Detection of Intestinal Protozoa in the Clinical Laboratory

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Despite recent advances in diagnostic technology, microscopic examination of stool specimens remains central to the diagnosis of most pathogenic intestinal protozoa. Microscopy is, however, labor-intensive and requires a skilled technologist. New, highly sensitive diagnostic methods have been developed for protozoa endemic to developed countries, including *Giardia lamblia* (syn. *G. intestinalis/G. duodenalis*) and *Cryptosporidium* spp., using technologies that, if expanded, could effectively complement or even replace microscopic approaches. To date, the scope of such novel technologies is limited and may not include common protozoa such as *Dientamoeba fragilis*, *Entamoeba histolytica*, or *Cyclospora cayetanensis*. This minireview describes canonical approaches for the detection of pathogenic intestinal protozoa, while highlighting recent developments and FDA-approved tools for clinical diagnosis of common intestinal protozoa.

Protozoan infections significantly contribute to the burden of gastrointestinal illness worldwide. While the prevalence of these infections is low in the United States, sporadic outbreaks, including the 2013 outbreak of cyclosporiasis in the United States, underscore the continued burden of disease these organisms present in developed countries. *Giardia*, *Cryptosporidium* spp., *Dientamoeba fragilis*, *Entamoeba* spp. (including nonpathogenic species), *Blastocystis* spp., and *Cyclospora cayetanensis* are the most common pathogenic protozoa reported in developed settings (1). However, accurate determination of the incidence of these infections is hampered by infrequent testing of stool for protozoa when patients present with gastroenteritis (1), by inappropriate test ordering by physicians (1, 2, 3), and by the lack of sensitive techniques by which to identify pathogenic protozoa in stool specimens.

The microscopic ova and parasite examination (O&P) is the traditional method for stool parasite testing. Although the O&P is labor-intensive and requires a high level of skill for optimal interpretation, this test remains the cornerstone of diagnostic testing for the intestinal protozoa. At present, most clinical microbiology laboratories in the United States struggle with the ability to provide quality O&P results within a clinically significant time frame (Table 1). A pressing concern for these laboratories is the shortage of skilled technologists capable of reliably evaluating O&P. As the baby boomer generation retires from the workforce, inexperienced technologists, who in some instances are inadequately trained in parasitology, are left to fill the void. Few laboratories in the United States encounter a sufficient number of specimens that harbor intestinal protozoa to maintain technologist proficiency, let alone to allow for robust training of new technologists. As such, laboratories may be unable to accurately identify pathogenic protozoa, differentiate these from nonpathogenic species, and discriminate artifacts on O&P examinations. Further, in understaffed laboratories, the labor-intensive O&P is performed only once other laboratory tasks are completed, yielding long turnaround times and limiting this test’s clinical utility.

To address competency issues, some laboratories have developed affiliations with organizations that conduct parasitology surveillance in regions of disease endemicity around the world and have unique access to clinical specimens for teaching and training purposes. Examples of such organizations are the Walter Reed Army Institute of Research, the Naval Medical Research Center, the Joint Pathology Center (previously the Armed Forces Institute of Pathology [AFIP]), and the Centers for Disease Control and Prevention (CDC) DPDx laboratories. Laboratories may also consider pooling resources on a local level, both for training purposes and to share specimens for competency. In the authors’ laboratories, positive specimens are reviewed by all trained technologists to maximize staff competency.

Long-term solutions to these challenges include lessening laboratory reliance on the O&P for the diagnosis of intestinal protozoa; indeed, some people have already suggested limiting the use of the O&P in routine clinical practice (4). Antigen detection tests for *Giardia*, *Cryptosporidium* spp., and *Entamoeba histolytica* have been cleared by the U.S. FDA (Table 2) and are associated with significant improvements in the detection of these organisms in stool. Unfortunately, no FDA-cleared antigen test detects *D. fragilis*, which is a pathogenic protozoa frequently detected in many U.S. laboratories (R. M. Humphries and M. R. Couturier, unpublished observations). Regardless, some have suggested the use of algorithmic testing that involves front-line antigen testing for *Giardia* and *Cryptosporidium*. If the results of such testing are negative, traditional microscopic approaches are used (5). Successful implementation of such a system would likely require developing a physician guidance tool to aid in appropriate ordering, as the laboratory very rarely receives the information required to determine if the test is requested in the clinical context of gastrointestinal complaints or as part of the evaluation of a returning traveler, immigrant, or patient prior to transplantation. Furthermore, such algorithmic testing delays diagnosis of pathogens for which the laboratory has not initially tested.

