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Improving the Specificity of *Plasmodium falciparum* Malaria Diagnosis in High-Transmission Settings with a Two-Step Rapid Diagnostic Test and Microscopy Algorithm

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ABSTRACT Poor specificity may negatively impact rapid diagnostic test (RDT)-based diagnostic strategies for malaria. We performed real-time PCR on a subset of subjects who had undergone diagnostic testing with a multiple-antigen (histidine-rich protein 2 and *pan*-lactate dehydrogenase pLDH [HRP2/pLDH]) RDT and microscopy. We determined the sensitivity and specificity of the RDT in comparison to results of PCR for the detection of *Plasmodium falciparum* malaria. We developed and evaluated a two-step algorithm utilizing the multiple-antigen RDT to screen patients, followed by confirmatory microscopy for those individuals with HRP2-positive ($HRP2^+$)/pLDH-negative ($pLDH^-$) results. In total, dried blood spots (DBS) were collected from 276 individuals. There were 124 (44.9%) individuals with an $HRP2^+/pLDH^+$ result, 94 (34.1%) with an $HRP2^+/pLDH^-$ result, and 58 (21%) with a negative RDT result. The sensitivity and specificity of the RDT compared to results with real-time PCR were 99.4% (95% confidence interval [CI], 95.9 to 100.0%) and 46.7% (95% CI, 37.7 to 55.9%), respectively. Of the 94 $HRP2^+/pLDH^-$ results, only 32 (34.0%) and 35 (37.2%) were positive by microscopy and PCR, respectively. The sensitivity and specificity of the two-step algorithm compared to results with real-time PCR were 95.5% (95% CI, 90.5 to 98.0%) and 91.0% (95% CI, 84.1 to 95.2), respectively. HRP2 antigen bands demonstrated poor specificity for the diagnosis of malaria compared to that of real-time PCR in a high-transmission setting. The most likely explanation for this finding is the persistence of HRP2 antigenemia following treatment of an acute infection. The two-step diagnostic algorithm utilizing microscopy as a confirmatory test for indeterminate $HRP2^+/pLDH^-$ results showed significantly improved specificity with little loss of sensitivity in a high-transmission setting.

KEYWORDS *Plasmodium falciparum*, antigen specificity, diagnostics, epidemiology, malaria, rapid tests

Since 2010, the World Health Organization (WHO) has recommended that all suspected malaria cases have a parasite-based diagnosis, either by microscopy or a rapid diagnostic test (RDT), prior to treatment with artemisinin-based combination therapy (ACT) (1). While light microscopy is the traditional reference standard for the diagnosis of malaria, RDTs are increasingly employed because they require minimal

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TABLE 1 Baseline demographic and clinical characteristics stratified by RDT result

Parameter[a]	Value for the parameter by RDT result				
	All	HRP2$^+$/pLDH$^+$	HRP2$^+$/pLDH$^-$	Negative	*P* value
Baseline characteristics					
No. of patients (%)	276	124 (44.9)	94 (34.1)	58 (21.0)	
Median age (yr [IQR])	13 (7–22)	12 (7–17.5)	14.5 (8–27)	18 (4–40)	0.002
Age (no. of patients [IQR])					
>15 yr	106 (39.0)	36 (29.0)	42 (44.7)	28 (51.9)	
5–15 yr	122 (48.9)	72 (58.1)	40 (42.6)	10 (18.5)	<0.001
<5 yr	44 (16.2)	16 (12.9)	12 (12.8)	16 (29.6)	
No. of male subjects (%)	114 (41.9)	62 (50.0)	33 (35.1)	19 (35.1)	0.047
Reported symptoms (no. of patients [IQR])					
Fever	241 (89.6)	119 (92.3)	84 (90.3)	38 (80.9)	0.09
Cough	132 (49.1)	54 (41.9)	55 (59.1)	23 (48.9)	0.04
Rhinorrhea	59 (21.9)	19 (14.7)	30 (32.3)	10 (21.3)	0.008
Vital signs (no. of patients [IQR])					
Febrile	35 (14.5)	26 (22.6)	7 (7.8)	2 (5.4)	0.003
Tachycardic	64 (25.5)	47 (38.8)	16 (17.2)	1 (2.7)	<0.001
Tachypneic	39 (15.4)	24 (19.7)	4 (4.4)	11 (26.8)	0.001

[a]IQR, interquartile range.

infrastructure, can be used by nonprofessional health workers, and provide an easy-to-interpret result in only a few minutes (2, 3).

