



OBSERVATION ARTICLE

Plasmodium falciparum infection rates for some *Anopheles* spp. from Guinea-Bissau, West Africa [v1; ref status: indexed, <http://f1000r.es/4in>]

Michelle R. Sanford¹, Anthony J. Cornel^{1,3}, Catelyn C. Nieman¹, Joao Dinis², Clare D. Marsden¹, Allison M. Weakley¹, Sarah Han¹, Amabelia Rodrigues², Gregory C. Lanzaro¹, Yoosook Lee¹

¹Vector Genetics Laboratory, Department of Pathology, Microbiology, and Immunology, School of Veterinary Medicine, University of California, Davis, 95616, USA

²National Institute of Public Health (INASA), Bissau, Guinea-Bissau

³Department of Entomology and Nematology, University of California, Davis, 95616, USA

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Abstract

Presence of *Plasmodium falciparum* circumsporozoite protein (CSP) was detected by enzyme linked immunosorbent assay (ELISA) in a sample of *Anopheles gambiae* s.s., *A. melas* and *A. pharoensis* collected in Guinea-Bissau during October and November 2009. The percentage of *P. falciparum* infected samples (10.2% overall) was comparable to earlier studies from other sites in Guinea-Bissau (9.6-12.4%). The majority of the specimens collected were identified as *A. gambiae* which had an individual infection rate of 12.6 % across collection sites. A small number of specimens of *A. coluzzii*, *A. coluzzii* x *A. gambiae* hybrids, *A. melas* and *A. pharoensis* were collected and had infection rates of 4.3%, 4.1%, 11.1% and 33.3% respectively. Despite being present in low numbers in indoor collections, the exophilic feeding behaviors of *A. melas* (N=18) and *A. pharoensis* (N=6) and high infection rates observed in this survey suggest *falciparum*-malaria transmission potential outside of the protection of bed nets.

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- 1 J Derek Charwood, London School of Hygiene & Tropical Medicine UK
- 2 Guido Favia, University of Camerino Italy

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Corresponding author: Yoosook Lee (yoslee@ucdavis.edu)

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Introduction

Malaria is among the leading causes of childhood mortality in Guinea-Bissau, comprising 18% of mortality of children less than five years of age as of 2010 (WHO, 2010). However, the human malaria incidence rate in Guinea Bissau varies considerably from year to year with a general decrease in recent years to about 3 children (<5 yrs of age) per thousand in some locations (Ursing *et al.*, 2014). *Plasmodium falciparum* predominates, causing 98% cases, followed by a few cases of *Plasmodium malariae* and *Plasmodium ovale*. Mixed infections of *P. malariae*, and to a lesser extent *P. ovale*, have been recorded but appear to be rare and highly variable in both Guinea-Bissau (Snounou *et al.*, 1993) and neighboring Senegal (Fontenille *et al.*, 1997a; Fontenille *et al.* 1997b).

Limited research has been conducted on the vectors and malaria parasite infection rates in Guinea-Bissau populations of *Anopheles* species in general and there is no data on comparative infection rates between *A. gambiae* and *A. coluzzii* and members of *A. gambiae* complex. Variability is also high among the *Anopheles* spp. implicated as vectors in this region of West Africa in terms of both their temporal population dynamics as well as species composition among study sites (Carnevale *et al.*, 2010; Fontenille *et al.*, 1997a; Jaenson *et al.*, 1994; Snounou *et al.*, 1993).

Here we present much needed data on *P. falciparum* infection of *Anopheles* spp. specimens collected from inside and around associated human habitations at eight sites in Guinea-Bissau (Table 1).

