

Supplemental Material for:

Prevalence of Asymptomatic non-Falciparum and Falciparum Malaria in the 2014-15 Rwanda Demographic Health Survey

Claudia Gaither¹, Camille Morgan², Rebecca Kirby³, Corine Karema⁴, Pierre Gashema⁵, Sam White¹, Hillary Topazian⁶, David Geibrecht³, Kyaw Thwai¹, Koby Boyter¹, Tharcisse Munyaneza⁷, Claude Mambo⁸, Jean De Dieu Butera⁷, Jeffrey A. Bailey^{3*}, Jean-Baptiste Mazarati^{4,5*}, Jonathan J. Juliano^{1,2,9,10*#}

1: Institute for Global Health and Infectious Diseases, University of North Carolina, Chapel Hill, NC, USA

2: Department of Epidemiology, Gillings School of Global Public Health, University of North Carolina, Chapel Hill, NC, USA

3: Department of Pathology, Brown University, Providence, RI, USA

4: Quality Equity Health Care, Kigali, Rwanda

5: INES-Ruhengeri, Ruhengeri, Rwanda

6: Imperial College, London, UK

7: National Reference Laboratory, Rwanda Biomedical Center, Kigali, Rwanda

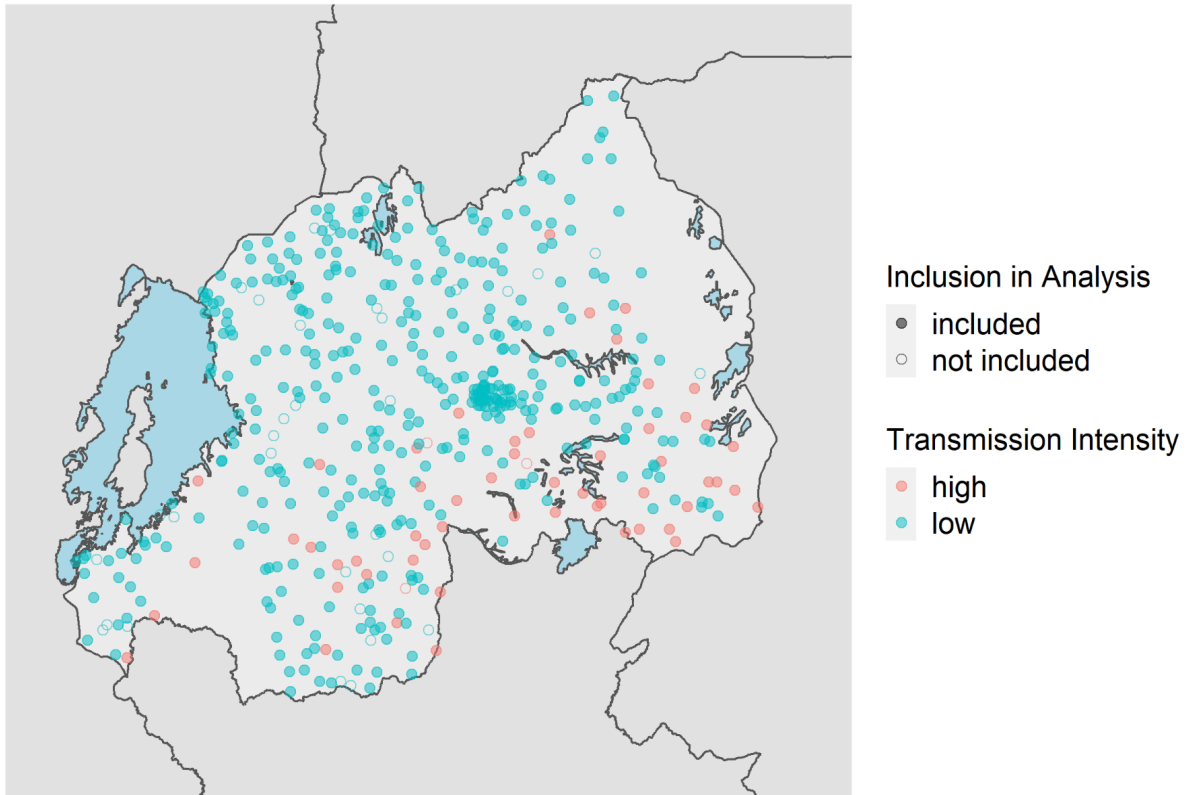
8: Rwanda Biomedical Centre, Kigali, Rwanda

9: Division of Infectious Diseases, School of Medicine, University of North Carolina, Chapel Hill, NC, USA

