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Research Article

Evaluation of Chemical acaricides and their Cytotoxic effect on Midgut of *Hyalomma anatolicum* -A Multi Host Tick

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Abstract

Hyalomma anatolicum, the multi host tick is the commonest species genus *Hyalomma* in India and cattle act as the main definitive host. The present study was conducted to assess the efficacy of cypermethrin, deltamethrin and fipronil against the *H. anatolicum* ticks collected from an unorganised cross-breed cattle farm and role of housing of cattle to the tick infestation.

Adult immersion test (AIT) was performed and efficacy of different acaricides were assessed. The results obtained by the AIT showed a low level of resistance (Level-I) to deltamethrin, while in case of cypermethrin and fipronil, ticks were found susceptible. The RR50 values were obtained as 0.32, 1.67 and 1.09 for cypermethrin, deltamethrin and fipronil. On cytological examination, small to large vacuoles were observed in digestive cells. Degeneration in digestive cells were also observed. On observation of cattle barn it was seen that the owner not maintained the farm in a organised manner and animals were kept with feeding and bedding materials which was form suitable conditions for tick propagation in barn. The *H. anatolicumis* a multi-host tick, So chances of exposed to repeated application of acaricides are very less, this may be the reason behind the susceptibility of ticks against these most commonly used chemicals to control the tick infestation.

Keywords: *Hyalomma anatolicum*; Cypermethrin; Deltamethrin; Fipronil; Resistance

Introduction

Globally, ticks and tick-borne diseases (TTBDs) are considered as the major hurdles to enhance livestock productivity with the threshold being much higher in developing countries. The cumulative global losses incurred for the management of TTBDs are estimated at the tune of US $ 22–30 billion per annum [1,2]. The direct effect of tick infestations on hosts includes pyemia, toxicosis and paralysis which cause a cumulative projected loss of about US $ 500 million annually [1,3]. They cause significant loss to the farming community by causing direct loss in terms of anaemia and indirectly by serving as vector for several viral (CCHF), rickettsial and protozoan diseases. Tick genera *Rhipicephalus* and *Hyalomma* are most widely distributed in India. *Hyalomma anatolicum* species are reported to be dispersed in 20 states of India, respectively [4]. *H. anatolicum* are predominantly found cattle tick in aridand semiarid regions of India [5]. Being a multi host tick species, *H. anatolicum* principally transmits bovine tropical theileriosis and equine piroplasmosis in India.

The natural distribution of *Hyalomma* species is limited to Asian, African and European countries [6]. Among the 27 species of *Hyalomma* [7], 05 species are commonly distributed and recorded in all the three continents, 07 species are restricted in Asia, 05 in Africa, 09 in Asia-Africa and 01 in Africa-Europe. About 50% of the species can infest and transmit pathogens, most significantly, the Crimean Congo Haemorrhagic Fever (CCHF) virus in humans and *Theileriaannulata* in cattle [8]. Apart from that, *Hyalomma* species are also known for the maintenance and transmission of many other viruses, bacteria and protozoan pathogens to animals and humans being also. Recently, due to climate change and other risk factors, TTBDs have risen insidiously, triggering heightened attention about their impact on human health. In concurrence to this, the Centre for Disease Control and Prevention (CDC) reported nearly doubling of TTBDs cases over 13 years [9].

For tick control, integrated tick management (ITM) manly focussed on use of chemical acaricides. Repeated applications of chemical acaricides are the backbone of tick management having limited efficacy [10]. The approach based on acaricide application suffers from many drawbacks such as repetition of applications, environmental pollution, acaricide residues in livestock products, selection of resistant ticks, non-availability of new generation acaricides in near future and high cost of development of new generation acaricides [11]. It has been estimated that the cost of discovering and developing a novel product is around the US $ 100 million, with an average time requirement is 10 years [12]. These issues enforced the scientific community to develop and introduce alternatives to acaricides that are consistent with the principles of sustainable TTBDs management [13]. The alternative approaches are strategic use of effective acaricides; adoption of the resistance monitoring system for identification of zone-specific effective acaricides; biological control; use of Phyto formulation and vaccines.

