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REVIEW

# Laboratory Detection of Malaria Antigens: a Strong Tool for Malaria Research, Diagnosis, and Epidemiology

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**SUMMARY** The identification and characterization of proteins produced during human infection with *Plasmodium* spp. have guided the malaria community in research, diagnosis, epidemiology, and other efforts. Recently developed methods for the detection of these proteins (antigens) in the laboratory have provided new types of data that can inform the evaluation of malaria diagnostics, epidemiological investigations, and overall malaria control strategies. Here, the focus is primarily on antigens that are currently known to be detectable in human specimens and on their impact on the understanding of malaria in human populations. We highlight historical and contemporary laboratory assays for malaria antigen detection, the concept of an antigen profile for a biospecimen, and ways in which binary results for a panel of antigens could be interpreted and utilized for different analyses. Particular emphasis is given to the direct comparison of field-level malaria diagnostics and laboratory antigen detection for the development of an external evaluation scheme. The current limitations of laboratory antigen detection are considered, and the future of this developing field is discussed.

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## INTRODUCTION

Malaria as a disease has been documented since the early Bronze Age (ca. 2000 BC) (1), although tools to correctly identify the causative agent were not available until much later. Even after Charles Laveran's observation in 1880 of *Plasmodium*-infected red blood cells (RBCs) in the blood of malarious patients, this etiology was not widely accepted until more-refined staining techniques were available for light microscopy (1, 2). Subsequent decades saw tremendous advances in malariology due to the capability for clear visual identification of the *Plasmodium* parasite and microstructures formed during infection in the *Anopheles* vector and the human host. By 2010, the World Health Organization (WHO) had recommended that in countries where malaria is endemic, all patients suspected to have malaria should be tested by laboratory parasitological confirmation (3). In addition to simple visual identification, the controlled environment of a laboratory setting offers multiple advantages for identifying components of, or products from, a malaria parasite and for conducting scientific investigations. Principal among these are the use of advanced machinery, the capacity for cold storage, and the ability to reduce the introduction of contaminants into a reaction system. For the purposes of this review, the focus on the detection of *Plasmodium* antigens will be limited primarily to the laboratory setting, although reference to the important applications for point-of-care (POC) diagnostics will also be made.

## HISTORY OF LABORATORY DETECTION OF *PLASMODIUM* ANTIGENS

### Evolution of Laboratory Assays throughout the 20th Century

Interest in malaria antigens was initially directed to vaccine development and the understanding of host-parasite interactions. Crude preparations from infected animal or human blood containing malaria antigen medleys were in usage by the early 20th century (4–6). Such preparations consisted of parasitized red blood cells and the antigenic components within them, and these seminal experiments allowed the investigation of the humoral response to *Plasmodium* infection and clearly showed recognition by the human immune system, with substantial antibody titers in convalescent patients and in persons from areas of malaria endemicity (5, 7). The second half of the 20th century provided an expanded laboratory toolkit, with the use of native antigen for serological investigations by hemagglutination tests and immunofluorescent microscopy (8–11). Human serum was observed to react most vigorously with the predominant *Plasmodium* species in the area (12–14), suggesting that different species of malaria parasites produce unique isoforms of antigens, or completely separate proteins altogether. Advances in gel filtration techniques further allowed researchers to separate crude antigen mixtures into broad groups on the basis of biochemical properties (15–17), leading to the identification of a group of stable (S) antigens seen in abundance during *Plasmodium falciparum* infection. These antigens showed strong concordance with higher prevalence in the population during the rainy season, more-frequent occurrence in patients with severe malaria, and a positive correlation between parasite density and antigen presence (18, 19). Well before the identification of *P. falciparum* ligands involved in sequestration, the isolation of malaria antigens by gel diffusion from the human brain (20) and placenta (21, 22) provided early evidence of *P. falciparum* pathogenicity in the human host. Enzyme-linked immunosorbent assays (ELISAs) utilizing a conjugate/substrate colorimetric system were developed in the early 1980s, with parallel publications by Bidwell and Voller (23) and Mackey et al. (24) providing a fundamental step forward, and allowed the practical quantitative detection of malaria antigens. Even these early experiments found flexibility in sample types with different fractions of blood; plasma, serum, whole blood, blood cell pellets, and blood dried on filter paper provided appropriate samples for data collection. Simultaneously, solid-phase radioimmunoassays (RIAs) to allow quantitative detection were created and published in the early 1980s (25–27), although these radiological

assays were largely abandoned by the end of the decade in favor of ELISAs, which do not require radioactive isotopes. Advances in recombinant DNA and cloning techniques around the same time facilitated the production of specific malaria antigens in larger quantities and in purer forms (28, 29), and mouse hybridomas producing anti-*Plasmodium* monoclonal antibodies (MAbs) allowed the development of highly specific immunological reagents (30, 31).

### Recognition of *Plasmodium* Antigens for Malaria Diagnosis

The diagnostic potential for malaria antigen detection in humans was initially realized with the discovery of large amounts of soluble antigen in children seeking treatment for acute malarial illness (32). As identified through Ouchterlony's technique of double diffusion in an agar gel (33), lines of precipitation were identified in high-parasite-density *P. falciparum* infections and, interestingly, were found occasionally to persist for many days following a negative malaria microscopy result (32). Though initially developed by measuring rodent *Plasmodium berghei* antigens (25, 34), the RIA published by Mackey et al. in 1980 moved toward quantitative detection of *P. falciparum* antigen by determining the amounts of purified anti-*P. falciparum* IgG absorbed by the RBCs of known infected persons (26). Good concordance was observed between RIA positivity and microscopy-confirmed infection, and higher inhibition was directly correlated with higher-parasite-density infections. Although early laboratory-based ELISAs provided diagnostic potential even in settings where malaria was endemic (23, 24), practical diagnostic antigen detection in field settings matured with the advent of deployable lateral flow tests in the early 1990s. These tests were transportable without cold-chain considerations, did not require machinery in order to be performed, and could be interpreted with the simple visual presence or absence of a test band (35, 36). By the late 1990s, multiple prototypes were being evaluated in both clinical and field settings (37–41). By this point, various MAbs against histidine-rich protein 2 (HRP2) had been standardized for the ELISA platform (42, 43), and in the following years, this assay served as an important comparator between the qualitative lateral flow test and more-sensitive laboratory detection.

