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Diagnostic Performance of Conventional and Ultrasensitive Rapid Diagnostic Tests for Malaria in Febrile Outpatients in Tanzania

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Background. A novel ultrasensitive malaria rapid diagnostic test (us-RDT) has been developed for improved active *Plasmodium falciparum* infection detection. The usefulness of this us-RDT in clinical diagnosis and fever management has not been evaluated.

Methods. Diagnostic performance of us-RDT was compared retrospectively to that of conventional RDT (co-RDT) in 3000 children and 515 adults presenting with fever to Tanzanian outpatient clinics. The parasite density was measured by an ultrasensitive qPCR (us-qPCR), and the HRP2 concentration was measured by an enzyme-linked immunosorbent assay.

Results. us-RDT identified few additional *P. falciparum*–positive patients as compared to co-RDT (276 vs 265 parasite-positive patients detected), with only a marginally greater sensitivity (75% vs 73%), using us-qPCR as the gold standard (357 parasite-positive patients detected). The specificity of both RDTs was >99%. Five of 11 additional patients testing positive by us-RDT had negative results by us-qPCR. The HRP2 concentration was above the limit of detection for co-RDT (>3653 pg of HRP2 per mL of blood) in almost all infections (99% [236 of 239]) with a parasite density >100 parasites per µL of blood. At parasite densities <100 parasites/µL, the HRP2 concentration was above the limits of detection of us-RDT (>793 pg/mL) and co-RDT in 29 (25%) and 24 (20%) of 118 patients, respectively.

Conclusion. There is neither an advantage nor a risk of using us-RDT, rather than co-RDT, for clinical malaria diagnosis. In febrile patients, only a small proportion of infections are characterized by a parasite density or an HRP2 concentration in the range where use of us-RDT would confer a meaningful advantage over co-RDT.

Keywords. Malaria; fever; diagnosis; ultrasensitive; RDT; quantitative; PCR; HRP2; Tanzania.

Prompt detection and treatment of symptomatic and asymptomatic malaria parasite infections is considered one of the key strategies to reduce transmission and accelerate malaria elimination. The large-scale deployment of conventional rapid diagnostic tests (co-RDTs) for detection of *Plasmodium falciparum* has considerably improved clinical case management of febrile patients [1]. Among other factors, such as age and previous exposure to malaria parasite infection, the development of malaria symptoms is mainly dependent on the parasite density in the blood of *P. falciparum*–infected individuals. The fraction of malaria-attributable fevers therefore increases with increasing parasite density [2]. Low-density *P. falciparum* parasitemia may be incidentally found in the blood of febrile patients but might not necessarily be the cause of fever in areas of high endemicity.

In contrast to malaria case management in clinical practice, intervention efforts for malaria elimination aim to identify asymptomatic parasite carriers. Asymptomatic carriers often harbor parasite densities below the limit of detection (LOD) of co-RDT or microscopy but contribute to maintaining malaria transmission, particularly in areas of low endemicity [3]. Detection of asymptomatic carriers in research surveys therefore uses highly sensitive molecular diagnostic assays; however, molecular diagnostic methods require highly trained personnel and sophisticated laboratory infrastructure and are often not available in field settings.

To facilitate the detection of asymptomatic *P. falciparum* infections in a point-of-contact manner, a novel ultrasensitive malaria RDT (us-RDT) was recently developed (Alere Malaria Ag P.f., Abbott Diagnostics). The analytical LOD of the us-RDT, at 80 pg of HRP2 per mL of blood, is 10-fold lower than that of co-RDT (800 pg/mL [4]) [5]. In community surveys in high-transmission (Uganda) and low-transmission (Myanmar)

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settings, us-RDT identified 84% and 44% of polymerase chain reaction (PCR)–detected *P. falciparum* infections, respectively, compared with 62% and 0%, respectively, for co-RDT [6]. A substantial gain in diagnostic sensitivity, from 25% for co-RDT to 50%–51% for us-RDT, was observed also in other community surveys, in Myanmar [7] and Papua New Guinea [8], which used ultrasensitive molecular diagnostic assays as a gold standard.

The intended use of us-RDT is the detection of asymptomatic infections during active case detection interventions supporting elimination campaigns; however, there is a possibility that its use could be extended to case management of febrile patients. While maximizing the detection and treatment of all possible malaria parasite infections within the clinical surveillance system, this off-label approach bears the risks of (1) misdiagnosis (ie, nonmalarial fevers might be attributed to a low-density incidental *P. falciparum* infection, owing to a positive test result) and, thus, inadequate treatment of the patient; (2) an increased number of false-positive test results due to the increased detection of residual HRP2 antigen, which persists >1 month in the circulation after clearance of a previous infection [9]; and (3) diversion of resources from management of true malaria cases, resulting in suboptimal patient care.

