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1 # Prevalence and characteristics of *Plasmodium vivax* Gametocytes in
2 Duffy-positive and Duffy-negative populations across Ethiopia

3

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18 *Abstract: Plasmodium* parasites replicate asexually in the human host. The proportion of
19 infections that carries gametocytes is a proxy for human-to-mosquito transmissibility. It is
20 unclear what proportion of *P. vivax* infections in Duffy-negatives carries gametocytes. This study
21 aims to determine the prevalence of *P. vivax* in Duffy-negatives across broad regions of Ethiopia
22 and characterize parasite stages. Finger-prick blood samples were collected for microscopic and
23 molecular screening of *Plasmodium* parasites and Duffy status of individuals. Molecular

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24 screening of plasmodium species and Duffy blood group genotyping was done using SYBR
25 green and Taqman qPCR method. Among the total 447 samples, 414 (92.6%) were *P. vivax*
26 confirmed and, 16 (3.9%) of them were from Duffy-negatives. Of these, 5/16 (31.3%) Duffy-
27 negative *P. vivax*-infected samples were detected with gametocytes. Of the 398 Duffy-positive
28 *P. vivax*-infected samples, 150 (37.7%) were detected with gametocytes, slightly higher than that
29 in Duffy-negatives. This study highlights the presence of *P. vivax* gametocytes in Duffy-negative
30 infections, suggestive of human-to-mosquito transmissibility. Although *P. vivax* infections in
31 Duffy-negatives are commonly associated with low parasitemia, some of these infections were
32 shown with relatively high parasitemia and may represent better erythrocyte invasion capability
33 of *P. vivax* and hidden reservoirs that can contribute to transmission. A better understanding of
34 *P. vivax* transmission biology and gametocyte function particularly in Duffy-negative
35 populations would aid future treatment and management of vivax malaria in Africa

36 **Keywords:** *Plasmodium vivax*, Duffy-negatives, Malaria transmission, Gametocytes, Ethiopia

37 **Running title**: Plasmodium vivax gametocyte in Duffy-negative population

## 38 Introduction

39 Yearly, there are about 619,000 malaria-related deaths and ~247 million malaria cases
40 reported globally.[1] Out of the five malaria *Plasmodium* species, *P. vivax* is the most widespread.[2]
41 Duffy negative individuals were thought to be resistant to *P. vivax*. However, a growing number
42 of *P. vivax* cases reported throughout Africa where Duffy-negative individuals predominate,[3]
43 demonstrated that *P. vivax* can infect Duffy-negative individuals[4,5] and could potentially spread
44 and transmit across populations.[5,6] Due to epidemiological and ethnic differences, the prevalence
45 of *P. vivax* in Duffy-negative individuals varies across Africa.[4] Considering *P. vivax* can infect
46 and adapt to Duffy-negative individuals, it is possible that these infections can produce

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47 gametocytes leading to transmission.[7] The extent of transmission may vary by environmental and
48 host factors.[8]

49 During the *Plasmodium* life cycle, the parasites undergo multiple asexual replicative
50 cycles in the human host, and in each erythrocytic replication cycle, a small portion (□0.1%-5%)
51 of the asexual stages develops into sexual gametocytes. The proportion of infections that carries
52 gametocytes is a proxy for human-to-mosquito transmissibility.[9] Within the mosquito's midgut,
53 male and female gametocytes undertake gametogenesis.[10] After the gametes have fertilized, a
54 zygote is created, which later transforms into a motile ookinete. Under the basal lamina,
55 ookinetes form an oocyst by crossing the midgut epithelium.[10,11] Numerous thousands of
56 sporozoites develop in the oocyst, and as the oocyst wall ruptures, sporozoites enter the
57 hemolymph and infect the salivary gland. The intricate life cycle of the parasite is then
58 completed when sporozoites are inoculated into another person through mosquito bites.[12]
59 Gametocytogenesis is influenced by epigenetic, ecological, and heritable factors associated with
60 the parasite.[13] The occurrence of gametocytogenesis is also influenced by factors associated with
61 human hosts such as immunity status, antimalaria drug treatment and genetic factors.[14,15]

62 The distribution of *P. vivax* in Duffy-negatives across Ethiopia as well as the parasite
63 stages of these infections remain largely unclear. The presence of gametocytes in symptomatic or
64 asymptomatic individuals can lead to onward transmission in the communities. Knowledge of
65 gametocyte reservoirs allows for prioritizing transmission blocking vaccines against *P. vivax* in
66 Africa.[16,17] This study aims to 1) compare the distribution of *P. vivax* in Duffy-positive and
67 Duffy-negative across Ethiopia; 2) determine the different stages of *P. vivax* in Duffy-positive
68 and Duffy-negative infections; and 3) examine demographic and clinical features of Duffy-

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69 negative *P. vivax* infections. These findings will advance current knowledge of *vivax* malaria
70 distribution and transmission in Africa.
71

## 72 Material & Methods

### 73 Study sites

74 A total of 447 febrile patient samples were collected in twenty-seven (27) health facilities
75 from seven major regions of Ethiopia including Afar, Amhara, Benishangul/Gumuz, Gambella,
76 Oromia, Sidama, and Southern Nations Nationalities and People's Region (SNNPR) (**Figure 1**)
77 from 2020-2021. These seven regions also vary in elevation: Afar is in the northeastern part of
78 the country with altitude 379 m (Lat, Long: 11.568, 41.438),[18] Amhara is in the north with
79 altitude 1268 m (Lat, Long: 11.66334, 338.821903),[19] Gambella is in the west bordering Sudan
80 with altitude 447 m (Lat, Long: 8.24999, 34.5833),[20] Oromia is in the east with altitude 959 m
81 (Lat, Long: 7.98906, 39.38118),[21] Sidama is in the southeast with altitude 1742 m (Lat, Long:
82 6.7372,38,4008)[22] and the SNNPR is in the south with altitude 1200 m (Lat, Long: 6.05862,
83 36.7273).[23]

84 **Figure 1.** A map showing the study sites in Ethiopia with malaria incidence ranges from high in
85 the western part to low in the eastern part of the country. These sites represent seven major
86 regions including Afar, Amhara, Benishangul/Gumuz, Gambella, Oromia, Sidama, and Southern
87 Nations, Nationalities, and People's Region (SNNPR) with diverse ethnic groups.
88

### 89 Blood sample collection and microscopic examination

90 Finger-prick blood samples were collected from individuals with at least two clinical
91 symptoms, had been suspected for malaria infection and visiting health facilities for malaria
92 diagnosis. All samples were selectively collected from *P. vivax* microscopic confirmed patients.

