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Infections by Intestinal Coccidia and *Giardia duodenalis*

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INTRODUCTION

The protozoa, as typically delineated in public health, are a nonmonophyletic conglomerate of unicellular eukaryotic organisms that are characterized by having animallike affinities. Most protozoa that infect the human enteric tract are characterized by having an environmentally stable stage such as a cyst or oocyst. Cysts and oocysts confer protection from environmental factors, allowing these parasites to infect other susceptible hosts through either the water or food-borne routes.

There are several parasitic protozoa that can cause enteric infections in humans, the focus here being *Cryptosporidium* spp, *Cyclospora cayetanensis*, *Giardia duodenalis* (syn. *Giardia lamblia*, *Giardia intestinalis*), and *Cystoisospora belli* (previously *Isospora belli*). Infections are usually characterized by gastrointestinal clinical manifestations that may include diarrhea, vomiting, abdominal cramps, and general malaise.[1,2] Three of these protozoa, *Cryptosporidium*, *Cyclospora*, and *Cystoisospora*, were previously classified as coccidian parasites because of their intracellular location (these parasite infect enterocytes) and a complex life cycle that includes asexual (meronts) and sexual (microgametocytes and macrogametocytes) reproductive stages.[3] *Giardia* is a flagellate, does not invade epithelial cells, and reproduces only asexually by binary fission.

The classification of eukaryotic parasites is in frequent revision because of modern systematics that incorporate bioinformatic data and cladistics classification into the traditional morphometric-based taxonomy. As of 2012, the formerly known coccidians are classified in the subgroup Apicomplexa, with *Cyclospora* and *Cystoisospora* being classified as Eimeriorinas (the sporozoites, which are the infective stages, are always enclosed in

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sporocysts within an oocyst) and *Cryptosporidium* grouped alone as a single clade (oocysts without sporocysts, containing 4 naked sporozoites).[4]

These parasites produce resistant stages (cysts in *Giardia*,[5] oocysts in the coccidia), which are released into the environment. The excretion intensity of these parasites can vary significantly, from very high to low, and can be sporadic.[6] Therefore, the diagnostic success of a single stool sample can be suboptimal.[7] It is currently recommended to test 3 samples,[8] ideally collected every other day, over a period of at least 1 week.[9]

Samples have to be properly preserved to assure success of the assays to be conducted. The most widely used method relies on a 2-vial collection system with sodium acetate-acetic acid-formalin (SAF); 10% buffered formalin; polyvinyl alcohol (PVA) containing fixatives such as mercury, zinc, or copper (Zn PVA, Cu PVA); or Schaudinn fluid. However, there is a trend to minimize the use of formalin (because of toxicity) and mercury (environmental impact)[10]; however, those alternatives may not always have high parasite recovery rates and not are always compatible with immunoassays.[7]

See Appendix 1 for laboratory procedures for the microscopic detection of coccidian parasites (*Cryptosporidium* spp, *C cayetanensis*, and *C belli*).

CRYPTOSPORIDIUM SPP

The genus *Cryptosporidium* was first described in 1910 by Tyzzer,[11] who in 1912 also described *Cryptosporidium parvum* in the small intestine of mice.[12] For several decades, human cryptosporidiosis was considered a benign self-resolving infection that was caused by *C parvum*.[13] This parasite was considered to have the potential to infect a broad range of mammalian species. With the advent of the human immunodeficiency virus (HIV)/AIDS epidemic, cryptosporidiosis became an important infection, where immunocompromised patients developed nonretractable life-threatening diarrhea.[14,15] Cryptosporidiosis was classified as opportunistic infection and also as an AIDS-defining illness.[16] Given its public health importance, numerous studies were conducted to better understand its pathogenesis, transmission routes, therapeutic approaches, and disease-prevention strategies. The renewed interest led to important discoveries that highlighted the importance of the immune system, more specifically CD4 cells, in the clearance of infections.[17,18] It was also confirmed that its primary route of transmission was waterborne and that people with immune deficiencies other than HIV were also at risk of severe disease.[19,20]

Data from studies using DNA-based methods started to provide evidence that not all isolates of *Cryptosporidium* previously identified as *C parvum* had the same DNA signatures.[21] Further studies showed that the parasites previously described as *C parvum* may actually encompass several species that were morphologically identical; however, DNA data showed distinct genetic signatures and epidemiologic and biological data showed that several isolates had defined host specificities.[22]

Differences in DNA patterns between human and animal isolates were reported using whole DNA extracts[21] and further substantiated using polymerase chain reaction (PCR)-amplified regions of the 18s small subunit ribosomal RNA gene.[22–24] The systematic use of DNA-

based methods, in conjunction with biological and epidemiologic studies, have led to a major revision of the genus *Cryptosporidium*.[25–28]

At present, there are 26 different species of *Cryptosporidium*.[29] The species most frequently detected in humans are *Cryptosporidium hominis*, an anthroponotic parasite (infecting only humans), and *C parvum*, a zoonotic species. It should be noted that *C parvum* is most frequently detected in weaned calves but not in heifers or older cattle.[30] These 2 species of *Cryptosporidium* have different epidemiologic distributions. Both parasites are frequent in European countries, whereas *C parvum* has been the species more frequently reported in the Middle East. In the United States, other industrialized nations, and other developing countries, *C hominis* is the parasite most frequently detected in people. Species less frequently reported are *Cryptosporidium ubiquitum* (previously described as cervine genotype) mainly in industrialized nations, *Cryptosporidium canis* and *Cryptosporidium felis* in nonindustrialized countries, and *Cryptosporidium cuniculus*, primarily in the United Kingdom. Other species with limited number of cases reported are *Cryptosporidium meleagridis*, *Cryptosporidium viatorum*, *Cryptosporidium suis*, *Cryptosporidium muris*, *Cryptosporidium fayeri*, *Cryptosporidium andersoni*, *Cryptosporidium bovis*, *Cryptosporidium tyzzeri*, *Cryptosporidium erinacei*, *Cryptosporidium scrofarum*, and *Cryptosporidium xiaoi*.[31]

