

1 Analysis of nucleic acids extracted from rapid diagnostic
2 tests reveals a significant proportion of false positive test
3 results associated with recent malaria treatment

4 Running title: Risk factors related to false positive malaria RDTs

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6 Salome Hosch^{a,b}, Charlene Aya Yoboue^{a,b,g}, Olivier Tresor Donfack^c, Etienne A. Guirou^{a,b},
7 Jean-Pierre Dangy^{a,b}, Maxmillian Mpina^{d,e}, Elizabeth Nyakurungu^{d,e}, Koranan Blöchlinger^{a,b},
8 Carlos A. Guerra^c, Wonder P. Phiri^c, Mitoha Ondo'o Ayekaba^f, Guillermo A. García^c, Marcel
9 Tanner^{a,b}, Claudia Daubenberger^{a,b,¶,#} & Tobias Schindler^{a,b,e,¶,#}

10

11 ^a Swiss Tropical and Public Health Institute, Basel, Switzerland

12 ^b University of Basel, Basel, Switzerland

13 ^c Medical Care Development International, Malabo, Equatorial Guinea

14 ^d Ifakara Health Institute, United Republic of Tanzania

15 ^e Laboratorio de Investigaciones de Baney, Baney, Equatorial Guinea

16 ^f Ministry of Health and Social Welfare, Malabo, Equatorial Guinea

17 ^g Centre Suisse de Recherches Scientifiques en Côte d'Ivoire, Abidjan, Côte d'Ivoire

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19 ¶ These authors contributed equally to this work.

20 # Corresponding authors: Tobias Schindler, tobias.schindler@swisstph.ch; Claudia

21 Daubenberger, claudia.daubenberger@swisstph.ch

22 Abstract

23

24 Surveillance programs often use malaria rapid diagnostic tests (RDTs) to determine the
25 proportion of the population carrying parasites in their peripheral blood to assess the malaria
26 transmission intensity. Despite an increasing number of reports on false-negative and false-
27 positive RDT results, there is a lack of systematic quality control activities for RDTs deployed
28 in malaria surveillance programs. Our study provides a larger scale comparative evaluation of
29 RDTs used in the 2018 Malaria Indicator Survey (MIS) conducted on Bioko Island, Equatorial
30 Guinea. We conducted a molecular analysis by extraction of nucleic acids from 1,800 negative
31 and 1,065 positive RDTs followed by qPCR analysis. These results were combined with a
32 dataset collected in a comprehensive questionnaire from each MIS participant. Of the 2,865
33 RDTs that were collected in 2018 on Bioko Island and analysed in our study, 4.7% had a false-
34 negative result. These false-negative RDT results were associated with low parasite density
35 infections. In a substantial proportion of samples, we identified masked *pfhrp2* and *pfhrp3* gene
36 deletions in which at least one *P. falciparum* strain carried a gene deletion. Among all positive
37 RDTs analysed, 28.4% were tested negative by qPCR and therefore considered to be false-
38 positive. Analysing the questionnaire data collected from the participants, this high proportion
39 of false-positive RDT results could be explained by PfHRP2 antigen persistence after recent
40 malaria treatment. We conclude that malaria surveillance depending solely on RDTs needs
41 well-integrated quality control procedures assessing the extend and impact of reduced
42 sensitivity and specificity of RDTs on malaria control programs.

43 Key words

44

45 Molecular malaria surveillance; false-positive malaria rapid diagnostic test; PfHRP2
46 persistence; *pfhrp2* gene deletion

47

48 Abbreviations

49

50 Pf (*Plasmodium falciparum*), RDT (rapid diagnostic test), PfHRP2/3 (histidine rich protein
51 2/3), ENAR (Extraction of nucleic acids from RDTs), MIS (Malaria Indicator Survey), RT-
52 qPCR (reverse transcription quantitative polymerase chain reaction), Cq (quantification cycle),
53 sSA (sub-Saharan Africa), ACT (artemisinin combination therapy), SES (socio-economic
54 status), NA (nucleic acid)

55 Introduction

56

57 According to the World Health Organization, more than 409,000 malaria deaths were reported
58 in 2019, most of them in children below the age of five years ¹. The majority of malaria
59 infections (94%) and malaria related deaths (95%) occurred in sub-Saharan Africa (sSA), where
60 *Plasmodium falciparum* (*Pf*) is the dominant malaria parasite ¹. The test-treat-track strategy
61 advised by WHO is one of the backbones of current malaria control and elimination programs
62 ². This strategy entails that every suspected malaria case should be tested, every confirmed case
63 should be treated, and the disease should be tracked through surveillance systems ³. Testing
64 relies heavily on rapid diagnostic tests (RDTs), exemplified by the more than 348 million RDTs
65 distributed globally in 2019 ¹. In sSA, RDTs have almost completely replaced light microscopy
66 for malaria diagnosis, accounting for an estimated 75% of all malaria tests conducted in 2017
67 ⁴. RDTs are point-of-care tests that detect circulating antigens like the *Pf* specific histidine rich
68 protein 2 (PfHRP2) or histidine rich protein 3 (PfHRP3) and the pan-*Plasmodium* spp. enzymes
69 lactate dehydrogenase (pLDH) or aldolase ⁵. More than 90% of RDTs currently in use target
70 the PfHRP2 antigen because of its higher sensitivity compared to non-PfHRP2 antigens ⁶.
71 PfHRP2-based RDTs used for the diagnosis of febrile patients that suffer from malaria infection
72 are highly sensitive and specific ⁷. RDTs are often used by national malaria surveillance
73 programs. However, when individuals are asymptomatic with low parasite densities, RDTs
74 often fail to detect the parasites due to low antigen concentrations ^{8,9}. A recent study showed
75 that false-negative RDTs (FN-RDT) are more common in lower malaria transmission settings,
76 younger subjects, and in urban areas in sSA ¹⁰. Reduced diagnostic performance of RDTs has
77 also been attributed to genetic diversity of the *pfhrp2* gene ¹¹, differences in expression levels
78 of PfHRP2 antigen in parasite field strains ¹², or deletion of *pfhrp2* and *pfhrp3* genes in isolates
79 ¹³. *Pfhrp2* gene deletions appear to be common and therefore are relevant as they might be a

80 threat to malaria control programs based on monitoring of malaria prevalence through RDT ¹⁴,
81 ¹⁵.

82 Less attention has been given to the specificity of malaria RDTs used in malaria surveys that
83 potentially result in false positive results. False-positive RDTs (FP-RDT) have been associated
84 with high levels of circulating rheumatoid factor ¹⁶⁻¹⁸ or acute typhoid fever ¹⁹. There is evidence
85 of FP-RDTs in patients infected with *Schistosoma mekongi* ²⁰ or human African
86 trypanosomiasis ²¹. FP-RDTs are also caused by persisting antigen circulation in peripheral
87 blood after malaria drug treatment. A meta-analysis revealed that half of the PfHRP2-detecting
88 RDTs remain positive 15 (95% CI: 5-32) days post malaria treatment, 13 days longer than RDTs
89 targeting the pLDH antigen ²². The latter study also reported a higher persistent RDT positivity
90 among individuals treated with artemisinin combination therapy (ACT) than those treated with
91 other anti-malarial drugs. RDTs are instrumental to malaria surveillance programs, and
92 therefore their diagnostic performance should be systematically monitored over time using
93 molecular methods detecting *Plasmodium* spp. genomic markers. We describe here an approach
94 for quality control of field-deployed malaria RDTs by retrospective molecular analysis of the
95 parasite DNA retained on them using qPCR.

