Comparison of Lateral Flow Immunoassays to Current Stool Evaluation Methods for the Detection of *Giardia* and *Cryptosporidium*

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1. INTRODUCTION

1.1. Diarrheal Disease and Intestinal Parasites

The World Health Organization (WHO) estimates that approximately two billion cases of diarrheal illness occur worldwide each year. Diarrheal illness is a considerable cause of morbidity and mortality, particularly in the developing world (28). A wide variety of pathogens are responsible for diarrheal illness including bacteria, viruses and parasites. The cost associated with these infections as a result of the medical treatment and lost wages is significant: It is estimated that food-borne illness costs the United States (U.S.) economy $6 billion dollars a year (29). Infections with intestinal parasites are an important cause of diarrheal illness (see Table 1), with at least one-third of the world's population is believed to be infected with intestinal parasites. In the United States, infection with intestinal parasites is more common in newly arrived refugees than in those born in the U.S. (26). In particular, *Giardia* and *Cryptosporidium* spp. account for the majority of intestinal parasite infections originating in the U.S. Approximately 20,000 *Giardia* and 9,000 *Cryptosporidium* infections were reported in 2010 (27). These parasites are highly infectious and outbreaks can spread quickly through institutional facilities, daycares or in communities with a contaminated water source. Nearly 300 outbreaks were attributed to *Cryptosporidium* and approximately 180 were attributed to *Giardia* in 2010 (27). Thus, the ability of clinical laboratories to quickly and accurately diagnose infections with these parasites is crucial for the treatment of patients and to the prevention of further spread of disease.

1.2. Intestinal Parasites *Giardia intestinalis* and *Cryptosporidium* spp.

1.2.1. Giardiasis

Giardiasis is an intestinal illness characterized by diarrhea, abdominal cramping, bloating, weight loss, marked flatulence and malabsorption. Although infection is usually self-limiting and can be asymptomatic, chronic intestinal disorders, allergies and reactive arthritis can occur (1). Cases of asymptomatic carriage of *Giardia* in immunocompetent individuals are generally rare in industrialized nations, with reported rates of between 1-7%. However, this frequency can vary depending on parasite prevalence in the geographical area, age of those tested, and incidence of gastrointestinal illness (46). Malabsorption and malnutrition are thought to be the result of the direct attachment of *Giardia* to the intestinal wall which is thought to result in disruption of intestinal enzymes production and stimulation of cytokines from the host immune system causing inflammation of the host intestinal mucosa (45). While these symptoms can be more severe in immunocompromised patients who have giardiasis, marked T-cell deficiency does not make these patients more susceptible to infection than immune competent individuals as B-cell dependent host defenses are known to play a more central role in mediating immunity to giardiasis (31).

Giardiasis is highly contagious and it is estimated that ingestion of as few as ten parasites can cause infection. Generally symptoms can last for 1-2 weeks, however once infected, a person can shed 10^8-10^9 cysts per day for several months after symptoms subside. The large number of cysts excreted, combined with the low infectious dose, contributes to the high infectivity of Giardiasis. (3). Several effective antimicrobial therapies exist for the treatment of this infection with metronidazole being most commonly employed. Metronidazole both damages the DNA structure and inhibits DNA synthesis (45). Nitazoxanide, quinacrine, furazolidone, tinidazole, and paromomycin are among the less commonly employed antimicrobial therapies that can also be used (1).

Giardia intestinalis is the most commonly detected parasite found in humans (27). The incidence of giardiasis in Washington State is estimated at 7.7 cases/100,000 people (27). Outbreaks of *Giardia* are usually associated with recreational water venues, such as public pools and water parks, due to poor filtration systems and the parasite possessing a moderate tolerance to chlorine. The CDC has included *Giardia* on the list of reportable diseases since 2002. Children ages 1-9 and adults ages 35-44 are most commonly infected with this organism, with most cases in the U.S. occurring in the months of June to October. This is believed to result from an increase in outdoor activities in areas with inadequately or untreated water supplies. Child and adult care facilities are also sources of outbreaks because once introduced, the highly infectious nature of *Giardia* allows the parasite to spread quickly in these populations. (1) *Giardia intestinalis* was first described in the late 17th century and has undergone changes in nomenclature from *G. lamblia* to *G. duodenalis* to its current name (*Giardia intestinalis*).

<u>Lifecycle of *Giardia sp.*</u>

Giardia are flagellated intestinal protozoans possessing both cyst and trophozoite forms, shown in Figure 1 (4). The lifecycle is shown in Figure 2 (4). Cysts are the infectious form of *Giardia* and are excreted in feces (Stage 1). They are in turn ingested from fecally contaminated food or water sources or through human-human contact (fecal-oral) (Stage 2). Once ingested, the cysts make their way to the duodenum where excystation occurs resulting in the release of two trophozoites (Stage 3). The trophozoites attach themselves to the surrounding mucosa of the small intestine and reproduce asexually by binary fission. The resulting trophozoites can either remain in the small intestine or travel towards the colon where they undergo encystation (Stage 4). Trophozoites that do not undergo encystation can occasionally be excreted in the feces but are themselves not infectious. In contrast, cysts are commonly excreted in the feces and are immediately infectious. Importantly, the detection of excreted cysts and trophozoites in feces is considered diagnostic for giardiasis. (4)

1.2.2. Cryptosporidiosis

Cryptosporidiosis is an intestinal illness characterized by profuse watery diarrhea, abdominal cramping, loss of appetite, low-grade fever, nausea, vomiting, and weight loss (1). The infectious dose is very low and has been estimated at 10-30 oocysts. Once infected, a person can shed 10^8 to 10^9 cysts/bowel movements for up to 50 days (1,3). With the discovery of AIDS in the 1980's, the role of *Cryptosporidium* as an opportunistic agent of infection in the immunocompromised focused much attention on an organism previously considered to be rare in humans (3). Immunocompromised patients can experience 6-25 bowel movements/day and can excrete 1- 17L of fluid/day, which can lead to severe dehydration, hypovolemic shock and death (3). Thus, immunocompromised individuals are at risk for the development of life-threatening cryptosporidiosis. In contrast, the disease is generally self-limiting in immunocompetent individuals with symptoms generally lasting for 1-10 days. Infection can also be asymptomatic (3), although this is generally believed to be low in prevalence in industrialized nations (approximately 0.2%) (46). Chronic infection is common in immunocompromised patients because the absence of cell-mediated immunity prevents these individuals from developing a significant immune response to the parasite (1, 3, 46). Permanent injury and destruction of the intestinal villi can cause long-term malabsorption syndrome and pancreatitis as a result of chronic inflammation of the mucosal surfaces (47). With the recent introduction of new antiretroviral therapies, cryptosporidiosis is better managed in these populations. Until recently, treatment of cryptosporidiosis using antimicrobials such as spiramycin, paromomycin and azithromycin was only marginally effective (3). The introduction of nitazoxanide in 2002 marked the first broad spectrum anti-parasitical approved for cryptosporidiosis. Nitazoxanide inhibits the growth of sporozoites through the reduction of metabolic enzymes and has proven effective in in the treatment of Cryptosporidosis in immunocompetent children and adults (48). However, according to the 2009-2010 Morbidity and Mortality Report from the CDC, nitazoxanide does not have proven efficacy for the treatment of disease in the immunocompromised patients (27).

Cryptosporidium spp. are waterborne parasites and were responsible for over 9,000 cases of diarrheal illness in the US in 2010 (27). The CDC included *Cryptosporidium* spp. on the list of reportable diseases since 1994 (1) and seventy-three cases were reported in Washington State in 2010 (2). Those most commonly infected are children, adults 25-39 and immunocompromised patients. The peak in incidence for adults likely reflects the high level of transmissibility from child to parent. Similar to giardiasis, cases of cryptosporidiosis increase in the months of June to October due to increased outdoor activities, especially those associated with recreational water venues. This is compounded by the high chlorine tolerance of the *Cryptosporidium spp.* and the tendency of those infected to shed oocysts for up to 50 days after symptoms subside (1). Two species of *Cryptosporidium* are most commonly seen in human infections: *C. parvum* and *C. hominis*. However, many other species of *Cryptosporidium* exist in a wide variety of animals.

Cases of *C. felis* (associated with cats), *C. canis* (associated with dogs), *C. meleagridis* (associated with birds), *C. suis* (associated with pigs), *C. muris* (associated with rodents), and *C. andersoni* (associated with cattle) have also been reported in humans on rare occasion (27).

Lifecycle of *Cryptosporidium* spp.

Cryptosporidium spp. are obligate intracellular parasites originally defined as primarily zoonotic in nature (Figure 3) (4). The lifecycle of *Cryptosporidium* is shown in Figure 4 and consists of both an asexual and sexual cycle of reproduction (4). Thick-walled oocysts are excreted in feces and then ingested from fecally contaminated food or water sources or through fecal-oral contamination (Stages 1-3). Oocytes are detected when excreted in the feces. However, the CDC has also recently reported that respiratory secretions may be another source of infection as the parasite can infect the epithelial brush border of both the intestinal and respiratory tracts. The ingested (or possibly inhaled) oocysts contain sporozoites that, once released, infect the brush border of the epithelial cells and develop as trophozoites (Stage a-c). The trophozoites mature into Type I meronts that divide to produce merozoites through asexual reproduction. These merozoites can either re-infect the epithelial cells continuing an asexual cycle as a Type I meront or transform into a Type II meront to undergo sexual reproduction (Stages d-f). In the sexual cycle, the merozoites from the Type II meront differentiate into either micro- or macrogamonts (Stages g-h). The gametes produced from the microgamont fertilize the macrogamont resulting in a sexually produced zygote (Stage i). This newly formed zygote can take one of two paths: The first path leads to the development of a thick-walled oocyst that exits the current host to infect another (Stage j), where as the second path results in a thin-walled oocyst containing the sporozoites needed to auto-infect the current host (Stage k) (1, 4).

1.3. Methods for the Diagnosis of Intestinal Parasites

A variety of methods are available for the detection of intestinal parasites in stool specimens. Wet mounts, stained slide preparations, and fluorescent staining using either indirect staining or direct fluorescent antibody staining are among the microscopic methods that can be employed. In addition to microscopic identification of parasites, a number of immunological assays are available. These methods generally fall into two categories. The first category consists of traditional enzyme immunoassays (EIA) with microplate wells that are pre-labeled with anti-parasite antibodies. The second category contains rapid lateral flow devices that function as solid-phase qualitative immuno-chromatographic assays. A comparison between methods and the advantages and disadvantages of methods are shown in Tables 2 and 3.

The (OAPP) is traditionally considered the gold-standard for intestinal parasite detection. Ranges for sensitivity and specificity of 58-100% and 93.2-99.9%, respectively, have been reported and are affected by the number of stools submitted (25, 30). Examination of three independent stool specimens, or the so-called the "three-stool-rule," is recommended to attain maximal diagnostic sensitivity (See Table 4). Concentrated stool is viewed by both wet mount and permanent staining after fixation. A large variety of fixatives are commercially available, including ECOFIX (Meridian Bioscience, Inc., Cincinnati, OH), PARASAFE (Scientific Devices Laboratory, Inc., Des Plaines, IL), Proto-Fix (Alpha-Tec Systems, Inc., Vancouver, WA), and low-viscosity PVA fixative(Meridian Bioscience Inc.). ECOFIX is the primary fixative utilized in the University of Washington Clinical Microbiology Laboratory and is reported to have the fewest processing steps, performs comparably to traditional fixatives in terms of parasite preservation and is environmentally safe when compared to other mercury-containing products

(49). Permanent stains such as trichrome and iron hematoxylin stain are most commonly used and are available commercially (50). Permanent stains provide contrast for both the background debris and parasites present using multiple stain colors, which allows for the detailed examination and recognition of organism. However, larger parasites, such as helminth eggs and larvae, can retain too much stain for proper visualization, which is why wet mount preparation is also utilized for parasite examination (50).

Advantages of OAPP Testing

The major advantage of the OAPP method is the ability to detect multiple and unusual organisms. Various stages in the parasite life cycle including cysts, trophozoites, eggs and adult worms can all be observed with a standard light microscope. OAPP is considered the gold standard for the microscopic detection of most intestinal parasites. Another advantage is that of specimen storage. Commercially available preservatives most commonly contain a 10% buffered formalin plus an acid/alcohol solution that allows for stool to be stored for extended periods while maintaining structural integrity of the parasites present (7). The storage of specimens allows for the future visualization of parasites which is beneficial for the training of new employees and for maintaining competency of current staff.

