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

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23 **SUMMARY**

24 BACKGROUND: Submicrocopic malaria infections contribute to transmission in exposed populations
25 but their extent is underestimated even by standard molecular diagnostics. Sophisticated sampling and
26 ultra-sensitive molecular methods can maximize test sensitivity but are not feasible in routine
27 surveillance. Here we investigate the gains achievable by using increasingly sensitive methods with the
28 aim to understand what diagnostic sensitivity is necessary to guide malaria interventions.

29 METHODS: Using ultra-sensitive qPCR (us-qPCR) on concentrated high-volume blood samples (2ml)
30 as reference, we quantified the proportion of *P. falciparum* (*Pf*) and *P. vivax* (*Pv*) infections and
31 gametocyte carriers detectable in finger-prick blood volumes (200µl) by standard 18SrRNA qPCR, us-
32 qPCR, RDT, and ultra-sensitive *Pf*-RDT in 300 cross-sectional venous blood samples from Papua New
33 Guinea.

34 FINDINGS: Standard qPCR identified 54% (87/161) and 51% (73/143) of *Pf* and *Pv* infections detected
35 by the reference method. Us-qPCR identified an additional 7% (11/161) and 10% (14/143). The vast
36 majority of gametocyte carriers (*Pf*: 86%, 80/93; *Pv*, 91%, 75/82) were found among infections

37 detectable by us-qPCR. Ultra-sensitive RDT missed half of *Pf* infections positive in standard qPCR,
38 including high gametocytemic infections. Epidemiological patterns corresponded well between
39 standard qPCR and the reference method. As the prevalence of *Pv* decreased with increasing age, the
40 proportion of *Pv* infections undetectable by standard qPCR increased.

41 INTERPRETATION: Virtually all potentially transmitting parasite carriers are identified using us-
42 qPCR on finger-prick blood volumes. Analysing larger blood volumes revealed a large pool of ultra-
43 low density *Pf* and *Pv* infections, which will unlikely transmit. Current RDTs cannot replace
44 molecular diagnostics for identifying potential *Pf* transmitters.

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48 **Evidence before this study**

49 We searched PubMed for publications until Mar 1, 2018 using the search terms: "plasmodium" AND
50 ("falciparum" OR "vivax") AND ("sub-microscopic" OR "submicroscopic" OR "ultra-sensitive" OR
51 "ultra-sensitive") AND ("pcr" OR "polymerase chain reaction"). We retrieved 135 studies, which were
52 screened for the sample type (venous blood versus finger prick), sample volume, and type of (molecular)
53 analysis method used for detection of malaria infection. At the Thai/Myanmar border and in Vietnam,
54 few studies investigating ultra-low parasitemias in asymptomatic carriers applied a detection method
55 by Imwong e*t al.* that uses venous blood combined with standard qPCR. However, this method does
56 not allow species determination of the lowest *Plasmodium* parasitemias, and no direct comparisons
57 were made to standard sampling and molecular detection methods used by the vast majority of malaria
58 epidemiological studies. One study by Das *et al* assessed the performance of a novel ultra-sensitive
59 lateral flow *P. falciparum* rapid diagnostic test (*Pf*-usRDT) in Myanmar and Uganda. We found no
60 studies investigating the presence of gametocytes among ultra-low density malaria infections, which
61 serves as a surrogate marker of their potential to contribute to malaria transmission.

62

63 **Added value of this study**

64 In many endemic areas the aim of anti-malarial interventions has shifted from just treating clinical cases
65 to also reducing or eliminating malaria transmission. This entails the identification and treatment of
66 asymptomatic parasite carriers that are characterized by low parasite densities, but still can maintain
67 malaria transmission. Improved diagnostic techniques have revealed a large reservoir of such infections
68 below the microscopic detection threshold, and even below the limit of detection of standard molecular
69 techniques. However, the venous sampling required for detection of the lowest parasitemias is not
70 feasible in routine surveillance and intervention monitoring. Our study therefore addresses the question

of how many *P. falciparum* and *P. vivax* infections are missed in population-based studies using standard molecular malaria diagnostics or a novel ultra-sensitive *Pf*-usRDT. Our study aims to evaluate the relevance of these "hidden 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. In contrast, *Pf*-usRDT missed a large number of *P. falciparum* infections with high gametocyte densities. Our findings thus relax the pressure to apply venous blood sampling for ultra-sensitive molecular diagnostics, while casting doubt on the effectiveness of implementing the *Pf*-usRDT in interventions aiming at reducing malaria transmission.

86 **INTRODUCTION**

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

94 Maximal detection of low-density malaria infections is thus often considered important for countries
95 aiming at malaria elimination; however, this is challenging in the context of routine surveillance
96 strategies. The detection of low-density infections requires active surveillance of entire populations with
97 molecular diagnostics, which are most commonly based on amplification of the *Plasmodium 18S rRNA*
98 gene from finger-prick blood samples (7). Recently, a first ultra-sensitive *P. falciparum* RDT (us-RDT)
99 was launched for simplified detection of low-density malaria infections in surveillance screens (8).

100 In the last years, improved nucleic acid amplification techniques have set increasingly high standards
101 in test sensitivity by using multi-copy target genes (9) or increasing the blood volumes processed (10).
102 In Tanzania and South East Asia these approaches have revealed low-density infections that would not
103 be detected by standard molecular malaria diagnosis, i.e. 18S rRNA quantitative polymerase chain
104 reaction (qPCR) on finger-prick samples (9,11). The extent, epidemiology and relevance of these
105 "hidden" ultra low-density *P. falciparum* and *P. vivax* infections requires more awareness in the context
106 of efforts towards malaria elimination and for discovery of remaining pockets of transmission.

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

119

120 **METHODS**

121 **Study design**

122 Venous blood samples were collected from 300 participants in a cross-sectional survey between
123 November 2016 and February 2017, i.e. during peak rainy season, in two coastal medium-endemic
124 villages in Madang province, Papua New Guinea (PNG) (12). Sample collection was embedded in a
125 larger census-based cross-sectional survey, during which participants aged 5 years and older (excluding
126 pregnant women) could volunteer for venous sampling. After informed consent, health status
127 assessment, a standard electronic prevalence questionnaire (http://malariasurveys.org/toolkit.cfm) and
128 a brief interview, 5 ml of venous blood were collected in sodium-heparin coated vacutainers (BD
129 Biosciences). 800µl of blood were immediately stabilized in RNAprotect Cell Reagent (Qiagen).

130 Participants presenting with signs and symptoms of malaria infection (>37·5°C axillary or reported
131 fever in the previous two days) were tested using the CareStart HRP2/pLDH (Pf/PAN) Combo RDT
132 (AccessBio). Test-positive participants were treated according to national guidelines.

133 Demographics of the study population were comparable between the two study villages. In Megiar
134 (n=163) and Mirap (n=137) villages, mean participant age was 30 years (median, 31; interquartile range
135 [IQR], 14-43) and 28 years (median, 24; IQR, 14-40). 48% and 55% of participants in Megiar and
136 Mirap were male and 76% and 90% reported having slept under a bednet in the preceding night. 20
137 participants presented with fever or reported fever within the two preceding days, and 24 participants
138 reported antimalarial treatment within the last month.

