

RESEARCH

Open Access



Malaria rapid diagnostic tests reliably detect asymptomatic *Plasmodium falciparum* infections in school-aged children that are infectious to mosquitoes

Lorenz M. Hofer^{1,2,3} , Prisca A. Kweyamba³, Rajabu M. Sayi³, Mohamed S. Chabo³, Sonali L. Maitra^{1,2,3}, Sarah J. Moore^{1,2,3,4}  and Mgeni M. Tambwe^{3*} 

Abstract

Background Asymptomatic malaria infections (*Plasmodium falciparum*) are common in school-aged children and represent a disease transmission reservoir as they are potentially infectious to mosquitoes. To detect and treat such infections, convenient, rapid and reliable diagnostic tools are needed. In this study, malaria rapid diagnostic tests (mRDT), light microscopy (LM) and quantitative polymerase chain reaction (qPCR) were used to evaluate their performance detecting asymptomatic malaria infections that are infectious to mosquitoes.

Methods One hundred seventy asymptomatic school-aged children (6–14 years old) from the Bagamoyo district in Tanzania were screened for *Plasmodium* spp. infections using mRDT (SD BIOLINE), LM and qPCR. In addition, gametocytes were detected using reverse transcription quantitative polymerase chain reaction (RT-qPCR) for all qPCR-positive children. Venous blood from all *P. falciparum* positive children was fed to female *Anopheles gambiae* sensu stricto mosquitoes via direct membrane feeding assays (DMFAs) after serum replacement. Mosquitoes were dissected for oocyst infections on day 8 post-infection.

Results The *P. falciparum* prevalence in study participants was 31.7% by qPCR, 18.2% by mRDT and 9.4% by LM. Approximately one-third (31.2%) of asymptomatic malaria infections were infectious to mosquitoes in DMFAs. In total, 297 infected mosquitoes were recorded after dissections, from which 94.9% (282/297) were derived from infections detected by mRDT and 5.1% (15/297) from subpatent mRDT infections.

Conclusion The mRDT can be used reliably to detect children carrying gametocyte densities sufficient to infect high numbers of mosquitoes. Subpatent mRDT infections contributed marginally to the pool of oocysts-infected mosquitoes.

Keywords Malaria diagnostics, *Plasmodium falciparum*, Asymptomatic malaria, Malaria transmission, *Anopheles gambiae* sensu stricto, Gametocytes

*Correspondence:

Mgeni M. Tambwe

mmohamed@ihi.or.tz

Full list of author information is available at the end of the article



© The Author(s) 2023. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated in a credit line to the data.

Introduction

Better knowledge on treatment strategies targeting the infectious reservoir of *Plasmodium falciparum* opens new avenues to tackle malaria infections that are asymptomatic and infectious to mosquitoes [1]. Asymptomatic malaria infections are common among school-aged children (6–15 years old) [2] and are characterised by higher complexity of infection [3, 4] and higher gametocyte prevalence than those with symptomatic infections [5]. Some proportions of school-aged children with asymptomatic malaria infections are particularly infectious to mosquitoes [6, 7] and consequently serve as a reservoir for *Plasmodium* transmission due to persistent asymptomatic infections [5, 8]. A mass test-and-treat (MTAT) approach for school-aged children may be a promising tool to target asymptomatic *Plasmodium* infections and reduce the transmission reservoir [9, 10], especially as school-aged children tend to be underserved by current control tools such as insecticide-treated nets (ITNs) [11].

In previous studies, mRDTs have been used for detection of asymptomatic malaria infections. For example, in community studies, a limited reduction in malaria transmission was reported from Zambia [12] while in Burkina Faso [13] no significant reductions in clinical malaria cases were observed. In Kenya, screening and treatment of school-aged children had no impact on their health or educational performance [14]. One possible explanation was the limited sensitivity of standard mRDTs in detecting low-density asymptomatic infections suggesting the need for using molecular techniques for future interventions [15, 16]. On the other hand, school-based interventions using standard mRDTs in Malawi found that MTAT reduced gametocyte prevalence and density [10].

Low-density asymptomatic infections—including those with gametocyte densities potentially infectious to mosquitoes—are often undetected by LM and mRDT, the standard diagnostic tools in malaria diagnostics. When compared to more sensitive molecular tools such as qPCR, LM and mRDT have repeatedly shown inferior performance [16–18]. In general, the likelihood of a mosquito becoming infected is dependent on gametocyte density as well as on male-to-female sex ratio of gametocytes [19]. Nonetheless, microscopic and submicroscopic gametocyte densities do infect mosquitoes [20, 21], with the latter being rarely detected by mRDTs. The question remains whether malaria infections subpatent to commercially available mRDTs contribute substantially to malaria transmission especially as low-density infections are unlikely to infect mosquitoes [5, 16].

Here, we investigated the diagnostic performance of a commercially available mRDT (SD BIOLINE) compared with LM and qPCR for detecting infectious and

asymptomatic malaria infections among school-aged children. To confirm onward transmission to mosquitoes DMFAs were performed. Our aim was to investigate whether parasite densities subpatent to mRDTs are infectious to mosquitoes in DMFAs. Using qPCR and RT-qPCR, we measured the infectiousness of microscopic and submicroscopic gametocyte densities from humans to mosquitoes.

Methods

Study design, participants and ethics

This cross-sectional study was conducted in two primary schools of Buma and Yombo located in the Bagamoyo district in coastal Tanzania. After the long rainy season that lasts from March to June, we recruited 170 asymptomatic school-aged children over a period of 10 weeks between June and the end of August 2020. Children aged 6–14 years were eligible for enrolment when written informed consent from their caregivers and assent from the children was obtained. All children screened positive for *Plasmodium* infection were enrolled for blood-drawing for DMFAs and then treated with artemether-lumefantrine (ALU). The treatment was done within 24 h of diagnosis according to national guidelines in Tanzania [22]. An overview of all study procedures is given in Fig. 1.

Sample preservation for nucleic acid extraction

All participants were finger pricked using a safety lancet (MiniCollect® Safety-Lancets 2 mm, Greiner, Austria) to obtain a capillary blood sample into an EDTA containing collection tube (Mini Collect® Tube 0.25/0.5 ml K3EDTA, Greiner, Austria). Then, 180 µL whole blood was transferred into Eppendorf tubes containing 540 µL of 1×DNA/RNA Shield™ (Zymo Research, Irvine, USA) for nucleic acid preservation until extraction of DNA (same day of preservation).

