Assessment of ultra-sensitive malaria diagnosis versus standard molecular diagnostics for malaria elimination: an in-depth molecular community cross-sectional study

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Summary

Background **Submicroscopic malaria infections contribute to transmission in exposed populations but their extent is underestimated even by standard molecular diagnostics. Sophisticated sampling and ultra-sensitive molecular methods can maximise test sensitivity but are not feasible in routine surveillance. Here we investigate the gains achievable by using increasingly sensitive methods with the aim to understand what diagnostic sensitivity is necessary to guide malaria interventions.**

Methods **Venous blood samples were collected from participants in a cross-sectional survey in two coastal medium-endemic villages in Madang province, Papua New Guinea. Using ultra-sensitive quantitative PCR (us-qPCR) on concentrated high-volume blood samples (2 mL) as reference, we quantified the proportion of *Plasmodium falciparum* and *Plasmodium vivax* infections and gametocyte carriers detectable in fingerprick blood volumes (200 µL) by standard 18S rRNA qPCR, us-qPCR, rapid diagnostic test (RDT), and ultra-sensitive *P falciparum* RDT. We further compared the epidemiological patterns observed with each diagnostic approach in the study population.**

Findings **Venous blood samples were collected from 300 participants between Dec 5, 2016, and Feb 24, 2017 (ie, during peak rainy season). Standard qPCR identified 87 (54%) of 161 *P falciparum* infections and 73 (52%) of 141 *P vivax* infections detected by the reference method. us-qPCR identified an additional 11 (7%) *P falciparum* infections and 14 (10%) *P vivax* infections. 80 (86%) of 93 *P falciparum* gametocyte carriers and 75 (91%) of 82 *P vivax* gametocyte carriers were found among infections detectable by us-qPCR. Ultra-sensitive RDT missed half of *P falciparum* infections detected by standard qPCR, including high gametocytaemic infections. Epidemiological patterns corresponded well between standard qPCR and the reference method. As the prevalence of *P vivax* decreased with increasing age, the proportion of *P vivax* infections undetectable by standard qPCR increased.**

Interpretation **Almost all potentially transmitting parasite carriers were identified with us-qPCR on fingerprick blood volumes. Analysing larger blood volumes revealed a large pool of ultra-low-density *P falciparum* and *P vivax* infections, which are unlikely to be transmitted. Therefore, current RDTs cannot replace molecular diagnostics for identifying potential *P falciparum* transmitters.**

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Introduction

During the past decade, malaria epidemiological studies have increasingly applied molecular methods for diagnosis of infections. This approach has revealed that a large proportion of malaria infections in naturally exposed populations is characterised by low parasite densities that are undetectable by light microscopy or a rapid diagnostic test (RDT).[1,2] Although chronic low-density infections are associated with negative clinical consequences in the long term,[3] they have no acute pathological impact and might even confer protection against severe malaria episodes.[4] In the context of malaria control, the main relevance of chronic low-density infections is their contribution to maintaining malaria transmission.[5,6]

Therefore, maximal detection of low-density malaria infections is often considered important for countries aiming to eliminate malaria; however, this goal is challenging in the context of routine surveillance strategies. The detection of low-density infections requires active surveillance of entire populations with use of molecular diagnostic tests, which are most commonly based on amplification of the plasmodium 18S rRNA gene from fingerprick blood samples.[7] In 2017, the first ultra-sensitive *Plasmodium falciparum* RDT (us-RDT) was launched for simplified detection of low-density malaria infections in surveillance screens.[8]

In the past decade, improved nucleic acid amplification techniques have set increasingly high standards in test sensitivity by use of multi-copy target genes[9] or increasing the blood volumes processed.[10] In Tanzania and southeast Asia, these approaches have revealed low-density infections that would not be detected by standard

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Research in context

Evidence before this study

We searched PubMed for publications, without any language restriction, until March 1, 2018, using the search terms "plasmodium" AND ("falciparum" OR "vivax") AND ("sub-microscopic" OR "submicroscopic" OR "ultra-sensitive" OR "ultrasensitive") AND ("pcr" OR "polymerase chain reaction"). We retrieved 135 studies, which were screened for the sample type (venous blood *vs* fingerprick), sample volume, and type of (molecular) analysis method used for detection of malaria infection. At the Thailand–Myanmar border and in Vietnam, a few studies investigating ultra-low parasitaemias in asymptomatic carriers applied a detection method by Imwong and colleagues that uses venous blood combined with standard quantitative PCR (qPCR). However, this method does not allow species determination of the lowest plasmodium parasitaemias, and no direct comparisons were made to standard sampling and molecular detection methods used by the vast majority of malaria epidemiological studies. One study by Das and colleagues assessed the performance of a new ultra-sensitive lateral flow *Plasmodium falciparum* rapid diagnostic test (us-RDT) in Myanmar and Uganda. We found no studies investigating the presence of gametocytes among ultra-low-density malaria infections, which serves as a surrogate marker of their potential to contribute to malaria transmission.

Added value of this study

In many endemic areas, the aim of antimalarial interventions has shifted from just treating clinical cases to also reducing or eliminating malaria transmission. This goal entails the identification and treatment of asymptomatic parasite carriers who are characterised by low parasite densities, but still can maintain malaria transmission. Improved diagnostic techniques have revealed a large reservoir of such infections below the microscopic detection threshold, and even below the limit of detection of standard molecular techniques. However, the venous sampling required for detection of the lowest parasitaemias is not feasible in routine surveillance and intervention monitoring. Our study therefore addresses the question of how many *P falciparum* and *Plasmodium vivax* infections are missed in population-based studies using standard molecular malaria diagnostics or a new *P falciparum* us-RDT. Our study evaluated the relevance of these missed infections in the context of malaria interventions by detecting gametocytes (transmission stages) in high-volume samples.

Implications of all the available evidence

Our findings show that a large proportion (up to 50%) of *P vivax* and *P falciparum* infections are undetected by standard molecular diagnostics using finger-prick blood volumes in cross-sectional studies. Despite this large number of missed detections, standard molecular malaria diagnostics suffice to investigate the epidemiological patterns in the population and to identify virtually all parasite carriers with gametocyte densities that are meaningful for onwards transmission. By contrast, us-RDT missed a large number of *P falciparum* infections with high gametocyte densities. Our findings thus reduce the pressure to apply venous blood sampling for ultra-sensitive molecular diagnostics, while casting doubt on the effectiveness of implementing the us-RDT in interventions aiming at reducing malaria transmission.

molecular malaria diagnosis (ie, 18S rRNA quantitative PCR [qPCR] on fingerprick samples).[9,11] More awareness of the extent, epidemiology, and relevance of these missed ultra-low-density *P falciparum* and *Plasmodium vivax* infections is required in the context of efforts towards malaria elimination and for discovery of remaining pockets of transmission.

