First report of the presence of L1014S Knockdown-resistance mutation in Anopheles gambiae s.s and Anopheles coluzzii from Togo, West Africa [version 1; peer review: 2 approved, 1 approved with reservations]

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Abstract

Background: To optimize the success of insecticide-based malaria control intervention, knowledge of the distribution of Anopheles gambiae species and insecticide resistance mechanisms is necessary. This paper reported an updated data on pyrethroids/DDT resistance in the An. gambiae s.l population from Togo.

Methods: From December 2013 to April 2015, females of indoor-resting An. gambiae s.l were captured in three locations belonging to three different ecological zones. Resistance to DDT, permethrin and deltamethrin was screened in F1 progeny of collected mosquitoes using WHO susceptibility tests. The identification of species of An. gambiae complex and the detection of kdr and ace1R allele were carried out using DNA-based molecular techniques.

Results: An. gambiae from Kovié and Nangbeto were highly resistant to DDT and permethrin with mortality rate ranging from 0.83% to 1.58% for DDT and zero to 8.54% for permethrin. Mosquitoes collected in Nangbeto displayed 81.53% mortality with deltamethrin. An. coluzzii and An. gambiae s.s were found in sympatry in Nangbeto and Mango. The allelic frequency of L1014F was high, ranging from 66 to 100% in both An. gambiae s.s and An. coluzzii. For the first time we detected the L1014S allele in both An. gambiae s.s and An. coluzzii from Togo at the frequency ranging from 5% to 13% in all the sites. The kdr N1575Y was present at various frequencies in both species ranging from 10% to 45%. Both An. gambiae s.s and An. coluzzii shared the ace1R mutation in all investigated sites with allelic frequency ranging from 4% to 16%.

Conclusion: These results showed that multiple mutations are involved in insecticides resistance in populations from Togo including the An. gambiae s.s and An. coluzzii.
insecticides resistance in *An. gambiae* populations from Togo including the kdr L1014F, L1014S, and N1575Y and *ace1* R G119S mutations.

**Keywords**

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**Author roles:** Djègbè I: Data Curation, Formal Analysis, Methodology, Writing – Original Draft Preparation; Akoton R: Data Curation, Formal Analysis, Methodology, Writing – Original Draft Preparation; Tchigossou GM: Data Curation, Formal Analysis, Methodology, Writing – Original Draft Preparation; Ahadji-Dabla KM: Data Curation, Formal Analysis, Methodology; Adéoti R: Data Curation, Formal Analysis, Methodology; Zeukeng F: Data Curation, Formal Analysis, Methodology; Ketoh GK: Data Curation, Formal Analysis, Methodology, Validation; Djouaka R: Conceptualization, Funding Acquisition, Investigation, Project Administration, Supervision, Validation, Visualization, Writing – Review & Editing

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

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Introduction

Despite a reported decline in infection and mortality, malaria remains the fourth leading cause of mortality in children under-five in the sub-region. In Togo, the malaria control strategy is based on universal access to Long Lasting Insecticidal Nets (LLINs) as recommended by the World Health Organization (WHO). However, malaria vectors were found resistant to all insecticide classes used in public health interventions in West Africa. Mosquito resistance to insecticides stands as a serious obstacle to the effectiveness of LLINs. Since 2010, reports of pyrethroids and dichlorodiphenyltrichloroethane (DDT) resistance have been widespread,[3-6] with further reports of carbamate resistance.[7-9] The resistance to pyrethroids/DDT is conferred by two main physiological mechanisms including metabolic resistance and the target site insensitivity.[10] The target site resistance remains the most studied to date, a result of the easier means of its assessment.[11] In Anopheles gambiae complex, kdr gene mutations, including the substitutions of leucine to phenylalanine (L1014F) and leucine to serine (L1014S), are the two mutations involved in the target site resistance.[12,13] These two target site mutations are largely distributed across the African Continent, yet differences in the allelic frequency have been reported between An. gambiae s.l. species and between breeding sites.[14,15] In several studies, clear associations have been shown between DDT/pyrethroids resistance and the presence of kdr mutations.[16] Recently, a new mutation named N1575Y has emerged within the linker between domains III-IV of the voltage gate sodium channel (VGSC) in An. gambiae s.s., An coluzzii and An. arabiensis.[17] Studies have demonstrated the appearance of the N1575Y mutation as an additional resistance mechanism that appears with the L1014F kdr mutation. N1575Y mutation is therefore being suggested as a secondary selective sweep, associated with resistance to pyrethroids/DDT in the West African region.[18,19] The development of new resistance mechanisms among Anopheles populations highlights a failure in pyrethroids-based control strategies, and could jeopardize the mosquito control efforts.[20,21] It is therefore seminal to establish the epidemiological consequences of pyrethroids resistance, and develop new intervention strategies for the management of insecticides resistance in West Africa.