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

142

143 **Sample processing and nucleic acid extraction**

144 Whole blood aliquots of 200µl (chosen to mimic finger-prick blood samples) and 2 ml were separated
145 into red blood cell (RBC) pellet and plasma. RBC pellets from the 2 ml blood aliquots were depleted
146 of white blood cells by Ficoll Paque Plus (GE healthcare) gradient centrifugation. RBC pellets,
147 RNAprotect samples, and whole blood aliquots of samples with sufficient volume (N=247) were stored
148 at -20°C.

149 DNA was extracted from the RBC pellets within three months using the QIAamp 96 DNA Blood Kit
150 (Qiagen) for small RBC volumes and QIAamp DNA Blood Midi Kit (Qiagen) for large RBC volumes
151 according to the manufacturer's instruction. DNA was eluted in 100µl and 400µl, respectively, yielding

152 2-fold or 5-fold template concentration with respect to the original blood sample. For samples that were
153 qPCR-negative for *P. falciparum* or for *P. vivax* when analysing DNA from small and large blood
154 volumes, a 200µl aliquot of DNA from the large blood volume was further concentrated 10-fold by
155 sodium acetate/ethanol precipitation, yielding a final 50-fold concentrated template. RNA was extracted
156 from the pelleted RNAprotect samples within six months using the RNEasy Mini Kit (Qiagen)
157 according to the manufacturer's protocol, including an on-column DNAse digest (13). RNA was eluted
158 in 80µl, yielding a 10-fold template concentration compared to the original blood sample.

159

160 **Detection of malaria infections**

161 Standard qPCR for detection of *P. falciparum* and *P. vivax* used previously published 18S rRNA assays
162 (13,14) with a modified *P. falciparum* reverse primer (PFS18S_revMAO: 5'–
163 TATTCCATGCTGTAGTATTCAAACACAA–3' (15)). Ultra-sensitive qPCRs with increased limit of
164 detection compared to standard qPCR (10, Appendix, page 1-2) targeted the *P. falciparum var* gene
165 acidic terminal sequence (*Pf*-varATS) (9) or the *P. vivax* mitochondrial *cox1* gene (*Pv*-mtCox1) (16).
166 Presence of gametocytes was investigated in all *P. falciparum* or *P. vivax*-positive samples using
167 previously published *pfs25* and *pvs25* qRT-PCR assays (13).

168 All molecular assays used 4µl of template material, hence the blood volume equivalent per reaction
169 ranged between 8µl and 200µl whole blood (Appendix, page 3). Parasitemia or gametocytemia was
170 quantified in relation to a standard row of target-specific plasmid (13) and adjusted according to the
171 concentration factor of DNA template with respect to whole blood.

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

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

181 Parasite densities correlated well between different molecular detection methods, with stronger
182 correlations observed for *P. falciparum* compared to *P. vivax* (*P. falciparum*, Spearman's rho=0·86-
183 0·92; *P. vivax*, rho=0·.80-0·86, Appendix, page 4)

184 Samples for which frozen whole blood was available were tested with *P. falciparum/P. vivax* st-RDT
185 (Malaria Ag P.f/P.v, SD Bioline) and *P. falciparum* us-RDT (Malaria Ag Pf Ultra-Sensitive, Alere)
186 using 5µl of thawed whole blood. Mean *P. falciparum* and *P. vivax* parasite densities in samples tested
187 by RDT were not significantly different from the full set of samples or samples not tested by RDT.

188

189 **Statistical analysis**

190 We aimed to evaluate whether certain population subgroups harbour more ultra-low density infections
191 than others and to compare the epidemiological patterns observed with the different diagnostics. To this
192 aim, the effect of covariates on the odds of detecting a *P. falciparum*/*P. vivax* infection or
193 gametocytemia was modeled using multivariable logistic linear regression. Covariates were selected a
194 priori on the basis of previous knowledge. Univariate factors were calculated for RDT-diagnosed *P.*
195 *falciparum* infections due to the low number of positive detect ions. All analyses were performed in
196 R version 3.4.1. Packages *plyr* and *reshape2* were used for structuring of data; packages *limma, gplots,*
197 *beeswarm* and *forestplot* for production of graphics; package *zoo* was used to calculate a rolling mean
198 of diagnostic sensitivity.

199

200 **Role of the funding source**

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

204

205 **RESULTS**

206 **The effect of test sensitivity on *P. falciparum* and *P. vivax* prevalence estimates**

207 Using large blood volumes and hv-us-qPCR, *P. falciparum* and *P. vivax* infections were detected in
208 53% (CI_{95}: 48-59) and 45% (CI_{95}: 39-51) of study participants (Table 1). Half of these infections were
209 missed using st-qPCR (on small blood volumes), resulting in almost two-fold lower prevalence rates of
210 29% (CI_{95}: 24-35) and 24% (CI_{95}: 20-30) for *P. falciparum* and *P. vivax* (Table 1).

211 Performing us-qPCR on small blood volumes increased parasite prevalence estimates slightly (*P.*
212 *falciparum*, 33%, CI_{95}: 27-38; *P. vivax*, 29%, CI_{95}: 24-35; Table 1) compared to st-qPCR. Parasite
213 densities in these additionally positive infections were similar to the lowest parasite densities detected
214 by st-qPCR (Figure 1B&C), with a median of 1·01 (IQR: 0·86-1·76) estimated *P. falciparum*

215 parasites/µl blood and 0·08 (IQR 0·03-0·16) estimated *P. vivax* parasites/µl blood (based on a
216 conversion formula in the Appendix, pages 6-10). In other words, detection of infections with few or a
217 single parasite in the small blood volume was more reliable using us-qPCR compared to st-qPCR, as
218 the higher number of DNA sequences targeted in us-qPCR reduces the effect of chance.

219 Detection of the lowest parasitemias was only achieved by hv-us-qPCR, in which a larger blood volume
220 equivalent is examined (Figure 1B&C). However even at such maximized sensitivity a chance effect
221 remained in detecting low-density infections, which was apparent from an imperfect overlap of
222 positivity between the molecular detection methods (Appendix, page 11).
223

224 In the 247 samples that were tested using RDT, st-RDT detected 15% (20/135) of all *P. falciparum*
225 infections (Figure 2A). us-RDT detected 27% (36/135) of all *P. falciparum* infections, corresponding
226 to 51% (36/70) of st-qPCR-detectable *P. falciparum* infections (Figure 2A). us-RDT detected *P.*
227 *falciparum* infections with lower parasitemia compared to st-RDT (Figure 1A) and showed improved
228 diagnostic performance over the whole range of *P. falciparum* densities (Figure 2B).

229 A single *P. vivax* infection was identified by st-RDT, which was in stark contrast to the 118 *P. vivax*
230 infections that were detected by qPCR methods in the subset of samples that were tested with RDT.

231

232 **Prevalence and density of gametocytes in infections detectable by different diagnostics**

233 Parasite and gametocyte densities correlated better for *P. vivax* (r=0·69) than for *P. falciparum* (r=0·42,
234 Appendix, page 13). For both species, parasite density was the single most important predictor for
235 gametocyte carriage (Appendix, page 14).