To quantify the potential effects of using us-RDT rather than co-RDT for case management of febrile patients, we retrospectively compared the diagnostic performances of us-RDT and co-RDT in febrile children and adults attending outpatient clinics in Dar es Salaam, Tanzania. Using molecular quantification, we defined the parasite density range that yields a positive RDT result and compared the limits of detection of us-RDT and co-RDT. Finally, we investigated the correlation of *P. falciparum* parasitemia level and HRP2 concentration in febrile individuals to explain the observed patterns in RDT positivity.

METHODS

Samples Collection and Study Design

Samples were obtained from 2 cohort studies in Dar es Salaam and analyzed retrospectively in a cross-sectional study design. Briefly, the cohorts included 3192 children aged 2–59 months presenting with acute febrile illness (axillary temperature, ≥37.5°C) to 9 outpatient clinics between December 2014 and February 2016 [10] and 519 adults aged 18–80 years presenting with acute febrile illness (tympanic temperature, ≥38°C) to 4 outpatient clinics between December 2013 and July 2014 [11]. Participants were managed using electronic and paper algorithms derived from the integrated management of childhood or adolescent and adult illness. In both cohorts, participants were treated with antimalarial medicines on the basis of a positive co-RDT result, according to national guidelines.

Written informed consent was obtained from each caretaker or patient. The studies were approved by the Institutional Review Board of the Ifakara Health Institute (Dar Es Salaam, Tanzania), the National Institute for Medical Research Review Board (Dar Es Salaam, Tanzania), and the Ethikkommission Beider Basel (Basel, Switzerland); an additional approval was obtained from the Boston Children's Hospital Ethical Review Board (Boston, MA) approved the study of the electronic algorithm [10].

Detection of *P. falciparum* Infections

Frozen venous whole blood specimens, stabilized in ethylenediaminetetraacetic acid, aliquoted, and stored at −80°C on the day of sampling, were available for 3004 children and 515 adults. After shipment while frozen, samples were analyzed retrospectively in the Swiss TPH laboratory, using 5 µL of whole blood for testing by co-RDT (Malaria Ag P.f., Abbott Diagnostics; reference 05FK50; lot 05CDB228A) and us-RDT (Alere Malaria Ag P.f., Abbott Diagnostics; reference 05FK140; lot 05LDB004A) according to the manufacturer's instructions. RDTs were initially read, scored, and photographed by a laboratory technician. A second independent reader reviewed the photographs for double data entry. Discordant entries were resolved by a third reader, using photographs.

Molecular analysis of samples was performed using an ultra-sensitive quantitative PCR (us-qPCR) targeting the conserved C-terminal region of the multiple-copy *var* gene family, which has a LOD <0.1 parasites per µL of blood [12]. DNA was extracted from 100 µL of whole blood by means of the DNeasy 96 Blood and Tissue Kit (Qiagen), using an overnight proteinase K digest followed by loading and washing steps, according to the supplier's instructions. DNA was eluted in 50 µL of elution buffer. Each 96-well extraction plate included 5 negative phosphate-buffered saline controls. A total of 4 µL of DNA was used per us-qPCR reaction, corresponding to 8 µL of whole blood. Parasite density was determined using a 10-fold dilution row of the World Health Organization first international standard for *P. falciparum* DNA nucleic amplification techniques (National Institute for Biological Standard and Control). For all samples with a parasitemia level <10 parasites/µL, DNA extraction was repeated, followed by us-qPCR analysis in duplicate. Samples with a parasite density <10 parasites/µL were scored as positive for parasites only if they were confirmed as such during this second round of DNA extraction and us-qPCR analysis. For samples with confirmed positive test results, the geometric mean density based on all positive us-qPCR runs was reported as the final parasitemia level.

Quantification of HRP2

The concentration of HRP2 antigen was quantified in all us-qPCR- and/or RDT-positive samples and in 200 randomly selected us-qPCR- and RDT-negative samples. HRP2 quantification was performed using a recently developed Q-plex array enzyme-linked immunosorbent assay (ELISA) kit, which was expanded from an earlier version of a multiplex ELISA (Quansys Biosciences [6]). Briefly, 12.5 µL of whole blood and 37.5 µL of sample diluent (containing heterophilic antibodies

and rheumatoid factor blockers) were added to each plate well and incubated for 2 hours at room temperature while shaking at 500 rpm. After 3 washes, the plate was incubated with detection mix (50 µL/well, containing biotinylated detector antibodies) for 1 hour while shaking. Following 3 washes, the plate was incubated with streptavidin–horseradish peroxidase (1×; 50 µL/well) for 20 minutes while shaking. The plate was then washed 6 times, and the detection substrate mix (50 µL/well, consisting of equal volumes of stabilized hydrogen peroxide and stabilized signal enhancer) was added. The plate was imaged using a Q-view imager (Quansys Biosciences).

The upper limit of quantification, defined as the concentration above which HRP2 protein can be detected but not accurately quantified, was 16 500 pg/mL. The lower analytical limit of quantification, defined as the concentration below which infections are considered HRP2 negative in this analysis, was 1.07 pg/mL.

