Silva Martins, WF, Silva Pereira, BN, Vieira Alves, AT, Murphy, A, Silva Martins, PG, Weetman, D, Wilding, CS and Donnelly, MJ

Development and application of a tri-allelic PCR assay for screening Vgsc-L1014F kdr mutations associated with pyrethroid and organochlorine resistance in the mosquito Culex quinquefasciatus

Parasites & Vectors

RESEARCH Open Access

Development and application of a tri-allelic PCR assay for screening *Vgsc*-L1014F *kdr* mutations associated with pyrethroid and organochlorine resistance in the mosquito *Culex quinquefasciatus*

Walter Fabricio Silva Martins[1,2*], Bárbara Natieli Silva Pereira[3], Ana Thayse Vieira Alves[1,4], Annabel Murphy[2], Paulo Geovani Silva Martins[5], David Weetman[2], Craig Stephen Wilding[6] and Martin James Donnelly[2]

Abstract

Background: *Culex quinquefasciatus* has a widespread distribution across tropical and sub-tropical regions, and plays an important role in the transmission of vector-borne diseases of public health importance, including lymphatic filariasis (LF) and multiple arboviruses. Increased resistance to insecticides threatens the efficacy and sustainability of insecticide-based anti-vector interventions which mitigate the burden of mosquito transmitted diseases in endemic regions. In *C. quinquefasciatus* two non-synonymous voltage gated sodium channel (*Vgsc*) variants, both resulting in a leucine to phenylalanine change at codon 1014, are associated with resistance to pyrethroids and DDT. This tri-allelic variation has compromised the ability to perform high-throughput single-assay screening. To facilitate the detection and monitoring of the *Vgsc*-1014 locus in field-caught mosquitoes, an Engineered-Tail Allele-Specific-PCR (ETAS-PCR) diagnostic assay was developed and applied to wild mosquitoes from Brazil, Tanzania and Uganda.

Results: This new cost-effective, single-tube assay was compared to two, well-established, genotyping approaches, pyrosequencing and TaqMan. The ETAS-PCR assay showed high specificity for discriminating the three alleles at *Vgsc*-L1014F, with genotyping results strongly correlated with pyrosequencing and TaqMan results (98.64% and 100% respectively).

Conclusions: Our results support the utility of the ETAS-PCR/*Vgsc*-1014 diagnostic assay, which stands as an effective alternative for genotyping tri-allelic variants.

Keywords: Target-site mutations, Vector-borne diseases, Allelic-specific PCR

Background

The mosquito *Culex pipiens quinquefasciatus* (hereafter *C. quinquefasciatus*) acts as a vector of several pathogens in both tropical and temperate environments [1]. In many tropical/sub-tropical regions *C. quinquefasciatus* is the primary vector of lymphatic filariasis (LF) [2–4] whilst in subtropical locations, it is a vector of potentially fatal arboviruses e.g. West Nile virus (WNV) and St. Louis encephalitis virus (SLEV) [5–7].

Vaccination and mass drug administration (MDA) are the primary methods for reducing the burden of diseases transmitted by *C. quinquefasciatus*, but insecticide-based vector control is advocated as an important adjunct. For example, vector control has assisted in mitigating the burden of transmission of arboviruses where immunization campaigns are challenging, such as in rural settlements [8–10].

*Correspondence: fabricio.martins@lstmed.ac.uk
[2] Department of Vector Biology, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, UK
Full list of author information is available at the end of the article

The effectiveness of insecticide-based vector control is threatened by the increased insecticide resistance detected across the globe [11, 12] with the increased resistance to pyrethroids especially worrying as it is the most common active ingredient used to control adult mosquitoes through indoor sprays, outdoor fogs and treated bednets [13].

In *C. quinquefasciatus* as well as in other mosquitoes of public health importance pyrethroid and DDT-resistance has been associated with two major mechanisms; overexpression of detoxification genes [14–16] and a variety of alleles in the voltage-gated sodium channel (*Vgsc*) gene, called knockdown resistance (*kdr*) mutations, such as the *Vgsc*-1014F [17–19]. Consequently, developing molecular tools to detect and monitor resistance-alleles at this locus is imperative for the study of the evolution of resistance, and may also assist programme managers in the rational deployment of insecticides.

