

Survey of Resistance to Permethrin and Diazinon and the Use of a Multiplex Polymerase Chain Reaction Assay to Detect Resistance Alleles in the Horn Fly, *Haematobia irritans irritans* (L.)

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ABSTRACT A field survey was conducted in 2001 to evaluate resistance to pyrethroid and organophosphate (OP) insecticides on horn flies, *Haematobia irritans irritans* (L.), from seven ranches in the state of Tamaulipas, Mexico, and from three locations in central Texas. Filter papers impregnated with either technical permethrin or diazinon were used to measure the levels of resistance to pyrethroids and OPs. A multiplex polymerase chain reaction (PCR) assay was used on individual horn flies from these field populations to detect the presence of the *kdr* and *super-kdr* alleles associated with pyrethroid resistance, and a mutated $\alpha E7$ esterase allele associated with OP resistance. Relative to a susceptible laboratory (Kerrville) strain, horn flies from Mexico exhibited 5.1- to 28.3-fold resistance to permethrin at the LC₅₀, and 23.8- to 136-fold resistance at the LC₉₀. Horn flies from Texas ranches exhibited only two- to five-fold resistance. All field populations of the horn fly were highly susceptible to diazinon, and no mutant $\alpha E7$ esterase alleles were detected. The *super-kdr* allele was found only in a single fly from a ranch in Mexico. Results of PCR assays showed that the *kdr* allele was present at various frequencies in field populations of horn flies. A gender-related bias in distribution of *kdr* genotypes was found in horn flies from Mexico, but not in horn flies from Texas. The overall *kdr* allelic frequencies in horn flies from Mexico were 23.2-37.8% higher in females than in males. Regression analysis revealed a significant correlation between *kdr* allelic frequencies and the levels of knockdown resistance to permethrin among the horn fly populations studied. The results validate the role of the PCR-based molecular assay as a diagnostic tool in monitoring resistance to pyrethroids and also provide useful information on population genetics of horn fly resistance to pyrethroids and OPs.

KEY WORDS insecticide resistance, permethrin, diazinon, *kdr*, the horn fly

THE HORN FLY, *Haematobia irritans irritans* (L.) remains one of the most important ectoparasites of pastured cattle throughout the American continents. The control of this pest relies heavily on chemical insecticides, particularly synthetic pyrethroids and organophosphates (OPs) (Kunz and Kemp 1994). In the United States, resistance to pyrethroid insecticides developed in several states soon after the introduction of pyrethroid-impregnated ear tags in the mid-1970s (Quisenberry et al. 1984, Kunz and Schmidt 1985, Sparks et al. 1985, Crosby et al. 1991). Resistance to pyrethroid insecticides in horn flies has also been reported in recent years in other countries, including Mexico (Kunz et al. 1995), Argentina (Sheppard and Torres 1998, Guglielmone et al. 1999), and Brazil (Barros et al. 2002). Various resistance mitigation strategies, such

as insecticide mixtures, synergists, or rotation with OPs, have been tried to alleviate problems of resistance to pyrethroids in horn flies (Byford et al. 1987, Sparks and Byford 1988, McKenzie and Byford 1993, Barros et al. 1999, Guglielmone et al. 1999). However, early detection of resistance in field populations of pests is the key for successful mitigation of resistance problems (Brent 1986). Several very helpful bioassay methods, including the filter paper bioassay, have been developed and used for testing horn fly susceptibility to pyrethroid insecticides (Schmidt and Miller 1987, Sheppard and Hinkle 1987).

Studies on mechanisms of resistance to pyrethroid insecticides in the horn fly indicate the contribution of both insensitive sodium channel, the target of pyrethroid action, and enhanced activity of metabolic enzymes, particularly mixed function oxidases (MFOs), in resistant horn fly populations (Bull et al. 1988, Sheppard 1995, Guerrero et al. 1997). Bioassays are important in measuring the levels of resistance and cross-resistance in pest populations, and in determining the possible role of metabolic enzymes in resistance (By-

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ford et al. 1985, Sheppard 1995). However, bioassay data give no information on the genotypic composition or the frequency of resistance genes in a population. Sodium channel gene mutations, including *kdr* and *super-kdr*, have been reported as major mechanisms of pyrethroid resistance in several insect species, including the fruit fly *Drosophila melanogaster* (Meigen) (Amichot et al. 1992), the house fly, *Musca domestica* L. (Williamson et al. 1993), tobacco budworm, *Heliothis virescens* (F.) (Taylor et al. 1993), German cockroach, *Blattella germanica* (L.) (Dong 1997), tobacco whitefly, *Bemisia tabaci* (Gennadius) (Morin et al. 2002), and the horn fly (Guerrero et al. 1997). A polymerase chain reaction (PCR)-based technique has been developed to detect the *kdr* and *super-kdr* mutations in individual horn flies (Guerrero et al. 1998). This technique has been successfully used to investigate field dynamics of pyrethroid target site-based resistance in horn flies (Guerrero et al. 2002, Guglielmo et al. 2002).