Malaria rapid diagnostic test (mRDT)

Examination of the presence of *Plasmodium* using mRDT (SD BIOLINE Malaria Ag Pf./Pan (HRPII/pLDH), Standard Diagnostic, South Korea) was executed according to the manufacturer's instructions. The mRDT detects the *P. falciparum*-specific histidine-rich protein 2 (HRPII) antigen targeting the parasites asexual blood stages and *Plasmodium* species in general via the parasite lactate dehydrogenase (pLDH), including sexual stages (gametocytes). One drop (~ 50 µL) of capillary blood was applied to the test and run with four drops of a delivered standard buffer. The test was kept on a flat surface while running and read by the laboratory technician after 15 min. The

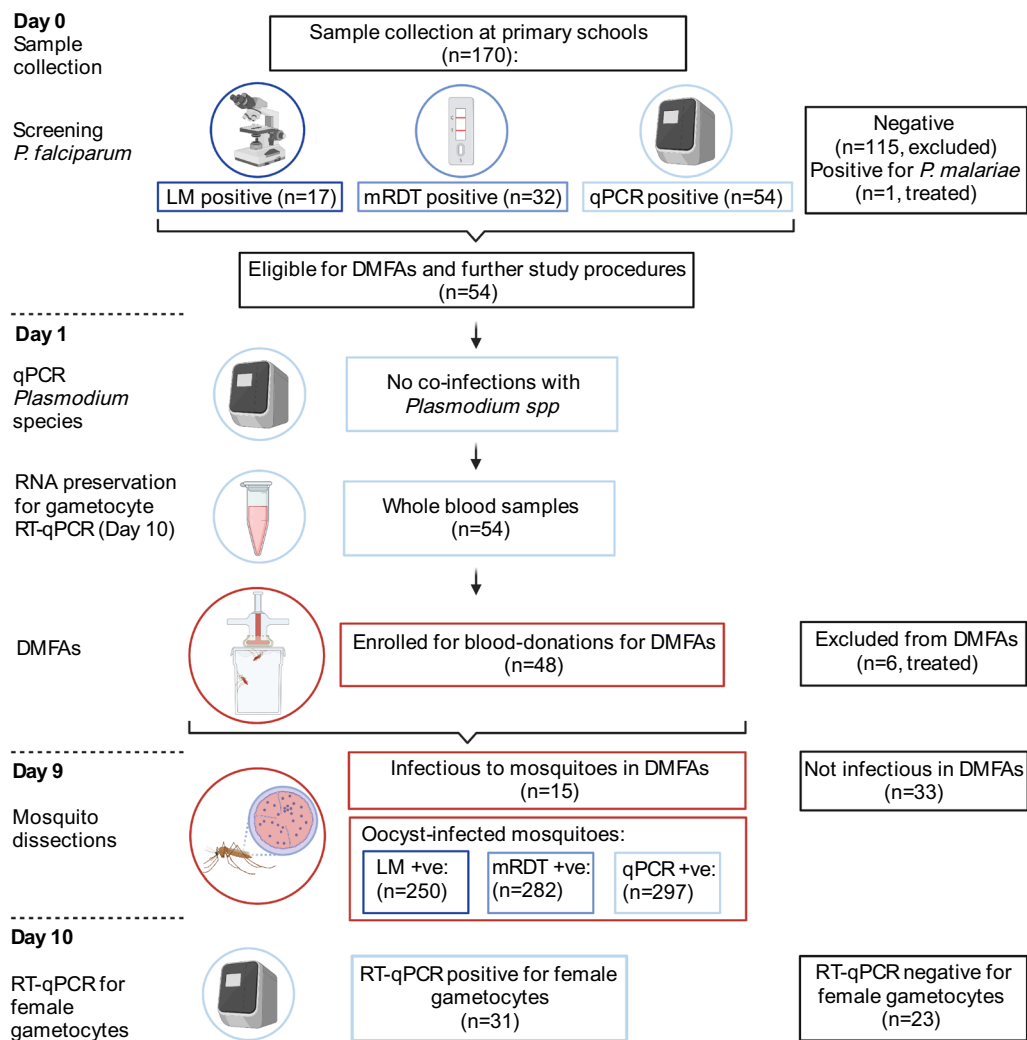


Fig. 1 Overview of the study procedures. Participants were screened systematically for *Plasmodium* infections using qPCR, mRDT, LM and RT-qPCR. All *Plasmodium falciparum* qPCR-positive children were invited to participate in DMFAs. Infectiousness of children participating in DMFAs was assessed by mosquito dissection and oocyst detection by microscopy 8 days post-infection. Numbers of infected mosquitoes were attributed to the diagnostic method, which detected the infectious participants

result was confirmed and recorded by a second member of the field team trained on reading the mRDT.

Light microscopy (LM)

Thick smears were prepared using capillary blood from the finger prick. Dried thick smears were stained for 45 min with 10% Giemsa stain and examined for the presence of *Plasmodium* parasites under a light microscope (100× magnification, oil immersion). Slides were considered negative if no *Plasmodium* parasites were detected after examination of 100 consecutive fields. After detection of the first parasites, asexual parasites (ring stages) were counted per 200 white blood

cells (WBCs) and gametocytes were counted per 500 WBCs.

Molecular detection and quantification of malaria infections by qPCR

The DNA for molecular diagnosis and quantification of *Plasmodium* infections was manually extracted from 180 µL whole blood preserved as described above using the Quick-DNA™ Miniprep kit (Zymo Research) according to the manufacturer's instructions. DNA was eluted in 50 µL elution buffer and stored at −20 °C.

Plasmodium falciparum infections were detected and quantified using the PlasQ qPCR assay [23, 24]. To

quantify parasite blood-stage parasites, the WHO international standard for *P. falciparum* quantification [23] was used. Serial dilutions ranging from 100,000 parasites/ μL —0.0001 parasites/ μL were prepared and run in triplicate. All primers and probes are listed in Additional file 1: Table S1. Amplification and qPCR measurements were performed using the Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, USA). The thermal profile used for the assay was as follows: 60 s at 95 °C; 45 cycles of 15 s at 95 °C and 45 s at 57 °C. Each reaction contained 2 μL DNA and 8 μL reaction master mix containing 1 \times Luna Universal Probe qPCR Master Mix (New England Biolabs, USA). All qPCR assays were run in duplicate with appropriate controls including Non-Template Control (NTC) and *P. falciparum* NF54 DNA as positive control (PC).

To investigate *P. falciparum* co-infections with other relevant parasite species in the Bagamoyo district [25, 26], all *Plasmodium*-positive samples were subjected to further analysis using the PlasID qPCR assay as previously described [27]. Samples were analysed with reagents, volumes and instruments as described above. Appropriate controls for all *Plasmodium* species were included. The thermal profile used for the assay was as follows: 5 min at 95 °C; 45 cycles of 15 s at 95 °C and 60 s at 55 °C.

