



RESEARCH ARTICLE

First detection of N1575Y mutation in pyrethroid resistant *Anopheles gambiae* in Southern Côte d'Ivoire [version 1; referees: 2 approved]

Ako Victorien Constant Edi ^{1*}, Bedjou Prisca N'Dri ^{1-3*}, Mouhamadou Chouaibou¹, Fondjo Behi Kouadio¹, Patricia Pignatelli⁴, Giovanna Raso^{2,3}, David Weetman⁴, Bassirou Bonfoh^{1,2}

¹Research and Development Department, Centre Suisse de Recherches Scientifiques en Côte d'Ivoire, Abidjan, 01 BP 1303, Cote d'Ivoire

²Swiss Tropical and Public Health Institute, Basel, CH-4051, Switzerland

³University of Basel, Basel, CH-4002, Switzerland

⁴Vector Biology Department, Liverpool School of Tropical Medicine, Liverpool, L3 5QA, UK

* Equal contributors

v1 First published: 24 Aug 2017, 2:71 (doi: [10.12688/wellcomeopenres.12246.1](https://doi.org/10.12688/wellcomeopenres.12246.1))
Latest published: 24 Aug 2017, 2:71 (doi: [10.12688/wellcomeopenres.12246.1](https://doi.org/10.12688/wellcomeopenres.12246.1))

Abstract

Background. The intensification of insecticide use for both public health and agriculture in Africa has contributed to growing insecticide resistance. Today, resistance to World Health Organization (WHO)-approved insecticide classes is widespread. In an agricultural area of Southern Côte d'Ivoire, the main malaria vector *Anopheles coluzzii* shows multiple resistance across insecticides mediated by both target site mutation and metabolic mechanisms. To plan new vector control strategies and avert future resistance liabilities caused by cross-resistance mechanisms extant within populations, it is crucial to monitor the development and spread of both resistance and mechanisms.

Methods. Larvae of *Anopheles gambiae* were collected from natural breeding sites in Tiassalé and Elibou, between April and November 2016 and raised to adults. Adult female non-blood fed mosquitoes, three to five days old, were exposed to deltamethrin in WHO bioassays. Extracted DNA samples from exposed mosquitoes were used for species characterisation and genotyping.

Results. Most adult *An. gambiae* tested were resistant to deltamethrin, with mortality rates of only 25% in Tiassalé and 4.4% in Elibou. Molecular analysis of DNA from samples tested showed the presence of both *An. coluzzii* and *An. gambiae* s.s in Elibou and only *An. coluzzii* for Tiassalé. As previously, the L1014F *kdr* mutation was present at high frequency (79%) in Tiassalé and the L1014S mutation was absent. The N1575Y mutation, which amplifies resistance conferred by L1014F was detected in a single unique individual from a Tiassalé *An. coluzzii* female whereas in Elibou 1575Y was present in 10 *An. gambiae* s.s, but not in *An. coluzzii*.

Conclusion. This is the first report of the N1575Y mutation in Côte d'Ivoire, and as in other populations, it is found in both dominant West African malaria vector species. Continued monitoring of N1575Y is underway, as are studies to elucidate its contribution to the resistance of local vector populations.

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Referee Status:

	Invited Referees	
	1	2
version 1 published 24 Aug 2017	 report	 report
1 Kobié H. Toe , National Center for Research and Training on Malaria, Burkina Faso		
2 Jacob Williams , Tropical Public Health Practice LLC, USA		

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Corresponding author: Ako Victorien Constant Edi (edi.constant@hotmail.com)

Author roles: **Edi AVC:** Formal Analysis, Investigation, Methodology, Writing – Original Draft Preparation; **N'Dri BP:** Investigation, Methodology; **Chouaibou M:** Formal Analysis, Supervision; **Kouadio FB:** Investigation; **Pignatelli P:** Formal Analysis; **Raso G:** Supervision; **Weetman D:** Formal Analysis, Supervision; **Bonfoh B:** Supervision

Competing interests: No competing interests were disclosed.