236 Gametocytes were detected in 95% (19/20; CI_{95}: 73-100) of *P. falciparum* infections identified by st-
237 RDT and in 75% (12/16; CI_{95}: 47-92) of infections additionally identified by us-RDT (Figure 3A).
238 Gametocytes were also detected in 44% (44/99; CI_{95}: 35-55) of us-RDT-negative/qPCR-positive *P.*
239 *falciparum* infections (Figure 3A). Of all *P. falciparum* gametocyte carriers, 59% (44/75) were not
240 detected by us-RDT. The range of gametocyte densities in us-RDT-negative gametocyte carriers was
241 comparable to that in us-RDT- and st-RDT-positive gametocyte carriers (Figure 3D).
242

243 When using molecular diagnosis, gametocytes were most common in st-qPCR-detectable *P. falciparum*
244 and *P. vivax* infections (gametocyte positive: *P. falciparum*, 82%, 71/87, CI_{95}: 72-89%; *P. vivax*, 92%,
245 67/73, CI_{95}: 82-97%, Figure 3B&C). More than half of infections additionally detected by us-qPCR
246 also carried gametocytes (*P. falciparum*, 67%, 10/15, CI_{95}: 39-87%; *P. vivax*, 58%, 11/19, CI_{95}: 34-
247 79%; Figure 3B&C). The proportion of gametocyte carriers was considerably lower in infections only
248 detectable in hv-us-qPCR (*P. falciparum*, 20%, 12/59, CI_{95}: 11-33%; *P. vivax*, 8%, 4/49, CI_{95}: 3-20%).

249 As a result, diagnosis of infections using st-qPCR missed 24% and 18% of all *P. falciparum* and *P.*
250 *vivax* gametocyte carriers in the population (Table 1). Using us-qPCR, only 14% and 9% of *P.*
251 *falciparum* and *P. vivax* gametocyte carriers were missed.
252
253 Mean *P. falciparum* and *P. vivax* gametocyte densities were significantly lower in infections that were
254 not detected by st-qPCR compared to those that were (geometric mean; *P. falciparum*, 1·0 vs 31·6 *pfs25*
255 transcripts/µl blood, $p<0{\cdot}001$; *P. vivax*, 0·3 vs 5·6 *pvs25* transcripts/µl blood, $p<0{\cdot}001$). In infections
256 that were only detected by hv-us-qPCR, estimated gametocyte densities did not exceed 1 gametocyte/µl
257 blood (based on previously published conversion formulas (13,17) (Figure 3E&F; Appendix, pages 6-
258 10). Also in infections that were detected by us-qPCR but not by st-qPCR, estimated gametocyte
259 densities were below 1 gametocyte/µl blood in all but one infection (Figure 3E&F).
260
261
262 **Identification of risk factors for malaria infection by different diagnostic methods**
263 The same main risk factors for malaria infection were identified by st-qPCR and hv-us-qPCR (Figure
264 4, Appendix, page 15). Age was the only significant predictor for the odds of a *P. vivax* infection. The
265 odds of a *P. falciparum* infection was significantly associated with village of residence and
266 haemoglobin level. Patterns in the odds of RDT-diagnosed *P. falciparum* infections were similar to
267 those of molecular *P. falciparum* diagnosis; however, the power of risk analysis was low due to the low
268 number of RDT-positive detections (Appendix, page 16-17).

269 The proportion of ultra-low density infections among all infections was up to 2-fold higher in population
270 subgroups with low parasite prevalence compared to subgroups with high prevalence. For example, as
271 *P. vivax* prevalence dropped from 63% (30/48, CI_{95}: 47-76%) in 11-15 year old children to 31% (14/45,
272 CI_{95}: 19-47%) in adults older than 50 years (Figure 5B), the proportion of ultra-low-density *P. vivax*
273 infections rose from 30% (9/30, CI_{95}: 15-50%) in the 11-15 years old children to 64% (9/14, CI_{95}: 36-
274 86%; Figure 5B) in the oldest age group. Overall, *P. vivax* density decreased with increasing age (Figure
275 5D, Anova $p<0.001$), while no clear trends with age were observed for *P. falciparum* (Figure 5A). For
276 *P. falciparum*, parasite prevalence differed between villages and was inversely related to the proportion
277 of ultra-low density infections per village (Appendix, page 18). However, these differences between
278 villages were not statistically significant.
279
280 **DISCUSSION AND CONCLUSION**
281 In this study we applied multiple molecular diagnostic methods with maximized sensitivity to explore
282 the true prevalence of *P. falciparum* and *P. vivax* in an endemic population in PNG. This revealed an

283 unexpectedly large reservoir of infections below the limit of detection of standard molecular diagnosis.
284 Main limiting factors were the blood volume sampled and the blood equivalent added to the detection
285 assay. However, complex laboratory procedures are necessary when using large blood volumes, which
286 are not feasible for routine malaria surveillance or intervention monitoring. This raises the questions
287 whether malaria interventions aimed at reducing transmission can benefit from detecting these ultra-
288 low-density residual infections.

289 In cross-sectional surveys, the density of gametocytes in the host's blood is often used as a surrogate
290 marker for the transmission potential to mosquitoes. Directly measuring infectivity in cross-sectional
291 surveys is challenging as it would require feeding of colony mosquitoes by direct exposure of the
292 infected individual or by membrane blood feeding. Although gametocyte density is positively
293 associated with infection success in membrane feeding experiments (18–20), measuring gametocyte
294 densities in the hosts's blood may provide only a limited picture of the true probability of onwards
295 transmission. This may rather depend on the density of mature gametocytes in the subcutaneous tissue,
296 where gametocytes may aggregate to facilitate transmission to mosquitoes (21).

297 In our study gametocyte densities were estimated from the number of *pfs25* or *pvs25* transcripts, both
298 highly expressed in mature female gametocytes. High-volume RNA sampling maximized the limit of
299 gametocyte detection to below 1 *P. falciparum* or 11 *P. vivax* gametocytes per 800µl blood (detailed
300 discussion of molecular quantification, Appendix, pages 6-10). Estimated gametocyte densities in our
301 study were often below 1 gametocyte per 1µl blood, a threshold below which mosquito infection is rare
302 in membrane feeding experiments (18–20). In fact, with one exception, estimated gametocyte densities
303 were below 1 gametocyte/µl blood in all infections undetected by st-qPCR, suggesting that those are
304 unlikely infective to mosquitoes. However, if parasitemia in infections undetectable by st-qPCR at the
305 time of sampling would increase at a later time point, the likelihood of transmission would increase.
306 Studies on the longitudinal dynamics of chronic *P. falciparum* infections revealed fluctuations in clonal
307 densities by transient absence and later re-appearance of clones (22,23). Large fluctuations in
308 *Plasmodium* densities over time have been described in Vietnam (24); however, in absence of parasite
309 genotyping it cannot be evaluated whether the observed density peaks represent new infections. In a
310 cohort of PNG children, 70% of febrile malaria episodes showed a new genotype (25). Low-density
311 clones persisting around the levels of qPCR detection thus seem to be under density control (with
312 fluctuations) and, in absence of superinfection, asymptomatic individuals are unlikely becoming highly
313 effective transmitters.

