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Use of the Larval Tarsal Test to determine acaricide resistance in *Rhipicephalus* (*Boophilus*) *microplus* Brazilian field populations

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ABSTRACT

Acaricide resistance of the cattle tick *Rhipicephalus* (*Boophilus*) *microplus* is widespread in most of the countries where this parasite is present. Bioassays are used to diagnose the level and pattern of resistance in tick populations. In the present study, we describe a detailed protocol of the Larval Tarsal Test (LTT) using simplified equipment and data on the resistance of 17 tick field populations originating from 5 Brazilian states. Nine acaricidal compounds from 5 major classes were tested: organophosphates (OP), synthetic pyrethroids (SP), macrocyclic lactones (ML), phenylpyrazols (PYZ) and amidines. For comparison, four of the tick populations were also tested with the Larval Packet Test (LPT) with one compound per class. The most common resistances were to SP, amitraz and OP, with frequencies of 94%, 88% and 82%, respectively. Resistance to PYZ was also found to be widespread (65%), suggesting a rapid development of fipronil resistance in Brazil. One case of ML resistance and 2 cases of suspected ML resistance were identified with the LTT. The LTT led to higher resistance ratios to all compounds than the LPT, reflecting its high sensitivity to detect resistance. Finally, the LTT allowed testing a larger number of compounds and doses with reduced labour in comparison to the LPT and turned out to be a reliable bioassay to detect resistance in field populations.

1. Introduction

The one-host cattle tick *Rhipicephalus* (*Boophilus*) *microplus* is a pest of major economic importance in tropical and subtropical countries. Treatments nearly exclusively rely on acaricides and multi-drug resistance has become widespread (Alonso-diaz et al., 2006; Jonsson and Hope, 2007; Martins et al., 2008; Castro-Janer et al., 2011). In Brazil, *R.* (*B.*) *microplus* is the most important ectoparasite of cattle and its economic impact on the Brazilian cattle industry was estimated at 2 billion US dollars per year (Grisi et al., 2002). This amount includes losses due to the increased mortality caused by tick-borne parasites, losses due to decreased milk production and decreased weight gain, damage to the leather, and treatment costs to control infestations. In Brazil resistance successively emerged to arsenic in 1950 (Freire, 1953), to organophosphates (OP) in 1974 (Amaral et al., 1974) and to synthetic pyrethroids (SP) in 1988 (Leite, 1988; Laranja et al., 1989). At the end of the 1990s, Farias (1999) pointed out that the widespread resistance to SP was a big issue in Brazil, especially considering that 90% of the acaricides available on the market at that time belonged to SP. In parallel, amitraz became an important alternative to control OP

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and SP-resistant populations but resistance to this compound was already reported in 1999 (Furlong, 1999; Farias, 1999). As a result, macrocyclic lactones (ML) were extensively used, and the first case of avermectin-resistance was observed in 2001 (Martins and Furlong, 2001), followed by other cases of ivermectin-resistance (Klafke et al., 2006, 2012). Recently, resistance to fipronil, a phenylpyrazol (PYZ) compound, was also detected (Castro-Janer et al., 2010). Nowadays, reports of resistance to OP, SP and amitraz are very common in Brazil (Farias et al., 2008; Martins et al., 2008; Mendes et al., 2011; Andreotti et al., 2011) while resistance to ivermectin and fipronil is still limited (Castro-Janer et al., 2010; Klafke et al., 2012). The newest acaricide classes are the benzoylureas (growth regulators) and the spinosyns, against which resistance has not been reported in the literature yet.

Farmers are currently facing many issues to control multi-drug resistant tick populations. It is essential that they obtain some information on the resistance profile of these populations in order to help them choosing the most suitable compounds to enhance treatment efficacy. In this context, bioassays are used to determine the resistance of tick populations to specific acaricides. Various *in vitro* tests are available, each of them with their own advantages and disadvantages. The FAO currently recommends the Adult Immersion Test (AIT) and the Larval Packet Test (LPT) (2004). The Larval Immersion Test (LIT) (Shaw, 1966) modified by Sabatini et al. (2001) is also currently used, mainly to test ivermectin and fipronil, and was shown to be more suitable to identify resistance to these 2 compounds (Castro-Janer et al., 2009; Klafke et al., 2012). In 2011, a new bioassay, the Larval Tarsal Test (LTT), was described and compared to the LPT (Lovis et al., 2011). The LTT is performed in microplates pre-treated with acaricides in which eggs are distributed, avoiding the handling of larvae and thus allowing testing a larger number of compounds and doses. The distribution of the eggs in the plates and the evaluation of the tests with the LTT required approximately 10-fold less time than the loading of the larvae in the packets and the evaluation of the tests with the LPT (Lovis et al., 2011).

Resistance status of tick populations can be determined by exposing ticks to a unique dose based on the data of a susceptible reference strain, and survival to this discriminating dose (DD) is considered as an indicator of resistance (FAO, 2004). In contrast, ticks can be exposed to several doses of acaricides in order to establish the doses which induce 50% or 90% mortality and compare them to a susceptible reference strain to determine the corresponding resistance ratios (RR50 and RR90). If the dose–response curves of the field populations and the reference strain are parallel, then these two values are similar. However, in absence of parallelism, two scenarios can be observed and the comparison of RR50 with RR90 reflects them: either the slope of the field population is greater than the reference strain, which leads to a RR90 smaller than the RR50, or the slope of the field population is smaller. Thereby, RR50 may be close to 1, whereas RR90 are much higher, allowing the detection of resistance. Hence, the comparison of RR50 and RR90 and observation of the slope of the response provides valuable information on emerging resistance.

In this paper, we provide a detailed protocol using simplified equipment for the LTT and evaluate this test for the detection of acaricidal resistance in field tick populations. The LTT was carried out in two laboratories in Brazil using 17 tick populations originating from 5 states of Brazil with 9 acaricidal compounds from 5 major classes (OP, SP, ML, PYZ and amidines). In addition, the resistance status of 4 field populations was also tested with the LPT using 5 compounds for comparison with the LTT.

2. Material and methods

2.1. Ticks

2.1.1. Susceptible strains

The Mozo strain was used as susceptible reference strain for OP, ML and PYZ while the Muñoz strain was used as susceptible reference strain for SP and amitraz. The Mozo strain was from the Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF), Eldorado do Sul, Brazil, obtained in November 2010 from the Centro de Investigaciones Veterinarias Miguel C. Rubino where it had been reared without acaricide pressure since collection from the field in 1973 in Uruguay. Some resistance to SP and amitraz was observed in the IPVDF isolate. The Muñoz strain was from the Novartis Animal Health Research Center (CRA), St-Aubin, Switzerland, obtained in 2010 from the Cattle Fever Tick Research Laboratory (CFTRL), Edinburg, TX, where it had been reared without acaricide selection since collection from the field in 1999 in Zapata County, TX, USA.

2.1.2. Field populations

In January and February 2011, *R.* (*B.*) *microplus* engorged females were collected in 17 Brazilian beef cattle ranches where farmers had observed some lack of treatment efficacy. Tick samples (ST) originated from the following 5 states: São Paulo (7), Rio Grande do Sul (RS, 4), Mato Grosso do Sul (MS, 4), Paraná (PR, 1), Espírito Santo (ES, 1). Samples included at least 20 fully engorged females collected from a minimum of 6 cows.