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93 As soon as possible, the DBS samples were also done at the site of the sample collection for
94 further molecular screening of plasmodium species. The thick and thin blood films were
95 prepared for microscopic screening of *Plasmodium* parasites. Blood smears were stained for 10
96 minutes with 10% Giemsa staining solution (pH 7.2). The parasite species, the developmental
97 stages of the parasites, and the density of asexual parasites and sexual gametocytes were
98 examined by microscopy. A minimum of 200 microscopic fields were examined at a
99 magnification of 1,000X using oil immersion optics before a slide was declared negative for
100 malaria parasites by the light microscope. Parasitemia/µl of blood was estimated from the thick
101 films as follows: the number of parasites per 200 white blood cells was multiplied by 8,000 (an
102 average white blood cell count/µl) and then divided by 200. Slides were read twice by the
103 primary readers at the site of the study and the secondary readers at EPHI. Discordant results
104 were confirmed by a third slide expert microscope readers. Final species diagnosis was decided
105 by the expert readers. Rapid diagnostic test (RDT) was also conducted for malaria detection.[24,25]
106

107 **Molecular screening of *Plasmodium species***

108 Parasite DNA was isolated from a dried blood spot using the Saponin/Chelex method.[26]
109 *P. vivax* and *P. falciparum* were detected by the SYBR Green qPCR detection method [2] using the
110 published primers (forward: 5′-GAATTTTCTCTTCGGAGTTTATTCTTAGATTGC-3′;
111 reverse: 5′-GCCGCAAGCTCCACGCCTGGTGGTGC-3′) specific to *P. vivax*[27,28] and *P.*
112 *falciparum* 18S rRNA (forward: 5′-AGTCATCTTTCGAGGTGACTTTTAGATTGCT-3′;
113 reverse: 5′-GCCGCAAGCTCCACGCCTGGTGGTGC-3′).[29] Amplification was conducted in a
114 20 µl reaction mixture containing 2 µl of genomic DNA, 10 µl SYBR Green qPCR Master Mix
115 (Thermo Scientific), and 0.5 uM primer. The reactions were performed in QuantStudio Real-

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116 Time PCR Detection System (Thermo Fisher), with an initial denaturation at 95°C for 3 min,
117 followed by 45 cycles at 94°C for 30 sec, 55°C for 30 sec, and 68°C for 1 min with a final 95°C
118 for 10 sec. This was followed by a melting curve step of temperature ranging from 65°C to 95°C
119 with 0.5°C increments to determine the melting temperature of each amplified product. Each
120 assay included positive controls of *P. vivax* Pakchong (MRA-342G) and Nicaragua (MRA-
121 340G) isolates, *P. falciparum* isolates 7G8 (MRA-926) and HB3 (MRA-155), in addition to
122 negative controls including uninfected samples and water. A standard curve was produced from a
123 ten-fold dilution series of the *P. vivax* and *P. falciparum* control plasmid to determine the
124 amplification efficiency of the qPCR. Melting curve analyses were performed for each amplified
125 sample to confirm specific amplifications of the target sequence. The slope of the linear
126 regression of threshold cycle number (*Ct*) versus log10 (gene copy number) was used to
127 calculate amplification efficiency of each plate run based on internal standard controls. For the
128 measure of reproducibility of the threshold cycle number, the mean *Ct* value and standard error
129 was calculated from three independent assays of each sample. A cut-off threshold of 0.02
130 fluorescence units that robustly represented the threshold cycle at the log-linear phase of the
131 amplification and above the background noise was set to determine *Ct* value for each assay.
132 Samples yielding *Ct* values higher than 40 (as indicated in the negative controls) were considered
133 negative for *Plasmodium* species. Parasite density in a sample was quantified by converting the
134 *Ct* values into gene copy number (GCN) using the following equation: $GCN_{sample} = 2^{E\times(40-Ct_{sample})}$
135 ; where GCN stands for gene copy number, *Ct* for the threshold cycle of the sample, and E
136 for amplification efficiency. The differences in the log-transformed parasite GCN between
137 samples among the study sites were assessed for significance at the level of 0.05.[30,31]
138

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139 **Duffy blood group genotyping**

140 For all DBS samples, we employed qPCR-based TaqMan assay to examine the point
141 mutation (c.1-67T>C; rs2814778) in the GATA-1 transcription factor binding site of the *DARC*
142 gene. The following primers (forward: 5'-GGCCTGAGGCTTGTGCAGGCAG-3'; reverse: 5'-
143 CATACTCACCCTGTGCAGACAG-3') and dye-labeled probes (FAM-
144 CCTTGGCTCTTA[*C*]CTTGGAAGCACAGG-BHQ; HEX-
145 CCTTGGCTCTTA[*T*]CTTGGAAGCACAGG-BHQ) were used. Each PCR contained 5µl
146 TaqMan Fast Advanced Master mix (Thermo), 1µl DNA template, and 0.5µl of each primer
147 (10nM), and 0.5µl of each probe (10nM). The reactions were performed with an initial
148 denaturation at 95°C for 2 min, followed by 45 cycles at 95°C for 3 sec and 58°C for 30 sec. A
149 no-template control was used in each assay. The *Fy* genotypes were determined by the allelic
150 discrimination plot based on the fluorescent signal emitted from the allele-specific probes. For *P.*
151 *vivax* positive samples, a 1,100-bp fragment of the *DARC* gene was further amplified using
152 previously published primers.[3] Each PCR contained 20µl DreamTaq PCR Mastermix, 1µl DNA
153 template, and 0.5µl each primer. PCR conditions were 94°C for 2-min, followed by 35 cycles of
154 94°C for 20s, 58°C for 30s, and 68°C for 60s, followed by a 4-min extension. PCR products
155 were purified, and Sanger sequenced. Chromatograms were visually inspected to determine and
156 confirm the *Fy* genotypes based on the TaqMan assays.[31]