314 While molecular methods are required to detect very low gametocyte densities, the associated asexual
315 parasite densities are approximately 10 to 100-fold higher and are thus detectable with less sensitive
316 methods. In a recent multi-country trial, high-quality research-grade microscopy identified >90% of
317 infectious *P. falciparum* carriers in high-transmission settings and two of three infectious carriers in a

318 low-transmission setting (26). In the same study, all infectious carriers were detectable by standard
319 molecular methods using finger-prick blood volumes (26). These results support our finding that little
320 can be gained by laborious sampling and processsing of larger blood volumes when diagnosis aims at
321 identifying infectious individuals.

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

330 In a previous comparative diagnostic study, the us-RDT missed 16% and 56% of PCR-detectable *P.*
331 *falciparum* infections in a high endemic (Uganda) and low endemic (Myanmar) setting (8). In PNG, us-
332 RDT missed 50% of *P. falciparum* infections that were detectable using st-qPCR, including samples
333 with high gametocyte densities. Although the effect on us-RDT sensitivity of using frozen-thawed
334 venous blood rather than fresh finger-prick blood in both studies is unknown, us-RDT seems a
335 suboptimal substitute for molecular diagnosis in anti-malarial interventions such as screen-and-treat
336 interventions for reducing or eliminating malaria transmission in PNG.

337 Although PNG currently does not represent a low-endemic or pre-elimination setting, where detecting
338 very-low density infections is considered particularly relevant, its unique local epidemiology resembles
339 that of other *P. falciparum*-*P. vivax*-endemic settings with declining transmission: Corresponding to
340 global trends of an increasing proportion of submicroscopic infections with decreasing parasite
341 prevalence (1), also in PNG parasite densities declined over the last decade alongside a decline in
342 clinical incidence and prevalence of malaria (12). Furthermore, malaria transmission in PNG is highly
343 heterogeneous over small spatial scales (25), which is considered a hallmark of declining transmission
344 and has been described in a variety of settings such as western Kenya (27), Thailand (28), and the
345 Peruvian Amazon (29).

346 A main limitation of our study was the exclusion of children younger than five years for ethical reasons.
347 Young children carry the main burden of malaria infection and disease, however, it is thought that their
348 contribution to mosquito infections is smaller than that of adolescents and adults (30). As parasite
349 densities are higher in young PNG children compared to adolescents and adults (17), ultra-low-density
350 infections may be less common in young children, and therefore also the gain by applying ultra-sensitive
351 diagnostics would be lower.

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

358 In conclusion, we have shown that the extent of both *P. falciparum* and *P. vivax* infections below the
359 limit of detection of standard molecular malaria diagnostics is substantial. Yet, gametocyte densities in
360 infections undetected by standard molecular diagnostics were very low and potentially not infective.
361 The us-RDT did not achieve this level of sensitivity and missed infections with high gametocyte
362 densities. Our findings relax the pressure to identify the very last parasite and advocate against the need
363 for venous sampling in malaria control and elimination interventions.

364

365 **CONTRIBUTORS**

366 Hofmann N.E, Data collection, data curation, data analysis, data interpretation, methodology, writing—
367 original draft; Gruenberg M., Data collection, data curation, data analysis, methodology, writing—
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375 Conceptualization, project administration, supervision, funding acquisition, data interpretation,
376 writing—review and editing.

377

378 **CONFLICT OF INTEREST**

379 We declare that we have no conflicts of interest.

380

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391
392
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479 falciparum malaria: past and future. Trends Parasitol. 2015 Jul;31(7):287–96.

480

481

482 **FIGURE LEGENDS**

483 **Figure 1. Parasite density distributions in *P. falciparum* (A,B) and *P. vivax* (C) infections detected
484 by RDT and molecular methods with different sensitivity.** Parasite density by hv-us-qPCR is plotted
485 (underlying histograms in the Appendix, page 5), therefore only samples positive in hv-us-qPCR are
486 shown. Samples were categorized according to their positivity by the specific detection methods.
487 Categories are: A, st-RDT positive, st-RDT negative/us-RDT positive, st-RDT and us-RDT-
488 negative/qPCR positive; in B&C, st-qPCR positive, st-qPCR negative/us-qPCR positive, st-PCR and
489 us-qPCR negative/hv-us-qPCR positive. Different colours represent the different categories. An
490 unknown number of target sequences is amplified in *P. falciparum* and *P. vivax* ultra-sensitive qPCR,
491 hence parasite densities cannot be directly compared between the two species (see discussion on
492 quantifying parasitemia by molecular methods in the Appendix, pages 6-10).

493

494 **Figure 2. Diagnostic performance of *P. falciparum* RDTs compared to qPCR methods in a subset
495 of 247 samples.** Frozen whole blood for RDT analysis was only available for 247/300 samples. (A)
496 Venn diagram of *P. falciparum* positivity by st-RDT, us-RDT and molecular detection methods. Five
497 samples were positive by st-RDT and/or us-RDT but negative by st-qPCR, and would thus have been
498 considered false positive by RDT. However, *P. falciparum* parasites were detected in all RDT-positive
499 samples using hv-us-qPCR. (B) Diagnostic sensitivity of st-qPCR, us-RDT and st-RDT in relation to

500 parasite densitiy (by hv-us-qPCR). Diagnostic sensitivity was calculated as a rolling mean of 10
501 observations using combined detections by any qPCR as reference, and is shown with 95% CI (shaded
502 areas). Curves were smoothed using lowess function (span=0.16). An assessment of overall RDT
503 diagnostic performance (sensitivity and specificity) is shown in the Appendix, page 12.

504

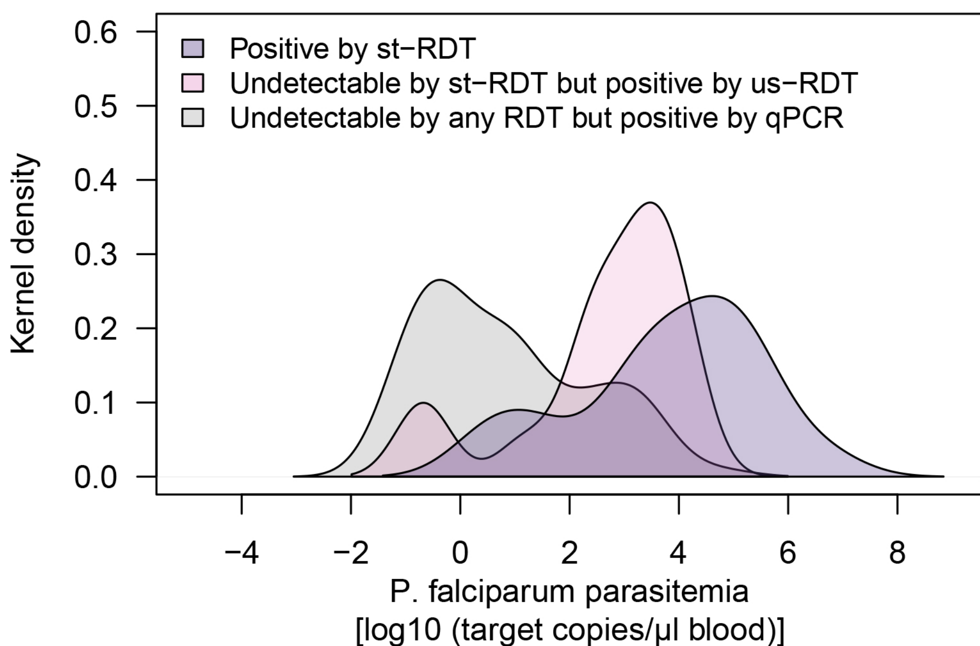
505 **Figure 3. Proportion of gametocyte-positive infections (A-C) and gametocyte density (D-F) in**
506 **infections detected by RDT (A,D) and molecular methods with different sensitivity (B,C,E,F). (A-**
507 **C).** Samples were categorized according to their positivity by the different diagnostic methods as
508 specified under each bar (corresponding to Figure 1). (A-C) The proportion of gametocyte positive
509 samples in each category is shown. (D-F) For each category, the concentration of gametocytes-specific
510 transcripts in the corresponding samples is displayed, with each dot representing one sample. For each
511 category, summary lines are displayed: thick line, median; thin lines, IQR. A different number of *pfs25*
512 and *pvs25* transcripts is amplified per *P. falciparum* and *P. vivax* gametocyte, hence gametocyte
513 densities cannot be directly compared between the two species (see discussion on quantifying
514 gametocytes by molecular methods in the Appendix, pages 6-10). *pfs25* and *pvs25* copy numbers
515 corresponding to one gametocyte (within the confidence range, based on previously published
516 correlations in (13,17)) are delineated with a horizontal line.

