DETERMINATION OF LD_{50} AND LD_{99} IN TWO SUSCEPTIBLE STRAINS OF *BOOPHILUS MICROPLUS* FOR LARVAL RESISTANCE TESTS

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SUMMARY: Two susceptible strains of the cattle tick *Boophilus microplus* were tested with several ixodicides, intending to characterize then as reference standards for resistance diagnosis tests. The larval test was performed using a standard kit supplied by FAO. Batches of 108,9 ± 29 larvae were placed into packets prepared with paper impregnated with the following ixodicides: cipermethrin, deltamethrin, cyalothrin, flumethrin, coumaphos and diazinon. After an incubation time of 24 hours under controlled environment (27 °C and 85% r.h.) larval mortality was assessed. Mortality rates were calculated as stated by FAO, and transformed by means of probit analysis. A field strain from the Paraíba river valley area, with reports of treatment failure, was also tested in order to confirm if the reference values obtained could be used for the calculation of the resistance index. The data presented here strongly suggests a serious resistance problem. The two susceptible strains were found to be adequate for use as reference standards, but the concentrations available in the FAO kits should be lower if greater accuracy in the determination for susceptible strains is to be expected.

KEY WORDS: Resistance tests, *Boophilus microplus*, ixodicides.

INTRODUCTION

Ixodicide resistance is a major concern for the cattle raising industry. Its appearance has followed the use of virtually all chemical compounds employed for tick control (ROULSTON & WHARTON, 1981). Several efforts have been directed toward the development of management strategies to deal with such problem. Is common sense that, since susceptibility once lost seldom can be restored, we should handle such problem attempting to avoid the resistance establishment. To do so, we need reliable diagnosis techniques, which would allow to early identify resistant strains in the field as well as mapping problem areas (AMARAL, 1993; CARDOZO, *et alii*, 1984a).

First attempts to standardize such a methodology included immersion tests with engorged females (MENDES *et alii*, 1999).

Larval tests, as stated by STONE & HAYDOCK (1962), appear as the widely used and recommended technique, being the one chosen as standard by FAO. Such methodology requires the use of a susceptible reference strain, by means of which the resistance factor can be determined. Among the advantages of this technique one could list the need of a small number of engorged females if compared to immersion tests (what can be a huge problem when dealing with field strains), making possible for only one group to analyze large areas through samples received from the field. The "dead or alive" response of larval tests offers also a more accurate assessment of reference doses, when compared to the nearly subjective larval hatching percentage data from immersion tests with adult parasites.

The present work was designed with two different purposes: 1) Characterization of two susceptible strains of *Boophilus microplus*, through the determination of their 50% and 99% lethal doses (LD_{50} and LD_{99}) values for the ixodicides tested, intending to use them as reference values for resistance evaluation tests; and 2) Use of the same methodology with one field strain that has a history of treatment failure with several ixodicides, in order to test the adequacy of using these two susceptible strains as a reference for resistance tests.

MATERIALS AND METHODS

Tick strains

Susceptible strains

The two susceptible strains used were the Mozo (from the DILAVE "Miguel C. Rubino", Montevideo, Uruguay, which has been kept in laboratory since 1973 with no contact with ixodicide products, and since 1994 kept in the "Departamento de Parasitologia - ICB/USP") and another Argentine susceptible strain named "Salta".

For the Mozo strain, larvae used came from engorged females obtained after natural infestation of calves raised free from parasites since birth. The females were collected after natural detachment and kept for two weeks in the incubator (B.O.D.) at 27 °C and 85% r.h. to allow oviposition. The eggs were placed in glass vials, and incubated under the same conditions for 14 days. The larvae used in the tests were 7 - 14 days old.

Engorged females of the Salta strain were sent to us by the staff from "Instituto de Pesquisa Veterinárias Desidério Finamor", Eldorado do Sul, RS, Brazil. The females were collected after natural detachment from donor calves, placed in thermic plastic boxes with a moistened cotton balls to provide humidity, and sent by mail. At receipt, any female had already started oviposition. They were then kept under the same previously described conditions to complete oviposition.

