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REVIEW

# Membrane Technology for Rapid Point-of-Care Diagnostics for Parasitic Neglected Tropical Diseases

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**SUMMARY** Parasitic neglected tropical diseases (NTDs) affect over one billion people worldwide, with individuals from communities in low-socioeconomic areas being most at risk and suffering the most. Disease management programs are hindered by the lack of infrastructure and resources for clinical sample collection, storage, and transport and a dearth of sensitive diagnostic methods that are inexpensive as well as accurate. Many diagnostic tests and tools have been developed for the parasitic NTDs, but the collection and storage of clinical samples for molecular and immunological diagnosis can be expensive due to storage, transport, and reagent costs, making these procedures untenable in most areas of endemicity. The application of membrane technology, which involves the use of specific membranes for either sample collection and storage or diagnostic procedures, can streamline this process, allowing for long-term sample storage at room temperature. Membrane technology can be used in serology-based diagnostic assays and for nucleic acid purification prior to molecular analysis. This facilitates the development of relatively simple and rapid procedures, although some of these methods, mainly due to costs, lack accessibility in low-socioeconomic regions of endemicity. New immunological procedures

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and nucleic acid storage, purification, and diagnostics protocols that are simple, rapid, accurate, and cost-effective must be developed as countries progress control efforts toward the elimination of the parasitic NTDs.

**KEYWORDS** neglected tropical diseases, parasites, point-of-care diagnostics, rapid diagnostics, helminths, protozoa

## INTRODUCTION

The disease burden due to parasitic infections is considerable, with over 1 billion people affected by neglected tropical diseases (NTDs) due to these pathogens (1, 2). Parasites causing NTDs include helminth, protozoan, and arthropod infections and predominantly afflict people in low-socioeconomic areas of the tropics and subtropics, although several also occur in more temperate regions (3, 4) (Table 1). These pathogens cost local economies billions of dollars each year due to medical treatment costs and loss of production in infected animals; nonetheless, they generally receive little public attention (1). Assessment of the years of life lost (YLL) and the years of life lived with disability (YLD) rather than mortality caused by various diseases led to the development of the term disability-adjusted life years (DALYs), allowing for a new way to illustrate the health impacts of NTDs (5, 6). Globally, NTDs caused by parasites result in the loss of over 25 million DALYs (5, 6). Management of these diseases involves treatment through chemotherapy, vector control (when applicable), improving hygiene and sanitation in areas of endemicity, education programs, and accurate diagnosis for surveillance (1, 7–9).

While some success has been achieved in NTD control, for example the near elimination of Guinea worm (*Dracunculus medinensis*) infection in Asia and some African countries (10), and schistosomiasis (due to *Schistosoma japonicum*) in China (11), disease management programs exhibit some limitations. The majority of these infections occur in rural, low-socioeconomic communities with poor sanitation and limited road access (12–16), and these areas generally lack the infrastructure necessary for accurate parasite diagnosis such as reliable electricity, making diagnostic tests that require complicated equipment and an adequate power supply nonviable. This lack of resources also makes collection, storage, and transport of clinical samples (blood, serum, feces, urine, skin scrapings, and saliva) to in-country or external laboratories capable of undertaking the relevant diagnostic procedures difficult and costly (Table 2). Furthermore, the absence of reliable electricity makes refrigeration of samples before and during transport difficult if not impossible (17). Another factor affecting parasite control is climate change, which has significant effects on parasite distribution and control, causing changes in the environment and animals available for zoonosis (18). Increasing temperatures have caused an expansion of the tropical and subtropical zones, giving ideal soil conditions for soil-transmitted helminth (STH) survival, allowing increased vector distribution, and encouraging expansion of parasitic disease distribution to areas that previously had not been regions of endemicity (18–21). Globalization has caused in an increased risk of foodborne helminths due to immigration and global exportation and importation of food and live animals. Furthermore, urbanization has caused increased contact between wild animals and humans, allowing wildlife-maintained parasites to infect humans and domestic animals (22–24).

Accurate diagnosis of NTDs is essential for the treatment and epidemiological monitoring of disease burdens. Mass drug administration (MDA) is the gold standard for large-scale treatment of parasitic diseases in areas of endemicity. However, due to a lack of education in many regions of endemicity, patients may not understand why they must take the drug, increasing mistrust and decreasing compliance and leading to low population coverage and little impact on overall prevalence (25). Furthermore, up to 80% of patients will experience transient side effects, causing these and others in the community to refuse taking part in future MDA due to apprehension about taking the drug (25, 26). Furthermore, since chemotherapy does not prevent reinfection,

**TABLE 1** Parasitic NTDs and worldwide case numbers[a]

| Condition | Organism(s) | No. of cases worldwide |
|---|---|---|
| Protozoa | | |
| Human African trypanosomiasis (HAT) | *Trypanosoma brucei* | <1,000 |
| Chagas disease | *Trypanosoma cruzi* | 6–7 M |
| Cutaneous leishmaniasis | *Leishmania tropica*, *Leishmania mexicana*, *Leishmania major* | 30,000 |
| Mucocutaneous leishmaniasis | *Leishmania braziliensis* | |
| Visceral leishmaniasis | *Leishmania donovani* | |
| Filariasis | *Wuchereria bancrofti*, *Brugia malayi*, *Brugia timori*, *Loa loa* | 120 M |
| Helminths | | |
| Soil-transmitted helminthiasis (STH) | *Ancylostoma duodenale*, *Necator americanus*, *Ascaris lumbrcoides*, *Trichuris trichiura*, *Strongyloides stercoralis* | 1.5 B |
| Onchocerciasis | *Onchocerca volvulus* | 20.9 M |
| Drancunculiasis | *Dracunculis medinensis* | 54 |
| Echinococcosis | *Echinococcus granulosus*, *Echinococcus multilocularis* | 1 M |
| Taeniasis/cysticercosis | *Taenia solium* | 2.79 M |
| Fascioliasis | *Fasciola hepatica*, *F. gigantica* | 10,635 |
| Schistosomiasis | *Schistosoma japonicum*, *Schistosoma mansoni*, *Schistosoma aematobium* | 240 M |
| Arthropods | | |
| Scabies | *Sarcoptes scabiei* | 200 M |

[a]Worldwide cases based on information from the WHO (332–334) and from the Global Burden of Disease Study 2016 (335) and other sources (336, 337). M, million; B, billion.

individuals can become reinfected almost immediately after treatment (26–28). Education programs, such as the Magic Glasses intervention for STHs, successfully tested in China, aim to change behavior and prevent infection by increasing knowledge about locally acquired parasite infections and correct hygiene practices, thereby decreasing reinfection rates after MDA (8, 29, 30). As such, community engagement is an essential component of improved MDA compliance (26, 31, 32).

Diagnostic tests for NTDs caused by parasites include laboratory-based methods, such as molecular and immunology-based assays. Samples are either sent to a central laboratory for analysis, or point-of-care (POC) testing is undertaken, where the assays are performed close to or near the patient. POC testing can result in decreased costs of diagnosis, improved efficiency, and optimized treatment regimens useful in resource-poor endemic countries with deficient infrastructure (33).

In 2015, the World Health Organization (WHO), The Foundation for Innovative Diagnostics (FIND), and the Centers for Disease Control and Prevention (CDC) defined criteria to be used as a benchmark for the use of POC diagnostic tests known as ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable to end-users) applicable in resource-poor endemic regions (34). The acceptability of a suitable diagnostic test must conform to this six-step guide (Table 3). This method of selection has been successfully employed in the implementation of a number of diagnostic tests for malaria (35), but few accurate and accessible POC diagnostics and monitoring tests for the NTDs have been developed (36, 37). Recently, the ASSURED criteria have been reassessed and improved to give REASSURED (Real-time connectivity, Ease of collection, Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment free/simple and environmentally friendly, and Deliverable to end-user), aiming to improve disease control, strengthen health systems, and improve patient outcomes (38, 39) (Table 4).

The WHO has recently put out a call for expert advice on diagnostics targeting schistosomiasis and STH, highlighting issues with currently available diagnostics and looking toward the development of POC diagnostics, particularly for schistosomiasis.