How to cite this article: Edi AVC, N'Dri BP, Chouaibou M *et al.* **First detection of N1575Y mutation in pyrethroid resistant *Anopheles gambiae* in Southern Côte d'Ivoire [version 1; referees: 2 approved]** Wellcome Open Research 2017, 2:71 (doi: [10.12688/wellcomeopenres.12246.1](https://doi.org/10.12688/wellcomeopenres.12246.1))

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Grant information: This work was supported by the Wellcome Trust [110430] to AVCE and [103995] to CM; Swiss Government Excellence Scholarships for Foreign Scholars and Artists(EKAS) to BPN'D; DELTAS Africa (Wellcome Trust, AAS, NEPAD, UKAID) [107753] to BB. *The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

First published: 24 Aug 2017, 2:71 (doi: [10.12688/wellcomeopenres.12246.1](https://doi.org/10.12688/wellcomeopenres.12246.1))

Introduction

Malaria remains an important tropical disease that requires a global effort for eradication (WHO malaria report, 2016). Scale-up of existing control measures and development of new tools for the markets (2016–2030) are key components of the global control and elimination strategies (WHO Global Malaria programme, 2016). The development of new tools is of utmost importance, since resistance in the major *Anopheles* malaria vectors of sub-Saharan Africa to WHO-approved insecticides is now widespread and increasing (Ranson & Lissenden, 2016). Increasingly, populations of mosquitoes that are resistant to more than one class of insecticides are being reported (Djouaka *et al.*, 2016; Edi *et al.*, 2014a; Nwane *et al.*, 2013).

Among insecticides, pyrethroids remain the only class approved for long-lasting insecticide treated nets, and major resistance mechanisms are metabolic detoxification, especially by P450 enzymes (David *et al.*, 2013), and mutations in the *para* voltage gated sodium channel (VGSC), the target site of pyrethroids and DDT. VGSCs are transmembrane proteins that transfer sodium ions inside the cell in order to achieve the depolarizing phase of action potentials, an essential phase of nervous impulses (Catterall *et al.*, 2005). Mutations in the VGSC cause a phenotype known as knock down resistance (*kdr*). The most common *kdr* mutations in *Anopheles* are substitutions at the 1014 leucine codon to either phenylalanine (Martinez Torres *et al.*, 1998) or serine (Ranson *et al.*, 2000). Both are now widely distributed across Africa, and sometimes co-occur (Fryxell *et al.*, 2012; Nwane *et al.*, 2011; Pinto *et al.*, 2006; Reimer *et al.*, 2008; Tripet *et al.*, 2007). Moreover, their frequency could differ

from a mosquito population tested with insecticide to another (Antonio-Nkondjio *et al.*, 2015). An additional asparagine-to-tyrosine mutation at codon 1575 within the linker between domains III-IV of the VGSC has also been documented in *An. gambiae* and/or *An. coluzzii* from Burkina Faso, Ghana, Benin and Cameroon (Jones *et al.*, 2012; Fossog Tene *et al.*, 2013). The N1575Y mutation provides a synergistic effect on pyrethroid and DDT resistance by elevating the insensitivity of the sodium channel gates produced by the 1014F and 1014S mutations (Wang, 2013), although to date it has only been found on the 1014F haplotype (Jones *et al.*, 2012).

Vector control requires improved management of insecticides and identification of mechanisms of resistance, which can be readily screened to forewarn increases in resistance or represent possible cross-resistance liabilities to new insecticides when they become available. Previous studies in Southern Cote d'Ivoire have documented resistance to multiple insecticides, mediated by multiple mechanisms (Edi *et al.*, 2012; Edi *et al.*, 2014a); however, all samples screened were wild type at the 1575 codon (Edi *et al.*, 2012). Here we report the first detection of N1575Y mutation in deltamethrin resistant populations of *An. gambiae* and *An. coluzzii* from Southern Côte d'Ivoire.

