

Control of the mosquito *Anopheles pseudopunctipennis* (Diptera: Culicidae) with *Romanomermis iyengari* (Nematoda: Mermithidae) in Oaxaca, Mexico

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Abstract

We evaluated the effect of monthly applications of *Romanomermis iyengari* on populations of larval stages of the mosquito *Anopheles pseudopunctipennis*, in an endemic area of malaria in Pochutla, Oaxaca, Mexico. In 1999, applications of *R. iyengari* were made for 9 months on four natural breeding sites of *An. pseudopunctipennis* with a total area of approximately 30,000 m². An application rate of 3000 nematodes m⁻² was used. Parasitism of mosquito larvae ranged from 46 to 100%, and the population reduction of the mosquito larvae varied from 38.1 to 99.8%. In two breeding sites *R. iyengari* was able to recycle and persisted for 5 months. The monthly applications of *R. iyengari* efficiently controlled the populations of larval *An. pseudopunctipennis*, thus reducing the risk of malaria transmission to people living nearby breeding sites.

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1. Introduction

Many species of mosquitoes in the genus *Anopheles* are vectors of malaria and have been traditionally controlled with synthetic pesticides. This type of control can be hazardous to humans, result in pesticide resistance in mosquitoes, damage fauna and flora; contaminate the air, water and soil, and be expensive (Fondo Mundial para la Naturaleza, 1998). In spite of the continuous use of synthetic pesticides, mosquito populations have not been reduced significantly, and anophelines retain their potential to support the outbreak and development of

malaria in endemic areas. Such a condition is found in the state of Oaxaca, Mexico, where during the past 30 years, numerous cases of malaria have been observed (Secretaría de Salud, 1996). Therefore, it is necessary to evaluate other control methods.

The mermithid nematode *Romanomermis iyengari* Welch is one of several natural control alternatives to synthetic pesticides for mosquito suppression. This parasite of mosquito larvae was first reported in 1927 in the Lower Bengal Delta (India) in *Anopheles* and *Culex* larvae (Gajanana et al., 1978; Iyengar, 1927). This mermithid develops inside mosquito larvae that die when the parasite emerges (Chandrasah and Rajagopalan, 1979).

Several research groups have evaluated the control potential of *R. iyengari* in a number of species of *Aedes*, *Anopheles* and *Culex* larvae under laboratory conditions

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and in small field trials (Chandrabhas and Rajagopalan, 1979; Gajanana et al., 1978; Pridantseva et al., 1990; Santamarina, 1994; Santamarina et al., 1992, 1996a; Vladimirova et al., 1990). Recently, Santamarina et al. (1999) demonstrated the effectiveness of *Romanomermis* for mosquito control in Oaxaca, Mexico, where malaria is a major concern in coastal regions.

The objective of this investigation was to evaluate the effectiveness of monthly applications of *R. iyengari* (3000 nematodes m^{-2}) to reduce the *Anopheles pseudopunctipennis* Theobald larval populations on four mosquito breeding sites in El Tomatal, Santa Maria Colotepec, Pochutla, Oaxaca, México. No previous field study has addressed the impact of periodical applications of *R. iyengari* on anopheline larvae in backwater areas of streams.

2. Methods

2.1. Selection of breeding sites of mosquito larvae

The study was initiated during December 1998. Four natural breeding sites with high *An. pseudopunctipennis* larval populations were selected in the community El Tomatal, Santa Maria Colotepec, Pochutla, Oaxaca, Mexico. The sites were numbered in order of importance according to their potential production of *An. pseudopunctipennis* mosquitoes and probable effects on the local population's health. Site 1 consisted of an area of 15,000 m^2 in a permanent stream that passed through the center of the community and consisted partly of flowing water and partly backwater that was stagnant and non-flowing. Site 2 occupied an area of 10,000 m^2 and passed 500 m to the northwest of Site 1. Site 3 consisted of an area of 4000 m^2 and was located 6 km to the east of the town center. Site 4 occupied an area of 4500 m^2 and was located 8 km northeast of the town center. The total surface area for all four breeding sites was approximately 30,000 m^2 .