2.1.3. Preparation of ticks

Engorged females were brought to the Instituto Biológico (IB), São Paulo, Brazil or to the IPVDF and kept at $28 \pm 1\,^{\circ}C$ and 65–85% relative humidity (RH) to complete oviposition. Two to three weeks after collection of the females, eggs were used for testing with the LTT and some were transferred to glass vials closed with humidified cotton plugs for the LPT. Larvae used for the LPT (ST40, ST41, ST42 and ST44) were 14–21 days old. The Mozo strain was tested at the IB and the IPVDF while the Muñoz strain was tested at the CRA. They were stored at the same conditions than the field populations.

2.2. Acaricides

Technical grade chlorpyriphos (OP) (Sigma–Aldrich, Fluka, Germany), amitraz, coumaphos (OP), cypermethrin (SP), fipronil (PYZ), flumethrin (SP), ivermectin (ML), moxidectin (ML) and pyriprol (PYZ) were used with the LTT. Details on the latter compounds are available in

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Table 1
Lethal concentrations 50 and LC_{90} and their 95% CI obtained for the 17 Brazilian field strains of *R.* (*B.*) *microplus* as well as their RR in comparison to the susceptible reference strain (Muñoz) and their survival rates at the DD when tested with cypermethrin and flumethrin. Concentrations are expressed in mg/m^2.

		CYPERMETHRIN									FLUMETHRIN								
strain	origin	LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)	PR**	LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)	PR**
Muñoz		0.5	(0.4-0.6)			2.2	(1.5-3)				0.01	(0.01-0.01)			0.03	(0.02-0.04)			
ST40	SãP	53.2	(47.9-58.4)	104.8	(83.2-127)	161.2	(119-204)	71.3	(36.6-106)	97.1	0.70	(0.5-0.9)	57.9	(41.7-74.1)	0.91	(0.1-1.7)	29.5	(-3-62.1)	99.9
ST41	SãP	71.9	(66.6-77.2)	141.0	(108-174)	211.7*	(164-260)	95.4	(32.8-158)	99.0	1.57	(1.4-1.7)	129.2	(102-156)	2.77	(1.9-3.7)	92.4	(42.1-143)	100.0
ST42	SãP	101.9	(75-129)	201.0	(144-258)	918.5*	(130-1707)	407.5	(59.1-756)	92.6	1.08	(0.9-1.2)	88.7	(63.2-114)	1.93	(1.5-2.3)	64.8	(31.3-98.4)	99.8
ST43	SãP	93.0	(65.5-121)	183.0	(133-233)	427.6*	(-20.2-875)	190.4	(24.2-356)	88.4	na	na	na	na	na	na	na	na	na
ST44	SãP	84.9	(65.2-105)	167.3	(127-207)	459.4*	(140-779)	203.4	(72.3-335)	95.7	1.80	(1.4-2.2)	147.3	(111-184)	3.10	(1.9-4.3)	105.1	(48.7-161)	99.9
ST45	MGS	4.0	(3.2-4.9)	8.0	(5.2-10.7)	25.9	(12.9-38.8)	11.5	(2.5-20.5)	13.4	0.01	(0.01-0.02)	1.1	(0.7-1.4)	0.25	(0.1-0.4)	8.2	(1.9-14.5)	17.8
ST46	ES	55.8	(46.5-65.1)	109.9	(82.5-137)	176.8	(106-248)	78.7	(33.9-124)	85.5	1.12	(0.8-1.5)	92.2	(67.4-117)	2.31	(0.9-3.7)	78.1	(33.7-123)	99.2
ST47	RGS	1.8	(1.6-2.1)	3.6	(2.8-4.5)	4.8	(3.5-6.2)	2.2	(1.1-3.3)	0.5	0.01	(0.01-0.01)	0.8	(0.6-0.9)	0.02	(0.02-0.02)	0.6	(0.3-0.9)	0.1
ST48	SãP	103.5	(84.2-123)	203.9	(154-254)	270.7*	(137-405)	120.4	(47.7-193)	95.4	0.77	(0.7-0.9)	63.3	(48.9-77.6)	1.65	(0.9-2.3)	55.5	(24.1-86.8)	100.0
ST49	MGS	79.1	(71.9-86.2)	155.7	(120-191)	165.3	(131-200)	73.6	(36.6-111)	98.2	0.91	(0.7-1.2)	74.8	(56.4-93.3)	1.40	(0.7-2.1)	46.9	(23.6-70.3)	99.9
ST50	MGS	36.2	(21.2-51.1)	71.3	(49.4-93.2)	192.7	(-32-417)	85.3	(16-155)	68.0	0.49	(0.4-0.6)	40.0	(30.1-50)	1.14	(0.8-1.5)	37.5	(6.8-68.2)	85.4
ST51	MGS	52.7	(45.1-60.3)	103.7	(77.9-130)	145.6	(98.7-193)	64.9	(29.9-99.9)	80.5	0.50	(0.4-0.6)	41.5	(31.4-51.7)	1.41	(1-1.8)	47.3	(29.9-64.7)	81.0
ST52	PA	112.8	(85.3-140)	221.5	(161-282)	757.2*	(158-1357)	339.4	(69-608)	95.7	1.42	(1.3-1.6)	116.1	(87.8-144)	2.66	(1.9-3.4)	91.1	(30.5-152)	100.0
ST53	RGS	62.4	(47.2-77.5)	122.8	(90.5-155)	307.4*	(70.7-544)	136.8	(34.7-239)	85.8	0.96	(0.8-1.1)	79.2	(62.4-96)	1.59	(1.4-1.8)	53.4	(35-71.8)	96.8
ST55	RGS	5.6	(3.7-7.4)	11.0	(7.8-14.1)	35.5	(3.9-67.1)	15.8	(4.5-27.2)	19.4	0.01	(0.01-0.01)	0.8	(0.6-1.1)	0.07	(0.01-0.13)	2.3	(0.8-3.9)	7.8
ST57	SãP	157.0	(107.9-206)	309.3	(200-419)	1651.0*	(-45.5-3348)	735.5	(-70.1-1541)	96.2	1.01	(0.9-1.1)	83.5	(68.1-98.9)	1.96	(1.5-2.4)	65.6	(37.3-94)	100.0
ST58	RGS	73.0	(64.2-81.8)	143.1	(113-173)	163.3	(114-213)	72.8	(37.3-108)	97.2	0.80	(0.7-0.8)	65.4	(52.5-78.3)	1.23	(1-1.5)	41.5	(20.9-62.1)	99.8

na: not available due to insufficient number of engorged females obtained. Colour code: RR ≤ 4.0 are represented on a light grey background; 4.0 < RR ≤ 10.0 are represented on a dark grey background; RR > 10.0 are represented on a black background.
*Estimates based on extrapolation (highest dose tested 200 mg/m^2).
**Predicted survival rates at the potential discriminating doses (2× LC_{99} of the susceptible reference strain).

Lovis et al. (2011) (Table 1). Compounds were dissolved in dimethyl sulfoxide (DMSO; Fluka) to prepare stock solutions of 20 mg/ml. For the LPT, technical grade chlorpyriphos (Sigma–Aldrich), cypermethrin (Sigma–Aldrich), ivermectin (Agromen Chemicals Co. Ltd., China), fipronil (Agromen Chemicals Co. Ltd., China) and formulated amitraz (12.5%, Schering Plough Saúde Animal Indústria e Comércio Ltd., Brazil) were used.

