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A Comprehensive Evaluation Of Diagnostic Techniques For Cryptosporidium Species With Special Emphasis To *Cryptosporidium Parvum*

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Abstract

Cryptosporidium parvum is a significant cause of gastrointestinal disease in humans and animals. Many diagnostic techniques are available for its detection. Most of these include- Microscopic methods, Serological methods, and Molecular methods. Current review aims in describing various diagnostic techniques available along with their advantages and limitations.

conclusion : Many diagnostic tests are available for the diagnosis of Cryptosporidium parvum. Recent advances look promising in diagnosis of C.parvum infections. Till now, no single diagnostic test is fool proof and many of diagnostic tests require a set up and expertise.

Keywords: Cryptosporidium parvum, diagnostic techniques, diarrhea

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Introduction

Cryptosporidium species are increasingly recognized as important enteric pathogens [1, 2] . Cryptosporidiosis was initially recognized as a cause of diarrhea in compromised hosts. Shortly thereafter, zoonotic and waterborne transmission of the parasite was identified[1-3]. Cryptosporidium is now considered one of the major causes of childhood diarrhea[2]. In addition, *Cryptosporidium* has been documented as a key component of the vicious cycle of infection and malnutrition that are major contributors to childhood morbidity and mortality worldwide[4]. The majority of human *Cryptosporidium* infections are attributed to two species: *C. hominis* and *C. parvum* [5, 6] . However, at least 13 other species may infect humans[6-8] .Clinically, cryptosporidiosis causes watery diarrhea in healthy patients. In contrast to other causes, diarrhea caused by cryptosporidiosis tends to be more prolonged and can be chronic in compromised hosts, such as children with malnutrition [9].Like other causative agent of diarrhea , the host immune response to Cryptosporidium involves components of both the innate and adaptive immune systems[10].

Cryptosporidium develop within the microvillus layer of intestinal epithelial cells, mainly found in the small intestines in immunocompetent hosts, but may be found throughout the GI tract and even the respiratory tract. Persistent infection is associated with villus atrophy, crypt hyperplasia, and variable increases in leucocytes in the lamina propria[11] . The symptoms of watery diarrhea and malabsorption are thought to be related to sodium malabsorption, electrogenic chloride secretion, and increased intestinal permeability, and severity of disease correlates with altered intestinal permeability[8, 11] .

Cryptosporidium Detection Methods

1.Clinical Diagnosis of cryptosporidiosis

Diagnosis of Cryptosporidium infections in diarrhoeal patients is not a routine laboratory test in both developed and developing countries because rehydration and electrolyte balance is more crucial [12] .Acute cryptosporidiosis is self-limiting and identification would be necessary in the event of an outbreak or if diarrhoea persist or becomes chronic. Reliance on the quality of stool samples, experience and skills of the microscopists and the resources available for stool evaluation is crucial in effective diagnosis of diarrhoea [13] .

2.Microscopic techniques

I. Concentration methods

Stool concentration techniques include

***a*) Flotation methods :** Flotation of oocysts performed in Sheather's sugar solution, zinc sulfate (1.18 or 1.20 specific gravity), saturated NaCl (1.27 specific gravity)[14, 15]. Sheather sucrose flotation (SSF) technique is simple ,cheap and easy to accomplish . The oocysts with pink color clearly demonstrated under high power magnification[16]

b) Sedimentation methods: Formalin-ether and formalin-ethyl acetate were used for recovery of cryptosporidium oocysts [17]. Preparation of the acid fast smear form the same sediment may reduce cost and technical time.

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II. Iodine-saline wet mount: Shedding of cryptosporidium in low number as well as the experience of laboratory worker have direct effect on the diagnostic sensitivity of this method. This procedure recommended as a routine screening procedure [18]. Lack of permanent record and the need to examine the prepared wet mount with less than fifteen minutes are the major restrictions for this procedure [19].If delay in examination of the prepared samples, collapse of oocysts takes place [9, 12].