Molecular detection of female gametocytes by RT-qPCR

Samples for ribonucleic acid (RNA) extraction were taken from all qPCR-positive (PlasQ assay) children and 150 μL of whole blood was transferred into an Eppendorf tube containing 450 μL 1 \times DNA/RNA Shield™. The tubes were mixed by inversion, spun down for 30 s and incubated for 60 min at room temperature before subsequent storage at -20 °C until further use. RNA was manually extracted using the Quick-RNA™ MiniPrep Plus kit (Zymo Research). The protocol was adapted as per manufacturer's suggestions: (i) after thawing of frozen samples, 100 μL of 1 \times DNA/RNA Shield™ was added and vortexed for 60 s at full speed; (ii) Proteinase K digestion was extended to 60 min at room temperature; (iii) after Proteinase K digestion, the samples were centrifuged for 5 min at 16,000 \times rcf to pellet all debris of red blood cells (RBCs). On-column DNase I digestion of gDNA was performed on all samples according to the manufacturer's instructions [28]. RNA was eluted in 50 μL of prewarmed (95 °C) DNase/RNase-free water and stored at -80 °C until further use.

Female gametocytes were detected using a RT-qPCR assay designed by Wampfler et al. [29, 30]. Reverse transcription, amplification and qPCR measurements were performed using the same instrument as above with the following thermal profile: 10 min at 50 °C; 60 s at 95 °C;

40 cycles of 10 s 95 °C and 60 s at 58 °C. Each reaction contained 4 μL RNA and 10 μL reaction master mix containing Luna Universal 1 \times One-Step RT-qPCR Kit (New England Biolabs, USA).

Mosquito rearing

We used the *Anopheles gambiae* sensu stricto (Ifakara strain) colony, which is reared at IHI, Bagamoyo Branch Insectary, Kingani. The colony is maintained under standard conditions at a 12:12 h dark:light cycle, $70 \pm 20\%$ humidity and 27 ± 2 °C. Larvae are fed on TetraMin® fish flakes (Tetra Ltd., UK) and reared at a density of 200 larvae (from L2 larval stage) per 1 L deionized water. Adult mosquitoes are kept in 30 \times 30 \times 30 cm containers and allowed to feed on 10% sucrose solution. Adult mosquitoes are routinely checked for microsporidia infections [31, 32]. Cages with microsporidia-infected mosquitoes are discarded and strict hygiene measures are maintained in the insectary.

Direct membrane feeding assays and dissection of mosquitoes

Unfed female mosquitoes (3–5 days old, mated) were sugar starved overnight with access to water only. The next morning, a batch of 50 female mosquitoes was transferred into paper cups. For each participant, six cups were prepared. Blood from *P. falciparum*-positive, asymptomatic school-aged children was drawn for DMFAs in lithium-heparin-coated vacutainers. Whole blood was transferred in pre-warmed 1.5 mL Eppendorf tubes centrifuged for 3 min at 300 rcf and 37 °C [33]. Autologous serum was removed and replaced with pre-warmed malaria-naïve AB serum [34] (37 °C). Mosquitoes were fed from water-jacketed glass feeders (39 °C) covered with a Parafilm® membrane. Each feeder was filled with 200 μL of blood and mosquitoes were allowed to feed for 10–15 min in the dark [35]. After the infectious blood meal, mosquitoes were kept at $75 \pm 2\%$ humidity and 27 ± 1 °C at 12:12 h dark:light cycle in a climatic chamber (AraLab, Portugal). The next morning, unfed mosquitoes were removed and allowed to feed on 10% sucrose solution. Eight days post-infection, mosquitoes were dissected and midguts stained for 10 min using 1% mercurochrome solution (Sigma, Switzerland) before examination under a compound microscope at 40 \times magnification for the presence of oocysts.

Statistical analysis

Data was entered in Excel (Microsoft, 2016) and analysed using the statistical software R and the R studio (Version 1.3.1093) interface. Sensitivity of each diagnostic test was manually calculated using 2 \times 2 contingency tables.

Proportions of positive and negative events of diagnostic tests including 95% confidence intervals (CIs) were calculated by one-sample z-tests. Positive predictive values of diagnostic tests in predicting infected mosquitoes deriving from infectious asymptomatic individuals were calculated as percentages of infected mosquitoes of the respective diagnostic tool, divided by all infected mosquitoes detected with qPCR.

Results

Study population

A total of 170 children participated in the study from Buma ($N=80$) and Yombo ($N=90$) primary schools. Mean age among study participants was 10.3 years and participants were 64% female ($N=109$) and 36% male ($N=61$).

Malaria prevalence detected by molecular and standard diagnostic tools

We screened 170 asymptomatic school-aged children for malaria infection using three different diagnostic methods (qPCR, mRDT, LM). Of 170, 54 were qPCR-positive for *P. falciparum* infection (31.7%, PlasQ assay, Fig. 2A) and one was positive for *Plasmodium malariae* (0.6%, PlasID assay) infection. No mixed infections were detected. Asexual blood-stage densities of *P. falciparum* molecularly quantified by qPCR ranged from 0.1 parasites/ μL to 275,000 parasites/ μL with a median parasite density of 6 parasites/ μL . About 18.2% of study participants were mRDT positive (Fig. 2A) with median parasite density of 35 parasites/ μL as quantified by qPCR and 9.4% were LM positive (Fig. 2A) with a median parasite density of 1493 parasites/ μL . Approximately eighteen percent (18.2%) of the study participants were tested positive on gametocyte carriage using RT-qPCR and 2.3% of all study participants had microscopically detectable gametocytes. Gametocyte densities as quantified by LM ranged from 16 gametocytes/ μL to 128 gametocytes/ μL , with median gametocyte density of 48 gametocytes/ μL . All LM-positive participants were also positive for mRDT and qPCR. These results are summarized in Table 1. Sensitivity of mRDT and LM compared to qPCR was 56.3% and 30.9% respectively and both methods showed >99% specificity (Table 2).

