# A Comparison of Three Bioassay Techniques to Determine Amitraz Susceptibility in *Boophilus microplus* (Acari: Ixodidae)

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**ABSTRACT** The ability of the Miller, Soberanes, and White bioassay techniques to describe amitraz susceptibility in three different strains of *Boophilus microplus* (Canestrini) (Acari: Ixodidae) was compared. For a susceptible strain, all techniques adequately described amitraz susceptibility by producing a full range of mortality that corresponded with increasing concentration of amitraz. However, when resistant strains were evaluated, only the Miller and the Soberanes techniques adequately estimated the dose–response relationship. Lethal concentrations were not precisely estimated when all the data were included in the analyses for every strain and technique tested. Better estimates were obtained when subsets of data around the range of interest were subjected to probit analysis. For the Soberanes technique, the slope of the probit regression was steeper for the Brazilian resistant and Texan susceptible strains compared with the heterozygous Mexican strain. The pattern was different when the same strains were tested with the Miller technique. The slopes of the regressions for the Mexican and the Texan strains did not differ significantly, but the Brazilian strain had a steeper slope than the other strains. Resistance ratios were much greater when the Soberanes technique was used than when the Miller technique was used on the same strains. However, neither technique produced enough separation between susceptible and resistant strains to develop a traditional discriminating dose (DD) test that required a concentration of $2\times$ $LC_{99.9}$ estimate. A DD test at the $LC_{99}$ would be possible for both techniques. We discuss the strengths and weaknesses of the three techniques, including potential improvements to the White technique. The White technique has the greatest potential to determine the mechanisms of amitraz resistance in detailed synergist studies. Currently, only the Miller method can fulfill this task. The Miller and Soberanes techniques are well suited for the study of the epidemiology of resistance worldwide, because they use commercially available, formulated amitraz that is easy and inexpensive to obtain.

**KEY WORDS** resistance, *Rhipicephalus* (*Boophilus*) *microplus*, southern cattle tick, amitraz

Amitraz has been used for the control of cattle ticks, *Boophilus* spp., for >40 yr. However, resistance to amitraz in cattle ticks has been reported in the literature only recently. This delay has partially been due to an inability to quantify amitraz resistance (Kemp et al. 1998). In 2002, two bioassay techniques were developed that produced dose–response relationships in southern cattle tick, *Boophilus microplus* (Canestrini) (Acari: Ixodidae), larvae exposed to amitraz. One technique was a modification of the Shaw test (Shaw 1966). This method was used to define and report the first case report of amitraz resistance in *B. microplus* from Mexico (Soberanes-Céspedes et al. 2002). The other method was a modification of the Food and Agriculture Organization Larval Packet Test originally developed by Stone and Haydock (1962). This technique was used to detect resistance in *B. microplus* and *Rhipicephalus sanguineus* (Latreille) and was used in synergist studies at the Cattle Fever Tick Research Laboratory (CFTRL) to determine the mechanisms of amitraz resistance in *B. microplus* (Miller et al. 2002, Li 2004). A third bioassay technique, the larval immersion microassay, was developed in 2004. This technique was developed to screen many tick species to numerous experimental compounds and has been shown to produce reliable dose–response relationships to amitraz in *B. microplus* and the lone star tick, *Amblyomma americanum* (L.) (White et al. 2004).

These three types of bioassays have a great potential for use in laboratories throughout the world, but fur-

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ther evaluation must be done so that the strengths and weaknesses of each test are known. Our study was done to compare the precision of the three techniques with both susceptible and resistant strains of *B. microplus.* We discuss the strengths and weaknesses of each technique, and we suggest improvements.

## Materials and Methods

**Ticks.** All tick strains used in this study were maintained at the CFTRL. The reference strain Muñoz was collected from Zapata County, TX, in 1999. It is susceptible to pyrethroid, organophosphorous, and amidine acaricides and has been reared in the laboratory without acaricide selection. The amitraz-resistant strain San Alfonso was collected in 2001 in Tabasco, México, and was provided to the CFTRL by the Cento Nacional de Servicios de Constatacion en Salud Animal (CNSCSA) Jiutepec, Morelos, Mexico. The Santa Luiza strain was originally collected in an area of southern Brazil (Alegrete, Rio Grande do Sul) where amitraz resistance was suspected. A colony was established at the CNSCSA and subsequently shipped to the CFTRL in November 2000. Rearing conditions for *Boophilus* at the CFTRL have been described by Davey et al. (1980). In conducting the research described in this report, the investigators adhered to the Guide for the Care and Use of Laboratory Animals, as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association of Laboratory Animal Care.

**Bioassay Techniques.** The Soberanes technique was done as described by Soberanes-Céspedes et al. (2002). Formulated amitraz (Taktic 12.5% EC, Nor-am, Wilmington, DE) was diluted to specific concentrations with distilled water ($dH_2O$). Ten milliliters of each concentration was put into a 10-cm-diameter glass petri dish containing a 9-cm-diameter piece of Whatman no. 1 filter paper (Whatman, Maidstone, United Kingdom). Approximately 400 larvae (14 d old) were placed onto the wet filter paper, and a second piece of filter paper (9 cm in diameter) was placed on top. The larvae were held between these pieces of filter paper for 10 min, and then they were removed in groups of ≈100 into three untreated dry packets made of pieces of Whatman no. 1 filter paper (7.6 by 8.9 cm) sealed on the sides with steel paper clips (Bulldog, Boston Clip No. 2, Hunt Manufacturing Co., Statesville, NC) to form a packet. After the larvae were introduced to the packet, a third clip was used to seal the top. The larvae were held in an environmental chamber at 27°C, 85–90% RH, and a photoperiod of 12:12 (L:D) h. After 72 h, the packets were removed from the environmental chamber, opened, and numbers of live and dead larvae were recorded. Each concentration of the treatment was replicated three times.