Several diagnostic tests for typing *kdr* mutations have already been developed and applied in *Culex*, *Anopheles* and *Aedes* species [20–22]. Whilst many rely on standard PCR methods [23–26], over the past few years there has been an increase in the use of high-throughput methods such as quantitative PCR (e.g. TaqMan allele-specific and melt curve analysis) and pyrosequencing [18, 21, 27].

Regardless of methodology, most assays are designed to perform bi-allelic discrimination, and there are a limited number of low-cost approaches for typing tri-allelic mutations (e.g. [20, 24, 28]) such as the alternative codons (TTA/TTT/TTC) at locus *Vgsc*-1014 already reported in *C. quinquefasciatus* populations from Africa and Asia [18, 21, 29].

To address this limitation, we designed and applied a new diagnostic assay, ETAS-PCR (Engineered-Tail Allele-Specific-PCR), which runs under standard PCR conditions, to type tri-allelic variation at *Vgsc*-1014. Samples of field-caught mosquitoes from Brazil, Tanzania and Uganda were genotyped using the ETAS-PCR/*Vgsc*-1014 assay along with pyrosequencing and TaqMan allele-discrimination to assess concordance.

Methods

Sampling

A total of 183 field-caught and pyrethroid- or DDT-selected *C. quinquefasciatus* individuals from Brazil, Tanzania and Uganda were genotyped. Mosquitoes from Campina Grande (7°13′50″S, 35°52′52″W), Paraíba, Brazil were collected in 2016 using 50 ovitraps, one per dwelling randomly distributed within a single neighbourhood. Blood-fed mosquitoes from Mwanza, Tanzania (02°28′S, 32°55′E) were collected from inside houses using a manual aspirator in 2013. Field-caught mosquitoes from Tororo (0°40′41.62″N, 34°11′11.64″E) and Jinja (0°26′ 52.285″N, 33°12′9.403″E), Uganda, were collected in 2012 while Tororo insecticide exposed mosquitoes were phenotyped using insecticide bioassays as described previously [14, 21]. Additionally, 12 specimens from Uganda, genotyped by Sanger sequencing were used as a control [27].

Genomic DNA of individual mosquitoes was isolated using the DNeasy Kit (Qiagen, United Kingdom) and samples were confirmed as *C. quinquefasciatus* through a diagnostic PCR assay [30].

ETAS-PCR assay design

Primer design

Genetic variation in primer-binding sites could result in null alleles and thereby erroneous PCR genotyping. Therefore, conserved regions in the vicinity of the *Vgsc*-1014 locus were selected from an alignment of 10 *C. quinquefasciatus* sequences from distinct geographic regions (Additional file 1: Figure S1), and 6 sequences from other *Culex pipiens* subspecies: *C. pipiens quinquefasciatus*, Saudi Arabia, Thailand, Uganda and USA; and *C. pipiens pallens* and *C. pipiens pipiens* from China and USA, respectively (see Additional file 1: Figure S2 for GenBank accession numbers).

Development of the ETAS-PCR/Vgsc1014 assay

The ETAS-PCR/*Vgsc*-1014 multiplex-assay is a single PCR reaction, followed by an endonuclease digestion. The strategy for amplification and allelic-discrimination of the three alternative alleles is depicted in Fig. 1a.

To amplify a genomic region of 178 bp encompassing the *Vgsc*-1014 locus by multiplex-PCR reaction, four primers were designed based on a conserved sequence region (Additional file 1: Figure S1) using Primer 3 software [31]; with a universal reverse primer and three specific forward primers (Fig. 1a). For each allele-specific primer, the 3′-end terminus was designed to match exclusively one of the three SNP alleles (TTA, TTT, TTC) at the last base of the *Vgsc*-1014 codon. To enhance specificity, a deliberate mismatch was also introduced within the last three bases (see Fig. 1a).