Data Analysis

Four children were excluded from the analysis because us-qPCR or HRP2 data could not be reproduced during quality control. The diagnostic performance of co-RDT and us-RDT were calculated using us-qPCR as the reference standard. Logistic regression was used to estimate the lowest parasite density and HRP2 concentration at which an infection would be detected with 95% probability by co-RDT and us-RDT. For calculation of diagnostic sensitivity across the range of parasite densities and HRP2 concentrations, all observations were assigned a status of true positive, false positive, true negative, or false negative. Observations were sorted by parasite density or HRP2 concentration, and a point estimate of sensitivity per parasite density or HRP2 concentration was calculated as a rolling mean in moving intervals of 18 observations, using the R-package *zoo*. All analyses were performed in R, version 3.4.3.

RESULTS

Diagnostic Performance of co-RDT and us-RDT

A positive co-RDT result was observed in 230 of 3000 children (7.7%) and 35 of 515 adults (6.8%; Table 1). In comparison, us-qPCR detected *P. falciparum* infections in 309 of 3000 children (10.3%) and 48 of 515 adults (9.3%). us-RDT identified all patients with positive co-RDT results plus 9 additional children with positive co-RDT results (0.3% [9 of 3000]) and 2 additional adults (0.4% [2 of 515]). More than half of patients positive by us-RDT but negative by co-RDT (55% [6 of 11]) were also positive by us-qPCR. The remainder (45% [5 of 11]) were negative by

Table 1. Positivity Counts and Diagnostic Performance of a Conventional Rapid Diagnostic Test (co-RDT) and an Ultrasensitive RDT (us-RDT) in Febrile Children and Adults from Tanzania

co-RDT			Children (n = 3000)	Adults (n = 515)
Status	co-RDT Result	us-qPCR Result[a]	No.	No.
True positive	+	+	226	35
False positive	+	–	4	0
False negative	–	+	83	13
True negative	–	–	2687	467
Diagnostic performance			Value (95% CI)	Value (95% CI)
Sensitivity			73.1 (68.1–78.1)	72.9 (60.3–85.5)
Specificity			99.9 (99.7–1.00)	100.0 (100.0–100.0)
Positive predictive value			98.3 (96.6–1.00)	100.0 (100.0–100.0)
Negative predictive value			97.0 (96.4–97.6)	97.3 (95.8–98.7)
Positive likelihood ratio			492.0 (184.4–1313.1)	Infinity
Negative likelihood ratio			0.3 (0.2–0.3)	0.3 (0.2–0.4)
us-RDT				
Status	us-RDT Result	us-qPCR Result[a]	No.	No.
True positive	+	+	230	37
False positive	+	–	9	0
False negative	–	+	79	11
True negative	–	–	2682	467
Diagnostic performance			Value (95% CI)	Value (95% CI)
Sensitivity			74.4 (69.6–79.3)	77.1 (65.2–89.0)
Specificity			99.7 (99.4–99.9)	100.0 (100.0–100.0)
Positive predictive value			96.2 (93.8–98.6)	100.0 (100.0–100.0)
Negative predictive value			97.1 (96.5–97.8)	97.7 (96.4–99.0)
Positive likelihood ratio			222.6 (115.5–428.7)	Infinity
Negative likelihood ratio			0.26 (0.21–0.31)	0.2 (0.1–0.4)

Abbreviation: CI, confidence interval.

[a]Ultrasensitive quantitative polymerase chain reaction (us-qPCR) was the gold standard.

us-qPCR, suggesting detection of residual HRP2 after parasite clearance or parasite sequestration (Figure 1A). HRP2 ELISA confirmed the presence of HRP2 in these 5 samples (Figure 1B).

Owing to the low number of additional us-qPCR–positive specimens detected by us-RDT as compared to co-RDT, the gain in diagnostic sensitivity was low. The diagnostic sensitivity of co-RDT, using us-qPCR as the gold standard, was 73.1% (95% CI, 68.5%–77.7%) and did not differ between children (73.1% [95% CI, 68.1%–78.1%]) and adults (72.9% [95% CI, 60.3%–85.5%]; χ^2 P = .548; Table 1). For us-RDT, the sensitivity was 74.8% (95% CI, 70.3%–79.3%) overall, 74.4% (95% CI, 69.6%–79.3%) in children, and 77.1% (95% CI, 65.2%–89.0%) in adults (χ^2 P = .602).

