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Diagnosis of intestinal cryptosporidiosis in Africa: Prospects and Challenges

[REVIEW PAPER]

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ABSTRACT
Cryptosporidium parvum is an opportunistic parasite capable of causing gastrointestinal illness in both immunocompetent and immunocompromised patients as well as in children less than 2 years old particularly in developing countries. The parasite causes diarrhea that is self-limiting in immunocompetent individuals and another that is potentially life-threatening in immunosuppressed individuals. Transmission of the parasite could be direct through feacal-oral route or indirect via contaminated water supply, food or environment. Methods of diagnosis of infection due to the parasite, involve use of polymerase chain reaction (PCR), immunological – based detection methods, and microscopy. These diagnostic procedures in Africa are however confronted with several challenges ranging from constant lack of power supply and lack of experienced technologists, poor funding from government for periodic trainings, seminars and workshops for laboratory workers as well as technical complexity of the protocols. Given the poor state of health care infrastructure in Africa, brain drain of skilled personnel, low allocation of resources to health care among others the inclusion of immediate work-ups for the prompt diagnosis of Cryptosporidiosis is imperative
Key words*: Cryptosporidium, diagnosis, PCR*, Microscopy

INTRODUCTION
Protozoan parasites of the genus *Cryptosporidium*, members of the Phylum Apicomplexa, affect a wide variety of vertebrate hosts. In humans, *Cryptosporidium hominis* (anthroponotic origin) and *C. parvum* (zoonotic origin) are responsible in most areas for more than 90% of cases of cryptosporidiosis, a diarrheal disease in which progression and severity are closely linked to the immune status of infected persons (Fayer, 2004; Xiao and Ryan, 2004).
Cryptosporidiosis is a diarrheal disease caused by parasites of the genus *Cryptosporidium*, which measure between 4 – 6µm in size (Morgan *et al*., 2002; Robertson *et al.,* 2006; Gomez-Couso *et al.,* 2010). Diarrhea caused by parasites account for more than 3.1 million deaths each year among children less than 15 years of age, mostly in developing countries (Colford *et al*., 2005). Cryptosporidiosis causes chronic and life threatening diarrhea among immunocompromised individuals as well as self-limiting diarrhea in immunocompetent individuals (Paul and Gordon, 2002; Banwat *et al.,* 2004). Infection by the parasite accounts for up to 6% of all diarrheal

diseases in immunocompetent persons and presents in up to 24% of persons with both AIDS and diarrhea worldwide (Bialek *et al*., 2002; WHO/ UNICEF, 2007).

Cryptosporidiosis is normally initiated by ingestion of *Cryptosporidium* oocytes from contaminated drinking water, recreational water and contaminated food. *Cryptosporidium* oocytes are prevalent in surface waters, extremely resistant to commonly used disinfectants, and generally survive for several months in aquatic environments (Miler *et al*., 2006). *Cryptosporidium* is recognized as a major human waterborne pathogen worldwide (Clancy and Hargy, 2007). Following ingestion, oocytes excyst in the gastrointestinal tract and release infective sporozoites, which attach to the apical membrane of the host epithelial cell. When the sporozoites mature, they undergo asexual reproduction to produce merozoites which are released into the intestinal lumen. The merozoites can either infect other epithelial cells or mature into gametocytes which are the sexual forms of the parasite. The gametocytes later release the oocysts (infective stages) which are excreted via diarrheic stool into the environment to start another life cycle (Morgan *et al.,* 2002).

The major routes of transmission include drinking and recreational water (swimming pools). Other routes are through food handlers, animal handlers and day care centre attendants. Most instances of transmission occur by the feacal-oral route and several recreational outbreaks have been traced to individuals who had recently recovered from *Cryptosporidium*- related diarrhea and who were thought to be shedding oocysts at the time (Xiao *et al*., 2002; Karanis *et al*., 2007).

Clinically, cryptosporidiosis could result in life threatening diarrhea, while the entire population could be seriously affected due to the ability of the oocysts to survive the usual water treatment procedures. Severe outbreaks affecting a larger population have also been reported and the role of animals in contributing to the spread of the parasite has also been confirmed (Geurden *et al*., 2006). This clearly shows the immense role that accurate diagnosis could play both for patient management and safeguarding human population.

Diagnosis of cryptosporidiosis has progressed from histological identification in intestinal biopsies to microscopic examination of feacal specimens for infective oocysts, enzyme immunoassay (EIA) for parasite antigens and nucleic acid amplification assay as well as use of molecular techniques such as Polymerase Chain Reaction (PCR) (Garcia *et al*., 1997; Phillip *et al.,* 2008).