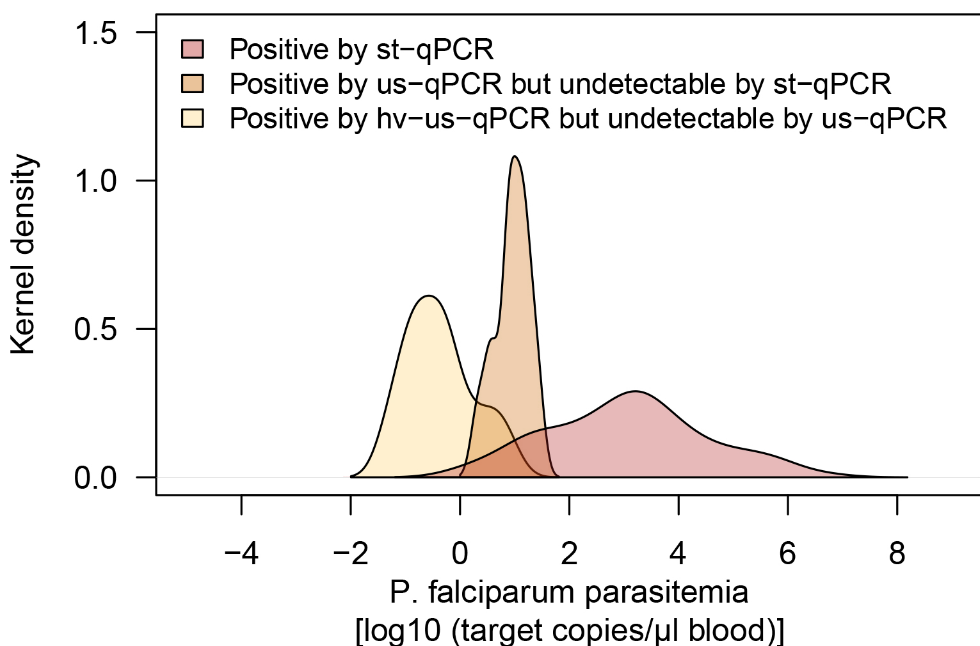
517

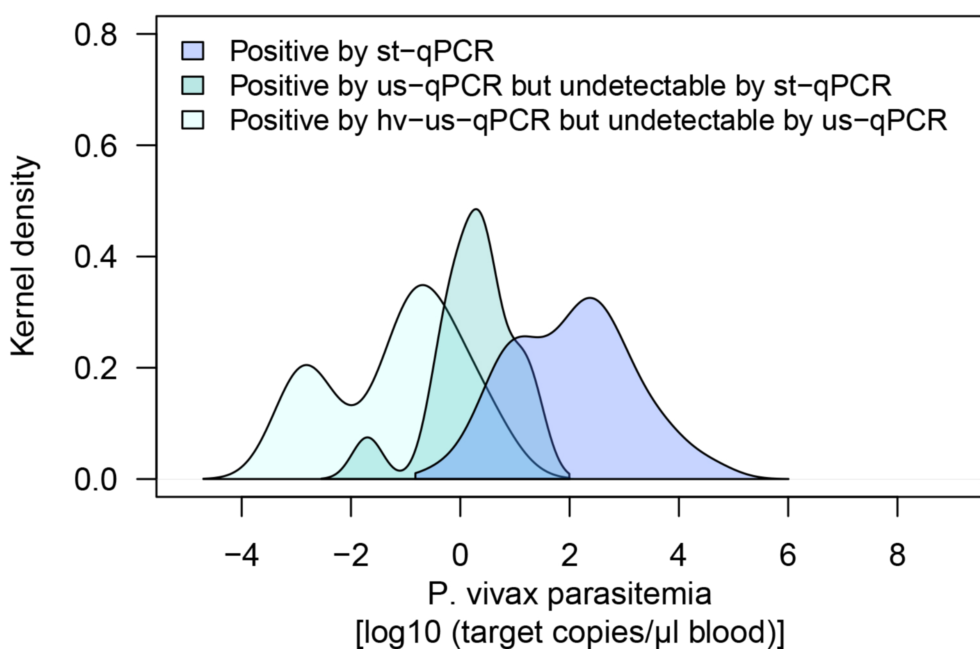
518 **Figure 4. Forest plot comparing the epidemiological patterns in *P. falciparum* (A) and *P. vivax* (B)**
519 **infections detected using molecular methods with different sensitivity.** Odds ratios were modeled
520 using logistic regression for infections detected using st-qPCR or using hv-us-qPCR. Detailed numeric
521 model results for qPCR diagnosis (as well as for RDT diagnosis) are shown in the Appendix, pages 15-
522 17.

