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Susceptibility status of field populations of *Rhipicephalus bursa* (Acari: Ixodidae) to pyrethroid insecticides

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Abstract. *Rhipicephalus bursa* is a two-host ixodid tick with wide distribution in north of Iran especially in Mazandaran province. Acaricide treatment is the main tick control measure; however, acaricide resistance occurs in hard ticks in many areas of the world including Iran. Comprehensive information on susceptibility status of *Rhipicephalus bursa* is lacking, therefore, this study is undertaken to determine the susceptibility status of the species to pyrethroid acaricides and probable biochemical underlying mechanisms of resistance. From May 2013 to March 2014, engorged females *Rhipicephalus bursa* were collected using standard entomological procedures from body surface of sheep, goat and cattle in different areas of Mazandaran province, northern Iran. Eleven and ten pooled tick populations were tested against cypermethrin and lambda-cyhalothrin, respectively using larval packet test. Population SC-16 showed a maximum resistance ratio of 5.79 against cypermethrin in Sari County when compared to the most susceptible population NH-16 and 63.64% of tick populations were resistant at LC_{99} level. With lambda-cyhalothrin, 30% of the tick populations were resistant with low level and NK-2 was the most resistant population with resistance ratio of 4.32 in Nowshahr County. The results of biochemical assays demonstrated elevated levels of monooxygenases, glutathione S-transferases and esterases in pyrethroid resistant populations tested.

INTRODUCTION

Ticks are obligatory ecto-parasites of wild and domesticated mammals (Sonenshine & Roe, 2014). Ticks have important etiological roles in veterinary and human health as they transmit serious pathogens to livestock and humans hence, causing economical losses as a result of decrease of milk and body weight of domestic animals (Jongejan & Uilenberg, 2004; Ginsberg, 2008).

Rhipicephalus bursa (Rh. bursa) is a two-host hard tick found in Mediterranean basins, southern parts of the Palearctic zone, Black and Caspian seas (Walker *et al.*, 2003). Three species of the genus *Rhipicephalus* (including *Rh. bursa*, *Rh. sanguineus*, and *Rh. turanicus*) have been reported from Iran known to transmit *Babesia*, *Theileria and Anaplasma* parasites to ruminants (Shayan *et al.*, 2007; Telmadarraiy *et al.*, 2012; Abdigoudarzi, 2013). These parasites were reported from domesticated animals from Mazandaran province (Zaeemi *et al.*, 2011; Ziapour *et al.*, 2011; Hosseini-Vasoukolaei *et al.*, 2014). Among the hard tick species reported from Iran, *Rh. bursa* is one of the most prevalent ticks in Mazandaran province, northern Iran (Razmi *et al.*, 2007; Asgarian *et al.*, 2011). *Rh. bursa* has been known as a vector of *Babesia ovis* in Iran (Shayan *et al.*, 2007; Esmaeilnejad *et al.*, 2014). This intracellular haemoprotozoa is the main causative agent of ovine Babesiosis in the study area (Motavalli Haghi *et al.*, 2013). Therefore, hard ticks control programs are needed to reduce tick-borne diseases occurrence in Mazandaran province (Ghosha *et al.*, 2007).

Ticks are managed mainly by acaricides treatment, however, at the same time their intensive or inadequate use increase the risk of tick resistance to acaricides leading to control failure (FAO, 2004). Detection of acaricide resistance in ticks is performed using reference methods including larval packet test (LPT) described by FAO (2004), however, it could not identify the mechanisms of resistance to insecticides. Elevated detoxifying enzymes have important roles in insecticide resistance (Lee *et al.*, 2014). Biochemical assays including quantification of enzyme activity/content in unprocessed insect homogenates using model substrates is a rapid detection of metabolic mechanisms involvement in insecticide resistance (Pethuan *et al.*, 2007). For example, biochemical studies detected the involvement of esterases, cytochrome P450 monooxygenases and glutathione S-transferases (GSTs) in the metabolic resistance to synthetic pyrethroids (SPs) (Enayati & Ladonni, 2006; Yang *et al.*, 2004; Baffi *et al.*, 2008).

Hard ticks resistance to SPs is reported from different geographical regions of the world demonstrating their tick control failure (Andreotti *et al.*, 2011; Fernández-Salas *et al.*, 2012; Kumar *et al.*, 2013). A variety of SPs have been used as acaricides for tick control in Iran including cypermethrin, lambda-cyhalothrin, flumethrin and deltamethrin (Khalaj *et al.*, 2009; Vatandoost *et al.*, 2012). Under the shadow of the lack of a comprehensive tick control policy in Iran, farmers adopt individual control practices which may exacerbate acaricide resistance. Although livestock farmers made complaints about the lack of efficacy of different acaricides against ticks, determination of acaricide resistance with bioassays on *Rh. bursa* for generating basic information has not been undertaken adequately. There is only one report on susceptibility status of *Rh. bursa* to pyrethroids in the literature that reported resistance in Iranian *Rh. bursa* populations from Sari, capital of Mazandaran province (Enayati *et al.*, 2010). Therefore, it is important to investigate the susceptibility status of *Rh. bursa* to commonly used acaricides in other areas of Mazandaran province, northern Iran where animal husbandry is the second major occupation of its people. The results of this study will be useful for developing rational tick control programs as well as acaricide resistance management strategies.

