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Recent Advances in Clinical Parasitology Diagnostics

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Abstract Microscopy has several limitations in the diagnosis of parasitic infection. New technologies have emerged to address some of these limitations. We review recent advances in three key areas. The detection of enteric protozoa is a commonly requested test, particularly with increasing travel to and migration from endemic countries. Microscopy is slow and labor intensive and requires a high level of technical expertise. It also lacks both sensitivity and specificity. Recently developed nucleic acid amplification tests are automated and rapid and show superior accuracy. Proteomics shows promise for both the diagnosis of infections where parasite detection is difficult and the potential for accurate assessment of cure in these cases. Finally, rapid and simple diagnostic tests suitable for use in low-resource settings are now allowing for improved study and control of infection in endemic regions.

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Introduction and Scope

The microscopic appearance of human intestinal parasites was first described and published in the seventeenth century, not long after the discovery of the microscope, by Francesco Redi, considered the grandfather of modern parasitology [1]. Although the instruments were refined, microscopy remained the principal tool for parasitological diagnosis over the next 350 years. Only in the past two decades have alternative technologies evolved to the stage of mainstream use. Microscopy is labor and time intensive, requires extensive training and technical expertise, and lacks sensitivity and reproducibility [2]. These new technologies afford improvement in all of these test parameters. In this review, we focus on three areas of innovation that are rapidly transforming clinical parasitology. The first is the use of nucleic acid amplification, particularly in the search for enteric pathogens. The second is proteomics, which allows the identification of disease states, rather than the simple documentation of the presence of a pathogen. The third is the development of rapid diagnostics suitable for use in low-resource settings under field conditions.

Nucleic Acid Amplification Tests for Enteric Parasites

The most promising alternative platform for the detection of pathogenic parasites in stool specimens is the nucleic acid amplification test (NAAT), with polymerase chain reaction (PCR) being the best described. Progress has been incremental but steady. Many "in-house" assays have been developed and

studied, but only in 2013 was the first commercial kit FDA approved for diagnostic use (Luminex, see succeeding text).

There are several explanations for this delay in NAAT applications. DNA extraction from stool is complicated by inhibition and cross-contamination problems, which have been mostly resolved [3]. Published in-house NAATs use various targets and extraction methods, hampering standardization. Automated "fluid-handling" instruments often perform poorly with stool matrices. Traditional stool collection in formalin or other common preservatives degrades the performance of NAATs, so ideal specimens need to be provided fresh or frozen. Clinical studies have been severely limited by the relatively small numbers of infected patients available in centers capable of developing the methodology, and studies of cost-effective algorithms are not yet available. Vasoo and Pritt have published an extensive overview of molecular methods in the diagnosis of parasitic infections [4••].

Demands in clinical parasitology have increased in step with growth in international travel and migration from endemic areas. There is an increasing interest in parasites as a cause of chronic diarrhea and other symptoms, as well as a cause of morbidity in the immunocompromised host. NAATs have the potential to reduce turnaround time and especially labor costs. It has been suggested that the number of specimens requiring microscopy could be reduced by 90 % [5].

The possibility of developing multiplex NAATs provides an opportunity for syndrome-based microbiological diagnosis. Since there is no inherent reason to limit the multiplex panel to parasites, it becomes possible to start with a syndrome, such as diarrhea, and probe for parasitic, bacterial, and viral pathogens at the same time [6]. At least in theory, efficient algorithms could be developed for clinical categories, and testing panels could be determined targeting etiologic agents, including some not commonly identified in most laboratories today. Coinfections will likely be found more frequently, which may challenge our understanding of the etiology of these syndromes. The presence of pathogens not previously recognized in some regions will become evident, and discrepancies between NAAT and conventional results will require explanation [7].