Initially, arsenic compounds were used for the management of cattle ticks. Later, after the development of resistance, the organophosphates replaced the use of organochlorines. Newer compounds having different modes of action were introduced over the years. However, due to misuse of the introduced compounds targeted populations have become resistant to the compounds. Reports of resistance to organophosphates and synthetic pyrethroids have been published over the years [14], whereas sporadic reports of resistance to amitraz, ivermectin and fipronil compounds were also reported [15]. It was determined that the effective mean life of most of the introduced acaricides is between 10 and 15 years and resistance has been reported within that period [16-18].

Resistance monitoring in tick populations is mainly focussed on bioassays, such as adult immersion test (AIT), larval immersion test (LIT) and larval packet test (LPT), using susceptible reference samples [19]. In India, altogether more than 28 reports of resistance against various compounds have been published against *R. microplus*. Resistance were reported against DZN, DLM, CYP, fenvalerate, IVM and AMZ from Uttar Pradesh, West Bengal, Bihar, Gujrat, Rajasthan, Haryana, Madhya Pradesh, Punjab, Assam and low level of resistance from Kerala states [20]. Alongwith the above mentioned compounds, resistance to ivermectin [21] has also been reported from Punjab, Uttar Pradesh and Madhya Pradesh. It was observed that *R. microplus* has developed multi-acaricides resistance in comparison to *H. anatolicum* and management of the population is the major challenge for productive maintenance of animal herds. In the present study, detection of efficacy of cypermethrin, deltamethrin and fipronil in *H. anatolicum* from a unorganised cross-breed cattle farm was attempted using FAO recommended adult immersion test (AIT) with effect of farm management on tick infestation.

Materials and Methods

Study area

The adult dropped female *Hyalomma anatolicum* ticks were collected from a cross-bred cattle farm situated in santer village of Mhow block, where intensive agricultural and animal husbandry activities were practiced. People were used to rear milking animals in their small houses. The geographic area has a pleasant climate, however the peak summer and winter periods may get extremely hot and cold, respectively. Temperature may go as high as 43 °C during summers and as low as 4 °C during winter.

Sampling and collection of data on acaricide use in studied area.

The crossbreed cattle in the study areas was predominantly (90%) dedicated to milk production. Samples were collected from unorganized farm and surveyed. In that farm, animal owner has reported tick infestations as a major problem and SP, organophosphates (OP), macrocyclic lactones (ML) and amitraz were commonly used for the tick control. The availability of green fodder was high in this studied area and farmers used to graze animals in agricultural fields. The animals were kept in semi concrete floor, where they were also kept fodder for animals. The frequency of application of SP and ML has reached at very high level at that farm. The animals were infested with both *H. anatolicum* and *R. microplus*. The tick infestations in animals were reported almost throughout all seasons of the year and SP and ML were the most commonly used chemicals for tick control.

Animal barn

Animal rearing site and farm management were also studied in this research to investigate the role of housing on population of ticks and the occurrences of haemoprozoan disease of cattle.

Acaricides

In this study, commercially available product of cypermethrin, deltamethrin and fipronil were used. For the experimental bioassay, different concentrations of the acaricides were prepared in distilled water from the stock solutions (5000ppm each) and tested against tick collected from the farm.

Bioassay

Adult immersion test (AIT)

The assay was conducted according to the methods of Sharma., *et al.* [22] with minor modifcations. A series of concentrations in ppm (100, 200, 300, 400 for cypermethrin; 15, 30, 45, 60 for deltamethrin; 1.25, 2.5, 5, 7.5 for fipronil) were prepared and each concentration was replicated 6 × with five adults per replication (n = 6 × 5). Preweighed engorged *Hyalomma anatolicum* were immersed for 2 min in each concentration and soaked in tissue paper before transferring into the Petri dishes. The control group of ticks were treated with solvent. After 24h, treated ticks were transferred to tick rearing tubes and were kept in desiccators placed in BOD incubator maintained at 28 °C and 85 ± 5% RH. Ticks that did not oviposit up to 14 days after treatment or oviposited non-viable eggs were considered as dead.