Life Cycle and Biology

The latest classification of eukaryotic organisms places *Cryptosporidium* in its own clade, which is outside the Coccidia proper.[4] *Cryptosporidium* spp are morphologically characterized by the presence of an attachment feeder organelle, location in the host's cells (intracellular, but extracytoplasmic), presence of 2 functional types of oocysts (thin and thick walled), presence of a gamontlike extracellular stage, and lack of sporocysts, microphyles, and polar granules.[32–35]

Only 1 host is required for *Cryptosporidium* to complete its life cycle. Fully sporulated, thick-walled oocysts are shed in the feces. Unlike other intestinal coccidia, oocysts of *Cryptosporidium* spp are immediately infective when shed from the host. Infection occurs after the ingestion of oocysts in fecal-contaminated food, water, and fomites. After ingestion by a suitable host, oocysts excyst in the small intestine, releasing sporozoites. The sporozoites parasitize the epithelial cells of the intestinal tract and produce a parasitophorous vacuole located between the host cell's cytoplasm and cell membrane. This unique intracellular but extracytoplasmatic location allows the parasite to derive nutrients via a feeder organelle.

Within the parasitophorous vacuole, sporozoites undergo asexual cycles of merogony. Mature meronts rupture from the infected host cell and take 1 of 2 pathways. Type I meronts give rise to merozoites that perpetuate the asexual cycle in the surrounding host cells. Type II meronts give rise to merozoites that initiate the sexual cycle by producing microgametes (males) or macrogametes (females). Fertilization of macrogametes by microgametes results in the formation of zygotes. Zygotes differentiate into 4 sporozoites and develop a cyst wall, becoming oocysts. Thin-walled oocysts rupture in the lumen of the intestine and perpetuate

autoinfection, whereas thick-walled oocysts are shed in stool where they are immediately infectious to a susceptible host.[36,37]

Epidemiology

Cryptosporidiosis is ubiquitous and is reported worldwide. It is primarily transmitted through the waterborne route[38]; however, food-borne[39–41] or direct-contact transmission can also occur.[42–44] Overall, the frequency of cryptosporidiosis has shown distinct patterns between areas with high endemicity, mainly associated with other enteropathogens, and areas with low endemicity, mainly from industrialized countries, where infections are usually in low levels. There are some patterns for seasonality. For example, in European countries and New Zealand, *C hominis* was more frequently detected in fall, whereas *C parvum* was more frequently reported in spring.[45]

In areas of high endemicity, first cryptosporidial infections usually occur in young children, mainly by age 2 years. Most first infections are symptomatic, primarily associated with diarrhea. Detectable infections decrease with age, and cases of cryptosporidiosis are highly infrequent in children. Meanwhile, the adults living in the same settings usually do not have detectable cryptosporidiosis. Some seasonal trends have been reported.[1]

In industrialized nations, infections have been reported in people of all ages. Cryptosporidiosis in the United States is a reportable disease. The number of cases is consistently greater during the summer months, when outbreaks associated with the use of recreational waters are reported, and more frequently among children aged 1 to 9 years, followed by young adults (aged about 25–39 years).[46–48] Foodborne outbreaks have been associated with the consumption of raw or undercooked foods[49] or unpasteurized drinks.[50]

Clinical Manifestations

Immunocompetent people and children usually present short-term, self-limited watery diarrhea that may be accompanied with nausea and vomiting, which can lead to dehydration.[1,51] People with immune deficiencies, especially with low CD4+ counts ($<140/mm^3$)[17] may present chronic and debilitating disease, which can lead to life-threatening syndromes.[52]

Treatment

Multiple drugs or immunotherapeutic compounds have been developed or tested for the treatment of human cryptosporidiosis. Very few of these compounds have shown therapeutic efficacy. Rehydration through the oral route is the most widely used intervention. Thus far, only nitazoxanide has been approved by the US Food and Drug Administration (FDA) for the treatment of *Cryptosporidium* infections in people.[53] This product has shown to shorten the duration of diarrhea and parasite excretion, although it is not highly efficacious in the treatment of cryptosporidiosis in people with immune deficiencies.[54] In people with HIV infections and low CD4 counts, the preferred treatment and preventative measure would be the administration of highly active antiretroviral therapies (HAART). Relapses are frequent when patients discontinue HAART.

Diagnosis

Microscopy—*Cryptosporidium* spp are not readily detected by routine O&P testing such as formalin-ethyl acetate (FEA) concentration or trichrome staining.[9] Visualization of oocysts require special staining, such as Kinyoun MAF, Zieh-Neelsen acid-fast, or safranin (Fig. 1). *Cryptosporidium* may also be detected by the aurimine O stain used for mycobacteria. Direct fluorescent antibody (DFA) microscopy kits are also helpful for detection and specimen screening. Unlike several other coccidians, *Cryptosporidium* spp do not autofluoresce under ultraviolet (UV) light.[7,9,55]

Antigen detection and rapid/point-of-care diagnosis—These assays are based on the detection of parasite antigens that can be detected in stool specimens. They have the advantage of simplicity and not requiring specialized trained personnel, and several products are already approved for clinical diagnosis (Table 1).[56] Disadvantages are that the species identification or quantification of parasite loads is not possible.