Monitoring the molecular and physiological markers of pyrethroids resistance has significant advantages for the management of insecticides resistance. In Togo, despite the huge investments in LLINs, information is still lacking on the routine monitoring of insecticides resistance in malaria vectors. An entomological survey was carried out since 2009 in two localities of the Southern coastal zone in the North (zone II), the Central plains zone (zone III), the Mountains Togo meridional zone (zone IV), and the Southern coastal zone (zone V). Table 1 describes the characteristics and geographic coordinates (GPS) of the investigated sites.

Methods

Study sites

The study was carried out in three rural sites from different ecological zones of Togo (Figure 1, Table 1). Togo is a coastal country located in West Africa, with a population of ~7 million inhabitants. The country has two tropical climates: the Sudanian in the North, characterized by one rainy season and one dry season; and the subequatorial in the South, characterized by two dry seasons (from December to March and from August to September) and two rainy seasons (from April to July and from October to November). According to Ahadj-Dabla et al.[21], the Republic of Togo covers five ecological zones: the plains zone in the North (zone I), the mountains zone in the North (zone II), the Central plains zone (zone III), the Mounts Togo meridional zone (zone IV), and the Southern coastal zone (zone V). Table 1 describes the characteristics and geographic coordinates (GPS) of the investigated sites.

Mosquito collection and rearing

Mosquito sampling was conducted from December 2013 to April 2015 using electric aspirators. In each study sites, the households were randomly selected for mosquito aspirations. Verbal and written consents of the household heads were sought prior to insect collection in their houses. Indoor resting blood fed adult female Anopheles mosquitoes (F0), were captured between 06:00 and 10:00 am, kept in cool boxes and brought to the insectary of the AgroEcoHealth Platform of the International Institute of Tropical Agriculture (IITA-Benin). A forced-egg laying method was used to induce the females to lay eggs as previously described.[22] The egg batches were then allowed to hatch in a small paper cup and later transferred to larvae bowls for rearing as previously described.[22,23]

Insecticide susceptibility test

F1 female progeny of wild An. gambiae and laboratory susceptible Kisumu strain aged 3–5 days were exposed to impregnated papers at diagnostic concentrations of insecticides according to WHO protocol.[24] Insecticide papers were obtained from the WHO reference centre at the Vector Control Research Unit, University Sains Malaysia. The impregnated papers included 4% DDT, 0.75% permethrin and 0.05% deltamethrin. Briefly, for each tested insecticide, batches of 20–25 unfed females were exposed to an impregnated paper for 60 min, after which they were transferred into tubes containing untreated papers and placed under observation at 25°C and 80% relative humidity (RH) with 10% sugar solution. Mortality rate was recorded 24h post-exposure. Tube tests containing untreated papers were run in parallel as a control.