Field strain

This strain was collected in a dairy farm of Caçapava, São Paulo State, Brazil. The farmer reported several cases of treatment failure with different ixodicides. Adult engorged females were gently collected directly from cows. They were then rinsed with distilled water, dried, sorted by color, weight and size. Females showing any damage were discarded. They were then put under the same previously described conditions to allow oviposition.

Impregnated papers

FAO World Acaricide Resistance Reference Centre (FAO - WARRC), Berlin, Germany, supplied the papers impregnated with several ixodicides. The products tested were cipermethrin, deltamethrin, flumethrin, cyalothrin, coumaphos, and diazinon. The concentrations used are shown in Table 1. Two replicates were prepared for each concentration. A non impregnated paper was used in the control group.

Preparation of packets

Batches of 108,9 ± 29 larvae were collected from the vials with the help of a fine brush, and placed in packets prepared with the FAO papers, following the instructions of the kit supplied by WARRC - FAO. Packets with larvae were kept in the incubator (B.O.D.) at 27 °C and 85% r.h. for the following 24 hours, then the percentages of dead and live larvae were recorded.

Statistical analysis

The mortality rates were calculated as stated by WARCC - FAO. The results were analyzed by probit analysis. Resistance Index (R.I.) was calculated as follows (AMARAL, 1993; CARDOZO *et alii*, 1984b):

$$R.I. = \frac{LD_{50} \text{ of field strain}}{LD_{50} \text{ of reference strain}}$$

RESULTS

The 50% and 99% lethal doses (LD_{50} and LD_{99}) values for the ixodicides tested in susceptible strains are presented in Tables 2 and 3. The LD_{50} and LD_{99} could be determined for all ixodicides, except for Flumethrin (for both strains). The results for the field strain, together with the resistance indexes are presented in Table 4.

A comparison between all three strains used is presented in Figures 1 to 3, showing respectively the probit transformed mortality versus dose logarithms for each ixodicide tested, the LD_{50} and LD_{99} values with their confidence limits.

Table 1 - Concentrations available (%) in the impregnated papers of FAO standard test for resistance assessment.

Product	Concentrations used (%)								
Coumaphos	0.00625;	0.0125;	0.025;	0.05;	0.1;	0.2;	0.4*;	0.8*	1.6*
Diazinon		0.0125;	0.025;	0.05;	0.1;	0.2*;	0.4*;	0.8*	
Cipermethrin		0.0125;	0.025;	0.05;	0.1;	0.2;	0.4*;	0.8*	1.6*
Cyalothrin	0.00625;	0.0125;	0.025;	0.05;	0.1;	0.2*;	0.4*;	0.8*	1.6*
Deltamethrin		0.0125;	0.025;	0.05;	0.1*;	0.2*;	0.4*		
Flumethrin	0.0009;	0.0018;	0.0037;	0.0075;	0.015;	0.03*;	0.06*;	0.12*	

* Concentrations used only for the field strain.

Table 2 - LD_{50} and LD_{99} values for the Mozo strain.

Product	LD_{50}	Confidence Limits		LD_{99}	Confidence Limits	
	(%)	Min	Max	(%)	Min	Max
Cipermethrin	0.03291	0.02863	0.03782	0.11146	0.08614	0.16503
Cyalothrin	0.02797	0.01855	0.04595	0.11538	0.06259	0.66064
Deltamethrin	0.01124	0.01017	0.01208	0.02827	0.02341	0.03601
Coumaphos	0.03857	0.02365	0.06284	0.35833	0.16424	2.37404
Diazinon	0.01346	0.00655	0.01889	0.10415	0.05797	0.57435

Table 3 - LD_{50} and LD_{99} values for the Salta strain.

Product	LD_{50}	Confidence Limits		LD_{99}	Confidence Limits	
	(%)	Min	Max	(%)	Min	Max
Cipermethrin	0.04306	0.02428	0.06931	0.19527	0.10448	1.58395
Cyalothrin	0.02141	0.01455	0.03015	0.08806	0.05306	0.31812
Deltamethrin	0.01075	0.00958	0.01149	0.02303	0.01979	0.03144
Coumaphos	0.05922	0.03797	0.1003	0.39271	0.1863	2.79577
Diazinon	0.01917	0.01585	0,02243	0.08013	0.0598	0.12972

Table 4 - LD_{50}, LD_{99} and R.I. values for the field strain.