Infectiousness of asymptomatic individuals through DMFAs relative to detection by molecular or standard diagnostic tools

Of the 54 qPCR (PlasQ) *P. falciparum*-positive children, six were not enrolled for DMFAs and were treated directly with ALU. Reasons for exclusion were developing

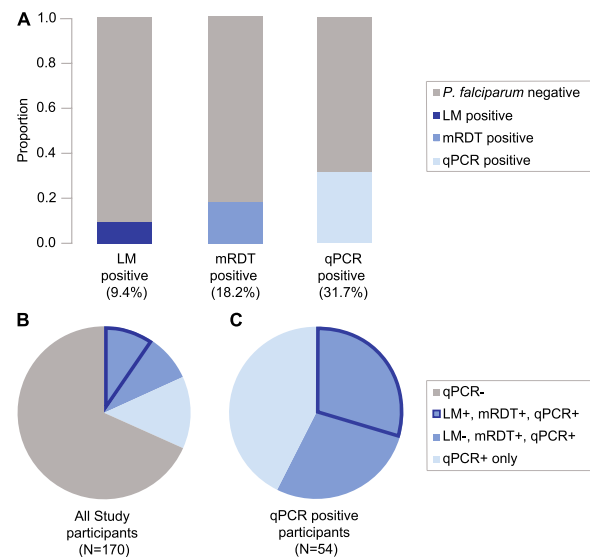


Fig. 2 Malaria prevalence detected separately by each of the three diagnostic methods **A**; LM (9.4%), mRDT (18.2%) and qPCR (31.7%). Proportions **B** of all study participants ($N=170$) either negative (68.3%, grey) or as detected by all three methods together (9.4%, dark blue margin), by mRDT and qPCR but not LM (8.8%, medium blue) or qPCR only (13.5%, light blue). Proportions **C** of all qPCR-positive participants ($N=54$) as detected by all three methods together (29.6%, dark blue margin), by mRDT and qPCR but not LM (27.8%, medium blue) or qPCR only (42.6%, light blue). LM light microscopy; mRDT malaria rapid diagnostic test; qPCR quantitative polymerase chain reaction

symptoms ($n=2$), withdrawn from the study by caregiver ($n=1$), fear of needle pricking for blood drawing ($n=2$) and insufficient mosquito numbers for DMFAs ($n=1$). The remaining 48 qPCR-positive children were enrolled for DMFAs. From those, 15 (31.2%, Fig. 3A) were infectious to mosquitoes and infected at least one mosquito. Results of DMFAs are summarized in Table 3. In total, we dissected 5022 mosquitoes on day 8 post-infection with an average of 105 dissected mosquitoes per DMFA. We recorded 297 oocyst-infected mosquitoes from the 15 infectious DMFAs with median oocyst intensity of two oocysts per infected mosquito. The highest oocyst number in a single midgut reached 29. Only four participants infected 253 out of 297 oocyst-infected mosquitoes (85%, Fig. 3C). The remaining 15% of oocyst-infected mosquitoes were infected from 11 infectious participants.

All infected mosquitoes derived from infectious participants detected by qPCR and RT-qPCR. (Table 3 and Fig. 3B). Nine participants detected by mRDT infected 94.9% (282) of all infected mosquitoes. Consequently, subpatent mRDT infections accounted for 5.1% (15) of all infected mosquitoes only. Eight participants detected by LM-infected 84.2% (250) of all infected

Table 1 *Plasmodium* infections in 170 asymptomatic school-aged children detected by qPCR assays, mRDT and LM

Diagnostics	<i>n</i> ^a	<i>n</i> / <i>N</i> ^b (%) ^c	95% CI (%) ^d	Parasite densities per diagnostic method
qPCR				
<i>P. falciparum</i> (PlasQ assay)	54	31.7	25.2–39.1	6 (0.1–275,000)
<i>P. malariae</i> (PlasID assay)	1	0.6	0.001–3.3	–
mRDT	31	18.2	13.2–24.7	35 (0.1–275,000) ^e
LM				
<i>P. falciparum</i> all stages	16	9.4	5.9–14.7	1439 (133–173,000) ^f
<i>P. falciparum</i> asexual stages	10	5.8	3.2–10.5	–
<i>P. falciparum</i> gametocytes	6	2.3	0.1–5.9	48 (16–128) ^g
<i>P. malariae</i> all stages	1	0.6	0.001–3.3	–
Gametocyte-specific RT-qPCR				
<i>P. falciparum</i> female gametocytes (pfs25)	31	18.2	13.2–24.7	–

qPCR quantitative polymerase chain technique; mRDT malaria rapid diagnostic test; LM light microscopy; RT-qPCR reverse transcriptase quantitative polymerase chain technique

^a *n* = number of positive participants

^b *N* = 170 participants tested in total

^c *n*/*N* (%) = percent positive

^d 95% CI = 95% confidence interval

^e Median parasite densities quantified by PlasQ as detected by qPCR and mRDT in parasites/μL. Range of densities in brackets

^f Median asexual parasite densities quantified by LM in parasites/μL. Range of densities in brackets

^g Median sexual parasite densities quantified by LM, in gametocytes/μL. Range of densities in brackets

Table 2 Sensitivity and specificity of mRDT and LM compared to qPCR in 170 study participants

Diagnostics	qPCR positive (<i>n</i> = 55)	qPCR negative (<i>n</i> = 115)	Sensitivity % (95% CI)	Specificity % (95% CI)
mRDT positive (<i>n</i> = 32)	31	1	56.3	99.1
mRDT negative (<i>n</i> = 138)	24	114		
LM positive (<i>n</i> = 17)	17	0	30.9	100
LM negative (<i>n</i> = 153)	38	115		

qPCR quantitative polymerase chain reaction; mRDT malaria rapid diagnostic test; LM light microscopy

mosquitoes. This also represents the contributions of microscopic (84.2%) and sub-microscopic (15.8%) infections from human to mosquito transmission as recorded by this study (Fig. 3D).

Discussion

The aim of this study was to compare the performance of standard diagnostic tools (mRDT and LM) to a sensitive molecular tool (qPCR) in detecting asymptomatic malaria infections that are infectious to mosquitoes. To

our knowledge, this is the first study that specifically assesses the performance of an mRDT including the onward transmission of detected infections to mosquitoes using DMFAs.

We observed that nearly one-third (31.2%, 15/48, Fig. 3A) of all asymptomatic malaria infections were infectious to mosquitoes in DMFAs. Of those, 60% were detected by mRDT and 53% by LM (Fig. 3B). The finding that most asymptomatic *P. falciparum* infections that are infectious to mosquitoes are detected by standard diagnostic tools is consistent with evidence from other African countries [5, 6, 36]. When comparing the performance of the mRDT and LM regarding infectiousness to mosquitoes, only one infectious participant was detected by mRDT but subpatent to microscopy (Fig. 3C), suggesting that with improved microscopy (e.g. using a second microscopist for proof-reading) the performance of mRDT and LM would be nearly equal. Of all infected mosquitoes, 95% derived from infections that were detected by mRDT and 85% of infections detected by LM. A small proportion of infected mosquitoes (5%) derived from infections subpatent to both standard diagnostic tools and was detected by qPCR only.

