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Direct Estimation of Sensitivity of *Plasmodium falciparum* Rapid Diagnostic Test for Active Case Detection in a High-Transmission Community Setting

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Abstract. Community-based active case detection of malaria parasites with conventional rapid diagnostic tests (cRDTs) is a strategy used most commonly in low-transmission settings. We estimated the sensitivity of this approach in a high-transmission setting in Western Kenya. We tested 3,547 members of 912 households identified in 2013–2014 by index children with (case) and without (control) cRDT-positive malaria. All were tested for *Plasmodium falciparum* with both a cRDT targeting histidine-rich protein 2 and with an ultrasensitive real-time polymerase chain reaction (PCR). We computed cRDT sensitivity against PCR as the referent, compared prevalence between participant types, and estimated cRDT detectability as a function of PCR-estimated parasite density. Parasite prevalence was 22.9% by cRDTs and 61.5% by PCR. Compared with children aged < 5 years or adults aged > 15 years, geometric mean parasite densities (95% CI) were highest in school-age children aged 5–15 years (8.4 p/uL; 6.6–10.6). The overall sensitivity of cRDT was 36%; among asymptomatic household members, cRDT sensitivity was 25.5% and lowest in adults aged > 15 years (15.8%). When modeled as a function of parasite density, relative to school-age children, the probability of cRDT positivity was reduced in both children aged < 5 years (odds ratio [OR] 0.48; 95% CI: 0.34–0.69) and in adults aged > 15 years (OR: 0.35; 95% CI: 0.27–0.47). An HRP2-detecting cRDT had poor sensitivity for active *P. falciparum* case detection in asymptomatic community members, and sensitivity was lowest in highly prevalent low-density infections and in adults. Future studies can model the incremental effects of high-sensitivity rapid diagnostic tests and the impacts on transmission.

INTRODUCTION

Between 2000 and 2015, malaria cases in Africa have declined by 40%[1,2] and the proportion of Africans who live in hyper- or holoendemic transmission settings has declined by more than 70%.[1] These successes have enabled fresh approaches to enhanced control measures, some of which rely on active case detection (ACD) in asymptomatic individuals in community settings.[3] Active case detection is typically deployed either in response to an index case as reactive case detection (RACD) or in high-risk populations as proactive case detection (PACD).[4] These ACD programs are common strategies of malaria control programs in low-transmission settings in Asia.[5] In African studies, PACD and RACD identify large numbers of additional cases,[6–12] but commonly cited barriers to ACD in African settings are operational barriers, limited spatial aggregation, high parasite prevalence, and uncertain suitability of common malaria rapid diagnostic tests (RDTs) to detect abundant low-density infections in community settings.[13]

Conventional rapid diagnostic tests (cRDTs) enhance clinical diagnosis and case management[14] but are increasingly used for community-based detection in asymptomatic people.[15] Because of their operability and availability, cRDTs have been repurposed for community-based studies for detection of "hot spots,"[16] for RACD,[12] and mass screen and treat programs.[17] Despite these applications, the sensitivity of cRDTs in such settings and how this varies between groups remain unclear. Generally, cRDTs that detect the *Plasmodium falciparum* histidine-rich protein 2 (HRP2) are benchmarked to detect parasitemias at a density of 200 parasites/μL (p/μL) of whole blood[18] but are believed to have a limit of detection of approximately 100 p/μL.[15] Many studies have compared cRDT detection with molecular detection as a reference,[12,19–21] but few[19,22] have used PCR estimates of parasite density by which to explore the quantitative limit of detection of cRDT. Therefore, there exists a paucity of direct estimates of the probability of cRDT positivity as a function of parasite density among asymptomatic individuals of all ages in high-transmission settings.

In this study, we developed and applied a sensitive molecular *P. falciparum* detection assay that allows for infections to be quantified and then used this assay to directly estimate the sensitivity of a conventional HRP2-based RDT applied in a case–control study of malaria prevention in Western Kenya. Our field study enrolled children as cases (cRDT-positive) or controls (cRDT-negative), along with their household members; we hypothesized that, in this high-transmission setting, this HRP2-detecting cRDT would detect a large proportion of PCR-positive infections and that detectability would be highest in children.

MATERIALS AND METHODS

Ethical review. The study was approved by the Ethical Review Boards of Moi University (000778) and Duke University (Pro00044098). All participants provided written informed consent; assent was obtained from children older than 8 years.

