



Resistance of *Rhipicephalus (Boophilus) annulatus* populations against Commercial Preparation of Deltamethrin, Beni-Suef, Egypt.

Abstract

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A field survey was conducted to evaluate susceptibility of *Rhipicephalus (Boophilus) annulatus* to commercial deltamethrin 5% on different localities in Beni-suef province using different bioassays (Adult immersion test AIT, Larval Packet test LPT and Esterase test). Two concentrations of deltamethrin 5% (200 and 400 ppm) were tested for their acaricidal efficacy against adult *R. (B.) annulatus* collected from different localities. Adult tick mortality at 400 ppm was higher than 200ppm and the survived females' oviposited with complete eclosion blocking for eggs. While 200 ppm allowed eggs to hatch in all populations. The larval packet test was used to evaluate the larval mortality at six deltamethrin concentrations (0.000625, 0.00125, 0.0025, 0.005, 0.010 and 0.020). Lethal concentrations (LC50–LC90), confidence intervals (CI) and slope (S) were estimated by probit analysis and the highest LC50 appeared in Ehanasia locality (0.01) but the lowest LC50 appeared in Alfashin (0.0006). Esterase assay with substrate α naphthylacetate applied on *R. (B.) annulatus* larvae and mean enzyme activity was determined; Ehnsaia locality appeared with the highest activity 2.373 ± 0.058 while Alfashin was the lowest 1.024 ± 0.014 . From the results of this work, it was concluded that one tick population of the investigated locations was of possible susceptible, one of possible tolerant and one resistant to deltamethrin. The data generated on deltamethrin resistance status will help in formulating tick control strategy in the region.

Keywords: *Rhipicephalus (Boophilus) annulatus*, Adult immersion test, larval packet test, deltamethrin, Resistance-susceptibility, Egypt.

Introduction:

Acaricides have played an important role in the control of this tick species, however, as a consequence of extensive exposures to acaricides, ticks have developed resistance to all major classes of acaricides (Jonsson et al., 1998; Rodriguez-Vivas et al., 2006; Chevillon et al., 2007). Among these acaricides the synthetic pyrethroids (deltamethrin) which is commercially available in Beni-Suef and presently the predominant acaricide used to control tick in the province. Tick resistance is defined as a significant increase in the number of individuals within a tick population that can tolerate doses of drug(s) that have proved to be lethal for most individuals of the same species (FAO, 2004). This phenomenon may be divided into four categories: behavioral, cuticular, metabolic and target site resistance. Target site resistance exists when an allele of the gene (2006). Bioassays are very useful tools to monitor resistance. The Food and Agriculture Organization of the United Nations (FAO 2004) recommends two in vitro tests to evaluate resistance against pyrethroids (deltamethrin): Larval Packet Test (LPT) and Adult Immersion Test (AIT). Each test has its own advantages and disadvantages: LPT is a laborious and time-consuming test while AIT requires many ticks for testing (Lovis et al.,

coding for the target molecule attacked by the acaricide has an amino acid mutation that confers resistance to the acaricide. This resistance mechanism is common in the case of pyrethroid class of acaricides (target site resistance) (Rodríguez-Vivas, et al. 2014). While the increased ability to detoxify insecticides is one of the main types of resistance (metabolic resistance). It takes place when the activity of detoxifying enzymes (esterase enzymes) is enhanced, impeding the insecticide to reach its target and evaluated by esterase assay (kumar et al., 2013). Early detection of resistance is essential in order to avoid further selection of resistant ticks using the same active ingredient and to delay the spread of resistance (Rodriguez-Vivas, 2003; Foil et al., 2004; Klafke et al., 2012). While biochemically esterase assay which measure the level of esterase enzymes evaluate resistance mainly against organophosphates (kumar et al., 2013). In this paper, Adult immersion and larval packet tests were used to evaluate resistance of *R. (B.) annulatus* populations in Beni-Suef province to deltamethrin (SP). Also esterase test was applied on the larvae to measure the level of esterase enzymes.