To allow allelic discrimination by agarose gel electrophoresis, three 56 bp engineered-tails were synthesized at the 5′-end of the AS forward primer, differing from each other by the engineered position of an *Eco*32I restriction site (5′-GATATC-3′), which was introduced at distinct points of each tail (Fig. 1a). Thus, after digestion of the 234 bp ETAS-PCR amplified products, each alternative allele has a distinct fragment length (Fig. 1b): TTT (181 bp), TTA (206 bp) and TTC (231 bp).

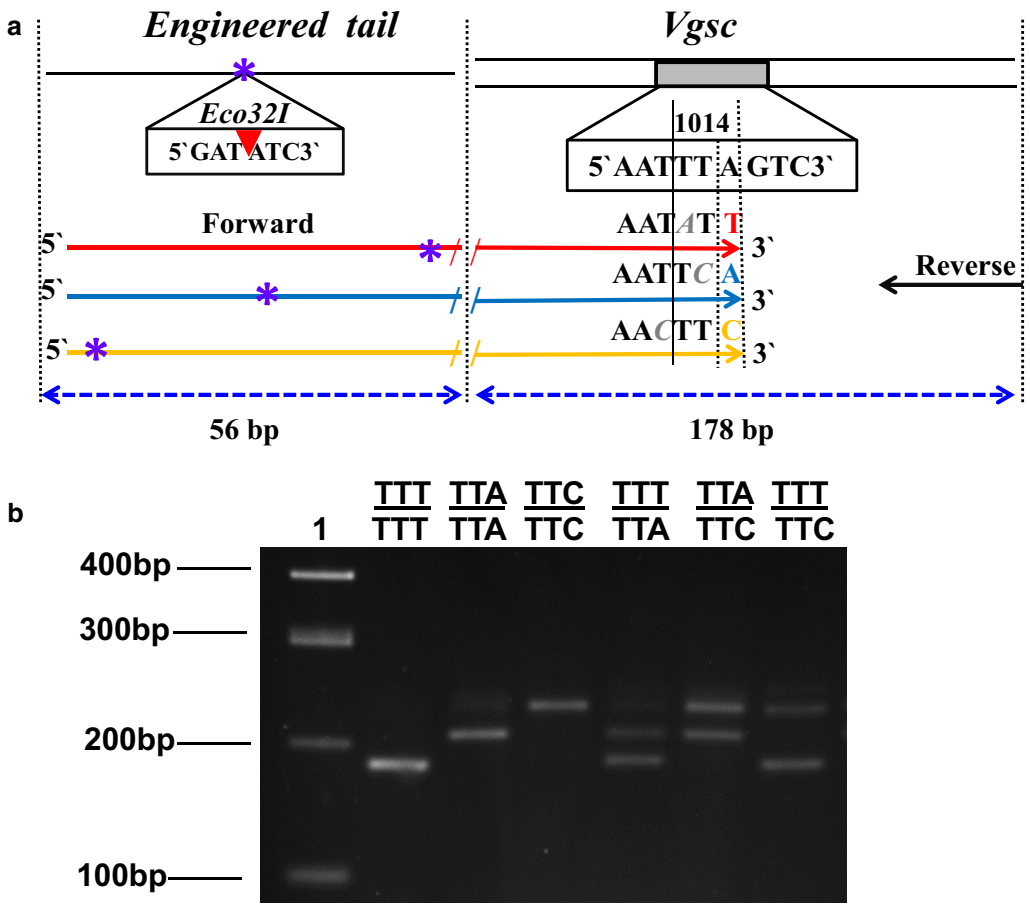

Fig. 1 Allele-specific PCR (AS-PCR) for genotyping tri-allelic *Vgsc*-L1014F variation in *C. quinquefasciatus*. **a** Schematic representation of the ETAS-PCR/*Vgsc*-1014 assay design with primer locations and predicted size of PCR products. Arrows indicate the location of PCR primers; black arrows indicate the universal reverse primer and red, blue and yellow arrows indicate each allele-specific primer (codons TTT, TTA and TTC, respectively). Bases highlighted in grey at the 3′-ends of each specific primer are deliberate mismatches. Blue-asterisks represent the restriction-site of the *Eco*32I enzyme. Full-length primer sequences are reported in Table 1. **b** An example of the ETAS-PCR/*Vgsc*-1014 gel electrophoresis. Lane 1: 100 bp DNA ladder; Lanes 2–4: homozygote for each codon: TTT, TTA and TTC, respectively; Lanes 5–6: heterozygous individuals TTT/TTA, TTA/TTC and TTT/TTC, respectively

