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Comparison of Molecular and Conventional Methods of Detecting Cryptosporidium Parvum in Seropositive HIV Patient's Fecal Specimens

John Godsplan Uchenna[1*], Ekejindu Ifeoma Mercy[1], Smith Stella[3], Oshim Ifeanyi Onyema[1], Urama Evelyn Ukamaka[1], Okeke –Nwolisa Benedictta Chinweoke[1], Olise Nkechi Augustina [2]

*Corresponding author: John Godsplan Uchechukwu

Email: ifeanyioshim@gmail.com, +2348064542656.

[1] Department of Medical Laboratory Science, Faculty of Health Sciences and Technology, Nnamdi Azikiwe University, Anambra, Nigeria.

[2]Department of Medical Laboratory Science, School of Basic Medical Science, University of Benin,Benin-city, Nigeria.

[3]Nigerian Institute of Medical Research Yaba, Lagos, Nigeria.

Abstract

This study was conducted to compare a conventional method of detecting the parasite in faecal samples and comparing it with molecular methods with reference to multiplex PCR to determine the difference in the level of sensitivity between them and to characterize the parasite detected to aid epidemiological studies. This cross–sectional study was carried out to detect Cryptosporidium parasites in HIV positive patients using Modified Ziehl Neelsen (MZN) Microscopy and Multiplex Polymerase Chain Reaction (Multiplex PCR). 49 fecal samples comprising 19 MZN –Positive samples and 30 MZN – negative diarrheic samples were analyzed using three (3) Polymerase Chain Reaction (PCR) approaches. A total of 15 samples that were positive by routine microscopy at University of Port Harcourt Teaching Hospital but negative by PCR and by microscopy in the laboratory were treated as false positives. Microscopy therefore exhibited 64% sensitivity and 72% specificity compared to PCR. Sensitivity, specificity, ability to genotype, ease of use, and adaptability to batch testing make PCR a useful tool for future diagnosis and studies on the molecular epidemiology of Cryptosporidium infections.

Keywords: *Cryptosporidium parvum*, PCR, Microscopy, HIV patients ,Sensitivity,Specificity

Introduction

The name *Cryptosporidium*, pronounced "Krip-to-sporidiəm"[1] is a Latin word meaning "Hidden Spore" and was first discovered in mice in 1907 by a Harvard Biologist (Parlange, 2015) called Ernest Edward Tyzzer [2,3] which he described as coccidian [4]. The First case of cryptospordium infection in humans was associated with enterocolitis [5] reported in 1976 using electron microscope [6] with severe but, self limiting watery diarohoea in a healthy child of three years old [7]. Cryptosporidiosis was documented as major human pathogen by CDC in 1982 due to reports of outbreaks of Cryptosporidial diarrhoea in homosexual AIDS patients as opportunistic infection and as a result cryptosporidiosis was included as AIDS defining illness and a Protozoan of Public Health importance. In 2004 WHO included it in the list of diseases that exhibit a considerable and increasing global burden with impairment ability [8]. An

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important advantage of the PCR test over conventional method is that, its ability to directly differentiate between different Cryptosporidium genotypes, will assist in determining the source of cryptosporidial outbreaks.

Microscopic Techniques

The various microscopic techniques for the detection of *Cryptosporidium oocysts* in faecal samples utilize various staining techniques which include: Modified Ziehi Neelsen, Acid Fast kinyon, Auramine Phenol, Giemsa, Jenner, Hot Saffranin and Fluorescent Staining Techniques. Other techniques include: [9]. The sensitivity and specificity vary according to the technique employed. However, microscopy is laborious and time consuming and result depends on the efficiency and the experience of the microscopist [10]. Microscopic examination of *Cryptosporidium* in stool sample must be specifically requested as the oocysts are not detected in routine staining microscopy for ova and parasite [11].

