In-vitro trials to ascertain sustained release efficacy of assembly pheromone micro particles for the control of brown dog tick, *Rhipicephalus sanguineus*

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Abstract Sustained release micro particles were prepared incorporating assembly pheromone and deltamethrin. Two natural polymers, namely, chitosan and calcium alginate and a synthetic polymer, poly-ε-caprolactone were used for encapsulating the assembly pheromone–acaricide combination. The micro particles were subjected to in vitro evaluation freshly after preparation and then at monthly intervals to assess their sustained release efficacy. The response of the unfed stages of dog tick, *Rhipicephalus sanguineus* to fresh and aged micro particles was assessed and results were recorded. The micro particles were found to release assembly pheromone in a sustained manner up to 2 months of study period.

Keywords Assembly pheromone · Micro particles · Sustained-release · Tick control

Introduction

Pheromones are biologically active compounds that fall within the broad category of semiochemicals. With the parasitic ticks developing resistance to acaricides and the hazardous nature of chemical acaricides, the search for alternate tick control strategies has become more intense.

Agricultural scientists are familiar with semiochemical assisted control measures for the control of crop pests while this strategy is still in its infancy in control of pests of veterinary importance. The exploitation of pheromones in field conditions however warrants a sustained/slow release as these compounds are relatively unstable. Champion et al. (1978) pointed out that controlled release of pheromones is essential, for reasons of both, economy and biological function if they are to be used effectively in the field. Hence the current study was designed to prepare sustained release pheromone–acaricide formulations for tick control.

Assembly pheromone of ticks possess a wide spectrum of action as they are capable of attracting all post-embryonic stages namely, the unfed larvae, unfed nymph and unfed adult stages (Sonenshine 1985). Due to its efficacy to elicit responses among different stages of ticks, assembly pheromone was selected in the current study.

Materials and methods

Preparation of micro particles

The micro particles were prepared using three polymers: calcium alginate, chitosan and poly-ε-caprolactone. The quantity of assembly pheromone utilized was in the ratio of 25:1:1 of guanine, adenine and xanthine (Sonenshine 2004). Synthetic analogues of these compounds were procured from Sigma-Aldrich, USA. A total of 102.6 mg of assembly pheromone (95 mg of guanine + 3.8 mg of adenine + 3.8 mg of xanthine) was incorporated into micro particles along with acaricide. The optimum lethal dose of acaricide, Deltamethrin (Butox[®], Intas Pvt. Ltd.,

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India) utilized was 250 µl based on the trials conducted by Ranju (2011).

Calcium alginate beads were prepared by extrusion method by cross-linking 2% sodium alginate suspension containing assembly pheromone and acaricide with 1.5% following the procedure of Anwar et al. (2009). Chitosan beads were also prepared by similar extrusion method. Four percent chitosan suspension with assembly pheromone and acaricide was cross-linked with 2% sodium tripolyphosphate. The cross-linking time was 20 min for calcium alginate beads and 45–60 min for chitosan beads. The beads were then dried for 24–48 h and stored in air-tight containers.

Poly-ε-caprolactone (PCL) micro particles were prepared by following the water-in-oil-in-water double emulsion protocol of Mukerjee et al. (2007).

In-vitro evaluation of micro particles

Brown dog tick, *Rhipicephalus sanguineus* was collected from infested dogs presented at Small Animal Clinics in Madras Veterinary College Teaching Hospital, Chennai. The collected engorged stages (female, larvae and nymph) were maintained in the laboratory in trays filled with moist sand to maintain a relative humidity of >85% until they hatch/moult to unfed stages required for the trials. The ticks were used on the same day of hatch/moult for the in vitro trials.

In-vitro trials were carried out by modified petridish bioassay (Yoder and Stevens 2000) as employed by Ranju (2011). 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 and also to avoid response of the ticks to carbon-dioxide emitted by the investigator which may lead to erroneous results. Ticks were handled only with camel hair brushes. The test was replicated until N = 500 for unfed larvae, N = 50 for unfed nymphs and N = 100 for unfed adults for each polymer type. The test was conducted with fresh micro particles, then 1-month old

micro particles and 2-month old micro particles. The ticks were handled only with camel hair brushes so as to avoid the human lipids interfering with the response of ticks. Utmost care was taken so as to avoid damage to the first pair of legs. All the tests were conducted at room temperature and observations were recorded at 2 and 24 h post-exposure. Statistical analysis of the mean dead stages was done with One-way ANOVA and the F-values obtained were compared.

Results

The response of the unfed larvae is as shown in Table 1. Among the larvae exposed to plain calcium alginate test beads, 58.4% mortality was recorded with 2-month old beads as against 65 with fresh test beads and 62.2 with 1-month old beads at 2 h post-exposure. However, 24 h post-exposure the mortality was almost cent percent even with 2-month old beads.

