

## COMPARISON OF HRP2- AND pLDH-BASED RAPID DIAGNOSTIC TESTS FOR MALARIA WITH LONGITUDINAL FOLLOW-UP IN KAMPALA, UGANDA

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**Abstract.** Presumptive treatment of malaria results in significant overuse of antimalarials. Malaria rapid diagnostic tests (RDTs) may offer a reliable alternative for case management, but the optimal RDT strategy is uncertain. We compared the diagnostic accuracy of histidine-rich protein 2 (HRP2)- and plasmodium lactate dehydrogenase (pLDH)-based RDTs, using expert microscopy as the gold standard, in a longitudinal study of 918 fever episodes over an 8-month period in a cohort of children in Kampala, Uganda. Sensitivity was 92% for HRP2 and 85% for pLDH, with differences primarily due to better detection with HRP2 at low parasite densities. Specificity was 93% for HRP2 and 100% for pLDH, with differences primarily due to rapid clearance of pLDH antigenemia after treatment of a previous malaria episode. RDTs may provide an effective strategy for improving rational delivery of antimalarial therapy; in Kampala, either test could dramatically decrease inappropriate presumptive treatments.

### INTRODUCTION

Diagnostic capabilities are limited in Africa, and in most cases fevers are treated presumptively as malaria without laboratory-confirmed diagnosis. In many settings, presumptive treatment of all fevers as malaria results in extensive overuse of antimalarials and delays the diagnosis of other causes of fever.<sup>1–4</sup> With older antimalarial drugs, which were inexpensive, safe, and widely available, the potential benefits of early treatment of all fevers supported presumptive antimalarial therapy. However, in the era of increasing drug resistance, new combination therapies are being deployed that are much more expensive and have less established safety records.<sup>5,6</sup> In this setting, improved ability to diagnose malaria may prevent many unnecessary antimalarial treatments and should also allow prompt attention to other causes of fever when malaria is ruled out. Light microscopy, for decades the gold standard for malaria diagnosis, remains unavailable to most patients in Africa.<sup>7,8</sup> Malaria rapid diagnostic tests (RDTs), newer diagnostic modalities that identify circulating antigens of malaria parasites, may offer a reliable alternative for case management.

The most studied malaria RDTs offer simple identification of two parasite antigens: histidine-rich protein 2 (HRP2) and plasmodium lactate dehydrogenase (pLDH). HRP2 was the first antigen targeted by an RDT,<sup>9</sup> has been available in various commercial formats for several years, has shown good sensitivity in a variety of field settings, and is increasingly advocated as a diagnostic test where reliable microscopy is not available. A potential problem for HRP2-based assays is persistence of detectable circulating antigen for up to several weeks after parasites have been eradicated.<sup>10–12</sup> Persistent HRP2 antigenemia has not correlated with treatment failure, suggesting that this finding is not representative of persistent infection.<sup>10,12</sup> Persistent antigenemia thus may limit the usefulness of HRP2-based assays in areas of intense malaria transmission, where positive tests may commonly be due to

prior infections that are no longer clinically relevant. pLDH-based RDTs appear to be slightly less sensitive than those detecting HRP2, but the antigen is rapidly cleared from the bloodstream, becoming undetectable at about the same time blood smears become negative after antimalarial therapy.<sup>13–15</sup> Thus, if sensitivity is adequate, the increased specificity of pLDH-based assays for acute malaria suggests that they may be better-suited for high-transmission areas, such as much of sub-Saharan Africa. With increasing advocacy for the implementation of RDTs, it is critical that optimal diagnostic strategies are identified. The true impact of the varied sensitivity and specificity of different tests is best compared with long-term follow-up to consider the impacts of prior infections and persistent antigenemia on test results. For this reason, we compared the diagnostic accuracy of HRP2- and pLDH-based RDTs, using expert microscopy as the gold standard, in a longitudinal cohort of children in Kampala, Uganda.