2.3. Larval Tarsal Test

The LTT was conducted at the IB and at the IPVDF following the protocol described previously in Lovis et al. (2011) with some modifications. Ready-to-use treated microtiter plates were prepared in advance in the CRA. Briefly, 20 μl of a coating solution (100% ethanol, olive oil (Sigma–Aldrich, Fluka), 400:1) was dispensed in the wells of flat bottom 96-well polystyrene plates (NUNC, Catalogue No. 260836, Denmark) and ethanol was allowed to evaporate overnight. Then 5 μl of acaricidal compounds diluted in DMSO to obtain 12 two-fold dilutions were distributed in the appropriate wells of the plates. The upper and lower rows as well as one of the inner rows contained DMSO only, and the inner row with DMSO was used as control. Plates were placed for 1 h in an N_2 sampler concentrator (Techne DB-3 Dri-Block, Witec AG, Switzerland) or for 2 h in a centrifugal vacuum concentrator (SC21017 SpeedVac® Plus, ThermoSavant) for complete DMSO evaporation. In order to avoid potential oxidation of the compounds, plates were placed in airproof plastic bags (ZU3605, Severin) and sealed (Folio bag sealer FS 3602, Severin) under N_2 atmosphere using an anaerobic chamber (Bactron anaerobic chamber model II, Shel Lab). In addition, the treated plates were kept with silica gel and were not exposed to direct light to optimise their preservation. Plates were shipped to the IB and IPVDF, kept at room temperature (20–28 °C) and used for testing within 5 weeks after preparation.

Since eggs aggregate, they were separated by the use of glass beads in order to facilitate their distribution in the wells. In details, a portion (40 ml-volume) of 3 mm diameter glass beads was placed in a 100 ml glass bottle. A small amount (~30 mg) of talc (Fluka, Catalogue No. 86255) was added and mixed thoroughly with the beads to ensure that the surface of the beads was covered with talc. Egg clusters (300–1000 mg) were added to the beads and the bottle was closed. To separate the eggs, the bottle was smoothly turned to mix the eggs and the beads and egg clusters were disrupted. At the beginning of the egg separation process, it helps to open the glass bottle to break the egg masses of big size with a spatula. If necessary, additional talc (~30 mg) was added to the beads to ensure that the eggs did not stick to the beads or to the walls of the glass bottle. When the separation was completed and in order to extract the eggs from the beads, the content of the bottle was poured in a sieve (mesh width: 0.9 mm) which allowed the eggs to pass through but not the beads. Eggs were collected in a glass Petri dish. Around 50 eggs (mean: 54.6; standard deviation: 4.6) were distributed per well by using a 2.5 mm-diameter spoon, corresponding to a 4 mm^3 volume (Meyerhoefer Chalazion Curette, Size 3, RUMEX, Catalogue No. 16066).

After distribution of the eggs, uncovered plates were kept for 24 h at 28 ± 2 °C at ~95% RH. Then plates were sealed with a transparent sealing film (VIEWseal, Greiner bio-one, Catalogue No. 676070, Switzerland) and held at 28 ± 2 °C and 80–90% RH for 3–4 additional weeks. The sealing of the plates as well as egg distribution were performed on a static control mat (157 KIT, elme) to remove electrostatic charges. After incubation (*i.e.* 40–42 days after the collection of the females), plates were removed from the environmental chamber and larval mortality was evaluated by counting dead or surviving larvae using a stereomicroscope. Larval motility and general appearance were used as criteria to assess mortality.

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The following concentrations were tested: flumethrin, fipronil, pyriprol: 0.003–6.25 mg/m^2; moxidectin: 0.05–100 mg/m^2; amitraz, cypermethrin, ivermectin: 0.1–200 mg/m^2; chlorpyrifos: 0.2–400 mg/m^2; coumaphos: 0.4–800 mg/m^2. Each dilution was tested in triplicates in separate plates.

2.4. Larval Packet Test

The LPT was carried out at the IB as previously described (FAO, 2004). Briefly, technical grade acaricides were dissolved in a mixture of trichloroethylene (Synth, Diadema-SP, Brazil) and olive oil (Sigma–Aldrich) (2:1) to prepare 1% active ingredient (AI) stock solutions which were subsequently diluted in trichloroethylene:olive oil to prepare 6–12 concentrations per compound. A volume of 670 μl of each dilution was used to impregnate 7.5 × 8.5 cm filter papers (Whatman No. 1, Whatman International Ltd., Maidstone, United Kingdom) and trichloroethylene was allowed to evaporate at room conditions for a minimum of 24 h. Each concentration was tested in triplicates and controls contained the diluent only. Treated papers were stored in a fridge and used within 3 weeks. For testing, they were folded in half and sealed on the sides with metal clips forming an open-ended packet. Then, around 100 larvae were collected with a paintbrush from the glass vials and inserted in each treated packet. Packets were then sealed with a third clip and incubated at 28 ± 2 °C and 80–90% RH for 24 h. Packets were removed from the incubator, opened and larval mortality assessed by counting dead and surviving larvae. Larvae that moved their legs but did not walk were counted as if dead.

For amitraz, the LPT protocol modified by Miller et al. (2002) was followed. Nylon fabric (Type 2320, Cerex Advanced Fabrics, Pensacola, FL, USA) was therefore used instead of filter papers and formulated amitraz was used instead of technical grade amitraz. In addition, impregnated nylon fabric was not stored in the fridge but used directly once the evaporation was completed.

Tested concentrations varied among strains and were included in the following ranges: amitraz: 0.0002–0.02% AI; fipronil: 0.0031–0.1% AI; ivermectin: 0.03–0.3% AI; chlorpyriphos: 0.0005–1% AI; cypermethrin: 0.13–5% AI.

2.5. Statistical analysis

Data were entered in Excel software (Microsoft Office 2003) and transferred to Intercooled STATA release 11.0 (StataCorp, College Station, TX, USA). Abbott's formula (Abbott, 1987) was used to normalize mortality values by the mortality of the control wells. Outer wells of the plates with increased mortality due to occasional edge effect in microplates were removed (Lovis et al., 2011). Statistical analysis was performed on the R software (version 2.12.0) using the drc package (version 2.0-1), specific for modelling dose–response curves (Ritz and Streibig, 2005). A five-parameter log-logistic function with the bottom and top limits fixed at 0 and 100 respectively was used to model the dose-mortality data (drm command). Lethal concentrations at 50% and 90% mortality (LC_{50}, LC_{90}, respectively) and their corresponding resistance ratios (RR) (RR50 and RR90) as well as their 95% confidence interval (CI) were calculated with the ED and SI commands and the Delta options. Populations were considered to be susceptible to a specific compound when the RR was smaller or equal to 4, moderately resistant for $4 < RR \leq 10$ and highly resistant for RR greater than 10. Potential discriminating doses (DD) were calculated as 2× the LC_{99} of the susceptible strains (Jonsson et al., 2007). The survival rates of the field strains at the DD were estimated with the PR command. Discriminating doses were not generated for amitraz as the use of a single DD is not recommended for this compound (FAO, 2004; Jonsson et al., 2007; Lovis et al., 2011).