Specimen collection. The field study was conducted in Webuye, Kenya, in 2013–2014, and described elsewhere.[23] Webuye has perennial *P. falciparum* transmission with a seasonal peak in May–June, and the principal vectors are *Anopheles arabiensis* and *Anopheles gambiae* s.s. Briefly, index case and control children and their households were enrolled: case children aged 1–10 years were consecutively identified when admitted to Webuye Sub-County Hospital with cRDT-confirmed *falciparum* malaria; on discharge, these

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case children were age-, village-, and gender-matched to community-dwelling control children. Control children were cRDT negative and excluded if they were unwell or had taken antimalarials in the prior month. All household members of index children were sampled within 1 week of case child hospital discharge. All index and household participants were tested at enrollment with the SD Bioline Malaria Ag P.f. (HRPII) cRDT (Standard Diagnostics, Inc., Suwon city, Korea). Participants with positive cRDT results were treated: case children were treated with usual care as per local guidelines—typically parenteral quinine—and community participants with artemether–lumefantrine. At the time of cRDT testing, capillary blood was stored on a filter paper as a dried blood spot (DBS) in plastic bags with a desiccant.

Parasite detection assay development. We designed a duplex TaqMan real-time PCR assay targeting the *P. falciparum* multicopy motif *pfr364*[24] and the human gene β-tubulin.[25] We aligned 41 separate sequences of *pfr364* in the *P. falciparum* reference genome 3D7 in ClustalW[26] and used Primer3 to design primers to amplify a 126-nt segment from 22 of these sequences and to design a TaqMan probe targeting a conserved segment of this amplicon with the reporter FAM and the quencher QSY; the β-tubulin TaqMan probe was synthesized with the VIC reporter and a QSY quencher (Supplemental Table 1).

We first amplified purified genomic DNA (gDNA) templates of reference *P. falciparum* lines. These individual 12 μL reactions consisted of 250 nM of each *pfr364* primer, 300 nM of *pfr364* probe, 300 nM of each β-tubulin primer and probe, 6 μL of TaqMan Environmental MasterMix (Applied Biosystems, Foster City, CA), 1 μL of template gDNA, and water, and were amplified on an ABI QuantStudio 6 platform. Reference parasite lines were 3D7, Dd2, K1, FCR3/FMG, and 7G8, each obtained from MR4, and were quantified using the Qubit dsDNA HS Assay Kit (ThermoFisher, Waltham, MA). We then prepared quantitative mocked DBSs of *P. falciparum* at known densities of 2,000, 1,000, 200, 100, 20, 10, 2, 1, 0.2, and 0.1 p/μL with which to test the lower limit of detection of *P. falciparum*. To do so, we cultivated *P. falciparum* strain 3D7 using standard methods and pelleted erythrocytes, and diluted these 1:20 into uninfected whole blood; we measured % parasitemia by Giemsa-stained thin smear and erythrocytes/μL using a hemocytometer. Serial dilutions of this source material were made into uninfected whole blood, and these were each preserved in duplicate as DBSs. We first tested the duplex *pfr364*/β-tubulin assay using these templates in six technical replicates; we then used these templates to directly compare in parallel the performance of this assay with that of reported real-time assays targeting *var*ATS[27] and *pfldh*[28]; for these tests, cycle threshold (Ct) lines were set manually for each assay at identical ΔRn values above background fluorescence and in the exponential phase of amplification. Reaction mixtures were as reported for these assays, excepting for the enzyme (TaqMan Environmental MasterMix), the platform (QuantStudio 6), and the number of cycles (45, as reported for the *var*ATS assay).

Parasite detection procedures. Field-collected DBSs were stored at room temperature for up to 2 years and then singly punched into individual wells of a 96-well deepwell plate; each plate also contained a punch from a mock, nonquantitative DBS containing 3D7 gDNA as an extraction control. In this study, gDNA was extracted from DBSs in a plate format using Chelex-100.[29] Each gDNA specimen was tested with the duplex *pfr364*/β-tubulin real-time PCR assay in duplicate in 384-well reaction plates, with reaction conditions as described previously. Each reaction plate included 10 positive controls in duplicate as a standard curve, at parasite densities of 2,000, 1,000, 200, 100, 20, 10, 2, 1, 0.2, and 0.1 p/μL. Negative controls were included on each reaction plate, and all reaction plates were prepared in dedicated workspaces and with filtered tips. Cycle threshold lines were set manually above baseline fluorescence.

Statistical analyses. We compared between assays test positivity with chi-squared tests and Ct values by computing pairwise correlation coefficients. For testing of clinical samples, the analytical population was defined as nonmissing specimens from participants with nonmissing individual clinical/demographic data who were from households where the index child (case or control) was non-missing. Specimens positive for *P. falciparum* met the following criteria: 1) positive amplification of human β-tubulin and 2) amplification of *pfr364* in either both replicates or in a single replicate with a Ct value ≤ 38. We assigned parasite densities based on mean estimated quantities using a standard curve generated from standards from 2,000 to 1 p/μL. Prevalence ratios (PRs) were computed with Poisson regression. We summarized parasite densities by computing geometric means (geomean) and compared densities using analysis of variance (ANOVA) of $\log_{10}$-transformed densities. We modeled the probability of cRDT positivity as a function of $\log_{10}$-transformed parasite density with logistic regression. We repeated these analyses in adjusted models including indicator variables for age to estimate age-specific odds ratios (ORs) controlling for parasite density. Statistical analyses were computed in Stata/SE v14.2 (Stata Corp, College Station, TX).