The specificity of co-RDT and us-RDT, using us-qPCR as the gold standard, was >99% (Table 1), as the number of samples with a negative us-qPCR result and a positive co-RDT (n = 4) or us-RDT (n = 9) result was low. No difference in specificity was observed between adults and children. The positive predictive value was slightly higher for co-RDT (98.5% [95% CI, 97.0%–100%]) as compared to us-RDT (96.7% [95% CI, 94.6%–98.8%]), while the negative predictive values were equal because of the high number of true-negative results (co-RDT, 97.0% [95% CI, 96.4%–97.6%]; us-RDT, 97.2% [95% CI, 96.7%–97.8%]). Agreement between the both RDTs was almost perfect, with a Cohen κ of 0.98. Both RDTs also agreed well with us-qPCR (Cohen κ, 0.82 for agreement with co-RDT and 0.83 for agreement with us-RDT).

Sensitivity of co-RDT and us-RDT in Relation to Parasite Density and HRP2 Concentration

When comparing the LOD between the both RDTs, the parasite density detected by us-RDT was 2 times lower than that detected by co-RDT (Figure 2A). The LOD, defined as the lowest parasite density that could be detected with 95% probability, decreased from 626 parasites/μL (95% CI, 255–2066) for co-RDT to 346 parasites/μL (95% CI, 142–1173) for us-RDT. When comparing the lowest reliably detected HRP2 concentration (Figure 2B), there was a larger (5-fold) difference between the two RDTs. The LOD was 793 pg HRP2/mL (95% CI, 363–1640) for us-RDT, compared with 3653 pg/mL (95% CI, 2527–4735) for co-RDT.

Owing to the low difference in the parasite density LOD between the two RDTs, the diagnostic sensitivity was comparable across a wide parasite density range (Figure 2C). A gain in diagnostic sensitivity by using us-RDT was mainly observed for patients with a parasite density of 10–100 parasites/μL, reaching a maximal gain of 12% (32% vs 44%) at 42 parasites/μL (Figure 2C). In contrast, when analyzing diagnostic sensitivity in relation to HRP2 concentration, a clear gain over the whole range of HRP2 concentrations was observed by using us-RDT as compared to co-RDT (Figure 2D), reaching a maximal gain of 43% (33% vs 76%) at 1317 pg/mL.

Distributions and Correlation of Parasite Density and HRP2 Concentration

The parasite density (detected by us-qPCR) and the HRP2 concentration (detected by ELISA) in febrile children and febrile adults followed a bimodal distribution (Figure 3). The distribution of the parasite density was characterized by 2 pronounced peaks and a trough at 100–1000 parasites/μL (Figure 3A and 3B). In contrast, the distribution of the HRP2 concentration exhibited 1 major peak, at 10 000 pg/mL, and 1 minor peak, at 1–100 pg/mL (Figure 3C and 3D). No linear correlation was observed between parasite density and HRP2 concentration in children or adults (Spearman ρ, 0.36 for both groups; Figure 4).

The parasite density was lower in adults (geometric mean, 1102 parasites/μL; median, 1691 parasites/μL [IQR, 27–87 812 parasites/μL]) as compared to children (geometric mean, 3844 parasites/μL; median, 54 742 parasites/μL [IQR, 13–385 514 parasites/μL]; Mann-Whitney U P = .042). Also, the HRP2 concentration was lower in adults as compared to children, although there was little difference in the median HRP2

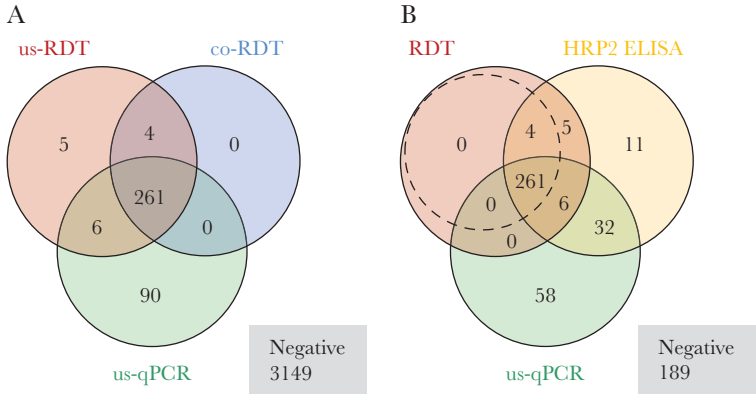

Figure 1. Venn diagram of *Plasmodium falciparum* positivity by different diagnostic methods. *A*, Positivity by a conventional rapid diagnostic test (co-RDT), an ultrasensitive RDT (us-RDT), and an ultrasensitive quantitative polymerase chain reaction (us-qPCR) in 3515 febrile children and adults attending outpatient clinics in Dar es Salaam, Tanzania. *B*, Positivity by RDT, us-qPCR, and an HRP2 enzyme-specific immunosorbent assay (ELISA) in the subset of 566 children and adults who were tested by HRP2 ELISA. For RDT positivity, the larger, continuous circle delineates positivity by us-RDT, and the smaller, dashed circle delineates positivity by co-RDT.