Species identification, kdr L1014F, L1014S, N1575Y and ace.1\textsuperscript{a} genotyping

Genomic DNA from respectively 88, 70 and 58 females (F0) collected in Ković, Nangbéto and Mango of bioassay control was individually extracted using the Livak DNA extraction method.[25] The species of An. gambiae mosquitoes were identified using polymerase chain reaction techniques. The kdr
Figure 1. Map of Togo showing the study sites.

<table>
<thead>
<tr>
<th>Localities</th>
<th>Main agriculture practices</th>
<th>Ecological zones</th>
<th>Periods of collection</th>
<th>Geographic coordinates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kovié</td>
<td>Rice, vegetables</td>
<td>(Coastal)</td>
<td>December 2013</td>
<td>N 06°20.305’ 001°07.425’ E</td>
</tr>
<tr>
<td>Nangbéto</td>
<td>Cereals, tubers</td>
<td>(Forest)</td>
<td>April 2015</td>
<td>N 07°25.802’ 001°26.822’ E</td>
</tr>
<tr>
<td>Mango</td>
<td>Cereals, cotton</td>
<td>(Savannah)</td>
<td>April 2015</td>
<td>N 10°21’17.9’ 0°28’21.7’ E</td>
</tr>
</tbody>
</table>
mutations L1014F, L1014S and N1575Y were screened using TaqMan real time PCR assays as previously described. The presence of G119S-Ace1 allele was also screened in An. gambiae populations as describe by Bass et al.

**Statistical analysis**
The resistance profile of An. gambiae s.l was determined using WHO criteria:
- Mortality rate > 98% = susceptible mosquito population
- Mortality rate between 90–98% = suspected resistance in the mosquito population
- Mortality rate < 90% = resistant mosquito population

According to Hardy-Weinberg equilibrium using the Had2know online statistical software, calculated genotype frequencies of L1014F, L1014S, N1575Y and G119S were confirmed and compared between An. coluzzii and An. gambiae s.s. with Chi-square test.

**Results**

Species composition of An. gambiae s.l.
The PCR analysis on the F0 females for identification of sibling species among An. gambiae complex revealed the presence of only two species in the study sites as An. coluzzii and An. gambiae s.s. Both An. gambiae s.s. and An. coluzzii were found in sympatry in Nangbéto (59% versus 41%) and Mango (97% versus 3%). An. coluzzii was predominant (100%) in Kovié (rice field) (Figure 2).

![Species composition in study sites](image)

**Insecticide resistance status**
Figure 3 describes the insecticide resistance profile of three An. gambiae s.l populations collected in Togo. The laboratory strain Kisumu exhibited very high susceptibility to the insecticides tested: 99% mortality to 100% mortality to the organochlorine and permethrin respectively, and 99.5% mortality to deltamethrin. In control groups (untreated papers) the mortality rates recorded with the wild An. gambiae populations were below 5% at 24 hours post-exposure.

An. gambiae population from Kovié and Nangbéto were highly resistant to DDT and permethrin with mortality rates ranging from 0.83% to 1.58% for DDT and from zero to 8.54% for permethrin. Mosquitoes collected in Mango and Nangbéto displayed high resistance to deltamethrin with mortality rates of 28.67% and 81.53% for permethrin and deltamethrin respectively.

Detection of resistance genes
Mosquitoes from each study site were used for kdr and ace.l\(^{\text{a}}\) screening. Table 2 presents the distribution of knock-down (L1014F, L1014S and N1575Y) and ace.l\(^{\text{a}}\) (G119S) mutations in An. gambiae s.s. and An. coluzzii. The L1014F kdr mutation was found at various allelic frequencies in An. gambiae s.s. and An. coluzzii in the three sites. The 1014F allelic frequency was high in both species ranging from 66% to 100% in An. coluzzii and from 80% to 83.96% in An. gambiae s.s. The 1014S kdr allele was found for the first time in Anopheles mosquitoes from Togo (Kovié and Nangbéto). The allelic
Table 2. Resistant allele frequencies in Anopheles coluzzii and Anopheles gambiae s.s in study sites.