Methods

Study site

All collections were carried out in Tiassalé (latitude 5.89839, longitude -4.82293) and Elibou (latitude 5.69000, longitude -4.50000), Southern Côte d'Ivoire (Figure 1). The study sites are located in the evergreen forest zone. The primary agricultural activity is cash

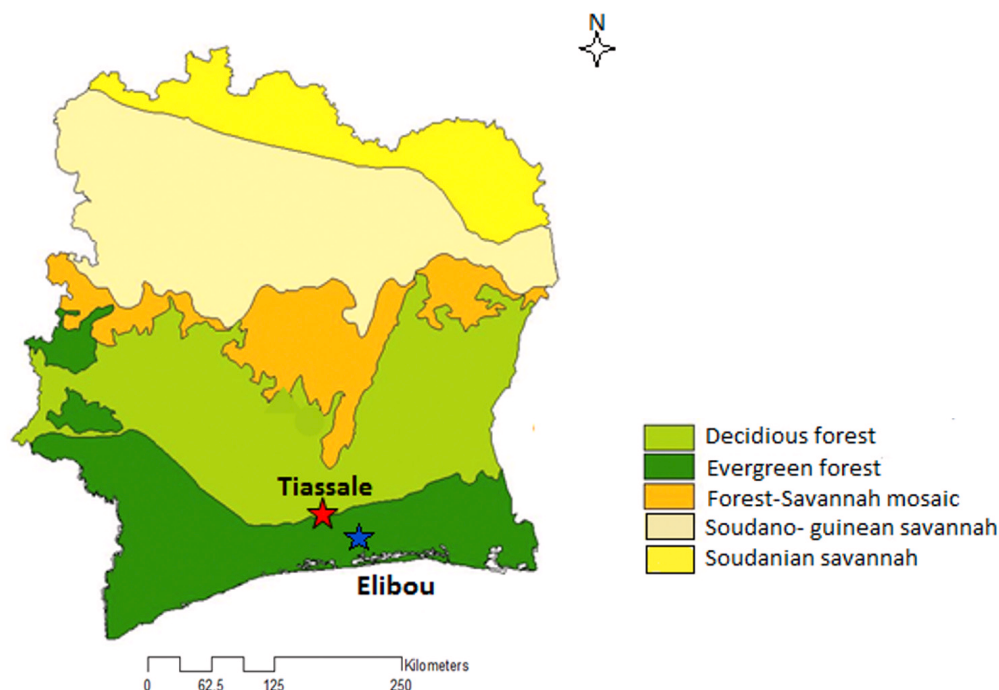


Figure 1. Vegetation map of Côte d'Ivoire, showing the study areas Tiassalé and Elibou.

crop farming of vegetables in Elibou and irrigated rice fields in Tiassalé. High malaria transmission occurs during the rainy seasons, between May and November.

Larval collections

Larval collections of *An. gambiae s.l.* were obtained by dipping from breeding sites in each study location during the peak malaria transmission periods between April and November 2016. All larvae were provided a diet of Tetra Mikromin fish food (Tetra; Melle, Germany) until adult emergence. All mosquito rearing was performed under controlled ambient environmental conditions (27°C±2°C, 80% ±4% relative humidity). Adult mosquitoes were given access to 10% sucrose solution.

WHO diagnostic bioassays

Adult female non-blood fed mosquitoes, two to five days old, were exposed to 0.05% deltamethrin for one hour, using WHO tubes and criteria (WHO, 2013). Mosquitoes surviving insecticide exposure were given access to 10% sucrose solution in WHO holding tubes. Mortality was assessed after 24 hours. For each replicate, fifty mosquitoes were exposed to non-treated filter papers as a control in two separate tubes. Exposed mosquitoes were then stored with silica gel until DNA extraction. Levels of mortality between study locations were compared using Chi-square test run on the open source software package R, version 3.4.0 (R Core Team, 2008). For statistical testing, the level of significance was set at $\alpha = 0.05$.

DNA extraction

Genomic DNA was extracted according to the LIVAK method (Livak, 1984) from individual *An. gambiae s.l.* mosquitoes from Tiassalé (n=92) and Elibou. In Tiassalé, extraction was made on individuals surviving exposure to deltamethrin. In Elibou, additional 110 untested females (control samples) obtained from larval collection were considered. Mosquitoes were individually ground in 100µl of preheated grind buffer made with 1.6 ml 5M NaCl, 5.48 g sucrose, 1.57g Tris, 10.16 ml 0.5M EDTA and 2.5 ml 20% SDS (Thermo Fisher Scientific). Mixed buffer-mosquito solution was incubated at 65°C for 30 minutes and then 14 µl 8M K-acetate was incorporated and gently mixed. The resulting thicker mixture was incubated for 30 min on ice and then centrifuged at 13,000 rpm for 20 min (4°C). 200 µl 100% EtOH was then added to the supernatant (later transferred in new Eppendorf tube 1.5 ml) and the mixture was centrifuge again at 13,000 rpm for 15 min (4°C). The supernatant was discarded and the pellet was rinsed with 100 µl ice cold 70% EtOH. Dried pellets were re-suspended in 100 µl TAE buffer. The DNA was used for species ID and target site genotyping.