Earlier studies focused on protozoa, where PCR typically was more sensitive and specific compared to microscopy and also superior to antigen detection assays and serology [8–10]. Performance remains strong with multiplex assays using many targets [11]. Validation has been provided by limited studies in a general practice setting [12] and in a travel clinic [13]. Most studies demonstrate a higher analytic sensitivity for NAATs compared to microscopy. This likely minimizes the problem of day-to-day variability in parasite shedding. A study of a TaqMan assay reported a limit of detection of 10^3 to 10^5 copies/g of feces for protozoa [14••]. One study showed higher sensitivity for protozoa using PCR on a single unpreserved specimen than traditional fecal concentration and microscopy on three stained specimens [5]. NAATs can also differentiate morphologically identical organisms, such as *Entamoeba histolytica* and *Entamoeba dispar*. In theory, specific strains of a species which might be more pathogenic could be targeted, as may be relevant for species such as *Dientamoeba fragilis*.

The modularity of NAATs offers the possibility of creating panels that are tailored to pathogens common in particular patient groups. For the diagnosis of diarrhea in high-income settings, *Giardia* and *Cryptosporidium*, perhaps with the addition of *Dientamoeba*, would be sufficient. Travelers' panels might also include *Entamoeba* and *Cyclospora*. For the immunocompromised patient, *Microsporidia* species could be added, as well as *Cystoisospora* (formerly *Isospora*) *belli*. *Strongyloides*, a particularly problematic organism, might also be added for those with "dyspeptic" symptoms. This patient-centered approach is discussed by van Lieshout and Verweij [15]. Using PCR-Luminex bead technology, it is possible to build a modular methodology, where reagents are added or withheld based on the need to identify particular pathogens, species, or strains in a given situation [16]. This assay, which also targets several helminths, was studied in an endemic setting for use as a research tool.

A major advantage of microscopy is that it is not necessary to predefine the particular parasites being sought, and thus, we are unlikely to completely abandon this venerable technique. In a study of patients from a travel clinic, an assay targeting *E. histolytica*, *Giardia*, *Cryptosporidium*, and *Strongyloides* had equal or higher sensitivity than microscopy for all targets, but missed *Cyclospora* and a number of helminths. However, these "missed" cases would have increased the overall prevalence of positive specimens by only 0.5 % [13]. Unfortunately, it was not possible to use the clinical history to identify a subgroup containing these additional cases. Microscopy also identified many specimens with protozoa considered to be nonpathogenic, although the clinical relevance of these findings is unclear.

At this time, the only FDA-approved commercial assay is the xTAG® Gastrointestinal Pathogen Panel (xTAG GPP, Luminex, Austin, TX), a qualitative bead-based multiplex PCR assay, based on xMAP technology, targeting 15 GI pathogens (nine bacterial, three viral, three parasitic: *Giardia*, *E. histolytica*, *Cryptosporidium*). Several clinical studies have been performed, although the total number of cases of parasitic infection is fairly small [17, 6, 18–21]. Commercial assays currently on the market in North America or Europe are listed in Table 1.

The FilmArray GI (BioFire Diagnostics, Salt Lake City, UT) is expecting FDA approval in 2014. This is a nested PCR array, targeting 23 enteric pathogens (including *Giardia*, *Cryptosporidium*, *E. histolytica*, and *Cyclospora cayetanensis*). Clinical studies are in progress, and published data are expected soon.

Table 1 Selected commercialized nucleic acid amplification tests for enteric parasitic infections

NAAT	Parasites detected	Separate DNA extraction step required?	Platform used	Specimens per run
xTAG GPP (Luminex, Austin, TX)	*Cryptosporidium* sp. *Giardia* sp. *Entamoeba histolytica*	Yes	Luminex only	96
RIDA®GENE Parasitic Stool Panel (R-Biopharm AG, Darmstadt, Germany)	*Cryptosporidium* sp. *Giardia* sp. *Entamoeba histolytica* *Dientamoeba fragilis*	Yes	Several	Typically up to 96, depending on platform used
FilmArray GI (BioFire Diagnostics, Salt Lake City, UT)	*Cryptosporidium* sp. *Giardia* sp. *Entamoeba histolytica* *Cyclospora cayatanensis*	No	BioFire only	1
FTD Stool parasites (Fast-track Diagnostics, Sliema, Malta)	*Cryptosporidium* sp. *Giardia* sp. *Entamoeba histolytica*	Yes	Several	Typically up to 96, depending on platform used
Gastroenteritis/parasite panel (Diagenode, Liege, Belgium)	*Cryptosporidium* sp. *Giardia* sp. *Entamoeba histolytica*	Yes	Several	Typically up to 96, depending on platform used