### METHODS

**Study population and longitudinal drug-efficacy trial.** We evaluated two RDTs in a cohort of 601 children enrolled in an on-going longitudinal antimalarial treatment efficacy trial in Kampala. The trial began in November 2004, and is based at Mulago Hospital, Uganda's main public hospital. Participating children are residents of Mulago III parish, located within 2 km of Mulago Hospital. Households were randomly selected for enrollment into the trial after a census of the parish.<sup>16</sup> Children in the study cohort receive all their medical care free of charge at our study clinic. Participants are encouraged to come to the clinic promptly for any illness and to avoid any medications not administered by study clinic staff. Participants are seen at least monthly, either at the study clinic for evaluation of illness or for routine follow-up visits, or during home visits. Each time a participant presents to the study clinic with fever (documented tympanic temperature  $\geq 38^{\circ}\text{C}$  or history of fever within the previous 24 hours), a fingerprick blood sample is obtained for thick and thin smears and storage on filter paper. If the blood smear is positive, the child is treated with antimalarials and followed for 28 days; if the smear is negative, the child does not receive antimalarials and is treated according to standard clinical algorithms and

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the study physician's judgment. Parents/guardians gave informed consent for all study procedures, and the study was approved by the Uganda National Council of Science & Technology and by the institutional review boards of Makerere University and the University of California, San Francisco.

**RDT study methods.** At the time of the RDT evaluation, children in the cohort ranged in age from 1.5 to 11.5 years. From October 2005 to May 2006, each time a blood smear was done to evaluate fever in a study participant, except when the fever occurred within 3 days of a confirmed episode of malaria, a fingerprick blood sample was obtained for the two RDTs in addition to thick and thin smears and storage on filter paper (Figure 1). If a participant presented with repeated episodes of fever after diagnosis of a non-malarial illness, the RDT was repeated at the study physician's discretion. Clinical management was guided by microscopy results; RDT results did not influence patient care.

Thick and thin smears were stained with 2% Giemsa for 30 minutes and read by experienced laboratory technologists. Parasite densities were calculated from thick smears by counting the number of asexual parasites per 200 leukocytes (or per 500 leukocytes, if the count was < 10 asexual parasites/200 leukocytes), assuming a leukocyte count of 8,000/ $\mu$ L. Smears were considered negative if the examination of 100 high-power fields did not reveal asexual parasites. Gametocytemia was determined from thick smears and parasite species from thin smears. All smears were read a second time by study laboratory staff to confirm results, and discrepant readings

were resolved by a third reader. If the first and second readers both reported a positive smear, but the second density report differed from the first by  $\geq 2000/\mu$ L, the final density recorded was that of the third reader.

RDTs were selected for evaluation on the basis of ease of use (relatively few preparation steps and clear distinction between positive and negative results), safety (minimal exposure to blood during test preparation), completeness of packaging and labeling, appropriate packaging for transport and storage in tropical environments (each test individually wrapped in foil with plastic liner and desiccant), low market price, and reliability of supply. The RDTs studied were Paracheck (detection of HRP2, Orchid Biomedical Systems, Goa, India) and Parabank (detection of pLDH, Zephyr Biomedicals, Goa, India). RDTs were obtained directly from the manufacturers and stored in their original packaging at room temperature in the study clinic. Temperature and humidity of the storage area were monitored, but not controlled. Over the course of the study period, the temperature in the storage area ranged from 19 to 29°C, with a mean low of 24°C and a mean high of 27°C. The relative humidity ranged from 31% to 82%, with a mean low of 53% and a mean high of 69%. Prior to the beginning of the study, positive and negative control blood samples were obtained, and stored at -80°C for quality-control testing of RDTs throughout the study. Each batch of RDTs underwent quality-control testing when opened and at 8- to 12-week intervals thereafter. The two positive control samples had densities 84/ $\mu$ L and 5000/ $\mu$ L, respectively. All

