Different Detection Methods of Cryptosporidiosis

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Abstract

Background: Cryptosporidium is a coccidian protozoan parasite infecting humans and animals worldwide. It was so named because of the absence of sporocysts within the oocysts (Hidden sporozoites). The first Cryptosporidium spp. was described by Tyzzer in 1907 in lab mice gastric mucosa and was named Cryptosporidium muris (C. muris). A few years later, another smaller species was identified in the small intestine of mice and named C. parvum. It is now recognized that Cryptosporidium causes self-limiting diarrhea in immunocompetent case, but chronic life threatening diarrhea in immunocompromised individuals and it was probably been a human pathogen since the beginning of humanity. The diagnosis of cryptosporidiosis is essential for proper interventions and proper management of patients. Environmental samples are required for source tracking, risk factors assessment, and detection of outbreaks. Most of the laboratories may not look for Cryptosporidium unless specifically asked for, and so some selection criteria can be used to look for Cryptosporidium such as immunocompromised individuals, children <5 years, farm visitors, travelers from non-endemic to endemic countries, and local outbreaks. Stool sample is the most commonly examined sample in the diagnosis of cryptosporidiosis, while sometimes small bowel aspirates, biopsies or tissue samples may be available: Cryptosporidium can be diagnosed by a number of techniques including: 1- Microscopic examination either by the wet mount preparation or stained smears with modified acid-fast stain or by fluorescent stains. 2- Immunological methods detecting either antigen or antibody.3- Histological examination of tissue biopsy. 4- Molecular methods for detection of parasite DNA

Key Words: Cryptosporidiosis, Diagnosis

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Introduction:

Cryptosporidium is a coccidian protozoan parasite infecting humans and animals worldwide. It was so named because of the absence of sporocysts within the oocysts (Hidden sporozoites). The first Cryptosporidium spp. was described by **Tyzzer (1)** in lab mice gastric mucosa and was named Cryptosporidium muris (C. muris). A few years later**,** another smaller species was identified in the small intestine of mice and named C. parvum **(1)**. Since then, Cryptosporidia have been identified in all vertebrate classes **(2).**

Cryptosporidia spp. was considered commensals until their association with diarrhea in young turkeys, Cryptosporidium meleagridis, in the 1955. Although the first 2 human case was reported in 1976**,** it had not gained importance in causing human infection until 1982, when the first case of cryptosporidiosis was reported in AIDS patient. It is now recognized that Cryptosporidium causes self-limiting diarrhea in immunocompetent case, but chronic life threatening diarrhea in immunocompromised individuals and it was probably being a human pathogen since the beginning of humanity **(3).**

Taxonomy:

The genus Cryptosporidium has been classified with other enteric coccidian parasites in the phylum Apicomplexa, class Sporozoasida, subclass Coccidiasina, order Eucoccidiida, suborder Eimeriina and family Cryptosporidiidae on the basis of many similarities in their morphological characteristics and life cycles. The latest molecular and biological studies found that Cryptosporidium parasite is more closely related to gregarine than to coccidia due to their ability to complete its life cycle in the absence of host cells and the identification of gregarine like gamont stages **(4).**

Currently, Cryptosporidium is classified in the sub-class Cryptogregaria, and the Class Gregarinomorphea, that is characterized as epicellular containing organisms, a food organelle very similar to that of the gregarines (epimerite) but it does not present apicoplast.The diagnosis of cryptosporidiosis is essential for proper interventions and proper management of patients. Environmental samples are required for source tracking, risk factors assessment, and detection of outbreaks. Most of the laboratories may not look for Cryptosporidium unless specifically asked for, and so some selection criteria can be used to look for Cryptosporidium such as immunocompromised individuals, children <5 years, farm visitors, travelers from non-endemic to endemic countries, and local outbreaks **(5).**

Stool sample is the most commonly examined sample in the diagnosis of cryptosporidiosis, while sometimes small bowel aspirates, biopsies or tissue samples may be available.

Cryptosporidium can be diagnosed by a number of techniques including:

1- Microscopic examination either by the wet mount preparation or stained smears with modified acid-fast stain or by fluorescent stains.