MATERIALS AND METHODS

Study area

Mazandaran province is located in northern Iran between 50º 342 - 54º 102 E and 35º 472-36º 352 N encompassing an area of 23 756 km^2 (1.46% of the mainland Iran) (Mesgari *et al.*, 2014) (Figure 1). Sheep and cattle husbandry is one of the most economically important occupations in Mazandaran province. There are approximately 759 880 cattle, 2 289 349 sheep and goats in Mazandaran province (Data were obtained from Mazandaran provincial veterinary department). The sampling was undertaken

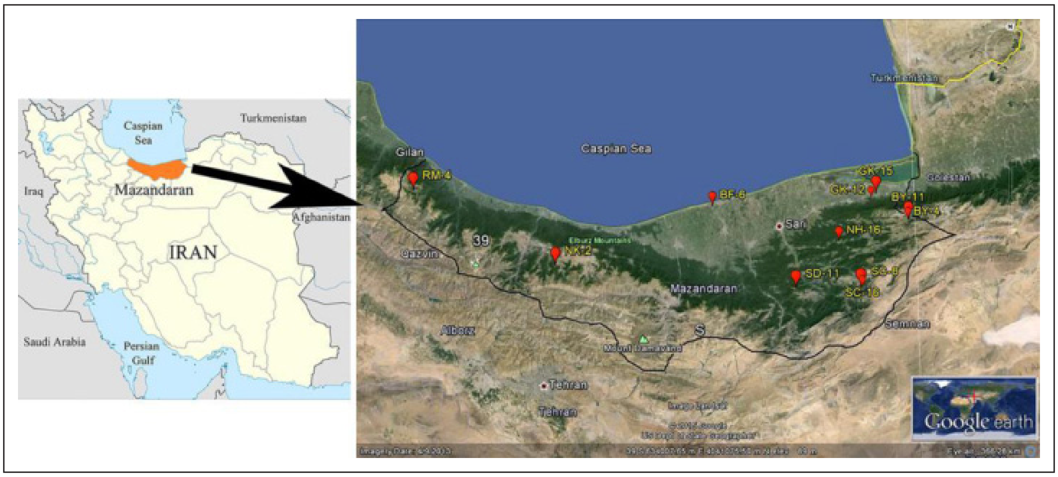

Figure 1. Map of Iran (left) and study area in Mazandaran province, northern Iran (right) with red icons and names of tick populations indicating where fully engorged female *Rhipicephalus bursa* were collected.

in 24 sentinel sites in three geo-ecological areas of plain, woodland and highland of Mazandaran province. Most studied villages are in the vicinity of the towns. The three study areas are characterized by a moderate climate with high humidity in plain areas up to a maximum altitude of 150 meters; woodland areas with altitude between 151 to 1 800 meters; and moderate to semi-arid climate highlands with an altitude of more than 1 200 meters with medium to low humidity and low temperature. The mean monthly temperature is about 15.6°C ranging from 6°C to 25.5°C. The mean monthly precipitation is about 65 mm ranging from 4.5 mm to 161 mm, most of which occur between fall and winter (Mesgari *et al.*, 2014). Herds of ruminants graze on vegetation of these areas from spring until winter in plain and woodland areas and spring to summer in highland areas but some herds graze in fall and winter in sunny days in highland areas.

Ticks sampling

In a large scale field study, multistage cluster randomized sampling method was used to identify twenty-four sentinel sites located in three geo-ecological regions of highlands, woodlands and plain/coastal areas of all 19 counties of Mazandaran province. Sampling took place in each season from domesticated ruminants from May 2013 to March 2014. Collected specimens of hard ticks were placed into separate 50 ml well labeled falcon tubes. Fine pores were punched into the lid of the tubes to allow air and moisture exchange for survival of ticks. The tubes were transferred to the Laboratory of Insect Biology and Pesticides, Department of Medical Entomology and Vector Control, School of Public Health, Mazandaran University of Medical Sciences. Engorged adult female ticks were placed in fresh well labeled falcon tubes containing ladder shaped filter paper. Species were identified using tick key manuals under a stereomicroscope (Axiume®, Spain) (Walker *et al.*, 2000; Walker *et al.*, 2003). Then engorged female *Rh. bursa* ticks were kept in controlled insectary under 27±2ºC temperature, 80±5% relative humidity and 12:12 h (L:D) periodicity. Engorged adult female ticks oviposited after 5-10 days; they hatched in 20-40 days and 12-18 days old larvae were used for LPT. Eleven populations of *Rh. bursa* were tested against cypermethrin and ten populations against lambda-cyhalothrin. The locations where fully engorged females of *Rh. bursa* were captured on naturally infested sheep, goat and cattle herds are shown in Figure 1. All engorged female *Rh. bursa* were collected in spring and summer and no engorged specimens were captured in fall and winter.

Bioassays

larval packet test was carried out according the standard method (Stone & Haydock, 1962; FAO, 2004; Enayati *et al.*, 2010) with some modifications including the use of paper staples and adhesive paper tapes instead of bulldog clips in sealing of treated packets and use of enameled surgical tray with an adhesive paper tape on its surrounding edge for preventing the larvae from escape.

