Review Article: Malaria Diagnostics in Clinical Trials

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Abstract. Malaria diagnostics are widely used in epidemiologic studies to investigate natural history of disease and in drug and vaccine clinical trials to exclude participants or evaluate efficacy. The Malaria Laboratory Network (MLN), managed by the Office of HIV/AIDS Network Coordination, is an international working group with mutual interests in malaria disease and diagnosis and in human immunodeficiency virus/acquired immunodeficiency syndrome clinical trials. The MLN considered and studied the wide array of available malaria diagnostic tests for their suitability for screening trial participants and/or obtaining study endpoints for malaria clinical trials, including studies of HIV/malaria co-infection and other malaria natural history studies. The MLN provides recommendations on microscopy, rapid diagnostic tests, serologic tests, and molecular assays to guide selection of the most appropriate test(s) for specific research objectives. In addition, this report provides recommendations regarding quality management to ensure reproducibility across sites in clinical trials. Performance evaluation, quality control, and external quality assessment are critical processes that must be implemented in all clinical trials using malaria tests.

INTRODUCTION

The burden of malaria infections is continually changing and not clearly understood, but leads to more than 500,000 deaths annually, mostly among children in sub-Saharan Africa.[1,2] The effort to eradicate this disease is based on comprehensive interventions, including antimalarial drugs, insecticide-treated bed nets, indoor residual spraying, and elimination of vector-breeding sites. Although currently unavailable, a vaccine to prevent morbidity and mortality in at-risk populations is one ultimate goal. It is also increasingly recognized that co-infections with other diseases such as infection with human immunodeficiency virus-1 (HIV-1), tuberculosis, and helminthiases, can further modify and complicate malaria and the other co-infection.[3–5]

Plasmodium parasites are transmitted to humans from mosquitoes and cause the wide range of disease manifestations known as malaria. Upon transmission from mosquitoes, motile sporozoite-stage parasites enter the bloodstream and migrate to the liver, where they invade hepatocytes and undergo massive asexual proliferation over the course of several days. The host is asymptomatic during liver-stage infection and there are no reported diagnostic tests to routinely detect human infection at this stage. After approximately 6–10 days, infected hepatocytes rupture and release tens of thousands of merozoites into the bloodstream, and each merozoite is capable of infecting an erythrocyte. Depending on the *Plasmodium* species, blood stage malaria parasites undergo an asexual 1–3-day life cycle of erythrocyte invasion, intracellular growth (as trophozoites), and mitosis (as schizonts), rupture from the erythrocyte as a new generation of merozoites and re-invasion of new erythrocytes. The cyclical rupture of infected erythrocytes, the release of parasite antigens and toxins, and the species-specific endothelial sequestration of growing *P. falciparum* trophozoites lead to most of the clinical manifestations associated with malaria, including severe anemia, endothelial pathology, and end organ failure.

In general, for malaria-naive persons, there is an imperfect positive correlation between level of parasitemia and the signs and symptoms experienced. Male and female gametocytes arise during the blood stage of infection and are transmitted back to a mosquito if present in the insect's blood meal. These forms of the parasite continue the sexual cycle in the mosquito midgut and ultimately produce infectious sporozoites that can continue the cycle.

The manifestations of malaria vary in different patient populations and different epidemiologic settings, which complicates diagnosis.[6,7] Non-immune persons are susceptible to rapid expansion of erythrocytic parasites and mount highly pro-inflammatory cytokine and parasite-endothelial adhesion cascades, responsible for much of the immunopathogenesis of disease. As a person experiences repeated infections with malaria, increasing tolerance to the presence of parasites gradually develops because of a broadening antibody repertoire, which better controls parasitemia,[8] and poorly understood T cell and cytokine responses.[6] The end result is commonly referred to as the semi-immunity of malaria, in which the frequency and severity of malaria episodes are reduced with age and exposure in most malaria-endemic settings. Therefore, in malaria-endemic settings, most adult malaria infections are subclinical and show intermittent asymptomatic parasitemia and gametocytemia serving as significant reservoirs of infection for mosquitoes. Patients can also be infected by multiple strains of the same species or more than one species of malaria parasite, although the impact of mixed infections on clinical disease risk and outcomes is not well understood.[9,10] Patient factors such as concurrent HIV-1 infection or pregnancy can also alter the risk of clinical malaria in exposed populations.[11] Understanding this complexity is key to understanding the utility and limitations of all diagnostic methods.

In malaria-endemic regions, many patients are presumptively treated for malaria on the basis of febrile symptoms alone. However, malaria is not the only cause of fever in these settings. As such, this practice encourages over-use of anti-malarial drugs and development of drug resistance,

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TABLE 1
Considerations for initiating malaria diagnostics for clinical trials

Basic clinical trial considerations
    What clinical and research question(s) are being asked?
    What is the size and scope of the trial? Phase I, II, or III?
    What are the diagnostic purposes of the assay?
    Is qualitative or quantitative detection required (i.e., positive/negative vs. enumeration of parasites)?
    Is there a need to detect and quantify any parasite infection, whether subclinical or not? Is there a need to identify the species?
        Or is there only a need to diagnose and document clinical episodes?
    Is the assay used for clinical diagnosis (targeted vs. screening), to measure response to treatment, or for another experimental question,
        or epidemiologic endpoint (e.g., surveillance)?
    Which parasite stages are of interest?
    Is the sample collected in a resource-limited or resource-rich setting?
    Is the laboratory testing performed on-site or in a core laboratory in the same country or overseas?
Basic epidemiologic considerations
    Region of high, low or epidemic transmission intensity? Travelers' malaria?
    What is the human population being studied? What is known about host variability (genetics, cultural practices, exposures, epidemiology,
        age, rates of co-infection, reproductive status/parity)?
    What is the diversity of local/endemic *Plasmodium* populations? (intra- and inter-species variability, drug resistance)?

and fails to adequately diagnose and treat those with other significant infections.[12] Recognizing this problem, the World Health Organization (WHO) is trying to eliminate presumptive drug treatment of malaria. As part of this effort, the 2010 malaria treatment guidelines recommend that all suspected malaria cases be confirmed with a parasite-based diagnostic test prior to therapy, unless parasitologic diagnostic capabilities are not available.[13] Diagnosis of malaria is primarily made by demonstrating the presence of parasites in erythrocytes, and many techniques have been developed for this purpose. Although not all tests are appropriate for every clinical or research application, the current malaria diagnostic portfolio includes microscopy, serologic analysis, molecular diagnostics, and other modalities, some of which are intended for bedside or point-of-care (POC) use. In clinical settings, false-positive results can lead to unnecessary treatments with associated increased financial costs, side effects, and selection pressure for development of resistance in malaria parasite populations.[14–16] Conversely, false-negative results lead to excess morbidity and mortality and further transmission. Thus, quality diagnostics are essential.