There is a pressing need for newer diagnostic test options to

**Table 1. Turnaround Times**

<table>
<thead>
<tr>
<th>Technique</th>
<th>Response Time</th>
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<tbody>
<tr>
<td>O&amp;P</td>
<td>7–10 days</td>
</tr>
<tr>
<td>Antigen</td>
<td>2–4 hours</td>
</tr>
<tr>
<td>Nucleic Acid</td>
<td>2–4 hours</td>
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TABLE 1 Top five challenges faced by the clinical laboratory in the detection of intestinal protozoa, as identified by the authors

<table>
<thead>
<tr>
<th>Challenges</th>
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<tbody>
<tr>
<td>1. Reliance on labor-intensive, technically demanding tests (e.g., O&amp;P)</td>
</tr>
<tr>
<td>• O&amp;P testing is left until other laboratory testing is completed, yielding long turnaround times, due to the misguided notion this testing is “less critical” than others</td>
</tr>
<tr>
<td>• Many laboratories do not have technologists that can reliably identify pathogens and differentiate these from nonpathogenic species or artifacts</td>
</tr>
<tr>
<td>2. Reliance on insensitive tests</td>
</tr>
<tr>
<td>• O&amp;P is associated with a sensitivity of 20 to 90% compared to molecular assays</td>
</tr>
<tr>
<td>• Some antigen detection tests, e.g., those for Cryptosporidium spp., are insensitive</td>
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<tr>
<td>3. Shortage of clinical specimens positive for intestinal protozoa</td>
</tr>
<tr>
<td>• Limits the opportunities for adequate training</td>
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<tr>
<td>• Limits ability of technologists to maintain proficiency</td>
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<tr>
<td>• Limits validation of new testing platforms and transport medium</td>
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<tr>
<td>4. Shortage of training programs/resources for parasitology</td>
</tr>
<tr>
<td>• Confounded by the retirement of experienced technologists who would otherwise perform training</td>
</tr>
<tr>
<td>5. Suboptimal physician ordering practices</td>
</tr>
<tr>
<td>• Few physicians will order organism-specific tests, even during outbreaks</td>
</tr>
<tr>
<td>• Inadequate access to patient information by laboratory prevents implementation of algorithmic testing</td>
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</table>

replace the O&P. Such tests should broadly detect most, if not all, pathogens commonly identified microscopically. Multiplexed PCR has the potential to meet this need. However, only one such assay has been cleared by the U.S. FDA to date, the highly multiplexed Luminex xTAG GPP, which detects Giardia and Cryptosporidium in addition to numerous bacterial and viral targets. Such molecular assays, depending on their design, may require a laboratory with proficiency in molecular testing, which would limit their use to major academic hospitals and reference laboratories. Alternatively, sample-to-answer solutions, which provide direct diagnosis from unprocessed samples, such as the BioFire Diagnostics FilmArray platform, could be used in virtually any laboratory setting.

Despite the challenges outlined in Table 1, detection of intestinal protozoa is still almost exclusively based on O&P microscopic examination. This article will thus review optimal diagnostic approaches and the microscopic morphology of key pathogenic protozoa. The pathogenesis of some protozoa discussed is controversial, including that of Blastocystis hominis and Dientamoeba fragilis. Other common protozoa, such as Endolimax nana, are not discussed herein, as less is known about their potential virulence. Antigen and molecular-based detection methods are also summarized.

SPECIMEN COLLECTION

Optimal recovery and microscopic identification of protozoa from patients with intestinal infections is dependent on proper collection and preservation of fecal specimens. Well-recognized factors that influence the sensitivity of parasite examinations include patient medications, specimen collection interval, and the preservation of stool prior to testing (6). The diagnostic yield of the O&P is also significantly impacted by the number of stool specimens collected and submitted to the laboratory for testing. Many intestinal protozoa are irregularly shed, and data suggest that a single stool specimen submitted for microscopic examination will detect 58 to 72% of protozoa present (4, 7). Hiatt and colleagues found that evaluating three specimens, as opposed to one, resulted in an increased yield of 22.7% for E. histolytica, 11.3% for Giardia, and 31.1% for D. fragilis (8). As such, many laboratories continue to request 3 specimens be collected and submitted for testing; specimen collection is made, optimally, every other day, over a period of up to 10 days (6). However, alternative approaches have been proposed to help curtail unnecessary testing, including application of an algorithm that requires a negative specimen and persistence of symptoms before a second or third specimen is analyzed by the laboratory (4). Specimens may also be pooled prior to screening based on microscopy. In contrast, the enhanced sensitivity of molecular detection methods may require only 1 specimen for testing to achieve sensitivity equal to, if not greater than, microscopy. One study demonstrated a 14% increase in yield for gastrointestinal protozoa when a real-time PCR was performed on a single stool specimen, compared to microscopy on three specimens (5).