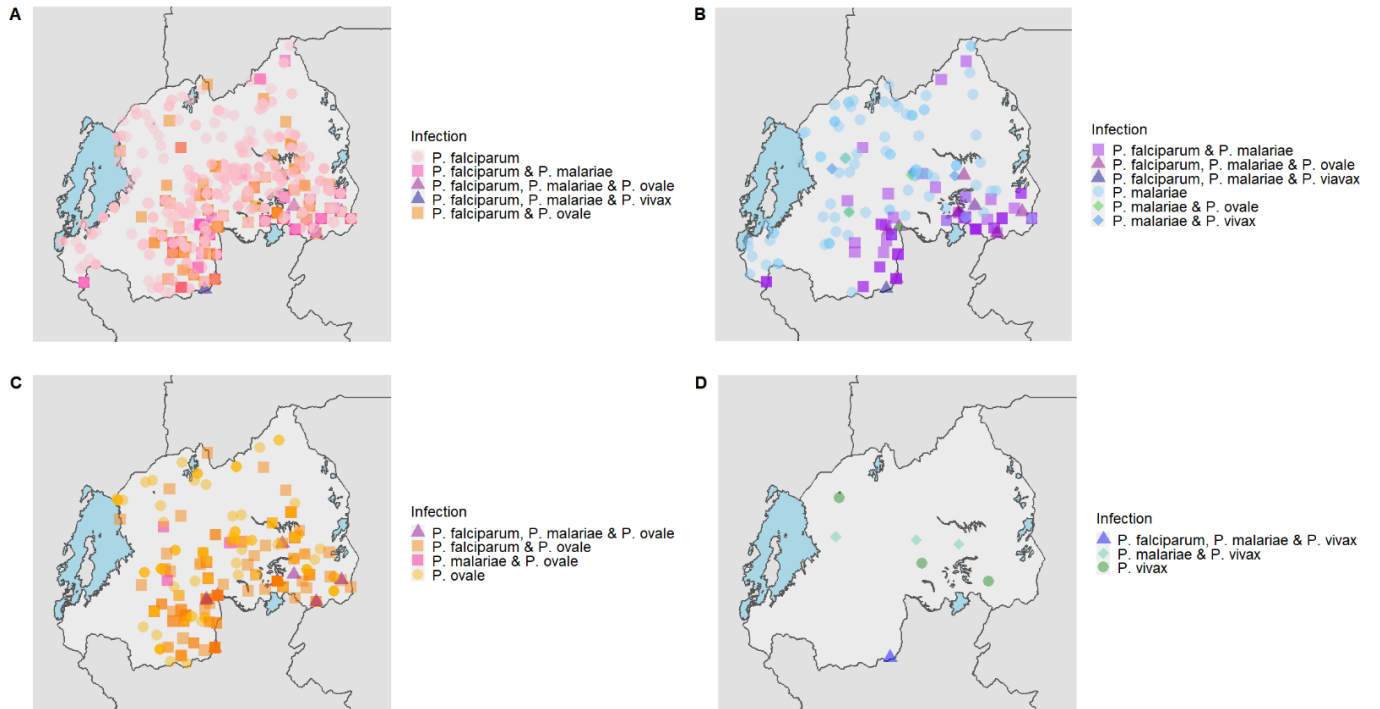
10: Curriculum in Genetics and Molecular Biology, School of Medicine, University of North Carolina, Chapel Hill, NC, USA

Supplemental Figure 1. Distribution of 2014-2015 Rwanda Demographic Health Survey Clusters.

Clusters are color coded based upon malaria transmission intensity based on DHS malaria testing results (high represents >15% positive by RDT or microscopy). Shape fill is based on the cluster's inclusion in this analysis.



Supplemental Figure 2. Distribution of clusters where malaria species were Identified. Panel A, B, C and D represent clusters where *P. falciparum*, *P. malariae*, *P. ovale* spp, and *P. vivax* malaria were identified, respectively. Mixed infections are denoted.



Supplemental Table 1. PCR Primers, Probes and Reaction Condition

Plasmodium falciparum (varATS)

Adapted from: Hoffman, N, et. al. PLOS Medicine. 2015.