NAAT nucleic acid amplification tests

There are several kits currently being sold in Europe, with clinical study data available from the manufacturers. These include the FTD Stool parasites (Fast-track Diagnostics, Sliema, Malta), which uses a one tube multiplex real-time PCR for the diagnosis of *E. histolytica*, *Giardia lamblia*, and *Cryptosporidium*; Diagenode (Liege, Belgium) also markets a gastroenteritis/parasite panel targeting the same parasites; and the RIDA®GENE Parasitic Stool Panel (R-Biopharm AG, Darmstadt, Germany), a multiplex PCR kit targeting *G. lamblia*, *Cryptosporidium parvum*, *E. histolytica*, and *D. fragilis*.

Development of assays for the detection of helminths has been more challenging than for protozoa. In theory, the excretion and distribution of helminth DNA should be less variable than the excretion of eggs or larvae, which might give NAATs better sensitivity than microscopy. In practice, studies using samples from endemic settings, targeting between one and seven different helminths, have been promising. Progress has been made in the identification of optimal DNA extraction protocols and suitable DNA target selection. Comparison with traditional methods is particularly complicated in the case of *Strongyloides*, where multiple concentration and culture methods have been described, all of which have limitations. Some of these methods have now been compared with PCR diagnostics, in a variety of different ways and settings [22–26]. In general, sensitivity is high for high and moderate larval burdens detectable by standard stool concentration methods. For low-burden infections, sensitivity remains problematic and highly dependent on DNA extraction methods, when compared to culture.

Finally, in addition to enteric pathogens, NAATs are well studied for the diagnosis of blood and tissue parasites including malaria, *Leishmania*, American and African trypanosomes, filaria, and *Babesia* [4••]. It is also probable that as the technology becomes simpler (such as isothermal NAATs) and machinery becomes more robust and less expensive, these tests may become more practical in resource-poor settings.

NAATs will likely bring even more changes to diagnostic parasitology in the near future. Many assays allow for reliable quantitative results, although the clinical relevance is largely unknown at this time. Several groups have shown excellent results with the use of rectal swabs, compared to cumbersome bulk stool collection [27]. Sad as it may be for many microbiologists, it might not be long before the microscopes in clinical parasitology labs are finally relegated to history.

Proteomics in Clinical Parasiotology

Proteomic approaches have been at the forefront of development of novel diagnostics methods in recent years. One important reason for studying the proteomes of hosts and parasites is to identify proteins that can be utilized as biomarkers to differentiate between healthy and diseased individuals. Biomarkers can originate from parasites or various host tissues, cell types, and developmental stages. They can be used as potential targets for drug discovery and interventional strategies in addition to differentiating between disease states. In the latter case, well-characterized biomarkers could form the basis of novel diagnostic tests to assess disease but also to confirm cure following treatment.

In recent years, the identification of novel biomarkers in parasite diagnostics has relied on the use of mass spectrometry (MS) platforms. Such instruments include matrix-associated

laser desorption ionization time-of-flight MS (MALDI-TOF MS), surface-enhanced laser desorption ionization time-of-flight MS (SELDI-TOF MS), liquid chromatography combined with mass spectrometry (LC-MS), isotope-coded affinity tags (ICAT), and isobaric tags for relative and absolute quantification (iTRAQ) [28].