Materials and Methods:**Preparation of Acaricide:**

Commercial available deltamethrin 5% was used and purchased from local veterinary stores (Formulated deltamethrin (Butox®, EC; 5% active ingredient<AI>). For experimental bioassay (LPT) stock solution of deltamethrin 5% prepared in two parts of trichloroethylene and one part of olive oil and serial dilutions of deltamethrin were made in these diluents to generate testing doses (0.000625, 0.00125, 0.0025, 0.005, 0.010 and 0.020) and tested against the various field isolates of *Rhipicephalus (Boophilus) annulatus* larvae (Aguirre et al., 2000). While for adult immersion test commercial deltamethrin was diluted in distilled water to prepare two concentrations 200 and 400ppm to test against the various field isolates of *R. (B.) annulatus* adult (Ravindran et al., 2014).

Collection of Ticks:

Live engorged adult female *R. (B.) annulatus* ticks were collected from different localities in Beni-Suef governorate at the time from March 2014 to July 2015. After restraining of animals, engorged females tick were collected (Rodriguez-vivas et al., 2012) directly from infested animals in each locality, placed in identified carton boxes and

transported to the laboratory of Parasitology at the Faculty of Veterinary medicine, Beni-Suef University.

Preparation of ticks and production of *R. (B.) annulatus* larvae:

Samples collected from particular area were designated as an isolate or population and washed thoroughly in distilled water and allowed to dry in paper towels, then weighted and labeled. Part of samples was kept at 27-28°C and 80-90 relative humidity for laying eggs. After oviposition (14-18d), pooled eggs from all females were randomly divided into 3ml glass vials and plugged with a cotton cap to allow air and moisture exchange. At 7-14d after larval eclosion. One vial of larvae was used for bioassays testing Larval Packet Test (LPT) (Kemp et al., 1998) and the other was frozen at -70 °C for esterase assay (Rodriguez-Vivas et al., 2012). The remaining engorged adult female ticks were used to estimate the acaricidal effects of respective concentration of chemical acaricide (deltamethrin) by AIT (Shyma et al., 2015).

Adult immersion test (AIT):

The Adult Immersion Test (AIT) was conducted as per the protocol described by Drummond et al. (1973). AIT has been used by various workers for evaluation of efficacy of various acaricides against *Rhipicephalus (Boophilus) microplus* (Jonsson et al., 2010; Gonçalves et al.,

2007; kumar et al., 2011; Sharma et al., 2012; Ravindran et al., 2014). Briefly, the engorged females of *R. (B.) annulatus* were immersed in two concentrations of deltamethrin (200 and 400ppm) for two min and then dried with filter paper before transferring into the petri dishes. After 24 h, ticks were transferred to plastic tubes covered with muslin cloth and were kept in BOD incubator maintained at 28 ± 1 °C and 80 ± 5 % RH. The control group was treated in similar manner with distilled water (Reghu Ravindran et al., 2014). Each concentration was replicated three times with ten adult ticks each ($n = 30$) and the following parameters were compared:

- a) **Mortality:** recorded up to 14 days post treatment.
- b) **The egg mass** weight laid by the live ticks.
- c) **Reproductive index (RI)** = egg mass weight/live tick weight.
- d) **Percentage inhibition of oviposition (%IO)** = $[(RI_{\text{control}} - RI_{\text{treated}}) / RI_{\text{control}} \times 100]$.

Laboratory bioassays (Larval Packet Test - LPT):

The LPT was performed on 12 to 14 day-old larvae as prescribed by (FAO, 2004) with some minor modifications i.e. instead of technical grade deltamethrin, commercially available formulation was used. Deltamethrin was diluted in two parts

of trichloroethylene and one part of olive oil. Serial dilutions of deltamethrin (0.000625, 0.00125, 0.0025, 0.005, 0.010 and 0.020) were made in these diluents to generate testing doses. Diluent alone used as control. Packets were prepared by depositing 1ml of testing dose on a 7.5 cm * 8.5 cm piece of whatman filter paper 1; then the acaricide impregnated papers were allowed to dry for 2 h to allow trichloroethylene to evaporate. Treated Papers were folded into half and sealed on the sides with bulldog clips to form packets, which were then sealed along the top with additional bulldog clip. The packets were placed in an incubator ($26-28^{\circ}\text{C}$ and $85-92\text{RH}$) for 24 h. After which the papers were taken out and opened. Control packets were opened first and examined for larval mortality. Then the packets were opened in order of increasing concentration of the acaricide. Each testing dose was tested in triplicate and the average of dead and live larvae was scored. The larval mortality in larval packet test at a given testing dose was expressed as percentage from the total number of larvae (Aguirre et al., 2000).

