Screening for Pfhrp2/3-Deleted Plasmodium falciparum, Non-falciparum, and Low-Density Malaria Infections by a Multiplex Antigen Assay

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Background. Detection of Plasmodium antigens provides evidence of malaria infection status and is the basis for most malaria diagnosis.

Methods. We developed a sensitive bead-based multiplex assay for laboratory use, which simultaneously detects pan-Plasmodium aldolase (pAldo), pan-Plasmodium lactate dehydrogenase (pLDH), and P. falciparum histidine-rich protein 2 (PfHRP2) antigens. The assay was validated against purified recombinant antigens, monospecies malaria infections, and noninfected blood samples. To test against samples collected in an endemic setting, Angolan outpatient samples (n = 1267) were assayed.

Results. Of 466 Angolan samples positive for at least 1 antigen, the most common antigen profiles were PfHRP2+/pAldo+/pLDH+ (167, 36%), PfHRP2+/pAldo+/pLDH− (163, 35%), and PfHRP2+/pAldo+/pLDH− (129, 28%). Antigen profile was predictive of polymerase chain reaction (PCR) positivity and parasite density. Eight Angolan samples (1.7%) had no or very low PfHRP2 but were positive for 1 or both of the other antigens. PCR analysis confirmed 3 (0.6%) were positive for PfHRP2 and/or Pfhrp3.

Conclusions. These are the first reports of Pfhrp2/3 deletion mutants in Angola. High-throughput multiplex antigen detection can inexpensively screen for low-density P. falciparum, non-falciparum, and Pfhrp2/3-deleted parasites to provide population-level antigen estimates and identify specimens requiring further molecular characterization.

Keywords. malaria; antigen detection; multiplex immunoassay; Pfhrp2 deletion; non-falciparum malaria.

Detection of Plasmodium antigens in blood samples is an invaluable tool for malaria control and research. It has been employed for in vitro antimalarial drug sensitivity assays [1, 2], as a marker of clinical severity [3], a test to verify posttreatment parasite clearance [4], and, most importantly, as a field-deployable diagnostic tool for suspected malaria patients [5, 6]. The use of antigen detection for field diagnosis has greatly expanded in the past decade, with over 300 million rapid diagnostic tests (RDTs) used annually, at a cost of hundreds of millions of dollars [7]. Laboratory tests for Plasmodium antigens have also become a useful tool in malaria research, providing a sensitive measure for malaria protein detection and supplementing simple detection with the ability to quantify antigen concentrations [8]. Recently, a novel immunoassay for the Plasmodium falciparum histidine rich protein 2 (PfHRP2) has been developed. The assay utilizes a microbead capture and detection system on the Luminex platform, and has shown improvement in the limit of detection over other quantitative immunoassays [9]. As most RDTs currently detect the PfHRP2 antigen, this novel laboratory assay can compare RDT test results with actual blood PfHRP2 concentrations for the same individual—providing estimates for false positivity and false negativity RDT rates and estimating the in situ RDT limit of detection for the survey in which they were deployed [9–11].

The PfHRP2 antigen produced by P. falciparum is a unique protein in malaria biology, provides a species-specific marker for this parasite [12], and lingers in the blood circulation for weeks after successful clearance of parasites [13, 14]. The Plasmodium antigens aldolase (pAldo) and lactate dehydrogenase (pLDH) are also used for diagnostic purposes, but are thought to be cleared from the human host soon after resolution of infection [13, 15], thus potentially providing a more specific marker for active infection than PfHRP2. In addition, as antibodies recognizing pan-Plasmodium epitopes on pAldo and pLDH have been identified, detection of these 2 antigens in conjunction with PfHRP2 could allow estimation of non-falciparum malaria prevalence in coendemic areas of the world.
Studies describing concentrations of all these 3 antigens within a single infection, or at a population level, have been lacking, in part due to the requirement to perform an immunoassay 3 times to obtain 3 unique assay signals.