Venous blood sampling and sophisticated sample processing are required for the most sensitive molecular diagnostic tests, which are feasible in research studies but not in large-scale surveillance. Therefore, in this study, we aimed to address the question of whether the use of highly sophisticated molecular detection methods provides more useful information for design and monitoring of malaria interventions compared with standard molecular detection. To this end, we systematically validated the proportion of *P falciparum* and *P vivax* infections as well as gametocyte carriers detected in samples from a community survey using different blood volumes, different molecular diagnostics, standard RDT (st-RDT), and a novel us-RDT.[8] We compared the epidemiological patterns observed with each diagnostic approach to investigate whether certain subgroups of the human host population are of greater importance than others for harbouring of low-density malaria infections. The knowledge gained could be used as a benchmark for the design of surveillance strategies, in which maximisation of test sensitivity has to be balanced against the feasibility of venous bleeding.

For more on the **electronic prevalence questionnaire (Malaria Indicator Survey Toolkit)** see http://malariasurveys.org/toolkit.cfm

Methods

Study design and participants

Venous blood samples were collected from participants in a cross-sectional survey in two coastal medium-endemic villages in Madang province, Papua New Guinea.[12] Sample collection was embedded in a larger census-based cross-sectional survey, during which participants aged 5 years and older (excluding pregnant women) could volunteer for venous sampling. After written informed consent was obtained, a health status assessment was undertaken and a standard electronic prevalence questionnaire was completed, followed by a brief interview. 5 mL of venous blood were collected in sodium-heparin-coated vacutainers (BD Biosciences, Franklin Lakes, NJ, USA). 800 µL of blood were immediately stabilised in RNAprotect Cell Reagent (Qiagen, Hilden, Germany).

Participants presenting with signs and symptoms of malaria infection (>37·5°C axillary or reported fever

in the previous 2 days) were tested using the CareStart HRP2/pLDH (Pf/PAN) Combo RDT (AccessBio, Somerset, NJ, USA). Test-positive participants were treated according to national guidelines.

Ethical approval for the study was obtained from the Papua New Guinea Institute of Medical Research Institutional Review Board (PNGIMR IRB number 1516) and the Medical Research Advisory Committee of the Papua New Guinea Ministry of Health (MRAC number 16.01).

Sample processing and nucleic acid extraction

Whole-blood aliquots of 200 µL (chosen to mimic fingerprick blood samples) and 2 mL were separated into red blood cell (RBC) pellet and plasma. RBC pellets from the 2 mL blood aliquots were depleted of white blood cells by Ficoll Paque Plus (GE Healthcare, Chicago, IL, USA) gradient centrifugation. RBC pellets, RNAprotect samples, and whole-blood aliquots of samples with sufficient volume were stored at –20°C.

DNA was extracted from the RBC pellets within 3 months using the QIAamp 96 DNA Blood Kit (Qiagen) for small RBC volumes and QIAamp DNA Blood Midi Kit (Qiagen) for large RBC volumes according to the manufacturer's instruction. DNA obtained from small RBC volumes was eluted in 100 µL, yielding twofold template concentration with respect to the original blood sample. DNA from large RBC volumes was eluted in 400 µL, yielding fivefold template concentration. For samples that were qPCR-negative for *P falciparum* or for *P vivax* when analysing DNA from small and large blood volumes, a 200 µL aliquot of DNA from the large blood volume was further concentrated tenfold by sodium acetate and ethanol precipitation, yielding a final 50-fold concentrated template. RNA was extracted from the pelleted RNAprotect samples within 6 months using the RNEasy Mini Kit (Qiagen) according to the manufacturer's protocol, including an on-column DNAse digest.[13] RNA was eluted in 80 µL, yielding a tenfold template concentration compared with the original blood sample.

Detection of malaria infections

Standard qPCR for detection of *P falciparum* and *P vivax* used previously published 18S rRNA assays[13,14] with a modified *P falciparum* reverse primer (PFS18S_revMAO 5′–TATTCCATGCTGTAGTATTCAAACACAA–3′).[15] Ultra-sensitive qPCRs with increased limit of detection compared with standard qPCR[9] (appendix pp 1–2) targeted the *P falciparum var* gene acidic terminal sequence (*Pf*-varATS)[9] or the *P vivax* mitochondrial *cox1* gene (*Pv*-mtCox1).[16] Presence of gametocytes was investigated in all *P falciparum*-positive or *P vivax*-positive samples using previously published *pfs25* and *pvs25* qRT-PCR assays.[13]

All molecular assays used 4 µL of template material; hence, the blood volume equivalent per reaction ranged between 8 µL and 200 µL whole blood (appendix p 3). Parasitaemia or gametocytaemia was quantified in relation to a standard row of target-specific plasmid[13] and adjusted according to the concentration factor of DNA template with respect to whole blood.

All small blood volume DNA samples were tested using *P falciparum* and *P vivax* 18S rRNA, *Pf*-varATS, and *Pv*-mtCox1 qPCRs. Throughout this Article, 18S rRNA qPCRs on small blood volume DNA samples are referred to as standard qPCR (st-qPCR) and *Pf*-varATS and *Pv*-mtCox1 qPCRs on small blood volume DNA samples as ultra-sensitive qPCR (us-qPCR).

Eluted high-volume DNA samples were tested using *Pf*-varATS and *Pv*-mtCox1 qPCRs. Samples negative for *P falciparum* or *P vivax* on eluted DNA from both small and large blood volumes were further tested in *Pf*-varATS and *Pv*-mtCox1 qPCRs using concentrated large-volume DNA. Results obtained by *Pf*-varATS or *Pv*-mtCox1 qPCRs on eluted and on concentrated high-volume DNA were combined and are further referred to as high-volume us-qPCR (hv-us-qPCR).

Parasite densities correlated well between different molecular detection methods, with stronger correlations observed for *P falciparum* than *P vivax* (Spearman's r=0·86–0·92 for *P falciparum*; r=0·80–0·86 for *P vivax*; appendix p 4).

Samples for which frozen whole blood was available were tested with a *P falciparum*/*P vivax* st-RDT (Malaria Ag P.f/P.v, Standard Diagnostics, Yongin-si, South Korea) and a *P falciparum* us-RDT (Malaria Ag Pf Ultra-Sensitive, Standard Diagnostics) using 5 µL of thawed whole blood. Mean *P falciparum* and *P vivax* parasite densities in samples tested by RDT were not significantly different from the full set of samples or samples not tested by RDT.