RESULTS

Parasite detection assay performance. When tested against purified gDNA from *P. falciparum* reference strains, the duplex *pfr364* TaqMan real-time PCR assay reported lower limits of detection, ranging from 2.2×10^{-5} ng/μL to 1×10^{-2} ng/μL of gDNA. When tested initially on gDNA extracted from mock quantitative DBSs prepared with known densities of parasites, the assay was positive down to 0.1 p/μL (Supplemental Table 2). A standard curve fit to Ct measurements from densities of 1–2,000 p/μL returned an R^2 of 0.9624 with an efficiency of 90%.

These templates were then used to directly compare the performance of this *pfr364* assay with that of real-time PCR assays targeting *pfldh* and *var*ATS (Figure 1). In 12 replicates at densities of 1 p/μL or less, detection was successful (defined as Ct ≤ 40) less often for *pfldh* (5/12) than for *varATS* (11/12) or *pfr364* (10/12) ($P < 0.05$ for each comparison with *pfldh*). When compared with *var*ATS and *pfldh* parasite detection assays, Ct values among successful tests for *pfr364* were highly correlated with those for both *pfldh* (correlation efficient 0.9392, $P < 0.001$) and *var*ATS (0.9833, $P < 0.001$).

Parasite prevalence. The parent field study enrolled 4,377 participants (2,203 in case households and 2,154 in control households). From these, DBS samples were available for 4,116 participants; of these, 4,075 were able to be matched to clinical data and tested for malaria parasites with real-time PCR. We further restricted analyses only to those households for which clinical and molecular data were available for the index child, resulting in 3,547 participants in 912 households.

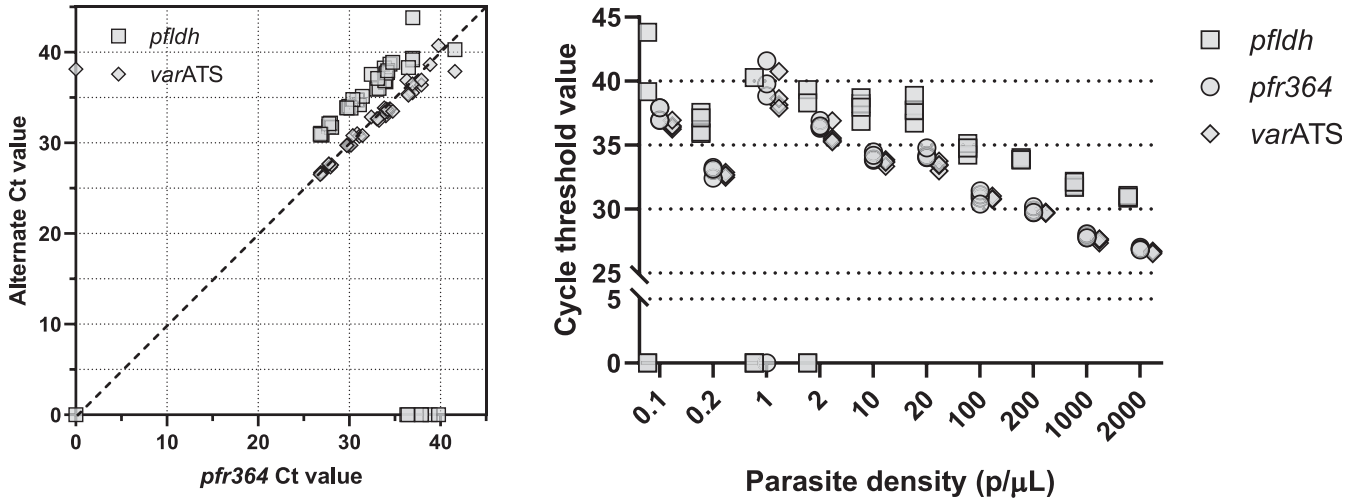

FIGURE 1. Comparison of cycle threshold (Ct) values of real-time PCR *Plasmodium falciparum* detection assays genomic DNA (gDNA) extracted from dried blood spots (DBSs) prepared by spiking into whole blood *P. falciparum* 3D7 parasites at densities of 0.1 to 2,000 parasites/µL (p/µL) were tested in parallel in real-time PCR assays targeting *pfr364*, *pfldh*, and *varATS*. Each template was tested in quadruplicate in each assay, and Ct lines were manually set for each assay at identical ΔRn values above background fluorescence and in the exponential phase of amplification. A Ct value of 0 indicates failed amplification. Left: scatterplot of Ct values of *pfr364* (*x* axis) and corresponding Ct values of *pfldh* or *var*ATS (*y* axis) during amplification of gDNA of mocked DBSs across a range of parasite densities. Dotted line indicates equality. Right: Ct values for *pfldh* (squares), *pfr364* (circles), and *var*ATS (diamonds) as a function of parasite density.