2.2. Production and preparation of nematodes

The nematodes were obtained from the mass production plant located at the CIIDIR (Interdisciplinary Center of Investigation for Integrated Regional Development) campus Oaxaca of The National Polytechnic Institute (IPN), where approximately 360 million pre-parasitic nematodes were produced each month. Nematode cultures kept in plastic containers (21 × 14 × 6 cm) containing river sand, were stored for 6 weeks at $25 \pm 2^\circ C$ (Pérez et al., 2003). As needed, the containers were filled with distilled water to induce the hatching of eggs and the emergence of the infectious pre-parasitic nematodes. To determine the appropriate concentration for the application sprayers, the water from the cultures was collected after 4 h at $27 \pm 2^\circ C$,

and the concentration of nematodes in suspension was calculated by means of the volumetric dilution method (Petersen and Willis, 1972).

2.3. Application of nematodes

The nematode applications were carried out on a monthly basis as follows. Seven and eight applications were made at Sites 1 and 2, respectively, since they were the primary sources of vectors for malaria due to their proximity to the town center and it was necessary to achieve adequate control in the least possible amount of time. Sites 3 and 4 were considered relatively low-risk, due to their distance to the town center (6–8 km), and they received only four and two applications, respectively.

The applications were carried out between January and November, 1999, using Knapsacks sprayers (Swiss Mex-82 brand, 15 liter capacity) and a concentration of 3000 nematodes m^{-2} . Long spray wands (1.5 m long) were used to obtain a good coverage of the treated area.

2.4. Evaluation of parasitism, infestation, and reduction of larval populations

One day before application, each site was sampled to determine the pre-treatment density of mosquito larvae. The sampling procedure followed one suggested by the Health Secretariat (Secretaría de Salud, 1992), where 90 ml samples (calados) of water are taken from the surface and the captured larvae counted. For each breeding site, 100 “calados” were taken in different areas, and the average larval concentration (ALC) was determined. These pre-treatment samples were labeled as ALCpre.

To determine the percentage of parasitism (PP) of mosquito larvae and the number of nematodes per larva (mean infection or MI), 100 larvae/treatment site were collected 3 days after the infestation and dissected under a stereoscopic microscope. Using the same methodology, recycling of the nematodes was also estimated, since parasites were detected in the ALCpre samples in May, 1999, at Sites 1 and 2.

To determine the density of surviving mosquito larvae, 100 samples were taken 5 days after each application from each treatment site, as described above. This variable was named the ALC post-treatment (ALCpos) and was used to estimate the effectiveness of control as reduction percentage (RP) according to the modified formula of Lacey and Mulla (1979):

$$RP = (ALC_{pre} - ALC_{pos} / ALC_{pre}) \times 100.$$

To determine the PP and MI present before the treatments were applied again, 100 larvae of third and fourth instars were taken from the breeding sites. These instars were chosen to guarantee the capture of larvae that had been exposed to the maximum concentration during the previous application.

2.5. Statistical analysis

The collected mosquito larvae were subdivided into five sub-samples of 20 larvae for each site to get five replicates per application, and a total of 105 observations. Assuming a completely randomized treatment design, analysis of variance on these data was carried out and the PP and MI averages were calculated. A comparison of means by the least significant difference (LSD) was considered significantly different at $P < 0.05$. To determine the relationship between PP and MI, correlation coefficients were calculated between these data across all treatments. The ALCpre and ALCpos at all treatment sites were compared using the Wilcoxon signed rank test ($P = 0.01$).

3. Results

3.1. Percentage of parasitism

The PP of the *R. iyengari* nematode on *An. pseudopunctipennis* larvae are shown in Table 1. The overall

average value was 68% (range = 46–100%). Site No. 3 showed the highest PP (77%), but it was not significantly different from the lowest one (65%). However, the analysis of variance carried out on the data of all 21 applications was highly significant ($F_{20,84} = 5.56, P < 0.01$).