Multiple studies have demonstrated the cost-effectiveness of RDT-based diagnostic strategies in all but the most extreme transmission scenarios (4–6). From a public health perspective, RDTs have the potential to improve the case management of febrile illness and slow the development of resistance to ACT by decreasing the number of inappropriate prescriptions (7, 8). Additionally, the diagnostic accuracy of RDTs should conceivably motivate recognition and treatment of alternative causes of febrile illness (9) as well as improve the quality of communicable disease surveillance (10).

The value of RDTs in practice, however, is dependent on a number of factors, including the diagnostic validity of the RDT under field conditions, which can vary by transmission setting. Modeling studies often assume that histidine-rich protein 2 (HRP2) RDTs will perform with a sensitivity and specificity of approximately 95% (5), an estimate derived from results of WHO product testing (11) and a previous meta-analysis (12). However, in routine practice, test performance may vary significantly from these assumptions. Sensitivity can be affected by low parasite density (13, 14) although the significance of any missed cases is of unclear clinical consequence outside elimination programs targeting asymptomatic parasite reservoirs. In contrast, poor specificity is more likely to negatively impact the economic and public health benefits of RDT-based diagnostic strategies. Frequent false-positive test results will lead to overtreatment and diminish the intended value of RDTs.

We conducted a large, prospective study of severe malaria in western Uganda that included multiple testing modalities for suspected malaria cases. All participants underwent testing with an RDT and microscopy, and we performed real-time PCR on a subset of subjects. In the manuscript, we report on the diagnostic validity of a multiple-antigen RDT assay compared to that of PCR and microscopy and propose a novel diagnostic algorithm, with the intent of improving the diagnostic specificity for malaria diagnosis in resource-limited, peripheral health centers with limited laboratory capacities.

RESULTS

Dried blood spots (DBS) were collected from 276 individuals. In total, there were 124 (44.9%) individuals with an HRP2-positive/*pan*-lactate dehydrogenase-positive (HRP2$^+$/pLDH$^+$) result, 94 (34.1%) with an HRP2$^+$/pLDH-negative (pLDH$^-$) result, and 58 (21%) with a negative RDT result (see Fig. S1 in the supplemental material). Demographic and clinical characteristics of the cohort are shown in Table 1. Notably, subjects with

TABLE 2 *Plasmodium* species distribution by diagnostic assay type and microscopy and PCR positive results stratified by RDT result

Test type(s) and group or result	No. (%) of positive results by:		
	RDT	Microscopy	PCR
All assays for *Plasmodium*			
Total	218 (79.0)	149 (54.0)	156 (56.5)
P. falciparum		145 (52.5)	150 (54.4)
P. malariae		1 (0.4)	0 (0)
P. ovale		2 (0.7)	2 (0.7)
P. falciparum and *P. malariae*		1 (0.4)	1 (0.4)
P. falciparum and *P. ovale*		0 (0)	3 (1.5)
RDT for *P. falciparum* malaria			
Total	218 (79.0)	146 (52.9)	154 (55.8)
$HRP2^+/pLDH^+$	124 (44.9)	114 (91.9)[a]	118 (95.2)[b]
$HRP2^+/pLDH^-$	94 (34.1)	32 (34.0)[c]	35 (37.2)[d]
Negative	58 (21.0)	0 (0.0)	1 (1.7)[e]

[a]Does not include one *P. malariae* infection.
[b]Includes three *P. falciparum-P. ovale* mixed infections.
[c]Includes one *P. falciparum-P. malariae* mixed infection; does not include two *P. ovale* infections.
[d]Includes one *P. falciparum-P. malariae* mixed infection; does not include one *P. ovale* infection.
[e]Does not include one *P. ovale* infection.

$HRP2^+/pLDH^+$ results were significantly younger, disproportionately male, and more likely to be febrile and tachycardic at presentation than other patients.