Molecular diagnosis—DNA-based methods are primarily used for species identification and molecular typing. PCR and PCR-related methods have been developed for the detection and identification of species within *Cryptosporidium* spp. Several protocols for PCR detection of *Cryptosporidium* DNA have been made available; however, none of these methods has received approval from FDA or for use of the European Conformity (CE) logo. These protocols allow the identification of *Cryptosporidium* that are of public health importance. Methods using PCR amplification followed by restriction fragment length polymorphism have been developed for the detection and differentiation of *Cryptosporidium* at the species level. Most of these techniques are based on the amplification of the small-subunit (SSU) rRNA gene.[57] There are other PCR-based protocols that have been designed for the detection and differentiation of *C parvum* and *C hominis*, the 2 most frequent species affecting humans. However, these protocols cannot detect or differentiate other *Cryptosporidium* spp or genotypes.[45]

Subtype analyses, which are based on PCR amplification followed by DNA sequence analysis, is a powerful tool for outbreak investigations. Several subtyping tools have also been developed to characterize the diversity within *C parvum* or *C hominis*.[45] This method is based on the sequence polymorphism of the GP-60 locus (also known as gp15/45/60, gp40/15), which has shown to be a robust tool in outbreak investigations.[44]

CYCLOSPORA CAYETANENSIS

This coccidian parasite was described in 1993.[2] It is recognized as an important cause of food-borne outbreaks, both in the United States as well as in other industrialized nations. The name *C cayetanensis* was first proposed in 1992; however, previous studies reported organisms that later have been considered to be similar to *Cyclospora*.[58] However, earlier reports hypothesized that those new organisms could belong to a new species of *Isospora.*[58]

In 1993, the description of oocysts, which after sporulation had 2 clearly defined sporocysts, each with 2 sporozoites, led to the organism's classification as *Cyclospora* and to the species name *C cayetanensis*. The genus *Cyclospora* is currently placed among the Eimeriorina.[4,59]

Molecular data suggest that *Cyclospora* may actually belong nestled within the genus *Eimeria*.[60,61] At present, *C cayetanensis* is the only species known to infect humans. Three other species are known to infect nonhuman primates, all parasites of monkeys in Africa.[62]

Biology

Cyclospora cayetanensis (*Cyclospora*) is an anthroponotic parasite. Attempts to infect other animal species have proved unsuccessful[63]; thus, there is no animal model to better understand its biology. The live stages of *Cyclospora* were described from jejunal biopsies.[64] Infections start when a susceptible person ingests sporulated oocysts, a stage that is environmentally resistant. Oocysts are broken in the upper gastrointestinal tract because of partial digestion with gastric juices and digestive enzymes, leading to the release of the 2 internal cysts, called sporocysts, each with 2 infectious stages called sporozoites. On their release in the small intestine, the sporozoites infect epithelial cells where they transform into merozoites and replicate asexually, also infecting other enterocytes. After asexual replications, the merozoites differentiate into sexual stages called microgametocytes and macrogametocytes. New oocysts are formed as result of sexual reproduction between the microgametocytes and macrogametocytes. Unsporulated (and therefore noninfectious) oocysts are eventually released into the environment. Sporulation occurs in the environment. The precise factors that cause sporulation are not known, but it is estimated that it occurs in about 2 weeks.[2,65]

Epidemiology

Cyclospora infections have been reported in several areas of the world. It is endemic mainly in nonindustrialized nations, whereas sporadic reports associated with outbreaks have been frequently reported in industrialized countries.[66] In endemic settings, cyclosporiasis is more frequent in children between the ages of 2 and 5 years, showing a marked seasonal pattern.[67] For example, in Nepal, there were higher rates of infection during the summer and rainy seasons,[68] whereas in Peru, most cases were detected between December and May, which are warmer months but without rain.[67]

In industrialized nations, cyclosporiasis has been more frequently reported in the summer months and most outbreaks have been traced back to imported fresh products. In the 1990s, most reported outbreaks in the United States were linked to imported berries. This trend has changed, and during the past 14 years, most outbreaks have been linked to leafy greens consumed raw, such as basil and herbs.

Clinical Manifestations and Treatment

Gastrointestinal manifestations associated with cyclosporiasis include diarrhea, fatigue, and abdominal cramps, which are most likely reported between 1 and 2 weeks after infection. The duration of the patent period and the severity of symptoms are different between people living in endemic and nonendemic areas. Infections in endemic settings occur mainly in children older than 2 years and are almost never detected after 10 years of age. In these cases, infections may resolve spontaneously, and as high as 50% of infected people may not show any clinical symptoms. It seems that immunity plays a significant role.

In industrialized nations, where the parasite is not endemic, most people are likely to be naive and infections are almost always symptomatic, lasting 1 to 2 weeks or more. The main symptom is diarrhea, which persists if untreated.[69] Other symptoms are general malaise, lack of energy, loss of appetite, mild fever, nausea, flatulence, and abdominal cramps.[67,70]

In the case of people with deficits in their immune system, there are anecdotal reports of infections with a longer duration, although cyclosporiasis is not considered an opportunistic infection in HIV-infected people.[71] Treatment is highly effective and is based on the administration of sulfamethoxazole trimethoprim (TMP/SMX) orally, twice daily for 7 to 10 days.[72] However, no specific treatment is recommended for people who are allergic to sulfa drugs.[55]

Diagnostic Testing

Microscopy—As with other coccidia, *C cayetanensis* is not easily detected with traditional O&P procedures such as FEA concentration and trichrome stain.[55] Oocysts can be better visualized in FEA concentrates if viewed under differential interference contrast or phase contrast microscopy.[2,65] Oocysts can be more easily detected with permanent staining methods such as safranin or MAF staining. Oocysts stain red with both stains; however, characteristic nonuniform staining is observed with MAF and unstained oocysts are usually white and are referred to as ghost forms (see Fig. 1). In the case of safranin, oocysts stain more uniformly.[55]

Another characteristic of *C cayetanensis* is the autofluorescence of the oocyst wall under UV light, using excitation filters of 330 to 365 nm and less intense autofluorescense with filters of 450 to 490 nm.[2,9,65] Autofluorescence plus morphometric characteristics are helpful when detecting *Cyclospora* (Fig. 2). There are currently no DFA or molecular procedures that are approved by the FDA for routine clinical diagnosis of cyclosporiasis.

