21-25	2	12	14
26-30	2	10	12
31-35	0	12	12
36-40	2	4	6
41-45	3	0	3
46-50	4	6	10
51-55	2	3	5
	16	**50**	**66**

TABLE 2: Age / Gender distribution of diarrhoeic HIV negative individuals

Age Range	Males	Females	Total
16-20	0	2	2
21-25	1	3	4
26-30	2	3	5
31-35	0	3	3
36-40	0	2	2
41-45	1	2	3
46-50	1	1	2
51-55	1	0	1
	6	**16**	**22**

Table 3: Gender distribution between Diarrhoeic HIV +ve and control population

	Males	Females	Total
Diarrhoeic HIV +ve	16(18%)	50(57%)	**66(75%)**
Non-diarrhoeic HIV –ve (control)	6(7%)	16(18%)	**22(25%)**
Total	**22(25%)**	**66(75%)**	**88(100%)**

Fisher's exact test (two sided): $p>0.05$; there was no significant association in the gender distribution between diarrhoeic HIV +ve and non diarrhoeic HIV –ve (control) population.

Table 4: Gender distribution between Diarrhoeic and non diarrhoiec HIV positive population

	Males	Females	Total
Diarrhoeic HIV +ve	**16(16%)**	50 (51%)	**66(67%)**
Non-diarrhoeic HIV +ve	14(14%)	18(18%)	**32(33%)**
Total	**30(31%)**	**68(69%)**	**98(100%)**

Fisher's exact test (two sided): $p>0.05$; there was no significant association in the gender distribution between diarrhoeic and non diarrhoeic HIV +ve individuals.

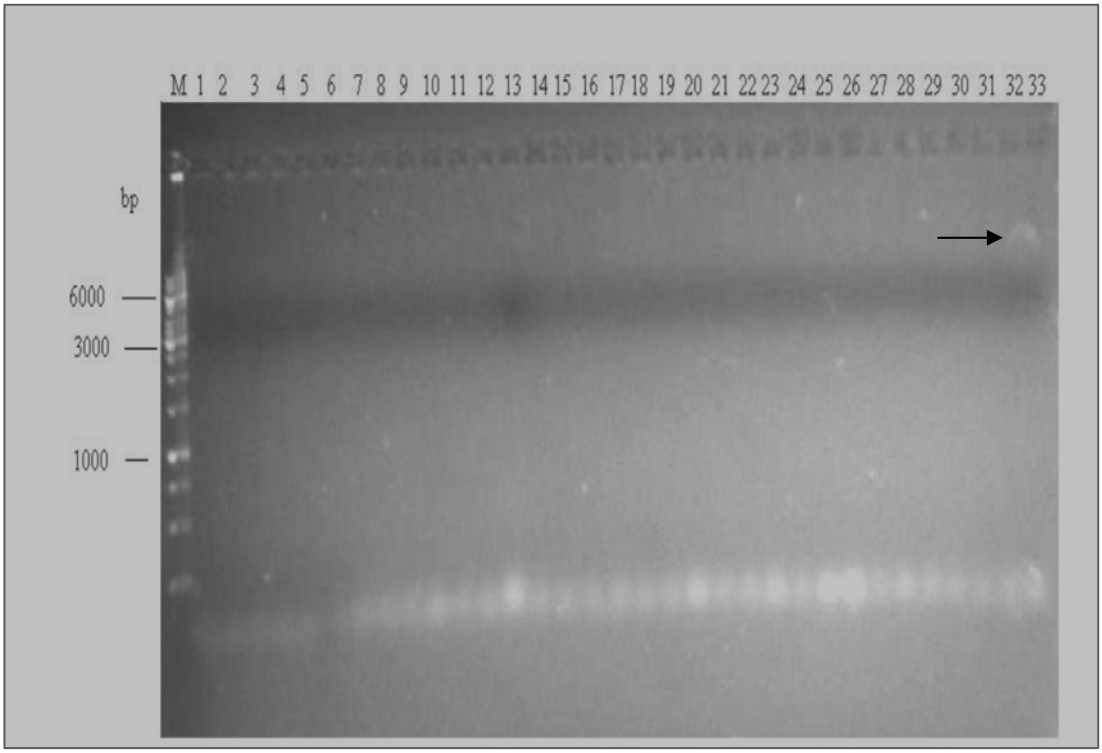

Plate 1: Agarose gel. Arrow showing identical *C. parvum* positive band at columns 32.