Most specifically, studies published on parasitic diseases, such as human African trypanosiomasis (HAT), fascioliasis, cysticercosis, and Chagas disease, have all focused on the use of SELDI-TOF MS [28, 29]. Unlike MALDI spotting plates, samples for SELDI are spotted on ProteinChip™ arrays exhibiting various chemical surfaces. This permits selective binding of proteins according to hydrophobicity, metal-binding properties, or by charge. In turn, this allows for the profiling of several complex biological samples, most commonly serum, in a high-throughput top-down approach. SELDI-TOF MS has facilitated the analysis of large sample groups for the discovery of biomarkers that, alone or in combination, can distinguish between diseased or healthy groups. The output is a spectrum of mass-to-charge ratios (m/z values) with their corresponding relative abundance intensities. Like any other technique, the SELDI has its limitations, one being its lower resolution and lower mass accuracy compared to the MALDI. In addition, SELDI is unsuitable for high molecular weight proteins (>100 kDa) and is limited to the detection of proteins bound onto the ProteinChip™ array. Luckily, with recent advances in the field, newer mass spectrometry instruments have been engineered to address these limitations.

Given these limitations, the SELDI has nonetheless proven its utility for discovering biomarkers of interest, especially for Chagas disease. In a 2010 report [30], several biomarkers for Chagas disease were identified using the SELDI platform by comparing large cohorts of serum samples from healthy and chagasic individuals from Venezuela. The data generated was submitted to thorough statistical analysis resulting in the discovery of several protein peaks of interest that were further characterized using other proteomics tools, including immunoprecipitation, MALDI, and immunoblotting. They were all identified as being human host proteins, more specifically apolipoprotein AI and fragments thereof, as well as one fragment of fibronectin. This was the first study highlighting a distinctive pattern or configuration of circulating serum proteins in Chagas disease. This pattern is also known as "proteomic fingerprinting."

In a recently completed study on Chagas disease (Ndao et al., submitted), the proteomic fingerprint identified in the previous study [30] was tested for its potential to assess cure in Chagas patients treated with nifurtimox. Serum samples from Bolivian chagasic participants were collected as well as follow-up samples 3 years post-nifurtimox treatment. Healthy controls matched by age and sex were also included in the study (see Jackson et al. [31]). All three groups (healthy, chagasic, and follow-up) were analyzed by SELDI-TOF MS. The same key biomarkers identified previously, namely the fragments of apolipoprotein AI and fibronectin, were successfully characterized in this study and by Miao et al. [32]. The proteomic pattern of these biomarkers in healthy and chagasic patients was then compared and validated the pattern found in the first study. A unique feature of this study was that the patterns of healthy and follow-up samples could also be compared. Interestingly, it was noted that biomarker levels returned to the levels observed in healthy patients in 46 % (17/37) of the nifurtimox-treated patients. These results suggest that these key biomarkers could potentially be used to predict cure.

These initial studies demonstrate the utility of mass spectrometry approaches, especially SELDI, in the discovery phase of potential biomarkers and the use of proteomic fingerprinting in the development of diagnostic tests for disease and/or assessment of cure. It is unlikely that proteomic platforms will emerge as routine primary diagnostic tools in most settings due to their high cost and complexity of integration in the field. However, their power lies in their ability to help in the discovery of novel biomarkers that can then be incorporated into simple and affordable diagnostic tests based on immunological assays such as ELISAs, immunoblots, or dipsticks. Screening of large complex biological samples for individual protein and peptide biomarkers would simply not be possible without mass spectrometry. Thus, proteomic platforms currently play an increasing role in diagnostics development and—in some settings—may be used in routine clinical laboratories in the future.

Rapid Diagnostic Tests for Remote or Low-Resource Settings

Following decades of neglect of funding for diagnostic laboratory infrastructure in much of the world [33], accurate diagnosis has been recognized as a principal factor limiting access to lifesaving care for many severe but treatable parasitic infections [34]. The pertinence and availability of rapid diagnostic tests (RDT) for such infections has been extensively reviewed [35••, 36••, 37, 38, 39•]. For this section, RDTs are defined as any test yielding results within minutes and that can be performed in health centers with basic infrastructure and minimally trained personnel. Requirements for simplicity, thermostability, and affordability mean that, for the moment, available RDTs consist mostly of immunoassays detecting either host antibodies or parasite antigens. The RDTs briefly presented below have achieved a high degree of validation. A myriad of other RDTs not mentioned here are sold in many countries, usually with minimal registration requirements and variable or poorly established accuracy.

