DETERMINATION OF MINIMAL IMMERSION TIMES FOR USE IN *IN VITRO* RESISTENCE TESTS WITH *BOOPHILUS MICROPLUS* (CANESTRINI, 1887) ENGORGED FEMALES AND PYRETHROID ACARICIDES

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SUMMARY: The minimal immersion time to be used in *in vitro* tests with engorged females of *Boophilus microplus* was determined. The following ixodicides were used: flumethrin, deltamethrin, cipermethrin and cifluthrin. A total of 840 engorged females from a sensitive reference strain (Mozo) were dipped into decreasing concentrations of the products tested, in order to determine their 50% effective concentration (EC_{50}) and 99% effective concentration (EC_{99}). Different immersion times were used. Ticks were grouped in batches of 10 individuals each, 3 replicates per concentration evaluated. Six dilutions were prepared for each compound, using acetone as solvent. Females were dipped for 1, 5, 10 or 25 minutes. Female and egg weights were recorded and allowed the calculation of efficacy rates for each immersion time and concentration. EC_{50} and EC_{99} were calculated through probit analyses. Based upon this data (EC_{50} mainly) the minimal immersion times were settled as follows: 5 minutes to flumethrin, deltamethrin and cifluthrin and 10 minutes for cipermethrin. The EC_{50} values presented here can be used to calculate resistance indexes of field strains, once they were stated for a sensitive reference strain.

KEY WORDS: *Boophilus microplus*, pyrethroids, flumethrin, deltamethrin, cipermethrin, cifluthrin, *in vitro* tests, ixodicides, resistance.

INTRODUCTION

The cattle tick *Boophilus microplus* (Canestrini, 1887) is an Asian native ectoparasite. Neverthless, it has nowadays has a worldwide occurrence, being present in almost all cattle herds located between parallels 32° north and 32° south, where the best conditions for its development can be found.

Damages due *B. microplus* can be both direct and indirect. Direct damages are those caused by the action of tick bite it, i.e., blood sucking, irritant effects causing host stress, and allergic and toxic effects of saliva. Indirect damages are those caused by the tick-borne diseases (*Babesia* and *Anaplasma* mainly) and losses on leather quality (CORDOVÉS, 1996).

Parasitized animals present lower feed conversion rates leading to reduction of their productive performances. Heavily infested host can be present anemia among other disorders. Economic damages in Brazil sum 1 billion US$ per year (SPATH, 1989).

Such losses make the adoption of control programs an imperative. Every and each control strategy or program must be based upon the precise knowledge of tick biology and epidemiology, not forgetting to taking in account the social and economic conditions of each area to be addressed.

Chemical Control and Resistance

Chemical control, using ixodicides, is so far the most efficient method available for tick control. Nevertheless, their continuous and sometimes not responsible use allowed the appearance of resistance.

Since once resistance is already established, is very hard to revert it, its appearance should be delayed as long as possible. To do so, we need reliable and practical monitoring methods that allow us to early diagnose resistance problems. Several *in vitro* techniques were developed to check resistance status of field strains and/or to confirm susceptibility of tick to new compounds. Among them, methods using engorged females and non-fed larvae appear as the more indicated and used.

According to NARI *et alii* (1984) *in vitro* methods are better than *in vivo* ones, cause they allow to process large numbers of samples, easing epidemiological surveillance.

In Brazil, the most widely used technique is the immersion test, where engorged females are dipped into the product to be

Table 1 - Chemical groups of tickcides used in the trials.

Concentration (ppm)	Flumethrin	Cifluthrin	Deltamethrin	Cipermethrin
IS	*1.2	10	20	35
A	0.24	2	4	7
B	0.048	0.4	0.8	2.8
C	0.0096	0.08	0.16	1.4
D	0.00192	0.016	0.032	0.28
E	0.000384	0.0032	0.0064	0.056

IS = Initial solution (1/5 of recommended dose)
* = Initial solution (1/25 of recommended dose)

tested. Unfortunately, there is huge variation from one study to another in factors such as immersion time, making hard to compare different results. PALMER (1965) tested the influence of several factors on the results of immersion tests with engorged females, including tick size and age, temperature of the immersion solution, temperature during the incubation period and immersion time. Among these, variations on immersion time were the only one to present some effect on final results such as egg mass weight and hatching rates.

