Monitoring of acaricide resistance in *Rhipicephalus bursa* ticks collected from sheep on the island of Lesvos, Greece

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1. Abstract

Chemical acaricides are widely used to control ticks on animals to avoid heavy tick burdens, which cause economic loses. In tick populations certain individuals are more tolerant for a specific acaricide than other individuals. Previous studies have shown that continued exposure to an acaricide results in removal of the susceptible part of a tick population and an increase in the proportion of resistant individuals.

Tick control strategies in Greece rely heavily on synthetic pyrethroids. Therefore, this study was designed to test the susceptibility of *Rhipicephalus bursa* ticks, collected from sheep of the island Lesvos, for the synthetic pyrethroid, alpha-cypermethrin. The Larval Packet Test was carried out for definitive confirmation of a diagnosis of resistance. For *Rhipicephalus bursa* ticks, the lethal concentration (LC) to kill 50% of the ticks was $3{,}816 \cdot 10^{-3}$ mg/mL alpha-cypermethrin. In future studies, this LC_{50} can be used as a baseline to compare and/or confirm the resistance status of field populations of *Rhipicephalus bursa* ticks.

It is recommended to closely monitor possible changes in susceptibility to acaricides at an early stage in order to adapt the tick control policies. Additional baseline data are required for other ticks found on livestock in Greece using the same methods employed in this study.

2. Introduction

Ticks belong to the class arachnida together with mites and spiders. Compounds that are used to control ticks are called acaricides. Different compounds have different acaricidal properties and in tick populations certain individuals are more tolerant for a specific acaricide than others (1). It may be difficult to differentiate between resistance and tolerance that may exist in every population of ticks (2). Acaricide resistance has been reported in cattle ticks, whereas resistance in ticks feeding on dogs and cats is rare (1). Acaricides provide farmers a method to keep their stock protected from ticks, in a low labour-input and cost-effective way (3). Previous studies showed that continued exposure to an acaricide results in removal of the susceptible part of a tick population with an increase in the proportion of the resistant individuals, in other words a process of selection for resistance (4). Heavy tick burdens cause huge economic losses through different ways, but the long-term use of acaricides has generated acaricide resistance in many tick species nowadays, thereby reducing the ability to control ticks (4).

According to the literature, there are three necessary conditions for evolution of resistance to occur:

- individuals in the population must differ genetically,
- genetic differences must produce a phenotypic difference
- and the phenotypic difference must enhance survivability, transferring the resistance to the next generation (1).

The definition of resistance has changed with time, which should be kept in mind as historical reports of 'resistance' are reviewed. In 1957 the World Health Organization (WHO) (5) defined resistance as: "the development of an ability to tolerate toxicants which would prove lethal to the majority of individuals in a normal population of the same species". Later, in 1992, the WHO (6) defined resistance in arthropods as: "an inherited characteristic that imparts an increased tolerance to a pesticide, or group of pesticides, such that the resistant individuals survive a concentration of the compound(s) that would normally be lethal to the species". In a review of Coles et al. (1) this latter definition is still called problematic because it includes the term 'tolerance'.

As resistance has developed, there are three different types of resistance mechanisms that can be distinguished; acquired resistance, cross-resistance and multiple resistance. Acquired resistance results from heritable decreases in sensitivity to drugs with the passage of time. Cross-resistance is the sharing of resistance among different acaricides with a similar mode of action and multiple resistance means resistance to more than one drug, even though they have different modes of action (4).

Ticks can be resistant to different acaricides like organochlorides, organophosphates, carbamates, macrocyclic lactones, formamidines and pyrethrins/pyrethroids. Pyrethroids are synthetic forms of pyrethrins, naturally-occuring compounds derived from members of the chrysanthemum family that have a quick "knock down" effect against arachnids, designed to be more stable and have a longer lasting effect as neurotoxins (4). Cyphenothrin, permethrin, flumethrin and deltamethrin belong to the group of second generation synthetic pyrethroids. Pyrethroids act on sodium ion channels. Closing these channels leaves the nerve cell membrane in a permanent state of depolarization, resulting in a sudden "knock down" effect in ticks (7).

Resistance in cattle ticks against acaricides like pyrethroids has been reported in India, Brazil, Colombia, Mexico, USA, Australia and Iran (4). At least two different mechanisms were found to confer resistance to pyrethroids (8). Target site mediated resistance was confirmed by He et al. (9) in 1999 who discovered a mutation on the Na^+-channel. In the second pyrethroids resistance a metabolic mechanism was responsible. Jamroz et al. (10) confirmed this with discovery that CzEst9 esterase activity was much higher in the resistant population. This mechanism involving overexpression of the esterase, appears to facilitate pyrethroids resistance. Thus, an assay to specifically quantitate CzEst9 protein activity in tick populations seems most appropriate. This would be a fast way for determination of a pyrethroids resistance mechanism (8).

