Efficacy of entomopathogenic nematodes (Nematoda : Rhabditida) against *Culex gelidus* (Diptera : Culicidae) larvae

Wongdyan Pandii[1], Sornpet Maharmart[1], Supawadee Boonchuen[1], Suthep Silapanuntakul[2], Vacharee Somsook [3]
[1]Department of Parasitology, Faculty of Public Health, Mahidol University
[2]Department of Environmental Health Sciences, Faculty of Public Health, Mahidol University
[3]Entomology and Zoology Division, Plant Protection Research and Development Office, Department of Agriculture, Ministry of Agriculture and Co-operative

ABSTRACT

The breeding sites of *Culex gelidus*, a secondary vector of Japanese Encephalitis, are close to agricultural areas and homes, such as temporary and semi-permanent fresh ground water from pig farms and rice fields. Entomopathogenic nematodes (EPN) are an alternative bio-control for insects. Therefore, the application of EPN to control *Cx. gelidus* larvae was studied with the objectives of 1) determining the efficacy of EPN between 2 genera against 3rd–4th instars larvae of *Cx. gelidus* under laboratory conditions and 2) determining the dosages of EPN effective against *Cx. gelidus* larvae. The experiment was carried out in the laboratory under room temperature of 29 ± 2°C and relative humidity (RH) 70–80 %.

Results indicated that mortality rates of 3rd–4th instars *Cx. gelidus* larvae caused by *Steinernema carpocapsae* (Weiser) EPN were greater than *Heterorhabditis* indica (Local Thai strain) 63% and 13%, respectively. The mortality of both control groups was 5%. Infection rates between the 2 genera were 14.5% and 2%, respectively. The thorax of dead *Cx. gelidus* larvae were the site where EPN were mostly found, more than other parts of their bodies. Comparing mean difference for mortality rates of *Cx. gelidus* larvae between 2 genera at 48 and 72 hours post exposure found significant difference by T-Test (p-value < 0.05). *S. carpocapsae* (Weiser) kills more than 50% at dosage 2000 and 4000 IJs per larvae, but there was no significant difference in number of 3rd–4th instars larvae *Cx. gelidus* killed at either dosage. There was significant interaction between the 2 genera at the various dosages (p-value < 0.01, analysis by 2 way ANOVA).

The results showed that under laboratory conditions, *S. carpocapsae* (Weiser) EPN have potential as a bio-control against 3rd–4th instars *Cx. gelidus* larvae. Further study should involve water depth, temperature, pH of water and feeding behavior of target host prior to use in field trials.

KEY WORDS : ENTOMOPATHOGENIC NEMATODE / *STEINERNEMA CARPOCAPSAE* (WEISER) / HETERORHABDITIS INDICA (LOCAL THAI STRAIN) / *CULEX GELIDUS* LARVAE

ประสิทธิภาพของไส้เดือนฝอย (Nematoda: Rhaditida) ต่อการเข้าทำลายลูกน้ำยุงรำคาญ *Culex gelidus* (Diptera: Culicidae)

[1]วงเดือน ปั้นดี, [1]ศรเพชร มหามาตย์, [1]สุภาวดี บุญชื่น, [2]สุเทพ ศิลปานันทกุล, [3]วัชรี สมสุข
[1]ภาควิชาปรสิตวิทยา คณะสาธารณสุขศาสตร์ มหาวิทยาลัยมหิดล
[2]ภาควิชาวิทยาศาสตร์อนามัยสิ่งแวดล้อม คณะสาธารณสุขศาสตร์ มหาวิทยาลัยมหิดล
[3]กลุ่มกีฏและสัตววิทยา สำนักวิจัยและพัฒนาอารักขาพันธุ์พืช กรมวิชาการเกษตร กระทรวงเกษตรและสหกรณ์