AMARAL (1993) points the need to use standard values of 50% effective concentration (EC_{50}) and 99% effective concentration (EC_{99}) in order to make the comparison between different results possible, through the calculation of a resistance index. Such reference EC_{50} and EC_{99} values must be settled for a sensitive reference strain.

So, in the present work, we compared four different immersion times - 1, 5, 10 and 25 minutes - with four different pyrethroids: flumethrin, deltamethrin, cipermethrin and cifluthrin. Through the determination and analyses of their respective EC_{50} and EC_{99} we choose the minimal immersion time to be used.

MATERIALS AND METHODS

Boophilus microplus Strain

The Strain used is know as "Mozo" and it was first isolated at the Veterinary Research Center "Miguel C. Rubino", Uruguay. Since May 1973 this strain has been kept in laboratory without any contact of ixodicides, i.e., it only had contact with chlorinated and organophosphorated products.

Since 1994, this strain has been kept at the Veterinary Parasitology Laboratory at the Parasitology Department, Biomedical Sciences Institute, São Paulo University, Brazil.

Donor Animals

Male Holstein-Friesian calves aged more than 4 months were used. Donors were raised worm free since birth. After infestation they were moved to metabolic cages with meshed floors, in order to easy collection of naturally detached engorged females. Circa 20,000 larvae was used in each infestation.

Table 2 - Recommended doses and purity of products used.

Product	Recommended Dose (ppm)	Purity (%)
Flumethrin	30	61.39
Cifluthrin	50	94.2
Deltamethrin	100	97.1
Cipermethrin	175	94.9

Experimental design

A total of 840 naturally detached engorged females were used. Ticks were grouped in batches with 10 females each, constituting a replicate. These replicates were distributed to the different treatments, i.e., the several concentrations of ixodicides to be tested as shown in Table 1. Control groups were dipped into 40% acetone. Recommended doses and purity of products used are listed in Table 2.

Immersion Tests

After collection, females were washed in distilled water, dried with absorbent paper and kept at room temperature for 24 hours. A selection was performed then, based on vitality, color, size and physical damages. Individuals that present any abnormality were discarded. Replicates of 10 ticks each were formed.

Each replicate was weighted and placed into vials where 30 ml of tickcide solution was added. According to the each treatment, females were left into solution for 1, 5, 10 or 25 minutes, and then dried with the help of a ventilator. Three replicates were used for each treatment (immersion time, concentration and product).

After immersion, each replicate was placed in a Petri dish, identified by product used, dilution, immersion time and replicate number. Petri dishes were kept in a BOD stove (27 °C and 85% relative humidity) for two weeks to allow oviposition. After this period, the total amount of eggs laid by the females of each replicate was weighted.

The egg mass from each Petri dish was disposed in a plastic syringe with its end cut of. Eggs were carefully spread over one side in order to easy the assessment of hatching rate. Syringe end was sealed with moistened cotton balls. After new identification by product used, dilution, immersion time and

replicate number, samples were incubated in the same BOD stove, under the same conditions described above for other two weeks. After 15 days of egg incubation, hatching rates were assessed by visual evaluation under stereo microscope.

Calculation of Reproductive Efficiency and Product Efficacy

Treatment efficiency was evaluated through the calculation of reproductive efficiency (RE) and product efficacy (PE), according to DRUMOND (1973).

$$RE = \frac{\text{egg weight x hatching rate (\%)}}{\text{female weight}} \times 20.000$$

$$PE = \frac{\text{RE (control)} - \text{RE (treated)}}{\text{RE (control)}} \times 100$$

Statistic Analyses

The Product Efficacy (PE) data was compared through variance analysis and then the Tuckey test. The EC_{50} and EC_{99} concentration were determined by probit analyses (FINNEY, 1971).

RESULTS

The product efficacy results for the different products are shown in Tables 3 to 6.