### Detection of Malaria Antigens Expressed throughout the Parasite Life Cycle

Although the human malaria species express >5,000 genes in their large genomes (>23 Mb) (44), a vanishingly small number of targets have been identified for specific antigen detection from human specimens. The focus here will be on the targets for which mono- or polyclonal detection reagents have been developed. As *Plasmodium* moves through its definitive and secondary hosts, the expression of different proteins allows the parasite to modulate, survive, and propagate itself in different settings. Upon hepatocyte release of merozoites into the systemic circulation, the rapid proliferation of the parasite produces high quantities of multiple antigen types: secreted, membrane bound, and utilized for metabolic activity. For falciparum malaria, the paralogs *P. falciparum hrp2* and *hrp3* (*pfhrp2/3*) form the histidine-rich proteins, which are highly expressed and secreted during blood-stage infection (42, 45), although expression has also been observed in early-sexual-stage parasites (46). Interestingly, the HRP2 and HRP3 antigens remain in the human bloodstream for weeks to months after *P. falciparum* has been cleared (47), meaning that detection of these antigens is not necessarily a diagnostic qualifier for acute infection. The *Plasmodium* glycolytic enzymes aldolase, lactate dehydrogenase (LDH), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are also highly expressed during blood-stage infection (48, 49), but aldolase and LDH are known to clear from circulation quickly following the resolution of infection (50). Homology can be observed among *Plasmodium* species to the extent that unique detection reagents have not yet been identified for many of these antigens (51, 52). However, the LDH antigen is known to have both *P. falciparum*- and *Plasmodium vivax*-specific regions, allowing MAbs to be raised against both the species-specific and pan-*Plasmodium* epitopes (51, 53). Unique regions have been identified within GAPDH that make it possible to raise antibodies with pan-*Plasmodium* specificity and to

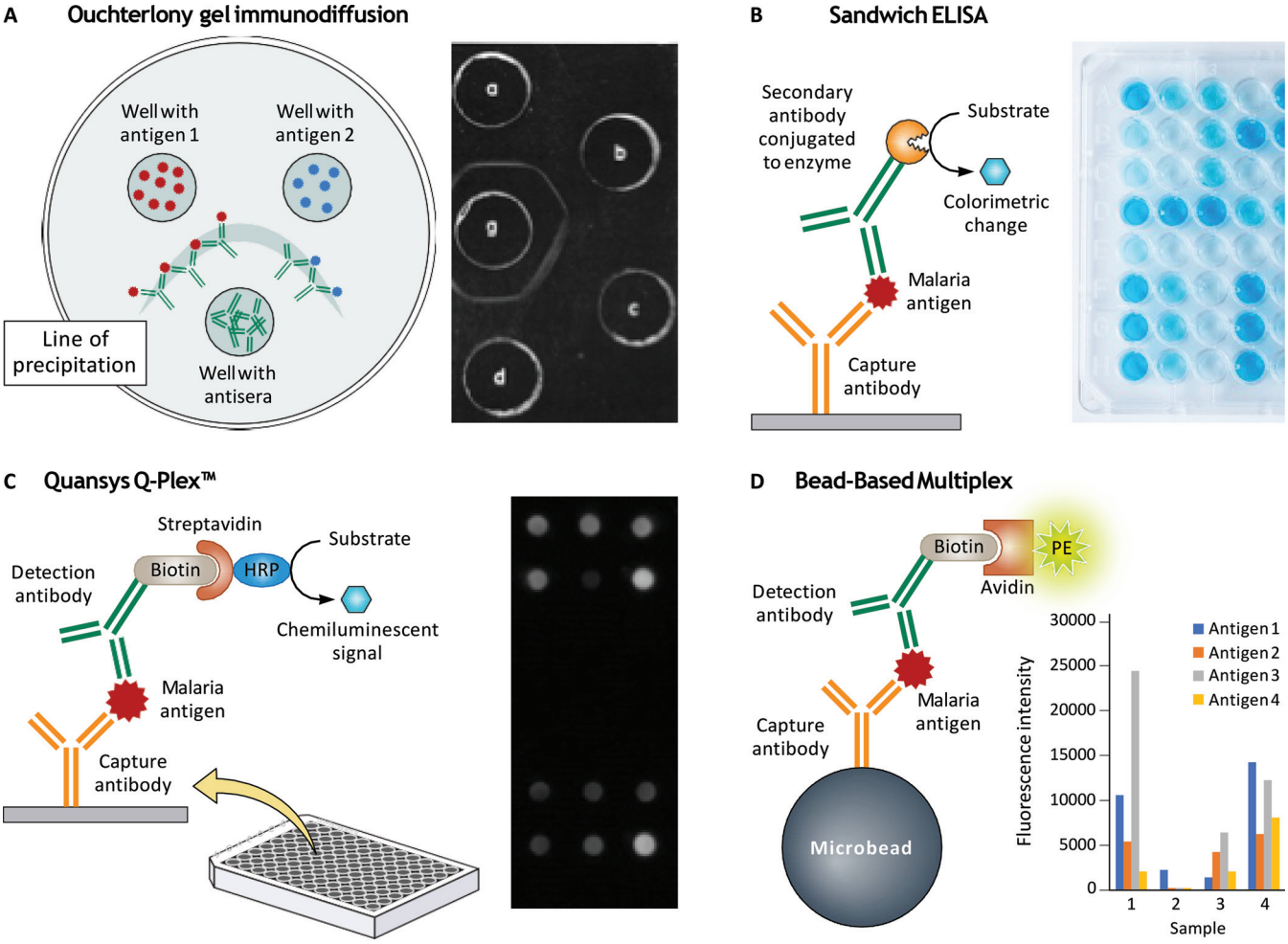


**FIG 1** Laboratory-based immunoassays for the detection of malaria antigens. For each of the four immunoassays presented, a diagram on the left shows the antibody-antigen binding mechanism that would create an assay signal, and the picture or graph immediately to the right shows an example of what the laboratorian would see. (A) Ouchterlony's agar gel double diffusion with an agarose gel containing lines of precipitation for four samples (132). (Gel image in panel A reproduced from reference 132 with permission of the publisher.) (B) Colorimetric sandwich ELISA with assay wells showing the intensity of color proportional to the amount of antigen in the sample. (C) Quansys Q-Plex Human Malaria assay showing chemiluminescence signals for six antigen targets for two samples. (D) Bead-based assay on the Luminex xMAP platform showing fluorescence intensity signals for four antigen targets for four samples (130).

detect *P. falciparum* or rodent malarias specifically (49, 54), but species-specific detection for the GAPDH of other human malarias has not been reported yet.