Disadvantages of OAPP Testing

Although microscopic examination can reveal the presence of different parasitic organisms, the method has a number of disadvantages. Firstly, parasites have to be present to be visualized. It is possible for a patient to be infected but to have limited shedding of parasites in the stool. Consistent with this, the CDC Morbidity and Mortality Review for giardiasis from 2011 estimated that, while approximately 12,000 cases of *Giardia* infection are diagnosed per year, closer to 1.2 million could be going undiagnosed annually (2). Although not all patients will necessarily seek medical care for intestinal symptoms, the known intermittent shedding of *Giardia* along with the high variability in parasite quantity observed in stool specimens could contribute to the under diagnosis of infection with this parasite by OAPP (1). Detection rates vary between patients for a number of reasons including diminished immune function, nutritional status, bowel motility, and decreased stomach acid. Due to the difficulty in the detection of parasites by OAPP, it has been recommended that multiple stool specimens be submitted for testing i.e. the so-called the “three-stool-rule”. It is recommended that patients suspected of having an intestinal parasitic illness submit three stool specimens over a three to ten day period to achieve maximum sensitivity (7, 30). As shown in Table 5, sensitivity of a single specimen has been reported as being as low as approximately 58%, with submission of a second specimen increasing sensitivity an additional 21% and a third a further 21% (30). A sensitivity of close to 100% for three stool examinations reduces the need to submit additional specimens. However, obtaining multiple specimens across multiple days does not always occur as it is burdensome to patients. Consequently, false negative OAPP results are likely to be observed due to insufficient sampling.

It is important to note that OAPP testing is not suitable for all intestinal parasites. In particular, *Cryptosporidium* spp. are often confused with yeast or red blood cells when visualized by OAPP due to their similar size of 4-6um. Thus, alternative methods, such as immunofluorescence and acid-fast staining, must be used to microscopically detect this reportable stool parasite. (1, 3)

A further disadvantage of the OAPP method is the significant amount of processing and hands-on time required to perform the testing. Preparation of stool concentrates and staining are labor intensive. In addition, both a wet mount and stained slide must be analyzed by a trained technologist. The microscopic time involved per slide is lengthy because the visualization of two to three hundred fields is recommended (32, 50). The extensive pre-analytical and analytical time required therefore makes routine OAPP an expensive method for the detection of stool parasites.

1.3.1. Direct Fluorescent Antibody (DFA) Testing

Direct fluorescent antibody (DFA) testing is commercially available for the detection of both *Giardia* and *Cryptosporidium* species. Merifluor (Meridian Bioscience Inc., Cincinnati, OH) and PARA-TECT (Medical Chemical Corporation, Los Angeles, CA) are two commonly employed products currently on the market for this method (11). DFA is a microscopic test that utilizes fluorescently labeled monoclonal antibodies directed against parasite cell-wall antigens. Due to the difficulties associated with *Cryptosporidium* detection by OAPP, DFA is considered the test of choice for this parasite (11). Visualization of the *Giardia* and *Cryptosporidium* cysts is accomplished by fixing the stool specimen on a slide followed by staining with fluorescent monoclonal antibody and subsequent analysis using a fluorescent microscope (10). When present, *Giardia* and *Cryptosporidium* cysts demonstrate a bright apple-green fluorescence (10). Sensitivity of this method for the detection of *Giardia* ranges from 96-100%, with specificity ranging from 99.8-100% (10-13). The sensitivity of *Cryptosporidium* detection for this method ranges from 96-100%, with specificity ranging from 99.8-100% (10-13). Performance data is shown in Table 4.

<u>DFA Advantages</u>

Two major advantages of DFA are its excellent specificity and increased sensitivity over OAPP (12). With OAPP, both parasites and stool debris are stained making visualization of parasites difficult particularly if they are present in low quantities. However, the specific nature of the fluorescence with DFA dramatically reduces the amount of fecal material stained. Virtually none of the stool debris fluoresces allowing the parasites to be easily visualized even when only a few organisms are present (13).

DFA Disadvantages

As a microscopic method, DFA is associated with visualizing parasites present in the specimen. However, the cyclical nature of cyst shedding is one limitation for any staining method, including direct fluorescent staining (27). A secondary limitation is the requirement of a fluorescent scope, which may not be available in all clinical laboratories. DFA is also limited by detecting only *Giardia* and *Cryptosporidium* making it unsuitable for patients who are infected with other parasites.

1.3.2. Acid-Fast Staining

Acid-fast staining can be used for the detection of parasites and uses nonspecific stains like Auramine-O or Kinyoun stain. Both stains take advantage of parasite resistance to acid decolorizing in the staining methods, which allows the parasites to be differentiated from other organisms. Auramine-O is a fluorescent stain that when applied to *Cryptosporidium,* causes the cysts to appear apple green on a dark background. Reported sensitivities and specificities for the Auramine-O stain are 78.4% and 100%, respectively (13). Kinyoun stain utilizes two stains, carbol-fuchsin as the primary stain and methylene blue as the counterstain. When viewed under a light microscope, *Cryptosporidium* cysts appear red in a blue background. Reported sensitivities and specificities for this method are 54.8-97% and 94-100%, respectively (10, 12, 18, 21). Performance data for both methods is shown in Table 4.

Acid-Fast Stain Advantages

The non-specific nature of acid-fast staining has an advantage of detecting multiple parasites. Where DFA is specifically directed against cell-surface antigens of *Giardia* and *Cryptosporidium*, Auramine-O can be used to detect other stool parasites like *Cystoisospora* and *Cyclospora* in the same slide preparation saving time and materials. If present, size and shape of the organism can be used to help differentiate parasites. Both Auramine-O and Kinyoun stain can be used for identification of other microbial organisms (18). Stains with multiple applications reduce ordering costs (17). An additional advantage of Kinyoun stain is that slides are viewed under the light microscope so a specialized scope is not required for identification.

Acid-Fast Staining Disadvantages

Acid-fast staining has similar disadvantages to other microscopic methods in terms of parasite shedding (6, 7, 18). Auramine-O, like DFA, requires the use of a fluorescent scope. In addition, because Auramine-O is an indirect fluorescent stain, it is not specific. Background material can sometimes retain stain and multiple parasites can fluoresce, and therefore requires analysis by an experienced technologist (7). The Kinyoun staining method is reported as being both time-consuming and requiring an experienced eye as acid-fast staining lacks specificity for parasites. As a more general use stain, Kinyoun staining will appear positive in the presence of debris, bacteria and a variety of parasites. The small size and variably staining nature of the *Cryptosporidium* oocytes makes detection of these organisms difficult (12). Careful consideration of morphology must be employed with the use of both stains. (6)

1.3.3. Standard Enzyme Immunoassays (EIA)

Enzyme immunoassays provide a non-microscopic based method to detect the presence of parasite surface antigens rather than the parasites themselves (i.e. cysts and trophozoites). This is important as parasite antigens can be present without the visualization of infecting organism. A variety of assays are commercially from multiple manufacturers. ProSpecT (Remel, Lenexa, KS), *Giardia/Cryptosporidium* Chek (TechLab Inc., Blacksburg, VA), and can detect *Giardia* and *Cryptosporidium* antigens simultaneously. EIA's come as microplate wells labeled with anti-parasite antibodies. If antigen is present, it is captured by the antibodies present in the wells and a labeled conjugate is added forming a complex. After the addition of a substrate, a color change indicates the presence of the antibody-antigen complex. This change in color can be measured spectrophotometrically or by visual read.

Sensitivities for EIA methods vary by manufacturer but have reported ranges for *Giardia* and *Cryptosporidia* of 88-99.2% and 88-100%, respectively (6, 11, 14-16). Specificities for *Giardia* and *Cryptosporidia* have been reported as between 99-100% and 88-100%, respectively (6, 11, 14-16). See Table 4.

<u>EIA Advantages</u>

Since EIA's detect the presence of antigens instead of organism itself, the cyclical nature of parasite shedding does not interfere with diagnosis. It has been reported that infection can be diagnosed in patients with low parasite burden and that EIA sensitivity can exceed that of OAPP (1, 5, 8). Although, additional reports suggest that detection of *Cryptosporidium* is low in asymptomatic patients (19). Unlike microscopic methods, performance of the test does not require an experienced parasitologist to identify organism morphology. Furthermore, EIA kits routinely come with 96 well microplates allowing testing of multiple specimens simultaneously. (11, 14-16).

EIA Disadvantages

The performance of traditional EIA is time-consuming requiring multiple wash and incubation steps. In addition, the proximity and size of wells on the microplate can lead to carry-over of grossly positive specimens into neighboring patient wells. Reading of results can be done visually but is more accurately performed using a spectrophotometer, which may not be available in all laboratories (14, 15). Currently, available EIA's for the detection of both *Giardia* and *Cryptosporidium* fail to distinguish between the two parasites. Thus, a positive result for the dual testing method requires additional testing to differentiate between *Giardia* and *Cryptosporidium*. (11, 14-16). In comparison with DFA, there is some question about the limit of detection for *Cryptosporidia*. (12). Specimens with low numbers of *Cryptosporidium* oocytes have been reported to give false negative results by EIA (12, 18). In asymptomatic patients with low parasite numbers, there has been concern about using EIA as the sole detection method because of false negative reporting (12, 18).

1.3.4. Rapid Lateral Flow Immunoassays

Rapid chromatographic methods used to detect the presence of antigen are commercially available from a variety of manufacturers. In this study, three manufacturer's assays were evaluated: 1) ImmunoCard STAT! *Cryptosporidium/Giardia* Rapid Assay (Meridian Bioscience Inc.), 2) Xpect *Giardia/Cryptosporidium* (Remel), and 3) *Cryptosporidium/Giardia* Quik Chek (TechLab Inc.). All three assays simultaneously detect the presence of *Giardia* and *Cryptosporidium* antigens. Rapid immunological methods utilize a solid-phase qualitative immuno-chromatographic assay to binds antigen specific for each parasite using an antibody-conjugate complex. With the addition of substrate, colored bars indicate a positive result when antigen is present. Testing cartridges are single use and come with internal controls.

Reported sensitivities for rapid lateral flow immunoassay methods range from 56-100% for *Giardia* and from 58-98.8% for *Cryptosporidium* (11-16, 21). Specificities for *Giardia* range from 44% to 100% with *Cryptosporidium* specificities ranging from 50% to 100% as shown in Table 4 (11-16, 21). In addition, performance data for the individual manufacturers is shown in Table 6. The large variability seen in sensitivity and specificity testing was generally reported for assays when testing was done by competing manufacturers or when PCR was used as the study's reference method (16, 21). Performance data for the specific manufacturers are listed in Table 6.

<u>Rapid Lateral Flow Assay Advantages</u>

As their name suggests, rapid methods can be performed quickly. One advantage in rapid testing is decreased testing time compared with current methods. As noted above, some assays require as little as ten minutes of hands-on time per specimen. Since, testing cartridges are single use and come with internal controls, batching of multiple specimens is not required enabling STAT ordering if time sensitive results are required in the case of potential outbreaks. As with traditional EIA's, rapid methods do not rely on parasite visualization for diagnosis, and thus these methods do not require expertise in parasitology. A spectrophotometer is not required for these rapid immunoassays because unlike standard EIA, visually reading of results is sufficient.

<u>Rapid Lateral Flow Assay Disadvantages</u>

A significant disadvantage of the rapid detection methods compared with traditional EIA's is cost: Test kits come with individually wrapped test cartridges rather than the 96-well plates seen with traditional EIA's. Although these cartridges are single use only and are often double the expense of an EIA, the testing costs are comparable to OAPP because of decreased testing time per specimen.

In comparison with DFA there is some concern about the limit of detection, which could be significant in asymptomatic populations (12). When low parasite numbers are present, antigen levels fall below the detection limit of these methods, which causes the reporting of false negative results (12). In a study by Johnston *et. al*, when *Cryptosporidium* cysts were seen only rarely by DFA, rapid methods did not detect the presence of antigen. (12 Specifically for *Giardia*, the presence of trophozoites alone can also lead to false negative reporting by rapid methods (13). Furthermore, rapid methods are specifically designed to detect the two most common species of *Cryptosporidium* reported in humans, *C. parvum* and *C. hominis*. As mentioned earlier, other species of *Cryptosporidium* also cause disease in humans (27). According to reports by the manufacturers, there is some question as to whether the rapid methods would be able to detect these species (22-24) although this is an area that requires additional research. When immunoassay methods have been tested against species other than *C. parvum* and *C. hominis* a decrease in the sensitivity of the assays has been observed (19).

1.4. Statement of Purpose

1.4.1. Site Information

The University of Washington Medical Center (UWMC) in Seattle, Washington is a 500-bed tertiary care academic medical center located in Seattle, WA. The Clinical Microbiology laboratory at UWMC receives specimens for testing from regional and national hospitals, and serves as a reference laboratory in the areas of molecular microbiology, mycology, bacteriology and parasitology.