See Online for appendix

	Plasmodium falciparum			*Plasmodium vivax*		
	$n_{positive}$	Prevalence (95% CI)	Proportion detected*	$n_{positive}$	Prevalence (95% CI)	Proportion detected*
Standard qPCR						
All positive	87	29% (24–35)	54%	73	24% (20–30)	52%
Gametocyte carriers†	71	24% (19–29)	76%	67	22% (18–28)	82%
Ultra-sensitive qPCR						
All positive	98	33% (27–38)	61%	87	29% (24–35)	62%
Gametocyte carriers†	80	27% (22–32)	86%	75	25% (20–30)	91%
High-volume ultra-sensitive qPCR						
All positive	159	53% (48–59)	99%	135	45% (39–51)	96%
Gametocyte carriers†	93	31% (26–37)	100%	81	27% (22–32)	99%
Any PCR (reference)*						
All positive	161	54% (48–59)	NA	141	48% (42–53)	NA
Gametocyte carriers†	93	31% (26–37)	NA	82	27% (22–33)	NA

$n_{positive}$=number of positive samples. qPCR=quantitative PCR. NA=non-applicable. *The proportion of parasite carriers detected by each method was calculated against the combined detections by any qPCR (defined as reference). The overlap between diagnostic methods, as well as an assessment of diagnostic performance of each assay (sensitivity and specificity), is shown in the appendix (p11–12). †Gametocyte carriers among all positives detected by the respective qPCR method.

Table: **Comparison of molecular methods with increasing sensitivity for the detection of *Plasmodium falciparum* and *Plasmodium vivax* infections and gametocyte carriers**

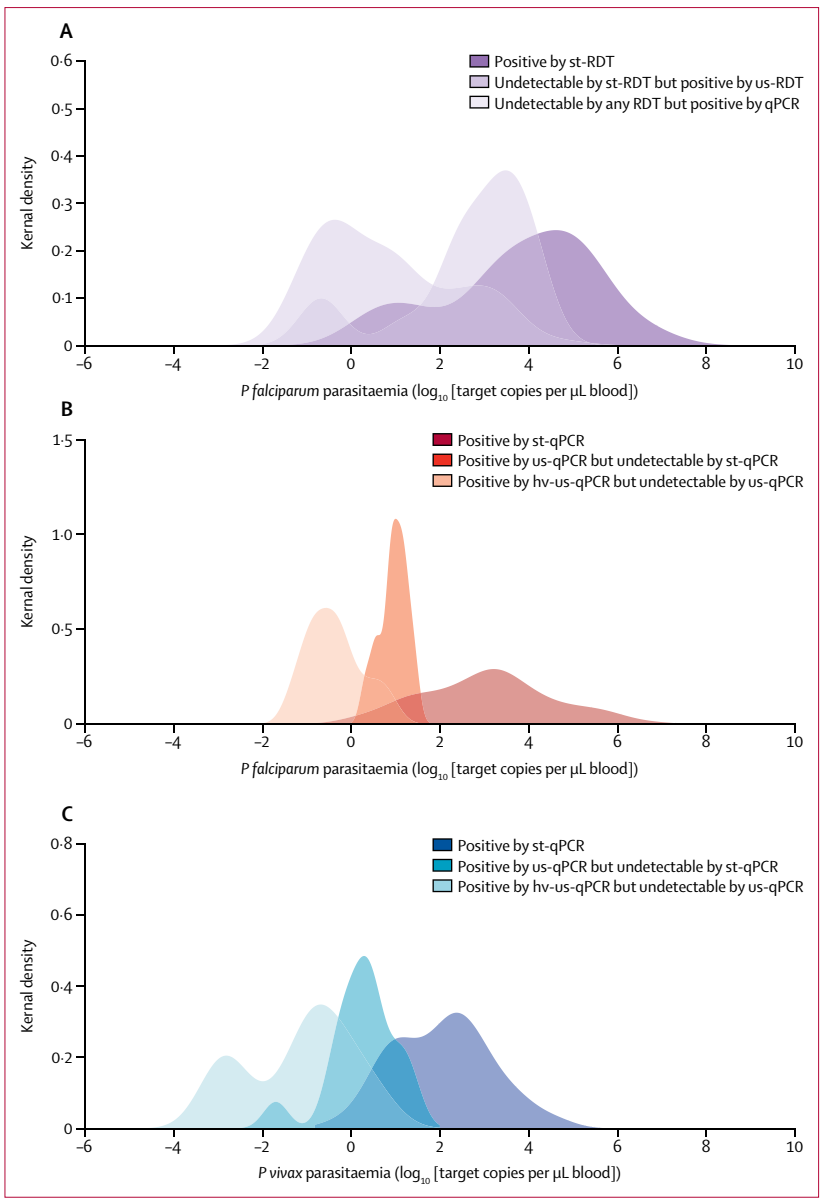

Figure 1: **Parasite density distributions in *Plasmodium falciparum* (A,B) and *Plasmodium vivax* (C) infections detected by RDTs and molecular methods with different sensitivity**
Parasite density by hv-us-qPCR is plotted (see underlying histograms in the appendix p 5), so only samples positive in hv-us-qPCR are shown. Samples were categorised according to their positivity by the specific detection methods. An unknown number of target sequences is amplified in *P falciparum* and *P vivax* us-qPCR, so parasite densities cannot be directly compared between the two species (see discussion on quantifying parasitaemia by molecular methods in the appendix pp 6–10). hv-us-qPCR=high-volume ultra-sensitive qPCR. qPCR=quantitative PCR. RDT=rapid diagnostic test. st-qPCR=standard qPCR. st-RDT=standard RDT. us-qPCR=ultra-sensitive qPCR. us-RDT=ultra-sensitive *P falciparum* RDT.

Statistical analysis

We aimed to evaluate whether certain population subgroups harbour more ultra-low-density infections than others and to compare the epidemiological patterns observed with the different diagnostics. To this end, we modelled the effect of covariates on the odds of detecting a *P falciparum* or *P vivax* infection or gametocytaemia using multivariable logistic linear regression. We selected covariates a priori on the basis of previous knowledge. We calculated univariate factors for RDT-diagnosed *P falciparum* infections resulting from the low number of positive detections. We used R version 3.4.1 for all analyses. Packages plyr and reshape2 were used for structuring of data; packages limma, gplots, beeswarm, and forestplot for production of graphics; package *zoo* was used to calculate a rolling mean of diagnostic sensitivity.

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Venous blood samples were collected from 300 participants between Dec 5, 2016, and Feb 24, 2017 (ie, during peak rainy season). Whole blood was available for 247 of 300 samples after RNAprotect samples had been prepared and DNA extractions from small and large blood volumes had been done. Demographics of the study population were comparable between the two study villages. In Megiar (n=163), mean participant age was 30 years (SD 16·7; median 31 years [IQR 14–43]), and in Mirap (n=137) it was 28 years (16·1; median 24 years [14–40]). 78 (48%) participants in Megiar and 76 (55%) participants in Mirap were male. 124 (76%) participants in Megiar and 123 (90%) in Mirap reported having slept under a bednet in the preceding night. 20 (7%) participants presented with fever or reported fever within the 2 preceding days, and 24 (8%) participants reported antimalarial treatment within the past month.