Corresponding thin and thick blood films were available for all patients. *P. falciparum* was the predominant *Plasmodium* species identified by microscopy, accounting for more than 97% of infections (Table 2). The geometric mean parasite density (GMPD) was 4,410 parasites/μl (95% confidence interval [CI], 3,120 to 6,449 parasites/μl) or approximately 0.1% (Fig. S2). The overall sensitivity of the RDT compared to that of microscopy was 100% (95% CI, 96.8 to 100.0%), while the specificity was 44.3% (95% CI, 35.7 to 53.2%) (Table 3). The sensitivity of the pLDH antigen band was 78.6% (95% CI, 70.9 to 84.8%), and the specificity was 92.4% (95% CI, 86.1 to 96.1%). The pLDH antigen

TABLE 3 Summary of test performance data for different diagnostic testing strategies for all patients and for different age categories[a]

Test strategy and age group	Sensitivity (%)	Specificity (%)	Positive LR[b]	Negative LR
HRP2/pLDH RDT				
All patients	99.4 (95.9–100)	46.7 (37.7–55.9)	1.9 (1.6–2.2)	0.01 (0.002−0.1)
>15 yr	98.1 (88.6–99.9)	50.9 (37.0–64.8)	2.0 (1.5–2.6)	0.04 (0.005–0.3)
5–15 yr	100 (94.5–100)	25.6 (13.6–42.4)	1.4 (1.1–1.6)	0
<5 yr	100 (78.1–100)	61.6 (40.7–79.0)	2.6 (1.6–4.2)	0
pLDH RDT				
All patients	76.6 (69.0–82.9)	95.1 (89.2–98.0)	15.6 (7.1–34.2)	0.3 (0.2–0.3)
>15 yr	66.0 (56.1–74.8)	98.1 (88.6–99.9)	35 (5.0–246.2)	0.4 (0.2–0.5)
5–15 yr	80.7 (70.2–88.3)	87.1 (71.8–95.2)	6.3 (2.8–14.4)	0.2 (0.1–0.5)
<5 yr	88.9 (63.9–98.1)	100 (84.0–100)		0.1 (0.03–0.4)
Expert microscopy				
All patients	92.2 (86.4–95.7)	95.9 (90.3–98.5)	22.7 (9.6–53.6)	0.08 (0.05–0.1)
>15 yr	83.0 (69.7–91.5)	96.2 (85.9–99.3)	22 (5.6–86.1)	0.2 (0.1–0.3)
5–15 yr	95.2 (87.5–98.4)	92.3 (78.0–98.0)	12.4 (4.2–36.7)	0.05 (0.02–0.1)
<5 yr	100 (78.1–100)	100 (84.0–100)		0
Two-step algorithm				
All patients	95.5 (90.5–98.0)	91.0 (84.1–95.2)	10.6 (6.0–18.6)	0.05 (0.02–0.1)
>15 yr	90.6 (78.6–96.5)	94.3 (83.4–98.5)	16.0 (5.3–48.2)	0.1 (0.04–0.2)
5–15 yr	97.5 (90.8–99.6)	84.6 (68.8–93.6)	6.3 (3.0–13.3)	0.03 (0.007–0.1)
<5 yr	100 (78.1–100)	100 (84.0–100)		0

[a]Values in parentheses are 95% confidence intervals.
[b]LR, likelihood ratio.