ETAS-PCR/Vgsc-1014 amplification, endonuclease digestion and genotyping

The ETAS-PCR/*Vgsc*-1014 multiplex reaction was performed in a total volume of 25 µl containing 10 ng of gDNA, 200 µM each dNTP, 1× PCR buffer, 0.2 units HotStartTaq DNA polymerase (Qiagen), 0.6 µM universal reverse primer *Vgsc*-1014/R, 0.35 µM of the specific forward primers (*Vgsc*-1014/F-T and *Vgsc*-1014/F-C) and 0.3 µM of the *Vgsc*-1014/F-A primer (Table 1). After initial denaturation at 95 °C for 15 min, amplification was performed for 40 cycles of 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s, followed by a final extension step of 72 °C for 10 min.

After amplification, the ETAS-PCR/*Vgsc*-1014 multiplex products were digested with FastDigest *Eco*32I restriction enzyme (Thermo Scientific, United Kingdom) in a final reaction volume of 30 µl including 10 µl of the PCR product, 2 µl of 10× FastDigest Green buffer, 1.5 µl of FastDigest enzyme and 17 µl distilled water. The reaction was incubated at 37 °C for 10 min. Finally, 10 µl of digestion reaction was loaded on a 3% agarose gel.

Vgsc-1014 Screening by pyrosequencing assay

For pyrosequencing genotyping, PCR reactions were performed in a total of 25 µl containing 10 ng of gDNA, 200 µM of each dNTP, 1× PCR buffer, 2.0 mM $MgCl_2$, 0.6 units of HotStartTaq DNA polymerase (Qiagen) and 0.4 µM of primers pyr-*Vgsc*-F and pyr-*Vgsc*-R (Table 1) [21]. After initial denaturation at 95 °C for 15 min, PCR

Table 1 Primers used in the ETAS-PCR/*Vgsc*-1014, pyrosequencing and *Taq*Man assays for genotyping the *Vgsc*-L1014F in *C. quinquefasciatus* mosquitoes. The *Eco*32I site is underlined

Primer	Sequence (5′–3′)	Fragment size (bp)
Vgsc-1014/F-T	AGTAGCGGATAACAATTTCACACAGGAAGGGTTTTCCCAGTCACGACGTTGATATCGCCACCGTAGTGATAGGAAATATT	181
Vgsc-1014/F-A	AGTAGCGGATAACAATTTCACACAGGATATCGAAGGGTTTTCCCAGTCACGACGTTGCCACCGTAGTGATAGGAAATTCA	206
Vgsc-1014/F-C	GATATCAGTAGCGGATAACAATTTCACACAGGAAGGGTTTTCCCAGTCACGACGTTGCCACCGTAGTGATAGGAAACTTC	231
Vgsc-1014/R	GATCGGTATGAACTGTTTGTTTACATC	
Pyrosequencing assay		
pyr-*Vgsc*-F	CTTGGCCACCGTAGTGATAGG	105
pyr-*Vgsc*-R	Biotin-GCTGTTGGCGATGTTTTGACA	
pyrSeq. primer	CCGTAGTGATAGGAAATTT	
Dispensation	(A/C/T)GTCGTGAGTATTCCAGCGTGAAGTC	
TaqMan assay		
*Taq*Man-F	CTTGGCCACCGTAGTGATAGG	
*Taq*Man-R	GCTGTTGGCGATGTTTTGACA	
*Taq*Man-Probe-TTC	FAM-CACGACGAAATTT	
*Taq*ManProbe-TTT	FAM-TCACGACAAAATTT	
*Taq*ManProbe-TTA	VIC-ACTCACGACTAAATTT	

amplification was performed for 40 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, followed by a final extension step at 72 °C for 10 min.