1.4.2. <u>Evaluation of Current Testing Methods and Consideration of Alternative Testing Methods for the Detection of Intestinal Parasites</u>

The UWMC Clinical Microbiology laboratory is currently evaluating testing methods for the detection of intestinal parasites. The two parasites of specific interest are *Giardia intestinalis* and *Cryptosporidium* spp. because of their regional prevalence. In a 2010 report by the CDC on *Giardia* and *Cryptosporidium*, providers were encouraged to order testing specifically for both *Giardia* and *Cryptosporidium* when gastrointestinal illness was suspected in their patients (1). Current methods for evaluation of stool for parasites at the UWMC Clinical Microbiology Laboratory include standard Ova and Parasite exam (OAPP) by concentrate and trichrome stain, *Cryptosporidium* (CYCLOP) exam by Auramine-O fluorescent stain and a *Giardia* EIA (SGRDAG) utilizing a standard EIA kit.

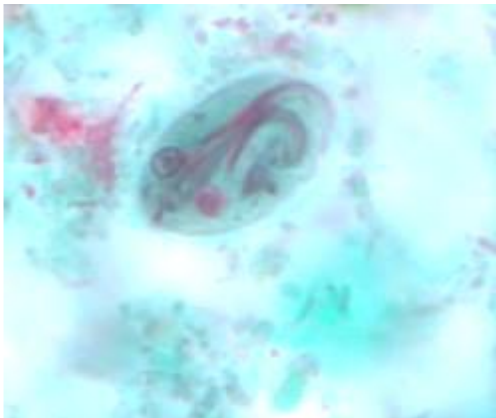
Alternative methods under consideration for the detection of *Giardia* and *Cryptosporidium* included three rapid lateral flow immunoassays. Evaluation and comparison of both current and proposed methods was based on the following criteria: 1) Evaluation of provider ordering practices for current methods using retrospective data, 2) Evaluation of proposed methods usability by comparison of the three rapid lateral flow immunoassays with respect to ease of use and interpretation of results, 3) Comparison of the labor costs, turn-around-time (TAT) and total processing time (TPT) between the current and proposed methods, and 4) Evaluation of the performance of proposed methods. Furthermore, selection of a rapid lateral flow immunoassay for additional validation and plans for the implementation of a new ordering scheme for stool parasites were also established.

TABLE 1. Geographic Distribution of Common Intestinal Parasites Detected in International Refugee Populations Currently Living in the United States

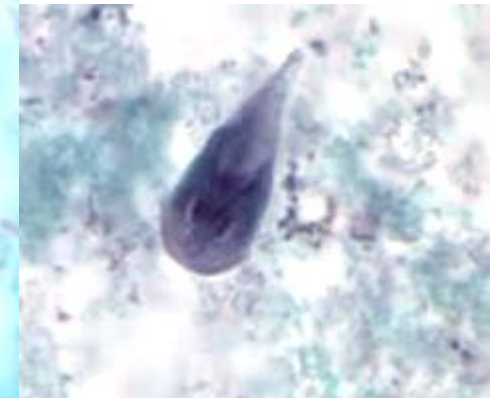
Global	**Africa**	**Asia**	**Latin America**	**Middle East**	**Eastern Europe**
Ascaris lumbricoides *Trichuris trichiura* *Strongyloides stercoralis* *Enterobius Vermicularis* *Fasciola* *Hymenolepis* *Cryptosporidium* Most protozoa, especially *Giardia intestinalis*	*Schistosoma mansoni haematobium intercalatum*	*Fasciolopsis buski*	*Taenia solium* *Schistosoma mansoni*	*Echinococcus* spp. *Giardia* spp.	*Diphyllobothrium latum* *Opisthorchis felineus*
	Ethiopia and Eritrea:	Southeast Asia:	Ecuador:		
	Taenia saginata	*Opisthorchis viverrini* *Clonorchis sinensis* *Schistosoma japonicum mekongi*	*Opisthorchis guayaquilensis*		
		South Asia:			
		Taenia solium			

Adapted from the Centers for Disease Control, “Intestinal parasite guidelines for domestic medical examination for newly arrived refugees.” Organisms listed are either unique to that particular location or, particularly common or over-represented in refugee populations. (27)

FIGURE 1. CDC Images of *Giardia intestinalis* (5)



Giardia intestinalis cyst stained with a trichrome stain.



Giardia intestinalis trophozoite stained with a trichrome stain.

FIGURE 2. CDC Lifecycle for *Giardia intestinalis* (5)

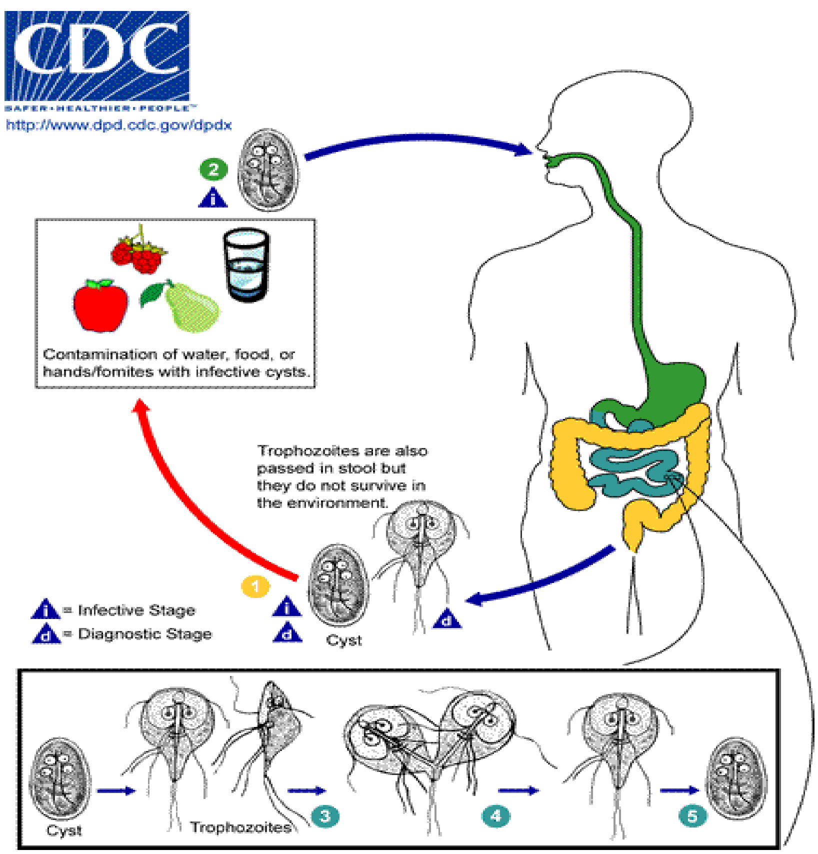


FIGURE 3. Image of *Cryptosporidium* Oocytes Stained with Auramine-O Fluorescent Stain (51)

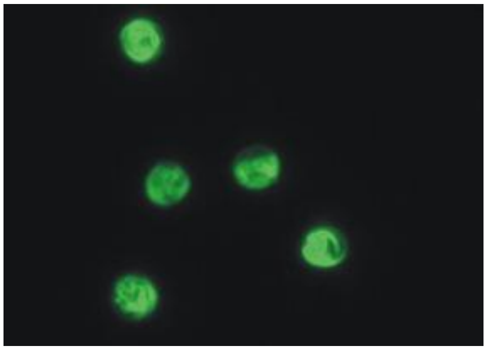


FIGURE 4. CDC Lifecycle of *Cryptosporidium* spp. (5)

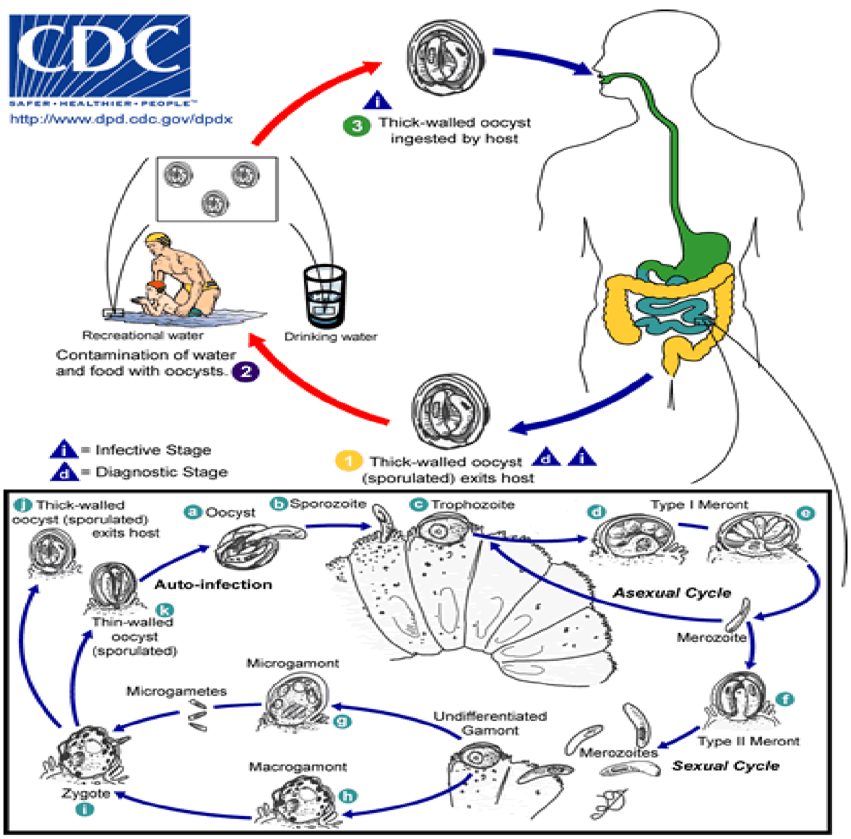


TABLE 2. Comparison of Commonly Used Detection Methods in the Diagnosis of Intestinal Parasites

Detection Method	Type of Detection	Stool Parasites Commonly Detected
Ova and Parasite Exam	Microscopic	Cestodes, Nematodes, Trematodes, Flagellates, Protozoans, Amebae, Microsporidiae, Protists
Direct Fluorescent Antibody	Microscopic Immunodetection	*Giardia*, *Cryptosporidia*
Acid-Fast Staining:	Microscopic:	
Auramine-O Stain	Fluorescent	*Cryptosporidia*, *Cyclospora*, *Cystoisospora*
Kinyoun Stain	Light	*Cryptosporidia*, *Cyclospora*, *Cystoisospora*
Enzyme Immunoassays	Immunoassay	*Giardia*, *Cryptosporidia*, *Entamoeba sp.*
Rapid Lateral Flow Assays	Immunoassay	*Giardia*, *Cryptosporidia*, *Entamoeba sp.*

TABLE 3. Advantages and Disadvantages of Parasite Detection Methods

Detection Method	Advantages	Disadvantages
Ova and Parasite Exam	- Detects multiple parasites - Allows for long-term storage of specimen - Uses standard light microscope	- Detection limited by parasite shedding - Not suitable for *Cryptosporidium* detection - High labor costs for intensive processing and analysis -Requires experienced technologist
Direct Fluorescent Antibody	- Excellent sensitivity and specificity reported - Can detect both *Giardia* and *Cryptosporidium*	- Detection limited by parasite shedding - Requires fluorescent scope - Limited to only detecting *Giardia* and *Cryptosporidium* -Requires experienced technologist
Auramine-O Stain	- Detects multiple parasites - Stain can be used for other organism types (*Mycobacterium* spp.)	- Detection limited by parasite shedding - Requires fluorescent scope - Fluorescence is non-specific - Requires experienced technologist
Kinyoun Stain	- Stain can be used for other organism types (*Mycobacterium* spp.) - Uses standard light microscope	- Detection limited by parasite shedding - Poor sensitivity and specificity reported - Staining is time-consuming - Requires experienced technologist
Enzyme Immunoassays	- Detects antigen, thus not limited by parasite shedding - Does not require an experienced technologist - Can detect both *Giardia* and *Cryptosporidium* - Can analyze multiple specimens at once	- Labor intensive processing - Requires additional testing to differentiate positive result - Carry-over of specimen can yield false positive results - Limited to only detecting *Giardia* and *Cryptosporidium*
Rapid Lateral Flow Assays	- Detects antigen, not limited by parasite shedding -Does not require an experienced technologist -Can detect both *Giardia* and *Cryptosporidium* -Hands-on time as little as 10 min.	- Single test cartridges increase materials cost - Limited to only detecting *Giardia* and *Cryptosporidium* - May not detect all species of *Cryptosporidium*

