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Exploiting assembly pheromone for the control of ixodid ticks

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ABSTRACT

The effect of an Assembly Pheromone – Deltamethrin complex (AP – D complex) on the larvae of five ixodid tick species viz. *Rhipicephalus sanguineus*, *R. microplus*, *R. haemaphysaloides*, *Hyalomma marginatum* and *Haemaphysalis bispinosa*, as well as the adults of *R. sanguineus* and *R. microplus*, was assessed by in vitro bioassays. All the larval as well as adult stages of ticks exposed to the AP – D complex were lured and killed within 24 hours of exposure, except *H. bispinosa*. Exposure to the AP – D complex for an hour resulted in 70%, 95%, 90%, 90%, 95% mortality of the larval stages of *Rhipicephalus sanguineus*, *R. microplus*, *R. haemaphysaloides*, *H. bispinosa* and *H. marginatum* respectively. No mortality was observed when the larval stages of the five tick species were exposed to AP alone (positive control). Exposure of adults to the AP – D complex for an hour resulted 92% and 90% mortality of *R. microplus* and *R. sanguineus*, respectively. Negligible mortality was recorded in adult ticks exposed to deltamethrin alone (Negative control), while no mortality was recorded when exposed only to AP (Positive control). Hence, the AP – D complex is better in luring and killing ticks than AP or D alone, regardless of the life stage. Our results suggest that the development of a sustained release AP – D complex device may be an effective means of controlling ticks in India.

Keywords Assembly pheromones, Ixodid ticks, tick control, deltamethrin, Guanine, Adenine

Introduction

Ticks have attracted a great deal of scientific attention primarily because of their considerable medical and veterinary importance. They have a key role in disease transmission and impose a major constraint on the development and improvement of the livestock industry. In India, *Rhipicephalus sanguineus* is the most common ixodid tick parasitizing dogs, while *Haemaphysalis bispinosa*, *R. haemaphysaloides* and *Hyalomma marginatum* are seen infesting small and large ruminants, in addition to *R. microplus* infesting large ruminants. These ticks act as important vectors of many diseases. In India, the economic losses due to tick and tick-borne diseases were estimated to be US\$ 498.7 million per annum (Ghosh *et al.*, 2007), with a global annual loss of \$109 billion (Jabbar *et al.*, 2007).

In India, the mainstay of tick control is the use of chemical acaricides. However, with its well-known drawbacks, research is actively working toward an alternative tick control strategy. One alternative control measure, not commonly exploited in India, is the use of semiochemicals. Semiochemicals are chemical signal vehicles of host/tick origin which are secreted into the external environment to mediate tick behaviour. These information-containing compounds include pheromones (used for conspecific communication), allomones (defense secretions) and kairomones (used for host identification and location) (Regnier, 1970). Pheromones are the best known, intensively studied and include arrestment (assembly) pheromones, attraction-aggregation-attachment (AAA) pheromones and sex pheromones (Attachment sex pheromone,

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Mounting sex pheromone, Genital sex pheromone). Amongst semiochemicals, assembly / arrestment pheromone (AP) has been used with considerable success (Sonenshine, 2004). A patented device incorporating purines from the faecal wastes of the prostriate tick, *I. scapularis* into oily droplets released from a pump sprayer was designed for delivery to vegetation. The oily droplets adhered to vegetation where *I. scapularis* quest for hosts (Allan *et al.*, 2002). The assembly pheromone components, including guanine and xanthine along with an acaricide (permethrin), caused the ticks that encountered the droplets to cling to the contaminated surfaces where they acquired a lethal dose of the acaricide (Sonenshine, 2006). An increased mortality rate from 70 per cent for the device with acaricide alone to 95 per cent for the device with an arrestment pheromone – acaricide mixture was observed in laboratory studies using *I. scapularis* ticks (Sonenshine, 2004, 2006).

Here, we explore the effect of an assembly pheromone – deltamethrin complex (AP – D complex) on five ixodid ticks which are known to commonly parasitize animals in the Indian sub continent. The trial made use of deltamethrin as an acaricide of choice, rather than permethrin, since deltamethrin is main drug presently used for tick control in India. Moreover, unlike permethrin, deltamethrin is not a repellent and therefore will not interfere with the response of ticks to AP.