Tick control strategies in Greece rely heavily on synthetic pyrethroids. A question that arises regarding acaricide resistance is the susceptibility of one of the most common tick species in Greece, *Rhipicephalus bursa*, to synthetic pyrethroids. *Rhipicephalus bursa* has developed acaricide resistance for pyrethroids (11) and propetamphos, an acaricide that belongs to the group of organophosphate, in Iran (12).

The purpose of this study was to test the susceptibility of *Rhipicephalus bursa* ticks for pyrethroids. To achieve this, larvae of *Rhipicephalus bursa* ticks were tested using the Larval Packet Test. The acaricide which was used in this study is alpha-cypermethrin, a synthetic pyrethroid available on the market as Alfapor®.

3. Main research question

3.1 Main research question

What is the susceptibility of *Rhipicephalus bursa* ticks, collected from sheep on the island of Lesvos, Greece, for synthetic pyrethroids by using the Larval Packet Test?

3.2 Hypothesis

H_0; *Rhipicephalus bursa* ticks collected from sheep on the island of Lesvos, Greece, are susceptible to pyrethroids.

H_1; *Rhipicephalus bursa* ticks collected from sheep on the island of Lesvos, Greece, are resistant to pyrethroids.

4. Materials and methods

The standard FAO-recommended research methodology, the Larval Packet Test, will be applied, in order to create valuable and comparable results.

4.1 Conditions ticks during testing

Rhipicephalus bursa engorged female ticks were previously collected from sheep in Greece and used to start a laboratory collection in the UCTD. Larvae from this laboratory colony of exactly the same age were used for the acaricide test. In a climate-controlled chamber the engorged females will be placed under a temperature of 27-28 °C and relative humidity (RH) of 85-95 %, for egg laying. After the eggs are collected they were kept under the same humidity and temperature as the engorged females, until the eggs hatch (13). Then the larvae were held under the same conditions as mentioned above.

4.2 Acaricide resistance testing

Larvae of *Rhipicephalus bursa* ticks were used for this test. The susceptibility for alpha-cypermethrin, a synthetic pyrethroid (Alfapor®) was tested (*see table 1*).

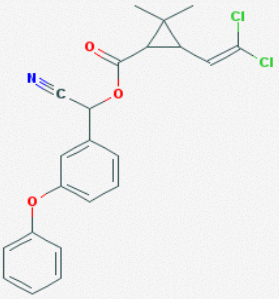
Scientific name	Chemical group	Molecule	Veterinary medicine	Spectrum
Alpha-cypermethrin	Synthetic pyrethroids		Alfapor	Ectoparasites

Table 1. Feature of acaricide used in this study (7,14)

The dilutions were based on the concentrations between the lowest concentration that kills all ticks and the highest concentration in which all ticks were alive (i.e. 0-100 % mortality series). The used dilutions are showed below in *table 2*.

Tick species Rhipicephalus bursa	
Dilution	**Concentration alpha-cypermethrin**
1	**$0{,}1 \cdot 10^{-4}$ mg/mL**
2	**$0{,}1 \cdot 10^{-3}$ mg/mL**
3	**$0{,}1 \cdot 10^{-2}$ mg/mL**
4	**$0{,}1 \cdot 10^{-1}$ mg/mL**
5	**0,1 mg/mL**
6	**1,0 mg/mL**
7	**10 mg/mL**

Table 2. Used dilution series

Larval Packet Test

This test was used for definitive confirmation of a diagnosis of resistance. For the Larval Packet Test, there are sheets of filter paper (10x5 cm) folded and secured with clips on the edges and in the center. The packet was moistened with 1800 µL of the acaricide to be tested (synthetic pyrethroid alpha-cypermethrin), in different dilutions in olive oil and trichloroethylene (1:2), in triplicate for each dilution per test. Three control packets were also moistened, using only olive oil and thrichloroethylene (1:2). On each sheet ± 100 larvae were placed and the packets were placed in a climate-controlled chamber for 24 hours. To prevent contamination, all packets were stored separately. So eight different containers were used, one for each of the seven different dilutions and one for the control, containing three packets. After opening the packets 24 hours later, the larvae were counted, dead and alive. For a more detailed protocol description, see *Appendix A*.

4.3 Statistics

After counting the larvae, dead and alive, the numbers were entered in to an EXCEL spreadsheet and a percentage of mortality for each dilution was calculated. If the mortality among the larvae in the control packets was below 5 %, then the direct mortality figures were used. If the mortality was found to be between 5 % and 10 % in the control packets, then the percentage mortality in all of the dilutions were corrected by using Abbotts's formula (*see figure 1*). If mortality was found to be higher than 10 % in the control packets, the results were disregarded and the test was repeated.

$$\text{Corrected percent mortality} = \frac{\text{\% test mortality - \% control mortality}}{\text{100 - \% control mortality}} \times 100$$

Figure 1. Abbotts's formula

Results were plotted with percent concentration (x-axis) by Probit mortality (y-axis) for the acaricide and tick specie, hereby determining the LC_{50} and LC_{99}. After that the factor of resistance can be determined (*see figure 2*).

$$\text{Factor of Resistance} = \frac{LC_{50} \text{ of acaricide read from graph}}{LC_{50} \text{ for susceptible strain}}$$

Figure 2. Formula to determine factor of resistance

If the population is homogeneously susceptible, a straight line should be obtained for all tests.