3. Staining techniques

Several widely used techniques for demonstrating Cryptosporidium oocysts in fecal specimens from humans and other animals are:

a) **Hematoxylin *And Eosin Stain***

In intestinal tissue section , cryptosporidium oocysts demonstrate 2-5 µm basophilic spherical structures adhere to the apical brush border of the absorptive epithelial cells of the intestine. [12].

b) Romanowsky stains (Methylene Blue & Eosin Y Stain)

These neutral stains consist of a mixture of basic dye which is an oxidized methylene blue that have the ability to attach with nucleus and the result reflected as blue to purple color . The second component is the acidic dye ,Eosin Y which has the ability to bind with alkaline cytoplasm giving red color. Cryptosporidium oocysts stained with blue to azure with a crescentic pattern. This staining procedure is simple and relatively cheap . [20-23].

c) Giemsa stain

Staining with Giemsa method ,first used determine Cryptosporidium in murine gut mucosa [24, 25] , cattle faeces [26] ,and human feces [4, 17, 27] .

d) Modified Acid-fast stain (mZN)

Cryptosporidium oocysts seems as pink round to oval stained structures with diameter (3- 6 µm) [28].Acid-fast stains methods usually either hot or cold [29, 30]. By using modified cold Kinyoun stain the oocysts demonstrated as dark red structures(3 - 5 µm) in diameter with background of green or blue color according to counterstain [12]. The mZN stain characterized by its low cost, constant stain , suitable for examination of large number of clinical specimens [31] . On the other hand such ;low sensitivity and specificity due to indiscrimination between oocysts , moulds and yeast ; time-consuming , requires experts for interpretation of the results[20, 32, 33] .

e) Safranin- Methylene blue (SMB) staining

Cryptosporidium oocysts are appear as spherical or slightly ovoid ,orange to pink structures with 5 µm diameter and sporozoites within the oocyst stain slightly darker [20]. SMB characterized by its rapidness and simplicity and have the ability to differentiate between the yeasts and oocysts [29]. On the other hand ,the requirement for acid-methanol treatment before the addition of safranin and strong heating during the safranin addition represent the main disadvantages[12, 22, 23].

f) Leishman's stain

This stain efficient in making a persuasive diagnosis of cryptosporidiosis by laboratory personnel little experience but it is not available and used for flexible examination in most of the laboratories [21].

g) Negative stains

Negative stains represent the first choice for examining of slides for detection of cryptosporidium spp [12]. For negative staining ,

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2-5% light green ; Malachite green , nigrosin ,Carbolfuchsin, Safranin, which stain background while oocysts appear as unstained, strongly refractive, round to oval structures of (3 - 6 µm) in diameter. Although negative staining techniques faster but are variable in sensitivity from 100%[4] to less than conventional staining procedures [12, 34].

By using malachite green stain, yeasts were distinctly differentiated from cryptosporidial oocysts as they capture the stain. [35]. The malachite green staining procedure is a workable, safer and sensitive than other staining methods required less experienced microscopists for accurate detection of cryptosporidium oocysts in stool samples [29].

Yeasts did not permanently capture the light green as well as nigrosin stain that extremely confusing the diagnoses[20]. On the other hand , nigrosin staining technique is relatively of low cost , and easy to perform with minimum time requirement for staining. The sensitivity increased by using phase-contrast microscopy or examination at x400 magnification . The limited knowledge about usefulness, phase-contrast microscope requirement constitute the major drawbacks [12].

h) Dimethyl Sulfoxide Modified Acid Fast Stain (DMSO-mAFS)

By using DMSO-mAFS, The oocysts stained with brilliant pink to fuchsia and the background stained pale green. The Cryptosporidium-typical internal vacuole and material clumped to one side of the 4-5 um cyst under 100x[36]. The internal morphology was well preserved in contrary to routine acid fast staining[12, 37]. DMSO -mAFS characterized by its simplicity and expedites starting of treatment and minimize the possibility of contamination [36].On the other hand the main disadvantage was the variability in capturing of stain due to the stain itself or prolonged storage of oocysts [36]

i) Fluorescent stains

One inexpensive approach is to use non-specific fluorescent stains such as auramine-rhodamine or Auramine-phenol (AP) [12, 38, 39], Cryptosporidium oocysts appropriately sized (4-6 µm) round or slightly oval structures give yellow fluorescence [39],when examined at (×200 and ×400) of fluorescent microscope. Auramine-Carbolfuchsin staining utilized for investigation of cryptosporidium oocysts in animals and human stool specimens [40] .Cryptosporidia appear as bright fluorescent discs against a dark red background[41, 42] . Acridine orange stain also used [38]. Advantages include, rapid screening, higher detection efficacy [39]. Limitations include, low sensitivity and specificity ; high cost, considerable experience required .