We detected *P falciparum* in 159 (53%; 95% CI 48–59) and *P vivax* in 135 (45%; 39–51) participants using large blood volumes and hv-us-qPCR (table). Using st-qPCR (on small volumes), we identified 87 *P falciparum* infections and 73 *P vivax* infections, which corresponded to 54% of 161 *P falciparum* infections detected by any qPCR method, and 52% of 141 *P vivax* infections detected by any qPCR method. Parasite prevalence in the population was thus two times lower by st-qPCR (29% [95% CI 24–35] for *P falciparum* and 24% [20–30] for *P vivax*) than by hv-us-qPCR (table).

us-qPCR on small blood volumes identified an additional 11 (7%) of 161 *P falciparum* infections and an additional 14 (10%) of 141 *P vivax* infections. Use of us-qPCR therefore increased parasite prevalence estimates in the population slightly (33% [95% CI 27–38] for *P falciparum*; 29% [24–35] for *P vivax*; table) compared with st-qPCR. Parasite densities in these additionally positive infections were similar to the lowest parasite densities detected by st-qPCR (figure 1B,C), with a median of 1·01 (IQR 0·86–1·76) estimated *P falciparum* parasites

per µL of blood and 0·08 (0·03–0·16) estimated *P vivax* parasites per µL of blood (based on a conversion formula in the appendix pp 6–10). In other words, detection of infections with few or a single parasite in the small blood volume is more reliable with us-qPCR than st-qPCR, since the higher number of DNA sequences targeted in us-qPCR reduces the effect of chance.

Detection of the lowest parasitaemias was only achieved by hv-us-qPCR, in which a larger blood volume equivalent is examined (figure 1B,C). However even at such maximised sensitivity, a chance effect remained in detecting low-density infections, which was apparent from an imperfect overlap of positivity between the molecular detection methods (appendix p 11).

In the 247 samples that were tested using RDT, st-RDT detected 20 (15%) of all 135 *P falciparum* infections detected by any qPCR method in this subset of samples (figure 2A). us-RDT detected 36 (27%) *P falciparum* infections, corresponding to 51% of the 70 st-qPCR-detectable *P falciparum* infections in this sample subset (figure 2A). us-RDT detected *P falciparum* infections with reduced parasitaemia compared with st-RDT (figure 1A) and showed improved diagnostic performance over the whole range of *P falciparum* densities (figure 2B).

One *P vivax* infection was identified by st-RDT, and 118 *P vivax* infections were detected by qPCR methods in the subset of samples that were tested with RDT.

Gametocytes were detected in 19 (95% [95% CI 73–100]) of 20 *P falciparum* infections identified by st-RDT and in 12 (75% [47–92]) of 16 infections additionally identified by us-RDT (figure 3A). Gametocytes were also detected in 44 (44% [95% CI 35–55]) of 99 us-RDT-negative and qPCR-positive *P falciparum* infections (figure 3A). Of all 75 *P falciparum* gametocyte carriers, 44 (59%) were not detected by us-RDT. The range of gametocyte densities in us-RDT-negative gametocyte carriers was similar to that in us-RDT-positive or st-RDT-positive gametocyte carriers (figure 3D).

Parasite and gametocyte densities correlated better for *P vivax* (r=0·69) than for *P falciparum* (r=0·42; appendix p 13). For both species, parasite density was the single most important predictor for gametocyte carriage (appendix p 14). Therefore, when using molecular diagnosis, gametocytes were most common in st-qPCR-detectable *P falciparum* and *P vivax* infections. 71 (82%; 95% CI 72–89) of 87 st-qPCR-detectable *P falciparum* infections were gametocyte positive, and 67 (92%; 82–97) of 73 *P vivax* infections were gametocyte positive (figure 3B,C). More than half of infections additionally detected by us-qPCR also carried gametocytes (ten [67%; 95% CI 39–87] of 15 *P falciparum* infections; 11 [58%; 34–79] of 19 *P vivax* infections; figure 3B,C). The proportion of gametocyte carriers was considerably lower in infections only detectable in hv-us-qPCR (12 [20%; 95% CI 11–33] of 59 *P falciparum* infections; four [8%; 3–20] of 49 *P vivax* infections). As a result, diagnosis of infections using st-qPCR identified 71 (76%) of all 93 *P falciparum* and 67 (82%) of all 82 *P vivax* gametocyte carriers in the population (table). Using us-qPCR, 80 (86%) of *P falciparum* gametocyte carriers and 75 (91%) of *P vivax* gametocyte carriers were identified.

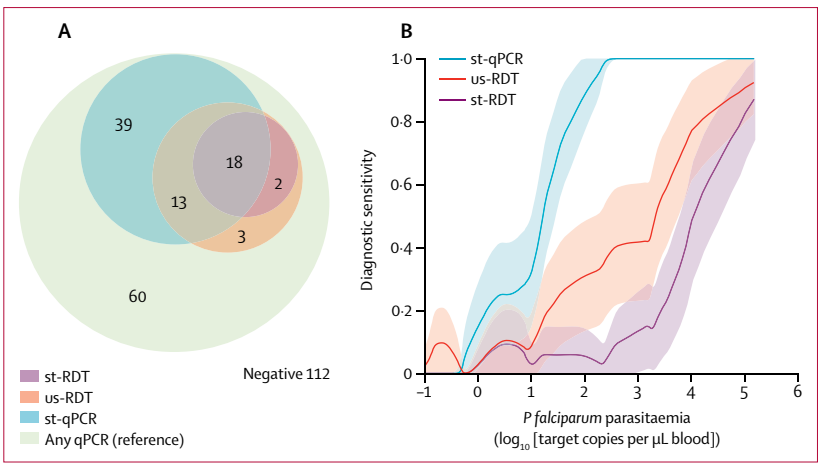

Figure 2: **Diagnostic performance of *Plasmodium falciparum* RDTs compared with qPCR methods in a subset of 247 samples**

Frozen whole-blood for RDT analysis was only available for 247 of 300 samples. (A) Venn diagram of *P falciparum* positivity by st-RDT, us-RDT, and molecular detection methods. Five samples were positive with us-RDT, or us-RDT and st-RDT, but negative with st-qPCR, and would thus have been considered false positive by RDT. However, *P falciparum* parasites were detected in all RDT-positive samples with hv-us-qPCR. (B) Diagnostic sensitivity of st-qPCR, us-RDT, and st-RDT in relation to parasite densitiy (by hv-us-qPCR). Diagnostic sensitivity was calculated as a rolling mean of ten observations using combined detections by any qPCR as reference, and is shown with 95% CI (shaded areas). Curves were smoothed using locally weighted scatterplot smoothing function (span=0·16). An assessment of overall RDT diagnostic performance (sensitivity and specificity) is shown in the appendix (p 12). hv-us-qPCR=high-volume ultra-sensitive qPCR. qPCR=quantitative PCR. RDT=rapid diagnostic test. st-qPCR=standard qPCR. st-RDT=standard RDT. us-RDT=ultra-sensitive *P falciparum* RDT.