5. Results

According to Robertson et al. (15), as long as the experiment is replicated three times with five doses in each replication, the test is valid.

All results are showed in *figure 3*. In *table 3*, two lethal concentrations were given to kill 50% (LC_{50}) and 99% (LC_{99}) of all ticks. A total of five (A,B,C,D,E) Larval Packet tests were carried out, with seven dilutions in each replication, to determine a reliable LC_{50}.

The lethal concentration to kill 50% of all *Rhipicephalus bursa* ticks was $3{,}816 \cdot 10^{-3}$ mg/mL alpha-cypermethrin from the product Alfapor®. This LC_{50} is quite accurate, thus can be used for baseline data to compare and/or confirm the resistance status of field populations of *Rhipicephalus bursa* in the future. The LC_{99} lethal concentration is not reliable, due to the sample size and must be disregarded (*see Discussion, page 11*)(15).

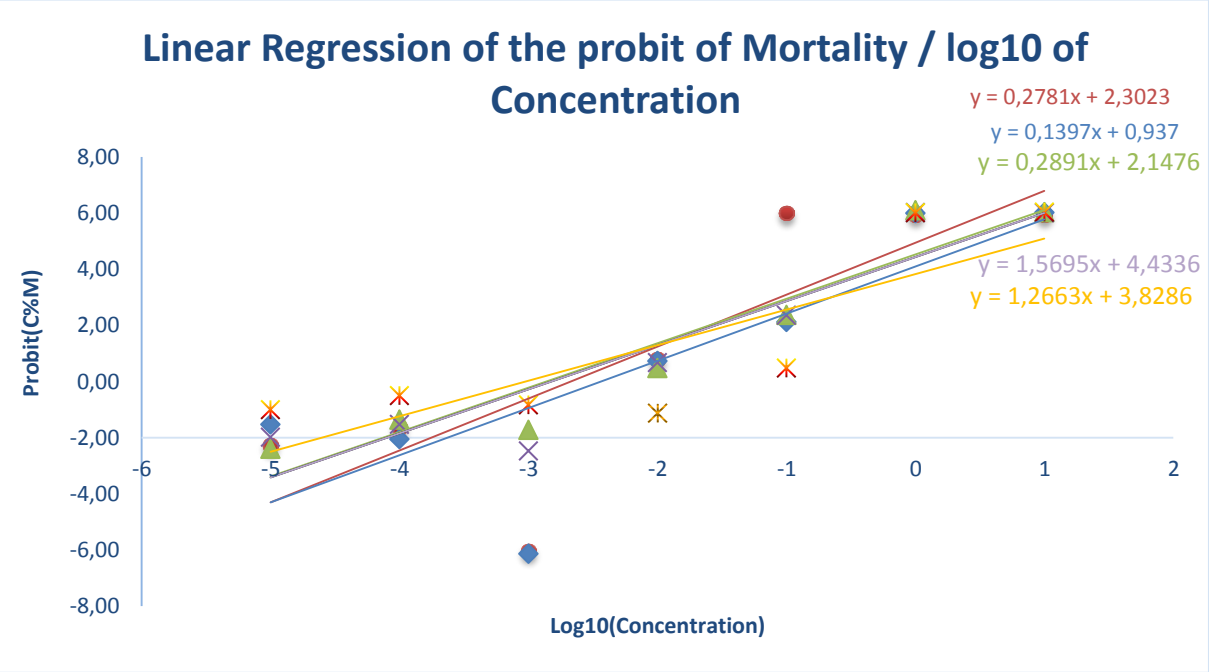


Figure 3. Results of a total of five LPT for Rhipicephalus bursa

	Equation y=ax+b		Log concentration			LC	
	a	b	log50	log99		LC50	LC99
A	2.014479621	4.403533	-2.18594094	-1.03113		0.006517	0.09308
B	1.590751831	3.649678	-2.29431014	-0.83189		0.005078	0.14727
C	1.575128539	3.618737	-2.29742318	-0.8205		0.005042	0.15118
D	1.569457002	4.433608	-2.82493134	-1.34267		0.001496	0.04543
E	1.266305507	3.828634	-3.02346802	-1.18635		0.000947	0.06511
MEAN						0.003816	0.10041

Table 3. Mean lethal concentration of alpha-cypermethrin to kill 50% and 99% of all ticks, extracted from *figure 3*

Because these five Larval Packet Tests with alpha-cypermethrin were so successful with the used dilution series, another Larval Packet Test was performed but with the active ingredient amitraz

(from the product Milbitraz®) instead of alpha-cypermethrin. For this test an adjustment on the protocol was necessary; standard incubation time of 24 h was replaced by 48 h, because of the mode of action of the active ingredient amitraz (*see Discussion, page 12*). For the result of this Larval Packet Test see *figure 4*. *Table 4* gives the tentative lethal concentrations for *Rhipicephalus bursa* ticks with amitraz.