Recent reports have emerged of *P. falciparum* parasites that do not produce the PfHRP2 and/or PfHRP3 antigens due to complete or partial genomic deletions of the *Pfrhp2/3* gene(s) [16]. The potential for selection and spread of these parasites is a major emerging threat for future malaria diagnosis by HRP2-based RDT, as most RDTs deployed in malaria-endemic countries with predominant *P. falciparum* transmission detect only the PfHRP2 antigen. *P. falciparum* strains with partial or complete *Pfrhp2/3* deletions in natural malaria infections were first reported in South America [17], but have now been reported in South Asia [18] and multiple countries in sub-Saharan Africa [19–22]. Systematic monitoring for *Pfrhp2/3*-deleted parasites in patient specimens is currently not applied globally, and discovery of mutant parasites has typically been triggered by a discordance between positive microscopy results and negative HRP2-based RDT results in patients undergoing both tests [17].

Genomic confirmation of complete or partial deletions of the *Pfrhp2/3* gene requires time-intensive multireaction polymerase chain reaction (PCR) assays [23], and does not assay for the parasite-expressed antigen, which is the target of RDTs. Among other factors, the onerous nature of the current PCR assays hinders large-scale and timely screening of sample sets for *Pfrhp2/3* deletions. The suitability of multiantigen detection for screening large sample sets in a high-throughput manner suggests a potential application for population screening for *Pfrhp2/3* deletions in the laboratory.

Here, we describe an extension of our single-antigen bead-based immunoassay [9] to allow for multiantigen capture and detection, simultaneously assaying for the pan-*Plasmodium* antigens pAldo and pLDH, as well as *P. falciparum*-specific PfHRP2. The assay was validated using a panel of well-characterized single-species *Plasmodium* infections representing all 4 human malaria. To test its utility in an endemic setting, it was employed to assay dried blood samples from surveys of health facility patients in 2 provinces in Angola. The samples' different multiantigen profiles were used to infer the presence of non-*falciparum* malaria parasites, low-density *P. falciparum* infections, and *P. falciparum* infections not expressing the PfHRP2 antigen.

**METHODS**

**Study Design**

A multiantigen assay for the simultaneous detection of PfHRP2, pAldo, and pLDH was developed and validated with purified recombinant antigens and on a panel of 239 known monoinfections in anonymized samples from returning US resident travelers with each of the 4 species of human malaria, as previously confirmed by PCR. The signal intensity cutoff for antigen positivity by the bead assay was determined using a panel of 73 malaria-negative US blood donors without travel to malaria endemic countries. The multiantigen assay was then used to screen 1267 dried blood spot samples previously collected during a 2016 health facility survey in Huambo and Uíge Provinces in Angola [24]. A subset of samples was chosen based on multiantigen profile and further assayed by quantitative reverse transcription PCR (qRT-PCR) for ultrasensitive detection of parasite nucleic acid to characterize malaria infection status. Separately, samples with an antigen profile showing no detected PfHRP2 but positive for pAldo and/or pLDH had another blood spot eluted and assay repeated to confirm antigen detection results. DNA was then extracted from these target samples and assayed by molecular methods for the presence of *Plasmodium* DNA and to determine species. Of these, samples that were confirmed to harbor *P. falciparum* infections were then analyzed using the standard confirmatory assay for *Pfrhp2/3* gene deletions. See Supplementary Methods 1–4 for detailed sample collection and analysis methods.

**Ethics Statement**

All persons involved in the study provided informed consent before blood sample collection. For the samples from both the United States and Angola, testing was approved as research not involving identifiable human subjects by the Office of the Associate Director for Science in the Center for Global Health at the Centers for Disease Control and Prevention. For the Angola samples, collection and antigen detection was approved as part of the health facility survey by the Angolan Ministry of Health.

**Statistical Analysis**

In order to determine a signal intensity of the bead assay that would indicate a true positive signal for detecting a given antigen, 73 blood samples of US residents with no reported history of travel to malaria endemic countries (living in a malaria non-endemic setting) were tested at a 1:10 dilution. For each of the 3 antigens assayed in this sample set, the median fluorescence intensity minus background (MFI-bg) lognormal mean plus 3 standard deviations of this population was used as the positivity cutoff value to indicate which MFI-bg signal represented a true positive value when testing human blood samples [9], and these signals and estimated antigen detection limits of the assay are shown in Supplementary Table 1.

For the Angola survey, characteristics for each patient whose sample was profile positive for at least 1 antigen were compared to characteristics of patients testing negative for all 3 antigens. The proportion of patients with recorded fever or reported history of fever in the last 24 hours was compared using a X² test. Differences in mean patient age were assessed using a 2-sample Student t test. The proportion of *Pfrhp2/3*-deleted parasites was calculated as the number of samples with genomically
confirmed Pfhrp2/3 deletions divided by the total number of samples positive for any Plasmodium antigen.