<table>
<thead>
<tr>
<th>Localities</th>
<th>Species</th>
<th>N</th>
<th>f (1014F)</th>
<th>N</th>
<th>f (1014S)</th>
<th>N</th>
<th>f (1575Y)</th>
<th>N</th>
<th>f (119S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kovié</td>
<td><em>An. coluzzii</em></td>
<td>79</td>
<td>67.72%±10.31</td>
<td>87</td>
<td>5.17%±4.65</td>
<td>81</td>
<td>45.06%±10.84</td>
<td>63</td>
<td>16.66%±10.76</td>
</tr>
<tr>
<td>Nangbéto</td>
<td><em>An. coluzzii</em></td>
<td>27</td>
<td>66.67%±17.78</td>
<td>26</td>
<td>13.46%±7.56</td>
<td>29</td>
<td>10.34%±7.76</td>
<td>28</td>
<td>-</td>
</tr>
<tr>
<td>Mango</td>
<td></td>
<td>2</td>
<td>100%</td>
<td>2</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Nangbéto</td>
<td><em>An. gambiae s.s</em></td>
<td>40</td>
<td>80%±12.4</td>
<td>31</td>
<td>6.45%±4.38</td>
<td>41</td>
<td>17.07%±11.66</td>
<td>41</td>
<td>4.87%±2.67</td>
</tr>
<tr>
<td>Mango</td>
<td><em>An. gambiae s.s</em></td>
<td>53</td>
<td>83.96%±9.88</td>
<td>53</td>
<td>-</td>
<td>52</td>
<td>11.53%±8.6</td>
<td>49</td>
<td>7.14%±6.93</td>
</tr>
</tbody>
</table>

NB: Allelic frequencies (f) are given in means ± Standard deviation (SD)

The frequency of this kdr mutation ranged from 5.17 to 13.46% in both *An. gambiae s.s* and *An. coluzzii*. The kdr N1575Y were also detected in the two species with the allelic frequencies ranging from 10.34 to 45.06% in all the sites investigated. *ace.1R* mutation was mainly found in *An. gambiae s.s* with allelic frequency ranging from 4.87 to 16.66%.

Discussion

This study provides an update on current levels of resistance to permethrin and deltamethrin and frequencies of the kdr and *ace.1R* mutation in *An. coluzzii* and *An. gambiae s.s* in rural areas of Togo.

*An. coluzzii* and *An. gambiae s.s* were the only *Anopheles* species observed in this study and lived in sympaty at varying frequencies. *An. gambiae s.s* was the most abundant species at the two cereal cultivation sites of Mango and Nangbéto whereas *An. coluzzii* is far more predominant in the rice field of Kovié, supporting findings from previous research in Togo and Benin. This heterogeneous composition of *An. gambiae* population observed in these localities could be due to a competitive exclusion between the two subspecies. This study provides updated information on the insecticide resistance profile of *Anopheles gambiae* and the underlying mechanisms involved in rural areas of Togo.