Furthermore, we report that 85% of all infected mosquitoes derived from only four children; all of them were detected by mRDT and three were LM-positive for gametocytes (Fig. 3C). Our finding that a very small

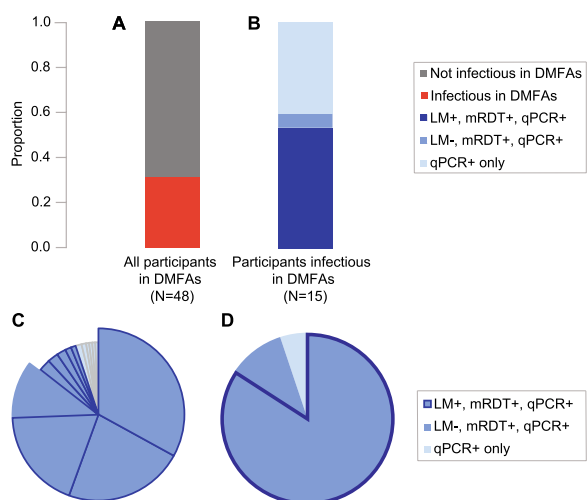


Fig. 3 **A** Proportion of study participants infectious to at least one mosquito in DMFAs (15/48, 31.2%) from all qPCR-positive study participants enrolled for DMFAs (N=48). **B** Participants infectious to mosquitoes (N=15) as detected with all three methods (eight participants, dark blue margin), mRDT and qPCR but not LM (nine participants, medium blue) and qPCR only (six participants, light blue). **C** Oocyst-infected mosquitoes per participant, majority of mosquitoes infected by only four participants (big slices, 85%). **D** Oocyst-infected mosquitoes from blood sources as detected with all three methods (84.2%, N=250, dark blue margin), mRDT and qPCR positive but not LM (94.9%, N=282 medium blue) and qPCR only (5.1%, N=15 light blue). LM light microscopy; mRDT malaria rapid diagnostic test; qPCR quantitative polymerase chain reaction; DMFA direct membrane feeding assay

proportion of our study population accounts for the vast majority of mosquito infections supports recent findings from Uganda [5]. Also, exclusively from these four participants, we observed mosquitoes with higher oocyst densities that are more likely to develop higher densities of sporozoites [37]. These mosquitoes remain infectious for a longer time than their counterparts with mostly one or rarely two oocysts per midgut, as observed in mosquito infections from only qPCR-positive participants [19].

We have demonstrated that mRDTs identified a slightly higher number of the qPCR-positive infections compared to LM. While LM performance may be further improved by including a second microscopist, the success of the mRDT was encouraging and indicates its potential to be used alone for detection of infectious asymptomatic individuals. The advantage of using mRDTs over LM is that it is convenient and does not need highly skilled experts to interpret the results. Our findings on sensitivity and specificity concur with a previous study that evaluated the same mRDT product in Tanzania [38]. Also similar differences in sensitivity between LM (30.9%) and qPCR (100%) as reported here have been reported from low transmission settings before [39]. The proportion of sub-patent infections in standard diagnostic tools that are only detected with molecular assays is very similar to previous findings from a low-transmission area in coastal Kenya [6].

Table 3 Results of DMFAs and positive predictive value for oocyst-infected mosquitoes per diagnostic method

Diagnostics	DMFAs resulting in mosquito infection ^a		Proportion of oocyst-infected mosquitoes ^b		Positive predictive value ^c % (n/qPCR)
	n/N	% (95% CI)	n/N	% (95% CI)	
qPCR					
<i>Plasmodium falciparum</i> (PlasQ)	15/48	31.2 (19.9–45.3)	297/1610	18.4 (16.6–20.4)	100 (297/297)
mRDT					
<i>P. falciparum</i>	9/31	29.0 (16.1–46.5)	282/1005	28.1 (25.3–30.9)	95.0 (282/297)
LM					
<i>P. falciparum</i> asexuals only	3/10	30 (10.8–60.3)	18/391	4.6 (2.9–7.1)	6.1 (18/297)
<i>P. falciparum</i> gametocytes	5/6	83.3 (43.6–96.9)	232/643	36.0 (32.5–39.9)	78.1 (232/297)
<i>P. falciparum</i> all stages	8/16	50 (27.9–72)	250/930	26.8 (24.0–29.8)	84.2 (250/297)
RT-qPCR					
<i>P. falciparum</i> female gametocytes (pfs25)	15/31	48.4 (32.0–65.1)	297/1610	18.4 (16.6–20.4)	100 (297/297)

qPCR quantitative polymerase chain reaction; mRDT malaria rapid diagnostic test; LM light microscopy; RT-qPCR reverse transcription quantitative polymerase chain reaction; DMFA direct membrane feeding assay

^a All 48 participants enrolled for DMFAs were qPCR positive for *P. falciparum*; n/N is the proportion of DMFAs resulting in at least one infected mosquito (n) out of all infections detected by the corresponding diagnostic method (N)

^b Proportion of mosquitoes scored positive by microscopy for oocysts on day 8 post-infection (n) out of total mosquitoes dissected of all DMFAs that resulted in at least one infected mosquito with the corresponding diagnostic method (N)

^c Positive predictive value for infected mosquitoes by diagnostic method compared to qPCR, which predicted 100% of all infected mosquitoes resulting from DMFAs

The reported malaria prevalence places the Bagamoyo district as a moderate transmission area [40], similar to other coastal regions in East Africa, e.g. in Kenya [6]. Results presented in this study are in line with broad scientific consensus regarding the performance of all three diagnostic methods in asymptomatic malaria infections as reported elsewhere [20, 41, 42].

In our study, a small proportion of children was LM positive for gametocytes, but when applying molecular diagnostics, we found that nearly every fifth child carried female gametocytes as detected by RT-qPCR. These results were not surprising and comparable to gametocyte prevalence detected by LM and RT-qPCR in previous studies from Tanzania [2, 43] and other low and moderate transmission areas in sub-Saharan Africa [6, 36, 42]. Results presented here are limited because we have not screened for male gametocytes or quantified both female and male gametocytes using molecular methods.

Measuring the transmissibility of malaria infections using DMFAs has several limitations [34]. Here, we conducted DMFAs with serum replacement to remove transmission-blocking attributes and to increase the transmission potential of parasite isolates in the blood samples fed to mosquitoes [44]. Thereby, loss of infectiousness of gametocytes when fed through membranes [45] can be partly compensated. We observed mostly low intensities of oocyst infections with median oocyst density of two in infected midguts. This is what is commonly observed in natural infections and xenodiagnostic surveys [46]. While oocyst detection is regarded as standard end point for measuring parasite transmission in membrane feeding assays (MFAs) [46], dissection for oocysts does not reflect the true onward-transmission potential of mosquitoes. Including the detection of sporozoites would help to better understand the infectiousness of mosquitoes.