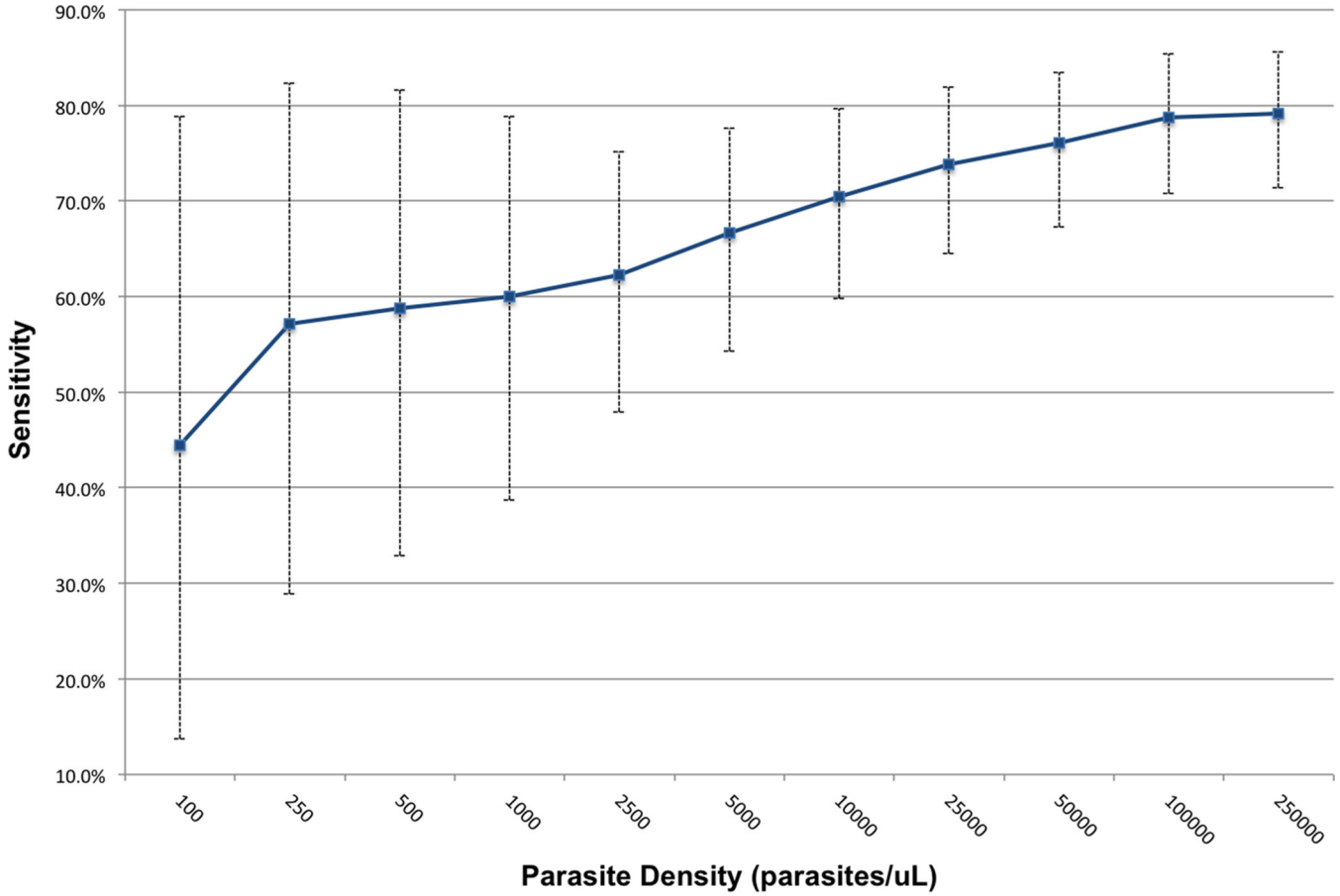

FIG 1 Graph demonstrating that the sensitivity of the pLDH antigen band increases with higher parasite density infections, plateauing near 80% when patients with parasitemia of 100,000 parasites/μl or more were considered.

band did demonstrate improved sensitivity with increasing parasite density (Fig. 1), but the sensitivity remained less than 60% below a threshold of 2,500 parasites/μl.

In the primary analysis, we found that the sensitivity and specificity of the RDT compared to results with real-time PCR were 99.4% (95% CI, 95.9 to 100.0%) and 46.7% (95% CI, 37.7 to 55.9%), respectively. The HRP2$^+$/pLDH$^-$ results were responsible for 59 of 65 (90.8%) false-positive tests (Table 2). The sensitivity of the pLDH antigen band was 76.6% (95% CI, 69.0 to 82.9%), and the specificity was 95.1% (95% CI, 89.2 to 98.0%). Notably, the pLDH band performed well in children of $<$5 years of age, where the sensitivity and specificity were 88.9% (95% CI, 63.9 to 98.1%) and 100% (95% CI, 84.0 to 100.0%), respectively (Table 3).

We estimated the test performance of a two-step diagnostic algorithm in which patients with HRP2$^+$/pLDH$^+$ results would be treated without further testing, while those with HRP2$^+$/pLDH$^-$ results were considered indeterminate and thus subject to confirmatory testing via microscopy (Fig. 2). After this algorithm was applied to our cohort, the overall sensitivity compared to that of real-time PCR was 95.5% (95% CI, 90.5 to 98.0%), and the specificity was 91.0% (95% CI, 84.1 to 95.2%). The specificity was similar and significantly better than that of the multiple-antigen RDT across each of the age strata (Table 3).