CYSTOISOSPORA BELLI (PREVIOUSLY *ISOSPORA BELLI*)

Biology and Taxonomy

Cystoisospora belli is placed among the Eimeriorina along with *Cyclospora.*[4] For several decades, *C belli* was placed in the genus *Isospora* until morphologic and molecular data were used to support its proper classification in the genus *Cystoisospora*.[73,74] This genus now includes all previously classified *Isospora* spp that infect mammals, whereas *Isospora* contains only parasites that infect passeriform birds.[74]

Life Cycle and Biology

Cystoisospora belli is known to infect only humans and requires only the one host for completion of its life cycle, although paratenic hosts may be involved.[75] Typically, partially sporulated oocysts containing 1 (rarely 2) sporoblast are shed in feces. In the environment, the sporoblast divides into 2 sporoblasts, each of which secretes a cell wall to become sporocysts. After sporulation, each sporocyst contains 4 sporozoites. Humans become infected after ingestion of fully sporulated oocysts, through contaminated food, water, and fomites. On ingestion, the sporocysts excyst in the small intestine and release the

sporozoites, which invade the host epithelial cells. The sporozoites undergo asexual replication called schizogony. Mature schizonts rupture, releasing merozoites that parasitize surrounding epithelial cells, perpetuating asexual multiplication. Eventually, multinucleate meronts are formed and sexual stages develop. Macrogametocytes are fertilized by microgametocytes resulting in formation of the oocyst, which can persist in the environment for several weeks or months.[55,75,76]

Epidemiology

Cystoisospora belli has a worldwide distribution; however, several studies from AIDS patients with diarrhea have shown a higher prevalence of cystoisosporiasis among people from tropical or subtropical areas.[77–79]

Clinical Manifestations and Treatment

In immunocompetent people, *C belli* has been associated with diarrhea, usually lasting 6 to 10 days, and infections self-resolving in 2 to 3 weeks, although intermittent shedding may continue for an additional 2 to −3 weeks. Severe symptoms were reported among people with immunocompromised systems, which can lead to life-threatening chronic profuse diarrhea.[52,80] Treatment is based on TMP/SMX.[81]

Microscopic Diagnosis

Because of their large size, oocysts of *C belli* are usually detected during route O&P examinations. However, because oocysts tend to be shed in small numbers, repeated stool examinations and concentration procedures are recommended. Coccidian-specific stains, such as MAF and safranin, are preferred if permanent stains are used for diagnosis (see Fig. 1). As with *C cayetanensis*, screening of wet mounts can be enhanced by using UV microscopy, with both oocyst and sporoblast/sporocyst walls capable of autofluorescence.[7,9,75] There are currently no DFA or molecular procedures approved for routine clinical diagnosis of cystoisosporiasis.

GIARDIA DUODENALIS

Biology

The protozoan flagellate parasite *G duodenalis* is a common cause of human diarrheal disease worldwide.[82] It is transmitted through the fecal-oral route, frequently through ingestion of contaminated water and food.[6,76,82] This parasite has a direct life cycle, and the cysts passed in the feces are immediately infectious. These cysts can remain infectious for long periods in moist and cool environments.

Epidemiology

Giardia has a worldwide distribution.[83] It affects people of all ages and has an important impact on public health. In the United States, it is more frequently reported in children aged 1 to 9 years.[84] This parasite has been associated with major outbreaks and can also be present in domestic animals, such as household pets and farm animals. Giardiasis is highly underreported, with data from the United States showing that the number of annually

reported cases remained steady for several years at around 20,000, whereas the estimated number of cases was 2 million.[85]

Through molecular genotyping methods, *G duodenalis* has been classified in distinct assemblages or genotypes. Assemblages A and B are the most frequently reported in humans, either in industrialized or nonindustrialized nations. These assemblages have also been reported in cattle, dogs, and cats from different countries around the world. However, other assemblages of *Giardia* have been reported almost exclusively in animal species: assemblages C and D in domestic and wild canids, assemblage F in cats, and assemblage E in ruminants. Therefore, *G duodenalis* is considered a multispecies complex, where assemblages A and B are considered to have broad host specificity and zoonotic potential.[86–89]

Clinical Manifestations

Approximately 40% of *Giardia* infections may be symptomatic, depending on the population. Symptoms may include diarrhea, cramps, bloating, nausea, and vomiting[6] and may be prolonged. Infections are normally self-limiting, but chronic diarrhea may occur in children[90] and a low proportion of immunocompromised people.[91] There is a report that prolonged giardiasis from early childhood has been associated with poor cognitive function later in life.[92]

Treatment of infections is recommended only when clinical manifestations affect the well-being of the infected person. Drug of choice is metronidazole, although search for alternative therapies is ongoing.[93]

Diagnostic Testing

Microscopy—Because cysts are shed sporadically during an infection, detection of *Giardia* may require several stool samples to be examined.[7,9] *Giardia* may be detected by microscopy, immunologic, or molecular methods. *Giardia* cysts and trophozoites can be readily detected via traditional methods such as trichrome staining and FEA concentrations (Fig. 3), although microscopy of whole organisms requires trained technicians as well as time to prepare and examine smears. The formalin-ether (Ritchie) concentration method is another common method used for the concentration of stool samples.[55]

Antigen detection and rapid/point-of-care diagnosis—These assays are standardized and can generate results in a short time. Tests more widely used are antigen detection immunoassays such as DFA tests that detect whole organisms and enzyme immunoassays that detect antigens in stool, which can be completed in 1 to 2 hours. They have been reported to be highly sensitive and specific (see Table 1B).[94,95]

PCR assays for *Giardia* have become more common; however, PCR amplification and sequence analysis are more frequently used for genotype/assemblage classification and are not routinely used for diagnosis. The SSU rRNA fragment, commonly amplified by PCR for other enteric parasites, is rich in GC content and thus requires special PCR conditions. Therefore, PCR-based methods have been designed to amplify other informative loci, such

as the triose phosphate isomerase, glutamate dehydrogenase, and β-giardin, which are used for the taxonomic or epidemiologic classification into assemblages.[88,89]