บทคัดย่อ

ยุงรำคาญ (*Culex gelidus*) เป็นพาหะรองของโรคไข้สมองอักเสบ แหล่งเพาะพันธุ์ส่วนใหญ่อยู่ใกล้กับบ้านเรือนที่เป็นลักษณะเชิงเกษตรกรรม โดยเฉพาะน้ำขังบริเวณรอบคอกสัตว์และน้ำขังในนาข้าว ปัจจุบันมีการใช้ไส้เดือนฝอยในการควบคุมแมลงทางชีวภาพ ดังนั้นจึงได้มีการประยุกต์ใช้ไส้เดือนฝอยในการควบคุมลูกน้ำยุงรำคาญ โดยมีวัตถุประสงค์ คือ เปรียบเทียบประสิทธิภาพระหว่างไส้เดือนฝอย 2 สกุล คือ *Steinernema carpocapsae* (Weiser) และ *Heterorhabditis indica* (Local Thai strain) ที่มีต่อการควบคุมลูกน้ำยุงรำคาญ ในระยะที่ 3-4 และปริมาณของไส้เดือนฝอยที่เหมาะสมต่อการควบคุมลูกน้ำยุงรำคาญ ทั้งนี้เป็นการทดลองภายในห้องปฏิบัติการที่อุณหภูมิห้อง 29 ± 2 องศาเซลเซียส และความชื้นสัมพัทธ์ 70–80 %

ผลการทดลองพบว่า อัตราการตายของลูกน้ำยุงรำคาญ ระยะที่ 3-4 จากไส้เดือนฝอยสกุล *S. carpocapsae* (Weiser) สูงกว่า *H. indica* (Local Thai strain) กล่าวคือ 63% และ 13% ตามลำดับ ในขณะที่กลุ่มควบคุม อัตราการตายอยู่ที่ 5% ทั้ง 2 สกุล และพบว่าอัตราการติดเชื้ออยู่ที่ 14.5% และ 2% ตามลำดับ ส่วนใหญ่แล้วลูกน้ำที่ตายจะพบไส้เดือนฝอยอยู่บริเวณช่องอกมากกว่าบริเวณอื่น และเมื่อเปรียบเทียบค่าเฉลี่ยการตายของลูกน้ำยุงรำคาญ ระยะที่ 3-4 ในเวลา 48 และ 72 ชั่วโมง พบว่าลูกน้ำที่ตายจาก *S. carpocapsae* (Weiser) สูงกว่าลูกน้ำที่ตายจาก *H. indica* (Local Thai strain) อย่างมีนัยสำคัญทางสถิติ ($p\text{-value} < 0.05$) ปริมาณของไส้เดือนฝอยต่อลูกน้ำยุงรำคาญ สกุล *S. carpocapsae* (Weiser) ที่ทำให้อัตราตายของลูกน้ำมากกว่า 50% คือ ที่ 2000:1 ซึ่งไม่แตกต่างกับที่ 4000:1 จากการวิเคราะห์โดยใช้ 2 way ANOVA พบว่า มีปฏิกิริยาร่วมระหว่างไส้เดือนฝอย 2 สกุล ($p\text{-value} < 0.001$)

ผลการทดลองแสดงให้เห็นว่า ไส้เดือนฝอยสกุล *S. carpocapsae* (Weiser) มีความเป็นไปได้ในการนำมาใช้ควบคุมลูกน้ำยุงรำคาญ *Cx. gelidus* ระยะที่ 3–4 แต่ถึงอย่างไรควรมีการศึกษาถึงระดับความลึกของน้ำ อุณหภูมิของน้ำ ความเป็นกรดด่าง และพฤติกรรมการกินของลูกน้ำยุงรำคาญก่อนที่จะนำไปใช้ทดลองในภาคสนามต่อไป

คำหัส : ไส้เดือนฝอย, *STEINERNEMA CARPOCAPSAE* (WEISER), HETERORHABDITIS INDICA (LOCAL THAI STRAIN), ลูกน้ำยุงรำคาญ

1. Introduction

Japanese encephalitis (JE), a central nervous system (CNS) infection caused by a flavivirus, is the most important public health problem among other encephalitis in Thailand (1). The vector of the disease is Culicine mosquitoes which in Thailand they are *Culex tritaeniorhynchus Cx. gelidus* and *Cx. fuscocephala*. Among these three species, *Cx. tritaeniorhynchus* is the main vector of the disease while *Culex gelidus* Theobald is secondary vector (1).

