RESEARCH NOTE

Rapid spread of double East- and West-African kdr mutations in wild Anopheles coluzzi from Côte d’Ivoire [version 1; peer review: 2 approved, 1 approved with reservations]

Chouaïbou Seïdou Mouhamadou¹,², Prisca Bédjou N’Dri¹,³, Behi Kouadio Fodjo¹, Christabelle Gba Sadia¹, France-Paraudie Kouadio Affoue¹, Benjamin Guibehi Koudou¹

¹Centre Suisse de Recherches Scientifiques, Abidjan, Cote d’Ivoire
²Department of Entomology and Plant Pathology, North Carolina State University, Raleigh, NC, 27695-7508, USA
³Department of Epidemiology and Public Health, Swiss Tropical and Public Health Institute, University of Basel Switzerland, Basel, Switzerland

Abstract
Malaria morbidity and mortality rates in Sub-Saharan Africa are increasing. The scale-up of long-lasting insecticidal nets and indoor residual spraying have been the major contributors to the decrease of malaria burden. These tools are now threatened by insecticide resistance in malaria vectors, which is spreading dramatically. After two different real-time polymerase chain reaction molecular characterizations carried out on 70 mosquitoes sampled in the locality of Elibou in southern Côte d’Ivoire, results revealed that 9 mosquitoes from Anopheles coluzzi harbored the double East- and West-African knockdown resistance mutations. In the previous year, only 1 mosquito out of 150 sampled from 10 regions of the country had the same genotype. These results show the rapid spread of insecticide resistance in malaria vectors and highlight the urgent need to diversify the methods of vector control in order to avoid the failure of insecticide-based vector control tools which may favor malaria fatalities.

Keywords
Vector control, Insecticides, Long lasting insecticidal bednet, Indoor residual spraying, insecticide resistance, knockdown resistance
Background

Malaria morbidity and mortality rates in Sub-Saharan Africa are increasing, with the number of World Health Organization (WHO)-estimated cases reaching 219 million, with 435,000 associated deaths, in 2017 compared to the 216 million scored in 2016 which had already increased for about 5 million cases over 2015. Malaria prevention relies on vector control using insecticides, either by indoor residual spraying (IRS) or in long-lasting insecticide-impregnated mosquito bed nets (LLINs). The efficacy of these measures depends primarily on the susceptibility of the malaria vectors to insecticides. An estimated 663 million cases of malaria have been averted in sub-Saharan Africa between 2000 and 2015 as a result of the scale-up of malaria control interventions, of which 68%, 22% and 10% were attributed to LLINs, artemisinin-based combination therapy (ACT) and IRS, respectively. Unfortunately, despite these efforts, the number of resistant mosquito populations is increasing dramatically, and the efficacy of pyrethroids (the most commonly used insecticide class) is decreasing, which translates to an increase in malaria cases.

The two major causes of resistance to pyrethroids include alterations in the target site (knockdown resistance (kdr)) and increases in the rate of insecticide metabolism by enzymes in various P450 families. This kdr occurs due to mutations in the para-gated sodium channel gene. In Anopheles gambiae, two kdr mutations (1014F and 1014S) have been identified at the same codon.

Studies aimed at estimating the frequency of these mutations across Africa have shown that the 1014F kdr mutation has spread from West Africa to East Africa and the 1014S kdr mutation has spread from East Africa to Central and West Africa. In Côte d’Ivoire, resistance to insecticides used for vector control is prevalent, involving multiple mechanisms. So far, only two cases of East African kdr (1014S) have been reported, both a few years ago, in Côte d’Ivoire; the first by Chouaibou et al., 2004 and the second by Fodjo et al., 2005, each on individual An. gambiae mosquitoes.

Further follow-up studies carried out recently helped us to describe the recent and rapid spread of 1014F mutation in Côte d’Ivoire. Interestingly, our key findings demonstrated that development of the 1014S mutation occurs exclusively in mosquitoes that already have the 1014F mutation. An. gambiae mosquitoes bearing both kdr mutations are described in detail below.

Methods

Mosquitoes

The mosquitoes used in this study were collected as part of the large bionomic study of malaria transmission in the locality of Elbou (5°40'57"N; 4°30'30"W) in South Côte d’Ivoire. Sampling was done in the larval stage in several breeding sites. Larvae were evenly pooled together and reared to the adult stage at the Centre Suisse de Recherches Scientifiques (CSRS) insectarium under standard conditions (temperature of 25–27°C and 70–90% relative humidity).

