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Effect of synergists on ivermectin resistance in field populations of *Rhipicephalus* (*Boophilus*) *microplus* from Punjab districts, India

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ABSTRACT

The status of ivermectin resistance in *Rhipicephalus* (*Boophilus*) *microplus* collected from various districts of the Punjab state, India was determined using larval immersion test (LIT). Regression graphs of probit mortality of larval ticks of various field isolates were plotted against log values of increasing concentrations of technical grade ivermectin for determination of slopes of mortality, lethal concentrations for 50% (LC_{50}) and resistance factors (RF). Values for the coefficient of determination (R^2) in LIT assay ranged from 0.82 to 0.98 indicating the model to be a good fit. The RF values against ivermectin ranged from 1.65 to 9.07 revealing resistance status in all the field isolates. Pre-exposure to a single pre-determined sub-lethal concentration of ATP-binding cassette transporter inhibitors (cyclosporin-A, MK571) and *p*-glycoprotein inhibitor (verapamil) lead to reduction in LC_{50} values of ivermectin in different field tick isolates. Among the various field isolates, the highest synergistic factor for MK571 and verapamil was recorded in the Moga isolate as 4.97 and 3.21, respectively whereas for cyclosporin-A, the highest value was recorded in the Mansa isolate as 2.81. Among the three synergists used in the current study, MK571 caused the highest increase in toxicity against ivermectin in the field ticks. Therefore, combination products of ivermectin with the above synergists could prolong the useful life of this drug for effective control of ticks.

1. Introduction

The one host cattle tick, *Rhipicephalus* (*Boophilus*) *microplus* commonly known as the “Cattle tick” or the “Tropical cattle tick” is an endemic ectoparasite of cattle particularly in tropical and sub-tropical regions of the world. These ticks are responsible for causing anaemia, loss of milk, meat and leather production and act as vector of the causative agents of bovine babesiosis and anaplasmosis, leading to huge economic losses to the livestock farmers (Jonsson, 2006; Jonsson et al., 2008). The geo-climatic conditions of the Punjab state, India, characterized by high humidity and ambient temperature have been reported to be favourable for development and propagation of the ticks, resulting in heavy tick infestations in the dairy animals (Singh and Rath, 2013).

Chemical acaricides, like organophosphates, synthetic pyrethroids, formamidines and macrocyclic lactones (ML) continue to be the foremost tick control strategy employed by the livestock farmers due to their abundance in the market and ease of use on the infested animals. However, large scale and repeated applications had limited their efficacy in reducing tick infestations and are often accompanied by serious drawbacks, including the development of acaricide resistant ticks, environmental contamination, and even contamination of milk and meat products with insecticide residues (Graf et al., 2004). Presently, ivermectin is the most commonly used acaricide in the dairy sector of Punjab state, India for controlling the tick infestation (Sharma et al., 2012; Singh et al., 2015). However, in recent past, resistance development in *R.* (*B.*) *microplus* against ivermectin had been reported from various regions of India (Singh et al., 2015; Nandi et al., 2018).

Insensitivity of the glutamate gated chloride channel (Glu-Cl) receptor, which prevents the binding of drug to its target site, has been associated with ivermectin resistance development in certain nematodes and arthropods (Dent et al., 2000; McCavera et al., 2009; Kwon et al., 2010). In arthropods, the ML resistance has also been associated with an increase in the oxidative metabolism (Scott, 1989; Argentine et al., 1992) and decrease in the drug penetration (Scott, 1989). Recently, it has been observed that ATP-binding cassette (ABC) transporter proteins are the most important molecules involved in these processes (Lespine et al., 2008; Bourguinat et al., 2011). The ABC transporter proteins have been found to perform very important function in cellular defence as they actively pump a broad range of structurally and chemically different compounds out of the cell against their concentration gradients in an ATP-dependent process, thereby

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mediating multidrug resistance mechanisms (Holland and Blight, 1999; Leslie et al., 2005).

Chemosensitizers or synergists when administered together with a toxicant or drug, have been found to restore the toxicity or efficacy both *in vivo* and *in vitro* (Dermauw and Van Leeuwen, 2014). Cyclosporin A (CsA), an ABC transporter inhibitor at sub-lethal dose has shown to cause an increase in the toxicity against ivermectin in cattle tick, *R.* (*B.*) *microplus* (Pohl et al., 2011, 2012, 2014). Similarly, compound MK571, another ABC-transporter inhibitor at sub-lethal doses lead to significant reduction in the lethal concentration (LC) values of ivermectin against multi-acaricide resistant strain of *R.* (*B.*) *microplus* (Pohl et al., 2012). Verapamil (VR), a *p*-glycoprotein (P-gp) inhibitor, is also known to increase the toxicity level of some insecticides, namely, cypermethrin, endosulfan and ivermectin in mosquitoes of the *Culex pipiens* complex (Buss et al., 2002). Therefore, the present study was undertaken to investigate the ivermectin resistance levels in *R.* (*B.*) *microplus* field isolates from Punjab state, India and study the possible role of synergists like CsA, MK571 and VR to enhance the susceptibility of ivermectin resistant tick populations.