Product	LD_{50}	Confidence Limits		LD_{99}	Confidence Limits		R.I.	R.I.
	(%)	Min	Max	(%)	Min	Max	Mozo	Salta
Cipermethrin	0.40448	0.25170	0.72194	4.94472	2.01337	37.80495	12.29	9.39
Cyalothrin	0.14075	0.07548	0.25067	4.71455	1.60915	58.06035	5.03	6.57
Deltamethrin	0.12402	0.10046	0.15573	0.7066	0.4599	1.43097	11.03	11.53
Flumethrin	0.01462	0.00803	0.03174	0.41208	0.12136	7.29587	-	-
Coumaphos	0.3605	0.23013	0.63878	23.3628	7.24844	191.8472	9.34	6.08
Diazinon	0.29872	0.20282	0.51889	19.1275	5.77932	170.6445	22.19	15.58

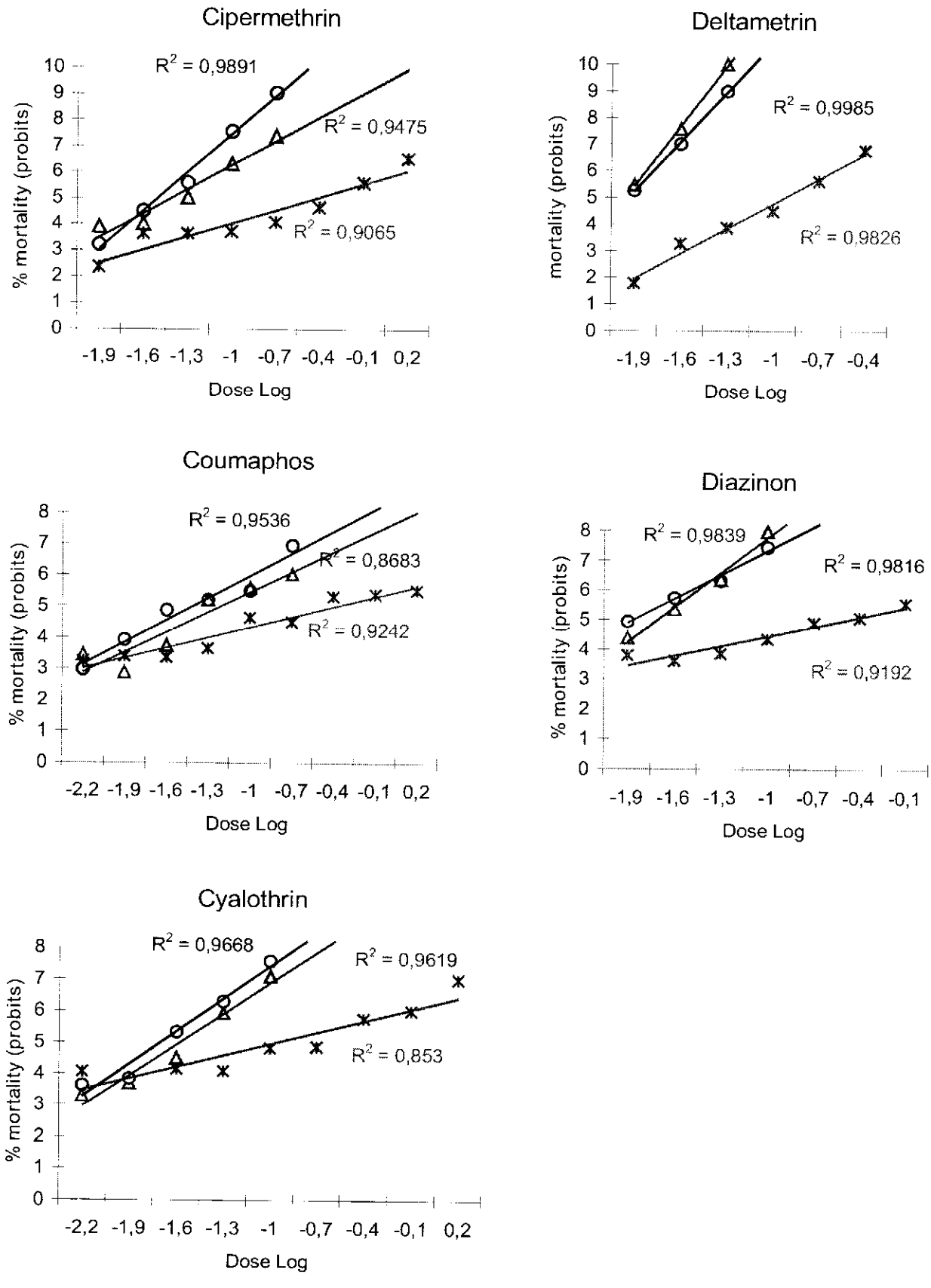

Fig.1 - Comparison of probit transformed mortality rates for all ixodicides testes in three strains.