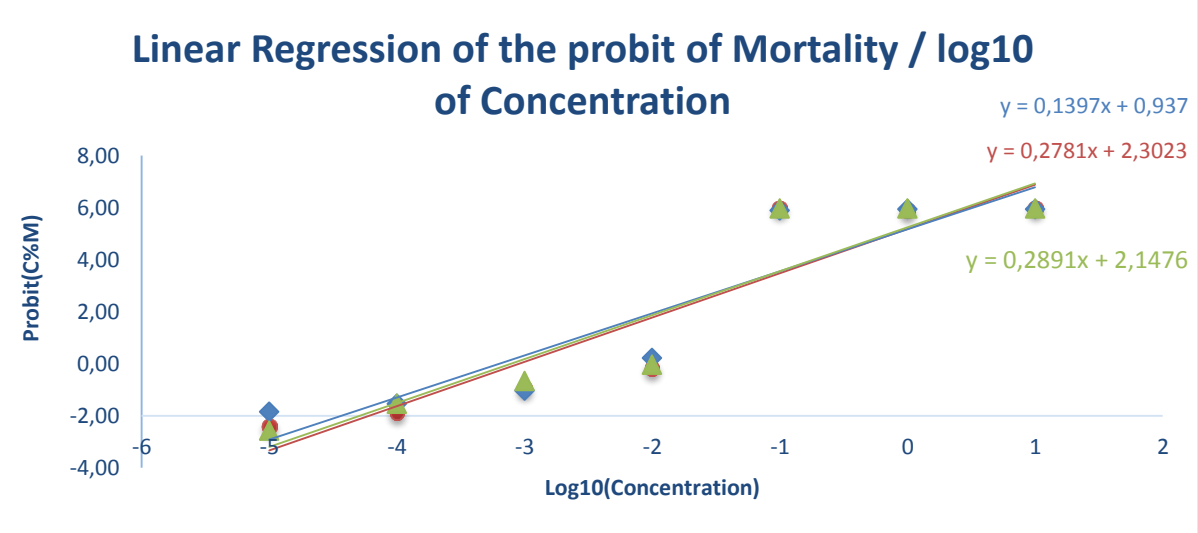


Figure 4. Result Larval Packet Test with amitraz.

	Equation y=ax+b		Log concentration			LC	
	a	b	log50	log99		LC50	LC99
A	1,701024419	5,17577	-3,04273684	-1,67512		0,000906	0,02113
B	1,616951542	5,172704	-3,19904715	-1,76032		0,000632	0,01737
C	1,687826512	5,240367	-3,10480169	-1,72649		0,000786	0,01877
MEAN						0,000775	0,01909

Table 4. Mean lethal concentration of amitraz to kill 50% and 99% of all ticks, extracted from *figure 4*

6. Discussion

6.1 Dilution series

Before performing the Larval Packet Test, dose selection must be performed. Normally the definitive dilution series are based on a serial dilution of seven doses which are randomly chosen to determine a narrower range of effective concentrations between the lowest concentration of the active ingredient that kills all ticks and the highest concentration in which all ticks stayed alive (i.e. 0 -100% mortality series). This is called the dose-fixing phase, in which only about ten larvae per dose are used to determine the mortality (15). In this study, the dilution series are based on previous studies from the UCTD with *Rhipicephalus bursa* larvae that showed the susceptibility of the tick specie to fipronil from the product Frontline® and the pyrethroid insecticide resistance review article from Iran (11). The dilution series that are used in this study appeared to be very successful, covering the entire range between 0 – 100% mortality as mentioned above, so no adjustments were made (*see table 2*).

6.2 Product

Tick control strategies in Greece rely heavily on synthetic pyrethroids. The question that arises regarding acaricide resistance is the susceptibility of one of the most common tick species in Greece, *Rhipicephalus bursa.* Ectopor® is an synthetic pyrethroid available on the Greek market. In the ideal situation this product was used in the Larval Packet Test to determine susceptibility baseline date for *Rhipicephalus bursa*. Unfortunately it was not possible to obtain this product, so instead the product Alfapor® was used in this study. Alfapor® is an synthetic pyrethroid with the same active ingredient, alpha-cypermethrin. This product is most commonly used in Uganda in tick control strategies.