2- Immunological methods detecting either antigen or antibody.

3- Histological examination of tissue biopsy.

4- Molecular methods for detection of parasite DNA.

Firstly, macroscopic examination should be done to look for the consistency of the stool sample. Usually, it looks watery with mucus. The number of oocysts is usually related to the consistency of the stool. Oocysts will be seen in the watery stool more than the solid one.

Microscopic examination:

Microscopic examination is an important and essential method of diagnosis because of the low cost of reagents, but both of good staining and visual skills are necessary. Microscopic examination of Cryptosporidium oocysts in stool samples is the conventional method of diagnosis. It can be performed by wet mount, staining with a special dye such as acid-fast dye, fluorescence, or immunofluorescence to enhance the sensitivity of oocysts detection **(6)**.

Sample collection:

Since the oocysts show fluctuation in their fecal shedding, a minimum of three stool samples has to be collected and at least five to six stained smears should be examined before it is considered as negative sample. However, some studies have shown that the first sample is enough to provide accurate diagnosis in nearly 90% of the cases **(7).**

Cryptosporidium can be demonstrated in the preserved as well as unpreserved stool samples. The samples suspected to be delayed should be preserved in either 10% formalin, sodium acetate formalin, or polyvinyl alcohol. However, some staining techniques may not be suitable with PVA preserved samples. Formalin-preserved samples is not suitable for molecular diagnosis.

Concentration techniques should be done to increase the yield of the oocyst by reducing the debris in the sample and this will increase the sensitivity of microscopy. So, it should be done as a part of the routine diagnostic procedure **(8).**

Concentration techniques:

The detection limit for unconcentrated stool sample by microscopy has been reported to be 1×10^4 to 5×10^4 while concentration increases the sensitivity by 10 folds **(9).**

The different conc. Techniques :

a- Sedimentation technique

Formol ether and formalin ethyl acetate are the sedimentation concentration methods that is commonly used. It is used for the preparation of acid-fast stains **(10).**

b- Floatation techniques

Sheather's sucrose, zinc sulfate, and saturated sodium chloride are different floatation concentration techniques. Sheather's solution gives the best result, but the oocysts get collapsed and lose their distinct shape when Sheather's solution is kept for more than 15 min. **(11).**

Iodine-saline wet mount:

The wet mount can be made from the concentrated stool sample to look for the oocyst that is highly refractile, smooth, spherical or slightly ovoid, colorless, double walled bodies, with size ranging from 3 to 8 µm. It can be used for the routine screening procedure. However, its limitation is that it couldn't be maintained as a permanent record **(9).**

Staining methods:

Cryptosporidium is very small which makes its detection in the presence of fecal debris, a challenge. So, stains are very essential to facilitate its detection.

- **Romanowsky stains:**

Romanowsky stains such as Giemsa and Jenner's stain were the first to be used for Cryptosporidium oocysts identification. Oocysts appear as semi-translucent bodies with a narrow clear halo around it and stained blue to azure with four to six red or purple eosinophilic granules appearing as dots.sometimes, "Ghost" forms may also be found without granules.the technique was easy and noninvasive, but it lacked both sensitivity and specificity **(9).**

- **Hematoxylin and eosin stains:**

Hematoxylin and eosin stains are used to describe the developmental stages of Cryptosporidium which appear as small, spherical, and basophilic bodies within the microvillous region of the intestinal mucosa **(12).**

- **Modified acid fast stain:**

Cryptosporidium oocysts appears red with acid fast stains, with size 4-6 µm and contain 4 crescent-shaped sporozoites. The familiar acid-fast stains used are the modified Ziehl–Neelsen technique, modified dimethyl sulfoxide, safranin-methylene blue, and modified Koster. But these stains are not universally recommended for the detection of C. parvum oocysts in fecal samples because of low specificity and sensitivity **(13).**

