Clinical Laboratory Diagnosis of Intestinal Protozoa

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Abstract

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 compliment or even replace microscopic approaches. To date, the scope of such novel technology 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.
Introduction

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 non-pathogenic 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), inappropriate test ordering by physicians (1,2,3), and 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 (Table1). A pressing concern for these laboratories is the shortage of skilled technologists capable of reliably evaluating O&Ps. 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 many 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 endemic regions of 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 Unit,
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 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 United States 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
protozoon frequently encountered in many US laboratories (RMH, MRC unpublished
observations). Regardless, some have suggested the use of algorithmic testing that involves front-line antigen testing for *Giardia* and *Cryptosporidium* which, when negative, can be reflexed to traditional microscopic approaches (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 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 not initially tested for.

There is a pressing need for newer diagnostic test options, to 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 United States Food and Drug Administration (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 Table1, 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 *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 58-72% 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 optimally every other day, over 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 by 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, as compared to microscopy on three (5).

**Stool Preservation**

While visualizing motility in unpreserved 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. 30-60 minutes). 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 the 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 5-10% 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 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 by zinc sulfate-PVA versus mercuric chloride-PVA with the 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; PARASAFE® (Cruinn Diagnostic, Dublin, Ireland) which also contains neither mercury nor formalin. A study conducted 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 morphologic quality to the preserved parasites on concentrated wet mounts as compared to formalin fixed specimens. ECOFIX® alone yielded satisfactory protozoan morphology on the permanent stained smears, when 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 when 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 relatively new, FDA-approved mercury-, formalin- and PVA-free fixative. Similar to the ECOFIX®, specimens prepared by 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 as 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 by 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 would need to be collected in both fixatives, which may require pre-approval 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 have been used in our laboratories, including a 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 with 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 US cases of giardiasis are reported to the CDC, but are estimated to comprise as little as 1-10% 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 concentration or permanent stained smear, although the latter is associated with 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 10-20 μm in length, 9-12 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 11-14 μm in length and 7-10 μm in width (Figure 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 performed 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 with 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 fluorescent microscope, 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 capacity 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\textsuperscript{®} (Remel, Lenexa, KS) and CELISA (Cellabs, Brookvale, NSW, Australia) assays, found sensitivities that ranged from 63-91%, and specificities of $\geq$95% for all assays (16). A second study demonstrated 94-100% 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 abdominal discomfort; while others are asymptomatic. Accordingly, as with the case of *B. hominis*, described further 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 – 20% in patients in the developed world with diarrhea, but 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 5-15 $\mu$m in length and 9-12 $\mu$m in width and contain 1-2 characteristically fragmented nuclei. While well-preserved specimens might contain cells with the classically described tetrad nuclei (Figure 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 as 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 and 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 (4-6 μm) containing four crescent shaped sporozoites (which may or may not be seen in all oocysts) (Figure 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 (Table 2) is used. Multiple studies have evaluated the sensitivity and specificity of the available kits and found overall similar performance 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 - 86.7% sensitivity, dependent on 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 of DFA at minimum, and molecular-based assays, if available, for patients with suspect cryptosporidiosis.

Giardia or 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 and EIA tests for *Giardia* and *Cryptosporidium* revealed that: A) DFA tests tended to have slightly higher sensitivity for both organisms; B) the Merifluor® *Cryptosporidium/Giardia* test had the highest sensitivity of the DFAs; and C) the specificity 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 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 8-10 μm refractile spheres with a central morula, resembling wrinkled cellophane (Figure 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). It is important to recognize that *Cryptosporidium* does not consistently stain by modified safranin, and as a result this stain should not replace a MAF stain in general practice. In addition to the modified safranin stain, oocysts of *C. cayetanensis* in a standard concentrated wet-mount intrinsically
... autofluoresce white-blue under UV light using a 330-365 nm excitation filter. Less intense, blue-green autofluorescence can be seen using a 450-490 nm excitation filter. 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).