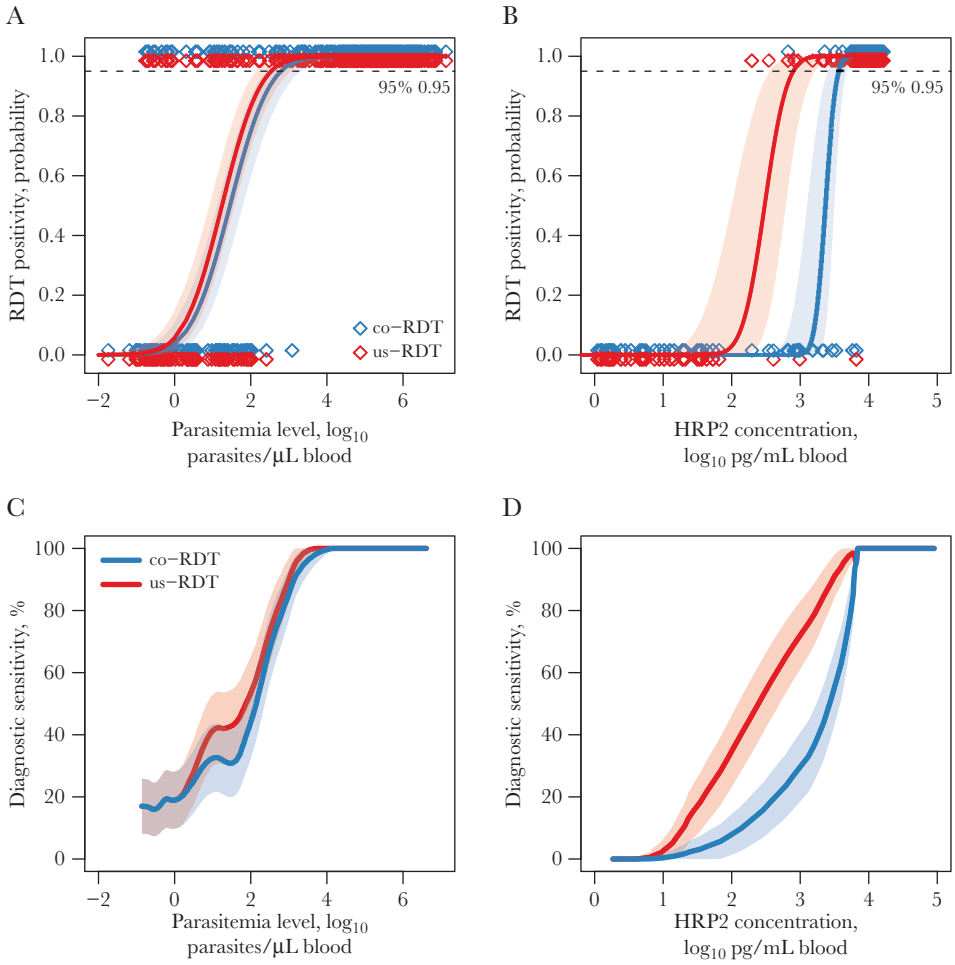

Figure 2. Limit of detection and diagnostic sensitivity of a conventional rapid diagnostic test (co-RDT) and an ultrasensitive RDT (us-RDT) in relation to parasite density and HRP2 concentration. *A* and *B*, Relationship between the probability of testing positive for *Plasmodium falciparum* by co-RDT and us-RDT and parasite density (*A*) or HRP2 concentration (*B*). Shaded areas represent 95% confidence intervals (CIs) of the logistic regression model. *C* and *D*, Diagnostic sensitivity of co-RDT and us-RDT in relation to parasite density (*C*) and HRP2 concentration (*D*). Diagnostic sensitivities were calculated as a rolling means of 18 observations, using us-qPCR as a gold standard, and are shown with 95% CIs (shaded areas). Curves were smoothed using the lowess function (span, 0.05).

concentration between the 2 groups (adults, 6472 pg/mL [IQR, 86–7853 pg/mL]; children, 7933 pg/mL [IQR, 6653–9924 pg/mL]; Mann-Whitney *U* $P < .001$).

RDT Positivity in Relation to HRP2 Concentration and Parasitemia Level in Children Versus Adults

The concentration of HRP2 was >3653 pg/mL, which is above the co-RDT LOD, in practically all children and adults (99% [207 of 210] and 100% [29 of 29], respectively) with a parasitemia level of >100 parasites/μL (Figure 4). These infections were thus generally detected by co-RDT (98% of patients [235 of 239] tested positive), and the gain in positivity by using us-RDT was marginal (99% [236 of 239] tested positive; McNemar χ^2 $P = 1.000$).

Among 118 febrile children and adults with a parasite density <100 parasites/μL, the HRP2 concentration varied substantially, ranging from 1.1 to >16 500 pg/mL (median, 409 pg/mL [IQR, 8–9991 pg/mL]; Figure 4). The HRP2 concentration was above the co-RDT LOD in 20% of patients (24 of 118) and above the us-RDT LOD in an additional 4% (5 of 118). Hence, in infections with parasitemia <100 parasites/μL there was only a small and nonsignificant increase in the proportion of patients with RDT-detectable infections detected by us-RDT (26% of patients [31 of 118] tested positive) as compared to co-RDT (22% [26 of 118] tested positive; McNemar χ^2 $P = .074$).