The Miller technique was done as described by Miller et al. (2002). Formulated amitraz (Taktic 12.5% EC, Nor-am) was diluted to specific concentrations in 2 parts trichloroethylene (TChE; Sigma-Aldrich, St. Louis, MO) and 1 part olive oil (Sigma-Aldrich, St. Louis, MO). A volume of 0.7 ml of each concentration was applied to a piece of nylon fabric (7.6 by 8.9 cm; type 2320, Cerex Advanced Fabrics, Pensacola, FL). The trichloroethylene was allowed to evaporate from the filter paper for 2 h under a fume hood. After the trichloroethylene evaporated, the treated papers were folded in half and sealed on the sides with steel paper clips (Bulldog, Boston Clip No. 2, Hunt Manufacturing Co.). This formed a packet into which 100 larvae (14 d old) were placed. Once the larvae were inside the packet, the top was sealed with a third clip. The packets containing larvae were held in an environmental chamber at 27°C, 85–90% RH, and a photoperiod of 12:12 (L:D) h. After 24 h, the packets were removed from the environmental chamber, opened, and the number of live and dead larvae was recorded. Larvae that could walk across the treated substrate after the incubation period were scored as alive, but larvae that did not move or could only move legs without walking were scored as dead. Each concentration of the treatment was replicated three times.

The White larval immersion microassay was performed by as described by White et al. (2004). Stock solutions were prepared by dissolving ultrapure amitraz (98% pure; catalog no. PS-1005, Chem. Service, West Chester, PA) in dimethyl sulfoxide (DMSO; Sigma-Aldrich). Ninety-seven microliters of an aqueous vehicle solution ($dH_2O$, 0.2% Triton X-100, filter sterilized) was dispensed into each well of a round bottom 96-well microtiter plate (polystyrene). Amitraz was serially diluted in DMSO, and 3 $\mu$l of each acaricide dilution was dispensed to the appropriate well to yield a desired final concentration of acaricide while maintaining a constant solvent concentration in each well. The vehicle solution containing solvent alone was used as a negative control in all experiments.

After bioassay plates were prepared, ≈150 tick larvae were transferred into each well. Larvae were immersed into the acaricide solution at room temperature for 30 min. A P-200 pipet with a wide-bore tip was used to aspirate 50 $\mu$l of solution containing the larvae from each well and dispense them into the open end of a Whatman no. 1 filter paper packet (described above in the Soberanes technique). The packets containing larvae were held in an environmental chamber at 27°C, 85–90% RH, and a photoperiod of 12:12 (L:D) h. After 24 h, the packets were removed from the environmental chamber, opened, and the number of live and dead larvae was recorded.

**Statistical Analysis.** Probit analyses were done with PoloPlus (LeOra Software 2003). We first used all data (12 concentrations, replicated three times for each bioassay) to identify experimental concentrations in ranges that would estimate each $LC_{50}$, $LC_{90}$, $LC_{99}$, and $LC_{99.9}$ most precisely. In the second set of probit analyses, we used at least four consecutive concentrations that produced 10–90% mortality to estimate a more precise $LC_{50}$. Lethal concentrations ≥90 were estimated with at least four consecutive concentrations that caused 50–100% mortality and one concen-

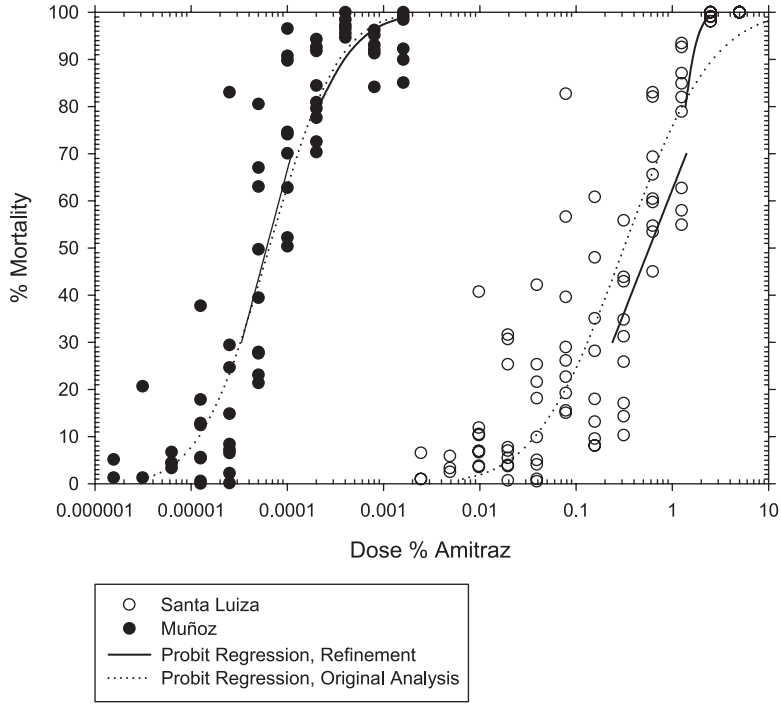


**Fig. 1.** Santa Luiza and Muñoz *B. microplus* larvae exposed to amitraz using the Soberanes technique. Circles represent observed mortality data. Dotted lines represent the original probit regression with the entire range of dose–mortality data included in the analysis. Solid lines represent the refined probit regression. Only data from the range of interest was included in the analysis.