Flumethrin

Significative differences in comparison between the different immersion times only could be found for two concentrations (Table 3). For the solution with 0.048 ppm, the mean PE for 1 minute was different of those found for all other times (which did not differ from each other). At the concentration of 0.0096 ppm, 5 and 10 minutes presented the same PE, but differ from 1 and 25 minutes, which by its turn, differ from each other.

Deltamethrin

Again, significative differences in comparison between the different immersion times only could be found for two concentrations (Table 4). For the 4 ppm concentration, only 1 and 25 minutes differ from each other. For the 0.8 ppm concentration the same occurred for 5 and 25 minutes.

Cipermethrin

A statistically significant effect of immersion time on PE only could be found for the concentration of 2.8 ppm (Table 5). The 10 minutes immersion time was different form 1 and 5 minutes and equal to 25 minutes.

Cifluthrin

Once again, a statistically significant effect of immersion time on PE only could be found for one concentration (0.04 ppm), where 1 and 5 minutes differ from each other (Table 6).

Looking at results alltogether, we can see that significative variations caused by different immersion times, only could be found for intermediate concentrations.

Tables 7, 8, 9 and 10 shows the EC_{50} and EC_{99} for the different products tested. These values can be used to calculate the resistance index as follows (CARDOZO *et alii*, 1984b):

$$RE = \frac{EC_{50} \text{ of a field strain}}{EC_{50} \text{ of a reference strain}}$$

Figures 1 to 4 present EC_{50} values and their confidence limits, obtained through probit analyses. Based on these results we choose the following times as the minimal immersion times to be used in *in vitro* trials: 5 minutes to flumethrin, deltamethrin and cifluthrin and 10 minutes for cipermethrin.

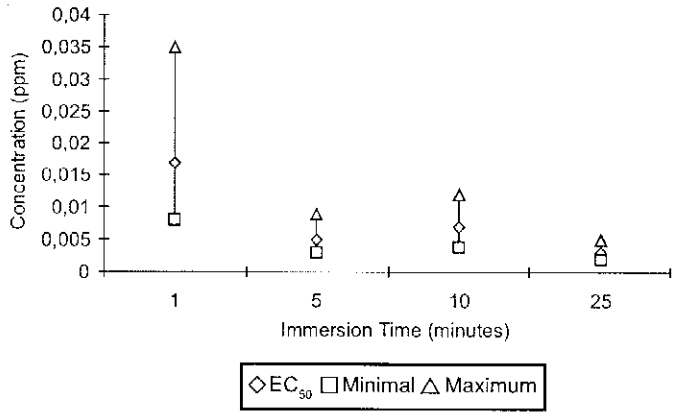

Fig. 1- EC50 values and confidence limits for Flumethrin.

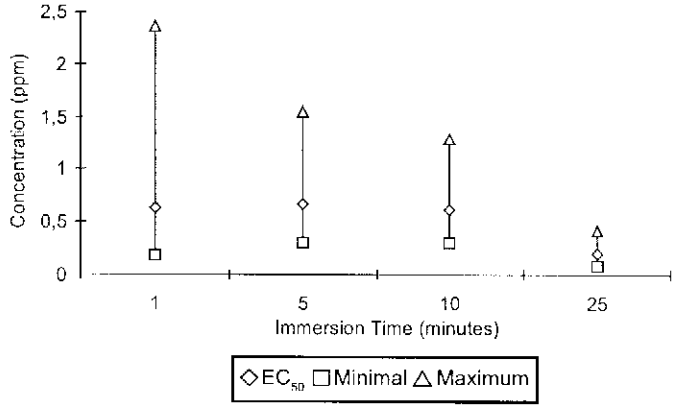

Fig. 2 - EC_{50} values and confidence limits for Deltamethrin.