The resistance factors (RF) for field tick isolates were calculated as the quotient between LC50 of field ticks and LC50 of a susceptible one of *R. (B.) annulatus* (Gopalan et al., 1996). On the basis of RF, the resistance levels (RL) of *R. (B.)*

annulatus were classified as susceptible (RF < 1.4), level I resistance (RF = 1.5-5.0), level II resistance (RF = 5.1–25.0), level III resistance (RF = 25.1– 40) and level IV resistance (RF > 40) (**Kumar Sachin et al., 2011, Sharma et al., 2012**).

Esterase assay:

Esterase activities with the substrate α naphthylacetate was determined in tick larvae according to the method of **Hemingway (1998)** with some modifications. Twenty deep frozen larvae from each population were homogenized in a precooled glass pestle in 200 μ l of distilled water. The homogenates were spun at 1100 \times g in a refrigerated centrifuge at 4°C for 15min and resulting supernatant was used for assay. Reaction mixtures contained 20 μ l of the homogenate in wells of microtitre plate (each sample applied in triplicate) and 200 μ l of α - naphthylacetate solution (250 μ l of 30mM stock in 25ml of phosphate buffer 0.02M, pH7.2.). The action mixtures were incubated at room temperature for 30min before addition of 50 μ l of fast blue solution (0.023g fast blue salt dissolved in 2.25ml distilled water and 5.25ml of 5%SDS in 0.1M sodium phosphate buffer, pH7.2) to each well. The plates were incubated for five min at room temperature and absorbance was measured at 570nm in a microtitre plate reader (Tecan,Austria) operated by a personal computer using Magellan 6 software

(**kumar et al., 2013**).

Statistical analysis:

For the AIT all data were expressed as the mean \pm SD. using SPSS software (IBM, USA) (**Reghu Ravindran et al., 2014**). Probit Analysis was applied for LPT and dose response data were analyzed by probit method (**Finney, 1952**) with determination of lethal concentrations (LC50 and LC90) and their respective 95 % confidence intervals (CI).

Results:

The questionnaire results revealed that most of farmers in Beni-Suef governorate depend mainly on deltamethrin spraying at concentration 200ppm and ivermectin injection for treatment of tick infestation. The majority of the cattle owners have reported treatment inefficiency of these chemicals in field conditions and complained repeated infestation with short intervals of application of the acaricide. The results of AIT showed in **Table (1) and (2)** where commercial deltamethrin 5% used with two different concentrations 200 ppm (the field concentration applied by farmers) and 400 ppm tested for their acaricidal activities on adult engorged female ticks *Rhipicephalus (Boophilus) annulatus* collected from different localities in Beni-Suef governorate. It was observed that deltamethrin at 200 ppm produced higher level of adult mortality after 14 days in Alfashin locality only

83.33±4.714, while the lowest adult mortality recorded in Ehnasia was 33.33±4.714. Moreover, the inhibition percent of oviposition reached 100% (no oviposition occurred) in Alfashin while in Ehnasia was 47.54±11.69. Also 200 ppm concentration allowed hatching of the eggs laid by the treated ticks to 100 percent in all populations. While at 400ppm the adult mortality after 14 days was higher in Alfashin 86.66±4.714 and lower in Ehnasia 66.66±4.714, the percent inhibition of oviposition was higher than 90% in most populations and reached 100% in Alfashin but in Ehnasia population was 89.62±0.562. It was observed complete eclosion blocking to the eggs occurred in all populations (no hatching occurred) except in Ehnasia the level of hatching was 3.33±4.714%. In the present study, different populations of *R. (B.) annulatus* were surveyed by LPT showed RF (Resistance Factor) between 1 and 16.6 to deltamethrin showed in (Table 3) and the LC₅₀/LC₉₀ values for deltamethrin with their respective 95%CI and their T slopes for each population were calculated. It was observed that the lowest LC₅₀ appeared in Alfashin where the farmer did not depend on deltamethrin in control (0.0006) with 95%CI (0.0005 to 0.0007 and LC₉₀(0.002) with 95%CI (0.001 to 0.002). So, it is considered to be useful as a susceptible reference strain but the highest LC₅₀