Acaricides used were commercial cypermethrin 10% EC (MAC TOMIEL®) and lambda-cyhalothrin 5% EC (MAC SILAT®) rather than analytical grade acaricides (Chevillon *et al.*, 2007; Jonsson *et al.*, 2007; Enayati *et al.*, 2010). These products were manufactured by Melli Agrochemical Co., Iran. A 1:1000 dilution of these formulations is used by farmers in the field according to the manufacturer's recommendations. In order to obtain 0.4% (4 g/L) stock solutions of cypermethrin and lambda-cyhalothrin, the formulations were diluted by 1:25 and 1:12.5, respectively in olive oil (Chevillon *et al.*, 2007). Then 0.4% stock solution of both chemicals were serially diluted in two parts of trichloroethylene (Merck®, Germany) and one part of olive oil to obtain doses that kill 5-95% of the larvae based on the literature and our unpublished pilot study. These doses for cypermethrin were: 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.01563, 0.00781, 0.00391 g/L and for lambda-cyhalothrin were: 0.125, 0.0625, 0.03125, 0.01563, 0.00781, 0.00391, 0.00195 g/L (Chevillon *et al.*, 2007; Enayati *et al.*, 2010). A volume of 0.67 ml of each dilution was evenly distributed onto a 7.5 x 8.5 cm filter paper (Whatman® No. 541, Maidstone, UK) and trichloroethylene was allowed to evaporate under a fume cabinet for 2 hours before the papers were stored at 4°C until use. All bioassays were done in duplicates coupled with a pair of control replicates.

For LPT, treated filter papers were put on the lab bench at room temperature for 20 minutes and then folded in half and the sides were sealed with paper staples and adhesive paper tape forming a packet. About one hundred larvae were then placed into each packet using a paintbrush, which was immediately sealed with staples and adhesive paper tape. Sealed packets were individually placed into glass Petri dishes that were put into an enameled surgical tray containing tap water (to prevent larvae from scape) and incubated under insectary condition for 24 h before the mortality was scored. To score the mortality results, the packets containing treated larvae were emptied into an 11 cm glass Petri dish in fresh tray with no water in it and with adhesive paper tape round its edges for preventing live larvae from escape. Larvae that could not walk or were only able to move their legs were considered dead.

Metabolic enzyme assays

Larvae homogenization

Seventy five 12-18 days old deep frozen larvae of each tick population were placed into each well of 96-well flat-bottom microtiter plate (Maxwell®, China) using a paintbrush, 25 µl of cold distilled water was added and the larvae were homogenized using a handheld homogenizer on ice. Then another 25 µl of cold distilled water was added and homogenizing process was repeated to ensure complete homogenization. Two hundred µl of cold distilled water was added and the plate was centrifuged at 1 109 x g in a refrigerated centrifuge (Beckman Coulter®, Inc., California, USA) at 4°C for 15 min and the supernatants were used as enzyme source in biochemical assays.

Enzyme assays

Cytochrome P450 monooxygenase (mixed function oxidase (MFO)), glutathione S-transferase (GST), general esterases (including α- and β-esterases) and protein assays were performed according to Enayati *et al.* (2010) with minor modifications. The *p*-NPA esterase assay was also performed as described by Penilla *et al.* (1998). Two control replicates with distilled water instead of the enzyme source were prepared with the same method per plate. The absorbance of each enzyme mixture was measured at specific wavelength as end point or kinetic method by microtiter plate reader system (Bio-Tek® Instruments, Inc., Model: ELX 808, USA), operated by a personal computer using KC-Junior software.

Statistical analyses

The results of LPT with mortality in the controls of more than 20% were discarded and repeated but the results of those tests with mortality in the controls of less than 20% were corrected using Abbott formula (Abbott, 1925). The LPT data were entered into the EPA Probit analysis software (Ver. 1.5, USA) for calculating LC_{50} and LC_{99} values, slope, intercept of the regression lines and Chi-square test for heterogeneity according to Finney formula for Probit analysis (Finney, 1971). A regression line of all populations was drawn and interpreted by SigmaPlot software (Ver. 12, 2012). Slope and intercept of regression lines of all populations were compared with general linear model analysis by Minitab software (Ver. 14). Relative Resistance Ratios (RRs) were calculated based on a formula that was described previously (Nolan, 1985; Enayati *et al.*, 2010). As a standard susceptible indigenous strain of *Rh. bursa* was lacking in Iran, one field tick population with the lowest LC_{50} and LC_{99} and a history of no acaricide use was selected as susceptible tick population for calculating relative RR. The criteria defined by Mendes *et al.* (2007) was used to classify RR_{50} and RR_{99} in susceptible level (S, RR $\leq$ 2.4), resistance level (RL) I (RI, RR between 2.5- and 5.4-fold), resistance level II (RII, RR between 5.5- and 50-fold) and resistance level III (RIII, RR > 50-fold).

The enzyme activities/contents were calculated as equivalent units of cytochrome P450/min/mg protein for MFOs, mM CDNB conjugated/min/mg protein for GSTs, µM product/min/mg protein for α- and β-esterases and *p*-NPA esterases in the Microsoft® Excel program. The enzyme activities were expressed as enzyme ratio (ER; mean activity of enzyme in field population divided by mean activity of the same enzyme in the most susceptible field population). ANOVA analysis coupled with post-hoc Tukey test was used to compare the enzyme activity/content of field populations and the most susceptible field population (NH-16). A p value $\leq$ 0.05 was considered as significant difference.

RESULTS

Susceptibility bioassays

Cypermethrin

The results of LPT on 11 populations of *Rh. bursa* for cypermethrin are shown in Table 1. LC_{50} varied from 0.086 to 0.223 and LC_{99} from 0.238 to 1.376 g/L. Population NH-16 showed the least LC_{50} and LC_{99} of 0.086 and 0.238 g/L, respectively and with no history of pyrethroid acaricide use was considered as the most susceptible field population. SC-16 was the most resistant population to cypermethrin with LC_{50} and LC_{99} of 0.223 and 1.376 g/L, respectively. Most of the tick populations (90.91%) were susceptible to cypermethrin where RR_{50}s varied from 1 to 2.22 (Table 2). Only SC-16 population was classified as RI at LC_{50} level. However, when RR_{99} is considered, 63.64% of tick populations were resistant. Almost half of the tick populations (45.5%) were RI for cypermethrin at LC_{99} level, with values ranging from 2.51 to 4.67 leaving four populations (36.4%) as susceptible. Two populations including GK-12 and SC-16 (18.2%) were RII. The SC-16 population showed the highest RR_{99} of 5.77 (Table 2). These data clearly explain the resistance status of this species. Besides, LC_{99} of the most resistant SC-16 population is 13.8 times higher than the concentration of cypermethrin recommended by the formulating company.

