Explor Anim Med Res,
Vol. 8, Issue - 1, 2018, p. 33-39

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

Research Article

AN ECO-FRIENDLY APPROACH TO CONTROL *RHIPICEPHALUS SANGUINEUS* BY USING SUSTAINED RELEASE POROUS CALCIUM ALGINATE BEADS CONTAINING ASSEMBLY PHEROMONE

Dhivya Bhoopathy[1*], Bhaskaran Ravi Latha[2]

Received 16 January 2018, revised 26 February 2018

ABSTRACT: A novel attempt was made to encapsulate assembly pheromone of ticks in porous calcium alginate beads. The study was carried out using test beads containing assembly pheromone and acaricides and three control beads- plain alginate beads, pheromone control and acaricide control respectively. *In-vitro* trials were carried out by petridish bioassay and the unfed larvae, unfed nymph and unfed adult of *Rhipicephalus sanguineus* were utilized for the assay. Cent percent mortality was recorded with test beads amongst all the three unfed stages in *in-vitro* bioassay. Field trials were carried out in kennels with the test and control beads suspended in specially designed lures to assess their efficacy in attracting and killing pre-parasitic environmental stages of *R. sanguineus*.

Key words: Pheromone-assisted tick control, Assembly pheromone, Sustained-release, Porous calcium alginate beads.

INTRODUCTION

The eight-legged hematophagous ticks gain medical and veterinary significance due to their vector potentiality in transmitting diseases of varied aetiology. Next to mosquitoes, ticks are thus ranked as important vectors of human diseases (Sonenshine 2004).The control of these blood-sucking parasites thus become necessary. Since the early 1840's when the first sheep dip was introduced, it is the acaricides which have been used for effective tick control (Wall 2007). With the reports of development of resistance among ticks (Miller *et al.* 2001) and residues of acaricides in the environment and animal products, the continued application of acaricides became questionable thus pressing upon the need for alternate control strategies. Pheromone assisted tick control measure is one such alternate measure. Assembly pheromone is present in the excreta of ticks and its chemical composition has been elucidated to contain adenine guanine, xanthine and hematin in the ratio 1:25:1:1 (Sonenshine 2004). Allan *et al.* (2002) carried out trials using a patented device termed Last Call™ (IPM technology, Portland, USA) for the control of *Ixodes scapularis* by utilizing this assembly pheromone in the form of oily droplets.

Pheromone assisted pest control is unpopular because of the attributes namely, volatility and lack of stability of these biologically active compounds in field conditions. Sustained/controlled release formulations will thus be a key-stone to prevent quick environmental biodegradation and also to prolong the activity of volatile pheromones.

Rhipicephalus sanguineus is the common tick known to infest dogs in Tamilnadu (Koshy *et al.* 1983). As it is a three-host tick, to achieve an effective control, the environmental stages should also be targeted simultaneously while treating an infested animal as pointed out by Lord (2011). As an eco-friendly approach, the current study attempted in designing sustained release of assembly pheromone device for the control of pre-parasitic environmental stages of *R. sanguineus*. The ultimate goal of this research was to successfully encapsulate assembly pheromone of ticks using economically feasible polymer to prevent rapid environmental degradation.

MATERIALS AND METHODS

i) Ticks for *in-vitro* assays

Rhipicephalus sanguineus was collected from the dogs presented at the small animal clinics of Madras Veterinary

[1]*Assistant Professor, Department of Veterinary Parasitology, Veterinary College and Research Institute, Orathanadu-614 625, Thanjavur, India.*
[2]*Professor and Head, Department of Veterinary Parasitology, Madras Veterinary College, Chennai-600 007, India.*
**Corresponding author. e-mail: dhivvet@gmail.com*

College Teaching Hospital and those housed in Blue Cross of India, Velachery. Engorged female ticks of *R. sanguineus* were collected from infested dogs. These ticks were maintained in the laboratory for oviposition by placing at a relative humidity (RH) of ≥ 80 per cent. They were left undisturbed until they oviposited and the larvae hatched out. Engorged larvae and engorged nymph were also maintained in similar conditions until they moulted to unfed nymph and unfed adults.

ii) Assembly pheromone

The synthetic analogues of assembly pheromone (AP) *viz*., adenine, guanine, xanthine and hematin were procured from Sigma-Aldrich, USA. Pure synthetic compounds were utilized in this study as they were tested earlier and found to be effective by Ranju *et al.* (2012). The ratio utilized was according to that indicated by Sonenshine (2004).