Demographic and clinical characteristics of the seven patients with a positive PCR who were not identified by the algorithm are shown in Table 4. We attempted to quantify the parasite density of these submicroscopic infections using an LDH-based quantitative PCR (qPCR) from DNA extracted from the RDT, but the level of parasitemia was below the level of detection, which is generally considered 10 parasites/μl (15). However, we were able to confirm the initial real-time PCR results using the highly

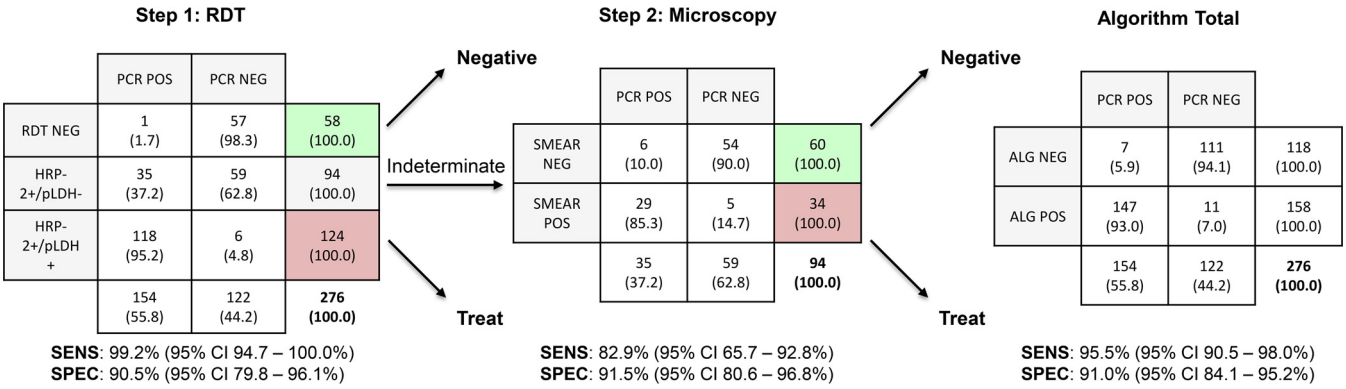

FIG 2 Test performance of proposed two-step diagnostic algorithm using HRP2/pLDH rapid diagnostic tests as the initial screen and microscopy as confirmatory testing for HRP2$^+$/pLDH$^-$ results, which show a high rate of false positivity. Sens, sensitivity; Spec, specificity.

sensitive *var* gene acidic terminal sequence (ATS) assay, results of which were again positive for *P. falciparum* DNA (Table 4). Notably, none of these individuals met the criteria for severe malaria, and five of six for whom records were available were discharged with oral antimalarial therapy.

DISCUSSION

A multiple-antigen (HRP2/pLDH) rapid diagnostic test showed high sensitivity (99.4%) but poor specificity (46.7%) for the diagnosis of malaria at a peripheral health center in western Uganda. Nearly two-thirds (59 of 94) of HRP2$^+$/pLDH$^-$ RDT results were false positives. These findings suggest that the use of HRP2-based RDTs in high-transmission settings will lead to the overestimation of malaria incidence and the inappropriate prescription of antimalarial therapies, while possibly contributing to the undertreatment of other causes of febrile illness (9).

While other studies have reported ranges of HRP2 specificity from 55% to 88% at high-transmission sites in Uganda (16–20), our results suggest that the specificity may be even lower than previously described. The most likely explanation for these findings is the persistence of HRP2 antigenemia following treatment of an acute infection. A recent study from southwestern Uganda demonstrated that the median time to a negative HRP2 RDT after treatment was between 35 and 42 days, with more than a quarter of individuals remaining positive at the end of the 6-week study period (21). Another study from eastern Uganda reported HRP2 persistence in approximately half of cases 1 month after treatment (18).

An alternative explanation, at least when microscopy is utilized as the reference standard, is the presence of submicroscopic parasitemia. For example, a study looking at the performance of RDTs across six sites in Uganda found that approximately 56% of

TABLE 4 Demographic and clinical characteristics of patients misdiagnosed by the two-step diagnostic testing algorithm

Patient age (yr)	Patient sex[a]	Diagnostic result					Patient clinical data[d]					
		RDT[b]	Microscopy	18S rRNA PCR	*var* ATS PCR[c]	C_T	Temp (°C)	HR	SBP (mm of Hg)	SpO_2 (%)	Hb (g/dl)	Lactate (mmol/liter)
14	F	HRP2$^+$/pLDH$^-$	Negative	*P. falciparum*	*P. falciparum*	35.0	37.3	**123**	104	98	12.8	0.8
15	M	HRP2$^+$/pLDH$^-$	Negative	*P. falciparum*	*P. falciparum*	36.2	**38.0**	**101**	**84**	98	12.6	1.4
20	M	HRP2$^+$/pLDH$^-$	Negative	*P. falciparum*	*P. falciparum*	37.4	36.4	67	116	**93**	14.4	1.2
21	M	HRP2$^+$/pLDH$^-$	Negative	*P. falciparum*	*P. falciparum*	40.2	36.3	78	138	98	15.2	1.5
25	F	HRP2$^+$/pLDH$^-$	Negative	*P. falciparum*	*P. falciparum*	41.9	37.4	**116**	119	96	11.9	1.9
48	F	Negative	Negative	*P. falciparum*				78	114	99		
77	F	HRP2$^+$/pLDH$^-$	Negative	*P. falciparum*	*P. falciparum*	37.6	35.9	95	141	95	14.6	1.8