157

158 **Statistical analyses**

159 SPSS version 21.0 was used for analyzing the socio-demographic information of the
160 participants using descriptive statistics. To test the association between malaria infection and
161 factors including gender, age, ethnicity, and clinical symptoms, bivariate and multivariate

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162 logistic regression was performed. The odds ratio and associated 95% confidence interval (CI)
163 were computed to assess the strength of association. *P*-values under 0.05 were considered as
164 significant.
165

166 **Ethics statement**

167 Scientific and ethical clearance was obtained from the institutional scientific and ethical
168 review boards of Ethiopian Public Health Institute, Ethiopia and Drexel University, USA.
169 Written informed consent/assent for study participation was obtained from all participants of the
170 study and parents/guardians (for minors under 18 years old).
171

172 **Results**

173 **Socio-demographic characteristics of study participants**

174 For the 447 study participants, the age ranged from 6 months to 70 years old. The mean
175 age was 20.87; and 269 (60.1%) of the respondents were males and 171 (38.2%) were females.
176 The greatest proportion of the samples were from SNNPR (34.8%) followed by Oromia (31.3%)
177 and Amhara (23.9%). Afar (1.1%) and Sidama (0.4%) had the smallest sample size (**Table 1**).

178 **Table 1.** Socio-demographic characteristics of the study participants in seven regional states
179 across Ethiopia.
180

181 **Distribution of the Duffy genotypes and prevalence of gametocyte across Ethiopia**

182 Of the 447 study participants, 422 (94.4%) were confirmed with *Plasmodium* infections.
183 About 72% of the cases were *P. vivax* infection (322 out of 447), 1.8% were *P. falciparum*
184 infection (8 out of 447), and 20.6% were *P. vivax-P. falciparum* mixed infection (92 out of 447).
185 A total of 20 out of the 447 (4.5%) study participants were Duffy-negative. Eleven (11/20) of the

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186 Duffy-negatives were infected with *P. vivax*, five (5/20) were infected with both *P. vivax* and *P.*
187 *falciparum*, and four (4/20) were not infected. Duffy-negative infections by *P. vivax* were
188 observed in different sites across Ethiopia, specifically in the Amhara, Oromia,
189 Benishangul/gumuz and SNNPR regions but not in Afar, Gambella, and Sidama regions. This
190 could be due to the small sample size in these study sites.

191 The gametocyte prevalence in Duffy-negative individuals was 31.3% (5 out of 16), with
192 one of them detected in a *P. vivax-P. falciparum* mixed infection. This proportion had no
193 significant difference from Duffy-positive samples which showed 37.7% (150 out of 398), with
194 26 of them detected in *P. vivax-P. falciparum* mixed infections. Gametocyte stages of *P. vivax*
195 infections were mostly found in the SNNPR, 46.2% (70 out of 155) and Amhara, 30.3% (47 out
196 of 155) followed by Oromia, 12.9% (20 out of 155). There was no gametocyte detected in *P.*
197 *falciparum* infections. Five gametocyte-positive *P. vivax* infections were detected in Duffy-
198 negatives including two from Amhara, two from SNNPR, and one from Oromia regions (**Table**
199 **2**).

200 **Table 2.** Distribution of the Duffy genotypes among *Plasmodium* species infections, mixed (*Pf*
201 and *Pv*) infections, and gametocyte carriers across Ethiopia.
202

203 **Asexual parasitemia and parasite stage comparisons**

204 No significant difference was detected in parasitemia among the *P. vivax* samples
205 collected from southwestern, southern, and eastern regions of Ethiopia, except for samples in
206 Amhara, which is in the northwest. While previous studies indicated that parasitemia in Duffy-
207 negative individuals are expected to be low, our data showed that *P. vivax* parasitemia in Duffy
208 negatives widely vary among infections, with relatively low parasitemia observed in Oromia and
209 SNNPR regions (**Figure 2**).

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210 **Figure 2.** Asexual parasitemia comparison by qPCR among Duffy-positive and Duffy-negative
211 malaria patients in different major regions of Ethiopia.
212
213 Most of the infections had mixed parasite stages and the proportion of parasite stages vary
214 among regions. In SNNPR, 71 out of 140 (50.7%) *P. vivax* samples had trophozoites, 66 (47.1%)
215 with mixed trophozoite, schizont, and gametocyte stages, and three (2.1%) with gametocytes
216 only. In Oromia, 99 of the 118 (83.9%) *P. vivax* samples had trophozoites, followed by 16
217 (13.6%) with mixed trophozoite, schizont, and gametocyte stages, and three (2.5%) with
218 gametocytes only. In Amhara, similar proportion was observed where 55 of 107 (51.4%) *P. vivax*
219 samples had mixed trophozoite, schizont, and gametocyte stages, followed by 45 (42.1%) with
220 trophozoites, and three (2.8%) with gametocytes. In Benshangul/gumuz, 17 of 22 (77.3%) *P.*
221 *vivax* samples had trophozoites and five (22.7%) had mixed trophozoite, schizont, and
222 gametocytes. In Gambella, 11/15 (73.3%) *P. vivax* samples had trophozoites with gametocyte
223 mixed and gametocyte only. In Afar, all five mixed *P. vivax* and *P. falciparum* samples were
224 detected trophozoites. In Sidama, the two *P. vivax* samples had mixed trophozoite, schizont, and
225 gametocyte stages. Overall, almost all samples had trophozoites and mixed stages across study
226 sites **(Figure 3).**
227 **Figure 3.** Comparison of parasite stages of *P. vivax*-infected samples by microscopic
228 examination across broad regions of Ethiopia.
229
230 The gametocyte count of *P. vivax* species was done with a light microscope against 200
231 WBCs and calculated using an average WBC count value per µl of blood. The highest
232 gametocyte count was 2856 gametocyte/µl, detected in homozygous Duffy-negative individual
233 and the lowest gametocyte count was 15 gametocytes/µl, detected in heterozygous Duffy-