The overall prevalence of parasites was 22.9% (813/3,547) by cRDT and 61.5% (2,180/3,547) by real-time PCR (Table 1); overall, the prevalence of subpatent infections, defined as cRDT-negative and PCR-positive, was 39.3% (1,395/3,547). The prevalence of parasites in members of case households was higher than that in members of control households by both cRDT (PR: 2.21; 95% CI: 1.82–2.70) and PCR (PR: 1.74; 95% CI: 1.58–1.92). Compared with children aged < 5 years, the prevalence of parasites in adults aged > 15 years was lower by cRDT (PR: 0.65; 95% CI: 0.49–0.87) but not by PCR (PR: 1.0; 95% CI: 0.88–1.15). We observed similar patterns in both case and control households, in that adults had lower prevalence by cRDT but similar prevalence to children by PCR (Supplemental Table 3).

Parasite density distribution. The geomean (95% CI) parasite density was 12.1 p/µL (10.4–14.0) (Table 2). Predictably, geomean (95% CI) densities were highest among case children (1,717 p/µL; 1,172–2,514) and lowest among control children, who were defined as cRDT-negative (1.3 p/µL; 1.0–1.8); surprisingly, among asymptomatic household members, geomean (95% CI) densities were higher in case (5.9 p/µL; 5.0–6.9) than in control households (4.0 p/uL; 3.2–4.9) (P = 0.005 by ANOVA). Excluding index children, geomean (95% CI) densities were highest in school-age children aged 5–15 years (8.4 p/µL; 6.6–10.6) compared with younger (5.2 p/uL; 3.7–7.2) and older (3.4 p/uL; 2.9–4.0) participants (P < 0.001 by ANOVA).

Sensitivity of parasite detection by cRDT. Among the 2,180 PCR-positive infections, we computed cRDT sensitivity against PCR as the reference standard after binning PCR-estimated densities (Table 3). The overall sensitivity of cRDT was 36% (785/2,180); this declined from 98.2% (54/55) at densities in excess of 100,000 p/µL to 8.5% (55/650) at densities < 1 p/µL; above the conventional limit of detection of 100 p/µL, the aggregate sensitivity was 84% (436/517); less than 100 p/µL, the sensitivity was 21% (349/1,663). By age, the overall sensitivity was highest in children aged < 5 years (49.3%; 322/653) and lowest in adults aged > 15 years (15.9%;

TABLE 1
Parasite prevalence by detection method and subgroup

	Conventional malaria rapid diagnostic test–positive, % (*n*/*N*)	Prevalence ratio (95% CI)	Real-time PCR positive, % (*n*/*N*)	Prevalence ratio (95% CI)	Subpatent infections,* % (*n*/*N*)	Prevalence ratio (95% CI)
Overall	22.9 (813/3,547)	NA	61.5 (2,180/3,547)	NA	39.3 (1,395/3,547)	NA
Index children						
Controls	0 (0/419)	REF	33.7 (141/419)	REF	33.7 (141/419)	REF
Cases	100 (360/360)	NA	98.9 (356/360)	2.94 (2.42–3.57)	0	NA
Household members†	16.4 (453/2,768)	–	60.8 (1,683/2,768)	–	45.3 (1,254/2,768)	–
Controls	10.3 (144/1,405)	REF	44.6 (626/1,405)	REF	35.1 (493/1,405)	REF
Cases	22.7 (309/1,363)	2.21 (1.82–2.70)	77.6 (1,057/1,363)	1.74 (1.58–1.92)	55.8 (761/1,363)	1.59 (1.42–1.78)
Age (years)						
< 5	14.6 (75/513)	REF	58.1 (298/513)	REF	44.6 (229/513)	REF
5–15	26.6 (254/955)	1.82 (1.41–2.35)	65.3 (624/955)	1.12 (0.98–1.29)	40.2 (384/955)	0.90 (0.76–1.06)
≥ 15	9.5 (124/1,300)	0.65 (0.49–0.87)	58.5 (761/1,300)	1.0 (0.88–1.15)	49.3 (641/1,300)	1.10 (0.95–1.28)

NA = not applicable; REF = referent category.
* Defined as the proportion of real-time PCR-positive infections that were negative by cRDT.
† Excluding index children.