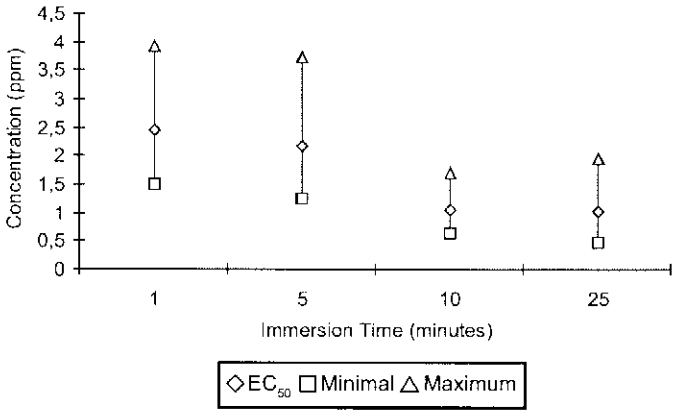

Fig. 3 - EC_{50} values and confidence limits for Cipermethrin.

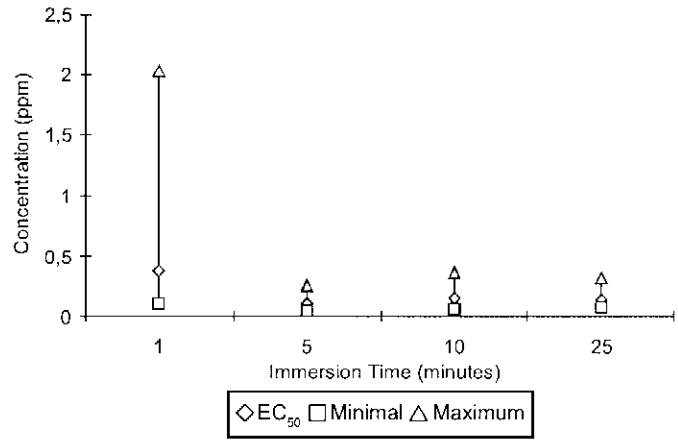

Fig. 4 - EC_{50} values and confidence limits for Cifluthrin.

Table 3 - Mean efficacy for Flumethrin in the different concentrations and immersion times tested.

Concentration (ppm)	Immersion Time (minutes)			
	1	5	10	25
1.2	100 ± 0	100 ± 0	100 ± 0	100 ± 0
0.24	100 ± 0	100 ± 0	100 ± 0	100 ± 0
0.048	46.13^{b*} ± 6.82	83.1^{a} ± 3.23	74.48^{a} ± 11.25	97.28^{a} ± 2
0.0096	27.72b ± 7.23	49.92^{ab} ± 15.24	48.33^{ab} ± 12.88	74.44^{a} ± 19
0.00192	26.79 ± 18.09	28.67 ± 8.76	23.3 ± 8.96	28.07 ± 12.28
0.000384	11.58 ± 12.13	20.59 ± 6.64	22.48 ± 4.76	23.27 ± 8.22

* Different letters indicate significative statistical difference ($p<0.05$) in the comparison between times for the same concentration.

Table 4 - Mean efficacy for Deltamethrin in the different concentrations and immersion times tested.

Concentration (ppm)	Immersion Time (minutes)			
	1	5	10	25
20	95.12 ± 7.68	100 ± 0	100 ± 0	100 ± 0
4	54.64^{a*} ±.7.41	76.22^{ab} ± 13.18	72.89^{ab} ± 17.29	93.94^{b} ± 9.94
0.8	39.14^{ab} ± 17.37	27.08^{a} ± 4.97	38.31^{ab} ± 6.38	59.5^{b} ± 23.32
0.16	25.79 ± 8.26	26.29 ± 8.02	18.71 ± 3.84	36.49 ± 8.75
0.032	22.42 ± 12.39	13.08 ± 3.4	17.51 ± 7.50	25.2 ± 7.83
0.0064	23.61 ± 17.03	12.5 ± 4.05	14.06 ± 3.61	18.83 ± 8.14

* Different letters indicate significative statistical difference ($p<0.05$) in the comparison between times for the same concentration.

Table 5 - Mean efficacy for Cipermethrin in the different concentrations and immersion times tested.