Mean *P falciparum* and *P vivax* gametocyte densities were significantly lower in infections that were not detected by st-qPCR than in those that were (geometric mean 1·0 *vs* 31·6 *pfs25* transcripts per µL of blood, p<0·001 for *P falciparum*; 0·3 *vs* 5·6 *pvs25* transcripts per µL blood, p<0·001 for *P vivax*). In infections that were only detected by hv-us-qPCR, estimated gametocyte densities did not exceed one gametocyte per µL of blood (based on previously published conversion formulas;[13,17] figure 3E,F; appendix pp 6–10). Similarly, in infections that were detected by us-qPCR but not by st-qPCR, estimated gametocyte densities were lower than one gametocyte per µL of blood in all but one infection (figure 3E,F).

The same main risk factors for malaria infection were identified by st-qPCR and hv-us-qPCR (figure 4; appendix p 15). Age was the only significant predictor for the odds of a *P vivax* infection. The odds of a *P falciparum* infection was significantly associated with village of residence and haemoglobin level. Patterns in the odds of RDT-diagnosed *P falciparum* infections were similar to those of molecular *P falciparum* diagnosis; however, the power of risk analysis was low because of the low number of RDT-positive detections (appendix pp 16–17).

The proportion of ultra-low-density infections among all infections was up to two times higher in population

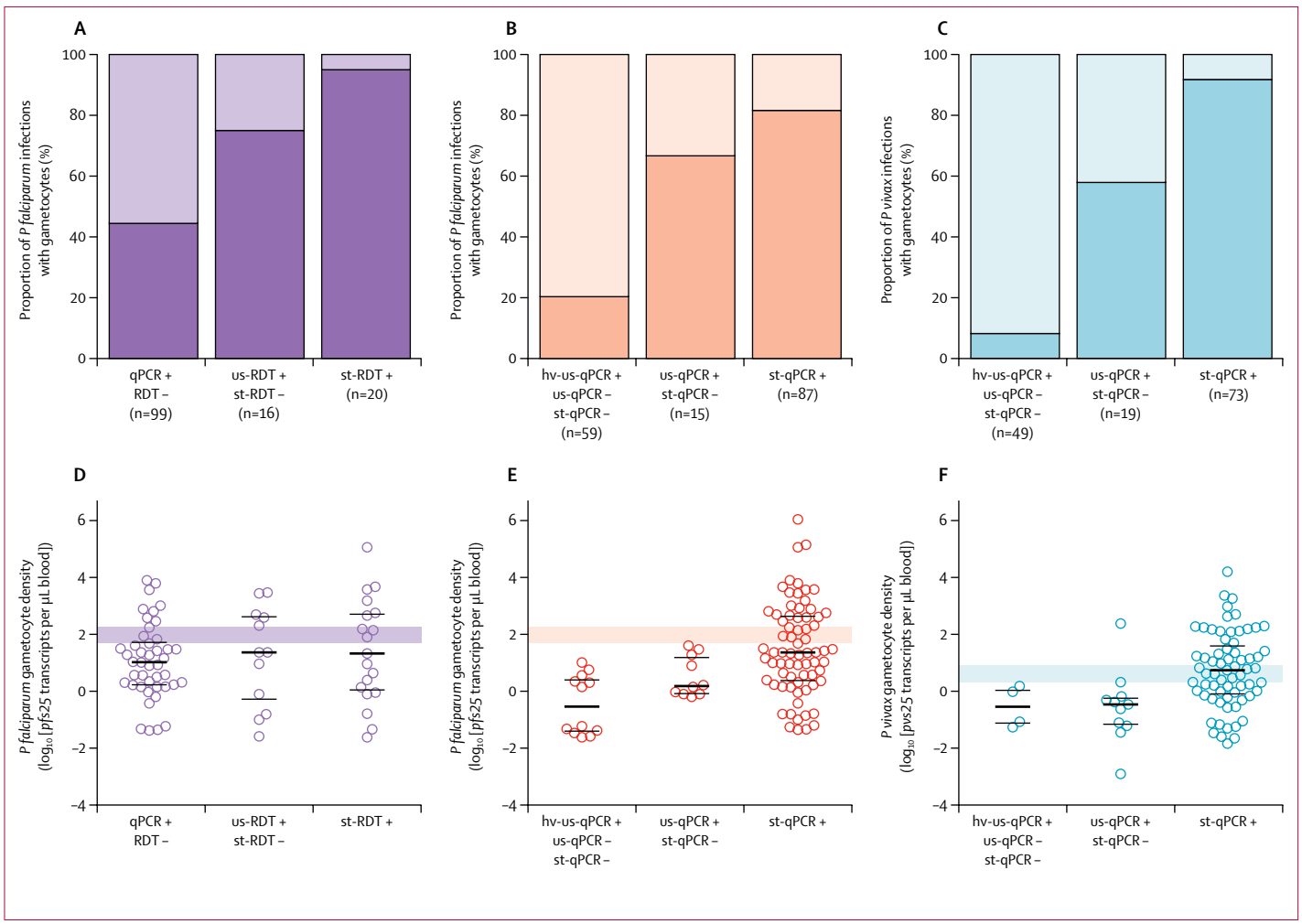

Figure 3: **Proportion of gametocyte-positive infections (A–C) and gametocyte density (D–F) in infections detected by RDTs (A,D) and molecular methods with different sensitivity (B,C,E,F)**
Samples were categorised according to their positivity by the different diagnostic methods as specified under each bar (corresponding to figure 1). (A–C) The proportion of gametocyte positive samples in each category is shown. (D–F) For each category, the concentration of gametocyte-specific transcripts in the corresponding samples is displayed, with each circle representing one sample. For each category, summary lines are displayed: thick black lines indicate the median, and thin black lines indicate the IQR. A different number of *pfs25* and *pvs25* transcripts is amplified per *Plasmodium falciparum* and *Plasmodium vivax* gametocyte; hence, gametocyte densities cannot be directly compared between the two species (see discussion on quantifying gametocytes by molecular methods in the appendix pp 6–10). *pfs25* and *pvs25* copy numbers corresponding to one gametocyte (within the confidence range, based on previously published correlations[13,17]) are delineated with a horizontal coloured line. hv-us-qPCR=high-volume ultra-sensitive qPCR. qPCR=quantitative PCR. RDT=rapid diagnostic test. st-qPCR=standard qPCR. st-RDT=standard RDT. us-qPCR=ultra-sensitive qPCR. us-RDT=ultra-sensitive *P falciparum* RDT.

subgroups with low parasite prevalence than in subgroups with high prevalence. For example, as *P vivax* prevalence decreased from 63% (30 of 48 [95% CI 47–76) in 11–15-year-old children to 31% (14 of 45 [19–47]) in adults older than 50 years (figure 5B), the proportion of ultra-low-density *P vivax* infections rose from 30% (nine of 30 [15–50]) in the 11–15-year-old children to 64% (nine of 14 [36–86]; figure 5B) in the oldest age group. Overall, *P vivax* density decreased with increasing age (figure 5D; Anova p<0·001), whereas no clear trends with age were observed for *P falciparum* (figure 5A). For *P falciparum*, parasite prevalence differed between villages and was inversely related to the proportion of ultra-low-density infections per village (appendix p 18). However, these differences between villages were not statistically significant.