Guanine	-	95 mg
Xanthine	-	3.8 mg
Adenine	-	3.8 mg
Hematin	-	3.8 mg
Total		106.4 mg

iii) Acaricide

A commercially available preparation of the synthetic pyrethroid (Butox®, Intas-Pvt. Ltd - Deltamethrin 1.25 per cent containing 12.5 mg/ml), was used as acaricide in the current study. The optimum lethal dose of 250 µl was utilized based on trials conducted earlier (Ranju *et al*. 2012) was used. Deltamethrin was directly incorporated into micro particles along with assembly pheromone.

iv) Preparation of porous calcium alginate beads

Porous calcium alginate beads were prepared by the reaction between calcium carbonate added to the alginate matrix and acetic acid in calcium chloride which produce carbon dioxide gas. The protocol utilized was as followed by Krishnan *et al.* (2010).

Reagents used and procedure

10 percent acetic acid (Rankem Chemicals, India) was prepared in distilled water. 1.5 g of calcium chloride was dissolved in 100 ml of this solution and the resulting solution was utilized as cross-linking agent. 1:4 ratio of calcium carbonate and sodium alginate was dissolved in distilled water and the assembly pheromone and/or acaricides will be dissolved in this solution.

Polymer control beads were prepared by cross-linking alginate solution with calcium chloride in a beaker by extrusion method. Pheromone control beads containing assembly pheromone in the standard ratio was dispersed directly in 2 per cent sodium alginate by continuous stirring for another 30 minutes. Assembly pheromone and 3.125 mg deltamethrin was also dispersed in the alginate matrix and cross-linked in the same way to prepare test

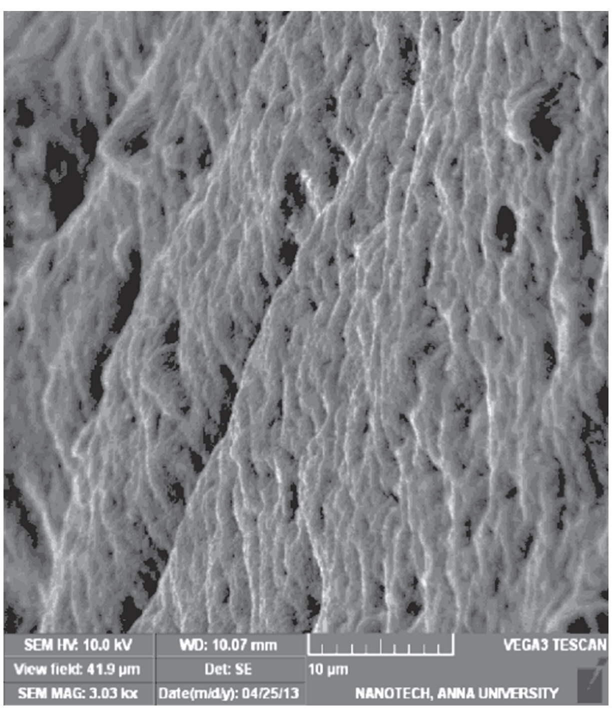

Fig. 1. SEM analysis revealing pores on the surface of beads.