In 81 patients with very low-density infections (parasitemia level, <10 parasites/μL), a similar variation in HRP2 concentration was observed in children (median, 37 pg/mL [IQR, 3–13 800 pg/mL]; n = 73; Figure 4A) but not in adults (Figure 4B). Instead, very low-density infections in adults were characterized by a low HRP2 concentration (median, 14 pg/mL [IQR, 6–42 pg/mL]; n = 8). As a result, very low-density infections with a positive co-RDT or us-RDT result were almost exclusively found in children (93% [14 of 15]). Coherently, a high HRP2 concentration and positive co-RDT or us-RDT results among

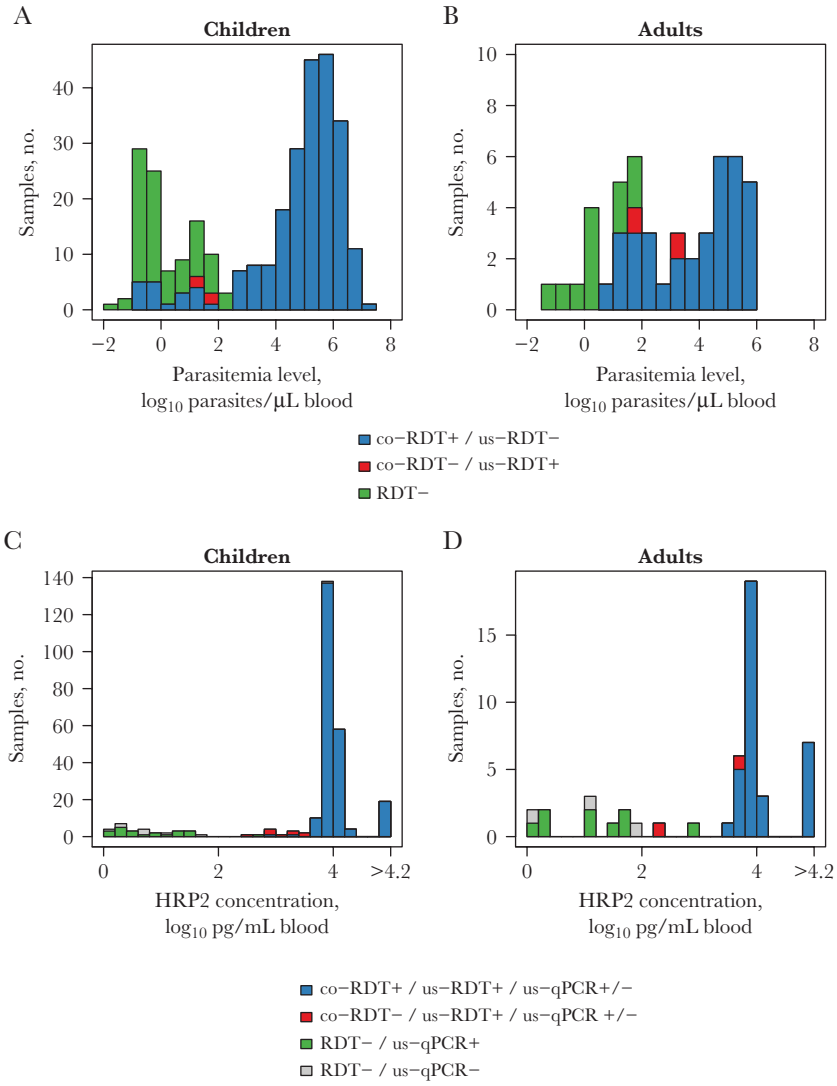

Figure 3. Distributions of parasite density and HRP2 concentration in febrile children and adults attending outpatient clinics in Dar es Salaam, Tanzania. *A* and *B*, Distribution of parasite densities in 309 of 3000 children (*A*) and 48 of 515 adults (*B*) with positive results of an ultrasensitive quantitative polymerase chain reaction (us-qPCR). *C* and *D*, Distribution of HRP2 concentrations in 270 of 3000 children (*C*) and 49 of 515 adults (*D*) with positive us-qPCR, conventional rapid diagnostic test (co-RDT), or ultrasensitive RDT (us-RDT) results who had a positive HRP2 enzyme-linked immunosorbent assay (ELISA) result. In addition, of 200 randomly selected samples with negative us-qPCR, co-RDT, and us-RDT results that were tested by HRP2 ELISA, 11 HRP2-positive samples are shown. –, negative; +, positive.

us-qPCR–negative samples were exclusively found in children (median, 3336 pg/mL [IQR, 2112–8550 pg/mL]; n = 9).