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234 positive individual. The average number of *P. vivax* gametocyte stages among all samples
235 detected with gametocyte stages and all Duffy blood group status was 449 gametocytes/µl of
236 blood, of which the average gametocyte counts of homozygous Duffy negatives (CC),
237 heterozygous Duffy positives (TC) and homozygous Duffy positives (TT) were 1060
238 gametocytes/µl, 425 gametocytes/µl and 395 gametocytes/µl, respectively **(Figure 4)**.
239 **Figure 4**. The gametocyte counts among homozygous Duffy-negatives (CC), heterozygous
240 Duffy-positives (TC) and homozygous Duffy-positives (TT) isolates of *P. vivax* species.
241
242 **Duffy blood group and other factors associated with *Plasmodium* infections**
243 The bivariate analysis was done to show the association of *P. vivax* infection with
244 independent factors. The prevalence of *P. vivax* infections in Duffy positive individuals were
245 about four times more likely than the Duffy negatives (OR = 4.6, 95% CI 1.4, 14.96, $p = 0.011$).
246 *P. vivax* infection was not significantly different among males and females. Although the
247 prevalence of *P. vivax* infection was recorded in all age groups, a relatively higher in the age
248 group < 15 years old, three times more likely than the age group > 45 years old (OR = 2.9, 95%
249 CI 0.45-1.98, $p = 0.98$). The *Plasmodium* infections were significantly different among various
250 clinical symptoms of the study participants. The odds of infection among patients with headache
251 was three times more likely than without headache (OR = 3.0, 95% CI 0.81-11.14, $p = 0.09$),
252 patients who had sweating is three times more likely than those who did not have sweating (OR
253 = 3.5, 95% CI 1.7-7.44, $p = 0.0009$) and among patients with chills were over two times more
254 likely than without chills (OR = 2.5, 95% CI 1.05-4.8, $p = 0.037$). No significant difference was
255 found in malaria symptoms such as fever, muscle and joint pain, nausea, and vomiting between
256 plasmodium infected and non-infected individuals ($p > 0.05$) (**Table 3**).
257 **Table 3.** Results of bivariate odds ratio to determine main predictors of *Plasmodium* infections
258 across Ethiopia. Asterisk represents significance at level of 0.05.

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259

260 ## Discussion

261 In Sub-Saharan Africa where Duffy-negative individuals are predominant, *vivax* malaria
262 has been reported but whether these infections can transmit among individuals is poorly
263 documented. This study indicates that *P. vivax* infections in Duffy-negative individuals distribute
264 across broad regions of Ethiopia. In our study, the prevalence of *P. vivax* among Duffy-negatives
265 was 3.8% (16/421). The result is in line with previous studies in the country revealed the
266 prevalence of *P. vivax* among Duffy-negatives 2.9%[32] and 4.4%,[33] on the contrary our result is
267 lower than the result found in Sudan (17.9).[34] In the general populations, Duffy negativity varies
268 from 20–36% in East Africa to 84% in Southern Africa.[4] Compared to Duffy-positive infections,
269 the average parasite density is much lower in Duffy-negative infections.[4] Nonetheless, a few
270 Duffy-negative *P. vivax* infections in Amhara were detected with relatively high parasitemia,
271 suggestive of certain *P. vivax* strains can invade Duffy-negative erythrocytes efficiently. The
272 exact mechanisms of Duffy-negative erythrocyte invasion by *P. vivax* are still unclear and merit
273 further investigation. For instance, *P. vivax* glycosylphosphatidylinositol-anchored micronemal
274 antigen (*Pv*GAMA) and merozoite surface protein-1 paralog (*Pv*MSP1P) have been recently
275 shown to bind to both Duffy-positive and negative red blood cells, suggesting their possible
276 involvement in Duffy-independent invasion pathway.[35-37] The reticulocyte binding protein
277 (*Pv*RBP2b) of *P. vivax* has been shown to bind to transferrin receptor 1 (TfR1) to invade Duffy-
278 positive RBCs and thus, present alternative pathways for Duffy-negative erythrocyte invasion.[5,38]
279 Such findings are critical to the development of blood-stage vaccine against the parasites.[39-41]
280 Amongst regions, the difference in *P. vivax* gametocyte production in Duffy-positive and
281 Duffy-negative individuals was not significant. However, the mean number of gametocyte count

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282 among Duffy-negatives was higher compared to Duffy-positives, 1060 gametocytes/µl and 425
283 gametocytes/ µl, respectively. This result indicates the dominance of sexual stages of *P. vivax*
284 parasites that contributes for the occurrence of asymptomatic infections in Duffy-negative
285 individuals. The detection of *P. vivax* gametocytes in Duffy-negative infections in Amhara,
286 Oromia, and SNNPR raises concern that these infections not only cause clinical symptoms but
287 can also contribute to transmission, despite its lower prevalence than in Duffy-positive infected
288 individuals. Given Duffy-negative and Duffy-positive individuals co-exist in Ethiopia, the extent
289 of transmission remains uncertain. It is possible that the asexual parasites converted into
290 gametocytes and spread from Duffy-negative to other Duffy-negative or Duffy-positive
291 individuals.[4,32,42] This finding lends support to earlier study showing that the parasites detected in
292 Duffy-negative and Duffy-positive populations were not genetically different.[4] Based on
293 computation modeling, Duffy-negatives in Ethiopia can serve as both the source and sink of
294 infections, though transmission is likely more frequent in Duffy-positives.[4,5]
295 Given that *P. vivax* has been widely reported in West and Central Africa where > 90% of
296 the populations are Duffy-negatives, these infections can certainly serve as reservoirs for
297 transmission both at the local and regional level.[5,33] Due to previously being exposed, the host
298 may have acquired immunity against symptomatic blood-stage parasitemia; however, due to the
299 early gametocyte development of *P. vivax*, long lasting sub-clinical illnesses may still contribute
300 to continuous transmission.[43,44] In this study, almost all gametocytes detected among the mixed
301 infections were *P. vivax*. This result supports the notion that the development of *P. vivax*
302 gametocytes is much faster than the *P. falciparum* ones at the onset of symptoms in febrile
303 malaria patients, and that *P. falciparum* gametocytes are seldomly detected in routine
304 microscopic examination of febrile malaria patients.[45]