In Thailand, JE virus was reported to be isolated from *Cx. gelidus* in 1972 (2) and this virus was also found in *Cx. gelidus* in suburban area of Bangkok (3). This mosquito is close to man and domestic animals such as pigs, cows and buffaloes in rural and urban communities of Thailand. Their larvae have been collected from various habitats such as temporary and semi-permanent fresh ground water from pig farms and rice fields.

Biological control, a natural control method, is one of the alternative means that has been used to control pests and one example of this method used pathogen from nature like bacteria, protozoa, virus, fungi and nematode(4). Entomopathogenic nematodes (EPN), which are insect pathogens, have become increasingly popular in insect control since the infective juveniles kill insects in 24-48 hr. and they are proven to be safe for plants, animals and environment by The United States Environmental Protection Agency(5).

In Thailand, most of the studies regarding the efficacy of EPN against mosquito larvae are for control of *Aedes aegypti* (L.) and the results show they are significantly effective(6). The aim of this study is to determine the efficacy of EPN, i.e. *Steinernema carpocapsae* (Weiser) and *Heterorhabditis indica* (Local Thai strain), under laboratory conditions, as to whether they can be used as an alternative mean in controlling *Cx. gelidus* larvae or not.

Objectives of Study

General objective

To determine the efficacy of entomopathogenic nematodes against *Cx. gelidus* larvae under laboratory conditions.

Specific objectives

To compare the efficacy of entomopathogenic nematodes of the genera *Steinernema carpocapsae* (Weiser) and *Heterorhabditis indica* (Local Thai strain) against *Cx. gelidus* larvae.

To determine the dosages of entomopathogenic nematodes which is effective against *Cx. gelidus* larvae.

Materials and methods

Research Design

Experimental 2 x 4 factorial research designs were used in this study. Each experiment had 4 replications. Mortality of 3^{rd}–4^{th} instar larvae of *Culex gelidus* by entomopathogenic nematodes, *S. carpocapsae* (Weiser) and *H. indica* (Local Thai strain), were then determined. Factors A are two genera of entomopathogenic nematodes and factors B are different dosage of nematode (numbers of EPN per *Cx. gelidus larva*) which is 500, 1000, 2000 and 4000.

Experimental Place

The experiments were carried out at :

1. Laboratory of Parasitology Department, Faculty of Public Health, Mahidol University, Bangkok and

2. Entomology and Zoology Division, Plant Protection Research and Development Office, Department of Agriculture, Ministry of Agriculture and Co-operative, Bangkok.

The study was divided into three parts as follows :

2.1 Preparation of the entomopathogenic nematodes, *Steinernema carpocapsae* (Weiser) and *Heterorhabditis indica* (Local Thai strain)

The procedure for preparing nematodes of the two genera were followed the manual recommended by the Entomology and Zoology Division, Plant Protection Research and Development Office, Department of Agriculture. Using dilution counting technique they were identified under microscope. Four million nematodes are stored in synthetic sponge sealed in plastic bag. Rinse these nematodes off synthetic sponge using dechlorinated tap water.

2.2 Preparation of 3^{rd}–4^{th} instar larvae of *Cx. gelidus.*

Engorged females of *Cx. gelidus* were collected at rice fields in Bangbuathong district, Nonthaburi province using aspirator from man baits and light trap. The mosquitoes were then transferred into paper cups (approximately 100 individuals per cup). Cotton wool soaked with 5% sugar solution was provided as the source of food during transportation. At the laboratory, female mosquitoes were released into 30 x 30 x 30 cm cages. Cotton wool soaked with a mixture of 5% sugar solution and multivitamin syrup (1:1) was supplied as food.