Discussion

In this study, we applied multiple molecular diagnostic methods with maximised sensitivity to explore the true prevalence of *P falciparum* and *P vivax* in an endemic population in Papua New Guinea. This approach revealed an unexpectedly large reservoir of infections below the limit of detection of standard molecular diagnosis. Main limiting factors were the blood volume sampled and the blood equivalent added to the detection assay. However, complex laboratory procedures are necessary when using large blood volumes, which are not feasible for routine malaria surveillance or intervention monitoring. This issue raises the question of whether malaria interventions aimed at reducing transmission can benefit from detecting these ultra-low-density residual infections.

In cross-sectional surveys, the density of gametocytes in the host's blood is often used as a surrogate marker for the transmission potential to mosquitoes. Directly measuring infectivity in cross-sectional surveys is challenging because it would require feeding of colony mosquitoes by direct exposure of the infected individual or by membrane blood feeding. Although gametocyte density is positively associated with infection success in membrane feeding experiments,[18–20] measuring gametocyte densities in the hosts's blood could provide only a restricted picture of the true probability of onward transmission. This onward transmission might instead depend on the density of mature gametocytes in the subcutaneous tissue, where gametocytes might aggregate to facilitate transmission to mosquitoes.[21]

In our study, gametocyte densities were estimated from the number of *pfs25* or *pvs25* transcripts, which are highly expressed in mature female gametocytes. High-volume RNA sampling maximised the limit of gametocyte detection to below one *P falciparum* or 11 *P vivax* gametocytes per 800 µL of blood (for a detailed discussion of molecular quantification, see appendix pp 6–10). Estimated gametocyte densities in our study were often below one gametocyte per 1 µL of blood, a threshold below which mosquito infection is rare in membrane feeding experiments.[18–20] In fact, with one exception, estimated gametocyte densities were below one gametocyte per µL of blood in all infections undetected by st-qPCR, suggesting that those densities are unlikely to be infective to mosquitoes. However, if parasitaemia in infections undetectable by st-qPCR at the time of sampling increased at a later timepoint, the likelihood of transmission would increase. Studies[22,23] on the longitudinal dynamics of chronic *P falciparum* infections revealed fluctuations in clonal densities by transient absence and later reappearance of clones. Large fluctuations in plasmodium densities over time have been described in Vietnam;[24] however, in the absence of parasite genotyping, it cannot be evaluated whether the observed density peaks represent new infections. In a cohort of children in Papua New Guinea, 70% of febrile malaria episodes showed a new genotype.[25] Low-density clones persisting around the levels of qPCR detection thus seem to be under density control (with fluctuations) and, in the absence of superinfection, asymptomatic individuals are unlikely to become highly effective transmitters.

Although molecular methods are required to detect very low gametocyte densities, the associated asexual parasite densities are approximately ten to 100 times higher and are thus detectable with less sensitive methods. In a multi-country trial,[26] high-quality research-grade microscopy identified more than 90% of infectious *P falciparum* carriers in high-transmission settings and two of three infectious carriers in a low-transmission setting. In the same study, all infectious carriers were detectable by standard molecular methods and fingerprick blood volumes. These results support our finding that little can be gained by laborious sampling and processing of larger blood volumes when diagnosis aims at identifying infectious individuals.

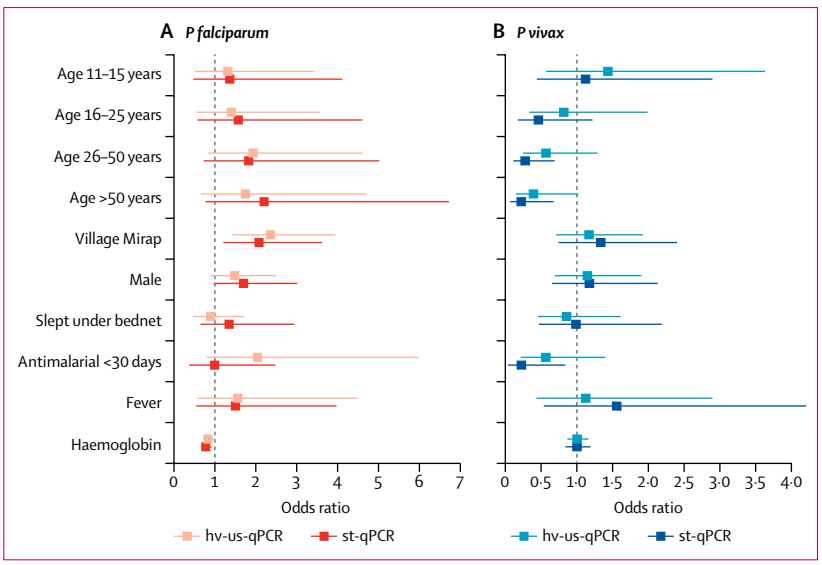

Figure 4: **Forest plot comparing the epidemiological patterns in *Plasmodium falciparum* (A) and *Plasmodium vivax* (B) infections detected using molecular methods with different sensitivity**
Odds ratios were modelled using logistic regression for infections detected using st-qPCR or using hv-us-qPCR. Error bars indicate 95% CIs. Detailed numeric model results for qPCR diagnosis (as well as for RDT diagnosis) are shown in the appendix pp 15–17. hv-us-qPCR=high-volume ultra-sensitive qPCR. qPCR=quantitative PCR. RDT=rapid diagnostic test. st-qPCR=standard qPCR.

The relevance of maximising molecular diagnostic sensitivity in malaria surveillance surveys was further investigated by analysing the predictors of infection in cross-sectional data. If ultra-low-density infections accumulate in certain demographic pockets, these population subgroups would require specific targeting with improved detection methods. The same epidemiological patterns were observed with st-qPCR and hv-us-qPCR, supporting the view that standard molecular methods are adequate for investigating the relative distribution of malaria infections in populations. By contrast, the extent of undetected ultra-low-density infections should be considered when absolute parameters such as parasite prevalence are to be measured.