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305 Most *P. vivax* infections in Amhara, Gambella, Sidama, and SNNPR had mixed parasite
306 stages including gametocytes, whereas in Oromia and SNNPR, trophozoites were prominent in
307 most samples. This variation in the parasite developmental stages could be associated with
308 environmental, host and parasite factors among different study districts. The epidemiology of
309 malaria within each district may also be a determining factor. For instance, while the general
310 proportion of *P. falciparum* and *P. vivax* in Ethiopia is 60% and 40%, respectively, considerable
311 regional difference exists.[46]

312 Warmer temperatures and higher rainfall/humidity in lowland than highland areas may
313 allow parasites to develop faster and greater production of gametocytes, which result in majority
314 mixed stages among *P. vivax* infections and enhance transmission. This might be supported by
315 the ability of the parasite to develop the asexual stage into gametocytes faster, within 48 hours
316 after generation of the first merozoites in the blood. The *P. vivax* infected and swollen RBCs due
317 to gametocyte development are flexible and can pass splenic filtration helps to stay all stages
318 together in the peripheral blood.[13] The unique biological features and genetic variability of the *P.*
319 *vivax* parasites certainly present a challenge in eradicating malaria in Ethiopia.[47,48]

320 For all *P. vivax* confirmed infections, typical symptoms were fever as well as headache
321 and fatigue. Other symptoms including muscle and joint pain, chills, sweating, and vomiting
322 vary by individuals across the seven study regions. Interestingly, our analyses revealed that *P.*
323 *vivax* cases were more likely to occur in individuals aged $\leq$ 15 years old followed by $<$ 45 and $>$
324 15 years old age group. Such demographic pattern could be explained as the host immunity in
325 old individuals might be higher compared to the younger and children. The mosquito vector
326 feeding time and behavior (outdoor or indoor resting/biting), the form of occupation (outdoor or

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327 indoor), environment (rural or remote populations), and economic status (poverty) also have
328 contribution.[49] These factors are critical when identifying disease trends or at-risk population.[5]
329 To conclude, the prevalence of Duffy-negative individuals among *P. vivax* malaria
330 patients varies across Ethiopia. This study confirms that Duffy-negativity does not completely
331 protect against *P. vivax* infection and these infections are frequently associated with low
332 parasitemia, which may represent hidden reservoirs that can contribute to transmission.
333 Understanding *P. vivax* transmission biology and gametocyte function via infectivity studies and
334 *in vitro* assays especially in Duffy-negative populations would enhance the treatment and control
335 strategies of *vivax* malaria in Africa. Further study is needed to quantify *Pvs*25 transcripts by
336 qRT-PCR for gametocyte density in Duffy-negative infected samples and to expand sample size
337 that will allow fair comparisons of gametocyte carriage between Duffy-positive and Duffy-
338 negative infections. A deeper comprehension of the association between Duffy-negativity and
339 the invasion processes of *P. vivax* would aid the development of *P. vivax* specific eradication
340 tactics, including substitute antimalarial immunizations other than a Duffy-binding protein-based
341 vaccine.
342

343 **Acknowledgements**

344 We thank the laboratory staffs at each sample collection health facilities for assisting sample
345 collection and preliminary laboratory work; Adama malaria center expert microscopists for
346 microscopic examinations; and all study participants for their willingness to provide blood
347 sample and information. We also thank Alfred Hubbard for creating the map for Figure 1.
348

349 **Funding**

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350 This research is supported by NIH R01 AI162947 and R01 AI173171.

351 **Disclosure**: The study was conducted with permission from the institutional scientific and ethical
352 review boards of Ethiopian Public Health Institute, Ethiopia and Drexel University, USA.
353 Written informed consent/assent for study participation was obtained from all participants.
354
355 All data produced in the present study are available upon reasonable request made to the
356 corresponding author, as per institutional and national legal norms and procedures.
357

358 **Authors' contributions**

359 TTS, EL, SM and EL Conceptualization and designed the study; TTS, MTN, and AA collected
360 sample and perform preliminary laboratory tests; EL, BRA and EL performed molecular
361 laboratory test, analysis, and interpretation; TTS, EL, BRA, JP and EL wrote and reviewed the
362 paper. All authors read and approved the final manuscript.
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501 **Table 1.** Socio-demographic characteristics of the study participants in seven regional states
502 across Ethiopia.

| Characteristics | | Number of participants (%) |
|---|---|---|
| **Gender** | | |
| | Male | 269 (60.1%) |
| | Female | 171 (38.2%) |
| **Age (years old)** | | |
| | < 15 | 150 (33.5%) |
| | ≥ 16 and < 45 | 273 (61.0%) |
| | ≥ 45 | 16 (3.5%) |
| **Region** | | |
| | Afar | 5 (1.1%) |
| | Amhara | 107 (23.9%) |
| | Benishangual/Gumuz | 22 (4.9%) |
| | Gambella | 15 (3.3%) |
| | Oromia | 140 (31.3%) |
| | Sidama | 2 (0.4%) |
| | SNNPR | 156 (34.8%) |

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514 515 **Table 2.** Distribution of the Duffy genotypes among *Plasmodium* species infections, mixed (*Pf* and *Pv*) infections, and gametocyte carriers across Ethiopia.

