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Short communication

Acetylcholinesterase cDNA sequencing and identification of mutations associated with organophosphate resistance in *Cochliomyia hominivorax* (Diptera: Calliphoridae)

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ABSTRACT

Altered acetylcholinesterase (AChE) has been identified in numerous arthropod species resistant to organophosphate (OP) and carbamate insecticides. The New World screwworm (NWS) *Cochliomyia hominivorax* (Coquerel), one of the most important myiasis-causing flies in the Neotropics, has been controlled mainly by the application of OP insecticides in its current geographical distribution. However, few studies have investigated insecticide resistance in this species. Based on previous studies about mutations conferring OP resistance in related dipteran species, AChE cDNA was sequenced allowing a survey for mutations (I298V, G401A, F466Y) in NWS populations. In addition, the G137D mutation in the carboxylesterase E3 gene, also associated with OP resistance, was analyzed in the same NWS populations. Only 2/135 individuals presented an altered AChE gene (F466Y). In contrast, a high frequency of the G137D mutation in the E3 gene was found in some localities of Brazil and Uruguay, while the mutant allele was not found in Cuba, Venezuela or Colombia. These findings suggest that the alteration in the carboxylesterase E3 gene may be one of the main resistance mechanisms selected in this ectoparasite. The knowledge of the frequency of these resistance-associated mutations in the NWS natural populations may contribute to the selection of appropriate chemicals for control as part of pest management strategies.

1. Introduction

Acetylcholinesterase (AChE; EC 3.1.1.7) is a key enzyme in the nervous system, responsible for the rapid hydrolysis of the neurotransmitter acetylcholine at cholinergic synapses (Rosenberry, 1975). Organophosphate compounds (OP) target the AChE enzyme as its primary site of action, phosphorylating the active site serine to block the hydrolysis of acetylcholine, leading to the death of the insect (Menozzi et al., 2004). Point mutations in the AChE gene have been described for resistant strains of different dipteran species (Mutero et al., 1994; Walsh et al., 2001; Vontas et al., 2002; Temeyer et al., 2008). Most of these mutations in the AChE gene are conserved in these species and combinations of several point mutations in this enzyme have already been found in several alleles, where they induced higher levels of organophosphate resistance (Mutero et al., 1994).

The New World screwworm (NWS), *Cochliomyia hominivorax*, is one of the most important myiasis-causing

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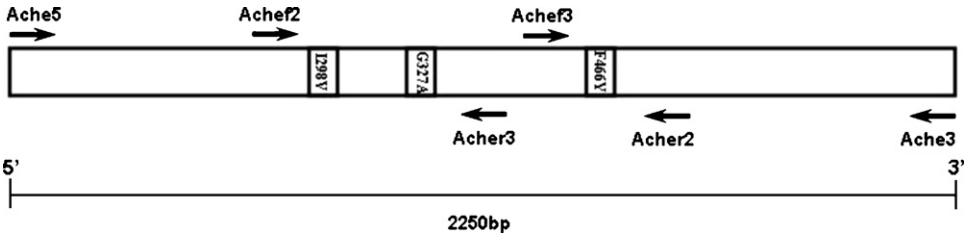

Fig. 1. Schematic view of AChE cDNA from *Cochliomyia hominivorax* showing the mutation positions (I298V, G401A, F466Y) and the hybridization sites of primers.

flies in the Neotropics, characterized by the ability of its larvae to develop in the flesh of vertebrates, causing severe economic losses to livestock industry (Hall and Wall, 1995). Although the Sterile Insect Technique (SIT) was successful for NWS eradication in North and Central America (Galvin and Wyss, 1996), throughout its current geographical distribution the control of this species has relied on the application of chemical insecticides, which normally leads to the selection of resistant individuals. Although there are few reports regarding resistance in NWS (Veríssimo, 2003; Coronado and Kowalski, 2009; Robinson et al., 2009), mutations in the carboxylesterase E3 gene are shown to involve a general form of OP resistance in *Lucilia cuprina* (Newcomb et al., 1997) and *Musca domestica* (Claudianos et al., 1999) and have been described in NWS (Carvalho et al., 2006, 2009; Silva and Azeredo-Espin, 2009), indicating a putative selective pressure by OP compounds.