Data obtained through quality diagnostics in clinical trials may be used for malaria epidemiology and modeling studies, evaluation of experimental medicines or vaccines, study of malaria pathogenesis, or assessing the interaction between malaria and other co-infections such as HIV-1, tuberculosis, and helminthiases. Inaccurate diagnostics may lead investigators to inappropriately and unknowingly reject viable drug or vaccine candidates or draw other erroneous conclusions.[17] Such outcomes can have disastrous results. False-positive results in drug or vaccine trials may lead to the withdrawal of a good candidate from further evaluation. In vaccine trials, vaccine-induced seropositivity can also result, an outcome well known in the HIV/acquired immuno-deficiency syndrome literature.[18] In persons with vaccine-induced seropositivity, serial testing or multi-test algorithms are required to maintain the specificity of true infection endpoints for the trial data and for the participants.[19] Thus, the first step toward obtaining accurate malaria diagnostics is to ensure that the appropriate assay is selected. The appropriate assay will depend on trial-specific research/clinical question(s) and the epidemiologic environment at the study site(s). Some endpoints of trials will require more than one diagnostic method for confirmatory testing.

Multiple method characteristics, such as sensitivity, specificity, cost, and feasibility, must all be taken into account during test selection. In general, medium-to-large field trials (Phase II and III) require assays that are medium-to-high throughput to accommodate a larger testing population, are reproducible across multiple sites, and specifically answer the clinical question(s) under investigation (Table 1). Phase I trials in healthy volunteers may accommodate diagnostic methods that are more expensive, require more time and expertise, and/or are limited to a single location to elucidate details about early infection events and immune responses. Depending on where testing is performed, minimal to moderate training (i.e., for POC testing) or more advanced training (i.e., for tests performed in core facilities) may be required.

For more than 100 years, microscopy has been the gold standard test for malaria. Microscopy remains in wide use and can be useful in clinical trials. Beyond microscopy, other techniques are commonly applied to malaria studies, including a variety of rapid diagnostic tests (RDTs), serologic assays, and molecular assays. All of these techniques are reviewed below. In addition to such mainstream technologies, novel diagnostic tests continue to be developed and we touch on some promising future technologies.

THE GOLD STANDARD: MICROSCOPY

General considerations. Microscopic examination of blood smears has many advantages, including accuracy, availability, low cost, and ability to quantify parasites and monitor parasite clearance (Table 2). The blood may be prepared in two ways for a microscopic examination for malaria: the so-called thick blood smear and the thin blood smear. For a thick blood smear, a drop (approximately 6–10 μL) is spread on a clean dry microscope slide. After drying, the thick blood smear is stained with a Romanovsky stain such as Giemsa. During the 10–60 minutes of staining in this aqueous medium, the erythrocytes lyse because of osmotic swelling, leaving the leukocytes and parasites, if present, largely (but not entirely[20]) intact. Thick blood smears can diagnose infection in most persons with symptomatic malaria and even some with asymptomatic infections, depending on

TABLE 2
Attributes of microscopy for diagnosis of malaria

Advantages
  Low-tech, simple, inexpensive
  Field friendly
  Tests for the parasite itself, not a correlate
  Identifies presence/absence of parasites (sensitivity 4–100/μL depending on setting), species identification, and parasite stage
  Provides quantitative information (useful to quantify parasite clearance rate in settings where artemisinin resistance is of concern)
  Assesses hyperparasitemia (a clinical classification of severe malaria that may serve as an exclusion criterion or an adverse event in trials)
  Stained slides can serve as permanent records
  Recommended to monitor patients during prophylactic or curative trials
Disadvantages
  Requires expertise, including microscopist training, proficiency/competency assessments, internal quality control, and standard slide sets
  External quality assurance is costly to set up and maintain
  Buffered slide staining reagents critical for good staining quality
  Reading slides is labor intensive and operator fatigue can make reliability variable
  Low- to medium-throughput depending on microscopist expertise, degree of parasitemia, and whether qualitative or quantitative diagnosis
    is needed
  Requires microscope maintenance and stable electrical source
  Proficiency requires practice; difficult to maintain proficiency in non-endemic areas
  Sensitivity varies with microscopist expertise; less sensitive than molecular assays
  Parasite loss from thick blood smear during wash steps impacts quantification and sensitivity

the parasitemia, the number of high-powered fields (hpfs at 1,000× magnification) examined, and the skill of the microscopist.[7,17] The advantage of lysing erythrocytes is that more blood may be examined and thick blood smears are therefore considered the more sensitive of the two blood smear preparations. The reported limit of detection for a thick blood smear is 4–20 parasites/μL,[21,22] but this limit is higher (50–100 parasites/μL) under field conditions[23,24] with many factors contributing variation and reduced sensitivity as recently summarized.[25]

The disadvantages of thick blood smears are that the parasites are not viewed *in situ* within the erythrocyte, are bunched up and less morphologically recognizable, may be hidden behind or above leukocytes, and may be more easily confused with artifact. If a smaller volume (approximately 2 μL) of blood is spread into a monolayer in the preparation of a traditional blood smear and briefly immersed in methanol, the erythrocytes are fixed and will not subsequently lyse during staining. The resulting preparation is commonly referred to as a thin blood smear. Although the thin blood smear limits the examination to less blood, it provides the microscopist the ability to discern morphologic details of the parasite within an erythrocyte and facilitates parasite species identification. Many, if not most, diagnosticians therefore combine the use of both smears, thereby optimizing the probability of finding a parasite by using the thick blood smear, and identifying it by using the thin blood smear. Species identification and quantification may be clinically important, (e.g., treatment of hypnozoite-producing *P. vivax* and *P. ovale* differs from that of *P. falciparum*). The advantages and disadvantages of microscopy are summarized in Table 2.

Considerations for use of microscopy in clinical trials. We reviewed current practices in malaria microscopy and describe aspects that may affect qualitative and quantitative endpoints. Specific protocol details can be found elsewhere.[26] Other investigators are also developing guidelines for the use of microscopy in controlled human malaria infection (CHMI) trials where subjects are infected with malaria parasites through mosquito bites or needle injections to evaluate malaria vaccines or prophylactic drugs.[27]

When using blood smears, parasites at lower densities may be missed by microscopy for a variety of reasons.[20,28,29] A typical thick blood smear (an approximately 15-mm diameter circle) contains more than 2,800 hpfs and a thin blood smear contains many more hpfs (Prescott WR, unpublished data), but only a fraction of these hpfs are typically evaluated during clinical trial slide reading. Thus, blood smears from persons with low parasite densities may actually contain parasites but in parts of the slide that are never even viewed by the slide reader. Beyond such sampling issues, poorly performed malaria microscopy is also a well-known cause of error in clinical field trials, which can result in adverse clinical outcomes and costly trial delays.[17] In addition to false-positive and false-negative errors, species identification and particularly the accuracy and precision in quantifying the parasite density (parasites/microliter of blood) can be difficult.

The variation in results among unskilled slide readers can be high, particularly at low parasitemias.[30] However, it should be noted that even among proficient certified slide readers, a coefficient of variation (SD/mean) in quantifying the parasites in the same blood sample can be as high as 30% (Prescott WR, unpublished data).

Inter-sample comparisons often do not align perfectly for a number of reasons beyond operator inaccuracy. In an early study, a significant portion of parasites were reported as lost during routine processing of thick blood smears.[26] Although such losses during thick blood smear preparation may be constant and permit comparison between positive slides, loss of parasites become much more critical when processing leads to false-negative slide results despite a true low-level parasitemia. Manual leukocyte counts are used as the multiplier in the calculation of parasitemia in thick blood smears, and either leukocyte or erythrocyte counts can be used for thin blood smear quantitation, depending on the method. For all these reasons, quantification by thin and thick blood smears may yield different results for the same sample.[30] In some cases, reliable complete blood count data (e.g., to provide accurate leukocyte quantification) is available to improve the accuracy of the calculation, especially useful at the extremes of age.[31] However, in many

cases, standard estimates of these values are used as multipliers to calculate either parasites/microliter or percent parasitemia. These inaccuracies are relatively minor for most purposes because the range of potential parasitemias spans more than five logs. However, if accurate quantitation is an important endpoint of an interventional trial, these assumptions may have more impact.