Collection and processing of midgut

Toxicity of deltamethrin and fipronil on survival of *H. anatolicum* was determined using LC95 concentrations by adult immersion test (AIT). After 24h of treatment, about 10 ticks from each group (deltamethrin and fipronil) were dissected in 0.1 mol/L PBS (phosphate buffered saline) at pH 7.2 and the midguts were collected. The samples were fixed in 10% formaldehyde for 24 h, rinsed with 0.1M phosphate buffer for 15 min (3 times). The samples were passed twice each through series of 30%, 50%, 70%, 80%, 90%, and 100% ethanol for 15 min. After they have dried, the materials were embedded in pure paraffin wax and sections (4-6 µ) were cut with the help of microtome and stained with routine H and E stain and slides were examined under light microscope.

Statistical analysis

The dose-response data were subjected to probit analysis using GraphPad Prism v.5.00 for Windows (GraphPad Software, San Diego, CA, USA) [23]. The resistance factor off ipronil was estimated by dividing the LC50 for field isolate by LC50 for the susceptible reference strain (IVRI-I) [24]. For calculation of Resistance ratio, data on reference susceptible tick line IVRI-II was used [25] for cypermethrin and deltamethrin and for fipronil data available on *R. microplus* reference susceptible IVRI-I strain was used as reference value pertaining to *H. anatolicum* is not yet available for this compound. On the basis of RF, the resistance status of field population of ticks was classified as susceptible (RF < 1.4), level I resistance (1.5 < RF < 10.0), level II resistance (10.1 < RF < 25.0), level resistance (26 < RF < 40), and level IV resistance (RF > 41).

Results

The data collected from cross-breed cattle farm owner indicated that cypermethrin, deltamethrin and amitraz were commonly used for regular basis for management of ticks. Sometimes the farm owner also treated animal without consulting the veterinarian with under or overdose of acaricide. This kind of practices facilitates the development of resistance and made difficult to manage off host tick population.

Susceptibility of ticks to acaricides

All collected *Hyalomma anatolicum* females from crossbreed cattle farm were tested in AIT format. In this test against cypermethrin, the slope value was 3.115 ± 1.00 which is comparatively lower than the slope value of 4.08 ± 1.14 in IVRI-I, the susceptible reference strain, against cypermethrin, indicating the heterogeneity in the tested *Hyalomma anatolicum* females (Table 1). The resistance ratio (RR) was observed as 0.32. The RR value was showed susceptibility in adult females. The LC50 value was also observed much lower (78.87ppm) than the reference susceptible IVRI-II strain (245.91). The all ticks were died in maximum concentration of 400ppm used in the experiment. The co-efficient of determination (R2) value was calculated as 0.83 indicating a good fit of data in the statistical model and 83% correlated response with log doses of cypermethrin (Figure 1). Because the tick is a three-host tick, the chemical compound was proven to be effective against adult female *Hyalomma anatolicum*. There is a potential of escaping the acaricide treatment if all life stages of the female are not present over the animal's body. They weren't exposed to chemicals on a regular basis in that state; therefore they didn't build resistance against acaricides easily.

Concentration in ppm	Number of tick used	Number of tick live	Number of tick died	Mortality % (Mean ± SE)	Slope ± SE	R^2	LC_{50}	95%CI	RR_{50}	RL
100	30	20	10	33.3 ± 1.57	3.115 ± 1.00	0.83	78.87	73.06-85.13	0.32	S
200	30	12	18	60.0 ± 5.77						
300	30	04	26	73.3 ± 2.57						
400	30	00	30	100.0 ± 0.00						
control	30	30	00	0.00 ± 0.00						

Table 1: Effect of different concentrations of cypermethrin on adult female of *Hyalomma anatolicum.*

RR_{50} Resistance raito; RL Resistance level; S susceptible;

Susceptible = RF < 1.4; Level I = 1.5 ≤ RF ≤ 5; level II = 5.1 ≤ RF ≤ 25; level III = 25.1 ≤ RF ≤ 40; level IV = RF > 40.