Lambda-cyhalothrin

The results of LPT bioassays on 10 different *Rh. bursa* populations to lambda-cyhalothrin are presented in Table 1. The NH-16 population with the least LC_{50} and narrower range of 95% CI for LC_{99} with no history of acaricide exposure was considered as the most susceptible field population. The LC_{50} and LC_{99} of the tested populations varied from 0.011-0.032 and 0.035-0.176 g/L, respectively. Most of the tick populations (80%) were susceptible to lambda-cyhalothrin at LC_{50} level, with resistance ratios ranging from 1 to 2.32 but GK-12 and BF-6 populations (20%) were classified as RI. When LC_{99} is

Table 1. Results of cypermethrin and lambda-cyhalothrin larval packet test (LPT) on *Rhipicephalus bursa* in Mazandaran province, northern Iran

			Bioassays									
			Cypermethrin					Lambda-cyhalothrin				
Populations	**Counties**	**Animals**	**Slopes ± SE**	**Intercepts ± SE**	χ^{2a}	**LC_{50} (95% CI) (g/L)**	**LC_{99} (95% CI) (g/L)**	**Slopes ± SE**	**Intercepts ± SE**	χ^{2a}	**LC_{50} (95% CI) (g/L)**	**LC_{99} (95% CI) (g/L)**
GK-12	Galugah	Sheep	8.46 ± 0.81	-30.56 ± 3.42	0.64	0.160 (0.149-0.171)	0.302 (0.271-0.350)	3.60 ± 0.38	-7.65 ± 1.36	4.33	0.032 (0.028-0.037)	0.143 (0.114-0.200)
GK-15	Galugah	Sheep	2.50 ± 0.28	-5.53 ± 1.18	10.02	0.160 (0.135-0.193)	1.364 (0.883-2.654)	N/A[b]	N/A[b]	N/A[b]	N/A[b]	N/A[b]
BY-4	Behshahr	Sheep	5.74 ± 0.75	-19.59 ± 3.20	9.42	0.192 (0.175-0.209)	0.487 (0.398-0.676)	6.08 ± 0.67	-15.11 ± 2.24	0.79	0.020 (0.018-0.022)	0.049 (0.041-0.062)
BY-11	Behshahr	Sheep	3.61 ± 0.34	-9.95 ± 1.40	7.53	0.138 (0.122-0.155)	0.609 (0.480-0.851)	3.92 ± 0.35	-7.34 ± 1.13	1.03	0.014 (0.013-0.015)	0.055 (0.044-0.074)
NH-16[c]	Neka	Sheep	5.28 ± 0.54	-15.78 ± 2.14	3.60	0.086 (0.079-0.094)	0.238 (0.197-0.312)	4.09 ± 0.83	-7.45 ± 2.55	6.86	0.011 (0.002-0.021)	0.041 (0.021-93.75)
SC-8	Sari	Sheep & goat	3.88 ± 0.33	-11.22 ± 1.37	6.23	0.150 (0.136-0.165)	0.597 (0.487-0.786)	6.07 ± 0.65	-14.15 ± 2.07	2.53	0.014 (0.013-0.016)	0.035 (0.029-0.044)
SC-16	Sari	Cattle	2.95 ± 0.30	-7.82 ± 1.34	1.40	0.223 (0.194-0.259)	1.376 (0.981-2.262)	3.32 ± 0.28	-5.41 ± 0.88	2.70	0.014 (0.012-0.015)	0.069 (0.054-0.096)
SD-11	Sari	Sheep	2.77 ± 0.21	-6.34 ± 0.87	5.97	0.125 (0.111-0.141)	0.867 (0.66-1.242)	5.59 ± 0.58	-13.01 ± 1.89	2.33	0.017 (0.015-0.018)	0.044 (0.037-0.056)
BF-6	Fereydunkenar	Goat	8.14 ± 2.75	-28.61 ± 11.39	21.29	0.135 (0.065-0.235)	0.261 (0.183-6240.2)	3.98 ± 0.73	-8.80 ± 2.62	10.16	0.029 (0.016-0.041)	0.112 (0.070-0.557)
NK-2	Nowshahr	Goat	2.66 ± 0.40	-6.08 ± 1.65	2.81	0.148 (0.123-0.180)	1.109 (0.669-2.759)	2.78 ± 0.33	-4.46 ± 1.17	4.30	0.026 (0.021-0.031)	0.176 (0.124-0.300)
RM-4	Ramsar	Sheep & goat	3.03 ± 0.22	-7.47 ± 0.91	8.60	0.130 (0.119-0.143)	0.763 (0.590-1.069)	5.67 ± 1.24	-13.11 ± 4.01	19.00	0.016 (0.011-0.019)	0.040 (0.028-0.121)

[a]Pearson chi-square, goodness-of-fit test.
[b]N/A, Not applicable due to inadequate number of larvae.
[c]The most susceptible field population.

considered, most of the tick populations (70%) were susceptible to lambda-cyhalothrin, with RR_{99} values ranging from 0.85 to 1.70 leaving three populations (30%) classified as RI. NK-2 population showed the highest resistance to lambda-cyhalothrin (RR_{99} = 4.32) (See Table 2). The LC_{99} of the population NK-2 was 3.5-fold higher than the maximum dose recommended by the formulating manufacture.