Quality management of malaria microscopy. We present key quality recommendations, including 1) rigorous training of microscopy personnel, 2) ongoing proficiency and competency assessments, 3) ongoing internal quality control (QC) and external quality assessment (EQA), 4) adoption of standardized slide preparation protocols (including automated stainers if possible), and 5) acquisition of automated slide reading instruments (when available) to increase throughput and consistency.

Several resources are already available to investigators using microscopy as study endpoints. The WHO published a number of useful references for many years, including a guide for QA programs that includes guidance for developing and validating slide sets for operator testing.[32] In sub-Saharan Africa, the U.S. National Institute of Allergy and Infectious Disease (NIAID) Mali International Center for Excellence in Research at the University of Science, Techniques and Technologies in Bamako, Mali, and the Malaria Diagnostics Center at the Kenya Medical Research Institute in Kisumu, Kenya are two well-established training centers that have demonstrated superb execution of a rigorous qualification process.[33,34] Both sites have successfully trained and qualified hundreds of microscopists across Africa. This process involves didactic and hands-on intensive microscopy training on established slide sets comprised of hundreds of unique slides. Each site can create qualification slide sets with known parasite densities, single- or mixed-species infections, and blood spiked with artifacts that may be confused with parasites (e.g., Howell-Jolly bodies, reticulocytes, platelets overlaid on erythrocytes) and can engage with trainee follow-up and EQA programs as available.

In addition to microscopy training centers, the creation of slide archives has also been increasing. The Malaria Research and Reference Reagent Resource Center Malaria Microcopy Reference Slide Set was the first such archive and is a well-known standard set of 1,200 slides.[30] This effort also included standard procedures for preparation of high-quality slides. In addition, several sub-Saharan countries, including Equatorial Guinea, Ghana, and Ethiopia, are creating National Archives of Malaria Slides, which may be available for use by investigators planning clinical trials in that country, such as the effort in Equatorial Guinea.[35] One reason for this increase in resources over the past 10–15 years has been the increased interest in improving the diagnosis of malaria among local healthcare workers. In many malaria-endemic settings, experience alone has often been relied upon to qualify technicians to read malaria slides. Unfortunately, anecdotal proficiency based on experience has been shown to be an unreliable predictor of objectively-tested proficiency,[30] and deliberate proficiency testing is highly recommended and is likely to be required by protocols in clinical trials.

Qualification of staff performing malaria blood smear microscopy should be conducted at each site to provide

assurance that timely diagnosis is feasible and as accurate as possible. In multi-center trials, the qualification process should be carried out across sites and be comparable in degree of difficulty to ensure consistency in diagnostic accuracy. For qualification, specificity and sensitivity of the technician should be assessed. The exercise to evaluate these two classical diagnostic testing parameters challenges a technician's ability to detect, quantitate, and determine the species of *Plasmodium* parasites. The importance of species identification varies depending on study objectives, malaria endemicity, and clinical needs. Overwhelming infection by one parasite species can make it more difficult to detect mixed infections with other species. Recommendations have been put forth by the WHO regarding the degree of difficulty of the slides and the size of the tests when examining diagnosticians at different competency levels,[32] such as the 55-slide test for national core reference level microscopists and a 24-slide test for health center level- or district-level microscopists. Adherence to the established objective standards enables unambiguous results and scores that are directly comparable regardless of when or where competency tests are administered. The published WHO guidelines, designed to address various levels of diagnosis in the public health arena, note that accreditation of expert microscopists for clinical trials usually requires a more stringent assessment geared to the specific requirements of the trial.[32] Thus, each trial site may need to determine its own acceptable qualification requirements, which should be a careful deliberate discussion between clinical trial and laboratory staff. One such site, the NIAID Mali International Center for Excellence in Research, recommends that a site determine criteria and assess performance by using a validated slide set and requires performance at a threshold of > 90% sensitivity and specificity with a demonstrated competence in species identification to conduct the trial.[33] Each site should determine their qualification process and adhere to standard operating procedures (SOPs) and study protocols for ensuring consistency, such as performing qualification before each new study and at two-year intervals for technical staff performing microscopy. In addition to verifying laboratory diagnostic capacity, all qualification processes should include a documented verification of competency in the form of a wet-laboratory examination.

As part of continuous process improvement of any clinical trial laboratory, EQA to verify proper internal QC (laboratory level) and technical skill should be considered essential, even in malaria-endemic settings. In addition to those programs already mentioned, the College of American Pathologists (CAP) provides proficiency testing (PT) panels of blood parasite microscopy slides and infected blood for RDTs three times annually to member laboratories. Participants analyze the specimens and return the results to the CAP for evaluation. In turn, each participating laboratory receives a report of their performance, as well as a report summarizing the results of all participating laboratories. A second EQA provider, DigitalPT (Oneworld Accuracy, Westford, MA), which began in 2012, also provides PT panels of blood parasite microscopy slides. They provide five thin and thick blood smear slides three times a year with their panel. Although such efforts are commendable and largely adequate for clinical diagnosis, we agree that these should not be considered rigorous enough on their own for

clinical trial endpoints. Refresher training courses can significantly improve all aspects of malaria diagnostic testing by laboratory personnel.[36] Each program or institute will need to ascertain which combination of availability, stringency, and cost of EQA programs best fits their requirements. Finally, even the best microscopist will require adequate and reliable equipment. Therefore, it is critical to choose an appropriate microscope, maintain it according to the manufacturer's instructions, and ensure a stable source of electricity. Recommendations for microscopes and slides are provided by the WHO.[32]

Future of microscopy: standardized automated slide preparation and reading. Automated slide staining machines have been developed and could aid in standardizing the preparation of slides for blood smear microscopy in clinical trial settings by improving the consistency of the staining procedure. The HemaTechnologies (Cardinal Health, Dublin, OH) models of automated slide staining machines use Romanowsky staining principles of acidic and basic dyes mixed accurately and uniformly with buffering solutions to obtain a consistent, readable blood smear. These machines feature a wide variety of staining requirements to suit the precise laboratory need, and consistently generate high-quality slides for diagnosis. For example, the HemaTechnologies models use 1 μL of blood to make a 1 cm^2 slide that can be read as a thin blood smear. Although they greatly increase the consistency and quality of the slides, automated slide preparers are expensive and are not likely to become widely available in resource-limited settings.