3. Results

3.1. Larval Tarsal Test

Lethal concentrations inducing 50% and 90% mortality and their respective 95% CI are presented for the 17 field strains in comparison with the reference strains (Tables 1–5). The RR50, RR90 and their respective 95% CI, as well as the survival rates of the field populations at the potential DD are also presented.

The 95% CI of LC_{90} and of RR90 were wider than those calculated for LC_{50} and RR50. The resistance status was therefore based on RR50, while the RR90 was used for comparison reasons. Some discrepancy between RR50 and RR90 in the identification of resistance was observed in case of absence of parallelism between the dose–response curves of the field populations and the reference strain as illustrated in Fig. 1 for pyriprol and amitraz.

3.1.1. Resistance status of the field populations based on RR50

Sixteen (94%) field populations showed evidence of resistance to SP with RR50 to cypermethrin ranging from 8.0 to 309.3 and RR50 to flumethrin ranging from 40.0 to 147.3. Three SP-resistant populations demonstrated resistance to cypermethrin only. Resistance ratios at 50% mortality appeared to be systematically higher when testing cypermethrin than when testing flumethrin.

Fourteen (82%) populations were found to be resistant to coumaphos, with RR50 between 4.9 and 72.9, and 11 (65%) populations were resistant to chlorpyriphos, with RR50 between 4.4 and 179.7. Five of these strains were considered as highly resistant to both OP compounds.

Amitraz resistance was detected in 88% (15/17) of the populations with RR50 ranging from 4.2 to 32.9. Six populations were considered as moderately resistant and 9 as highly resistant.

Eleven populations (65%) were resistant to fipronil, with RR50 ranging from 6.6 to 55.7, among which 8 populations were also resistant to pyriprol, with RR50 ranging from 4.3 to 43.9. Four populations were highly resistant to both PYZ compounds.

Finally, RR50 to ivermectin varied between 0.9 and 4.2. The population possessing the RR50 value of 4.2 (ST53) was considered as the single case of resistance to ML but two other populations (ST44 and ST55) had RR50 very close to the threshold value (3.6 and 4.0, respectively). No

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Table 2
Lethal concentrations 50 and LC_{90} and their 95% CI obtained for the 17 Brazilian field strains of *R.* (*B.*) *microplus* as well as their RR in comparison to the susceptible reference strain (Mozo) and their survival rates at the DD when tested with chlorpyriphos and coumaphos. Concentrations are expressed in mg/m^2.

		CHLORPYRIPHOS									COUMAPHOS								
strain	origin	LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)	PR**	LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)	PR**
Mozo		2.7	(2.6-2.9)			5.9	(5-6.8)				2.3	(2.2-2.4)			4.0	(3.6-4.3)			
ST40	SãP	53.9	(41-66.8)	19.8	(15.5-24.1)	188.0	(96.3-280)	32.3	(16.9-47.7)	65.6	28.6	(21.6-35.5)	12.7	(9.7-15.6)	74.8	(37.3-112)	18.8	(10.1-27.6)	79.3
ST41	SãP	20.6	(17.5-23.6)	7.6	(6.3-8.8)	103.0	(54.7-151)	17.6	(8.6-26.6)	35.6	35.3	(31.8-38.7)	15.5	(11.7-19.3)	120.6	(93.1-148)	30.8	(13.8-47.9)	79.2
ST42	SãP	13.7	(11.6-15.9)	5.1	(4.1-6)	70.3	(37.6-103)	11.9	(5.6-18.1)	24.5	30.4	(25.6-35.2)	13.4	(9.9-16.8)	65.0	(47.2-82.9)	16.6	(8.9-24.3)	75.1
ST43	SãP	na	na	na	na	na	na	na	na	na	67.6	(54.7-80.5)	29.9	(22.6-37.2)	231.4	(161-302)	58.0	(35.1-81)	75.3
ST44	SãP	77.8	(61.1-94.4)	28.7	(22.5-34.8)	1105.9*	(403-1809)	185.3	(65.6-305)	73.4	165.2	(130-201)	72.9	(53.7-92)	461.9	(220-704)	117.2	(45.2-189)	97.8
ST45	MGS	4.2	(2.6-5.7)	1.6	na	6.8	(3.9-9.7)	1.2	(1-1.3)	0.0	3.9	(3.5-4.4)	1.7	(1.3-2.2)	11.4	(6.9-15.9)	2.9	(0.4-5.4)	5.6
ST46	ES	442.3*	(281-603)	162.1	(114-210)	1187.7*	(-2013.6-4389)	210.3	(-158-578)	92.6	102.9	(78.9-127)	45.4	(32.7-58.1)	525.5	(251-801)	133.4	(51.4-216)	86.8
ST47	RGS	3.8	(3.4-4.2)	1.4	(1.2-1.6)	8.6	(5.9-11.3)	1.5	(0.9-2.1)	0.6	5.6	(4.9-6.3)	2.5	(2.1-2.9)	9.5	(5.2-13.8)	2.4	(1.1-3.7)	1.0
ST48	SãP	20.1	(18.3-21.8)	7.3	(6.1-8.6)	43.6	(32.9-54.3)	7.6	(4-11.1)	23.3	29.8	(21.7-37.9)	13.5	(8.7-18.3)	86.1	(49.5-123)	21.0	(8.6-33.5)	69.4
ST49	MGS	10.5	(9.8-11.2)	3.9	(3.4-4.4)	15.3	(11.9-18.6)	2.6	(1.6-3.6)	0.0	26.5	(19.8-33.2)	11.7	(8.8-14.7)	57.8	(36-79.7)	14.6	(8.6-20.6)	71.0
ST50	MGS	11.9	(10.2-13.6)	4.4	(3.7-5.1)	42.3	(27-57.5)	7.3	(4.2-10.3)	15.9	19.5	(13.1-25.9)	8.6	(5.8-11.4)	101.5	(41.3-162)	25.7	(10.5-40.9)	56.5
ST51	MGS	10.8	(9.2-12.4)	4.0	(3.4-4.5)	19.1	(9.5-28.6)	3.3	(1.9-4.7)	1.7	11.5	(9.1-14)	4.9	(3.3-6.5)	49.3	(28.1-70.5)	13.0	(5.3-20.7)	37.7
ST52	PA	484.6*	(272-697)	179.7	(97.3-262)	3763.8*	(-7330.2-14857.8)	790.4	(-1531-3112)	91.3	82.2	(65-99.5)	35.1	(26.4-43.8)	244.1	(96.6-392)	64.5	(30.3-98.7)	100.0
ST53	RGS	19.7	(15.9-23.6)	7.2	(5.7-8.8)	329.6	(139-521)	57.0	(21.9-92.1)	40.7	15.8	(10.9-20.7)	6.7	(4.5-9)	82.3	(28-137)	21.8	(7.4-36.2)	49.7
ST55	RGS	2.6	(2.3-2.9)	1.0	(0.8-1.1)	4.8	(3.5-6.2)	0.8	(0.5-1.1)	0.0	2.8	(2.4-3.2)	1.2	(0.9-1.5)	4.9	(3.1-6.7)	1.2	(-0.5-2.9)	0.0
ST57	SãP	> 400†	-	> 146.9	-	> 400†	-	> 26.3	-	98.9	107.6	(82.8-132)	45.7	(34.6-56.8)	278.0	(84.4-472)	75.1	(35.6-115)	100.0
ST58	RGS	23.2	(19.1-27.4)	8.5	(7-10.1)	144.9	(76.8-213)	24.8	(13-36.6)	41.0	20.7	(18.4-23)	8.8	(6.7-10.9)	49.4	(33.6-65.2)	13.0	(6.2-19.9)	70.2

na: not available due to insufficient number of engorged females obtained. Colour code: RR ≤ 4.0 are represented on a light grey background; 4.0 < RR ≤ 10.0 are represented on a dark grey background; RR > 10.0 are represented on a black background.
*Estimates based on extrapolation (highest dose tested 400 mg/m^2).
**Predicted survival rates at the potential discriminating doses (2× LC_{99} of the susceptible reference strain).
†Mortality of 13.6% at the highest dose tested (400 mg/m^2). LC estimates not generated because of too much uncertainty.

resistance at all to moxidectin was observed, with RR50 varying between 0.6 and 2.1.