6.3 LC_{50} and LC_{99}

As mentioned in the chapter Results, the LC_{50} can be assumed as quite accurate, in contrast to the LC_{90}. Due to the sample size, the LC_{90} is not reliable and must be disregarded, according to Robertsen et al. (15). Robertsen et al. (15) determined the required sample size for bioassays with arthropods to obtain a reliable LC_{50} and LC_{90} by comparing different designs for estimation of a LC_{50} and LC_{90}. Each lethal concentration (a.o. LC_{50}, LC_{90}, LC_{95} and LC_{99}) requires a different sample size. They also found that precision increases as the number of doses increased and of course estimates of LC_{50} and LC_{90} become more precise as total sample size increases.
The optimal test setting contains of, if possible, eight doses and a total sample size of 500 test subjects (larvae), which means approximately 63 larvae per dose. However, it is noted that when seven to twelve doses are tested, most estimates of LC_{50} are precise. In this study eight doses are used with approximately 100 larvae per dose, so the LC_{50} can be assumed pretty accurate. For precise estimation of LC_{99}, none of the designs of four to six doses is acceptable, at least seven doses are necessary. This study meets this requirement by the use of eight different doses, but the required sample size for this estimation is 100.000 larvae, which means a minimum of 12.500 larvae per dose, is nearly impossible in practice, so the estimation of LC_{99} is unreliable.
Sample sizes are, even on a mass scale, limited by the realities of insect collection and/or production. For experiments in which comparisons are involved; screening, population responses, natural

variation in response, a basic design for estimation of the LC_{50} will suffice and comparisons at the LC_{99} should be avoided (15).

6.4 Comparison with literature

According to Bardosh et al. (16) and the instructions which have been added to the product Alfapor®, the recommended dilution is; 1 mL Alfapor® product in 1 L water. The product Alfapor® contains 50 mg/mL active ingredient alpha-cypermethrin. The solution which can be used in the field, contains $50 \cdot 10^{-3}$ mg/mL alpha-cypermethrin in 1 L dilution (0,05 mg/mL alpha-cypermethrin).
In this study, the lethal concentration to kill 50% of all ticks, was calculated on $3,8 \cdot 10^{-3}$ mg/mL alpha-cypermethrin (0,0038 mg/mL alpha-cypermethrin). Less active ingredient is needed to kill 50% of all ticks, so there can be assumed that the concentration of alpha-cypermethrin in the recommended solution in the field, kills the majority part of the ticks. Unfortunately, the LC_{99} is not reliable, otherwise the lethal concentration was known to kill 99% of all ticks. Which is ultimately what the manufacturer wants to achieve in the field with their recommended dilution, otherwise acaricide resistance is actually promoted.

The instructions which are added to the product Alfapor® is contradictory as regards the replication of the treatment. Two different replications are mentioned; after 4-6 weeks as need may be and after 2 weeks, even one week in case of heavy tick infestations or in tsetse infested areas.

As mentioned above, you can assume that the manufacturer of Alfapor® wants to kill all ticks with their recommended solution. If not all ticks are killed and still the same acaricide is applied on the animals, resistance will develop. According to Johnsson et al. (17) greater than five treatments per season is a positive risk factor for acaricide resistance. Suggesting that high treatment frequency with the same acaricide predisposes cattle ticks to selection for resistance.

6.5 LPT with amitraz

Amitraz is a formamidine that is selective towards mites and ticks and has been used for the last fifty years to control ticks. Amitraz induces an alteration of the behavior, which has been studied in ticks. Amitraz binds to the octapamine receptors on the nerve cell membrane, which leads to the stimulation of monoamine oxidases (adenylate cyclase activity) and the G protein. This chain reaction has various intracellular actions, due to the synthesis of cAMP and cGMP. Treatment with amitraz will cause the attached ticks to fall off their host. Tick that infest a host do not attach nor feed. At sublethal doses, reproduction is impaired, prolificity is reduced and most of the eggs do not hatch (7). In a review of Ducornez et al. (18) it came forward that the standard LPT does not produce dose-mortality relationships that can be used to discriminate between susceptible and resistant individuals. Miller et al. (19) said the cause for this lack of a dose-mortality relationship had been attributed to an inadequate exposure time, possible interaction of technical amitraz and the paper substrate, and the instability of technical amitraz which maybe degrading during the bioassay. Therefore, the bioassays used for amitraz involved increasing in the exposure time from 24 to 48 h and replacing technical amitraz with formulated amitraz (20), so the LPT was adapted. Furthermore, the standard Larval Packet Test procedure was followed in the test with amitraz.

As mentioned above, the lethal concentrations that are given in *table 4* are preliminary. Result of one Larval Packet Test is not enough to define as baseline data for the resistance status of *Rhipicephalus bursa* ticks for amitraz. Unfortunately, there were not enough larvae available in order to perform two more tests. Two more Larval Packet Tests need to be done in the future to define an accurate LC_{50} for amitraz which can be used as baseline data.

The regression line of the graph in *figure 4* requires further fine tuning as followed. This can be done by using additional concentrations which will result in additional points that can be used to create a more suitable graph. In the Larval Packet Test with amitraz in this study the dilution was 1:10. In *table 5* more possibilities are given to change the dilution series in 1:5 or even 1:4 to create a more suitable regression line.