A few 2-3 days later, plastic cups containing rice straw infusion water were placed in cage for oviposition. The female mosquitoes lay egg rafts in plastic cups. About 200-300 hatched larvae were introduced into an enamel tray containing about 1.5 liters of 3 days fermented rice straw infusion water. Add homogenized dog biscuit which were provided as food for larvae. (7)

The larvae which were suitable for the test are 3rd–4th instars larvae. This is done by observation of their body length which were 0.4-0.5 cm and 0.8-1.0 cm respectively.

2.3 Determination of the efficacy of EPN against *Cx. gelidus* larvae. Four dosages of EPN per larva, i.e. 500, 1000, 2000 and 4000 were tested against *Cx. gelidus* larvae, each dosage consisted of four replications and control group. The total volume of water filled in each cup was 38.5 cm^2 and water depth was 2.5 cm. The observations time for mortality and infection were done at 18, 24, 48, 72 and 96 hours after the larvae are exposed to EPN. Dead larvae (no movement and no feed) were dissected in saline water under microscope. Record and take photographs of dissected larvae.

2.4 Data Analysis

The results of four replicates in exposure time observed was calculated by mean ($\overline{X}$), percentage (%) and standard deviation (S.D.). The comparatives mean percentage difference of *Culex gelidus* larvae mortalities by using entomopathogenic nematodes between two genera, *S. carpocapsae* (Weiser) and *H. indica* (Local Thai strain) and the mean percentage difference level of nematode per larvae was analyzed by Analysis of Variance 2–way ANOVA. The determination of significant difference p-value level was at 0.05.

3. Results

3.1 The mortality of the 3rd–4th instars *Culex gelidus* larvae by two genera of entomopathogenic nematodes.

The mortality of the third and fourth instars *Cx. gelidus* larvae caused by *S. carpocapsae* (Weiser). The mortality rates of either dosages were different between 4000, 2000, 1000, 500 and 0 IJs per larvae and were 63.0%, 53.0%, 31.0%, 29.0% and 5.0%, respectively (Table 1).

The mortality in the third and fourth instars *Cx. gelidus* larvae in part of *H. indica* (Local Thai strain) had a least difference in each level of nematodes per larvae and exposure times. The mortality rates of either dosage were different at 4000, 2000, 1000, 500 and 0 IJs per larvae and were 13.0%, 8.0%, 5.0%, 4.0% and 5.0%, respectively (Table 1).

The data indicated that the mortality rates in highest dosage were difference between two genera, *S. carpocapsae* (Weiser) and *H. indica* (Local Thai strain) namely 63% and 13% and the mortality of both control groups were 5%.

Table 1 Mean and standard deviation mortality of the 3rd–4th instars *Culex gelidus* larvae infected by two genera of entomopathogenic nematodes.

Species of nematodes	Dosage of nemato-des per larva	n	Cumulative No. of dead larvae in duration of time (Hours)					Mortality rates (%)
			18	24	48	72	96	
S. carpocapsae (Weiser)	4000	4	0.0±0.0	1.0±0.8	7.3±1.9	13.0±1.4	15.8±1.7	63.0
	2000	4	0.0±0.0	0.5±0.6	5.8±3.1	11.3±2.8	13.3±2.2	53.0
	1000	4	0.0±0.0	0.0±0.0	4.3±1.0	6.0±1.4	7.8±1.7	31.0
	500	4	0.0±0.0	0.3±0.5	3.3±2.2	5.3±2.5	7.3±1.9	29.0
	0	4	0.0±0.0	0.0±0.0	0.0±0.0	0.5±0.6	1.3±0.5	5.0
H. indica (Local Thai strain)	4000	4	0.0±0.0	0.0±0.0	0.5±0.6	1.8±1.0	3.3±2.1	13.0
	2000	4	0.0±0.0	0.0±0.0	0.0±0.0	0.8±0.5	2.0±0.8	8.0
	1000	4	0.0±0.0	0.0±0.0	0.5±0.6	0.8±1.0	1.3±1.0	5.0
	500	4	0.0±0.0	0.0±0.0	0.3±0.5	0.5±0.6	1.0±0.8	4.0
	0	4	0.0±0.0	0.0±0.0	0.0±0.0	0.3±0.5	1.3±1.0	5.0