### Antigen Detection by Current Laboratory Assays

Figure 1 illustrates examples of the historical and more-contemporary assays for malaria antigen detection in the laboratory with schematics of how antigen is captured/detected and photographs of the actual assay readouts that the laboratorian would observe. The development of novel assays has greatly benefited from the aforementioned improvements throughout the decades. Some of the most important advances came in the development of stable hybridomas as sources of MAbs and improvements in immunoassay platforms. Using the sandwich ELISA format (which has been in use for decades), Jang et al. have recently reported reliable detection of HRP2 at levels under 100 pg/ml (55), a dramatic improvement over previously published malaria antigen ELISAs. Two novel immunoassay platforms capable of multiplexing analyte detection that have been formatted for malaria diagnostic purposes have recently emerged: the Luminex xMAP bead-based assay (56) and the Quansys Q-Plex Human Malaria assay (57). Currently, each assay has different panels of antigen targets,

**TABLE 1** Interpretation of antigen profiles from a specimen using HRP2 with other hypothetical targets

| Antigen target | | | | |
|---|---|---|---|---|
| HRP2 | Non-HRP2, *P. falciparum* specific | *P. vivax* specific | Pan-*Plasmodium* | Reasonable interpretation[a] |
| – | – | – | – | No malaria infection |
| + | – | – | – | Low-density *P. falciparum* or past *P. falciparum* infection |
| + | + | – | – | Higher-density *P. falciparum* infection |
| + | + | – | + | |
| + | – | – | + | |
| – | + | – | + | Infection with *P. falciparum* not producing HRP2 and HRP3 antigens |
| – | + | – | – | |
| – | + | + | + | Mixed *P. falciparum*/*P. vivax* infection, with *P. falciparum* not producing HRP2 and HRP3 antigens |
| – | + | + | – | |
| + | – | + | – | Mixed *P. falciparum*/*P. vivax* infection, with *P. falciparum* producing HRP2 and/or HRP3 antigen |
| + | – | + | + | |
| + | + | + | – | |
| + | + | + | + | |
| – | – | + | – | *P. vivax* infection |
| – | – | + | + | |
| – | – | – | + | *Plasmodium ovale* or *Plasmodium malariae* infection[b] |

[a]Assuming all four human malarias could be present. Appropriate consideration of endemic *Plasmodium* species in a study population could appropriately reduce potential interpretations. HRP2, histidine-rich protein 2; HRP3, histidine-rich protein 3.
[b]*Plasmodium knowlesi* or other endemic zoonotic *Plasmodium* species may also be suspected here.

but both offer detection of HRP2 as well as additional antigens with pan-*Plasmodium* specificity. Even when structured in a multiplex format, these assays are able to detect low concentrations of malaria antigens; HRP2 capture and detection are the most successful, in the single-digit picogram-per-milliliter range. Nontraditional immunoassay and aptamer-based antigen detection systems have been developed for laboratory use as well, with promising initial findings regarding sensitivity and specificity (58–62), but these have not yet been tested in multiple studies.

## ANTIGEN PROFILES TO INTERPRET MALARIA INFECTION STATUS

### Categories of Malaria Infection Status Based on Multiple Antigen Targets

For the identification of current infection, the simple qualitative detection of any *Plasmodium* antigens in a blood sample yields a simple interpretation: evidence of active *Plasmodium* blood-stage infection. However, in assessing the status of the biospecimen against multiple antigen targets, and even the concentrations of these targets, the concept of an "antigen profile" for a person is quickly expanded to include much more information and interpretations outside of a simple (and single) binary result. The improved sensitivities of current laboratory assays and the capacity for multiplexing antigen detection pose a quandary for the malaria community: now that very low levels of these different antigens can be reliably detected in a biospecimen, what does it mean to be malaria antigen positive? Furthermore, how can an individual's antigen profile be appropriately interpreted, and what does antigen carriage tell us about malaria in a study population? Although the contemporary laboratory assays mentioned above generally provide quantitative data, the simplest interpretation and understanding of antigen carriage remains the binary classification: is that specific antigen present in a specimen? Table 1 provides combinations of antigen positivity for a hypothetical assay with four antigen targets: *P. falciparum*-specific HRP2, a non-HRP2 *P. falciparum* marker, a *Plasmodium vivax* marker, and a pan-*Plasmodium* marker. No assumptions are made here regarding which immunoassay platform or detection system is used; we assume only that these four targets could be measured and assay results dichotomized for each antigen. Since this speculative antigen detection panel has 4 targets, each with a yes/no answer, there are 16 ($2^4$) possible combinations of antigen positivity when one is assessing binary results (63). This table does not provide an exhaustive list of all potential interpretations for these 16 combinations but does

afford the reasonable interpretations for the malaria status of an individual at time of sampling based on the antigen profile. Additionally, with the addition of each new target, this framework has the ability to expand to 32, 64, or more ($2^{\text{number of antigens}}$) combinations, with each antigen positivity profile having a specific interpretation. It should be noted here that interpretation would also be contingent on the global source of the specimens or study population. In parts of South America (64, 65), Southeast Asia, Indonesia, and Oceania (66–68) (among other worldwide settings), carriage of a zoonotic *Plasmodium* parasite is a completely reasonable possibility. Additionally, if a certain human *Plasmodium* species has never been reported in an area, interpretation categories could be logically constricted.

### Refined Estimates and Further Studies Needed To Better Interpret Antigen Profiles

Ultimately, in most applications, clinicians, malaria researchers, and surveillance officials are interested primarily in the simplest answer regarding an individual's infection status—specifically, whether the individual currently has active infection, and with what species. This has typically meant using either relatively insensitive and laborious microscopy or expensive nucleic acid assays. However, the ability to determine the presence or absence of multiple antigens in a high-throughput manner in the laboratory opens the possibility of generating data to help more accurately determine infection status than categories based solely on antigen presence or absence (Table 1). Especially for the evaluation of different concentrations of unique antigens in a specimen, the use of advanced statistical tools such as machine learning to analyze data sets with both antigen profile data and known infection status will build the evidence base around using the antigen profile alone to predict infection status. These machine-learning approaches have already been utilized for other *Plasmodium* antibody studies that collected data on multiple targets of potential value for exposure classification (69, 70). Multiple validation studies will allow investigators to infer infection status and categories of antigen profiles with increasing confidence from antigen data alone.