We conducted a field survey in 2001 in the state of Tamaulipas, Mexico, and several ranches in central Texas to test for horn fly susceptibility to permethrin and diazinon. An additional goal of the study was to test whether there was a correlation between the *kdr* allelic frequency with the level of target site resistance to permethrin. We used a multiplex PCR assay to assess the role of *kdr* and *super-kdr* alleles in pyrethroid resistance. This assay was also capable of detecting the presence of an amino acid substitution at Gly 137 of the metabolic esterase $\alpha E7$. This amino acid substitution has been associated with OP resistance in the blow fly *Lucilia cuprina* Weidemann (Newcomb et al. 1997) and *M. domestica* (Claudianos et al. 1999). The susceptible wild-type allele of $\alpha E7$ has been cloned from the horn fly (Guerrero 2000). However, an extensive survey of horn fly populations for the presence of mutant alleles has never been performed. This article reports the results of permethrin and diazinon bioassays, and the multiplex PCR assays.

Materials and Methods

Horn Flies. The field study was conducted from May through September 2001 using adult horn flies from seven ranches in the state of Tamaulipas, Mexico, and three ranches in central Texas in the United States. The Laboritas, La Negra, and Guadalupe ranches were located near the city of Victoria, and the La Gaviota, El Rayo, La Mesa, and Santa Martha ranches were located in the Aldama area of Tamaulipas. The Santos and Rogers ranches were located near Kerrville, TX. Camp Stanley was located west of San Antonio, TX. A laboratory susceptible horn fly strain maintained at the USDA-ARS Knippling-Bushland U.S. Livestock Insects Research Laboratory in Kerrville, TX, was used as a reference strain.

Bioassays. A filter paper method (Sheppard and Hinkle 1987) was used to test the susceptibility of horn flies to permethrin and diazinon. Technical grade permethrin with 92.21% active ingredient ([AI]; *cis:trans* ratio = 1:3) and diazinon (87.7% [AI]) were obtained

from FMC (Philadelphia, PA) and ECTO Development Corporation (Excelsior Springs, MO), respectively. Serial dilutions of each insecticide were made in acetone yielding six to nine concentrations of active ingredient for each bioassay. One milliliter of each dilution was applied evenly to a filter paper circle (Whatman no. 1, Whatman, Maidstone, England) in a fume hood. Three filter papers were prepared for each dilution. Filter papers treated only with acetone were used as controls. Acetone was allowed to evaporate from the filter paper for 2 h. The treated filter papers were used either immediately in bioassays or wrapped in aluminum foil and stored at -20°C .

Horn flies were collected from penned cattle with a sweep net and transferred to a small holding cage. The cage was then immediately taken to a shaded site where flies were transferred to petri dishes containing filter paper circles treated with various concentrations of permethrin or diazinon. Each petri dish contained 15–20 unsexed, mixed age flies. Mortality of horn flies in each petri dish was determined 2 h after the introduction of flies. The immobile flies and those unable to crawl on the filter paper were considered dead. Bioassays on the susceptible reference strain were conducted at room temperature in a laboratory at Knippling-Bushland U.S. Livestock Insects Research Laboratory.