appeared in Ehanasia, where the farmers/farm owners reported frequent applications of higher doses of deltamethrin due to very low efficacy of the most aggressively marketed product, (0.01) with 95%CI (0.005 to 0.01) and LC₉₀ (0.02) with 95%CI (0.01 to 0.02). According to the results appeared in LPT bioassay and classification described by **Kumar Sachin et al. (2011)** one locality can be considered susceptible (Alfashin) to deltamethrin with RF = 1 and one locality considered resistant **level I** or **tolerant** to deltamethrin with RF =3.3 (Beni-Suef) in addition to, one locality considered resistant **level II** with RF varied from 16.6. Esterase activities using a general esterase substrate (α naphthyl acetate) were determined within tick populations of different localities in Beni-Suef province and summarized in **Table 4**. The mean enzyme activity appeared in lower level in Alfashin (1.024±0.014) (susceptible population) but appeared in higher level in Ehnasia (2.373±0.058) (resistant population).

Discussion:

The results of the current study revealed the presence of a widespread resistance to deltamethrin in different locality field isolates of *R. (B.) annulatus* collected from Beni-suef province, Egypt. So it appeared from the results that adult tick populations *R. (B.) annulatus* in Beni-Suef governorate

showed resistance to deltamethrin so, to avoid development of any resistant generations of tick's in future, deltamethrin preparations should be used at or above 400 ppm that agreed with **Reghu Ravindran et al. (2014)** who reported above 300 ppm.

The resistance status to deltamethrin was established in different localities in Beni-Suef province using LPT and the resistance factor (RF) was varied from 1.00 to 16.6 in larval progenies of engorged female ticks. One population showed RF below 1.4 and was designated a **susceptible** population, one locality showed RF from 1.5 to 5.0 and was designated as **resistance level I** or **tolerant** and one locality showed RF from 5.1 to 25.0 and was designated as **resistance level II** according to a classification described by **Kumar Sachin et al. (2011)**. This may be due to differences between the products used at each locality. While populations which showed RF varied from 1.6 to 8.3 were collected in area where macrocyclic lactones used beside pyrethroids, the population showed the highest RF 16.6 depended mainly on deltamethrin. Similar results reported by **Kumar et al. (2013)**.

In the present study, higher levels of esterase enzymes were detected in the resistant populations of *R. (B.) annulatus* in Beni-suef governorate and that agreed with (**Jamroz et al., 2000; Baffi et al.,**

2008; Kumar et al., 2013; Li et al., 2004; Chevillon et al., 2007), (Rosario-Cruz et al., 2009; Jonsson et al., 2000) (Rodriguez-Vivas et al., 2006).

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Table (1) Results of adult immersion test in different localities in Beni-Suef province, at dose 200ppm of commercial deltamethrin

Location	Concentration	Mean Adult Tick weight (gm) \pm SD	Mean Adult mortality within 14 days (%) \pm SD	Mean Mass of Egg weight (gm) \pm SD	Reproductive index (RI%) \pm SD	Percentage of inhibition of oviposition (%) \pm SD	Hatching (%) visual
Ehnasia	Control	1.18 \pm 0.005	0 \pm 0	0.651 \pm 0.045	0.551 \pm 0.036	0 \pm 0	100
	200ppm	1.109 \pm 0.031	33.33 \pm 4.714	0.316 \pm 0.053	0.285 \pm 0.047	47.54 \pm 11.69	100
Beni-Suef	Control	1.802 \pm 0.058	0 \pm 0	0.665 \pm 0.056	0.368 \pm 0.019	0 \pm 0	100
	200ppm	1.654 \pm 0.019	36.66 \pm 4.714	0.071 \pm 0.016	0.043 \pm 0.009	87.53 \pm 3.59	100
Alfashin	Control	1.605 \pm 0.046	0 \pm 0	0.766 \pm 0.09	0.479 \pm 0.067	0 \pm 0	100
	200ppm	1.486 \pm 0.038	83.33 \pm 4.714	0 \pm 0	0 \pm 0	100 \pm 0	0

Table (2) Results of adult immersion test in different localities in Beni-Suef province, at dose 400ppm of commercial deltamethrin