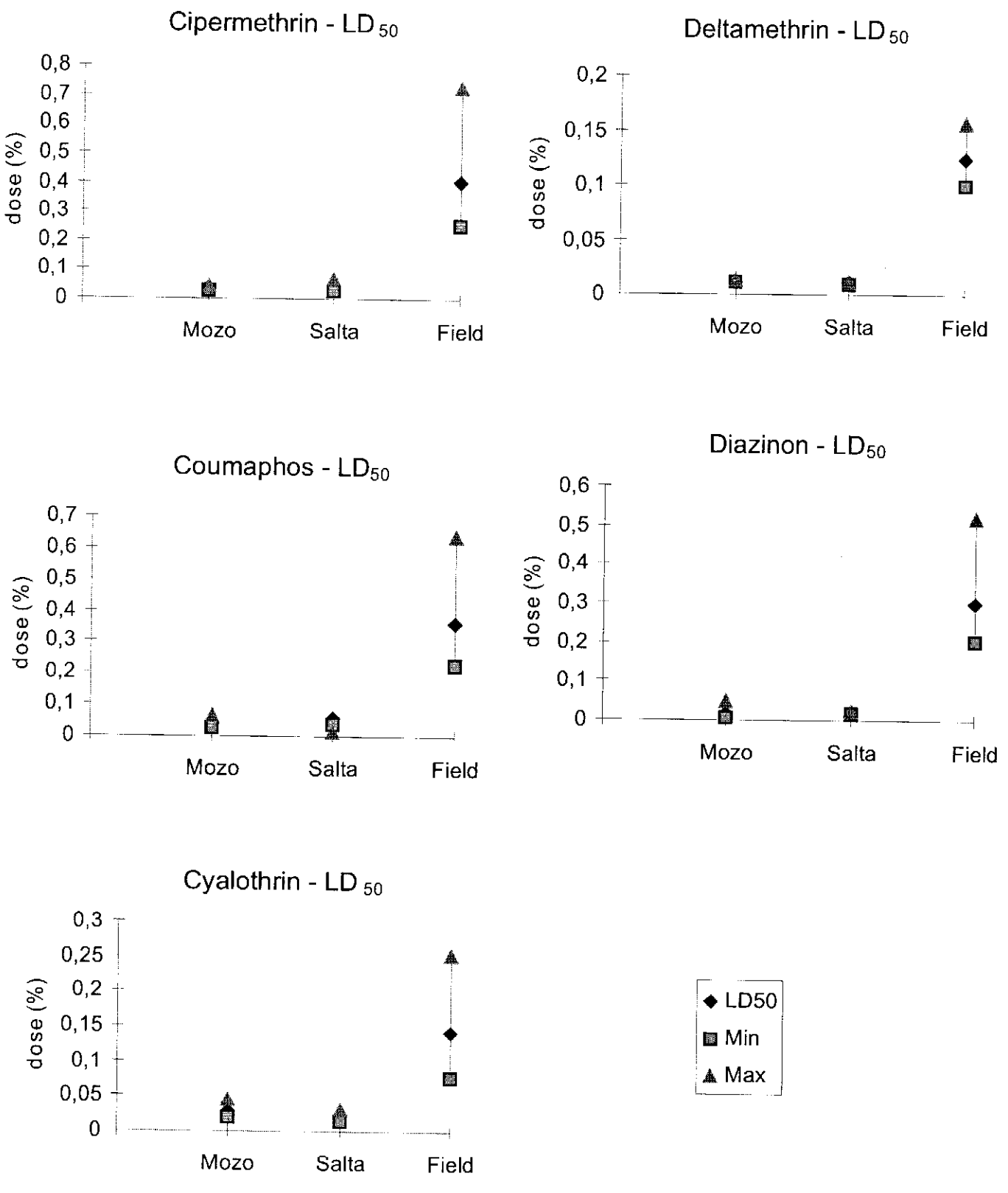

Fig.2 - Comparison of LD_{50} values for all ixodicides testes in three strains (Confidence limits 95%).