516

| Region | Total samples | Duffy-positives | | | | | Duffy-negatives | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ***Pv*** | ***Pf*** | **Mixed *Pv-Pf*** | **Malaria-negative** | **Pv with gametocytes** | ***Pv*** | ***Pf*** | **Mixed *Pv-Pf*** | **Malaria-negative** | **Pv with gametocytes** |
| **Afar** | 5 | 0 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Amhara** | 107 | 65 | 4 | 18 | 15 | 45 | 5 | 0 | 0 | 0 | 2 |
| **Benishangul/Gumuz** | 22 | 6 | 0 | 13 | 0 | 5 | 2 | 0 | 0 | 1 | 0 |
| **Gambella** | 15 | 7 | 0 | 8 | 0 | 11 | 0 | 0 | 0 | 0 | 0 |
| **Oromia** | 140 | 116 | 3 | 13 | 1 | 19 | 3 | 0 | 2 | 3 | 1 |
| **Sidama** | 2 | 2 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| **SNNPR** | 156 | 120 | 0 | 26 | 6 | 68 | 1 | 0 | 3 | 0 | 2 |
| **Total** | **447** | **315** | **7** | **83** | **22** | **150** | **11** | **0** | **5** | **4** | **5** |

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517

518 **Table 3.** Results of bivariate odds ratio to determine main predictors of *Plasmodium* infections

519 across Ethiopia. Asterisk represents significance at level of 0.05.

| **Parameters** | | **Infection rate by 18s qPCR** | | | |
|---|---|---|---|---|---|
| | | **Total samples** | **Infection** | **Non-infection** | **Odds ratio (95% CI)** |
| **Duffy status** | Duffy+ | 427 | 405 | 22 | 4.6 (1.4, 14.96), *P*= 0.011* |
| | Duffy- | 20 | 16 | 4 | 1 |
| **Gender** | Female | 171 | 159 | 12 | 1 |
| | Male | 269 | 249 | 20 | 0.94 (0.45, 1.98) *P*= 0.87 |
| **Age (years old)** | ≤ 15 | 150 | 143 | 7 | 2.9 (0.55, 15.4) *P*= 0.21 |
| | > 15 and < 45 | 273 | 250 | 23 | 1.55 (0.33, 7.26) *P*= 0.58 |
| | ≥ 45 | 16 | 14 | 2 | 1 |
| **Symptoms** | | | | | |
| **Fever** | Yes | 398 | 369 | 29 | 0.59 (0.03, 10.43) *P*= 0.72 |
| | No | 10 | 10 | 0 | 1 |
| **Headache** | Yes | 391 | 365 | 26 | 3.0 (0.81-11.14) *P*=0.099 |
| | No | 17 | 14 | 3 | 1 |
| **Fatigue** | Yes | 257 | 238 | 19 | 0.89 (0.4, 1.96) *P*=0.77 |
| | No | 151 | 141 | 10 | 1 |
| **Muscle and Joint Pain** | Yes | 257 | 240 | 17 | 1.12 (0.52, 2.41) *P*= 0.77 |
| | No | 163 | 151 | 12 | 1 |
| **Chills** | Yes | 258 | 245 | 13 | 2.25 (1.05, 4.8) *P*=0.037* |
| | No | 150 | 134 | 16 | 1 |

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| | | | | | |
|---|---|---|---|---|---|
| **Sweating** | Yes | 218 | 208 | 10 | 3.5 (1.7, 7.44) *P*= 0.0009* |
| | No | 200 | 171 | 29 | 1 |
| **Nausea and Vomiting** | Yes | 177 | 163 | 14 | 0.81 (0.38, 1.73) *P*=0.59 |
| | No | 230 | 215 | 15 | 1 |

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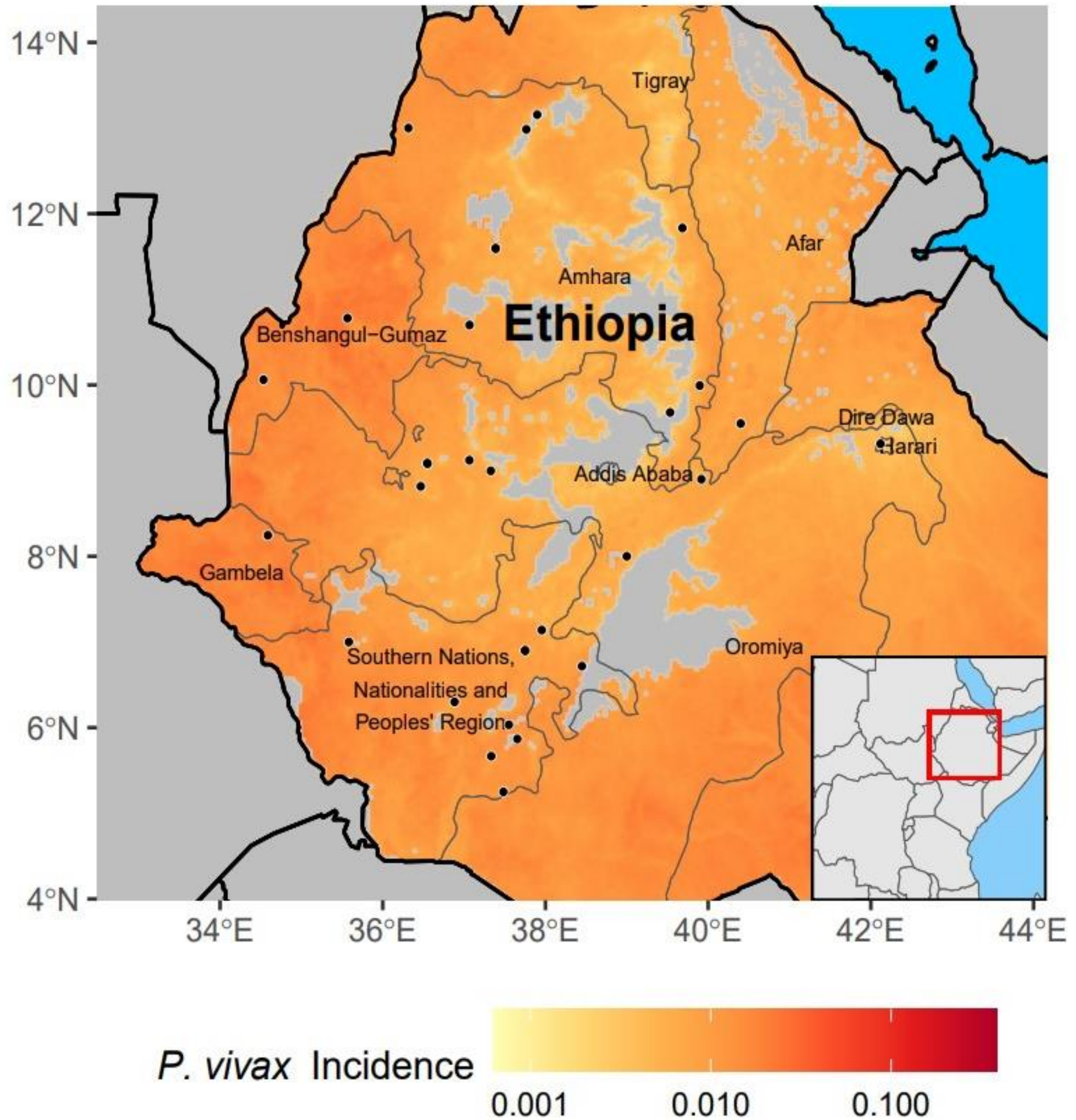