SUMMARY ON DETECTION OF PARASITES

Microscopy continues to be the primary method for detection of the parasites covered in this review (Table 2), highlighting the importance of specimen processing and staining (see Table 2) and morphometric characteristics (Table 3). Rapid diagnostic assays (lateral flow/immunochromatographic cards) are also available for cryptosporidiosis or giardiasis, including approved devices for their simultaneous detection (see Table 1C). At present, there are molecular-based assays for the simultaneous detection of multiple enteric pathogens, which include virus, bacteria, and parasites. Two of these assays have been cleared by FDA and have been reported to have high sensitivity and specificity, including the detection of coinfections (see Table 1D).[96,97]

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APPENDIX 1: LABORATORY PROCEDURES FOR THE MICROSCOPIC DETECTION OF COCCIDIAN PARASITES: CRYPTOSPORIDIUM SPP, C CAYETANENSIS, AND C BELLI

Modified Kinyoun Acid-Fast Stain

Specimen requirements: unfixed stool or stool preserved in 10% formalin (including FEA concentrates), SAF, or EcoFix.
Procedure

1. Prepare a thin film of stool on a glass microscope slide. Allow to completely air dry (do not use heat blocks to speed up drying).
2. Fix in absolute methanol for 30 seconds.
3. Stain with carbol-fuschin for 1 minute.
4. Rinse and drain with distilled water.

5 Decolorize in 10% sulfuric acid (10 mL sulfuric acid in 90 mL absolute ethanol) for 2 minutes.

6 Rinse quickly and drain with distilled water.

7 Counterstain with 3% malachite green (3 g malachite green in 100 mL distilled water) or methylene blue for 2 minutes.

8 Allow to dry (air dry or on a slide warmer at 60°C).

9 Mount with no. 1 thickness coverslip using Permount (or other sealing reagent).

Procedural notes

- Do not use a slide warmer for the first step (drying of specimen); it cooks the oocysts of *Cryptosporidium*, and they do not retain stain.
- Drain slides between each reagent.
- All reagents should be changed monthly or earlier as needed.

Hot Safranin Stain

Specimen requirements: unfixed stool or stool preserved in 10% formalin (including FEA concentrates), SAF, or EcoFix.
Procedure

1 Prepare a thin film of stool on a glass microscope slide. Allow to completely air dry (do not use heat blocks to speed up drying).

2 Fix in acid alcohol (3 mL hydrochloric acid in 97 mL absolute methanol) for 5 minutes.

3 Boil in safranin (1% aqueous solution in distilled water) for 1 minute.

4 Counterstain with 3% malachite green (3 g malachite green in 100 mL distilled water) for 1 minute.

5 Allow to dry (air dry or on a slide warmer at 60°C).

6 Mount with no. 1 thickness coverslip using Permount (or other sealing reagent).

Procedural notes

- Do not use a slide warmer for the first step (drying of specimen); it cooks the oocysts of *Cryptosporidium*, and they do not retain stain.
- Drain slides between each reagent.
- All reagents should be changed monthly or earlier as needed.
- Safranin stain should be boiling when slides are added.

KEY POINTS

- Sample collection and preservation are important steps, and examination of 3 specimens from different days increases the accuracy of diagnosis.
- *Giardia* can be detected by light microscopy during ova-and-parasite (O&P) examination, antigen detection methods (laboratory and rapid diagnostic devices), or fluorescent microscopy.
- *Cryptosporidium* and *Cyclospora* are not easily detectable by O&P examination, and parasite-specific tests must be requested, such as modified acid-fast (MAF) microscopy. For *Cryptosporidium*, there are antigen detection assays (laboratory or rapid diagnostic devices) or antibody-based fluorescent microscopy. Properly equipped fluorescent microscopes can be used in research laboratories for confirmation of *Cyclospora*, as this parasite autofluoresces with the appropriate excitation wavelength.
- *Cystoisospora* can be detected by light microscopy, and confirmation is accomplished through morphometric characteristics of samples that have been stained with safranin or acid-fast stain and also by autofluorescence.