Relman *et. al* developed a nested PCR assay that targets the 18S rRNA gene that has been used 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 U.S. Biofire (Salt Lake City, UT) FilmArray® GI Panel includes *C. cayetanensis* and is currently available in the U.S. with research use only (RUO) status, but is in clinical trials with the FDA.

**Cystoisospora belli**

Cystoisosporiasis is a relatively uncommon gastroenteritis caused by the coccidian *C. belli* that can result in cholera-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 of O&P exams. Importantly, oocyst maturation continues post-defecation and thus morphology depends upon the duration between specimen collection and preservation. If placed immediately into preservative, long oval-shaped *C. belli* oocysts (20-33 µm in length and 10-19 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 1-2 circular sporoblasts. While detection is relatively straight-forward from concentrated wet-mounts, modified acid-fast, safranin or auramine rhodamine stains can be used to increase contrast and simplify detection, though staining may interfere with sporoblast visualization (Figure 1B) (26, 27). Similar to Cyclospora, the oocysts of Cystoisopora 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 extra-intestinal involvement. Despite their microscopic morphological similarity to Entamoeba dispar and Entamoeba moshkovskii, intestinal infections with E. histolytica in non-endemic 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 12-60 μm in diameter and contain a single, well-defined nucleus (Figure 1C). Spherical cysts measure 12-15 μm, contain 2-4 nuclei and occasionally have cigar-shaped, cytoplasmic chromatoidal 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 *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 80-94%, as compared to PCR; but one study found the TechLab ELISA to be less sensitive than microscopy (29). Examples of FDA-approved EIA tests for *Entamoeba* spp. are included in Table 2 along with their sensitivity and specificity, as defined by their package inserts.

Diagnosis of disseminated amebiasis caused by *E. histolytica* is challenging because the 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). Sigmoidoscopy material may also be submitted to the laboratory for permanent stained smear evaluation. In patients with liver abscesses, serological
assays are informative due to the concomitant systemic exposure to amoebic antigens (1); 95% of patients with extraintestinal disease will be positive by serology. When evaluating patients from endemic areas, it is important to be aware that modern serological assays, which employ recombinant *E. histolytica* antigens, will turn negative following abscess treatment earlier than did the traditional indirect hemagglutination based tests, which remained 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 non-symptomatic individuals. Some experts hypothesize that *B. hominis* should be split into multiple species, some being 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 6-40 μm in diameter with a large central body surrounded by up to six small nuclei (Figure 1F). The large central body often stains a characteristic red, green, or blue in trichrome stained samples. Non-microscopic and molecular strategies for diagnosis will likely hinge on whether studies can effectively differentiate pathogenic versus non-pathogenic strains.
(33). When observed on routine O&P, *B. hominis* should be reported, along with a semi-quantitative assessment.

**Balantidium coli**

Balantidiasis is an intestinal parasitic disease associated with ciliated *B. coli* trophozoites that typically only affects immunocompromised or malnourished individuals and has 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, masking its characteristic features.

*B. coli* is the largest infectious intestinal protozoan at 50-100 microns in length and 40-70 microns in width. Trophozoites have fine, visible cilia and a large, kidney-bean shaped macronucleus (Figure 1A). A single, polar cystosome, or oral groove, can also be detected on some cells. The cyst form also has a visible macronucleus, but are smaller (50-70 µm long, 40-60 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 US.
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 technologies for the clinical microbiology laboratory, diagnostic development for intestinal protozoan parasites has 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 non-pathogenic protozoa is lost. In particular, some physicians interpret the presence of non-pathogenic 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 (5-20 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% sensitive and 89% specific)
The FDA-approved version of the assay does not include *E. histolytica*, but the reagents for this analyte are available as research use only (RUO).

