Explor Anim Med Res,
Vol.11, Issue - 1, 2021, p. 49-54
DOI : 10.52635/EAMR/11.1.49-54

ISSN 2277- 470X (Print), ISSN 2319-247X (Online)
Website: www.animalmedicalresearch.org

Research Article

ADULT BIOASSAY BASED AMITRAZ RESISTANCE STATUS IN *RHIPICEPHALUS MICROPLUS* POPULATIONS OF PUNJAB, INDIA

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Received 17.03.2021, revised 03.06.2021

ABSTRACT: Amitraz resistance status was estimated in cattle tick, *Rhipicephalus microplus* populations collected from fifteen districts of the Punjab state using adult immersion test. A two-minute immersion protocol with various working concentrations (1-500 ppm) of formulated amitraz (Taktic 12.5% EC) in distilled water was adopted. The regression graphs of probit mortality of engorged female ticks against log concentrations of amitraz were utilized for estimation of slope of mortality, lethal concentration for 50% (LC_{50}), 99% (LC_{99}) and resistance factors (RF). The values of RF were found to be in the range of 0.20-4.55 indicating low level resistance status in eight field populations, whereas, seven were found susceptible to amitraz. Effects of amitraz on the various reproductive parameters of engorged *R. microplus viz.* egg mass weight, reproductive index, percentage inhibition of oviposition and hatchability percentage were assessed and found to be dose dependent and more discernible upon exposure with higher concentrations.

Key words: Amitraz, Resistance, Reproductive parameters, *Rhipicephalus microplus.*

INTRODUCTION

Ticks are among the most economically and medically important sanguinivorous ectoparasites of domestic animals posing a serious threat to the livestock industry throughout the tropical and subtropical regions of the world (Jongejan and Uilenberg 2004). Amongst the 106 valid tick species reported from India, *Rhipicephalus microplus* Canestrini (Acari: Ixodidae) is the most widely prevalent and damaging species infesting cattle in Punjab state (Singh and Rath 2013). These ticks cause severe economic losses through anorexia, tick toxicosis, decreased milk production and weight gain, damage to leather, increased mortality due to tick-borne parasites and treatment costs (Ghosh *et al*. 2007).

Presently, chemical acaricides *viz*. organophosphates (OP), synthetic pyrethroids (SP), formamidines and macrocyclic lactones (ML) are being used for the control of *R. microplus*. Several reports of resistance development against commonly used chemical acaricides like OP (Jyoti *et al*. 2016, 2020), SP (Singh *et al*. 2014a, Singh and Rath 2014, Prerna *et al*. 2019), ivermectin (Singh *et al*. 2015a; Khangembam *et al*. 2018) and multi-acaricide resistance (Singh *et al*. 2019) has been reported from Punjab state. Owing to the resistance reports, there is an increased usage of amitraz by the livestock owners/dairy farmers to control these resistant tick populations in the region. Recently, development of amitraz resistance has been reported in *R. microplus* (Singh *et al*. 2014b, Jyoti *et al*. 2021) and *Hyalomma anatolicum* (Jyoti *et al*. 2019) populations from the Punjab state.

The published reports from the region have revealed resistance status against amitraz but no information is available on its effect on the reproductive parameters of *R. microplus* which is of utmost importance to define the effectiveness of an acaricide. Therefore, it is important to generate comprehensive data on the effect of amitraz on the reproductive parameters of field tick populations to assess the commercial life span of the drug and contribute to effective tick control.

MATERIALS AND METHODS

Location, Geography and Climate of Study Area

Punjab state is located in the northwest region of India which extends from the latitudes 29.30°N to 32.32°N and longitudes 73.55°E to 76.50°E. It covers a geographical area of 50,362 km^2 and lies between altitudes 180 and

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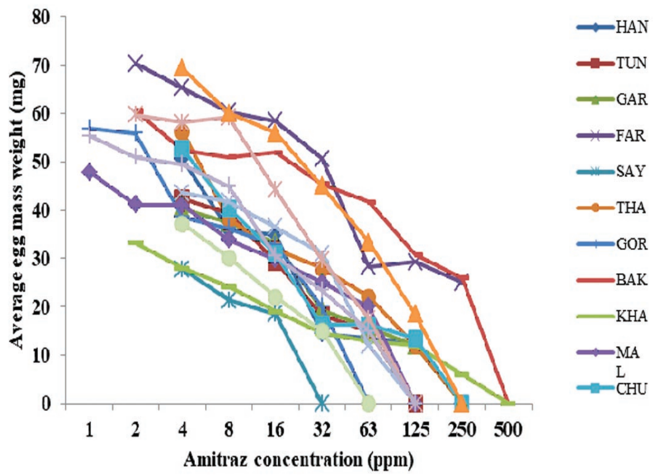

Fig. 1. Average egg mass weight laid by various field populations of *Rhipicephalus microplus* against amitraz.