It also provides baseline information on the susceptibility/resistance status of *An. coluzzii* and *An. gambiae s.s* in this location to permethrin and deltamethrin, the insecticides used to impregnate the bed nets freely distributed by NMCP of Togo. The WHO bioassay results indicated a high prevalence of resistance to pyrethroids/DDT in all study sites. This suggests that similar selection pressures are acting on these populations. Recently Ahadjı-Dabla et al. reported similar observation of resistance in *An. gambiae s.l* populations to DDT, deltamethrin and permethrin in Lomé. The proportion of *An. gambiae* surviving to permethrin exposure has increased slightly in Kovié compared with that in previous studies conducted in the same area: In 2009, Ahadjı-Dabla et al. reported mortality of 56%, while any mortality was recorded in this study. This observation suggests that pyrethroids resistance has significantly increased in this area over the last five years. It probably indicates that the selection pressure is not high but could change with the intensive and uncontrolled used of chemical and fertilizers in rice.
production and the mass-distributed of LLINs in this area. Moreover, the implantation of uncontrolled insecticides selling markets in the country especially around rice cultivation areas, contributed to the increase in the resistance\textsuperscript{20}. This can therefore explain the high frequencies of kdr resistant alleles found in our study areas. The knock-down resistance gene was the main resistance mechanism found in all assessed mosquito populations. The 1575Y allele was found in both An. coluzzii and An. gambiae s.s in all the study sites. The frequencies of this mutation were similar to those found in the nearby countries like Benin\textsuperscript{18} and Burkina-Faso\textsuperscript{37}. This allele was highly distributed in An. coluzzii in Kovié (0.45) as previously reported in the rice fields in Northern Benin\textsuperscript{15}. However, the prevalence of this mutation has rapidly increased in the West African region suggesting an ongoing strong selection of the L1014F-N1575Y haplotype in this region. The kdr (L1014F) mutation was found at higher frequencies in An. gambiae s.s than An. coluzzii at all sites. These results confirm those of Dabiré \textit{et al.}\textsuperscript{31} who found that An. gambiae s.s showed the highest levels of resistance than An. coluzzii.

This kdr mutation could be used for the high resistance observed to permethrin and deltamethrin in An. gambiae population from Togo. Hence, this is a serious problem for malaria control programs because the currently widespread distributed nets are impregnated by these insecticides. Fortunately, a recent paper published in Benin revealed that insecticide-treated nets provide protection against malaria to children in an area of insecticide resistance in southern Benin\textsuperscript{25}.

To our knowledge, this study is the first reporting the presence of the L1014S kdr mutation in wild An. gambiae s.l populations from Togo. The L1014S allele was detected in both An. coluzzii and An. gambiae s.s. This allele, originating from East Africa, was recently reported in Benin in An. arabiensis\textsuperscript{19} and in An. coluzzii and An. gambiae s.s in Burkina-Faso\textsuperscript{33}. It is possible that An. gambiae populations carrying the kdr L1014S mutation might have migrated, through active or/and passive ways, from bordering countries (e.g. Benin, Burkina-Faso) due to intense traffic and exchanges between these countries and Togo.

These findings therefore provide strong evidences on the increasing distribution of the kdr mutations among Anopheles mosquitoes across Africa, and could be used as baseline data for proper monitoring of this allele in West African countries. Further research should be implemented to provide knowledge on the geographical distribution of L1014S kdr allele in West Africa, its role in pyrethroids phenotypic resistance, as well as its impact on the efficacy of pyrethroids treated nets.

In the present study, the G119S mutation was identified in all investigated sites at a relatively low frequency. This is in contrast to previous findings that reported a high frequency of ace-1\textsuperscript{P} mutation in An. gambiae s.l from Lomé\textsuperscript{20}. This resistant allele was detected in both species with frequencies ranging from 4.8% to 16.66%. The presence of ace-1\textsuperscript{P} mutations in An. coluzzii and An. gambiae s.s has already been reported by Weill \textit{et al.}\textsuperscript{32} and Djogbenou \textit{et al.}\textsuperscript{19}. The incidence of the G119S mutation in the An. gambiae s.l population from Togo suggests a probable resistance to carbamates and organophosphates insecticides. However, this assertion needs to be proved by WHO toxicological tests\textsuperscript{26}. We cannot therefore exclude the possibility that besides the four mutations targeted in these study sites, other enzymes and genetic mechanisms could be contributed to the resistance, as suggested by previous studies in Benin\textsuperscript{18,37}.

Conclusion

The present study revealed the widespread of kdr 1014F, 1014S and 1575Y as well as the G119 alleles in Togo. For the first time, this gives the evidence of the presence of 1014S kdr allele in wild populations of An. gambiae s.l from Togo where entomological surveys are scanty. Hence confirming the expansion of pyrethroids resistance alleles in Africa. There is therefore a need for regular updating on the current entomological data for appropriate decision making and proper intervention strategies for malaria vector control in this country.