The sensitivity and the specificity of microscopic examination vary according to the technique used as no single staining technique can detect all positive stool sammples, but combination of methods does. For example the combination of sheather's sucrose floatation, auramine and rhadamine techniques can detect sample that are positive [12]. Modified Ziehl Neelson staining technique has been reported as showing low sensitivity when compared with auramine phenol and rhadanine but high er sensitivity is report in indirect immunofluorescence in terms of diagnosing *Cryptosporidium species* in many laboratories, but high cost keeps it out of intending users due to high cost of fluorescent micrococoscopy [12]. Oocysts can be detected with light microscope, while others stages of parasites can be detected with electron microscope [13,14]. The easiest way to confirm diagnosis of *Cryptosporidium species* is to identify and detect oocysts using the microscope analysis of faecal specimen in modified ZN stain the oocyst stains red [15] but requires much carefulness because it is the same size and shape with yeast, though yeast is not acid fast. Other protozoa that need to be differentiated are those that are acid fast such as *Cystoispora (Isospora) belli* and *Cyclospora*, but vary in size from *Cryptosporidium species* [15].

Zhiehl Neelsen stain was later improved upon as modified Ziehl Neelsen stain and adapted to the staining of coccidians particularly *Cryptosporidium* [16] in 1981. Other coccidian detectable using modified Ziehl Neelsen staining inclucle: *Isospora belli*, and *Cyclospora cayetanensis*. The coccidians can as well be referred to as acid fast protozoa. Both Ziehl Neelsen and the modified Ziehl Neelsen stains have the same working principle but differ in the nature of mordant and staining duration. The modified Ziehl Neelsen stain is also composed of basic dye (carbol fuchsin 3%) but with increased concentration, mordant (phenol with wetting agent chemical), and no heat is thereby required for penetration) but still performed cold with increased staining duration. Other components in modified ZN are 3% acid in 95% alcohol, (acid-alcohol) solution as decolourizer, and methylene blue and malachite green as counter stain[17].

The confirmation of cryptosporidium infection can be established by visualizing microscopically the protozoa in a faecal smear on microscopic glass slide prepared from unconcentrated faecal smear [18].

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Preparation Staining of Unconcentrated Faecal Smear.

- With applicator stick, a thick, but correct transparency was made on a grease free, one-end-frosted, labeled microscopic glass slide.
- The smear allowed to air dry and fixed with absolute methanol for about 3 minutes.
- The slide was flooded with cold strong carbol fuchsin with and stained for 15minutes.
- The slide was thoroughly rinsed in tap water and decolorized using 1% acid-methanol solution for 10min
- The slide was rinsed in tapwater and counter stained with methylene blue for 30sec and washed with tap water and air dried.
-

Preparation of Faccal Specimen by Concentration Techniques

Several methods of concentrating Cryptosporidium oocyst in faecal samples in order to improve detection and sensitivity via microscopy have been practiced in many laboratories. Although often desired, but concentration technique should be performed with carefulness as excess sucrose can reduce both oocyst attachment to glass slide and subsequent antibody binding. Prolonged exposure to NaCl can distort morphology and morphometry of the protozoa and formalin can reduce the sensitivity of PCR reactions [18]

Formol/Ether Centrifugation Concentration Sedimentation Technique

With an applicator stick, 500-1g faecal is put into a clean 12-15ml centrifuge tube containing 7ml of 10% formalin. But where the stool sample is watery, about 750 ul of the specimen is pipette into the centrifuge tube. But in case of formed sample, the sample is broken up properly and emulsified with applicator stick.

The suspension is filtered through a filter into a beaker and the filterate is poured back into the same centrifuge tube and 2ml of diethyl ether (ethyl acetate formalinized solution) and the neck of the tube sealed with a rubber bung or glove thumb over the top of the tube and the mixture shakened vigorously for 30 sec. The tube is the inverted a few time and the pressure developed is released gently by removing the rubber bung (or the thumb) slowly. The tube is then centrifuged at 1100g for 2 minutes. The fatty plug loosened by passing the stick between the inner part of the tube and the plug. The plug both above and below the tube are removed by inverting the tube, allowing only the last one or two drops to fall back into the tube. The fluid containing the diethyl-ether and formalin is discarded into a resealable liquid waste container. The pellet is then resuspended by agitation and the whole resuspended fluid is dropped on a microscope glass slide, or the resuspended content is transferred onto the microscope slide with a disposable pipette and air dry [18].