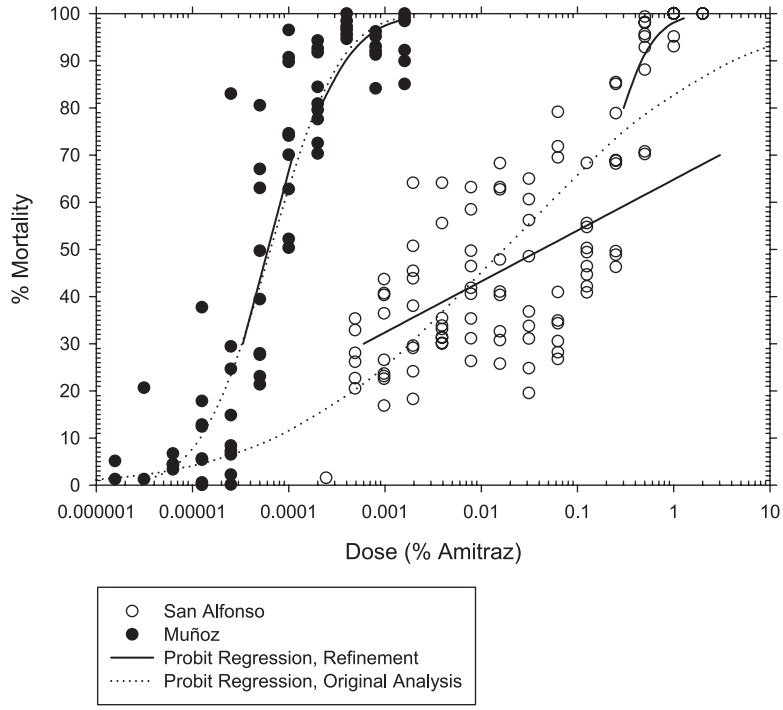


**Fig. 2.** San Alfonso and Muñoz *B. microplus* larvae exposed to amitraz using the Soberanes technique. Circles represent observed mortality data. Dotted lines represent the original probit regression with the entire range of dose–mortality data included in the analysis. Solid lines represent the refined probit regression. Only data from the range of interest was included in the analysis.

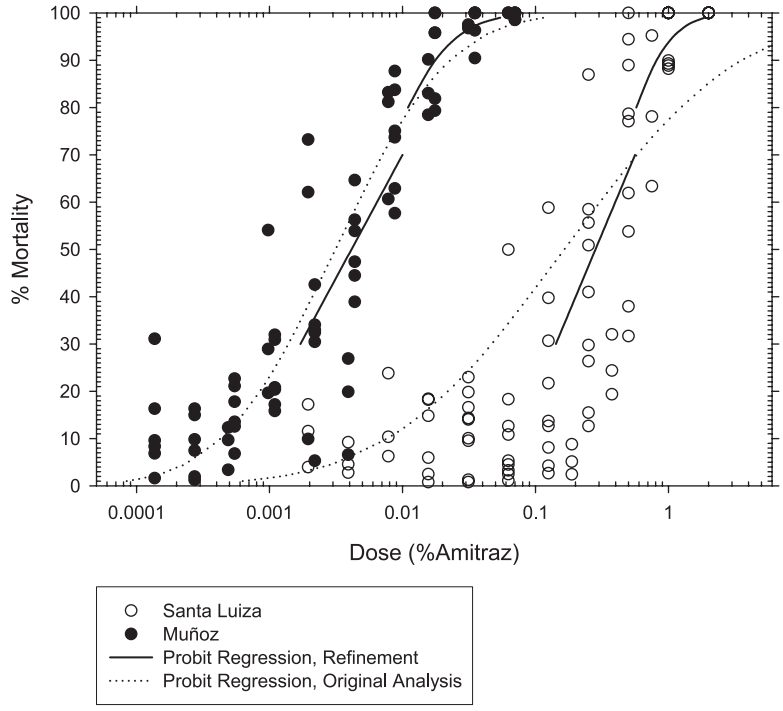


**Fig. 3.** Santa Luiza and Muñoz *B. microplus* larvae exposed to amitraz using the Miller technique. Circles represent observed mortality data. Dotted lines represent the original probit regression with the entire range of dose–mortality data included in the analysis. Solid lines represent the refined probit regression. Only data from the range of interest was included in the analysis.

tration that produced <10% mortality. This sequential procedure to increase precision was suggested by the discussion of dose placement by Robertson and Preisler (1992), although these authors did not directly describe use of data subsets from large data sets.

Goodness-of-fit of data for the reestimated, versus the original, regressions was assessed by the examination of plots of standardized residuals. A standardized residual is the difference between each observed value and its expected value, divided by the standard error of the difference. For good fit, residuals plotted against dose are randomly scattered around zero and within a band between −2 and 2 (Robertson and Preisler 1992). These values are automatically produced by PoloPlus (LeOra Software 2003).

Resistance ratios for amitraz susceptibility comparisons were calculated relative to the Muñoz strain. Significance of each comparison was determined when the number 1 was not contained in confidence interval of the resistance ratio (Robertson and Preisler 1992).

## Results

The Soberanes and Miller techniques produced dose–response relationships from 0 to 100% mortality for the susceptible, Muñoz, strain and the resistant, San Alfonso and Santa Luiza strains (Figs. 1–4). In contrast, the White procedure only produced dose–response relationships from 0 to 100% for the susceptible but not the resistant strains (Fig. 5).

Probit analysis that included the entire range of mortalities from ≈0 to 100% mortality from any strain produced a regression that closely described the observed data across this broad range of mortalities. Use of only the range of data close to the observed mortalities of interest produced regressions that described the observed data more closely (Figs. 1–4), especially in the analysis with the resistant strains. The standardized residuals from the refined analyses were closer to the estimated probit lines with less deviation (Figs. 6–9, b and c) than when the entire concentration–mortality curves were analyzed (Figs. 6–9, a).

The estimated slopes with the Soberanes technique differed significantly among strains ($\chi^2 = 1590$, df = 2, $P < 0.05$). Pairwise tests of parallelism indicated that the slope of the San Alfonso strain was significantly smaller than those observed for the Santa Luiza and Muñoz strains [slope (SE) = 0.78 (0.02), 1.36 (0.11), and 2.02 (0.07), respectively]. When the Miller method was used, a smaller slope was estimated for the San Alfonso compared with the Santa Luiza, but not when compared with the Muñoz strain ($\chi^2 = 24.67$, df = 2, $P < 0.05$; slope [SE] = 1.24[0.0], 1.76[0.0], and 1.37[0.1], respectively).