Location	Concentration	Mean Adult Tick weight (gm) \pm SD	Mean Adult mortality within 14 days (%) \pm SD	Mean Mass of Egg weight (gm) \pm SD	Reproductive index (RI%) \pm SD	Percentage of inhibition of oviposition (%) \pm SD	Hatching (%) visual
Ehnasia	Control	1.18 \pm 0.005	0 \pm 0	0.651 \pm 0.045	0.551 \pm 0.036	0 \pm 0	100
	400ppm	1.13 \pm 0.053	66.66 \pm 4.714	0.025 \pm 0.035	0.021 \pm 0.029	89.62 \pm 0.562	33.33 \pm 4.714
Beni-Suef	Control	1.802 \pm 0.058	0 \pm 0	0.665 \pm 0.056	0.368 \pm 0.019	0 \pm 0	100
	400ppm	1.595 \pm 0.039	66.66 \pm 4.714	0.007 \pm 0.01	0.004 \pm 0.006	98.75 \pm 1.76	0
Alfashin	Control	1.605 \pm 0.046	0 \pm 0	0.766 \pm 0.09	0.479 \pm 0.067	0 \pm 0	100
	400ppm	1.422 \pm 0.052	86.66 \pm 4.714	0 \pm 0	0 \pm 0	100 \pm 0	0

Table 3: Lethal concentrations 50 and 90% of 14-21days-old larvae of *R. (B) annulatus* populations from different localities in Beni-Suef province calculated from larval packet tests with a commercial formulation of deltamethrin 5%:

Location	Larvae	Slope (SE)	X2	t for slope	LC ₅₀ (95%CI)	LC ₉₀ (95%CI)	Resistance Factor(RF)	Resistance Level(RL)
Beni-Suef	100	1.93	5.91	13.14	0.004 (0.003- 0.004)	0.01 (0.008- 0.01)	3.3	I(T)
Alfashin	100	2.12	6.73	8.93	0.0006 (0.0005- 0.0007)	0.002 (0.001- 0.002)	1	S
Ehnasia	100	1.91	13.06	6.89	0.01 (0.005- 0.01)	0.02 (0.01- 0.02)	16.6	II

Table 4: Mean Esterase activity \pm SD of *R. (B). annulatus* larvae from different localities in Beni-suef province:

Locality	Mean of esterase activity \pm SD
Alfashin	1.024 \pm 0.014
Ehnasia	2.373 \pm 0.058
Beni-Suef	1.67 \pm 0.155

الملخص العربي

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تم عمل مسح حقلّي لتقييم قابلية قراد البيوفيلس انيولايّيس إلى الدلتاميثرين التجاري 5% في مناطق مختلفة في محافظة بنى سويف باستخدام اختبارات بيولوجية مختلفة مثل اختبار الغمر لانتى القراد اليافعة واختبار الباكيت لليرقات واختبار الاستريز. تم اختبار اثنين من تركيزات الدلتاميثرين 5% (2% و 4%) لقياس مدى فعالية المبيد ضد انتى القراد اليافعة التي تم جمعها من مناطق مختلفة في محافظة بنى سويف حيث تم تسجيل شكوى من فشل العلاج في كثير من الأحيان في هذه المناطق وكان معدل الوفيات عند تركيز 4% أعلى من 2% لكن لم تصل إلى 100% لكن عند تركيز 4% لم يفقس البيض ماعدا البيض الناتج لكن تركيز 2% سمح بفقس البيض. تم استخدام اختبار حزمة اليرقات لتقييم موت اليرقات في ستة تركيزات مختلفة للدلتاميثرين الدلتاميثرين. كما تم تقدير التركيزات القاتلة وفترات الثقة والمنحدر من خلال تحليل الاحتمالية وظهر أعلى تركيز قاتل لنصف القراد في اهناسيا (0.01) واقل تركيز في الفشن (0.0006). وتم قياس نشاط انزيم الاستريز في يرقات قراد البيوفيلس انيولايّيس باستخدام النافثيل اسيتيت وظهر أعلى نشاط للانزيم في اهناسيا $0.058 \pm 373,2$ واقل نشاط في الفشن 0.014 ± 1.024 . من نتائج هذا العمل تم التوصل إلى أن ثلاثة مناطق في محافظة بنى سويف لها مقاومة للدلتاميثرين وبناء على البيانات التي تم إنشاؤها على وضع المقاومة للدلتاميثرين هذا يساعد في وضع صياغة استراتيجية لمكافحة القراد في المنطقة.