**TABLE 2** Clinically relevant laboratory-based diagnostic methods for parasitic NTDs[a]

| Parasite and condition | Parameter | Microscopy | Immunodiagnostics | DNA-based | Other/notes |
|---|---|---|---|---|---|
| Protozoa | | | | | |
| Human African trypanosomiasis | Sample type(s) | Blood smear, CSF, lymph, chancre | Serum, blood | Blood/serum | Positive serology requires confirmatory testing via visualization of parasites. *T. rhodesiense* has no available rapid test and requires lab-based serology testing. PCR may complement serology and parasite visualization if available. |
| | Test name(s) | Direct or concentrated slide prep | Card agglutination, rapid lateral flow, ELISA, IFAT | PCR, qPCR | |
| | Reference(s) | 338–342 | 129, 343–345 | 346–348 | |
| Chagas | Sample type(s) | Blood, buffy coat | Serum | Blood/serum, skin | PCR can be used for monitoring patients for reactivation risk; sensitive detection in acute disease. Parallel antigen-based tests are required to improve the accuracy of diagnosis. |
| | Test name(s) | Direct blood smear or buffy coat | ELISA, IFA, TESA, IHA, Abbott TESA Chagas | PCR, qPCR | |
| | Reference(s) | 349 | 350–353 | 349, 353–358 | |
| Cutaneous/mucocutaneous leishmaniasis | Sample type(s) | Skin/lesion biopsies | | Skin/lesions | Parallel tests (histology, culture, PCR) for best diagnostic accuracy. RFLP-PCR distinguishes species. |
| | Test name(s) | Histology, NNN medium culture | | PCR, qPCR, RFLP-PCR | |
| | Reference(s) | 359–362 | | 361–364 | |
| Visceral leishmaniasis | Sample type(s) | Bone marrow, whole blood, lymph node, spleen | Serum | Blood/serum | Bone marrow aspirate is the preferred specimen. Consider serology for suspected VL with negative or inconclusive histopathology, culture, and PCR. Immunocompromised individuals may have lower sensitivity to serological tests; PCR and culture are recommended. SMART Leish PCR has been developed by the U.S. Department of Defense and approved by the FDA but is not commercially available (365). |
| | Test name(s) | Direct blood smear or buffy coat, histology, NNN media culture | ICT, IFA, ELISA, DAT | qPCR, PCR | |
| | Reference(s) | 359–361 | 189, 361, 366–369 | 361, 366, 370 | |
| Helminths | | | | | |
| Filariasis | Sample type(s) | Blood | Serum | | Midnight smears required for most species. However, *W. bancrofti* from Pacific islands and *B. malayi* in some Asian countries exhibit subperiodic periodicity and mf can always be found in peripheral blood (54, 371, 372). Negative antigen tests do not exclude infection or lymphatic damage in previous infection. |
| | Test name(s) | Thin/thick smears, concn (Knott's, Nuclepore) | CFA, ELISA, BinaxNOW, Alere filariasis test strip | | |
| | Reference(s) | 373–375 | 156, 209, 216, 373, 376–379 | | |
| Strongyloides | Sample type(s) | Sputum, stool, bowel biopsy | Serum | Stool, blood/serum | Larvae in stool and sputum. Clinical symptoms and epidemiological data key for initial evaluation. Microscopy often has low sensitivity; serology is recommended. A number of molecular diagnostics have been developed and are used in some diagnostic labs. |
| | Test name(s) | agar plate culture, Baermann, sputum smear, histopathology | IFA, Western blotting | qPCR, LAMP | |
| | Reference(s) | 100, 380–387 | 385, 388, 389 | 390–392 | |

(Continued on next page)

**TABLE 2** (Continued)

| Parasite and condition | Parameter | Microscopy | Immunodiagnostics | DNA-based | Other/notes |
|---|---|---|---|---|---|
| Soil-transmitted helminths | Sample type(s) | Stool | | Stool | Multiplex PCR panels often combine STH with intestinal protozoan detection |
| | Test name(s) | Thick/thin smear, concn technique, agar plate | | Multiplex PCR | |
| | Reference(s) | 100, 380, 393–395 | | 396, 397 | |
| Onchocerciasis | Sample type(s) | Skin | Serum | | Antibody testing is not reliable; high cross-reactivity and not specific for current infection. Antigen testing has a higher sensitivity. Ultrasonography of nodules can also be used to identify adult worms (398). Skin snip PCR is not available clinically. |
| | Test name(s) | Skin snips, biopsy of onchercomas | ELISA, Ov-16 card test, ICT | | |
| | Reference(s) | 399, 400 | 401–404 | | |
| Dracunculiasis | | | | | Clinical presentation; observation of adult female worm (405). |
| Echinococcosis | Sample type(s) | | Serum | | Visualization of cysts (CT, MRI, ultrasonography), confirmed with serology or antigen testing. ELISA appears to be the most sensitive and specific of available assays. Antibody detection is more sensitive than antigen detection in diagnosing *E. granulosus* (406) |
| | Test name(s) | Cyst aspirate or biopsy (if performed, risk of secondary seeding of infection) | IHA, ELISA, CIEP, RIA, TR-FLA, immunoblotting, latex agglutination, Western blotting | | |
| | Reference(s) | 407, 408 | 406, 409–413 | | |
| Taeniasis/cysticerocosis | Sample type(s) | Stool | Serum | Plasma, CSF | Taeniasis can be diagnosed as eggs in feces via microscopy or PCR. Cysticercosis diagnosed from imaging (cysts) via CT and MRI, with confirmation via serology. Poor performance is identified with commercially available ELISA (414). MAT available in Europe, limited availability in the USA. qPCR may be useful in diagnosing subarachnoid NCC. |
| | Test name(s) | Direct or concentrated slide prep | EITB, ELISA | qPCR | |
| | Reference(s) | 415 | 414, 416–418 | 419) | |
| Fascioliasis | Sample type(s) | Stool, bile, duodenal aspirates | Serum | | Serological diagnostics lack specificity, cross-react with other parasites. |
| | Test name(s) | | IHA, IFA, ELISA, immunoblotting | | |
| | Reference(s) | 420, 421 | 166, 420, 422–425 | | |
| Schistosomiasis | Sample type(s) | Stool, urine (species dependent) | Serum | Serum, urine, feces | Microscopy can be used for species differentiation. In MGS, eggs may also be identified in ejaculate (426) and in vaginal swabs in women with FGS (427, 428). The majority of commercially available antibody tests are not species specific. |
| | Test name(s) | Thin or thick smear, concentrated, urine filtrate, FLOTAC | ELISA, IHA, RIA, IFA, Western blotting, complement fixation | qPCR, PCR | |
| | Reference(s) | 44–47 | 12, 44, 168, 174, 429–431 | 12, 73, 432, 433 | |
| Arthropod | | | | | |
| Scabies | Sample type(s) | Skin scrapings/biopsy | | | Clinical diagnosis based on itching, plaque, and location (interdigital spaces, skin folds). |
| | Reference(s) | 434, 435 | | | |

[a]CIEP, countercurrent immunoelectrophoresis; CT, computed tomography; DAT, direct agglutination test; ELISA, enzyme-linked immunosorbent assay; EITB, enzyme-linked immunoelectrotransfer blot; FGS, female genital schistosomiasis; ICT, immunochromatographic test; IFA, immunofluorescent antibody test; IFAT, indirect fluorescent antibody test; IHA, indirect hemagglutination assay; mf, microfilariae; MGS, male genital schistosomiasis; MRI, magnetic resonance imaging; TR-FLA, time-resolved fluoroimmunoassay; TESA, trypomastigote excreted secreted antigens; RIA, radioimmunoassay.

**TABLE 3** WHO guidelines for evaluation, validation, and implementation of POC diagnostic tests[a]

| Step | Description |
|---|---|
| 1 | Define the purpose of the test (why, what, where, and who?) |
| 2 | Review the market |
| 3 | Review the regulatory approval by international and national bodies |
| 4 | Determine optimal diagnostic accuracy of test |
| 5 | Determine practical diagnostic accuracy of test |
| 6 | Monitor routine use of test |

[a]Source: World Health Organization (34).

The focus is on easy interpretation by field workers and low cost (less expensive than two to three rounds of MDA), and any necessary equipment should be highly transportable and battery powered if electricity is required at all; the whole procedure should take less than a day between sample collection and interpretation of result and treatment decision (40).

In this review, we provide an overview of the challenges posed by currently available collection, storage and diagnostic procedures for parasitic NTDs and emphasize how revolutionary membrane technologies can be used to streamline these processes. Specifically, we focus on (i) a description of the commonly used procedures used for clinical sample collection, storage, and an appraisal of the diagnostic methods available; (ii) the application of membrane technology for clinical sample collection, storage, and transport; and (iii) the uses of membrane technology for clinical sample preparation, including nucleic acid purification and antigen/antibody detection.

## OVERVIEW OF CURRENT DIAGNOSTIC METHODS

### Microscopy-Based Procedures

Accurate diagnosis of a parasitic infection involves the use of laboratory techniques, clinical history, geographic location, and travel history (28, 41). The current gold standard of diagnosis involves microscopic identification of parasitic stages in clinical samples, usually blood, urine, or feces (28, 41, 42). Low-cost and accessible microscopy-based procedures in areas of endemicity include the Kato-Katz thick-smear method (KK), the formalin-ether concentration technique (FECT), and urine filtration (for *Schistosoma haematobium*) (26–28, 43–47) (Table 2). However, these methods can be

**TABLE 4** REASSURED criteria and definitions

| Acronym | Criterion | Definition |
|---|---|---|
| R | Real-time connectivity | Tests can be connected and/or a mobile phone or reader can be used to power the reaction and/or read test results to provide required data to decision-makers. |
| E | Ease of specimen collection | Tests should be designed to use specimens collected by noninvasive procedures. |
| A | Affordable | Tests must be affordable to the end-users and healthcare system. |
| S | Sensitive | Avoid false negatives. |
| S | Specific | Avoid false positives. |
| U | User-friendly | Simple procedure for testing: the test can be performed in a few steps that require minimal training. |
| R | Rapid and robust | Results must be available rapidly following patients first visit (about 15 min to 2 h); tests are able to survive supply chains without the need for specialized transport and storage conditions. |
| E | Equipment free or simple and environmentally friendly | Tests do not require special equipment or equipment can be operated by simple devices that are powered by solar or battery power (completed tests should be manufactured from recycled materials that require simple disposal). |
| D | Deliverable to end-user | Accessible to those who require tests the most |

[a]Sources: Gordon et al. (3) and Machado et al. (389).

time-consuming and require skilled laboratory scientists that must pass regular competency testing to identify parasite eggs, larvae, and cysts (42). Furthermore, good-quality microscopes with light sources, thus requiring electricity, are necessary for parasite detection and also include correct objectives, including oil immersion objectives, calibration, and regular maintenance, making them problematic in resource-poor regions (42). Microscopy-based procedures also lack sensitivity and specificity due to the difficulty in identifying parasites to the species level, even with strict morphological criteria to follow (28, 48–50).