Resistance ratios were much greater for the Soberanes method compared with the Miller method (Tables 1 and 2). Resistance ratios ranged from 260 to

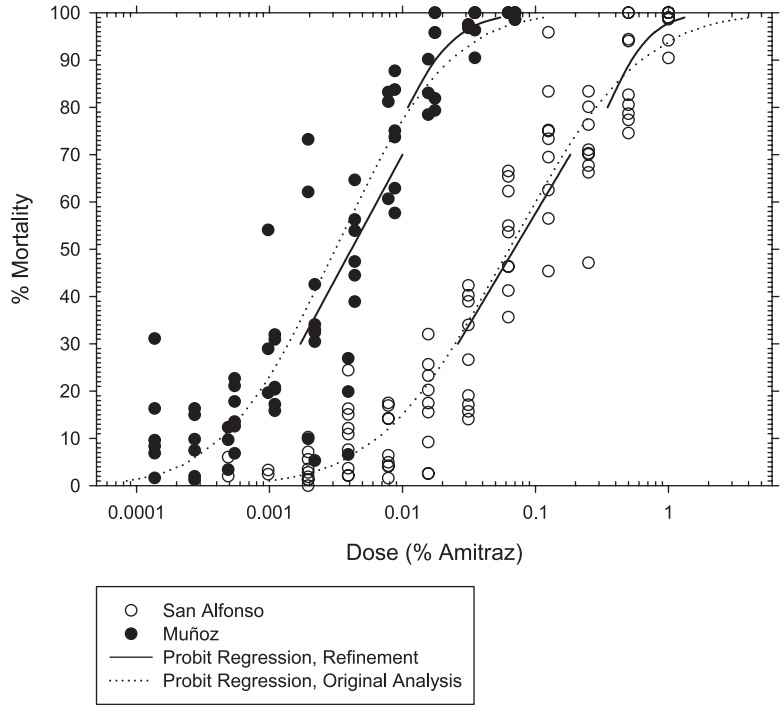


**Fig. 4.** Santa Alfonso and Muñoz *B. microplus* larvae exposed to amitraz using the Miller technique. Circles represent observed mortality data. Dotted lines represent the original probit regression with the entire range of dose-mortality data included in the analysis. Solid lines represent the refined probit regression. Only data from the range of interest was included in the analysis.

9,511 for the Soberanes method, whereas the RRs ranged only from 18 to 68 for the Miller method. However, neither method produced data that could completely discriminate between susceptible and resistant individuals at 2× the $LC_{99.9}$ as required by the standard FAO discriminating dose technique. The

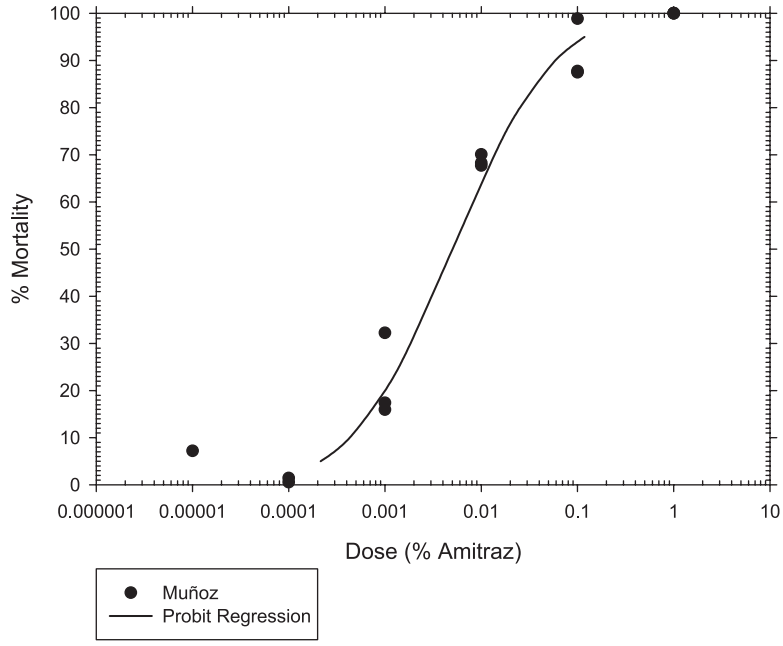


**Fig. 5.** Muñoz *B. microplus* larvae exposed to amitraz using the White technique. Circles represent observed mortality data. The solid line represents the probit regression. The entire range of dose–mortality data were included in the analysis.