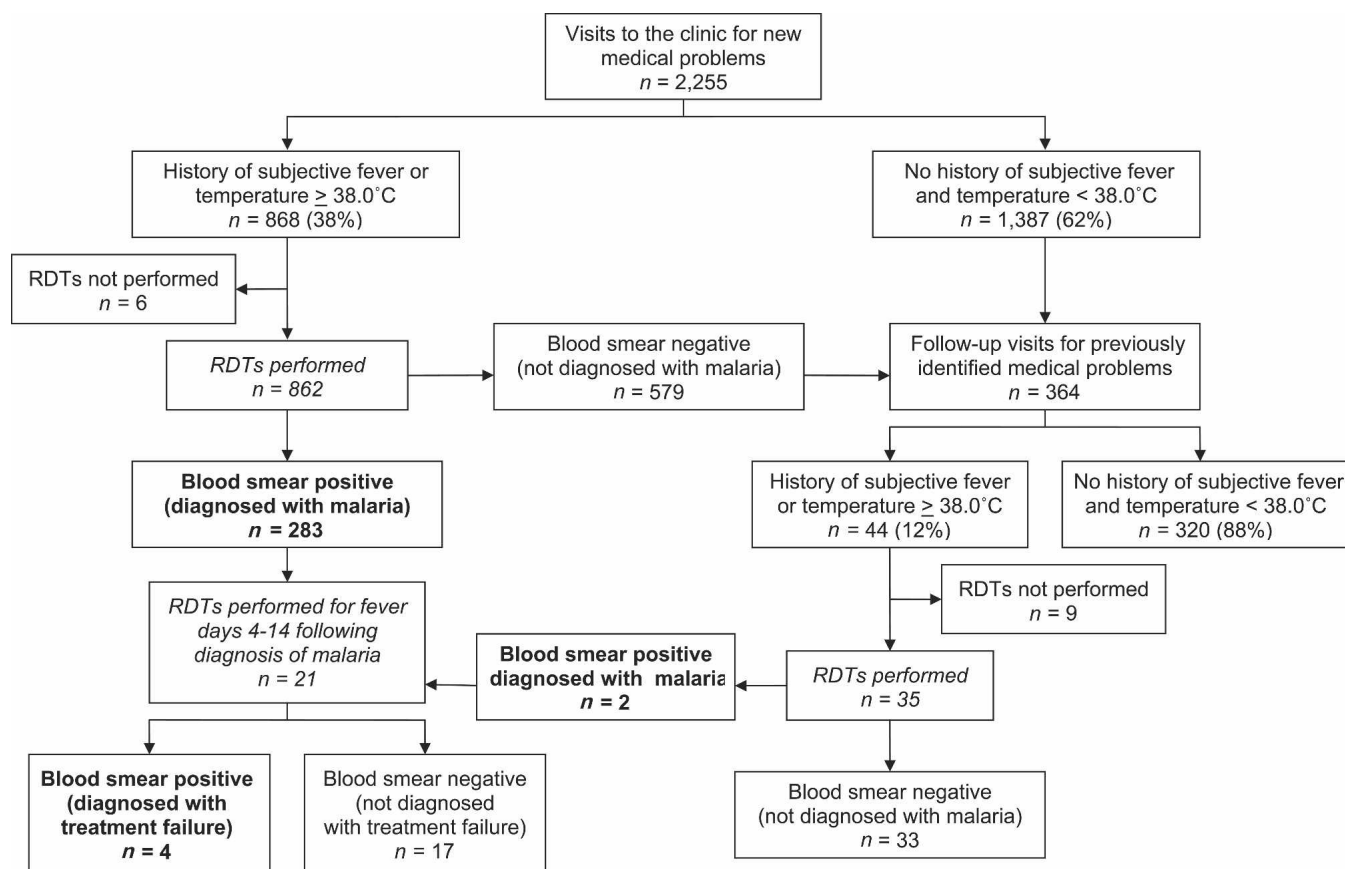


FIGURE 1. Trial profile showing clinic visits, blood smear results, rapid diagnostic tests (RDTs) performed (italics), and malaria episodes (bold). At the beginning of the RDT evaluation, 565 children were enrolled in the study cohort; 524 remained enrolled at the end of the evaluation.

HRP2 RDTs tested with quality-control samples were accurate; all pLDH RDTs tested were accurate for the negative and 5000/ $\mu$ L samples, but only 2 of 8 were accurate for the 84/ $\mu$ L sample.

RDTs were prepared and read by study physicians and then read by laboratory technicians. All readers were trained to perform the tests according to manufacturers' instructions. Study physicians interpreted and recorded RDT results after 15 minutes, at which time they were unaware of blood smear results. They were advised that if the background of the RDT test window remained pink (bloody) at the end of 15 minutes, they should allow the background to clear before reading the RDT. RDTs were then carried to the adjacent study laboratory, where they were re-read by laboratory technicians who were unaware of both the physician's interpretation and the patient's microscopy result. Readers recorded RDT results as either positive or negative; they were trained to consider faint test lines as positive.