As discussed earlier, even results obtained via expert microscopy can be inconsistent because of lack of process harmonization amongst microscopists. Given these challenges and inherent inconsistencies, there has long been a quest for automated malaria microscopy methods, and numerous tools have been developed and experimentally evaluated.[37–39] These instruments automate microscope focusing and scanning with preliminary parasite identification, which are tedious processes for persons. Although some of these devices present images to the microscopist for confirmation and/or image analysis, other instruments also attempt to identify parasites to make a diagnosis.[38] Depending on the speed at which the automated readers can scan the slides, the overall scanning time may be greatly reduced and a larger proportion of the slide can be scanned.[39] No automated slide reading device has been approved by any regulatory agency, but such devices are in development and may eventually be ideal instruments for malaria diagnosis in future clinical trials, especially because the digital record of scanned slides provides a permanent and infinitely viewable record of the result. The most rapid of the automated slide readers in development have the potential to read hundreds to thousands of fields per minute, thereby automating the most tedious aspect of microscopy, namely, locating any intracellular inclusions that might be parasites for evaluation by either an algorithm or the operator. Thus, they have the potential to substantially increase the sensitivity of microscopy, although as yet the actual increase in sensitivity is not documented. Theoretically, at least, the sensitivity should be equivalent to one parasite within the number of fields the machine is asked to examine; a reasonable expectation might be < 10 parasites/μL. As imaging technology and automated recognition software continue to improve, the diagnos-

tic applications are exciting and numerous. Remote mobile phone applications in health care are also expanding, permitting the potential for experts to review images remotely for a tie-breaker or expert opinion before making a final diagnosis.

RAPID DIAGNOSTIC TESTS

General considerations. In the past decade, RDTs have emerged as a promising alternative to microscopy for the diagnosis of malaria and have been listed as an acceptable means of diagnosis in recent WHO guidelines (Table 3).[13] Similar to at-home pregnancy tests, these immunochromatographic tests rely on the detection of parasite-specific antigens in blood samples using monoclonal antibodies immobilized to test strip membranes using capillary lateral flow technology. Results are based on the presence or absence of a colored line on test strips and are available in 5–20 minutes, depending on the product. An incorporated internal QC is included in each test to monitor quality and validity. The RDTs have the advantage over microscopy in that little infrastructure and comparatively less training are needed for their proper use in resource-limited settings (Table 3). However, their application is limited as more of a clinical screening assessment tool because they do not provide quantification. Although most RDTs were validated by using fresh whole venous or capillary blood, some can accept a variety of sample types, including EDTA-anticoagulated whole blood, refrigerated blood (stored ≤ 3 days), or frozen/thawed or lysed blood (even years later although sensitivity may wane). Specific performance criteria, accepted specimen types, and other limitations are listed in RDT product inserts. In general, RDTs perform well down to approximately 500 *P. falciparum* parasites/μL or 5,000 *P. vivax* parasites/μL.[25] However, there has been wide and alarming variation in RDT quality between manufacturers, and the WHO and its partners instituted an ongoing RDT evaluation program intended to enhance quality worldwide.[40]

The RDTs determine correlates of malaria infection because parasite antigens, not intact parasites, are detected.

TABLE 3
Attributes of rapid diagnostic tests for diagnosis of malaria

Advantages
  Suitable for field use
  Low cost (if used in clinical trial site capacity)
  Simple concept and use, increases workforce
  Requires comparatively less infrastructure
  Rapid results (5–20 minutes)
  Can detect *Plasmodium falciparum* and/or
    non-*P. falciparum* species
  Depending on the specific product, may accept
    EDTA-anticoagulated, refrigerated, and/or frozen blood
  Built-in internal quality control included with each assay
Disadvantages
  High cost (if used at population level)
  Humidity sensitive (requires desiccant and foil pouch)
  Difficult to manufacture at high quality
  Quality control is necessary and expensive
  Less sensitive (500–5,000/μL depending on species) than expert
    microscopy and molecular assays
  Not validated for quantification
  Cannot be used to monitor parasite clearance because of
    antigen persistence
  Definite shelf life, dependent on manufacturer and
    storage conditions

Antigen kinetics are not necessarily aligned to intact parasite kinetics, and many RDTs will give positive results at least seven days after treatment.[41] Current generation RDTs rely on the detection of 1–3 widely used *Plasmodium*-specific antigens: histidine-rich protein 2 (HRP2), lactate dehydrogenase (LDH), and aldolase. Histidine-rich protein 2 is expressed mainly by *P. falciparum* and is the most sensitive antigen tested for by RDTs. However, a small proportion of *P. falciparum* parasites appear to lack intact HRP2 or have altered forms, which can lead to false-negative results.[42,43] In addition, HRP2 antigen can persist in the blood for up to two weeks after resolution of blood-stage parasite infection,[44] partly because of carriage by gametocytes.[45] Thus, HRP2-based RDTs should not be used for patient follow-up for at least one month after treatment.[46] Lactate dehydrogenase is a less sensitive target, and LDH-specific assays can be designed to detect *P. falciparum* LDH or non-*P. falciparum* LDH. Lactate dehydrogenase circulates in the peripheral blood for a shorter time than HRP2. A study of HRP2- and LDH-based RDTs at a site of varying malaria transmission intensity in Uganda found that the negative predictive value of an HRP2-based test was relatively unaffected by rising transmission intensity, whereas negative predictive values for an LDH-based RDT and microscopy decreased as the rate of sub-patent parasitemia increased; the authors suggested that HRP2-based RDTs are a good choice in regions of Africa with medium-to-high malaria transmission rates.[47] The third malaria antigen targeted by some RDTs is parasite aldolase, which is a glycolytic enzyme found in all *Plasmodium* species.

Considerations for use of RDTs in clinical trials. The RDTs provide qualitative results for malaria detection and are well-suited for screening malaria-endemic populations to detect current or recent infections. Unfortunately, in many settings, healthy persons can have low-level parasitemias below the threshold of sensitivity for most RDTs (< 500 *P. falciparum* or 5,000 *P. vivax* parasites/μL as above). Before selecting a particular RDT, investigators should research the performance characteristics of the particular product, such as sample requirements, consistency, specificity, and sensitivity.[40] In addition, site-specific needs will further guide RDT selection. Many products can detect a difference between a *P. falciparum* infection and an infection with another species, but cannot identify which other species is present; in such cases, microscopy remains the most widely used backup diagnostic tool for making treatment decisions. If species other than *P. falciparum* are common in the study region, then HRP2 assays should not be used. In addition, clinical trial sites should develop policies for tracking RDT lot temperatures during shipment and storage and for tracking development time between test days and between reagents with different lot numbers. If not already in place in clinical trials, RDT method validation policies should be developed and implemented to ensure quality RDT performance and to understand the differences compared with other tests like microscopy or polymerase chain reaction (PCR).

The RDTs should be used according to the manufacturer's recommendations, and great care should be taken to interpret faint lines (including obtaining an immediate second opinion from another trained technician). When screening for clinical trials, there may be no need to repeat an RDT that gives a negative result. However, when there is a clinical suspicion of malaria illness in the setting of a negative RDT result or in regions where it is documented that false-negative results are an issue, testing can be repeated, either several times a day, with an RDT specific for a different antigen or, if the laboratory is qualified to perform microscopy, with a blood smear evaluation that may support or refute the original RDT result. Whether to institute the practice of repeating RDTs or switching to blood smears after a questionable negative result ultimately rests with the site, clinicians, and study objectives. In areas of low or only intermittent transmission, positive RDT results may be confirmed by a second RDT from a different manufacturer, by thick blood smear, or by PCR if capacity exists. In contrast, positive RDT results are seldom repeated in regions of high endemicity because it would be unduly burdensome and would be unlikely to substantially increase diagnostic accuracy.