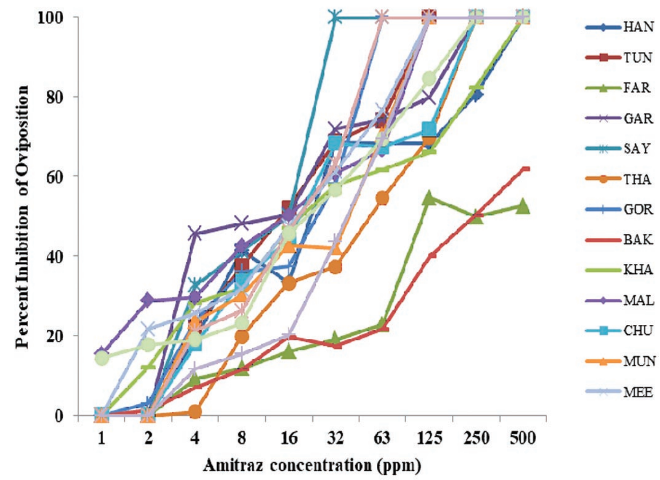

Fig. 2. Percent inhibition of oviposition (%IO) of various field populations of *Rhipicephalus microplus* against amitraz.

300 m above sea level. Average rainfall in state is 565.9 mm ranging from 915 mm in north to 102 mm in south with moderately humid climate (Singh and Rath 2014).

Collection and preparation of ticks

The ticks were collected from fifteen locations of the Punjab state, India *viz*., BAK (Bakhriana, Kapurthala), BAN (Banur, SAS Nagar), CHU (Chugawan, Moga), FAR (Faridkot City Bypass, Faridkot), GAR (Garolian, Fatehgarh Sahib), GOR (Goraya, Jalandhar), HAN (Handaya, Barnala), KHA (Khanpur, Ludhiana), MAL, (Malakpura, Mansa), MEE (Meemsa, Sangrur), MUN (Mundian, Rupnagar), REH (Rehipa, SBS Nagar), SAY (Sayianwala, Ferozepur), THA (Thakkarwal, Hoshiarpur) and TUN (Tungawali, Bathinda) using convenience sampling approach. Cracks, crevices, loose bricks and all other possible tick hiding places in the cattle sheds of selected locations were thoroughly searched and freshly dropped engorged adult female ticks were collected. The collected ticks were kept in plastic vials, closed with muslin cloth to allow air and moisture exchange and brought to the Entomology Laboratory, Department of Veterinary Parasitology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. The ticks were washed thoroughly in water, dried with paper towel and were used for bioassay. The ticks collected from village Sehra, of Patiala district, Punjab were used as reference susceptible isolate for estimation of resistance status in field isolates (Jyoti *et al*. 2021).

Adult Immersion Test

The Adult Immersion Test (AIT) was conducted according to the method of Drummond *et al*. (1973) using formulated amitraz (Taktic® 12.5% EC, MSD Animal Health, Pune, India) at concentrations in range of 1-500 ppm by serial dilution (1, 2, 4, 8, 16, 32, 64, 125, 250 and 500 ppm) in distilled water. Briefly, the collected engorged females from each location were randomly grouped into ten ticks each. Two replicates of ten engorged females of *R. microplus* were immersed in each working concentration of amitraz. The control ticks were immersed in distilled water for similar time period. After immersion, the ticks were dried on filter paper with the help of paper towels and placed in sterile Petri dishes for complete drying. The ticks were weighed individually, transferred to individual glass tubes covered with muslin cloth and kept in an incubator maintained at 28±1°C and 85±5% RH. Mortality in adult ticks was assessed for 14 days post treatment and ticks which did not oviposit even after 14 days post treatment were considered as dead. Reproductive Index (RI) was measured following the methods of Singh and Rath (2014). The Percent Inhibition of Oviposition (%IO) was calculated as: [(RI control - RI treated)/RI control×100]. After being weighed, eggs were retained in glass tubes and allowed to hatch for 21 days and visual estimation of hatchability was done (Singh *et al*. 2014c).