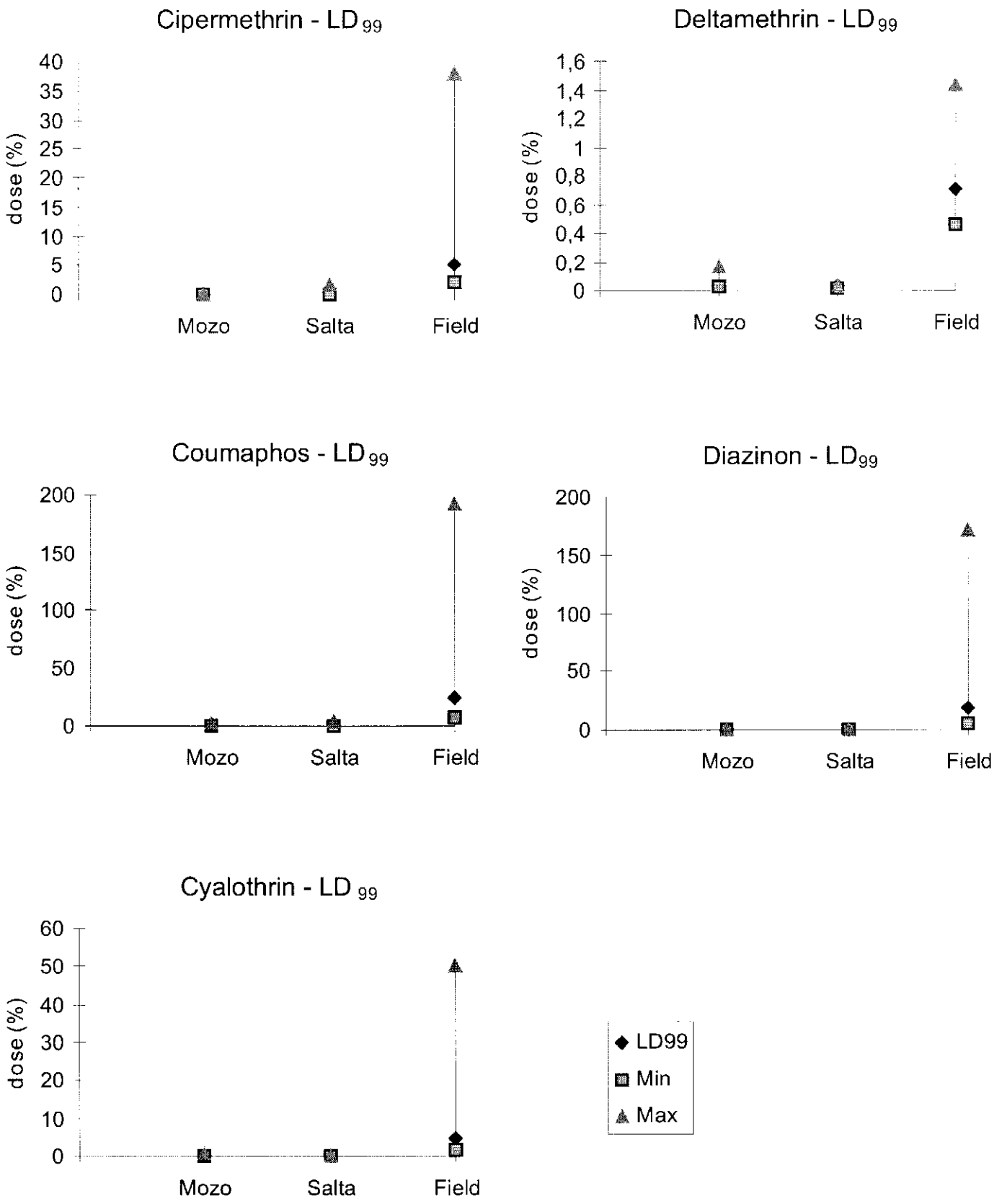

Fig.3 - Comparison of LD_{99} values for all ixodicides testes in three strains (Confidence limits 95%).