Genotyping of mosquitoes

Genomic DNA was extracted from 70 adult mosquitoes using the MegaZorb® DNA Mini-Prep Kit according to the manufacturer instructions (Promega Corporation, USA). The identification of An. gambiae complex members was made by short interspersed element (SINE)-PCR according to the methods described by Santolamazza et al. The East- and West-African knockdown resistance genes were characterized using a triplex assay, optimized by Mavridis et al. for simultaneously detecting the wildtype L1014 and the kdr mutations 1014F and 1014S in the same reaction. The reaction was performed on a Bio-Rad CFX96 Real-Time System using DNA extractions of individual mosquitoes in 10-µl reaction volumes. Each probe was labelled with a different fluorescent dye: HEX for wildtype L1014 (CTTACGACTAAATTTC), FAM for 1014F (ACGACAAATTTTC), and Atto for 1014S (ACGACTGAATTTC). The cycling conditions used were 50°C for 15 min, 95°C for 3 min, and 40 cycles of 95°C for 3 s and 60°C for 30 s, allowing a PCR run of approximately 70 min. Genotypes appeared during amplification as three-color curves.

At the end of this initial molecular analysis, nine mosquitoes presented the 1014S mutation. Surprisingly, these same mosquitoes also presented the 1014F mutation. To confirm genotyping results, the DNA extractions of the same mosquitoes were further used in TaqMan assays to characterize 1014F and 1014S mutations. In each of the East-kdr and West-kdr assay, two probes labelled with fluorochromes FAM (ID for Lifetech: AHGI2PM) and HEX (ID for Lifetech: AHAA5AD) were used to detect the mutant alleles and the wild type susceptible allele, respectively. The reaction was performed on the Bio-Rad CFX96 Real-Time qPCR thermal cycler in 10-µl reactions volumes, including master mix, primer/probe, and water. The thermal cycle parameters were 10 min at 95°C, then, 40 cycles of 10 s at 95°C and 45 s at 60°C. Genotypes were determined after real-time amplification from dual-color scatter plots using Bio-Rad CFX Manager 3.1 software.

Results

The results obtained with the TaqMan assays and the triplex PCR assays were in agreement. Nine out of 70 An. gambiae mosquitoes (12.85%) were found to harbor concurrently both the 1014S kdr mutation and the 1014F kdr mutation (Table 1 and Figure 2). All mosquitoes used in analyses were identified as An. gambiae coluzzi. None of the mosquitoes were found to have only the 1014S kdr mutation. Raw data are provided on OSF.

Discussion and conclusion

In the current study, 9 out of 70 the species of Anopheles coluzzi collected in one locality of Elbibou in Côte d’Ivoire appeared to have the double East- and West-African kdr mutations, whereas just 1 year ago, only 1 mosquito out of 150 sampled across country had the same genotype. These results could be found too preliminary as only one location has been sampled and the change is only observed between two time points. Nevertheless, this should be taken seriously since as the phenotypic consequences and thus importance of the combination of the
Figure 1. East African kdr genotype of wild Elibou Anopheles coluzzi population. The ‘A’, ‘C’, ‘D’ and ‘E’ on the figure are the positive controls respectively for the East-kdr homozygous mutant allele, heterozygous mutant/susceptible allele, homozygous susceptible allele and blank. Nine mosquitoes (‘B’) displayed the heterozygous.