Concentration (ppm)	Immersion Time (minutes)			
	1	5	10	25
35	99.86 ± 0.25	100 ± 0	100 ± 0	100 ± 0
7	77.35 ±.6.77	80.48 ± 13.39	81.73 ± 9.89	95.01 ± 4.60
2.8	35.81^{b*}± 7.69	33.78^{b} ± 7.22	72.17^{a} ± 13.11	56.85^{ab} ± 9.95
1.41	32.82 ± 9.69	33.32 ± 6.76	42.01 ± 1.72	48.93 ± 19.55
0.28	13.13 ± 2.6	16.32 ± 8.93	20.62 ± 1.83	20.2 ± 11.72
0.056	5.23 ± 3.9	9.12 ± 12.48	17.5 ± 2.44	19.4 ± 13.14

* Different letters indicate significative statistical difference ($p<0.05$) in the comparison between times for the same concentration.

Table 6 - Mean efficacy for Cifluthrin in the different concentrations and immersion times tested.

Concentration (ppm)	Immersion Time (minutes)			
	1	5	10	25
10	78.18 ± 11.15	91.25 ± 8.03	99.88 ± 0.22	100 ± 0
2	52.17 ± 5.38	67.66 ± 5.94	58.24 ± 8.84	68.99 ± 13.09
0.4	35.78^{b*} ± 14.12	64.4^{a} ± 11.54	52.82^{ab} ± 6.44	49.78^{ab} ± 12.82
0.08	29.88 ± 3.31	41.8 ± 9.22	29.42 ± 5.15	32.89 ± 7.98
0.016	28.61 ± 1.97	22.11 ± 6.84	22.06 ± 10.34	13.28 ± 8.94
0.0032	22.78 ± 11.83	15.62 ± 5.34	18.8 ± 11.30	14.92 ± 6.68

* Different letters indicate significative statistical difference ($p<0.05$) in the comparison between times for the same concentration.

Table 7 - EC_{50} and EC_{99} values for Flumethrin.

Time (minutes)	EC_{50} (ppm)	Fiducial Limits (95%)	EC_{99} (ppm)	Fiducial Limits (95%)	Regression Equation
1	0,0173	0,0082 - 0,0354	1,4512	0,3917 - 16,1281	Y = 6,424 + 0,833 x
5	0,0054	0,0030 - 0,0091	0,5498	0,2108 - 2,4686	Y = 7,252 + 0,989 x
10	0,0068	0,0037 - 0,0117	0,8469	0,3189 - 3,8156	Y = 6,948 + 0,898 x
25	0,0028	0,0017 - 0,0049	0,0917	0,0412 - 0,3386	Y = 8,209 + 1,251 x

Table 8 - EC_{50} and EC_{99} values for Deltamethrin.

Time (minutes)	EC_{50} (ppm)	Fiducial Limits (95%)	EC_{99} (ppm)	Fiducial Limits (95%)	Regression Equation
1	0,6294	0,1958 - 2,3686	258,9542	30,6499 - 39404,02	Y = 5,058 + 0,540 x
5	0,6731	0,3076 - 1,5476	94,8626	22,9456 - 1270,663	Y = 5,091 + 0,802 x
10	0,6149	0,3032 - 1,2926	113,5385	29,3104 - 1157,512	Y = 5,120 + 0,769 x
25	0,1966	0,0892 - 0,4147	24,1795	6,7925 - 232,2736	Y = 5,630 + 0,863 x

Table 9 - EC_{50} and EC_{99} values for Cipermethrin.

Time (minutes)	EC_{50} (ppm)	Fiducial Limits (95%)	EC_{99} (ppm)	Fiducial Limits (95%)	Regression Equation
1	2,4268	1,514 - 3,9206	67,8445	28,2815 - 316,6972	Y = 4,490 + 1,313 x
5	2,1622	1,2546 - 3,7463	47,5448	19,7627 - 228,9527	Y = 4,597 + 1,159 x
10	1,0605	0,6446 - 1,6788	69,0441	27,9086 - 301,6015	Y = 4,992 + 1,069 x
25	1,0172	0,4900 - 1,9555	31,7825	11,45 - 238,7414	Y = 5,040 + 1,110 x

Table 10 - EC_{50} and EC_{99} values for Cifluthrin.