96 Materials and Methods

97

98 **The 2018 malaria indicator survey conducted on Bioko Island as a biobank of RDTs for** 99 **molecular malaria surveillance**

100 A malaria indicator survey has been conducted annually since 2004 on the Island of Bioko,
101 Equatorial Guinea, to evaluate the impact of malaria control interventions²³. The survey uses a
102 standard questionnaire developed by the Roll Back Malaria initiative to gather information on
103 selected households and their occupants. The 2018 Bioko Island MIS covered 4,774 households
104 with 20,012 permanent residents, among whom 13,505 persons consented to storage and
105 molecular analysis of their RDT. Briefly, consenting individuals living in surveyed households
106 are tested for malaria and malaria-related anaemia. Malaria testing was done with the
107 CareStartTM Malaria HRP2/pLDH (Pf/PAN) combo test (ACCESS BIO, New Jersey, USA).
108 The haemoglobin level in peripheral blood was measured during the MIS using a battery-
109 operated portable HemoCue system (HemoCue AB, Ängelholm, Sweden). The anaemia status
110 (mild, moderate, severe) was categorized based on definitions published by the World Health
111 Organization²⁴ stratified by age, gender, and pregnancy status. Households were assigned
112 scores based on the type of assets and amenities they own to derive a surrogate of their socio-
113 economic status (SES), using principal component analysis (PCA). After ranking all households
114 based on their score, they were divided into five equal categories (quintiles), each with
115 approximately 20% of the households. The first quintile corresponded to the lowest wealth
116 index and the fifth to the highest wealth index. The household wealth index categories were
117 also assigned to permanent household members.

118 **Detection and quantification of *Plasmodium* spp. nucleic acids extracted from RDTs**

119 We developed an approach to extract nucleic acids (NA) from blood retained on used malaria
120 RDTs named “Extraction of Nucleic Acids from RDTs” (ENAR)²⁵. Briefly, RDTs were
121 barcoded, stored at room temperature, and shipped to Basel, Switzerland, for NA extraction and
122 detection. This approach simplifies small volume blood collection, transport and storage
123 logistics, and allows linking outcomes of molecular based detection of parasite derived NA with
124 the demographic and socio-economic information collected from each corresponding MIS
125 participant at high throughput.

126 In total, 2,865 RDTs (21.2%) collected during the 2018 MIS were included in this study. The
127 median age in this sample collection was 22 years (interquartile range 9 to 38 years), female
128 participants were slightly overrepresented (58.2%), and 97.8% of the participants were
129 asymptomatic, non-febrile individuals. More than two-thirds of the RDTs were collected in the
130 urban areas of the capital city Malabo on Bioko Island.

131 All 2,865 samples were initially screened with the PlasQ RT-qPCR assay²⁶. In this RT-qPCR
132 assay, the high copy number *Pf* specific varATS region²⁷ and the pan-*Plasmodium* 18S rDNA
133 gene were targeted^{28,29}. Samples with cycle of quantification (Cq) value < 45 in two replicates
134 of either of the two targets, varATS or 18S rDNA, were considered positive. *Pf* parasites were
135 quantified based on their Cq value for varATS²⁵. In addition, only samples with Cq value < 35
136 for amplification of the internal control gene, the human *masep* gene were included, to
137 demonstrate that the NA extracted from the RDTs is sufficient for reliable molecular analysis
138 of malaria parasites. Non-*falciparum* malaria species identification of samples positive for the
139 pan-*Plasmodium* target 18S rDNA was performed with a multiplex RT-qPCR assay based on
140 species-specific 18S rDNA sequences as described previously²⁵.

141

142 **Quality control and categorization of RDT outcomes**

143 A RDT was considered positive if the healthcare worker recorded a positive signal for the
144 PfHRP2, pLDH, or both targets during the MIS. Among these positive RDTs, a true-positive
145 RDT (TP-RDT) result was defined as a RDT with detectable *Plasmodium* spp. NA (two
146 replicates with varATS and/or 18S rDNA Cq < 45 and human *rnasep* Cq < 35). A false-positive
147 RDT (FP-RDT) result was defined as positively read and recorded RDT in the field but with a
148 negative outcome for *Plasmodium* spp. NA based on PlasQ RT-qPCR in the presence of human
149 *rnasep* Cq < 35. Negative RDTs were classified as being read as negative by the healthcare
150 worker during the MIS and recorded in the database. A true-negative RDT (TN-RDT) result
151 was defined as a RDT whose negative result collected in the field was confirmed by the PlasQ
152 RT-qPCR. A false-negative RDT (FN-RDT) result was defined as negatively read by the
153 healthcare worker in the field with a positive PlasQ RT-qPCR result based on two replicate
154 amplifications with varATS and/or 18S rDNA Cq < 45 and the human *rnasep* Cq < 35.

155 **qHRP2/3-del assay for detection of *pfhrp2* and *pfhrp3* deletions**

156 The previously published qHRP2/3-del assay that simultaneously amplifies the *pfhrp2* and
157 *pfhrp3* genes together with the internal control gene *pfrrnr2e2* was adapted to accommodate for
158 the lower input of NA³⁰. Briefly, the probe for the internal control gene *pfrrnr2e2* was labelled
159 with fluorescein (FAM) instead of Cy5 to improve its detectability. Additionally, the final
160 concentration of all primers was increased from 0.3 µM to 0.45 µM. Concentrations of 0.15 µM
161 were used for the *pfrrnr2e2* probe, and 0.225 µM for the *pfhrp2* and *pfhrp3* probes each. All
162 samples were run in triplicates and the number of amplification cycles was increased from 45
163 to 50. Every 96 well qPCR plate contained control DNA extracted from a known *pfhrp2*-deleted
164 Pf strain (Dd2), a *pfhrp3*-deleted Pf strain (HB3), and a Pf strain without *pfhrp2* and *pfhrp3*
165 gene deletions (NF54) as well as a non-template control (NTC). We defined successful
166 amplification as a mean Cq < 40 for *pfrrnr2e2* calculated from at least two replicates for each

167 sample. We ran the qHRP2/3-del assay only with NA extracted from RDTs that had displayed
168 a $Cq < 35$ for the *varATS* target in the PlasQ RT-qPCR.

169 *Pfrrn2e2*, *pfhrp2*, and *pfhrp3* are all single-copy genes and they show comparable performances
170 in the multiplex qPCR assay ³⁰. One approach to detect Pf strains with *pfhrp2* and/or *pfhrp3*
171 gene deletions in mixed Pf strain infections (herein defined as masked gene deletions) is to
172 calculate the difference in Cq values obtained between *pfhrp2* or *pfhrp3* and *pfrrn2e2*
173 amplifications (ΔCq values). This is done by subtracting the Cq value obtained during the
174 amplification of *pfrrn2e2* from the Cq value of *pfhrp2* or *pfhrp3*, respectively. Combining all
175 runs that were conducted, the mean ΔCq for *pfhrp2* in controls (NF54 and HB3) was 0.00 (SD
176 ± 0.52) and for *pfhrp3* the mean ΔCq in controls (NF54 and Dd2) was 1.19 (SD ± 0.83). For
177 *pfhrp2* a ΔCq cut-off value of 1.0 (mean + 2x SD for controls) was chosen to identify masked
178 gene deletions. For *pfhrp3* a ΔCq cut-off value of 2.9 (mean + 2x SD for controls) was chosen
179 to identify masked gene deletions.

180 **Genotyping of *P. falciparum* *pfmsp1* and *pfmsp2* genes**

181 Genotyping with *pfmsp1* and *pfmsp2* was performed following published procedures using
182 nested PCR ³¹. The first two PCR reactions amplify conserved sequences within the
183 polymorphic regions of *pfmsp1* and *pfmsp2*, respectively. The second, nested PCR targets
184 allele-specific sequences in five separate reactions. Samples were run in 20 μ L total volume
185 with 1x Hot Firepol Master Mix (Solys BioDyne, Estonia), 0.25 μ M of forward and reverse
186 primers and 2 μ L template DNA. The cycling conditions for the first PCR were 95°C for 12
187 minutes, 25 cycles of 95°C for 30 seconds, 58°C for 1 minute and 72°C for 2 minutes and 72°C
188 for 10 minutes. For the second PCR, the cycling conditions for the three allele-specific *pfmsp1*
189 primer pairs were 95°C for 12 minutes, 35 cycles of 95°C for 30 seconds, 56°C for 40 seconds
190 and 72°C for 40 seconds and 72°C for 10 minutes. For the two *pfmsp2* allele-specific reactions
191 the conditions were: 95°C for 12 minutes, 35 cycles of 95°C for 30 seconds, 58°C for 40

192 seconds and 72°C for 40 seconds and 72°C for 10 minutes. Presence and size of PCR products
193 was determined and documented visually on a 1% agarose gel with a 100bp DNA ladder.