Given the poor state of health infrastructure in Africa, brain drain of skilled personnel, low allocation of resources to health, among other factors, for accurate and prompt diagnosis of cryptosporidiosis is urgent. Furthermore as the success of managing cryptosporidiosis lies on accurate diagnosis, (both for samples from patients and/or environment), it is important to elucidate on the available diagnostic methods and recommend the inclusion of *Cryptosporidium* in routine testing for diarrheal pathogens (Robert *et al.,* 2005).

DIAGNOSIS OF CRYPTOSPORIDIOSIS

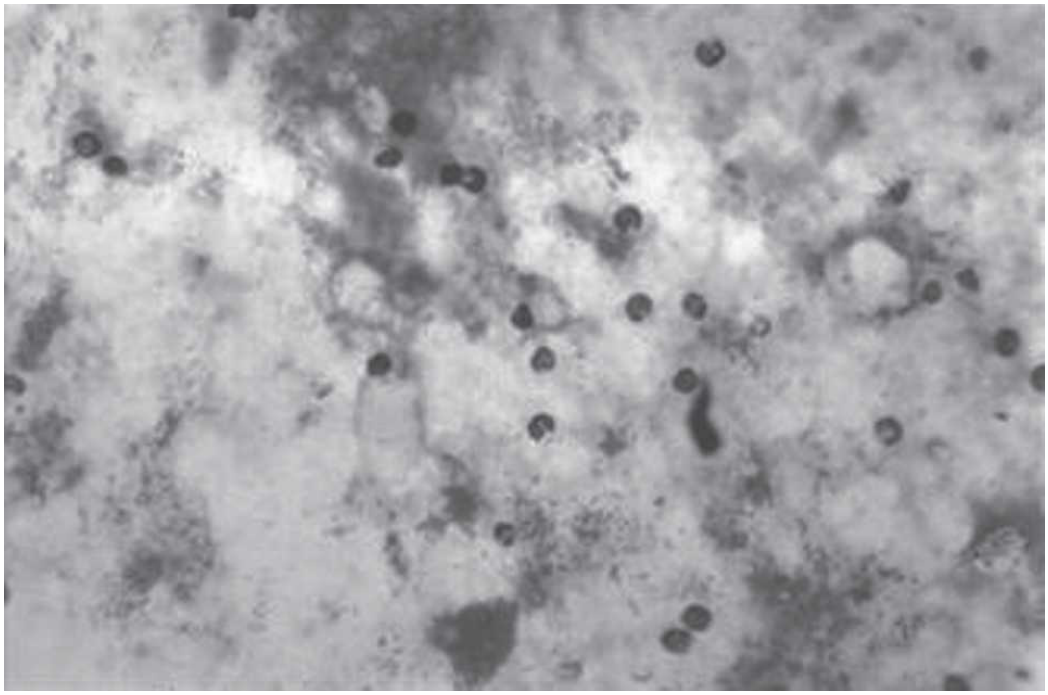
There are different methods used in detecting *C. parvum* oocysts in clinical and environmental samples. These include microscopic techniques, polymerase chain reaction (PCR) and enzyme immunoassay methods (Braiken *et al.,* 2003; Coupe *et al.,* 2005; Pelayo .*et al.,* 2008).

Microscopy : Historically, the use of electron or light microscope has been the method of choice in diagnosis of *Cryptosporidium* oocyst. Failure to recognize *Cryptosporidium* oocyst as a cause of human disease until 1976 was in large part associated with inadequacy of the applied staining techniques to identify oocysts in human and animal faeces and in environmental samples. Microscopic analysis of stained feacal smears is however the most widely used method for screening stool samples for *Cryptosporidium* in clinical diagnostic laboratories (Tzipori *et al.,* 2002).

Microscopy method involves concentration of the samples and staining to obtain accurate diagnosis. Microscopic diagnosis involves the morphological identification of the parasite in any specimen. This depends to a large extent on the skill, patience and experience of the microscopist. Currently, diagnosis is achieved by detecting the presence of oocysts, the

environmentally resistant forms, in stool samples. *C. parvum* oocysts in infected persons can be identified by staining of feacal smear with an acid-fast stain (Ziehl-Neelsen). The oocysts measures 4-6µm and stain bright red with refractile round thick capsules when observed under high power light microscope (Goodgame *et al.,* 1995). Acid fast stain technique is more widely used than other staining techniques such as Giemsa and Metheinamine silver stains (Casemore *et al.,* 1984; Xiao *et al.,* 2004; Pelayo *et al.,* 2008). Conventional microscopic method with acid fast stain was however found in a study to be a more reliable diagnostic tool than immunological methods because its affordability and ease of usage (Tzipori *et al*., 2002).