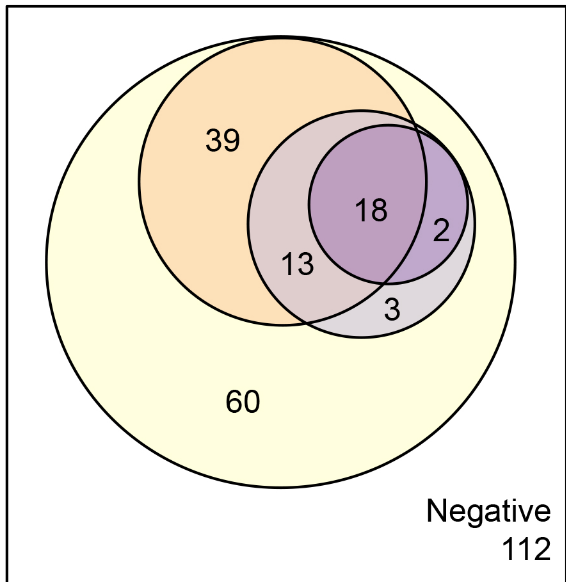
523

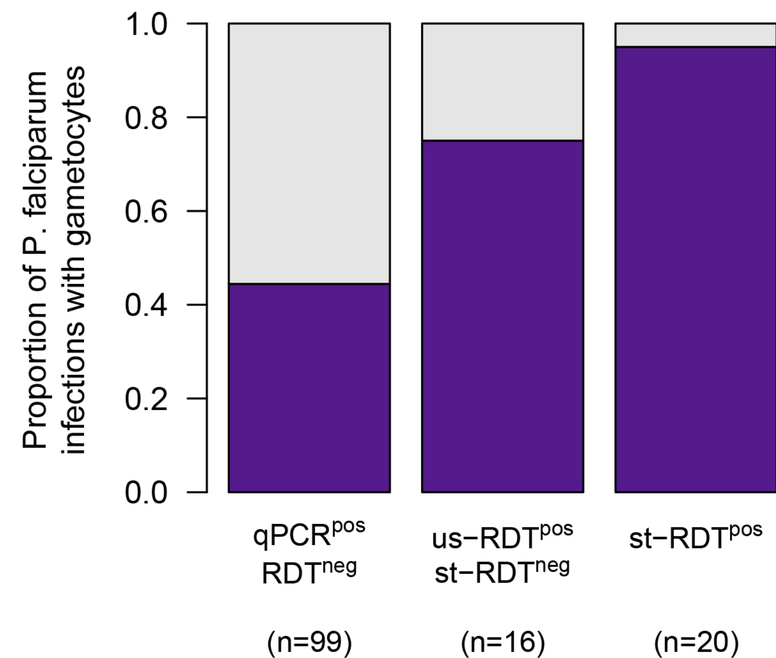
524 **Figure 5. Age patterns in *P. falciparum* (A, C) and *P. vivax* (B, D) infections.** (A,B) Age patterns in
525 parasite prevalence (by hv-us-qPCR) and in the proportion of infections undetectable by st-qPCR.
526 Shaded areas represent 95% confidence intervals. (C, D) Age patterns in parasite density (by hv-us-
527 qPCR). Each dot represents one sample in the respective age group, and summary lines are displayed
528 (thick line, median; thin lines, IQR). Parasite densities in infections undetectable by st-qPCR are plotted
529 in yellow (C) and light blue (D). An unknown number of target sequences is amplified in *P. falciparum*
530 and *P. vivax* ultra-sensitive qPCRs, hence parasite densities cannot be directly compared between the
531 two species (see discussion on quantifying parasitemia by molecular methods in the Appendix, pages
532 6-10).

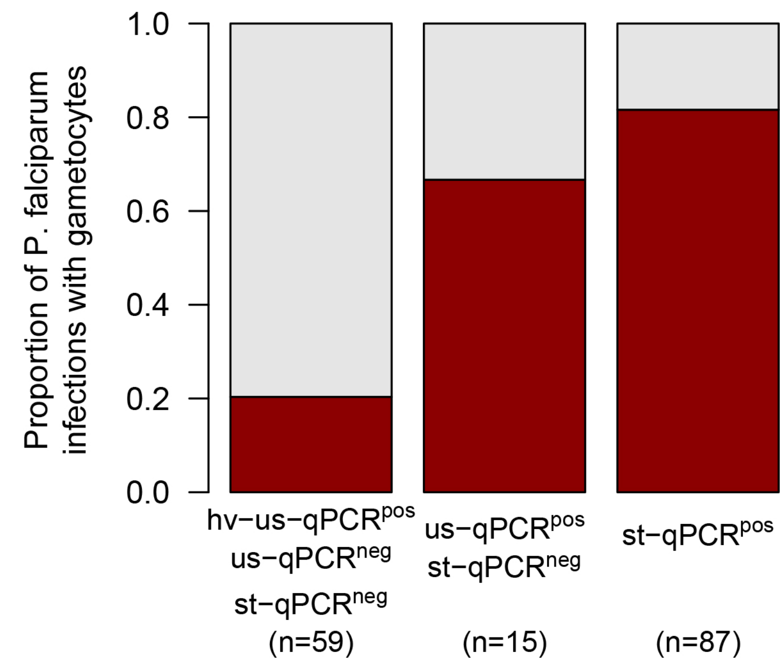

A
Positive by st−RDT
Undetectable by st−RDT but positive by us−RDT
Undetectable by any RDT but positive by qPCR
Kernel density
P. falciparum parasitemia
[log10 (target copies/µl blood)]

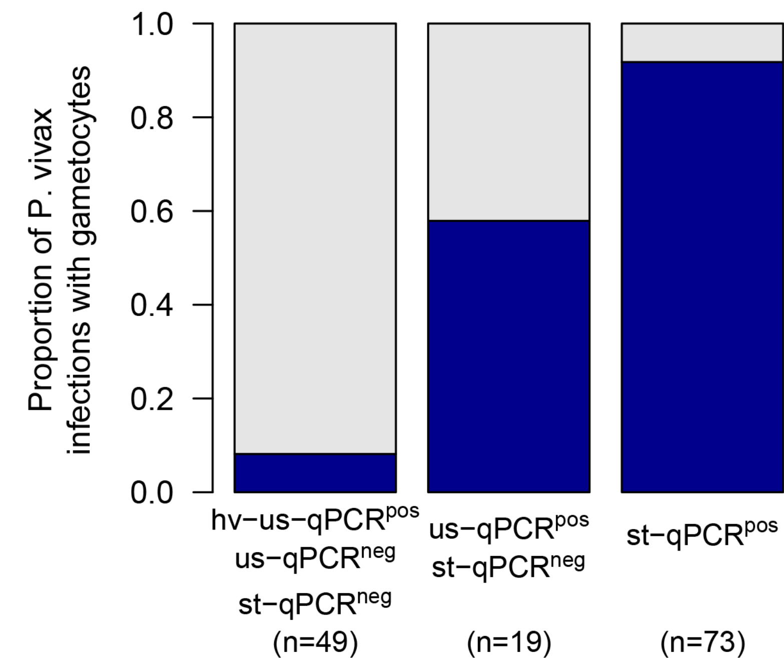

B
Positive by st−qPCR
Positive by us−qPCR but undetectable by st−qPCR
Positive by hv−us−qPCR but undetectable by us−qPCR
Kernel density
P. falciparum parasitemia
[log10 (target copies/µl blood)]

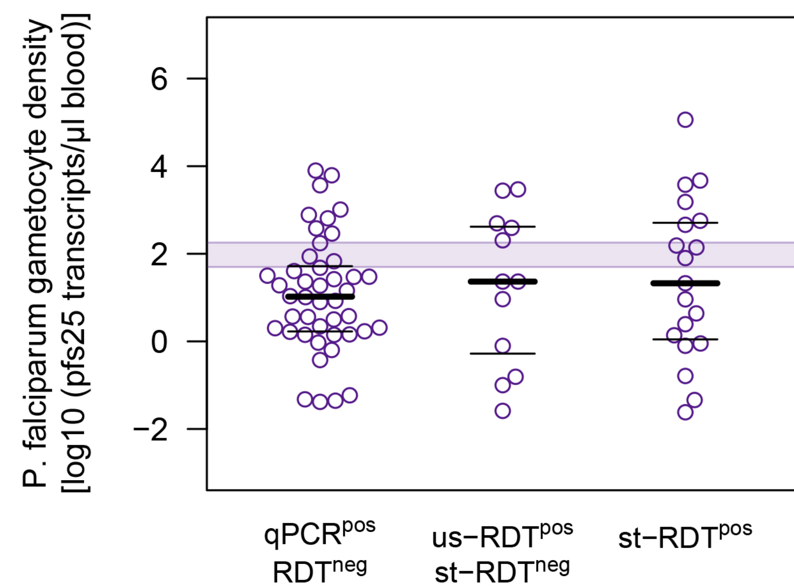

C
Positive by st−qPCR
Positive by us−qPCR but undetectable by st−qPCR
Positive by hv−us−qPCR but undetectable by us−qPCR
Kernel density
P. vivax parasitemia
[log10 (target copies/µl blood)]