4.Immunological detection methods

Immunological-based techniques include:

I. Antigen detection methods

a) Immunochromatographic dipstick test (ICT): Immunochromatographic dipstick test (lateral flow immunochromatographic tests) utilized for rapid detection of target in clinical specimens. the test kit always composed from a single-use strip or cartridge which develop a visible colored end products that evaluated either positive or negative[43]. ICT depends on the attachment with target antigen (or antibodies) found in stool specimens .The ICT assay use antibodies which were binds on a special paper strip or a nitrocellulose membrane as the immobile capture antibody for C.Parvum oocyst antigen in stool specimens. By utilizing a

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property of capillary flow , antibody conjugated with a colloidal gold or colored microparticle attaches to C.Parvum oocyst antigen in stool [44]. A positive test is produced by development of a colored line or pattern. Another control antibody to the conjugate binds the excess colloidal dye conjugate and acts as the control line [44] .

ICT have several features like its simple ,rapid, minimum time requirement , specific and sensitive , can be utilized for large scale diagnosis in rural and in segregated areas[45]. Evaluation of result is very easy and can be done by inexperience persons [46, 47].

b) Enzyme Immunoassays (EIAs)[48]

Over the past decade , a variety of highly sensitive and specific enzyme immuno-

d) ***Immunofluorescent Antibody (IFA) Assay:***

Immunofluorescent assay (IFA) , emplo say (IFA) , employing specific antibodies against cryptosporidium found in stool and environmental samples[51].

The IFA can either be direct or indirect. The direct IFA has highly specificity and

e) Latex agglutination

The quantitative latex agglutination test used for determination of *C. parvum* antigens in fecal samples characterized by its low cost ,rapidity (required just 20 minutes for establishment) , simplicity, sensitivity , do not required any washing or addition of susbstrate [53, 54].

II. Antibody detection methods

Specific antibodies have been detected in 95% of patients with *C.parvum* infected patients by ELISA , at the time of clinical examination and in 100% after two weeks of presentation[12] .The limited *C.parvum* seroprevalence data suggest that asymptomatic *C. parvum* that do not distinguished by assays (EIAs) have oped that detect the antigens of cryptosporidium species oocysts , thus removing the need for microsopic examination [49]. EIAs has less detection time, economical, lesser skill required [12]. Several limitations were reported such as cost factor, false- positive results are common, A negative result can occur from a species antigen level lower than the detection limits of this assay [50].

c) Reverse passive Hemagglutination (RPHA) technique

The assay involves an anti- Cryptosporidium oocyst monoclonal antibody coupled to stabilized sheep erythrocytes [20].False-positive results represent the main disadvantage[12] .

ly specificity and sensitivity [43]. Time consuming the necessity for e ing the necessity for experienced personnel which may not be available to interpret the results ,represent the main disadvantages [52]

cryptosporidium infections are more common than the infection rates registered in surveys depends on determination of fecal oocysts [29].

III. Flow cytometry

Cryptosporidium oocysts can be detected by flow cytometry (FC) [55]. Flow-cytometric assay developed by Luminex technology allows the detection of various targets simultaneously [12].The microsphere beads bound to antigens, antibodies, or oligonucleotides which serve as probes [56] . Thus the luminex assay could distinguish different parasites or genotypes of one organism at the same time [57]. Flow cytometry was applied to the distinguish *C. hominis and* usual antigen detection methods or serologi-

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cal techniques [58]. Advantages of flow cytometry ,it is a simple and efficient and sensitive method. The high cost and the need for technical experience represent important limitations was [59] .

5. Molecular methods

a) Polymerase Chain Reaction (PCR)

Different PCR-based procedures for diagnosis of cryptosporidium were utilized [60, 61]. The sensitivity of PCR were affected by fixation of stool samples in 10% buffered formalin and if stool samples kept frozen[12].