BioFire Diagnostics has in development a sample-to-answer gastrointestinal pathogen panel that includes detection of *Giardia, Cryptosporidium, E. histolytica* and *Cyclospora cayentensis*. Whether the company is able to collect sufficient number 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 multiplex panels 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 the perspective of these authors, 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 (Table1). There is increasing demand for low complexity, high-throughput, and cost effective complements to (or replacements for) the labor-intensive microscopy-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 laboratory’s ability to identify these
Acknowledgments: We thank Dr. David A Bruckner for his insightful review of this manuscript.


Table 1. Top 5 challenges faced by the clinical laboratory associated with the detection of intestinal protozoa, as identified by the authors

1. Reliance on labor-intensive, technically demanding tests (e.g., O&P)
   - *O&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*
   - *Many laboratories do not have technologists that can reliably identify pathogens, and differentiate these from non-pathogenic species, or artifacts*

2. Reliance on insensitive tests
   - *O&P is associated with a sensitivity of 20-90% compared to molecular assays*
   - *Some antigen detection tests, e.g., Cryptosporidium are insensitive*

3. Shortage of clinical specimens positive for intestinal protozoa
   - *Limits adequate training*
   - *Limits ability of technologists to maintain proficiency*
   - *Limits validation of new testing platforms and transport medium*

4. Shortage of training programs / resources for parasitology
   - *Confounded by the retirement of experienced technologists who may otherwise perform training*

5. Suboptimal physician ordering practices
   - *Few physicians will order organism-specific tests, even during outbreaks*
   - *Inadequate submission of patient information prevents implementing algorithmic testing*
Table 2. FDA-approved assays for molecular and serologic detection of intestinal protozoan parasites.

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<th>Target organism</th>
<th>Assay type</th>
<th>Sensitivity</th>
<th>Specificity</th>
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<td><strong>Entamoeba histolytica</strong></td>
<td>Stool enzyme immunoassay</td>
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<td>1) TechLab <em>Entamoeba histolytica</em> II</td>
<td>[100%</td>
<td>94.7%]</td>
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<td></td>
<td>2) Cellabs <em>Entamoeba</em> CELISA Path</td>
<td>[93-100%</td>
<td>93-100%]</td>
</tr>
<tr>
<td>E. histolytica (Possibly <em>E. dispers</em>)</td>
<td>1) Remel ProSpect <em>Entamoeba histolytica</em></td>
<td>[87%</td>
<td>99%]</td>
</tr>
<tr>
<td><strong>Giardia lamblia</strong></td>
<td>Immuno-chromatographic</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1) Remel Xpect <em>Giardia</em> (EZ)</td>
<td>[96-98%</td>
<td>98%]</td>
</tr>
<tr>
<td></td>
<td>2) Remel ProSpect <em>Giardia</em> IFU</td>
<td>[98-100%</td>
<td>98-100%]</td>
</tr>
<tr>
<td></td>
<td>3) Medical Chemical PARA-TECT <em>Giardia</em></td>
<td>[85%</td>
<td>95.9%]</td>
</tr>
<tr>
<td></td>
<td>4) Cellabs <em>Giardia-CELISA</em>²</td>
<td>[98-]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Direct fluorescent antibody</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1) Remel Xpect <em>Giardia</em> [97.9% - 97.1%]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1) Cellabs <em>Giardia-CEL²</em> [100%</td>
<td>100%]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1) Bordier Affinity <em>Entamoeba histolytica</em> IgG [100%</td>
<td>80-96%]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2) NovaTec <em>Entamoeba histolytica</em> IgG [95%</td>
<td>95%]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3) Sciemedx Corp <em>Entamoeba histolytica</em> antibody detection test [92%</td>
<td>100%]</td>
<td></td>
</tr>
<tr>
<td>Cryptosporidium spp.</td>
<td>1) Remel ProSpecT Cryptosporidium [97%</td>
<td>96-100%]</td>
<td>1) Cellabs Crypto-CEL [100%</td>
</tr>
<tr>
<td>---------------------</td>
<td>-------------------------------------------</td>
<td>-----------------------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2) Medical Chemical PARA-TECT Cryptosporidium [100%</td>
<td>97-100%]</td>
<td></td>
</tr>
</tbody>
</table>