Forward Primer 5' - CCCATACACAACCAAYTGGG - 3'

Reverse Primer 5' - TTCGCACATATCTCTATGTCTATCT - 3'

Probe 5' - 6-FAM-TRTCCATAAAATGGT-NFQ-MGB - 3'

Cycling conditions		45 cycles			
temp (degrees Celsius)		50	95	95	55
time		2 min	10 min	15 s	1 min

Roche FastStart Universal Probe Master

Fwd primer 800 nM

Rev primer 800 nM

Probe 400 nM

Template DNA 2.5 µl

Total volume 12.5 µl

Plasmodium ovale (18s)

Adapted from: Mitchell C, et. al. Journal of Infectious Diseases. 2021

Forward Primer 5' - CCRACTAGGTTTTGGATGAAVRTTTTT - 3'

Reverse Primer 5' - AACCCAAAGACTTTGATTTCTCATAA - 3'

Probe 5' - VIC/CRAAAGGAATTYTCTTATT - 3'

Cycling conditions		45 cycles			
temp (degrees Celsius)		50	95	95	52
time		2 min	10 min	15 s	1 min

Roche FastStart Universal Probe Master (Rox)

Fwd primer 400 nM

Rev primer 400 nM

Probe 200 nM

Template DNA 2 µl

Total volume 12 µl

Plasmodium malariae (18s)

Rougemont M, et. al. Journal of Clinical Microbiology. 2004.

5' - AGTTAAGGGAGTGAAGACGATCAGA - 3'

5' - CAACCCAAAGACTTTGATTTCTCATAA - 3'

5' - 6-FAM-ATGAGTGTTCCTTTTAGATAGC-NFQ-MGB - 3'

Cycling conditions		45 cycles			
temp (degrees Celsius)		50	95	95	60
time		2 min	10 min	15 s	1 min

Roche FastStart Universal Probe Master

Fwd primer 300 nM

Rev primer 300 nM

Probe 400 nM

Template DNA 2 µl

Total volume 12.5 µl

Plasmodium vivax (18s)

Brazeau N, et. al. Nature Communications. 2021

5' - ACGCTTCTAGCTTAATCCACATAACT - 3'

5' - ATTTACTCAAAGTAACAAGGACTTCCAAGC - 3'

5' - /56-FAM/TTCGTATCG/ZEN/ACTTTGTGCGCATTTTGC/3IABkFQ/ - 3'

Cycling conditions		45 cycles			
temp (degrees Celsius)		50	95	95	60
time		2 min	10 min	15 s	1 min

Roche FastStart Universal Probe Master (Rox)

Fwd primer 400 nM

Rev primer 400 nM

Probe 200 nM

Template DNA 5 µl

Total volume 18 µl

Supplemental Table 2. Cross reactivity of non-falciparum real time PCRs at 45 cycles. Each assay was run to 45 cycles against plasmids diluted to representative parasitemias (based on 6 copies per genomic equivalent).

Assay	Template	Genomic equivalent (GE)	# positive	Ct of positive
<i>P. vivax</i>	<i>P. falciparum</i>	212 GE/ul	0/20	ND
	<i>P. ovale</i>	110 GE/ul	0/20	ND
	<i>P. malariae</i>	100 GE/ul	1/20	42.1
<i>P. ovale</i>	<i>P. falciparum</i>	212 GE/ul	1/20	43.7
	<i>P. vivax</i>	76.5 GE/ul	0/20	ND
	<i>P. malariae</i>	100 GE/ul	1/20	42.5
<i>P. malariae</i>	<i>P. falciparum</i>	212 GE/ul	0/20	ND
	<i>P. vivax</i>	76.5 GE/ul	0/20	ND
	<i>P. ovale</i>	110 GE/ul	0/20	ND

Supplemental Table 3. Comparison of Population Used for Molecular Screening to Overall DHS Population (weighted counts)