[a]F, female; M, male.
[b]RDT, rapid diagnostic test.
[c]*var* ATS PCR was performed on DNA extracted from RDTs rather than DBS.
[d]C_T, threshold cycle; Temp, oral temperature; HR, heart rate in beats per min; SBP, systolic blood pressure; SpO_2, oxygen saturation as determined by pulse oximetry; Hb, hemoglobin; lactate, venous lactate. Values in bold represent abnormal findings.

HRP2-positive/pLDH-negative and microscopy-negative results were subsequently positive by PCR, suggesting submicroscopic parasitemia (16). However, in our cohort 90% (54 of 60) of these individuals were also PCR negative, which is more consistent with HRP2 persistence.

The specificity of the pLDH antigen band was significantly better than that of the HRP2 band (95.1 versus 46.7%; $P < 0.001$). However, the pLDH antigen band suffered from decreased sensitivity, especially at lower parasite densities. Given the high morbidity and mortality associated with untreated malaria, a sensitivity of 76.6% is likely insufficient for routine use. Our results, however, do suggest that pLDH-based RDTs may be a reasonable choice for children less than 5 years of age (sensitivity, 88.9%; specificity, 100%).

With two imperfect antigens, an RDT-based diagnostic strategy in a high-transmission setting represents largely a trade-off between sensitivity and specificity. A two-step diagnostic approach combining a multiple-antigen RDT and microscopy may mitigate these limitations while still taking advantage of the low cost and ease of use of the RDT. The overall sensitivity and specificity of this algorithm compared to PCR results were 95.5% and 91.0%, respectively. This estimate represents a large improvement in specificity (91.0% versus 46.6%; $P < 0.001$), with minimal loss of sensitivity (95.5% versus 99.4%; $P = 0.004$). Importantly, none of the misclassified cases showed signs or symptoms of severe malaria, and most harbored very low density infections.

One advantage of a two-step approach is that there would be little implementation cost as both RDT and microscopy are widely employed for the diagnosis of malaria. For peripheral health facilities without microscopy capabilities, the cost of such a strategy would be the additional time and resources required to implement microscopy for the subset of patients with indeterminate $HRP2^+/pLDH^-$ results. These costs would have to be weighed against the benefit of a reduction in artemisinin overtreatment. For example, among the larger RDT for Severe Malaria (RDTSM) cohort, 917 of 6,641 (13.8%) patients had an $HRP2^+/pLDH^-$ RDT result. If approximately two-thirds of these results are false positives that can be confirmed with microscopy, there is a reduction of about 600 artemisinin doses, which represents nearly a 25% decrease in the amount of antimalarial treatment given over the course of the study.

For facilities that currently utilize microscopy as the primary means of malaria diagnosis, the cost would be that of adding an HRP2/pLDH RDT screening step. Assuming that microscopy quality is reasonable, the benefit for these sites may not be a reduction in ACT overtreatment but, rather, a significant decrease in the number of smears performed, with a resulting increase in the proportion of parasitologically confirmed diagnoses and in overall laboratory throughput. For example, our previous work suggests that replacing microscopy with RDTs as the primary method of malaria diagnosis can result in a 2- to 3-fold increase in laboratory throughput, with a similar increase in the proportion of patients receiving a parasite-based diagnosis (22). Our model, however, which utilized expert microscopy with accuracy similar to that of PCR (sensitivity, 92.2% [95% CI, 86.5 to 95.7%]; specificity, 95.9% [95% CI, 90.2 to 98.5%]), may not accurately reflect real-world conditions (23, 24). Undoubtedly, with decreasing microscopy skill, especially with lower-density infections, the potential benefits could be lost. Prospective studies evaluating the accuracy and cost of this algorithm in routine practice are needed.