With porous alginate test beads, cent percent mortality was recorded 24 h post-exposure in 1 and 2-month old beads whereas a drop in mortality up to 30% was observed with 2 h post-exposure in 2-month old beads compared to fresh beads.

On exposure to chitosan test beads, a slow and steady decline in percent mortality was observed. Two hour post-exposure the percent mortality was 63, 62.8 and 59.4 with fresh, 1 and 2-month old beads. Seventy-two percent mortality was observed 24 h post-exposure with 2-month old beads as against 79 with fresh beads. Larvae exposed to PCL microspheres also resulted in similar variations in mortality as that of chitosan beads.

The response of nymphs to 1 and 2-month old beads has been tabulated in Table 2. A steady decline in percent mortality has been observed with all the polymer types with time. A decline in percent mortality ranging between 2 and 18% was observed with various polymer types. A notable observation is that the percent mortality 2 h post-exposure remained the same (40%) for fresh, 1 and 2-month old chitosan beads.

Table 1 Response of unfed larvae in sustained release studies

Test beads	Freshly prepared beads		1-month old beads		2-month old beads	
	Death in 2 h	24 h post exposure	Death in 2 h	24 h post exposure	Death in 2 h	24 h post exposure
Plain alginate beads	325 (65)	500 (100)	311 (62.2)	500 (100)	292 (58.4)	490 (98)
Porous alginate beads	488 (97.6)	500 (100)	420 (84)	500 (100)	340 (68)	500 (100)
Chitosan beads	315 (63)	395 (79)	314 (62.8)	389 (77.8)	297 (59.4)	360 (72)
PCL micro particles	281 (56.2)	478 (95.6)	278 (55.2)	462 (92.4)	270 (54)	464 (92.8)

Figure in the parenthesis indicates percent

Table 2 Response of unfed nymphs in sustained release studies

Test beads	Freshly prepared beads		1-month old beads		2-month old beads	
	Death in 2 h	24 h post exposure	Death in 2 h	24 h post exposure	Death in 2 h	24 h post exposure
Plain alginate beads	26 (52)	45 (90)	17 (34)	42 (84)	13 (26)	40 (80)
Porous alginate beads	47 (94)	50 (100)	40 (80)	50 (100)	39 (78)	48 (96)
Chitosan beads	20 (40)	44 (88)	20 (40)	44 (88)	20 (40)	43 (86)
PCL micro particles	9 (18)	31 (62)	8 (16)	29 (58)	7 (14)	28 (56)

Figure in the parenthesis indicate percent

Table 3 Response of unfed adults in sustained release studies

Test beads	Freshly prepared beads		1-month old beads		2-month old beads	
	Death in 2 h	24 h post exposure	Death in 2 h	24 h post exposure	Death in 2 h	24 h post exposure
Plain alginate beads	60 (60)	97 (97)	50 (50)	95 (95)	47 (47)	91 (91)
Porous alginate beads	100 (100)	100 (100)	80 (80)	100 (100)	70 (70)	100 (100)
Chitosan beads	50 (50)	61 (61)	50 (50)	60 (60)	50 (50)	59 (59)
PCL micro particles	25 (25)	44 (44)	22 (22)	43 (43)	18 (18)	39 (39)

Figure in the parenthesis indicate percent

Table 4 Mean dead and F-value of unfed larvae in sustained release studies

Test beads	Freshly prepared beads		1-month old beads		2-month old beads	
	Death in 2 h	24 h post exposure	Death in 2 h	24 h post exposure	Death in 2 h	24 h post exposure
Plain alginate beads	65 ^b ± 1.0	100 ^c ± 1.7	62.2 ^b ± .374	100 ^c ± 1.61	58.4 ^b ± .509	98 ^c ± 1
Porous alginate beads	97.6 ^c ± .509	100 ^c ± 1.61	84 ^c ± 1.41	100 ^c ± 1.7	68 ^c ± .632	72 ^a ± 1.41
Chitosan beads	63.0 ^b ± .89	79 ^b ± .316	62.8 ^b ± .663	77.8 ^a ± .374	59.4 ^b ± .6	72 ^a ± 1
PCL micro particles	56.2 ^a ± .66	95.6 ^a ± .509	55.6 ^a ± .509	92.4 ^b ± .509	54 ^a ± 1.41	92.8 ^b ± .374
F-value	546.875**	68.048**	214.437**	74.25**	45.435**	180.702**

** Highly significant ($P < .01$), values bearing same superscript do not vary significantly

Unfed adults responded in a similar manner as that of the nymphal stages. The variation in responses of adults is shown in Table 3. There was a decline in mortality percent ranging between 2 and 11, 24 h post-exposure with 2-month old beads. Similar to the nymphs, the percent mortality with chitosan 2 h post-exposure was constant at 50% with fresh test beads, 1 and 2-month old beads.