Another important factor affecting microscopic diagnosis is timing, since the parasite may not be identifiable if investigation is undertaken too early or too late during infection. For example, during *Schistosoma* spp. infections, diagnosis by microscopic identification of parasite eggs in fecal samples is not possible until the infection becomes patent, when eggs are being produced, 4 to 8 weeks postinfection (51, 52). Another example is lymphatic filariasis, where microfilariae may not be present in the patients with overt clinical symptoms or exhibit periodicity (53, 54). Damage to lymphatic systems which results in elephantiasis may occur years before the physical manifestation of edema, decreasing the utility of microscopy as a diagnostic in late infections or chronic diseases where antigen testing is preferred (54) (Table 2).

**Immunodiagnostics**

Serological assays use organism-specific antigens or antibodies to diagnose parasitic diseases. Antigens consist of shed parasite-derived products that are detectable in the blood, serum, urine, saliva, and vaginal fluids of infected individuals (12, 55–59), whereas antibodies are specific responses by the host to the invading parasite and can be primarily detected in blood and sera. Antibody detection can have issues with cross-reactivity, and interpretation can be difficult in some later flow assays; since the parasites are present long after the infection has cleared, however, these methods can be useful in areas of low endemicity or chronic infections, where parasitemia may be low in infected individuals (12, 60) (Table 2). The majority of RDTs are based on antibody detection.

Serological methods include the indirect hemagglutination test, direct or indirect immunofluorescent antibody tests (DFAs or IFAs), complement fixation (CF) tests, rapid diagnostics tests (RDTs), such as immunochromatographic antigen detection and the enzyme-linked immunosorbent assay (ELISA) (61–64) (Table 2). ELISAs use 96-well plates that have been coated to allow for protein binding (65–67). The wells are coated with the antigen of interest or primary antibody before being washed and blocked, using agents such as bovine serum albumen, ovalbumin, and other animal proteins to prevent nonspecific protein binding (65–67). The samples are then incubated with an enzyme-conjugated antibody (primary and/or secondary), followed by the addition of a substrate, such as horseradish peroxidase or alkaline phosphatase, which initiates a visible color change within 30 min (65–67). Replacing the plastic wells used for ELISA with a nitrocellulose membrane simplified the method significantly, giving rise to the Dot-ELISA (68, 69). This change also reduced cost the associated with the traditional ELISA (68, 69).

Most available POC tests are immunology based due to their accessibility and relative simplicity for use in the field and have been widely used in the past for diagnosis and control program monitoring (Tables 2 and 5). More details, as well as examples for RDT immunodiagnostics, are presented below in "RDT Immunodiagnostics."

**Molecular-Based Diagnostics**

Nucleic acid amplification tests (NAATs) exhibit high sensitivity and specificity in areas with both high and low intensity parasite infections and involve a number of PCR (PCR)-based methods, including conventional PCR, direct-PCR, real-time PCR (qPCR), droplet digital PCR, and loop-mediated isothermal amplification (LAMP) (Table 2) (70–72). NAATs have many advantages compared to other diagnostic methods, including increased sensitivity, specificity, and flexibility of testing (58, 71–73). One of the

**TABLE 5** Advantages and disadvantages of different protocols used in the diagnosis of parasitic NTDs[a]

| Diagnostic test/sample collection tool | Advantages | Disadvantages |
|---|---|---|
| Immunoassays[b] | | |
| ELISA | High sensitivity and specificity, high throughput, simple, rapid | Lab-based technique, requires specialized technicians, high reagent costs. |
| Dot-ELISA | Simple, rapid, results easy to interpret visually, require small sample volumes, can be performed external to a lab. | High reagent cost, samples must be stored below 4°C, sensitivity, specificity dependent on parasitemia. |
| LFIA | Rapid, simple, higher sensitivity and specificity than ELISA, can be interpreted visually, require small sample volumes | Samples must be stored below 4°C, sensitivity and specificity depend on parasitemia, cross-reactivity issues, some problems with false negatives/positives and trace bands. |
| Schistosomiasis POC-CCA cassette-based test | Antigen detection. Commercially available POC detection of *S. mansoni* and *S. haematobium* | Significant issues with detecting false positives, difficult test interpretation, cross reactivity noted with other helminth species. |
| Molecular | | |
| Whatman | Samples stable at room temp for 10 yrs, small sample volumes required, simple sample collection, low-cost transport. | Complex processing requirements, no research on use of Fusion 5 in parasitic NTD diagnosis. |
| Fusion 5 | Increased wicking speed, increased efficiency of DNA capture; effective for DNA extraction from blood, pig mucin, saliva, buccal swabs, and cigarette buds. | High cost, requires large sample volumes, only studied on saliva and blood, no research on use of AOMs for parasitic NTD diagnosis. |
| DNA dipstick | Low cost, can be combined with LAMP assay. Simple workflow. | Not tested on clinical samples, only tested on *S. japonicum* parasite material and infected snails. |
| AOM | Rigid membrane, increased wicking speed, high sensitivity for diagnosis of bacterial and viral infections | Complex processing requirements, no research on use of Fusion 5 in parasitic NTD diagnosis. |
| Djinni Chip | Low cost, LAMP assay on the chip. Simple workflow. | Not yet used for parasite NTD diagnosis. Contamination in a "mock field" trial. |
| SPS and microfluidic chip | SPS separates plasma for blood without electricity. LAMP occurs on the chip. | Has only been performed in laboratories and on blood spiked with *S. mansoni* DNA |

[a]ELISA, enzyme-linked immunosorbent assay; LFIA, lateral flow immunodiagnostic assays; AOM, aluminum oxide membrane; SPS, superhydrophobic plasma separator.
[b]In antibody-based ELISAs, it can be difficult to distinguish between past and present infections.

main factors preventing widespread use of NAATs as POC diagnostics is the cost of reagents equipment, the need for electricity, the requirement for a unidirectional workflow to minimize contamination, and the cost of the methods required to purify nucleic acids from clinical samples (74, 75). Recently, however, new molecular-based machines, including the Djinni chip (which we discuss below in "DNA-Based RDT") and GeneXpert (76, 77). GeneXpert is a module which runs and reads Xpert cartridges for various tests, including tuberculosis (TB) in sputum samples (78) and, more recently, COVID-19, for which emergency FDA approval has been granted (79). The GeneXpert units have been placed in diagnostic laboratories across Africa, and thus their presence could be leveraged for the molecular diagnosis of endemic NTDs, including parasitic diseases.

A disadvantage of NAATs is that the sensitivity and specificity of some methods can be decreased for certain parasite species and sample types. For example, during chronic Chagas disease, where the causative parasite, *Trypanosoma* spp., is not circulating in the blood, NAATs are unable to reliably detect infection (80). In these cases, serological tests are necessary to confirm infection; indeed, parallel antigen tests are suggested for improved diagnostic accuracy for Chagas disease (80) (Table 2). However, PCR can be useful in monitoring for reactivation of disease (Table 2). The three most commonly used nucleic acid purification methods are: (i) organic methods using phenol-chloroform; (ii) inorganic methods based on binding nucleic acids to silica/Chelex substrates in the presence of high-salt solutions; and (iii) solid-phase extraction methods, which act by binding DNA to paramagnetic or silica beads (81–84). A series of wash and centrifugation steps remove contaminants that may be present in the sample before the final elution step (81, 82, 85, 86). These methods are often laborious, involving complicated reaction steps and necessitating trained technicians and

electrical equipment, and expensive. Thus, these methods are problematic for incorporation in POC diagnosis in resource-poor areas of endemicity (87, 88).

### Clinical Samples and Parasite Locations

Parasites that cause NTDs have differing life cycles and infect different bodily tissues and fluids that can vary depending on the life cycle stage and infection status (acute versus chronic), and some, such as lymphatic filariasis, experience periodicity in peripheral blood; this affects the type of clinical sample harboring parasite material that can be utilized for diagnostic purposes (57, 89, 90). Parasites that invade the circulatory system as adults or larval stages directly release waste products, surface materials, eggs, and DNA into the circulatory system, which can be detected subsequently in host bodily fluids (57). For example, sloughed-off tegument from adults and schistosomula larvae and eggs, and DNA, including cell-free DNA, have been successfully detected in the feces, blood, serum, urine, saliva, and vaginal fluids of mammalian hosts harboring *Schistosoma* blood flukes in the blood vessels surrounding the gut (*S. mansoni* and *S. japonicum*) or the bladder (*S. haematobium*) (12, 57, 58, 70).

