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PARASITOLOGY

Assessing Performance of HRP2 Antigen Detection for Malaria Diagnosis in Mozambique

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ABSTRACT Rapid diagnostic tests (RDTs) that detect the *Plasmodium falciparum*-specific histidine-rich protein 2 (PfHRP2) antigen are the primary methods for malaria diagnosis in Mozambique. However, these tests do not detect infections with non-falciparum malaria or *Pfhrp2*- and *Pfhrp3*-deleted *P. falciparum* parasites. To assess the appropriateness of conventional PfHRP2-only RDTs for malaria diagnosis in Mozambique, samples collected during a health facility survey conducted in three provinces of Mozambique were screened using antigen detection methods and further characterized by molecular techniques. Samples from 1,861 outpatients of all ages and symptoms attending 117 randomly selected public health facilities in 2018 were analyzed with an ultrasensitive bead-based immunoassay for the presence of PfHRP2, pan-*Plasmodium* aldolase (pAldo), and pan-*Plasmodium* lactate dehydrogenase (pLDH). The presence of PfHRP2 in patient blood detected using the bead-based assay was compared to the results of PfHRP2-based RDTs performed during the routine health facility consult and during the survey reexamination at the exit interview. Samples with discordant antigen profiles (negative for PfHRP2 but positive for pAldo and/or pLDH) were further characterized by photoinduced electron transfer PCR (PET-PCR). Using the bead-based laboratory assay as the gold standard, the sensitivities of the conventional RDTs administered during the routine health facility consult and the exit interview were 90% and 83%, respectively, and the specificities were 91% and 97%, respectively. Of 710 samples positive for at least one antigen, 704 (99.2%) were positive for PfHRP2. Six (0.8% of total) discordant samples lacked PfHRP2 but were positive for pAldo and/or pLDH; 3 of these (0.4% of total) were *Plasmodium ovale* monoinfections or coinfections where *P. ovale* was the dominant species. The remaining 3 discordant samples were negative by PET-PCR. The sensitivity and specificity of the conventional RDTs performed in the routine health facility consults and survey exit interviews were acceptable, and there was no evidence of *Pfhrp2*- and *Pfhrp3*-deleted parasites. Monoinfections with non-falciparum malaria species comprised <1% of the total malaria infections. Nearly all malaria antigen-positive patients had detectable PfHRP2, confirming that this antigen remains an appropriate malaria diagnostic target in the surveyed provinces.

KEYWORDS HRP2 deletion, *Plasmodium ovale*, rapid diagnostic test

Diagnosis of malaria is a key step in the malaria case management pathway. Most malaria diagnoses in countries of endemicity are currently performed through the detection of malaria antigen using rapid diagnostic tests (RDTs). Most malaria RDTs rely

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on detection of the highly expressed *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2) antigen; detection antibodies to PfHRP2 also cross-react with the related *Plasmodium falciparum* antigen histidine-rich protein 3 (PfHRP3). Like most other sub-Saharan countries where *P. falciparum* is the predominant species of human malaria, Mozambique procures PfHRP2-only RDTs to provide the majority of malaria diagnostic capacity across the public health system.

Despite the prolific nature of PfHRP2 production that makes it an important diagnostic target, detection of PfHRP2 for malaria diagnosis has several potential limitations. First, the protein is unusually persistent in patients for several weeks after resolution of infection (1, 2). Second, it is specific to *P. falciparum* and is not produced by other human *Plasmodium* species. Third, unlike other diagnostic markers, it is not essential for parasite survival, and parasites can delete the gene and still reproduce and circulate (3). RDTs based on the detection of PfHRP2 only therefore do not detect non-falciparum malaria infections or *P. falciparum* infections with parasites that have deleted or mutated *Pfhrp2*- and *Pfhrp3* genes leading to nonexpression of the antigens (4). Rates of non-falciparum malaria are thought to be low in Mozambique, but there are limited data. However, serological studies have shown rates of antibody seropositivity ranging from 35% to 56% to *Plasmodium malariae* and *P. ovale* in the north of the country (5, 6). A previous study reported a single *Pfhrp2*-deleted parasite from Mozambique, found among 1,162 samples collected from a single site in the south of the country (7). Low but nonzero rates of *Pfhrp2* and/or *Pfhrp3* deletions have been reported from other sub-Saharan countries (8–11), with Eritrea being a notable outlier, reporting substantial rates (12).