Method

Mosquitoes were collected by mouth aspiration from both the island and inland areas of Guinea-Bissau (Figure 1) in 2009 between October and November, which corresponds with the time of year previously observed to have the highest infection rate in *Anopheles* species (Jaenson *et al.*, 1994). The mosquito was dissected and the head and thorax were preserved in 100% ethanol for subsequent ELISA. Genomic DNA was extracted using a DNeasy extraction kit

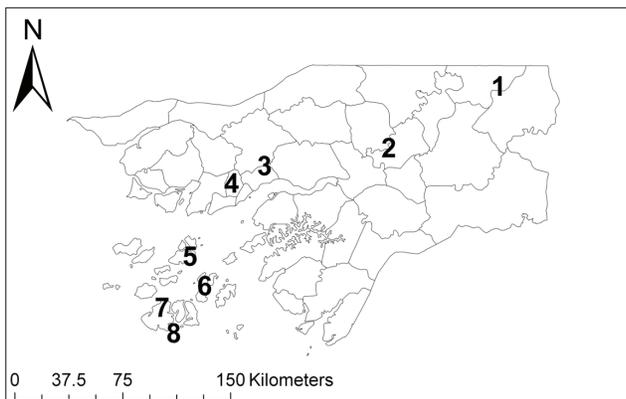


Figure 1. Collection sites in Guinea-Bissau. 1: Canjufa (12.43N, 14.13W), 2: Bambadinca (12.02N, 14.86W), 3: Antula (11.91N, 15.58W), 4: Prabis (11.80N, 15.74W), 5: Abu (11.46N, 15.91W), 6: Brus (11.23N, 15.88W), 7: Ponta Anabaca (11.18N, 16.14W) and 8: Eiticoga (11.16N, 16.14W).

Table 1. Sites, species and *Plasmodium falciparum* circumsporozoite protein (CSP) detection information from *Anopheles* spp. samples collected in Guinea-Bissau, October and November 2009. Numbers (#) indicate site locations on the map of Guinea-Bissau in Figure 1. All mosquitoes were collected indoors with a single exception; samples in Ponta Anabaca were opportunistically collected outside.

#	Site	<i>P. falciparum</i> infected	Uninfected	Total collected	Infection rate
<i>Anopheles coluzzii</i>					
1	Canjufa	0	1	1	0.0%
2	Bambadinca	2	16	18	11.1%
3	Antula	0	17	17	0.0%
4	Prabis	0	24	24	0.0%
5	Abu	1	7	8	12.5%
6	Brus	0	1	1	0.0%
8	Eiticoga	0	1	1	0.0%
	SUBTOTAL	3	67	70	4.3%
<i>Anopheles gambiae</i>					
1	Canjufa	1	1	2	50.0%
2	Bambadinca	0	1	1	0.0%
3	Antula	13	63	76	17.1%
4	Prabis	3	50	53	5.7%
5	Abu	1	30	31	3.2%
6	Brus	0	5	5	0.0%
7	Ponta Anabaca	8	46	54	14.8%
8	Eiticoga	3	5	8	37.5%
	SUBTOTAL	29	201	230	12.6%
<i>A. coluzzii</i> x <i>A. gambiae</i> hybrids					
1	Canjufa	1	0	1	100.0%
3	Antula	1	26	27	3.7%
4	Prabis	0	14	14	0.0%
5	Abu	0	5	5	0.0%
8	Eiticoga	0	2	2	0.0%
	SUBTOTAL	2	47	49	4.1%
<i>Anopheles melas</i>					
3	Antula	1	2	3	33.3%
4	Prabis	1	7	8	12.5%
5	Abu	0	2	2	0.0%
6	Brus	0	6	6	0.0%
8	Eiticoga	0	1	1	0.0%
	SUBTOTAL	2	16	18	11.1%
<i>Anopheles pharoensis</i>					
2	Bambadinca	2	4	6	33.3%
	Grand Total	38	337	375	10.2%

(Qiagen). Species determination of mosquitoes from the *A. gambiae* complex were made with the combination of species diagnostic assays (Fanello *et al.*, 2002; Favia *et al.*, 2001; Santolamazza *et al.*, 2008; Scott *et al.*, 1993) and a divergence island SNP (DIS) genotyping assay (Lee *et al.*, 2014) while other species were identified by morphology.