Microscopy has several advantages including provision of a single procedure for the detection of *C. parvum,* low cost; absence of false negative results; freedom from inhibitors; and technical simplicity. The method is however not without challenges. These include its time consuming procedures, tediousness, requirement of experienced microscopists for parasite identification, lack of constant power supply especially in developing countries hampering use of light microscopes, and insensitive to bulk processing since the Technologist is required to spend a minimum of five minutes per slide irrespective of the number of samples to be screened (Areeshi *et al.,* 2008; Essid *et al.,* 2008).



Cryptosporidium specie cyst in feaces (Kinyoun acid-fast stained, magnification x1000).
Source: Sanchez-Vega *et al*., 2006

Immunological-based detection methods

Immunofluorescence assay (IFA): Immunoflourescent assay (IFA) procedures that employ *Cryptosporidium* – specific polyclonal or monoclonal antibodies is developed to aid in the identification of oocysts in stool and environmental samples (Garcia *et al*, 1997). Antibodies specific to *Cryptosporidium* have been detected, using IFA procedure, in sera obtained from persons who recovered from confirmed infections (Campbell and Current, 1998). The IFA can either be direct or indirect. In direct IFA, the sample is fixed onto a microscope slide, then a drop of pathogen specific antibody labeled with a suitable fluorochrome e.g. fluoescein isothiocyanate (FITC) is applied and incubated. If the antibody binds to the antigen, the sample will fluoresce against a green background while any unbound conjugates would be rinsed off when washed with water; the slide is then examined under an epifluorescent microscope (Garcia *et al.,* 1997).

In indirect IFA, the pathogen's specific-antibody is detected by a second anti-immunoglobulin antibody labeled with FITC. IFA technique is not required to

distinguish between *C. parvum* oocyst and non-*C. parvum* oocysts, but all the commercially available diagnostic tests will give a positive result for non-*C. parvum oocysts* (Gosh,* 2001). This calls for a need for better diagnostic techniques that would reliably differentiate *C. parvum* species. The direct IFA has proved to be highly specific and reasonably sensitive for human clinical samples (Fraser *et al*., 2000).

IFA is important since it can be done in combination with membrane exclusion / permeability dyes to estimate oocysts viability (Campbell and Current, 1998). However, important challenges to the use of this method in Africa are that it is time consuming, allows only few samples to be examined per day, and the necessity for an expert or experienced personnel to interpret the results who may not be available, (Chappell and Okhuysen, 2002; Nair *et al*., 2008).

Enzyme immunoassay (antigen detection*):* Over the past decade, a variety of highly sensitive and specific enzyme immunoassays have been developed that detect the antigens of *Cryptosporidium* species (Shimizu, 2000). These enzyme immunoassays have become widely accepted as methods for screening stool samples to detect *Cryptosporidium* and other parasitic organisms (Pieniazek *et al*., 2002).

The two commercially available enzyme immunoassays are (i)Prospect *Cryptosporidium*-micro plate assay and (ii) Color PAC *Cryptosporidium* rapid assay. These two assays are used in detecting *C. parvum* in stool specimens fixed in 10% formalin. The techniques combine the specimen antibody with the amplification of antibody-antigen interaction by enzyme catalysis, and therefore, can detect very small amounts of pathogen. Many assays are done in wells of microtitre plates in which the reactants are immobilized. Antigen in the sample may or may not be bound by specific antibody immobilized on the surface (Marques *et al.,* 2005). Specific anti-*Cryptosporidium* IgG and/or IgM was detected by an enzyme-linked immunoabsorbent assay (ELISA), in the sera of 95% of patients with cryptosporidiosis at the time of medical presentation and in 100% of patients with cryptosporidiosis in 2 weeks of presentation (Ungar *et al*., 1988). Several serological surveys have reported that more than 50% of persons with no known infection may have anti-*Cryptosporidium* IgG, suggesting recent exposure to the parasite (Ungar *et al*., 1994; Koch *et al*., 1995). ELISA can be more sensitive in formalin fixed samples.

Antigen based ELISA have several significant advantages over other methods currently used for diagnosis of *Cryptosporidium* oocysts including excellent sensitivity and specificity, use of 96 well plate formats which enhances their potential as large scale screening tools in epidemiological studies such as water borne outbreak situations (Ungar *et al.,* 1994), wide use and simplicity performance with minimal labour (Papaventsis *et al.,* 2007). However the expensive nature of the enzyme immunoassay kits used for ELISA places them above the reach of many laboratories in developing countries and this may hamper their routine usage in diagnostic services (Warren *et al*., 2003; Miller *et al.,* 2006).