194 **Genotyping of *P. malariae* circumsporozoite protein (pmcsp)**

195 The *pmcsp* gene was amplified by semi-nested PCR for all samples with a positive signal for
196 *P. malariae* in the non-*falciparum* malaria species identification assay²⁵. The first PCR was
197 run with 3 µL of DNA template in a reaction volume of 20 µL. The reaction mix contained 1x
198 Hot Firepol Master Mix and 0.25 µM of each of the primers *csp_OF*³² and *csp-R*³³. The
199 conditions for the first PCR were: 95 °C for 12 minutes; 35 cycles of 95 °C for 15 seconds, 53
200 °C for 30 seconds and 65 °C for 90 seconds and final elongation at 65 °C for 10 minutes. The
201 second, semi-nested PCR used 1.5 µL of the product from the first reaction in a total volume of
202 15 µL. The reaction mix contained 1x Hot Firepol Master Mix and 0.33 µM of the primers
203 *csp_IF*³² and *csp-R*. The conditions for the second PCR were: 95 °C for 12 minutes; 35 cycles
204 of 95 °C for 15 seconds, 52 °C for 30 seconds and 62 °C for 90 seconds and final elongation at
205 62 °C for 10 minutes. The PCR product was sent to Microsynth (Microsynth AG, Switzerland)
206 for bidirectional sanger sequencing.

207 **Data analysis and statistics**

208 The generated (RT)-qPCR data was initially analysed with the CFX Maestro Software (Bio-
209 Rad Laboratories, California, USA). Thresholds for each fluorescence channel were set
210 manually and Cq values were then uploaded to the ELIMU-MDx platform for data storage and
211 analysis (23). Sequence analysis was performed using Geneious Prime 2019.1.1
212 (<https://www.geneious.com>). Statistical analysis and data visualisation was performed using the
213 R statistical language (version 4.0.3) based on packages *data.table*, *dplyr*, *epiDisplay*, *epitools*,
214 *ggplot2*, *ggpubr*, *ggridges*, *gridExtra*, *lme4*, *readxl*, *reshape2*, *scales*, *stringr*, *tidyr*, *tidyverse*.
215 Wilcoxon rank sum test was used for numeric values. Fisher's exact test (two-sided) was used

216 for contingency tables. A generalized linear mixed-effects model with fixed and random effects
217 was used for calculation of odds ratios and their confidence intervals.

218 **Data availability**

219 All data needed to evaluate the conclusions in the paper are present in the manuscript or the
220 Supplementary Materials. Further information will be made available to interested researchers.
221 The 15 sequences of *P. malariae* circumsporozoite protein from Bioko Island have been
222 deposited into GenBank under the accession numbers MW963324-MW963338.

223 **Ethical approval**

224 The Ministry of Health and Social Welfare of Equatorial Guinea and the Ethics Committee of
225 the London School of Hygiene & Tropical Medicine (Ref. No. LSHTM: 5556) approved the
226 2018 malaria indicator survey. Written informed consent was obtained from all adults and from
227 parents or guardians of children who agreed to participate. Only samples for which an additional
228 consent for molecular analysis was obtained were included in this study. We confirm that all
229 experiments were performed in accordance with relevant national and international guidelines
230 and regulations.

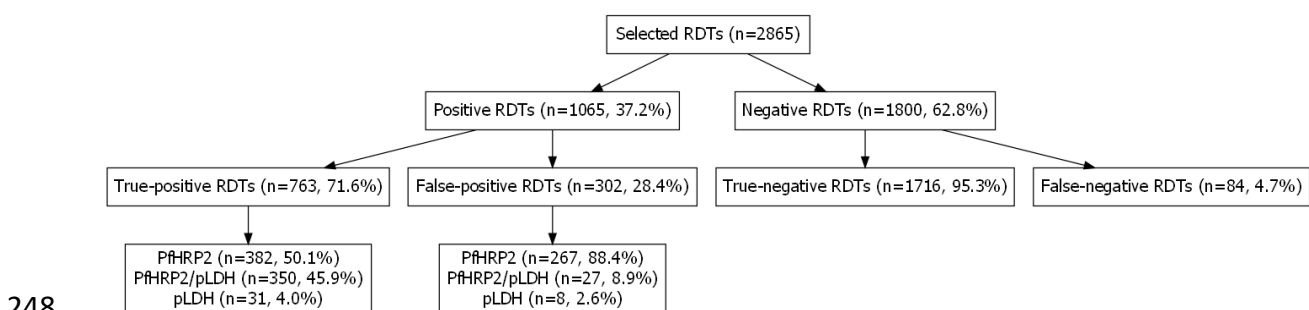
231 Results

232

233 Integration of molecular diagnostic methods into the national malaria control program to 234 assess the performance of malaria RDTs

235 Following NA extraction, a PlasQ RT-qPCR result was generated for 1,800 malaria negative
236 and 1,065 malaria positive RDTs, as collected in the MIS database. By comparison between
237 PlasQ RT-qPCR results and RDT results collected in the field, RDTs were grouped into four
238 categories, namely true-positive (TP), true-negative (TN), false-positive (FP), and false-
239 negative (FN), respectively (Figure 1). The PlasQ RT-qPCR was used as a gold standard to
240 evaluate the performance of the RDT, and this resulted in an overall sensitivity of 90.0% and
241 specificity of 85.0% of field-deployed RDTs used during the 2018 Bioko Island MIS.

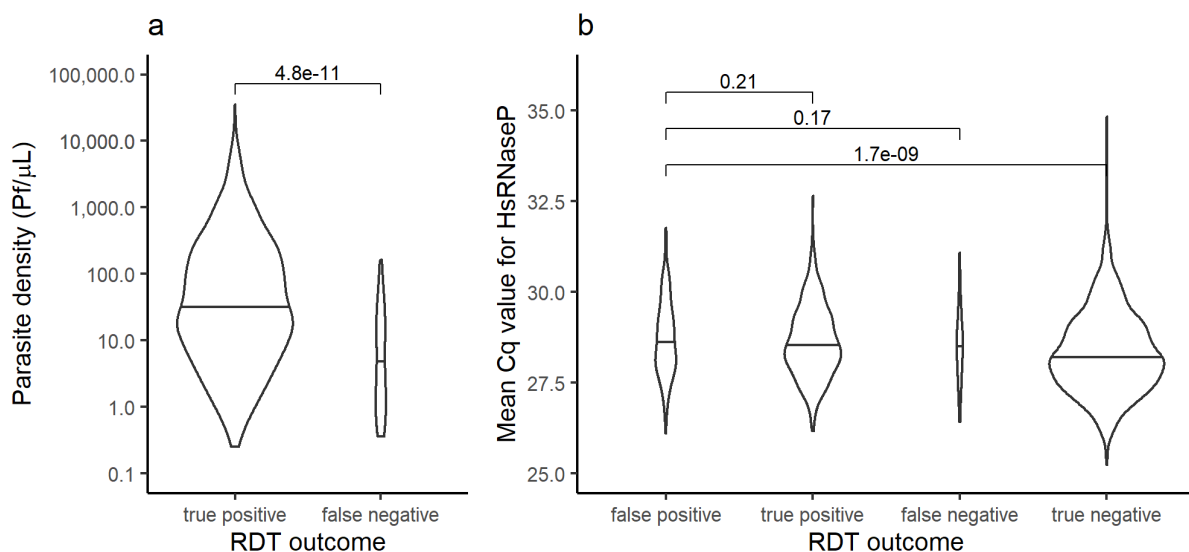
242 When stratified by the type of antigen, FP-RDTs were predominantly those that detected only
243 the PfHRP2 antigen (88.4%) during the MIS; whereas 8.9% and 2.6% of the FP-RDTs were
244 respectively those that detected both, the PfHRP2 plus the pLDH antigens, and those that
245 detected the pLDH antigen only. Around half of TP-RDTs were those that detected the PfHRP2
246 antigen only (50.1%), followed by those that detected both antigens (45.9%) and lastly, those
247 that detected the pLDH antigen only (4.0%) during the MIS.