Among 200 patients with negative results in us-qPCR, us-RDT, and co-RDT, HRP2 was detected in 11 (5.5%). The concentration of HRP2 in these patients ranged from 1.2 to 65.4 pg/mL (Figure 3C and 3D and Figure 4). Low levels of residual circulating HRP2 can therefore remain in the blood even in absence of us-qPCR–detectable parasitemia. However, parasite DNA was detected in 73% of samples (29 of 40) with a comparably low HRP2 concentration (<100 pg/mL), suggesting that in the majority of cases, low-level HRP2 in febrile children and adults originates from a concurrent low-density *P. falciparum* infection.

DISCUSSION

Only a few additional *P. falciparum*–positive febrile patients were detected by us-RDT compared to co-RDT in 2 age cohorts, comprising 3000 children and 515 adults, presenting to outpatient clinics in an urban setting in Tanzania. This resulted in a minimal gain of 2% in diagnostic sensitivity by using us-RDT, with no difference between adults and children. Such a small gain is explained by the small proportion of symptomatic malaria parasite infections (10% [37 of 357]) with a parasite density in the range of 10–100 parasites/μL, for which the use of us-RDT would confer a meaningful improvement over co-RDT. This implies that there is a limited increase in sensitivity associated with the use of us-RDT in the management of febrile cases

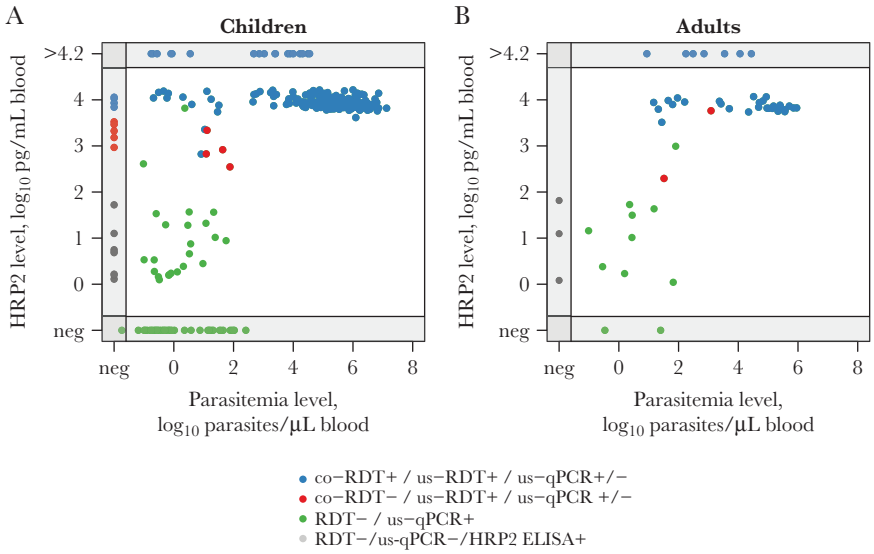

Figure 4. Correlation of parasite density and HRP2 concentration in febrile children (*A*) and febrile adults (*B*) attending outpatient clinics in Dar es Salaam, Tanzania. Data points are color-coded according to the sample's positivity by a conventional rapid diagnostic test (co-RDT) and an ultrasensitive RDT (us-RDT). ELISA, enzyme-linked immunosorbent assay; neg, negative; us-qPCR, ultrasensitive quantitative polymerase chain reaction; –, negative; +, positive.

but also a limited risk of a positive us-RDT result masking other underlying causes of fever and misleading clinicians.

Our results confirm a previous evaluation of the utility of us-RDT in different target populations, which estimated that improving the RDT LOD from 3000 pg/mL to 200 pg/mL would yield an additional 50%–60% of RDT-positive results among afebrile individuals but would only have a limited effect, with 10%–20% extra RDT-positive results, among febrile patients [13]. In our survey of febrile patients, where we estimated the LOD of co-RDT to be 3653 pg/mL and that of us-RDT to be 793 pg/mL, we found an additional 4% of parasite-positive patients (11 of 265) by using us-RDT as compared to co-RDT. The comparably high LOD estimates in the study presented here, compared with the previously described LOD of 80 pg/mL for us-RDT [5] and 800 pg/mL for co-RDT [4], may be explained by the choice of study population: in febrile patients, samples from only a few patients (10% [34 of 319] with positive HRP2 ELISA results) yielded an HRP2 concentration of 100–1000 pg/mL, while most samples were characterized by either very low (in patients with nonmalarial fever) or very high (in patients with malaria-attributable fever) HRP2 concentrations. This may have contributed to uncertainty in the LOD estimation.