**Figure 1.** A map showing the study sites in Ethiopia with malaria incidence ranges from high in the western part to low in the eastern part of the country. These sites represent seven major regions including Afar, Amhara, Benishangul/Gumuz, Gambella, Oromia, Sidama, and Southern Nations, Nationalities, and People's Region (SNNPR) with diverse ethnic groups.

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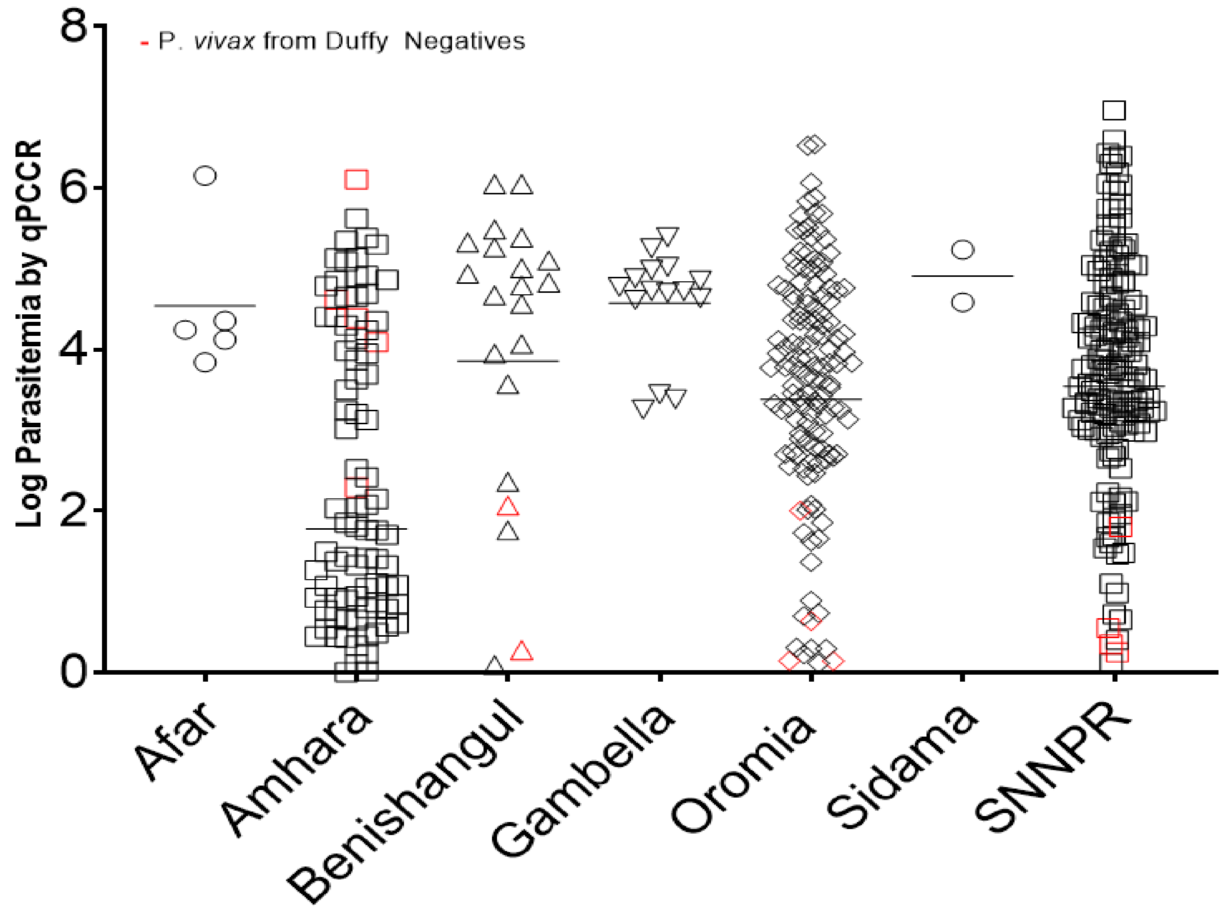


**Figure 2.** Asexual parasitemia comparison by qPCR among Duffy-positive and Duffy-negative malaria patients in different major regions of Ethiopia.