In a previous comparative diagnostic study,[8] the us-RDT missed 16% of PCR-detectable *P falciparum* infections in a high-endemic (Uganda) setting and 56% in a low-endemic (Myanmar) setting. In Papua New Guinea, us-RDT missed 50% of *P falciparum* infections that were detectable using st-qPCR, including samples with high gametocyte densities. Although the effect on us-RDT sensitivity of using frozen-thawed venous blood rather than fresh fingerprick blood in both studies is unknown, us-RDT seems to be a suboptimal substitute for molecular diagnosis in antimalarial interventions such as screen-and-treat interventions for reducing or eliminating malaria transmission in Papua New Guinea.

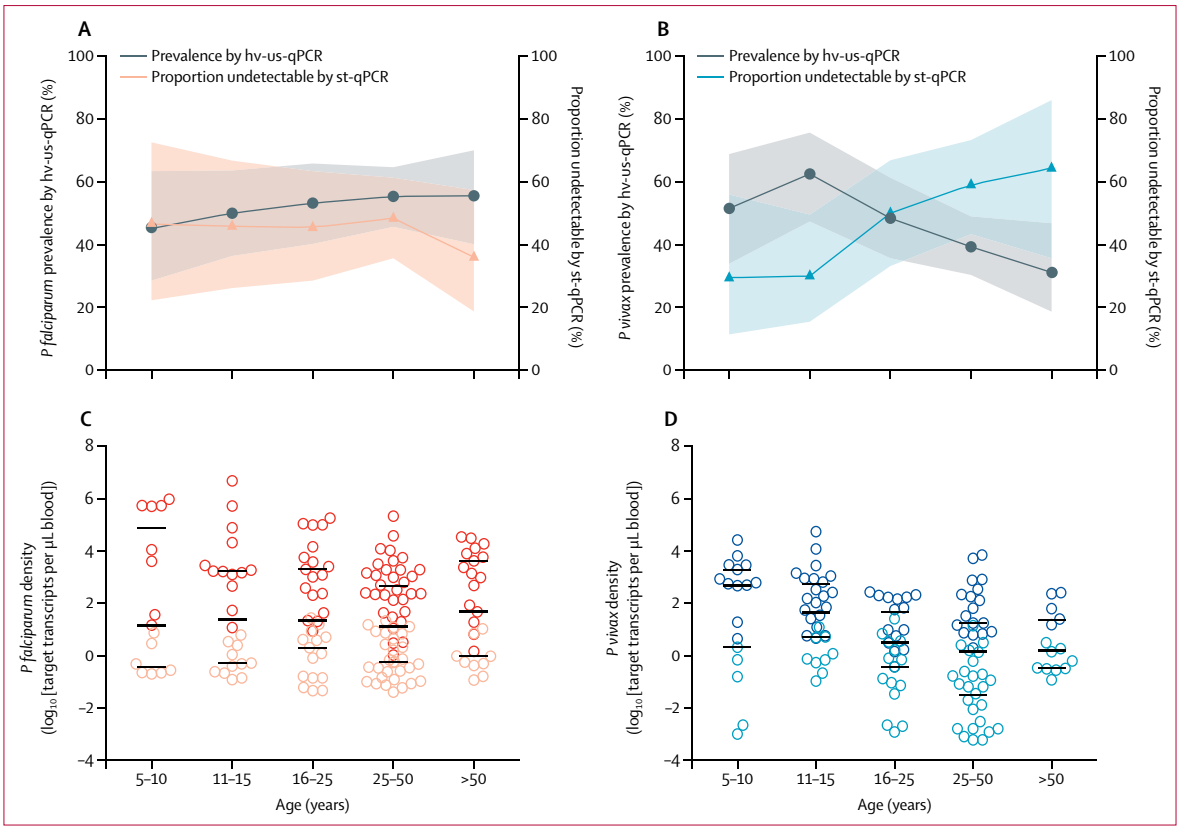

Figure 5: **Age patterns in *Plasmodium falciparum* (A,C) and *Plasmodium vivax* (B,D) infections**
(A,B) Age patterns in parasite prevalence (by hv-us-qPCR) and in the proportion of infections undetectable by st-qPCR. Shaded areas represent 95% CIs. (C,D) Age patterns in parasite density (by hv-us-qPCR). Each circle represents one sample in the respective age group, and summary lines are displayed (thick lines indicate the median; thin lines indicate the IQR). An unknown number of target sequences is amplified in *P falciparum* and *P vivax* ultra-sensitive qPCRs; hence, parasite densities cannot be directly compared between the two species (see discussion on quantifying parasitaemia by molecular methods in the appendix pp 6–10). hv-us-qPCR=high-volume ultra-sensitive qPCR. qPCR=quantitative PCR. st-qPCR=standard qPCR.

Although Papua New Guinea currently does not represent a low-endemic or pre-elimination setting, where detecting very-low-density infections is considered particularly relevant, its unique local epidemiology resembles that of other *P falciparum*-endemic and *P vivax*-endemic settings with declining transmission. Corresponding to global trends of an increasing proportion of submicroscopic infections with decreasing parasite prevalence,[1] parasite densities in Papua New Guinea declined over the past decade alongside a decline in clinical incidence and prevalence of malaria.[12] Furthermore, malaria transmission in Papua New Guinea is highly heterogeneous over small spatial scales,[25] which is considered a hallmark of declining transmission and has been described in various settings, such as western Kenya,[27] Thailand,[28] and the Peruvian Amazon.[29]

A main limitation of our study was the exclusion of children younger than 5 years for ethical reasons. Young children carry the main burden of malaria infection and disease; however, their contribution to mosquito infections is thought to be smaller than that of adolescents and adults.[30] Because parasite densities are higher in young children than adolescents and adults in Papua New Guinea,[17] ultra-low-density infections might be less common in young children, and therefore little would be gained applying ultra-sensitive diagnostics.

A technical limitation that applies to molecular malaria diagnostics, as well as microscopy, is the effect of chance in capturing a scarce parasite, which depends on the volume of blood or DNA solution investigated. In our study, some low-density infections were not detected by a supposedly more sensitive method but were positive by a supposedly less sensitive molecular method. The chance effect that is intrinsic to all malaria diagnostics can thus be lowered, but not abolished, by sampling of larger blood volumes and targeting of high-copy DNA sequences.

In conclusion, we have shown that the extent of both *P falciparum* and *P vivax* infections below the limit of detection of standard molecular malaria diagnostics is substantial. However, gametocyte densities in infections undetected by standard molecular diagnostics were very low and potentially not infective. The us-RDT did not achieve this level of sensitivity and missed infections with high gametocyte densities. Our findings reduce the

pressure to identify the very last parasite and advocate against the need for venous sampling in malaria control and elimination interventions.