Statistical analysis showed significant difference in the mortality rate of all the stages to four types of micro particles (Tables 4, 5, 6). Among the four types of beads compared, porous calcium alginate was highly effective as fresh beads with diminishing efficacy as they are aged with reference to larval and nymphal stages. The efficacy of 2-month old porous alginate beads is comparable to that of chitosan beads of same age as far as larval and nymphal responses are considered. However, with the adults, the response seems to be much similar to fresh micro particles of various types except for PCL 24 h post-treatment, but

with 2-month old beads, porous alginate is yet again found to be the most efficacious of all.

Discussion

In the sustained release study, the micro particles were found to be stable and possess slow-release capability. Dubnika et al. (2012) stated that though a natural polymer, chitosan released the encapsulated substance more slowly than alginate and over prolonged period of time. The slow release and hence the delayed response in ticks could be due to the higher polymerization of chitosan. Among the alginate beads, porous beads were found to be effective compared to plain beads. This could be attributed to the fact that there would have been rapid dissipation of the encapsulated substance through the porous beads when compared to beads that have a non-porous surface.

Table 5 Mean dead and F-value of unfed nymphs in sustained release studies

Test beads	Freshly prepared beads		1-month old beads		2-month old beads	
	Death in 2 h	24 h post exposure	Death in 2 h	24 h post exposure	Death in 2 h	24 h post exposure
Plain alginate beads	8.66 ^b ± .881	15 ^b ± .577	5.66 ^b ± .88	14 ^b ± .577	4.33 ^a ± .333	13.33 ^b ± .33
Porous alginate beads	15.6 ^c ± 1.2	16.66 ^b ± 1.2	13.3 ^c ± .66	16.66 ^c ± .66	13 ^c ± 1.15	16 ^c ± .577
Chitosan beads	6.66 ^b ± .66	14.66 ^b ± 1.2	6.66 ^b ± 1.2	14.66 ^b ± .88	6.66 ^b ± .666	14.33 ^b ± .881
PCL micro particles	3.0 ^a ± .57	10.33 ^a ± .88	2.66 ^a ± .33	9.66 ^a ± .881	2.33 ^a ± .333	9.33 ^a ± .33
F-value	37.778**	7.296*	29.16**	14.905**	42.87**	24.083**

* Significant ($P < .05$), ** Highly significant ($P < .01$), values bearing same superscript do not vary significantly

Table 6 Mean dead and F-value of unfed adults in sustained release studies

Test beads	Freshly prepared beads		1-month old beads		2-month old beads	
	Death in 2 h	24 h post exposure	Death in 2 h	24 h post exposure	Death in 2 h	24 h post exposure
Plain alginate beads	15 ^b ± 1.08	24.5 ^c ± 1.04	12.5 ^c ± .866	23.75 ^c ± 1.1	11.75 ^c ± .075	22.75 ^d ± .478
Porous alginate beads	25 ^b ± 1.29	25 ^c ± 1.08	20 ^b ± .912	25 ^c ± 2.19	17.5 ^b ± .645	25 ^c ± .408
Chitosan beads	12.5 ^b ± .86	15.25 ^b ± .629	12.5 ^b ± .866	15 ^b ± .816	12.5 ^b ± .86	14.75 ^b ± .478
PCL micro particles	6.25 ^a ± .853	11 ^a ± .408	5.5 ^a ± .645	10.75 ^a ± .629	4.5 ^a ± .86	9.75 ^a ± .85
F-value	56.401**	68.40**	51**	26.585**	46.277**	147.862**

** Highly significant ($P < .01$), values bearing same superscript do not vary significantly

Moreover in all the cases the attraction was never reduced but the rate mortality and sluggishness were only affected initially but was the same as compared to the fresh beads 24 h post-exposure. In addition, the responses such as mortality and sluggishness were affected to a small extent 2 h post-exposure with prolonged exposure for 24 h resulting in responses comparable to those evinced in the presence of fresh beads.

Also all the polymers used in the current study i.e., calcium alginate, chitosan and poly-ε-caprolactone are all biodegradable, environmentally safe and biocompatible (Quong 2003, Dubnika et al. 2012 and Lin et al. 1999). All these polymers are employed in sustained release oral drug formulations also. Moreover, calcium alginate (Quong 2003) and chitosan (Henderson et al. 1997) have already been employed for sustained release of pheromones and they have been found to be safe vehicles as they do not interact with the encapsulated substance. Hence this study forms the basis for an eco-friendly pheromone assisted tick control measure and the formulation of a commercially viable tick-control product utilizing pheromones will thus not be a distant dream anymore.

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