- **Modified Ziehl-Neelsen:**

,Henriksen and Pohlenz. (14) used acid-fast Ziehl–Neelsen (ZN) stain to identify Cryptosporidium oocysts, which was modified **by Casemore et al. (15)** Modified Ziehl- Neelsen" MZN" became the widely used method for oocysts detection, in which oocyst appears pink-colored, round, 4–6 µm in diameter, may contain four sporozoites against green or blue background **(16).** The degree of the stain taken by the individual oocyst varies and can be confused with other various structures such as fecal debris, yeast, and bacterial spores which also stained red but are comparatively smaller; other parasites such as Cyclospora and Isospora are also stainrd but are comparatively larger It is of low cost, permanent stain, and could screen a large number of samples, but it is time-consuming procedure, require intensive training, and experience However, most health care centers using MZN as a gold standard method for Cryptosporidium diagnosis **(17).**

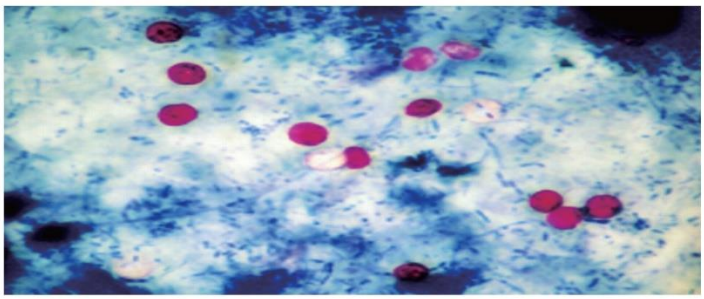

Fig (1): Cryptosporidium oocysts (MZN stain ×1,000) **(18).**

- **Negative staining:**

Negative staining method of Heine, in which the fecal sample is mixed with carbol fuschsin, spread as thin film, allowed to air dry, then immersion oil is added to the smear, and a cover slip is placed and examined under bright field microscopy (×400). The oocyst is seen as unstained, strongly refractile, rounded structure of 4–6 µm in diameter. It also can be done with safranin, 2–5% light green, malachite green, and nigrosin. They are rapid and simple, but need phase-contrast microscopy **(19).**

- **Fluorescent stains:**

Fluorescent stains such as auramine-rhodamine, auramine-phenol, auramine-carbol fuchsin, and acridine orange can be used. It is rapid screening and has higher detection efficacy than microscopy and enzyme-linked immunosorbent assay (ELISA), but low sensitivity, low specificity, and high cost are its main limitations. Auramine-phenol stain is considered as an alternative to MZN stain because it is simple, rapid and sensitive procedure**.** It can be used as a rapid screening technique and may be confirmed later by MZN. Many labs consider it as the gold standard method as it provides the highest combination of both sensitivity and specificity. Smears stained with auramine-phenol or MZN stain have an advantage that the stained oocyst can be scraped off from the slide for subsequent DNA extraction **(20).**

- **Immunofluorescence stain:**

The rate of detection of Cryptosporidium is increased using anti-Cryptosporidium specific fluorescent antibody staining over acid-fast staining. It is more sensitive and

specific and found to be the gold standard method in many labs. **De. (21)** found that DFA-Cryptosporidium had a sensitivity of 99% and specificity of 100% while DFA-Cryptosporidium/Giardia had a sensitivity of 88–98% and specificity of 87–100%. This test is limited by its expense and lack of availability of fluorescence microscope.

Electron microscopy:

This method was used for confirmation the infection in humans reported in the initial studies **(22).** Its major disadvantage is equipment cost, installation cost, complex processing, and inability to analyze large number of samples.

String test:

A string of certain lenghth with a weight in its end, is swallowed and travels to the upper part of the jejunum. Bile staining of the string indicates that the string has reached the jejunum. After a period of time about 4-6 hours, the string is pulled up and the fluid is examined. Oocysts can be detected in duodenal aspirate **(23).**

Serological diagnosis:

It can be based on detection of either antigen or antibody. These methods have reported to yield a good sensitivity and specificity. Antigen detection tests are useful for diagnosis of acute infection, while antibody detection ones are useful in seroepidemiological surveys. Immunological techniques for detection of antigens in stool samples have a positive impact on diagnosis sensitivity using monoclonal antibodies in ELISA or Immunochromatographic assays **(24).**

a- Antibody detection:

The detection of antibodies to Cryptosporidium-specific antigens in serum, saliva, or fecal samples is an indirect diagnostic method for the evidence of infection or exposure. Therefore, this is useful mainly for the seroepidemiological surveys of the disease.