Tick species Rhipicephalus bursa			
Dilution 1:10		**Dilution 1:5**	**Dilution 1:4**
Dilution	**Concentration amitraz**	**Concentration amitraz**	**Concentration amitraz**
1	**$0{,}1 \cdot 10^{-4}$ mg/mL**	**0,000064 mg/mL**	**0,0002441406 mg/mL**
2	**$0{,}1 \cdot 10^{-3}$ mg/mL**	**0,00032 mg/mL**	**0,0009765625 mg/mL**
3	**$0{,}1 \cdot 10^{-2}$ mg/mL**	**0,0016 mg/mL**	**0,00390625 mg/mL**
4	**$0{,}1 \cdot 10^{-1}$ mg/mL**	**0,008 mg/mL**	**0,015625 mg/mL**
5	**0,1 mg/mL**	**0,04 mg/mL**	**0,0625 mg/mL**
6	**1,0 mg/mL**	**0,2 mg/mL**	**0,25 mg/mL**
7	**10 mg/mL**	**1 mg/mL**	**1 mg/mL**

Table 5. Future possible dilution series

7. Conclusion

Resistance represents one extreme of response, compared with susceptibility, the other extreme. Various degrees of tolerance lie between the two extremes. Quantal response bioassays are useful to identify and monitor shifts in population tolerance. In this study the Larval Packet Test was used to define a diagnosis of resistance. For *Rhipicephalus bursa* ticks a LC_{50} of $3{,}816 \cdot 10^{-3}$ mg/mL alpha-cypermethrin was found. This LC_{50} can be used for baseline data to compare and/or confirm the resistance status of field populations of *Rhipicephalus bursa* in the future by determining the factor of resistance. The LC_{99} lethal concentration is not reliable and must be disregarded. In conclusion, *Rhipicephalus bursa* ticks collected from sheep on the island of Lesvos, Greece, are susceptible to pyrethroids. So, H_0 hypothesis is confirmed and the H_1 hypothesis should be rejected.

Not only genetic factors are involved in resistance development, but also operational factors and biological factors. Operational factors can be controlled by proper management by the operators. Education of the farmers and/or operators is very important. They should be educated regarding in their knowledge of the proper use and handling of acaricides. Close monitoring and resistance management strategies should be employed to delay the operational loss of pyrethroids for tick control not only in Greece, but actually all over the world.

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Appendices

Appendix A: Larval Packet Test Guideline

LARVAL PACKET TEST

Principle of the test

The larval packet test is considered to be the most repeatable, although it is limited by the length of time that it takes. Hence it remains the test of choice for surveys and for definitive confirmation of a diagnosis of resistance. In this test, tick larvae are exposed to chemically impregnated filter papers and their subsequent mortality is quantified after 24 hours. The FAO test kit contains standardized materials and procedures enabling data obtained from different parts of the world to be directly compared and discussed.

Collection

- Always collect as many undamaged, fully engorged female ticks as possible.
- 10-50^{+} fully engorged female ticks are needed per acaracide test. Test can be conducted with fewer ticks because sufficient larvae will hatch from the eggs of a few engorged female ticks. Where ticks are collected from animals recently treated with acaricides, the results might suggest a higher frequency of resistance than in the population of all ticks from the farm.
- Collect female ticks 3-8 and 14-17 days after acaracide treatment
    - Most engorged ticks drop off early in the morning.
    - If treated with MLs (amitraz) ticks should be collected 3 days after treatment.
    - If treated with fluazuron ticks should be collected at least 15 days after treatment.
- Each sample should be clearly identified, including time, date, place, group/animal number, treatment, and owner.

Storage

- Storage conditions are less critical for LPT than for AIT because the test is conducted on the progeny of the collected ticks rather than testing directly on the collected ticks. Factors influencing the viability of the engorged female will have a direct effect on AIT results but not on LPT results.
- In cases where sufficient numbers of ticks are not available, it is possible to collect them over several days, thus creating a pool of refrigerated (4°C) engorged female ticks.

Transport

- Ticks transported over small distances can be kept in cardboard boxes with a few small holes to allow circulation of air.
- If ticks are transported over long distances they should be placed between layers of slightly moistened paper towel in order to keep the environment humid and to protect the ticks from damage. Make sure the towels are not too wet!
- Do not transport ticks in airtight containers, plastic bags or glass tubes.
- Do not place the ticks in cotton wool.
- Do not expose the ticks or the transport medium to excessive heat/sunlight.

Laboratory handling

- Immediately upon arrivel in the lab wash female ticks in distilled water to remove any eggs laid during transport.
- The incubation condititions for all stages of Ixodid ticks before and during testing should be 27-28°C, 85-95% relative humidity, and no illumination.
- For all Ixodid tick larvae, the recommended age is 14 to 21 days. It may be found more convenient to use different ages for multi host tick species. However, it must be borne in mind that whatever the tick species or developmental stage, its age greatly affects its susceptibility to acaricides; standard ages for the testing of each species and stage should therefore be established.
- For all Ixodid tick larvae, the conditions for holding treated ticks should be 27 to 28°C and 85 to 95 percent RH and without illumination. The incubator should allow air exchange but does not require fan circulation.
- It is recommended that a different incubator be used for synthetic pyrethroid (SP) testing. If this is not possible, then SP testing could be conducted in the same incubator used for other acaricide groups, but at a different time. Following use with SP acaricides, the incubator should be cleaned with acetone and permitted to dry with adequate ventilation.