249 **Figure 1.** Comparison of RDT outcomes collected during 2018 MIS with PlasQ RT-qPCR
250 results obtained after NA extraction and amplification.

251 Low parasite density infections are likely to cause false-negative RDT results in the field

252 The ENAR approach used in this study detects 10-100x lower parasite densities than the
253 PfHRP2-based RDT itself²⁵. Here, we confirm that a clear association exists between FN-RDT,
254 TP-RDT, and *Pf* parasite densities assessed by the PlasQ RT-qPCR outcome. TP-RDT had
255 higher geometric mean parasite densities (35.0 Pf/ μ L, IQR: 7.2-166.0) compared to FN-RDTs
256 (4.6 Pf/ μ L, IQR: 1.1-20.0) (Figure 2a, Wilcoxon rank sum test, $p < 0.001$). Although *Pf* was the
257 most common (93.8%) *Plasmodium* spp. species among RT-qPCR positive RDTs, *P. malariae*
258 (4.0%) and *P. ovale* spp. (1.1%) were also identified. No *P. vivax* and *P. knowlesi* parasite NAs
259 were detected. The central repeat region of the *P. malariae* circumsporozoite protein (*pmcsp*)
260 was amplified by PCR and Sanger sequenced to confirm the presence of *P. malariae*
261 (Supplementary figure 1b). Nucleotide sequences were unique among all 15 *P. malariae*
262 isolates sequenced and also the number of NAAG and NDAG repeats varied between the
263 isolates. These results indicated high diversity of the *P. malariae* population on Bioko Island.
264 A slightly higher proportion, although not statistically significant, of non-*falciparum*
265 *Plasmodium* spp. parasites was found among FN-RDTs. *P. malariae* was found among 6.6%
266 of FN-RDTs compared to 3.8% among TP-RDTs. Similar, *P. ovale* spp. was more prevalent in
267 FN-RDTs (2.6%) than in TP-RDTs (0.9%).



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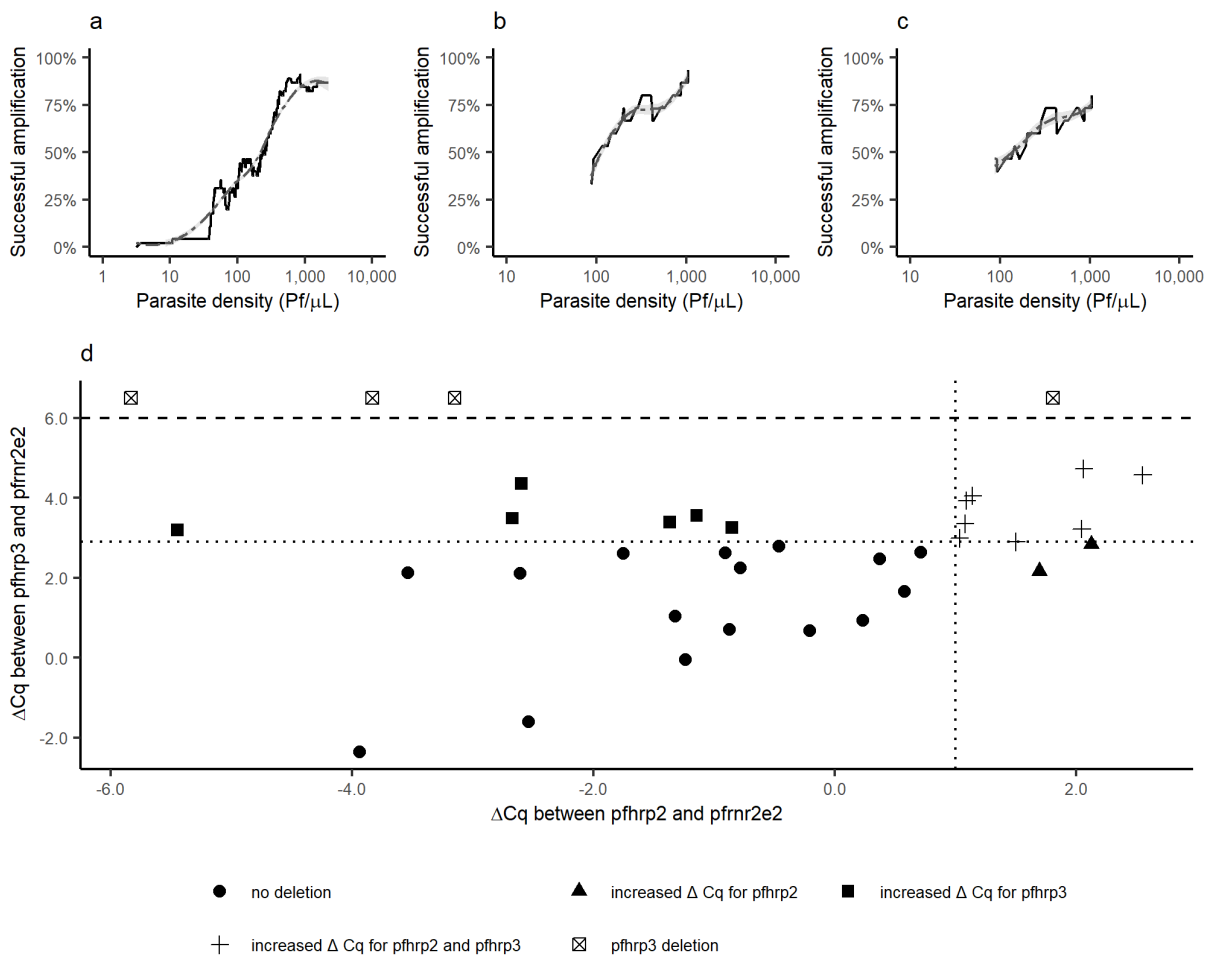
269 **Figure 2.** (a) *Pf* parasite densities compared between true positive and false negative RDT
270 outcomes. Parasite densities for *Pf* were estimated based on the varATS target of the PlasQ RT-
271 qPCR assay. Wilcoxon rank sum test was used to compare the two groups. (b) Comparison of
272 the Cq values obtained with the amplification of the human *rnasep* gene used as internal control
273 of the PlasQ RT-qPCR assay, across all samples stratified by RDT outcome. The group of RDTs
274 with a false-positive result was compared to the other RDT outcomes by Wilcoxon rank sum
275 test.

276

277 **False-negative RDT results are not associated with parasites carrying *pfhrp2* and *pfhrp3***
278 **gene deletions**

279 *Pf* strains were genotyped to identify *pfhrp2* and/or *pfhrp3* gene deletions. The number of
280 samples available was limited through the combination of low parasite density infections and
281 the limited amount of blood retained on RDTs as a source of NA. The single copy gene
282 *pfrnr2e2*, serving as the internal control of the qHRP2/3-del assay, was amplified with Cq < 40
283 in 184/406 (45.3%) samples. To avoid false reporting of *pfhrp2* and/or *pfhrp3* gene deletions,
284 the analysis was restricted to samples that had an additional amplification in either *pfmsp1*
285 (32/47, 68.1%) or *pfmsp2* (31/47, 66.0%). The success rate as a function of the parasite density
286 for amplifying each genotyping marker based on parasite density is shown in Figure 3a-c. At
287 least two out of three reference genes (*pfrnr2e2*, *pfmsp1* or *pfmsp2*) were amplified in thirty-six
288 samples, which were then included in the analysis of the *pfhrp2* and *pfhrp3* deletion status. No
289 evidence for parasites carrying a *pfhrp2* gene deletion was found in these 36 samples, but four
290 out of 36 samples (11.1%) were likely to carry *pfhrp3* gene deletions. All four samples with
291 *pfhrp3* deletion were recorded as positive for PfHRP2 by RDT.