Specific anti-Cryptosporidium IgG, IgM antibodies, or both can be detected by ELISA. 95% of infected patients are detected at the time of medical presentation and 100% within 2 weeks of presentation. It has an advantage of shorter detection time, more economic and detects many samples, but its limitations are the high cost of the kits and the need for special equipments **(25).**

a- Antigen detection:

Immunochromatographic assay is a popular procedure practiced in various labs involves the detection of Cryptosporidium/Giardia or triage panel. Four commercial rapid immunochromatographic assays were evaluated and found that the sensitivity and specificity depend onboth of the kit and the species. The mean sensitivities for all Cryptosporidium species were 70.6%, 68.8%, 62.4%, and 47.2% for ImmunoCard STAT, Remel-Xpect, RIDAQuick, and Crypto-Strip respectively. The specificity was 100% for ImmunoCard STAT, Remel-Xpect, Crypto-Strip and 98% for RIDAQuick. These tests are suitable to be performed on fresh, frozen, or formalin-preserved samples. Although it is a rapid method, but expensive **(26).**

Histopathological examination of biobsy:

Cryptosporidium oocysts diagnosis in biopsy specimen can be done by identification of their intracellular stages within specimens of human intestinal mucosa. In haematoxylin and eosin-stained sections, the developmental stages of cryptosporidium appear as small, spherical, basophilic bodies (2-5µm depending on the stage of the life cycle) within the microvillous region of the intestinal mucosa **(27).**

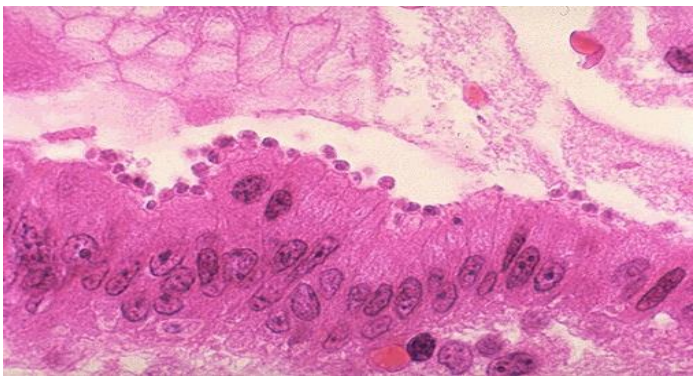

Fig (2): The little blue organisms lined up along the brush border of the small intestinal epithelium are Cryptosporidium parvum.

Molecular methods:

Molecular methods make a revolution in diagnosis with the advent of Polymerase chain reaction (PCR). It is the most sensitive of all the methods used for Cryptosporidium detection in both clinical and environmental samples with the detection range from 1 to 10^6 oocysts. It helps in both genotyping and subtyping of Cryptosporidium **(9).**

Various nucleic acid detection methods:

Various gene targets for the species identification are 18S rRNA, COWP, TRAP C1, Hsp 70, and DHFR genes. Further subtype determination can also be done using subtyping tools such as glycoprotein "gp60" gene, minisatellite, and microsatellite markers and also by analysis of extrachromosomal double-stranded RNA elements.

- **Nested polymerase chain reaction:**

It is a two-step PCR using two sets of primers. The common gene targets are gp60, hsp70, 18S rRNA, COWP, TRAP C1 and TRAP C2. Many studies found that nested PCR able to detect more positives specimens than microscopy, and ELISA. It is preferred for detecting small number of oocysts in the sample (<100), and it is the most popular technique which has been validated in numerous laboratories. Although it is sensitive and specific, its main drawback is that the species/genotypes can be identified only by sequencing or RFLP analysis and it takes a long time to complete the procedure **(28).**

- **Restriction fragment length polymorphism:**

RFLP helps in the analysis of PCR products after amplification of genomic DNA, and so genotyping, subtyping, and species identification can be done. But it is a time-consuming.