<i>Individual Level Covariates</i>					
Variable		This Study	%	Overall DHS	%
<i>Sex of respondent</i>	Male	2755	59.69%	8490	63.61%
	Female	1861	40.31%	4858	36.39%
<i>Age group (years)</i>	0-15	121	2.63%	255	1.91%
	15-24	658	14.25%	1614	12.09%
	25-34	1450	31.41%	4297	32.19%
	35-44	930	20.15%	2827	21.18%
	45-54	843	18.25%	2586	19.37%
	55+	614	13.31%	1770	13.26%
<i>Wealth Quintile</i>	Poorest	694	15.04%	2211	16.56%
	Poorer	820	17.77%	2470	18.51%
	Middle	828	17.94%	2641	19.78%
	Richer	1027	22.26%	2842	21.29%
	Richest	1246	26.98%	3184	23.86%
<i>Education</i>	None/preschool	907	19.66%	2741	20.56%
	Primary	2779	60.22%	8371	62.78%
	Secondary	724	15.68%	1663	12.47%
	Higher	205	4.44%	559	4.20%
<i>Owns livestock, herds, or farm animals</i>	No	2068	44.79%	5829	43.67%
	Yes	2548	55.21%	7519	56.33%
<i>Source of drinking water</i>	Unpiped	3912	84.79%	11719	87.84%
	Piped	702	15.21%	1622	12.16%
<i>Household bed net</i>	No	798	17.28%	2199	16.47%
	Yes	3818	82.72%	11150	83.53%
<i>Slept under LLIN last night</i>	No	1458	31.58%	4101	30.72%
	Yes	3158	68.42%	9248	69.28%
<i>Insecticide-treated household net</i>	No	7	0.14%	21	0.16%
	Yes	3527	76.41%	10414	78.02%
	Missing data	1082	23.45%	2913	21.83%
<i>1 bed net per 1.8 household members</i>	No	1905	41.37%	5238	39.32%
	Yes	2699	58.63%	8084	60.68%
<i>Cluster level covariates</i>					
<i>Region</i>	Kigali City	699	15.15%	1803	13.51%
	South	1176	25.48%	3248	24.33%
	West	1026	22.23%	2934	21.98%
	North	776	16.81%	2121	15.89%
	East	938	20.32%	3243	24.29%

<i>Place of residence</i>	Urban	1096	23.74%	2715	20.34%
	Rural	3520	76.26%	10634	79.66%
<i>Elevation (m)</i>	500-1000	15	0.33%	50	0.37%
	1001-1500	1263	27.37%	4013	30.06%
	1501-2000	2450	53.07%	7020	52.59%
	2001-2500	822	17.80%	2146	16.07%
	2500 <	66	1.43%	121	0.91%
<i>Month of data collection</i>	15-Jan	1067	23.11%	3117	23.35%
	15-Feb	780	16.90%	2432	18.22%
	15-Mar	1100	23.83%	2872	21.52%
	15-Apr	32	0.70%	94	0.70%
	14-Nov	457	9.90%	1884	14.11%
	14-Dec	1180	25.56%	2950	22.10%

Supplemental Table 4. Impact of potential false positivity on unadjusted prevalence. 3/160 replicates were false positive across assays: 1.9% (95%CI: 0.3%-5.4%) Binomial exact method. All adjustments rounded up to higher integer.

	Number tested	# Ct≤40	# Ct>40	Unweighted Prevalence	0.3% false positive in Ct>40	Adjusted unweighted prevalence	5.4% false positive in Ct>40	Adjusted unweighted prevalence
<i>P. falciparum</i>	4,595	1065	166	26.8%	165	26.8%	157	26.6%
<i>P. malariae</i>		147	21	3.7%	20	3.6%	19	3.6%
<i>P. ovale</i>		101	145	5.4%	144	5.3%	136	5.2%

Supplemental Table 5. Differences in District Level Prevalence by PCR Cutoff. The difference in prevalence for malaria for each district between the 45 and 40 PCR cycle threshold is shown for overall malaria burden, as well as for each species. Darker shading denotes larger differences in prevalence estimates, and districts are ordered by the difference in overall malaria prevalence from highest to lowest difference in prevalence.

<i>District</i>	<i>Difference in overall malaria prevalence</i>	<i>Difference in Pf prevalence</i>	<i>Difference in Pm prevalence</i>	<i>Difference in Po prevalence</i>	<i>Difference in Pv prevalence</i>
Nyarugenge	0.128	0.039	0.027	0.063	0.000
Gatsibo	0.120	0.047	0.000	0.112	0.000
Ngoma	0.102	0.054	0.002	0.091	0.002
Burera	0.090	0.037	0.018	0.034	0.000
Huye	0.089	0.073	0.002	0.033	0.000
Rwamagana	0.088	0.052	0.015	0.071	0.000
Kirehe	0.084	0.052	0.003	0.046	0.000
Gasabo	0.080	0.037	0.000	0.043	0.000
Nyamagabe	0.074	0.053	0.000	0.036	0.000
Rubavu	0.068	0.029	0.000	0.040	0.000
Kamonyi	0.063	0.024	0.007	0.047	0.000
Nyaruguru	0.063	0.049	0.000	0.039	0.000
Kayonza	0.061	0.048	0.000	0.020	0.000
Karongi	0.061	0.002	0.000	0.061	0.000
Gakenke	0.061	0.027	0.034	0.000	0.000
Gicumbi	0.060	0.017	0.009	0.033	0.000
Ruhango	0.059	0.030	0.021	0.038	0.000
Gisagara	0.058	0.052	0.003	0.068	0.000
Nyabihu	0.057	0.022	0.007	0.018	0.010
Muhanga	0.046	0.023	0.000	0.023	0.000
Nyamasheke	0.043	0.043	0.000	0.000	0.000
Nyanza	0.043	0.041	0.006	0.020	0.000
Nyagatare	0.041	0.041	0.000	0.018	0.000
Kicukiro	0.040	0.026	0.000	0.021	0.000
Musanze	0.036	0.022	0.006	0.008	0.000
Rutsiro	0.035	0.045	0.000	0.000	0.015
Bugesera	0.034	0.014	0.002	0.025	0.008
Ngororero	0.021	0.010	0.010	0.010	0.000
Rusizi	0.018	0.010	0.008	0.000	0.000
Rulindo	0.015	0.008	0.000	0.007	0.000