PCR Assays. A portion of horn flies collected from cattle at each ranch in Mexico was immediately put in 75% ethanol, and kept at 4°C in a refrigerator until assayed by PCR. Horn fly samples from ranches in Texas were frozen and stored at -70°C for use in PCR assays. Genomic DNA was isolated from individual adult flies by pulverizing in prechilled 1.5-ml microcentrifuge tubes kept on dry ice using disposable pellet pestles (Kontes, Vineland, NJ), followed by the addition of 25 μl of sample buffer (100 mM Tris, pH 8.3, 500 mM KCl) and continued grinding. After 15-s microcentrifugation, tubes were placed in a boiling water bath for 3 min. After a 5-min centrifugation at $15,000 \times g$, an aliquot was diluted 1:10 in water, and 1 μl of this diluted DNA solution was used for PCR. The entire male fly was used for DNA isolation, whereas only the heads of female flies were used to avoid DNA contamination from eggs. Multiplex PCR was performed in 0.5 ml of thin-walled microcentrifuge tubes (Bio-Rad, Hercules, CA) in a final reaction volume of 20 μl . Each reaction contained 1 μl of diluted genomic DNA solution from a single fly (20–150 ng), primers as described below, 14 mM Tris(hydroxymethyl)aminomethane hydrochloride, pH 8.3, 70 mM KCl, 0.05 mM each dNTP, 2.0 mM MgCl_2 , and 0.2 μl of a 1:1 (by volume) mix of *AmpliTaq* DNA polymerase (5 U/ μl stock, PerkinElmer Life Sciences, Foster City, CA) and *TaqStart* antibody (1.1 $\mu\text{g}/\mu\text{l}$ stock, BD Biosciences Clontech, Palo Alto, CA). Primers were used at the following concentrations: FG130, FG134, FG138, FG235, FG154, FG155, FG243, and FG234 at 1.0 μM ; and FG236, FG238, FG239, FG240, and FG241 at 0.5 μM (Table 1). Each complete PCR resistance genotyping assay requires two amplification reactions. To assay for the presence of susceptible alleles, only

Table 1. Sequences of PCR primers

| Primer | Sequence | Description |
|--------|----------------------|--|
| FG130 | TACTGTTGTCATCGGCAATC | Sus upstream <i>kdr</i> diagnostic |
| FG134 | TACTGTTGTCATCGGCAATT | Res upstream <i>kdr</i> diagnostic |
| FG138 | CAATATTACGTTTACCCAG | Sus/Res downstream <i>kdr</i> primer |
| FG235 | CTTCGGTATTCAAATGGCA | Sus/Res upstream <i>super-kdr</i> primer |
| FG154 | ACCCATTGTCCGGCCCA | Sus downstream <i>super-kdr</i> diagnostic |
| FG155 | ACCCATTGTCCGGCCCG | Res downstream <i>super-kdr</i> diagnostic |
| FG236 | TTGTTGTCATGTCGCCTCC | Sus/Res upstream $\alpha E7$ primer |
| FG238 | GCCACAAATGAAACC | Sus downstream $\alpha E7$ primer |
| FG239 | GCCACAAATGAAACG | Res downstream $\alpha E7$ primer |
| FG240 | GCCACAAATGAAACA | Res downstream $\alpha E7$ primer |
| FG241 | GCCACAAATGAAACT | Res downstream $\alpha E7$ primer |
| FG243 | GCCATGGCTTCCGTGTCC | GAPDH PCR positive control primer |
| FG234 | CTTCTTCATCGGTGATC | GAPDH PCR positive control primer |

primers FG236, FG238, FG138, FG235, FG154, FG243, FG234, and FG130 were included in the reaction mix. To assay for resistant alleles, only primers FG236, FG239, FG240, FG241, FG138, FG134, FG235, FG155, FG243, and FG234 were included in the reaction mix. Primers FG243 and FG234 were included in each reaction to amplify a 154-base pair (bp) horn fly glyceraldehyde-3-phosphate dehydrogenase (GAPDH) fragment, serving as a positive control in each PCR experiment. Amplification was carried out using a DNA Engine (MJ Research, Watertown, MA), programmed for 96°C for 2 min followed by 35 cycles, each consisting of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 1 min. The program also included a final extension step at 72°C for 7 min. Reaction products were fractionated on 3.5% NuSieve agarose (FMC BioProducts, Rockland, ME) Tris borate-EDTA gels, and DNA was visualized by staining with GelStar DNA staining dye (FMC BioProducts) and UV illumination. The sizes of the diagnostic PCR products for the *kdr*, *super-kdr*, and $\alpha E7$ alleles were 285, 72, and ≈ 250 bp, respectively.

Data Analysis. Probit analysis of dose-mortality data were conducted using POLO-PC (LeOra Software 1987). Resistance factors (RFs) were calculated using the method described by Robertson and Preisler

(1992) that takes account of the variance and covariance of the slope and intercept of each regression line at LC_{50} or LC_{90} . Resistance factors were calculated relative to the Kerrville laboratory susceptible reference strain. Chi-square tests were used to compare the genotype distributions in male and female flies of each horn fly population. The LC_{50} data were subjected to log transformation for use in analysis of correlation with *kdr* allelic frequency using JMP software (SAS Institute 2000).