3.2 The infection of *Cx. gelidus* larvae by two genera of entomopathogenic nematodes.

S. carpocapsae (Weiser), after dissected larvae the total infection rate was 14.5% and that found the nematodes infected mostly in thorax than head and abdomen of larvae mosquitoes 83.0%, 10.0% and 7.0%, respectively. While *H. indica* (Local Thai strain), after dissected larvae the total infection rate was 2.0% and the nematode genus *H. indica* (Local Thai strain) were found only in thorax of mosquito larvae (Figure 1).

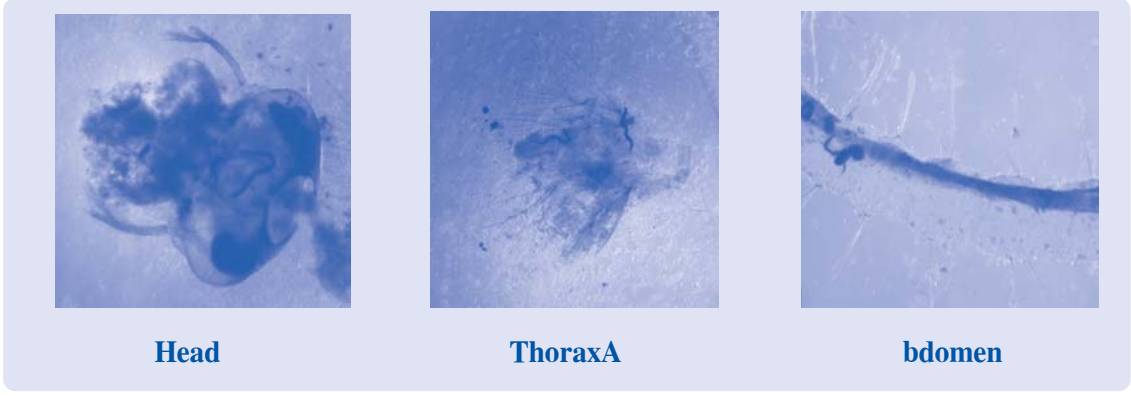

Figure 1 Infective stages of nematodes in different parts of *Cx. gelidus* larvae. ×100

3.3 Analytical mortality of odds ratio of EPN applied against the third and fourth instars *Cx. gelidus* larvae

The odds ratio of *S. carpocapsae* (Weiser) applied against larvae of *Cx. gelidus* in each dosage compared to control group (0 IJs per larvae) at 4000, 2000, 1000 and 500 were 32.35 (95% CI 11.32-99.62), 21.43 (95% CI 7.55-65.50), 8.54 (95% CI 2.96-26.44) and 7.76 (95% CI 2.68-24.13), respectively.

For genus *H. indica* (Local Thai strain) applied against larvae of *Cx. gelidus* in each dosage compared to control group (0 IJs per larvae) at 4000, 2000, 1000 and 500 were 2.84 (95% CI 0.89-9.56), 1.65 (95% CI 0.47-6.07), 1 (95% CI 0.24-4.14) and 0.79 (95% CI 0.17-3.53), respectively.

3.4 The comparative mean different of mortality between *S. carpocapsae* (Weiser) and *H. indica* (Local Thai strain) applied against the third to fourth instars *Cx. gelidus* larvae at either dosage.