Quality management of RDTs. RDT EQA should be implemented for clinical trial sites for excluding persons with malaria, and several centers are already moving in this direction.[48,49] As with microscopy, CAP provides three RDT panels twice a year, including one challenge each with a *P. falciparum* antigen, a non-*P. falciparum* antigen (positive for *P. vivax*, *P. malariae*, *P. ovale*, *P. knowlesi*, or a mixture), and a non-malaria infection (negative). More extensive EQA/PT is likely required for clinical trials, and efforts are underway in Addis Ababa and Manila by the WHO/Foundation for Innovative New Diagnostics/Centers for Disease Control and Prevention collaboration to develop lyophilized antigen panels that can be reconstituted and used to test lots of RDTs.[40] Until control antigens are available as EQA standards, an alternative may be to use standardized dried blood samples.[50] Laboratories could also create their own standards and controls by using patient samples that are well-characterized by microscopy and/or nucleic acid testing.

There have been many issues with failed RDT products, with inconsistency between lots and with poor reproducibility worldwide.[40] Because of a host of hurdles related to regulatory approval, intellectual property, and manufacturing, the best RDT products are not always present in the marketplace. In addition, false-positive and -negative results can be caused by numerous factors, many that are common for all antibody-based diagnostics. False-positive results can be caused by failure to follow the simple assay instructions (most commonly waiting too long to read the result), rheumatoid factor,[51,52] and antigen persistence as noted above. False-negative results can be caused by hyperparasitemia, leading to a prozone effect,[53] HRP2 deletion,[42,43] or allelic variation,[42] but are usually caused by infections at parasite densities below the RDT limit of detection.[54] Finally, faint lines on the assay strips are often confusing and can lead to different interpretations by different operators.

RDTs in special populations. *RDTs for pregnant women.* Pregnant women and their unborn children are particularly susceptible to complications from malaria infection.[55] Unfortunately, pregnancy also poses unique challenges to the diagnosis of malaria. Although parasites may be undetectable in peripheral blood, they can be present in the placenta in much larger numbers. In addition, even low-intensity infections can have profound impacts upon the pregnancy and the fetus.[56–58] Although this pathologic state decreases with

gravidity, 80% of all adult malaria deaths still occur in pregnant women.[59] A handful of pregnancy malaria vaccine candidates are in the clinical development pipeline, notably VAR2CSA, a member of the *P. falciparum* erythrocyte membrane protein family.[60,61] However, the topic of malaria diagnostic performance evaluations in this unique population has been largely inconsistent. The gold standard for diagnosis of placental malaria is the relatively impractical placental histologic analysis. However, peripheral blood RDTs and PCR may also be used to conclude a diagnosis of placental infection.

Malaria RDT performance varies when used in pregnant women because pregnancy affects the level of parasite sequestration and parasite frequency in peripheral blood, which in turn may affect the output of circulating antigen detected by RDTs.[62–66] Diagnosis by RDT is further complicated by persistence of the HRP2 antigen. During pregnancy, HRP2 can be detected in the circulation even when the parasite is sequestered in the placenta.[64,66] Nonetheless, few RDTs have been properly evaluated in pregnant women.[62,64,67] A study in Uganda recently evaluated the BinaxNOW® ICT RDT (HRP2-Aldolase) against placental histologic analysis and yielded an RDT sensitivity of 57% and a specificity of 90%.[62] However, a study in Ghana in pregnant women comparing the OptiMAL® (LDH) RDT against microscopy using peripheral blood reported an RDT sensitivity of 97% and a specificity 85%.[66] Furthermore, performance evaluations have typically been conducted at birth in conjunction with PCR, microscopy, and placental histologic anlaysis, which may yield different results than RDT testing at time points before birth. Most researchers have merely used an RDT that was locally purchased or already in use at a given site. Thus, there is a need to conduct additional quality performance evaluations on RDTs in women of varying gravidity and with pregnancies at different gestational ages and to compare the findings against microscopy on peripheral (before birth) and placental blood (at birth).

RDTs for HIV-positive populations. There is early evidence that antiretroviral therapy (ART) may reduce malaria in HIV-infected persons.[68] Because HIV and malaria endemicity overlap globally, and the overwhelming burden of HIV-malaria co-infection is found in sub-Saharan Africa,[11] RDTs could make an enormous impact. Presumptive treatment of fevers in HIV-positive persons with malaria drugs is extremely common, but laboratory confirmation of malaria infection is not uniformly conducted.[47,69,70] Even with a laboratory-confirmed negative blood smear, clinicians are inclined to still provide drugs to patients for assurance.[48] Although evidence supports a higher incidence of clinical malaria and severe malaria in HIV-infected children, recent studies have also reported a reduction in malaria rates in HIV-infected persons receiving trimethoprim-sulfamethoxazole prophylaxis, as well as reports of reduced rates of malaria for those children receiving certain ART regimens.[11,68] Thus, the selected diagnostic modality must be carefully monitored to ensure its continued effectiveness in the setting of this complex and evolving interaction.

In patients receiving ART, RDTs demonstrate > 95% sensitivity in detecting *P. falciparum* HRP2 antigenemia.[47,67,69] However, actual febrile illness caused by malaria appears to be low. In one study among febrile HIV-positive outpatients in Uganda attending rural ART clinics, malaria was only responsible for 5.7% of febrile illness when diagnosed by BinaxNOW® RDT and microscopy.[69] Furthermore, this RDT sensitivity was 85.7% and specificity was 97.8% compared with expert microscopy, indicating a favorable, although imperfect performance for using the BinaxNOW® RDT alone to exclude malaria as the cause of fevers among HIV-positive febrile patients in this setting. A cost-effective, operator-friendly, and robust RDT for POC use would greatly benefit patients requiring a laboratory diagnosis to permit clinicians to focus on the actual causative etiology of febrile illnesses among HIV-positive patients. It will be important for such assays to be evaluated across robust conditions with consideration for test operators (trial staff, counselors, lay persons, technicians), climate, HIV immune status (chronic versus acute infection), and various clinical manifestations of malaria infection.

Malaria RDTs in elimination settings. Malaria diagnosis in the setting of elimination presents unique challenges.[71] As parasitemia decreases to < 100 parasites/µL, the rate of false-negative RDT results increases. Therefore, RDTs may have limited success in the detection of asymptomatic cases, gametocytes, and other epidemiologic reservoirs in low-prevalence regions and in elimination efforts. Overall, there is a need to either identify or develop RDTs with the highest sensitivity at the lowest parasite densities for *P. falciparum* and *P. vivax* for use in the elimination setting. Molecular and serologic assays also offer promising approaches for detecting low-density *Plasmodium* parasite infections.[71]

MOLECULAR ASSAYS

General considerations. The use of nucleic acid amplification-based tests such as PCR and reverse transcription PCR (RT-PCR) has dramatically increased the analytical sensitivity of assays for many human pathogens, including malaria parasites. Over the past 20 years, many molecular assays for malaria have been developed.[72–91] Among published methods, > 65 primer sets have been reported with at least five molecular targets used to test for as many as five human malaria species. Methods include single-step PCR with electrophoresis gel-based detection or DNA probe-based real-time detection, nested PCR with gel-based or real-time SYBR Green dye-based detection, nucleic acid-based sequence amplification (NASBA) and real-time RT-PCR with probe-based detection.