Ethics statement and consent

No ethical clearance was required for this study according to the International Institute of Tropical Agriculture (IITA) Ethical Committee (IITA, 08 P.O. Box 0932, Tri-Postal, Cotonou, Benin). However, consent of the community leaders was sought prior to mosquito larva and adult collections in the community. We explained our study to the communities and household heads. Verbal and written consents of household heads were therefore obtained prior for mosquito collection.

Data availability

All data generated and analyzed during this study is included in the published article. Raw data are available from Open Science Framework: Dataset 1. First report of the presence of L1014S Knockdown-resistance mutation in Anopheles gambiae s.s and Anopheles coluzzii from Togo, West Africa, http://doi.org/10.17605/OSF.IO/M3G4P\textsuperscript{18}

Competing interests

No competing interests were disclosed.

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

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References


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Introduction
1- the sentence « However, malaria vectors were found resistant to all insecticide classes used in public health interventions in West Africa » need to be referenced.

2- Add a sentence in the introduction describing the G119S mutation causing resistance to organophosphates and carbamates as done for others target side modification mechanisms.

Materiel & Methods
The attempt was made by the authors to explain the methods used and analysis performed.

Results
The authors stated in their Statistical analysis that « According to Hardy-Weinberg equilibrium using the Had2know online statistical software, calculated genotype frequencies of L1014F, L1014S, N1575Y and G119S were confirmed and compared between An. coluzzii and An. gambiae s.s. with Chi-square test. ».

By analysing the table bearing the frequencies data, these statistical analysis results were not clear. Furthermore, it not easy to understand how the authors can infered the standard deviation (SD) from the calculations of resistant allele frequencies. All these remarks need to be clarified by the authors.

Discussion
The sentence need to be rephrased and be more specific « This study provides an update on current levels of resistance to permethrin and deltamethrin and frequencies of the kdr and ace.1R mutation in An. coluzzii and An. gambiae s.s in rural areas of Togo » It better to precise the location where the study was performed.
The sentence « The WHO bioassay results indicated a high prevalence of resistance to pyrethroids/DDT in all study sites » need to be rephrased using « high level of resistance » instead of « high prevalence of resistance »

The authors stated: « The WHO bioassay results indicated a high prevalence of resistance to pyrethroids/DDT in all study sites. This suggests that similar selection pressures are acting on these populations ». I have a concern with the logical between these two sentences. In my opinion, the high level of resistance displayed to pyrethroids cannot allow the authors to state « that similar selection pressures are acting on these populations »

The authors stated: « In 2009, Ahadji-Dabla et al.21 reported mortality of 56%, while any mortality was recorded in this study » this sentence need to be rephrased to « In 2009, Ahadji-Dabla et al.21 reported mortality of 56%, while no mortality was recorded in this study»

The authors stated in their discussion « The proportion of An. gambiae surviving to permethrin exposure has increased slightly in Kovié compared with that in previous studies conducted in the same area: In 2009, Ahadji-Dabla et al.21 reported mortality of 56%, while any mortality was recorded in this study. This observation suggests that pyrethroids resistance had significantly increased in this area over the last five years. It probably indicates that the selection pressure is not high but could change with the intensive and uncontrolled used of chemical and fertilizers in rice production and the mass-distributed of LLINs in this area. »

This part of the discussion appears to be confused for me.

Firstly, when they attempted to compare the mortality observed in this study to that of previous studies, the authors need to pay attention to the number of mosquitoes used for WHO bioassay compared to that which was used in the previous work. For me it seems that these numbers are largely different. They can attempt to speculate but not conform.