3.1.2. Survival rates at potential DD

Survival at DD was calculated to see the ability of the use of DD to differentiate resistant from susceptible populations. Considering all compounds on resistant strains (based on RR50), survival rates at the DD ranged between 13.1 and 100% in 98% (90/92) of the tests. The other 2 cases were ST53 with ivermectin (0% survival at DD) and ST47 tested with fipronil (2% survival at DD). For susceptible strains (based on RR50), survival rates at DD were below 10% in all but 3 cases: ST55 tested with fipronil (14.8%), ST48 with pyriprol (20.4%) and ST55 tested with pyriprol (28.0%).

3.2. Larval Packet Test

Four field populations were tested with the LPT. Table 6 summarizes the RR50 and the resulting resistance-classification when using the LPT in comparison to the LTT. Resistance ratios obtained with the LTT were higher than those obtained with the LPT in 95% (18/19) of the tests. As

Table 3
Lethal concentrations 50 and LC_{90} and their 95% CI obtained for the 17 Brazilian field strains of *R.* (*B.*) *microplus* as well as their RR in comparison to the susceptible reference strain (Muñoz) when tested with amitraz. Concentrations are expressed in mg/m^2.

		AMITRAZ							
strain	origin	LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)
Muñoz		1.1	(0.9-1.3)			18.5	(9-28)		
ST40	SãP	8.4	(6.5-10.3)	7.7	(5.4-10)	31.7	(14.6-48.7)	1.7	(0.5-3)
ST41	SãP	16.7	(15.2-18.1)	15.3	(12-18.5)	40.0	(30.6-49.4)	2.2	(0.9-3.5)
ST42	SãP	10.1	(8.3-12)	9.3	(6.7-11.9)	50.7	(28.1-73.3)	2.8	(0.8-4.8)
ST43	SãP	30.3	(23.2-37.3)	27.8	(19.3-36)	105.0	(45.7-164.3)	5.8	(1.4-10.1)
ST44	SãP	31.1	(25.4-36.8)	28.6	(21.7-35.2)	102.5	(53.7-151.2)	5.6	(2.1-9.2)
ST45	MGS	1.0	(0.6-1.4)	0.9	(0.6-1.2)	6.3	(1.1-11.6)	0.4	(0.1-0.6)
ST46	ES	9.4	(7.4-11.3)	8.6	(6.3-10.9)	30.2	(18.4-42)	1.7	(0.7-2.6)
ST47	RGS	3.7	(2-5.5)	3.4	(2.1-4.7)	13.7	(3.7-23.7)	0.8	(0.3-1.3)
ST48	SãP	35.2	(31.8-38.6)	32.3	(26.5-38.9)	56.8	(48.4-65.2)	3.2	(1.7-4.7)
ST49	MGS	6.0	(4.4-7.7)	5.5	(4.2-6.9)	19.0	(11.5-26.5)	1.0	(0.5-1.5)
ST50	MGS	12.6	(10.6-14.6)	11.6	(8.5-14.5)	37.3	(26.3-48.2)	2.0	(0.9-3.2)
ST51	MGS	4.6	(3.8-5.4)	4.2	(2.9-5.4)	16.8	(12.8-20.7)	0.9	(0.4-1.5)
ST52	PA	9.4	(7.5-11.4)	8.7	(6.1-11.1)	22.9	(14.5-31.3)	1.3	(0.5-2.1)
ST53	RGS	17.9	(12.4-23.5)	16.5	(10.8-22.1)	510.2*	(-13.2-1034)	27.3	(-2.8-57.5)
ST55	RGS	10.9	(9-12.9)	10.1	(7.6-12.4)	27.3	(14.9-39.6)	1.5	(0.6-2.5)
ST57	SãP	35.8	(26.5-45.1)	32.9	(23.3-42.1)	128.1	(47.9-208.4)	7.1	(2-12.2)
ST58	RGS	19.1	(15.1-23.1)	17.5	(12-22.8)	91.7	(45.4-138)	5.1	(1.1-9)

Colour code: RR ≤ 4.0 are represented on a light grey background; 4.0 < RR ≤ 10.0 are represented on a dark grey background; RR > 10.0 are represented on a black background.
*Estimates based on extrapolation (highest dose tested 200 mg/m^2).

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Table 4
Lethal concentrations 50 and LC_{90} and their 95% CI obtained for the 17 Brazilian field strains of *R.* (*B.*) *microplus* as well as their RR in comparison to the susceptible reference strain (Mozo) and their survival rates at the DD when tested with fipronil and pyriprol. Concentrations are expressed in mg/m^2.