292 Based on the available data from the *pfmsp1* and *pfmsp2* genotyping (Supplementary figure 1a),
293 polyclonal infections consisting of two or more distinct *Pf* clones were found in 63.0% (17/27)
294 of samples with successful amplification of *pfmsp1* and *pfmsp2*. The qHRP2/3-del assay was
295 used to identify *pfhrp2* and/or *pfhrp3* gene deletions in polyclonal *Pf* infections by calculating
296 the ΔCq values as the difference of Cq values between *pfhrp2* and *pfhrp3* gene amplification
297 and the *pfrnr2e2* internal control. Figure 3d shows the distribution of samples with their
298 respective ΔCq values for *pfhrp2* and *pfhrp3*. Of the 36 samples included, nine samples (25.0%)
299 had increased ΔCq values for both genes, two samples (5.6%) only for the *pfhrp2* gene and six
300 samples (16.7%) only for the *pfhrp3* gene, respectively. Importantly, the 11 samples which had
301 an increased ΔCq for the *pfhrp2* gene were positive for PfHRP2 by RDT.



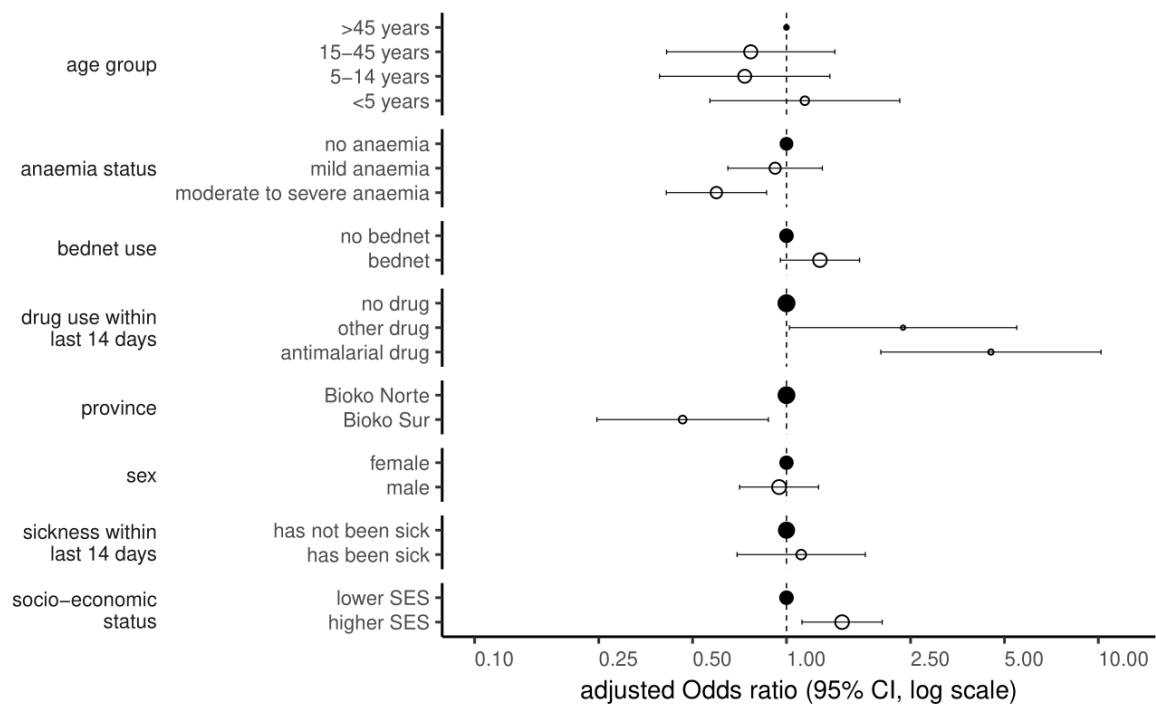
302

303 **Figure 3.** Amplification rate (rolling mean) for the genotyping reference genes (a) *pfmr2e2*,
304 (b) *pfmsp1* and (c) *pfmsp2* as a function of the parasite density of the sample. Parasite densities
305 for *Pf* were estimated based on the *varATS* target of the PlasQ assay. (d) The distribution of
306 ΔCq values between *pfhrp2* (x-axis) or *pfhrp3* (y-axis) and *pfmr2e2*. ΔCq thresholds (dashed
307 lines) were set at 1.0 for *pfhrp2* and 2.9 for *pfhrp3*. For samples with a *pfhrp3* deletion, the ΔCq
308 for *pfhrp3* was set arbitrarily at 6.5.

309 **False-positive RDT results are associated with recent use of antimalarial drugs**

310 The rate of FP-RDTs differed across age, level of anaemia, geographical location of residence,
311 and the socio-economic status (Supplementary figure 2). Interestingly, no study participant with
312 a FP-RDT had a fever ($>37.5^{\circ}$ C) at the time of the survey, while 1.7% (13/763) of those with
313 TP-RDTs were recorded with fever. Eight factors from the MIS were used to identify risk
314 factors associated with FP-RDTs through multivariate logistic regression analysis in which the
315 outcome of the test was set as the outcome variable (Supplementary table 1). FP-RDTs (n=297)
316 were compared to TP-RDTs (n=754). Because sample collection was clustered within
317 communities, community affiliation was introduced as a random effect to the model. The MIS
318 included 299 communities, of which 201 (67.2%) were represented in the dataset. The median
319 number of samples from a community was three. Survey participants belonging to higher socio-
320 economic classes (aOR 1.51 p=0.01) had increased odds of having a FP-RDT. Participants who
321 were reported to have been treated with an antimalarial drug in the two weeks preceding the
322 survey had more than four times the odds of a FP-RDT result than a TP-RDT (aOR 4.52,
323 p<0.001). In contrast, moderate to severe anaemia reduced the odds of having a FP-RDT (aOR
324 0.60, p=0.02). Those who reside in the rural Bioko Sur province had also decreased odds of
325 having a FP-RDT (aOR 0.44, p=0.01). Age, sex, bednet use, and reported sickness in the two
326 weeks preceding the survey were not significantly associated with FP-RDTs (Figure 4).

327



328

329 **Figure 4.** Risk factors associated with FP-RDT results by multivariate logistic regression
330 analysis. The size of the circles corresponds with the number of responses for each variable
331 outcome. The reference group is marked by filled circles and the other groups have open circles.
332 Higher socio-economic status (SES) included people from the 4th and 5th wealth quintiles.