In the same way that regular assessment of drug efficacy against falciparum parasites ensures that appropriate antimalarials are being utilized, periodic assessments of the field performance of RDTs might also be considered a key activity for monitoring malaria case management and informing national guidelines. In 2018, the Mozambique National Malaria Control Program (NMCP) and its partners conducted a health facility survey in three provinces to evaluate the quality of malaria case management in public health facilities (13). As part of this survey, blood samples were collected from study participants with the objectives of comparing RDT results from the survey with the concentration of *Plasmodium* antigens in individuals' blood samples and investigating presence and rates of non-falciparum and *Pfhrp2*- and *Pfhrp3*-deleted parasites in treatment-seeking persons.

MATERIALS AND METHODS

Study design. The study followed a previously described screening algorithm (14). In brief, dried blood specimens from outpatients attending health facilities in three geographically diverse provinces were assayed in the laboratory using a multiplex immunoassay for the presence of PfHRP2, pan-*Plasmodium* aldolase (pAldo), and pan-*Plasmodium* lactase dehydrogenase (pLDH). Antigen concentrations were calculated from fluorescence intensity values using standard curves. Conventional RDT results from the routine health facility consults and from retesting during the survey exit interview were compared against the PfHRP2 concentration measured in the laboratory (15). Samples with discordant antigen results (as defined by presence of pAldo and/or pLDH but no PfHRP2 antigen) and a random selection of other samples were analyzed by PCR to confirm the antigen findings.

Study population. Samples were analyzed from 1,861 patients of all ages and symptoms who were randomly selected from outpatients attending 117 health facilities in April to May 2018 (see Fig. S1 in the supplemental material) (13). The health facilities had been randomly selected from among all public health facilities in the Maputo, Zambézia, and Cabo Delgado provinces, representing the low-transmission south of the country and the high-transmission center and north of the country, respectively.

Sample collection and laboratory analysis. Study participants underwent an exit interview administered by survey staff immediately following their routine health facility consult. A subset of study participants had reported being tested by RDT during the routine health facility consult as part of routine clinical management. During exit interviews which followed the routine health facility consults, all study participants, regardless of symptoms, were also administered an RDT (SD Bioline *Pf* test; Yongin, Republic of Korea). While data on the brand of RDT used during routine health facility consult were not systematically collected, health facilities in Mozambique are supplied from the same supply chain from which the survey RDTs were sourced. Next, 1 to 2 drops of blood were collected on Whatman 903 filter paper, dried, and stored in individual plastic bags with desiccant. Samples were transported at ambient temperature to the U.S. Centers for Disease Control and Prevention (CDC) laboratories in Atlanta, GA.

TABLE 1 Performance of conventional PfHRP2-based RDTs compared to ultrasensitive laboratory confirmation of presence of PfHRP2 as assessed during health facility survey in Mozambique, 2018[a]

RDT result type	Result	PfHRP2 bead assay (gold standard) result (no.)		Sensitivity (%)	Specificity (%)
		−	+		
Exit interview[b]	−	1,024	110	83	97
	+	27	551		
Routine health facility[c]	−	279	45	90	91
	+	26	387		

[a]RDT, rapid diagnostic test; PfHRP2, *P. falciparum* histidine-rich protein 2.
[b]RDT performed by study team of all patients, regardless of symptoms, during exit interview.
[c]RDT performed by health care worker of symptomatic patients during routine consult, with result reported by patient during exit interview.