The highest PP was observed during June at Site 3, which also showed the largest value during August. Some of the lowest PP values were observed at Sites 1 and 2, especially during June, September and October. Overall, the first 3 months of the rainy season (June, July, and August) showed the highest PP values.

3.2. Infection of mosquito larvae

The values for MI rates of *R. iyengari* on *An. pseudopunctipennis* are shown in Table 2. The range was between 0.6 and 2.8 nematodes/larva with an overall average of 1.4 nematodes/larva. The analysis on MI showed a highly significant difference among treatments ($F_{20,84} = 15.58, P < 0.01$). The correlation between PP and MI across all 21 treatments in the four breeding sites was positive ($r = 0.75$) and statistically significant ($P < 0.01$).

Table 1
Percentage of parasitism of *Romanomermis iyengari* in *Anopheles pseudopunctipennis* larvae in Pochutla, Oaxaca, Mexico, 1999

Site	Jan	Feb	Apr	Jun	Jul	Aug	Sep	Oct	Nov	Average
1	61.0 C	46.0 D	72.0 BC	56.0 ^a CD	75.0 ^a BC	83.0 B	b	b	66.0 ^a C	65.6
2	70.0 BC	66.0 C	65.0 C	51.0 ^a D		78.0 ^a BC	57.0 ^a CD	55.0 ^a CD	78.0 BC	65.0
3	65.0 C		59.0 CD	100.0 A	b	84.0 B	b	b	b	77.0
4	72.0 BC		70.0 BC		b	b	b	b	b	71.0
Average	67.0	56.0	66.5	69.0	75.0	81.7	57.0	55.0	72.0	

Means followed by different uppercase letters in rows or columns are significantly different (LSD, $P < 0.05$).

^a Moderate rain flooded the treatment site.

^b Excessive rain modified the characteristics of the breeding sites and prevented applications during this interval.

Table 2
Mean infection rates of *Romanomermis iyengari* on *Anopheles pseudopunctipennis* larvae in Pochutla, Oaxaca, Mexico, 1999

Site	Jan	Feb	Apr	Jun	Jul	Aug	Sep	Oct	Nov	Average
1	1.4 CD	0.6 F	2.0 BC	1.1 ^a DE	0.9 ^a EF	2.0 BC	b	b	1.3 ^a DE	1.3
2	1.4 CD	0.9 EF	1.5 CD	1.2 ^a DE		1.6 ^a CD	0.8 ^a EF	1.3 ^a DE	1.8 C	1.3
3	0.9 EF		1.1 DE	2.3 B	b	2.8 A	b	b	b	1.8
4	1.1 DE		1.4 CD		b	b	b	b	b	1.3
Average	1.2	0.8	1.5	1.5	0.9	2.1	0.8	1.3	1.6	

Means followed by different uppercase letters in rows or columns are significantly different (LSD, $P < 0.05$).

^a Moderate rain flooded the treatment site.

^b Excessive rain modified the characteristics of the breeding sites and prevented applications during this interval.

Table 3

Reduction in the level of population of *Anopheles pseudopunctipennis* larvae due to the action of nematodes *Romanomermis iyengari* in Pochutla, Oaxaca, Mexico, 1999

Site		Jan	Feb	Apr	Jun	Jul	Aug	Sep	Oct	Nov	Mean
1	ALCpre ^c	4.6	2.1	6.3	0.3	0.4	1.1			0.5	2.2 A
	ALCpos ^d	1.9	0.7	0.1	0.16	0.2	0.02			0.2	0.47 B
	% reduction	58.7	66.7	98.4	46.7 ^a	50.0 ^a	98.3	b	b	60.0 ^a	68.4
2	ALCpre	3.0	2.1	2.2	1.0		3.9	4.0	3.0	6.4	3.2 A
	ALCpos	1.1	1.3	0.4	0.3		0.3	1.5	0.1	1.0	0.8 B
	% reduction	63.3	38.1	81.8	70.0 ^a		92.2 ^a	62.5 ^a	96.0 ^a	84.4	73.5
3	ALCpre	7.6		4.2	3.1		0.2				3.8 A
	ALCpos	2.1		0.7	0.1		0.1				0.8 B
	% reduction	72.1		99.8	96.8	b	50.0	b	b	b	79.7
4	ALCpre	5.5		17.0							11.3 A
	ALCpos	1.8		2.4							2.1 B
	% reduction	67.3		85.9		b	b	b	b	b	76.6
	Average (%)	65.4	52.4	91.5	71.2	50.0	80.2	62.5	96.0	72.4	