Malaria

RDTs for the detection of *Plasmodium falciparum* parasites in blood have been extensively field validated [38, 40] and their use is recommended by WHO [41]. They are based on the detection of either the HRP2 antigen or of *P. falciparum*-specific parasite lactate dehydrogenase (Pf-pLDH). While HRP2-based RDTs tend to have higher sensitivity [42], they remain positive for several weeks after infection resolves and can yield false-negative results in hyperparasitemic individuals [43, 44]. In contrast, Pf-pLDH rapidly becomes undetectable after successful treatment and is not subject to this "prozone" phenomenon. Despite high accuracy, several factors may lead to false-negative or false-positive results from malaria RDTs, as recently reviewed [45, 46••]. It is also important to note that current RDTs do not assess the degree of parasitemia and that this parameter is key for assessment of prognosis and response to treatment.

The limit of detection of expert microscopy is approximately 50 parasites/μl, roughly equivalent to a parasitemia of 0.001 % and a total body burden of 1×10^8 parasites. In routine laboratories where malaria is rarely encountered and microscopists have limited experience, the limit of microscopic detection is around 500 parasites/μl [47]. The overall sensitivity of RDTs for *P. falciparum* diagnosis reaches 95 % at parasite densities above 100/μl but falls below 75 % with less than 100 parasites/μl (roughly 0.002–0.01 % parasitemia), depending on the type of RDT used [46••]. In expert travel clinics, parasite densities below 500/μl are observed in about 10 % of the febrile patients diagnosed with *P. falciparum* malaria [46••]. Finally, it is important to note that although many malaria RDTs can detect other *Plasmodium* species via pan-specific lactate dehydrogenase (pLDH), the clinical sensitivity for these (fortunately rarely lethal) species is far lower than for *P. falciparum* and may frequently yield false-negative results.

Visceral Leishmaniasis

Visceral leishmaniasis (VL) is second only to malaria in a number of fatalities from a parasitic disease [48]. The clinical features of this infection lack specificity, rendering diagnosis difficult. RDTs detecting antibodies against rK39, a recombinant antigen from *Leishmania infantum*, were developed over 15 years ago [49]. A meta-analysis of 13 studies revealed an overall sensitivity of 93.7 % and a specificity of 95.3 %, with a trend toward decreased sensitivity in East Africa [50]. Lower sensitivity in East Africa and Brazil was confirmed in later studies [51, 52, 53•]. Only two rK39-based RDTs are sufficiently validated for clinical use: the Kalazar Detect™ (Inbios, Seattle, USA) and the IT-LEISH™ (BioRad, Marnes-la-Coquette, France). More recently, RDTs detecting antibodies to a synthetic peptide fusing three antigenic targets (rK28) showed promising results (sensitivity 95.9–98.1 %) in a small study of patients in Sudan and Bangladesh [54].

Unfortunately, VL relapses cannot be diagnosed using antibody detection tests because of long-term persistence of antibodies [55]. Work is underway to convert an existing urinary antigen detection test (KAtex™, Kalon Biological Ltd, Guildford, UK) into a more reliable lateral flow format. Identification of suitable antigen targets and development of field-suitable antigen detection tests for VL is a key priority for clinical care and diseases control.

In summary, the accuracy of a given RDT varies substantially across regions, but not within a given region [53•]. Kalazar Detect™ and IT-LEISH™ may be used to confirm a first episode of VL in clinical suspects, but they safely rule out disease only in South Asia. For the diagnosis of relapses—which occur in 60 % of HIV coinfected patients within the first year [56]—an accurate and easy to use RDT based on antigen detection is needed.