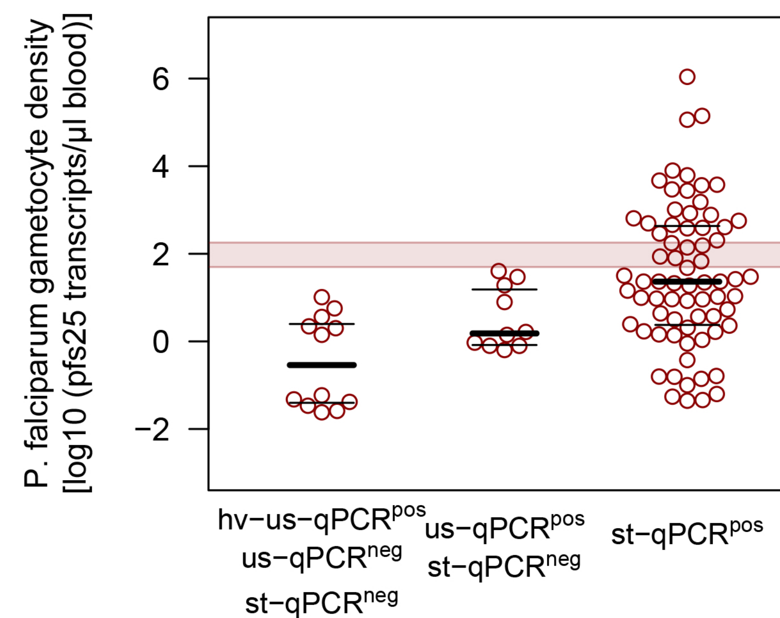

A
39
18
2
13
3
60
Negative
112
Standard RDT
Ultra-sensitive *Pf*-RDT
Standard qPCR
Any qPCR (reference)
B
st−qPCR
us−RDT
st−RDT
Diagnostic sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
−1
0
1
2
3
4
5
P. falciparum parasitemia
[log10 (target copies/μl blood)]

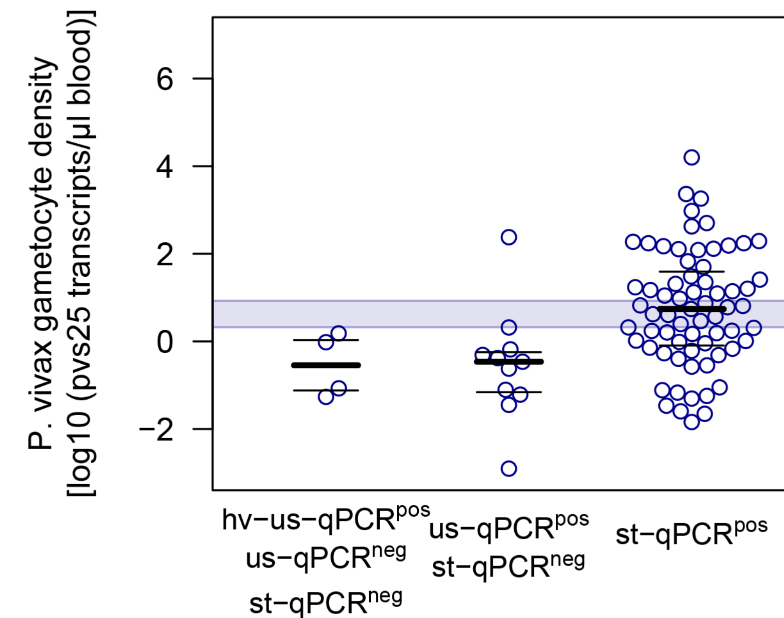

A
Proportion of P. falciparum infections with gametocytes
1.0
0.8
0.6
0.4
0.2
0.0
qPCR^{pos} RDT^{neg}
us−RDT^{pos} st−RDT^{neg}
st−RDT^{pos}
(n=99)
(n=16)
(n=20)


B
Proportion of P. falciparum infections with gametocytes
1.0
0.8
0.6
0.4
0.2
0.0
hv−us−qPCR^{pos} us−qPCR^{neg} st−qPCR^{neg}
us−qPCR^{pos} st−qPCR^{neg}
st−qPCR^{pos}
(n=59)
(n=15)
(n=87)


C
Proportion of P. vivax infections with gametocytes
1.0
0.8
0.6
0.4
0.2
0.0
hv−us−qPCR^{pos} us−qPCR^{neg} st−qPCR^{neg}
us−qPCR^{pos} st−qPCR^{neg}
st−qPCR^{pos}
(n=49)
(n=19)
(n=73)


D
P. falciparum gametocyte density [log10 (pfs25 transcripts/µl blood)]
6
4
2
0
−2
qPCR^{pos} RDT^{neg}
us−RDT^{pos} st−RDT^{neg}
st−RDT^{pos}


E
P. falciparum gametocyte density [log10 (pfs25 transcripts/µl blood)]
6
4
2
0
−2
hv−us−qPCR^{pos} us−qPCR^{neg} st−qPCR^{neg}
us−qPCR^{pos} st−qPCR^{neg}
st−qPCR^{pos}


F
P. vivax gametocyte density [log10 (pvs25 transcripts/µl blood)]
6
4
2
0
−2
hv−us−qPCR^{pos} us−qPCR^{neg} st−qPCR^{neg}
us−qPCR^{pos} st−qPCR^{neg}
st−qPCR^{pos}

A

High−volume ultra−sensitive qPCR
Standard qPCR

Age 11−15
Age 16−25
Age 26−50
Age >51
Village Mirap
Male gender
Slept under bednet
Antimalarial <30d
Fever
Haemoglobin