| Cryptosporidium spp. and Giardia intestinalis | 1) Remel ProSpecT Giardia/Cryptosporidium [97.7-99.2% | 99.6%] | 1) Meridian ImmunoCard STAT Crypto/Giardia [97.3-100% | 100%] |
|---------------------------------------------|-------------------------------------------|-----------------------------------|
|                                             | 2) TechLab Giardia/Cryptosporidium Chek² [97.6% | 100%] | 1) Meridian MERIFLUOR Cryptosporidium/Giardia² [97-100% | 94-100%] |
|                                             | 3) TechLab Giardia/Cryptosporidium Quick Chek² [98.9% | 100%] | 2) Medical Chemical PARA-TECT Cryptosporidium/Giardia² [100% | 100%] |
|                                             |                                           | 3) Cellabs Crypto/Giardia-CEL² [100% | 100%] | [Multiplex PCR] Luminex xTAG Gastrointestinal Pathogen Panel (GPP) [95 – 100% | 89-100%] |

| Giardia intestinalis | 1) Biosite Diagnostics Triage Parasite Panel [90.5% - 95.1% | 85 -88.4%] |                                   |
|---------------------|-------------------------------------------|-----------------------------------|
| (Possibly E. dispar) | Cryptosporidium parvum³ |                                   |
1 [% sensitivity | % specificity] taken from package inserts of each product. Performance characteristics for these tests in individual laboratories may vary from the data presented in the package inserts. 2 Only detects Giardia cysts. 3 The range 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>5% or 10% Formalin</td>
<td>Only Concentrated wet mount</td>
<td>EIA, FA, IC</td>
<td>Poor</td>
<td>Poor NAT potential and poor trophozoite preservation</td>
</tr>
<tr>
<td>SAF</td>
<td>Permanent stained smear &amp;</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 &amp;</td>
<td>NAT</td>
<td>Poor</td>
<td>Immunoassays not possible and fixative is highly toxic</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Method</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Modified Schaudinn’s</strong></td>
<td>Permanent stained smear and Concentrated wet mount (rare)</td>
</tr>
<tr>
<td><strong>Proprietary fixative formulations</strong></td>
<td>Permanent stained smear and Concentrated wet mount</td>
</tr>
<tr>
<td><strong>Concentrated wet mount (rare)</strong></td>
<td>Permanent stained smear and Concentrated wet mount</td>
</tr>
</tbody>
</table>

**Notes:**
- Immunoassays not possible and concentrated wet mounts are uncommonly performed. Suboptimal trophozoite morphology.
- Some immunoassays are possible. Most NATs.
- Suboptimal trophozoite morphology. Not all immunoassays are possible.

**Abbreviations:**
- EIA, enzyme immunoassay; FA, fluorescent antibody; IC, immunochromographic tests; NAT, nucleic acid amplification tests
Figure. Key microscopic morphology of the enteropathogenic protozoa. Organisms are ordered from largest to smallest, based on average cell size. (A) *Balantidium coli* trophozoite unstained on wet mount. (B) *Cystoisospora belli* oocyst, (G) *Cyclospora cayetanensis* oocyst and (H) *Cryptosporidium* spp. oocyst shown after modified acid fast staining. Trophozoite forms are shown stained with trichrome for (C) *E. histolytica*, (D) *D. fragilis*, and (F) *B. hominis*. (E) Cyst form of *Giardia* stained with trichrome.
Average cell size

- ~75x55μm
- ~25x15μm
- 12-60μm
- ~10μm
- ~7x10μm
- 6-40μm
- 8-10μm
- ~4-6μm

A B C D E F G H
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