The pyrosequencing genotyping was performed using single-stranded PCR products obtained using the PyroMark Q24 Vacuum Prep Workstation then used in pyrosequencing reactions performed using the PyroMark Gold Q96 reagent kit (Qiagen). The sequencing primer and dispensation order are described in Table 1.

Vgsc-1014 genotyping by TaqMan allelic discrimation

Due to the tri-allelic variation in the locus *Vgsc*-1014, two distinct TaqMan assays are applied in parallel for each sample; one to genotype TTA/TTC alleles and the other to detect TTA/TTT variants [27]. Primers and TaqMan probes are shown in Table 1.

Association between target-site alleles and resistant phenotypes

To verify the presence of *Vgsc*-1014 alleles in insecticide exposed mosquitoes, 3–5 day-old adult F1 female mosquitoes from Tororo, Uganda, were exposed to three insecticides using WHO papers impregnated with a diagnostic concentration: DDT (4%), lambda-cyhalothrin (0.05%) or deltamethrin (0.05%). Mosquitoes were exposed to DDT and lambda-cyhalothrin for 1 h and to deltamethrin for 4 h, in 4 replicates of 25 non-blood fed mosquitoes at an average temperature of 26 °C and relative humidity of 63%. Control bioassays were performed with 25 mosquitoes exposed to non-insecticide treated papers. Insecticide exposed mosquitoes were then transferred to clean holding tubes and provided with 10% glucose for a 24-h period after which mortality was recorded and alive and dead mosquitoes were collected and individually stored on silica gel.

Results and discussion

To determine if the newly-designed ETAS-PCR/*Vgsc*-1014 performed consistently across *C. quinquefasciatus* wild populations, genotyping was conducted on mosquitoes from different continents (Africa and South America). Samples from multiple locations in Africa ($n=75$); Uganda (Tororo and Jinja) and Tanzania (Mwanza) were genotyped by pyrosequencing and 74 (98.6%) agreed with the ETAS-PCR/*Vgsc*-1014 scores, with the single ETAS-PCR discordant result resulting from low PCR efficiency for that sample, which does not allow detection of PCR fragments after digestion. For the TaqMan assay, mosquitoes from Campina Grande, Brazil ($n=24$) and pyrethroid-exposed individuals from Tororo, Uganda ($n=84$) were genotyped, with 100% agreement to ETAS-PCR/*Vgsc*-1014.

The ETAS-PCR/*Vgsc*-1014 genotyping compares extremely well to the standard assays and represents a more convenient alternative for low technology laboratories because it uses relatively inexpensive laboratory methods (standard PCR and agarose-gel electrophoresis) yet allows genotyping of variation at this tri-allelic SNP in a single reaction. For instance, the genotyping cost for ETAS-PCR is around 4.6 and 3.12 times lower compared to TaqMan and pyrosequencing, respectively, based on the minimum chemistry required (e.g. DNA polymerase or master mix, either standard or biotinylated primers or fluorescence probes).

Table 2 Specificity of ETAS-PCR *Vgsc*-1014 in comparison to pyrosquencing and TaqMan genotyping

Country	Brazil				Uganda				Tanzania	
Location	Campina Grande		Tororo[a]		Tororo		Jinja		Mwanza	
Genotype	ETAS-PCR	TaqMan	ETAS-PCR	TaqMan	ETAS-PCR	Pyr	ETAS-PCR	Pyr	ETAS-PCR	Pyr
TTA/TTA	24	24	23	23	2	1	4	4	3	3
TTC/TTC			15	15	10	10	4	4	2	2
TTT/TTT			1	1			1	1	1	1
TTA/TTC			23	23	1	1	9	9	8	8
TTA/TTT			9	9			3	3	7	7
TTC/TTT			13	13	12	13	3	3	2	2
N (98)		24		84		28		24		23

[a] Mosquito exposed to insecticides (WHO—Tube Bioassay)