Polymerase chain reaction (PCR) based method: Genetic method for detecting *Cryptosporidium* oocysts have recently been developed. The method identifies and amplifies *Cryptosporidium* nucleic acid using PCR (Johnson *et al*., 1995; Abe *et al*., 2002). Oocysts can be detected by PCR in waste water, surface water, stool sample and drinking water; however the sensitivity of the PCR assay could be inhibited by uncharacterized components in the samples. A study carried out by Dixon *et al*, (2002), however, showed that integration of flow cytometry, dot blot and magnetic antibody methods could improve the sensitivity of the PCR assay. PCR is sensitive and has shown potential for accurate diagnosis in patients unaware of the cause of their diarrhea since it is highly sensitive

Several factors could complicate the PCR-based detection of *C. parvum* in stool samples. For example, standard fixation in 10% buffered formalin could reduce the sensitivity of PCR, particularly if fixation occurs over an extended period. Also, extended formalin fixation may alter the buoyancy of *C. parvum* oocysts from frozen stool.

Similarly the sensitivity of PCR could also be reduced, when *C. parvum* oocysts are examined in frozen stool, probably due to rupture of oocysts during thawing. A study carried out by Morgan *et al*. (1998) showed that feacal constituents such as bilirubin, bile salts and complex polysaccharides could reduce PCR sensitivity and inhibit it even when they are present at low concentrations. To eliminate these problems one method for oocyst purification from stool was developed for routine use in research laboratories, which involves density gradient centrifugation of stools (Tzipori, 1998; de Otazu *et al*., 2004)

Direct use of PCR does not distinguish between live and dead oocysts, since oocyst DNA is apparently preserved for at least a week after cell death. Viability

of oocysts is determined by detecting and amplifying a viable sporozoites DNA fragment. These protocols are adequate to detect low numbers of viable oocysts in drinking water and environmental samples (Semenza *et al*., 2007).

Morgan *et al*., (1998),in their contribution noted the importance of PCR in processing clinical as well as environmental specimens suspected to be contaminated with *Cryptosporidium* species. The workers posited that the developed PCR primers specific for *Cryptosporidium* and random amplified polymorphic DNA (RAPD) are simpler approaches for developing diagnostic primers, since most of the products generated by RAPD-PCR are frequently species specific. In a similar contribution Balatbat *et al;* (1996) developed a nested PCR assay for detection of *C. parvum* oocysts directly from stool specimens by using a nested primer. This test can detect few oocysts ranging from asymptomatic infections to monitoring response to therapy. For clinical as well as environmental samples, PCR can detect pathogens reliably in addition to the important function of determining the density of pathogens. PCR is sensitive and has the potential for accurate diagnosis in patients who do not presently know the cause of their diarrhea. This could have considerable advantage in the treatment of immunosuppressed individuals, allowing for early diagnosis before the onset of symptoms. PCR technique is rapid and accurately obtained results are easy to interpret (Keegan *et al.,* 2003). Furthermore PCR test is capable of directly differentiating between human and animal derived genotypes of *Cryptosporidium* on the basis of size of the PCR product (Morgan *et al*., 2000).

PCR technique cannot, however, be used for routine diagnosis of *Cryptosporidium* oocysts due to several challenges. These include its technical complexity and interference of results by inhibitors, its expensive nature, and time-consuming procedures (Sulaiman *et al.,* 2005; Gatei *et al.,* 2006).

Concentration techniques for detection of oocysts in water

Flow cytometry method: Flow-cytometry is very sensitive in detecting *Cryptosporidium* oocysts in environmental samples (Dixon *et al.,* 2002). Flow-cytometry with fluorescence activated cell sorter (FACS) method is used routinely in the U.K and Australia for detection of *Cryptosporidium spp* and *Giardia spp* in environmental samples (Vessey *et al*., 1993). Flow cytometry is a laser-based instrument that analyzes particles in a liquid suspension on a particle-by-particle basis. It can differentiate and physically separate particles based on their size, internal complexity and fluorescence. Its advantages are varied amongst which are increased sensitivity, less labor and time demanding processes. On the other hand, initial expense of the instrument and the level of expertise required can hamper the usage of this diagnostic procedure in African countries (WHO, 2005).