		FIPRONIL									PYRIPROL								
strain	Origin	LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)	PR**	LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)	PR**
Mozo		0.008	(0.01-0.01)			0.02	(0.01-0.03)				0.02	(0.02-0.02)			0.05	(0-0.1)			
ST40	SãP	0.026	(0.02-0.03)	3.1	(2.5-3.7)	0.07	(0.05-0.08)	3.2	(1.6-4.8)	2.8	0.04	(0.04-0.05)	2.1	(1.6-2.6)	0.10	(0.1-0.1)	2.2	(1-3.4)	1.0
ST41	SãP	0.165	(0.14-0.19)	20.1	(15.3-25)	2.10	(1.2-3)	100.8	(33.6-167.9)	57.0	0.16	(0.1-0.2)	8.0	(5.9-10)	1.59	(0.9-2.3)	33.9	(11.4-56.4)	39.8
ST42	SãP	0.028	(0.02-0.03)	3.4	(2.7-4)	0.08	(0.05-0.12)	4.0	(1.9-6.1)	5.2	0.03	(0.03-0.04)	1.6	(1.3-2)	0.10	(0.1-0.1)	2.1	(1-3.2)	2.5
ST43	SãP	0.077	(0.07-0.09)	9.4	(7.7-11.2)	0.14	(0.09-0.19)	6.8	(3.2-10.4)	15.2	na	na	na	na		na	na	na	na
ST44	SãP	0.095	(0.07-0.12)	11.8	(8.8-14.7)	0.21	(0.1-0.3)	9.7	(5.1-14.3)	34.8	0.10	(0.1-0.1)	5.1	(3.8-6.4)	0.54	(0.4-0.7)	11.8	(4.7-18.9)	24.2
ST45	MGS	0.029	(0.03-0.03)	3.6	(2.7-4.5)	0.05	(0.04-0.06)	2.4	(1.3-3.5)	0.1	0.03	(0.02-0.03)	1.4	(1-1.7)	0.13	(0.1-0.2)	2.8	(0.9-4.7)	5.0
ST46	ES	0.139	(0.11-0.17)	17.0	(12.6-21.5)	0.62	(0.3-1)	29.5	(10.3-48.8)	53.5	0.12	(0.1-0.1)	6.2	(4.6-7.7)	0.46	(0.2-0.7)	9.9	(3.1-16.7)	26.8
ST47	RGS	0.078	(0.07-0.08)	9.6	(8-11.2)	0.11	(0.1-0.13)	5.4	(2.8-8)	2.0	0.08	(0.1-0.1)	3.9	(2.8-5)	0.28	(0.2-0.4)	6.0	(2.4-9.5)	13.1
ST48	SãP	0.054	(0.04-0.07)	6.6	(5-8.2)	0.63	(0.3-1)	29.8	(10.7-49)	30.6	0.06	(0.05-0.08)	3.2	(2.3-4.1)	0.54	(0.2-0.9)	11.6	(3.5-19.8)	20.4
ST49	MGS	0.022	(0.02-0.03)	2.8	(2.1-3.4)	0.09	(0.06-0.12)	4.5	(1.7-7.3)	6.8	0.03	(0.03-0.04)	1.6	(1.2-2.1)	0.20	(0.1-0.3)	4.2	(1.5-6.9)	8.4
ST50	MGS	0.315	(0.23-0.41)	38.4	(27.3-49.6)	14.5*	(2.7-26.2)	702.9	(129-1276)	65.6	0.44	(0.3-0.6)	21.8	(14.6-29.1)	32.8*	(-5.8-71.3)	698.0	(32.7-1363)	59.4
ST51	MGS	0.067	(0.04-0.1)	8.2	(5.5-10.8)	0.68	(-0.4-1.8)	33.3	(-0.7-67.3)	35.0	0.09	(0.05-0.12)	4.3	(2.9-5.7)	1.69	(-0.6-4)	36.2	(2.1-70.2)	31.1
ST52	PA	0.459	(0.32-0.6)	55.7	(38.3-73.1)	5.30	(0.7-9.9)	258.4	(33.5-483)	80.2	0.87	(0.6-1.2)	43.9	(26.1-61.7)	6.03	(0.8-11.2)	129.5	(4.1-254.9)	73.1
ST53	RGS	0.033	(0.03-0.04)	4.0	(3.1-4.8)	0.05	(0.04-0.06)	2.5	(1.4-3.6)	0.0	0.03	(0.03-0.04)	1.7	(1.3-2.2)	0.14	(0.1-0.2)	3.0	(1-5.1)	5.3
ST55	RGS	0.019	(0.01-0.02)	2.3	(1.6-2.9)	0.21	(0.07-0.36)	10.5	(2.8-18.1)	14.8	0.08	(0.04-0.11)	3.9	nd	1.08	(-0.1-2.3)	22.9	nd	28.0
ST57	SãP	0.307	(0.26-0.35)	37.2	(28.9-45.6)	0.82	(0.3-1.3)	39.7	(10.2-69.3)	97.4	0.41	(0.3-0.5)	20.8	(15.7-26)	1.33	(0.6-2)	28.3	(8.5-48)	82.3
ST58	RGS	0.179	(0.14-0.22)	21.6	(15.6-27.6)	1.04	(0.4-1.7)	51.3	(10.9-91.7)	61.8	0.37	(0.2-0.5)	18.8	(10-27.7)	3.97	(0.5-7.5)	82.6	(-16.6-182)	61.0

na: not available due to insufficient number of engorged females obtained, nd: not defined. Colour code: RR ≤ 4.0 are represented on a light grey background; 4.0 < RR ≤ 10.0 are represented on a dark grey background; RR > 10.0 are represented on a black background.
*Estimates based on extrapolation (highest dose tested 6.25 mg/m^2).
**Predicted survival rates at the potential discriminating doses (2× LC_{99} of the susceptible reference strain).

a consequence, the LPT has failed to identify resistance in 6 cases where the LTT showed RR50 values clearly above 4.

4. Discussion

The LTT is a time-effective test which relies on the distribution of tick eggs in the wells of pre-treated 96-well plates, allowing testing 12 doses of 5 compounds in a single plate. It was shown previously to be equally sensitive and much more time effective than the LPT (Lovis et al., 2011). In this article, we present some additional information to facilitate the completion of the LTT and some alternatives to the equipment presented in Lovis et al. (2011). The use of glass beads and talc allows individualising eggs in an extremely effective way and with very basic material. In addition, the use of a curette to measure the quantity of eggs and to distribute the eggs instead of the seed counter avoids investing in cumbersome and costly equipment. Furthermore, static electricity can be removed during distribution and the sealing of the plates by using a static control mat which is a simple alternative to other discharging systems. For the moment, DMSO evaporation after the coating of the plates with the acaricides still requires some particular equipment. We suggest here two possibilities (N_2 sampler concentrator, centrifugal vacuum concentrator) but a simplified system would be desirable. A possibility might be to consider using a different solvent which evaporates more easily while at the same time ensuring satisfactory dissolution of all tested compounds without damages to the polystyrene plates. In the setup of our study, plates treated with all compounds could be stored for at least 5 weeks without losing activity (data not shown). Finally, the

Table 5
Lethal concentrations 50 and LC_{90} and their 95% CI obtained for the 17 Brazilian field strains of *R.* (*B.*) *microplus* as well as their RR in comparison to the susceptible reference strain (Mozo) and their survival rates at the DD when tested with ivermectin and moxidectin. Concentrations are expressed in mg/m^2.