333

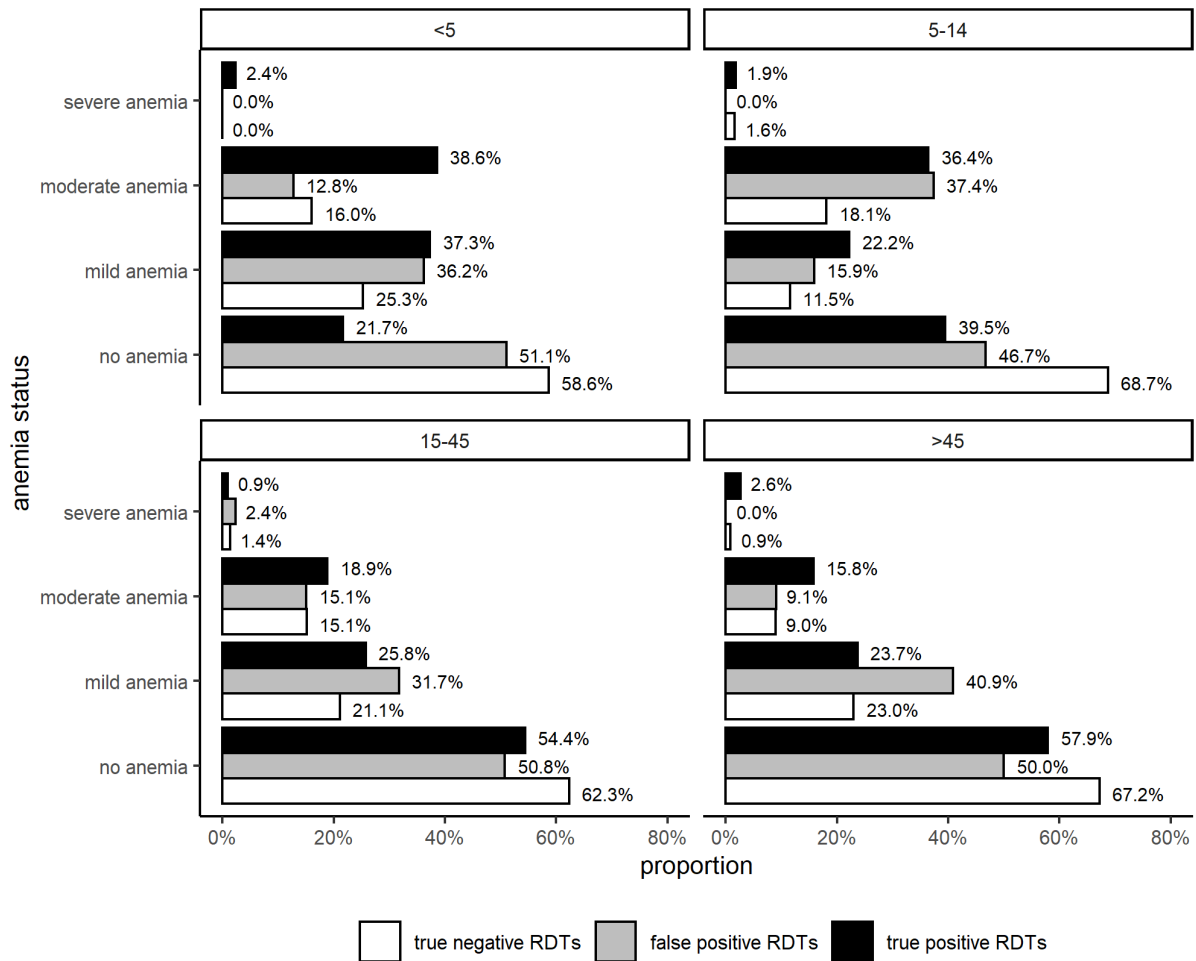
334 To exclude the possibility that FP-RDTs are the consequence of failed amplification related to
335 the degradation of NA retained on the RDTs, an additional analysis was carried out. During the
336 PlasQ RT-qPCR, the human *rnasep* gene was used as an internal control to monitor the amount
337 of NA extracted from each RDT. On average, the human *rnasep* was amplified with a Cq value
338 of 28.5 (SD \pm 1.0). There was no significant difference in the Cq values of the human *rnasep*
339 gene amplification among RDTs, which were categorized as false positive (28.6, SD \pm 1.0), true
340 positive (28.5, SD \pm 1.0), or false negative (28.6, SD \pm 1.0). TN-RDTs had a significantly lower
341 median Cq value (28.2, SD \pm 1.1) (Figure 2b). These results indicate that the lack of detectable

342 *Pf*NA in the blood retained on FP-RDTs is not related to poor NA extraction performance or a
343 failure in detecting NAs.

344

345 **The impact of asymptomatic malaria infections on anaemia status might be**
346 **underestimated by false-positive RDT results**

347 We hypothesised that high rates of FP-RDTs are likely to lead to underestimating the impact of
348 asymptomatic malaria infections on anaemia status. Among malaria infected children aged <5
349 years, the prevalence of anemia was 67.7% if malaria status was assessed by RDT. Stratification
350 by RT-qPCR-based correction revealed that the proportion of anemic children with a FP-RDT
351 result (48.9%) is similar to children with a TN-RDT result (41.4%), whereas children with a
352 TP-RDT result are more likely to suffer from anemia (78.3%) (Figure 5). This significant effect
353 is even more pronounced among children with moderate and severe anemia if compared to mild
354 anemia. Removing all FP-RDTs in this association between malaria infection status and anemia
355 levels in children < 5 years reveals that the association between asymptomatic malaria and
356 moderate or severe anaemia might be even higher than if malaria infection status is assessed by
357 RDT only. In older children and adults, the impact of FP-RDTs on assessing the anaemia status
358 is negligible.



359

360 **Figure 5.** Anaemia status compared between true positive, false positive, and true negative

361 RDT outcomes and stratified by age.

362 Discussion

363

364 Malaria control programs rely on continuous and systematic collection of surveillance data for
365 decision making and resource allocation ³⁴. A critical measure that closely reflects malaria
366 transmission intensity is the parasite rate, the proportion of the population found to carry
367 parasites in their peripheral blood ³⁵. RDTs, more specifically PfHRP2-based RDTs, are the
368 most widely used test to measure parasite rates in endemic countries and are a cornerstone of
369 malaria control. However, here we have identified diagnostic performance issues, particularly
370 related to limited specificity. Therefore, malaria surveillance depending solely on RDTs needs
371 well integrated quality control procedures assessing the impact of reduced sensitivity and
372 specificity of the tests used in malaria control programs. In this report, we present an efficient
373 approach to assess the performance of field-deployed RDTs used for malaria surveillance on
374 large scale based on molecular analysis of NA retained on the RDTs.

375 In 4.7% (84/1800) of the negative RDTs we found *Plasmodium* spp. NA and identified these
376 as FN-RDT. The low proportion of FN-RDT can be explained by the low parasite densities of
377 these asymptomatic individuals (geometric mean of 5.4 Pf/ μ L) and the low amount of blood
378 (one drop corresponds to approximately 5 μ L) used as starting material for the molecular
379 analysis. This is a limitation of our approach. Therefore, the true proportion of FN-RDTs in a
380 high prevalence setting such as Bioko Island is likely to be higher.

381 We identified *Pf* isolates with potential *pfhrp3* deletions but not a single isolate with a
382 confirmed *pfhrp2* deletion. Given the overall high frequency of polyclonal *Pf* infections in this
383 setting (63% by *pfmsp1/pfmsp2* genotyping), we assumed that if *Pf* carrying *pfhrp2* deletions
384 exist, then they would be most likely masked by co-infecting *Pf* isolates without *pfhrp2* gene
385 deletions. Of all the samples included for final analysis, 30.6% had an increased Δ Cq value for

386 *pfhrp2* and 50.0% for *pfhrp3* amplification, indicating for the first time that there are likely *Pf*
387 strains circulating on Bioko Island carrying deletions in their *pfhrp2* and/or *pfhrp3* genes. So
388 far, one report described *Pf* strains carrying *pfhrp2* and *pfhrp3* deletions in blood samples
389 collected on the continental region of Equatorial Guinea ³⁶. However, since the travel activity
390 between Bioko Island and the mainland of Equatorial Guinea is high, it can be assumed that
391 parasite strains are exchanged frequently between these locations ³⁷. Most importantly, blood
392 samples with *Pf* clones indicative for masked *pfhrp2* and *pfhrp3* gene deletions were recorded
393 as PfHRP2 positive by RDT. Likely, the co-circulating *Pf* clones compensate for the lack of
394 PfHRP2 expression resulting in RDT positive testing.

395 Our data support the notion that in malaria medium to high transmission settings, where
396 polyclonal *Pf* infections are common, only assays with the ability to identify masked *pfhrp2*
397 and/or *pfhrp3* gene-deleted parasites should be used ³⁸. Importantly, to avoid false reporting of
398 *pfhrp2* and/or *pfhrp3* gene deletions, we used a robust and multi-layered approach by which
399 only samples with a pre-defined parasite density, successful amplification of the assays' internal
400 control, and additional, independent amplification of either *pfmsp1* or *pfmsp2*, were included
401 into the analysis.