Results

Levels of Resistance. Results of permethrin bioassays on field populations of the horn fly are summarized in Table 2. Horn flies collected from ranches in Texas had low levels of resistance to permethrin. Two- to three-fold resistance was detected based on LC_{50} estimates, and up to five-fold resistance was detected based on LC_{90} estimates. Mexican horn fly populations had much higher resistance to permethrin, ranging from 5.1- to 28.3-fold at the LC_{50} and 23.8- to 136.2-fold at the LC_{90} . The reference strain from the Kerrville laboratory was highly susceptible. The high slope (=6.5) of its probit line suggests a homogeneous population (Table 2). Less steep slopes of the probit lines for three populations of horn flies from Texas indicate

Table 2. Results of permethrin bioassays with field populations of horn flies from Texas and Mexico

| Horn fly population ^a | Dose-mortality responses | | | | | | |
|----------------------------------|--------------------------|-------------|----------|--|------------------|--|-------------------|
| | n | Slope (SE) | χ^2 | LC_{50} (95% CI) $\mu g(AI)/cm^2$ | RF (95% CI) | LC_{90} (95% CI) $\mu g(AI)/cm^2$ | RF (95% CI) |
| Laboratory | | | | | | | |
| Kerrville | 520 | 6.50 (0.52) | 23.72* | 1.45 (1.35-1.55) | 1 | 2.28 (2.07-2.60) | 1 |
| Texas | | | | | | | |
| Rogers | 629 | 3.15 (0.68) | 49.19 | 2.81 (1.17-3.82) | 1.9 (1.5-2.6) | 7.18 (5.34-15.64) | 3.2 (2.4-4.1) |
| Camp Stanley | 450 | 2.80 (0.35) | 7.95* | 3.27 (2.90-3.71) | 2.3 (2.0-2.5) | 9.38 (7.38-13.68) | 4.1 (3.1-5.5) |
| Santos | 640 | 3.27 (0.48) | 39.8* | 4.4 (3.6-5.4) | 3.1 (2.7-3.5) | 10.98 (8.33-18.40) | 4.8 (3.6-6.4) |
| Mexico | | | | | | | |
| La Negra | 234 | 1.22 (0.30) | 8.60* | 7.37 (4.81-12.34) | 5.1 (3.3-7.7) | 82.04 (33.55-911.97) | 36.0 (9.7-133.5) |
| La Gaviota | 167 | 1.64 (0.33) | 18.18 | 9.01 (4.54-17.11) | 6.2 (4.6-8.5) | 54.36 (24.32-1635.7) | 23.8 (10.9-51.9) |
| El Rayo | 196 | 1.51 (0.33) | 41.32 | 11.65 (—) | 8.1 (5.7-11.3) | 81.75 (—) | 35.8 (12.8-99.9) |
| Santa Martha | 167 | 2.62 (0.66) | 12.56* | 20.55 (13.66-60.15) | 14.2 (9.9-20.4) | 63.51 (31.69-1589) | 27.9 (12.2-63.6) |
| Guadalupe | 307 | 1.36 (0.44) | 8.10* | 23.01 (13.45-60.35) | 15.9 (9.0-28.0) | 201.49 (70.56-16379) | 88.2 (16.3-479.1) |
| Laborcitas | 303 | 1.46 (0.48) | 17.45* | 41.00 (—) | 28.3 (13.1-61.2) | 311.00 (—) | 136.2 (18.3-1013) |

^a No permethrin bioassay was conducted on horn flies from the La Mesa ranch.

*, not significantly different from the probit model ($P > 0.05$); —, probit analysis program failed to generate CI with existing field data.

Table 3. Results of diazinon bioassays with field populations of horn flies from Texas and Mexico