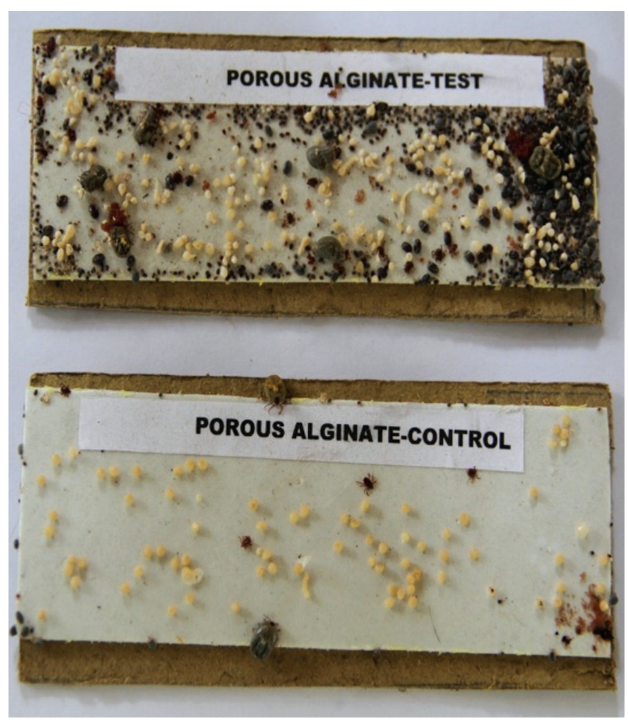

Fig. 2. Results of field trials - lure revealing the presence of attracted and dead ticks.

Table 1A. Response of unfed larvae to porous calcium alginate beads in *in-vitro* bioassay (10 – 30 min).

Combinations	No. of larvae that reacted in 10 minutes				No. of larvae that reacted in 30 minutes			
	Active	Akinetic	Sluggish	Dead	Active	Akinetic	Sluggish	Dead
Polymer Control	500	-	-	-	500	-	-	-
Pheromone control	458 (91.6)	42 (8.4)	-	-	399 (79.8)	101 (20.2)	-	-
Test beads	254 (50.8)	-	151 (30.2)	95 (19)	201 (40.2)	-	164 (32.8)	135 (27)
Acaricide control	356 (71.2)	-	58 (11.6)	86 (17.2)	324 (64.8)	-	82 (16.4)	94 (18.8)
Chi-square value	-	43.84**	58.35**	6.66**	-	75.35**	54.12**	27.57**

AP-Assembly pheromone; Pheromone control = Alginate + AP; Test beads = Alginate +AP + Deltamethrin; Acaricide control = Alginate + Deltamethrin.
* Significant (p< 0.05), ** Highly significant (p<0.01), Figure in the parenthesis indicates percentage.

Table 1B. Response of unfed larvae to porous calcium alginate beads in *in-vitro* bioassay (2 – 24 hrs).

Combinations	No. of larvae that reacted in 2 hours				Status of larvae 24 hours post exposure			
	Active	Akinetic	Sluggish	Dead	Active	Akinetic	Sluggish	Dead
Polymer Control	500	-	-	-	500	-	-	-
Pheromone control	361 (72.2)	139 (27.8)	-	-	340 (68)	160 (32)	-	-
Test beads	86 (17.2)	-	86 (17.2)	328 (65.6)	-	-	-	500 (100)
Acaricide control	289 (57.8)	-	97 (19.4)	114 (22.8)	195 (39)	-	97 (19.4)	208 (41.6)
Chi-square value	-	161.44**	33.39**	213.39**	-	190.48**	-	308.57**

AP-Assembly pheromone; Pheromone control = Alginate + AP;Test beads = Alginate +AP + Deltamethrin; Acaricide control = Alginate + Deltamethrin.
* Significant (p< 0.05), ** Highly significant (p<0.01), Figure in the parenthesis indicates percentage.

beads. Acaricide control beads containing only 3.125mg of deltamethrin in alginate matrix was also prepared in a similar manner. The cross-linking time was 20 minutes. Beads were washed twice with distilled water and then strained. The beads were then air dried for 24 hours at room temperature.

***v) In-vitro* bioassay and Statistical analysis**

A modified method of Yoder and Stevens (2000) as adopted by Ranju *et al.* (2012) was employed in the current study. The assembly pheromone encapsulated micro particles were placed in one quadrant of the petridish. Unfed larvae, nymph and adult stages were placed in the opposite quadrant. The petridish was covered with another petridish and sealed with laboratory grade parafilm in order to prevent escape of ticks. All tests were conducted at room temperature and results read after 10 minutes, 30 minutes, 2 hours and 24 hours. The mortality of the ticks was confirmed by checking the pedal reflex. Freshly prepared micro particles were used for each trial. Fresh, unexposed ticks were used for each trial.

Table 2A. Response of unfed nymphs to porous calcium alginate beads in *in-vitro* bioassay (10 – 30 min).