Species and molecular form identification

The SINE-PCR method was used to identify *An. gambiae s.l.* to species (Santolamazza *et al.*, 2008). A volume of 24.75 µl of master mix was considered per reaction. Overall the master mix (Applied Biosystems) contained 18.83 µl DNase free water, 2.5 µl buffer 10X, 0.75 µl MgCl₂ (25mM), 1 µl dNTP (10mM) and respective 1 µl of Sine 6.1a (10 µM) 5'-CGCTTCAAGAATTCGAG

ATAC-3' and Sine 6.1b (10 µM) 5'-TCGCCTTAGA CCTT-GCGTTA-3' and 0.17 µl Kappa Taq. Each PCR product containing 23 µl of mix and 2 µl of genomic DNA was amplified for 3 min at 94°C, followed by 35 cycles of 94°C, 62°C, and 72°C for 30 s respectively. The last cycle of was 5 min at 72°C. Products were run on 1.5% agarose gels. In Elibou, untested female samples were used for species and molecular form identification. In Tiassalé, only individual surviving exposure to deltamethrin were considered.

Genotyping assays

TaqMan assays with two labelled Fluorochromes probes FAM and HEX were used to screen for the L1014F and L1014S *kdr* mutations (Bass *et al.*, 2007) and the N1575Y mutation (Jones *et al.*, 2012). A total volume of 9 µl per reaction was used for the mix, containing DNase free water (3.875 µl), Bioline sensimix (5 µl) and specific primer/probe (0.125 µl) (*kdr*-Forward 5'-CATTTCCTTGCCACTGTAGTGAT-3', *kdr*-Reverse 5'-CGATCTTGGTCCATG TTAATTTGCA-3') for *kdr* 1014 and (3'NFQ-ATTTTTTTCATTGCATTATAGTAC-5' for N1575 and 3'NFQ-TTTTTCATTGCATAATAGTAC-5' for 1575Y, respectively. The mix was centrifuged at 2000 rpm for approximately 10 seconds. 9 µl of the mix with 1 µl of each gDNA were added to each TaqMan PCR (Applied Biosystems), and centrifuged at 2000 rpm for 15 seconds. Reactions were performed on the Agilent MX3005P qPCR system (Agilent Technologies). The genotype was determined from the fluorescence profiles and bi-directional scatter plots generated in the MX3005P software. The PCR condition was 95°C for 10 minutes (1 cycle) following by 40 cycles of 95°C at 10 seconds and 60°C at 45 seconds, respectively for *Kdr* genotyping. For N1575 Y mutation, PCR conditions of 10 min at 95°C followed by 40 cycles of 15 s at 92°C and 1 min at 60°C were considered (Jones *et al.*, 2012).

Results

A total of 291 mosquitoes were tested with deltamethrin in Tiassalé (n=200) and Elibou (n=91) during the rainy season, using standard WHO susceptibility assays. Mortality rates were 25% in Tiassalé and 4.4% in Elibou. The prevalence of *An. gambiae* resistance was different between study locations (Chisq=13, p=0.0003).

All mosquitoes were collected at larval stage. In Elibou, the first emerged (n= 91) were used for bioassay, as previously described, and a sample of 110 from the remaining untested ones were used for molecular form and species identification. All these mosquitoes were from the same batch. Thus, from a subset of DNA samples analysed in Elibou (n= 110) and Tiassalé (n=92), no *An. arabiensis* were detected. All individuals were found to be *An. coluzzii* (100%) in Tiassalé. In Elibou, both *An. coluzzii* (53.6%, n= 59) and *An. gambiae* ss (35.5%, n= 39) and additional hybrid individuals (10.9%, n= 12) were present.