The result of experiment with deltamethrin in AIT format showed a slope value of 1.583 ± 0.38 in ticks which is comparatively much lower than the slope value of 4.51 ± 0.28in, the susceptible reference strain IVRI-II against this compound, indicating the heterogeneity in the tested ticks (Table 2). The LC50 value was observed slight higher (19.52ppm) than the reference susceptible IVRI-I strain (11.7). The resistance ratio (RR) was calculated as 1.67 which is an indicative of development of low level of against this compound in tested *Hyalomma anatolicum* female ticks. The all tested ticks were died at a maximum concentration of 60 ppm. The co-efficient of determination (R2) value was observed as 0.89, indicating a good fit of data in the statistical model and 89 % correlated response with log doses of deltamethrin (Figure 1). Deltamethrin has been used extensively for tick control in this farm for many years, consequently, ticks acquired resistance over time.

Concentration in ppm	Number of tick used	Number of tick live	Number of tick died	Mortality % (Mean ± SE)	Slope ± SE	R^2	LC_{50}	95%CI	RR_{50}	RL
15	30	16	14	46.6 ± 2.40	1.583 ± 0.38	0.89	19.52	16.75-22.73	1.67	I
30	30	14	16	53.3 ± 3.57						
45	30	08	22	73.3 ± 2.58						
60	30	00	30	100.0 ± 0.00						
control	30	30	00	0.00 ± 0.00						

Table 2: Effect of different concentrations of deltamethrin on adult female of *Hyalomma anatolicum.*

RR_{50} Resistance raito; RL Resistance level; S susceptible;

Susceptible = RF < 1.4; Level I = 1.5 ≤ RF ≤ 5; level II = 5.1 ≤ RF ≤ 25; level III = 25.1 ≤ RF ≤ 40; level IV = RF > 40.

In case of fipronil, the slope value was recorded slight lower (3.003 ± 0.63) than the susceptible reference strain (3.49 ± 0.46) (Table 2), indicating some extent of heterogeneity in the tested tick population (Table 3). The LC50 value was observed as 1.82ppm, which was slight, higher than reference susceptible IVRI-I strain (1.62ppm). All the tested ticks died at a maximum concentration of 7.5ppm used in this experiment, hence the resistance ratio (RR) was obtained as 1.09. On the basis of RR the tested tick showed susceptibility in adult stage. The co-efficient of determination (R2) value was calculated as 0.92, indicating a good fit of observed data in the statistical model and 92 % correlated response with log doses of fipronil (Figure 1).

Concentration in ppm	Number of tick used	Number of tick live	Number of tick died	Mortality % (Mean ± SE)	Slope ± SE	R^2	LC_{50}	95%CI	RR_{50}	RL
1.25	30	18	12	40.0 ± 2.70	3.003 ± 0.63	0.92	1.82	1.68-1.96	1.09	S
2.50	30	10	20	53.3 ± 3.57						
5.00	30	04	26	86.6 ± 3.54						
7.50	30	00	30	100.0 ± 0.00						
Control	30	30	00	00.0 ± 0.00						

Table 3: Effect of different concentrations of fipronil on adult female of *Hyalomma anatolicum.*

RR_{50} Resistance raito; RL Resistance level; S susceptible;

Susceptible = RF < 1.4; Level I = 1.5 ≤ RF ≤ 5; level II = 5.1 ≤ RF ≤ 25; level III = 25.1≤RF≤40; level IV = RF > 40.