Estimation of resistance status

The regression curves of probit mortality plotted against log values of amitraz concentrations were drawn (Finney 1962) using GraphPad Prism 4. The lethal concentration values (LC_{50} and LC_{99}) of amitraz for different field populations were calculated from the regression equations. The resistance factors (RF) for

Table 1. Adult immersion test based amitraz resistance status in field populations of *Rhipicephalus microplus*.

Tick isolate	Slope (95% CL)	R^2	LC_{50} (95% CL)	LC_{99} (95% CL)	RF (RL)
BAK	2.53 ± 0.38 (1.54 to 3.52)	0.897	71.13 (65.57-77.17)	593.93 (468.92-752.26)	4.55 (I)
BAN	2.65 ± 0.33 (1.80 to 3.51)	0.927	9.93 (9.35-10.55)	47.88 (40.19-57.04)	0.37 (S)
CHU	3.10 ± 0.43 (1.90 to 4.30)	0.928	40.23 (37.64-42.99)	227.05 (187.26-275.30)	1.74 (I)
FAR	2.24 ± 0.27 (1.59 to 2.89)	0.922	50.89 (46.43-55.78)	556.45 (426.33-726.30)	4.26 (I)
GAR	2.55 ± 0.29 (1.80 to 3.08)	0.938	26.80 (24.73-29.05)	218.83 (173.21-276.48)	1.67 (I)
GOR	3.17 ± 0.41 (2.03 to 4.33)	0.936	9.57 (8.97-10.21)	51.84 (42.84-62.72)	0.40 (S)
HAN	2.49 ± 0.34 (1.61 to 3.37)	0.914	26.69 (24.57-28.99)	229.98 (180.95-292.31)	1.76 (I)
KHA	1.66 ± 0.19 (1.05 to 2.28)	0.962	10.54 (9.31-11.93)	264.90 (184.98-379.33)	2.01 (I)
MAL	2.59 ± 0.27 (1.91 to 3.28)	0.949	12.89 (11.91-13.95)	102.06 (81.00-128.59)	0.78 (S)
MEE	2.56 ± 0.36 (1.64 to 3.48)	0.911	17.31 (15.97-18.75)	140.6 (111.35-177.54)	1.08 (S)
MUN	3.05 ± 0.48 (1.70 to 4.39)	0.908	18.87 (17.64-20.18)	109.57 (90.10-133.23)	0.84 (S)
REH	2.66 ± 0.24 (2.03 to 3.29)	0.959	28.49 (26.37-30.78)	214.27 (193.57-237.17)	1.64 (I)
SAY	4.63 ± 0.66 (1.75 to 7.51)	0.959	7.95 (7.61-8.31)	25.31 (22.26-28.78)	0.20 (S)
THA	2.69 ± 0.22 (2.14 to 3.26)	0.969	27.43 (25.42-29.61)	200.53 (160.69-250.26)	1.54 (I)
TUN	3.37 ± 0.24 (2.72 to 4.03)	0.981	19.66 (18.50-20.90)	96.45 (80.80-115.13)	0.74 (S)
Patiala (Susceptible)	3.26 ± 0.43 (2.06 to 4.45)	0.935	25.12 (23.58-26.76)	130.56 (108.67-156.86)	1.0

different populations were worked out by the quotient between the LC_{99} of field populations and the LC_{99} of the susceptible population (Jyoti *et al*. 2021). The resistance status was classified on the basis of RF as susceptible (RF < 1.5), level I (1.5 < RF < 5), level II (5 < RF < 25), level III (25 < RF < 40) and level IV (RF > 40) (Sharma *et al*. 2012).

RESULTS AND DISCUSSION

Resistance status against amitraz

The AIT based values of LC_{50}, LC_{99}, slope, RF and resistance level (RL) against amitraz in the field populations of *R. microplus* are presented in Table 1. A concentration-dependent mortality response was observed for all populations and no mortality was recorded on control groups treated with distilled water. Values of the coefficient of determination (R^2) for AIT in the field isolates ranged from 0.897 to 0.981 indicating that the statistical model was a good fit. The RF values against amitraz ranged from 0.20-4.55 indicating variable resistance status among the field populations. Based on the RF values, level I resistance status was detectedagainst amitraz in eight field populations (HAN, FAR, GAR, THA, BAK, KHA, CHU and REH) whereas, the remaining seven (BAN, GOR, MAL, MEE, MUN, SAY and TUN) were susceptible (Table 1).

