HHS Public Access

Author manuscript

J Am Mosq Control Assoc. Author manuscript; available in PMC 2015 September 08.

Published in final edited form as:

J Am Mosq Control Assoc. 2012 December ; 28(4): 323–326. doi:10.2987/12-6280R.1.

COMPARISON OF BIOCHEMICAL AND MOLECULAR TESTS FOR DETECTING INSECTICIDE RESISTANCE DUE TO INSENSITIVE ACETYLCHOLINESTERASE IN *CULEX QUINQUEFASCIATUS*[1]

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Abstract

Insecticide resistance to organophosphates and carbamates can be the result of changes in acetylcholinesterase activity conferred by the ACE-1 mutation. Detection of this altered target site mutation is important in guiding informed decisions for resistance management. In this study we compared a competitive enzyme assay with a polymerase chain reaction assay utilizing a restriction enzyme. Both assays detected the ACE-1 mutation in *Culex quinquefasciatus* and agreement was 100%. The costs and benefits of each assay are presented.

Keywords

Culex quinquefasciatus; acetylcholinesterase; organophosphate resistance; ACE-1 mutation

Because of biological fitness costs usually associated with insensitivity of target sites to insecticides, insects carrying modified target site gene(s) generally remain at a low frequency in the absence of selection pressure. Remarkably, it is only minor changes in the gene(s), often a single base change, which make target proteins (enzyme or receptor) insensitive to insecticides (Chaudhry and MacNicoll 1998). The gene, ACE-1, is present worldwide and causes organophosphate (OP) and carbamate resistance. A high level of acetylcholinesterase (AChE)-1 protein insensitivity or resistance displayed in *Culex pipiens* L. is due to a single amino acid substitution, G119S, a mutation in the 3rd exon of the ACE-1 gene, leading to the replacement of a glycine (GGC, susceptible allele) by a serine (AGC) (Weill et al. 2003, 2004). This mutation is associated with reduced susceptibility to OP insecticides, modifications of the catalytic properties of AChE-1, and a high fitness cost (Weill et al. 2003). Understanding the underlying resistance mechanisms is important in making informed decisions on alternative control strategies. In our current economic situation, with funding and programs being cut, it is important to have access to a variety of different testing methods for detecting mechanisms of resistance depending on one's budget, personnel, and time. In this study we compare a biochemical assay used in our laboratory

[1]The views of the authors do not necessarily reflect the position of the Centers for Disease Control and Prevention.

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with a molecular assay, both of which detect resistance due to insensitive acetylcholinesterase. We evaluated not only the efficacy of each assay, but also calculated the time, cost, and skill level each assay required.

Culex quinquefasciatus

Say egg rafts were collected in Harris County, TX from 2004 through 2008. Mosquitoes were reared in incubators at 27.5°C, 80–85% RH, and 14 : 10 light : dark cycle. They were aspirated into 1.5-ml microtubes 1–2 wk after they had emerged and stored in a −80°C freezer (Thermo Scientific, Waltham, MA) for future use. Fifteen mosquitoes from each collection site throughout Harris County were tested, for a total sample size of 300. In order to compare the competitive enzyme assay to the polymerase chain reaction (PCR) assay, the same mosquitoes were used for both tests. For the PCR assay and sequencing, all 6 legs were removed from the females, and the genomic DNA was extracted with the use of DNAzol® (Molecular Research Center Inc., Cincinnati, OH). The head and body were homogenized in potassium phosphate (KPO_4) buffer for the enzyme assay.

The insensitive acetylcholinesterase assay followed the procedures described by Brogdon (1988), and outlined in McAllister et al. (2012). The authors previously found that 10 min was not a sufficient amount of time to see the necessary color change (McAllister and Scott, unpublished data). The protocol was modified, and the microplate was read immediately (T_0) on a spectrophotometer with the use of a 414-nm filter, and then the microplate was stored in the refrigerator and read again after 24 h (T_{24}) (McAllister et al. 2012). The T_0 reading was subtracted from the T_{24} reading for statistical analysis. Each mosquito sample was run in triplicate on the microplate.