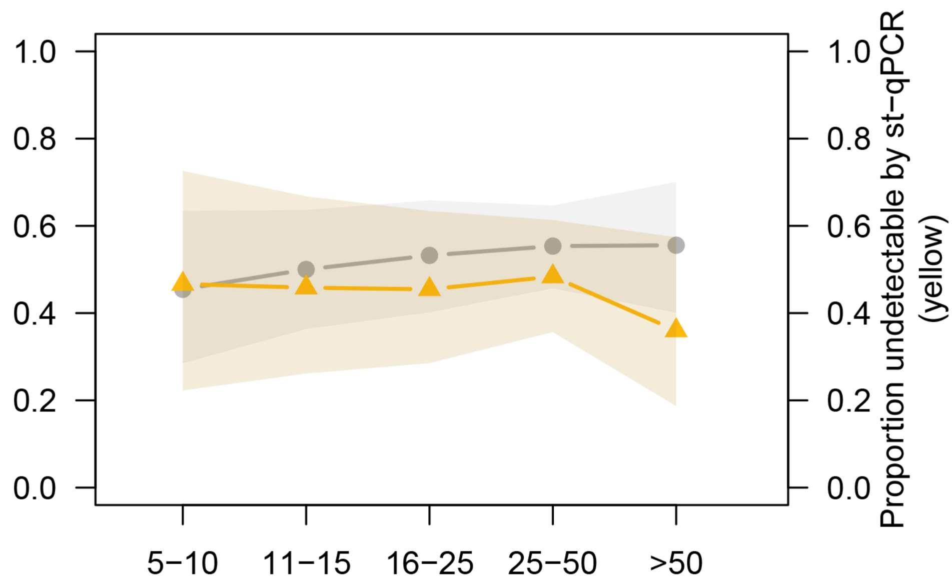
0 1 2 3 4 5 6 7
Odds Ratio

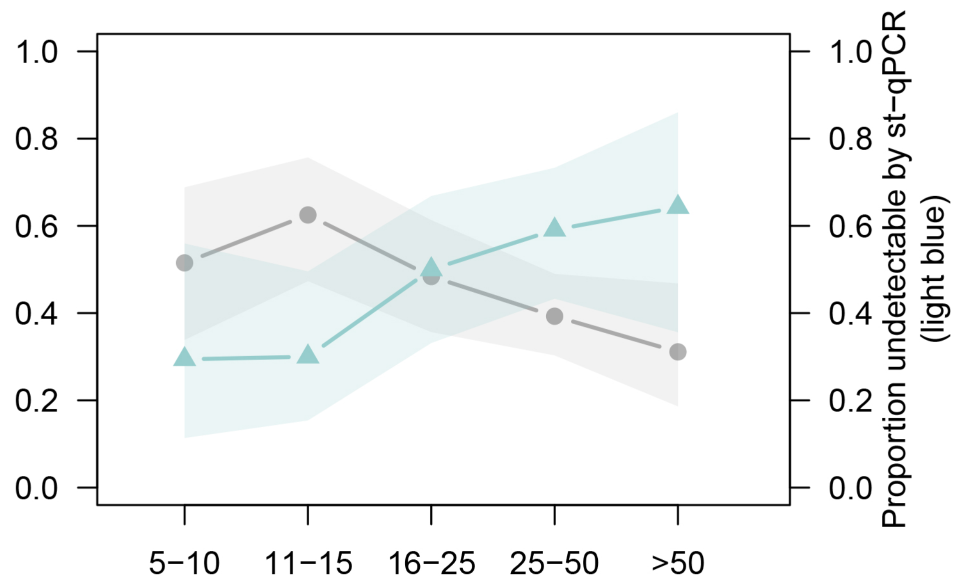
B

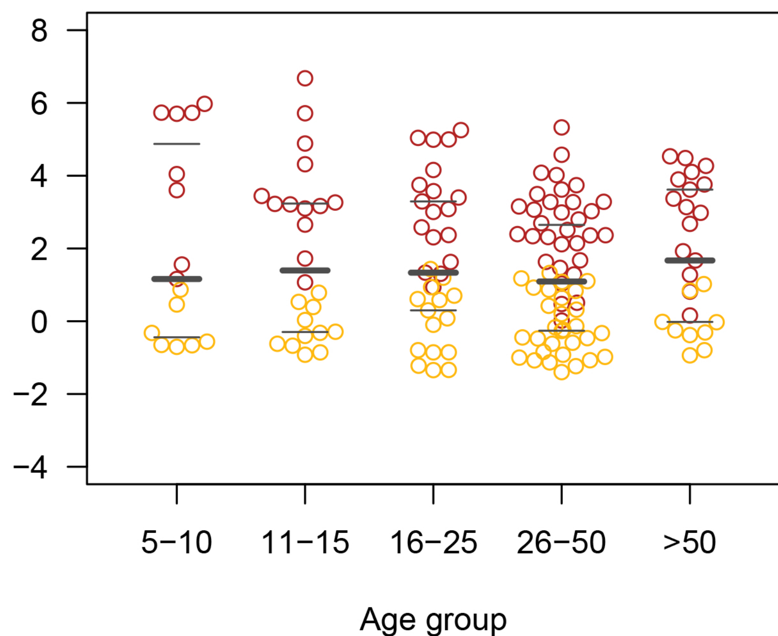
High−volume ultra−sensitive qPCR
Standard qPCR

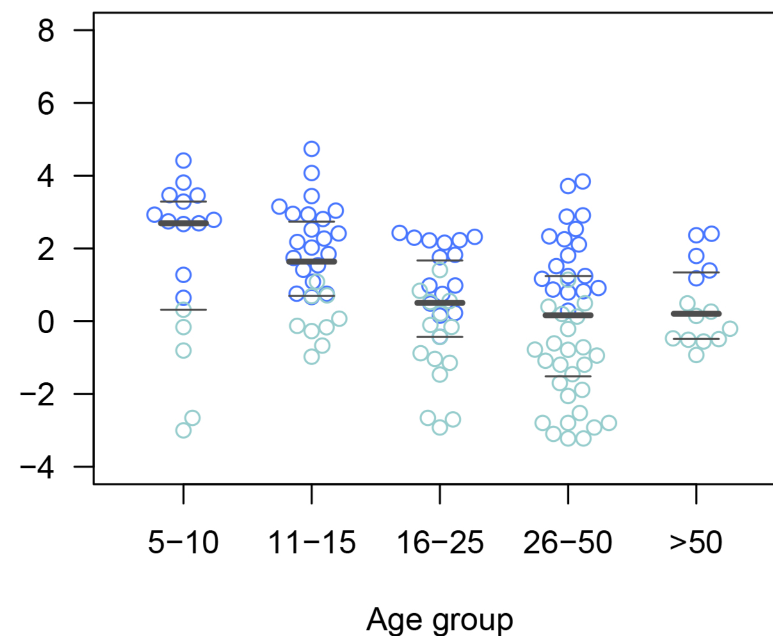
Age 11−15
Age 16−25
Age 26−50
Age >51
Village Mirap
Male gender
Slept under bednet
Antimalarial <30d
Fever
Haemoglobin

0 0.5 1 1.5 2 2.5 3 3.5 4
Odds Ratio


A
P. falciparum prevalence by hv−us−qPCR (grey)
Proportion undetectable by st−qPCR (yellow)
1.0
0.8
0.6
0.4
0.2
0.0
5−10
11−15
16−25
25−50
>50


B
P. vivax prevalence by hv−us−qPCR (grey)
Proportion undetectable by st−qPCR (light blue)
1.0
0.8
0.6
0.4
0.2
0.0
5−10
11−15
16−25
25−50
>50


C
P. falciparum density [log10 (target transcripts/µl blood)]
8
6
4
2
0
-2
-4
5−10
11−15
16−25
26−50
>50
Age group


D
P. vivax density [log10 (target transcripts/µl blood)]
8
6
4
2
0
-2
-4
5−10
11−15
16−25
26−50
>50
Age group