| Horn fly population | Dose-mortality responses | | | | | | |
|----------------------|--------------------------|-------------|----------|--|---------------|--|---------------|
| | <i>n</i> | Slope (SE) | χ^2 | LC ₅₀ (95% CI) μg (AI)/cm ² | RF (95% CI) | LC ₉₀ (95% CI) μg (AI)/cm ² | RF (95% CI) |
| Laboratory Kerrville | 450 | 9.37 (0.76) | 46.32 | 2.45 (2.25–2.69) | 1 | 3.36 (3.00–4.09) | 1 |
| Texas Rogers | 540 | 5.03 (0.50) | 26.75* | 2.83 (2.53–3.10) | 1.1 (1.0–1.3) | 5.08 (4.52–6.01) | 1.5 (1.3–1.7) |
| Camp Stanley | 450 | 4.49 (0.55) | 21.28* | 1.79 (1.43–2.06) | 0.7 (0.6–0.8) | 3.45 (2.99–4.35) | 1.0 (0.9–1.2) |
| Santos | 660 | 4.87 (0.51) | 32.41* | 2.89 (2.55–3.20) | 1.2 (1.1–1.3) | 5.30 (4.72–6.20) | 1.6 (1.4–1.8) |
| Mexico La Negra | 395 | 4.96 (0.76) | 6.64* | 1.23 (1.00–1.40) | 0.5 (0.4–0.6) | 2.23 (2.01–2.56) | 0.7 (0.6–0.8) |
| La Gaviota | 269 | 4.83 (0.89) | 8.46* | 1.39 (1.06–1.64) | 0.6 (0.5–0.7) | 2.57 (2.22–3.19) | 0.8 (0.6–0.9) |
| El Rayo | 282 | 4.95 (0.83) | 18.79* | 1.30 (0.96–1.51) | 0.5 (0.4–0.6) | 2.35 (2.09–2.87) | 0.7 (0.6–0.8) |
| Santa Martha | 240 | 5.10 (1.21) | 40.50 | 1.21 (—) | 0.5 (0.4–0.6) | 2.15 (—) | 0.6 (0.5–0.8) |
| La Mesa | 194 | 4.62 (1.99) | 8.77* | 0.79 (—) | 0.3 (0.2–0.6) | 1.49 (—) | 0.4 (0.4–0.6) |
| Guadalupe | 203 | 6.79 (1.24) | 14.98* | 1.85 (1.55–2.08) | 0.6 (0.5–0.7) | 2.85 (2.48–3.73) | 0.6 (0.5–0.8) |
| Laborcitas | 443 | 2.89 (0.57) | 53.03 | 0.79 (0.12–1.26) | 0.3 (0.2–0.5) | 2.19 (1.47–3.51) | 0.7 (0.5–0.8) |

*, not significantly different from the probit model ($P > 0.05$). —, probit analysis program failed to generate CI with existing field data.

the heterogeneity of these populations. All horn fly populations from Mexico were highly heterogeneous, as suggested by very low slope values of the probit lines (Table 2).

Susceptibility of horn flies from ranches in Texas to diazinon was similar to that of the susceptible reference strain (Table 3). Horn flies from ranches in Mexico generally exhibited a higher susceptibility to dia-

zidon, as indicated by lower LC₅₀ estimates. Regression analysis using LC₅₀ estimates showed a negative cross-resistance between diazinon and permethrin among those horn fly populations ($F = 12.77$; $df = 1, 8$; $r^2 = 0.62$; $P < 0.01$).

Genotypes of Horn Fly Samples. Table 4 summarizes the results of the multiplex PCR assays. Based on presence or absence of the *kdr* allele, individual flies

Table 4. Summary of PCR assays of adult horn flies from ranches in Texas and Mexico