Incubation of engorged female ticks

- A maximum of engorged female ticks are placed in a 150 mm glass rearing tube, which is then closed firmly with a ventilated stopper and placed in an incubator maintained at 27-28°C and 85-95% relative humidity.
- All eggs should be collected 7 days from commencement of incubation. Each tube containing the first week's egg production should be labelled with the date, to enable the selection of more uniform larvae for each LPT.
- Under optimal rearing conditions, the engorged female ticks of most species will begin to lay eggs within 2 to 7 days. Boophilus spp. begin to lay eggs after 2 to 3 days and will continue for 12 to 15 days. After 21 to 28 days, Boophilus spp. larvae begin to hatch.

Storage and handling of acaricide–impregnated papers

The papers should be kept in their original, individual isolating aluminium foil envelopes in a refrigerator and protected from high humidity, high temperature and light (especially direct sunlight) and opened only immediately before use. Individual papers should be handled with forceps and used only once for a test. After removing a paper, its foil envelope should be immediately resealed with adhesive tape. Self-protective, disposable gloves and facemasks must be worn.

Guidance notes before commencing a test

1. Two control packets and two for each of the concentrations per acaricide active ingredient (AI) are used for each tick sample suspected of having developed resistance.
2. The control papers are always prepared first, followed by the acaricide impregnated papers per active principal, each of these series being handled in ascending order of concentration.
3. The use of a white tray enables any accidentally fallen larvae to be seen and subsequently trapped on adhesive tape.
4. Instructions for the introduction of tick larvae into the acaricide impregnated paper packets should be read carefully before commencing, including a practice run first without any tick larvae being involved.

Composition of the FAO test kit

The kit contains sealed, aluminium foil envelopes containing equal numbers of impregnated papers for each of the following acaricide active ingredients (AIs), and standardized FAO data report forms.

Chemical		Percentage active ingredient in formulation on paper
BHC		
	dieldrin	0.20
OPs		
	chlorfenvinphos	0.20
	coumaphos	0.20
	diazinon	0.10, 0.20
SPs		
	cypermethrin	0.20
	cyfluthrin	0.03
	deltamethrin	0.06
	flumethrin	0.0036
	cyhalothrin	0.05, 0.1
Amidine		
	amitraz	0.4, 0.1 and 0.025
MLs		
	cydectin	1.0
Control		
	solvent only	

Additional equipment required, but NOT supplied with the FAO kit, consists of:

Tubular plastic clips	Log/probit graph paper (one per assay)
Plastic stands	40 mm rubber bung
Fine paint brushes	One glass Petri dish, 90 mm diameter
Pointed glass rods	One glass Petri dish, 150 mm diameter
Glass conical flask	Two glass beakers
Polystyrene blocks	White enamel tray
Needles	Two forceps
Cotton wool	Scissors
Adhesive tape	Disposable gloves and masks
Double sided adhesive tape	Tally counter
Small aerated cardboard boxes for tick collection, 150mm deep glass tubes with ventilated but larva-proof stoppers for tick rearing	Magnifying glass (×2)

Some modification to the LPT is required for diagnosis of amitraz resistance because resistant strains do not show a linear relationship between probit mortality and log concentration of the acaricide. (The reasons for this are unknown.) The test follows exactly the LPT protocol but the packets are enclosed in plastic Petri dishes (with each replicate of packets for one concentration in a separate dish) and the exposure time is extended to 48 hours.