Pyr; Pyrosequencing

The ETAS-PCR/*Vgsc*-1014 also addresses limitations reported in other Allelic-Specific PCR (AS-PCR) assays designed for typing triple variants within a codon. For instance, the AS-PCR applied for genotyping *Vgsc*-L1014F and *Vgsc*-L1014S in *Culex pipiens pallens*, can differentiate wild-type from resistance-alleles but lacks the resolution to discriminate between the two resistance alleles [25]. Additionally, the AS-PCR of Chamnanya [32] designed to type *Vgsc*-1014 in *C. quinquefasciatus* differentiates only two (TTA and TTT) of the three alleles but can not detect the TTC resistant allele, which we found to be common in Tanzania and Uganda (Table 2) and has been reported elsewhere [29]. Indeed, in mosquitoes from Tororo, Uganda we did detect a high frequency of heterozygous wild-type/resistance-alleles among the phenotypically resistant mosquitoes exposed to either deltamethrin, lambda-cyhalothrin or DDT, for which was recorded a frequency of genotypes harboring at least one resistance-allele ranging from 70.37% to 85.78% (Additional file 2: Table S1).

Conclusions

Developing tools which are easy to apply, such as the ETAS-PCR/*Vgsc*-1014, is imperative to detect and monitor resistance-alleles while may also assist decision-making for resistance management.

Additional files

Additional file 1: Figure S1. Multiple sequence alignment of the partial fragment of the *Vgsc* gene across *Culex pipiens pipiens, Culex p. quinquefasciatus* and *Culex p. pallens*. **Figure S2.** Neighbor-joining tree of the partial fragment of the *Vgsc* gene.

Additional file 2: Table S1. Allelic and genotypic frequency of *Vgsc*-1014F in relation to mosquito survival phenotype by deltamethrin, lambda-cyhalothrin or DDT in *C. quinquefasciatus* from Tororo, Uganda.

Abbreviations

LF: lymphatic filariasis; *Vgsc*: voltage gated sodium channel; ETAS-PCR: engineered-tail allele-specific-PCR; WNV: West Nile virus; SLEV: St. Louis encephalitis virus; MDA: mass drug administration; AS-PCR: allelic-specific PCR.

Acknowledgments

We would like to thank Henry Mawejje for supporting the field-collections in Uganda. The authors also thank Dr Triantafillos Liloglou and Keith Steen for supporting the pyrosequencing genotyping.

Authors' contributions

WFSM conceived, designed the experiments and wrote the paper. WFSM, BNSP, ATVA, AM and PGSM performed the experiments. WFSM and BNSP analysed the data. BNSP, ATVA, AM, PSM, DW and MJD contributed reagents/materials/sample collections. DW, CSW and MJD reviewed the final manuscript. All authors read and approved the final manuscript.

Funding

This study was supported by The Coordination for the Improvement of Higher Education Personnel, CAPES (Grant: BEX-6193). Partial funding for this work came from UEPB/PROPESQ (Grant: 2.08.04.00-8-389/2017-1) and Wellcome Trust Training fellowship in Public Health and Tropical Medicine (Grant: 209305/Z/17/Z) to WFSM.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its additional files.

Ethics approval and consent to participate

No specific permits were required for the described field work as no human participants were involved. Oral consent was obtained from householders prior to collections and no personal details were recorded.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1] Laboratório de Entomologia Médica e Molecular, Departamento de Biologia, CCBS, Universidade Estadual da Paraíba, Campina Grande, Paraíba, Brasil. [2] Department of Vector Biology, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, UK. [3] Departamento de Genética, CB, Universidade Federal de Pernambuco, Recife, Brasil. [4] Departamento de Farmácia, CCBS, Universidade Estadual da Paraíba, Campina Grande, Paraíba, Brasil. [5] Laboratório de Interações Insetos-Tóxicos, Departamento de Agronomia e Fitossanidade, Universidade Federal Rural de Pernambuco, Recife, Brasil. [6] School of Natural Sciences and Psychology, Liverpool John Moores University, Liverpool, UK.