STOOL PRESERVATION

While visualization of motility in unprocessed specimens may facilitate diagnosis, this technique is impractical for most laboratories, as transport of fresh stool to the laboratory for testing is rarely within the requisite time frame for examination (i.e., 30 to 60 min). A variety of stool fixatives have been developed and modified in recent decades for use with traditional microscopic examination. Those that remain widely used and commercially available include formalin, sodium acetate-acetic acid-formalin (SAF), Schaudinn’s fluid, polyvinyl alcohol-containing fixatives (mercury, copper, or zinc based), and mercury-free/formalin-free fixatives. A two-vial collection system, consisting of one vial containing 5 to 10% buffered formalin for use in concentrated wet mounts and a second vial containing a polyvinyl alcohol-based preservative for permanent stained smears, is considered the “gold standard.” However, concern over working with toxic formalin in the laboratory and the environmental impact and disposal costs associated with the use of mercury-based fixatives have led many to consider alternate preservatives and single-tube collection systems (9). SAF may be used to achieve this goal, if coupled with iron hematoxylin for the permanent stained smear; however, for laboratories desiring to maintain the trichrome stain, SAF is not a valid option, as poor-quality results have been documented with this combination.

Alternative stool preservatives. Zinc- and copper-based polyvinyl alcohol (PVA) formulations have been developed and are commercially available to replace the mercury-based fixatives (10, 11). In a paired study that evaluated 106 specimens prepared using zinc sulfate-PVA versus mercuric chloride-PVA with trichrome stain, 92.5% overall agreement was reported in the overall morphology and numbers of organisms detected between the two methods (11); in contrast, a study by the same group noted poor preservation of protozoa morphology when a copper-based PVA formulation was evaluated (10). Examples of commercial specimen collection kits using modification to the mercuric chloride PVA include ProtoFix (AlphaTec, Vancouver, WA), which contains no mercury and minimal formalin; EcoFix (Meridian Bioscience, Cincinnati, OH), which contains neither mercury nor formalin; and ParaSafe (Cruinn Diagnostic, Dublin, Ireland), which also does not contain mercury or formalin. A study con-
<table>
<thead>
<tr>
<th>Target organism</th>
<th>Assay type (% sensitivity/% specificity)</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entamoeba histolytica</td>
<td>TechLab Entamoeba histolytica II (100/94.7); Cellabs Entamoeba CELISA Path (93–100/93–100)</td>
<td>Serum-based ELAs: Bordier Affinity Entamoeba histolytica IgG (100/80–96); NovaTec Entamoeba histolytica IgG (95/95); Sciimedx Corp. Entamoeba histolytica antibody detection test (92/100)</td>
</tr>
<tr>
<td>Entamoeba histolytica (possibly E. dispar)</td>
<td>Remel ProSpect Entamoeba histolytica (87/99)</td>
<td></td>
</tr>
<tr>
<td>Giardia lamblia</td>
<td>Remel ProSpect Giardia (EZ) (96–98/98); Remel ProSpect Giardia IFU (98–100/98–100); Medical Chemical Para-Tect Giardia (85/95.9); Cellabs Giardia-CELISA® (98–100/100); TechLab Giardia II® (100/100)</td>
<td>Remel Xspect Giardia (97.9/97.1) Cellabs Giardia-CEL® (100/100)</td>
</tr>
<tr>
<td>Cryptosporidium spp.</td>
<td>Remel ProSpect Cryptosporidium (97/96–10); Medical Chemical Para-Tect Cryptosporidium (100/97–100)</td>
<td>Cellabs Crypto-CEL (100/100)</td>
</tr>
<tr>
<td>Cryptosporidium spp. and Giardia intestinalis</td>
<td>Remel ProSpect Giardia/Cryptosporidium (97.7–99.2/99.6); TechLab Giardia/Cryptosporidium Chek® (97.6/100)</td>
<td>Meridian ImmunoCard Stat Crypto/Giardia (97.3–100/100); Remel Xpect Giardia/Cryptosporidium (95.8–96.4/98.5); TechLab Giardia/Cryptosporidium Quick Chek® (98.9/100) Meridian Merifluor Cryptosporidium/Giardia® (97–100/94–100); Medical Chemical Para-Tect Cryptosporidium/Giardia® (100/100); Cellabs Crypto/Giardia-CEL® (100/100) Multiplex PCR Luminex xTAG gastrointestinal pathogen panel (GPP) (95–100/89–100)</td>
</tr>
<tr>
<td>Giardia intestinalis E. histolytica (possibly E. dispar)</td>
<td>Biosite Diagnostics’ Triage parasite panel (90.5–95.1/85–88.4)</td>
<td></td>
</tr>
<tr>
<td>Cryptosporidium parvum</td>
<td></td>
<td></td>
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</tbody>
</table>

a Sensitivity and specificity values were obtained from package inserts of each product. Performance characteristics for these tests in individual laboratories may vary from the data presented in the package inserts.

b Only detects Giardia cysts.

c The ranges of sensitivity and specificity for all targets are listed.