Conclusion

Commercially available mRDTs may be a suitable and convenient tool to identify asymptomatic malaria infections that are infectious to mosquitoes. Although mRDTs failed to detect a substantial number of asymptomatic malaria infections compared to the total infections detected by qPCR, the mRDT detected infections that accounted for 95% of all oocyst-infected mosquitoes after DMFAs. Many low-density infections that are subpatent to mRDTs could become infectious to mosquitoes at a later time point. Therefore, repeated testing with mRDTs and treatment may be required if implemented as an intervention to reduce the reservoir of transmission and numbers of infectious mosquitoes in a community efficiently [5, 47]. Finally, as our study was comparatively

small, we would recommend testing larger cohorts of school-aged children in additional settings to increase statistical power and if possible include more sensitive mRDTs for the evaluation of diagnostic tools [48].

Abbreviations

mRDT	Malaria rapid diagnostic test
qPCR	Quantitative polymerase chain reaction
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
LM	Light microscopy
DMFA	Direct membrane feeding assay
MTAT	Mass test and treat
IPT	Intermittent preventive treatment
ALU	Artemether–lumefantrine
DNA	Deoxyribonucleic acid
HRPII	Histidine-rich protein 2
pLDH	Parasite lactate dehydrogenase
WBC	White blood cell
RBC	Red blood cell
RNA	Ribonucleic acid
18 s rDNA	<i>Pan-plasmodium</i> 18S ribosomal DNA sequence
VarATS	<i>P. falciparum</i> -specific acidic terminal sequence of the var genes
IHI	Ifakara Health Institute
NIBSC	National Institute for Biological Standards and Control
CI	Confidence interval

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13071-023-05761-w>.

Additional file 1: Oligos used in this study.

Additional file 2: Complete data set of the present study.

Acknowledgements

The authors thank all children, caregivers and teachers from Yombo, Buma, Chamgoi and Chasimba for their participation in the study. We thank Prof. Claudia Daubenberger and Dr. Tobias Schindler for help with molecular assays and molecular quantification of parasites. We thank Dr. Tibebe Habtewold for help with optimizing mosquito colonies and husbandry and Profs. George Christophides and Nikolai Windbichler for their support and advice. Also, we are grateful to Dr. Amanda Ross for critical reading of the statistical section and to Dr. Nadja Wipf for critical reading of the whole manuscript.

Author contributions

LMH, MMT and SJM designed the study. LMH, PAK, MMT, RMS, SLM conducted data collection in the laboratory and insectary. MMT and SM coordinated field activities and MMT, SLM, MSC conducted screenings in the field. SLM coordinated data collection and storage. RMS reared mosquitoes for DMFAs. LMH and SJM analysed the data. LMH wrote the initial draft manuscript. SJM, MMT, PAK, RMS, SLM and MSC provided critical comments on progressive drafts. All authors read and approved the final manuscript.

Funding

Funding for LMH was received from Rudolf Geigy Foundation (RGF) Switzerland and Freiwillige Akademische Gesellschaft Basel (FAG), Switzerland. Laboratory expenses were partially funded by Novartis Foundation for Biomedical Research, Basel, Switzerland, grant no. #18C189. Funding for MMT, PAK, SJM was received from Bill and Melinda Gates Foundation (BMGF), grant no. OPP1158151.

Availability of data and materials

All data generated or analysed during this study are included in this published article as Additional file 2.

Declarations

Ethics approval and consent to participate

This study received ethical clearance by the Ethical Commission for North-western Switzerland (EKNZ, Req-2018–01019), Institutional Review Board (IRB) of IHI (IHI/IRB/No: 11–2019), and by the National Institute for Medical Research (NIMR), Tanzania (NIMR/HQ/R.8a/Vol. IX/3069). All participants orally assented to participate in the study and written consent was obtained for all participants from their caregivers.

Consent for publication

Permission to publish was granted by the National Institute for Medical Research (NIMR), Tanzania under Ref. No: NIMR/HQ/P.12 VOL XXXV/75.

Competing interests

All authors declare no competing interests.

Author details

¹Vector Biology Unit, Department of Epidemiology and Public Health, Swiss Tropical and Public Health, Institute, Kreuzstrasse 2, 4123 Allschwil, Basel, Switzerland. ²University of Basel, Petersplatz 1, 4001 Basel, Switzerland. ³Vector Control Product Testing Unit (VCPTU) Ifakara Health Institute, Environmental Health, and Ecological Sciences, 74, Bagamoyo, Tanzania. ⁴The Nelson Mandela African Institution of Science and Technology (NM-AIST), 447, Tengeru, Arusha, Tanzania.