Figure 2. West African kdr genotype of wild Elibou Anopheles coluzzi population. The ‘A’, ‘C’, ‘D’ and ‘E’ on the figure are the positive controls respectively for the East-kdr homozygous mutant allele, heterozygous mutant/susceptible allele, homozygous susceptible allele and blank. The same nine mosquitoes of Figure 1 displayed the heterozygous (‘B’) genotype. We have not quantified the DNA in extracted samples. A low quantity of DNA in the West-kdr homozygous positive control might explain the low signal observed for A.
mutations in the populations is unknown. We have not provided data for the kdr 1014F as this is part of another study. Overall, the present study confirms the general trend of intensification and propagation of resistance phenomena. The risks and consequences for vector control and malaria burden are widely documented. WHO\(^2\) reported in 2012 that coverage with LLINs and IRS in the WHO African Region was estimated to avert approximately 220,000 deaths among children under 5 years annually. If pyrethroids were to lose most of their efficacy, more than 55% of the benefits of vector control would be lost, leading to approximately 120,000 deaths that could not be averted. To remedy to this, it was found necessary by some companies to reformulate some active ingredients previously used exclusively in agriculture. This is the case for the neonicotinoids, reformulated by Bayer under the name of Fludora, composed of clothianidin and deltamethrin, or by Sumitomo under the name of Sumishield, composed of clothianidin. Neonicotinoids exhibit a mode of action that is completely different to the one appearing when public health insecticides are used. Neonicotinoids act by selectively targeting the invertebrate nicotinic acetylcholine receptor (nAChR) and disrupting excitatory cholinergic neurotransmission leading to paralysis and death.\(^3\) Given the massive use of neonicotinoids in agriculture\(^4\)\(^5\)\(^6\), their medium-to-long-term efficacy on vector populations remains questionable. An upcoming study have shown that wild populations of An. gambiae from agricultural areas of Cote d’Ivoire are already resistant to neonicotinoids (Chouaibou et al., Submitted). This dilemma reinforces the idea that the reformulation of agricultural insecticides for public health application is not necessarily the right solution for vector control, although it may appear as a transitional solution. Other solutions include the development of new insecticidal molecules dedicated exclusively to vector control in the public health sector, as described in the goal of the Innovative Vectors Control Consortium for the next 3 to 5 years. Other non-chemical methods of vector control should also be considered for the future. Given the huge emphasis on chemical-based control tools, people may think that chemical-based control strategies are the only way to overcome malaria vectors, while we are confident that the best approach is the integrated vector management strategy that includes all effective and available methods. The current study highlights the rapid spread of insecticide resistance in malaria vectors, which can lead to insecticide-based vector control failure and huge fatalities on malaria burden. Thus, collective awareness is essential. Vector control interventions must be rethought and reviewed overall; reflections must be made at all stages, and chemical control must not be seen as the ultimate solution.

**Data availability**

Output data from the genotyping of mosquitoes is available on OSF. DOI: https://doi.org/10.17605/OSF.IO/9BDT7\(^2\)\(^4\).

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

**Authors contributions**

CSM conceived and designed the initial study, interpreted the data and drafted the manuscript. PBN, BKF, CGS and FPKA carried out the field sampling, mosquito rearing, and laboratory analysis. BGK edited and improved the manuscript. All authors read and approved the final manuscript.

**Grant information**

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**Acknowledgments**

We thank CSRS laboratory staff

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Version 1

Reviewer Report 10 May 2019

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Penelope A. Hancock
Li Ka Shing Centre for Health Information and Discovery, University of Oxford, Oxford, UK

The paper is clearly written and the data, analytical methods and results are well explained. I agree that the observation of the double mutation in individual mosquitoes is interesting and warrants further study. However, I think the interpretation drawn from the results presented needs to be re-thought, because the paper does not present sufficient evidence that the frequency of the double East and West African kdr mutation is increasing. Referring to the statement in the first section of the Discussion and conclusion “In the current study, 9 out of 70 of the species of *Anopheles coluzzi* collected in one locality of Elibou in Cote d'Ivoire appeared to have the double East and West African kdr mutations, whereas just 1 year ago, only 1 mosquito out of 150 sampled across the country had the same genotype”. Can the authors be more specific about where the 150 mosquitoes from the previous year were collected and what species they were? A robust statement about whether resistance is spreading would really require more samples, both longitudinally and from multiple locations.

At present, the results need to be stated in a more qualitative sense, explicating the reasons why a proportion of 9 out of 70 can be considered as alarming, given prior observations. I should add that I am not sufficiently qualified to assess the soundness of the molecular analyses presented.

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

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

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

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

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

Are the conclusions drawn adequately supported by the results?
No

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Epidemiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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**Reviewer Report 16 April 2019**

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**Lizette L. Koekemoer**

Wits Research Institute for Malaria, Faculty of Health Sciences, School of Pathology, University of the Witwatersrand, Johannesburg, South Africa

This study reports on the spread of the kdr-resistant mutations in Cote d'Ivoire. The work is clearly written and the following are more specific comments to the authors.

Title is misleading as this is mainly a pilot study with a small sample size and although the frequency of the mutation seems to have increased, the "rapid spread" would need additional sampling from multiple sites in the country. Title should be revised.