Intestinal helminths release eggs, immature and adult life cycle stages, waste products, surface material, and DNA directly into the gastrointestinal tract, which can be detected in fecal samples. Material from intestinal worm infections can also be detected in blood and serum, urine, and saliva (12, 91, 92). This may be due to mucosal changes leading to "leaky gut" syndrome caused by some parasites, such as *Strongyloides stercoralis* (65, 93), or due to migrating larval stages such as is the case for hookworm species, *S. stercoralis* and *Ascaris lumbricoides*, which all enter the bloodstream at various stages of their life cycle (94–97). *A. lumbricoides* and hookworm also undergo tracheal migration, which accounts for parasite material detectable in saliva (96, 97). The main gastrointestinal parasites are the soil-transmitted helminths (STHs), which include hookworms (*Ancylostoma duodenale*, *Necator americanus*), whipworms (*Trichuris trichiura*), and roundworms (*A. lumbricoides*) and are estimated to infect over 1.5 billion people globally (98, 99). These parasites reside in the gastrointestinal tract, releasing eggs into the external environment in the feces, which develop into infectious larvae or mature eggs, ready to continue the life cycle (99, 100). DNA, antigens and whole parasite stages have been successfully detected in feces, blood, and serum of hosts infected with STHs (100).

The quality and quantity of parasite material in clinical samples are integral for precise diagnosis of NTDs (101–103). Accurate parasite diagnostics for human and animal hosts are key to ensuring both effective treatment and in surveillance, thereby helping to reduce rebound infections in areas where treatment is curtailed and where less-sensitive diagnostic tools may have been applied so as to confirm that parasite control measures leading to elimination have been successful (8, 12, 104). Key to this are the collection, storage, and transport of clinical samples to designated laboratories for NTD diagnosis.

### Storage and Transport of Clinical Samples

Clinical samples that can be used in parasite diagnosis are feces, urine, skin scrapings, blood and sera, and saliva; each of these present different issues for collection, storage, and processing. Subjects can collect saliva, urine, and stool samples themselves when provided with appropriate containers, whereas trained professionals are required to collect blood, sera, and skin scrapings, thereby increasing labor costs (105, 106). After collection, these samples must be stored correctly, often by refrigeration or freezing, and rapidly transported to a laboratory for analysis.

The cost of storage and transport depends on the sample (Table 1). Blood and serum must be stored in individual EDTA tubes, which themselves have a high cost, but are also expensive in terms of transport and shipping due to their weight (107). Fecal storage containers themselves can be lower in cost, but samples must undergo fixation, which increases costs. Which fixatives are used depends on whether it is a clinical setting or a field setting and on the downstream application—molecular, microscopy, or immunodiagnostics. Fixation often takes place in 10% formalin, 80% ethanol,

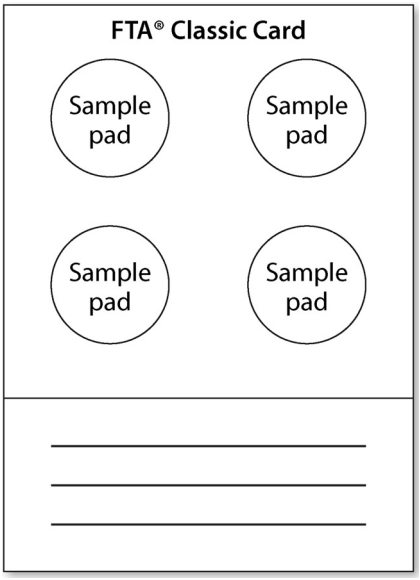


**FIG 1** Whatman FTA classic card. Sample pads contain cellulose matrix for DNA sample capture.

polyvinyl-alcohol (PVA) solutions, sodium acetate acetic acid and formalin, zinc sulfite-polyvinyl alcohol, or fresh, with costs ranging from $2 to $5 (AUD) per sample in addition to the transport costs (108–110). Some of these fixative methods can impact the method of detection; for instance, killing trophozoites or larvae mean that they are no longer motile and cannot be seen on a wet mount, but the dead parasites can be stained and viewed as a fixed mount. Other fixatives impact DNA integrity, and thus downstream detection needs to be considered. A clinical setting, where samples will be sent to a lab for testing quite quickly, will not have the same requirements as in the field, where times between defecation and fixing the sample can vary and long distances between field collection and processing in a laboratory can be quite lengthy and often without refrigeration (109, 111, 112). Urine and saliva collection containers are also low cost but must be refrigerated or frozen until used in diagnostic processing (113, 114). Saliva samples can also be fixed in PVA and 10% formalin and stored at room temperature in specially designed collection kits, again resulting in increased cost (113).

Parasite NTDs primarily occur in remote communities located in predominantly lower socioeconomic regions of the tropics and subtropics (12–16). Accordingly, collected samples may remain at room temperature for prolonged periods before transport to a testing facility (101–103). Furthermore, they are often exposed to high temperatures during transportation, which can result in the disintegration of protozoan trophozoites and cysts, as well as increased lysing of some helminth eggs (hookworm) and the disintegration of larvae (*Strongyloides*) (101–103). Consequently, storage and transportation procedures must be effective, accessible, and affordable.

**Whatman FTA cards: sample collection and storage.** Whatman Flinders Technology Associates (FTA) matrix cards (Fig. 1) consist of cellulose and contain a proprietary mix of chemicals that lyse cells on contact and physically entrap and stabilize DNA (105, 115). This occurs when the long DNA molecules become entangled with fibers of the filter paper, while other cell debris and contaminants are washed through the paper (116). This protects nucleic acids in most clinical samples, including blood, tissues, and buccal scrapes, from degradation by oxidation, ultraviolet light, and fungi at room temperature for up to 10 years (117–121).

The compact nature of Whatman filter paper technology permits their transport via standard mail, while still maintaining DNA stability, which allows for cost-effective and

reliable storage and transport (122). Furthermore, this technology can also help streamline sample collection. For example, a small finger prick is all that is necessary to obtain a blood sample, instead of collection by venipuncture, which requires the presence of a trained professional (105). FTA cards can be stored at the collection location at room temperature as long as required instead of the immediate transportation to a reference laboratory or the immediate refrigeration that is normally required for analysis of clinical samples (117–120). Whatman FTA cards have been utilized to store DNA from forensic, bacterial, and viral samples, cytological materials, and parasites (115, 123–127). Parasite entrapment has mainly focused on protozoa, including *Plasmodium*, *Trypanosoma*, *Leishmania*, and *Babesia* (Table 2), with studies successfully utilizing FTA cards for short- and long-term storage of clinical samples containing parasite DNA.

## RAPID DIAGNOSTIC TESTS

Rapid diagnostic tests (RDTs) are compact and affordable POC diagnostic tools that give rapid results. RDTs of varying efficacy have been developed for all NTDs except for scabies, STH, and dracunculiasis (128–147). Many available RDTs for NTDs utilize immunochromatography to detect circulating parasite-specific antigens (148) (Table 2). RDTs can come in dipstick or cassette form and can give results in approximately 20 min (149). RDTs that are based on immunochromatographic methods and utilize nitrocellulose membrane, which is a porous membrane with a high affinity for the hydrophobic binding of proteins (150, 151). The nitrocellulose allows wicking when the clinical sample is added, resulting in the formation of a single-colored control line for a negative result or two-colored lines (one the control line) for a positive result (152). Absence of the control line invalidates the test, and it must be repeated. These lines appear due the binding of parasite antigens present in the clinical sample to specific antibodies present on the nitrocellulose, which will be bound to gold nanoparticles, dye-loaded polymeric beads, fluorescent beads, magnetic nanoparticles, or carbon-based nanoparticles (153–158).

Molecular based RDTs are rarer; however, there are a few in early development stages that utilize LAMP in conjunction with a rapid DNA extraction step (76, 77, 159–162). These methods to date have also included the use of nitrocellulose membranes for DNA capture, with one molecular based RDT, the DjinniChip (76, 77), giving a similar read out to immunochromatographic diagnostics, with a test and control line appearing as the reaction completes, while others rely on fluorescence, turbidity, or other visual changes in the reaction mix to determine positivity.

### RDT Immunodiagnostics

**Dot-ELISA.** The ELISA is a solid-phase enzyme immunoassay that acts by the complexing of antibodies and antigens with substrates to give qualitative results that are visible to the naked eye (66, 67, 163). There are four types of ELISA: direct ELISA, indirect ELISA, sandwich ELISA, and competitive ELISA (163). Direct ELISA involves the direct binding of enzyme-linked antibodies to the antigen of interest (163) Indirect, sandwich, and competitive ELISAs work in multiple processes, involving the action of a primary antibody and a secondary enzyme-linked antibody (163).

Dot-ELISA (Fig. 2) utilizes a sandwich ELISA, capturing the antigen of interest between a primary and secondary antibody (164). To begin the process, the appropriately labeled primary antibody is incubated on pieces of nitrocellulose paper and allowed to dry. The nitrocellulose is then incubated with blocking solution for 15 min to prevent nonspecific protein binding (150, 151). Next, the nitrocellulose is incubated with the clinical test sample for 30 min, followed by incubation with a conjugated antibody (69, 150). Finally, the nitrocellulose is incubated with an enzyme substrate for 30 min, which reacts with the enzyme and allows for the formation of a colored dot if parasite antigens are present (69, 150). A positive clinical test sample is observed as a color change on the paper, allowing direct visualization of the result (150, 151). The use of nitrocellulose reduces the nonspecific binding of proteins usually observed with plastic plates, increases the sensitivity and specificity, and requires substantially less parasite

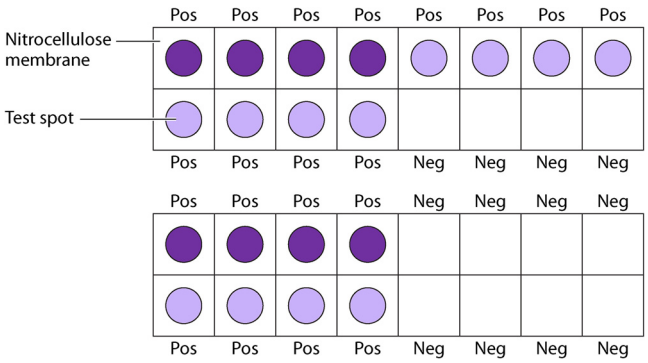


**FIG 2** Diagram showing Dot-ELISA diagnostics, showing interpretation of positive and negative samples.

reagent and clinical test sample than the standard ELISA (150, 151). However, the stability of many of these antibodies can decrease significantly if not stored below 4°C (165).