| Horn fly population ^a | Sex | Total no. of flies | Genotype* | | | Genotype frequency (%) | | | <i>kdr</i> allele freq. (%) |
|----------------------------------|------|--------------------|-----------|----|----|------------------------|------|------|-----------------------------|
| | | | SS | SR | RR | SS | SR | RR | |
| Kerrville | Both | 40 | 40 | 0 | 0 | 100.0 | 0.0 | 0.0 | 0.0 |
| | M | 20 | 20 | 0 | 0 | 100.0 | 0.0 | 0.0 | 0.0 |
| | F | 20 | 20 | 0 | 0 | 100.0 | 0.0 | 0.0 | 0.0 |
| Rogers | Both | 31 | 24 | 7 | 0 | 77.4 | 22.6 | 0.0 | 11.3 |
| | M | 13 | 8 | 5 | 0 | 61.5 | 38.5 | 0.0 | 19.2 |
| | F | 18 | 16 | 2 | 0 | 88.9 | 11.1 | 0.0 | 5.6 |
| Camp Stanley | Both | 36 | 25 | 11 | 0 | 69.4 | 30.6 | 0.0 | 15.3 |
| | M | 18 | 13 | 5 | 0 | 72.2 | 27.8 | 0.0 | 13.9 |
| | F | 18 | 12 | 6 | 0 | 66.7 | 33.3 | 0.0 | 16.7 |
| Santos | Both | 38 | 28 | 9 | 1 | 73.7 | 23.7 | 2.6 | 14.5 |
| | M | 18 | 14 | 3 | 1 | 77.8 | 16.7 | 5.6 | 13.9 |
| | F | 20 | 14 | 6 | 0 | 70.0 | 30.0 | 0.0 | 15.0 |
| La Negra | Both | 38 | 5 | 24 | 9 | 13.2 | 63.2 | 23.7 | 55.3 |
| | M | 18 | 3 | 15 | 0 | 16.7 | 83.3 | 0.0 | 41.7 |
| | F* | 20 | 2 | 9 | 9 | 10.0 | 45.0 | 45.0 | 67.5 |
| La Gaviota | Both | 38 | 7 | 18 | 13 | 18.4 | 47.4 | 34.2 | 57.9 |
| | M | 20 | 6 | 12 | 2 | 30.0 | 60.0 | 10.0 | 40.0 |
| | F* | 18 | 1 | 6 | 11 | 5.6 | 33.3 | 61.1 | 77.8 |
| El Rayo | Both | 35 | 2 | 11 | 22 | 5.7 | 31.4 | 62.9 | 78.6 |
| | M | 15 | 1 | 9 | 5 | 6.7 | 60.0 | 33.3 | 63.3 |
| | F* | 20 | 1 | 2 | 17 | 5.0 | 10.0 | 85.0 | 90.0 |
| Santa Martha | Both | 38 | 12 | 19 | 7 | 31.6 | 50.0 | 18.4 | 43.4 |
| | M | 20 | 9 | 9 | 2 | 45.0 | 45.0 | 10.0 | 32.5 |
| | F | 18 | 3 | 10 | 5 | 16.7 | 55.6 | 27.8 | 55.6 |
| Guadalupe | Both | 39 | 5 | 17 | 17 | 12.8 | 43.6 | 43.6 | 65.4 |
| | M | 20 | 5 | 10 | 5 | 25.0 | 50.0 | 25.0 | 50.0 |
| | F* | 19 | 0 | 7 | 12 | 0.0 | 36.8 | 63.2 | 81.6 |
| Laborcitas | Both | 40 | 1 | 15 | 24 | 2.5 | 37.5 | 60.0 | 78.8 |
| | M | 20 | 1 | 12 | 7 | 5.0 | 60.0 | 35.0 | 65.0 |
| | F* | 20 | 0 | 3 | 17 | 0.0 | 15.0 | 85.0 | 92.5 |

^a No flies from the La Mesa ranch were saved for PCR assays.

*, chi-square test statistics for comparing genotype distribution in male and female horn flies: La Negra ($\chi^2 = 10.62$, $df = 2$, $P < 0.01$); La Gaviota ($\chi^2 = 11.73$, $df = 2$, $P < 0.01$); El Rayo ($\chi^2 = 10.50$, $df = 2$, $P < 0.01$); Guadalupe ($\chi^2 = 8.39$, $df = 2$, $P < 0.05$); and Laborcitas ($\chi^2 = 10.57$, $df = 2$, $P < 0.01$).

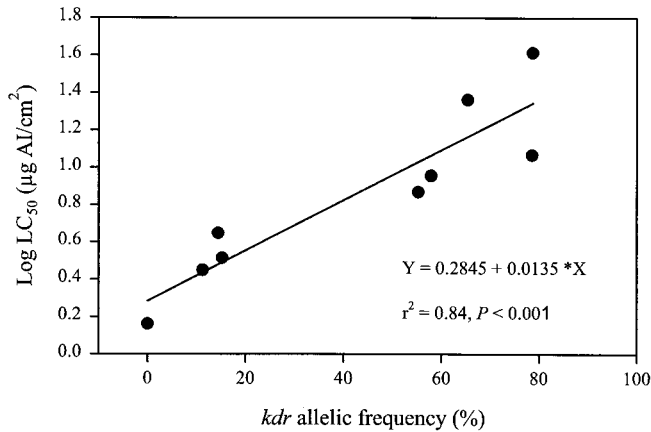


Fig. 1. Correlation between *kdr* allelic frequency and log LC₅₀ of horn flies from ranches in Texas and Mexico.