Diagnostic Features of *Cryptosporidium Species*

The degree and the proportion of staining varies with individual oocyst. The internal structures take up the stain in various degrees. Some may appear amorphous, while others may contain a characteristic crescent form of the sporozoite. *Cryptosporidium parvum* oocyst appears as disc 4-

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6µm in diameter. Yeast and faecal debris stain a dull red. Some bacteria spores may as well stain red, but too small to be confused with *Cryptosporidium* [18].

Microscopic Examination of Stained Smear:
For the examination of stained faecal sample, a little immersion oil was dropped on the stained smear and mounted without coverslipe. The iris diaphragm was fully opened and x100 objective put in place and the slide focused and the slide viewed scanning for the red oval shaped protozoa which stands out on a blue background

Smear (slide) in which no oocyst was seen is reported "No *Cryptosporidium* oocyst seen. But positive smear is reported as follows:

Positve Rating

- Less than 5 oocysts per slide = +
- 1-10 oocysts per slide field = ++
- 11- or more oocysts per field = +++

Detection of *Cryptosporidium Species* UsingPolymerase Chain Reaction

An important technique applied to molecular diagnosis in modern laboratories especially reference laboratories is polymerase chain Reaction (PCR). PCR is an invitro process based on natural process of DNA replication with an exponential reaction that can result in the generation of more than a billion copies of original or target DNA with 30-40 PCR cycles [19].

It is a diagnostic or cloning technique based on single nucleotide variation. Four basic elements are critical in PCR process and they are the template DNA, DNA polymerase, primers and nucleotides [19].It is an amplification technique that can generate an ample supply of a specific segment of a DNA (an amplicon) from a small amount of starting material DNA template or target sequence [20]. The template DNA from specimen that contains the sequence of interest to be amplified for instance *Cryptosporidium genotype*. The DNA polymerase is a heat stable enzyme that synthesizes fresh strands of DNA complementary to the target sequence. The primers helps the enzymes focus on a particular section of DNA to avoid copying the whole DNA [21] usually copied in pairs of short pieces of single strands of DNA that binds specifically to the complementary 3' end of the target gene that allow the polymerase to synthesize new DNA, while the nucleotide (dNTPs or deoxynucleotide triphosphates) single units of the bases A, T, C and G act as ingredients for the formation on new strands because DNA is made up of 4 bases. However, in the event of RNA instead of DNA as template material, the use of transcriptase (reversed transcriptase becomes inevitable in first converting the RNA to DNA as in the case of reversed transcriptase (RT) PCR [22]. In Africa, molecular identification of *Cryptosporidium species* is generally lacking. Species data have been collected in only five countries – Kenya, Malawi, Uganda, Equatorial Guinea and South Africa and PCR as a diagnostic or cloning technique based on single nucleotide variation [23].

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Molecular Diagnosis of *Cryptosporidium*

Molecular methods for diagnosing *Cryptosporidium* identify the protozoa to the species level and usually performed in reference laboratories [24] and used to specifically detect *Cryptosporidium* spp., differentiate between closely related *C. parvum* isolates and *Cryptosporidium* species, and differentiate between principal genotypes known to infect humans[25]. It has been recommended to be specifically requested by health care providers due to its necessity in the accurate diagnosis of *Cryptosporidium* [26]. The molecular method of diagnosis such as polymerase chain reaction (PCR) is reported to be more sensitive compared to other methods of diagnosis and easier to interpret, but more personnels are needed in performing the test at the same time[27].