Means followed by different uppercase letters are significantly different (Wilcoxon's signed rank test, $P < 0.01$).

^a Moderate rain that partially flooded the breeding sites.

^b Excessive rain that modified the characteristics of the breeding, which stopped the sprayings from being made.

^c Average larval concentration pre-treatment application.

^d Average larval concentration post-treatment application.

Table 4

Percentage of parasitism and infestation means as indicators of the capacity of the nematode *Romanomermis iyengari* to survive in Pochutla, Oaxaca, Mexico, 1999

Site	Month	Parasitism (%)	Average (%)	Mean infestation	Average
1	May	45.0		0.8	
	Jun	30.0	53.4	0.5	1.1
	Jul	66.7		1.3	
	Aug	72.0		1.7	
2	Jun	59.0		1.6	
	Jul	83.0	60.0	1.6	1.1
	Aug	72.0		0.9	
	Sep	26.1		0.4	
Average		56.7		1.1	

June, July, and August, the months with higher PP values, showed also the largest MI rates.

3.3. Mosquito larvae population reduction

The reduction of *An. pseudopunctipennis* larval populations by *R. iyengari* in the four breeding sites is shown in Table 3. The mermithid treatment showed a significant reduction in larvae from 38 to 99.8%, and the overall reduction averaged 73.3%. Site No. 3 showed the highest percentage reduction value, but also the lowest value in August. In contrast, Site 1 had the lowest average control percentage, but some of the highest observed values during April and August. On average, only April, August, and October had a reduction percentage above 80%, which was more related to MI rates than to PP in these months.

The calculated statistic for the Wilcoxon signed rank test was 115.00, indicating the existence of a highly sig-

nificant difference ($P < 0.01$) between ALCpre and ALCpos, which had overall mean values of 5.1 and 1.0 larvae, respectively.

3.4. Nematode survival

In areas with stagnant water, such as those found in Sites 1 and 2, *R. iyengari* was found parasitizing mosquito larvae after 4 months of the last application, thus demonstrating its potential for persistence. In Site 1, between May and August, a PP between 30 and 72% and an MI of 0.5–1.7 nematodes/larva were observed. In Site 2, PP values ranged between 26.1 and 83%, whereas MI rates varied between 0.4 and 1.6 nematodes/larva from June to September (Table 4). During the rainy season, the water flow increased, causing the larvae and nematodes to be swept away, which drastically affected the survival of the bio-control organism.

4. Discussion

The results obtained over an estimated treatment area of 30,000 m² showed that all monthly sprayings of *R. iyengari* at a rate of 3000 nematodes m⁻², effectively reduced *An. pseudopunctipennis* population levels during 1999.

The high variability of PP, MI, and RP (parasitism of 46–100%, mean infection values of 0.6–2.8 nematodes/larva and reduction percentages ranging between 38.1 and 99.8%) is probably mostly due to the dilution of the inoculum by rain and partly to the inconsistency of applicator's performance. Dilution of the inoculum was produced by the water flow in the central area of the breeding site, which decreased the density of nematodes per m² and hence its effectiveness. A similar condition was described by Vladimirova et al. (1990), who made several applications of *R. iyengari* and *Romanomermis culicivorax* Ross and Smith in breeding sites of *Anopheles superpictus* Grassi, *An. pulcherrimus* Theobald and *An. hyrcanus* Pallas. They obtained average parasitism levels between 46 and 98%, and attributed the variability of infection levels to the effects of flowing water, density of aquatic vegetation, elevated salinity, and lowered nematode densities.