## ANTIGEN POSITIVE VERSUS MALARIA NUCLEIC ACID OR MICROSCOPY POSITIVE: WHAT'S THE DIFFERENCE?

### Different Parts of a Parasite

In assigning positivity to a particular assay, the attribute of the *Plasmodium* parasite that elicits the positive call should be taken into consideration for the most appropriate interpretation. Light microscopy directly detects the presence of stained parasite components in human blood, and this landmark test has diagnosed untold numbers of malaria cases over the past century. The limitations of even expert microscopy are well known, especially for individuals with low-parasite-density infections (71), and relative to that of other tests, sensitivity is sacrificed, even though specificity for microscopy remains close to 100% (72). Detection of *Plasmodium* nucleic acids has been in use since the early 1990s, with initial assays outperforming microscopy detection limits by an order of magnitude (73–75). Nucleic acid tests (NATs) can provide a simple a yes-or-no answer on parasite presence, although real-time assays are able to calculate estimated parasite densities (76, 77). As the protein product of transcription and translation, the proteinaceous antigen is a fundamentally unique biomolecule to assay. Different antigens are expressed by the *Plasmodium* parasite during different life stages and in different quantities, making the interpretation of antigen detection results unique for each target. Furthermore, not all *Plasmodium* antigens will remain in the systemic circulation for the same period of time, and assay reagents for detection are better for antigens with numerous epitopes (such as HRP2). Typically, no information is available as to when a person would have been inoculated with sporozoites from an infected mosquito, so the duration of *Plasmodium* antigen expression and accumulation in the systemic circulation would be unknown for an individual at any one point in time. For these reasons and others, overall interpretation is not always as simple as positivity or negativity for a malaria antigen(s), and a biospecimen could

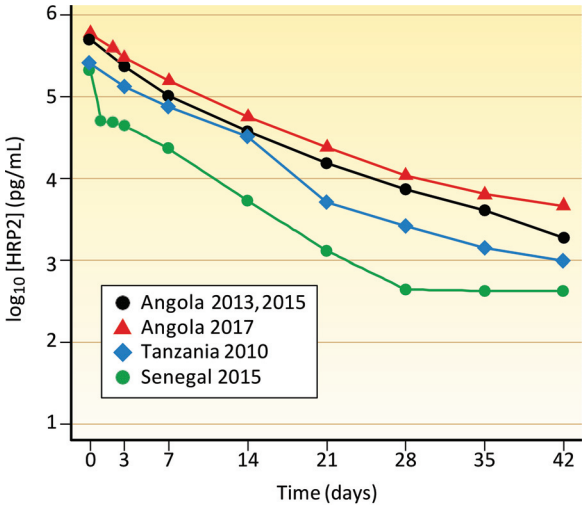


**FIG 2** Clearance of HRP2 antigen from participant blood up to 42 days after antimalarial treatment in clinical efficacy trials in Angola, Tanzania, and Senegal. The plot displays the mean concentration for all participants in a particular study as sampled at time points after the initiation of an antimalarial regimen. Studies with follow-up to 42 days were intentionally chosen to represent the ability of HRP2 to induce a positive malaria test (lab assay or RDT) result weeks after *P. falciparum* has been eliminated. (Data obtained from references 47 and 50, and used with permission.)

have various profiles for positivity or negativity for an antigen detection panel, as described in Table 1.

### HRP2 Remains in Human Blood following Parasite Elimination

The persistence of HRP2 antigenemia has been noted since the initial rapid diagnostic test (RDT) field trials (78), and the lower detection limits of the new generation of laboratory tests have revealed a long duration of persistence of HRP2 in human blood. To date, HRP2 has been directly observed in whole-blood samples for as long as 70 days following a successful antimalarial regimen (79), with models extrapolating persistence exceeding 100 days for some persons successfully clearing symptomatic and higher-parasite-density *P. falciparum* infections (47, 50). The long persistence of HRP2 is a direct consequence of the large quantity produced by the parasite combined with the fact that it is secreted outside the parasitized cell (80), as well as antigen retention in once-infected red blood cells (50, 81). The purpose of the parasite's large investment in HRP2 production, even though it is not essential for establishing human infection, has yet to be fully understood. There is some evidence that HRP2 may act as a decoy antigen that simulates the immune system to focus antibody production away from more-vulnerable and immunologically relevant parasite antigens (82). Extracellular secretion of antigen by the parasite and the emergence of *pfhrp2/3* gene deletions in low-transmission areas, where acquired immunity imposes less selection pressure, support this decoy antigen hypothesis.

Levels of HRP2 have been modeled for both first-order (47) and biphasic (79) clearance kinetics across multiple global populations. For the biphasic process, very quick clearance of HRP2 from plasma, within a few days of parasite clearance, and a second, slower phase of HRP2 decay due to once-infected RBCs have been estimated (79, 81). Figure 2 summarizes the results of four studies monitoring malaria patients for 42 days following successful *P. falciparum* treatment, which have found HRP2 still present in a large percentage of all study participants: 100% (Angola 2013–2015), 92% (Angola 2017), 45% (Senegal 2015), and 99% (Tanzania 2010).

Assessment of HRP2 clearance from a patient after treatment with artemisinin combination therapy (ACT) has been utilized to predict future clinical outcomes. HRP2-based RDT positivity 3 days after the initiation of antimalarial chemotherapy at whole-blood dilutions of 1:500 and 1:1,000 was shown to be strongly predictive of

postartesunate delayed hemolysis (81). The long-term decay dynamics of HRP2 from whole blood are so consistent that deviations from the first-order clearance pattern have been found to be indicative of treatment failure and inadequate clearance of *P. falciparum* parasites (47, 83), suggesting that monitoring of antigen clearance can potentially be used to assess the response to antimalarial treatment. In the antimalarial resistance field, resolution of malaria infection has traditionally been measured by serial microscopy, starting with daily follow-up visits for 3 days to verify the decline and clearance of parasitemia according to well-defined minimal clearance criteria (84). Trial participants are then followed weekly for 4 to 6 weeks to verify the absence of recurrent episodes of parasitemia. Complementing the microscopy data with antigen measurement data may allow investigators to identify future recurrent episodes of parasitemia based on delayed clearance of antigen during the daily follow-up phase and may help differentiate recrudescence from new infection when parasitemia episodes recur.