TABLE 2
Parasite density by participant type and age

	No. of PCR-positive infections	*Plasmodium falciparum* density, p/μL, geometric mean (95% CI)	*P*-value*
Overall	2,180	12.1 (10.4–14.0)	Not applicable
Type of participant			
Control child	141	1.3 (1.0–1.8)	< 0.001
Case child	356	1,717 (1,172–2,514)	
Control household member	626	4.0 (3.2–4.9)	
Case household member	1,057	5.9 (5.0–6.9)	
Age (years)†			
< 5	298	5.2 (3.7–7.2)	< 0.001
5–15	624	8.4 (6.6–10.6)	
≥ 15	761	3.4 (2.9–4.0)	

* Computed with analysis of variance comparing $\log_{10}$-transformed densities.
† Excluding case children.

121/762). Among only 1,683 asymptomatic household members, the overall sensitivity of cRDT was 25.5% (429/1,683); this was lower in adults aged > 15 years (15.8%; 120/761) than in children aged < 5 years (23.2%; 69/298) and with school-age children (38.5%; 240/624) ($P < 0.01$ for each).

Influence of parasite density on cRDT detection. We used logistic regression to estimate the probability of cRDT positivity as a function of PCR-estimated density. The overall distribution of densities remained right-skewed, and this was largely driven by cRDT-negative infections; the distribution of cRDT-positive infections was nearly normal (Figure 2A). The probability of cRDT detection was directly related to the $\log_{10}$-transformed density. Using this model, the probability of cRDT positivity at the conventional density threshold of 100 p/μL was 60.1% (95% CI: 56.6–63.6). The lowest parasite density below which the 95% CI of the probability of cRDT detection no longer crossed 0.95 was 7,377 p/μL (probability 93.1%; 95% CI: 90.9–94.8). We conditioned this model on age-groups (Figure 2B); controlling for parasite density, relative to school-age children, the probability of cRDT positivity was unchanged in children aged < 5 years (OR: 0.93; 95% CI: 0.71–1.23) but significantly lower in adults aged > 15 years (OR: 0.32; 95% CI: 0.24–0.43).

Finally, we used logistic regression to estimate the probability of cRDT positivity as a function of PCR-estimated density in models conditioned on age (Figure 2C and D) only among asymptomatic household members. Among asymptomatic household members, the lowest parasite density for which the sensitivity CI included 0.95 was 36,790 p/uL (probability of cRDT positive 91.9%; 95% CI: 88.2–94.5). When conditioned on age-group, relative to school-age children, the probability of cRDT detection was reduced in both children younger than 5 years (OR: 0.48; 95% CI: 0.34–0.69) and adults aged > 15 years (OR: 0.35; 95% CI: 0.27–0.47).

DISCUSSION

In this molecular epidemiologic study of malaria parasites in Kenya, we developed and used an ultrasensitive real-time PCR assay for *P. falciparum* parasites to describe the distribution of infections and directly measure the sensitivity of a common cRDT. We report that, among asymptomatic household members of index children who were admitted to hospital with cRDT-positive malaria, most infections were undetectable by cRDT and the overall sensitivity of the HRP2-detecting cRDT was very poor. This poor sensitivity was partially owing to a large abundance of low-density infections that was unexpected in this high-transmission setting. Finally, cRDT sensitivity was consistently lower in adults aged > 15 years, irrespective of parasite density. Collectively, these findings suggest that cRDTs are not suitable tools for effective malaria parasite case detection in asymptomatic residents of high-transmission communities.

The sensitivity of a widely used cRDT was very low. The cRDT we used detects HRP2, achieved a 95% detection rate against samples at 200 p/μL in rigorous testing,[18] and was a WHO-prequalified product. Despite this, the overall sensitivity in asymptomatic household members was 25.5%, similar to that of recent studies in Ethiopia[30] and Eswatini.[12] Quantitatively, in our study, only at densities greater than 36,790 p/μL did the probability of cRDT positivity exceed 95% in asymptomatic participants. We observed this poor performance despite cRDT testing in a high-transmission setting in which HRP2 persistence would be expected to enhance the detectability of parasites. It is unlikely that parasites with HRP2 deletions contributed to this poor performance because these parasites are rare in Western Kenya,[31] and, given that most infections in our study area are polyclonal,[32] this would require the co-occurrence of more than one HRP2-deleted (and HRP3-deleted) parasite to remain undetected. The low sensitivity and the resulting inability to identify a large proportion of low-density infections in asymptomatic, community members suggest cRDTs in high-transmission settings would be an ineffective approach to case detection.