Secondly, it is very hard to understand how they can infer from the sentence “This observation suggests that pyrethroids resistance had significantly increased in this area over the last five years” that “the selection pressure is not high but could change with the intensive and uncontrolled used of chemical ........). I’m confused when referring to the statement of the authors in the previous sentences.

The title of the manuscript is “First report of the presence of L1014S Knockdown-resistance mutation in Anopheles gambiae s.s and Anopheles coluzzii from Togo, West Africa” By analysing the discussion length, we can observe that only one paragraph was devoted for the discussion related to the presence of L1014S Knockdown-resistance mutation in Anopheles gambiae s.s and Anopheles coluzzii from Togo.

I strongly recommend the authors to rewrite the discussion and focus their discussion on idea related to the title or they can change the title of the manuscript according the discussion content.

**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?
No

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:** Medical Entomologist

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

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**Nafomon Sogoba**

International Center of Excellence in Research (ICER-Mali), Faculty of Medicine and Odontostomatology (FMOS), University of Sciences, Techniques and Technologies of Bamako, Bamako, Mali

The manuscript “First report of the presence of L1014S Knockdown-resistance mutation in *Anopheles gambiae s.s* and *Anopheles coluzzii* from Togo, West Africa” has updated data on pyrethroids/DDT resistance in the *An. gambiae s.l* population from Togo. In particular, it investigated the distribution of the kdr L1014F, L1014S and N1575Y alleles and ace.1R mutations in different ecological settings of Togo.

General comments

Overall the manuscript was very well written and I have only few comments:

1. Is it appropriate to compare results from mosquitoes collected in 2013 to those collected 2 years later (2015) when we know that insecticide resistance is evolving quickly?
2. How can you explain the high difference between the mortality rates of Deltamethrin and Permethrin in Nangbéto?

**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 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.
“Malaria remains the fourth leading cause mortality ….” Change to “Malaria remains the fourth leading cause of mortality ….”

“However, malaria vectors were found resistant to all insecticide classes used in public health interventions in West Africa.” Please add a reference at the end of this sentence.

The authors also need to provide information on the situation of malaria (morbidity, mortality) and insecticides resistance in Togo. What is the level of insecticide resistance in Togo, what is the level of coverage of the population by treated nets (this information could be extracted from the annual world malaria report)

**Method**

Mosquito collection and rearing

The authors say mosquito collections were undertaken from December 2013 to April 2015. This sentence is misleading they authors have to say as presented in table 1 that, mosquitoes were collected in Kovié in December 2013 and in Nangbéto and Mango in April 2015.

Collections were conducted during how many days/site/sampling period? Please add the information

The progeny of how many female were used for insecticide susceptibility tests because the result could be biased if just a few female progeny were used

Figure 1 please add the position of Togo in Africa

**Results**

Can the number of mosquito collected using electric aspirators during each period in each site be added the number who successfully laid eggs and the total number of mosquito who emerged from the females.

The author say in the statistical analysis section “According to Hardy-Weinberg equilibrium using the Had2know online statistical software, calculated genotype frequencies of L1014F, L1014S, N1575Y and G119S were confirmed and compared between An. coluzzii and An. gambiae s.s. with Chi-square test.” In the result section, no data of the comparison of genotypes between An. gambiae and An. coluzzii is provided please add these data.

**Discussion**

Mosquito used for bioassay were the progeny of females collected resting indoor it is possible that mosquitoes resting indoor may be more tolerant to insecticide than the general population. Following this it is possible that increased level of resistance recorded could be due to the use of this fraction of the population this need to be clearly stated in the discussion.

Also the fact that no *An. arabiensis* was recorded during the study could be due to the fact that the sampling concerned mosquitoes resting indoor whereas this mosquito is known to be highly exophilic the absence of *An. arabiensis* could result from a sampling bias this has to be included

**Source of data underlying the results available**

The authors need to include information on the genotypes to the kdr gene in the different sites

**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?**

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?
Partly

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

Are the conclusions drawn adequately supported by the results?
Yes

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

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.