TABLE 4. Reported Sensitivities and Specificities of Available Parasite Detection Methods

METHOD	Sensitivity	Specificity	Sensitivity	Specificity
	Giardia		*Cryptosporidium*	
OAPP	58-100%	93.2-99.9%	N/A	N/A
DFA	96-100	99.8-100	96-100	99.8-100
Auramine-O	N/A	N/A	78.4%	100%
Kinyoun	N/A	N/A	54.8-97%	94-100%
EIA	88-99.2%	99-100%	88-98.9%	88-100%
Rapid Assays	56-95.8%	44-100%	58-100%	50-100%

Reported performances (9, 11, 13-14, 16-17, 25, 30)

TABLE 5. Sensitivity of the Ova and Parasite Examination as a Function of Number of Stool Specimens Tested

# of Stools	Sensitivity
1	~58%
2	~79%
3	~100%

Adapted from, "The need for three stool specimens in routine laboratory examinations for intestinal parasites." Nazer, *et. al*, 1993. (30)

TABLE 6. Reported Sensitivities and Specificities of Available Rapid Lateral Flow Assays

	Giardia		*Cryptosporidium*	
Manufacturer	Sensitivity	Specificity	Sensitivity	Specificity
Meridian	78-97.3%	44-100%	67.6-100%	50-100%
Remel	56-95.8%	78-98.5%	58-96.4%	97-100%
TechLab	78-98.9%	100%	99.8-100%	99.8-100%

Reported performances (11-16, 21)

2. Retrospective Analysis of Provider Ordering Practices for Intestinal Parasite Testing

2.1. Goals of the Retrospective Analysis

The ordering practices of providers for intestinal parasite testing at UWMC were previously unknown. We therefore intended to assess the following by examining retrospective testing data over a three year period (1/2009 to 12/2011): 1) Whether providers were following CDC recommendations to order specific fecal immunoassays for *Giardia* and routine ordering of testing for *Cryptosporidium*, and 2) To ascertain whether providers were following the "three-stool-rule" recommendation when ordering OAPP for suspicion of diarrheal disease.

2.2. Materials and Methods

2.2.1. Current Testing Methods

Testing methods available for ordering by providers during the retrospective study included standard Ova and Parasite exam (OAPP) by concentrate and trichrome stain, *Cryptosporidium* (CYCLOP) exam by Auramine-O fluorescent stain and a *Giardia* EIA (SGRDAG) utilizing a standard EIA kit. The procedures for diagnostic testing by these methods follows:

Ova and Parasite (OAPP) Microscopic Method

Two methods were used for the microscopic examination of stool specimens for intestinal parasites: 1) Stool concentration for wet mount and 2) Permanent staining. Concentration of the stool for wet mount was accomplished using formalin-ethyl acetate sedimentation. Concentration allows for the recovery of protozoans, ova and larvae in the specimen, and specimens suitable for concentration include stools preserved in either ECOFIX or formalin. Fresh specimens were placed into ECOFIX prior to concentration. Importantly, formalin preserved specimens can be used only for stool concentration for wet mount and not for trichrome staining. Wet mounts were prepared on 3x2 inch glass slides by placing two drops of concentrated specimen on the slide. Saline was added to the first drop and either iodine or methylene blue was added to the second drop. Cover slips were placed on each drop prior to reading the slide on a light microscope. The slide was scanned first using the 10x objective. Larger parasites such as ova and larvae were viewed more easily at this lower magnification. Then the slide was observed under a light microscope at 40X magnification. If any organisms suspicious for protozoans were observed, the area of interest was then examined the higher magnification. (32)

Permanent staining was accomplished utilizing trichrome staining. Trichrome staining allowed for the visualization of parasites and their intracellular components. The PARA-PAK ECOSTAIN (Meridian Bioscience Inc.) was the trichrome stain used. Specimens suitable for trichrome staining include stools preserved in ECOFIX and non-mercury based polyvinyl alcohol (PVA) fixatives. Fresh specimens were first placed into ECOFIX before staining. PVA fixed specimens can only be used for trichrome staining and not for stool concentration for wet mount. Trichrome stained slides were examined microscopically with the 100X objective, with two to three hundred microscopic fields observed for each specimen. A control slide made from a specimen previously positive for *Giardia* was also prepared using the trichrome permanent stain method (33).

Auramine-O Fluorescent Staining Method (CYCLOP) for the Detection of *Cryptosporidium*:

CYCLOP testing was performed on fresh or formalin-fixed stools for the detection of *Cryptosporidium* spp. To perform this testing, stool was first thinly applied to a microscopic slide and then dried on a 55-65^{o}C heating block for fifteen minutes. Auramine-O stain (Medical

Chemical Corporation, Los Angeles, CA) was applied to the slide and was allowed to incubate for fifteen minutes at room temperature. The slide was then rinsed with tap water and acid alcohol decolorizer (Medical Chemical Corporation) was applied. After two minutes, the slide was rinsed again with tap water. A counter stain of potassium permanganate (Medical Chemical Corporation) was applied for three minutes before the final tap water rinse step. The slide was air dried and examined using an epifluorescent microscope equipped with an excitation filter that transmits at 250-400nm through a high emission light barrier filter. A control slide using acid-fast bacilli as the positive organism was prepared alongside patient slides using the same procedure (36).

<u>Remel Prospect Giardia Enzyme Immunoassay Method (SGRDAG):</u>

The Remel Prospect Giardia EIA kit (Remel, Lenexa, KS) was used in accordance with the manufacturer's directions. Solid stool was diluted with specimen dilution buffer. Liquid and preserved stool did not require dilution. This assay comes with a 96 well microplate coated with rabbit anti-GSA 65 antibody. After specimens and controls were added to individual wells, multiple wash and incubation steps were required. An enzyme conjugate containing horseradish peroxidase labeled mouse monoclonal anti-GSA was first added to each well and then a colored substrate. At the end of the last incubation, a stop solution was added and the reactions in the wells were read spectrophotometrically by an ELISA reader at 450nm within 10 minutes. Absorbance readings of 0.050AU and above indicated the presence of GSA 65 corresponding to the detection of *Giardia* antigen. Absorbance readings less than 0.050 indicated the contained no detectable presence of *Giardia* antigen. Use of positive and negative controls was required to confirm successful assay results (35).

2.2.2. Collection of Retrospective Data for the Evaluation of Provider Ordering Trends

Data for a three-year period from1/2009-12/2011 was collected using the laboratory's information system (LIS). The LIS was queried for the results of all OAPP, CYCLOP and SGRDAG ordered during the stated time period. Patients included in the study were from inpatient and outpatient locations. Providers ordering testing included medical doctors, physician's assistants and registered nurse practitioners. The number of positive and negative results were tallied for each order category. For OAPP, additional variables of specific parasites present and pathogenicity of parasites as classified by the CDC were also tallied. Data for a two-year period from 1/2009-12/2010 was also collected from patients for whom both OAPP and SGDRAG were ordered in order to compare detection of *Giardia*. Specimens positive for *Giardia* should be positive by both methods and discrepant test results were tallied.

2.3. Results

In a three-year period from 1/2009 to 12/2011, over 5400 specimens were received for OAPP, 1160 specimens were received for CYCLOP and over 3280 specimens were received for SGRDAG. The results of this retrospective analysis are shown in Tables 7 and 8. Of those specimens received for OAPP, approximately 11% were noted to have parasites present. However, only ~4% of specimens were positive for parasites that are classified as pathogenic by the CDC and thus are considered clinically significant (4). Of the clinically significant parasites, 3% were positive with *Giardia* infections. Other clinically significant parasites such as *Entamoeba histolytica/dispar* and intestinal worms represented 1.2% of all positive exams. *Dientamoeba fragilis,* which is known to be clinically significant in children, represented 26% of the positives with the majority (67%) of those being submitted by the pediatric clinic. Interestingly, only 0.5% of specimens received for CYCLOP testing were positive for *Cryptosporidium*. For *Giardia* testing by EIA, about 6% of specimens submitted were positive.

In a two-year period, from 1/2009-12/2010, 109 specimens were received for both OAPP and SGRDAG testing. Forty-one were positive for *Giardia* by one or both methods. Eighty-five percent of the positives were detected by both methods and were tallied as single stool submissions. One specimen was found to be positive by OAPP and negative by SGRADG and 5 specimens were found to be positive by SGRDAG but negative by OAPP, as shown in Table 9.

2.4. Discussion

When the retrospective data was evaluated, it became evident that the CDC recommendation in regards to ordering tests specifically and routinely for the detection of *Giardia* and *Cryptosporidium* is not being followed by providers. For *Giardia* detection, of the over 5400 OAPP's ordered during the three year period only 60% of orders had tests specifically for *Giardia*. Data from the two-year period, points to the importance of fecal immunoassays for Giardia detection because of the 41 positive specimens, 12% were positive by SGRDAG and negative by OAPP, while only 4% positive by OAPP were negative by SGRDAG.

Our findings are significant because of the debate in the literature regarding the number of stools that should be submitted for EIA testing for intestinal parasites. Similar to the recommendation of the "three-stool-rule" for OAPP, it is recommended by some that two stools be submitted for immunoassay testing in order to increase sensitivities in these methods (25, 37, 38). These recommendations are based on a study by Hanson and Cartwright that looked at specimens submitted for testing with EIA (38). The authors concluded that even though EIA sensitivity exceeded OAPP for a single specimen in symptomatic patients (83 versus 75%, respectively), performance was poorer in asymptomatic patients (77 versus 61%, respectively). In neither case did sensitivity exceed 90% (38). The reduced sensitivity observed in their study raised the question as to whether a single stool specimen is sufficient to rule out the presence of *Giardia* or *Cryptosporidium*. Thus, the recommendation to submit two stool specimens for EIA testing was made because the sensitivity of a single EIA, whether patients are symptomatic or asymptomatic (80% combined), is not comparable to the sensitivity of paired OAPP specimens (>90%).

Interestingly, a study performed by Mank *et. al* disputes Hanson and Cartwright's findings, stating that a single stool submission for EIA is more sensitive (92.7%) than a single stool submitted for OAPP (80%) and is almost as sensitive (92.7%) as two OAPP submissions (96.4%). Therefore, Mank et *al* argue that a single EIA is sufficient for the detection of *Giardia* antigen given the increase in cost-effectiveness and reduced treatment time that EIA affords even when compared to two stool submissions by OAPP (8).

The conflicting performance data observed in the two aforementioned studies may be the result of differing patient populations. Specifically, over half of the specimens tested by Hanson and Cartwright were from asymptomatic patients receiving health screens because of recent immigration to the U.S. (39). The recommendation stated by Hanson and Cartwright is conditional on the population served by a laboratory. Patient populations of asymptomatic individuals are served best when two stools are submitted for testing. In contrast, for those laboratories for whom the patient population has a low prevalence of clinically significant parasites overall where *Giardia* accounts for the majority of parasites detected, they state that the use of EIA rather than OAPP may be sufficient (38). Mank et *al* further contend that testing by EIA may be preferable for these populations because of reduced TAT and labor costs (38). The patient populations seen in this study follows this criteria for low prevalence and this laboratory have thus found success in implementing EIA as a primary testing method (8). Our retrospective data is in agreement with the sensitivities reported by Mank et *al*, which is somewhat expected given the low prevalence for clinically significant parasites in our patient population, with *Giardia* being the parasite most commonly detected.

Another ordering recommendation not being followed by providers is that of routine ordering for *Cryptosporidium* testing. Even though 5400 stools were received for OAPP, only 1160 specimens were received for *Cryptosporidium* testing. This is significant considering the importance of these parasites to our region and particularly the large number of immunocompromised patients seen in our institution. The incidence of *Cryptosporidium* observed in the UWMC Clinical Microbiology Laboratory are very low, far lower than that seen by the Washington state as a whole (2). While this may simply be a reflection of the regional differences in our state, there is therefore some concern that the dearth of ordering may also be influencing this trend. Testing for *Cryptosporidium* is currently offered separately from OAPP testing and is not performed for every stool specimen received for testing. As the CDC recommends more comprehensive testing for *Cryptosporidium* due to concerns that cryptosporidiosis is being missed with common testing methods, the investigation of the retrospective data made evident our need to evaluate our current testing options and to consider alternative methods to help providers better follow this recommendation.

Evaluation of compliance with the "three-stool-rule" was also conducted using the retrospective data. Of the over 5400 stools submitted for testing by OAPP during the three-year period, 70% submissions were of a single stool specimen, 8% were two stool submissions and 22% were three stool submissions, as shown in Figure 5. Given the sensitivity data for a single stool submission reported by Nazer et *al*, the lack of three stool submissions for our patients represents the potential for vast underreporting of intestinal parasites. As stated earlier, the number of pathogenic parasites seen in the retrospective data was low at only ~4%. The rate of positivity versus the number of specimens submitted may be explained by 70% of specimens being single stool submissions. Considering that the majority of pathogenic parasites detected are *Giardia,* implementation of testing more specific for this parasite maybe help in an environment where OAPPs are not being ordered correctly.