The Dot-ELISA has simplified the ELISA procedure and made it more accessible for POC diagnosis, giving sensitive results without the requirement of electrically powered equipment (69, 166). It is also rapid, can be completed at room temperature, and gives results that can be readily interpreted visually (151). However, due to the requirement for costly reagents and the high cost of storage to maintain sample viability, this assay remains generally inaccessible in resource poor regions (Table 4) (165). Nevertheless, to date, this method has been used, with a sensitivity and specificity similar to that of traditional ELISA, for the diagnosis of many parasitic diseases including: malaria, trypanosomiasis, leishmaniasis, toxoplasmosis, theileriosis, schistosomiasis, echinococcosis, fascioliasis, toxocariasis, taeniasis, and trichinosis (69, 150, 167–176) (Table 1).

**Dot-ELISA dipsticks.** Nitrocellulose-based dipsticks were first developed to improve the accessibility of the Dot-ELISA (177). Dipsticks are prepared before going to the field by cutting nitrocellulose paper into small strips impregnated with a specific antigen or antibody for the parasite species to be identified to give test and control lines and glued to acetate strips. In the field, the strips are incubated with patient serum for 7 min, washed, and incubated with a conjugated antibody for 7 min before incubation with a specific enzyme substrate for a further 2 min (153, 154). Positive results are observed as the appearance of control and test bands in the test cell, which occurs due to a sandwich immunoassay in the presence of the targeted biomarker (153, 154). Nitrocellulose paper can also act as a blood separator, allowing for the separation of serum from blood cells by capillary action. The separated serum then travels up the strip, and the used blood separator can be removed and discarded, allowing for POC testing using whole blood samples (156–158). The BinaxNOW malaria assay and Diamed OptiMAL are RDTs that can successfully diagnose *Plasmodium falciparum* and *P. vivax* infections—and likely *P. ovale*, *P. malariae*, and *P. knowlesi* infections as well; however, sufficient testing has not been performed to determine the sensitivity and specificity of these species (178–180). Furthermore, RDTs cannot detect low parasitemia, leading to false negatives (181) (Table 4). Nitrocellulose-based dipsticks have since been improved to allow for higher sensitivity, specificity, and applicability for POC diagnostics, giving rise to lateral-flow immunochromatographic assays (LFIAs).

**Lateral-flow immunochromatographic assays.** LFIAs are promising POC diagnostic devices due to their simplicity, low cost, robustness, and ease of implementation in remote regions of endemicity (182). The inclusion of nanotechnology in LFIAs has significantly improved disease detection by spectroscopic, optical, fluorescent, and electrochemical readouts, allowing for improved sensitivity and lower costs (152). Dipstick-based LFIAs, also known as test strips (Fig. 3), utilize chromatographic methods to

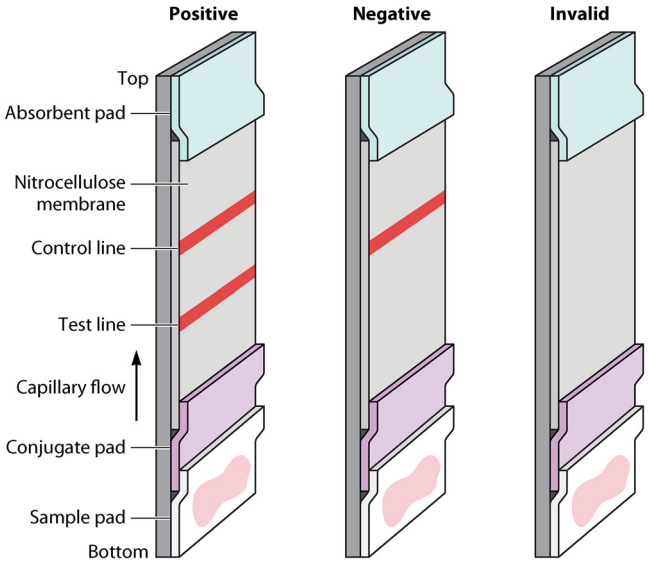


**FIG 3** Diagram of a nitrocellulose based lateral flow assay showing interpretations for positive, negative, and invalid results.

separate different sample components based on longitudinal or transverse flow through a carrier and immunochemical reactions to detect positive infections; the immunoreagents are immobilized on the carrier through which the sample fluid flows (183). A sample is dropped on the base of the strip, usually made of nitrocellulose or glass fibers, and eluted using a small amount of buffer that contains the immunoreagent. This dye-labeled complex is present on the nitrocellulose strip in a small test line, along with a control line (184). The control line contains antibodies specific for the indicator-labeled antibody complex and will appear in all tests, including negative results, due to a color change upon binding of the antibody complex. Test lines are embedded areas of target indicator-labeled antibodies and change color upon binding of target antigens present in a sample (178). Immunochromatography is rapid, stable at temperatures up to 40°C, and reagent and energy conservative, and hundreds of samples can be processed simultaneously, making it ideal for POC diagnosis and epidemiological monitoring (154). Furthermore, capture antibodies can be placed onto the membrane in advance and then blocked, dried, and stored for up to 3 months at 4°C, while maintaining stability for use (185). LFIAs have been studied extensively for the diagnosis of malaria (*P. falciparum* and *P. vivax* primarily), visceral leishmaniasis, filariasis, trichomoniasis, toxoplasmosis, piroplasmosis, amoebiasis, cryptosporidiosis, giardiasis, trypanosomiasis, schistosomiasis, taeniasis, fasciolosis, trichinellosis, and echinococcosis (154, 156–158, 178–180, 186–213) (Table 1). Currently, however, there are only a small number of approved LFIAs for POC diagnosis of malaria, visceral leishmaniasis, giardiasis, amoebiasis, cryptosporidiosis, and schistosomiasis (74).

Nitrocellulose-based LFIAs appear to be a promising tool for POC diagnosis of parasitic diseases, with research demonstrating that LFIAs exhibit higher sensitivity and specificity than other immunodiagnostic methods—such as ELISA, immunofluorescence antibody tests (IFATs), and direct antiglobulin tests (DATs)—and microscopy-based diagnostic methods (210, 214). However, as shown with studies on malaria, the sensitivity and specificity of LFIAs depend on infection intensity (195, 203, 214) (Table 4). Low parasitemia ($<$250 parasites/$\mu$l) fails to reach the threshold of detection and can result in false negatives. False negatives can also be seen in individuals with very high levels of parasitemia, the prozone effect, due to excess antigens binding to the detecting antibody, leaving no epitopes available for capture or the test band antibody, which results in no band occurring for the test band (215). LFIAs cannot quantify

infection burdens and, like other immunodiagnostic tests, generally fail to detect low parasitemia or low intensity infections; however, due to their relative simplicity, they could be used by untrained technicians in areas of endemicity where laboratories are not accessible or are poorly equipped (203, 206). However, despite ease of use, the interpretation of results can cause difficulty due to trace bands or multiple bands (216). In parasite-endemic areas, low levels of parasitemia may lead to asymptomatic infections, and thus a positive RDT with fever may indicate a plasmodium infection but not the cause of the fever (215); thus, sole reliance on RDT diagnostics can be problematic. Instead, RDTs can be one of several diagnostics, along with clinical symptoms and patient history, to determine patient status which is suggested for a number of parasitic NTDs (215) (Table 2). In malaria, changes to parasite antigens may also result in false negatives, such as variations in Pf-HRP2 or pLDH in *Plasmodium falciparum* (215, 217).

The timing of reading a lateral flow assay can also affect interpretation. A lateral flow assay for Chagas disease, PATH-Lemos, had optimal sensitivity and specificity (99.5 and 96.8%) after 20 min, which decreased slightly at 25 min (98.9 and 94.0%) (131). Longer times were not tested, but it might be expected to drop, and thus it may be critical for this, and other POC immunoassays, to read results at the right time. In addition to these issues, incorrect and length of storage can affect test reliability. While the inclusion of nanotechnology in immunoassays has greatly improved the rapidity and usability of LFIAs, there are several issues that are often overlooked. This includes nonspecific absorption of protein and gold nanoparticles, which can have serious effects on readability and sensitivity and result in high rates of false positives and negatives (152). Furthermore, LFIAs exhibit problems with cross-reactivity when blood samples are used instead of serum, causing decreased sensitivity (Table 4). This is due to the presence of cellular enzymes in whole blood or of fibrinogen and paraproteins in unclotted plasma, which prevents increased viscosity and hinders accurate sample pipetting (74).