Supplemental Table 6. District Level Malaria Prevalence at Different Cycle Cutoffs. The district level weighted prevalence for all malaria, all non-Pf malaria and reach species is shown for a cycle cut off of 45 cycles and 40 cycles.

<i>District</i>	<i>n</i>	45 cycles						40 cycles					
		<i>all malaria</i>	<i>non-Pf</i>	<i>Pf</i>	<i>Pm</i>	<i>Po</i>	<i>Pv</i>	<i>all malaria</i>	<i>non-Pf</i>	<i>Pf</i>	<i>Pm</i>	<i>Po</i>	<i>Pv</i>
Bugesera	244	36.6	7.2	32.6	1.8	4.6	0.8	33.2	3.7	31.2	1.5	2.2	0.0
Burera	117	16.0	10.9	6.1	4.5	6.4	0.0	7.1	5.7	2.4	2.7	3.0	0.0
Gakenke	99	8.2	4.7	3.4	4.7	0.0	0.0	2.1	1.4	0.7	1.4	0.0	0.0
Gasabo	151	16.5	4.3	12.2	0.0	4.3	0.0	8.5	0.0	8.5	0.0	0.0	0.0
Gatsibo	150	37.3	14.8	27.4	0.0	14.8	0.0	25.2	3.5	22.8	0.0	3.5	0.0
Gicumbi	104	13.3	8.1	5.2	4.8	3.3	0.0	7.3	3.8	3.5	3.8	0.0	0.0
Gisagara	188	45.3	13.3	41.5	3.2	9.2	0.9	39.5	6.2	36.2	2.9	2.4	0.9
Huye	151	45.0	11.2	39.8	0.2	10.9	0.0	36.0	7.6	32.5	0.0	7.6	0.0
Kamonyi	139	25.7	7.1	21.0	0.7	6.4	0.0	19.4	1.7	18.6	0.0	1.7	0.0
Karongi	142	13.9	9.4	5.6	1.5	7.9	0.0	7.8	3.3	5.5	1.5	1.8	0.0
Kayonza	211	35.1	7.3	29.1	2.2	5.1	0.0	28.9	5.4	24.3	2.2	3.1	0.0
Kicukiro	161	12.5	6.6	7.1	3.8	2.8	0.0	8.5	4.5	4.6	3.8	0.7	0.0
Kirehe	281	54.8	11.6	50.8	5.3	6.3	0.0	46.4	6.7	45.6	5.0	1.6	0.0
Muhanga	122	14.9	3.8	12.5	0.7	3.1	0.0	10.3	1.5	10.3	0.7	0.7	0.0
Musanze	119	9.6	4.8	4.8	4.0	0.8	0.0	5.9	3.4	2.6	3.4	0.0	0.0
Ngoma	257	56.0	21.4	46.5	6.9	14.3	0.2	45.8	11.9	41.0	6.7	5.2	0.0
Ngororero	100	11.2	9.1	4.0	6.2	3.0	0.0	9.1	7.0	2.9	5.1	1.9	0.0
Nyabihu	120	9.8	6.7	3.7	3.3	2.5	1.0	4.1	3.2	1.5	2.5	0.7	0.0
Nyagatare	113	21.6	9.0	15.4	6.3	2.6	0.0	17.6	7.1	11.3	6.3	0.8	0.0
Nyamagabe	203	25.5	10.8	16.7	4.7	6.1	0.0	18.0	7.2	11.5	4.7	2.5	0.0
Nyamasheke	142	16.9	3.1	13.8	3.1	0.0	0.0	12.6	3.1	9.6	3.1	0.0	0.0
Nyanza	193	56.9	9.3	54.7	4.1	5.1	0.0	52.6	6.6	50.6	3.5	3.1	0.0
Nyarugenge	183	19.9	11.7	9.3	3.7	7.3	0.6	7.0	2.7	5.4	1.1	1.0	0.6
Nyaruguru	141	24.3	9.2	20.2	3.1	6.1	0.0	18.0	5.3	15.2	3.1	2.2	0.0
Rubavu	105	16.5	8.1	8.5	1.8	6.3	0.0	9.7	4.1	5.6	1.8	2.3	0.0
Ruhango	147	34.2	10.2	28.4	4.4	5.7	0.0	28.3	4.2	25.4	2.3	1.9	0.0
Rulindo	116	10.3	2.5	7.8	0.7	1.8	0.0	8.8	1.8	7.0	0.7	1.1	0.0
Rusizi	165	13.9	5.6	8.7	5.6	0.0	0.0	12.1	4.9	7.7	4.9	0.0	0.0
Rutsiro	98	12.7	7.5	7.7	5.0	1.0	1.5	9.2	6.0	3.1	5.0	1.0	0.0
Rwamagana	134	33.5	13.7	25.6	4.4	8.6	0.7	24.6	5.1	20.4	2.9	1.5	0.7