For the Scott PCR (Scott *et al.*, 1993) and the Fanello RFLP (Fanello *et al.*, 2002), we used four primers (UN [5'-GTG TGG CCC TTC CTC GAT GT-3'], GA [5'-CTG GTT TGG TCG GCA CGT TT-3'], ME [5'-TGA CCA ACC CAC TCC CTT GA-3'] and AR [5'-AAG TGT CCT TCT CCA TCC TA-3']). We excluded QD primer (Scott *et al.*, 1993) because our study site is well outside of the geographic range of this species (East Africa). A 25 μ L PCR reaction containing 1X GeneAmp PCR Buffer (Applied Biosystems), 1mM MgCl₂, 0.2mM of each dNTP, 0.12 μ M of each primer and 0.05U AmpliTaq DNA polymerase (Applied Biosystems) was carried out for each individual. Scott PCR products were digested using HhaI enzyme (New England Biosystems) following the protocol stated in (Fanello *et al.*, 2002). Thermocycler conditions were 95°C for 5 min followed by thirty-five cycles of 95°C for 45 s, 50°C for 30 s and 72°C for 45 s, with a final elongation at 72°C for 7 min, and a 4°C hold.

For the Favia PCR (Favia *et al.*, 2001), we used four primers (R5 [5'-GCC AAT CCG AGC TGA TAG CGC-3'], R3 [5'-CGA ATT CTA GGG AGC TCC AG-3'], Mopint [5'-GCC CCT TCC TCG ATG GCA T-3'] and B/S int [5'-ACC AAG ATG GTT CGT TGC-3']). A 25 μ L PCR reaction containing 1X PCR Buffer (Applied Biosystems), 1.5mM MgCl₂, 0.2mM of each dNTP, 0.2 μ M of primer R5, 0.2 μ M of primer R3, 0.16 μ M of primer Mopint, 0.1 μ M of primer B/S int and 0.02U DNA polymerase AmpliTaq (Applied Biosystems) was carried out for each individual. Thermocycler conditions were 95°C for 5 min followed by thirty-five cycles of 95°C for 30 s, 64°C for 30 s and 72°C for 30 s, with a final elongation at 72°C for 7 min, and a 4°C hold.

For the SINEX PCR (Santolamazza *et al.*, 2008), we used S200 X6.1 forward [5'-TCG CCT TAG ACC TTG CGT TA-3'] and reverse [5'-CGC TTC AAG AAT TCG AGA TAC-3'] primers. A 25 μ L PCR reaction containing 1X PCR Buffer (Applied Biosystems), 2mM MgCl₂, 0.4mM of each dNTP, 0.2 μ M of each primer and 0.1U DNA polymerase AmpliTaq (Applied Biosystems) was carried out for each individual. Thermocycler conditions were 95°C for 5 min followed by thirty-five cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, with a final elongation at 72°C for 10 min, and a 4°C hold.

The resulting PCR products were analyzed on a Qiaxcel capillary electrophoresis instrument (Qiagen) using a DNA Screening Cartridge (Qiagen).

For DIS genotyping, we used Sequenom iPLEX Gold Genotyping Reagent Set (Catalog number: Sequenom 10158) and ran on MassArray (Sequenom) mass spectrometer at UC Davis Veterinary Genetics Laboratory. A mosquito was considered a hybrid if at least 5 out of 7 DIS on the X chromosome were in a heterozygous state.

P. falciparum infection was determined by enzyme linked immunosorbent assay (ELISA) of circumsporozoite protein (CSP) (Burkot *et al.*, 1984; Wirtz *et al.*, 1987) from the head and thorax of mosquito specimens in an attempt to capture the parts of the mosquito that would indicate they were infective mosquitoes. All chemicals except for substrate solutions (Item 5 on page 5 of the supplemental ELSA protocol document) were ordered from Sigma-Aldrich. Monoclonal antibodies (capture and conjugate) were obtained from Kirkegaard & Perry Laboratories. *P. falciparum* sporozoite protein for positive control was ordered from the Centers for Disease Control and Prevention (CDC). We followed the Sporozoite ELISA directions provided by the CDC (Sep, 2009 version) with a few modifications (see supplemental document for the modified ELISA protocol). Samples were considered positive if absorbance values were three or more standard deviations from the negative control samples (99% CI) on each ELISA plate (Beier *et al.*, 1987; De Arruda *et al.*, 2004).