The relationship between patient age and antigen concentration was modeled through locally weighted scatterplot smoothing (LOESS) regression in SAS v9.4 (Cary, NC).

RESULTS

Assay Validation

The MFI-bg assay signal titrated below 1000 pg/mL for all 3 antigens regardless of differences in the Plasmodium species isoforms of the recombinant proteins (Figure 1). Greater absolute detection capacity was shown when assaying for the pAldo and PfHRP2 antigens, with appreciable MFI-bg signals below 100 pg/mL and 10 pg/mL, respectively. For each recombinant antigen, signal cutoff values and limits of antigen detection appropriate for reporting (as described in Methods) are displayed in Supplementary Table 1.

The assay was also found to reliably detect antigen from samples from known monospecies malaria infection. For all 4 major species of human malaria, the bead assay was able to detect 1 or more expressed antigens in 98.7% (236/239) of patient samples (Figure 2A and Supplementary Figure 1). Detection of antigens in P. vivax infection appeared to be most reliable, with all 54 P. vivax-infected patient samples testing positive for either pAldo or pLDH, and 81.5% (44/54) testing positive for both. All patient samples with P. falciparum infection were positive for the PfHRP2 antigen, with most P. falciparum-infected patient samples giving a high PfHRP2 assay signal (Figure 2A).

For all 3 antigens, positive correlation was observed between antigen concentration and parasite density at the time of patient sampling with R² correlation values ranging from 0.10 to 0.40 (Figure 2B and Supplementary Table 2). Using these regression estimates, and the detection capacity of the bead assay (Supplementary Table 1), it would be predicted that assaying for Plasmodium antigens would allow identification of active infections of less than 1 parasite/μL (p/μL) blood by using detection of any of the 3 antigens. The only exception to this finding was pLDH expression in P. falciparum infections, which modeled a substantially lower intercept. For all species, the absolute quantity of pLDH detected in an individual sample was on average greater than pAldo by a factor of 2- to 10-fold, with P. vivax infections showing the highest ratio and P. falciparum the lowest (Figure 2C and Supplementary Table 2).

Screening of Angola Samples for Different Antigen Profiles

Of 1267 samples from the Angola health facility survey, 302 (24%) were found to be positive for pAldo, 171 (13%) positive for pLDH, and 459 (36%) positive for PfHRP2, with a wide range of antigen levels (Supplementary Figure 2A). For antigen-positive samples, an inverse relationship was observed between antigen concentration and increasing patient age (Supplementary Figure 3). In the same manner as the confirmed monospecies infections in Figure 2, a direct correlation was seen between antigen concentration and parasite density in the Angola samples (Supplementary Figure 4), with the performance for detection of each antigen compared with qRT-PCR as the gold standard (Supplementary Table 3). A total of 801 (63%) samples were negative for all 3 antigens (Table 1). Of the 466 samples positive for at least 1 antigen, 167 (36%) of these were positive for both. A similar number (163, 35%) were positive only for PfHRP2. The next most frequent antigen profile was PfHRP2+/pAldo+/pLDH−, observed in 129 (28%) antigen-positive patients. No samples with the PfHRP2+/pAldo+/pLDH+ profile were observed. Different combinations of the multiantigen profile were predictive of P. falciparum nucleic
acid carriage and mean parasite density as estimated by qRT-PCR (Figure 3). For samples with no antigen detected, 16% were positive for *P. falciparum* by qRT-PCR with a median parasite density amongst nucleic acid-positive samples of 0.41 p/μL (Table 1). Of samples positive only for the PfHRP2 antigen, 43% were qRT-PCR positive with a median parasite density of 2.0 p/μL. Amongst samples with the PfHRP2+/pAldo+/pLDH− profile, 96% were qRT-PCR positive, and the median parasite density in positive samples was 208 p/μL. All samples positive for all 3 antigens were qRT-PCR positive, with a median parasite density of 3104 p/μL. The prevalence of fever increased with cumulative antigen positivity, with 55%, 62%, 80%, and 88% of persons reporting fever for the 4 categories above, respectively (Table 1). Both PfHRP2+/pAldo+/pLDH+ and PfHRP2+/pAldo+/pLDH− patients were younger than patients negative for all 3 antigens, with mean ages of 9.4 and 17.3 years, respectively, compared to 23.8 years for the all-antigen–negative patients (*P* value < .01). The mean age of PfHRP2+/pAldo−/pLDH− patients (23.8 years) was not statistically different from the mean age of patients negative for all 3 antigens. The mean PfHRP2 concentration of individuals became reduced as persons were positive for fewer antigens (Supplementary Figure 2B).