**TABLE 3 Common fixatives used to preserve ova and parasites in stool**

<table>
<thead>
<tr>
<th>Preservative</th>
<th>Downstream preparations</th>
<th>Downstream assays</th>
<th>Potential for single-vial use</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formalin, 3% or 10%</td>
<td>Only concentrated wet mount</td>
<td>EIA, FA, IC</td>
<td>Poor</td>
<td>Poor NAT potential, poor trophozoite preservation</td>
</tr>
<tr>
<td>SAF</td>
<td>Permanent stained smear</td>
<td>EIA, FA, IC</td>
<td>Fair</td>
<td>Poor NAT potential, suboptimal trophozoite morphology</td>
</tr>
<tr>
<td>Mercury-based fixative with PVA</td>
<td>Permanent stained smear</td>
<td>EIA, FA, IC</td>
<td>Fair</td>
<td>Immunoassays not possible, fixative is highly toxic</td>
</tr>
<tr>
<td>Modified Schaudinn’s (copper, zinc, or other fixative with PVA)</td>
<td>Permanent stained smear</td>
<td>NAT</td>
<td>Poor</td>
<td>Concentrated wet mounts are uncommonly performed, suboptimal trophozoite morphology</td>
</tr>
<tr>
<td>Single -vial proprietary fixative formulations</td>
<td>Permanent stained smear</td>
<td>Some immunoassays are possible; most NATs</td>
<td>Good</td>
<td>Suboptimal trophozoite morphology, not all immunoassays are possible</td>
</tr>
</tbody>
</table>

*Abbreviations: EIA, enzyme immunoassay; FA, fluorescent antibody; IC, immunochromographic test; NAT, nucleic acid amplification test.*

Ducted by the CDC evaluated the performance of these preservatives head to head with the traditional two-vial set of formalin and mercuric chloride-PVA. This study found EcoFix and ProtoFix, but not ParaSafe, yielded an acceptable morphological quality to the preserved parasites on concentrated wet mounts compared to formalin-fixed specimens. EcoFix alone yielded satisfactory protozoan morphology on the permanent stained smears, compared with stool preserved in mercuric chloride-PVA (9). In contrast, a separate study found significantly (P < 0.001) reduced recovery of B. hominis and Endolimax nana in 261 EcoFix-preserved concentrates compared to formalin-fixed stool concentrates (12). Although the manufacturer of EcoFix has developed a proprietary stain, EcoStain, the conventional trichrome stain can be used with EcoFix and has been shown to produce comparable protozoan morphology (12). Total-Fix (Medical Chemical Corporation, Torrance, CA) is a relatively new, FDA-approved mercury-, formalin-, and PVA-free fixative. Similar to EcoFix, specimens prepared by using Total-Fix can be used for concentration, permanent stain, and a variety of immunoassays for detecting protozoa, though there have been no published reports describing the performance of this fixative compared to others to date. Table 3 summarizes many available fixatives used by clinical laboratories and highlights possible preparations and downstream assays for each.

A major impediment to replacing the traditional two-vial systems of laboratories in the United States is the requirement for laboratories to perform a verification study to confirm the performance specifications of these products. Few institutions encounter a sufficient number of positive clinical specimens to allow robust evaluation of these preservatives. Furthermore, in order to perform a method comparison study, specimens need to be collected in both fixatives, which may require preapproval or exemption status by local institutional review boards. Laboratories may thus need to develop creative means by which to evaluate these fixatives prior to clinical use. A combination of approaches has been used in our laboratories, including comparison of the morphology of white cells present in stool preserved in both fixatives, seeding fresh stool specimens with cultured protozoa, obtaining veterinary specimens for testing, and consulting the published literature (if available) on the performance of these products.

**DETECTION OF SPECIFIC INTESTINAL PROTOZOA**

*Giardia lamblia (syn. Giardia intestinalis and Giardia duodenalis).* Giardiasis is a common gastrointestinal parasitic infection associated with diarrhea, stomach cramps, upset stomach, and excessive gas. Annually, roughly 20,000 U.S. cases of giardiasis are reported to the CDC, but these are estimated to comprise as little as 1 to 10% of the total infection burden, despite being a nationally notifiable disease (13). While numerous diagnostic tests are available for *Giardia*, its highly distinctive morphology facilitates microscopic diagnosis. *Giardia* cysts can be observed in fresh smears, on formalin-ethyl acetate or permanent stained smear, although the latter is associated with a higher sensitivity for identification. Trophozoites are not always found in stool, as encystation begins before passage through the colon. In cases where *Giardia* is suspected but not detected in stool, duodenal specimens, such as those collected by a string test, may be used for permanent stains and concentrated wet mounts. Tear drop-shaped trophozoites range from 10 to 20 μm in length, 9 to 12 μm in width, and contain two nuclei, a sucking disk, 4 pairs of flagella, 2 axonemes, and 2 median bodies. Cysts contain 4 nuclei, 4 axonemes, and 4 median bodies and range from 11 to 14 μm in length and 7 to 10 μm in width (Fig. 1E).