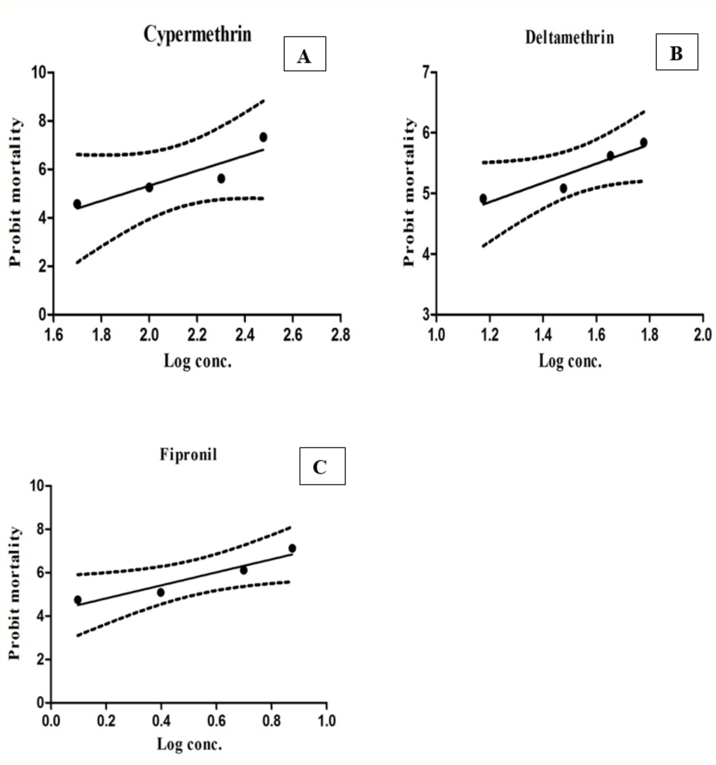

Figure 1: Individual regression curve showing probit mortality in AIT against log concentration of cypermethrin (A), deltamethrin (B) and fipronil (C) in Hyalomma anatolicum ticks collected from a Unorganised farm of Kishangang, Mhow.

Cytotoxicity

After 24 h of exposure to deltamethrin and fipronil, normal color of the cuticle was changed to bluish or blackish with shrunken cuticle n case of deltamethrin, where as in case of fipronil, normal color of the cuticle was changed to yellowish with shrunken cuticle. Exposure of deltamethrin and fipronil resulted in the damage of tick mid gut at many places with hemorrhages at several places. Ultimately it resulted in leakage of intestinal contents. Small to large vacuoles were observed in digestive cells. Degeneration in digestive cells were also observed (Figure 4).

Observations on cattle barn

During the sample collections from the cross-breed cattle farm the authors observed that all animals were kept on semi-concrete floor and roof of the cattle barn was made up of tin sheets (Figure 2). The floor of the barn was made up of concrete and mud and walls were also having many cracks and crevices. There was no cement plaster on wall and they were in very bad condition. The owner was also applied cow dung on walls, so ticks were easily hide behind the dung. The animals were kept close to each other and not maintained proper space for free movement. All animals were heavily infested with the different stages of *Hyalomma anatolicum* (Figure 2) and adults females were hiding in cracks and crevices. During the tick collection authors were also observed that adult's females were present in/on materials of cattle barn. The ticks were also present below the cow dung, on bedding and wheat straw placed in cattle barn (Figure 3). The authors also observed that, the condition of barn was highly favourable for ticks especially for Hyalomma which has eggs, larvae, nymph and unfed adult stages found off the host to establish reinfestation on animals. This kind of practice was also responsible for the tick and tick-borne diseases (TTBDs) in farm animals.

Figure 2: Rearing of animals on semi-concrete floor (A and B); Hyalomma anatolicum infested animals (C and D).