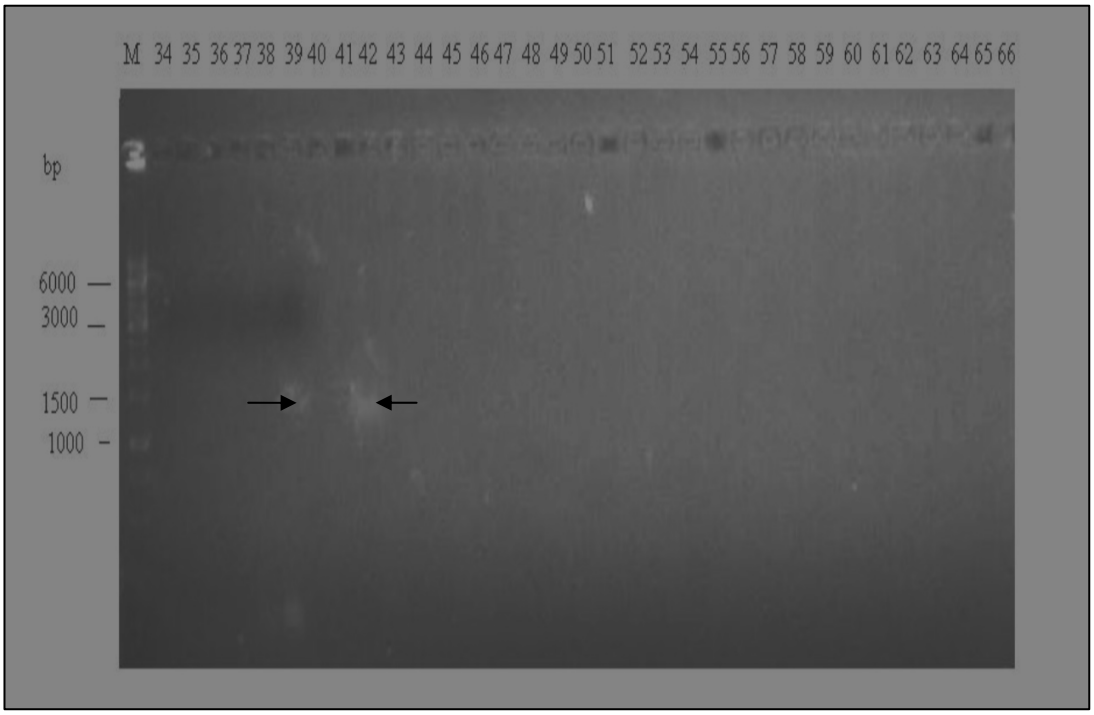

Plate2:Agarose gel. Arrows showing identical *C. parvum* positive bands at columns 39 & 42.

DISCUSSION

Women presented with more diarrhoiec HIV positive stool samples at much earlier ages 21-35(mean=28yrs) as compared to the masculine gender at ages 46-50 (mean=48yrs) in this study. While PCR indicated a higher diagnostic sensitivity (4.5%) to *C. parvum* (characteristically revealing different genetic composition in the three *C. parvum* isolated) as compared to other laboratory diagnostic techniques under investigation (FM, 3%; modified ZN, 3 %), which seems to be of no significant statistical difference. However, the limitation herein---a very few sample size of individuals presenting with watery diarrhoiec stool samples--- might have been responsible for the outcome. In most research investigations such as documented by Essid (2008), it was established that microscopy was used for the initial screening to identify positive samples while nested polymerase chain reaction and restriction fragment length polymorphism analysis was adopted as a confirmatory technique sensitive to determine various *Cryptosporidium species* also indicating the significant difference between samples collected from immunocompromised patients and those collected from healthy children (10.7% versus 1.7%) and a significant difference prevalence was also higher in diarrheal specimens than in formed specimens (6.3% versus 1.6%). A remarkable number of cases of cryptosporidiosis reported in HIV-infected persons from different countries like Brazil, Chile, Colombia, Peru and Venezuela with prevalent rates ranging from 4% to 22.8% (De Oliveira-Silva, *et al* 2007); Cama, *et al* 2003; Gatei *et al* (2002); Júnior and Souza (2007) compared to higher prevalence obtainable in the developing nations. Despite this difference, there also appears to be seasonal differences in *Cryptosporidium* infection, regardless of variable geographical factors, with more infections occurring in warmer or more humid months (De Oliveira-Silva, *et al* 2007). Prevalences of *Cryptosporidium* infection in seven studies conducted in different regions in Brazil on both