While *Giardia* cysts are easily recognizable on permanent stained smears, they are shed sporadically, and O&P examinations are often insufficient to demonstrate the presence of this organism (14). Alles and colleagues demonstrated a sensitivity of 66.4% for the detection of *Giardia* via a permanent stained smear, albeit chlorazol black stain was used as opposed to the more standard trichrome, and the number of specimens tested per patient was not taken into account (15). Regardless, detection of *Giardia* is improved through the use of antigen detection assays, several of which are commercially available and widely used in clinical laboratories across the United States. For example, in the aforementioned study by Alleles and colleagues, a sensitivity of 99.2% for the detection of *Giardia* was observed via a commercial, direct fluorescent antibody (DFA) test. Both the permanent stained smear and the DFA were 100% specific for *Giardia* in the 2,696 stool specimens examined by this study (15). In addition to the DFA, which requires laboratory access to a fluorescence micro-
FIG 1 Key microscopic morphology of the enteropathogenic protozoa. Organisms are ordered from largest to smallest, based on average cell size. (A) *Balanidium coli* trophozoite unstained, wet mount. (B) *Cystoispora belli* oocyst. (C, D, and F) Trophozoite forms are shown stained with trichrome for *E. histolytica* (C), *D. fragilis* (D), and *B. hominis* (F). (E) Cyst form of *Giardia* stained with trichrome. (G and H) *Cyclospora cayetanensis* oocyst (G) and *Cryptosporidium* spp. oocyst (H) after modified acid-fast staining.

scope, immunochromatographic (IC) tests, and enzyme immunoassays (EIAs) are commercially available for the detection of *Giardia* (Table 2). IC tests are optimally suited for laboratories with lower capacities for diagnostic complexity, while EIA-based tests may be more appropriate for high-throughput screening in high-prevalence areas. A study comparing four EIAs, including the FDA-approved ProSpecT (Remel, Lenexa, KS) and CELISA (Cellabs, Brookvale, NSW, Australia) assays, found sensitivities that ranged from 63 to 91% and specificities of ≥95% for all assays (16). A second study demonstrated 94 to 100% sensitivity and 100% specificity when 5 *Giardia* EIAs were evaluated with 100 positive and 50 negative specimens (17). Table 2 provides an overview of many of the available FDA-approved EIAs and their respective sensitivities and specificities, as determined by the manufacturer, for detection of *Giardia* either alone or in combination with other pathogenic protozoa.

**Dientamoeba fragilis.** Dientamoebiasis is an enteric infection caused by the flagellate *D. fragilis*. Symptoms associated with infection vary dramatically, with some individuals suffering nausea, vomiting, and diarrhea containing mucous and including abdominal discomfort, while others are asymptomatic. Accordingly, as with the case of *B. hominis*, described below, there is some uncertainty about the pathogenesis of *D. fragilis*. However, the morbidity associated with some infections justifies its inclusion as a definitive pathogen (18). The prevalence of *D. fragilis* has been estimated in many studies and ranges from 1.1 to 20% in patients in the developed world with diarrhea, but its prevalence may be higher in select populations or if molecular methods are used for detection (19).

Despite this relatively high prevalence, no antigen-based, molecular, or serologic diagnostics have been commercially developed to aid with laboratory identification. As such, detection of *D. fragilis* on the permanent stained smear is the current standard. Unfortunately, *D. fragilis* is difficult to identify morphologically. No cyst stage has been observed in humans, although a cyst stage has been recently observed in mice (20). Trophozoites range from 5 to 15 μm in length, 9 to 12 μm in width, and contain 1 to 2 characteristically fragmented nuclei. While well-preserved specimens might contain cells with the classically described tetrad nuclei (Fig. 1D), in general practice nuclei will only have visible holes through the center of the nucleus. Given its indistinct appearance, diagnosis is often only possible by experienced technologists, leading to many potentially missed infections. Even under ideal conditions, with prompt preservation of stool and evaluation by a skilled technologist, permanent stained smears are only 34% sensitive compared to molecular methods (21).