**Molecular methods.** PCR was performed to identify parasite species in samples positive by microscopy but negative by RDT, as well as to detect subpatent infections in samples negative by microscopy but positive by RDT, and in a random sample of microscopy-negative and RDT-negative samples. DNA was extracted from filter paper samples using Chelex resin<sup>17</sup> and stored at  $-20^{\circ}\text{C}$  until use. To detect *Plasmodium falciparum*, the block-3 region of merozoite surface protein-2 (msp-2) was amplified by nested PCR with primers corresponding to conserved sequences flanking this region<sup>18</sup> followed by primers to amplify the *IC3D7* and *FC27* allelic families, using conditions described previously.<sup>19</sup> In addition, to detect *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*, genus-specific followed by nested species-specific PCR of 18S small subunit ribosomal RNA<sup>20</sup> (ssu rRNA) for the four species (Malaria Research and Reference Reagent Resource Center, Manassas, VA) was performed, using oligonucleotide primers and conditions as described previously.<sup>21</sup> PCR products were analyzed by electrophoresis using 2% agarose gels.

**Statistical methods.** Data were entered using Epi-Info version 6.04 (Centers for Disease Control and Prevention, Atlanta, GA) and analyzed with Stata version 8.0 (Stata Corporation, College Station, TX). Sensitivity, specificity, and positive and negative predictive values were calculated by comparing the proportion of positive and negative results for each RDT with expert microscopy. Categorical variables were compared using  $\chi^2$  or Fisher's exact test. A *P* value of  $< 0.05$  was considered statistically significant.

## RESULTS

**Overall RDT accuracy.** We evaluated 918 episodes of fever over an 8-month interval in children from our cohort in Kampala (Figure 1). Over the 8-month period, 868 fevers were new fevers in a previously well child, 21 occurred 4–14 days after a diagnosis of malaria, and 44 occurred during follow-up of a non-malarial illness. RDTs were not performed in 15 episodes, in 9 at the discretion of the physician during follow-up of a non-malarial febrile illness, and in 6 because of protocol errors. Light microscopy identified positive malaria smears in 289 episodes (31%). Blood smear results served as the gold standard for comparison with RDT results. As RDT results are dependent on reader accuracy, we compared readings by two groups of clinic personnel: study physicians and

laboratory technicians. In both cases, the sensitivity ( $> 92\%$ ) and negative predictive value ( $> 96\%$ ) were higher for the HRP2 assay, and specificity ( $> 99\%$ ) and positive predictive value ( $> 99\%$ ) were higher for the pLDH assay (Figure 2). First readers interpreted RDT results an average of 15 minutes after preparation, and second readers interpreted results an average of 7 minutes later. First and second test readings agreed in 98% of readings; they disagreed for 16 HRP2 tests and 13 pLDH tests. For 14/16 (88%) discordant HRP2 readings and 10/13 (77%) discordant pLDH readings, only second readings were positive.

**Evaluation of false-negative results.** Possible reasons for false-negative RDT results include low parasite density, non-falciparum parasite species, and interpreting the RDT before the test line has fully developed. HRP2 is produced only by *P. falciparum* parasites, while the pLDH assay evaluated here detects antigen from all human malaria parasites, although some reports suggest pLDH may be less sensitive for non-falciparum species.<sup>22,23</sup>

Of the 22 false-negative HRP2 results (based on first reading), 15 (68%) occurred in non-falciparum infections (Figure 3). Of the remaining 7 false negatives, 5 were interpreted as positive by the second reader. The 2 remaining false negatives occurred in a *P. falciparum* mono-infection with parasite density 48/ $\mu$ L, and a *P. falciparum* and *P. vivax* mixed infection with density 680/ $\mu$ L. Considering only *P. falciparum* infections, the sensitivity of the HRP2 assay at the second reading was 99% (272/274).