Generally higher resistance levels to cypermethrin were observed compared to lambda-cyhalothrin in all populations tested except BF-6 and GK-12 at LC_{50} and LC_{99} levels and SD-11 at LC_{50} level (Table 2). Based on general linear model analysis, the slopes of the dose–response regression lines in all field populations for cypermethrin were not significantly different from each other. This indicates that the populations are not different in responding to pesticide doses and are homogenous (F = 0.36, P = 0.959). Tick populations showed more homogeneity of resistance in response to cypermethrin than against lambda-cyhalothrin (Figures 2, 3). The slopes of the dose–response regression lines in all field populations for lambda-cyhalothrin were significantly different from each other. This means that tested populations are different in responding

Table 2. Comparison of relative Resistance Ratios (RRs) and enzyme ratios (ERs) of *Rhipicephalus bursa* populations collected from Mazandaran province, northern Iran

	Bioassays										
	Cypermethrin			Lambda-cyhalothrin			Biochemical assays				
Population	RR_{50} [a]	RR_{99} [a]	RL_{99} [b]	RR_{50} [a]	RR_{99} [a]	RL_{99} [b]	ER_{MFO}	ER_{GST}	$ER_{\alpha\text{-esterase}}$	$ER_{\beta\text{-esterase}}$	$ER_{p\text{-NPA}}$
GK-12	1.86	1.27	S	2.94	3.52	RI	1.39	1.14	2.02***	1.94**	1.07
GK-15	1.86	5.74	RII	N/A [c]	N/A [c]	N/A [c]	1.60*	1.94***	1.81**	1.69*	0.81
BY-4	2.22	2.05	S	1.84	1.20	S	1.65*	1.58*	1.15	1.10	1.02
BY-11	1.60	2.56	RI	1.27	1.35	S	2.43***	2.35***	1.31	1.30	0.87
NH-16 [d]	1	1	SS	1	1	SS	1	1	1	1	1
SC-8	1.74	2.51	RI	1.30	0.85	S	2.06***	1.38	1.42	1.32	0.99
SC-16	2.59	5.79	RII	1.25	1.70	S	2.66***	2.17***	1.38	1.29	1.23
SD-11	1.45	3.65	RI	1.52	1.07	S	0.76	0.43*	1.08	1.03	1.13
BF-6	1.57	1.10	S	2.66	2.76	RI	0.96	1.07	2.47***	2.57***	1.29
NK-2	1.72	4.67	RI	2.32	4.32	RI	1.10	0.45*	1.19	1.21	0.83
RM-4	1.51	3.21	RI	1.41	0.98	S	2.38***	2.27***	1.89**	1.76*	1.02

[a]Resistance ratio between $LC_{50/99}$ of each population and the most susceptible population (NH-16). [b]Levels of resistance (RL) or calculated relative Resistance Ratio (RR) of tick populations categorized at LC_{99} level as: SS = the most susceptible field population, S = susceptible level, RI = Resistant level I, RII = Resistant level II upon Mendes *et al.* (2007) criteria. [c]Not applicable due to inadequate number of larvae. [d]The most susceptible field population. *P-value significantly was different in comparison with enzyme activity/content of the most susceptible field population (NH-16) by ANOVA-Tukey post hoc test (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

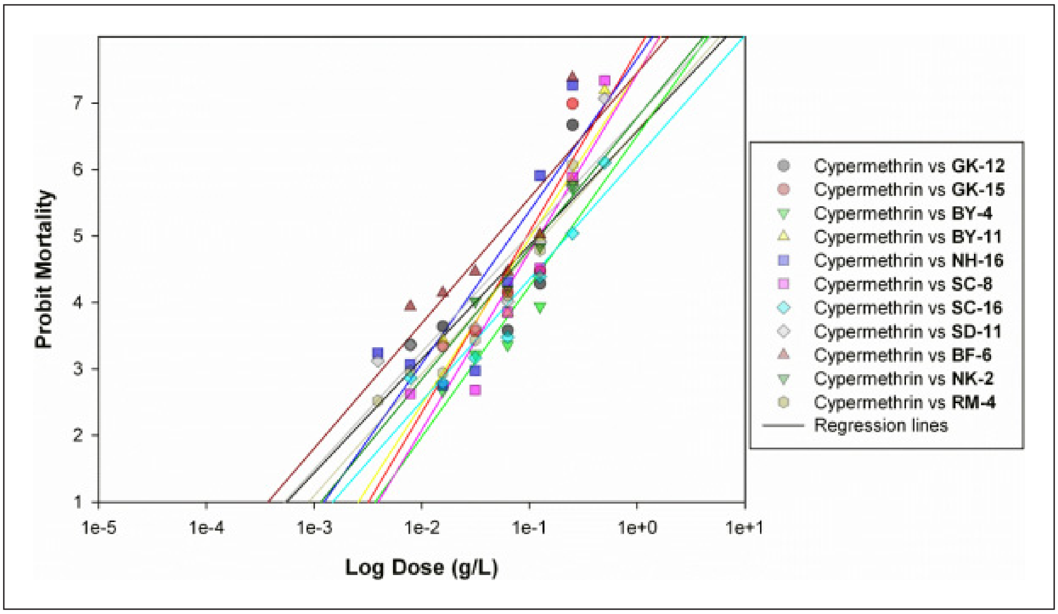

Figure 2. Comparison of regression lines from eleven field populations *Rhipicephalus bursa* bioassayed with cypermethrin in Mazandaran province, northern Iran.