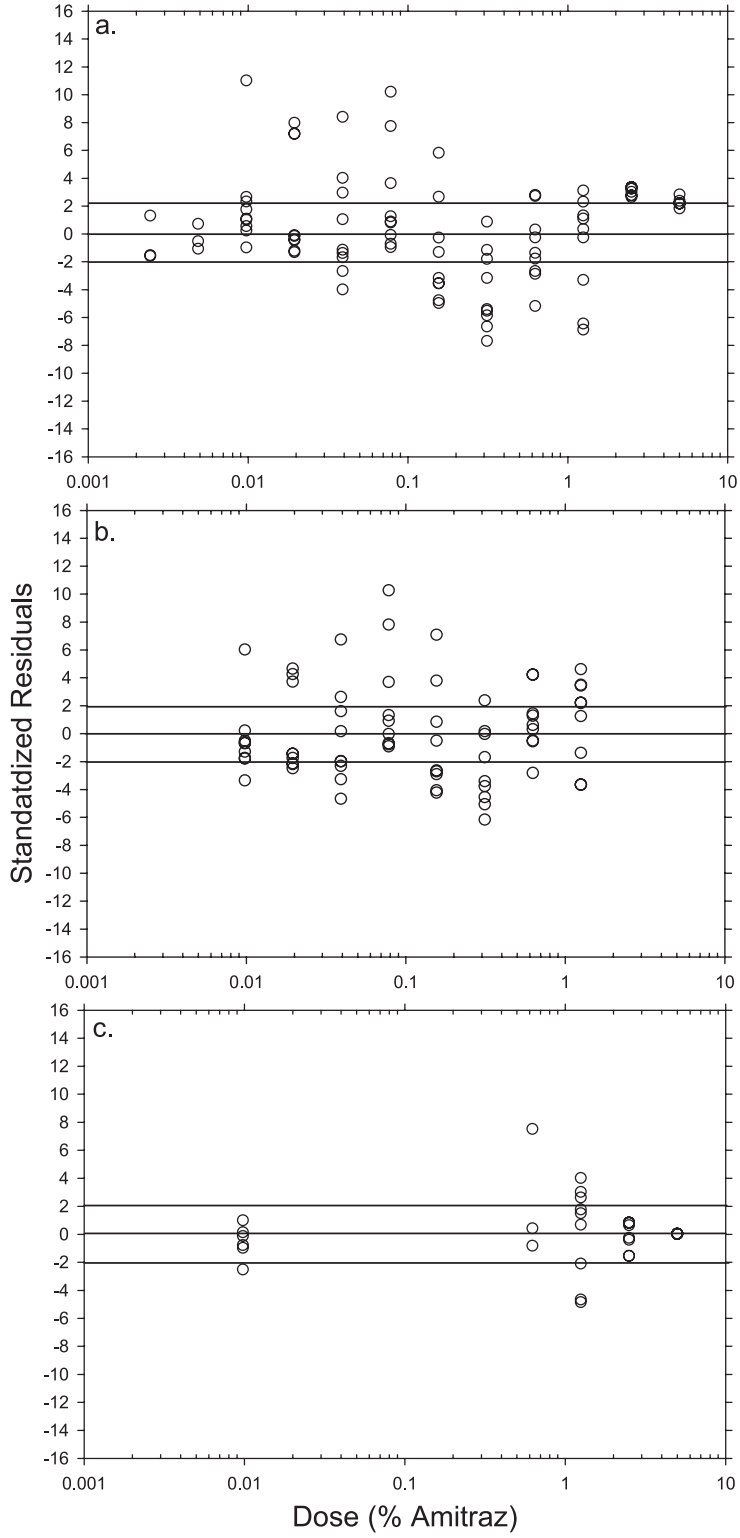


**Fig. 6.** Standardized residuals of data represented in Fig. 1 for the Santa Luiza strain exposed to amitraz by using the Soberanes technique. (a) Original probit analysis using data from the entire dose–mortality range. (b) Refinement analysis including data from concentrations that produced from 10 to 90% mortality. (c) Refinement analysis including data from concentrations that produced from 50 to 100% mortality and one concentration that produced <10% mortality.

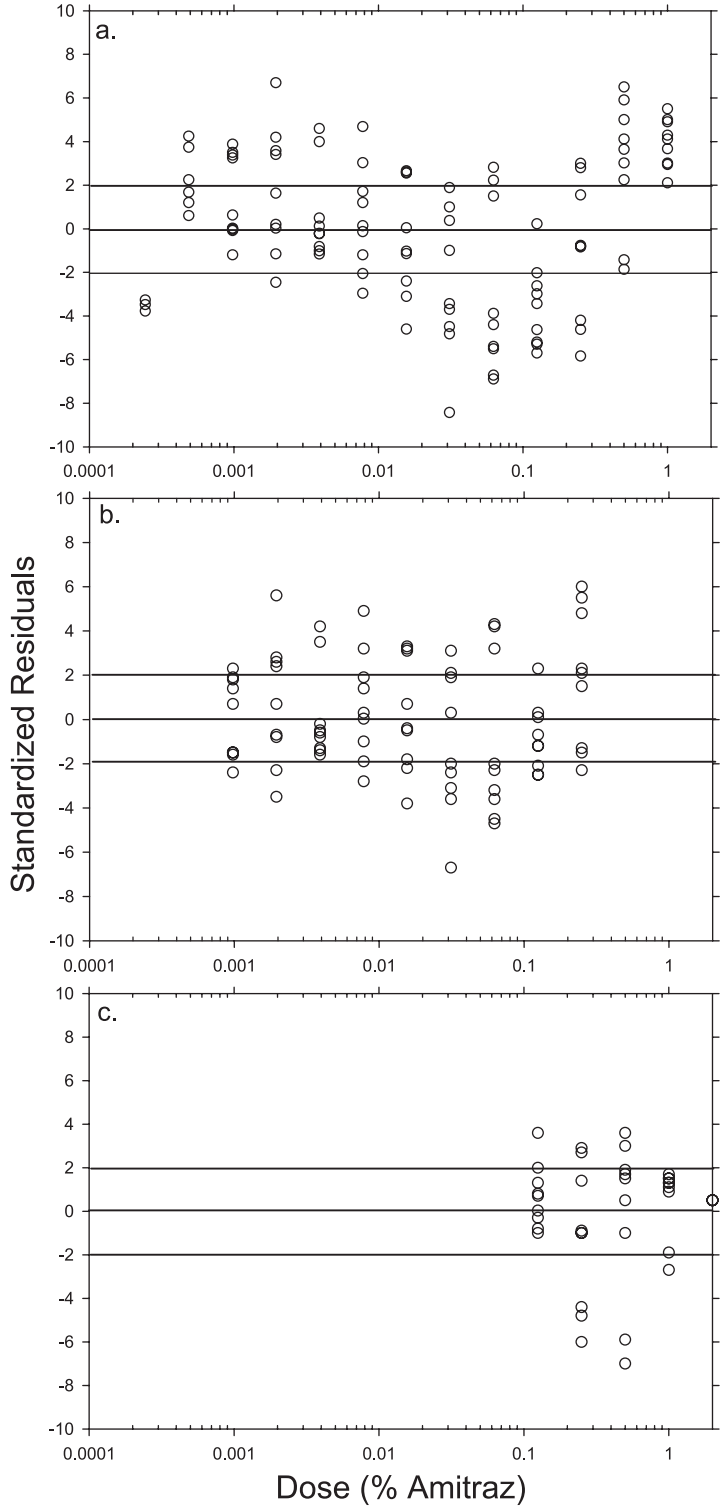


**Fig. 7.** Standardized residuals of data represented in Fig. 2 for the San Alfonso strain exposed to amitraz using the Soberanes technique. (a) Original probit analysis using data from the entire dose-mortality range. (b) Refinement analysis including data from concentrations that produced from 10 to 90% mortality. (c) Refinement analysis including data from concentrations that produced from 50 to 100% mortality.