PCR is marked with high sensitivity, specificity [28,29], fastness, reproducibility and recognized for its high accuracy and very crucial for the analysis of a large number of samples as with multiplex PCR [30]. And due to the increasing reliance upon genetic tests for identification and differentiation, the low concentration or number of organisms required to cause disease, and the often-found presence of multiple protozoan species in a single environmental or clinical sample, such methods would be ideally suited for the detection of the protozoan parasites [31]. The targeted loci in DNA diagnostic taxonomy for *Cryptosporidium* are the small subunit (SSU)-rRNA also known to be the 18S- rRNA, heat shock protein – 70 (HSP-70) gene, *Cryptosporidium* outer wall protein (COWP) Crypto-1 (TRAP) C-1 gene and actin gene [32]. Amid the various PCR diagnostic approaches, the multiplex PCR stands out as a rapid, convenient [33]. Multiplex analytical systems also allows for detection of multiple nucleic acid targets in one assay can provide rapid characterization of a sample while still saving cost and resources [34].

Preparing Specimen for PCR

PCR assay must first be preceded by sample preparation in order to have a yield of pure pathogen DNA (Template DNA) in a process of DNA extraction [35] fresh or frozen or preserved specimen may be used. DNA extraction is a multi step process that may be performed manually or with automation for instance the use of COBAS ampli pre instrument. The first strument that prepares samples for PCR automatically without human intervention [35].

PCR Principle

The PCR thermal cyclers rapidly heat and cool the reaction mixture allowing for heat-induced denaturation of duplex DNA strand separation, annealing of primers to the plus and minus strands of the DNA template and elongation of the PCR product. Cycling times calculated based on the size of the template and GC content of the DNA[35].

PCR Phases

Three basic phases complete each PCR cycle and each cycle approximately doubles the amount of the target DNA [35]. And the key processes involved are denaturation, annealing and synthesis or elongation.

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- **Denaturation Phase**: This is the process of separating target DNA. At this stage, the PCR tube containing the sample DNA is heated to more than 90^0C (194^0F) to dissociate the double stranded DNA into separate strand and with high temperature, the weak nucleotide bond between the nucleotides is broken [35]. Denaturation at 94^0C should not be allowed for more than 3 minutes to avoid inactivation of the DNA polymerase and its enzymatic activity destroyed. However, with hot start PCR in which the DNA polymerase is added after the initial exaggerated denaturation time extends from 3 minutes top to 9 minutes.

- **Annealing Phase**: The term annealing describes the process of the binding of primers, the synthetic nucleotides to the DNA sequence[35]. The primers bind to the plus and minus strands of the DNA template. The binding is only on the target sequence (specific genetic code) targeted by the PCR primer and not all the DNA in the sample. For example in detecting *Cryptosporidium*, *Cryptosporidium* primer incorporated will be copied. The primers are two and each binds to one of the newly separated single DNA marking off the sequence for the next step of the PCR cycle (synthesis). During the process of annealing, the PCR tube with the mixture is cooled and binding occurs between 40^0C-60^0C 35]. The primers due to Brownian motion giggle and ionic bonds are constantly formed and broken between the primer and the single stranded DNA template until the polymerase is attached and few bases built in and strong ionic bond which can no longer break results[36].
  DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction condensing the 5- phosphate group of the dNTP with the strand [37].
  - DNA template contains the DNA region (target) to be amplified
  - Two primers that are complementary to the 3 (three main) ends of each of the sense and anti sense strand of the DNA target.
  - DNA Polymerase does the synthesizing
  - Deoxynucleotide triphosphates (DNTPs nucleotide contain triphosphate group, the building block from which the DNA polymerase synthesizes a new DNA strand)
  - Buffer solution provides a suitable chemical environment for optimum activity and stability of the DNA polymerase.
  - Divalent cation (Mg^{2+}) utilized for PCR- mediated DNA mutagenesis [37].