In *Drosophila melanogaster*-resistant strains, the G265A mutation and the triple mutant I161V/G265A/F330Y in the AChE gene were found to be the most frequently encountered mutations (Menozzi et al., 2004). These three point mutations, also analyzed by *in vitro* site-directed mutagenesis in *L. cuprina* AChE, cause, singly and in combination, considerable insensitivity to OP (Chen et al., 2001). Based on the intensive use of OP insecticide for NWS control and its economic impact in livestock activity, in this study we sequenced a cDNA encoding AChE and surveyed the presence of these AChE mutations in NWS populations. In addition, we verified the frequency of the G137D mutation in the carboxylesterase E3 gene in the same populations. AChE sequencing will allow further studies associating NWS resistant phenotypes with altered sites in the enzyme, providing important information for NWS control.

2. Materials and methods

2.1. Samples and DNA/RNA extraction

C. hominivorax samples were collected from wounds of infested animals between 2003 and 2006 from regions throughout Brazil, including Caiapônia (BCA, 16° 57S/51° 48W), Estiva (BES, 22° 27S/46° 01W), Santa Maria das Barreiras (BSM, 08° 52S/49° 42W), Carambeí (BCI, 24° 55S/50° 05W) and Pinheiro Machado (BPM, 31° 34S/53° 23W). Samples from outside Brazil were also collected and these include Encontrados/Venezuela (VEN, 09° 03N/72° 14W); Bañado de Medina/Uruguay (UBM, 32° 23S/54° 21W); Turbo/Colombia (COT, 8° 05N/76° 43W); Ciego de Ávila/Cuba (CCA, 21° 50N/78° 46W). Ten individuals from each locality (one per wound) were used to analyze the frequency of E3 mutants, whereas for the AChE test, 15 individuals from each locality were analyzed (from at least 10 wounds). DNA was extracted from NWS larvae using the phenol-chloroform method (Infante-Vargas and Azeredo-Espin, 1995). For AChE cDNA sequencing, total RNA was extracted from NWS larvae using Trizol (Invitrogen) and the cDNA was synthesized using the SMART cDNA PCR synthesis kit (Clontech Laboratories), according to the manufacturer's instructions.

2.2. AChE cDNA sequencing

Two sets of primers, based on the *L. cuprina* AChE nucleotide sequence (Chen et al., 2001), were used for AChE amplification: Ache5 (5′ CGTCTACTATTATGGCTCG 3′) and AcheR2 (5′ CCTCATCCTTGACATTTCC 3′), Ache3 (5′ TTGAAAAATGCATGTGACC 3′) and AcheF2 (5′ CGATCCTGATCATTTAATCC 3′) (Fig. 1). The 50 μl PCR mix contained approximately 100 ng of double strand cDNA, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2 units of *Taq* polymerase (Invitrogen), 70 μM of each dNTP, 3.5 mM $MgCl_2$, 0.5 mg/ml BSA and 0.5 μM of each primer. After an initial denaturing step of 3 min at 96 °C, 35 cycles were performed, each one consisting of 1 min at 95 °C, 1 min at 52 °C and 2 min at 72 °C, with a final step of 10 min at 72 °C to fully extend all amplicons. PCR products were cloned into the pGEM-T plasmid vector (Promega) and sequenced (three clones for each fragment) using forward and reverse primers. Sequencing was performed with the Big Dye™ Terminator Cycle Sequencing Ready Kit, version 3.0 (ABI Prism™, Perkin Elmer) and an ABI 3700 Applied Biosystems Model automated DNA sequencer. Nucleotide sequences of NWS were analyzed by BLASTN (Altschul et al., 1997) to search for similarities, and sequence alignments were carried out using ClustalX (Thompson et al., 1997). The prediction of the signal peptide was performed using Signal P v.3.0 (Bendtsen et al., 2004).

2.3. E3 and AChE genotyping

To genotype the E3 gene, PCR-RFLP reactions were performed according to Carvalho et al. (2006). Based on the AChE sequence obtained in this work, new primers were designed, Achef3 (5′ AATCCCCAATCGGTTATG 3′) and Acher3 (5′ TTGCAATCATTTATCAAAGC 3′), to analyze the occurrence of the three point mutations associated with OP resistance (I298V, G401A, F466Y), avoiding the amplifica-