Test protocol		Done
1	An aluminium envelope containing the control papers (impregnated with solvent only) is opened and a single paper removed with forceps. The envelope is resealed. The paper is folded in half horizontally, with its identification mark (AI and concentration) on the inside.	
2	A single tubular plastic clip is slid up each short side of the paper, starting from the folded end. Alternatively, bulldog clips can be used. The packet formed, with its unfastened end upwards, is then put on a stand by pushing the side clips down over the stand's two nails. Pushing the side clips gently towards the middle of the packet forces it to open slightly. The process is repeated to make a second control paper packet that is also set up on a stand.	
3	Control packets (with the solvent only) are prepared with tick larvae first, followed by those with acaricide active ingredients and for each acaricide in order of increasing concentration. The glass rearing tube is freshly removed from the incubator, opened and tick larvae permitted to aggregate freely at its top rim. A small cluster that will contain approximately 100 larvae is picked up from the rim of the open tube using the fine brush and, with the aid of a glass rod, eased into the control packet. Care should be taken to ensure brush and rod do not come into contact with the packets, which should only be handled by the clips.	
4	The closed packet is laid on a tray ready for subsequent placement in the incubator. This entire procedure is repeated for the second control packet. The packets are arranged on trays or in racks, without contact with each other. The procedure is repeated with two packets for each concentration of each acaricide, always working in ascending order of their concentration per acaricide.	
5	The packets are placed in the incubator at a temperature of 27 to 28°C and 85 to 95 percent RH for 24h. Packets impregnated with amitraz should be enclosed in plastic Petrie dishes (with each replicate of packets for one concentration in a separate dish) and the exposure time is extended to 48 hours.	
6	The packets are examined in the same order as they were prepared and filled with tick larvae. This is an attempt to minimise variation in duration of exposure to test acaricide. The recommended mortality criterion is the inability of tick larvae to walk. Only those larvae capable of walking are considered to be alive. For assessment of walking ability, a magnifying glass and lamp should be used. Ticks can be stimulated by gently breathing directly onto them. All other larvae, including those that move their appendages but do not walk, are counted as if dead	
7	A control packet is opened by holding it by one side clip and lying it on the polystyrene block with the top opening to one side of the block, The top clip is removed and the bottom side of the paper packet secured to the block with a pin. The remaining clips are removed and the packet secured to the block in the open position with the other pin. The live larvae are removed with a paintbrush and immobilized on cotton wool moistened with a wetting agent (detergent) in water. The dead larvae remaining are then counted and recorded, followed by the living larvae that have been trapped in the cotton wool.	

Procedure done:

By: **On:** **Signature:**

Results

If counting reveals larval mortality to be "very low" (<5%), then the direct mortality figures can be utilized. If they are found to be "low" (5 to 10%) in the control, then the percentage mortality in all of the experimental batches of larvae will have to be corrected by applying Abbott's formula:

$$\text{Corrected percent mortality} = \frac{\%\text{ test mortality} - \%\text{ control mortality}}{100 - \%\text{ control mortality}} x100$$

Tabulation of results: the mean value for each of the two results for control and each concentration of each acaricide are recorded on the standard results forms provided in the FAO kit. (see table 1)

If a full dose-mortality test has been undertaken, results should be plotted: percent concentration (x-axis) by Probit mortality (y-axis) for each acaricide in the kit using log/probit graph paper. Alternatively, the data can be submitted to *Polo-PC* for analysis.

Results similar to (a), (b) or (c) (Figure 1) can be found in a complete dosage mortality test. If the population is homogeneously susceptible, a straight line will be obtained as in (a). If, on the other hand, a line similar to (b) is obtained, it indicates that the population is a mixture of susceptible and resistant individuals. The horizontal portion of this line (b) will vary in position depending on the proportion of resistant ticks in the sample. If the resistance factor is very low, the flat portion may be difficult to distinguish as the displacement of the susceptible line to the right will be small.

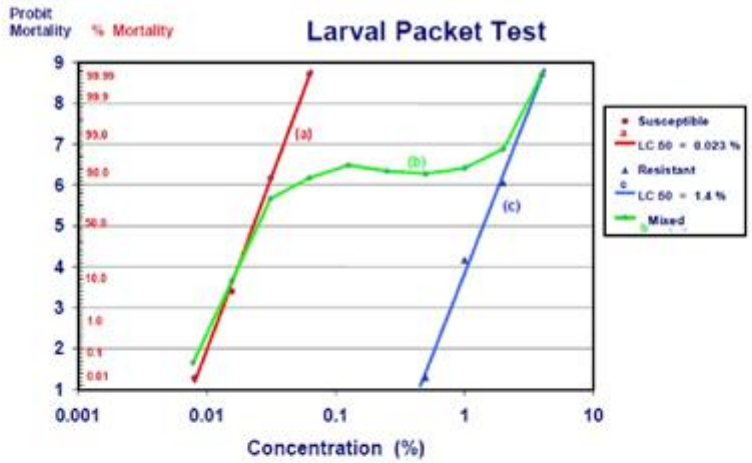

Figure 1. Examples of probit mortalities for samples of three populations (a, b and c) of the cattle tick, *Boophilus microplus*, subjected to a complete dosage mortality test for an acaricide. (LC = lowest concentration needed to provide a particular mortality rate)

Table 1: Larval Packet Test Results

Tick species on which tests are conducted:	

Product name:	Active ingredient:
Test date:	Signature:
Read date:	Signature:

Concentrations		Test a				Duplicate test b				Calculations	
		Total	Alive	Dead		Total	Alive	Dead		Mean	% Mortality

Product name:	Active ingredient:
Test date:	Signature:
Read date:	Signature:

Concentrations		Test a				Duplicate test b				Calculations	
		Total	Alive	Dead		Total	Alive	Dead		Mean	% Mortality

Product name:	Active ingredient:
Test date:	Signature:
Read date:	Signature:

Concentrations		Test a				Duplicate test b				Calculations	
		Total	Alive	Dead		Total	Alive	Dead		Mean	% Mortality