Mosquito genomic DNA was extracted from the legs with the use of DNAzol. The legs from individual mosquitoes were homogenized in 25 μl of DNAzol with the use of a Kontes pellet pestle cordless motor with disposable pestles and the pestles were rinsed with 25 μl of DNAzol. The homogenate was centrifuged for 2 min at 10,621 × *g* to remove insoluble tissue. A volume of 48 μl of the resulting viscous supernatant was transferred to a new 1.5-ml microtube. The DNA was precipitated from the homogenate by adding 25 μl of 100% ethanol (CAS 64-17-5). The samples were mixed by inversion to ensure a homogenous solution and incubated at room temperature for 3 min. The samples were centrifuged for 4 min at 14,000 rpm. The DNA precipitate was washed twice with 500 μl of 75% ethanol, allowed to air dry for 5–15 sec, and then resuspended in 25 μl of dH_2O (CAS 7789-20-0).

The genomic DNA extracted from the legs was PCR amplified with the degenerated primers Moustdir1 5′-CCG GGN GCS ACY ATG TGG AA-3′ and Moustrev1 5′-ACG ATM ACG TTC TCY TCC GA-3′ for 30 cycles of amplification (94°C for 30 sec, 52°C for 30 sec, and 72°C for 1 min), a procedure developed by Weill et al. (2004).

The PCR fragments were digested with *Alu*I restriction enzyme according to the manufacturer's instructions and fractionated on a 2% agarose gel. The 194 base-pair (bp) fragment amplified by PCR on genomic DNA is cut by the *Alu*1 restriction enzyme only in resistant mosquitoes. Frequencies of ACE-1 alleles were calculated as described in McAllister et al. (2012).

In order to check the identity of the amplified fragments, sequences were performed directly on PCR products of all 300 mosquitoes using the Big Dye® terminator kit (Applied Biosystems, Carlsbad, CA). The sequencing procedure outlined by Labbe et al. (2007) was followed. Two specific primers: CpEx3dir 5′-CGA CTC GGA CCC ACT CGT-3′ and CpEx3rev 5′-GAC TTG CGA CAC GGT ACT GCA-3′ generated a 457-bp fragment that amplified part of exon 3 of the ACE-1 gene, including position 119.

The concordance between insensitive AChE and the presence of the G119S mutation, as detected by the PCR test and enzyme assay, was 100% (n = 300). This was confirmed by sequencing of the PCR product. The AChE activity measured biochemically in adults is therefore due to the ACE-1 mutation. Genomic DNA amplified a 194-bp fragment that is undigested by *Alu*1 for susceptible homozygous mosquitoes, and cut into 2 fragments (74 bp and 120 bp) for resistant homozygous individuals. Heterozygous individuals displayed a combined pattern, with 3 fragments (74 bp, 120 bp, and 194 bp).

For the competitive enzyme assay, absorbance values were designated that signified susceptible homozygous, resistant homozygous, and heterozygous individuals by comparing the results to the restriction enzyme and the sequenced product. An absorbance reading of 0–0.44 nm indicated a susceptible homozygous individual, >2.45 nm a resistant homozygous individual, and a reading from 0.45–2.44 nm specified a heterozygous individual.

As seen in Table 1, of the 300 mosquitoes tested, 13 (4%) were resistant homozygous, 80 (27%) were susceptible homozygous, and 207 (69%) were heterozygous. Figure 1 shows absorbance (nm) levels detected in the tested mosquitoes. There was a wide range, 1.9 nm, of absorbance values for the heterozygous mosquitoes with the homozygous mosquito values spanning a range of 0.4 nm.

Competitive biochemical assays help detect specific resistance mechanisms in individual insects and can be used to estimate the frequency of resistance genes in populations. Propoxur is used in this assay to inhibit the activity of the sensitive (i.e., susceptible) AChE, allowing the detection of the altered enzyme when it is present. In resistant mosquitoes, the insecticide fails to inhibit AChE. The number of alleles of insensitive AChE is greater as the yellow color darkens; however, it can be difficult to determine exact cut-off points with the naked eye.