**Use of Antigen Profile to Identify Non-falciparum and Pfhrp2/3 Deletions in Angola Samples**

Of 466 Angolan samples positive for any antigens, 7 (1.5%) were negative for PfHRP2, but positive for at least 1 of the other
antigens: 3 PfHRP2−/pAldo+/pLDH−, 3 PfHRP2−/pAldo+/pLDH+, and 1 PfHRP2−/pAldo−/pLDH+ (Figure 4 and Figure 5). There was 1 additional sample with an exceptionally low concentration of PfHRP2 (541 pg/mL) despite considerable concentrations of pAldo (140 609 pg/mL) and pLDH (195 205 pg/mL), and was clearly identified on scatterplots of antigen concentrations (Figure 4B). All 8 samples were pronounced outliers that did not show the characteristic relationship between PfHRP2 concentration and pAldo and pLDH concentration (Figure 4B).

### Figure 1

**Figure 3.** Relationship of survey participants’ individual antigen profile with *Plasmodium falciparum* infection status and parasite density. Persons presenting to health facilities in Uige and Huambo, Angola had antigen levels for *Plasmodium aldolase* (pAldo), *Plasmodium lactate dehydrogenase* (pLDH), and *P. falciparum* histidine-rich protein 2 (PfHRP2) estimated by the multiplex bead assay, and had *P. falciparum* parasite density estimated by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Ratios on x axis for each antigen profile category indicate persons positive for *P. falciparum* nucleic acids divided by total number tested by qRT-PCR. Horizontal lines show mean parasite density for parasite-positive persons in each antigen category.
chain reaction (PET-PCR) assays confirmed 3 of these samples to be monoinfections with \textit{Plasmodium ovale}, all 3 from Uíge Province (Table 2). The remaining 5 (1.1%) antigen-positive samples were confirmed to be \textit{P. falciparum} monoinfections and were assayed for \textit{Pfhrp2/3} deletions. One sample from Huambo was confirmed to be an infection with a \textit{P. falciparum} parasite with a deletion of the \textit{Pfhrp2} gene, but positive for the \textit{Pfhrp3} gene. In 2 samples from Uíge, the \textit{Pfhrp2} and \textit{Pfhrp3} gene targets were successfully amplified, despite the absence of detectable PfHRP2 antigen. Finally, the \textit{Pfmsp1} and \textit{Pfmsp2} single-copy genes could not be amplified in 1 sample from Uíge, and thus cannot be reported on for verification of \textit{Pfhrp2} and \textit{Pfhrp3} genotype [23]. The workflow algorithm and summary of results for the non-\textit{falciparum} and \textit{Pfhrp2/3} screening are summarized in Figure 5 and Table 2.

**DISCUSSION**

Multiplexing detection of malaria antigens by the laboratory assay reported here allows for simultaneous detection of the pan-\textit{Plasmodium} proteins aldolase and LDH, as well as the \textit{P. falciparum}-specific protein PfHRP2. Once the appropriate platform has been procured (www.luminexcorp.com), at less than $1 USD per sample for reagents and supplies to assay for malaria antigens, this test can be used to quickly and inexpensively screen large sample sets for \textit{Plasmodium} antigens to characterize the study population by antigen positivity. The assay was found to perform well with blood dried on filter paper, allowing for use of a practical sample type for collection of large numbers of field samples. Sample preparation simply includes dilution of blood sample in blocking buffer, so a single laboratory worker could process and collect data on upwards of 250 samples per day. Automated systems could increase this throughput even further.

The ability to characterize the multiantigen profiles (combinations of antigen positivity and concentrations) in individuals allows for a more nuanced characterization of malaria burden in an area. The finding that antigen profiles were also predictive of carriage and quantity of \textit{P. falciparum} nucleic acids implies that low density (and more likely asymptomatic) parasite carriage in a population could be estimated simply by categorizing the study population by multiantigen profile. As currently formatted to be used in a laboratory setting, the utility of this assay does not likely extend to clinical use, but as a tool to provide epidemiological inference about circulating malaria in a population.