The results of the ELISA were analyzed for both CSP concentration, adjusted for plate-to-plate variation, with an analysis of variance and for a binary outcome using a χ^2 test implemented in SPSS 16.0 (SPSS, 2007). The data were analyzed for differences between species and among collection sites, using G-test implemented in Deducer library under R software (<http://www.r-project.org/>). Species and *P. falciparum* infection state and CSP concentration for each individual is provided in Dataset 1.

Results & discussion

ELISA results identifying *Plasmodium falciparum* infection status in *Anopheles* spp. collected in Guinea-Bissau

<http://dx.doi.org/10.6084/m9.figshare.1200058>

Mosquitoes were collected at eight different sites in Guinea-Bissau between October and November 2009. All mosquitoes were collected indoors except those collected at Ponta Anabaca which were collected outdoors. See associated article for methods.

Four species were collected during sampling; *A. coluzzii*, *A. gambiae*, *A. melas*, *A. pharoensis* and *A. coluzzi* x *A. gambiae* hybrids were observed. All mosquitoes were collected indoors with a single exception; samples in Ponta Anabaca were opportunistically collected outside of a human habitation while apparently host-seeking immediately after sunset at about 18:00 hr, which is earlier than reported observations for members of the *A. gambiae* complex in The Gambia (West Africa) (Lindsay *et al.*, 1989; Snow *et al.*, 1988). All species were collected at multiple sites except *A. pharoensis*, which was only collected at the more inland site of Bambadinca. *A. pharoensis* is not generally considered a significant vector in West Africa but the distribution observed in this study matches the previously observed pattern in Senegal (Carrara *et al.*, 1990). *Anopheles arabiensis* was absent from collections.

No significant differences were observed for CSP concentration or in the analysis of positive samples with χ^2 . This is probably due to the variation in the distribution of vector species and *P. falciparum* in the environment at the time of sampling. Table 1 presents CSP rate data and the total number of each individual species collected at each site.

The percentage of *P. falciparum* positive samples from members of the *A. gambiae* species complex observed in this study (overall 10.2%) were similar to earlier studies in other regions in Guinea-Bissau (12.0% (Snounou *et al.*, 1993) and 9.6–12.4% (Jaenson *et al.*, 1994)). The overall CSP positive rate for *A. gambiae* was 12.6% and 11.1% for *A. melas*. Previously published CSP positive rates for *A. gambiae* s.s. (= *A. gambiae* and *A. coluzzii*) range between 2.24% in Guinea (Carnevale *et al.*, 2010) to 9.6% in Guinea-Bissau (Jaenson *et al.*, 1994). Earlier studies when individual species within the *A. gambiae* complex were not identified, infection rate of *A. gambiae* s.l. ranged from as high of 17.73% in the eastern regions of The Gambia (Thomson *et al.*, 1994) to 12% in Guinea-Bissau (Jaenson *et al.*, 1994; Snounou *et al.*, 1993). The CSP positive rate was significantly higher in *A. gambiae* (12.6%) than *A. coluzzii* (4.3%) (Wilcoxon rank sum test P-value=0.0384). This is consistent with the earlier study in Senegal (Ndiath *et al.*, 2011) but differs from a recent survey conducted in Mali (Fryxell *et al.*, 2012). The study site in Senegal located in the village of Dielmo (13°43'N, 16°24'W) (Ndiath *et al.*, 2012)) was geographically closer (200km) than Mali sites (>800km) to our collection sites in Guinea-Bissau. The Senegal study site at Dielmo and nine of our study sites were proximal (<50km) to the Atlantic Ocean, while Mali is a land-locked country at least 500km away from the Atlantic Ocean. Therefore, the discrepancy among studies may be due to climatic and environmental pressure on the different genetic backgrounds of *A. gambiae* observed in this area of West Africa (Lee *et al.*, 2013). More robust sampling over a larger number of collection sites would help in confirming this trend.