Extension (Elongation) Phase: This is the third phase of PCR and it is basically the phase for making copies of DNA (that is the phase for DNA synthesis of only the annealed (bound or marked) DNA sequence and primer in the process that get no exact match is loosened due to high temperature and does not give an extension of fragment the synthesizing temperature is 72^0C.

However, the temperature for synthesizing may vary due to the polymerase selected for the experiment. (Taq polymerase, requires a temperature of between 70^0C-80^0C for 1 min to elongate the first 2kb and 1 extra minute for 1 each addition of 1 kb. Pfv DNA polymerase seems to be prefered in PCR and requires 2 minute for 1kb to be amplified.

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At the end of the synthesis, two (2) identical copies of the original DNA is made, and with the two copies made, the PCR cycle starts afresh using now the newly duplicated DNA for more synthesis and with 30-40 PCR cycles about a billion copies of the original DNA segment shall have been made.

Termination

This marks the end of the reaction or the experiment. It is achieved by chilling the mixture to 4^0C and/or by addition of EDTA to the final concentration of 10mM. This is also refered to as the final hold in which the reaction is held at 4-15^0C for an indefinite time, but usually utilized for short term storage of the reaction.

BASIC PCR PROTOCOL

- The PCR tubes were maintained in ice pack and PCR reagents added into cold PCR tube in reaction cabinent in order to avoid nuclease activity and non-specific priming.
- The negative control was prepared in a separate PCR tube containing all the reagents except the template DNA. The Positive Control was also prepared in another separate tube containing all the reagents with a previously determined template or primer known to amplify in the same present experimental condition.
- The reagents were added in order in a separate tube as follows: Sterile water, PCR buffer, dNTPS, $MgCl_2$, Primers and the test sample (containing the template DNA).
- Taq DNA polymerase was added to the test mixture in the ratio ½ vol of polymerase is to 1 Vol of master mix and properly mixed with up and down pipetting several times for at least 20 times.
- The test tube was capped and put into ependorf thermal cycler and programmed.
- The PCR tubes were removed and stored at 4^0C at the end of the reaction.
- Aliquot of the reaction was then load into well of an agarose gel stained with ethidium bromide.
- **Result:** DNA stained with the ethidium bromide migrate by electrophoresis and the DNA product that is present intercalated between the bases of the DNA strands by the ethidium by allowing bands to be visualized with UV illuminator.

Nested PCR

Is a modified PCR and usually performed with two sets of primers and in two successive PCR. Its use is necessary because, during the first PCR (Conventional or Primary PCR) with one pair of primers, the primers may generate DNA products, besides the intended target, which may consists of non-specifically amplified DNA fragments, hence the need for a second PCR set of primers with binding site absolutely or partially different from and located 3' end of each of the primers and reduces non-specific binding product due to amplification of unexpected primer binding site. Therefore, nested PCR provides for extra level selectivity of DNA target sequence and having two pairs of primers acts as a double check and reduces non-specific binding product due to amplification of unexpected primer binding site [38].

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Multiplex PCR

It was first described by chamberlain and co-workers in 1988 [38] and is another PCR variant in which multiple sets of primers are mixed together in single PCR test tube at the same time aimed at producing amplicons of varying sizes that are specific to different DNA Sequences [38]. Critical to multiplex PCR are the relative concentration of the primers, concentration of the PCR buffer, balance between the magnesium chloride and deoxynucleotide concentrations, cycling temperatures, amount of template DNA and Taq DNA polymerase which must all be precisely and accurately measured and properly mixed[39]. And more crucial is that the annealing temperatures for each of the set primers must be optimized to work correctly within a single reaction and amplicon sizes; meaning that their base-pair length should adequately vary so that the band formed can be differentiated when viewed by gel electrophoresis. An optimal combination of annealing temperature and buffer concentration is essential in Multiplex PCR in obtaining highly specific amplification product [39]. It has been proven to be simple, rapid and cost effective [40].