Contributors

NEH was involved in data collection, data curation, data analysis, data interpretation, methodology, and writing of the original draft of the article. MG was involved in data collection, data curation, data analysis, methodology, and reviewing of the manuscript. EN, AU, DR-R, and MS were invoved in patient recruitment, data collection, data curation, and reviewing of the manuscript. IM and TAS were responsible for data analysis, and reviewing and editing of the manuscript. ML was involved in patient recruitment, project administration, and reviewing of the manuscript. LJR was involved in conceptualisation, ethical clearance, project administration, supervision, data interpretation, and reviewing and editing of the manuscript. IF was involved in conceptualisation, project administration, supervision, funding acquisition, data interpretation,and reviewing and editing of the manuscript.

Declaration of interests

We declare no competing interests.

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References

1 Okell LC, Ghani AC, Lyons E, Drakeley CJ. Submicroscopic infection in *Plasmodium falciparum*-endemic populations: a systematic review and meta-analysis. *J Infect Dis* 2009; **200:** 1509–17.

2 Cheng Q, Cunningham J, Gatton ML. Systematic review of sub-microscopic *P. vivax* infections: prevalence and determining factors. *PLoS Negl Trop Dis* 2015; **9:** e3413.

3 Chen I, Clarke SE, Gosling R, et al. "Asymptomatic" malaria: a chronic and debilitating infection that should be treated. *PLoS Med* 2016; **13:** e1001942.

4 Smith T, Felger I, Tanner M, Beck HP. Premunition in *Plasmodium falciparum* infection: insights from the epidemiology of multiple infections. *Trans R Soc Trop Med Hyg* 1999; **93** (suppl 1)**:** 59–64.

5 Bousema T, Okell L, Felger I, Drakeley C. Asymptomatic malaria infections: detectability, transmissibility and public health relevance. *Nat Rev Microbiol* 2014; **12:** 833–40.

6 Lin JT, Saunders DL, Meshnick SR. The role of submicroscopic parasitemia in malaria transmission: what is the evidence? *Trends Parasitol* 2014; **30:** 183–90.

7 Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown KN. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. *Mol Biochem Parasitol* 1993; **58:** 283–92.

8 Das S, Jang IK, Barney B, 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.

9 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.

10 Imwong M, Hanchana S, Malleret B, et al. High-throughput ultrasensitive molecular techniques for quantifying low-density malaria parasitemias. *J Clin Microbiol* 2014; **52:** 3303–09.

11 Imwong M, Stepniewska K, Tripura R, et al. Numerical distributions of parasite densities during asymptomatic malaria. *J Infect Dis* 2016; **213:** 1322–29.

12 Koepfli C, Ome-Kaius M, Jally S, et al. Sustained malaria control over an 8-year period in Papua New Guinea: the challenge of low-density asymptomatic *Plasmodium* infections. *J Infect Dis* 2017; **216:** 1434–43.

13 Wampfler R, Mwingira F, Javati S, et al. Strategies for detection of *Plasmodium* species gametocytes. *PLoS One* 2013; **8:** e76316.

14 Rosanas-Urgell A, Mueller D, Betuela I, et al. Comparison of diagnostic methods for the detection and quantification of the four sympatric *Plasmodium* species in field samples from Papua New Guinea. *Malar J* 2010; **9:** 361.

15 Perandin F, Manca N, Calderaro A, et al. Development of a real-time PCR assay for detection of *Plasmodium falciparum*, *Plasmodium vivax*, and *Plasmodium ovale* for routine clinical diagnosis. *J Clin Microbiol* 2004; **42:** 1214–19.

16 Gruenberg M, Moniz CA, Hofmann NE, et al. *Plasmodium vivax* molecular diagnostics in community surveys: pitfalls and solutions. *Malar J* 2018; **17:** 55.

17 Koepfli C, Robinson LJ, Rarau P, et al. Blood-stage parasitaemia and age determine *Plasmodium falciparum* and *P. vivax* gametocytaemia in Papua New Guinea. *PLoS One* 2015; **10:** e0126747.

18 Da DF, Churcher TS, Yerbanga RS, et al. Experimental study of the relationship between *Plasmodium* gametocyte density and infection success in mosquitoes; implications for the evaluation of malaria transmission-reducing interventions. *Exp Parasitol* 2015; **149:** 74–83.

19 Kiattibutr K, Roobsoong W, Sriwichai P, et al. Infectivity of symptomatic and asymptomatic *Plasmodium vivax* infections to a Southeast Asian vector, *Anopheles dirus*. *Int J Parasitol* 2017; **47:** 163–70.

20 Tadesse FG, Slater HC, Chali W, 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.

21 Pichon G, Awono-Ambene HP, Robert V. High heterogeneity in the number of *Plasmodium falciparum* gametocytes in the bloodmeal of mosquitoes fed on the same host. *Parasitology* 2000; **121:** 115–20.

22 Farnert A, Snounou G, Rooth I, Bjorkman A. Daily dynamics of *Plasmodium falciparum* subpopulations in asymptomatic children in a holoendemic area. *Am J Trop Med Hyg* 1997; **56:** 538–47.

23 Felger I, Maire M, Bretscher MT, et al. The dynamics of natural *Plasmodium falciparum* infections. *PLoS One* 2012; **7:** e45542.

24 Nguyen T-N, von Seidlein L, Nguyen T-V, et al. The persistence and oscillations of submicroscopic *Plasmodium falciparum* and *Plasmodium vivax* infections over time in Vietnam: an open cohort study. *Lancet Infect Dis* 2018; **18:** 565–72.

25 Hofmann NE, Karl S, Wampfler R, et al. The complex relationship of exposure to new *Plasmodium* infections and incidence of clinical malaria in Papua New Guinea. *eLife* 2017; **6:** e23708.

26 Gonçalves BP, Kapulu MC, Sawa P, et al. Examining the human infectious reservoir for *Plasmodium falciparum* malaria in areas of differing transmission intensity. *Nat Commun* 2017; **8:** 1133.

27 Baidjoe AY, Stevenson J, Knight P, et al. Factors associated with high heterogeneity of malaria at fine spatial scale in the Western Kenyan highlands. *Malar J* 2016; **15:** 307.

28 Parker DM, Matthews SA, Yan G, et al. Microgeography and molecular epidemiology of malaria at the Thailand–Myanmar border in the malaria pre-elimination phase. *Malar J* 2015; **14:** 198.

29 Rosas-Aguirre A, Guzman-Guzman M, Gamboa D, et al. Micro-heterogeneity of malaria transmission in the Peruvian Amazon: a baseline assessment underlying a population-based cohort study. *Malar J* 2017; **16:** 312.

30 Stone W, Gonçalves BP, Bousema T, Drakeley C. Assessing the infectious reservoir of falciparum malaria: past and future. *Trends Parasitol* 2015; **31:** 287–96.