402 In our study, we discovered a significant proportion of FP-RDTs that were declared as malaria
403 positive in the field. Our findings are not unique to Bioko Island. In a study conducted in
404 Tanzania, 22% of malaria positive RDTs were negative by molecular analysis for *Pf*³⁹. A study
405 performed in Guinea-Bissau reported 26% FP-RDTs ⁴⁰, and in Western Kenya, approximately
406 one-third of positive RDTs were negative by molecular detection methods for *Pf*⁴¹. With the
407 wider access to novel “ultra-sensitive” RDTs, which are detecting lower concentrations of the
408 PfHRP2 antigens, the problem of FP-RDT results is expected to become even greater, as already
409 shown in a recently published study ⁴².

410 The PfHRP2 antigen (97.4%) was much more often detected among FP-RDTs than the pLDH
411 antigen (11.2%). We were able to associate the false positivity of PfHRP2-RDTs with the recent
412 use of antimalarial drugs. It has been well established that antimalarial treatment leads to false
413 positive PfHRP2-RDT results because the PfHRP2 antigen persists in the blood days to weeks
414 after parasite clearance ^{22, 43-46}.

415 Remarkably, we found not only an association between the recent use of antimalarial drugs and
416 FP-RDTs, but also an indirect association with accessibility to antimalarial drugs. A higher
417 socio-economic status and living in the urban part of the Island directs towards increased
418 accessibility to antimalarial drugs.

419 The impact of FP-RDTs differs greatly depending on the setting in which RDTs are deployed.
420 In clinical settings, FP-RDTs might be less common, but the consequences are more serious
421 since unnecessary prescription of antimalarials might increase the risk of overlooking other,
422 life-threatening diseases causing fever or inducing side-effects caused by the antimalarial drugs
423 ⁴⁷. However, if RDTs are used for epidemiological surveys, a high proportion of FP-RDTs due
424 to PfHRP2 antigen persistence might lead to an overestimation of the malaria prevalence in
425 regions with good access to antimalarial treatment (urban regions) or in populations which are
426 more likely to receive antimalarial treatments (higher socio-economic status). One striking
427 example shown here is that the use of RDTs to assess the relationship between asymptomatic
428 malaria infection and anemia status in children < 5 years will lead to an underestimation of the
429 severe consequence of asymptomatic malaria infections on hemoglobin levels.

430 The benefits and the challenges that come with large-scale deployment of molecular techniques
431 in malaria endemic regions have been discussed elsewhere ⁴⁸. And now, the COVID-19
432 pandemic has renewed the discussion regarding the importance of molecular testing as part of
433 public health systems across Africa ⁴⁹ and will likely accelerate efforts to integrate molecular
434 tools for larger scale genomic surveillance of malaria into control programs.

435 Conclusion

436 Malaria surveillance programs based on RDT assessments of malaria prevalence should be
437 strengthened by the integration of molecular epidemiological data in the same setting. These
438 data will serve as an early warning system for (i) spread of *Pf* strains evading widely used
439 diagnostic tests, (ii) understanding overuse of malaria drugs, (iii) help with identifying fever
440 causing diseases beyond malaria, and (iv) help to clarify the burden of asymptomatic malaria
441 as a cause of severe to moderate anemia, particularly in children < 5 years.

442 Acknowledgments

443 The authors would like to thank all MIS participants for their contribution and the BIMCP staff
444 for their commitment and support during sample collection. We would like to thank Christin
445 Gumpff, Christian Scheurer and Sergio Wittlin from the Swiss TPH Malaria Drug Discovery
446 Group for their help with cultivating PfNF54, PfDD2 and PfHB3 parasites, whose DNA was
447 used as controls for the qHRP2/3-del assay. We would like to acknowledge Amanda Ross for
448 her support and guidance with the statistical analysis used in this manuscript.

449 Funding sources

450 This study was funded by a public–private partnership, the Bioko Island Malaria Elimination
451 Project (BIMEP), composed of the Government of Equatorial Guinea, Marathon EG Production
452 Limited, Noble Energy, and Atlantic Methanol Production Company. Etienne A. Guirou and
453 Charlene Aya Yoboue are recipients of Swiss Government Excellence Scholarships (Number
454 2016.1250 and 2017.0748, respectively) granted by the State Secretariat for Education,
455 Research and Innovation. The funding sources had no role in the study design, the collection,
456 analysis, and interpretation of data, as well as in writing this manuscript and in the decision to
457 submit the paper for publication.

458 Author contributions

459 Conceptualization: SH, CD, TS. Data curation and validation: SH, TS, OTD. Formal analysis
460 and visualization: SH. Funding acquisition: CD, MT, GAG, WPP. Investigation: OTD, GAG,
461 WPP, MOA, CAG. Methodology: SH, CAY, EAG, JPD, KB. Resources: MM, EN, OTD,
462 GAG, WPP, CAG. Project administration and supervision: CD, TS. Writing original draft: SH,
463 TS, CD. All authors reviewed the manuscript.