The LFIA used for diagnosis of echinococcosis (VIRapid HYDATIDOSIS; Vircell, Granada, Spain) (serum) exhibits cross-reactivity when *Taenia solium* coinfections occur (218), while a dipstick LFIA for *Schistosoma japonicum* diagnosis (serum) exhibits cross-reactivity with *Clonorchis*, *Paragonimus*, or *Angiostrongylus cantanensis* coinfections (219, 220). An LFIA developed for *Wucheria bancrofti* infection detection (serum) exhibited high specificity and sensitivity, with little cross-reactivity observed in patients with *Onchocera*, *Loa Loa*, or *Strongyloides* infections (221), further suggesting that LFIAs may function more optimally when used on serum samples, which requires a centrifuge, thereby increasing costs (194, 195, 206). However, an LFIA developed for *O. volvulus* detection was shown to work as well with blood as with sera (222). In addition, a superhydrophobic plasma separator and microfluidic chip (162), which we discuss in more detail below, can separate serum from blood without electricity and may in future be combined with LFIAs to produce an optimal RDT for POC diagnostics in areas of endemicity. In clinical settings in developed nations, LFIAs may be used as part of a diagnostic process that often requires parallel testing with different diagnostics to improve sensitivity (Table 2).

**Schistosomiasis POC-CCA cassette-based test.** The POC-CCA cassette assay is the only commercially available (Rapid Medical Diagnostics) POC for schistosomiasis detection, detecting *S. mansoni* primarily, although can detect other schistosome species, *S. haematobium* and *S. japonicum*, with less sensitivity (223). While treatment is the same for all species, species determination can be important when considering disease and damage caused by infection, for example, risk of bladder cancer with *S. haematobium* infection (224). The cassette uses urine to determine infection. There have been two iterations of this cassette, with an earlier version requiring a buffer addition, with the latest no longer requiring it. However, a high rate of false positives, low sensitivity or low reactivity in individuals not excreting eggs, and interpretation of trace results are barriers to successful implementation of this diagnostic (225). The test is also much

more sensitive in detecting *S. mansoni* infections compared to *S. haematobium*, and there may be issues with use in areas with only *S. haematobium* present, or where the two schistosomes are coendemic. This obviously limits its potential use, particularly in Africa where both species are present, often in the same geographical setting. The presence of hybrid schistosome species in Africa may also be confounding factors for test accuracy (226–229). There has also been cross-reactivity identified with other helminth parasites (230). Interpretation of trace results remains a major stumbling block for replacement of KK with the POC-CCA (225, 231, 232). As part of the REASSURED (Tables 2 and 3) criteria that POCs should meet, usability and interpretation are key for field personnel with minimal training, as well as avoiding false negatives and positives (40).

The WHO is currently seeking expert feedback for target product profiles (TPPs) in concordance with the new 2021-2030 roadmap where diagnostics are highlighted as a crucial target. Within these TPPs the POC-CCA test, previously recommended by the WHO for control programs, has been highlighted as problematic due to low sensitivity in low-prevalence areas, with high false positivity in areas where prevalence is $<$10% (40). Manufacturing issues were also highlighted that resulted in variable performance in different product lots, including very high false-positive rates. A recent study in Brazil tested the POC-CCA RDT in an area where schistosomiasis was not endemic (233). All study participants were negative by microscopic methods (KK and helmintix); however, 37.9% were positive by POC_CCA, giving a specificity of 62.1% (233). Furthermore, there were differences in test results in fresh urine compared to the same urine samples stored at –20°C for 1 year, as per the manufacturer's instructions. The reported difference was more than could be accounted for different batches of test were used. We highlight here specificity issues with the test that need to be addressed. There is an ongoing clinical trial into the sensitivity and specificity of a POC-CCA for *S. japonicum* that is due to finish in early 2021 and may shed more light on the sensitivity and specificity of the POC-CCA (234).

### DNA-Based RDT

**Whatman FTA cards: DNA-based diagnostics.** Standard filter papers or qualitative filter papers differ in content from Whatman FTA cards since they do not contain the proprietary chemicals for DNA capture and storage (119). Standard filter paper technologies have been used widely for genetic and protein analyses; however, improvements in whole-genome amplification, single-nucleotide polymorphism analyses, and DNA extraction methods are required (235–244). Studies utilizing dried blood spots (DBS) from newborn screening found that samples stored below –20°C for up to 20 years maintained viability equal to that of fresh DBS; however, DNA recovery declined in samples stored at room temperature for over 10 years, particularly for amplification of larger DNA fragments (236, 243). Serological detection of parasite antigen eluted from DBS on conventional Whatman filter paper has been successfully applied in the diagnosis of strongyloidiasis and lymphatic filariasis, but samples still require storage in a 4°C refrigerator at collection locations and at –20°C until analysis (245–247). This means that while filter papers can be useful for large-scale sampling (248), diagnostics, and rapid DNA purification and extraction, they cannot maintain DNA stability at room temperature as efficiently as Whatman FTA cards (119, 161, 249, 250). As such, samples stored on filter papers would either require storage at –20°C during transport, or they would need to be transported and processed rapidly at room temperature.

Limited research has been undertaken to determine the efficacy of Whatman FTA cards for storing parasite DNA; however, parasite samples stored on FTA cards have been used successfully in PCR and LAMP assays for diagnosis, genotyping, and population screening (102, 105, 251–261) (Table 1). Recent studies utilizing qPCR and direct PCR for detecting *Dirofilaria repens* microfilariae in blood stored on FTA cards suggest that loss of parasite DNA during extraction may be an issue due to the heterogeneous distribution of parasites and the lower volumes of blood used in spots compared to that of normal whole-blood samples used for conventional DNA extraction (262–264).

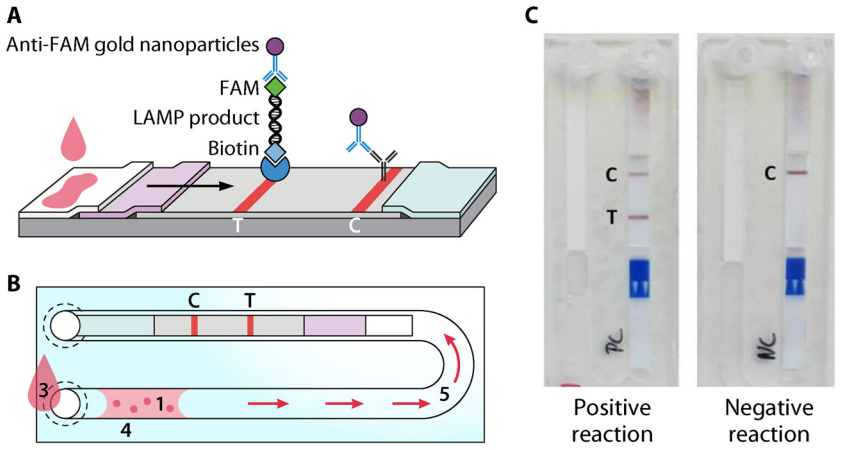


**FIG 4** Schematic principle of the DjinniChip. (A) LAMP product on lateral flow strip. (B) Components and processes. The DjinniChip contains lyophilized reagents (1) and a lateral flow test strip (2). A liquid sample (3) is loaded onto the chip through its inlet. The liquid sample reconstitutes the reagents (4). *Chlamydia trachomatis* DNA is amplified at 65°C. The reaction mix is pumped gently with a syringe to the lateral flow test (5). The appearance of the control band (labeled "C") indicates the correct function of the lateral flow test. The appearance of the test band (labeled "T") indicates the presence of *C. trachomatis* in the sample. (C) Detection on DjinniChips. (Reproduced from reference 77.)

However, these studies also found that although in low quantity, sufficient DNA could be recovered from FTA cards for conventional PCR analysis (264, 265). A study of different preservative methods for hookworm (*Necator americanus*)-spiked fecal samples suggested that FTA cards maintain DNA stability for long periods at 32°C compared to other storage methods, including 5% potassium dichromate, 90% ethanol, Formalternate, PAXgene, RNAlater, two-step desiccation ethanol-silica gel, and storage at −20°C (101). Despite the relatively high cost (AUD $2.20/sample) of FTA cards (101, 266), this initial outlay is compensated by savings in transport and storage costs (101, 266). By combining FTA cards with a comparatively low-cost diagnostic method, such as LAMP or direct PCR, diagnosis could become straightforward and accessible in remote, resource-poor areas (267–272).

**DNA dipstick.** The DNA dipstick is a rapid method for DNA extraction and is made from Whatman filter paper number 1 that is dipped in paraplast, leaving a small section for DNA binding (159, 161). The dipstick was initially developed for extracting DNA from plants, and plant pathogens but has a universal application, and the method could be performed by lay persons in the field since the workflow is quite simple and, when combined with a LAMP assay, it is a potential low-cost POC diagnostic. The sample DNA is being extracted from is placed in a tube with ball bearings, shaken vigorously, and then the DNA dipstick is inserted three times into the lysate. The DNA dipstick is the dipped three times into a tube containing wash buffer and then three times into the tube containing LAMP reagents. The LAMP is run, and the result is visualized by a color change or flocculation. To date, the dipstick has been tested on *S. japonicum* parasite material (adult worms and eggs) and infected snails (273). The method needs to be tested on clinical samples to determine whether the method would be able to extract—and then detect with a subsequent LAMP assay—parasite DNA from clinical samples.