Conventional rapid diagnostic test sensitivity was lowest in adults, in whom only 15.9% of PCR-positive infections were detected. Although this partially resulted from a more skewed density distribution in adults toward low densities, it is notable that sensitivity was poorer in adults even at similar densities. Specifically, among asymptomatic household members in models controlling for parasite density, compared with school-age children, the odds of detection with cRDT in adults was reduced by 65% (OR: 0.35; 95% CI: 0.27–0.47). Epidemiologically, enhanced detectability in children could result from HRP2 antigen accumulation in school-age children resulting from preceding infections, but this is not supported by the similar parasite point prevalence by PCR we measured in each age-group. The influence of preceding infections on cRDT sensitivity could be mediated by differential clearance rates of HRP2 by age, specifically here if HRP2 is cleared more rapidly in adults; this idea is supported by models indicating that HRP2 persistence after treatment is more prolonged in

TABLE 3
Sensitivity of conventional malaria rapid diagnostic test by parasite density and age

	PCR-estimated parasite density, p/μL										
	Any density	≥ 100,000	100,000–10,000	10,000–5,000	5,000–1,000	1,000–500	500–200	200–100	100–10	10–1	< 1
Age of all participants (years)	36.0 (785/2,180)	98.2 (54/55)	96.8 (119/123)	79.3 (23/29)	86.7 (65/75)	75.6 (31/41)	76.5 (65/85)	72.5 (79/109)	41.6 (159/382)	21.4 (135/631)	8.5 (55/650)
< 5	49.3 (322/653)	100 (37/37)	98.6 (72/73)	83.3 (10/12)	85.2 (23/27)	76.9 (10/13)	88.6 (31/35)	84.1 (37/44)	44.2 (38/86)	31.3 (51/163)	8.0 (13/163)
5–15	44.7 (342/765)	100 (17/17)	95.8 (46/48)	92.3 (12/13)	94.4 (34/36)	85.0 (17/20)	71.4 (25/35)	82.1 (32/39)	49.0 (72/147)	27.3 (56/205)	15.1 (31/205)
≥ 15	15.9 (121/762)	0 (0/1)	50 (1/2)	25 (1/4)	66.7 (8/12)	50 (4/8)	60 (9/15)	38.5 (10/26)	32.9 (49/149)	10.7 (28/263)	3.9 (11/282)
P-value	< 0.001	< 0.001	0.001	0.013	0.048	0.149	0.061	< 0.001	0.017	< 0.001	< 0.001
Age of asymptomatic household members (years)	25.5 429/1,683)	50.0 (1/2)	76.5 (13/17)	64.7 (11/17)	82.4 (42/51)	71.0 (22/31)	66.7 (38/57)	58.2 (39/67)	35.9 (117/326)	17.1 (93/545)	9.3 (53/570)
< 5	23.2 (69/298)		NA	75.0 (3/4)	66.7 (4/6)	70.0 (7/10)	50.0 (2/4)	63.6 (7/11)	45.5 (5/11)	19.2 (9/47)	20.2 (20/99)	11.3 (12/106)
5–15	38.5 (240/624)	1 (1/1)	83.3 (10/12)	85.7 (6/7)	93.1 (27/29)	84.2 (16/19)	71.0 (22/31)	80.0 (24/30)	45.4 (59/130)	24.6 (45/183)	16.5 (30/182)
≥ 15	15.8 (120/761)	0 (0/1)	0 (0/1)	25.0 (1/4)	66.7 (8/12)	50.0 (4/8)	60.0 (9/15)	38.5 (10/26)	32.9 (49/149)	10.7 (28/263)	3.9 (11/282)
P-value	< 0.001	0.157	0.168	0.127	0.068	0.124	0.740	0.005	0.003	< 0.001	< 0.001

children than in adults,[33] but it is unclear if this observation is the result of higher starting densities in children. Alternatively, HRP2 may be rendered less detectable in adults by anti-HRP2 IgG, which can be present in measurable quantities in clinical samples[34] but are of uncertain diagnostic impact.[35] Because the PCR-estimated parasite prevalence was similar in adults to other age-groups and because these represent a large proportion of prevalent, likely transmissible infections, the low sensitivity in adults further renders cRDTs unsuitable as a tool by which to identify and treat infections, and thereby efficiently reduce transmission.