464 Declaration of interest

465 The authors declare no conflicts of interest.

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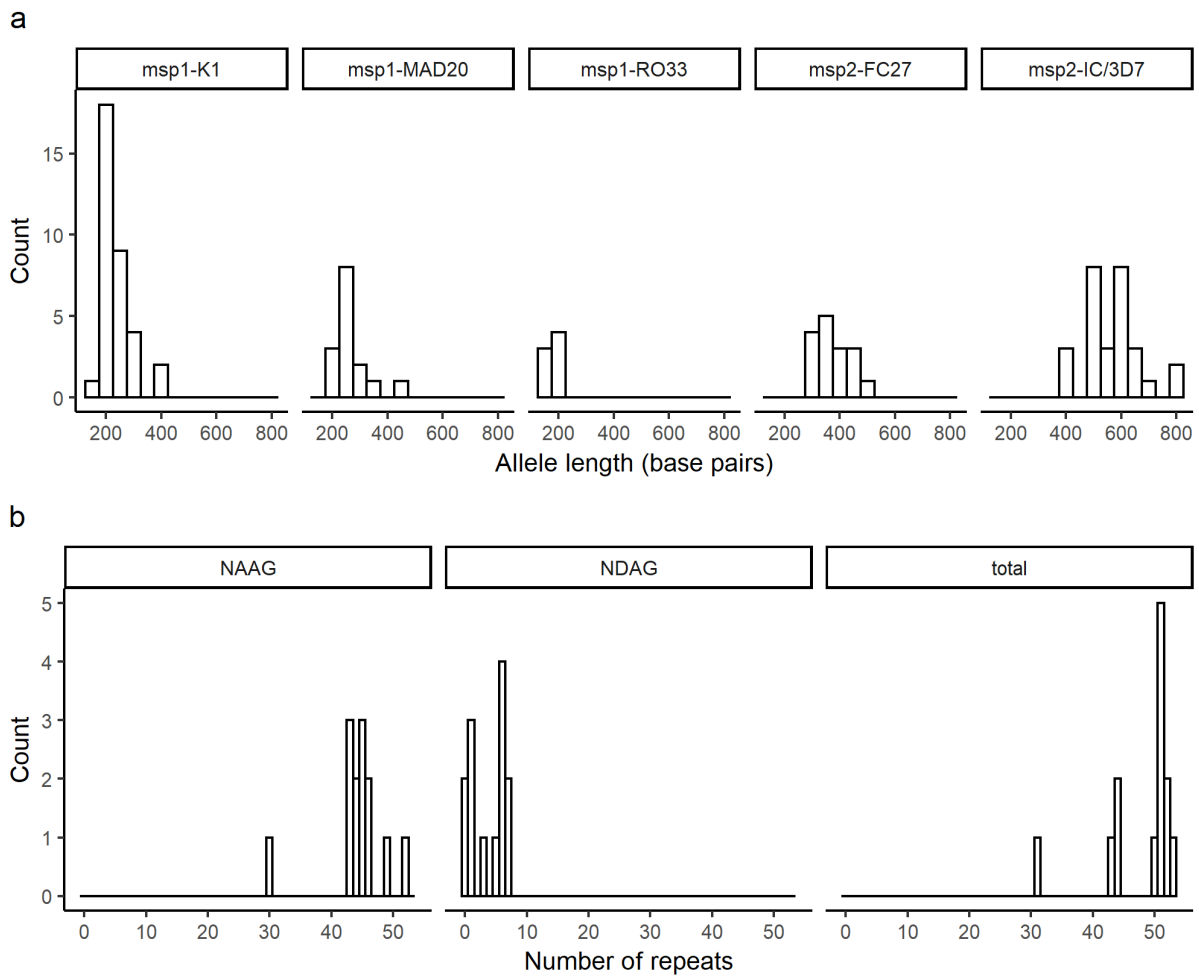
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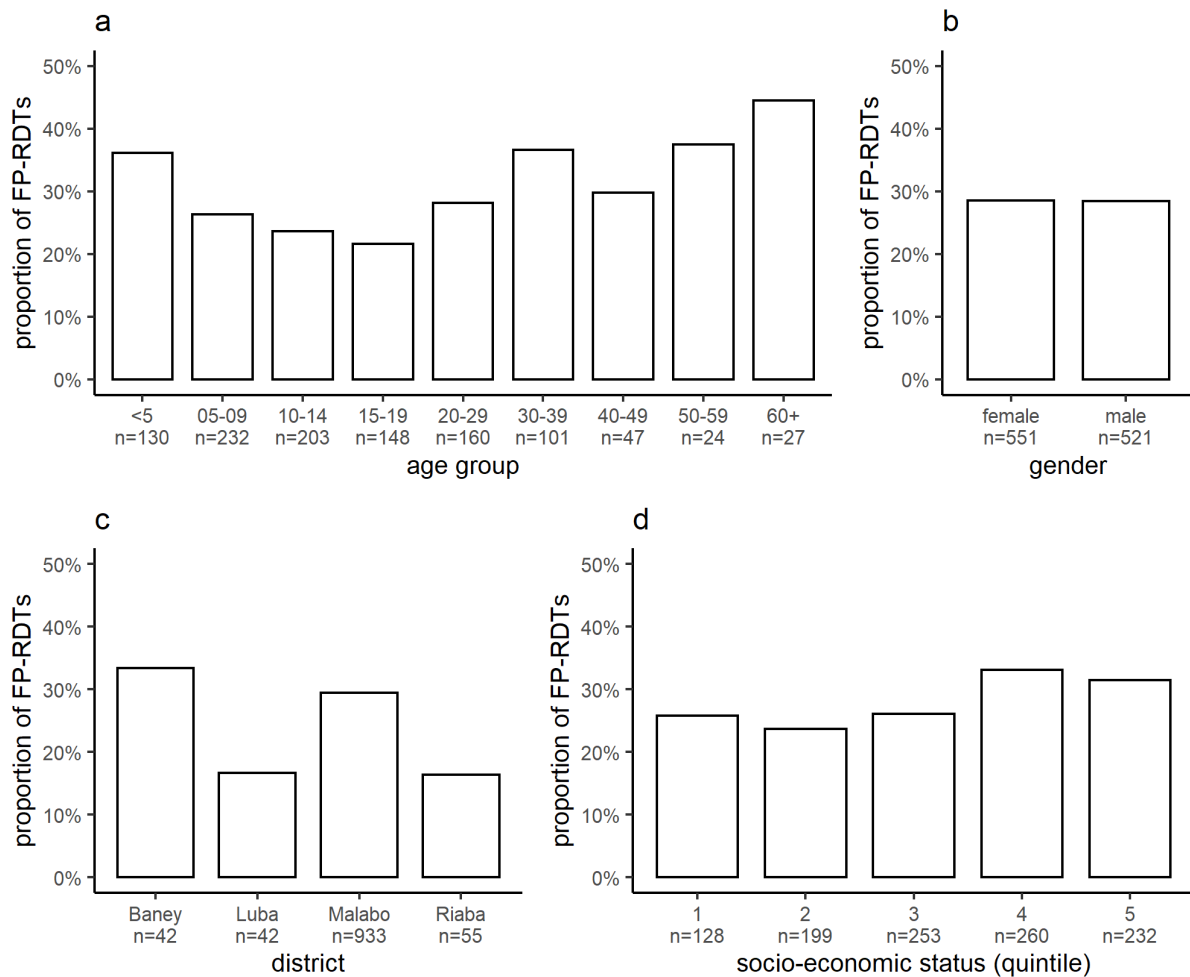
603 Supplementary Materials



604

605 **Supplementary figure 1.** (a) Genetic diversity of *Pf* determined by size polymorphism of
606 *pfmsp1* and *pfmsp2*. (b) Genetic diversity of *P. malariae* determined by the number of NAAG
607 and NDAG repeats in the *pmcsp* gene.

608



609

610 **Supplementary figure 2.** FP-RDTs as a proportion of all positive RDTs stratified by age (a),
611 gender (b), district (c) and socio-economic status (d). Quintile 1 refers to the lowest, while
612 quintile 5 to the highest socio-economic status.

613

Factor	Level	N	FP-RDT N (%)	Crude OR (95% CI)	Crude P value	Adjusted OR (95% CI)	P (LR test)
Age group	>45 years (ref)	59	21 (35.6%)				0.23
	15-45 years	436	106 (24.8%)	0.73 (0.41, 1.29)	0.28	0.77 (0.41, 1.43)	
	5-14 yea	428	125 (28.7%)	0.6 (0.33, 1.06)	0.08	0.73 (0.29, 1.38)	
	<5 years	128	45 (35.2%)	0.98 (0.51, 1.87)	0.72	1.15 (0.57, 2.31)	
Anaemia status	Non anaemia (ref)	484	147 (30.4%)				0.02
	Mild anaemia	272	81 (29.8%)	0.97 (0.7, 1.34)	0.87	0.92 (0.65, 1.30)	
	Moderate to severe anaemia	295	69 (23.4%)	0.7 (0.5, 0.98)	0.04	0.6 (0.41, 0.86)	
Bednet use	No bednet (ref)	568	145 (25.5%)				0.10
	Bednet	483	152 (31.5%)	1.34 (1.02, 1.75)	0.03	1.28 (0.96, 1.72)	
Drug use within last 14 days	No drug (ref)	976	257 (26.3%)				< 0.001
	Other drug	35	16 (45.7%)	2.36 (1.19, 4.65)	0.01	2.37 (1.02, 5.48)	
	Antimalarial drug	40	24 (60.0%)	4.2 (2.19, 8.03)	<0.001	4.52 (2.01, 10.2)	
Province	Bioko Norte (ref)	955	282 (29.5%)				0.01
	Bioko Sur	96	15 (15.6%)	0.44 (0.25, 0.78)	0.01	0.46 (0.25, 0.87)	
Sex	Female (ref)	543	154 (28.4%)				0.71
	Male	508	143 (28.1%)	0.99 (0.76, 1.29)	0.94	0.95 (0.71, 1.27)	
Socio-economic status	Lower SES (ref)	566	139 (24.6%)				0.01
	Higher SES	485	158 (32.6%)	1.48 (1.13, 1.94)	0.004	1.51 (1.12, 2.03)	
Sickness within last 14 days	Not sick (ref)	870	227 (26.1%)				0.65
	Sick	181	70 (38.7%)	1.79 (1.28, 2.5)	<0.001	1.11 (0.69, 1.79)	

615 **Supplementary table 1:** Multivariable logistic regression analysis of risk factors associated
616 with FP-RDTs using community as a random effect. Crude and adjusted odds ratios and their
617 respective 95% confidence intervals were calculated based on comparison between FP-RDT
618 and TP-RDT.