Using Reference Quantitative Polymerase Chain Reaction to Assess the Clinical Performance of the Paracheck-Pf® Rapid Diagnostic Test in a Field Setting in Uganda

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Abstract. Malaria rapid diagnostic tests (RDTs) are widely used in clinical and surveillance settings. However, the performance of most RDTs has not been characterized at parasite densities below detection by microscopy. We present findings from Uganda, where RDT results from 491 participants with suspected malaria were correlated with quantitative polymerase chain reaction (qPCR)-defined parasitemia. Compared with qPCR, the sensitivity and specificity of the RDT for *Plasmodium falciparum* mono-infections were 76% (95% confidence interval [CI]: 68–83%) and 95% (95% CI: 92–97%), respectively. The sensitivity of the RDT at parasite densities between 0.2 and 200 parasites/μL was surprisingly high (87%, 95% CI: 74–94%). The high sensitivity of the RDT is likely because of histidine-rich protein 2 from sub-microscopic infections, gametocytes, or sequestered parasites. These findings underscore the importance of evaluating different RDTs in field studies against qPCR reference testing to better define the sensitivity and specificity, particularly at low parasite densities.

Antigen-based malaria rapid diagnostic tests (RDTs) are widely used in both clinical and surveillance settings. The performance of RDTs is well characterized at parasite concentrations greater than 200 parasites/μL, which is the minimum parasite density used to estimate RDT performance in product testing by the World Health Organization (WHO).¹ However, the field performance of RDTs at lower parasite concentrations is poorly described. As many regions approach elimination and submicroscopic infections predominate, the ability of RDTs to detect these lower level malaria infections must be evaluated. Many field studies determine the sensitivity and specificity of RDTs relative to microscopy^{2–4} or quantitative polymerase chain reaction (qPCR)^{5–7} over wide ranges of parasitemias. However, few studies describe the ability of RDTs to detect submicroscopic infections. Recent studies have highlighted the importance of using sensitive reference tests to provide empirical data on the performance of RDTs at parasite densities below 200 parasites/μL.^{8,9} Here, we report the findings of a study in which we used qPCR to determine the malaria status of 491 patients compared with their RDT results.

Venous blood samples were collected in EDTA tubes from participants over 1 year of age with suspected uncomplicated malaria. The samples were collected as part of a larger diagnostics study conducted between February and April 2016 in Bugiri, Uganda, which is an area of high intensity transmission.¹⁰ The clinical officer identified patients with suspected malaria based on fever and/or other symptoms associated with malaria. Informed consent was obtained from adult participants and from minors' legal guardians or parents. Ethical approval was granted by the Health Research Ethics Board of the University of Alberta and the Higher Degrees, Research and Ethics Committee in Uganda.

Anticoagulated blood was used for histidine-rich protein 2 (HRP2)-based RDT testing, which was performed onsite using

Paracheck-Pf® (Catalog no. 30301100; Orchid Biomedical Systems, Verna, India). Patients testing positive were treated according to national guidelines. Blood samples were stored at –80°C and reference testing was performed at the end of the study. DNA was extracted from the blood samples using the DNEasy Blood & Tissue Kit (Catalog no. 69581; Qiagen, Toronto, Canada) and tested by qPCR using genus-wide primers and then species-specific primers, as described previously.¹¹ Only samples with positive genus and species results were considered true positives. The parasitemia was calculated based on a standard curve of *Plasmodium falciparum* (3D7) parasite dilutions run in triplicate using genus-wide primers. The efficiency of this reaction was 88.5%. The standard curve was also used to calculate the lower limit of quantification (0.2 parasites/μL) for the qPCR assay, which was defined as the lowest parasite dilution with 100% detection in three independent experiments and within the linear range of quantification of the standard curve.

Of the 491 participants, 154 (31%) were positive by qPCR with parasitemias ranging from 165,000 parasites/μL to fewer than 0.2 parasites/μL. Thirty samples were below the limit of quantification for the qPCR assay. However, these samples were considered positive, as they were positive in replicate tests with both the genus- and species-specific qPCR assays and were, therefore, included in the overall sensitivity and specificity calculations. Among the malaria-positive participants, 122 (79%) had *P. falciparum* mono-infections, five (3.2%) had *Plasmodium ovale* mono-infections, and six (3.9%) had *Plasmodium malariae* mono-infections. Twenty-one mixed infections were also identified: two (1.3%) infections with *P. falciparum* and *P. ovale*, 18 (12%) with *P. falciparum* and *P. malariae*, and one (0.7%) with *P. falciparum*, *P. ovale*, and *P. malariae*.

Of the 154 participants who tested positive for malaria, 42 (27%) had a body temperature more than 37.5°C at the time of testing, compared with 60 (18%) of the 337 participants who tested negative for malaria ($P = 0.016$) (Table 1). Also, fewer participants who tested positive for malaria reported sleeping under an insecticide-treated net the previous night (56/154, 36%) compared with those who tested negative for malaria (160/337, 48%) (odds ratio: 1.6, 95% confidence interval [CI]:

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TABLE 1
Participant characteristics

Participant characteristic	<i>Plasmodium</i> spp. PCR-positive (N = 154)	<i>Plasmodium</i> spp. PCR-negative (N = 337)	P value*
Age in years, median (IQR)	15 (4–28)	20 (4–34)	0.22
Female, n (%)†	95 (61.7)	237 (70)	0.058
Report of fever, n (%)	135 (87.7)	285 (85)	0.37
Axillary temperature > 37.5°C, n (%)‡	42 (27.3)	60 (18)	0.016
ITN use during previous night, n (%)	56 (36.4)	160 (48)	0.021
IRS during last 3 months, n (%)	130 (84.4)	280 (83)	0.71
History of antimalarial use, n (%)§	48 (31.2)	116 (34)	0.48

IQR = interquartile range; IRS = indoor residual spraying; ITN = insecticide-treated net; PCR = polymerase chain reaction. Bold type denotes P values less than 0.05.