A 6-mm punch from a dried blood spot from each individual was eluted in the laboratory to a 1:20 dilution of whole blood. Using previously described methodology, the presence and concentrations of PfHRP2, pAldo, and pLDH were measured using a bead-based multiplex immunoassay (14, 16). Fluorescence intensity values were converted to antigen concentration using standard curves prepared from purified recombinant antigens, as described previously (14, 16). Samples with discordant results ($n = 6$) plus a representative convenience selection of samples with different antigen profiles ($n = 66$ [4% of total]) were assayed by genus- and species-level *Plasmodium* photoinduced electron transfer PCR (PET-PCR) (17). A threshold cycle (C_T) value of <40 was considered a positive PCR result.

Statistical analysis. The RDT results from the routine health facility consult and the exit interview were compared to the presence of PfHRP2 measured in the bead-based assay (16). Sensitivity and specificity were calculated using the bead-based result as the gold standard. A logistic model was fit to estimate the level of detection at the 50% (LOD_{50}), 75%, 90%, and 95% levels (15), by province and separately for the RDT from the routine health facility consult and the exit interview. These estimates represent the PfHRP2 concentration at which 50%, 75%, 90%, and 95% of RDTs would be positive in a given site/test combination.

Samples were categorized by antigen profile, and the fever rates, ages, and PET-PCR positivity rates were estimated for each category. Differences in fever rates and ages were assessed using a chi-square test and a *t* test, respectively. Based on previous work (14), samples positive just for PfHRP2 were categorized as representing recent or very low-density *P. falciparum* infections, samples positive for PfHRP2 and either pAldo or pLDH were classified as those indicative of low-density *P. falciparum* infections, and samples positive for all three antigens were classified as representing acute infection with *P. falciparum*. Samples with discordant antigen results (presence of pAldo and/or pLDH but no PfHRP2 antigen) were classified based on the PET-PCR results. Rates of *Pfhrp2*- and *Pfhrp3*-deleted *P. falciparum* infections and non-falciparum infections were calculated using the total number of samples positive for at least one antigen as the denominator.

All data were analyzed using R version 3.3.2 (R Foundation for Statistical Computing, Vienna, Austria).

Ethics review. Adult participants and parents or guardians of children participants provided written informed consent. The study was reviewed and approved by the Mozambique National Health Bioethics Committee (338/CNBS/17) and the Office of the Associate Director for Science in the Center for Global Health at the CDC (CGH2017-517).

RESULTS

The results of an RDT performed during exit interviews by survey investigators were available for 1,712/1,861 (92%) of patients providing samples after accounting for cross-matching of biological samples with the study database. In addition, 737/1,861 (40%) patients reported having been tested by RDT during the routine health facility consult and reported the result of that test to the surveyor. Data on antigen concentrations from the bead-based immunoassay were available for 1,861 samples. After accounting for the sample dilution, the reportable level of detection (LOD) of the bead-based assay was 569 pg/ml for PfHRP2, 475 pg/ml for pAldo, and 224,749 pg/ml for pLDH.

Using the lab-confirmed presence of HRP2 as a gold standard, the sensitivity was 83% for the RDTs administered during the exit interview and 90% for RDTs administered during the routine health facility consult (Table 1). Specificities were 97% and 91%, respectively. For all three provinces together, the estimate of the LOD_{50} was 3.6 ng/ml (2.8 to 4.7 ng/ml) for the RDT administered during the exit interview and

TABLE 2 Estimated *in situ* level of detection of PfHRP2-based RDTs assessed during health facility survey in Mozambique, 2018[a]