- **Real-time polymerase chain reaction:**

It is a real-time detection of DNA using hybridization probes. This assay is sensitive, specific, but the equipment is expensive which is the only limitation to the utilization of this method **(29).**

- **Multiplex real-time polymerase chain reaction:**

This assay combines different targets into one assay. Its main drawback is the difficulty in the extraction of DNA from fecal sample and their possibility of contamination. However, RT-PCR with fluorescent detection probes were designed to reduces the risk of contamination, labor time, and reagent cost. The only problem is that it needs technical expertise **(30).**

- **Microsatellite analysis:**

Microsatellites or simple sequence repeats can serve as polymorphic makers. It helps distinguish between different isolates of the same species **(28).**

- **Fluorescent in situ hybridization:**

It uses rRNA-targeted oligonucleotide probe helps in hybridizing the synthetic oligonucleotide probes to particular regions within rRNA of the organism. It identifies the species both in clinical and environmental samples within 3 hours, so it is considered as a reliable alternative to PCR and RFLP **(31).**

- **Loop-mediated isothermal amplification:**

This method depends on the amplification of the target sequence of DNA using a constant temperature of 60–65°C which is in contrast to PCR that needs different temperature for each step. It can use four different primers to identify the six distinct regions of the target gene and this accelerate the reaction. All these increase the specificity of LAMP. The amplification product can be detected visually by the naked eye as turbidity formed due to magnesium pyrophosphate or using fluorescent dyes. It is a simple procedure, low cost since it does not require expensive equipment such as thermocyclers and shortens the time when compared to PCR-based method. LAMP has become a practical diagnostic tool for many organisms due to its simplicity and specificity **(32).**

Detection of Cryptosporidium oocysts in environmental samples:

The presence of a low number of parasites in most of the environmental samples represents a challenge for their detection. Another challenge of detection is the difficulty of differentiation the main human pathogenic Cryptosporidia from other species commonly found in the environment. The standard method for parasitic detection in water samples is large volume sampling (10–1000 L), concentration, then detection is made usually by indirect fluorescent microscopy or molecular techniques such as PCR**.**The food products are processed similarly by elution and detection **(9).** However, all these are tedious and time-consuming, and some automated technologies are being developed to detect these parasites for their widespread applicability for environmental samples.

Diagnosis of extraintestinal cryptosporidiosis:

Biliary cryptosporidiosis (33):

1. Ultrasonography: look for the bile duct wall thickening and the gallbladder dilatation.
2. Computed tomography
3. Endoscopic retrograde cholangiopancreatography (ERCP): It is the most sensitive method of diagnosis. It should be performed when there is high suspicion of biliary disease, with normal ultrasound. ERCP may show a papillary stenosis with intrahepatic sclerosing cholangitis.
4. Serum aminotransferases and alkaline phosphatase levels will be elevated.

Pulmonary cryptosporidiosis (33):

The samples collected are sputum, tracheal aspirate, bronchoalveolar lavage, and brush biopsy specimens. The presence of acid-fast organism indicates pulmonary cryptosporidiosis.

Nanotechnology:

In the last few years, several types of nanoparticles (NPs) have been used for infectious diseases diagnosis,

particularly flourescent, metalic and magnetic NPs. The semiconductor quantum dots have been used for immunoflourescent labeling of C.parvum oocysts in water samples and provided a relable and excellent results. Gold NPs (Au NPs) was conjugated with heat shock protien 70 (HSP-70) to target C.parvum HSP-70 mRNA**.** It has been used to detect C.parvum oocyst nucleic acid in the feacal amples**.**It also has been used in several assays to increase the sensetivity of immuno-detection method through coupling of alkaline phosphatase on Au NPs with anti-cyst antibody for C.parvum. The sensetivity of immunodo blot was enhanced by about 500 times better than the conventional method **(34).**

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