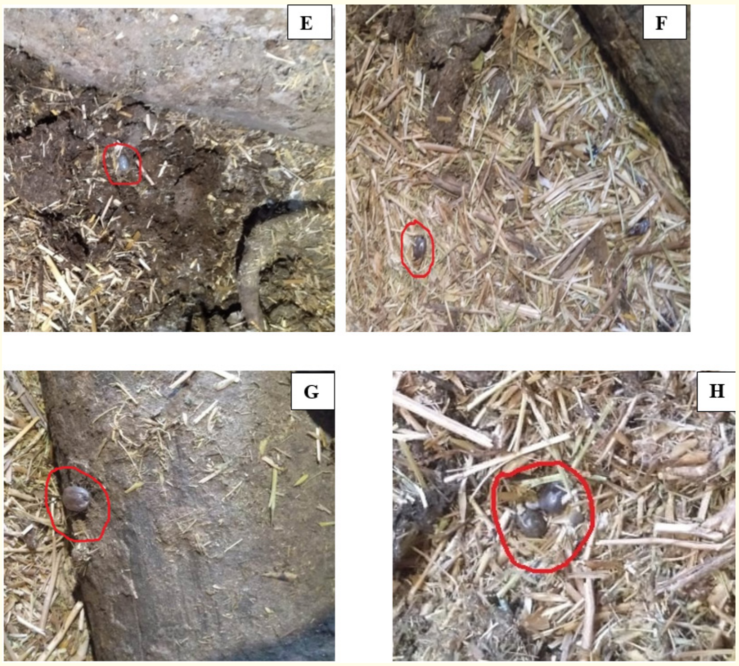

Figure 3: Collection of dropped females of Hyalomma anatolicum from different sites of farm (A-below the dung, B to D-from bedding and wheat straw present in same place where animals were kept.

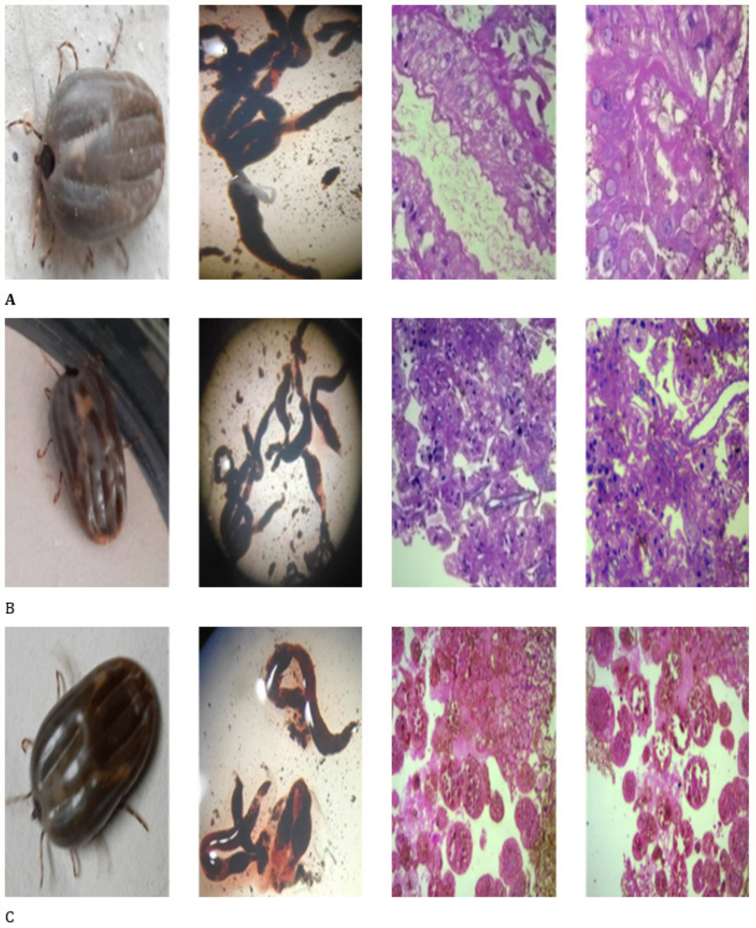

Figure 4: Photograph showing female ticks; and photomicrograph showing midgut of untreated ticks (A), deltamethrin treated ticks (B) and fipronil trated ticks (C)

Discussion

The problem of ticks and tick borne diseases (TTBDs) is particularly relevant in India because of the tropical and subtropical conditions present here which favours for tick survival throughout the year in most parts of the country and maintenance of susceptible cross bred animals to improve the production of milk and milk product. In India, the most widely accepted method as integrated tick management system for the control of ticks is the direct application of acaricides to host animals and thus the consumption of acaricides has been increased manifold during last decades. The use of acaricides as the principal means for tick control resulted in the selection of multiacaricide resistant tick populations along with contamination of the environment and animal products.