Materials and methods

Ticks

Rhipicephalus sanguineus were collected from dogs presented in the Small Animal Clinic of the Madras Veterinary College, Chennai. The common ticks on ruminants, namely *R. microplus*, *R. haemaphysaloides*, *Ha. bispinosa* and *Hy. marginatum* were collected from animals presented in the Large Animal Clinics of the Madras Veterinary College and the PGRIAS, Kattupakkam. Engorged female ixodid ticks were used to initiate laboratory colonies. Unfed larval stages from the colony and the partially fed male and female ticks collected from the animals were used for the trials. Only ticks with the first pair of legs intact were selected for the bioassays since pheromones are perceived by the Haller's organ found on this appendage (Sonenshine, 2006).

Filter paper

Whatman® qualitative filter paper (grade 3, diameter = 11 cm ; Whatman International Ltd., Maidstone, England) was used to impregnate both the assembly pheromone and the acaricide (Sonenshine, 2003). Filter paper discs of 2x2 cm in size were used in petri dish bioassays. The discs were handled with a gloved hand. This ensured that the filter paper discs did not come into contact with human skin lipids which were found attractive to ticks (Yoder *et al.*, 1998).

Acaricide

Butox®, a commercially available preparation of synthetic pyrethroid, containing 1.25 % deltamethrin (Intas Pvt. Ltd., Gujarat) was the acaricide used. An initial trial was conducted using different dilutions of butox to determine the optimum lethal dose to be used in the bioassay. Different dilutions of butox solutions were made using 10, 20, 30, 40, 50, 100, 250 and 500 µl of butox in 2.5 ml of diethyl ether (Fischer Chemic Ltd., Chennai). The filter paper discs were impregnated with the acaricide solution for 24 hours and were dried before use. Based on the pilot study, 250 µl of butox solution was selected as the minimum effective lethal concentration to be utilized in the current study. With 250 µl of butox solution, death of the ticks was rapid and 100 %.

Assembly pheromones (AP)

The synthetic analogues of the AP namely guanine, xanthine, adenine and hematin (Sigma Alderich, Germany) were used in a ratio 25:1:1:1 (Sonenshine, 2004) with slight modifications. The mixture was diluted in 4 ml of 0.95 % saline and stored at room temperature for 3 hours before use. A preliminary study using 50% of the ticks revealed that 200 µl of the AP mixture was suitable for attracting the ticks. Deltamethrin (D) impregnated filter paper discs were used for AP – D complex trials. Positive controls were maintained with AP whereas D alone served as a negative control. Ticks were also exposed to filter paper discs impregnated with the solvents used in the trials namely saline and diethyl ether individually.

Petri dish Bioassays

Petri dish bioassays were carried out as per Yoder and Stevens (2000) with slight modifications. Glass petri dishes of 9 cm diameter were used for the assays. Filter paper discs of size 2×2 cm were pasted in one quadrant of the petri dish with laboratory grade parafilm. Larvae and adult ticks were placed 10 at a time in the bioassay arena, opposite the filter paper discs. The petri dish was covered with another petri dish of the same diameter and sealed with laboratory grade parafilm in order to prevent the escape of ticks, as well as to avoid responses by the tick to the carbon dioxide emitted by the investigator. Care was taken while handling the ticks in order to avoid damaging the first pair of legs. Controls were performed in separate petri dishes. All tests were conducted at room temperature (37°C). Fresh ticks, chemicals and plastic wares were used for each trial. The number of ticks that were attracted to the disc, or to the quadrant with the disc, were counted and considered attracted to the AP- D complex. Observations were made after 10 minutes, 1 hour and 24 hours. The effect of AP on the larval stages of all five species of ixodid ticks and the adults of *R. sanguineus* and *R. microplus* were evaluated. Tests were replicated until N=100 for the larvae of each tick species and N=50 for the adults of *R. sanguineus* and *R. microplus*. The data obtained was analysed by chi- square test using IBM SPSS version 20.0 software for windows.