The two-step diagnostic algorithm combining a multiple-antigen RDT and microscopy is only suitable for high-transmission areas where *P. falciparum* is the predominant malaria species. As transmission intensity declines, there will be fewer infections, and the prevalence of posttreatment HRP2 antigenemia within the population should decline. Under these conditions, HRP2 RDTs perform with both high sensitivity and specificity, and the addition of a pLDH antigen band contributes very little to improved test performance. The proposed algorithm would also be confounded in areas where non-*P. falciparum* infections are common as the pLDH antigen band could not reliably serve as a semiquantitative marker of parasitemia.

The strengths of the study include the use of highly sensitive molecular measures as

the reference standard, the incorporation of demographic and clinical data in the analysis, and the modeling of a two-step algorithm to improve malaria diagnosis. The study also has a number of limitations. Our sample size was relatively small ($n = 276$), and the method of sample selection was purposeful rather than random, which may have introduced an element of selection bias and limits the generalizability. We are reassured that, by using data from the larger study, we did not find significant differences in baseline characteristics, including age ($P = 0.56$), sex ($P = 0.86$), and the proportion of individuals who were febrile at presentation ($P = 0.91$), between those patients who were sampled and those who were not.

Additionally, we deferred initial testing decisions to the clinical staff rather than having strict inclusion criteria. This approach better reflects current practice in Uganda but may have at times resulted in inappropriate testing decisions. We also did not confirm the presence of alternative causes of illness, such as viral or bacterial infections, which leaves us unable to discriminate clinical from subclinical malaria. We attempted to account for these limitations by using rigorous statistical methods and considering every patient with confirmed parasitemia to be a case for the purposes of diagnosis. While our results show lower specificity than other studies, the results are not far from previous estimates, and we have no reason to believe there were technical or storage issues with the RDT.

Conclusions. HRP2 antigen bands demonstrated poor specificity for the diagnosis of malaria compared to results with both microscopy and real-time PCR in a high-transmission setting. The most likely explanation for this finding is the persistence of HRP2 antigenemia following treatment of an acute infection. Potential implications include overtreatment with ACT and undertreatment of other causes of febrile illness. The two-step diagnostic algorithm utilizing microscopy as a confirmatory test for indeterminate HRP2$^+$/pLDH$^-$ results showed significantly improved specificity with little loss of sensitivity. Implementation of such an approach would require little additional investment beyond currently employed resources. Prospective studies evaluating this algorithm in routine practice, however, are needed.

MATERIALS AND METHODS

Study setting. The Bugoye Level III Health Center (BHC) in the Kasese District of western Uganda (0°18′N, 30°5′E) functions as the referral center for the Bugoye subcounty, serving a rural population of approximately 50,000 residents. Clinical officers, nurses, midwives, and laboratory technicians employed by the Ugandan Ministry of Health staff the health center, and care is provided at no cost to patients. Malaria RDTs were first introduced at BHC in 2011 (22). The climate in Bugoye permits year-round malaria transmission marked by semiannual transmission peaks, typically following the end of the rainy seasons. The two most recent malaria indicator surveys undertaken in the region found parasite rates of 48.4% in 2009 and 17.6% in 2014 (25, 26), while our previous facility-based work has found test positivity rates that can reach upwards of 60% (27).

Study design. The Rapid Diagnostic Tests for Severe Malaria (RDTSM) study was a prospective, observational cohort study of patients presenting to a rural health center with fever or other symptoms suspicious for malaria as determined by the clinical staff. The objective of the study was to assess the accuracy of a multiple-antigen RDT compared to WHO-defined clinical and laboratory criteria to identify patients with severe malaria (28, 29).

Initial testing for malaria was performed using the Standard Diagnostics 05FK60 Malaria Ag P.f/Pan assay (Standard Diagnostics, Hagal-Dong, South Korea). The RDT is a validated, antigen detection test with three individual bands signifying the control, the histidine-rich protein 2 (HRP2), and the *pan*-lactate dehydrogenase (pLDH) antigens (30–32). The monoclonal HRP2 antibody coated on the RDT membrane is specific to *P. falciparum*, while the pLDH antibody will complex with LDH antigen of *P. falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium vivax*.

The presence of a single HRP2 line (here referred to as a HRP2$^+$/pLDH$^-$ result) is intended to denote infection with *P. falciparum*, whereas a single pLDH line (HRP2$^-$/pLDH$^+$) is designed to indicate infection with one or more of the other *Plasmodium* species. The presence of a positive HRP2 line together with a pLDH line (HRP2$^+$/pLDH$^+$) indicates either *P. falciparum* infection or a mixed-species infection. All RDTs were obtained directly from the manufacturer, stored in the original packaging at ambient temperature, and utilized prior to the expiry date. RDTs were performed in accordance with the manufacturer's instructions.