Time (minutes)	EC_{50} (ppm)	Fiducial Limits (95%)	EC_{99} (ppm)	Fiducial Limits (95%)	Regression Equation
1	0,3767	0,1056 - 2,0317	19060,24	496,9316 - 1.13 x 108	Y = 5,088 + 0,350 x
5	0,1043	0,04393 - 0,2478	378,3213	53,1225 - 11524,56	Y = 5,544 + 0,557 x
10	0,1504	0,0655 - 0,3580	188,7477	32,4009 - 3835,351	Y = 5,458 + 0,576 x
25	0,1442	0,0686 - 0,3095	105,5169	23,1872 - 1216,236	Y = 5,513 + 0,642 x

DISCUSSION

Despite the considerable research efforts that are being carry out on alternative control methods, our tick control programs relay almost exclusively on chemical control. Thus, we should address the resistance issue very carefully, or we will have to face, in a short time, a critical situation.

Sooner or later, resistance reports have followed the introduction in the market of each and every chemical group used for tick control. According to BEUGNET *et alii* (1995), the same is supposed to occur with pyrethroids, and if it really does, it will be once again a "family problem".

NOLAN *et alii* (1989) in Australia did the first pyrethroid resistance report. Three resistant strains were found: the Marmor,

resistant to cyalothrin and cipermethrin; the Lamington, resistant to flumethrin and the Parkhurst, resistant to cyalothrin, deltamethrin and flumethrin. Again BEUGNET *et alii* (1995) found a strain resistant to deltamethrin.

In Brazil, COSTA (1986) found no alterations in the product efficiency rates when tested an organophosphate resistant strain with 4 different pyrethroids. All compounds tested presented efficacy rates close to 100%.

PEREIRA & LUCAS (1987) tested a field strain from the Jacaraí municipality, São Paulo state, founding a oviposition inhibition rates from 43.6 up to 48.98%, what indicates a resistance problem. ALVES BRANCO *et alii* (1992) reported *B. microplus* strains resistant to deltamethrin, founding oviposition inhibition rates up to 50% in certain farms.

The great variation of techniques used, practically exclude any possibility of comparison between results. The great majority of authors do not use technical preparations, preferring to use trade mark products. That is a tremendous source of bias since each manufacturer uses its on solvents and different pharmacological "devices", leading to misinterpretation of research results.

It interesting to notice, that among all authors and papers quoted here, none has performed a comparison with a reference sensitive strain, and hence, the "resistance" report is quite questionable.

So, we intend to supply all the researchers that dealt with tick resistance with reference data. Since ours results were obtained with an well know and well accepted sensitive strain, the EC_{50} and EC_{99} values presented here, can and should be used as standards for calculation of resistance indexes. And based on the calculation of resistance indexes, data comparison gets easier, and, a national research program to map the resistance status of Brazilian herds becomes possible.

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SUMÁRIO

Foi determinado o tempo mínimo de imersão a ser utilizado em testes *in vitro* com teleóginas de *Boophilus microplus*, para os seguintes piretróides: flumetrin, deltametrin, cipermetrin e ciflutrin. Para tanto, 840 fêmeas engorgitadas de uma cepa sensível de referência (Mozo) foram banhadas com diluições decrescentes dos princípios citados, para a determinação das concentrações eficazes CE_{50} e CE_{99}, em diferentes tempos de imersão. As teleóginas foram divididas em grupos de 10 constituindo 3 réplicas para cada concentração utilizada. Para cada produto foram feitas 6 diluições em acetona(40%) e utilizados os tempo de imersão de 1,5,10 e 25 minutos. Com base no peso das fêmeas e dos ovos foram calculadas as porcentagens de eficácia do produto para cada tempo e concentração. As CE_{50} e CE_{99} para cada tempo foram determinadas pelo método dos probitos. A análise destes resultados, em particular os das variações da CE_{50} em função do tempo, permitiu concluir que o tempo de 5 minutos foi o mais indicado para os testes utilizando o flumethrin, deltamethrin e o cifluthrin e o de 10 minutos para o cipermethrin. Os valores de CE_{50} encontrados para esta cepa sensível podem ser utilizados para o cálculo do fator de resistência de uma cepa de campo.

PALAVRAS-CHAVE: *Boophilus microplus*, piretróides, flumethrin, deltamethrin, cipermethrin, cifluthrin, teste "in vitro", acaricidas, resistência.

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