Combinations	No. of nymphs that reacted in 10 minutes				No. of nymphs that reacted in 30 minutes			
	Active	Akinetic	Sluggish	Dead	Active	Akinetic	Sluggish	Dead
Polymer Control	50	-	-	-	50	-	-	-
Pheromone control	47 (94)	3 (6)	-	-	35 (70)	15 (30)	-	-
Test beads	38 (76)	-	12 (24)	-	20 (40)	-	25 (50)	5 (10)
Acaricide control	42 (84)	-	5 (10)	3 (6)	30 (60)	-	8 (16)	12 (24)
Chi-square value	-	3.09^{NS}	2.99^{NS}	2.63^{NS}	-	17.65**	10.24**	0.61^{NS}

AP = Assembly pheromone; Pheromone control = Alginate + AP;Test beads = Alginate +AP + Deltamethrin; Acaricide control = Alginate + Deltamethrin.
^{NS} Non-significant, * Significant (p< 0.05), ** Highly significant (p<0.01), Figure in the parenthesis indicates percentage.

Table 2B. Response of unfed nymphs to porous calcium alginate beads in *in-vitro* bioassay (2 – 24 hrs).

Combinations	No. of nymphs that reacted in 2 hours				Status of nymphs 24 hours post exposure			
	Active	Akinetic	Sluggish	Dead	Active	Akinetic	Sluggish	Dead
Polymer Control	50	-	-	-	50	-	-	-
Pheromone control	27 (54)	23 (46)	-	-	20 (40)	30 (60)	-	-
Test beads	4 (8)	-	4 (8)	42 (84)	-	-	2 (4)	48 (96)
Acaricide control	25 (50)	-	5 (10)	20 (40)	13 (26)	-	10 (20)	27 (54)
Chi-square value	-	29.87**	3.88*	23.01**	-	42.86**	2.36^{NS}	18.30**

AP = Assembly pheromone; Pheromone control = Alginate + AP;Test beads = Alginate +AP + Deltamethrin; Acaricide control = Alginate + Deltamethrin.
^{NS} Non-significant, * Significant (p< 0.05), ** Highly significant (p<0.01), Figure in the parenthesis indicates percentage.

Tests were replicated with N=500 for larvae, N=50 for nymph and N=100 for adults for each combination of beads. Statistical analysis was carried out using chi-square test.

a) SEM and HPLC analysis

Particle size and surface morphology of the micro particles was analysed using scanning electron microscope at Nanotech Centre, Anna University, Chennai. HPLC analysis was done at PLAFFS, TANUVAS, Madhavaram, Chennai to confirm the encapsulation of assembly pheromone in calcium alginate beads.

b) Field trials

Field trials were carried out using specially designed lures. It consisted of a cardboard base measuring 5'X2' onto which a double sided adhesive tape was stuck. 1g of porous alginate test and control beads were sprinkled on to the tape and these were placed in tick infested kennels and observed after 10 days. These lures were mounted on walls (at a height of 2 ft), on window sills (3 ft from ground). Six replicates each for test and control were placed. The number and stages of ticks that were attached and dead were counted and recorded.

Table 3A. Response of unfed adults to porous calcium alginate beads in *in-vitro* bioassay (10 – 30 min).

Combinations	No. of adult ticks that reacted in 10 minutes				No. of adult ticks that reacted in 30 minutes			
	Active	Akinetic	Sluggish	Dead	Active	Akinetic	Sluggish	Dead
Polymer Control	100	-	-	-	100	-	-	-
Pheromone control	76 (76)	24 (24)	-	-	50 (50)	50 (50)	-	-
Test beads	51 (51)	-	36 (36)	13 (13)	34 (34)	-	31 (31)	35 (35)
Acaricide control	70 (70)	-	20 (20)	10 (10)	54 (54)	-	18 (18)	28 (28)
Chi-square value	-	27.27**	7.51**	1.62^{NS}	-	66.67**	7.66**	4.24*

AP = Assembly pheromone; Pheromone control = Alginate + AP;Test beads = Alginate +AP + Deltamethrin; Acaricide control = Alginate + Deltamethrin.
^{NS} Non-significant, * Significant (p< 0.05), ** Highly significant (p<0.01), Figure in the parenthesis indicates percentage.