can be genotyped as homozygous susceptible (SS), homozygous resistant (RR), or heterozygous (SR) genotype. No mutant *αE7* allele was detected in any of the horn fly samples tested, and only one male fly from El Negra ranch from Mexico was found to carry the *super-kdr* allele. Therefore, *super-kdr* and *αE7* data were not listed in this table. Although the mutant *αE7* allele associated with OP resistance was not found in these samples, the *αE7* reactions served as an additional positive control for these PCRs. None of these three resistant alleles was present in the Kerrville reference strain. PCR assays revealed clear differences in overall *kdr* allelic frequency between resistant and susceptible horn fly populations. Relatively low *kdr* allelic frequencies (11.3–15.3%) were detected in horn flies collected from Texas ranches. The *kdr* allele existed exclusively in heterozygous form on the Rogers and Camp Stanley ranches, and only a small portion (2.6%) of the flies possessed homozygous (RR) *kdr* alleles on the Santos ranch. Between 18.4 and 62.9% of the horn flies from Mexico possessed homozygous (RR) *kdr* alleles. *Kdr* allelic frequency ranged from 43.4 to 78.8% in the Mexican horn fly populations. Regression analysis revealed a significant correlation between *kdr* allelic frequency and the log transformations of the LC₅₀ estimates ($P < 0.001$; Fig. 1). In addition, the *kdr* allelic frequencies in females were 23.15–37.8% higher than that of the male flies in populations from Mexico. Chi-square tests indicated significant differences in genotype distribution between male and female flies in all Mexican horn fly populations except in the Santa Martha population (Table 4). The genotype distributions were not significantly different between male and female horn flies in populations from Texas.

Discussion

Pyrethroids, along with OP pesticides, have been used extensively in Mexico to control cattle ticks and the horn fly. Among OPs used there, diazinon was not used widely due to its high toxicity to cattle when used

as dipping or pour-on formulations. Ticks were the primary target of chemical treatments, and horn flies were exposed to pesticides and controlled as well at the same time (Kunz 1991, 1995). High levels of resistance to fenvalerate, a pyrethroid insecticide, were reported earlier in horn fly populations in several northwestern states of Mexico (Kunz et al. 1995). Horn flies resistant to cypermethrin were also found recently in several states in Mexico (C.A.G., unpublished data). Permethrin is a type I pyrethroid that possesses a negatively correlated temperature-reversible knockdown property (Matsumura 1985). The field bioassays were conducted at outdoor temperatures generally higher than room temperature. Therefore, the permethrin LCs, and consequently the RFs, may have been somewhat overestimated when the Kerrville strain, which was tested at room temperature, was used as a susceptible reference strain. Nevertheless, the results from our study confirm that resistance to pyrethroids continues to be a major problem in Mexico. However, despite the exposure of horn flies to OPs in Mexico, none of the horn fly populations we tested showed resistance to diazinon (Table 3). Indeed, populations from Mexico were slightly more susceptible to diazinon compared with populations from Texas and the Kerrville susceptible strain. It is not surprising that the *αE7* mutant allele was not present in those populations of the horn fly because they were highly susceptible to diazinon.

Our results agree with previous findings of a negative cross-resistance relationship between pyrethroid and organophosphate insecticides in horn flies in the United States (Cilek and Knapp 1993, Cilek et al. 1995). The mechanisms of resistance to pyrethroids in horn flies include both the sodium channel mutation (*kdr*) and enhanced detoxification by MFOs (Guerrero et al. 1997). The increased sensitivity to diazinon in Mexican horn flies found in this study is probably due to enhanced activity of MFOs that activate OPs, including diazinon, as reported in previous studies (Cilek and Knapp 1993, Cilek et al. 1995). Diazinon ear tags have been demonstrated to be ef-

fective in the control of pyrethroid-resistant horn flies in the United States (Byford et al. 1988, Barros et al. 1999) and could be a very useful tool in controlling pyrethroid-resistant horn flies in Mexico.

Previous reports have noted that resistance to pyrethroids was inherited as an incomplete recessive trait, and the heterozygotes (SR) were found to be 3 to 10 times less susceptible than the homozygous (SS) genotype (Roush et al. 1986, McDonald and Schmidt 1987). We observed significant differences in the distribution of resistant genotypes (SR and RR) between male and female flies in all but one horn fly population from Mexico. The female flies had higher proportions of homozygous (RR) individuals than did males, and most male flies with resistant alleles were heterozygous (SR). This could be a result of differential selection pressures on male and female flies due to differences in their feeding behaviors. In a laboratory study on steers by Harris et al. (1974), females were found to feed more frequently (38.4 feedings per day) and spend longer time feeding (163 min per day) than males (24 feedings per day and 96 min/d), therefore potentially facing a higher selection pressure on a treated animal. In addition, male horn flies were demonstrated to have a behavioral trait that allows less exposure to insecticides than females (Witherspoon and Burns 1967). In their study, nearly 100% of horn flies that inhabited the feet and lower legs of cattle were males, and very little pesticide was present on those parts of the cattle when topical treatments were applied. Therefore, under the field conditions in Mexico, female horn flies may have experienced a higher insecticide selection pressure, resulting in higher numbers of females with RR genotype surviving an insecticide treatment. In contrast, selection pressure on males may be less intense due to the behavioral differences, and males of SR genotype may have been able to survive treatment, not requiring the RR genotype.