Results

The response of larvae of *R. sanguineus*, *R. microplus*, *R. haemaphysaloides*, *Ha. bispinosa* and *Hy. marginatum* and the adult ticks of *R. sanguineus* and *R. microplus* to the different experimental assays are shown in Tables 1, 2, 3, 4,5,6 and 7, respectively.

Table 1 Effect of the assembly pheromones on the larvae of *Rhipicephalus sanguineus* (N=100 larvae)

Trials	No. of larvae attracted/ dead within 10 minutes				No. of larvae attracted / dead within 1 hour				No. of larvae attracted/ dead after 24 hours			
	On disc	Same quadrant	Total attracted	Death of larvae	On disc	Same quadrant	Total attracted	Death of larvae	On disc	Same quadrant	Total attracted	Death of larvae
Assembly + Deltamethrin	19 (30)	45 (70)	64 (64)	19 (19)	19 (27)	51 (73)	70 (70)	70 (70)	25 (34)	49 (66)	74 (74)	100 (100)
Assembly alone (Positive control)	8 (17)	38 (83)	46 (46)	Nil	14 (31)	31 (69)	45 (45)	Nil	17 (31)	37 (69)	54 (54)	Nil
Deltamethrin alone (Negative control)	6 (35)	11 (65)	17 (17)	Nil	4 (13)	27 (87)	31 (31)	25 (25)	7 (24)	22 (76)	29 (29)	100 (100)
Chi-square value and significance			46.07**	20.99**			31.25**	40.60**			40.76**	

** Highly significant (p≤ 0.01), Numbers in parenthesis indicate percentage over total number of ticks used in the assays

Table 2 Effect of the assembly pheromones on the adults of *Rhipicephalus sanguineus* (N=50 Adults)

Trials	No. of adult ticks attracted/ dead within 10 minutes				No. of adult ticks attracted / dead within 1 hour				No. of adult ticks attracted/ dead after 24 hours			
	On disc	Same quadrant	Total attracted	Death of adult ticks	On disc	Same quadrant	Total attracted	Death of adult ticks	On disc	Same quadrant	Total attracted	Death of adult ticks
Assembly + Deltamethrin	11 (33)	22 (67)	33 (66)	11 (22)	11 (34)	21 (66)	32 (64)	45 (90)	12 (36)	21 (64)	33 (66)	50 (100)
Assembly alone (Positive control)	4 (17)	20 (83)	24 (48)	Nil	9 (45)	11 (55)	20 (40)	Nil	8 (26)	23 (74)	31 (62)	Nil
Deltamethrin alone (Negative control)	2 (50)	2 (50)	4 (8)	2 (4)	2 (100)	Nil	2 (4)	2 (4)	2 (100)	Nil	2 (4)	50 (100)
Chi-square value and significance			36.53**	7.16**			39.58**	74.23**			48.86**	

** Highly significant ($p \leq 0.01$), Numbers in parenthesis indicate percentage over total number of ticks used in the assays

Table 3 Effect of the assembly pheromones on the larvae of *Hyalomma marginatum* (N=100 larvae)

Trials	No. of larvae attracted/ dead within 10 minutes				No. of larvae attracted / dead within 1 hour				No. of larvae attracted/ dead after 24 hours			
	On disc	Same quadrant	Total attracted	Death of larvae	On disc	Same quadrant	Total attracted	Death of larvae	On disc	Same quadrant	Total attracted	Death of larvae
Assembly + Deltamethrin	19 (36)	34 (64)	53 (53)	19 (19)	19 (36)	34 (64)	53 (53)	95 (95)	20 (37)	34 (63)	54 (54)	100 (100)
Assembly alone (Positive control)	15 (52)	14 (48)	29 (29)	Nil	15 (52)	14 (48)	29 (29)	Nil	34 (48)	37 (52)	71 (71)	Nil
Deltamethrin alone (Negative control)	Nil	29 (100)	29 (29)	Nil	Nil	29 (29)	29 (29)	29 (29)	4 (10)	37 (90)	41 (41)	100 (100)
Chi-square value and significance			16.47**	20.99**			16.47**	92.44**			23.27**	