Human African Trypanosomiasis

The card agglutination test for trypanosomiasis (CATT) has been used for mass screening and diagnosis of HAT from *Trypanosoma brucei gambiense* for decades [57]. Unfortunately, the CATT requires electricity and equipment, has variable sensitivity across endemic foci, and is formatted for mass testing. Two lateral flow immunochromatographic RDTs for the individual serodiagnosis of *T. brucei gambiense* HAT have been developed: (1) the "Immunochromatographic HAT-RDT" (Standard Diagnostics, Kionggi, Korea), in collaboration with the Foundation for Innovative New Diagnostics (FIND), and (2) the "*Gambiense*-Sero-K-set" (Coris BioConcept, Gembloux, Belgium); both were in collaboration with the Institute of Tropical Medicine, Antwerp. Both products were launched in 2013 following successful phase 2 validation [58], and phase 3 studies among clinical suspects presenting for care are ongoing.

No existing or projected blood-based test for *T. brucei gambiense* HAT can distinguish hemolymphatic (stage 1) from meningoencephalitic (stage 2) disease, which is necessary to determine appropriate treatment. This means that direct identification of parasites and white blood cells in cerebrospinal fluid is still required for people with positive screening tests.

For HAT caused by *Trypanosoma brucei rhodesiense* (so-called East African HAT), no RDT is in development. However, diagnosis is relatively straightforward with microscopy since parasite load is usually high in the blood of symptomatic individuals.

Schistosomiasis

Recent reports from East and West Africa have shown that a commercially available circulating cathodic antigen (CCA) cassette test using urine is more sensitive than multiple Kato-Katz thick smears derived from stool samples for the diagnosis of *Schistosoma mansoni* infection [59, 60]. Given the known limitations of stool microscopy as a reference standard, latent class analysis was used to determine a sensitivity of 96.3 % and a specificity of 74.7 % for the CCA cassette. These findings were confirmed in a subsequent multicountry field study [61]. Work on a diagnostic test for detection of all *Schistosoma* species has led to the development of a promising assay with very high sensitivity based on the serum circulating anionic antigen (CAA) [62].

Finally, although a specific parasitological diagnosis is optimal, a recent meta-analysis underscores the utility of urine heme "dipsticks" for the diagnosis of *Schistosoma haematobium* infections, with sensitivities ranging from 81 to 92 % and specificities of 89–97 %, depending on the population tested [63].

Other Pathogens and Special Considerations

Immunochromatographic assays are available for several enteric protozoa found in stool. Many of these perform acceptably compared to microscopy in nonspecialized laboratories and have been reviewed elsewhere [64].

Finally, coinfections can dramatically decrease the accuracy of well-characterized RDTs, and this effect varies according to the brand and model of RDT used. Known examples include coinfections involving HAT, malaria, HIV, and VL [65–67]. These are associated with decreased specificity of various RDTs, and this is likely related to high levels of circulating immunoglobulins.

Conclusions

Adequate diagnosis of many parasitic infections using microscopy is limited by many factors. Fortunately, the field of clinical parasitology is finally seeing the development of many new technologies and joining several other sections of the clinical laboratory in their application. Nucleic acid amplification for enteric protozoa is the most advanced, with several sophisticated assays already on the commercial market in many countries. Tests for the diagnosis of other protozoa and helminths are not far behind. Proteomics, while still requiring major capital investments for equipment, has opened up a new world of protein "fingerprints" for the diagnosis of both infection and successful recovery, especially for highly problematic diseases where parasite detection has been elusive, such as Chagas. Finally, rapid and simple tests are now allowing for the proper study and control of a variety of parasite infections in low-resource areas, helping to overcome the diagnostic hurdles which have stymied these efforts in so many parts of the world.

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Compliance with Ethics Guidelines

Conflict of Interest Michael Libman, Joanna Merckx, Momar Ndao, and Cedric Yansouni have no conflicts of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by the author.

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- • Of importance
- •• Of major importance

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