To a greater degree than other studies in sub-Saharan Africa, we report a large number of infections that are below the limits of detection of conventional diagnostics. We found that 74.5% (1,254/1,683) of infections in asymptomatic household members were cRDT-negative and 33.7% of these (570/1,683) were below the putative lower limit of detection of 1 p/μL for the high-sensitivity RDT (HS-RDT).[36] Similarly skewed distributions of parasite densities are more typically reported from low-transmission sites,[37–39] which are typically defined on the basis of a far lower PCR-estimated parasite prevalence (typically less than 5%). By contrast, our PCR prevalence was 60.8%, and reports in other settings with this degree of transmission have typically reported less right-skewed distributions of parasite densities with a much larger proportion of parasites at densities greater than 100 p/μL.[19,40,41] Even though our study differed by enrolling two parallel groups of household members with different levels of risk, the prevalence was high enough in both control (44.6%) and case (77.6%) households to expect that larger proportions would have been detectable by cRDTs.[42] The reasons for this unexpectedly high prevalence of very low-density infections may include the high levels of ownership and use of insecticide-treated nets.[23] In addition, the proportion of participants who were adults and therefore most likely to harbor low-density infections was higher in our study (47%) than in Tanzania (35% > 20 years)[41] or Burkina Faso (25% > 15 years), potentially skewing density profiles.[40] These directly measured density distributions support the use in high-transmission settings of newly available HS-RDT, which has demonstrated enhanced analytical sensitivity for low-density parasitemias in Uganda,[19] Papua New Guinea,[22] and Myanmar.[43] This enhanced detectability of HRP2 should enable the identification of a substantially higher proportion of low-density infections in asymptomatic community members.

The ability to capture these low-density infections in a range of transmission settings will be enhanced by the expanding use of ultrasensitive, high-throughput, real-time PCR detection assays like our duplex *pfr364* assay, which offers a new option for robust and scalable parasite detection in molecular epidemiologic studies. Like the parasite detection assay targeting *var*ATS,[27] this assay targets a multicopy motif in the *P. falciparum* genome, specifically annealing to 22 identical sequences in the parasite genome. In direct comparisons, Ct values obtained by *pfr364* and *var*ATS assays on identical templates were highly correlated and nearly identical (Figure 1). The assay amplified a wide range of reference parasite genomes from diverse settings, and, when applied to quantitative standards during the production phase of testing, returned positive results in 51/52 replicate tests of a standard at 0.1 p/μL. In addition, the assay includes an internal human control β-tubulin to confirm gDNA extraction, and, similar to