RDT result type by province	PfHRP2 concn (ng/ml) by LOD[b]			
	50%	75%	90%	95%
Exit interview[c]				
Maputo	21.3 (8.7–63)	78.4 (24–234)	292.6 (52–930)	708.0 (78–2,439)
Zambézia	4.2 (2.8–6.5)	25.1 (15–42)	148.7 (74–281)	497.4 (203–1,037)
Cabo Delgado	1.8 (1.3–2.6)	5.6 (3.6–8.6)	17.3 (9.2–29)	37.1 (17–66)
Total	3.6 (2.8–4.7)	15.1 (11–21)	62.2 (40–93)	163.1 (97–255)
Routine health facility[d]				
Maputo	10.4 (2.4–75)	78.4 (10–584)	583.9 (28–U)	2324.2 (45–U)
Zambézia	1.6 (0.9–3)	10.6 (5.3–21)	70.2 (27–167)	254.7 (74–689)
Cabo Delgado	1.2 (0.7–2)	5.7 (3–11)	28.0 (11–60)	82.5 (26–199)
Total	1.6 (1.1–2.3)	8.9 (5.6–14)	50.1 (27–88)	160.4 (73–312)

[a]RDT, rapid diagnostic test; PfHRP2, *P. falciparum* histidine-rich protein 2; LOD, level of detection.
[b]PfHRP2 concentrations at which 50%, 75%, 90%, and 95% of RDTs would be positive in a given site/test combination. Numbers in parentheses represent 95% confidence intervals. U, upper confidence interval undefined.
[c]RDT performed by study team of all patients, regardless of symptoms, during exit interview.
[d]RDT performed by health care worker of symptomatic patients during routine consult, with result reported by patient during exit interview.

1.6 ng/ml (1.1 to 2.3 ng/ml) for the RDT administered during the routine health facility consult (Table 2 and Fig. S2 and S3). For both, the LODs were generally lower in Zambézia and Cabo Delgado than in Maputo Province. The LOD estimates for Maputo Province were largely driven by three RDT outliers with high PfHRP2 concentrations. The exclusion of these data points brought the LOD estimates closer to the estimates from the other two provinces (Table S1).

Of the 1,861 samples analyzed using the bead-based assay, 710 (38%) were positive for at least one antigen (Fig. 1 and Table 3), and 704 (99%) of these 710 tested positive for the presence of PfHRP2. These were further divided into 244 (34%) samples positive just for PfHRP2 (recent or very low-density *P. falciparum* infections), 169 (24%) samples positive for PfHRP2 and either pAldo or pLDH (low-density *P. falciparum* infections), and 291 (41%) samples positive for all three antigens (acute infection with *P. falciparum*) (Table 3). As the number of antigens for which samples were positive increased, there were statistically significant increases in fever rates and decreases in mean participant age (Table 3).

A total of 66 representative samples with different antigen profiles were further analyzed by PET-PCR; 20 samples were negative for all three antigens, 15 samples were only positive for PfHRP2, and 31 samples were positive for at least two antigens. Of the 20 samples negative for all three antigens and tested by PET-PCR, 4 (20%) were positive by PET-PCR: 1 was positive for *P. ovale* (C_T, 37.7), and 3 were positive for *P. falciparum* (C_T values, 30.4, 38.43, and 39.8). Similarly, 40% (6/15) of the samples only positive for PfHRP2 were PET-PCR positive, all for *P. falciparum* with high C_T values (median, 36.3; range, 32.4 to 39.7). In contrast, 97% (30/31) of samples tested by PET-PCR that were positive for at least two antigens were positive for parasite nucleic acids.

Of the 6 (0.8% of samples positive for at least one antigen) discordant samples that were negative for PfHRP2 but positive for pAldo and/or pLDH, 2 (0.3%) were confirmed by PET-PCR to be *P. ovale* infections (1 from Maputo Province and 1 from Zambézia). One additional sample (0.1%) from Zambézia tested positive by PET-PCR for both *P. ovale* and *P. falciparum*; in this sample, the *P. ovale* DNA signal (C_T value, 35.9) was substantially stronger than the *P. falciparum* signal (C_T value, 39.2), and the absence of measureable PfHRP2 could be attributed to the very low-density *P. falciparum* infection. The remaining 3 discordant samples were negative by genus-level *Plasmodium* PET-PCR and possibly represented cross-reactivity, false positives on the bead-based assay, or a recently cleared malaria infection.