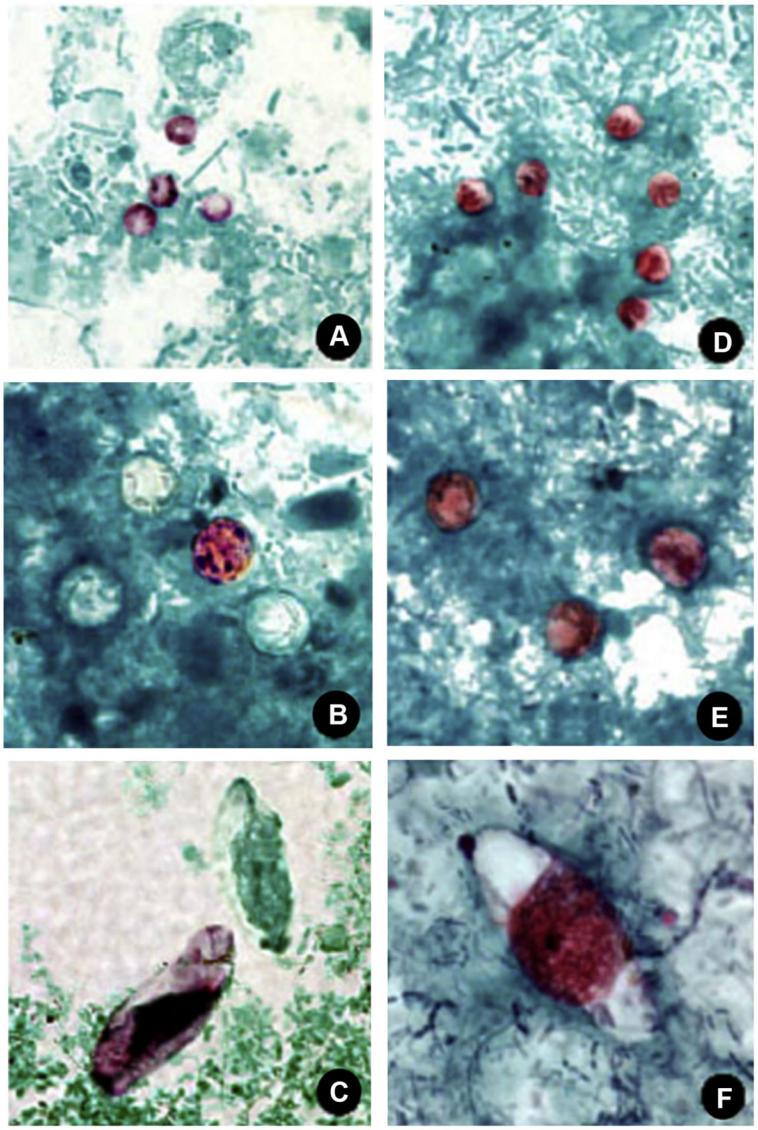

Fig. 1.

Comparison of (*A, D*) *Cryptosporidium* spp, (*B, E*) *Cyclospora cayetanensis*, and (*C, F*) *Cystoisospora belli* stained with modified acid-fast and safranin stains, original magnification × 1000. (Public domain images, *courtesy of* DPDx, Centers for Disease Control and Prevention, Atlanta, USA).

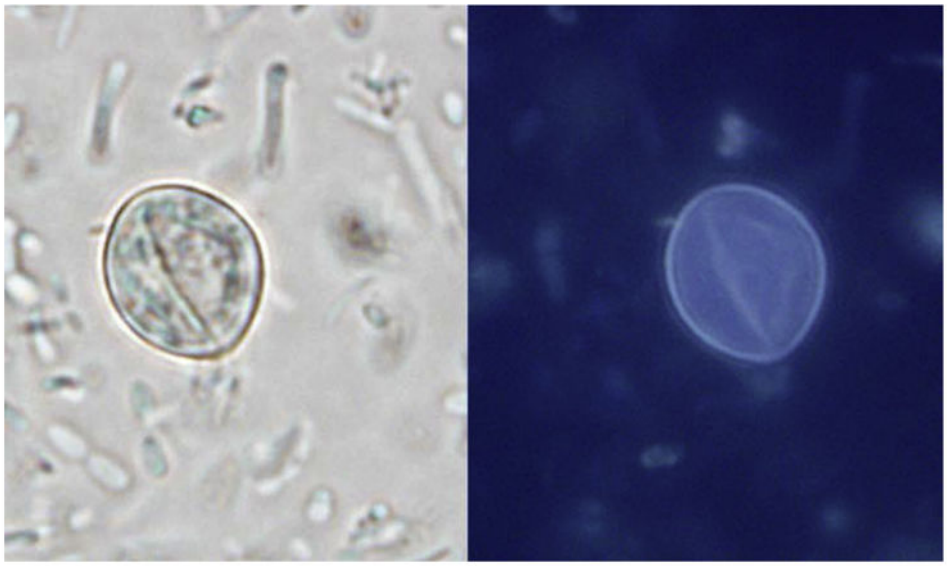

Fig. 2.

Cyclospora cayetanensis oocyst viewed under normal light (*left*) and ultraviolet light (*right*) in an unstained wet mount. (Original magnification × 1000). (*Courtesy of* DPDx, Centers for Disease Control and Prevention, Atlanta, USA).

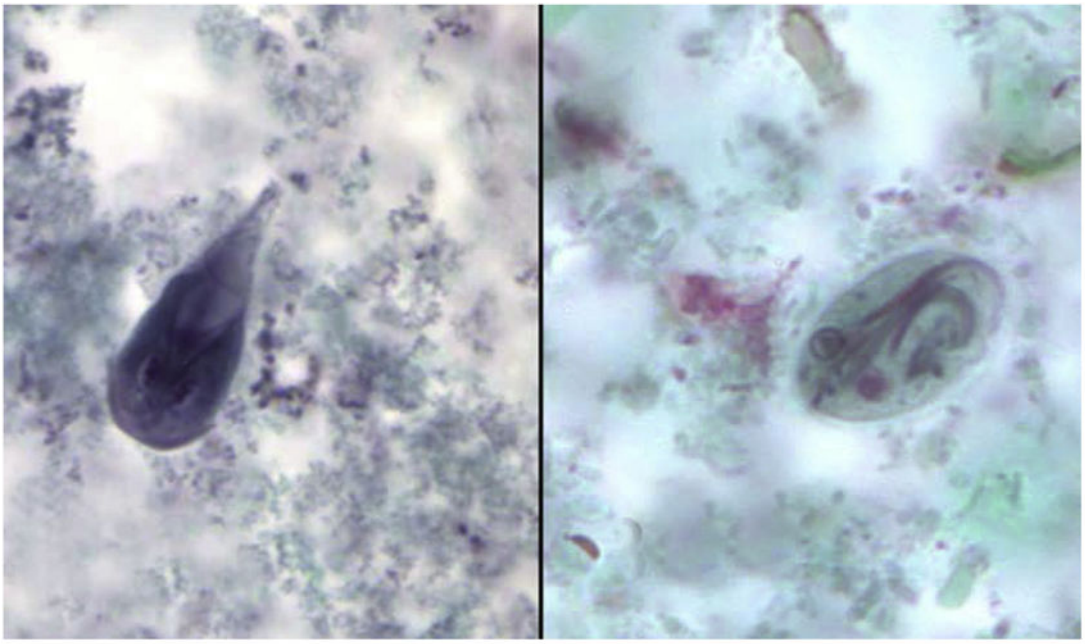

Fig. 3.
Trophozoite (*left*) and cyst (*right*) of *Giardia duodenalis* (trichrome, original magnification × 1000). (*Courtesy of* DPDx, Centers for Disease Control and Prevention, Atlanta, USA.)