Of the two potential substitutions screened at codon 1014, 1014S was absent, whereas the 1014F *kdr* allele was observed in Tiassalé at high frequency (79.3%). In Elibou, the frequencies

of L1014 mutation were not investigated. From the 92 surviving *An. coluzzii* mosquitoes exposed to deltamethrin, a single individual was found to carry the N1575Y mutation (in heterozygous form) in Tiassalé (Table 1). In Elibou, both heterozygous (20.4%) and a single homozygous resistant individual (2.2%) were detected in *An. gambiae* s.s (Figure 2). The overall 1575Y allele frequency in Elibou was 25% (Table 1).

First detection of N1575Y mutation in pyrethroid resistant *Anopheles gambiae* in Southern Côte d'Ivoire

3 Data Files

<https://dx.doi.org/10.6084/m9.figshare.5319250.v1>

Dataset 1: Raw data for mortality rates from WHO bioassays in Tiassalé.

Dataset 2: Raw data for mortality rates from WHO bioassays in Elibou.

Dataset 3: Raw data for N1575Y genotypes.

Discussion

The aim of this study was to investigate the level of resistance to the pyrethroid deltamethrin in Southern Cote d'Ivoire. It was found that the prevalence of *An. gambiae* resistance was different between study locations. In Tiassalé, the mortality rate to deltamethrin was 25%, while this was six-folds lower in Elibou. Resistance to deltamethrin in the Southern region could be explained by the insecticide pressure due to development of agriculture and use of insecticide in the region Reid & McKenzie, 2016. Indeed, deltamethrin insecticides are largely used by farmers for both pest control and increase of yield. A recent finding also showed that among the main pyrethroid insecticides used in Tiassalé, deltamethrin was the most common (46.9%) followed by lambda-cyhalothrin (35.2%) and cypermethrin (10.5%) (Chouaïbou et al., 2016). To our knowledge, there is no cultivation in the Elibou village, but in the surroundings, there is intense rubber (hevea) farming, where insecticides are also applied. Thus, potential contamination of existing breeding sites could occur given the

Table 1. Prevalence of N1575Y allele in Tiassalé and Elibou, Côte d'Ivoire, 2016.

Strains	Species	Phenotype	No. tested	No. per genotype			Frequency (%)
				NN	NY	YY	
Tiassalé	<i>An. coluzzii</i>	Alive	92	91	1	0	0.5
Elibou	<i>An. coluzzii</i>	Alive	22	22	0	0	0
	<i>An. gambiae</i> s.s	Alive	22	12	9	1	25

Y and N represent mutant resistant (tyrosine) and wild types alleles (asparagine), respectively.