The low gain in sensitivity by using us-RDT seems specific for diagnosing clinical malaria in febrile patients. In contrast, in previous cross-sectional surveys in asymptomatic individuals, a much higher percentage of additional positives (range, 35%–100%) was found [6–8]. The difference in the proportion of additional positive results among afebrile individuals as compared to febrile patients reflects the difference in parasite density distributions in the 2 populations. While parasite densities were normally distributed, with a maximum at 100–1000 parasites/μL, in the community surveys of afebrile individuals [6–8], in this survey of febrile patients we observed a bimodal distribution, with a trough at 100–1000 parasites/μL. Also, a recent study in pregnant Columbian women found that the use of us-RDT increased the diagnostic sensitivity (using nested PCR as the gold standard) in afebrile participants but not symptomatic participants when compared to co-RDT or microscopy.

Similarly, the correlation of parasite density and HRP2 concentration and, thus, RDT positivity seems to differ between different target populations and endemicity levels. While the parasite density and HRP2 concentration were linearly correlated (after $\log_{10}$ transformation) [7] or exhibited similar distributions [6] in 2 community surveys in areas of Myanmar with low malaria endemicities, this was not the case in our survey of febrile Tanzanian patients and in a community survey in an area of high endemicity in Uganda [6]. Instead, in the latter 2 populations, very high concentrations of HRP2 (>10 000 pg/mL) were the most common observations, and a steep, stepwise increase in the HRP2 concentration was observed at parasite densities >100 parasites/μL. In the high-transmission setting of Uganda, high levels of HRP2 in asymptomatic individuals are likely due to residual circulating HRP2 in frequently infected individuals. HRP2 is stable in the blood, with a half-life of 3–15 days after treatment, and can be detected for >1 month after antimalarial treatment [14, 15]. It is possible that residual HRP2 originating from a previous but cleared malaria parasite infection would also be detectable by RDTs in febrile patients presenting to clinics in high-transmission settings. This could potentially give rise to a greater increase in the detection of parasite-positive patients with us-RDT use in a clinical setting, compared with the minimal gain in low-transmission settings.

In the survey of febrile Tanzanian patients presented here, a high HRP2 concentration in patients with low parasitemia levels or even negative results of us-qPCR were observed in only a few children and no adults. This could be explained either by the much larger sample size of children as compared to adults or by biological factors related to antimalarial immunity in children. First, *P. falciparum* sequestration is more pronounced in children as compared to adults, leading to intermittent absence of the parasite from the peripheral blood in synchronous infections [16] while HRP2 remains in the circulation. Second, young children experience a higher peak parasitemia level upon infection as compared to adults, because their acquired immunity to malaria is lower and, thus, their parasite density is less controlled [17]. As the initial peak parasitemia level is the main determining factor influencing the duration of persistent RDT positivity after antimalarial treatment [15], young children remain RDT positive longer after antimalarial treatment, compared with adults [14].

Increased detection of residual HRP2 by us-RDT as compared to co-RDT is considered as one of the main risks associated with the use of us-RDT in clinical case management. In our survey, use of us-RDT doubled the number RDT-positive but us-qPCR–negative patients in comparison to co-RDT (4 vs 9). However, RDT-positive, us-qPCR–negative patients were rare, compared with the number of RDT-positive patients with malaria confirmed by us-qPCR (co-RDT, 261; us-RDT, 267). Also, diagnostic specificity was high (>99%) for us-RDT, owing to the very large number of correctly identified negatives (co-RDT, 3154; us-RDT, 3149).

A limitation of our study is the use of frozen venous blood samples. These may perform differently when analyzed by RDT, compared with fresh capillary blood specimens [18], but are nevertheless frequently used in diagnostic assessment of RDTs [4, 18], such as in previous studies evaluating us-RDT [6, 19]. Aliquoted at collection, our samples underwent ≤2 freeze-thaw cycles before analysis. In previous studies, such a limited number of freeze-thaw cycles did not affect the performance of HRP2 ELISA [20, 21] or HRP2 dipstick analysis [22].

The present article does not focus on the relevance of detecting and treating co-RDT–negative *P. falciparum* infections in febrile patients, which is discussed elsewhere (Hartley et al, unpublished data). Our previous studies showed an absence of clinical complications due to untreated malaria parasite infection after a negative co-RDT result in febrile Tanzanian children [23] and returning travelers [24]. This suggests that using ultrasensitive tests is not required in clinical case management.

In conclusion, our results show that the potential advantage and harm are minimal from the off-label use of us-RDT as a diagnostic assay in clinical practice and for malaria case management in areas of low endemicity, such as Dar es Salaam (prevalence, 1.1% in children aged 6–59 months [25]). us-RDT increases diagnostic sensitivity mainly around a parasite density of 100 parasites/μL; however, in low-transmission settings, infections with this parasite density are rarely found in febrile patients. Conversely, the risk of an increased frequency of malaria misdiagnosis is low when using us-RDT rather than co-RDT for detection of *P. falciparum* in clinical practice in areas of low endemicity. While our results are reassuring for clinical practitioners, they require validation in different transmission settings, including an evaluation of clinical outcomes in *P. falciparum*–positive patients whose infection is not detected by co-RDT.

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest.

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