Advantages of PCR methods ,it is sensitive technique; permits genotyping of recovered pathogen. While its main limitations , take too much time, high cost ,it was nonstandardized method , specialized training and equipment required, DNA extraction from stool required special preparations; possibility of DNA contamination is common [12]

b) Real-Time PCR for detection of Cryptosporidium spp. and genotypes

RT-PCR system depends on the utilization of different fluorescent chemistries, such as Sybergreen, Taqman probes, fluorescence resonance energy transfer (FRET), and Scorpion primers for quantitative determination of the original template's concentration [62] .This abolishes the requirement for gel electrophoresis hence minimizing the possibility of contamination and false-positive results [62] . The use of RT-PCR to determine low number of cryptosporidium oocysts in stool by amplifying a region which can be directly sequenced to identify species/genotype [63]

The main advantages of real time PCR ,minimum time requirement compared to conventional type, simplicity ,efficiency ,high sensitivity and specificity ,reproducibility, low possibility of contamination ,permit to discrimination between nonhuman from human pathogens[12, 64]

c) Multiplex Real-time PCR

The multiple real-time PCR with fluorescent probes of different sequences permits analysis of numerous cryptosporidium spp. in single reaction. The main advantage is a sensitivity ;specificity and save the time and cost by detection of more than one target in single reaction [12, 62, 65]. Several limitations for multiplex PCR that may leads to diversification in results ,such as requirement for technical expertise ,differences in DNA extraction protocols, choice of primers and use of various amplification protocols[59] .

d) Fluorescence In Situ Hybridization (FISH) Using rRNA-Targeted Oligonucleotide Probes

This method depends on the utilization of synthetic oligonucleotide probes for hybridization with specific regions within the rRNA of cryptosporidium [66]. Differentiation between *C. parvum* and *C. hominis*. was possible by using species-specific probes with two-color FISH [12, 67]. The main advantages of FISH, ability to identify Cryptosporidium species in different samples within a 3-hours which represent a suitable alternative for PCR [12].

e) Loop Mediated Isothermal Amplification (LAMP) : LAMP technology was used for detection of Cryptosporidium spp[68].Loop-mediated isothermal amplification (LAMP) is a unique amplification method with extremely high specificity and sensitivity able to discriminate between a single nucleotide difference [69]. It is characterized by the use of six

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different primers specifically designed to recognize eight distinct regions on a target gene, with amplification only occurring if all primers bind and form a product. LAMP reactions are easy to set up, and results can readily be assessed. The sample of interest is mixed with primers, substrates, and a DNA polymerase capable of strand displacement in a microcentrifuge tube. During the reaction, large amounts of pyrophosphate ions are produced, leading to the formation of a white precipitate [12]. This turbidity is proportional with the amount of DNA synthesized therefore one can assess the reaction by real-time measurement of turbidity or more importantly, simply through the naked-eye. Recently, parasitologists have adapted the LAMP approach for the detection of several parasitic diseases including Cryptosporidium. Unlike a regular PCR reaction, LAMP is carried out at a constant temperature (usually in the range of 60–65°C). The main advantages of LAMP ,higher yields, eliminates the need to buy a thermal cycler and shortens the reaction time by eliminating time lost during thermal changes [70].

Conclusion : Many diagnostic tests are available for the diagnosis of *Cryptosporidium parvum*. Recent advances look promising in diagnosis of *C.parvum* infections. Till now, no single diagnostic test is fool proof and many of diagnostic tests require a set up and expertise.

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Table(1): Diagnostic Techniques for Cryptosporidiosis

Microscopic Techniques	**[1]** Concentration techniques A. Floatation methods i. Sheater`s Sugar solution ii. Zinc sulfate iii. Saturated salt solution B. Sedimentation methods i. Formalin ether ii. Formalin ethyl-acetate
	[2] Iodine-saline wet mount
Staining methods	a) H & E (Hematoxylin & eosin) stain b) Romanowsky stains(methylene blue dyes and Eosin Y) c) Modified acid fast stain d) Safranine-methylene blue staining e) Negative staining i. Undiluted Carbol-Fuchsine ii. Safranin iii. 2-5% Light Green iv. Malachite Green v. Nigrosin f) DMSO modified acid fast staining g) Geimsa stains h) Leishman's stain i) periodic acid-Schiff (PAS) j) modified PAS k) silver methenamine l) Gomori's Trichrome
Immunological Techniques	A. Antigen detection methods i. Immunochromatographic dipstick test ii. Enzyme Immunoassays (EIAs) iii. Reverse passive Hemagglutination (RPHA) iv. Direct fluorescent antibody (DFA) assay v. Latex agglutination B. Antibody detection methods
Flow cytometry	
Molecular techniques	[1] Real time PCR [2] Multiplex real-time PCR [3] Fluorescence In Situ Hybridization (FISH) [4] Loop Mediated Isothermal Amplification (LAMP) [5] Recombinase Polymerase Amplification based Cryptosporidium(RPAC)

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