TABLE 7. Summary of Ova and Parasite Testing Practices at UWMC, 2009-2011

Testing Method	Specimens Received	Stools Positive by each Method	% Positive
OAPP	5406	576	11%
CYCLOP	1160	6	0.5%
SGRDAG	3287	187	6%

TABLE 8. Detailed Results of Ova and Parasite Testing at UWMC, 2009-2011

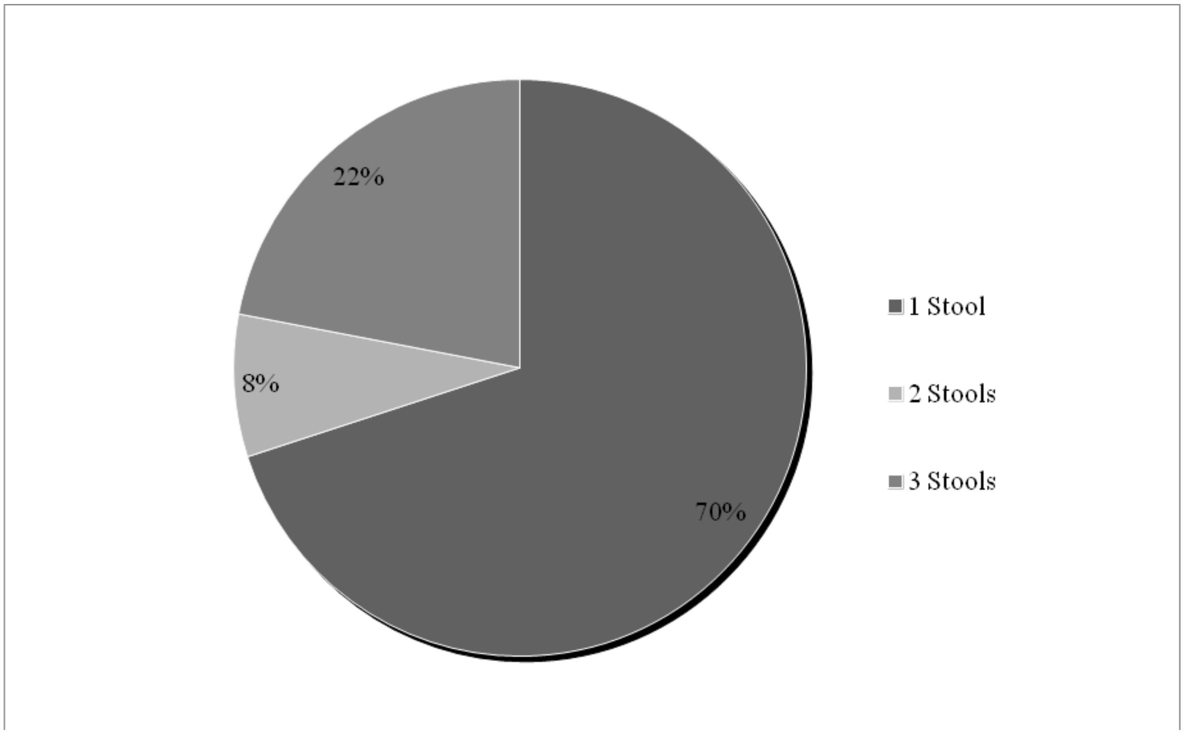
OAPP's Received	Total # Parasite Positive	Total # Pathogenic Parasites	*Giardia*	*Dientamoeba fragilis*	Worms*	*Entamoeba histolytica/dispar*
5406	576 (11%)	234 (4%)	160 (68%)	62 (26%)	10 (2%)	2 (1%)

*Worms: *Strongyloides*, *Taenia*, *Ascaris*
Pathogenic parasites were defined by CDC classification.

TABLE 9. *Giardia* Detection Results for Specimens Received for Both OAPP and SGRDAG Testing 2009-2010

METHODS	OAPP		
SGDRAG	Positive	Negative	
Positive	35	5	Total Specimens Tested
Negative	1	68	109

FIGURE 5. Stool Specimens Received for OAPP 2009-2011



3. Prospective Study Comparison of Current and Proposed Testing Methods

In an effort to be compliant with CDC testing recommendations, decrease labor costs and total processing time (TPT), and improve turn-around-time (TAT), alternative testing options for the detection of *Giardia* and *Cryptosporidium* were considered for the UWMC Clinical Microbiology Laboratory. After consideration of the many products on the market, the scope of this project has been narrowed to the testing of rapid lateral flow immunoassays currently available from manufacturers. These assays offer the potential to meet the aforementioned goals in a single test. Three separate kits from three different manufacturers were chosen for simultaneous evaluation. The three kits to be evaluated were the ImmunoCard STAT! *Cryptosporidium/Giardia* Rapid Assay from Meridian, the Xpect *Giardia/Cryptosporidium* from Remel, and the *Giardia/Cryptosporidium* Quik Chek from TechLab Inc. Images of the kit cartridges are shown in Figure 6.   Procedurally, all three kits differ in terms of testing time, ease of use and ease of interpretation. We therefore wished to monitor these differences and to determine which of the three kits would best suit the needs of the UWMC Clinical Microbiology laboratory. The rapid kit that performed the best would be considered for validation for use in our laboratory as a primary means of detecting *Giardia* and *Cryptosporidium*.

There are several potential benefits of rapid lateral flow immunoassays compared with the current testing being performed in the UWMC Clinical Microbiology Laboratory. Firstly, the availability of rapid assays would aid providers in following the CDC ordering recommendations for diarrheal illness because these tests simultaneously test for *Giardia* and *Cryptosporidium*. With our current ordering scheme, *Cryptosporidium* ordering is occurring less than 25% of the time that specimens are being sent for OAPP, and of those specimens sent for OAPP, only 22%

are following the "three-stool-rule" recommendation. Rapid assay testing would allow the laboratory to meet these guidelines. Another potential benefit is ease of testing and interpretation, defined as usability. The tests require far less processing and hands-on time than current methods and have results that are significantly more easily interpreted. This would allow for performance testing by technologists less experienced or lacking experience in the morphological identification of parasites.

An additional point of consideration is that of cost and time. Evaluation for *Giardia* and *Cryptosporidium* using the rapid kits is faster than current methods. The "three-stool-rule" recommended to increase sensitivity for OAPP testing is not necessary as most reported sensitivities for rapid methods are higher with a single specimen (11-14, 16, 21-24). While low numbers of parasites have been shown to lead to false negative reporting in these assays, the rapid assay could serve as primary testing with secondary testing being performed if patients are still symptomatic after a negative test result (12, 13). Faster TAT leads to faster reporting of results to the state lab, which is a significant consideration given that infection with either of these parasites is a reportable condition.

Finally, these cartridges are single use only and are often double the expense of the current *Giardia* EIA. However, the concomitant reduction in the amount of technologist time required to perform these assays may lead to a decrease in labor costs despite reagent costs. Therefore these methods may still be less expensive than current OAPP testing. Comparison of time and cost of these new methods was therefore included in our analysis.

Certainly, the most important consideration when evaluating these rapid assays is test performance. Firstly, do the proposed methods have comparable sensitivities and specificities to the current methods? The benefits of TAT and decreased technologist time are only valuable if the results are accurate. Despite their similar methodologies, the sensitivity and specificity for each kit varies with reported values ranging from 56-100% and from 44% -100%, respectively, as shown in Table 6 (11-14, 16, 21-24). The current reported ranges are wide, and therefore require extensive in-house verification and validation prior to routine use.

It is with these considerations that we elected to evaluate three new rapid lateral flow immunoassays. If these assays were to prove accurate, timely and cost effective, the best performing kit would be chosen for validation. This new testing method would be made available as an alternative testing option for providers who are seeking to rule-out the most regionally significant parasites in their patients who are suspected of having intestinal parasite infections.

3.1. Materials and Methods

3.1.1. Current Reference Testing Methods

The Ova and Parasite (OAPP) exam by concentrate and trichrome stain, *Cryptosporidium* exam by Auramine-O fluorescent stain (CYCLOP), and the Remel Prospect *Giardia* EIA utilizing a standard EIA kit (SGRDAG) are the current methods in use at the UWMC Clinical Microbiology Laboratory and were considered the reference methods for this study.

3.1.2. Proposed Testing Methods

All tests were performed with unconcentrated specimens either unpreserved fresh/frozen or preserved in 10% formalin or Cary Blair media (Becton, Dickinson and Company, Franklin Lakes NJ).

Meridian Bioscience Inc. ImmunoCard STAT! *Giardia/Cryptosporidium* Rapid Assay

The Meridian Bioscience Inc. ImmunoCard STAT! *Cryptosporidium/Giardia* Rapid Assay was used in accordance with the manufacturer's directions. Unpreserved stool specimens required a 1:4 dilution prior to testing. Diluted stools and those in manufacturer approved preservatives were added to a tube containing two drops of sample treatment buffer. Meridian Bioscience Inc. utilize two reagents to detect both *Giardia* and *Cryptosporidium* antigens. The first reagent is a biotinylated anti-*Giardia* capture antibodies and the second reagent contains monoclonal antibodies to both *Giardia* and *Cryptosporidium* labeled with a dye. This mixture was loaded onto the test cartridge and incubated for ten minutes. During that time parasite antigens were captured by the cartridge and lines corresponding to *Giardia* and *Cryptosporidium* appeared when antigen was present. The dye was captured by the internal control line indicating a successful test. Total testing time is approximately twenty minutes per specimen with ten minutes of hand-on time required. (22)

Remel Xpect *Giardia/Cryptosporidium* Rapid Assay

Testing with Remel *Xpect Giardia/Cryptosporidium* was performed in accordance with the manufacturer's directions. Unpreserved specimens required a 1:4 dilution before unspun stool specimen was added to a tube containing specimen dilution buffer. The Remel kit utilized a single conjugate reagent containing specific *Giardia* and *Cryptosporidium* colored micro particles linked to murine monoclonal antibodies. Conjugate was added to the specimen and the resulting solution was loaded onto the kit cartridge. After a fifteen minute incubation step, any *Giardia* or *Cryptosporidium* antigens present were captured on discreet test lines containing antibodies specific for each organism. An internal control line was present for reporting to occur.

Total testing time is approximately 25 minutes per specimen with ten minutes of hands-on time required. (23)

TechLab Inc. *Giardia/Cryptosporidium* Quik Chek Rapid Assay

Performance of TechLab Inc. *Giardia/Cryptosporidium* Quik Chek was in accordance with the directions of the manufacturer. To begin, diluent, conjugate and preserved or unpreserved unspun stool specimen was added to a testing tube. This mixture was added to the testing cartridge. The TechLab Inc. conjugate consisted of polyclonal antibodies. During the first incubation, the antigens in the specimen bound to the conjugate antibodies forming a complex that migrated across the test cartridge. The complexes were captured by *Giardia* and *Cryptosporidium* specific antibodies onto discreet test lines. After a wash step and the addition of substrate, another incubation occurred where a colored substrate bound to any captured complexes created a colored bar along the corresponding test line. The internal control line was also visible for test reporting. Total testing time is approximately 37 minutes per specimen with twelve minutes of hands-on testing required. (24)

3.1.3. Specimens (Prospective Study)

For a three month period in 2012, stool specimens that were submitted to the UWMC and Harborview Medical Center (HMC) Microbiology laboratories for clinical testing were prospectively saved for evaluation. These stool specimens were collected from both inpatient and outpatient locations within the University of Washington Medicine (UW Medicine) system and were obtained from patients of all ages in whom intestinal illness was suspected by the ordering clinician. Stool specimens were submitted either as unpreserved fresh-frozen specimens or preserved in 10% formalin, ECOFIX or Cary Blair medium. Nine specimens were obtained from patients for testing during this time period. Three of these specimens were positive for *Giardia* and 1 was positive for *Cryptosporidium.*

Additional specimens for evaluation were acquired from two commercially available challenge panels. Challenge panels consisted of stools preserved in formalin and were intended for kit validation. These panels were utilized in this study to increase the numbers of positive specimens available for testing in our evaluation given the low positivity rate encountered with patient specimens. Both the TechLab Inc. and Meridian Bioscience Inc. challenge panel specimens were used in this study. Twenty-two challenge panel specimens were tested by the proposed methods. Eleven were positive for *Giardia*, 9 were positive for *Cryptosporidium* and 2 specimens were positive for both organisms. Importantly, challenge panel specimens were not able to be tested using the laboratory's reference methods. The TechLab Inc. challenge panel specimens were unsuitable for OAPP or CYCLOP because they did not contain actual stool, but rather parasite antigens isolated from positive stools. The Meridian Bioscience Inc. challenge panel specimen volume was insufficient for OAPP or CYCLOP tests. In addition, the formalin preservative used by both manufacturers was inappropriate for both SGRDAG and trichrome staining for OAPP.