MATERIALS AND METHODS

The study Area

Samples were collected from HIV patients on ART and those not on ART at University of Port-Harcourt Teaching Hospital (UPTH) for testing for enteric parasites were used in the study. A total of 49 fecal samples were included in this study. Specimens were tested blindly by the Nigerian Institute for Medical Research (NIMR) Yaba, Lagos in appropriately sized batches for each procedure. Fecal samples were stored at 4°C without preservatives and were processed within 1 to 2 weeks.

Ethical approval

The study was approved by the institutional ethics committee at University of Port-Harcourt Teaching Hospital (UPTH) (the ethical approval number:UPTH/ADM/S.11/VOL.X/636) and Nigerian Institute for Medical Research (NIMR) Yaba, Nigeria (the ethical approval number: MR/GEN.AD/1032/IV/1056) respectively. The study design in November, 2017.

Microscopy

Thick stool smears were directly made on new clean frosted- end grease-free microscope slide labeled and allowed to air dry and fixed with absolute methanol. The fixed smears were allowed to air dry and stained with Modified Ziehl Neelsen staining technique. The stained smears were examined microscopically with oil immersion objective. The stool samples were preserved with 10% formol saline solution and stored in the refrigerator for PCR diagnosis remaining.

Protocols

a) Faecal smear was made on microscope slide and air dried at room temperature.
b) The smear was fixed with absolute alcohol (methanol) for 5min.
c) Then stained with carbol fuchsin with phenol for 15 mins.

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d) The stain was washed with tap water and;
e) Decolourized with 3% acid alcohol (3%HCl in ethanol) for 10 min.
f) And counter stained with 0.4% methlene blue solution for 1min, washed with tap water and air dried.
g) Slide Viewed with oil immersion microscope at x400 magnification. Ocyst of cryptosporidium appears as pink to red spherical to oval body in blue background.

Molecular Diagnosis

DNA Extraction: DNA was extracted from stool samples with QIAGEN DNA stool minikit, Helden Germany with strict adherence to the manufacturer's instructions.

Amplification: Based on the use of Jena Bioscience (Germany), 2016 PCR kit.

Primary (conventional) PCR

The PCR reaction concentration was determined by mixing 4ml of master mix, 0.4ml Forward Primer (SSU1), 0.4ml of the reversed primer (SSU2) and 5ml of template DNA all dissolved in 10.2ml of distilled water giving a total volume of 20ml. The Primary PCR was to test the presence or absence of the first target genes of *Cryptosporidium* (SSU rRNA). The first sets of Primers were: Forward Primer (SSU1)-(5'GAT TAA GCC ATG CAT GTC TAA G-3') and the Reversed Primer (SSU2) ('5 -TTC CAT GCT GGA GTA TTC AAG-3')

Secondary (Nested) PCR

This consists of two sets of primers and was intended to reduce non-specific binding product due to the amplification of unexpected primer binding site. The reactions concentration was also determined by mixing the master- mix 4ul, forward primer (SSU3-1ul), reversed primer SSU4-1ul, SSU 1-5ul, SSU2-5ml all dissolved in 9ml of distilled water giving also 20ml volume. SSU-3 (5'CAG TAA TAG TTT ACT TGA TAA TC-3), SSU-4 (5'CCT GCT TAA AGC CTA ATT TTC-3). The Cryptosporidium positive samples were amplified at 638 base-pair fragment. And the mixture 1PCR buffer, 2mM Mgcl2, 0.2mM of each(d NTP, d ATP, d GTP, d CTP ,d TTP), 40 pMol of each primer, 2ul Taq DNA polymerase and sterile water was used to make up the reactions mixture. The standard cycling condition were 24 cycles of 95^{o} C for 12sec, 95^{o}C, 50^{0} C , 55^{o}C for 50sec and 72^{0}C -1mm. The PCR was performed with Eppendorf PCR machine. While amplicons were analyzed using 1.5% Ethidium bromide stain and gel snapped using gel documentation machine.