** Highly significant ($p \leq 0.01$), Numbers in parenthesis indicate percentage over total number of ticks used in the assays

Table 4 Effect of the assembly pheromones on the larvae of *Haemaphysalis bispinosa* (N=100 larvae)

Trials	No. of larvae attracted/ dead within 10 minutes				No. of larvae attracted / dead within 1 hour				No. of larvae attracted/ dead after 24 hours			
	On disc	Same quadrant	Total attracted	Death of larvae	On disc	Same quadrant	Total attracted	Death of larvae	On disc	Same quadrant	Total attracted	Death of larvae
Assembly + Deltamethrin	12 (36)	21 (64)	33 (33)	12 (12)	12 (29)	30 (71)	42 (42)	90 (90)	15 (31)	33 (69)	48 (48)	100 (100)
Assembly alone (Positive control)	5 (12)	37 (88)	42 (42)	Nil	5 (12)	37 (88)	42 (42)	Nil	5 (12)	37 (88)	42 (42)	Nil
Deltamethrin alone (Negative control)	Nil	18 (100)	18 (18)	Nil	1 (6)	17 (94)	18 (18)	18 (18)	1 (4)	23 (96)	24 (24)	100 (100)
Chi-square value and significance			13.74**	12.77**			17.11**	104.35**			13.24**	

** Highly significant ($p \leq 0.01$), Numbers in parenthesis indicate percentage over total number of ticks used in the assays

Table 5 Effect of the assembly pheromones on the larvae of *Rhipicephalus haemaphysaloides* (N=100 larvae)

Trials	No. of larvae attracted/ dead within 10 minutes				No. of larvae attracted / dead within 1 hour				No. of larvae attracted/ dead after 24 hours			
	On disc	Same quadrant	Total attracted	Death of larvae	On disc	Same quadrant	Total attracted	Death of larvae	On disc	Same quadrant	Total attracted	Death of larvae
Assembly + Deltamethrin	14 (22)	51 (78)	65 (65)	14 (14)	16 (23)	54 (77)	70 (70)	90 (90)	16 (23)	55 (77)	71 (71)	100 (100)
Assembly alone (Positive control)	9 (19)	38 (81)	47 (47)	Nil	16 (31)	36 (69)	52 (52)	Nil	13 (25)	38 (75)	51 (51)	Nil
Deltamethrin alone (Negative control)	7 (25)	21 (75)	28 (28)	7 (7)	7 (22)	25 (78)	32 (32)	32 (32)	5 (12)	36 (88)	41 (41)	100 (100)
Chi-square value and significance			27.51**	15.05**			28.93**	70.70**			18.81**	

** Highly significant ($p \leq 0.01$), Numbers in parenthesis indicate percentage over total number of ticks used in the assays

Table 6 Effect of the assembly pheromones on the larvae of *Rhipicephalus microplus* (N=100 larvae)

Trials	No. of larvae attracted/ dead within 10 minutes				No. of larvae attracted / dead within 1 hour				No. of larvae attracted/ dead after 24 hours			
	On disc	Same quadrant	Total attracted	Death of larvae	On disc	Same quadrant	Total attracted	Death of larvae	On disc	Same quadrant	Total attracted	Death of larvae
Assembly + Deltamethrin	3 (5)	56 (95)	59 (59)	59 (59)	15 (20)	59 (80)	74 (74)	95 (95)	16 (21)	59 (79)	75 (75)	100 (100)
Assembly alone (Positive control)	3 (10)	28 (90)	31 (31)	Nil	3 (9)	31 (91)	34 (34)	Nil	3 (8)	33 (92)	36 (36)	Nil
Deltamethrin alone (Negative control)	Nil	18 (100)	18 (18)	Nil	Nil	23 (100)	23 (23)	24 (24)	Nil	19 (100)	19 (19)	100 (100)
Chi-square value and significance			33.37**	83.69**			58.57**	104.60**			67.14**	

** Highly significant ($p \leq 0.01$), Numbers in parenthesis indicate percentage over total number of ticks used in the assays