**Cryptosporidium** spp. Cryptosporidiosis is a gastrointestinal infection caused by various species of *Cryptosporidium*. Fecal-oral transmission via contaminated food, drinking water, or exposure in public swimming pools is responsible for most infections. Like all coccidian intestinal parasites, the small and poorly staining *Cryptosporidium* oocysts can be easily missed in routine O&P exams. Sensitivity of light microscopy is improved by performing modified acid-fast (MAF) stains, though even this modification has been shown to be associated with a sensitivity of only 54.8% (15). Furthermore, MAF staining is typically only performed upon physician request, or if the technologist detects structures suspicious for *Cryptosporidium* on the permanent stained smear. Unfortunately, many physicians assume that testing for *Cryptosporidium* is included with the routine O&P and infrequently order specialized stains or *Cryptosporidium* immunoassays, even in outbreak situations (3). Upon MAF staining, *Cryptosporidium* spp. oocysts appear as bright red spheres (4 to 6 μm) containing four crescent-shaped sporozoites (which may or may not be seen in all oocysts) (Fig. 1H). Additionally, oocysts may also occlude stain, resulting in transparent “ghost” cells.

As is the case for *Giardia*, sensitivity of detection is improved when an EIA or DFA is used (Table 2). Multiple studies have evaluated the sensitivities and specificities of the available kits and found overall similar performance levels for EIA- and DFA-based methods (sensitivity, >90%; specificity, >95% [17]). Rapid IC-based methods are significantly less sensitive, with one multi-institutional study reporting 50.1 to 86.7% sensitivity, dependent on the test manufacturer (22). Because HIV-infected and immunocompromised individuals are particularly at risk for severe complications due to infection with these coccidian parasites, physicians should consider routinely ordering DFA at a minimum and molecular-based assays, if available, for patients with suspect cryptosporidiosis.

*Giardia* and *Cryptosporidium* spp. are two of the most common protozoan infections in the United States, and multiple combined tests have been developed to facilitate rapid screening for both organisms simultaneously. Such tests include EIAs, IC assays, DFA assays, and multiplex PCR assays. A comparison between several DFA tests and EIAs for *Giardia* and *Cryptosporidium* revealed that (i) DFA tests tend to have slightly higher sensitivity for both organisms, (ii) the Merifluor *Cryptosporidium/Giardia* test had the highest sensitivity of the DFAs, and (iii) the specificities of
all tested EIA and DFA tests were 100% (17). However, these assays do not detect D. fragilis and, as such, these tests do not replace the O&P for routine testing.

**Cyclospora cayetanensis.** Cyclosporiasis is usually a self-limiting gastroenteritis caused by the coccidian *C. cayetanensis*. Due to poor uptake of most conventional stains by *C. cayetanensis* oocysts, microscopic detection can be challenging, but it remains the recommended diagnostic method (14). *C. cayetanensis* oocysts may stain irregularly by trichrome or the MAF stain. As is the case with *Cryptosporidium*, not all oocysts will take up these stains in a single smear, which may lead inexperienced technologists to overlook the organism. When observed, *Cyclospora* oocysts in stool are easily identified as 8- to 10-μm refractile spheres with a central morula, resembling wrinkled cellophane (Fig. 1G). If *Cyclospora* infection is specifically suspected (e.g., during established outbreaks), use of a modified safranin staining protocol provides consistent reddish-orange staining of oocysts and thus simplifies identification (23). In addition to the modified safranin stain, oocysts of *C. cayetanensis* in a standard concentrated wet mount intrinsically autofluoresce white-blue under UV light when a 330- to 365-nm excitation filter is used. Less-intense, blue-green autofluorescence can be seen when a 450- to 490-nm excitation filter is used. This property aids in the identification of *Cyclospora*; however, all fluorescent structures should be visualized by light microscopy to verify the morphology (http://www.asm.org/images/PSAB/CyclosporaWhitePaper2013.pdf).

Relm et al. developed a nested PCR assay that targets the 18S rRNA gene that has been in use in outbreak situations to confirm *Cyclospora* (23). Many other molecular techniques have been developed for the identification of *Cyclospora* (1), but there are no FDA-approved or analyte-specific reagents for *Cyclospora* available in the United States. The Biofire (Salt Lake City, UT) FilmArray GI panel includes *C. cayetanensis* and is currently available in the United States with research use only (RUO) status, but it is in clinical trials for the FDA.