The assay was developed using the bead-based Luminex system, which allows multiplexing based on the gating principles of flow cytometry and causes analyte detection to be highly specific [25]. Many potential advantages are provided by the ability to simultaneously detect several \textit{Plasmodium} antigens,
including time saved by not performing multiple single-analyte assays, and ability to use less sample to obtain the same data. Additionally, formatting antigen detection to the bead-based immunoassay system has been found to be more sensitive than enzyme-linked immunosorbent assay (ELISA) for the *P. falciparum* antigen PfHRP2 [9].

A multiplex diagnostic test strategy is similar to the multiple antigen detection approach used by many malaria rapid diagnostic tests that are deployed in areas of known multispecies endemicity [6, 15, 26]. Data on the presence and absence of multiple antigens and their concentrations provides additional resolution over single-antigen detection and provides the opportunity to classify individuals based on their antigen profiles. By the bead assay, positivity to all 3 antigens was shown to be 100% reliable in estimating current infection with *P. falciparum*, and isolates positive for all 3 antigens displayed the highest median levels of estimated parasite density by an order of magnitude. This study found samples positive for PfHRP2 and pAldo, but negative for pLDH, generally represented low-density acute *P. falciparum* infections with 96% of these persons with detectable *P. falciparum* infection at time of sampling. For the PfHRP2+/pAldo+/pLDH− antigen profile, higher mean age, lower parasite density, and lower antigen levels when compared to persons in the all-antigen–positive profile may represent adults who have sufficient acquired immunity to suppress parasite densities. Of samples only positive for PfHRP2 but negative for the other 2 antigens, 43% were *P. falciparum* nucleic acid-positive, meaning 57% of PfHRP2+ persons were not found to have any detectable *P. falciparum* nucleic acids and had potentially cleared a *P. falciparum* infection in the recent past [14].

In the Angolan setting, the rarest antigen profile was the presence of pAldo or pLDH with the complete absence or very low levels of PfHRP2. This particular profile could be explained by 2 likely scenarios: infection with a non-*falciparum* malaria or infection with a *P. falciparum* strain not producing the PfHRP2/3 antigens. Eight of these antigen profiles were found from the Angolan survey, and in looking at the clinical history of the persons providing these samples, no history of recent travel to other malaria-endemic areas was noted.

**Figure 5.** Screening algorithm for dried blood spots collected during health facility surveys in Angola, 2016. Percentages calculated using the total number of samples positive for any *Plasmodium* antigen as the denominator. *Includes 1 sample with abnormally low PfHRP2 concentration (<1000 pg/mL). **Absence of PfHRP2 protein despite successful amplification of Pfhrp2 and Pfhrp3 PCR targets. ***Does not meet World Health Organization reporting guidelines due to nonamplification of single-copy Pfmsp1 and Pfmsp2 genes. Abbreviations: pAldo, *Plasmodium* aldolase; PCR, polymerase chain reaction; PfHRP2, *Plasmodium* histidine-rich protein 2; pLDH, *Plasmodium* lactate dehydrogenase.
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Abbreviations: Ct, cycle threshold; LOD, limit of detection of bead-based assay; Neg, negative; pAldo, Plasmodium aldolase; PET-PCR, photoinduced electron transfer polymerase chain reaction; Pf, Plasmodium falciparum; PHR2, Plasmodium histidine-rich protein 2; pLDH, Plasmodium lactate dehydrogenase; Pm, P. malariae; Po, P. ovale; Pos, positive; Pv, P. vivax; RDT, rapid diagnostic test.

aDoes not meet World Health Organization reporting criteria due to nonamplification of single-copy Pfmsp1 and Pfmsp2 genes.

bAbsence of PHR2 protein despite successful amplification of Pfhrp2 and Pfhrp3 PCR targets.