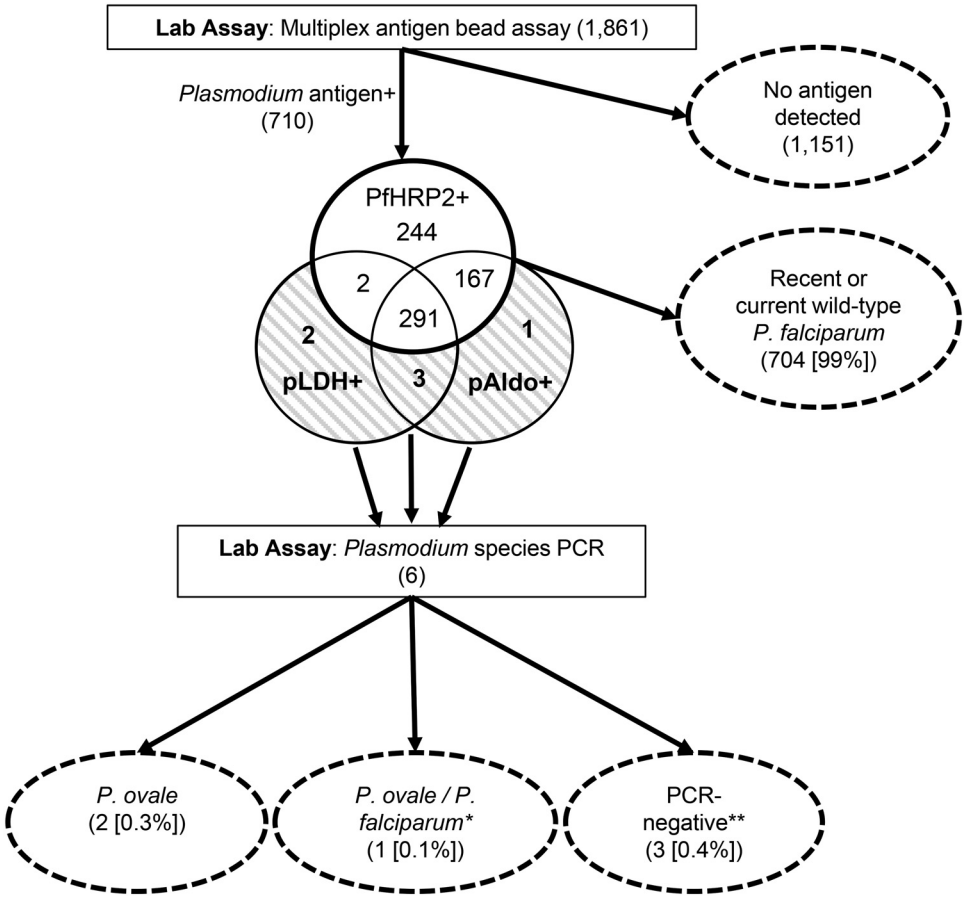

FIG 1 Results of screening algorithm for dried blood spots collected during health facility surveys in Mozambique in 2018. Percentages were calculated using the total number of samples positive for any *Plasmodium* antigen as the denominator. Dark border denotes the subset of samples with any PfHRP2 detected, and gray shading denotes the subset of samples with discordant antigen profiles (absence of PfHRP2 but presence of pLDH and/or pAldo). PfHRP2, *P. falciparum* histidine-rich protein 2; pAldo, pan-*Plasmodium* aldolase; pLDH, pan-*Plasmodium* lactate dehydrogenase. *, predominantly *P. ovale*, with *P. falciparum* positivity at a threshold of level of detection of PCR assay; **, false-positive antigen result or recent non-falciparum malaria infection.

DISCUSSION

This study confirmed good performance of conventional PfHRP2-only RDTs for laboratory confirmation of malaria infection in Mozambique, using a highly sensitive bead-based immunoassay as the gold standard. Previously, assessment of rapid diagnostic test performance in the field has been hindered by the lack of an appropriate gold standard, due to the limitations of comparing antigen detecting tests to diagnostic methods detecting presence of the parasite (microscopy) or parasite nucleic acids (PCR). In contrast, sensitive laboratory detection of parasite antigen allows for direct assessment of RDT performance.