Study staff prepared thin and thick blood smears for all individuals with a positive RDT result and approximately 15% of individuals with a negative RDT result. Smears were fixed with methanol and packaged with silica gel prior to transportation and stained in 10% Giemsa at the Epicentre Mbarara Research Centre. Slides were reviewed by experienced microscopists, who were blinded to the field

results. Asexual parasitemia of any level was reported as a positive smear. We examined 200 oil immersion fields with a 100× objective prior to reporting a negative result, following WHO/Tropical Disease Research (TDR) guidelines (33). Two independent microscopists read all slides. A third, senior microscopist resolved discrepancies between the first two reads.

As a substudy nested within RDTSM, we performed real-time PCR on approximately 5% of study participants in order to estimate the accuracy of slide reading. From 11 August to 10 September 2015, we collected dried blood spots (DBS) (Whatman, Chicago, IL) from study participants. DBS were generated by blotting 50 μl of blood on the filter paper and allowing it to dry at room temperature. DBS were individually packaged with desiccant and stored at room temperature until extraction. Sampling was based on RDT result to ensure a distribution of RDT results, with a goal of obtaining approximately twice the number of HRP2$^+$/pLDH$^+$ and HRP2$^+$/pLDH$^-$ results as negative results.

Molecular analysis of the DBS was completed at the Epicentre Mbarara Research Centre. DNA extraction was performed using a QIAamp DNA minikit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Real-time PCR primer and probe sequences detecting small-subunit 18S rRNA genes were selected from previously a published protocol using a Rotor-Gene Q cycler (Qiagen, Hilden, Germany) although we modified this protocol for DBS (34). *Plasmodium* species identification was accomplished by analysis of high-resolution melting curves and comparison of the thermal profile to that of positive controls (35).

As confirmation of the 18S rRNA PCR results for the individuals who were PCR positive but were not identified by the proposed diagnostic testing algorithm, we performed DNA extraction from the stored RDT using a previously described protocol (36). After confirmation of successful extraction via Qubit fluorescence (Thermo Fisher Scientific, Waltham, MA, USA), quantitative PCR was attempted to detect the *P. falciparum* LDH (PfLDH) loci (37). Upon amplification of positive controls but failure of experimental samples, the protocol was adjusted to accommodate an increase from the minimum 1 μl of DNA to a maximum of 5 μl. Following a second amplification failure, we changed assays to the ultrasensitive protocol targeting the *var* ATS sequence for qualitative detection to confirm the presence of *P. falciparum* DNA (38).

Statistical analysis. Data were entered into Microsoft Excel (Redmond, WA, USA) and analyzed with Stata, version 12.1 (College Station, TX, USA). We summarized patient characteristics and compared them between those with negative, HRP2$^+$/pLDH$^-$, and HRP2$^+$/pLDH$^+$ RDT results using linear regression for continuous variables and Pearson's chi-square testing for categorical variables. Parasite densities were log transformed and reported as geometric means. We graphically depicted the relationship between parasite density and age using local polynomial regression (LOESS) (39).

For our primary analysis, we determined the sensitivity and specificity of the multiple-antigen RDT in comparison to results with real-time PCR for the detection of *P. falciparum* malaria. Both HRP2$^+$/pLDH$^-$ and HRP2$^+$/pLDH$^+$ RDT results were considered positive in the primary analysis. We repeated this analysis using expert microscopy as the reference standard in order to evaluate the relationship between RDT performance and parasite density. Additionally, we examined the sensitivity and specificity among different age groups. As a secondary analysis, we examined the independent performance of the pLDH antigen band by considering HRP2$^+$/pLDH$^-$ RDT results as negatives while still using real-time PCR as the reference standard.

In a *post hoc* analysis attempting improve diagnostic specificity, we developed and tested a two-step algorithm utilizing the multiple-antigen RDT to screen patients, followed by confirmatory microscopy for those individuals with HRP2$^+$/pLDH$^-$ results. We estimated the sensitivity and specificity of this algorithm and described the demographic, laboratory, and clinical information for the patients who were positive by PCR but not identified by the two-step algorithm.

Ethics statement. Ethical approval of the study was provided by the institutional review boards of Partners Healthcare, the University of North Carolina at Chapel Hill, and the Mbarara University of Science and Technology. Informed consent was obtained from all study participants.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.00130-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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