**DjinniChip.** The DjinniChip (Fig. 4) is a novel LAMP-based molecular diagnostic assay that has been used for the diagnosis, surveillance, and control of trachoma, caused by *Chlamydia trachomatis* (274). The chips are assembled by injection-molding cyclic olefin copolymer, and the required LAMP reagents are freeze-dried into the channel (274). A lateral-flow test strip (LFS) is then placed into the chip, and the bottom of the chip is sealed. The clinical sample is added to the chip, and the sample

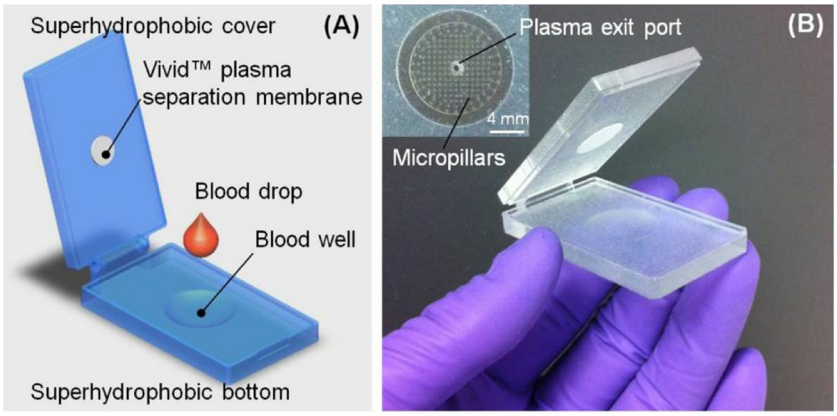


**FIG 5** Diagram (A) and images (B) of the superhydrophobic separator. (Reproduced from reference 162 with permission of the Royal Society of Chemistry.)

extract is driven by capillary action into the reaction channel, reconstituting the LAMP reagents, and the chip is further sealed (274). After a 35-min incubation at 65°C, the sample is then on placed on ice blocks to stop reactions, and chromatographic buffer is used to manually drive the amplified reaction toward the LFS, giving a visible result within 10 min due to the use of gold nanoparticles attached to anti-fluorescein-antibodies (274). The DjinniChip is low cost (<$2 [US] per test), is rapid, has a total test time of 50 min, requires only a portable heat block that can be powered with a car battery, and can be completed by untrained personnel, making it accessible in resource-poor regions (274). The requirement for ice, which was transported in a cool box in a "mock field" trial, will limit some usage in very remote areas (76, 77).

The DjinniChip has yet to be used for parasite diagnosis, but given the low price, simplicity, ease of assembly, lack of requirement for expensive laboratory equipment and trained personnel, rapid results visible to the naked eye, and stability in many environmental conditions, this chip provides an exciting opportunity for rapid accessible POC diagnosis of parasitic NTDs. However, the DjinniChip still requires further optimization to prevent contamination, which occurs due to the manual handling steps, to the improve the sensitivity and specificity of the test. A "mock field" trial on spiked samples resulted in a higher number of false positives (76, 77). Contamination in field settings will be an ongoing issue for all RTDs. At least some training may be required on proper aseptic techniques for those using the tests in field situations.

**Superhydrophobic plasma separator and microfluidic chip.** The superhydrophobic plasma separator (SPS) was developed to separate plasma from whole blood without electricity, making it ideal as part of an RDT (Fig. 5) (162). Whole blood often inhibits downstream applications such as PCR or LAMP, necessitating the removal of the blood cellular components or the separation of the blood by centrifugation with only the plasma section retained. In this case, the SPS was combined with a LAMP assay performed on a microfluidic chip with plasma separated from whole blood by the SPS (162, 275). Then, 200 $\mu$l of whole blood was spiked with *S. mansoni* DNA with a recovery rate of 84.5% compared to the more traditional method of centrifugation for separation of plasma from whole blood. As the amount of *S. mansoni* DNA increased, so also did the recovery rate, although not significantly, and the detection limit was identified as 0.5 fg of *S. mansoni* DNA (162).

The SPS is made up of a hinged slide coated in hydrophobic substrate, with the lower slide containing a small well for the blood to be placed, and a small membrane on the upper slide. After whole blood is placed in the well, the slides are closed together, forming a smear. The SPS relies on natural sedimentation, with the heavier red and white blood cells moving down toward the bottom of the well, leaving the top portion clearer and containing primarily plasma. The plasma is filtered through the

membrane for further cleaning of the sample. Once the plasma was separated, it was then removed and tested for *S. mansoni* using a real-time LAMP on a microfluidic chip (275).

This method has not been tested under field conditions or on "real-world"' samples and may require further optimization for the field. Any schistosome DNA detected in plasma will be cell-free DNA and therefore present in small amounts. This method only uses 200 $\mu$l of whole blood, and a minimum concentration of cell free schistosome DNA is required for detection. It would also be difficult to produce large numbers of the SPS device for field testing since it is currently produced by three-dimensional printing, nor is it clear whether the SPS or microfluidic chips could be re-used, which may increase costs. However, the SPS is a promising method that can also be used in conjunction with immunodiagnostics where plasma separation may also be beneficial.

**Fusion 5.** Fusion 5 technology is a silica-based filter (GE Healthcare, USA) designed to replace the solid materials that are currently used in lateral flow immunoassays and allows the manufacture of lateral-flow tests on a single sample (116). This technology reduces test times and costs and is naturally hydrophilic (116). Standard test strips are made of many different components laid over each other with an overlap, requiring the correct pressure between each layer to allow the sample to flow efficiently from one component to the next (116). Fusion 5 consists of glass fibers containing a plastic binder, which increases its mechanical strength, maintains hydrophilicity, and allows fast wicking speed, giving quicker test times (116). Fusion 5 technology has been shown to exhibit increased nucleic acid capture efficiency rates compared to that of other Whatman cellulose-based filters (116). These attributes allow Fusion 5 to have many functions, including a blood separator, a sample pad, a reaction substrate, and a conjugate release membrane (116).

In 2009, a nucleic acid extraction method was designed, called filtration isolation of nucleic acids (FINA), utilizing Fusion 5 technology as the nucleic acid capture membrane (276). Briefly, a Fusion 5 disk was sandwiched between 707 blotter paper and Parafilm, with a hole in the center for the disk where a clinical blood sample for testing was applied. This was then washed and placed directly in a PCR tube for amplification (276). Using this procedure, HIV proviral DNA was successfully extracted and provided 98.8% sensitivity and 100% specificity in all qPCR experiments compared to viral load data obtained through reverse transcription-PCR (276, 277). Importantly, dried blood modules stored at 37°C for up to 5 weeks maintained DNA stability of samples (276). Lysing the red blood cells with Triton-X and attaching the disk to the side of the PCR tube further optimized the method, yielding 100% sensitivity and specificity (278). These methods produced a low-cost device for DNA extraction; however, they are only applicable to blood samples. In 2015, a study created a novel paper-based micro-fluid device using Fusion 5 as the sample capture filter. For this method, the sample was loaded onto the capture filter and washed three times with water. NaOH, HCl, and water were then aspirated through the filter, which was then taken for PCR verification (116). This device was able to purify genomic DNA from more diverse clinical samples, including whole blood, dried blood spots, buccal swabs, saliva, and cigarette butts with high efficiency (116).

Fusion 5 has also been used as part of a simple paper microfluidic device for sample preparation and nucleic acid extraction. In this "microfluidic origami," a stack of flat polymer sheets is combined in a way that folding activates the DNA purification process (279). Fusion 5 membrane, cut to specific measurements by lasers, was used as the DNA capture filter with this device (279). The DNA capture filter was placed on the origami device, and a number of folding steps preceded placement of the sample on the filter, which was then washed, dried, and eluted using a low concentration of salt solution (279). This study successfully purified *Escherichia coli* DNA from pig mucin for conventional PCR amplification (279). The use of Fusion 5 technology for nucleic acid purification is still in its infancy; however, this technology presents an exciting opportunity for the development of simple and cost-effective purification of parasite

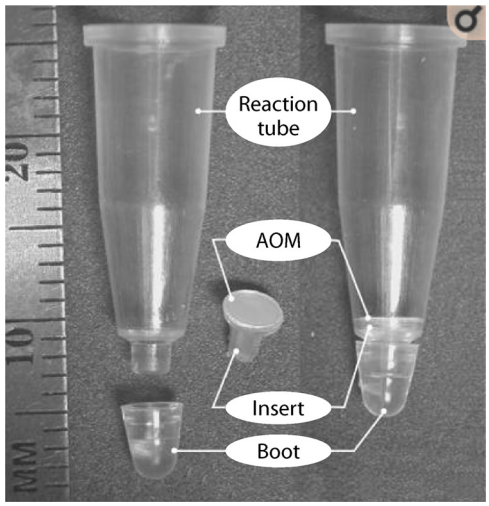


**FIG 6** PCR tube (0.2 ml) with AOM insert. (Reprinted from reference 286 with permission of Elsevier.)

genomic DNA, including parasite cell-free DNA that might be present particularly in blood, saliva, and urine, combined with isothermal amplification.

**Aluminum oxide membrane.** Aluminum oxide membranes (AOMs) form through the anodization of aluminum in acid electrolytes resulting in a semiordered parallel porous oxide membrane with a closed base (280, 281). The anodizing voltage and choice of electrolyte mediates AOM pore size, ultimately giving a rigid membrane that has 50% porosity, which allows a high rate of liquid flow (282). This has allowed AOMs to have many functions, including the filtration of biological materials (e.g., blood and bovine serum albumin), as a matrix for artificial tissue growth and cell culture, and as a template for nanodevice manufacture (255, 283, 284). An AOM, functioning as a filter, allows nucleic acid extraction, with the nucleic acids being localized to the surface (255, 283, 284).