		IVERMECTIN									MOXIDECTIN								
strain	Origin	LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)	PR**	LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)	PR**
Mozo		0.9	(0.7-1)			4.3	(1.8-6.7)				0.39	(0.3-0.4)			1.3	(1-1.7)			
ST40	SãP	1.7	(1.3-2.1)	2.0	(1.3-2.7)	6.7	(3.4-10.1)	1.6	(0.2-3)	0.3	0.43	(0.4-0.5)	1.1	(0.8-1.4)	1.3	(0.8-1.7)	1.0	(0.3-1.6)	0.2
ST41	SãP	1.4	(1.2-1.6)	1.7	(1.1-2.2)	8.6	(5.2-11.9)	2.0	(0.2-3.8)	1.3	0.64	(0.6-0.7)	1.7	(1.1-2.2)	2.2	(1.5-2.8)	1.7	(0.5-2.9)	0.6
ST42	SãP	1.4	(1.1-1.7)	1.6	(1-2.2)	5.1	(2.9-7.4)	1.2	(0.1-2.3)	0.2	0.62	(0.5-0.7)	1.6	(1.1-2.1)	2.7	(1.5-3.9)	2.0	(0.6-3.5)	1.4
ST43	SãP	na	na	na	na	na	na	na	na	na	0.35	(0.3-0.4)	0.9	(0.6-1.2)	1.7	(1-2.3)	1.3	(0.4-2.1)	1.0
ST44	SãP	3.1	(2.4-3.7)	3.6	(2.4-4.8)	11.6	(5.9-17.3)	2.6	(0.4-4.9)	0.8	0.66	(0.6-0.7)	1.7	(1.2-2.2)	2.3	(1.6-3)	1.7	(0.5-3)	0.8
ST45	MGS	0.8	(0.7-0.9)	0.9	(0.6-1.2)	3.0	(1.9-4.1)	0.7	(0.1-1.3)	0.1	0.26	(0.2-0.3)	0.7	(0.4-0.9)	0.6	(0.3-1)	0.5	(0.1-0.8)	0.0
ST46	ES	2.5	(2.3-2.8)	2.9	(2.1-3.8)	5.1	(3.6-6.6)	1.2	(0.2-2.1)	0.0	0.68	(0.5-0.8)	1.8	(1.2-2.3)	2.8	(1.5-4)	2.1	(0.5-3.7)	0.8
ST47	RGS	1.4	(1-1.7)	1.6	(1.1-2.1)	6.8	(1.1-12.5)	1.6	(0.1-3.1)	0.8	0.30	(0.3-0.3)	0.8	(0.6-1)	0.6	(0.4-0.9)	0.5	(0.2-0.8)	0.0
ST48	SãP	1.1	(0.9-1.3)	1.2	(0.8-1.7)	5.9	(3.2-8.6)	1.4	(0.1-2.7)	0.6	0.57	(0.4-0.8)	1.5	(0.9-2.1)	4.2	(1.4-7)	3.2	(0.6-5.8)	3.4
ST49	MGS	1.0	(0.9-1.1)	1.2	(0.8-1.6)	3.8	(2.7-5)	0.9	(0.1-1.7)	0.2	0.46	(0.4-0.5)	1.2	(0.8-1.6)	2.5	(1.5-3.5)	1.9	(0.4-3.4)	1.7
ST50	MGS	1.3	(1.1-1.5)	1.5	(1-2)	5.6	(2.8-8.3)	1.3	(0.2-2.5)	0.5	0.23	(0.2-0.3)	0.6	(0.4-0.8)	1.2	(0.4-2)	0.9	(0.3-1.6)	0.8
ST51	MGS	1.0	(0.9-1.1)	1.1	(0.7-1.5)	7.3	(5-9.6)	1.7	(0.2-3.3)	1.3	0.26	(0.2-0.3)	0.7	(0.4-0.9)	2.1	(1-3.2)	1.6	(0.3-2.8)	2.1
ST52	PA	1.4	(1.2-1.6)	1.6	(1-2.2)	7.4	(4.8-9.9)	1.8	(0.2-3.3)	0.7	0.64	(0.5-0.8)	1.7	(1.1-2.3)	2.1	(1.1-3.1)	1.6	(0.3-2.8)	0.4
ST53	RGS	3.6	(3-4.2)	4.2	(3-5.3)	6.6	(5.4-7.8)	1.6	(0.6-2.6)	0.0	0.80	(0.7-0.9)	2.1	(1.4-2.8)	2.4	(1.7-3.2)	1.8	(0.4-3.2)	0.5
ST55	RGS	3.4	(2.6-4.2)	4.0	(2.8-5.2)	5.8	(4.3-7.2)	1.4	(0.5-2.3)	0.0	0.83	(0.6-1)	2.1	(1.4-2.9)	1.6	(1.2-2)	1.2	(0.4-2)	0.0
ST57	SãP	2.3	(1.8-2.7)	2.6	(1.5-3.7)	8.5	(5.2-11.7)	2.0	(0.2-3.9)	0.2	0.56	(0.4-0.7)	1.5	(0.9-2)	2.9	(1.6-4.3)	2.2	(0.4-4)	1.7
ST58	RGS	1.8	(1.5-2.1)	2.1	(1.3-2.8)	8.8	(5.8-11.7)	2.1	(0.2-4)	1.0	0.80	(0.6-1)	2.1	(1.4-2.8)	1.8	(0.9-2.7)	1.3	(0.4-2.3)	0.0

na: not available due to insufficient number of engorged females obtained. Colour code: RR ≤ 4.0 are represented on a light grey background; 4.0 < RR ≤ 10.0 are represented on a dark grey background; RR > 10.0 are represented on a black background.
**Predicted survival rates at the potential discriminating doses (2× LC_{99} of the susceptible reference strain).

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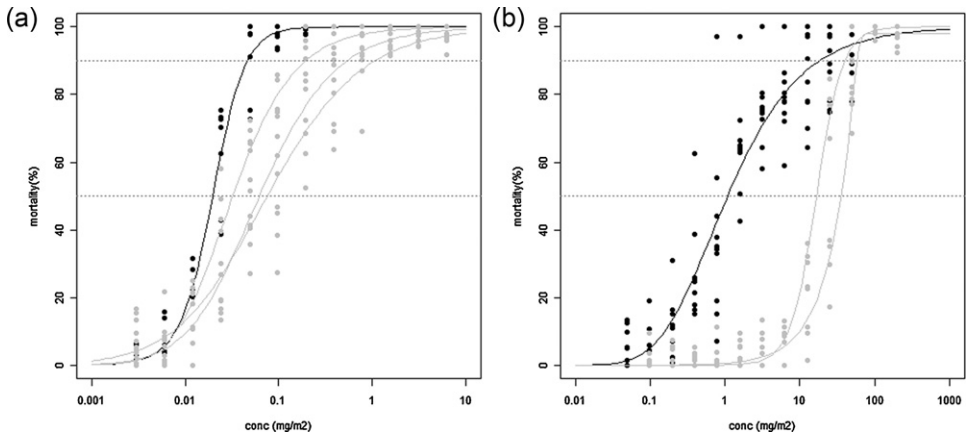

Fig. 1. Dose–response curves obtained with the LTT (a) when conducted with pyriprol: three field populations (ST48, ST49 and ST55, grey) in comparison to the susceptible reference Mozo strain (black) and (b) when conducted with amitraz: two field populations (ST41 and ST48, grey) in comparison to the susceptible reference Muñoz strain (black). The grey dotted horizontal lines indicate 50% and 90% mortalities.

incubation conditions of the plates should be kept as stable as possible to decrease the factors which could negatively impact the eggs between their distribution into the plates and their hatching.

The Mozo strain was meant to be used as susceptible reference strain for all compounds since it was tested in parallel to the field populations and in the same conditions. However, our Mozo isolate showed unexpectedly high resistance to both cypermethrin and flumethrin and moderate resistance to amitraz in comparison to the Muñoz strain (data not shown) and was for this reason replaced by the Muñoz strain for these compounds. The resistance of the Mozo strain is surprising since it has never been exposed to acaricides before and after its collection. Additionally, it has already been used as susceptible reference strain for SP (Mendes et al., 2011). Our isolate was established at the IPVDF in November 2010 and larvae used for testing were from the second generation. It is possible that the IPVDF isolate has been contaminated with ticks from a resistant isolate during that time.

The tested concentrations of the acaricidal compounds were suitable to calculate LC_{50} and LC_{90} of susceptible and resistant populations. Only in 5% and 10% of the tests, an extrapolation from the dose–response curve was necessary to estimate the LC_{50} and LC_{90}, respectively. For studies aiming to evaluate the susceptibility of field populations without prior knowledge on their resistance status, we recommend testing the same concentration ranges (as described in Section 2), with the following two modifications: chlorpyriphos, 0.4–800 mg/m^2 instead of 0.2–400 mg/m^2; cypermethrin, if the populations are expected to be resistant, 0.4–800 mg/m^2 instead of 0.1–200 mg/m^2. These ranges should minimize the cases where LC_{90} have to be extrapolated from the model.