These assays afford greatly improved detection and the ability to provide species identification using species-specific primers or probes and to precisely quantify parasites by comparison to standard curve materials. These assays have excellent sensitivity and quantification across a wider range of parasite densities than possible for microscopy. With highly sensitive assays, parasites may be detected in blood 1–4 days earlier than by microscopy.[29,83] However, it should be noted that not all molecular assays are highly sensitive and some report sensitivities more comparable to thick blood smears. In addition, quantification provided by some molecular methods makes it possible to determine the kinetics of asexual parasite growth in drug or vaccine trials. This feature is particularly useful in CHMI vaccine trials in which microscopic and molecular data can be

combined to examine pre-patent parasite kinetics to help prioritize blood-stage and imperfect liver-stage vaccine candidates based on efficacy; such data could be similarly useful for prophylactic drugs studies in challenge models. Molecular assays can also be used to quantitatively monitor parasite clearance kinetics after drug treatment, although there can be extremely low positive results obtained in the immediate post-treatment period despite clearance of all viable organisms by microscopy,[29] which is similar to the phenomena of low molecular positive results observed for other infectious disease molecular tests.[92,93]

Molecular assays are also amenable to higher throughput instrumentation and are less operator-dependent than microscopy. Assays for Phase I studies or epidemiologic studies with lower numbers of participants may not always warrant moderate-to-high throughput platforms, whereas Phase II–III studies and larger epidemiologic studies are likely to require such platforms. Testing for each study must be tailored to study-specific needs (i.e., qualitative or quantitative detection with or without species identification). The limit of detection (analytical sensitivity) should be appropriate for the setting and study type. The various advantages and disadvantages of these tests (Table 4) make molecular assays ideal for some but not all clinical trials.

It should be noted that parasite density (as determined by microscopy or molecular methods) generally and imperfectly correlates with the degree of illness, and persons with more parasites have more severe illnesses in some settings.[7] However, at a given parasitemia, the degree of illness can vary widely among persons because of age, endemicity, and genetic and immunologic resistance to malaria. Those persons with blood smear- or RDT-positive infections are more likely to have clinical illness, whereas many persons with low-level infections detected by molecular methods alone may remain asymptomatic. One study comparing PCR and microscopy found that although 47% of persons were PCR-positive for malaria, only the 17% who were smear-positive went on to develop symptomatic malaria.[94] Nonetheless, asymptomatic persons given a diagnosis of infection by molecular methods represent a significant population for eradication/elimination efforts because they may still transmit parasites to mosquitoes. Similarly, detection of pre-patent parasitemia may be critical for controlled human challenge vaccine and drug trials.

The following sections focus on the primary molecular assays that provide qualitative or quantitative parasite detection with or without species identification. Additional molecular assay types that may be useful for some trials also include those for determining the multiplicity of infection,[95] genotyping to determine recrudescence versus re-infection,[96] and those for detection of mutations that confer drug resistance[97]; such assays are beyond the scope of this report.

Considerations for use of molecular assays in clinical trials. *Target sequences and quantification.* Most molecular approaches focus on detection of conserved regions of *Plasmodium* genomic DNA encoding the 18S ribosomal RNA (rRNA) or the asexual 18S rRNA itself.[98] Sensitive detection and species identification can be attained by combining primers and/or probes specific for conserved and/or species-specific regions of the 18S rRNA genes. However, even within the 18S rRNA gene target, primer/probe design can greatly affect assay sensitivity and specificity. For instance, if RNA- or total nucleic acid–based tests such as RT-PCR are used, assay reagents must be specific for the A-type (asexual stage) 18S rRNAs because these RNAs, but not S-type (sexual-stage) 18S rRNAs, are predictably abundant during the erythrocyte stage of infection.[29] In addition, although most tests are based on publicly available sequence data, we are only now beginning to understand the complex genetic diversity in field settings. Thus, genetically variable parasites may be missed. False-negatives results may be caused by sub-species diversity if a molecular assay targets a sequence present in only a fraction of parasites within a given species, such as in *P. ovale* detection where it is known that two species of *P. ovale* co-exist as *P. ovale wallikeri* and *P. ovale curtesi*.[99,100] False-negative results for molecular assays caused by sub-species diversity may be detected when discordant microscopy (positive) and molecular (negative) findings highlight the likelihood of genetic variation, leading to failure of a sequence-specific molecular assay. Some groups use non-18S rRNA gene targets that are either single copy genes or are unique to a particular parasite species. The rationale for these approaches is that single copy genes afford more exact quantification and species-specific genes afford more definitive and specific species identification. The importance of species identification varies geographically and with the clinical situation. For instance, identification of *P. vivax* or *P. ovale* leads to other treatment modalities, and they could be missed by some assays in persons with higher density *P. falciparum* infections.

The density of parasitemia is typically reported as parasites/μL or as a percentage of erythrocytes. Infected erythrocytes usually, but not always, contain a single parasite as it develops through the erythrocyte life cycle; in microscopic analysis, these parasites are conventionally scored singly, but multiply infected erythrocytes cannot be identified by molecular assays. Because parasites multiply in erythrocytes, a parasite that starts with a single genome (i.e., ring-stage parasite) eventually becomes a multi-genome mature parasite (i.e., schizont stage). Thus, because schizont-stage infected erythrocytes may contain > 30 daughter merozoites, molecular assays can potentially overestimate the parasite burden compared with microscopy. For *P. falciparum*, where most circulating

TABLE 4
Attributes of molecular assays for diagnosis of malaria

Advantages
  Excellent sensitivity (0.01–1/μL depending on method and expertise)
  Earlier detection of new infections (up to four days before blood smears)
  Quantification across a wide range of parasite densities
  Species identification
  Potential for strain identification to distinguish new and recrudescent infections
  Samples can be batched for high throughput
Disadvantages
  Can be time consuming
  Expensive
  Extensive training required
  Mixed infections require more elaborate assay designs and can undermine some assays
  Standardization is complicated (across multiple sites, various assay platforms)
  Requires provisions to avoid cross-contamination of samples

parasites are likely to be rings, the correlation can be quite good. However, in non-*P. falciparum* species, molecular assays can estimate parasite genomes, but often cannot specify a parasitemia because of the ambiguity between ring, trophozoite, and schizont-stage parasites. Besides ongoing schizogony, other sources of error for conversion of PCR copy number results to parasites/μL are sequestration, multiple infection of single erythrocytes, and gametocytemias (because gametocytes have a different type of 18S rRNA). Thus, it may be useful to adopt the concept of genome equivalents for molecular malaria assays, similar to viral load data reported for HIV.

Blood collection and processing considerations for molecular assays. Blood collection procedures must account for the stability of the target nucleic acids. Depending on whether DNA and/or RNA are used, processing procedures can vary widely. Blood samples frozen and stored at –20°C and dried blood spot (DBS) cards are well suited for DNA-based assays. However, in some highly sensitive DNA-based assays, leukocytes are removed by filtration before freezing.[101] RNA or total nucleic acid-based tests require either that liquid whole blood be properly preserved with either an RNA-protective chemical (e.g., guanidinium-based lysis buffer, TRIzol® Reagent) or by rapid and careful preservation on properly-dried DBS cards, such as Whatman filter papers.[102] Guanidinium-treated samples must be tested within a few days or be frozen and transported at ultra-low temperatures before testing. In all cases, DBS cards are affected by humidity and must be properly stored with desiccant to ensure sample integrity. Malaria DBS cards for RNA-based assays are also more prone to cross-contamination compared with HIV DBS, although the risk of cross-contamination can be mitigated by using disposable cutting instruments or contact-free laser[102] cutting in lieu of traditional punch processing. Thus, great care in preparation, handling and storage should be observed.