Effect of amitraz on reproductive parameters

The regression analysis of egg mass weight (EMW) and reproductive index (RI) of treated ticks against log values of progressively increasing log concentrations of amitraz revealed a negative dose-dependent slope for both mean EMW and RI in all tick isolates. Progressively fewer eggs were laid by the survived treated ticks exposed to increasing concentrations of amitraz (Fig. 1). Results thus indicate that although the increase in concentration of amitraz may have not caused mortality but the survived ticks showed a significant decrease ($p>0.05$) in their efficiency to convert their live weight into egg mass. Thus, a dose-dependent significant increase in the mean percent inhibition of oviposition (%IO) of treated ticks was recorded (Fig. 2). The hatching percentage of eggs was determined by visual estimation and a dose dependent effect was recorded. A low hatching percentage was recorded in eggs laid by all amitraz treated female ticks in comparison to control ticks treated with distilled water. However, the survival of the hatched larvae was not affected by amitraz treatment and was similar to control group.

The geo-climatic conditions of the Punjab region characterized by high humidity and ambient temperature for most parts of the year are highly conducive for development and propagation of ticks and often result in heavy tick infestations (Singh and Rath 2013). Among the various acaricides used for the control of ticks in livestock, resistance has been reported against most of the acaricides in *R.microplus* (Nandi *et al*. 2015, Jyoti *et al*. 2016, Khangembam *et al*. 2018).The first case of amitraz resistance was reported in the San Alfonso strain of *R. microplus* collected from a ranch in the state of Tabasco, Mexico (Soberances *et al*. 2002). Later, reports of amitraz resistance against *R. microplus* ticks were published from various parts of world (Li *et al*.2004, Petermann *et al*. 2016, Klafke *et al*. 2017).

Few reports of amitraz resistance are available from India particularly from Punjab, probably because the use of amitraz for tick control started recently to control OP and SP resistant ticks (as stated by farmers). But now upon its indiscriminate and incessant use for past few years the problem of resistance is emerging and would soon be widespread if suitable measure is not taken. Sporadic reports of development of amitraz resistance in *R. microplus* from Gujarat (Singh *et al*. 2015b) and some northern and eastern states of India (Kumar *et al*. 2014, Dutta *et al*. 2017) have been published. After the first report of amitraz resistance in *R. microplus*from SBS Nagar in Punjab (Singh *et al*. 2014b), it was also recorded in multi-host tick, *Hyalomma anatolicum* (Jyoti *et al*. 2019). Recently, molecular genotyping of amitraz resistance in *R. microplus*targeting a partial segment of the octopamine/tyramine (OCT/Tyr) receptor gene conducted in our laboratory revealed 92.8% larval population as heterozygous (SR) with percentage of resistant alleles in the tick populations as 53.6 (range 50.0-57.2). Also, positive correlation between percent homozygous resistant (RR) larval population and its resistance factor value against amitraz estimated by modified larval packet test was recorded (Jyoti *et al*. 2021).

In the present study, a significant adverse effect of amitraz on various reproductive parameters (EMW, RI and hatching percentage) of treated ticks was recorded as reported earlier (Kumar *et al*. 2014, Dutta *et al*. 2017). Further, detection of low-level resistance in our study indicates that amitraz resistance in Punjab state is in initial or emerging phase of development. Therefore, an alert on its judicious use aiming for effective tick control is required to maintain the efficacy of the drug and enhance its commercial life.

ACKNOWLEDGEMENT

The authors are grateful to Department of Science and Technology, New Delhi for funding through Women Scientist Scheme (WOS-A) Project No. SR/WOS-A/LS-1120/2015 to the first author. Sincere thanks are also due to the Head, Department of Veterinary Parasitology and Director of Research, GADVASU for providing facilities.

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Cite this article as: Jyoti, Singh NK, Singh H. Rath SS (2021) Adult bioassay based amitraz resistance status in Rhipicephalus Microplus populations of Punjab, India. Explor Anim Med Res 11(1): 49-54. DOI : 10.52635/EAMR/11.1.49-54