## OVERVIEW OF CONTEMPORARY ANTIGEN DETECTION FOR DIAGNOSTIC USE

### Malaria Antigen Levels To Estimate Symptomatic Disease

The ability to determine antigen concentrations in the laboratory has allowed researchers to estimate the antigen levels (as a proxy for more-accurate parasite burdens) that are likely to cause symptomatic disease. Since *P. falciparum*-parasitized red blood cells are able to be sequestered in the microvasculature and organs of the host, microscopic estimation of parasite density from peripheral blood smears can substantially underestimate true parasite biomass, and this underestimation has been shown to be more pronounced with greater severity of disease (85). Building on previous work that determined the "pyrogenic threshold" for parasite density, defined as the threshold parasite density needed to cause fever in infected individuals, researchers have now quantified the "antigen pyrogenic threshold" in various settings (86, 87). These estimates, which represent the antigen concentration that would be expected to predict symptomatic disease, can be used by RDT manufacturers to adjust RDT levels of detection so as to accurately identify clinically relevant antigen loads. In addition, the antigen pyrogenic threshold can be used to estimate the proportion of antigen-positive febrile persons whose fever can be attributed to malaria. In several settings with different malaria transmission intensities, these analyses have suggested that most fever in antigen-positive persons is attributable to malaria (87), but further work to explore the robustness of these findings in other settings is warranted.

### The Malaria RDT

The 2010 WHO recommendation for universal testing of all suspected malaria cases and the subsequent introduction of malaria antigen-detecting RDTs have led to an exponential increase in malaria testing in countries where the disease is endemic (3). In 2018, approximately 412 million malaria RDTs were supplied to countries of endemicity, and their impact has been reflected in the increase observed in the confirmation rates of malaria cases, from about 35% in 2010 to >80% in 2018 (88). Most of the increase in diagnostic testing has been due to the use of antigen-detecting RDTs, which account for about 60% to 75% of all malaria testing worldwide (88, 89). *P. falciparum* diagnosis through antigen detection has been proposed to be a more-accurate indication of parasite biomass than microscopy, since sequestered parasites would not be visually present in peripheral blood samples but would still release antigens into the circulation (90, 91).

Malaria RDTs are inexpensive to procure and easily deployable, thus representing one of the most pragmatic tools for malaria control. In most of sub-Saharan Africa, RDTs have been deployed in small health care facilities that are unable to support malaria diagnosis by microscopy, and they are now frequently used by community health workers operating in communities without easy access to health care facilities. As a result of this widespread use of RDTs, rates of malaria confirmation before the administration of antimalarials have increased globally, and this has, in turn, led

to more-accurate estimations of disease burden and appropriate drug use. Due to cost and other associated complexities, molecular tests are not routinely used in most countries where malaria is endemic, and malaria is diagnosed largely by RDTs and microscopy. While the presence of parasites in blood is often associated with antigen detection by RDTs, there are circumstances under which antigen is detected in the absence of parasites, or vice versa (92). Understanding the dynamics of malaria antigen production and detection is therefore critical to the interpretation of test results irrespective of whether they are for primary diagnosis or for surveillance. This is especially true for the HRP2 antigen, which can be detected by RDTs for as long as 40 days post-parasite clearance (78, 93). Lingering posttreatment *Plasmodium* LDH (pLDH) antigenemia also occurs, but for a shorter time, up to approximately a week post-parasite clearance (50, 78). Parasite detection by microscopy in the absence of antigen detection by RDTs is often encountered when parasite density in the blood being tested is very low and the antigen concentration is below the limit of detection (LOD) of the RDT (94). A solution to the latter problem has been the development of an ultrasensitive (or high-sensitivity) HRP2-based RDT (uRDT) (79, 95, 96). However, the advantage of uRDTs over standard HRP2 RDTs in identifying clinical malaria cases is minimal, especially in regions of moderate to high endemicity, since most clinical cases are characterized by higher parasite densities, and therefore antigen concentrations, exceeding the limit of detection of conventional RDTs (97). In addition, the lower limit of detection of the uRDT means that the window of HRP2 detection following parasite clearance is most likely longer than that for conventional HRP2 RDTs (98). Therefore, the proposed use for uRDTs has been not for the diagnosis of clinical malaria in moderate- to high-transmission settings but rather for surveys or surveillance of asymptomatic, non-treatment-seeking populations or in elimination settings, where the proportion of low-parasite-density infections is expected to be greater (99, 100).

**False-negative RDT results due to deletion of genes encoding HRP2 and HRP3.** In rare but increasingly encountered situations (101–103), HRP2-based RDTs yield false-negative results when the *pfhrp2* and *pfhrp3* genes of the infecting *P. falciparum* parasites are deleted or harbor nonfunctional mutations. This phenomenon was first reported for natural parasite populations in South America, where some *P. falciparum* populations showed a >50% prevalence of deletions in one or both genes (104–106), although RDTs were not in widespread use at that time. In other situations, the inherent ability of a *P. falciparum* parasite to produce only low levels of HRP2 despite an intact *pfhrp2* gene could potentially result in a false-negative test result at a clinically relevant parasite density (107).

## Evaluation of RDT Results from the Field by an Appropriate Gold Standard

**The need for an external comparator.** As the use of RDTs in surveillance and as measures of outcome in malaria research trials expands, it will become crucial to develop methods for external validation of RDT results obtained in the field. As point-of-care diagnostic devices, malaria RDTs can be read only during a short time window (typically 15 to 30 min), with a risk of false-positive or false-negative results if they are read before or after this time. This is in sharp contrast to microscopy slides, which can be stored and read at a later date by different readers for concordance and validation of results. To date, there has been no corresponding way to externally validate malaria RDT results read in the field. Since RDTs detect *Plasmodium* antigens, validation of their results using blood slides or the presence of parasite nucleic acids from simultaneously collected dried blood spots (DBS) on filter paper has several limitations that make such comparisons inadequate. There are instances where RDT and microscopy results are so divergent that they point to likely RDT failure (101, 103, 108, 109). However, even though microscopy results can be verified later by (potentially multiple) expert microscopists, in the absence of an independent measure of antigen concentration, RDT-discordant cases have usually been left unexplored and unexplained.