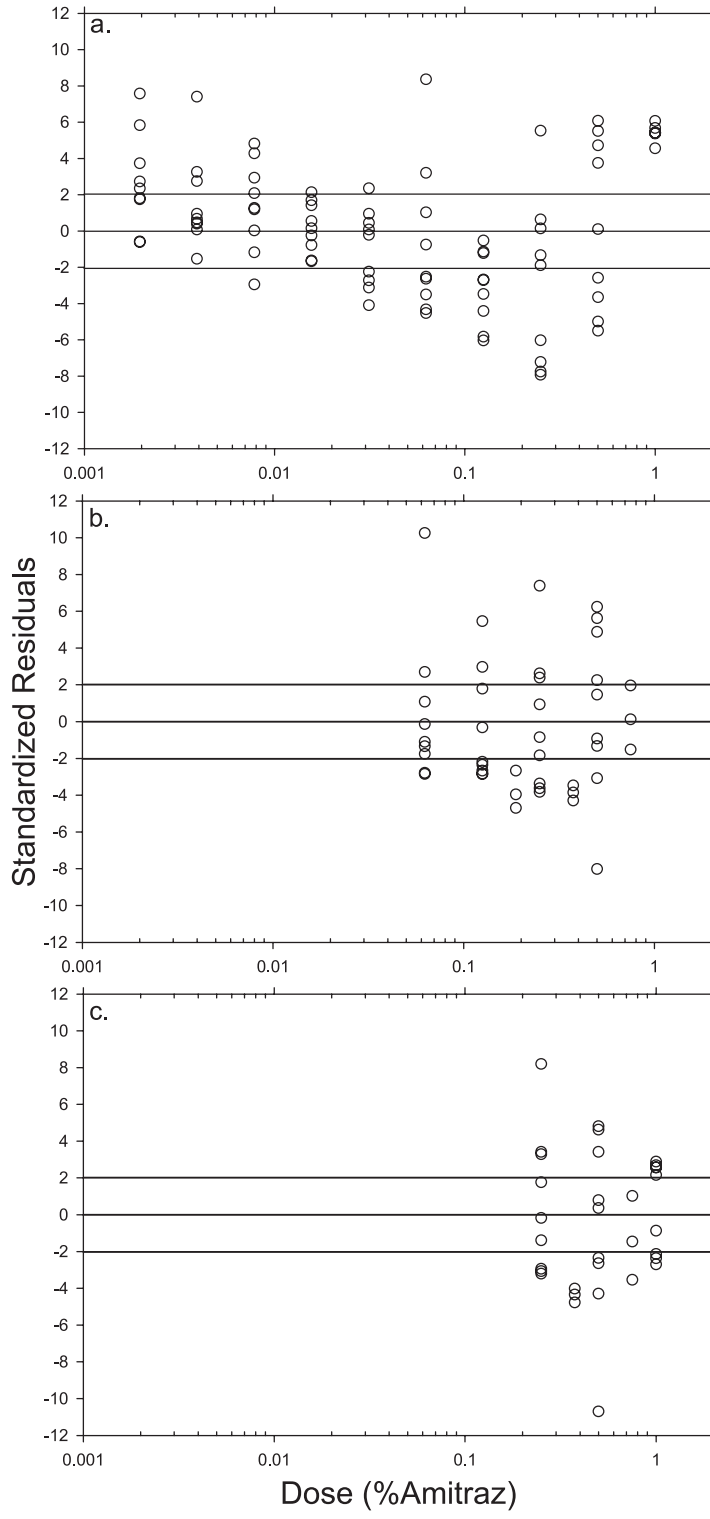


**Fig. 8.** Standardized residuals of data represented in Fig. 3 for the Santa Luiza strain exposed to amitraz by using the Miller technique. (a) Original probit analysis using data from the entire dose–mortality range. (b) Refinement analysis including data from concentrations that produced from 10 to 90% mortality. (c) Refinement analysis including data from concentrations that produced from 50 to 100% mortality.