Of the 43 false-negative pLDH results, 12 (28%) occurred in non-falciparum infections; the remaining 31 were all *P. falciparum* mono-infections. Of these 31 false negatives, 9 were interpreted as positive by the second reader. For the remaining 22 false negatives, the geometric mean parasite density was 352/ $\mu$ L (range 16 to 26,080/ $\mu$ L). Considering only *P. falciparum* infections, the overall sensitivity of the pLDH assay at the second reading was 91% (250/274). The sensitivity for *P. falciparum* infections decreased from 98% (217/222) to 88% (28/32) to 25% (5/20) for parasite densities  $> 5000/\mu\text{L}$ , between 1000 and 5000/ $\mu\text{L}$ , and  $\leq 1000/\mu\text{L}$ , respectively ( $P < 0.0001$ ).

**Evaluation of false-positive results.** Possible reasons for false-positive RDT results include persistent antigenemia after antimalarial treatment, detection of gametocytes when asexual forms are not present, RDT detection of low-density microscopy-negative infections, or presence of antigenemia early in infection before parasites are detected by microscopy.

Of the 42 false-positive HRP2 results, 12 (29%) occurred within 14 days of a prior diagnosis of malaria, 26 (62%) within 28 days, and 32 (76%) within 42 days. In contrast, negative HRP2 results occurred as early as 7 days after initial diagnosis of a previous episode of malaria.

Gametocytes were detected by microscopy in only 12 of the 918 cases (1.3%). No HRP2 result was positive in a case where the smear showed gametocytes but not asexual parasites.

PCR was conducted to assess whether false-positive RDT results may have been associated with subpatent parasitemia. Of 40 evaluable false-positive HRP2 results, PCR was positive for *P. falciparum* in 8 (20%), compared with PCR positivity in 5/66 (8%) of control HRP2- and microscopy-negative samples ( $P = 0.07$ ). Four of the 8 smear-negative, RDT- and