Table 3B. Response of unfed adults to porous calcium alginate beads in *in-vitro* bioassay (2 – 24 hrs).

Combinations	No. of adult ticks that reacted in 2 hours				Status of adult ticks 24 hours post exposure			
	Active	Akinetic	Sluggish	Dead	Active	Akinetic	Sluggish	Dead
Polymer Control	100	-	-	-	100	-	-	-
Pheromone control	41 (41)	59 (59)	-	-	46 (46)	64 (64)	-	-
Test beads	9 (9)	-	33 (33)	58 (58)	-	-	-	100 (100)
Acaricide control	50 (50)	-	15 (15)	35 (35)	31 (31)	-	13 (13)	56 (56)
Chi-square value	-	83.69**	31.77**	32.50**	-	83.69**	-	42.71**

AP = Assembly pheromone; Pheromone control = Alginate + AP;Test beads = Alginate +AP + Deltamethrin; Acaricide control = Alginate + Deltamethrin.
^{NS} Non-significant, * Significant (p< 0.05), ** Highly significant (p<0.01), Figure in the parenthesis indicates percentage.

RESULTS AND DISCUSSION

In the Scanning electron microscopy analysis the particle size was found to be 1.24 mm. The micro particles were spherical with distinct pores on the surface (Fig. 1). HPLC analysis confirmed the presence of assembly pheromone in the porous calcium alginate beads.

Ticks of unfed stages in *in-vitro* bioassay, when exposed to polymer control beads remained alive 24 hours of post-exposure. On exposure to the pheromone control beads certain behavioural responses were evident. The ticks contacted the beads after momentary questing and later assumed feeding posture (lowering of palps and raising of the posterior part of the body). They finally became akinetic and formed clusters. No mortality was recorded with pheromone control beads throughout the observation period.

With the test beads, there was rapid mortality among the exposed ticks. The ticks initially became sluggish after contacting the beads and eventually all the sluggish ticks died. The per cent mortality at various time intervals are shown in Table 1A and 1B, 2A and 2B, 3A and 3B, respectively. Cent percent mortality was observed among the unfed larvae and unfed adults exposed to test beads whereas 96 percent mortality was recorded in the unfed nymph, 24 hours post-exposure. On exposure to acaricide control beads, sluggishness followed by mortality was observed. Statistical analysis revealed the mortality with test beads to be highly significant ($p<0.01$) among all unfed stages as compared to the acaricide control beads.

In the field trials, the percent mortality in the lures containing test beads was as follows: 1 percent engorged female, 30 percent engorged larvae and 10 percent engorged nymph. Among the unfed stages that were lured and killed, unfed larvae constituted 53 per cent, unfed nymph 12 per cent and unfed adults 5 per cent (Fig. 2).While in the lures containing control beads, very few or no ticks were found to be attracted and killed.

This study is a pioneer attempt to encapsulate assembly pheromone using porous calcium alginate beads. Calcium alginate is a biodegradable component and hence is non-hazardous to both organisms and environment. The lures thus designed in the current study can be used as an effective eco-friendly tick control device. Although, similar sustained release assembly pheromone devices were designed using chitosan (Dhivya *et al*. 2014a) and poly-ε-caprolactone (Dhivya *et al.* 2014b), it was found that the porosity on the surface of the calcium alginate beads aided in effective release of the encapsulated assembly pheromone compared to the other variants tested.

The different stages of ticks becoming akinetic following contact with assembly pheromone as observed in the current study in the presence of assembly pheromone has also been recorded by Carde' and Baker (1984) and Grenacher *et al.* (2001). This cessation of ambulatory activity has been found to reduce distance between individuals, promotes clustering which in turn protects the stages from desiccation concentrating them in favourable microenvironment (Leahy 1979).

In the field trials conducted, various stages of ticks in the environment were found attracted towards the lure with test beads and killed, while there were negligible number of ticks in the lure with control beads. The unfed stages and the fully engorged stages were found attached/ stuck in the lure. The reason for the unfed stages being attracted could be attributed to the fact that assembly pheromone as a role in enhancing the host finding success (Hassanali *et al.* 1989). The engorged stages congregating in response to assembly pheromone is to tide over unfavourable conditions, thus minimizing desiccation and to moult successfully.