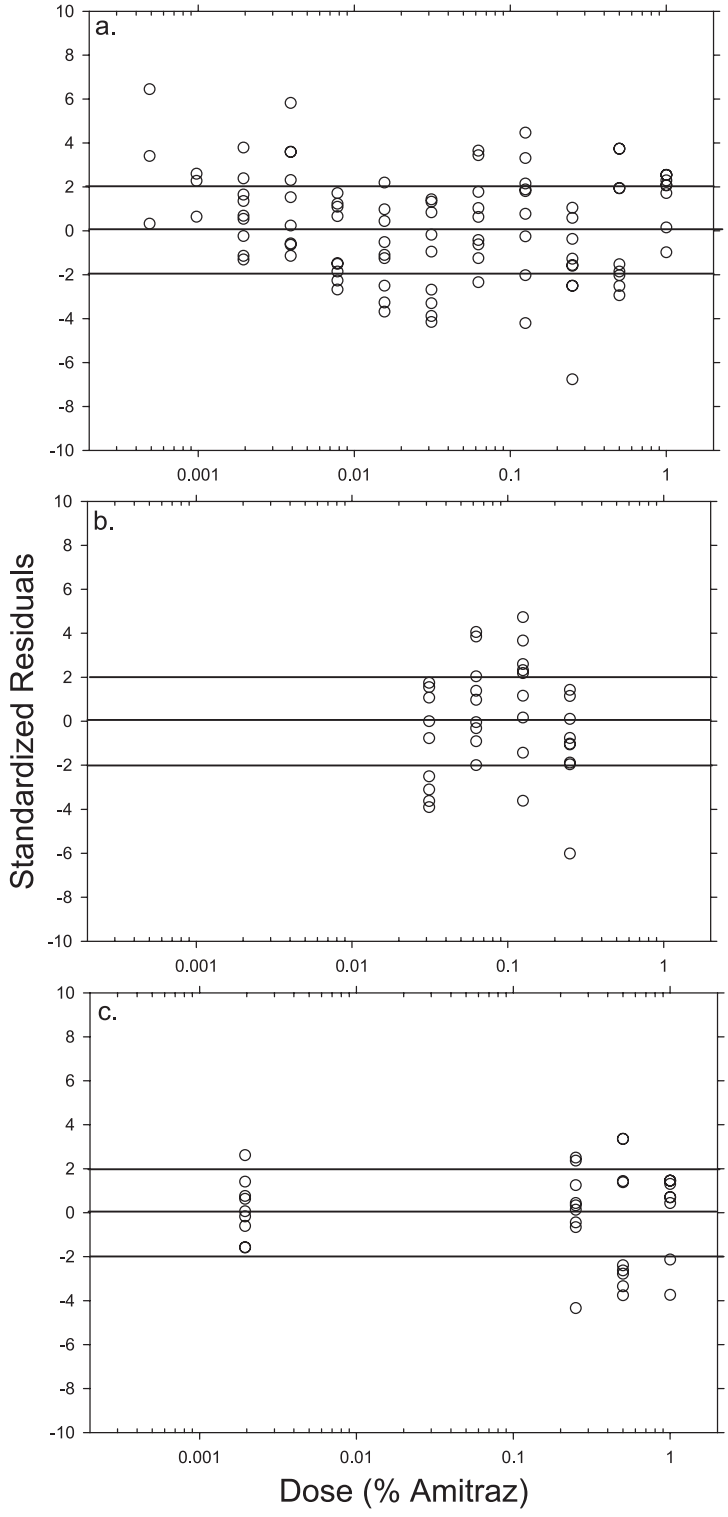


**Fig. 9.** Standardized residuals of data represented in Fig. 4 for the San Alfonso strain exposed to amitraz using the Miller technique. (a) Original probit analysis using data from the entire dose-mortality range. (b) Refinement analysis including data from concentrations that produced from 10 to 90% mortality. (c) Refinement analysis including data from concentrations that produced from 50 to 100% mortality and 1 concentration that produced <10% mortality.

**Table 1. Comparison of bioassay results at the $LC_{50}$ estimate by using the Soberanes and Miller techniques on *B. microplus***

| Strain | *n* | Slope (SE) | $LC_{50}$[a] (95% CL) | RR[b] (95% CI) | $\chi^2$ | df |
|---|---|---|---|---|---|---|
| Soberanes | | | | | | |
| Santa Luiza | 6,274 | 1.36 (0.11) | 0.58 (0.32–0.85) | 9,511 (8,318–10,875) | 798 | 22 |
| San Alfonso | 7,457 | 0.78 (0.02) | 0.04 (0.022–0.11) | 700 (530–924) | 59 | 25 |
| Muñoz[c] | 5,712 | 2.02 (0.07) | 0.000061 (0.000047–0.000076) | | 742 | 12 |
| Miller | | | | | | |
| Santa Luiza | 2,708 | 1.76 (0.08) | 0.28 (0.22–0.39) | 68 (54–86) | 597 | 19 |
| San Alfonso | 3,150 | 1.24 (0.07) | 0.069 (0.051–0.088) | 17 (13–21) | 214 | 10 |
| Muñoz[c] | 3,554 | 1.37 (0.14) | 0.0041 (0.0011–0.0067) | | 352 | 12 |

[a] Median lethal concentration estimates are presented as percentage of active ingredient.
[b] RR, resistance ratio relative to susceptible strain.
[c] Susceptible strain.

$LC_{99.9}$ estimate for the Muñoz strain was 0.017 (0.0065–0.097) and 0.32 (0.16–1.1) for the Soberanes and Miller methods, respectively. These values corresponded with the observed morality of the Santa Luiza strain from 1 to 30% and 1–40% and from 10 to 60% and 65–95% at 1× and 2× the Muñoz $LC_{99.9}$ estimate for the and Soberanes and Miller methods, respectively. Even if the $LC_{99}$ estimate was used as a discriminating dose with the Soberanes and Miller techniques, the actual mortality measured at this potential discriminating dose was between 0 and 7% and 0 and 19%, respectively, for the Santa Luiza strain.

## Discussion

The modified Soberanes and Miller tests detected and quantified amitraz susceptibility in susceptible and resistant strains of *B. microplus.* In addition, analyses of data specific to the $LC_{50}$ or the $LC_{90-99.9}$, improved the precision of LC estimates. However, the concentration–response relationships for the susceptible and resistant strains overlapped when both the Soberanes and Miller method was used. Therefore, it was not possible to determine a concentration that would perfectly discriminate between susceptible and resistant individuals to permit a traditional discriminating dose test. However, a discriminating dose test could still be done with using a concentration within the $LC_{90-99}$ range to take advantage of the greatest separation between susceptible and resistant individuals. This type of discriminating dose test would detect resistance if there were an observed deviation from the expected response.

The White test was able to quantify amitraz susceptibility in the reference strain but not for the resistant strains used in our study. At higher concentrations, technical amitraz precipitated, and we could not increase the amitraz concentration high enough to kill resistant ticks. Therefore, the White technique could be useful in the detection of resistance in a discriminating dose bioassay, but further quantification and description of resistance mechanisms would be impossible with this technique. However, the White technique was originally designed and optimized for