We observed particularly high resistance frequencies to OP, SP, amitraz and PYZ, most probably because farms were selected based on the observation of treatment failures. We reported 94% resistance to SP and 65% resistance to chlorpyriphos, which is comparable to the values determined by Martins et al. (2008) with the AIT and by Mendes et al. (2011) with the LPT. In contrast, the coumaphos and amitraz resistance frequencies we observed (82% and 88%, respectively) are higher than those of previous studies (Campos Júnior and Oliveira, 2005; Farias et al., 2008; Martins et al., 2008). Resistance to fipronil was detected in 65% of the farms we surveyed, which is very high considering that resistance to this compound was reported only recently in Brazil (Martins et al., 2008; Castro-Janer et al., 2010). In 2008, Martins et al. reported an average of efficacy of fipronil of 88.5% among 723 populations tested with the AIT between 1997 and 2006. More recently, Castro-Janer et al. (2010) also reported some resistance to fipronil with the LIT. The value obtained in the present study is worrying and suggests that resistance to fipronil is spreading rapidly in Brazil. The presence of four populations demonstrating dose–response mortality curves typical of heterogeneous populations also reflects that fipronil resistance is in process of development in field populations.

Table 6
Resistance ratios based on the LC_{50} when assessed with the LTT and LPT for 4 Brazilian field populations.

	Chlorpyriphos*		Cypermethrin**		Ivermectin*		Fipronil*		Amitraz**	
strain	LTT	LPT	LTT	LPT	LTT	LPT	LTT	LPT	LTT	LPT
ST40	19.8	3.1	104.8	65.6	2.0	1.3	3.1	1.5	7.7	2.4
ST41	7.6	1.9	141.0	69.2	1.7	1.5	20.1	2.1	15.3	7.9
ST42	5.1	na	201.0	106.6	1.6	1.2	3.4	0.8	9.3	27.8
ST44	28.7	3.4	167.3	45.5	3.6	1.4	11.8	1.4	28.6	12.8

na: not available because of insufficient data to generate the dose–response mortality curve. Colour code: RR ≤ 4.0 are represented on a light grey background; 4.0 < RR ≤ 10.0 are represented on a dark grey background; RR > 10.0 are represented on a black background.
*Mozo is the reference strain.
**Muñoz is the reference strain.

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Similarly, resistance to pyriprol was also found in nearly half of the populations surveyed whereas this compound is not used to treat cattle against ticks. Finally, resistance to ivermectin has also already been reported several times in Brazil using the LIT (Klafke et al., 2006, 2012).

In the present study, field populations were considered to be resistant when RR50 were greater than 4. This value allowed discriminating very well between SP susceptible and SP resistant populations since most of the RR50 estimates were greater than 40 for resistant, or around 1 for susceptible populations. In contrast, for OP, PYZ and amitraz, several populations possessed RR50 between 3 and 5, with some estimates smaller or equal to the threshold of 4.0, but having the upper 95% CI limit over 4.0. In these last cases, susceptibility can be argued, but this is inherent to the use of cut-off values. Likewise two populations (ST44 and ST55) demonstrated RR50 estimates to ivermectin of 3.5 and 4.0, respectively and resistance could therefore be suspected. Finally, if populations had been considered resistant when RR50 was statistically significant and greater or equal to 2, instead of using our cut-off value, as it is has been done in previous studies (Castro-Janer et al., 2011; Klafke et al., 2012), many additional populations would have been considered resistant. Thus, all the populations would have been considered resistant to fipronil and 44% resistant to ivermectin.

The resistance status of the field populations was based on RR50 because they possess smaller 95% CI than RR90 and are therefore more reliable estimates. However, as it is essential not to miss resistance at its emergence, resistance statuses based on RR50 were also compared to those based on RR90. Considering RR90 instead of RR50 would have led to similar conclusions to distinguish resistant from susceptible field populations in 94% (124/132) of the cases, excluding amitraz. The discrepancies were observed with flumethrin, fipronil and pyriprol, for which 1, 2 and 4 additional populations, respectively would have been considered resistant if based on RR90 instead of RR50 (Fig. 1a for pyriprol). This observation suggests that resistance is emerging in these populations. Inversely, the population considered as ivermectin-resistant based on RR50 would have been considered susceptible based on RR90, reflecting the steep dose–response curve of the field population. Amitraz situation (Fig. 1b) was particular since all the field populations demonstrated greater slopes than the reference strain. Thus, although a wide shift was observed between the response of the reference and the field populations at low concentrations, the curves intersect around 90–95% mortality. As a consequence, if the RR90 had been considered, only 5 out of the 15 amitraz resistant strains based on RR50 would have been diagnosed as resistant. To conclude, if the complete dose–response curve is obtained, we recommend generating both RR50 and RR90 estimates, considering RR50 as a priority and comparing them to RR90 to detect emerging resistance, with the exception of amitraz, for which the use of RR90 is clearly not indicated.

The use of DD to determine resistance has been recommended by the FAO (2004) but has been criticised (Jonsson et al., 2007). We therefore wanted to assess if DD would have been suitable in our study and found a wide agreement between the survival rates at the DD calculated as $2\times$ LC_{99} of the susceptible reference strain, and the RR50 or RR90. Survival rates at these DD exceeded 10% in all the populations diagnosed as resistant based on the RR50, with 3 exceptions. Additionally, the use of the DD would have allowed detecting 3 of the 7 cases of emerging resistance. However, since DD are particularly valuable to reduce the amount of work and of ticks needed to detect resistance, their interest is, to our opinion, limited in the case of the LTT and we would not recommend their use as a substitute of the full dose–response mortality curves.

The LPT and LTT have already been compared using the laboratory strains Muñoz and Ultimo and were shown to perform equally well in the detection of the resistance to diazinon, flumethrin, cypermethrin and amitraz (Lovis et al., 2011). In the present study, we repeated the comparison but limited it to four field populations due to the labour-intensive nature of the test. The LTT showed a higher sensitivity than the LPT to measure resistance, providing higher resistance ratios to all compounds. This was most visible for chlorpyriphos and fipronil, for which the LPT failed to detect resistance while the LTT detected 4 and 2 resistant populations, respectively. Finally, since the LIT is getting increasingly used for the detection of resistance to ivermectin and fipronil (Klafke et al., 2006; Castro-Janer et al., 2011) and has been shown to perform better than the LPT for the detection of fipronil resistance (Castro-Janer et al., 2009), it would be also relevant to compare the sensitivity of the LTT and the LIT to detect resistance to these compounds.

To conclude, the present study showed that the LTT is a reliable bioassay to diagnose acaricide resistance in *R.* (*B.*) *microplus* field populations of ticks. The original method as described by Lovis et al. (2011) was adapted to reduce the required lab-infrastructure for the test performance. A detailed protocol for the tick egg separation and the distribution into the microtiter plates is provided. With these modifications, the LTT can be carried out in laboratories without additional needs of expensive equipment and infrastructure. It allowed here to confirm the widespread resistance to OP, SP and amitraz, to identify a few cases of ivermectin resistance, but also to show the important on-going development of PYZ resistance in Brazil.

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