* Calculated using the Mann-Whitney test (age) or χ^2 test (all others).

† Gender was not recorded for one participant.

‡ Current fever was defined as a body temperature greater than 37.5°C.

§ Reported use of antimalarial treatment in the previous 2 weeks.

1.1–2.3, $P = 0.021$). There were no other significant differences between the two groups.

Using qPCR as the gold standard, the sensitivity of the RDT for *P. falciparum* mono-infections was 76% (95% CI: 68–83%) and the specificity was 95% (95% CI: 92–97%). The positive and negative predictive values were 84% (95% CI: 77–89%) and 92% (95% CI: 87–94%), respectively. The RDT specificity was surprisingly high in our study, suggesting that few false positives were detected. Previous studies showed that RDT specificity is hampered by false positives because of persistent HRP2 antigen following chemotherapy.^{12,13} However, we did not observe this in our study setting. Using logistic regression analysis, we found that self-reported antimalarial treatment was not a significant independent predictor of RDT positivity, after correcting for infectious status (PCR positivity) (MedCalc software, version 17.9; Ostend, Belgium).

The high specificity of the RDT in our study underscores the importance of using a sensitive reference test as the gold standard when evaluating the performance of a diagnostic test. This was also highlighted in an earlier study in which 92% of samples considered “RDT false positives” by microscopy were positive by PCR.¹⁴ High RDT specificity (99.9%, 95% CI: 99.1–100) was also observed in a study using ultrasensitive qPCR as the reference test.⁵ In our study, most RDT-positive

patients were also positive by qPCR, suggesting the RDT detected active infections, although we cannot rule out persistent HRP2 and parasite DNA following recent treatment.

Consistent with previous studies, the sensitivity of the RDT in children of 5 years and less than that (98%, 95% CI: 87–100%) was much higher than that in older participants (66%, 95% CI: 55–76%).⁸ This is likely a result of higher parasite densities in young children (median interquartile range [IQR]: 615.4 (3.0–14,250.0) parasites/ μ L) than in older children and adults (median [IQR]: 3.6 (0.2–91.1) parasites/ μ L, $P = 0.017$). However, the RDT detected infections well below the minimum parasite density used in the WHO product testing (200 parasites/ μ L) (Figure 1). Between 0.2 and 200 parasites/ μ L, the sensitivity was 87% (95% CI: 74–94%) (Table 2). The sensitivity greater than 200 parasites/ μ L was 100%, consistent with the WHO evaluation of this RDT.¹

We speculate that the relatively high sensitivity of the RDT below 200 parasites/ μ L is because of ongoing HRP2 production by sequestered parasites, submicroscopic infections, or circulating gametocytes. Rapid diagnostic tests detect HRP2 released into the patient's blood stream and studies showed that the levels of antigen in the blood are poorly correlated with parasite density.¹⁵ Infections with circulating parasite densities less than 1 parasite/ μ L can produce a broad range of HRP2 concentrations in blood (median of 430 pg/mL, range 6–12,570 pg/mL).¹⁵ Given that several leading RDTs can detect HRP2 concentrations as low as 800 pg/mL, many submicroscopic infections would likely be RDT positive.¹⁶ This is also consistent with a recent modeling study, which predicted that parasite densities as low as 7.8–64.9 parasites/ μ L would yield a positive RDT result.¹⁷ Several studies also showed that RDTs can detect HRP2 produced by circulating gametocytes.^{18,19} If chronic submicroscopic infections or sexual stages of *P. falciparum* can produce enough HRP2 to be detected by some RDTs, then these tests may play an important role in surveillance of asymptomatic infections and in pre-elimination/elimination settings. However, the clinical relevance of these infections should also be considered, as symptoms such as fever may be falsely attributed to these infections, and other etiologies may be neglected.

It is difficult to generalize our results to other study settings, as RDT diagnostic performance is known to vary with factors such as host immunity, HRP2 antigenemia, and transmission

FIGURE 1. (A) Rapid diagnostic test (RDT) and reference quantitative polymerase chain reaction (qPCR) results for 459 participants. Thirty-two mixed infections were not included in the analysis. (B) Rapid diagnostic test results for *Plasmodium falciparum* mono-infections quantified by qPCR. The parasite density in nine samples that were positive by RDT and 22 that were negative by RDT was below the lower limit of quantification for the qPCR assay (dashed line, 0.2 parasites/ μ L).

TABLE 2

RDT test performance characteristics at different qPCR-quantified *Plasmodium falciparum** parasite densities

Parasite density (μL)	N	RDT positive	RDT negative	Sensitivity % (95% CI)
< 0.2	30	8	22	27 (12, 46)
0.2–200	52	45	7	87 (74, 94)
> 200	40	40	0	100 (96, 100)
Overall	122	93	29	76 (68, 83)

CI = confidence interval; qPCR = quantitative polymerase chain reaction; RDT = rapid diagnostic test.

*Analysis only includes *P. falciparum* mono-infections.

intensity.⁸ Rapid diagnostic test performance can also vary between lots.²⁰ Furthermore, the frequency of Pfhpr2 and Pfhpr3 deletions in our study population is unknown, which may be responsible for the RDT “false negative” results. Given these variables, our results highlight the importance of using a reference standard to quantify submicroscopic parasite densities in field trials to accurately evaluate the performance of RDTs. Also, characterizing the nature of low-level infections that can be detected by different RDTs will be important to optimize their use as diagnostics.

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