A total of thirty-one patient and challenge specimens were tested by the proposed methods, as shown in Table 10. The results of the patient specimens and the manufacturer-submitted specimens were known prior to testing with the proposed methods. The testing data were nominal with nonparametric qualities. Descriptive statistics were used to analyze the data.

TABLE 10. Specimens Used for the Method Comparison Study

Origin of Specimens	**Positive Specimens**		**Negative Specimens**	**Total Number**
	Giardia	*Cryptosporidium*		
Patient	3	1	5	9
Challenge Panel	11	9	4	22*
Total # of Specimens Included				31

*Two Challenge Panel specimens were positive for both *Giardia* and *Cryptosporidium*

FIGURE 6. Rapid Lateral Flow Immunoassay Manufacturer's Images

Meridian Bioscience Inc. ImmunoCard STAT! *Cryptosporidium/Giardia* Rapid Assay (22)
Remel Xpect *Giardia/Cryptosporidium* (23)

TechLab Inc. *Giardia/Cryptosporidium* Quik Chek (24)

3.2. <u>Proposed Methods Usability Survey</u>

3.2.1. Goals of Usability Survey

As stated previously, usability was defined as the ease of testing and ease of interpretation of the proposed methods. Based on daily, clinical laboratory usage, it has been reported that the rapid lateral flow immunoassay kits perform similarly to one another, thus the criteria of usability by which to judge between the kits was established (25). With this goal in mind, a comparison of usability between the three kits was performed.

3.2.2. Materials and Methods

Ten laboratory technologists currently working in the UWMC Clinical Microbiology Laboratory agreed to participate in a survey designed to ascertain the ease of use for each of the three lateral flow kits to be evaluated. Seven of the ten participants are board certified American Society of Clinical Pathologists (ASCP) medical laboratory generalists capable of working in all areas of the medical laboratory. Two participants are ASCP medical laboratory specialists in microbiology. Participants had experience in the clinical laboratory ranging from 3-28 years with a median of 8 years.

All participants performed testing of a single challenge panel specimen on all three proposed method kits. After performing the requisite testing, participants then completed a usability survey addressing the ease of use and interpretation of results of the three kits. The survey used is shown in Table 11. They were also asked to rate the kits in the order of preference for daily use and to list any comments or concerns they had regarding any of the kits. The survey data were ordinal and were analyzed using descriptive statistics.

3.2.3. Results

Participation by members of the laboratory was elicited to compare usage preferences between the proposed methods. Each manufacturer's kit was subjectively rated according to three criteria by participating laboratorians: 1) *Ease of use*. This referred to the clarity of directions, the amount of specimen processing required, the simplicity of specimen testing, and the time required for testing for each kit individually, 2) *Ease of interpretation of results*. This addressed the visibility of result lines for each individual kit, and 3) *Choice for daily use*. This criterion required participants to rank the three kits by preference for daily use and took into account the preferences from the first two criteria. A comments section for routine testing was also included to capture specific comments from the participants regarding the three kits. Results were tabulated to give an individual percentage rating for ease of use and interpretation, and a cross-kit comparison percentage rating for choice for daily use. All three criteria were calculated to give an overall score and the comments received were recorded below, as shown in Table 12.

The Meridian Bioscience Inc. kit scored 93% for "ease of use" because although testing was simple and testing time was short, the directions were found to be unclear by some participants. "Ease of interpretation" scored 90% because the visibility of lines was good. The "choice for daily" use was highest (87%) between kits primarily because of the short testing time. This gave Meridian Bioscience Inc. lateral flow kit the highest overall score of 90%. Remel scored excellently on ease of use (100%) because the directions were very clear, testing was simple and test time was short. For ease of interpretation Remel scored poorly (63%) because of two false negative results. In addition, two other results had extremely faint that were difficult to interpret. Despite the high ease of use score, the difficulty interpreting results lowered the choice of daily use (50%) and Remel's overall score was 71%. TechLab Inc. scored lower in both ease of use (77%) and choice for daily use (40%) compared to the other kits primarily because of long testing time. Testing time for this kit is double what is required by Meridian. However, on ease of interpretation TechLab Inc. received the highest score (93%) because of consistently visible result lines. Even with clear results, the lengthy testing time put TechLab Inc. in third place in overall score at just 70%. Based on the preference of participants, the Meridian Bioscience Inc. kit was chosen as the most preferred proposed method.

3.2.4. Discussion

In choosing which manufacturer's assay to validate, we investigated usability as a point of comparison based on reports that have stated that the kits perform comparably to one another and that laboratories should choose the kit that suits their daily use best (25). With that recommendation in mind we developed the usability survey so that ease of testing and interpretation along with overall choice for daily use could be evaluated. When evaluated using the survey, the Meridian Bioscience Inc. kit scored highest by our laboratorians in overall choice for daily use. Even though directions for the Meridian Bioscience Inc. kit were considered less clear than the other two kits, the shorter incubation and processing time made it preferable for everyday use. As the Meridian Bioscience Inc. and TechLab Inc. had identical performances, it was the results of this survey that led to Meridian Bioscience Inc. being the kit with which validation was pursued.

TABLE 11. Stool Parasite Assay Usability Survey

<table>
<tr><td colspan="3">Date:
Technologist:
Specimen ID:
Please rate the Kits on the following (circle the corresponding number):</td></tr>
<tr><td>KIT A</td><td>KIT B</td><td>KIT C</td></tr>
<tr><td>Ease of use:

0. Very Difficult to Use
1. Somewhat Difficult to Use
2. Somewhat Easy to Use
3. Very Easy to Use</td><td>Ease of use:

0. Very Difficult to Use
1. Somewhat Difficult to Use
2. Somewhat Easy to Use
3. Very Easy to Use</td><td>Ease of use:

0. Very Difficult to Use
1. Somewhat Difficult to Use
2. Somewhat Easy to Use
3. Very Easy to Use</td></tr>
<tr><td>Ease of Result Interpretation:

0. Very Difficult to Interpret the Result
1. Somewhat Difficult to Interpret the Result
2. Somewhat Easy to Interpret the Result
3. Very Easy to Interpret the Result</td><td>Ease of Result Interpretation:

0. Very Difficult to Interpret the Result
1. Somewhat Difficult to Interpret the Result
2. Somewhat Easy to Interpret the Result
3. Very Easy to Interpret the Result</td><td>Ease of Result Interpretation:

0. Very Difficult to Interpret the Result
1. Somewhat Difficult to Interpret the Result
2. Somewhat Easy to Interpret the Result
3. Very Easy to Interpret the Result</td></tr>
<tr><td>When ranking the three kits Kit A is my:

0. 3rd Choice for Daily Use
1. Ties with Kit _____ for Daily Use
2. 2nd choice for Daily Use
3. 1st choice for Daily Use</td><td>When ranking the three kits Kit B is my:

0. 3rd Choice for Daily Use
1. Ties with Kit _____ for Daily Use
2. 2nd choice for Daily Use
3. 1st choice for Daily Use</td><td>When ranking the three kits Kit C is my:

0. 3rd Choice for Daily Use
1. Ties with Kit _____ for Daily Use
2. 2nd choice for Daily Use
3. 1st choice for Daily Use</td></tr>
<tr><td colspan="3">Other Comments or Concerns about the Kits:</td></tr>
</table>

TABLE 12. Participants' Preferences for Use of the Three Proposed Methods by Percentage

Kit	Ease of Use	Ease of Interpretation	Choice for Daily Use	Overall Score
Meridian	93%	90%	87%	90%
Remel	100%	63%	50%	71%
TechLab	77%	93%	40%	70%
Comments:				
Meridian	Fast, Directions not as clear			
Remel	Fast, 2 False Negative Results, Hard to interpret the faint lines			
TechLab	Time Consuming, Easiest to Interpret			

3.3. Labor Costs, Total Processing Time, and Turn-Around-Time Comparison of Current and Proposed Methods for Intestinal Parasite Testing

3.3.1. Goals of Labor Costs, Total Processing Time, and Turn-Around-Time Comparison

When considering alternative methods for intestinal parasite testing questions about cost and time were important factors to evaluate. To more clearly define our goals of decreasing cost and time for our laboratory, definitions were established to better compare current and proposed methods. Labor cost was defined as the cost associated with the technologist's hands-on testing time per specimen, plus the cost of testing materials, such as reagents, test cartridges, pipettes, etc. The cost of one hour of technologist time was calculated at $48.21 in our laboratory for 2011. Using that number labor cost is calculated in the equation:

Labor Cost= $48.21/hour + Testing Materials

Total processing time (TPT) was defined as the time required for the complete processing of the specimen, from the time it was ordered to when it was resulted. This time includes the hands-on time associated with labor costs but also takes into account any incubation, centrifugations or other aspects of test processing. TPT was considered an important measure for our laboratory because although it includes aspects of testing that do not directly impact labor costs, tests that require extensive TPT can affect laboratory efficiency and accuracy. Tests with multiple incubations, for example, require a technologist to be interrupt other tasks in order to attend to those tests. The repeated start and stop of activities is an inefficient use of technologist time. Also, interruptions to work flow can decrease concentration on the task at hand, thus leading to increased errors. By defining TPT, the differences in the complete processing of specimens between current and proposed methods can be evaluated.

Turn-round-time (TAT) is the total time between when specimens are submitted for testing and when results are reported to providers. Where labor costs and TPT both focus on laboratory operations, TAT has a greater emphasis on patient care. Treatment of illness in patients and reporting of notifiable diseases to governing agencies is hindered by tests with long TAT. In an effort to improve patient care and reporting requirements, an evaluation of current method TAT versus the TAT of proposed methods was made.

3.3.2. Results

We directly compared the labor costs, TPT and TAT between the current and proposed methods. Current methods in place for detection of *Giardia lamblia* and *Cryptosporidium* varied in their labor costs, TPT and TAT. Data for Labor costs and TPT for current and proposed methods are listed in Figure 7.

<u>Current Methods Labor Costs, Total Processing Time, and Turn-Around-Time</u>

The labor cost for an individual OAPP was ~$50/specimen, requiring 1 hour of hands-on testing time due to the interpretation of both concentrate and trichrome slides. OAPP has a TPT of 120 minutes because of extensive processing required prior to testing. The combination of lengthy processing and testing time led to a 48hr ±1 week variation in TAT for OAPP.

Labor costs for *Giardia* antigen (SGRDAG) were approximately $18/specimen requiring 10 minutes of hands-on technologist time. TPT was 110 minutes due to multiple incubation and wash steps. SGRDAG testing could have a TAT of as little as 24 hours but because testing is batched and not performed every day the actual time from receipt to reporting was 48 hrs.

Cryptosporidium detection by Auramine-O (CYCLOP) labor costs are approximately $34/specimen with 40 minutes of hands-on time. TPT is approximately 65 minutes because of stain incubation. Since CYCLOP specimen testing was batched, TAT was ~48hrs. Results are shown in Table and Figure 7.

Proposed Methods Labor Costs, Total Processing Time, and Turn-Around-Time

Because the proposed methods were not yet orderable by providers, the TAT was estimated at 24hrs for each kit to account for testing potentially being batched and performed only once per day. The Meridian Bioscience Inc. ImmunoCard STAT! *Cryptosporidium/Giardia* Rapid Assay had a labor cost of ~$38 per specimen, a hands-on testing time of 10 minutes, and a TPT of 20 minutes. The Remel Xpect *Giardia/Cryptosporidium* had a labor cost of ~$38 per specimen, a hands-on testing time of 15 minutes and a TPT of 25 minutes. The TechLab Inc. *Giardia/Cryptosporidium* Quik Chek had a cost of ~$50 per specimen, hands-on testing time of 20 minutes, and a TPT of 30 minutes. Results are shown in Table and Figure 7.

3.3.3. Discussion

In terms of labor costs, TPT, and TAT the proposed methods out-performed current methods. Labor costs and TPT associated with OAPP make this method an expensive choice for routine ordering. While batching of specimens occurs to help off-set processing time, TPT can still be lengthy if ordering recommendations are followed. This is a disadvantage to the "three-stool-rule" collection. Following this recommendation triples the cost to patients, and the very large number of specimens received delays the reporting of results. This is particularly the case during the summer months when *Giardia* and *Cryptosporidium* infections are most prevalent. While TAT for the processing of OAPP specimens is estimated to take only 48hrs, this time can often extend to 1 week when requests are numerous and three stools per patient are received. Long TAT's could potentially delay treatment or the reporting of these infectious diseases to the state laboratory. A positivity rate of ~4% for clinically significant parasites combined with high cost and slow TAT has led to the question of whether or not the OAPP method is the best choice for the majority of specimens being submitted for testing.