Received: 24 November 2022 Accepted: 29 March 2023

Published online: 30 June 2023

References

- Stone W, Mahamar A, Sanogo K, Sinaba Y, Niambale SM, Sacko A, et al. Pyronaridine-artesunate or dihydroartemisinin-piperaquine combined with single low-dose primaquine to prevent *Plasmodium falciparum* malaria transmission in Ouélessébougou, Mali: a four-arm, single-blind, phase 2/3, randomised trial. *Lancet Microbe*. 2022;3:e41–51. [https://doi.org/10.1016/s2666-5247\(21\)00192-0](https://doi.org/10.1016/s2666-5247(21)00192-0).
- Sumari D, Mwingira F, Selemeni M, Mugasa J, Mugittu K, Gwakisa P. Malaria prevalence in asymptomatic and symptomatic children in Kiwan-gwa, Bagamoyo district, Tanzania. *Malar J*. 2017;16:222. <https://doi.org/10.1186/s12936-017-1870-4>.
- Felger I, Maire M, Bretscher MT, Falk N, Tladen A, Sama W, et al. The dynamics of natural *Plasmodium falciparum* infections. *PLoS One*. 2012;7:e45542. <https://doi.org/10.1371/journal.pone.0045542>.
- Buchwald AG, Sorkin JD, Sixpence A, Chimenya M, Damson M, Wilson ML, et al. Association between age and *Plasmodium falciparum* infection dynamics. *Am J Epidemiol*. 2019;188:169–76. <https://doi.org/10.1093/aje/kwy213>.
- Andolina C, Rek JC, Briggs J, Okoth J, Musiime A, Ramjith J, et al. Sources of persistent malaria transmission in a setting with effective malaria control in eastern Uganda: a longitudinal, observational cohort study. *Lancet Infect Dis*. 2021;21:1568–78. [https://doi.org/10.1016/s1473-3099\(21\)00072-4](https://doi.org/10.1016/s1473-3099(21)00072-4).
- Gonçalves BP, Kapulu MC, Sawa P, Guelbéogo WM, Tiono AB, Grignard L, et al. Examining the human infectious reservoir for *Plasmodium falciparum* malaria in areas of differing transmission intensity. *Nat Commun*. 2017;8:1133. <https://doi.org/10.1038/s41467-017-01270-4>.
- Lin Ouédraogo A, Gonçalves BP, Gnémé A, Wenger EA, Guelbeogo MW, Ouédraogo A, et al. Dynamics of the human infectious reservoir for malaria determined by mosquito feeding assays and ultrasensitive malaria diagnosis in Burkina Faso. *J Infect Dis*. 2015;213:90–9. <https://doi.org/10.1093/infdis/jiv370>.
- Barry A, Bradley J, Stone W, Guelbeogo MW, Lanke K, Ouedraogo A, et al. Higher gametocyte production and mosquito infectivity in chronic compared to incident *Plasmodium falciparum* infections. *Nat Commun*. 2021;12:2443. <https://doi.org/10.1038/s41467-021-22573-7>.
- Cohee LM, Nankabirwa JI, Greenwood B, Djimde A, Mathanga DP. Time for malaria control in school-age children. *Lancet Child Adolesc Health*. 2021;5:537–8. [https://doi.org/10.1016/s2352-4642\(21\)00158-9](https://doi.org/10.1016/s2352-4642(21)00158-9).
- Cohee LM, Valim C, Coalson JE, Nyambalo A, Chilombe M, Ngwira A, et al. School-based screening and treatment may reduce *P. falciparum* transmission. *Sci Rep*. 2021;11:6905. <https://doi.org/10.1038/s41598-021-86450-5>.
- Olopeju B, Choiriyah I, Lynch M, Acosta A, Blaufuss S, Filemyr E, et al. Age and gender trends in insecticide-treated net use in sub-Saharan Africa: a multi-country analysis. *Malar J*. 2018;17:423. <https://doi.org/10.1186/s12936-018-2575-z>.
- Larsen DA, Bennett A, Silumbe K, Hamainza B, Yukich JO, Keating J, et al. Population-wide malaria testing and treatment with rapid diagnostic tests and artemether-lumefantrine in southern Zambia: a community randomized step-wedge control trial design. *Am J Trop Med Hyg*. 2015;92:913–21. <https://doi.org/10.4269/ajtmh.14-0347>.
- Tiono AB, Ouédraogo A, Ogutu B, Diarra A, Coulibaly S, Gansané A, et al. A controlled, parallel, cluster-randomized trial of community-wide screening and treatment of asymptomatic carriers of *Plasmodium falciparum* in Burkina Faso. *Malar J*. 2013;12:79. <https://doi.org/10.1186/1475-2875-12-79>.
- Halliday KE, Okello G, Turner EL, Njagi K, McHaro C, Kengo J, et al. Impact of intermittent screening and treatment for malaria among school children in Kenya: a cluster randomised trial. *PLoS medicine*. 2014;11:e1001594. <https://doi.org/10.1371/journal.pmed.1001594>.
- Cook J, Xu W, Msellem M, Vonk M, Bergström B, Gosling R, et al. Mass screening and treatment on the basis of results of a *Plasmodium falciparum*-specific rapid diagnostic test did not reduce malaria incidence in Zanzibar. *J Infect Dis*. 2015;211:1476–83. <https://doi.org/10.1093/infdis/jiu655>.
- Hofmann NE, Gruenberg M, Nate E, Ura A, Rodriguez-Rodriguez D, Salib M, et al. Assessment of ultra-sensitive malaria diagnosis versus standard molecular diagnostics for malaria elimination: an in-depth molecular community cross-sectional study. *Lancet Infect Dis*. 2018;18:1108–16. [https://doi.org/10.1016/s1473-3099\(18\)30411-0](https://doi.org/10.1016/s1473-3099(18)30411-0).
- Bousema T, Okell L, Felger I, Drakeley C. Asymptomatic malaria infections: detectability, transmissibility and public health relevance. *Nat Rev Microbiol*. 2014;12:833–40. <https://doi.org/10.1038/nrmicro3364>.
- Das S, Jang IK, Barney B, Peck R, Rek JC, Arinaitwe E, et al. Performance of a high-sensitivity rapid diagnostic test for plasmodium falciparum malaria in asymptomatic individuals from Uganda and Myanmar and naive human challenge infections. *Am J Trop Med Hyg*. 2017;97:1540–50. <https://doi.org/10.4269/ajtmh.17-0245>.
- Bradley J, Stone W, Da DF, Morlais I, Dicko A, Cohuet A, et al. Predicting the likelihood and intensity of mosquito infection from sex specific *Plasmodium falciparum* gametocyte density. *eLife*. 2018. <https://doi.org/10.7554/eLife.34463>.
- Okell LC, Bousema T, Griffin JT, Ouédraogo AL, Ghani AC, Drakeley CJ. Factors determining the occurrence of submicroscopic malaria infections and their relevance for control. *Nat Commun*. 2012;3:1237. <https://doi.org/10.1038/ncomms2241>.
- Lin JT, Saunders DL, Meshnick SR. The role of submicroscopic parasitemia in malaria transmission: what is the evidence? *Trends Parasitol*. 2014;30:183–90. <https://doi.org/10.1016/j.pt.2014.02.004>.
- MoHSW. National guidelines for diagnosis and treatment of malaria. Dar es Salaam: Ministry of Health and Social Welfare; 2006.
- Kamau E, Alemayehu S, Feghali KC, Saunders D, Ockenhouse CF. Multiplex qPCR for detection and absolute quantification of malaria. *PLOS ONE*. 2013;8:e71539. <https://doi.org/10.1371/journal.pone.0071539>.
- Hofmann N, Mwingira F, Shekalaghe S, Robinson LJ, Mueller I, Felger I. Ultra-sensitive detection of *Plasmodium falciparum* by amplification of multi-copy subtelomeric targets. *PLoS Med*. 2015;12:e1001788. <https://doi.org/10.1371/journal.pmed.1001788>.
- Schindler T, Jongo S, Studer F, Mpina M, Mwangoka G, Mswata S, et al. Two cases of long-lasting, sub-microscopic *Plasmodium malariae* infections in adults from coastal Tanzania. *Malar J*. 2019;18:149. <https://doi.org/10.1186/s12936-019-2787-x>.
- Tarimo BB, Nyasembe VO, Ngasala B, Basham C, Rutagi IJ, Muller M, et al. Seasonality and transmissibility of *Plasmodium ovale* in Bagamoyo District Tanzania. *Parasit Vector*. 2022;15:56. <https://doi.org/10.1186/s13071-022-05181-2>.
- Schindler T, Robaina T, Sax J, Bieri JR, Mpina M, Gondwe L, et al. Molecular monitoring of the diversity of human pathogenic malaria species in