In this study, a few *A. pharoensis* (N=6) were collected, half of which were CSP positive. Other studies in this region of West Africa have found that *A. funestus* and *A. arabiensis* may also be important vector species at different times in nearby Senegal (Fontenille *et al.*, 1997a; Fontenille *et al.*, 1997b). *A. arabiensis* was not collected in our study while a small number (N<10) of *A. funestus* were observed but not collected.

Recent studies on the prevalence of malaria parasites in humans have suggested that infection rates in Guinea-Bissau may be in decline due to widespread use of effective treatment and insecticide treated bed nets (ITNs and long lasting insecticide treated bed nets, LLINs) by the most high-risk groups (Rodrigues *et al.*, 2008; Ursing *et al.*, 2014). The malaria parasite life cycle is complicated and may not directly relate to the prevalence of human cases but it is possible that the lack of data during periods of political unrest has concealed a more stochastic pattern than was previously observed in Guinea-Bissau (Ursing *et al.*, 2014).

Outdoor mosquito collection was not the focus of this survey and was only made at Ponta Anabaca Hotel grounds when we fortuitously noted mosquitoes biting. Consequently no general comments about the degree of exophily of *A. gambiae* in Guinea-Bissau can be made. However, evidence of exophily by the major malaria vector *A. gambiae* in this study and by others in West Africa (Reddy *et al.*, 2011; Tchouassi *et al.*, 2012) raises the concern of the long term

effectiveness of Indoor Residual Spraying (IRS) and Long lasting Insecticide-treated Nets (LLINs) in reducing outdoor transmission of malaria especially before bedtime and by people sleeping outdoors. The relatively high infection rate of 11.1% of *A. melas* in Guinea-Bissau together with its tendencies to be both endophilic and exophilic and have a high human blood index (Sharp *et al.*, 2007; Tuno *et al.*, 2010) make the species a significant vector, which may also be hard to control by reliance on ITNs and LLINs.

The high CSP rate of 33.3 % in the 4 indoor collected *A. pharoensis* might implicate a significant role in malaria transmission in drier inland Guinea Bissau, however this should be viewed with caution due the small sample size. Very low infection rates and absence of malarial parasites, traditionally found in West and Central African populations of *A. pharoensis* has always led to the conclusion that this mosquito plays little role in malaria transmission despite its anthropophilic habits and that it can be easily experimentally infected (DeMeillon, 1947; Ndiath *et al.*, 2012; Tchouassi *et al.*, 2012). In drier Sahel regions of Africa where the major vectors of malaria are absent or very rare and irrigated rice and other crop lands are increasing, *A. pharoensis* is considered more important at maintaining low levels of malaria (Kerah-Hinzoumbe *et al.*, 2009; Kibret *et al.*, 2010).

Data availability

figshare: ELISA results identifying *Plasmodium falciparum* infection status in *Anopheles* spp. collected in Guinea-Bissau. doi: 10.6084/m9.figshare.1200058 (Sanford *et al.*, 2014).

Author contributions

YL, GCL and AJC conceived the study, designed experiments and conducted field collections. JD conducted field collection. AR provided logistical support and coordination for field collection in Guinea-Bissau. CCN, CDM, AMW and SH conducted DNA extraction, ELISA and PCR. MRS performed data analysis and wrote manuscript. All authors were involved in the revision of the draft manuscript and have agreed to the final content.

Competing interests

No competing interests were disclosed.