The costs, time, and skill set needed for each test vary significantly. For the enzyme assay, the cost per sample is $0.07 (assuming a 96-well plate); this includes the materials (acetone, ATCH, DTNB, propoxur, and KPO_4 buffer) and the consumables (microtiter plates, 1.5-ml tubes, and pestles). The initial equipment (spectrophotometer and pH meter) costs can range from $5,000 to $30,000. It is important to note that a spectrophotometer is not necessarily needed, as it is possible, using the naked eye, to distinguish roughly between resistant homozygous, heterozygous, and susceptible homozygous individuals by their discrete absorbance classes.

For the molecular assay, the cost per sample is $1.66 (assuming a 96-well plate), which includes the materials (Taq, DNAzol, ladder, primers, dNTPs, agarose, EtBR, ethanol, etc.) and the consumables (PCR plates, cap strips, 1.5-ml tubes, and pestles). The initial

equipment (centrifuge, thermal cycler, gel system, transilluminator, and hot water bath) costs are $40,000+.

The enzyme assay (30 mosquitoes per plate) requires 45 min, which includes making the stock chemicals; homogenizing the mosquitoes; loading the plates with homogenate, ATCH, and DNTB; and running the plates on the spectrophotometer. The plates must also be incubated for 24 h in the refrigerator in order to see the necessary color change. In total, the enzyme assay requires 45 min to complete, and a 24-h incubation period. For the molecular assay (96 mosquitoes total), one has to extract the DNA (2 h), run the PCR program (2 h), incubate the PCR product with the restriction enzyme (2–4 h), and run a gel (30–45 min), which totals 6½ to 7¾ h.

Training to conduct both assays is straightforward, but with the molecular assay it is advised that the newly trained individual be evaluated to assure proper technique. All samples are run in triplicate in the enzyme assay, so outlying data points due to pipetting errors or a large piece of homogenate can be disregarded. If an error arises when running the gel or sequencing the PCR product, it is necessary to start the assay over minus the extraction step. Also of importance is that the enzyme assay is not species specific, but can be run on any species of mosquito with no changes in chemicals or procedures. However, it is advised that the absorbance values indicating resistant/susceptible homozygous and heterozygous individuals on each species and on an individual-laboratory basis be determined.

The restriction enzyme assay was devised to detect the ACE-1 mutation in *Anopheles gambiae* Giles and *Cx. pipiens*, 2 mosquitoes belonging to different genera. The primers have also been shown to work on *An. albimanus* Wiedemann and *Cx. quinquefasciatus*. This indicates that it probably has a broad applicability within the Culicidae family (Weil et al. 2004). The assay has also been adapted to work on *An. funestus* Giles, *An. arabiensis* Patton, and *An. quadriannulatus* (Theobald) with the use of a different set of primers (Djegbe et al. 2011, Yewhalaw et al. 2011). Primers must be developed for each species of interest if not already available.

In conclusion, both assays detected ACE-1 genotypes with 100% agreement. The enzyme assay requires less time by personnel, costs less, employs a simpler technique than the PCR assay, and is not species specific. Either assay would be an invaluable option for any laboratory, and would greatly aid resistance management decisions.

Acknowledgments

The authors wish to thank the personnel of the Harris County Public Health and Environmental Services, Mosquito Control Division, who contributed to collecting and shipping eggs to the Centers for Disease Control and Prevention. In particular we wish to thank Pamela Stark and Nathan Vessey. They would also like to thank Michael Hayashi for his work on the study.

REFERENCES CITED

Brogdon WG. Microassay of acetylcholinesterase activity in small portions of single mosquito homogenates. Comp Biochem Physiol. 1988; 90C(1):145–150.

Chaudhry M, MacNicoll A. Mechanisms of insecticide resistance. Pestic Outlook. 1998; 9:23–28.

Djegbe I, Boussari O, Sidick A, Martin T, Ranson H, Chandre F, Akogbeto M, Corbel V. Dynamics of insecticide resistance in malaria vectors in Benin: first evidence of the presence of L1014S kdr mutation in *Anopheles gambiae* from West Africa. Malar J. 2011; 10:261. [PubMed: 21910856]

Labbe P, Berthomieu A, Berticat C, Alout H, Raymond M, Lenormand T, Weill M. Independent duplications of the acetylcholinesterase gene conferring insecticide resistance in the mosquito *Culex pipiens*. Mol Biol Evol. 2007; 24:1056–1067. [PubMed: 17283366]

McAllister JC, Godsey MS, Scott ML. Pyrethroid resistance in *Aedes aegypti* and *Aedes albopictus* from Port-au-Prince, Haiti. J Vector Ecol. 2012 in press.