In global basis, the incidence of acaricide resistance is the highest in one host ticks of the genus *Boophilus*. This may be due to a much larger fraction of the total population of this species remains under chemical exposure more than multi host ticks. In addition to it, a single generation of multi-host tick may extend over up to 3 years compared to 2–3 months in Boophilus. The immature stages of the multi host ticks often feed on small wild animals, even if the adults tend to prefer large domestic animals. These could also be contributory factors which protect these ticks from exposure to chemical acaricides. Early reports showed resistance to organochlorine acaricides like dieldrin in some isolates of *Rhipicephalusappendiculatus* in Zambia [26]. Similarly, isolates of *Amblyommalepidum* and *Hyalomma impeltatum* from Sudan showed resistance to lindane [27]. There are only few records of resistance in multi host ticks to OP compounds or synthetic pyrethroids which could be due to later introduction of these acaricides in field and slower development of acaricide resistance in multi host ticks. However, in view of the capacity of multi host ticksto develop resistance to the acaricides introduced earlier, the chances of resistance development to the newer acaricides must eventually be expected.

In present study, the collected *H. anatolicum* ticks were found susceptible to cypermethrin and fipronil (RR > 1.4) and a very low level of resistance was observed against deltamethrin (RF = 1.67) in spite of indiscriminate use for tick management in the field. In India, very few resistance reports on *H. anatolicum* tick being prevalent in more than 20 states were published from two three states in comparison to *R. microplus*. Many authors reported deltamethrin (SP) resistance in *R. microplus* ticks from different states of India such as; Gujarat [28], Bihar [29], Haryana and Rajasthan [30] and determined RR in varying range from 0.02 to 34.9. Similarly, resistance against deltamethrin has also been reported from other countries such as; Benin, West Africa [31], Mexico [32], Australia [33].

Sharma., *et al*. [34] reported resistance against cypermethrin in *R. microplus* ticks collected from Uttar Pradesh, Bihar, West Bengal, Punjab, Rajasthan and Tripura states of India and RR values from 0.02 to 9.88. Similarly, many authors characterized *R. microplus* populations collected from Punjab, Gujarat, Bihar, Rajasthan [29,35,36] and reported cypermethrin resistance pattern in adults and larvae and the level of resistance was comparatively lower than deltamethrin. The variation in the level of resistance to cypermethrin was also reported from Brazil, where RF was found in the range of 0.95 to 95.44 in 23 populations [37] and the RF was definitely higher than that observed in the present study and the earlier reports using isolates collected from other states of India.

In case of *H. anatolicum*, a low grade resistance (level-I, RF-5) reported to both deltamethrin and cypermethrin in 10 areas and higher grade resistance (level-II, RF-25) to deltamethrin in one area [38]. In another study, Shyma., *et al*. [39] reported development of resistance in *H. anatolicum* collected from Haryana against cypermethrin, deltamethrin and diazinon. The published data by Surbhi., *et al*. [40] revealed resistance development against deltamethrin and coumaphosin *H. anatolicum* collected from different locations of Haryana.

On the other hand, only few literatures is available on fipronil resistance in *R. microplus* ticks. Shakya., *et al*. [41] characterized twenty-five isolates of *R. microplus* collected from six states (Madhya Pradesh, Uttarakhand, Meghalaya, Assam, Gujarat and Haryana) of India and reported RF in larvae with the range of 0.39 to 10.9 while RF in adults with the range of 0.08 to 2.55. Recently, Gupta., *et al*. [42] reported Emergence of fipronil resistance in cattle ticks *R. microplus* and *H. anatolicum* collected from Haryana, India. Many workers from other countries reported resistance to fipronil in *R. microplus* tick [43]. However, in the present study, the *H. anatolicum* isolate was found susceptible to fipronil with RF 1.09.