Table 1

Commercial diagnostic assays for the detection of protozoa, either approved by the FDA or registered for selling in the European Union, having the CE seal of European Conformity or *Conformité Européenne*

Product Name	Manufacturer, Country	Sensitivity (%)	Specificity (%)	Approval
1.A. *Cryptosporidium*				
A. Immunofluorescence assays				
Crypto-Cel IF Test	Celllabs, AUS	100	100	FDA/CE
B. Enzyme immunoassays				
Cryptosporidium II TEST(direct Ag/spectrophotometric/visual)	Alere/Techlab, Inc USA	97	100	FDA
Prospect *Cryptosporidium* Rapid Assay	Thermo Fisher Scientific/REMEL, USA	100	No data	FDA
RIDASCREEN *Cryptosporidium*[a]	R-Biopharm, Germany	100	97.3	CE
PARA-TEC C*ryptosporidium*	Medical Chemical Corporation, USA	100	100	CE
C. Immunochromatographic/point-of-care assays				
UNI-GOLD *Cryptosporidium*	Trinity Biotech, Ireland/USA	100	100	FDA/CE
XPECT *Cryptosporidium* LATERAL FLOW ASSAY, MODEL 2451020	Thermo Scientific REMEL, USA	96.4	98.3	FDA
Cryptosporidium Fecal ELISA Test	Cortez Diagnostics, USA	100	100	FDA[a]/CE
Crypto-Strip C-1005 (CRYPTO UNI-STRIP, CRYPTO-CIT)[a]	Coris BioConcept, Belgium	95.7	100	CE
RIDA Quick *Cryptosporidium* (dipstick or cassette)[a]	R-Biopharm, Germany	93.8	100	CE
CRYPTO (card and blister formats)[a]	CerTest Biotec, Spain	99	99	CE
Stick Crypto[a]	Operon, Spain	79.3	99.5	CE
D. PCR-based assays				
KHCRYP (real-time PCR)	CEERAM S.A.S., France	No data	No data	CE
1.B. *Giardia duodenalis*				
A. Immunofluorescent assays				
Giardia-Cel IF Test	Cellabs, Australia	90.3	100	FDA/CE
B. Enzyme immunoassays (antigen detection)				
Giardia lamblia ANTIGEN DETECTION MICROWELL ELISA	Ivd Research, Inc USA	100	100	FDA
TechLab *Giardia* II TEST	Techlab Inc/Alere USA	100	100	FDA

Product Name	Manufacturer, Country	Sensitivity (%)	Specificity (%)	Approval
Giardia ELISA kit	Cortez Diagnostics, USA	100	100	FDA[a]/CE
ProSpecT *Giardia* Microplate Assay (direct Ag/spectrophotometric/visual)	Thermo Fisher Scientific/REMEL, USA	97	No data	FDA
ProSpecT Giardia EZ Microplate Assay	Thermo Fisher Scientific/REMEL, USA	97	No data	FDA
RIDASCREEN *Giardia*[a]	R-Biopharm, Germany	100	99.60	CE
PARA-TEC *Giardia lamblia*	Medical Chemical Corporation, USA	85	97.9	CE
C. Immunochromatographic/point-of-care diagnostics				
Uni-Gold *Giardia*	Trinity Biotech, Ireland	100	100	CE
Stick *Giardia*[a]	Operon, Spain	93.8	98.9	CE
Giardia lamblia (*Giardia*)[a]	CerTest Biotec, Spain	97	99	CE
Giardia (dipstick and cassete)	Coris BioConcept, Belgium	96.3	97.8	CE
D. Molecular assays				
Giardia Test Kit KHGIAR	CEERAM S.A.S., France	No data	No data	CE
1.C. Dual detection*: Cryptosporidium* spp (C) and *Giardia duodenalis*(G)				
A. Immunofluorescent microscopy assays				
Crypto/Giardia-Cel IF Test	Cellabs, Australia	100	100	FDA/CE
MERIFLUOR *Cryptosporidium/Giardia*	Meridian Biosciences, USA	100	100	FDA
PARA-TECT *Cryptosporidium/Giardia* DFA 75Test Kit	Medical Chemical Corporation, USA	100	100	CE
IVD Crypto/Giardia DFA	IVD Research, Inc, USA	100	100	FDA
B. Enzyme immunoassays (antigen detection)				
Giardia/Cryptosporidium QUIK CHEK test	Techlab Inc/Alere, USA	C-100, G-98.9	C-99.8, G-100	FDA
Giardia/Cryptosporidium CHEK	Techlab Inc/Alere, USA	97.6	100	FDA
Crypto/Giardia Ag Combo ELISA kit	Cortez Diagnostics, USA	99	100	CE/FDA[a]
ProSpecT *Giardia/Crypto* (spectrophotometric)	Thermo Fisher Scientific, USA	100	95	FDA
C. Immunochromatographic/point-of-care assays				
XPECT *Giardia/Cryptosporidium*	Thermo Scientific REMEL, USA	C-96.4, G-95.8	C-98.5, G-98.5	FDA
Biosite Triage Parasite Panel	Alere/Biosite Incorporated, USA	C-91.4, G-95.1	C-98.2, G-88.4	FDA
ColorPAC *Giardia*/*Cryptosporidium* Rapid Assay	Becton Dickinson, USA	C-97.3, G-100	C-100, G-100	FDA
Crypto/Giardia Duo-Strip[a]	Coris BioConcept, Belgium	C-95.7, G-96.3	C-100, G-97.8	CE