**Table 2. Comparison of bioassay results at the $LC_{90}$, $LC_{99}$, and $LC_{99.998}$ estimates by using the Soberanes and Miller techniques on *B. microplus***

| Strain | *n* | Slope (SE) | $\chi^2$ | df | LC[a] | Estimate | (95% CL) | RR[b] | (95% CI) |
|---|---|---|---|---|---|---|---|---|---|
| Soberanes | | | | | | | | | |
| Santa Luiza | 3,347 | 6.07 (0.52) | 165 | 12 | 90 | 1.62 | (1.48–1.89) | 4,152 | (3,709–4,648) |
| | | | | | 99 | 2.41 | (2.02–3.49) | 1,432 | (1,168–1,756) |
| | | | | | 99.9 | 4.54 | (3.22–9.64) | 260 | (175–387) |
| San Alfonso | 3,768 | 2.37 (0.09) | 267 | 12 | 90 | 0.46 | (0.39–0.59) | 1,186 | (1,046–1,344) |
| | | | | | 99 | 1.28 | (0.93–2.07) | 760 | (607–951) |
| | | | | | 99.9 | 6.49 | (3.58–16.20) | 372 | (242–571) |
| Muñoz[c] | 4,025 | 1.65 (0.06) | 494 | 12 | 90 | 0.00039 | (0.00027–0.00060) | | |
| | | | | | 99 | 0.0017 | (0.00099–0.0040) | | |
| | | | | | 99.9 | 0.017 | (0.0065–0.097) | | |
| Miller | | | | | | | | | |
| Santa Luiza | 3,015 | 2.88 (0.12) | 455 | 12 | 90 | 0.81 | (0.67–1.10) | 46 | (41–51) |
| | | | | | 99 | 1.87 | (1.32–3.45) | 35 | (26–44) |
| | | | | | 99.9 | 7.13 | (3.77–22.52) | 22 | (13–37) |
| San Alfonso | 2,923 | 2.58 (0.17) | 152 | 10 | 90 | 0.52 | (0.44–0.63) | 29 | (26–33) |
| | | | | | 99 | 1.31 | (0.96–2.24) | 24 | (18–32) |
| | | | | | 99.9 | 5.85 | (3.12–18.14) | 18 | (9–32) |
| Muñoz[c] | 3,523 | 2.14 (0.15) | 153 | 12 | 90 | 0.018 | (0.015–0.022) | | |
| | | | | | 99 | 0.054 | (0.039–0.095) | | |
| | | | | | 99.9 | 0.32 | (0.16–1.1) | | |

[a] Median lethal concentration estimates are presented as percentage of active ingredient.
[b] R, resistance ratio relative to susceptible strain.
[c] Susceptible strain.

the rapid screening of many potential acaricidal compounds, not for quantification and description of amitraz resistance. A change in the solvent and detergent concentration used in the technique might increase the solubility of amitraz. This could lead to the development of full concentration–mortality relationships for amitraz resistant strains and make the White test a very powerful technique for the study of amitraz resistance.

The advantage the White technique compared with the Soberanes and Miller techniques was definition of all of the chemical components of the White technique. The Soberanes and Miller techniques used a commercial formulation of amitraz diluted in water or TChE and olive oil, respectively, to make test concentrations. Because the components of the commercial formulation were proprietary, we did not know exactly what compounds to which the tick larvae were exposed nor could we hold their concentrations constant.

The White assay was completely defined. The only chemical components were ultrapure amitraz, Triton X-100, $dH_2O$, and DMSO. Therefore, all chemical components could be held at a constant concentration, whereas the amitraz concentration was changed. If the White method could be sufficiently modified to produce complete dose–mortality relationships with ticks resistant to amitraz, this defined method would be better suited for studies with synergists and other detailed studies of amitraz resistance compared with the Soberanes and Miller techniques.

Although the Soberanes and Miller techniques were well-suited for amitraz resistance detection and study, some differences were observed in the ease of execution of and quality of the information gained between the two methods. The main differences were test sensitivity, ease in scoring dead larvae, ease in the detection of a heterozygous strain, adaptability of the techniques to synergist studies, and time required to run the test.

The Soberanes technique was more sensitive in the sense that it produced much higher resistance ratios compared with the Miller technique. However, this sensitivity was offset by greater variability of the Soberanes method compared with the Miller method.

The Miller method took more time to score compared with the Soberanes method. Larvae in the Miller method were scored as dead when they could no longer walk in a coordinated manner, whereas larvae in the Soberanes technique were scored as dead when they no longer moved at all. Scoring the Miller technique took more time because this decision of live or dead needed to be made consistently on moving larvae. However, this difference in scoring criteria did not translate into higher variability in the data.

A smaller estimated slope leading to a difference in $LC_{50}$ and $LC_{90}$ estimates compared with a homozygous strain probably indicates that a strain is heterozygous for a particular resistance trait (Robertson and Preisler 1992). The San Alfonso strain produced a smaller slope compared with the Muñoz and Santa Luiza strains when the Soberanes method was used. This pattern was different when the Miller technique was used. The smallest slope was estimated for the San Alfonso strain, but it was not significantly different from the slope for the susceptible Muñoz strain. Like the results for the Soberanes technique, the estimated slope for the San Alfonso was significantly smaller than that for the Santa Luiza resistant strain. We expected this result because the San Alfonso strain had not been selected for amitraz resistance in the laboratory for as many generations as the Santa Luiza strain had been. The Soberanes technique seemed to describe the heterozygous strain more clearly than the Miller method.

The Miller technique was better suited for the study of the mechanisms of resistance. The solvent used in the test was TChE and will dissolve the synergists commonly used to implicate the presence of oxidases, esterases, and glutathione *S*-transferases in resistance. Li (2004) used the synergists piperonyl butoxide, triphenylphosphate, and diethyl maleate with the Miller technique to study the mechanisms of resistance in 15 strains of *B. microplus* collected from Mexico. Their study found that a combination of esterase and target site mechanisms were responsible for amitraz resistance in the Santa Luiza strain.

Finally, the Miller method required 24 h to complete, whereas the Soberanes technique required 72 h. Because both techniques required larvae to be 12–16 d-old at the beginning of the test, we could perform five tests with the Miller method, but only two tests with the Soberanes method within this time frame. In addition, testing at a 72-h interval only allowed for two tests to be completed within a week. The Miller technique with its 24-h incubation time allowed six bioassays to be done per week.

In conclusion, the Soberanes, Miller, and White techniques detected resistance to amitraz in *B. microplus*, but the Soberanes and Miller techniques were better suited for more detailed studies of the magnitude and mechanisms of resistance, respectively. Future scientific investigations using these techniques will lead to a better understanding of the epidemiology and mechanisms of amitraz resistance and to better management of *Boophilus* spp. worldwide.

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