2. Materials and methods

2.1. Tick collection

Fully engorged and dropped female ticks were collected during April, 2016 to March, 2017 from dairy sheds of fourteen districts of Punjab state, India *viz.* Amritsar (31.63°N, 74.87°E) (ASR), Barnala (30.38°N, 75.54°E) (BNN), Fatehgarh Sahib (30.68°N, 76.41°E) (FGSB), Fazilka (30.40°N, 74.02°E) (FKA), Jalandhar (31.32°N, 75.57°E) (JUC), Kapurthala (31.37°N, 75.37°E) (KXH), Ludhiana (30.9°N, 75.85°E) (LDH), Mansa (29.98°N, 75.37°E) (MSZ), Moga (30.81°N, 75.17°E) (MOGA), Patiala (30.34°N, 76.37°E) (PTA), Rupnagar (30.97°N, 76.52°E) (RPAR), Sahibzada Ajit Singh Nagar (31.70°N, 76.71°E) (SASN), Shaheed Bhagat Singh Nagar (31.09°N, 76.03°E) (NSS) and Sri Muktsar Sahib (30.47°N, 74.51°E) (MKS). All included dairy farms used ivermectin for the last five to eight years for tick control. The possible tick hiding places in cattle sheds, like cracks and crevices, loose bricks and other debris on the ground were thoroughly searched as the ticks are usually found hidden in these places.

2.2. Tick preparation

The sampled ticks collected from sheds were kept in separate 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. Adult ticks were identified as *Rhipicephalus* (*Boophilus*) *microplus* under a stereomicroscope, according to general identification keys as per Estrada-Pena et al. (2004). The ticks collected from a particular district were pooled, designated as an isolate, washed thoroughly in water, and kept individually in labeled glass tubes. Each of the sample tubes were covered with muslin cloth, kept in desiccator jars and placed in a refrigerated/humidity cabinet maintained at 28 ± 1 °C and 85 ± 5% relative humidity (RH) for oviposition. The eggs laid were allowed to hatch to larvae under similar temperature and RH conditions of incubation. Around 10–14 days old unfed larvae were utilized for the bioassay for detection of resistance status against ivermectin and to study the effect of synergists with ivermectin in resistant ticks, if any.

2.3. Acaricide preparation

Technical grade ivermectin (primarily ivermectin B1a, Sigma-Aldrich, St. Louis, MO, USA) was used for preparation of stock solution as per Singh et al. (2015). The various working concentrations of ivermectin used in the study were 5.0, 10.0, 20.0, 40.0, 80.0, 160.0 and 320.0 ppm in 1% ethanol-Triton X-100 solution (ethanol with Triton X-100 at 2%, diluted at 1% in distilled water) (1% Eth-TX).

2.4. Larval immersion test (LIT)

The LIT was conducted as described by Singh et al. (2015). Briefly, 0.5 mL volume of each concentration of ivermectin was transferred into a 1.75-mL micro-centrifuge tube. Thereafter, approximately 100 tick larvae were added, the tube was shaken vigorously for larval immersion and kept for 10 min. The tube lids were then opened and larvae were transferred with a paint-brush to a paper filter (Whatman No.1) for drying. Afterwards, dried larvae were transferred to triangular paper filter packets, sealed with bull-dog clips and incubated at 28 ± 1 °C and 85 ± 5% RH for 24 h. All the experiments were repeated in triplicates. The control groups were maintained by immersing the larvae in the diluent alone. After 24 h of drug exposure the larval mortality was calculated and larvae capable of locomotion were considered alive.

2.5. Synergist preparation

All the synergists used in present study *viz.* VR, CsA and MK571 were purchased from Sigma-Aldrich, St. Louis, MO, USA. Working concentrations of 5.0, 10.0, 20.0, 30.0, 40.0, 60.0, 80.0 and 160.0 µM prepared in 1% Eth-TX were used for determination of sub-lethal dose of synergists against 10–14 day old unfed larval ticks by LIT (data not shown). The sub-lethal concentrations of 100 µM, 30 µM and 30 µM of VR, CsA and MK571, respectively were used in the current study.

2.6. Bioassay with synergists

The LIT was performed with 10–14 day old unfed larval ticks pre-exposed to a single pre-determined sub-lethal concentration of respective synergist and control solution (diluent alone) as described above. Each experiment was repeated three times.

2.7. Estimation of resistance status

The dose response data for ivermectin alone as well as with different synergists was analyzed by probit method (Finney, 1962) using GraphPad Prism 4 software (La Jolla, CA, USA). This analysis included probit transformation of percentage mortality and natural logarithm transformation of concentration. The lethal concentrations at 50% (LC_{50}) with 95% confidence limits (CL) values of ivermectin against *R.* (*B.*) *microplus* were estimated. The resistance factors (RF) were worked out by the quotient between LC_{50} of various field tick isolates and LC_{50} of the susceptible isolate. The LC_{50} value of susceptible Kulgam isolate (3.41 ppm) as reported earlier from our laboratory was used in the current study for determination of RF in various field isolates (Singh et al., 2015).

2.8. Estimation of synergism factor (SF)

To estimate any alteration in the toxicity of ivermectin caused by the pre-exposure of various synergists at sub-lethal dose, synergism factors (SFs) were calculated for each synergist based on the ratio of LC_{50} of ivermectin alone to the LC_{50} of ivermectin with the respective synergist for the same isolate as per Pohl et al. (2011).

$$SF_{isolate} = \frac{LC_{50} \text{ of ivermectin alone for particuler isolate}}{LC_{50} \text{ of ivermectin and synergist together of the same isolate}}$$