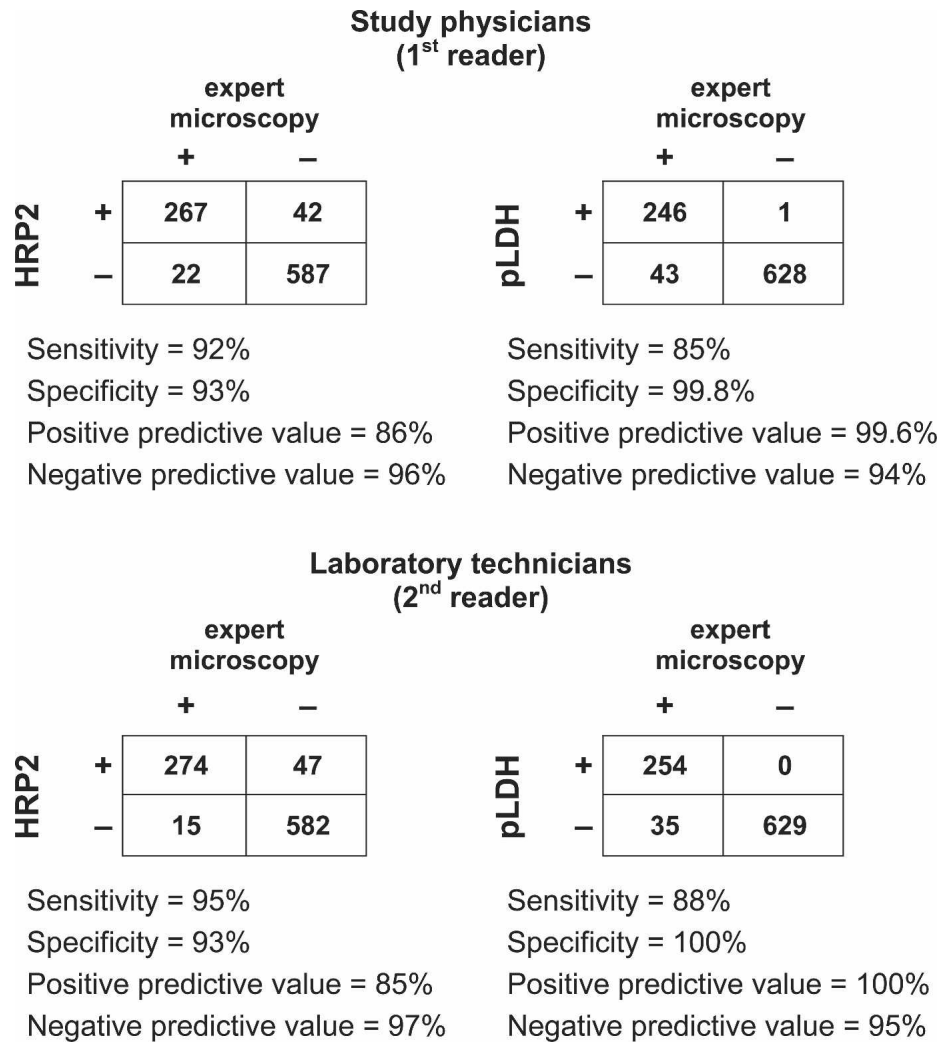


FIGURE 2. Point estimates of RDT accuracy. Blood smears were read by experienced microscopists in the study laboratory. All smears were read a second time by study laboratory staff to confirm results, and discrepant readings were resolved by a third reader. RDTs were read sequentially by study physicians and laboratory technicians, as described in Methods.

PCR-positive samples were obtained within 28 days of a prior episode of malaria.

Negative HRP2 results were recorded up to 3 days prior to an episode of malaria. Only one patient developed malaria within a week after a false-positive HRP2 result. The sample from the initial evaluation showed no asexual parasites or gametocytes but was positive for *P. falciparum* by PCR. The patient returned with persistent fever 5 days after initial evaluation, at which time the blood smear was positive, with parasite density 52,840/ $\mu$ L.

Only one pLDH test result was false-positive, in a patient who had no documented previous episode of malaria over 469 days of follow-up, and no malaria during the subsequent 2 months of study follow-up. No gametocytes were seen in the smear, the sample was negative by PCR for all four malaria species, and the second reading of the RDT was negative, strongly suggesting that this false positive was due to an error during the first test reading.

#### DISCUSSION

As compared with microscopy, both HRP2- and pLDH-based RDTs demonstrated acceptable sensitivity and speci-

ficity for the diagnosis of malaria in Kampala. The HRP2 assay showed superior sensitivity but inferior specificity compared with the pLDH assay. The longitudinal design of our study allowed us to clarify the relative importance of contributors to RDT false-negative and false-positive results. The difference in sensitivity between the tests was due mostly to better detection with HRP2 at low parasite densities. Non-falciparum infections contributed to false-negative results for both RDTs. In particular, in two-thirds of cases in which the HRP2 test was negative although microscopy detected parasites, the infection was caused by non-falciparum species. The higher specificity and positive predictive value of the pLDH assay was due to the fact that pLDH antigenemia closely mirrors parasitemia, while HRP2 commonly persists in the bloodstream weeks after successful treatment of malaria.<sup>10,12</sup> Subpatent parasitemia, as detected by PCR, pre-patent infections, and gametocytemia, did not appear to contribute importantly to false-positive results for either RDT. In summary, both studied RDTs accurately identified clinically relevant malaria infections but they differed importantly in sensitivity and specificity.