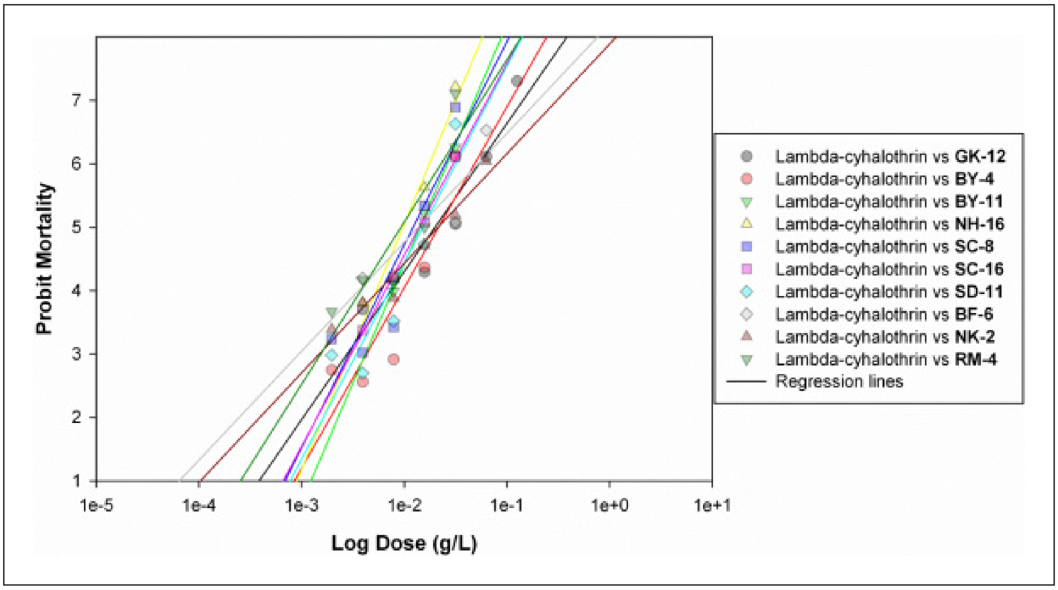

Figure 3. Comparison of regression lines from ten field populations *Rhipicephalus bursa* bioassayed with lambda-cyhalothrin in Mazandaran province, northern Iran.

to pesticide doses and are heterogeneous ($F = 2.90$, $P = 0.008$). Figure 3 depicts heterogeneity between populations and the initiation of development of resistance if selection pressure with lambda-cyhalothrin sustained.

Enzyme assays

The highest ER for α- and β-esterase, *p*-NPA, GST and MFO were 2.5, 2.6, 1.3, 2.4 and 2.7-fold, respectively. The mean enzyme activity of α- and β-esterase was the lowest in susceptible population NH-16. The most

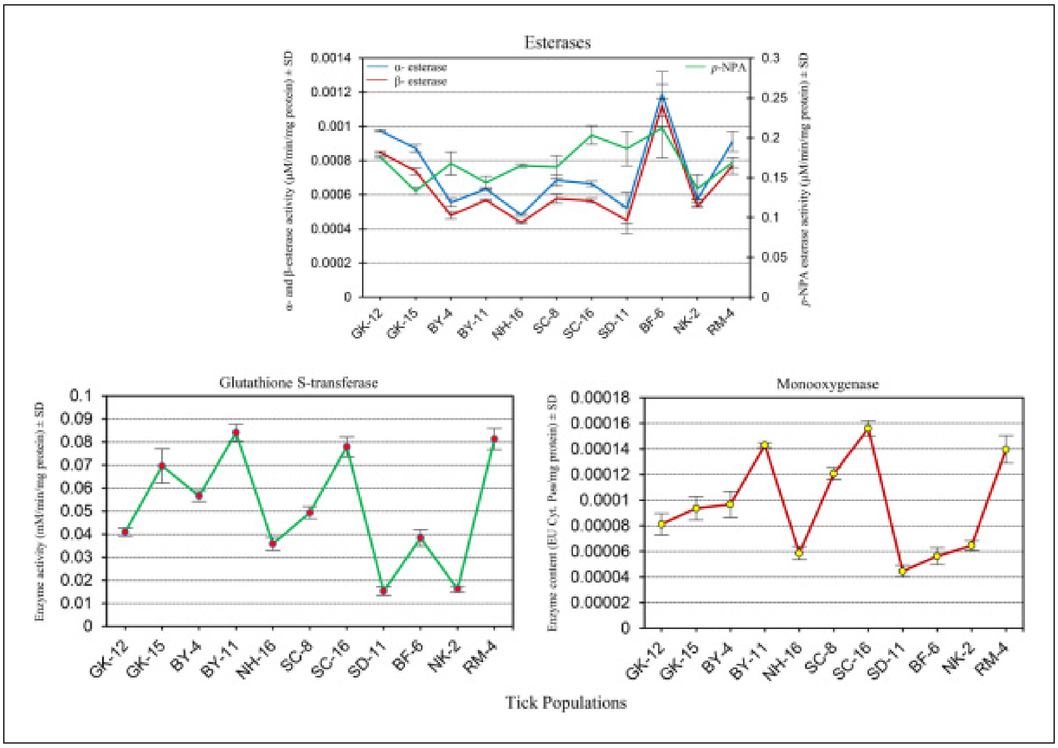

Figure 4. Mean enzyme activity/content of eleven populations of *Rhipicephalus bursa* in Mazandaran province, northern Iran.