cryptic absence of PHR2 protein.
samples (Table 2), 7 of these 8 individuals presented to a health facility with fever and all tested negative for an HRP2-based RDT, indicating they would not have been treated for malaria. Of these 8, we were able to confirm *P. ovale* infection in 3. This was not unexpected, as *P. ovale* infection has been confirmed in Angola [27] and in the contiguous nations of Zambia and Democratic Republic of the Congo (DRC), with population prevalence rates between 2.1% and 8.3% [28, 29]. Surveillance in nonendemic countries have traced *P. ovale curtisi* and *wallikeri* cases to migrant worker or tourist travel from Angola [30–33], and serological studies have provided evidence of substantial *P. ovale* transmission in other areas in Southern Africa [34]. However, the reliance on HRP2 RDTs for the majority of malaria diagnosis and the low sensitivity of microscopy in detecting non-*falciparum* infections and mixed infections precludes precise estimates of the rate of *P. ovale* infection in areas highly endemic for *P. falciparum*. Though this current study was not designed to estimate a point prevalence of malaria (*falciparum* or non-*falciparum*) in this region, the multiplex bead assay could be readily employed in large, population-based surveys to efficiently estimate antigen prevalence in a high-throughput manner, and specify samples requiring further molecular characterization and species identification. Of the 5 remaining samples with the PfHRP2−/pAldo+/pLDH+ antigen profile, 2 were confirmed to be mutant *P. falciparum* isolates lacking functional Pfhrp2 and/or Pfhrp3 genes. One sample did not have a sufficiently high parasite density to PCR amplify single-copy gene controls to infer presence or absence of the Pfhrp2/3 genes [23]. Interestingly, 2 additional samples did not have detectable PfHRP2 protein levels, but we were able to amplify regions from both the Pfhrp2 and Pfhrp3 genes, suggesting an unknown disruption in the pathway between the presence of the gene and the expression of the functional protein in these 2 cryptic cases.

The 2 confirmed Pfhrp2/3-deleted isolates represent the first report of Pfhrp2 and Pfhrp3 deletion in Angola. With recent reports of deletions in the south-west region of neighboring DRC [22], it may have been anticipated Pfhrp2/3 deleted parasites would be seen in Angola as well. Moreover, the identification of a parasite with a Pfhrp2 deletion but an intact Pfhrp3 gene in Uige matches the major Pfhrp2/3 deletion profile reported in DRC [22]. The 2 samples represent less than 1% of the active or recent *P. falciparum* infections detected during the survey. However, the true prevalence of Pfhrp2/3-deleted parasites could be different, because mixed infections with wild-type and Pfhrp2/3-deleted *P. falciparum* would still produce PfHRP2. This study was not designed to provide estimates for Pfhrp2/3 deletion prevalence in these study areas but suggests a low prevalence of these parasites and provides empirical evidence that HRP2-based RDTs remain a strong and appropriate diagnostic tool in this setting.

As with any immunoassay, a primary limitation to the existing capacity of this assay are the currently available antibodies that are specific for these 3 malaria antigens. Specifically, the limit of detection for the pLDH antigen in blood was seen to only be reliable at approximately 4.5 ng/mL, which would be comparable or higher than currently published reports of ELISA limits of detection [35, 36]. Additionally, use of this multiplex assay by itself to identify species of infection (or prediction of Pfhrp2/3 genotype) assumes a monospecies and monogenicome infection. Lingering PfHRP2 from a previous *P. falciparum* infection could lead to misidentifying a current non-*falciparum* infection simply because of the presence of PfHRP2, and a mixed (wild-type) *P. falciparum* infection with another *Plasmodium* species would mask the presence of the non-*falciparum* malaria, as is the case for PfHRP2-based RDTs in the field [37]. Additionally, dichotomizing the antigen signal using a threshold determined in a nonimmune population might be biased by the influence of anti-HRP2 antibodies in endemic populations [38]. In order to reduce these errors in identification, molecular characterization of samples showing any unexpected patterns of antigen expression is suggested. This multiplex assay allows high-throughput and relatively inexpensive screening for the presence and amount of *Plasmodium* antigens at an individual and population level, and allows testing at both greatly reduced cost and time compared to exclusive testing by Pfhrp2/3 PCR assays. The multiplex antigen assay can efficiently screen large sample sets by narrowing the focus of molecular testing to a small proportion of samples that deserve further characterization.

**Supplementary Data**

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

**Notes**

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**Disclaimer.** The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention (CDC) or the US President’s Malaria Initiative. Use of particular brand of instruments in this study does not mean CDC endorses such products.

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