**Cystoisospora belli.** Cystoisosporiasis is a relatively uncommon gastroenteritis caused by the coccidian *C. belli* that can result in choler-like symptoms in up to 1% of HIV-infected or otherwise-immunocompromised individuals (25). Detection of oocysts from stool or duodenal samples is simplified by their distinctive size and shape. However, *C. belli* oocysts are only easily recognizable in concentrated wet mounts from O&P exams. Importantly, oocyst maturation continues postdefecation, and thus morphology depends upon the duration between specimen collection and preservation. If placed immediately into preservative, long oval-shaped *C. belli* oocysts (20 to 33 μm in length and 10 to 19 μm in width) will contain a single circular immature sporoblast. If specimens are not quickly preserved, oocysts of roughly the same size and shape will contain 1 to 2 circular sporoblasts. While detection is relatively straightforward from concentrated wet mounts, modified acid-fast, safranin, or auramine rhodamine stains can be used to increase contrast and simplify detection, although staining may interfere with sporoblast visualization (Fig. 1B) (26, 27). Similar to *Cyclospora*, the oocysts of *Cystoisospora* will autofluoresce under the conditions described above. *C. belli* oocysts are not always found in stool, and examination of duodenal specimens collected by biopsy or string test may be necessary.

**Entamoeba histolytica.** Roughly 50 million worldwide cases of amoebic dysentery and 100,000 deaths are associated with *E. histolytica* annually (28). Despite the extreme morbidity associated with intestinal infections by *E. histolytica*, serological tests are not typically informative in uncomplicated cases because seroconversion is rare outside the context of extraintestinal involvement. Despite their microscopic morphological similarity to *Entamoeba dispar* and *Entamoeba moshkovskii*, intestinal infections with *E. histolytica* in nonendemic areas are still primarily diagnosed via microscopy on the permanent stained smear. Organisms may be accompanied by clubbed RBCs in cases of dysentery. On the permanent stained stool smear, *E. histolytica* trophozoites are 12 to 60 μm in diameter and contain a single, well-defined nucleus (Fig. 1C). Spherical cysts measure 12 to 15 μm, contain 2 to 4 nuclei, and occasionally have cigar-shaped, cytoplasmic chromatidal bars. Nuclei of both forms are surrounded by an obvious nuclear membrane, a compact, central karyosome, and evenly distributed peripheral chromatin. Without evidence of erythrophagocytosis (which is seen most often in tissue specimens), *E. histolytica* is indistinguishable from *E. dispar* and should be annotated as *E. histolytica/dispar* on the laboratory report. Ingested RBCs can only be definitively identified when concomitant extracellular RBCs are visible. In cases of chronic amebic infection, ingested RBCs are infrequently observed, making differentiation from *E. dispar* difficult.

In areas of the world where *E. histolytica* infection is endemic or if infection is specifically suspected by a physician, antigen-based tests can be performed, though these require unpreserved specimens. *E. histolytica* antigen tests that are specific for *E. histolytica* employ monoclonal antibodies against the Gal/GalNAc-specific lectin expressed by *E. histolytica*. Not all commercially available antigen tests differentiate between *E. histolytica* and *E. dispar* (Table 2). Sensitivity for the *E. histolytica* antigen detection tests has been shown in several studies to range from 80 to 94% compared to PCR, but one study found the TechLab enzyme-linked immunosorbent assay (ELISA) to be less sensitive than microscopy (29). Examples of FDA-approved EIAs for *Entamoeba* spp. are included in Table 2 along with their sensitivities and specificities, as defined in their package inserts.

Diagnosis of disseminated amebiasis caused by *E. histolytica* is challenging because stool O&P examinations are almost always negative for these patients. When such cases are suspected, cecal or colonic endoscopy to look for hallmark lesions followed by endoscopic biopsy to visualize the presence of *E. histolytica* trophozoites are quite helpful (30). This algorithm has been shown to be effective in differentiating amebic colitis from colon cancer and uncomplicated colitis (31, 32). Sigmodioscopy material may also be submitted to the laboratory for permanent stained smear evaluation. In patients with liver abscesses, serological assays are informative for the concomitant systemic exposure to amoebic antigens (1); 95% of patients with extraintestinal disease will be positive by serology. When evaluating patients from areas where *E. histolytica* infection is endemic, it is important to be aware that modern serological assays, which employ recombinant *E. histolytica* antigens, will turn negative following abscess treatment earlier than the traditional indirect hemagglutination-based tests, which remain positive for at least 6 months following treatment. Serum and liver abscess aspirates from patients with disseminated *E. histolytica* have been subjected to off-label antigenic testing, with varying sensitivity.