Even though the SGRDAG testing has low labor costs, several disadvantages exist for traditional EIA methods. Traditional EIA is a lengthy process taking approximately 110 minutes to complete the required wash, incubation and interpretation steps (36). Another disadvantage is the potential carry-over of grossly positive specimens into adjacent wells observed in our laboratory, necessitating the repeat of entire runs of specimens. This doubles the TPT, which is already significant for this method. Our laboratory has found that the length of testing and finesse required to reduce carry-over, makes this method less than ideal for everyday testing, which increases TAT to 48hrs for these orders.

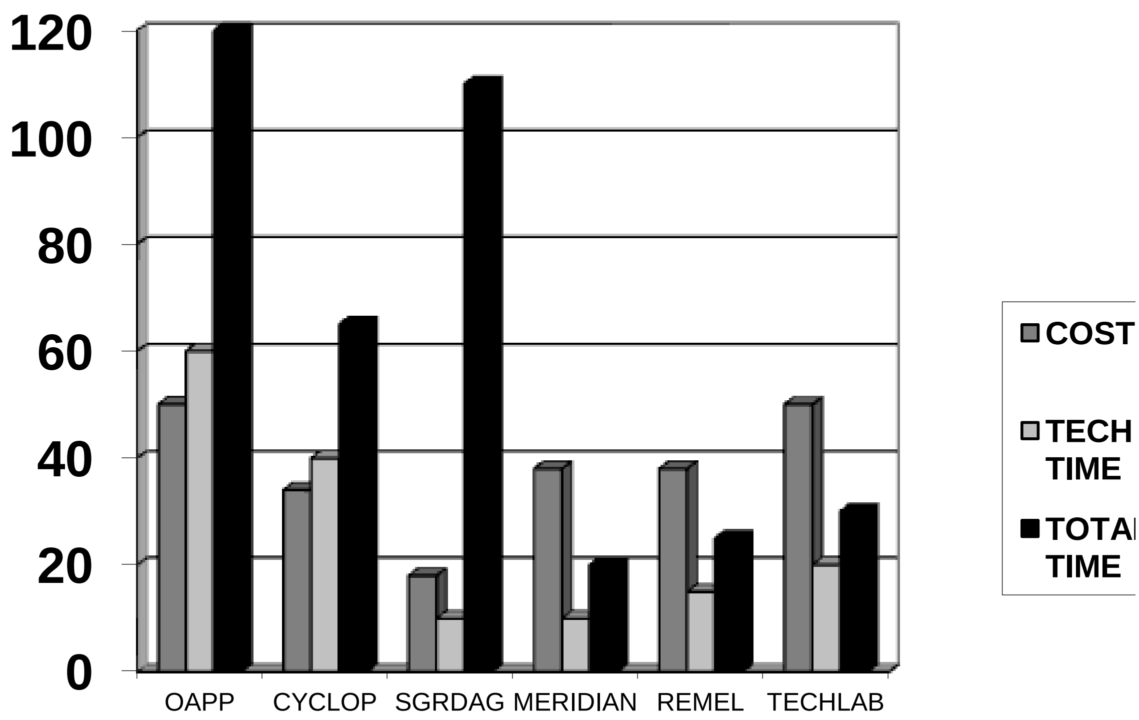
Auramine-O, the stain used for CYCLOP is an indirect fluorescent stain and requires the careful attention of an experienced technologist to properly distinguish *Cryptosporidium* from potential debris and other parasites. While labor costs are only around $34, the TPT is not insignificant at nearly 65 minutes per specimen from start to finish, necessitating that specimen processing and interpretation be batched, thus increasing TAT for these orders. The combined disadvantages of this method have brought into question its use as our primary method for detection of this parasite.

All three proposed methods performed better than current methods for TPT and TAT. The substantial decrease in TPT, resulting in a potential reduction in TAT, will allow us to report results to providers more quickly. The decreased hands-on time will improve labor costs even in the context of increased materials cost in comparison to OAPP, particularly when the “three-stool-rule" is in place. Of the three rapid kits evaluated, the Meridian Bioscience Inc. kit has the shortest TPT of 20 minutes, which is less than the other proposed methods and considerably less than current methods. The price of the Meridian Bioscience Inc. kit is comparable to the other two kits and requires slightly less technologist time than its competitors, which will make it less expensive than the other proposed methods overall.

TABLE 13. Labor Costs and Total Processing Time Comparison between Current and Proposed Methods

	CURRENT METHODS			PROPOSED METHODS		
VARIABLES	OAPP	CYCLOP	SGRDAG	MERIDIAN	REMEL	TECHLAB
Labor Costs ($)	50	34	18	38	38	50
Technologist Time (min.)	60	40	10	10	15	20
TPT (min.)	120	65	110	20	25	30

FIGURE 7. Graph of Labor Costs and Total Processing Time Comparison between Current and Proposed Methods



3.4. Proposed Method Performance in Prospective Study

3.4.1. Goals of Performance Evaluation

Performance of the proposed methods was the most significant variable when evaluating the rapid lateral flow immunoassays for use. Complying with ordering recommendations, ease of testing, and short TAT are only valuable attributes if the testing has sufficient sensitivity and specificity for the detection of *Giardia* and *Cryptosporidium*. As performance in the literature of these methods varied widely, evaluation of our in-house performance was an important goal of method comparison.

3.4.2. Results

Nine patient specimens and 22 challenge panel specimens were included in the prospective study (see Table 10). All 31 stool specimens were tested using the three proposed rapid method kits. In total, 71% (21/30) of all specimens included in the study were positive by the reference methods, as shown in Table 14.

Patient specimens included in the study were first tested by in-house methods: OAPP and SGRDAG for detecting *Giardia* and CYCLOP for detecting *Cryptosporidium*. Results from these methods were known prior to inclusion in the study and were considered the reference by which the rapid testing results for the patient specimens were compared. Of the 9 patient specimens 3 were positive for *Giardia* by OAPP and 1 was positive for *Cryptosporidium* by CYCLOP. All three proposed methods had a sensitivity of 100% for patient specimens positive for *Giardia*. The one patient specimen that tested positive for *Cryptosporidium* by the CYCLOP reference method was negative by all three proposed rapid methods. This specimen was tested by PCR and was found to be positive with *Cryptosporidium hominis*. Specificity for both parasites was 100% by all 3 proposed methods. Performance data for patient specimen testing is shown in Table 15.

All 22 challenge panel specimens were tested previously by the manufacturers. The results of this testing was known prior to our prospective testing and was considered the reference by which rapid testing results for the challenge panel specimens were compared. Of the challenge panel specimens, a total of 18 were positive: 11 were positive for *Giardia*, 9 positive for *Cryptosporidium*, and 2 were positive for both organisms by the manufacturers' reference methods. When tested against the proposed methods, all 9 challenge panel specimens were positive for *Cryptosporidium*. Of the 11 positive *Giardia* challenge panel specimens, the Meridian Bioscience Inc. and TechLab Inc. kits had a sensitivity of 100%. Two challenge panel specimens positive by the manufacturer's reference method tested negative for *Giardia* with the Remel kit, yielding a sensitivity of 81% for this assay. Specificity for both parasites was 100% by all 3 proposed methods (see Table 16). The performance data for challenge panel specimen testing is shown in Table 17.

3.4.3. Discussion

Comparison of Performance Between Rapid Lateral Flow Immunoassays

When comparing the performance of rapid assays to current methods, both the Meridian Bioscience Inc. and TechLab Inc. assays were equivalent to our current methods of *Giardia* detection, with the Remel kit having two false negative results. However, all three rapid assay kits failed to detect the positive *Cryptosporidium* detected by our current method. We believe this may be the result of sub-optimal specimen handling. Specifically, the positive specimen in question was unpreserved stool that had undergone at least two freeze-thaw cycles prior to evaluation. In the manufacturer's package insert it states that fresh specimens should be processed as soon as possible or placed in a suitable preservative prior to testing to increase specimen stability (22). We therefore hypothesize that the discrepancy in test results may have occurred due to multiple cycles of freeze-thawing. Because this specimen was the only patient positive for *Cryptosporidium* included in the study, further collection of specimens are needed before rapid assays are offered as primary testing.

The Meridian Bioscience Inc. kit was chosen as our kit of choice for further validation. Sensitivities and specificities for the Meridian Bioscience Inc. ImmunoCard STAT! *Cryptosporidium/Giardia* Rapid Assay were equivalent to the TechLab Inc. *Giardia/Cryptosporidium* Quik Chek and exceeded the Remel Xpect *Giardia/Cryptosporidium*. Other studies have also looked at performance between these three rapid immunoassays. The performance we observed of the three rapid assays is supported by the analysis of Minak et *al* where the same rapid lateral flow immunoassays were compared to EIA (21). Similar to our results, Meridian Bioscience Inc. and TechLab Inc. had identical sensitivities (94/100% for *Giardia/Cryptosporidium*) with Remel demonstrating poorer sensitivity (88/82% for *Giardia/Cryptosporidium*) (21). The specificities varied among the kits, with TechLab Inc. demonstrating superior specificity (100%) (21). In addition to using EIA as a reference method to compare performance of the three kits in the Minak *et. al* study, PCR was also used as a reference method. With PCR as the reference, they found that sensitivities of all three kits were greatly reduced. Only the TechLab Inc. specificity remained the same with Meridian Bioscience Inc. performing the most poorly (44/50% *Giardia/Cryptosporidium*) (21). No explanation was provided in that study as to why specificities varied between kits, however, the TechLab Inc.

Giardia/Cryptosporidium Quik Chek was selected for use. The study by Minak et *al* in which TechLab Inc. was ultimately chosen is cited on the TechLab Inc. website in support of their product. It is unclear whether this study was sponsored by the manufacturer or simply cited by them.

Consideration of Molecular Methods for the Detection of Intestinal Parasites

As the study by Minak et *al* utilized PCR as a means to detect *Giardia* and *Cryptosporidium*, the option to implement molecular techniques in the detection of intestinal parasites has also been considered in our laboratory. Multiple molecular platforms are available for intestinal parasite detection and are associated with a number of advantages over traditional and antigen-based methodologies. PCR has been reported as having excellent sensitivity and specificity with *Cryptosporidium* performance levels of 100% (40, 44). Sensitivities and specificities for *Giardia* have been reported as 98-100% and 92-100%, respectively (39, 44-45). PCR can detect small amounts of parasite DNA present in feces and therefore is not limited by parasite shedding. A variety of parasites can be detected simultaneously including important pathogenic organisms such as, *Giardia*, *Cryptosporidium spp.*, *Dientamoeba fragilis,* and *E. histolytica* using multiplex PCR assays. In addition, parasites can be indentified to the species level, which is particularly useful with *Cryptosporidium* where a number of different species have been reported in human infection (27). Despite these advantages, PCR has some significant disadvantages. While technology is changing quickly in the area of molecular testing, current PCR training and processing are extensive with long TAT's, and costs are very high. Our goals in this evaluation were to seek methods that require less technical skill and cost, and to decrease TAT, which is why molecular methods were not considered as a testing alternative to our current testing methods.

Investigation of Stool Submission Recommendations

Questions about the number of specimens submitted for rapid immunoassays have been proposed. As stated earlier in the evaluation of current EIA methods, some concern with regards to the number of specimens submitted has arisen for immunoassay detection methods. In presentations given separately by Garcia and Pritt regarding parasitology testing in the clinical laboratory (25, 37), the study by Hanson and Cartwright regarding two stool submissions for immunoassays have been mentioned (38) Although the study by Hanson and Cartwright is focused on the sensitivity of traditional EIA, their recommendation has been cited for all antigen based immunoassay testing for *Giardia* by Garcia and Pritt without a clear distinction being made between EIA and rapid antigen methods (25, 37). The lack of distinction between these two methods is a concern because in our study we are considering implementation of rapid lateral flow immunoassays as a primary testing method. If two stool submissions are required for these methods, the materials costs would be prohibitive to our laboratory. Interestingly, by a second publication by Garcia et *al* made no mention of the requirement for two stools the performance by the ImmunoCard STAT! *Cryptosporidium/Giardia* Rapid Assay demonstrated a sensitivity of *Giardia* and *Cryptosporidium* of 93.5% and 98.8%, respectively. Specificities for both parasites were 100%. The high performance of the rapid immunoassay seen by Garcia et *al* in the context of only one stool, brings into the question the earlier concern about two stool submissions. In our study, sensitivities were 100/90% for *Giardia/Cryptosporidium* for a single stool submission by the kit we intend to validate. Thus, we will not be implementing a two stool submission for the proposed rapid immunoassay.