DNA-based assays are sufficiently sensitive for many applications, especially if a larger extraction volume is used. However, the advantage of testing RNA is that the parasite 18S rRNA is biologically amplified by the living parasite, thereby maximizing assay sensitivity from a smaller blood sample. Although the average 18S rRNA content per parasite has been measured,[29] this measurement may vary when different extraction methods are used and should be independently verified for new assays. RNA-/total nucleic acid–based assays are typically ultrasensitive (i.e., limit of detection = 0.01–0.02 parasites/μL) and may be more sensitive than needed for some clinical trials, especially in field settings. At the lowest parasitemias, sampling error will be a common cause of a false-negative result, and the prevalence of infection at sub-molecular parasitemias cannot be estimated. Finally, although there are reports of molecular diagnosis of malaria infection from urine and saliva,[103–105] plasma,[106] and serum[107] (as described later in this report), whole blood remains the most common sample type used for definitive diagnosis because the parasites reside in erythrocytes.

Once collected, template extraction is achieved by a variety of lower-throughput manual (e.g., DNA spin columns, TRIzol® Reagent, boiling blood in 5% Chelex) and higher throughput automated (e.g., silica-based) extraction methods. Extractions generally eliminate contaminating hemoglobin, which can otherwise serve as a potent PCR inhibitor.[108]

The extracted DNA or total nucleic acids are then subjected to PCR, RT-PCR, or NASBA-type amplification and detection procedures.

Quality management of molecular assays. For clinical trial use, molecular malaria assays should be subjected to internal and external QC. Multiplexed internal controls can be easily incorporated into PCR, RT-PCR, and NASBA assays and enable detection of template loss and amplification inhibitors. Standardized controls (e.g., negative, high, and low positive samples) analyzed with each test run should be used to monitor ongoing assay performance by using Levey-Jennings charts in accordance with the molecular version of the so-called Westgard rules.[109] Quantitative assays are considerably more difficult to validate, calibrate, and control than qualitative tests. Quantitative DNA assays may use linearized DNA, but RNA-/total nucleic acid–based tests require *in vitro* transcribed RNA calibrators. Well-characterized malaria parasites obtained by culture or from patient samples can also be used to correlate copy number with parasite quantity. Awareness of the synchronicity of parasites to control for the life cycle stage (e.g., all ring-stage parasites or mixed stages) is critical when using these materials for assay calibration because one intact parasite may contain 1 to more than 30 genome equivalents as noted above.

At this time, a formal program established for malaria molecular assay EQA is lacking, yet we believe that there is a tremendous need. The Malaria Laboratory Network and the growing network of CHMI centers in collaboration with the European Vaccine Initiative are currently embarking on a first-generation program (Murphy S, unpublished data). Until such programs are formalized, laboratories are advised to exchange samples and SOPs and to conduct performance evaluations and compare results. Our group and others have exchanged samples as part of such interlaboratory comparisons and strongly advocate this quality management approach to these assays. Because of diversity of assays and molecular targets within the 18S rRNA sequence, it may be difficult to compare results between sites without a traceable international standard. With respect to 18S rRNA assays, we propose the joint development of a full-length 18S rRNA–encoding DNA plasmid and the corresponding RNase-protected *in vitro* transcribed full-length 18S rRNA for distribution as quantified, traceable calibrators. Such materials would enable malaria diagnostic laboratories to rigorously compare assays and participate in more meaningful EQA and PT efforts.

Molecular assays in special populations. *Molecular assays in pregnant women.* Placental blood PCR may also be useful in pregnant women. In a recent study in Mozambique, quantitative PCR of peripheral and/or placental blood had a higher sensitivity than peripheral blood microscopy; an HRP2 enzyme-linked immunosorbent assay (ELISA) and an HRP2 RDT from plasma was also more sensitive for detection of parasites in placental specimens than the gold standard (placental histologic analysis).[110] Therefore, depending on the population and trial aims, ultra-sensitive molecular assays may be more useful and should be considered.

Molecular assays in CHMI trials. The CHMI model has been routinely used as a safe and effective method for early stage testing of candidate vaccines.[111,112] In these studies, the usual trigger for drug treatment is blood

smear–confirmed parasitemia. Because highly sensitive and quantitative molecular assays detect pre-patent parasitemia up to four days before blood smears,[29,83,113,114] it is currently possible to estimate parasite replication kinetics based on molecular assay results obtained in the days before drug treatment.[29,114,115] The CHMI trials often enroll healthy volunteers in Phase I/IIa studies and are often conducted on malaria-naive persons in non-endemic regions. A key issue in this regard is whether earlier detection by a validated molecular assay in CHMI trials should replace microscopy as the gold standard, thereby enabling earlier diagnosis and treatment with the goal of decreasing clinical illness and risk in trial participants. Molecular assays are currently an indispensable tool for such studies. Implementation of EQA efforts among human challenge centers will ensure accurate comparison of trial results between sites and perhaps lead to gold standard molecular tests that can serve as primary endpoints.

Molecular assays for detection of gametocytes. Some investigators will also need to monitor gametocyte carriage as a measure of transmission interruption. However, molecular assays for infected erythrocytes (e.g., targeting parasite 18S rRNA) may not always detect gametocytes. Instead, sensitive stage–specific RT-PCRs that detect gametocyte-specific transcripts are useful for such studies. Several such gametocyte assays have been developed.[116–118]

SEROLOGIC ASSAYS

General considerations. Unlike assays that directly detect whole parasites (microscopy) or parasite components (RDTs, PCR), serologic assays for immune responses against *Plasmodium* spp. are less often used for acute disease diagnosis and management and more often used for evaluating exposure or immune responses to a candidate vaccine. Serologic assays (Table 5), such as ELISAs and bead-based Luminex® assays, can be useful for detecting seroconversion in treated patients in whom the original diagnosis of malaria was in doubt, or for questionable exposures to blood or other products. In vaccine studies, serologic assays clearly play an important role in monitoring immune responses, often against specific subunit antigens, and sometimes against a variety of parasite antigens after whole organism vaccination. Although beyond the scope of this report, newer ELISAs for mosquito salivary proteins may eventually be used to monitor putative parasite exposure in epidemiology studies and for future transmission-blocking vaccine efforts.[119,120]

TABLE 5
Attributes of serologic assays for diagnosis of malaria

Advantages
  Useful for detecting specific humoral immune responses and
    providing an estimate of past exposure
  High throughput for larger trials
  Computer software assists with conversion and expression
    of results
Disadvantages
  Not recommended for acute disease diagnosis, quantification,
    or treatment management
  Purified antigens may be restricted to particular species/strain
  Can be difficult to compare results across sites/studies
  Labor intensive with extensive training required

Quality management of serologic assays. An ELISA must be carefully controlled by using total quality management, including rigorous training, process controls, SOPs, and staff qualification. An ELISA can achieve low intra- and inter-site variation with total quality management in place to standardize accuracy regionally or across multi-site trials.[121] Each trial team or site must determine their acceptable level of staff training for qualification and have clear SOPs for defining qualification, setting acceptable coefficients of variation between replicates (within-run precision), between plates, and between technicians (between-run precision), and for determining when QC passes. In addition, process control of the batch preparation of antigen and antibody reagents and use of a reference serum curve in the assay greatly facilitate standardization, and in turn, improve accuracy and precision across multiple sites using the same technique.[33]

One such center that has implemented rigorous total quality management to establish a global reference laboratory is the ELISA Service Center at the Walter Reed Army Institute of Research. The PATH Malaria Vaccine Initiative and the U.S. Agency for International Development established the ELISA Service Center in 2005 in an effort to provide high-quality serologic assays for the research community. The assays offered by the center were initially developed to evaluate vaccine candidates produced by the Walter Reed Army Institute of Research, including the 3D7 strain of *P. falciparum* apical merozoite antigen-1, merozoite surface protein-1 (p42), liver-stage antigen-1, and the circumsporozoite surface protein.