DISCUSSION

Both Mozo and Salta strains confirmed their susceptibility to the ixodicides tested. The mortality rates reached 100% at doses much lower than those ones normally used under field conditions. It could be concluded that both strains can be used as reference susceptible strains.

Based upon these results, we speculate if the concentrations available in the kits provided by WARRC, FAO are appropriate to determine reference values in these two susceptible strains, since then for two ixodicides (Flumethrin and Deltamethrin), the smaller concentration available showed a mortality rate greater or very close to 50%, which could lead to misleading data. In order to determine reference values accurately, we should have lower concentrations available.

For coumaphos, both sensitive strains showed a huge variation in the confidence limits (95%) at probit analysis. Such findings could be explained as a memory of a previous contact of these strains with organophosphate ixodicides. This variation becomes less obvious when the field strain was included in the comparison.

The field strain LD_{50} values for all ixodicides were higher than for both sensitive strains. The resistance index (R.I.) values found strongly suggest a serious resistance problem. CARDOZO *et alii* (1984) observed treatment failure in cases when the R.I. values found were grater than 6.

The strain which we analyzed presented R.I. values higher than 20. Although only one sample was tested, such finding raise up a serious concern about the resistance problem in the area of Paraíba river valley. The strain test was found to be resistant to both organophosphates and pyrethroids. Further studies are necessary to accurately map the resistance status among São Paulo dairy herds.

The analysis of LD_{50} and LD_{99} values altogether with their confidence limits (95%) offers valuable data about the population profile regarding to the resistance status. The narrower is the range of maximal and minimal possible values for the LD_{50}, greater is the genetic homogeneity of the tested population. By analyzing these confidence limits one can estimate how far the frequency of the trait resistance is spread among the population. For the field strain tested in this case, we find individuals responding only with a one fold increase over the mean LD_{50} dose (which was already four times greater than for susceptible strains). If the LD_{99} was considered instead, the situation goes worst, and 200 % increases could be found. Such information, unfortunately does not give us quantitative data about how many individuals present such a resistance degree, but confirms that resistance is already well established among the population since at least few individuals present a high level of tolerance to the chemical compound used. Having this in mind, we should be very careful in the management of a herd with such a profile. Unaware use of compounds with a baseline resistance profile could quickly lead to the development of really serious resistance problem.

SUMÁRIO

Duas cepas suscetíveis do carrapato *Boophilus microplus* foram testadas com diferentes acaricidas, com o objetivo de caracterizá-las como padrões de referência para testes de diagnóstico de resistência. Os testes com larvas foram realizados usando um kit padrão fornecido pela FAO. Lotes de 108,9 ± 29 larvas foram colocados em envelopes preparados com papéis impregnados com os seguintes acaricidas: cipermetrin, deltametrin, cialotrin, flumetrin, coumafós e diazinon. Após um período de incubação de 24 horas em ambiente controlado (27 °C e 85% u.r.) a contagem de larvas vivas e mortas foi realizada. As taxas de mortalidade foram calculadas segundo recomendações da FAO, e transformadas pela análise de probitos. Uma cepa de campo originária do Vale do Paraíba e com histórico de falha de tratamento foi também examinada, a fim de confirmar se os valores de referência obtidos podem ser usados para o cálculo do índice de resistência. Os dados apresentados sugerem fortemente a ocorrência de um sério problema de resistência. As duas cepas suscetíveis foram consideradas adequadas para o uso com padrões de referência, porém concentrações menores deveriam estar disponíveis nos kits da FAO a fim de se obter maior acurácia nas determinações para estas cepas suscetíveis.

PALAVRAS-CHAVE: Testes de resistência, *Boophilus microplus*, acaricidas.

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