In Uganda, RDTs are increasingly available in the private

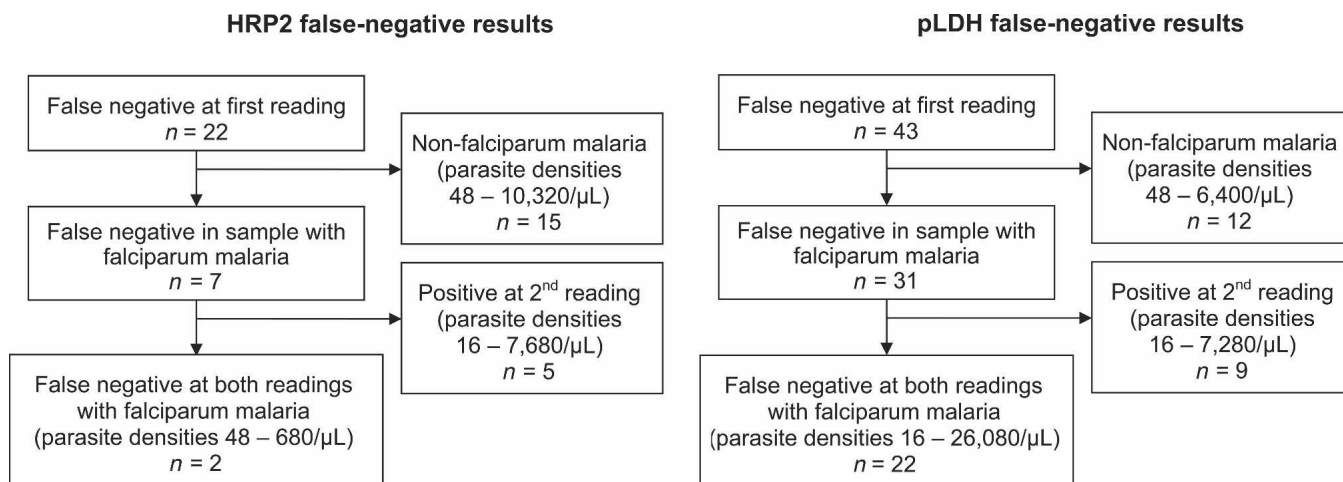


FIGURE 3. Factors associated with false-negative HRP2 and pLDH RDT results.

health care sector and are widely advocated for use in the public sector, though clear guidelines or algorithms for their use are lacking. In Kampala, both the HRP2 and pLDH tests showed a high negative predictive value and appeared to offer good reliability in ruling out malaria as the cause of a fever. Considering the potential values of RDTs, some limitations in both sensitivity and specificity may be acceptable. The lower specificity of the HRP2-based test, due to persistent antigenemia after recent infections, may lead to some inappropriate treatments, but many fewer than if all episodes of fever were treated as malaria. However, the lower specificity of HRP2 assays may be more problematic, with many more unnecessary malaria treatments, in regions with higher transmission intensity than Kampala. The lower sensitivity of the pLDH-based assay might also be a concern, but in Kampala, missed episodes were primarily of relatively low parasitemia, suggesting that, in immune populations, mostly mild or asymptomatic infections will be missed. Indeed, especially if technological innovations can improve the sensitivity of pLDH-based tests, they may well offer the optimal balance of sensitivity and specificity for the diagnosis of malaria in Africa.

To our knowledge, this study offers the first comparison of RDTs in a longitudinal format, allowing assessment of the importance of previous and future malaria infections in RDT accuracy. A number of other RDT evaluations have been conducted, though results have varied widely, likely due at least in part to different methodologies and locations. Two previous RDT studies in western Uganda compared HRP2-based tests with expert microscopy. One evaluation, using an older HRP2 assay, found a sensitivity of 99.6% for parasitemia  $> 500/\mu\text{L}$  and specificity of 92.7% in patients with fever.<sup>24</sup> The other study, using the same HRP2 test as in our evaluation, found a sensitivity of 97% and specificity of 88% for *P. falciparum* infections.<sup>25</sup> These estimates are similar to those for the HRP2-based test in our current evaluation. Our results also confirm the superior specificity of pLDH seen in a study in Tanzania,<sup>26</sup> although sensitivity of both tests was somewhat lower in our study.

Our results are not immediately applicable to fever case management across Africa. We obtained RDTs directly from manufacturers, and we used and stored kits as recommended

by manufacturers; adherence to these guidelines may be challenging in rural settings, and test quality is likely to deteriorate if kits are less well maintained.<sup>27</sup> Our evaluation was performed in an area with relatively low malaria transmission. Because of the location and design of our study, our patients likely presented to the clinic earlier in the course of malaria than in non-research settings. Our staff was carefully trained in use of the two RDTs before initiation of our study; test accuracy may be lower in field settings, although a number of reports indicate that village health workers with minimal training are able to satisfactorily prepare and interpret RDTs.<sup>28,29</sup> Considering these limitations, how should the results of this evaluation influence malaria treatment policy? For Kampala, our results suggest that, in settings without access to microscopy, use of either HRP2- or pLDH-based RDTs could dramatically lower the use of inappropriate antimalarial therapy without missing many episodes of clinical malaria. However, it will be necessary to perform similar analyses in areas with different epidemiology to determine the predictive values of different RDTs in various settings. In addition, the issue of cost and cost-effectiveness of RDTs, compared with presumptive treatment and with diagnosis with microscopy, must be considered. In the era of artemisinin combination therapies, using RDTs to target treatment to confirmed cases of malaria may help to maximize the impact of these valuable resources.

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