Weill M, Duron O, Labbe P, Berthomieu A, Raymond M. Molecular clues to the insecticide resistance of mosquitoes. Med Sci. 2003; 19:1190–1192.

Weill M, Malcolm C, Chandre F, Mogensen K, Berthomieu A, Marquine M, Raymond M. The unique mutation in ace-1 giving high insecticide resistance is easily detectable in mosquito vectors. Insect Mol Biol. 2004; 13:1–7. [PubMed: 14728661]

Yewhalaw D, Wassie F, Steurbaut W, Spanoghe P, Bortel WV, Denis L, Tessema DA, Getachew Y, Cossemans M, Duchateau L, Speybroeck N. Multiple insecticide resistance: an impediment to insecticide-based malaria vector control program. PLoS One. 2011; 6:e16066. [PubMed: 21264325]

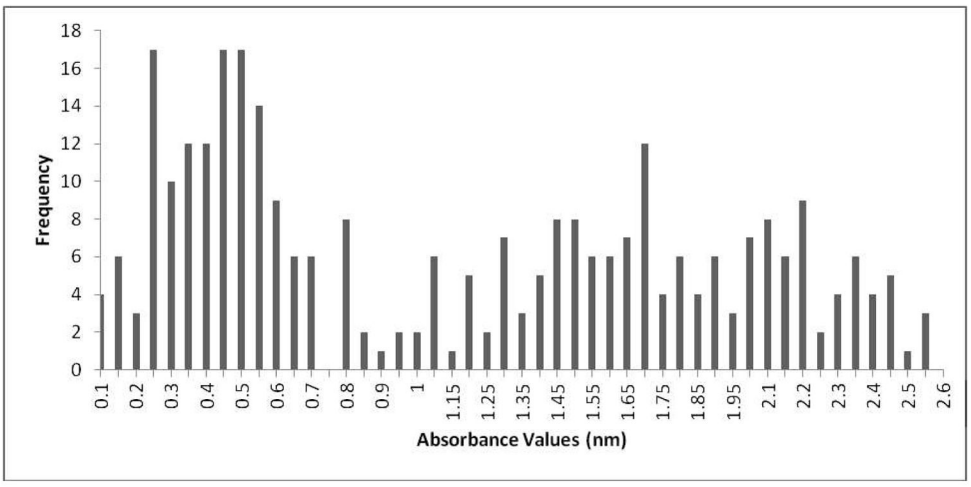

Fig. 1.

Frequency of absorbance values (nm) detected with competitive enzyme assay of *Culex quinquefasciatus* from Harris County, TX.

Table 1

Frequency of single-nucleotide polymorphism mutations associated with ACE-1 mutation in *Culex quinquefasciatus* from Harris County, TX. Susceptible homozygous individuals are Gly/Gly; resistant homozygous individuals are Ser/Ser, and heterozygotes are Gly/Ser.

Year	Sampled area	Gly/Gly	Gly/Ser	Ser/Ser	*N*	Frequency resistant allele (serine)	95% confidence index
2004	109	3	12	0	15	0.400	0.202
2005	205	4	11	0	15	0.367	0.186
	225	4	11	0	15	0.367	0.186
2006	51	1	13	1	15	0.500	0.253
	91	4	11	0	15	0.367	0.186
	93	2	13	0	15	0.433	0.219
	225	7	7	1	15	0.300	0.152
	904	3	12	0	15	0.400	0.202
	936	9	4	2	15	0.267	0.135
2007	51	5	9	1	15	0.367	0.186
	93	1	13	1	15	0.500	0.253
	205	7	8	0	15	0.267	0.135
	904	4	8	3	15	0.467	0.236
	936	4	10	1	15	0.400	0.202
2008	51	0	15	0	15	0.500	0.253
	55	0	14	1	15	0.533	0.270
	93	7	8	0	15	0.267	0.135
	109	10	5	0	15	0.167	0.084
	225	2	12	1	15	0.467	0.236
	604	3	11	1	15	0.433	0.219
Total		80	207	13	300	0.388	0.044