Supplemental Table 7. Mixed Species Infection Count. Weighted and unweighted counts for mono and coinfections, separated by species. Values for both 45 and 40 cycles as a cutoff are included.

<i>unweighted count</i>	4595
<i>weighted count</i>	4616

***P. falciparum* infection counts**

	<i>mono infection</i>	<i>total co-infection</i>	<i>pf_pm</i>	<i>pf_po</i>	<i>pf_pm_po</i>	<i>pf_pm_pv</i>	<i>total</i>
<i>45 cycles unweighted</i>	1069	162	56	96	9.0	1.0	1231
<i>45 cycles weighted</i>	710	103	28	69	4.7	1.2	813
<i>under 40 cycles unweighted</i>	964	101	50	47	4.0	0.0	1065
<i>under 40 cycles weighted</i>	604	54	25	27	1.2	0.0	658

***P. malariae* infection counts**

	<i>mono infection</i>	<i>total co-infection</i>	<i>pf_pm</i>	<i>pm_po</i>	<i>pm_pv</i>	<i>pf_pm_po</i>	<i>pf_pm_pv</i>	<i>total</i>
<i>45 cycles unweighted</i>	94	74	56	5.0	3.0	9.0	1.0	168
<i>45 cycles weighted</i>	109	43	28	5.4	3.6	4.7	1.2	152
<i>under 40 cycles unweighted</i>	84	63	50	1.0	2.0	9.0	1.0	147
<i>under 40 cycles weighted</i>	95	34	25	0.9	2.4	4.7	1.2	129

***P. ovale* infection counts**

	<i>mono infection</i>	<i>total co-infection</i>	<i>pf_po</i>	<i>pm_po</i>	<i>pf_pm_po</i>	<i>total</i>
<i>45 cycles unweighted</i>	136	110	96	5.0	9.0	246
<i>45 cycles weighted</i>	158	79	69	5.4	4.7	237
<i>under 40 cycles unweighted</i>	44	57	47	1.0	9.0	101
<i>under 40 cycles weighted</i>	47	33	27	0.9	4.7	79

***P. vivax* infection counts**

	<i>mono infection</i>	<i>total co-infection</i>	<i>pm_pv</i>	<i>pf_pm_pv</i>	<i>total</i>
<i>45 cycles unweighted</i>	3.0	4.0	3.0	1.0	7.0

<i>45 cycles weighted</i>	2.6	4.9	3.6	1.2	7.5
<i>under 40 cycles unweighted</i>	1.0	3.0	2.0	1.0	4.0
<i>under 40 cycles weighted</i>	1.0	3.6	2.4	1.2	4.6

All malaria counts

	<i>mono infection</i>	<i>co-infection</i>	<i>total Pf</i>	<i>total Pm</i>	<i>total Po</i>	<i>total Pv</i>	<i>total non-pf</i>	<i>total</i>
<i>unweighted count</i>	1302.0	350	1231	168	246	7.0	421.0	1652.0
<i>weighted count</i>	979	230	813	152	237	7	396	1209
<i>under 40 CT unweighted count</i>	1092	224.0	1019	128	93	4.0	225.0	1244.0
<i>weighted under 40 count</i>	746	125	658	129	79	5	213	871