- blood donations on Bioko island Equatorial Guinea. *Malar J.* 2019;18:9. <https://doi.org/10.1186/s12936-019-2639-8>.
28. Research Z: Quick-RNA™ Miniprep Plus Kit RNA from any sample. 2020.
 29. Wampfler R, Mwingira F, Javati S, Robinson L, Betuela I, Siba P, et al. Strategies for detection of *Plasmodium* species gametocytes. *PLoS One.* 2013;8:e76316. <https://doi.org/10.1371/journal.pone.0076316>.
 30. Gruenberg M, Hofmann NE, Nate E, Karl S, Robinson LJ, Lanke K, et al. qRT-PCR versus IFA-based quantification of male and female gametocytes in low-density *Plasmodium falciparum* infections and their relevance for transmission. *J Infect Dis.* 2019;221:598–607. <https://doi.org/10.1093/infdis/jiz420>.
 31. Herren JK, Mbaisi L, Mararo E, Makhulu EE, Mobegi VA, Butungi H, et al. A microsporidian impairs *Plasmodium falciparum* transmission in *Anopheles arabiensis* mosquitoes. *Nat Commun.* 2020;11:2187. <https://doi.org/10.1038/s41467-020-16121-y>.
 32. Musiime AK, Okoth J, Conrad M, Ayo D, Onyige I, ReK J, et al. Is that a real oocyst? Insectary establishment and identification of *Plasmodium falciparum* oocysts in midguts of *Anopheles* mosquitoes fed on infected human blood in Tororo, Uganda. *Malar J.* 2019;18:287. <https://doi.org/10.1186/s12936-019-2922-8>.
 33. Sangare I, Michalakis Y, Yameogo B, Dabire R, Morlais I, Cohuet A. Studying fitness cost of *Plasmodium falciparum* infection in malaria vectors: validation of an appropriate negative control. *Malar J.* 2013;12:2. <https://doi.org/10.1186/1475-2875-12-2>.
 34. Bousema T, Churcher TS, Morlais I, Dinglasan RR. Can field-based mosquito feeding assays be used for evaluating transmission-blocking interventions? *Trends Parasitol.* 2013;29:53–9. <https://doi.org/10.1016/j.pt.2012.11.004>.
 35. Habtewold T, Tapanelli S, Masters EKG, Hoermann A, Windbichler N, Christophides GK. Streamlined SMFA and mosquito dark-feeding regime significantly improve malaria transmission-blocking assay robustness and sensitivity. *Malar J.* 2019;18:24. <https://doi.org/10.1186/s12936-019-2663-8>.
 36. Tadesse FG, Slater HC, Chali W, Teelen K, Lanke K, Belachew M, et al. The relative contribution of symptomatic and asymptomatic *Plasmodium vivax* and *Plasmodium falciparum* infections to the infectious reservoir in a low-endemic setting in Ethiopia. *Clin Infect Dis.* 2018;66:1883–91. <https://doi.org/10.1093/cid/cix1123>.
 37. Stone WJ, Eldering M, van Gemert GJ, Lanke KH, Grignard L, van de Vegte-Bolmer MG, et al. The relevance and applicability of oocyst prevalence as a read-out for mosquito feeding assays. *Sci Rep.* 2013;3:3418. <https://doi.org/10.1038/srep03418>.
 38. Manjurano A, Omolo JJ, Lyimo E, Miyaye D, Kishamawe C, Matamba LE, et al. Performance evaluation of the highly sensitive histidine-rich protein 2 rapid test for *Plasmodium falciparum* malaria in North-West Tanzania. *Malar J.* 2021;20:58. <https://doi.org/10.1186/s12936-020-03568-z>.
 39. Satoguina J, Walther B, Drakeley C, Nwakanma D, Oriero EC, Correa S, et al. Comparison of surveillance methods applied to a situation of low malaria prevalence at rural sites in the Gambia and Guinea Bissau. *Malar J.* 2009;8:274. <https://doi.org/10.1186/1475-2875-8-274>.
 40. Thawer SG, Chacky F, Runge M, Reaves E, Mandike R, Lazaro S, et al. Sub-national stratification of malaria risk in mainland Tanzania: a simplified assembly of survey and routine data. *Malar J.* 2020;19:177. <https://doi.org/10.1186/s12936-020-03250-4>.
 41. Drakeley C, Reyburn H. Out with the old, in with the new: the utility of rapid diagnostic tests for malaria diagnosis in Africa. *Trans R Soc Trop Med Hyg.* 2009;103:333–7. <https://doi.org/10.1016/j.trstmh.2008.10.003>.
 42. Slater HC, Ross A, Felger I, Hofmann NE, Robinson L, Cook J, et al. The temporal dynamics and infectiousness of subpatent *Plasmodium falciparum* infections in relation to parasite density. *Nat Commun.* 2019;10:1433. <https://doi.org/10.1038/s41467-019-09441-1>.
 43. Mwingira F, Genton B, Kabanyanyi AN, Felger I. Comparison of detection methods to estimate asexual *Plasmodium falciparum* parasite prevalence and gametocyte carriage in a community survey in Tanzania. *Malar J.* 2014;13:433. <https://doi.org/10.1186/1475-2875-13-433>.
 44. Bousema T, Dinglasan RR, Morlais I, Gouagna LC, van Warmerdam T, Awono-Ambene PH, et al. Mosquito feeding assays to determine the infectiousness of naturally infected *Plasmodium falciparum* gametocyte carriers. *PLoS One.* 2012;7:e42821. <https://doi.org/10.1371/journal.pone.0042821>.
 45. Collins KA, Wang CY, Adams M, Mitchell H, Rampton M, Elliott S, et al. A controlled human malaria infection model enabling evaluation of transmission-blocking interventions. *J Clin Investig.* 2018;128:1551–62. <https://doi.org/10.1172/jci98012>.
 46. Graumans W, Jacobs E, Bousema T, Sinnis P. When is a plasmodium-infected mosquito an infectious mosquito? *Trends Parasitol.* 2020. <https://doi.org/10.1016/j.pt.2020.05.011>.
 47. Cohee LM, Valim C, Coalson JE, Nyambalo A, Chilombe M, Ngwira A, et al. School-based screening and treatment may reduce *P. falciparum* transmission. *Sci Rep.* 2021;11:6905. <https://doi.org/10.1038/s41598-021-86450-5>.
 48. Slater HC, Ding XC, Knudson S, Bridges DJ, Moonga H, Saad NJ, et al. Performance and utility of more highly sensitive malaria rapid diagnostic tests. *BMC Infect Dis.* 2022;22:121. <https://doi.org/10.1186/s12879-021-07023-5>.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