resistant population to cypermethrin (SC-16) showed the highest level of MFO and also very high GST levels, with significant difference when compared with NH-16 population. Populations BY-11 and RM-4 showed the highest levels of GST and very high levels of MFO. However, no elevation in all tested enzymes was observed in NK-2, the most resistant population to lambda-cyhalothrin, this phenomenon was also observed in SD-11 which was RI to cypermethrin. In other lambda-cyhalothrin resistant populations (GK-12 and BF-6) very high general esterases activities were measured. GK-15, the second high resistant population to cypermethrin showed different levels of elevation in GST, esterases and MFO. There are variations in enzyme levels in different susceptible and resistant populations presented in Table 2 and Figure 4.

DISCUSSION

Rhipicephalus bursa is widespread in different areas of Iran (Nabian *et al.*, 2007; Razmi *et al.*, 2007; Asgarian *et al.*, 2011; Shemshad *et al.*, 2012). This study is the second such report on this species susceptibility status to pyrethroids in the world (Enayati *et al.*, 2010). A relatively low to moderate resistance level to lambda-cyhalothrin and cypermethrin, respectively were detected in *Rh. bursa* populations of Mazandaran province, northern Iran. In the only available study addressing the susceptibility status of *Rh. bursa* similar degree of pyrethroid resistance was shown by LPT method in Sari (capital of Mazandaran province, Iran). Comparison of the RR_{99}s in both studies revealed that cypermethrin resistance is faster developed than lambda-cyhalothrin (Enayati *et al.*, 2010). This phenomenon is supported by other studies in bed bugs (Moore & Miller, 2006; Turner & Brigham, 2008). Besides, in other Iranian species, *Hyalomma anatolicum*, higher susceptibility to lambda-cyhalothrin than cypermethrin was reported (Khalaj *et al.*, 2009). These data indicate that lambda-cyhalothrin may provide more effective control of these species than cypermethrin. Susceptibility to lambda-cyhalothrin was reported in other hard ticks including *Ixodes ricinus* and *Dermacentor marginatus* in Serbia (Jurisic *et al.*, 2010). These results also are in accord with the results of a study reporting tick-control failures using alpha-cypermethrin based on interviews of 60 stockbreeders involved in traditional farming from Burkina Faso (Adakal *et al.*, 2012). Cypermethrin and deltamethrin resistance was also reported in *Rh. appendiculatus* in Zambia (Luguru, 1995) and *Rhipicephalus (Boophilus) microplus* in Brazil (Mendes *et al.*, 2001).

The slopes of dose-response regression lines to cypermethrin showed homogeneity of the tested tick populations compared with those tested with lambda-cyhalothrin. In addition, the slopes of the regression lines in susceptible populations to cypermethrin (NH-16, GK-12, BY-4 and BF-6) are higher than those for the resistant populations. It means that the heterogeneity in the resistant populations are higher than the susceptible populations and cypermethrin resistance will develop even further should the selection pressure maintained (Miller *et al.*, 2005; Telmadarraiy *et al.*, 2007). The slopes of the regression lines in some susceptible populations including BY-4, SC-8, SD-11 and RM-4 are higher than those of the resistant populations including GK-12, BF-6 and NK-2 in response to lambda-cyhalothrin. Other susceptible populations including NH-16, BY-11, SC-16 have low slopes which in turn indicates the possibility of building resistance in these susceptible populations if selection pressure remains high (Khalaj *et al.*, 2007). The fact that the characteristics of the regression lines of the more resistant populations are different from those of the susceptible populations implies that the populations are genetically divergent and higher degrees of resistance might be expected (see Table 1 and Figures 2, 3).

As LC_{99} is in the upper most part of the dose-response line with higher operational significance, comparison at this level is preferred (Brown & Pal, 1971). Population SC-16 showed RR_{99} = 5.79 indicating resistance to cypermethrin in *Rh. bursa* and the failure of tick control program using this acaricide whereas the same population showed RR_{99} = 1.70 to lambda-cyhalothrin which was categorized as susceptible. One possible reason for this higher RR_{99} to cypermethrin may well be because of higher use of this acaricide in tick control than lambda-cyhalothrin in the field, a fact that was reflected in the data collected in questionnaire. In a study on *Rh. bursa* in Sari County from north of Iran, the resistance levels to cypermethrin and lambda-cyhalothrin were 7- and 2-fold respectively higher than the doses recommended by the formulating company (Enayati *et al.*, 2010). This has increased to 13.8- and 3.5-fold in our study that indicates an increase of approximately 2x resistance in response to both pyrethroids after about 5 years in this tick species.

The development of cypermethrin resistance in *Rh. bursa* in the study area could possibly be due to an increase in the use of cypermethrin based acaricides in the past ten years. Acaricide use has not been consistent especially in traditional farms compared with industrial farms in Iran due to a number of factors including managerial as well as economic issues (Luguru *et al.*, 1985)

Rhipicephalus bursa is a two-host tick (Walker *et al.*, 2003) with longer generation time and fewer generations per year leading to less selection pressure. This means development of pesticide resistance will be slower than one-host tick (Nolan, 1990). Because of publishing of our data in April 2016 (acta tropica), the word "unpublished" must be delete and the current sentence must be change to "Our pilot bioassay data in Nur County of Mazandaran province on *Rhipicephalus (Boophilus) annulatus* (a one-host tick) in 2012, revealed 75-fold cypermethrin resistance in comparison with field recommended dose by the formulating company (Ziapour *et al.*, 2016) whereas in the current research 13.8-fold resistance was detected in *Rh. bursa* (Peter *et al.*, 2005). This finding is in accord with that of the Mekonnen *et al.* (2002) which showed one-host tick, *Rhipicephalus (Boophilus) decoloratus*, was more resistant to all of the acaricides tested than multi-host ticks including *Rh. everetsi everetsi*, *Rh. appendiculatus* and *Amblyomma hebraeum* populations.