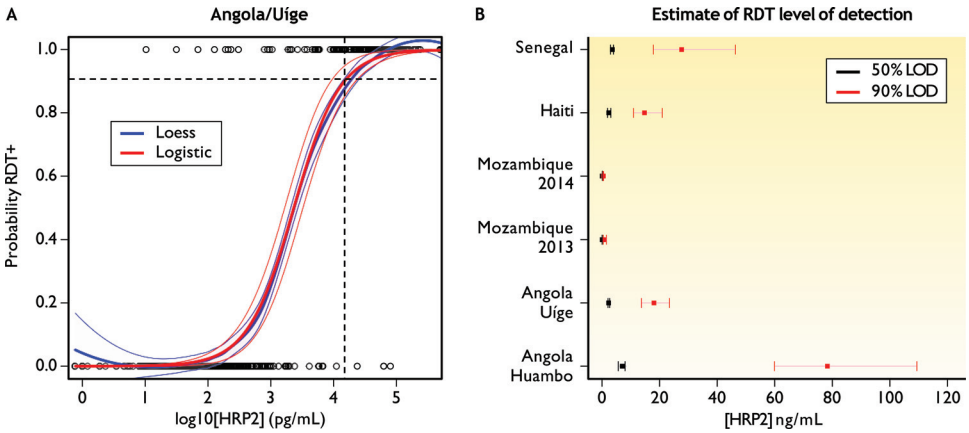


**FIG 3** Regression modeling for estimating the limit of detection of an antigen-based RDT used in a study. (A) Examples of logistic (parametric) (in red) and LOESS (nonparametric) (in blue) regression fitted to the binary RDT results by continuous HRP2 concentration (along the *x* axis; expressed in picograms per milliliter of blood), with dashed lines indicating the HRP2 concentration that provided positive tests 90% of the time in that particular survey. Investigators could choose any stringency level to their satisfaction (75%, 95%, etc.) to estimate reliable RDT detection of HRP2 from a particular survey. (Panel A adapted from reference 94.) (B) Point estimates and 95% confidence intervals for six separate surveys that utilized HRP2-based RDTs. Shown are estimates of the limits of detection of the RDTs for the HRP2 antigen where 50% and 90% of tests were positive in the study population, with the RDT result plotted as a function of the HRP2 concentration measured in the laboratory. One could easily perform such modeling for other RDT targets as well. (Data used from reference 94 with permission.)

***Post hoc* laboratory antigen measurement from filter paper samples.** The practicality of filter paper samples collected following the administration of RDTs during field surveys, combined with advances in laboratory detection of antigen, points to new approaches for external validation of field RDT results. Collecting dried blood spots on filter paper during surveys already administering RDTs (or preparing blood slides) is a straightforward additional task for field staff. Furthermore, this process does not incur additional costs beyond the (relatively low) cost of the filter paper, typically does not require repricking participants, and is becoming the standard for high-quality surveys. Comparison of antigen presence and concentration measured in the laboratory from these filter paper samples with RDT results can validate field RDT results retrospectively (94, 95, 100, 110). This process could also be performed with liquid blood samples, but practical limitations would make this a more cumbersome process for sample handling, storage, and transport. Since laboratory antigen detection methods provide a sensitive and appropriate gold-standard measure of antigen presence, the sensitivity and specificity of field RDT results can be directly estimated (98, 100, 111). Moreover, the quantitative antigen concentration measured in the laboratory can be compared to the dichotomous RDT result to estimate the empirical dose-response relationship (94) (Fig. 3). Fitting nonparametric (e.g., locally estimated scatterplot smoothing [LOESS]) or parametric (e.g., logistic) models can provide estimates of the LOD at any required sensitivity. In comparing the results from a binary test (RDT) to a quantitative continuous value (antigen concentration), dose-response modeling is limited in terms of appropriate regression models to employ. The nonparametric LOESS approach (94) fits polynomial equations to subsets of the data centered at each data point to create a smoothed visualization of the underlying trend. Since no comprehensive regression function yielding a mathematical formula is produced by this approach, LOESS provides a more appropriate type of regression for visual inspection and identification if the data are subject to influential deviation at any point along a range of antigen concentrations. The parametric logistic regression model (45, 94, 111) will force-fit a sigmoidal dose-response curve to a set of RDT binary results as a function of antigen concentration. As outlined in Fig. 3, the regression equation modeled to the relationship between these two tests yields a parameter output(s) that can be translated to

estimate RDT performance at different measured HRP2 concentrations in the study population. The utilization of this modeling scheme can allow the comparison of outputs among study sites, RDT manufacturers, and different years or transmission seasons (94). Overlap between the nonparametric and parametric models suggests that there is good convergence between the two modeling strategies and that the logistic model is well specified and provides a good approximation of the underlying true dose-response relationship for the RDT used in that particular study.

**Parametric modeling to estimate the RDT limit of detection during the field survey.** Estimation of these LODs has revealed substantial variability by RDT brand, setting, and type of study (94). The differences in LOD are likely due to a combination of internal differences in the tests themselves, such as differences in manufacturer (antibodies used and manufacturing quality) or storage conditions; host factors, such as the level of antibodies (host antibodies blocking the binding of diagnostic antibodies); parasite factors, such as differences in antigen structure or production among strains; and external factors, such as lighting conditions (112) or operator characteristics, such as visual acuity and general competency. The finding of differences in LODs sheds light on the risk of bias in comparisons of RDT results from surveys across time or space, since even the same brand can have different sensitivities in different settings in the same survey. Ultimately, the root cause is the "off-label" use of RDTs for nonclinical survey estimates, although RDTs were developed and calibrated to detect a minimum level of clinically relevant infections and have no defined lower LOD (3) (who.int/malaria/publications/rdt-manual/en/). The ability to measure antigen concentrations in a high-throughput manner in the laboratory presents an opportunity to externally validate RDT results from the field. The only requirement is the collection of a participant blood specimen directly following administration of the RDT. Comparison of laboratory-measured antigen concentrations and field RDT results provides a systematic method of validating field RDT results and can inform the comparison of those results with RDT results from other surveys. Since validation of microscopy slides is typically done on a subset of slides, even analysis of a representative subsample of survey specimens would be able to provide information on the validity of the RDT results for an entire survey.

## Methodology for Mass Screening

Laboratory antigen detection has a role to play in detecting a growing cause of false-negative RDT results: deletions in the *pfhrp2/3* genes. *P. falciparum* parasites with one or both genes deleted are less likely to be detected by HRP2-based RDTs (101, 113), and their emergence and spread pose a threat to recent progress in improving malaria case management. Confirmation of *pfhrp2/3* deletions requires molecular evidence of gene deletion, but differences in the sensitivities of these assays mean that molecular confirmation of deletions is subject to a high risk of bias (113–115). Due to these inherent complexities and the expense of molecular assays, *pfhrp2/3* molecular assays should preferably be performed on samples with phenotypic evidence of deletions, such as aberrant levels of the HRP2/3 antigens. In the WHO *pfhrp2/3* surveillance guidance (who.int/malaria/publications/atoz/information-note-hrp2-based-rdt/en/), evidence for suspected deletions is either a positive microscopy result with a negative HRP2-based RDT result or a positive non-HRP2-based (i.e., *Pf*LDH) RDT result with a negative HRP2-based RDT band. However, the more-sensitive laboratory assays allow alternative ways of determining the antigen expression phenotype. Detection of a non-HRP2 *P. falciparum* antigen(s) and HRP2 allows systematic, reproducible, and high-throughput identification of discordant samples for further downstream analysis. As shown in Fig. 4, laboratory antigen assays can quickly screen and triage a set of specimens into malaria-negative, HRP2-positive, and antigen-discordant (negative for HRP2 but positive for another *Plasmodium* antigen) categories. The last category of samples could then be prioritized for assaying using molecular methods to identify species and investigate *pfhrp2/3* deletions from the sample set. This algorithm ensures that samples that are molecularly typed meet strict criteria for potential deletion