Since their development, AOMs have been widely studied for detecting viral and bacterial infections from extracted genomic DNA (282, 285–290). Single-tube nucleic acid extraction methods using AOMs have been designed wherein the membrane is built into a 0.2-ml PCR tube (Fig. 6) (285, 286). This has successfully been used for hepatitis C virus detection with accuracy comparable to that of conventional extraction methods to which it was compared (285, 286). PCR amplification of DNA extracted using AOM filtration provided results that were close to 100% efficient (282). AOMs can be incorporated into microfluidic chips for DNA extraction from blood and saliva, a procedure that has been used to detect both methicillin-resistant and -susceptible strains of *Staphylococcus aureus* and HIV (282, 285–290). AOMs have yet to be utilized for parasite DNA extraction and, given the requirement for blood collected by venipuncture, these methods may not be ideal for use in parasite diagnosis due to cost (282). As such, for AOMs to be considered for wide-scale POC diagnostic application, further optimization would be required to allow DNA extraction from small sample volumes or from dried blood spots.

## ANIMAL HOSTS AND RDTs

Many of the parasitic NTDs are zoonotic, with animal reservoir hosts important in transmission and maintaining the parasite in the environment (18). *Schistosoma japonicum*, as an example, has 46 potential mammalian definitive hosts (291), as well as a molluscan intermediate host, that complicate control efforts. *Dracunculus medinensis*, otherwise known as guinea worm, has almost been eliminated. However, complications in Chad, one of the last remaining countries where the guinea worm is still

endemic, including potential paratenic hosts and zoonotic transmission in dogs, may set back elimination of this disease (292–294).

It is therefore highly important that animals are also included in control programs by testing and treating infected animals, as well as using other control interventions to prevent transmission of parasites. For example, in China, bovines have been identified as major reservoir hosts, and these animals have been removed from areas of endemicity, while in the Philippines interventions to prevent fascioliasis transmission from bovines, such as drying and storing animal feces for a set amount of time before using on fields and keeping bovines away from flooded fields, have been implemented (295–297). For some parasite-related diseases, such as schistosomiasis, fascioliasis, taeniasis, dracunculiasis, and STH, environmental monitoring of water and soil is appropriate and can indicate active transmission sites for formation of risk maps (298–305).

In control programs and field research studies of zoonotic parasites, a number of diagnostic methods, including microscopy, immunodiagnostics, and molecular assays, have been used to diagnose animal infections (71, 306–321). RDTs used on humans would ideally also be able to identify animal infections for rapid treatment and management of animal cases, as well as prevent transmission to humans from animal reservoirs of infection in cases of zoonotic transmission.

Several RDTs have been developed for canine visceral leishmaniasis. The Kalazar Detect Canine Rapid Test (Inbios; rK39) and the Canine and DPP CVL (BioManguinhos) RDTs both detect leishmaniasis from serum samples (146, 147). The sensitivity of the Inbios rK39 increases with increasing parasitemia, and it is therefore more sensitive in animals exhibiting clinical disease symptoms than in those who are asymptomatic—86.7 and 59.3%, respectively—from a meta-analysis on rK39 (146).

Animal African trypanosomiasis (AAT) is caused by species that generally do not cause infections in humans (*T. congolense*, *T. vivax*, and *T. brucei brucei*), although there are some cases of AAT causing human infections (322). AAT can cause significant economic losses in Africa (322). Two RDTs developed for detecting human African trypanosomiasis (HAT) have been tested in cattle for detecting AAT using fresh whole blood from cattle in Kenya and thawed serum samples from cattle in Uganda (323). The first RDT, the 1G RDT (SD BIOLINE HAT; FIND and Alere/Standard Diagnostics), is available commercially, while the second, p2G RDT, is a prototype. The specificity in animals from regions of endemicity was low for both tests (14.6 to 22.6%), while the specificity in areas of nonendemicity was high (97.5%) for the p2G RDT but low for the 1G RDT (57.9%). In both cases neither RDT is suggested for use in detecting AAT (323).

There are a number of immunochromatography (ICT) RDTs that detect *Toxoplasma*, a zoonotic protozoan parasite, in animals and humans (324). The majority detect antibodies in sera, with three of the available ICT tests commercially available for use in humans (324).

## REGULATORY APPROVAL

*In vitro* diagnostics (IVDs) utilize human clinical samples to diagnose diseases and can be in the form of commercial test kits or laboratory-developed tests (LDTs). Although the cost of diagnostic testing comprises a fraction, $<$5%, of overall of medical and hospital costs, IVDs contribute to up to 70% of treatment decisions (325, 326). This highlights the critical requirements for highly accurate IVDs that are reliable, effective, and accessible. IVDs are constantly evolving and advancing due to increasingly sophisticated scientific techniques, biomarkers, and available assays. Further, there is an increased demand for point-of-care and home-based testing kits that provide rapid results, aiming to reduce the diagnosis times and ensure that patients receive appropriate medical treatment as quickly as possible (325, 326). However, many barriers affect the adoption of new innovative diagnostic tests in routine medical practice.

The U.S. Food and Drug Administration (FDA), the Therapeutic Goods Administration (TGA; Australia), and the European Medicines Agency (EMA) regulate the development of IVDs. IVDs are divided into three classes: class I (low to moderate risk), class II (moderate

to high risk), and class III (high risk). There are three main approval routes available for IVDs, 510(k), premarket approval (PMA), and *de novo* reclassification, each of which requires the availability of samples and associated data, the use of relevant samples for the market, compliance with informed consent, and lack of any ethical issues (327, 328). The 510(k) is a regulatory pathway for tests that are shown to be equivalent to an existing test, while PMA is required when a test is not similar to an existing test. *De novo* reclassification allows for a low to moderate risk test that does not have a comparable device, which is usually required when 510(k) applications are rejected due to not being similar enough. These regulatory hurdles are complex, and the process of approval can be lengthy, taking up to 5 years from time of submission. Laboratories can avoid these hurdles by developing in-house diagnostic tests (LDTs); however, these tests have recently begun being more closely scrutinized by the FDA, which threatens to significantly slow down the development and use of innovative LDTs.

Of all the NTDs, only the following three have FDA-approved molecular diagnostics: Dengue (CDC DENV-1-4 Real-Time PCR Assay); *Leishmania* spp. (SMART Leish), which has limited availability (military use only); and Trachoma (multiple tests) (329). The EMA and TGA currently do not have lists of approved IVDs; however, the TGA indicates that one will be available by July 2022.

As part of the new roadmap for NTDs 2021 to 2030, the WHO has highlighted diagnostics as one of four key priority areas (330, 331). As part of this the WHO has established a diagnostics technical advisory group (DTAG) for collaborative development of new diagnostic tools. Key areas of responsibility for the DTAG include reviewing diagnostics needs in NTD programs, defining use cases and target product profiles, and linking with key partners to support test development and validation. A "silver lining" of the COVID-19 pandemic has been an increase in molecular testing capacity in countries that previously had low capacity, increasing the likelihood of adoption of molecular methods, at least in the short term. However, reagent costs may still place traditional molecular diagnostics for NTDs out of reach.

## CONCLUSION

The ideal diagnostic test should be simple, rapid, and low in cost; require few steps; and exhibit high sensitivity and specificity—features particularly relevant in areas of endemicity where infection intensity is reduced as the result of MDA programs operating. Although serological tests, especially dipstick assays, provide diagnostic simplicity, they can lack sensitivity and specificity and may not distinguish between past and current infections, since antibodies generally remain in circulation after drug treatment. Furthermore, antibody levels do not accurately correlate with parasite burden (61–64, 187). Moreover, the current costs associated with LFIAs prohibit their extensive use in resource-poor areas of endemicity.

Cellulose-based Whatman FTA cards have been utilized extensively for parasite diagnosis and in epidemiological studies. These cards can maintain nucleic acid stability under less-than-optimal conditions, providing low-cost storage and transport of clinical samples for subsequent nucleic acid purification. Fusion 5 technology, AOMs, and the DjinniChip have yet to be tested or utilized for parasite nucleic acid extraction, but with such promising results obtained for other pathogens using whole blood, saliva, and buccal swabs, they represent a promising advance (274, 276, 278). These methods allow for rapid and simple nucleic acid extraction and require only small sample volumes which can be stored at temperatures up to 37°C, while maintaining DNA stability (276, 278). Given their efficiency for DNA extraction from blood, these methods may be particularly useful for diagnosing blood-borne parasites.

A major consideration in molecular diagnosis remains the high cost of DNA extraction. Many NAATs, except for LAMP, remain expensive and complicated, making them generally inaccessible for POC diagnosis. Therefore, as countries look toward NTD elimination, new nucleic acid purification techniques that are simple, rapid, and more cost-effective must be developed. The use of membrane technology provides a novel

avenue for simple and cost-effective parasite DNA purification. Further optimization of this technology could pave the way for significant improvements in the accessibility of highly sensitive molecular diagnostic techniques applicable to the most affected regions of NTD endemicity globally.

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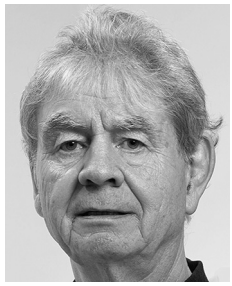

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