symptomatic and asymptomatic HIV-infected adults and children ranged from 4% to 19.1% (Gatei *et al* 2008; Chacin-Bonilla *et al*,1992; Botero *et al*,2003; Ribeiro *et al*,2004).

While Kaushik *et al* (2008) discovered that the staining techniques were less sensitive as compared to antigen detection and PCR for detection of Cryptosporidium in HIV seropositive patients with CD4 count >200cells/microl, Zaidah (2008) also recognized the importance of molecular diagnosis in determining the true prevalence and epidemiology of *C. Parvum* as compared to other microscopic methods. His nested PCR investigation identified a total of nine samples (16%) compared to microscopy, which identified only three samples. Furthermore, Kaushik (2008) established that none of the 50 apparently healthy control subjects in his investigation was found to be infected with Cryptosporidium by any of the PCR, ELISA, ZN and FM techniques. All these established our research findings that the immunocompetent individuals were not infected due to self limiting nature of the protozoa while PCR offers a better diagnostic sensitivity over microscopic techniques.

Further investigation using a larger sample size with watery diarrhoiec stool might justify the probable relative diagnostic benefits of PCR over the other studied conventional laboratory methods in diagnosing *C. parvum* among immunocompromised individuals according to Kaushik (2008) and Zaidah (2008) in order to avert unforeseen consequential morbidity or mortality.

The findings of 4.5% prevalence of *C. parvum* in this study was lower compared to 4.9% (Kaushik 2008) and 10.7% (Essid, 2008), 16.83% (Ibrahim, 2007) and 52.7% (Adesiji, 2007) of HIV-infected patients with chronic diarrhea. All these might infer the utilisation of a more sensitive/prompt diagnostic technique(s) and effective disease control measure achieved in Lagos state similar to findings in some developed countries (Júnior and Souza 2007); Kaushik (2008);

Zaidah (2008) as compared prevalence of *C .parvum* obtainable in other studied locations in the developing nations.

Cases of increased prevalence of *C. parvum* co-infection with HIV/AIDS in many countries may probably be due to increased susceptibility of this population to water-borne infections. The high burden of cryptosporidiosis recorded in Jos (Nigeria) and Nepal might also be due to their occupational lifestyles--farming, hunting and artisans which increase the possibility of its spread/transmission, while continuous re-exposure to the parasite contributes to its self-limitation and the differences in prevalence between various studied populations.

CONCLUSION

Watery stool are more likely to habour more of the parasitic agents than mucoid and non-diarrhoic stool samples. Also, immunosuppression associated with the diarrhoiec state of HIV positive individuals might be a contributory factor against the self-limiting nature of *C. parvum* in infected populations. Although, the financial implication of acquiring PCR machine and trained personnel are inevitable, the use of PCR on a substantial sample size of diarrhoiec HIV positive individuals might hold a greater promise towards prompt and effective diagnosis of *C. parvum* (Zaidah AR,2008) when compared earlier conventional laboratory diagnostic methods such as FM and ZN (Kaushik K,2008).

Finally, cryptosporidiosis, though predominant in this environment, are probably under studied or under-diagnosed in diarrhoeic HIV patients, particularly in the developing countries, for many reasons---infections often cause non-specific symptoms and poor diagnostic facilities. A PCR and or other improved molecular diagnostic methods over FM and ZN techniques are expected to

lead to a more fruitful and realistic assessments of cryptosporidiosis in various human population especially in the immunocompromised patients.

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