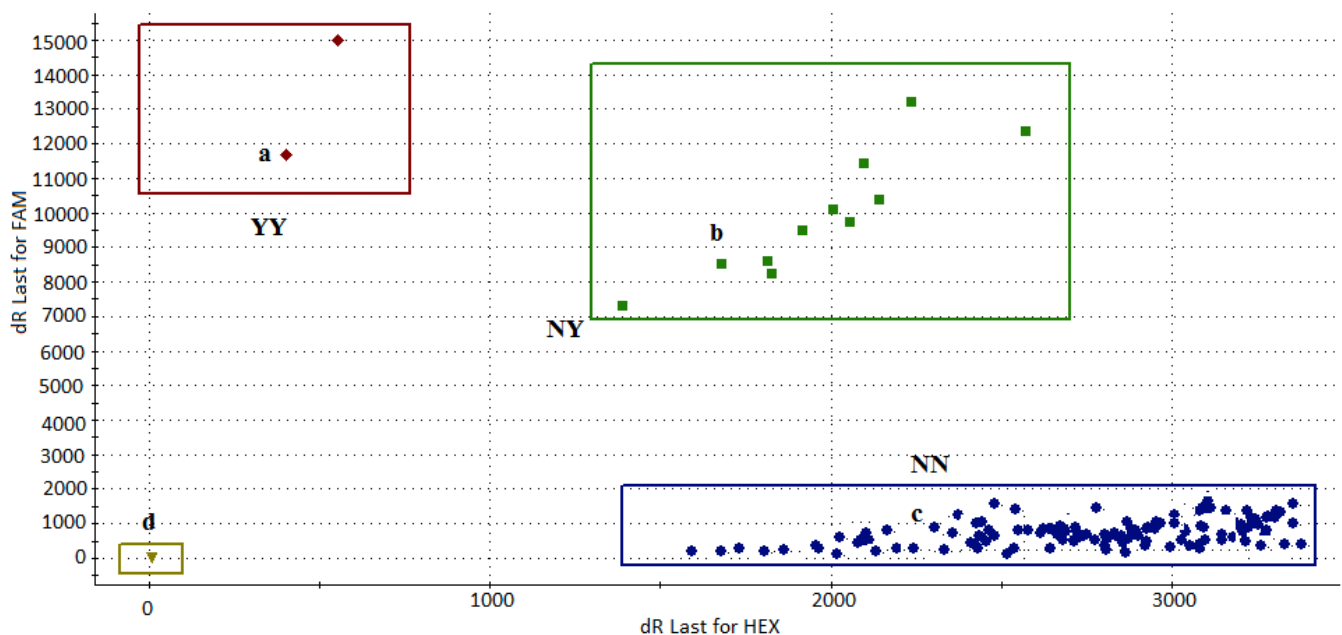


Figure 2. Distribution of N1575Y alleles in Southern Côte d'Ivoire. The letters a, b, c and d represent the positive controls for the homozygous mutant allele (Y), heterozygous mutant (NY) and the homozygous susceptible allele (N) and blank, respectively. Nine individual mosquitoes displayed the heterozygous mutant/susceptible genotype (NY), only one individual carried the homozygous resistant genotype (YY). All the other samples carried the susceptible genotype (NN).

ecology of the region (see description of study sites), and further investigations are required to better understand the emergence of resistance to deltamethrin in this area.

In Tiassalé, the presence of the 1575Y resistance allele was detected for the first time. This is surprising because previous consecutive data from 2011 (0%, n= 184) and 2012 (0%, n=92) did not report the N1575Y mutation (Edi *et al.*, 2012; Edi *et al.*, 2014b). In Elibou, our finding revealed the presence of both heterozygous and homozygous resistant tyrosine alleles in *An. gambiae s.s.* Elibou is located within a 100 km radius from Tiassalé and characterized by the presence of both *An. coluzzii* and *An. gambiae s.s.* The detection of N1575Y mutation in *An. coluzzii* in Tiassalé and *An. gambiae s.s.* in Elibou revealed the potential for both species to carry the asparagine-to-tyrosine resistance mechanisms, as previously documented in Burkina Faso (Jones *et al.*, 2012).

The detection of N1575Y mutation now in southern Côte d'Ivoire requires more investigation to better characterize its expected synergistic relationship with 1014F *kdr*. This resistance mechanism could spread very rapidly, and threaten the fragile gains that have been made in reducing the malaria burden in this region through vector control interventions.

Conclusion

The present study showed the presence of the N1575Y mutation in Côte d'Ivoire. The discovery of an additional mechanism that could further reduce insecticide efficacy in the already pyrethroid resistant mosquitoes in this region is concerning. Continued monitoring of N1575Y is underway to elucidate its contribution to the resistance of local vector populations.

Data availability

Figshare

First detection of N1575Y mutation in pyrethroid resistant *Anopheles gambiae* in Southern Côte d'Ivoire

Dataset 1: Raw data for mortality rates from WHO bioassays in Tiassalé.

Dataset 2: Raw data for mortality rates from WHO bioassays in Elibou.

Dataset 3: Raw data for N1575Y genotypes.

<https://doi.org/10.6084/m9.figshare.5319250.v1> (Edi *et al.*, 2017)

Competing interests

No competing interests were disclosed.

Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

We thank the Centre Suisse de Recherches Scientifiques en Côte d'Ivoire; Mr Jackson Kouamé, Mr Kassoum Bamogo, and Mr Yves Kadio for their technical assistance at Tiassalé. Finally, we would like to acknowledge the proof reading from Jasmina Saric at CSRS and thank the populations and the authorities of Tiassalé and Elibou for facilitating this study.