This study has made a pioneer attempt to encapsulate assembly pheromones using porous calcium alginate beads. The porosity of the beads aid in more effective release of the encapsulated substance compared to the earlier studies where chitosan (Dhivya *et al.* 2014a) and poly-ε-caprolactone (Dhivya *et al.* 2014b) were utilized as polymers for encapsulating assembly pheromones. Moreover, calcium alginate is a cheap and easily biodegradable polymer with no environmental or human hazard and this is more of a selected control approach wherein only the targeted organism is lured and killed. This study is thus, a step-stone to the manufacture of a commercially viable and cheap, eco-friendly pheromone assisted control measure for environmental stages of the three-host brown dog tick.

REFERENCES

Allan SA, Sonenshine DE, Burridge MJ (2002) Tick pheromones and uses thereof. United States Patent Office, Patent No. 6331297.

Carde RT, Baker TC (1984) Sexual communication with pheromones. In: Chemical ecology of insects. Sinaeur Associates, Suderland. 355-383.

Dhivya B, Latha BR, Raja MD, Sreekumar C, Leela V (2014a) Control of brown dog tick, *Rhipicephalus sanguineus* using assembly pheromone encapsulated in natural polymer, chitosan. Expl Appl Acarol 63(1): 85-92.

Dhivya B, Latha BR, Uma TS, Sreekumar C, Leela V (2014b) A novel approach to control brown dog tick, *Rhipicephalus sanguineus* using sustained release poly-ε-caprolactone-pheromone microspheres. Acta Parasitol 59(1): 153-157.

Grenacher S, Krober T, Guerin PM, Vlimant M (2001) Behavioural and chemoreceptor cell responses of the tick, *Ixodes ricinus* to its own faeces and faecal constituents. Expl Appl Acarol 25: 641- 660.

Hassanali A, Nyandat E, Obenchain FA, Otieno DA, Galun R (1989) Humidity effects on response of *Argas persicus* (Oken) to guanine, an assembly pheromone of ticks. J Chem Ecol 15(3): 791-797.

Koshy TJ, Rajavelu G, Lalitha CM (1983) On the lifecycle of *Rhipicephalus sanguineus.* Cheiron 12(6): 337-338.

Krishnan V, Sasikumar S, Dass FP, Vijayaraghavan R (2010) Effect of pore forming agents on the physical characteristics and release kinetics of levofloxacin hemihydrate from floating alginate drug delivery system - an *in vitro* study. Trends Biomater Artif Organs 24: 139-145.

Leahy MG (1979) Pheromones of Argasid ticks. Cited in: Recent Advances in Acarology, J.G. Rodriguez, Academic Press, New York. 2: 298-308.

Lord CC (2011) Brown Dog tick, *Rhipicephalus sanguineus* Latrielle (Arachnida: Acari: Ixodidae). Featured Creatures, University of Florida Institute of Food and agricultural sciences, Department of Entomology and Nematology, Gainesville, FL. (http://edis.ifas.ufl.edu/in378).

Miller RJ, George JE, Guerrero F, Carpenter L, Welch JB (2001) Characterization of acaricide resistance in *Rhipicephalus sanguineus* (latreille) (Acari: Ixodidae) collected from the Corozal army Veterinary quarantine center, Panama. J Med Entomol 38(2): 298-302.

Ranju RS, Latha BR, Leela V, Basith SA (2012) Effect of 1-octen-3-ol, the ruminant kairomone on ixodid tick larvae. Vet Arhiv 82(6): 609-615.

Sonenshine DE (2004) Pheromones and other semiochemicals of ticks and their use in tick control. Parasitol 129: 404-425.

Wall R (2007) Ectoparasites: Future challenges in a changing world. Vet Parasitol 148: 62-74.

Yoder JA, Stevens BW (2000) Attraction of immature stages of the American dog tick (*Dermacentor variabilis*) to 2, 6-dichlorophenol. Expl Appl Acarol 24: 159-164.

***Cite this article as:** Bhoopathy D, Latha BR (2018) An eco-friendly approach to control *Rhipicephalus sanguineus* by using sustained release porous calcium alginate beads containing assembly pheromone. Explor Anim Med Res 8(1): 33-39.