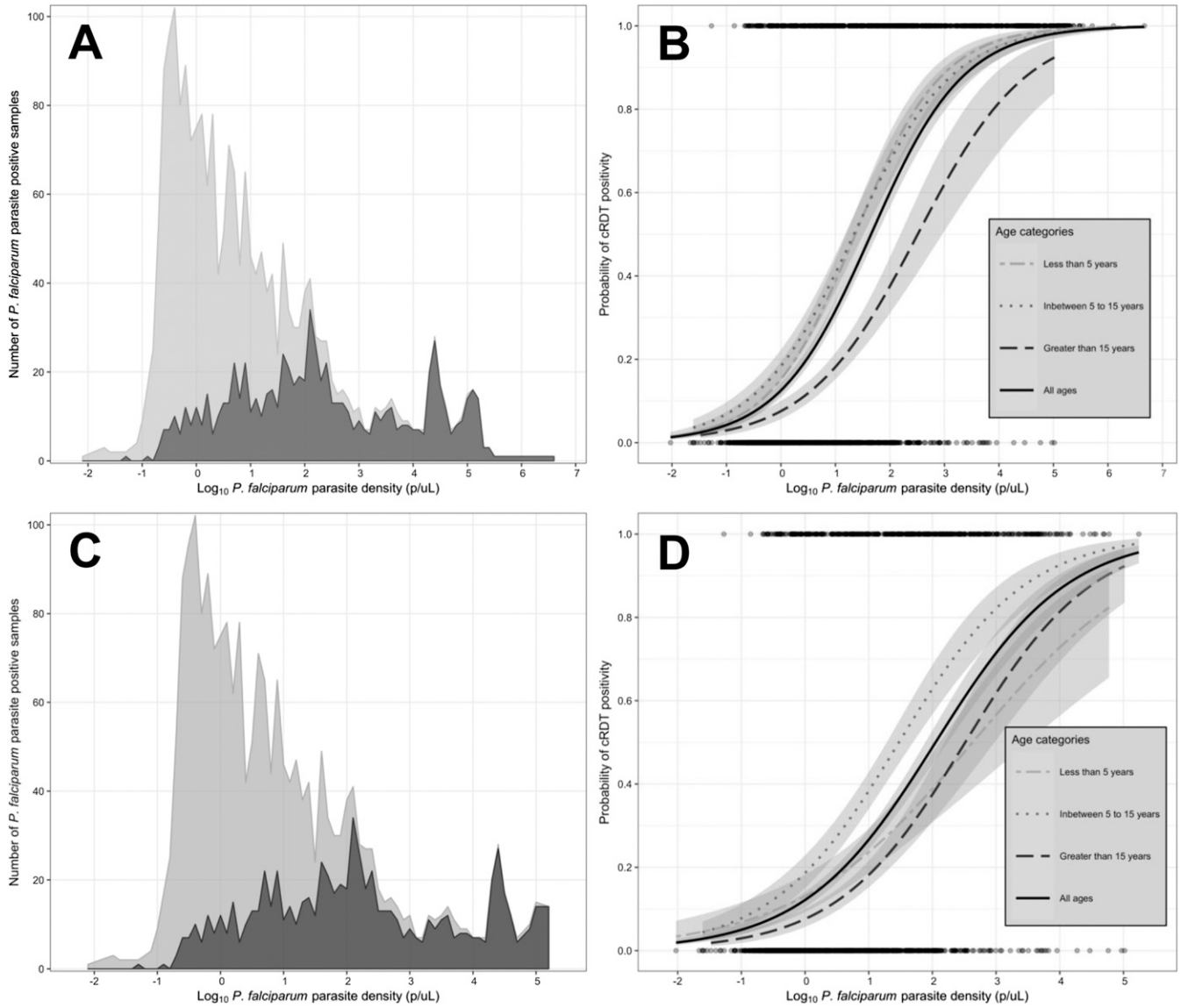

FIGURE 2. Parasite density distribution and probability of detection by conventional rapid diagnostic test (cRDT). (**A**) Distribution of parasite densities estimated by PCR among cRDT-positive (dark gray) and cRDT-negative (light gray) participants for all participants (case, control, and asymptomatic household members). (**B**) Probability of cRDT positivity as a function of PCR-estimated parasite density for all participants (case, control, and asymptomatic household members), modeled by logistic regression and stratified by age categories. Dots at top and bottom indicate positive and negative cRDT results, and gray shading indicates 95% CI of modeled probability. (**C**) Distribution of parasite densities estimated by PCR among cRDT-positive (dark gray) and cRDT-negative (light gray) participants for only asymptomatic household members. (**D**) Probability of cRDT positivity as a function of PCR-estimated parasite density for only asymptomatic household members, modeled by logistic regression and stratified by age categories. Dots at top and bottom indicate positive and negative cRDT results, and gray shading indicates 95% CI of modeled probability.

the *var*ATS assay but unlike other ultrasensitive PCR detection assays, does not require a large blood volume from venipuncture[37] or RNA extraction with reverse transcription,[44] two steps which limit application to large field studies owing to both cost and specialized sampling requirements. Given the demonstrated ability of the similarly ultrasensitive *var*ATS assay to identify gametocytemic infections[22] and therefore the likely contribution of these low-density infections to onward transmission,[42] future studies can adapt and improve these assays to better sample these cryptic infections in diverse settings.

As we previously reported with cRDT detection,[23] we observed with PCR detection the clustering of *P. falciparum* infections within the households of children with malaria. Notably, this household clustering has typically been reported from low-transmission areas, including in Southeast Asia,[45] Zambia,[46] Namibia,[47] Zanzibar,[48] Rwanda,[49] and coastal Kenya,[50] and less often investigated in high-transmission settings like ours. Potential reasons for the spatial aggregation of infections typically include shared risks of vector exposure, similar accessibility to health care, or participation in household-level chains of parasite transmission. Understanding these factors, and how they may be interrupted in high-transmission settings, could serve as a rationale for household-level interventions.

This study has some limitations. Because enrollment was triggered by hospitalized malaria cases, we sampled few

households during the low-transmission season, when parasite distributions may be quite different. Therefore, we could not compare detectability and parasite epidemiology between seasons. In addition, insofar as our observations may influence the design of RACD interventions, this approach may limit the generalizability of our findings to interventions which may use alternate triggers for screening, such as uncomplicated malaria. Our parasite density estimations could have been biased, owing to differential efficiency of gDNA extraction between samples or to stochastic variability between technical replicates at low densities. In mitigation, we observed consistent human β-tubulin amplification between samples within a very narrow range of Ct values, and we enforced additional criteria on parasite amplification output to reduce the risk of false-positives. In addition, the assay targets *P. falciparum* only and therefore would not be a suitable tool where non-*falciparum* species predominate. Finally, we did not have direct measurements of HRP2, the antigen detected by the cRDT, to better understand if HRP2 differences account for differences in parasite detectability.

We describe a large proportion of cRDT-negative infections in this high-transmission setting. Additional observations are that parasite densities in all participant subgroups were skewed toward very low densities, and therefore that the sensitivity was poor of a cRDT detecting HRP2. In addition, age mediated the probability of detecting an infection with cRDT, in that adults aged > 15 years had the lowest detectability irrespective of parasite density. These findings suggest that cRDT is not likely to be effective at detecting a large proportion of asymptomatic infections. Further steps include credible modeling of the impact on transmission of the detection and treatment of cRDT-positive infections, as well as of the incremental benefits on detectability and downstream impact of new HS-RDTs. These data and tools will enable us to test the efficiency and effectiveness of community-based detection strategies on malaria transmission in diverse settings.

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