TABLE 14. Reference Methods Results for Patient and Challenge Panels Specimens

Reference Method	**Total # Tested by this Method**	***Giardia* Positive**	***Cryptosporidium* Positive**	**Positive for *Giardia* and *Cryptosporidium***	**Negative for *Giardia* and *Cryptosporidium***
OAPP	9	3	N/A	N/A	5
SGRDAG	2	0	N/A	N/A	2
CYCLOP	2	N/A	1	N/A	1
Manufacturer	22	11	9	2	4

TABLE 15. Proposed Methods Testing Results for Patient Specimens

Proposed Rapid Methods	*Giardia* Positive	*Cryptosporidium* Positive	Negative for *Giardia* and *Cryptosporidium*	Total # Tested
Meridian	3	0	6	9
Remel	3	0	6	
TechLab	3	0	6	

TABLE 16. Proposed Method Testing Results for Challenge Panel Specimens

Proposed Rapid Methods	*Giardia* Positive	*Cryptosporidium* Positive	Positive for *Giardia* and *Cryptosporidium*	Negative for *Giardia* and *Cryptosporidium*	Total # Tested
Meridian	11	9	2	4	22
Remel	9	9	1	5	
TechLab	11	9	2	4	

TABLE 17. Performance Table for Proposed Methods

	Giardia		*Cryptosporidium*	
Method	Sensitivity	Specificity	Sensitivity	Specificity
Meridian	100%	100%	90%	100%
Remel	86%	100%	90%	100%
TechLab	100%	100%	90%	100%

4. CONCLUSION

4.1. Summary of Retrospective and Prospective Study Findings and Limitations

This thesis encompasses both a retrospective and a prospective study. In the retrospective study we investigated the ordering practices of providers for current testing methods. Ordering trends were compared with the CDC recommendations for the use of specific fecal immunoassays for *Giardia* and routine ordering of testing for *Cryptosporidium*. In addition, ordering trends were compared with the “three-stool-rule” recommendation for when providers were ordering OAPP for suspicion of diarrheal disease. Our results show that providers are not following ordering recommendations for current testing methods. In order to aid providers in complying with recommendations, alternative testing methods were evaluated in the prospective study.

In the prospective study, we investigated whether that use of rapid lateral flow immunoassays for the detection of *Giardia* and *Cryptosporidium* would be a valid primary testing method for stool specimens submitted to our laboratory for patients with diarrheal illness. A comparison between current methods in use and these proposed methods was completed. Test performance, labor costs, TPT, TAT, usability, and complying with CDC ordering recommendations were evaluated in this comparison. In addition, three different manufacturers' rapid assay kits were compared to one another to assess which one of these proposed assays would be most suitable for daily use based on test performance and ease of testing.

Our results show that rapid lateral flow immunoassays are adequately comparable to current methods in regards to performance, exceed current methods in TAT, labor costs and ease of testing, and with one order comply with CDC recommendations for the detection of *Giardia* and

Cryptosporidium. Of the three kits tested, the Meridian Bioscience Inc. ImmunoCard STAT! *Cryptosporidium/Giardia* Rapid Assay is the rapid lateral flow immunoassay that will undergo further validation by our laboratory.

<u>Limitations</u>

One significant limitation of this study was small sample number tested. During the three month study period, although 770 specimens were received for testing by current methods, only 17 specimens were positive for *Giardia* by OAPP. However. due to the submission of many specimens in the fixative ECOFIX, only four of these specimens were suitable for testing by rapid assays. Of the specimens received for testing with CYLOP, only 2 specimens were found to be positive for *Cryptosporidium* by Auramine-O. Due to insufficient volume of one of the submitted specimens, only one positive *Cryptosporidium* could be included in the study. No positive specimens were detected by SGRDAG during the specimen collection period. The limited numbers of positives received in our laboratory combined with the limitations of fixative methods resulted in a small sample size available for additional testing. Conducting a study such as this over a larger time frame would help insure a larger sample size and thus increase the robustness of observed results. As reported in an earlier section, only 5% of the stools tested in our laboratory for OAPP testing are positive for *Giardia* and only 0.5% are positive for *Cryptosporidium* by CYCLOP. Also, with both OAPP and CYCLOP testing being ordered less frequently than recommended, the number of samples having been received is limited from the start.

To help increase the numbers of positives tested in our analysis, manufacturer challenge panel specimens were incorporated into our study model. While helpful in evaluating the performance of the rapid assays, the challenge panel specimens were unable to be tested by our current methods due to specimen preservative and/or insufficient volume. Because the majority of our positive specimens were of this type, the purpose of our study, which was to evaluate current testing methods, was not supported by these specimens. Procuring positive patient specimens from other locations was extremely challenging because of the preservative requirements of the rapid assays. ECOFIX, a common preservative used in our region is not suitable for the rapid assays and thus limited our availability of specimens we could receive from outside laboratories. As we move forward with further testing of the Meridian Bioscience Inc. assay, education of providers as to appropriate media will be required and will accompany implementation of a new ordering scheme.

Another limitation in terms of specimen testing is the sensitivity of our current methods. While negative patient specimens tested by our current methods were included, the focus of our study was to test positives detected by current methods with proposed methods. As mentioned in earlier sections, sensitivity of OAPP, in particular, is low. The possibility of proposed methods detecting positive specimens missed by current methods was not addressed as none of the included specimens fell under that criterion. As we proceed with further testing of the Meridian Bioscience Inc. assay, this possibility may arise and means of confirmatory testing may be necessary.

4.2. Validation of Meridian Bioscience Inc. ImmunoCard STAT! *Cryptosporidium/Giardia* Rapid Assay

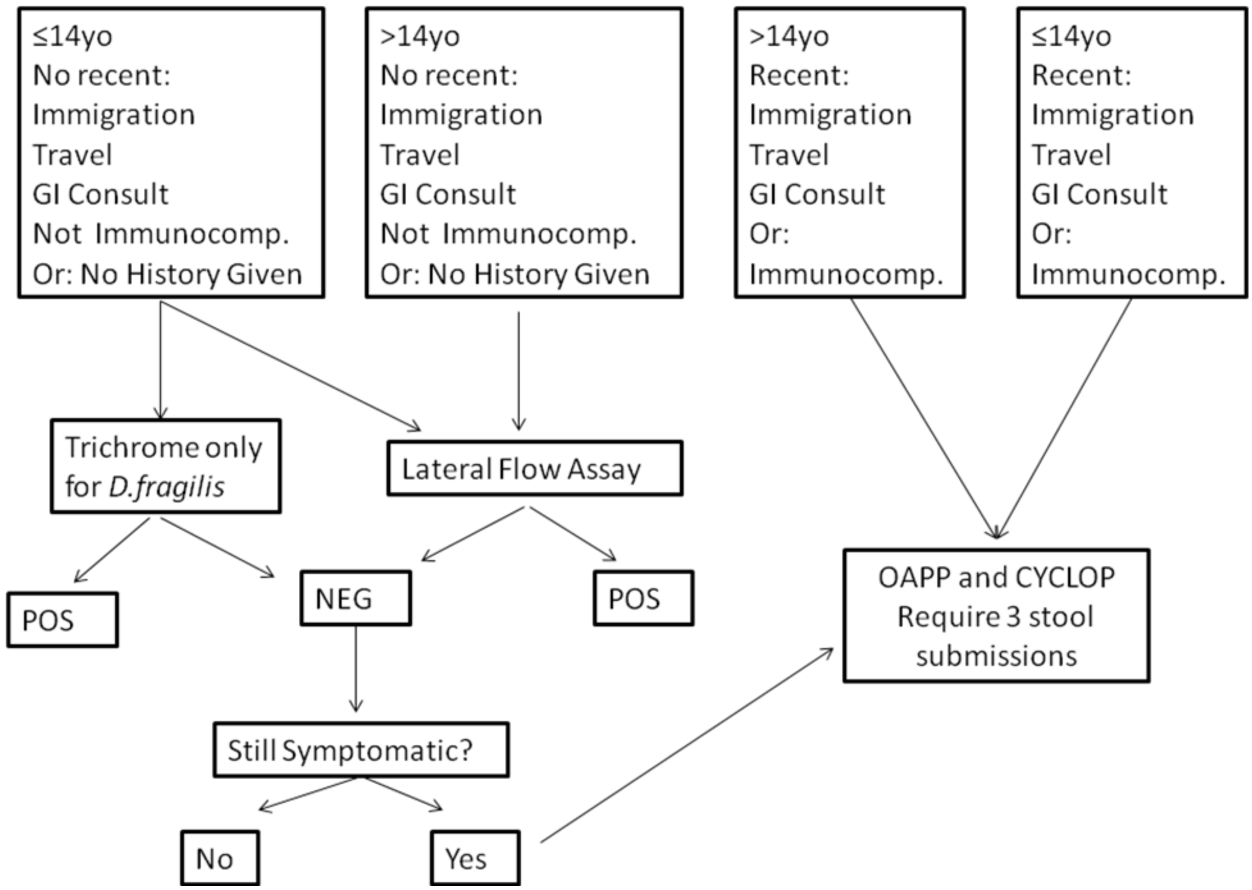
Future directions for this study include conducting an additional analysis of the Meridian Bioscience Inc. assay for validation. Our next step is to perform a prospective study utilizing 100 patient specimens that are submitted for testing with current methods. If this kit continues to perform well, it has the potential to completely replace the current EIA in use, and to serve as primary testing ahead of OAPP and CYCLOP for adult patient specimens submitted to our laboratory. With further validation and future implementation of the Meridian Bioscience Inc. ImmunoCard STAT! *Cryptosporidium/Giardia* Rapid Assay as our primary testing method, we will be able to comply with the routine testing recommendations by simultaneously testing for *Giardia* and *Cryptosporidium,* and will improve our TAT for test results. Both of these benefits improve patient care by decreasing the time individual patients receive results and decreasing potential outbreak situations by having more timely reporting of notifiable diseases to the state laboratory. In addition to the benefits to our patients, implementation of this rapid method will help us reduce technologist time and training decreasing cost to our laboratory overall.

4.3. Implementation of New Ordering Scheme

An ordering system will be established that will aid providers in selecting testing suitable for their patients, thus complying with CDC recommendations. In a study completed by Church et *al*, EIA was evaluated for both *Giardia* and *Cryptosporidium* detection (34). They implemented EIA as a primary method and developed a new ordering scheme that considered patient age and required information such as travel history, country of origin, or gastroenterology consult in order to decide whether EIA or OAPP would be performed. For adults with no history travel, recent immigration or GI consult, primary testing was by EIA. Children falling in that same category received EIA and a trichrome stain to rule-out *Dientamoeba fragilis,* which in children is considered potentially pathogenic by the CDC. For patients of any age with a history suggestive of needing further parasite detection, OAPP was performed. Utilization of this new ordering scheme replaced the duplicate and triplicate submissions for OAPP to a single stool specimen submission for EIA for the majority of patients. This change helped reduce TAT and labor costs for the laboratory. Despite the change in ordering practice to primarily testing with EIA which led to reduction in overall stool submissions, their detection rate of clinically significant parasites remained stable as compared to the previous ordering processes. By developing a new ordering scheme they were successful in aiding providers in the ordering of tests appropriate for their patients (34).

Notably, the laboratory in the Church et *al* study is similar in both in size and patient population to those patients served by the UWMC Clinical Microbiology Laboratory. Thus, in addition to implementing rapid immunoassay as a primary testing method for the majority of our patients we implement a new ordering scheme to aid providers in the ordering process utilizing the criteria stated in Church et *al* (shown in Figure 8). An additional criterion of immune competency will be added because our facility serves a large population of immunocompromised patients. This new ordering scheme will require patient history information be included if current testing methods are to be performed. Without the inclusion of patient history, testing will default to rapid assay for adults and will also include testing for *Dientamoeba fra*gilis for children. As with the study by Church et *al*, patients of any age with a history suggestive of needing further parasite detection will receive OAPP and CYCLOP testing. If a provider is unable to collect history information for a patient but has strong suspicion of a more extensive parasite infection, special request for OAPP and CYLCOP testing can be made through the laboratory's physicians on call. It is our hope that the implementation of this suggested ordering scheme will aid providers in the ordering process and support more suitable testing for patients as defined by CDC recommendations.

FIGURE 8. Proposed Ordering Scheme for Stool Parasite Testing



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