**Blastocystis hominis.** The pathogenicity of *B. hominis* is largely controversial, given that it is commonly identified in nonsymptomatic individuals. Some experts hypothesize that *B. hominis*
should be split into multiple species, some of which are more pathogenic than others, though few studies have been performed to confirm this hypothesis (33). The continuing uncertainty is primarily due to the fact that all isolates of Blastocystis are morphologically similar and are occasionally found in combination with other protozoan infections. However, in the absence of antigen detection or molecular diagnostics, the standard method for detection is still microscopy. While B. hominis is visible on wet mounts, definitive identification is easier with permanent stained smears. B. hominis is typically 6 to 40 μm in diameter with a large central body surrounded by up to six small nuclei (Fig. 1F). The large central body often stains a characteristic red, green, or blue in trichrome-stained samples. Development of nonmicroscopic and molecular strategies for diagnosis will likely hinge on whether studies can effectively differentiate pathogenic versus nonpathogenic strains (33). When observed on routine O&P, B. hominis should be reported, along with a semiquantitative assessment.

**Balantidium coli.** Balantidiasis is an intestinal parasitic disease that is associated with ciliated *B. coli* trophozoites, which typically only affect immunocompromised or malnourished individuals and have a worldwide distribution (34). Like many other intestinal protozoa, no established molecular or serologic tests are available for *B. coli*. Instead, microscopic diagnosis is facilitated by its distinctive size and morphology on concentrated wet mounts; diagnosis from permanent stains is not recommended, because trophozoites absorb large amounts of dye, which can mask its characteristic features.

*B. coli* is the largest infectious intestinal protozoan, at 50 to 100 μm in length and 40 to 70 μm in width. Trophozoites have fine, visible cilia and a large, kidney-bean-shaped macronucleus (Fig. 1A). A single, polar cystosome, or oral groove, can also be detected on some cells. The cyst form also has a visible macronucleus, but is smaller (50 to 70 μm long, 40 to 60 μm wide) and rounder than the trophozoites. Cysts have a thick cyst wall and often do not have visible cilia. While molecular or serologic-based diagnostics might improve detection sensitivity compared to microscopic diagnosis, development of such tests has been a low priority due to the relative simplicity of microscopic detection and infrequency of infection in the United States.

**IMPLICATIONS FOR FUTURE DIAGNOSTICS**

As discussed above and documented in recent studies, multiplex PCR assays are both more sensitive and specific than microscopy for the detection and identification of pathogenic protozoa (35). However, despite a rapidly growing field of molecular and genetic techniques for the clinical microbiology laboratory, diagnostic developments for intestinal protozoan parasites have remained relatively stagnant. Challenges associated with developing a replacement test for the O&P includes coverage of all pathogenic species and the potential for long-term, residual detection of previous infections. Furthermore, while analyte-specific approaches may yield enhanced sensitivity for pathogenic protozoa, documentation of the presence of human cells (white blood cells and erythrocytes), Charcot-Leyden crystals, and nonpathogenic protozoa is lost. In particular, some physicians interpret the presence of nonpathogenic protozoa as indicative of patient exposure to contaminated food or water, although there are no studies that have clearly demonstrated this to be fact.

The Luminex XTAG gastrointestinal pathogen panel has received FDA approval and can simultaneously detect 14 enteropathogens, including *Giardia* and *Cryptosporidium* spp. This assay is the first molecular method approved by the FDA for the detection of pathogenic protozoa. The analyte-specific reagents (ASRs) for the XTAG assay were recently evaluated; while the overall number of positive specimens was low in this study (5 to 20 positives), the ASRs were highly sensitive and specific for *Cryptosporidium* (95% sensitivity and 99% specificity), *Giardia* (95% sensitivity and 99% specificity), and *E. histolytica* (100% sensitivity and 89% specificity) (36). The FDA-approved version of the assay does not include *E. histolytica*, but the reagents for this analyte are available for research use only.

BioFire Diagnostics has in development a sample-to-answer gastrointestinal pathogen panel that includes detection of *Giardia, Cryptosporidium, E. histolytica*, and *Cyclospora cayetensis*. Whether the company will be able to collect sufficient numbers of specimens positive for each target to garner FDA clearance or if some will remain RUO remains to be seen. Like the Luminex panel, this platform does not include detection of *D. fragilis*, which is one of the most commonly encountered protozoa in the United States.

One major critique for these multiplexes is the cost per test, which is many times higher than the reagents associated with performing the O&P. However, if an assay were to replace the O&P examination, the savings in labor, from our perspective, would far outweigh the cost associated with performing a multiplex commercial test.

**SUMMARY**

In summary, adequate diagnosis of intestinal protozoa by the clinical laboratory is limited by many factors (Table 1). There is increasing demand for low-complexity, high-throughput, and cost-effective complements to (or replacements for) the labor-intensive microscopic- or serologic-based approaches to protozoan diagnosis. While efforts in this regard have been slow to come, many diagnostic manufacturers are rising to the challenge, including Luminex and BioFire. These efforts may restore or enhance the abilities of laboratories to identify these pathogens, yielding increased knowledge on the present state of these diseases in the United States and other countries.

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**REFERENCES**


Continued next page
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