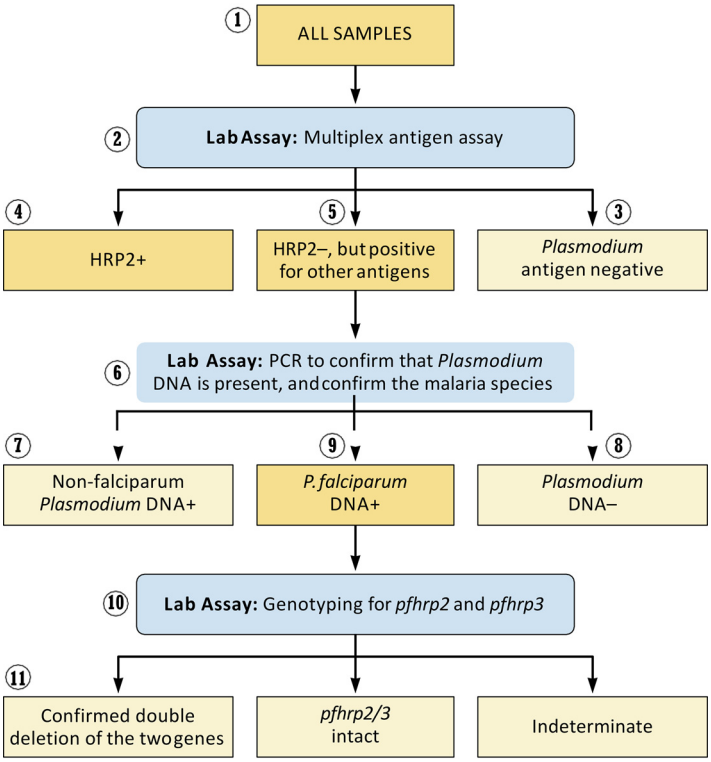


**FIG 4** Methodology for multiplex-based antigen screening of sample sets to identify specimens needing further molecular characterization. The entire set of specimens (1), ideally from a representative community- or health facility-based survey, is assayed by multiplex antigen detection assay in the laboratory (2). This multiplex assay will include, at a minimum, detection of HRP2 and one pan-*Plasmodium* antigen as targets but could also include other targets for further categorization. Samples negative for all antigens are classified as likely malaria negative (3); samples positive for HRP2 are classified as indicating active or recent *P. falciparum* infection (4); and samples without HRP2 but positive for pan-*Plasmodium* antigen are selected as a priority group for further molecular tests (5). DNA is extracted from these selected samples and is assayed by malaria species-specific PCR (6). Samples positive for non-falciparum *Plasmodium* DNA are considered to indicate non-falciparum malaria infections (7), and samples negative for any parasite DNA are considered to indicate very-low-density parasitemia or lingering antigenemia (8). Confirmed *P. falciparum* DNA specimens without HRP2 (9) then undergo genotyping for the *pfhrp2* and *pfhrp3* genes (10). Three possible results of genotyping are displayed in section 11: confirmed deletions, intact genes, and indeterminate results (inability to amplify other single-copy-number genes [133]).

and reduces the number of samples and the cost of deletion surveillance. This screening algorithm has been used for health care facility survey samples from Angola and Mozambique to show strong evidence for a lack of HRP2 deletions and low rates of non-falciparum *Plasmodium* monoinfections (45, 63). Specimens collected at health care facilities during routine care are particularly informative for these purposes, since they provide a representative sample of parasites in symptomatic patients seeking care, the parasite population most relevant for the evaluation of RDT performance.

### Characterizing Population Antigen Expression To Guide RDT Choice

The approach to antigen screening outlined above can address a current weakness in malaria control guidance—a limited evidence base for data-driven RDT procurement by countries. Currently, guidance for countries seeking to procure RDTs for malaria diagnosis focuses primarily on identifying RDTs that meet minimum performance criteria set by WHO and have undergone evaluation under the WHO Prequalification of *In Vitro* Diagnostics program (3). However, the process of selecting RDTs does not address the larger question of which antigen or combination of antigens is the appropriate diagnostic marker for a given country. More specifically, the advisability of procuring RDTs that detect a single target, HRP2, should be considered carefully. For most countries in

sub-Saharan Africa, this issue remains largely unaddressed. Many countries procure HRP2-only RDTs based on historical, and at times incomplete, data suggesting that most malaria infections are due to *P. falciparum*. However, these assumptions about the predominance of HRP2-producing *P. falciparum* do not always reflect the growing threat of *pfhrp2/3*-deleted *P. falciparum* or evidence of higher-than-expected non-falciparum malaria prevalence uncovered by surveys using sensitive molecular tools (116–119). Moreover, as countries rely increasingly on HRP2-only RDTs, there is a risk that evidence of non-HRP2-producing parasites may be hidden by the exclusive use of HRP2-only diagnostics. Ultimately, decisions on what RDT to procure for a country should be based on comprehensive and representative data on the profile of antigens produced by *Plasmodium* parasites circulating in the countries. Systematic surveys that characterize the diversity and distribution of these profiles are necessary to build this evidence base and are now possible given the advances in laboratory-based characterization of antigen profiles from large sample sets.