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Open Peer Review

Current Referee Status:  

Version 1

Referee Report 29 September 2017

doi:[10.21956/wellcomeopenres.13258.r25868](https://doi.org/10.21956/wellcomeopenres.13258.r25868)



Jacob Williams

Tropical Public Health Practice LLC, Washington, DC, USA

The primary purpose of reporting on the presence of N1575Y mutation in pyrethroid resistant *Anopheles gambiae* – is well demonstrated. This study provides crucial additional information to the body of knowledge on the evolving scenario of insecticide resistance in the West Africa sub-region. There are few issues requiring the attention of the authors:

1. Consider a slight modification of the title of the paper to indicate evaluation of *Anopheles gambiae sensu lato*, since both *An. coluzzii* and *An. gambiae s.s.*, were evaluated:

"First detection of N1575Y mutation in pyrethroid resistant *Anopheles gambiae (s.l.)*, in Southern Côte d'Ivoire"

2. While the mosquitoes tested were 2-5 days old and varies from the WHO recommendation of 3-5 days old test samples, in this case, the discrepancy does not present a significant problem since the objective of the study to detect the presence of the resistance gene mutation. However, comparison of phenotypic expression from this study (i.e. mortality rates from WHO bio-assay), with results obtained from other datasets elsewhere, may be constrained. This is because mortality rates have been shown to vary with the age of mosquito and the addition of 2-day old samples, will be a confounding factor.
3. It is unclear if the mosquito samples evaluated in Elibou ($n=44$, Table 1) were those surviving the WHO deltamethrin bio-assay, or a subsample from the additional 110 samples the authors indicated were "untested" but also evaluated. I am inclined to believe that the 44 mosquitoes evaluated for N1575Y, were survivals from the 91 samples tested via WHO bio-assay. Please clarify.

Authors indicate ongoing monitoring. They are encouraged to ensure robust concurrent evaluations of the progress/frequency of mutation and the resistance rate (phenotypic) among the various subspecies of *An. gambiae s.l.* in the area, to capture the very likely differences among the species.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

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.

Referee Report 18 September 2017

doi:[10.21956/wellcomeopenres.13258.r25343](https://doi.org/10.21956/wellcomeopenres.13258.r25343)



Kobié H. Toe

National Center for Research and Training on Malaria, Ouagadougou, Burkina Faso

The paper looks interesting, clear and well written. The report of the N1575Y mutation, which now contribute to pyrethroid resistance although only reported in some countries. This paper is the proof that it could be spread also in other countries. However I have noticed that some useful details which could lead to better understanding and avoid confusion of the work were missing. Please see comments to author.

Abstract: In the method paragraph it said that three to five days old mosquitoes were used for the bioassays. Which contradict what is mentioned in **the Methods-WHO diagnostic bioassays** part where it is mentioned that two to five day old mosquitoes were used. It is confusing. Give the right age of mosquitoes used for the bioassays. If it was two to five days mosquitoes used, explained why, as the WHO procedure you have cited recommends to use three to five days old mosquitoes. I do not know which is right.

Methods:

In Elibou untested mosquito were used for molecular analysis, and in Tiassalé surviving mosquito to deltamethrin exposure were used.

In Elibou the frequency of 1014F has not been investigated, there is a specific reason for that? It is known that the 1014F is linked to 1575Y. The data of the 1014F should be interesting. Providing this data should be important.

Table1: in this table presenting the results the frequencies of the 1575Y mutation in Tiassalé (n= 92) and Elibou (n= 44), I am a bit confused. 110 untested mosquitoes from Elibou were used for molecular. Why in the table the total number of mosquitoes from Elibou was only 44? These mosquitoes are from those surviving deltamethrin exposure or are subset of the 110 untested mosquitoes used for molecular form and species identification.

Of the most recent paper (<http://www.ncbi.nlm.nih.gov/pubmed/25766412>), reporting the presence of the 1575Y mutation in Tengrela area in the Burkina Faso, of the closest area from Côte d'Ivoire where this mutation is reported with a frequency around 0.25- is not cited anywhere in the paper. It could be interesting to report to this paper, as it is one of the most recent talking also about this mutation.

I have hope that the authors will continue the monitoring of the mutation as mentioned in the conclusion.

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Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Referee Expertise: Medical entomologist (field and lab works)

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.