Received: 21 January 2019 Accepted: 6 May 2019
Published online: 14 May 2019

References

1. Farajollahi A, Fonseca DM, Kramer LD, Kilpatrick AM. "Bird biting" mosquitoes and human disease: a review of the role of *Culex pipiens* complex mosquitoes in epidemiology. Infect Genet Evol. 2011;11:1577.
2. Simonsen PE, Mwakitalu ME. Urban lymphatic filariasis. Parasitol Res. 2013;112:35–44.
3. Bockarie MJ, Pedersen EM, White GB, Michael E. Role of vector control in the global program to eliminate lymphatic filariasis. Annu Rev Entomol. 2009;54:469–87.
4. Behura SK, Lobo NF, Haas B, deBruyn B, Lovin DD, Shumway MF, et al. Complete sequences of mitochondria genomes of *Aedes aegypti* and *Culex quinquefasciatus* and comparative analysis of mitochondrial DNA fragments inserted in the nuclear genomes. Insect Biochem Molec. 2011;41:770–7.
5. Cornel A, Lee Y, Fryxell RT, Siefert S, Nieman C, Lanzaro G. *Culex pipiens sensu lato* in California: a complex within a complex? J Am Mosq Control Assoc. 2012;28:113–21.
6. Molaei G, Huang S, Andreadis TG. Vector–host interactions of *Culex pipiens* complex in northeastern and southwestern USA. J Am Mosq Control Assoc. 2012;28:127–36.
7. Hamer GL, Kitron UD, Brawn JD, Loss SR, Ruiz MO, Goldberg TL, et al. *Culex pipiens* (Diptera: Culicidae): a bridge vector of West Nile virus to humans. J Med Entomol. 2008;45:125–8.
8. Hills SL, Phillips DC. Past, present, and future of Japanese encephalitis. Emerg Infect Dis. 2009;15:1333.
9. Addiss D. The 6th Meeting of the global alliance to eliminate lymphatic filariasis: a half-time review of lymphatic filariasis elimination and its integration with the control of other neglected tropical diseases. Parasit Vectors. 2010;3:100.
10. Rivero A, Vezilier J, Weill M, Read AF, Gandon S. Insecticide control of vector-borne diseases: when is insecticide resistance a problem? PLoS Pathog. 2010;6:e1001000.
11. Coleman M, Hemingway J, Gleave KA, Wiebe A, Gething PW, Moyes CL. Developing global maps of insecticide resistance risk to improve vector control. Malar J. 2017;16:86.
12. Moyes CL, Vontas J, Martins AJ, Ng LC, Koou SY, Dusfour I, et al. Contemporary status of insecticide resistance in the major *Aedes* vectors of arboviruses infecting humans. PLoS Negl Trop Dis. 2017;11:e0005625.
13. Kleinschmidt I, Bradley J, Knox TB, Mnzava AP, Kafy HT, Mbogo C, et al. Implications of insecticide resistance for malaria vector control with long-lasting insecticidal nets: a WHO-coordinated, prospective, international, observational cohort study. Lancet Infect Dis. 2018;18:640–9.
14. Martins WFS. Evolutionary genetics of insecticide resistance in *Culex quinquefasciatus*. PhD Thesis, University of Liverpool. 2015. https://livrepository.liverpool.ac.uk/2047820/. Accessed 5 Jan 2019.
15. Mitchell SN, Stevenson BJ, Müller P, Wilding CS, Egyir-Yawson A, Field SG, et al. Identification and validation of a gene causing cross-resistance between insecticide classes in *Anopheles gambiae* from Ghana. Proc Natl Acad Sci USA. 2012;109:6147–52.
16. David J-P, Ismail HM, Chandor-Proust A, Paine MJI. Role of cytochrome P450s in insecticide resistance: impact on the control of mosquito-borne diseases and use of insecticides on Earth. Philos Trans R Soc Lond B Biol Sci. 2013;368:20120429.
17. Scott JG, Yoshimizu MH, Kasai S. Pyrethroid resistance in *Culex pipiens* mosquitoes. Pestic Biochem Physiol. 2015;120:68–76.