On examination of sections of midgut, breakage and detachment of Generative cells from basal membrane with low to high severity, and some were with small vacuolation. Similar findings were reported by Chigure., *et al*. [44] after exposure of lead 3 natural compound on IVRI-I and resistance reference IVRI-V strains. Recently, Fular., *et al*. [45] reported similar changes after exposure of Phyto formulation and FIP to IVRI-I and IVRI-V strains. Similar results were also reported by many researchers after treatment of ticks with different herbal and synthetic acaricides compound [46-48].

In the present study, it was observed that the condition of animal farm was not adequate for management of tick infestation in animals. Estrada-Peña., *et al*. reported numbers of factor affecting tick infestation in farm animals such as poor management of farms, uncontrolled movements of domestic animals, abundance of wild animals, and absence of an adequate framework to capture the ecological plasticity of certain ticks may explain the complexity of the control measures. Soberanes-Céspedes., *et al*. [49] observed that feedlots, particularly in tropical and semitropical countries have more possibilities to increase tick infestation rates by maximizing host finding ability of the larvae but the risk of tick infestations in feedlot cattle might be reduced by making the environment unsuitable for the free-living stages of the tick. To the extent possible, cattle and buffalo sheds should be tick proof especially for the housing of purebred exotic and crossbred cattle, as they are more susceptible to the tick infestation than native cattle and buffaloes. There should be no cracks and crevices in the buildings (as the ticks hide and breed there). An acaricide channel should encircle the entire building. According to Muhammad., *et al*. [50], heaps of dung cakes and stacks of bricks may also provide breeding places to the ticks in animal sheds and should therefore be removed regularly. There should be proper of exposure sunlight in the shed and appropriate ventilation reducing the relative humidity which is one of the key factors for the hatching of tick's eggs. These all managemental malpractices make animal to more prone to tick infestation and TTBDs. By managing the cattle shed we can manage ticks infestation to farms upto some extent and reduced the occurrence of Haemoprotozoan diseases.

Conclusion

Based on the data obtained in this study, *H. anatolicum* ticks showed susceptibility to cypermethrin and fipronil. But indiscriminate use of chemical acaricides may raise the problem of resistance in *H. anatolicum.* The results signify that there is a need for continuous monitoring of acaricide resistance in field situation for strategic application of available acaricides and for maintaining the life span of the product.

Declarations

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- **Conflict of interest:** The authors declare that they have no conflict of interest.
- **Ethical Statement:** It is certified that the research manuscript entitled "Evaluation of commercial acaricides against *Hyalommaanatolicum* a multi host tick and role of animal house in tick infestation". The research work was not funded by any department. It was conducted by using facility in Department of Parasitology, College of Veterinary Science & Animal Husbandry, Mhow (NDVSU), Indore, Madhya Pradesh and the certify by animal ethical clearance committee of the University.
- **Consent to participate:** The authors consent is included.
- **Consent for publication:** All the given information is true and not submitted anywhere for publication. Authors are responsible for correctness of the statements provided in the manuscript
- **Data Availability:** The raw data were generated at Department of Parasitology, College of Veterinary Science and Animal Husbandry, Mhow (NDVSU), Indore, Madhya Pradesh. Derived data supporting the findings of this study are available from the corresponding authors on request.
- **Code availability:** Not Applicable
- **Author's contributions:** MKS and KG conceived and designed research. SJ, MS and VM conducted experiments. MKS contributed new reagents or analytical tools. VA, GPJ, AKS and SK analyzed data. MKS and SK wrote the manuscript. All authors read and approved the manuscript. This is approved by all authors of the manuscript.

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