Multiplex PCR

Samples which were positive for *Cryptosporidium* species DNA after PCR using SSU primers were further analyzed with the Multiplex PCR at the Heat Shock Protein (HSP-70) locus. The multiplex PCR was performed in cluster of primers specific for some *Cryptosporidia species* in order to identify differences in the HSP to gene sequence for *C. parvum* (human and bovine) genotype, *C. canis*, *C.felis*, *C. meagridis*. The reaction concentration was determined by mixing master mix-10ml, HSP1=0.25ml HSP13=0.2ml, HSP5=0.25ml, HSP8=0.25ml and DNA5ml all dissolved in 3.75ml of sterile water to give a total volume of 20ml. Multiplex PCR reaction was performed under a standard

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cycling condition at 45 cycles, 95ºC for 12mins, 95º C for 50^{O} C, 55º C for 50 sec and 72º C for 1min analyzed with eppendorf PRC machine. The reactions were performed with forward primer HSP-1 (5'GTT GGT GTA TGG AGA AAC GAT A-3')and reversed primers HSP-13 (human genotype)(5'CTG TGT TTT CTG GAT TTC TAG CAA CT-3) HSP-5 canis genotype (5' CAG GTA CGG TGA CGA CGG CGT TCT TAA)TTG=3, HSP-6, *C.felis* genotype (5' TGT ACC ACC TCC CAG ATC GAA AAT CAG G-3') and HSP-8,bovine genotype (5'CGC ATT GAG TTC TGA GTC TTC TTA AAG-3').

RESULTS

A total of 49 fecal samples were screened by both microscopy and PCR (Table (Table1).
49 fascal samples comprising 19 MZN –Positive samples and 30 MZN – negative diarrheic samples were analyzed using three (3) Polymerase Chain Reaction (PCR) approaches. The results showed that:The first round of PCR using conventional method did not yield amplification of any amplicon. But the second round of PCR using nested PCR 638 base-pair fragment of 18S rRNA of *Cryptosporidium species* were observed in 9 samples 9 (18.36%). Four (4) of these samples were originally positive with the modified Ziehl Neelsen Microscopy, but 5 MZN negative samples were positive with PCR. Microscopy therefore showed 64% sensitivity and 74% specificity compared to 100% sensitivity and specificity for PCR (Table (Table1).

Further analysis using multiplex PCR targeting Heat shock protein to (HSDP – 70) gene for characterization and speciation of the Cryptosporidium species failed to amplify any fragment and did not generate any PCR product (Figure1-4).

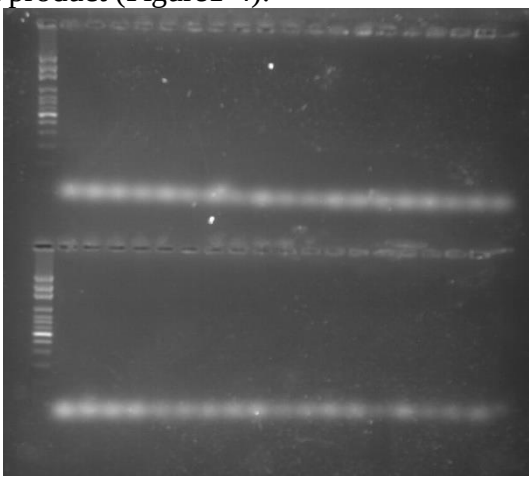

Figure 1

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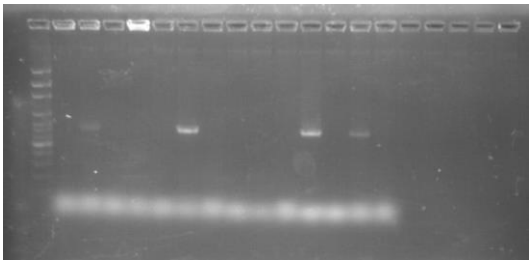