Product Name	Manufacturer, Country	Sensitivity (%)	Specificity (%)	Approval
ImmunoCard STAT! *Cryptosporidium/Giardia* Rapid Assay	Meridian Bioscience, USA	C-97.3, G-100	C-100, G-100	CE
RIDA Quick *Cryptosporidium/Giardia* Combi (dipstick or cassette)[a]	R-Biopharm, Germany	C-93.8, G-100	C-100, G-95.2	CE
RIDAQuick[b] *Cryptosporidium/Giardia/Entamoeba* Combi (dipstick or cassette)[a]	R-Biopharm, Germany	C-83, G-91.9	C-93.3, G-99.5	CE
CRYPTO-GIARDIA (card and blister)[a]	CerTest Biotec, Spain	C-99, G-97	C-99, G-99	CE
CRYPTO-GIARDIA-ENTAMOEBA[a,b]	CerTest Biotec, Spain	C-99, G-97	C-99, G-99	CE
Stick Crypto-Giardia[a]	Operon, Spain	C-79.3, G-93.8	C-99.5, G-98.9	CE
1.D. Molecular assays for detection of multiple enteric pathogens				
Film Array Instrument[c]	Biofire Diagnostics, USA			FDA/CE
xTAG Gastrointestinal Panel (GPP)[d]	Luminex, USA	C-100, G-100 *Cyclospora*-100	C-99.6, G-99.5 *Cyclospora*-100	FDA/CE

[a]Not available in the United States.

[b]Also for *Entamoeba* spp.

[c]Multiplex assay including bacterial and viral pathogens.

[d]Multiplex assay including the protozoa *Cyclospora cayetanensis*, plus bacterial and viral pathogens.

Data from Catalog of cleared and approved medical device information from FDA. Database of approved in-vitro diagnostic devices. 2014. Accessed October 20, 2014.

Table 2

Microscopy procedures for detection of *Cryptosporidium* spp, *Cyclospora cayetanensis*, and *Cystoisospora belli*

Procedure	Parasite	Advantages	Disadvantages
Wet mount (FEA concentrates)	*Cryptosporidium* spp	Concentration allows for better yield FEA concentrate can be used for DFA (see later in the table)	Oocysts may be confused for yeast and other nonparasitic elements Formalin waste
	Cyclospora cayetanensis	Concentration allows for better yield May be enhanced with differential interference contrast (DIC) or phase microscopy	Oocysts may be confused with nonparasitic elements Formalin waste
	Cystoisospora belli	Concentration allows for better yield May be enhanced with DIC or phase microscopy	Oocysts shed sporadically so multiple collections should be tested Formalin waste
Trichrome stain	*Cryptosporidium* spp	Not recommended	Oocysts do not stain well with trichrome; likely to be confused for yeast/fungal elements Specimens in PVA usually not concentrated
	Cyclospora cayetanensis	Not recommended	Oocysts do not stain well with trichrome Specimens in PVA usually not concentrated
	Cystoisospora belli	Not recommended	Not readily detected by trichrome Specimens in PVA usually not concentrated
Modified Kinyoun acid-fast (MAF) stain	*Cryptosporidium* spp	Uniform staining of oocysts; sporozoites often visible	Oocysts may be confused with yeast and other fungal elements that often stain red to purple with MAF
	Cyclospora cayetanensis	Oocysts can stain pink to red	Variability in staining; often many oocysts do not stain (ghost forms) and may be overlooked by inexperienced microscopists
	Cystoisospora belli	Sporoblasts stain red with MAF	Often there is shrinkage of oocyst wall, distorting form of oocysts
Hot safranin stain	*Cryptosporidium* spp	Uniform staining of oocysts	Requires heat; messy procedure
	Cyclospora cayetanensis	More uniform staining of oocysts	Requires heat; messy procedure
	Cystoisospora belli	Uniform staining of sporoblasts Oocyst wall less likely to collapse than with MAF	Requires heat; messy procedure
Ultraviolet microscopy	*Cryptosporidium* spp	Not available	Oocysts do not autofluoresce
	Cyclospora cayetanensis	Oocyst walls autofluoresce Allows for more rapid screening	Requires special filter with wavelength of 450–490 nm (not routine in most laboratories)
	Cystoisospora belli	Oocyst and sporoblast walls autofluoresce Allows for more rapid screening	Requires special filter with wavelength of 450–490 nm (not routine in most laboratories)
DFA	*Cryptosporidium* spp	Allows for rapid screening of *Cryptosporidium* spp Some allow for simultaneous screening of other organisms (eg, *Giardia*)	Requires microscopy with fluorescence capabilities Could be expensive if not used routinely
	Cyclospora cayetanensis	None available	None available
	Cystoisospora belli	None available	None available

Table 3

Comparative morphology of *Giardia duodenalis*, *Cryptosporidium* spp, *Cyclospora cayetanensis*, and *Cystoisospora belli*

Organism	Size	Other Morphologic Features	Preferred Morphologic Diagnostic Test
Giardia duodenalis	Trophozoites, 10–20 μm long Cysts 8.0–10 μm long	Trophozoites: pyriform shape; sucking disk; 2 nuclei; 2 median bodies; 8 flagella (4 lateral, 2 ventral, 2 posterior) Cysts: ovoid shape; 2–4 nuclei; fibrils and median bodies; no flagella	Trophozoites: direct wet mount; trichrome stain Cysts: FEA concentration wet mount; trichrome stain
Cryptosporidium spp	Oocysts: 4.0–6.0 μm	Oocysts: spherical shape; sporulated in feces (4 sporozoites present)	Modified acid-fast stain; Safranin stain
Cyclospora cayetanensis	Oocysts: 8.0–10 μm	Oocysts: spherical; unsporulated in fresh feces; refractile globules present	Modified acid-fast stain; Safranin stain; UV microscopy
Cystoisospora belli	Oocysts: 20–33 μm long	Oocysts: oval to ellipsoidal shape; unsporulated in fresh feces; double-layered hyaline cyst wall; single sporoblast usually present	Modified acid-fast stain; safranin stain; UV microscopy