The two way interaction test found that there were interactions between 2 factors ($p = 0.008$; $df = 4$; $F = 4.178$). Multiple comparison of interaction between two genera in each dosages of EPN per larvae that found significant difference interaction in varies dosages $\chi^2 = 48.435$, $df = 15$, $p < 0.001$ (Kruskal Willis Test) and multiple comparison to determine mean rank difference between groups namely (A,I) (A,J) (A,K) (A,L) (A,M) (A,N) (A,O) (A,P) (B,I) (B,J) (B,K) (B,L) (B,M) (B,N) (B,O) (B,P) (C,I)(C,J) (C,K) (C,L) (C,M) (C,N) (C,O) (C,P) (D,I) (D,J) (D,K) (D,L) (D,M) (D,N) (D,O) (D,P) (E,J) (E,K) (E,L) (E,M) (E,N) (E,O) (E,P) (F,L) (F,M) (F,N) (F,O) (F,P) (G,K) (G,L) (G,M) (G,N) (G,O) (G,P) (H,L) (H,M) (H,N) (H,O) (H,P) (I,O) (I,P) (J,P) and (K,P) (Figure 2).

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
A (12.0)									*	*	*	*	*	*	*	*
B (13.5)									*	*	*	*	*	*	*	*
C (14.5)									*	*	*	*	*	*	*	*
D (15.5)									*	*	*	*	*	*	*	*
E (16.75)										*	*	*	*	*	*	*
F (19.5)												*	*	*	*	*
G (21.5)											*	*	*	*	*	*
H (25.63)												*	*	*	*	*
I (38.13)	*	*	*	*											*	*
J (39.38)	*	*	*	*	*											*
K (42.88)	*	*	*	*	*		*									
L (48.38)	*	*	*	*	*	*	*	*								
M (49.25)	*	*	*	*	*	*	*	*								
N (50.5)	*	*	*	*	*	*	*	*								
O (53.88)	*	*	*	*	*	*	*	*	*							
P (58.75)	*	*	*	*	*	*	*	*	*	*						

* Significant difference (p-value < 0.05)

The numbers in parenthesis are mean rank of dead mosquito larvae after interaction dosages.

Figure 2 Difference and similar of interaction between two genera of EPN in each dosage of EPN per *Cx. gelidus* larvae. * Difference and similar of interaction between two genera of EPN in each dosage of EPN per *Cx. gelidus* larvae.

***(A = S500+H500, B = S500+H1000, C = S1000+H500, D = S1000+H1000, E = S500+H2000, F = S1000+H2000, G = S500+H4000, H = S1000+H4000, I = S2000+H500, J = S2000+H1000, K = S2000+H2000, L = S2000+H4000, M = S4000+H500, N = S4000+H1000, O = S4000+H2000 and P = S4000+H4000)**

4. Discussion

Efficacy of entomopathogenic nematodes of the genera *Steinernema carpocapsae* (Weiser) and *Heterorhabditis indica* (Local Thai strain) against *Cx. gelidus* larvae. This study has showed that the infective juveniles (IJs) of S. carpocapsae (Weiser) caused significant higher mortality than control ($P < 0.001$). The mortality rate at highest dosage of nematodes per larva was more than 60%. In contrast, the mortality of larvae by *H. indica* (Local Thai strain) compared to control was not significant difference. The mortality rate at highest dosage was lower than 15%. Comparative two genera had significant ($P < 0.05$). Infection rate from *S. carpocapsae* (Weiser) was higher than that from *H. indica* (Local Thai strain), being 14.5 and 2%, respectively. Mortality rates had strongly started at 48 hour in genera *S. carpocapsae* (Weiser) but not seem in genera *H. indica* (Local Thai sp). The reasons for these differences remain unclear and may be related to nematode behavior, efficiency of nematodes, and adaptation to a given host (attraction to the host of ability to overcome defense mechanism) (8). In study of Fallon et al., 2006 (9) found similarity studied our that efficacy of EPN against the cottonwood borer, *Plectrodera scalator* (Fabricius), *Steinernema feltiae* SN and *S. carpocapsae* all killed 58% and 50% of larvae, while *H. indica* MG-13 killed less than 10% of larva in both assays. The host diet was compared to that of *Anoplophora glabripennis*, a host against which *S. carpocapsae* Sal and *S. feltiae* produced 71–100% mortality in similar bioassay (10-11).