Immunomagnetic separation (IMS): Immunomagnetic separation is used to separate oocysts following a filtration step. IMS concentrates *Cryptosporidium* oocysts by using a magnetic bead coated with an *anti-Cryptosporidium* antibody. Following elution, the sample is incubated with magnetic beads that bind the oocysts. The solution is inserted into a magnetic particle concentrator that binds the magnetic bead – *Cryptosporidium* complex. After the supernatant is decanted, the beads are released from the magnet. Oocysts are dissociated from the magnetic particles using an acid wash, neutralized with base, and then discarded (Fontaine and Guillot, 2003). However, the efficiency of the IMS method could be decreased in highly turbid water samples. (Fontaine and Guillot, 2003).

Fluorescence activated cell sorter (FACS): Cells stained with fluorescein isothiocyanate (FITC) antibody conjugate can be identified and separated from other components of a suspension in an instrument known as 'fluorescence activated cells sorter' (FACS). The process involves passage of the suspension through a laser beam with the cells being separated on the basis of fluorescence intensity. The sorted cells could then be examined microscopically. This technique is mainly used to identify *Cryptosporidium* oocysts in water samples (Vessey *et al*., 1993). In another study by Vessey *et al.* (1993), it was observed that FACS detected greater than 92% of the *Cryptosporidium* in seeded and reservoir samples. Similarly, studies carried out by Hoffman *et al.,* (1997), with a variety of environmental samples showed that FACS detected almost three times more *Cryptosporidium*-positive samples than membrane immunofluorescence (94.1% versus 35.3%) respectively.

CHALLENGES TO CRYPTOSPORIDIOSIS DIAGNOSIS IN AFRICA

The current goal of the World Health Organization is to assist each country in Africa to develop her ability to provide laboratory diagnosis of diseases (WHO, 2004). Many serious diseases in developing countries go undetected due to improper diagnosis, due to lack of standard diagnostic tests available in developed countries. Consequently, African nations have been called upon to prepare for a more proactive system in which cryptosporidiosis diagnosis would be included in routine tests. To achieve this aim, there is need for African countries to train skilled public health workers, and nationalize regional laboratories for diagnosis and research communication systems (WHO, 2004). Below are some of the areas identified for invention to improve diagnosis of cryptosporidiosis. Below are some of the areas identified for intervention to improve cryptosporidiosis diagnosis.

(1) Capacity of technologists: for the technologist to acquire enough experience, funds have to be made available for the attendance of seminars, workshops and training courses to equip them with new techniques in the diagnosis of parasites. Lack of this exposure could eventually lead to inefficiency of the technologists and loss of confidence in the services rendered. There is need for African countries to be constantly upgraded in acquiring advanced technical skills applicable to modern scientific research (WHO, 2005; Anthen, 2006).

(2) Infrastructure: African countries are faced with the challenge of inadequate facilities and equipment for diagnosis of cryptosporidiosis. This makes research impracticable. The reason for this may not be unconnected with the expensive nature of diagnostic equipment such as a good microscope, which places them beyond the reach of the technologists. Furthermore, lack of good and conducive working environments are other factors contributing to the challenge of efficient and accurate diagnosis of Cryptosporidiosis in Africa (WHO, 2004). Scholars in African countries should invest in procuring equipments for the development of science and technology

(3) Inadequate and inefficient power supply: Common among African countries have contributed in no small way to the problem confronting reliable diagnosis of *C. parvum* infection in the continent. Inadequate power supply renders specimens and samples stored in laboratory fridges and freezers completely inadequate for future use (WHO, 2005).

(4) **Availability of materials such as kits, primers and reagents for diagnosis of cryptosporidiosis:** the use of kits and reagents enhances accurate diagnostic results. These kits are however not readily available in Africa due to their high cost and lack of knowledge to produce one. In many developing countries, the lack of proper equipment and kits makes it difficult to diagnose and treat patient's illness accurately (WHO, 2004; O' Hara *et al*., 2007).

(5) In addition to the above, **low technological development, poverty and lack of established reference laboratories** in Africa contributes further to the problem of accurate diagnosis of cryptosporidiosis in the continent (Okpala *et al*., 2005; Kojeku *et al.,* 2005). Government should assist researchers in developing countries financially to enable them carry-out their research activities effectively.

CONCLUSION

Governments should invest in reliable power supply so as to enable researchers and technologists carry-out proper diagnosis of cryptosporidiosis and other parasitic infections. In addition, training and periodic re-training of microscopists and other laboratory staff involved in diagnosis of cryptosporidiosis should be encouraged since this would enable them interpret laboratory results and findings correctly. Similarly, there is the need for establishment of reference laboratories that would assist to perform quality assurance testing on diagnostic tests and perform comprehensive surveillance for cryptosporidiosis. Finally, enhanced public education on simple health measures in developing countries should be instituted as adequate creation of awareness on the mode of transmission and severity of the disease could assist in reducing the incidence of cryptosporidiosis in Africa.

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