Biochemical assays are used to detect the mechanisms of metabolic resistance (Limoee *et al.*, 2011; Mendes *et al.*, 2013). A fairly straight relation was observed between RR_{99} and ER in the studied populations. SC-16, the most resistant tick population to cypermethrin, showed the highest ER of MFO and GST activities which indicate the possibility of involvement of these enzymes in metabolic resistance to cypermethrin ($P < 0.001$). Monooxygenase-mediated resistance is probably the most frequent type of metabolic resistance (Pethuan *et al.*, 2007), although esterases and glutathione S-transferases are also important (Abdullah *et al.*, 2012; Lee *et al.*, 2014; Xu *et al.*, 2015).

RM-4 and BY-11 populations which are level RI resistant to cypermethrin showed significantly increased monooxygenses contents and elevated GSTs activities compared with the most susceptible population ($P < 0.001$). In addition, the GK-15 population, with the second highest RR_{99} (5.74) against cypermethrin, showed elevated GST and general esterases activities which indicates metabolic resistance mechanism against cypermethrin. GSTs have fundamental roles in resistance to insecticides (Ketterman *et al.*, 2011). Increased GST activity has been associated with resistance to organophosphorus, organochlorine and pyrethroid insecticides (Penilla *et al.*, 1998; Enayati *et al.*, 2009; Enayati *et al.*, 2010). GSTs can protect against pyrethroids by binding and sequestering the insecticide and also protecting against oxidative stress when this is a by-product of insecticidal toxicity (Enayati *et al.*, 2005). Furthermore, GSTs are regulated by different mechanisms in response to insecticides in specific manner based upon species, sex, feeding and developmental stage and GST-based insecticide resistance mechanism must be considered for pest management (Tripathy & Kar, 2015).

The BF-6 population, with resistance level RI against lambda-cyhalothrin at $LC_{50/99}$, showed the highest ER of general esterases ($P < 0.001$) that probably indicates involvement of esterases in developing resistance to lambda-cyhalothrin in this population. This result is supported by other studies in hard ticks on pyrethroid resistance mechanisms (Baffi *et al.*, 2008; Abdullah *et al.*, 2012; Kumar *et al.*, 2013). Coincidently, similar to the study by Enayati *et al.* (2010) on *Rh. bursa* in Sari, maximum cypermethrin relative RR was also observed in the current study in Sari and the same patterns of GST and MFO activities were observed ($P < 0.001$). In addition, the authors suggested that lambda-cyhalothrin resistance in *Rh. bursa* might be due to elevated GST activities whereas resistance to this acaricide observed in the current study is probably related to higher activity of general esterases which shows different metabolic resistance pathways in two studies (Enayati *et al.*, 2010). As the current study showed, involvement of elevated esterases in cypermethrin resistance was also shown in multi-host tick, *Hyalomma anatolicum*, from India (Shyma *et al.*, 2012).

The NK-2 population, the most resistant population to lambda-cyhalothrin, with RR_{99} of 4.32 demonstrated cross-resistance to cypermethrin with RR_{99} of 4.67. To our surprise, this population showed no elevation in activities or contents of metabolic enzymes related to acaricide resistance. The SD-11 population with RR_{99} of 3.65 to cypermethrin had no elevation in detoxifying enzymes, too. This dictates the necessity to explore the possibility of involvement of other resistance mechanisms in NK-2 and SD-11 populations especially the kdr mechanism (Scott, 1999).

The lack of consistency between the resistance ratio and enzyme ratio is also reflected in the literature (Scott & Kasai, 2004; Pethuan *et al.*, 2007; Araujo *et al.*, 2013). There are multiple isozymes of the same metabolizing enzyme involved with insecticide resistance in insects and resistance could be due to an elevation in as few as one isozyme regulated independently from each other (Chien *et al.*, 1995). This elevation may not be enough to change the total amounts of the enzyme in question measured by universal substrates. Therefore, it would be necessary to isolate and characterize those isozymes independently (Scott, 1991). Accordingly it is highly recommended to perform biochemical assays along with bioassays to have a more comprehensive picture of the susceptibility status of the arthropod in question.

In order to save the efficacy of our current acaricides, insecticide resistance management (IRM) strategies e.g. regulating dose, adding a synergist, changing the insecticide, creating a refuge, targeting a specific stage of insect are essential to help lower selection pressure (Zhao *et al.*, 2010). This is usually executed through a number of strategies, including rotation, the use of insecticide mixtures, and mosaic applications of insecticides (Insecticide Resistance Action Committee (IRAC) Public Health Team, 2011).

In conclusion, pyrethroid resistance is confirmed in some *Rh. bursa* populations in Mazandaran province, northern Iran. Metabolic enzymes such as MFO, GST and esterases are involved in the acaricide resistance in this species. As in some tick populations, metabolic mechanisms of acaricide resistance were rolled out; possible involvement of other mechanisms should be investigated. As a resistance management strategy, use of acaricides with different mode of action including systemic insect growth regulators (IGRs) is recommended.

DISCLOSURE

The authors declared that they have no conflict of interests.

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