Spelling of *An. coluzzii*, should be corrected in the manuscript and there is no need to call the species *An. gambiae coluzzii*.

Background section: Authors' can also rephrase to indicate the personal protection the nets will provide in addition to the killing effect. The increase in malaria cases with an increase in pyrethroid resistance can be mitigated with the use of insecticide resistance management plan. This can be added to explain the sentence stated that it will translate to an increase in malaria cases.

The authors should use the formal naming of the mutations: L1014F and L1014S.

Methods section: indicate how many breeding sites are "several"? Difficult to interpret if the
sample size of 70 is from 2 or 50 breeding sites.

General comments: Fludora and Sumishield are trade names and should be indicated with a “®”

Are there indications that the population of An. coluzzii with two mutations have an increase in resistance intensity on pyrethroids? What was the resistance on pyrethroids from earlier years, has this changed?

**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?**
I cannot comment. A qualified statistician is required.

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

**Are the conclusions drawn adequately supported by the results?**
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Malaria vector biology and control

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

Reviewer Report 12 March 2019

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Stephen Magesa
VectorLink Mozambique Project, Abt Associates Inc. Maputo, Maputo, Mozambique

Title
The title sounds appropriate, though the initial word “Rapid” in the title referring to the pace at
which the double mutations are spreading has been measured and found to be high depicts a misrepresentation. A better word could be found to replace this one. Or else omitting it altogether does no harm to the title.

The work is very clearly and accurately presented using simple language. It also cites most of the current literature on the particular subject. Very pertinent being recent work in the same country and other neighbouring countries in the region. However, I have picked a few issues around the way the results are presented interpreted and need for further verification of the results. I would like therefore to raise the following concerns and some additional comments that the authors may wish to use for improving the article:

The work is technically sound since the laboratory techniques employed in the analysis includes the standard methodologies that are current in the subject. However, the design could have been better by sampling from more areas and if possible in all the ten regions where the initial study by Chouaïbou et al. (2017) were sampled. This study is based on samples from only one locality which we may not know whether is a stronghold for these combined gene mutations or not. This is stated with the understanding that the current study is a follow-on of earlier studies by Chouaïbou et al. (2017) and Fodjo et al. (2018).

The current research note has sufficient details of the employed methods and analysis. The details provided are enough to allow for replication of the same study by others. However, there is not enough information provided regarding data presented on Tables 1 and 2. Some abbreviations used are not explained at all. The text does not at any point call to the tables. This makes it extremely difficult for non-specialists to understand the data presented there.

The above caveat notwithstanding, most data seem to be available except for the kdr 1014F data that has not been provided ostensibly due to being part of another study. I am not sure this is good enough reason and whether or not the data could be made available at a later stage should one require them for the purpose.

Looking at the magnitude of the question that the study is trying to address, the study falls into a situation where the results presented here simply provides preliminary information that may not fully address whatever conclusions are being proposed.

- The epidemiological significance of the East African mutant gene, 1014S kdr is not addressed here. Based on previous observation that the West African mutant 1014F kdr confers greater phenotypic resistance, it remains to be seen as to what the East-kdr spread into West Africa adds to the current resistance status. The situation becomes more complex given that all such mutations were found to be paired resulting into double mutations in all nine occurrences observed.
- The statement that “If pyrethroids were to lose most of their efficacy, more than 55% of the benefits of vector control would be lost, leading to approximately 120,000 deaths that could not be averted” sounds unnecessarily too alarmist! Since pyrethroids are mainly used for net treatment, the role of the net physical barrier cannot be underestimated. Moreover, the portfolio of new insecticides that are coming up should be able to avert a good proportion of the malaria burden despite the observed shortcomings by the authors.

Contrary to what would have been expected from such a brief research note, the authors do not attempt to make a case for further surveillance to monitor the East-kdr prevalence in the area. This is an interesting area that is developing and has not been fully explored. Previous studies and
the current study have limitations in terms of time and scale, thus calling for a larger more elaborate study to establish the status of not only the spread of the East-kdr, but also its epidemiological significance in terms of its impact on phenotypic pyrethroid resistance in the country and whole of West Africa region.

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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?
I cannot comment. A qualified statistician is required.

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

Are the conclusions drawn adequately supported by the results?
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Vector control with a focus towards appropriate vector control technologies for Africa.

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