Table 7 Effect of the assembly pheromones on adults of *Rhipicephalus microplus* (N=50 Adults)

Trials	No. of adult ticks attracted/ dead within 10 minutes				No. of adult ticks attracted / dead within 1 hour				No. of adult ticks attracted/ dead after 24 hours			
	On disc	Same quadrant	Total attracted	Death of adult ticks	On disc	Same quadrant	Total attracted	Death of adult ticks	On disc	Same quadrant	Total attracted	Death of adult ticks
Assembly + Deltamethrin	8 (35)	15 (65)	23 (46)	8 (16)	8 (35)	15 (65)	23 (46)	46 (92)	9 (38)	15 (62)	24 (48)	50 (100)
Assembly alone (Positive control)	7 (25)	21 (75)	28 (56)	Nil	12 (41)	17 (59)	29 (58)	Nil	17 (57)	13 (43)	30 (60)	Nil
Deltamethrin alone (Negative control)	2 (29)	5 (71)	7 (14)	2 (4)	2 (40)	3 (60)	5 (10)	3 (6)	2 (50)	2 (50)	4 (8)	50 (100)
Chi-square value and significance			20.30**	4*			26.49**	73.99**			31.26**	

** Highly significant ($p \leq 0.01$), * significant, Numbers in parenthesis indicate percentage over total number of ticks used in the assays

Figure 1 Effect of assembly pheromone: A – Clustering of *Rhipicephalus sanguineus* larvae on assembly pheromone impregnated filter paper disc; B – Arrestment of *Rhipicephalus sanguineus* larvae on assembly pheromone impregnated filter paper disc; C – Behaviour of the adults of *Rhipicephalus sanguineus* in assembly pheromone-deltamethrin trial; D – Behaviour of the adults of *Rhipicephalus sanguineus* in positive control; E – Behaviour of the adults of *Rhipicephalus sanguineus* in negative control; F – Response of the adults of *Rhipicephalus sanguineus* to natural assembly pheromone.

Figure 2 Behavioural responses of ixodid tick larvae to assembly pheromone: A – Curled leg appearance of *Hyalomma marginatum* larvae; B – Feeding posture exhibited by *Rhipicephalus microplus* male ticks; C – Clustering of partially fed females of *Rhipicephalus sanguineus*

Figure 3 Comparison of the per cent death of ixodid tick larvae after 1 hour in Assembly pheromone-deltamethrin and deltamethrin treatment.

Figure 4 Comparison of the per cent death of adult ticks after 1 hour in Assembly pheromone-deltamethrin and deltamethrin treatment.

Figure 5 Comparison of the per cent attraction of ixodid tick larvae after 24 hours.

Figure 6 Comparison of the per cent attraction of adult ticks after 24 hours.

In vitro trials with larvae and adult ixodid ticks revealed attraction to the AP treated filter paper discs. The attracted adult ticks and larvae crawled onto the pheromone source, assembled either on the disc or in the same quadrant, became akinetic (Fig. 1) with a curled-leg appearance (Fig. 2A) and lowered mouth parts simulating a feeding posture (Fig. 2B). The ticks formed loose or tight groups near the AP source within 10 minutes of exposure (Fig. 2C). Larvae and adults which came into contact with the AP – D complex, died immediately, whereas in negative controls, ticks exhibited a slow death. Exposure to the AP source for an hour resulted in 45%, 34%, 52%, 42%, 29% attraction among the larval stages of *R. sanguineus*, *R. microplus*, *R. haemaphysaloides*, *Ha. bispinosa* and *Hy. marginatum*, respectively and exposure to AP – D complex for an hour resulted in 70%, 95%, 90%, 90%, 95% mortality of the larval stages of *R. sanguineus*, *R. microplus*, *R. haemaphysaloides*, *Ha. bispinosa* and *Hy. marginatum*, respectively (Fig. 3). One hour after exposure to the AP source observed, attraction among adult ticks was 40% and 58%, whereas AP – D complex mortality in this life stage was 90 %, and 92 % for *R. sanguineus* and *R. microplus*, respectively (Fig. 4). One hundred percent death was observed in the AP – D assays and in negative controls after 24 hours of exposure (Fig 5-6). Statistical analysis revealed significant differences in percent attraction and death between AP – D complex and the controls for all species considered ($p < 0.05$; see species and stage specific tables for tests statistics)