The dosage of entomopathogenic nematodes which is effective against *Cx. gelidus* larvae. This study has showed that the infective juveniles (IJs) of *S. carpocapsae* (Weiser) in the dosage had significant different mortalities of mosquito larvae than control ($P < 0.001$). Although between dosage 2000 and 4000 IJs per larva had highest mortality rates (more than 50%), but there was not significant different among the two dosage in the mortality. Therefore, the dosage suitable for control larvae is 2000 IJs per larvae. In contrast, the mortality of larvae by *H. indica* (Local Thai strain) was so low that it may be unsuitable for control *Cx. gelidus* larvae.

George, Pionar and Kaul, 1981(12) showed the similar results when the nematode concentration increases, a higher number are ingested and more nematodes reach the body cavity of host. The larval mortality rates of *Aedes aegypti* was a positive linear function of nematode dosage and exposure time. (13) Size (stage) of host, parasitism in general was highest in fourth-instar larvae. (12,14) Host reaction, *Cx. pipiens* larvae are able to melanize nematodes that have entered their hemocoels. The melanization process is mush more rapid and strong in the third and fourth-instar larva than the second. In the latter, a newly entered nematode is often able to initiate development and liberate the bacterium. Only after the maximum melanization number is reached can additional nematodes develop freely in the hemocoel and release their bacteria. (14) Then, involve behavior of larvae feeding (bottom

feeding). Level of depth water that was found related feeding behavior of larvae mosquito. Manit, Chaiyaporn and Anu, 2004 (6) showed that five nematodes had most effect in controlling the larvae of *Aedes aegypti* (L.) than other mosquito larvae because feeding behavior are "Collecting-gathering" that mean highly expose than other larvae. The aquatic habitat offers an excellent environment for nematode survival. However, steinernema and heterorhabditids are soil organisms and are not adapted for directed motility in the aquatic environment (15).

Mosquito feeding behavior and spatial of the nematodes also affect efficacy. The substrate type influences uptake of nematodes by the mosquito larvae. Nematodes settle quickly to the bottom. Mosquitoes will easily remove them from a smooth surface, but when debris is added, the nematodes are less available to the mosquitoes larvae (16,17). The result that found that larval mortality of *Aedes aegypti* was a positive linear function of nematode dosage and exposure time (13).

Responses interaction to *Cx. gelidus* larvae infected between two genera in combining each dosage of EPN. This study has showed multiple comparison had mean rank difference significant ($p < 0.001$) namely interaction between two genera of EPN in each dosage of EPN per larva. *S. carpocapsae* and *S. glaseri* co-invade *Galleria* larvae in the laboratory (18, 19). Koppenhofer et al. (1995)(18) found no effect on number of nematodes establishing in mixed versus single infections, whereas Wang and Ishibashi, 2006 (19) found that more *S. carpocapsae* invaded when mixed with *S. glaseri* than when they were used alone. But normally *Heterorhabditis* and *Steinernema* cannot co-exist within a host, though clearly they can co-infect (20). In addition, responses by *Heterorhabditis* to insects harboring heterospecifics have received less attention than those of steinernematids.

Pertersen and Willis, 1970 (14) studied the susceptibility of insects to control agents has generally been found to decline with increases insect size that has been demonstrated with mermithid nematodes against mosquitoes larvae.

Mosquitoes and black flies would appear to be prime candidates for control with nematodes because they readily ingest nematodes. However, a number of factors reduce efficacy, including damage to the nematode during ingestion, (21-22) host immune response and spatial separation of host and nematode. (17)

Gaugler and Molloy, 1981 (21) demonstrated that the nematodes were physically excluded during feeding of the first through third instars, rendering the host resistant to infection. Older instars were susceptible to infection. The principle regulating susceptibility was nematode injury caused by the larva mouthparts during ingestion, Dadd, 1971 (22) observed that larvae size excluded nematode ingestion by early instars and that some nematode injury occur during ingestion.

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