18. Jones CM, Machin C, Mohammed K, Majambere S, Ali AS, Khatib BO, et al. Insecticide resistance in *Culex quinquefasciatus* from Zanzibar: implications for vector control programmes. Parasit Vectors. 2012;5:78.
19. Li T, Zhang L, Reid WR, Xu Q, Dong K, Liu N. Multiple mutations and mutation combinations in the sodium channel of permethrin resistant mosquitoes, *Culex quinquefasciatus*. Sci Rep. 2012;2:781.
20. Unwin VT, Ainsworth S, Rippon EJ, Paine MJ, Weetman D, Adams ER. Development of a rapid field-applicable molecular diagnostic for knockdown resistance (kdr) markers in *An. gambiae*. Parasit Vectors. 2018;11:307.
21. Martins WFS, Wilding CS, Steen K, Mawejje H, Antão TR, Donnelly MJ. Local selection in the presence of high levels of gene flow: evidence of heterogeneous insecticide selection pressure across Ugandan *Culex quinquefasciatus* populations. PLoS Negl Trop Dis. 2017;11:e0005917.
22. De LMM, Martins AJ, Andrighetti MTM, Lima JBP, Valle D. Pyrethroid resistance persists after ten years without usage against *Aedes aegypti* in governmental campaigns: lessons from São Paulo State, Brazil. PLoS Negl Trop Dis. 2018;12:e0006390.
23. Singh OP, Bali P, Hemingway J, Subbarao SK, Dash AP, Adak T. PCR-based methods for the detection of L1014 kdr mutation in *Anopheles* culicifacies sensu lato. Malar J. 2009;8:154.
24. Zhong D, Chang X, Zhou G, He Z, Fu F, Yan Z, et al. Relationship between knockdown resistance, metabolic detoxification and organismal resistance to pyrethroids in *Anopheles sinensis*. PloS One. 2013;8:e55475.
25. Chen L, Zhong D, Zhang D, Shi L, Zhou G, Gong M, et al. Molecular ecology of pyrethroid knockdown resistance in *Culex pipiens pallens* mosquitoes. PloS One. 2010;5:e11681.
26. Linss JGB, Brito LP, Garcia GA, Araki AS, Bruno RV, Lima JBP, et al. Distribution and dissemination of the Val1016Ile and Phe1534Cys Kdr mutations in *Aedes aegypti* Brazilian natural populations. Parasit Vectors. 2014;7:25.
27. Martins WFS, Subramaniam K, Steen K, Mawejje H, Liloglou T, Donnelly MJ, et al. Detection and quantitation of copy number variation in the voltage-gated sodium channel gene of the mosquito *Culex quinquefasciatus*. Sci Rep. 2017;7:5821.
28. Tan WL, Li CX, Wang ZM, Liu MD, Dong YD, Feng XY, et al. First detection of multiple knockdown resistance (kdr)-like mutations in voltage-gated sodium channel using three new genotyping methods in *Anopheles sinensis* from Guangxi Province, China. J Med Entomol. 2014;49:1012–20.
29. Wondji CS, De Silva WAPP, Hemingway J, Ranson H, Karunaratne SHPP. Characterization of knockdown resistance in DDT- and pyrethroid-resistant *Culex quinquefasciatus* populations from Sri Lanka. Trop Med Int Health. 2008;13:548–55.
30. Smith JL, Fonseca DM. Rapid assays for identification of members of the *Culex (Culex) pipiens* complex, their hybrids, and other sibling species (Diptera: Culicidae). Am J Trop Med Hyg. 2004;70:339–45.
31. Rozen S, Skaletsky H. Primer3 on the www for general users and for biologist programmers. Methods Mol Biol. 2000;132:365–86.
32. Chamnanya S, Somboon P, Lumjuan N, Yanola J. Development of tetra-primer allele specific PCR (tetra-primer AS-PCR) for the detection of L1014F mutation in sodium channel gene associated with pyrethroid resistance in mosquito *Culex quinquefasciatus*. Am J Med Sci. 2016;49:389.

Publisher's Note