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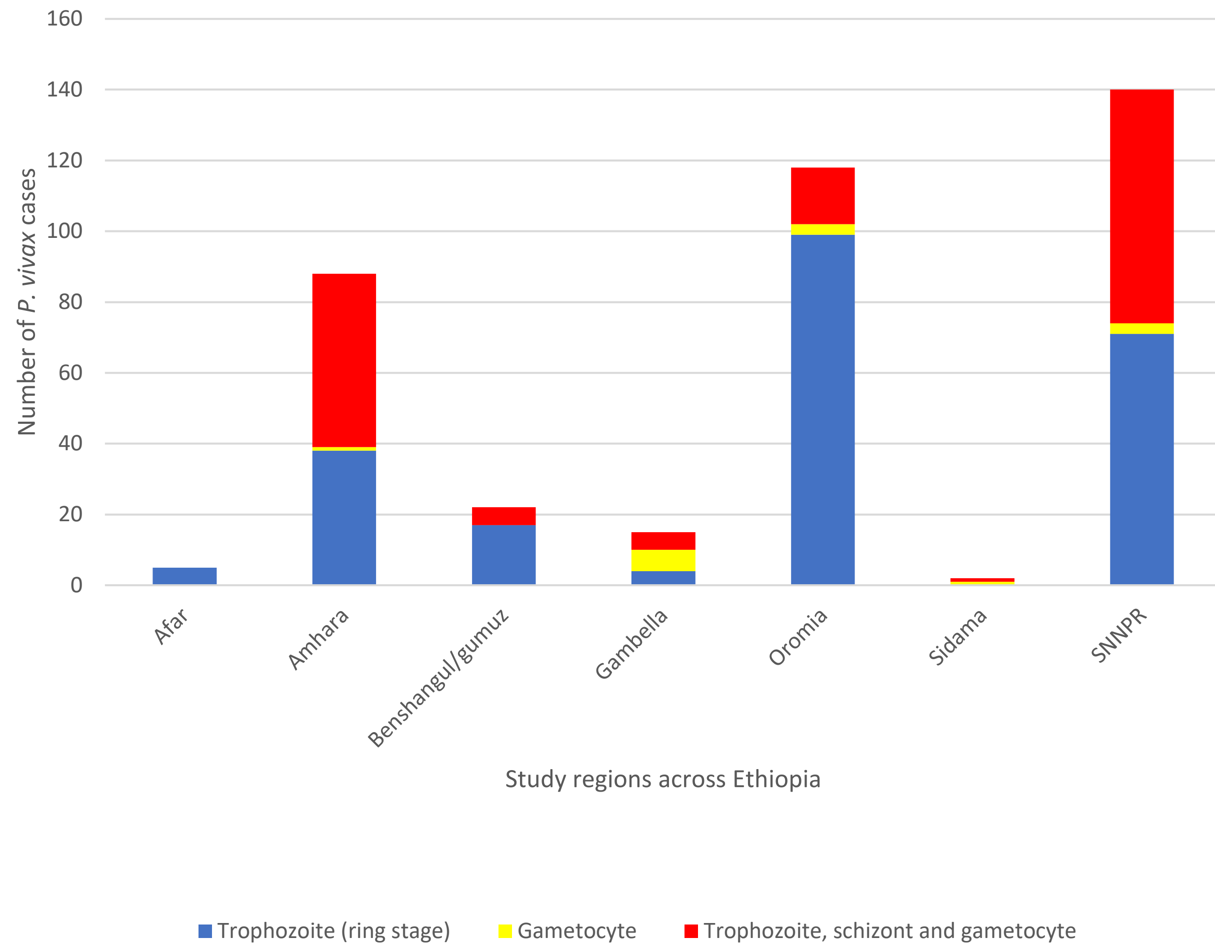


**Figure 3.** Comparison of parasite stages of *P. vivax*-infected samples by microscopic examination across broad regions of Ethiopia.

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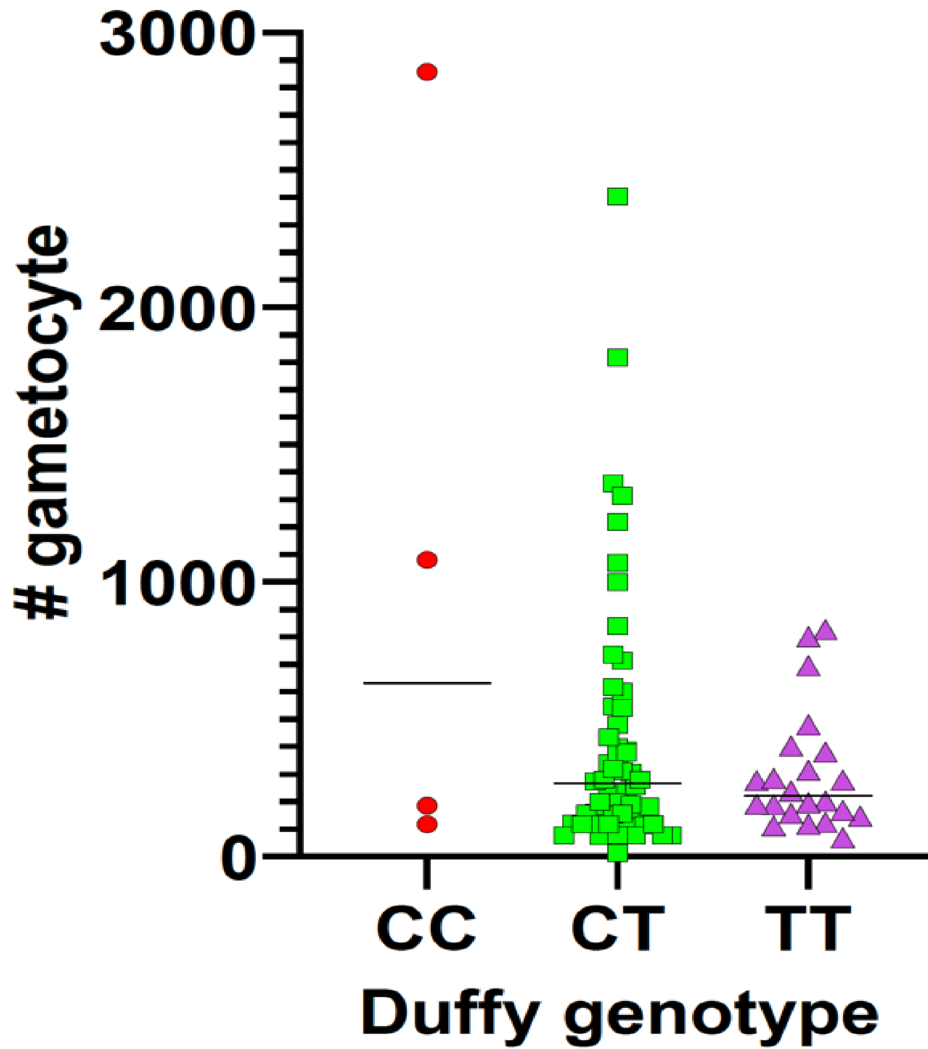


**Figure 4**. The gametocyte counts among homozygous Duffy-negatives (CC), heterozygous Duffy-positives (TC) and homozygous Duffy-positives (TT) isolates of *P. vivax* species.