Figure 2

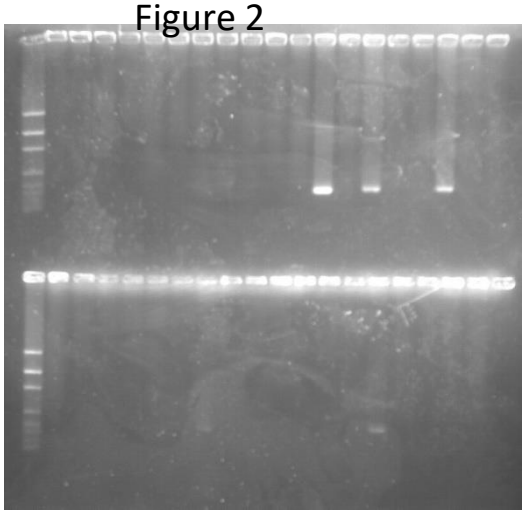

Figure 3

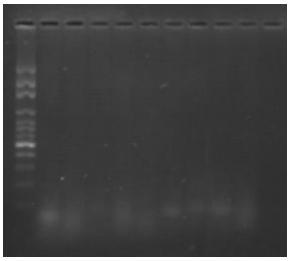

Figure 4

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Table 1
COMPARISON OF PCR VERSUS MICROSCOPIC DETECTION OF CRYTOSPORIDIUM

Method	NO of sample Examined	NO of Positive	Sensitivity (%)a	Specificity (%)b	Strain Distrimination
PCR	49	9	100	100	Yes c
Microscopy	49	19	64	72	No

aCalculated as follows: [number of true positives/(number of true positives + number of false negatives)] × 100.
bCalculated as follows: [number of true negatives/(number of true negatives + number of false positives)] × 100.
cThe PCR test was able not only to detect Cryptosporidium oocysts but also to identify isolate genotypes.

Discussion

Cryptosporidium protozoa still remains one of the most common enteric protozo parasites associated with *Human Immunodeficiency Virus (HIV)* positive patients due to their compromised immune system, presenting a chronic, life-threatening copious mucoid non-bloody watery diarrhoea. In this study, a significant prevalence of cryptosporidiosis with Modified Ziehl Neelsen technique (7.6%) and PCR (18.3%) has been observed. Indicating that *Cryptosporidium species* is still prevalent among HIV patients particularly in rural settings where personal and environmental sanitation receive the lowest attention interspersed with poverty index. This report agrees with Settawy and Fathy (2012) who detected more of the parasites in rural communities than in cities. Also Erhabor, *et aI* (2011) had reported a lower prevalence of 2.9% among HIV patients in UPTH, but in this study (0.0%) prevalence was recorded in UPTH. Also Morgan *et al* (1998) reported similar prevalence (5.6%) with MZN in which, 29 positive samples detected in 511 samples. Microscopy therefore exhibited 64% sensitivity and 72% specificity compared to PCR. PCR was more sensitive and easier to interpret but required more hands-on time to perform and was more expensive than microscopy. This report agrees with Morgan *et al* (1997) who compared conventional microscopy with acid-fast staining with a recently developed PCR test for the detection of Cryptosporidium and found microscopy to be considerably less sensitive and less specific than PCR analysis.

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Conclusion

PCR amplification is an obvious choice for improved detection of Cryptosporidium from feces.

Because of its sensitivity, the assay described in this study has the potential for accurate diagnosis in patients who do not presently know the reason for their diarrhea. This will have considerable advantages in the treatment of immunosuppressed individuals, allowing early diagnosis before the onset of symptoms. In addition, this PCR test is capable of directly differentiating between human- and animal-derived genotypes of Cryptosporidium on the basis of the size of the PCR product . The potential for zoonotic transmission from livestock and wild-animal reservoirs via environmental contamination is of increasing concern. Thus, the primers used in this study may be valuable in the predictive epidemiology of cryptosporidial infections in humans and livestock.

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