If malaria serologic analysis is selected for a clinical trial endpoint, we recommended either obtaining reagents from a centralized or sole source (i.e., antigens, primary/secondary antibodies, control reagents, chemicals, and procedures) and/or undergoing inter-site standardization for multi-center trials. Depending on costs and transport logistics, it may be more feasible and less costly to either ship samples to a central core laboratory or to ship reagents to trial sites. Finally, because short-term disappearance and the long-term persistence of antibody responses to various malaria antigens are well documented to occur in experimental and natural settings, and because serologic assays are highly susceptible to variations caused by parasite allelic complexity, these assays should be chosen with extreme caution and cognizance of their fallibility in interpretation if they are to be considered for diagnosis of infection.

TECHNOLOGIES IN DEVELOPMENT

There are many novel assays in development, and many aim to automate a fatiguing or tedious part of the assay process. For instance, in automated microscopy, some devices are intended to focus and scan fields while relying on the microscopist for confirmation and image analysis, and others are also attempting to automate the entire analysis step. Alternative molecular models are in development for use in resource-limited settings, such as loop-mediated isothermal amplification (LAMP),[75] as a low-cost way to bring molecular testing to the field. When used in a field setting in Uganda, LAMP had equivalent sensitivity to quantitative PCR (90% sensitivity) and substantially more sensitivity than thick blood smear microscopy (50% sensitivity compared with LAMP).[122] A portable tube

prototype of LAMP (LAMP-Tube) yielded an impressive 98.3% sensitivity and 100% specificity compared with thick blood smear microscopy for detection of *P. falciparum* and *P. vivax* in India.[123] Other diagnostic methods seek to improve performance and capabilities of RDTs. For example, two-dimensional paper networks use geometry and flow properties of reagents and paper to deliver multiple reagents to a detection region from a single activation step. Like traditional lateral flow devices, two-dimensional paper networks are relatively low in cost and easy to use. However, unlike RDTs currently available, two-dimensional networks enable signal amplification,[124] multiple dilutions,[125] and could eventually enable multiplexed assays for multiple malaria species, as well as for HIV-1 and other pathogens.

Malaria diagnosis from fluids other than whole blood and for POC use. The literature contains several reports of PCR performed on serum or plasma samples.[106,107] Although parasite DNA can be detected in these sample types, the extra-hepatic parasite lives in erythrocytes and prospective malaria clinical trials are advised to design trials to use whole blood if available. However, for studies using archival material or in which whole blood or DBS are unavailable, testing of serum or plasma may provide useful data, albeit with less sensitive detection, than for whole blood.[106]

Urine and saliva from malaria-infected persons contain detectable amounts of plasmodial DNA. Thus, this approach could be used as an alternative to microscopy or RDTs at sites that have established PCR capacity.[103,104] One group evaluated urinary and salivary DNA preparations and a nested PCR specific for the mitochondrial cytochrome b gene and compared this PCR with microscopy and 18S rRNA nested PCR.[105] Cytochrome b PCR was 16% and 40% more sensitive than 18S rRNA PCR and microscopy, respectively. In addition, 34% and 17% of *P. falciparum* and *P. vivax* mono-infections, respectively, detected by microscopy were actually mixed *P. falciparum* and non-*P. falciparum* infections. Similarly, *P. falciparum* HRP2 ELISA was recently evaluated with saliva and results agreed completely with microscopy.[126] More performance evaluations using these types of samples and systems are warranted to fully understand the accuracy and utility of such tests, particularly for screening or epidemiologic studies.

In other disease systems, use of blood alternatives to diagnose infection has been a focus when bringing the laboratory to the patient. The POC urine- and saliva-based RDTs have undergone performance evaluations compared with standard RDTs and with nucleic acid testing and shown promising results for HIV diagnosis in the field.[127–130] In addition, a urine-based *Mycobacterium tuberculosis* RDT using the pan-mycobacterial antigen lipoarabinomannan is being evaluated in field settings to determine its accuracy.[131,132] Evaluating prototype malaria RDTs based on this knowledge could potentially yield a POC device that could increase testing uptake by clinicians. This feature would be particularly attractive for testing children, if they and their parents have a favorable view of healthcare workers who use painless testing. Because *P. falciparum*, *P. vivax*, and *P. knowlesi* sequence data have been published, this knowledge could be harnessed to develop new prototype POC nucleic acid assays and RDTs for improved sensitivity by including newly identified targets. At least one such target

from each *Plasmodium* species (Pfr364, Pvr47, Pkr140) was found to be suitable for molecular diagnosis with some advantages over existing molecular method targets.[133] Another rapidly emerging development in microfluidics in a lab-on-a-chip approach aims to develop POC devices to improve diagnostic capacity, and is already is being optimized for implementation for sexually transmitted infections and HIV-1 detection.[134]

DISCUSSION

Malaria is a complex disease that variably manifests itself according to host, vector, and parasite genetics; co-infections; environmental and social factors; access to healthcare; and other issues. The variety of available tests and a lack of consensus guidelines for diagnosis in clinical trials complicate malaria clinical trial design. The historical gold standard, microscopy of thick and thin blood smears continues to offer many advantages-perhaps most notable is its utility for species identification, some quantification, and parasite clearance endpoints. Malaria clinical disease and malaria parasite positivity must be considered differently in the context of a given clinical trial and when considering diagnostic tests. Beyond microscopy, other techniques, such as RDTs, molecular assays, and serologic assays have been used successfully to diagnose malaria infections.

In areas of high malaria endemicity, an RDT is an extremely powerful tool, and a positive test result can be used for making treatment decisions. The RDTs have been widely deployed and once standardization and test performance is better understood, RDTs may become the principal modality in many settings for clinical diagnosis. However, the lack of sensitivity and persistence of antigen may make RDTs unsuitable for generating endpoint data in some clinical trials. The high sensitivity of molecular assays makes them well-suited for detecting and quantifying even low parasitemia infections, provided that laboratory finances, adequate quality control, and resources are available.

Serologic tests also have a place in some trials, especially for archival serum/plasma samples or for studies on the breadth of allelic exposure. In clinical trial design, it is critically important to select the most appropriate malaria diagnostic tests, to ensure proper quality for the tests and to interpret the results appropriately. Strong quality assurance systems are needed to support laboratory testing in clinical trials and to provide a framework for malaria EQA programs. Such systems will ensure the reproducibility of data within and between clinical trials.

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