Discussion

Like unfed tick life stages, partially-fed male and female ticks, once detached from the host, readily reattach to new host and resume feeding (Little et al., 2007). The current study used unfed larval stages of five ixodid tick species and the partially-fed adult stages of *R. sanguineus* and *R. microplus* for the *in vitro* trials. Ticks are purinotelic with guanine as the main product of nitrogen metabolism. Trace amounts of xanthine, hypoxanthine and adenine have been reported in the excretory and secretory products of ticks (Dusbabek et al., 1998). These nitrogenous waste products of metabolism serve as AP in most soft ticks and some hard ticks (Sonenshine, 2006). The reports of Allan and Sonenshine (2002) confirm that AP, when used as a mixture, gave the strongest response among ixodid ticks. The behavioural responses of ticks in response to the AP in the present study were characteristic. Individual ticks became akinetic and remained in close contact with one another forming clusters. Similar behavioural responses were reported in *Ornithodoros moubata* (Leahy et al., 1973). In the present study, the arrestment behaviour was so intense that large clusters of ticks could be lifted without individuals dispersing, a phenomenon also reported by Leahy et al. (1981) and Dusbabek et al. (1991). Assembly behaviour is believed to enhance host finding opportunities and possibly even protect soft ticks against desiccation (Yoder and Stevens, 2000). The effect of arrestment pheromone on hard ticks also seems to be similar (Hamdy, 1972, 1973; Otieno et al., 1985).

The assembly formation depends on the amount of guanine and xanthine in the excretory / secretory products (Dusbabek et al., 1991). Some environmental micro organisms like *Proteus*, *Pseudomonas*, *Aerobacter* and *Clostridium spp.* are responsible for the aerobic purine degradation of tick excreta (Dusbabek et al., 1991). Indeed, the fluid part of natural tick excreta, as well as synthetic purine analogues, are chemically unstable and get converted into uric acid by purine degrading micro organisms. When the guanine content drops down in AP samples, tick assembly decreases significantly (Dusbabek et al., 1998). Hence, in the current study, purines were diluted in 0.95 % normal saline in order to avoid the degradation of these products.

The effect of AP declines with time and, as a result, the lured ticks show a tendency to wander away from the pheromone source. This behaviour was avoided by the use of the AP – D complex, where a high percentage mortality was observed, likely due to the immediate lethal effect of deltamethrin on the lured ticks. Larvae of *R. haemaphysaloides* showed the highest attraction at the 10 minute interval in the AP-D complex treatment, with a range between

53-69 % in all species except *Ha. bispinosa*. After 24 hours, the range of attraction in AP-D treatment group was 71-75 % in all tick larvae except *Ha. bispinosa* and *Hy. marginatum*. Lower attraction in *Ha. bispinosa* could be due to interspecies variation in the ratio of purines. However *Hy. marginatum* larvae showed 71 % attraction to AP alone but failed to respond to the AP-D combination after 24 hours of observation. Whether the presence of deltamethrin deterred *Hy. marginatum* larvae has to be investigated. Another observation was the slow death among the ticks in the negative control bioassay (D alone). The reason attributed to this is that unlike in the AP – D complex bioassay where assembly / arrestment of the ticks occurred on the deltamethrin impregnated strip, in the negative control, ticks barely made contact with the lethal dose of deltamethrin and the dose they received was insufficient to instantaneously kill the tick.

In conclusion, a high level of attraction to AP was observed with all ixodid ticks, with the possible exception of *Ha. bispinosa*. The AP – D complex was most effective in controlling the larval stages of the five ixodid tick species, but also performed reasonably well for adults of *R. microplus* and *R. sanguineus*. Further standardisation of the concentration of purines for individual species would enable increased levels of attraction of tick stages and may thereby aid to optimize tick control. Our results suggest that the development of a sustained release AP – D complex device may be an effective means of controlling ticks in India.

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