## PRESENCE OF MALARIA ANTIGENS FOR EPIDEMIOLOGICAL PURPOSES

### The Value of Multiple Unique Malaria Indicators in a Population

In assessing the prevalence (or incidence) of malaria in a population, microscopy has remained the epidemiological foundation for more than 100 years (120). However, estimates have also been generated for active infection through splenomegaly, RDTs, and PCR-based tests (121–123) and for past malaria exposure through antibody detection tests (124). Although they identify "malaria" (in the broadest sense), the methods listed above identify different components of a parasite, or of a human's response to a parasite. Given the opportunity, combining different indicators of malaria infection/exposure for analyses provides a more nuanced view of malaria in a population (37, 71, 103, 108, 109, 125). However, practical and financial limitations often hinder the collection of different data types in a malaria survey, or detection of multiple malaria biomarkers is simply thought of as redundant and unnecessary. Epidemiological estimates are often based solely on one indicator as the only type of data with which to assess malaria in a population. This is further complicated by the need to distinguish infection from disease. Historically, these estimates have been done using microscopy, but more recently (especially on the African continent), many surveys have been relying on RDTs alone to detect malaria in a population (126–128). If a systematic problem were to lead to an identified failure of the only indicator available (or if the team was unaware of a failure), the population-level estimates provided would be completely inaccurate for the malaria situation in the target population. A previous analytical study found prevalence estimates generated by microscopy and RDT to be generally similar, although concordance between PCR and RDT estimates was much lower (129). Importantly, for surveys in settings of lower malaria prevalence ($<$20%), the positivity rates from an RDT and from another indicator were sometimes wildly different, by an order of magnitude or more—and occasionally, the prevalence estimates were 0% for one indicator and $>$10% for the other (129). For antigen detection in the laboratory to occur, a specimen of some type needs to be collected from participants. For participants who are already being lanced and providing a blood specimen for microscopy or RDT, blood dried on filter paper is a pragmatic sample type that has been effective in multiple previous studies (45, 56, 100, 101, 110, 130).

### Sensitive Antigen Detection To Identify More Malaria-Positive Individuals

Malaria RDTs and lab antigen assays will test for the same component of a parasite, although the lab test will allow the recognition of lower antigen concentrations (94, 95) and therefore will generally provide more positive results in a study population that has data for both tests (130). In addition to being comparable to RDT prevalence estimates, data from laboratory antigen detection have also shown typically higher malaria prevalence estimates for a study population than either microscopy or PCR (Table 2). Augmented prevalence estimates for different studies may be a factor of malaria transmission in the population but may also be a factor of the study design or

**TABLE 2** Laboratory detection of malaria antigen as an epidemiological indicator in comparison to other field and laboratory tests

| Study location (yr) | Participant enrollment | Ages included | Sample size[a] | Prevalence (%) by: | | | | Reference |
|---|---|---|---|---|---|---|---|---|
| | | | | Microscopy[b] | RDT[b] | PCR[b] | Lab antigen detection[c] | |
| Kenya (2007) | Health care facility | 5–9-yr-olds | 195 | 33.3 | –[d] | 95.9 | 33.3 | 134 |
| Senegal (2015) | Health care facility | All ages | 506 | 61.2 | 69.7 | 67.5 | 81.2 | 94 |
| Angola (2016) | Health care facility | All ages | 1,254 | – | 28.7 | – | 36.9 | 130 |
| Mozambique (2018) | Health care facility | All ages | 1,861 | – | 31.1 | – | 38.1 | 45 |
| Mozambique (2013) | Household | All ages | 1,137 | – | 55.1 | – | 62.5 | 94 |
| Mozambique (2014) | Household | All ages | 1,282 | – | 60.4 | – | 65.4 | 94 |
| Ethiopia (2015) | Household | All ages | 3,169 | 0.6 | 1.3 | 1.8 | 1.6 | 135 |
| Haiti (2015) | Household | All ages | 4,432 | – | 0.6 | – | 1.4 | 94 |
| Myanmar (2017)[e] | Household | Adults | 1,442 | 3.5 | 5.2 | 12.9 | 14.0 | 111 |
| Tanzania (2017) | Household | All ages | 7,313 | 15.8 | 33.3 | – | 32.0 | 136 |
| Haiti (2015) | School | 6–7-yr-olds | 1,231 | – | 0.6 | 0.7 | 2.1 | 125 |
| Haiti (2016) | School | 6–7-yr-olds | 1,629 | – | 1.9 | – | 1.4 | 125 |

[a]Includes the number of specimens with all tests performed for that survey.
[b]Positivity for any of the four human malaria organisms: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*.
[c]Positivity for any of the antigens assayed for in the study.
[d]–, test not used in the particular study.
[e]Only *P. falciparum* markers were assessed.

antigen variants being produced by the endemic parasites (107). Furthermore, as shown in Table 2, lab antigen detection does not always provide the highest prevalence estimates in comparison to other tests for malaria. However, prevalence estimates from some studies were found to be >2-fold higher by lab antigen detection than by microscopy or RDT. Especially as a region moves toward malaria elimination (71), more-accurate estimates of malaria prevalence in a population will provide the best data for epidemiological estimates, program planning, and resource mobilization (131).

## CONCLUDING REMARKS

As new tools continually supplement the malaria community's ability to interpret disease diagnosis, research, and epidemiology, constant reassessment will be needed to determine how novel data can be translated into real-world concepts that support disease control and prevention. Laboratory detection of malaria antigens has shown great potential to provide the appropriate "gold standard" for antigen-based field diagnostics, and further refinement is needed for systematic evaluation schemes. In addition, sensitive detection of malaria antigens in biospecimens provides yet another metric by which to estimate the prevalence and incidence of different malaria parasites in a population and to determine *pfhrp2/3* genotypes. With multiple laboratory groups working in this field to continually advance the technology and improve assays, new research questions will arise that have not been considered yet (see "Further Questions for Investigation" for a nonexhaustive list). Since detection of antigens in the laboratory allows for relatively inexpensive and high-throughput testing of samples, one area of particular importance will be overall acceptance of modifying malaria field studies to more routinely collect blood specimens for subsequent laboratory antigen detection. In addition, the implementation of laboratory antigen detection methodologies by laboratories in settings where malaria is endemic will be an important factor in determining whether the malaria community can capitalize on the possibilities opened up by the advances in technology.

### Further Questions for Investigation

What additional targets can be added to laboratory antigen assays, and how will this change the interpretation of a person's malaria antigen profile?

What are other species-specific, non-HRP2 targets for *P. falciparum* that would make the identification of *pfhrp2* and *pfhrp3* deletions (or nonexpression mutations) more sensitive?

Can data on antigen positivity and concentration accurately predict the presence and concentration of parasites or parasite nucleic acids across all settings where malaria is endemic?

Will *post hoc* laboratory detection of antigen gain wide acceptance and use for external validation of field RDT results?

Can monitoring of antigen positivity and concentration be enough to assess adequate response to antimalarial treatment, and if so, what would be the ideal timing for follow-up visits?

What does antigen carriage in the population mean for areas in the preelimination or elimination phase?

To what extent will the modification of study protocols to allow the collection of dried blood samples be accepted by malaria programs, and will this become the default practice?

What are the ideal sampling strategies for population-level antigen carriage surveys, including timing and geographic scope?

How can antigen measurements, by themselves or in conjunction with other malaria indicators, be used to refine prevalence estimates?

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