The periodic treatment of approximately 30,000 m² in the four breeding sites was a large undertaking and this may have influenced the differences in parasitism levels, as it was not possible to make uniform applications in the entire breeding site. Also, a number of topographical and hydrological factors increased the variability in the breeding sites. For example, pH (range 6.8–8.3) and temperature (range 28–33 °C) remained fairly constant, but salinity varied widely (range 115–280 mg l⁻¹). On average, Site 3 and August showed the lowest salinity values (Table 5).

Site No. 3 showed the highest PP and larval population reduction values. Thus, smaller and stagnant (non-flowing) water sites, may increase the effectiveness of *R. iyengari* mostly because of a more uniform and higher

pre-parasitic density, but water salinity capacity may also play a role. This agrees with results obtained by Pérez et al. (1998) and Santamarina et al. (1999), who under smaller, stagnant breeding sites, obtained better mosquito control. A uniform application of the nematodes at adequate concentrations is required for a higher effectiveness of control (Santamarina, 1994; Santamarina et al., 1992, 1996a,b, 1999; Pérez et al., 1998).

Our data showed that *R. iyengari* had the ability to recycle over a 4-month period in areas with stagnant water, such as Sites 1 and 2. This indicated that *R. iyengari* had good conditions for its reproduction. This means that post-parasitic nematodes were able to grow, molt to the adult stage, mate, lay eggs, and develop to the infectious pre-parasitic nematodes under these conditions. Similar results were obtained by Santamarina (1994) with the application of *R. iyengari* on *Anopheles albimanus* Wiedmann, *Culex nigripalpus* Theobald and *Psorophora* sp. larvae breeding sites.

Most of the monthly applications of the *R. iyengari* nematode, at a rate of 3000/m², significantly reduced *An. pseudopunctipennis* larvae populations in breeding sites with flowing water. The effectiveness of this nematode on other mosquito species has been demonstrated elsewhere (Chandahas and Rajagopalan, 1979; Santamarina, 1994; Santamarina et al., 1992, 1996a) as well as the susceptibility of *An. pseudopunctipennis* to other nematode species (Santamarina et al., 1996b).

Reductions in larval mosquito densities have been associated with decrease in malaria prevalence in human populations. Rojas et al. (1987) demonstrated significant reductions in malaria vectors and prevalence of malaria in El Valle, Colombia after introduction of *R. culicivorax* in mosquito breeding areas. Therefore, it is expected that in the Oaxacan coast, an endemic malaria area, the risk of malaria transmission may be reduced.

Our results show a high potential of this nematode species for controlling naturally *An. pseudopunctipennis* mosquito larvae populations in backwater regions of streams typical at malaria endemic areas. Because of the

Table 5
Salinity, pH, and temperature values observed at the breeding sites treated with *Romanomermis iyengari* in Pochutla, Oaxaca, 1999

Site	Parameter	Jan	Feb	Apr	Jun	Jul	Aug	Sep	Oct	Nov
1	pH	7.6	8.3	7.8	7.1	6.8	6.8			6.2
	Salinity, mg/liter	240	215	260	230	220	180			210
	°C	29	32	33	30	31	30			32
2	pH	7.1	7.8	7.4	8.0		7.2	6.8	6.9	7.4
	Salinity, mg/liter	270	280	249	270		180	210	160	220
	°C	30	33	29	32		31	29	30	31
3	pH	7.6		7.8	6.8		6.9			
	Salinity, mg/liter	160		140	120		115			
	°C	28		32	33		31			
4	pH	7.5		7.6						
	Salinity, mg/liter	180		185						
	°C	32		30						

advantages this nematode brings to the reduction in the use of chemical pesticides, it is important to consider this biological alternative in mosquito management programs.

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