The high sensitivity and specificity of the PfHRP2-based RDT results, together with the low proportion (<1%) of non-falciparum single-species malaria infections and lack of evidence of *Pfhrp2* gene deletions suggest that PfHRP2 remains a valuable diagnostic target antigen in Mozambique.

The rates of RDT sensitivity reported here (83% and 90%) should be interpreted taking into account the high sensitivity of the bead-based assay (16). Point-of-care tests should be able to detect clinically relevant antigenemias (18) but not necessarily all antigenemias. A recent study showed that a PfHRP2-based RDT with an LOD in the range of 3 to 10 ng/ml would provide ideal sensitivity and specificity in diagnosing malaria infections at densities high enough to be the cause of fever (19). From this

TABLE 3 Association between *Plasmodium* antigen status, fever rates, ages, and PCR positivity of 1,861 outpatients attending clinics in three provinces in Mozambique, 2018[a]

Presence of PfHRP2	Presence of pAldo	Presence of pLDH	Samples (no. [%])	Proportion with fever			Mean age (yr)	*P* value[b]	% PCR positive (no./total no.)[c]	Interpretation
				%	OR	*P* value[b]				
−	−	−	1,151 (62)	62	1.00	Ref	23.2	Ref	20 (4/20)[d]	No or very low-level malaria infection
+	−	−	244 (13)	73	1.17	<0.01	19.4	<0.01	40 (6/15)	Recent or very low-level *P. falciparum* infection
+	−	+	2 (0.1)	50			25.5		0 (0/1)	Low-level *P. falciparum* infection
+	+	−	167 (9)	92	1.48	<0.01	13.5	<0.01	100 (16/16)[e]	
+	+	+	291 (16)	96	1.55	<0.01	8.1	<0.01	100 (14/14)[f]	Acute *P. falciparum* infection
−	−	+	2 (0.1)	50			22.0		0 (0/2)	PfHRP2 and PfHRP3 mutant *P. falciparum* or non-falciparum malaria
−	+	−	1 (0.1)	100			45.0		100 (1/1)[g]	
−	+	+	3 (0.2)	50			11.6		67 (2/3)[h]	

[a]PfHRP2, *P. falciparum* histidine-rich protein 2; pAldo, pan-*Plasmodium* aldolase; pLDH, pan-*Plasmodium* lactate dehydrogenase; OR, odds ratio.
[b]Compared to reference (Ref) noninfected category (PfHRP2$^-$, pAldo$^-$, and pLDH$^-$).
[c]Positivity for *Plasmodium* of any species, all *P. falciparum* monoinfections, unless otherwise noted.
[d]Includes 1 *P. ovale* infection.
[e]Includes 1 *P. falciparum-P. ovale* coinfection.
[f]Includes 1 *P. falciparum-P. malariae* coinfection.
[g]*P. ovale-P. falciparum* coinfection.
[h]Both *P. ovale* infections.

study, the LOD_{50} estimates generally fall into this range, although the 75%, 90%, and 95% estimates exceed this range.

This study found substantial differences in LOD estimates between provinces, confirming earlier findings from other studies that showed estimated RDT LODs to be varied across settings, even within the same brand (15). Notably, while there were differences in the LODs between provinces, there generally was overlap between the LOD between the RDTs used during the routine health facility consult and those used in the exit interview. However, a comparison of the LODs and of the sensitivity and specificity estimates, is confounded by the fact that only a subset of patients (febrile persons) were tested during the routine health facility consult, whereas all patients, regardless of symptoms, were tested during the exit interview. Moreover, the possibility that the RDT brands used in the routine health consults were different from those used in the exit interviews cannot be excluded because data on RDT brand used in the routine health facility consult were not systematically collected. Future surveys of this type should systematically collect information on the RDT brand used during the original routine health facility consult. Finally, the discordance in estimates from Maputo Province and the other two provinces was largely driven by three outliers, samples from RDT-negative patients but with high PfHRP2 concentrations. Estimates of LOD are sensitive to outliers like these in settings with few RDT positives, and removal of these outliers shifted the LOD estimates for Maputo more in line with the other provinces.