Guerrero et al. (2002) reported higher *kdr* allele frequencies in females than males in their study of pyrethroid-resistant horn flies in Louisiana. Scott et al. (1997) demonstrated that a pyrethroid-resistant horn fly strain suffered some fitness costs regarding pupation success and fecundity in a laboratory study. Guerrero et al. (2002) also suggested that the declines in the resistance to pyrethroids and in the frequency of *kdr* alleles in the spring populations of horn flies on a Louisiana ranch could be a result of reduced survivorship of diapausing pupae of the resistant horn flies. Our data from Camp Stanley and from ranches in the Kerrville area demonstrate that the *kdr* allele can be sustained at a relatively low frequency in a population with little or no exposure to pyrethroids. Pyrethroid insecticides have been used occasionally on the Santos and Rogers ranches, and no insecticide had been used on Camp Stanley for several years. No homozygous (RR) resistant flies were detected on the Camp Stanley and Rogers ranches, and a very low proportion (2.6%) of flies at the Santos ranch possessed the RR genotype. Between 22.6 and 30.6% of the flies from those ranches were heterozygotes (SR).

A previous study by Jamroz et al. (1998) reported a high frequency (42%) of the *super-kdr* allele in a permethrin-resistant strain of the horn fly, which was under continuous selection pressure for many generations in the laboratory. We found in this study only a very low frequency (4–5%) of the *super-kdr* allele in the field populations of the horn fly with similar resistance levels to colonies studied by Jamroz et al. (1998). Guerrero et al. (2002) reported the presence of the *super-kdr* allele in horn flies on a ranch where PBO-synergized cyhalothrin ear tags were used every other year from 1991 to 1997. Ear tags impose a continuous selection pressure on horn flies, whereas spray treatments, applied several weeks apart, allow some generations of horn flies to be completed without insecticidal selection (Sheppard 1987). Ear tags have not been used widely in Mexico, which could explain the lack of *super-kdr* alleles.

PCR-based molecular techniques have been used to detect pyrethroid resistance conferred by mutated sodium channel alleles in many insects. The PCR-based genotyping technique has also been used to study field dynamics of pyrethroid resistance in the horn fly (Guerrero et al. 2002, Guglielmo et al. 2002). Our study established a positive correlation between overall *kdr* allelic frequency and permethrin resistance. To some extent this was expected. The horn fly is a relatively permanent ectoparasite that feeds very frequently (Harris et al. 1974), is somewhat difficult to colonize, and suffers high off-host mortality with bioassay periods >2–4 h. The necessarily short bioassay period thus reflects knockdown-type effects, supplemented by metabolic mechanisms. Although metabolic mechanisms, such as MFOs, are known to contribute to pyrethroid resistance in the horn fly, sodium channel mutation (*kdr*) has been suggested to be the major mechanism (Bull et al. 1988, Sparks et al. 1990). In addition, genetic linkage of insensitive sodium channel and MFO-mediated detoxification has been reported in *H. virescens* (Park and Brown 2001). No study has been conducted to test whether such linkage of genes controlling the *kdr* allele and MFOs exists in the horn fly. However, results of our synergist bioassays (data not shown) indicate that the activity of MFOs in the Mexican resistant populations was higher than the susceptible reference strain.

The correlation between *kdr* allelic frequency and the levels of resistance to pyrethroid insecticides demonstrated in this study validates the role of the PCR-based molecular assay as a diagnostic tool in detecting target site-based pyrethroid resistance in the horn fly. Due to the involvement of metabolic detoxification mechanisms in pyrethroid resistance, PCR data alone may not give an accurate estimate of the level of resistance in a population. Nevertheless, PCR-based assay is useful in detection of pyrethroid resistance from a large number of ethanol-preserved samples of field populations of the horn fly submitted by ranchers or extension agents.

It will be interesting to continue surveying pyrethroid-resistant horn fly populations with this PCR assay, synergist bioassays, and efficacy studies to de-

termine what level of target site resistance must accumulate before product failure occurs, and whether the PCR assay alone is sufficient to predict product failure. Although there are many literature reports of target site pyrethroid resistance occurring in conjunction with MFO-based resistance, there are few if any reports of metabolic-based resistance in the absence of target site-based resistance.

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