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Supplementary materials

Sporozoite ELISA Directions. [Click here to access the supplement.](#)

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Referee Report 23 October 2014

doi:[10.5256/f1000research.5855.r6412](https://doi.org/10.5256/f1000research.5855.r6412)



Guido Favia

School of Bioscience and Veterinary Medicine, University of Camerino, Camerino, Italy

This “observation article” is very well written in a format that is accessible to both general and specialist audience. It describes some novel observations about malaria infection rates in different vector species in Guinea-Bissau. In particular it reports the some how unexpected high infection rates in *Anopheles melas* and *A. pharoensis*, thus suggesting *Plasmodium falciparum*-malaria transmission potential outside of protection (i.e. bed nets).

Details about the circumstances of the finding and evidence of the observation are properly provided. The manuscript appropriately cites relevant bibliography in the field. Figures and tables are informative and helpful. Methods section is well organized and nicely descriptive.

As observational article it looks perfectly adequate to the journal purpose.

I have only a very minor concern: in the Introduction (line 8) *Plasmodium malaria* should be re-written as *Plasmodium malariae*.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Report 22 October 2014

doi:[10.5256/f1000research.5855.r6413](https://doi.org/10.5256/f1000research.5855.r6413)



J Derek Charlwood

London School of Hygiene & Tropical Medicine, London, UK

This paper provides information on sporozoite rates from a relatively unstudied area the mainland and islands of Guinea Bissau. It gives the impression that it is a spin off from another study that perhaps aimed at characterizing the genetics of populations of *Anopheles gambiae* from the mainland and the islands, perhaps for future genetic control efforts. Given the widespread use of mosquito bednets rates are exceptionally high and not, apparently different from rates recorded earlier. The authors do make the comment that this might be the result of civil strife in Guinea-Bissau but whatever the cause this is

disquieting and implies that the gains in reduction of malaria are going to be at best temporary.

The data are presented without confidence intervals but these should be added. Given the relatively small numbers involved either adjusted Wald confidence intervals (that can easily be calculated using the site www.measuringu.com/wald.htm or a routine in R) can, I think, be used. (But since I am signing this review everyone should know that my statistical abilities are limited!

The kind of collection undertaken needs to be explained in more detail. Were the mosquitoes collected resting or were they landing collections? I do not really want to be the person raising this issue but something on ethics should be included somewhere. (My own thoughts on ethics in general is that if the rule of 'first do no harm and second maybe do some good' is followed then a study – that may include even *ad hoc* landing collections – is not unethical.) This is especially important if the collections were landing collections.

To avoid possible misunderstanding, the sentence 'Four species were collected during sampling; *A. coluzzii*, *A. gambiae*, *A. melas*, *A. pharoensis* and *A. coluzzi* x *A. gambiae* hybrids were observed' should be rewritten (since it could be misinterpreted) perhaps as two sentences: 'Four species, *A. coluzzii*, *A. gambiae*, *A. melas*, *A. pharoensis* were collected during sampling. A number of *A. coluzzi* x *A. gambiae* hybrids were also collected'

There are a number of small errors in the paper that need to be rectified. For example in the last paragraph they state '33% of the 4 *Anopheles pharoensis* collected indoors when they either mean 33% of the six *Anopheles pharoensis* collected or 50% of the four collected indoors. With regard to this species it may be worth pointing out that in Mozambique none of the 4390 tested were positive for sporozoites (Charlwood *et al.*, 2013) but at the same time in Ghana, (Dzodzomenyo *et al.*, 1999) found that two of three specimens of *An. pharoensis* examined were infected (with Bancroftian filariasis) and one of these was infectious. Given the possibility of false positives among primarily zoophilic anophelines (that may also include *An. melas*) and given that the authors have access to a sophisticated laboratory it is a shame that they did not run a PCR on the sporozoite positive specimens to ensure that they were indeed human malarias.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Discuss this Article

Version 1

Author Response 16 Oct 2014

Yoosook Lee,

The infection rate is conservative (using 99% CI for calling uninfected samples). The infection rate estimates are higher with 95% CI.

Competing Interests: I am an author of this paper.