The work presented here confirms the utility of the antigen screening algorithm in efficiently and accurately classifying samples in a high-throughput manner. The results mirror earlier findings showing that an antigen positivity profile can be used to infer infection status (14, 20). Individuals positive for all three antigens in almost all cases were febrile and represented active *P. falciparum* infections, whereas individuals positive for PfHRP2 and one of the other antigens likely had low-density-active *P. falciparum* infections with high C_T values. In contrast, individuals positive just for PfHRP2 were mostly PCR negative, likely representing recent *P. falciparum* infections. Compared to a previous study using the multiplex antigen test (14), PCR positivity rates by an individual's antigen profile for malaria infection were strikingly close in this report. In cases where none of the three antigens were found in the sample, 20% of samples were found to be PCR$^+$, versus 16% in the previous study. Similar percentages were also seen for PfHRP2 positivity alone (40% PCR$^+$ in this report versus 43% previously), PfHRP2 and

pAldo positivity (100% versus 96%, respectively), and positivity to all three antigens (100% versus 100%, respectively). This relationship between antigen profile and PCR positivity should be further investigated in other epidemiologic settings, particularly outside the African context, to see if antigen profile can reliably provide a strong proxy for nucleic acid positivity, allowing more efficient selection of samples for downstream molecular analyses.

While the screening algorithm can categorize infections efficiently and accurately, the approach has limitations, particularly in the context of mixed (multiple strain and/or multiple species) infections. Mixed infections with both *P. falciparum* and non-falciparum parasites would generally be expected to have a PfHRP2 signal, so rates of non-falciparum infections would be expected to be underestimated in this approach. Similarly, mixed wild-type and *Pfhrp2*- and *Pfhrp3*-deleted *P. falciparum* infections would also generally be expected to produce a PfHRP2 signal, and thus, the population prevalence of *Pfhrp2*- and *Pfhrp3*-deleted strains would be underestimated. Future studies using this screening approach should consider the collection of data on the distribution of the multiplicity of infection in the samples to better quantify the likelihood of this "masking."

The low but nonzero proportion of samples negative for all three antigens but positive by PET-PCR suggests that the antigen assay is not completely sensitive in identifying all infections and these samples could in turn also represent *Pfhrp2*- and/or *Pfhrp3*-deleted strains, although likely at very low parasite densities. In particular, the lower sensitivity of pLDH detection compared to the pAldo and pHRP2 antigens combined with lower expression of pAldo (2) means that low-level non-falciparum and *Pfhrp2*- and/or *Pfhrp3*-deleted *P. falciparum* infections might be missed by this screening approach. This is particularly important in the non-falciparum context since infections with species such as *P. malariae* and *P. ovale* are notorious for persistent but very low parasite densities in the human host (21, 22).

Despite the limited ability of the screening assay to identify mixed infections, the primary goal of RDTs is to identify clinically relevant antigen levels to enable early *P. falciparum* diagnosis and appropriate clinical treatment. For this, the results reported here show that nearly all samples with laboratory-detectable malaria antigenemia contained PfHRP2, confirming PfHRP2 to be an appropriate diagnostic target in these three provinces in Mozambique. Although the results of this report cannot necessarily be extrapolated to the rest of the country, they do provide evidence that PfHRP2-only RDTs remain an appropriate diagnostic tool for the Mozambique context.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.00875-19.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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All data are available from the authors upon request.

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M.M.P., B.C., J.C., R.Z., and E.R. designed the study. B.C., M.D., and R.Z. oversaw the collection of samples. A.L., D.N., and E.R. performed laboratory analyses. M.M.P. and E.R. analyzed the data. M.M.P., B.C., M.D., J.C., A.L., D.N., R.Z., and E.R. read and approved the final draft. All authors read the final manuscript and provided consent for publication.

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