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The pathogenic potential of *Steinernema* sp. against the dengue virus vector *Aedes aegypti*

Santhana Bharathi N, Deepa A, Chithra P and Sujatha K

Abstract
The *Steinernema*– *Xenorhabdus* symbionts were tested for its pathogenicity against 1st to 4th instar larvae and pupae of *Aedes aegypti* under laboratory conditions. The larval mortality study was carried out for treatments T_1 to T_{10} with inoculum dosage of 100 to 1000 IJs/10 larvae and pupae of *A. aegypti*. After 24 and 48 h of application there was no mortality observed in 1st and 2nd instar larvae of *A. aegypti*. The maximum larval mortality of 63% and 60% were noted as 48 h of application in 4th instar and pupae respectively. Further biochemical studies showed a highly significant reduction when compared to the control groups. This may be due to the utilization of these biochemical compounds for the metabolism of EPNs. The present work has shown the efficiency of *Steinernema*– *Xenorhabdus* symbionts against dengue viral vector *A. aegypti*. The study paves a way to identify novel nematodes and bacterial toxins against *A. aegypti*.

Keywords: EPNs, *Aedes aegypti*, pathogenicity, bio chemical

Introduction
Nematodes are non-segmented roundworms, soft bodied, that are obligate or sometimes facultative parasites of insects. Nematodes come under the Phylum Nematoda found in nearly all environments throughout the world which occur naturally in soil environments (Kaya and Gaugler, 1993) [12]. The Entomopathogenic Nematodes (EPN) in the Steinernematidae and Heterorhabditidae families are considered interesting candidates for biological control programs since they have a mutualistically symbiotic association with enterobacteria *Xenorhabdus* sp. and *Photorhabdus* sp. that makes them strongly virulent to insects (Kaya, 1993) [13]. Infective juveniles (IJs) nematodes search and enter into the host, then release the symbiotic bacteria into the haemocoel; the bacteria is the primary agent responsible for killing the host usually within 24 to 48hours.The bacterium needs the nematode for protection from the external atmosphere and penetration into the host's haemocoel to inhibit host's antibacterial proteins (Hazir *et al.*, 2004) [11]. Once inside the host, the bacterial symbionts create a favorable environment for the nematode to propagate by suppressing the immune protein of the insect (Gotz *et al*., 1981) [9] and providing nutrition for the development and reproduction of nematodes (Poinar, 1983) [22]. The *Aedes aegypti* (yellow fever mosquito), spreads dengue fever, chikungunya, zika fever and yellow fever and other deadliest diseases. *A. egypti* is the main vector and usually lives near human habitation areas (Rueda *et al.*, 1990) [25]. Chikungunya virus has been reported in more than 45 countries with epidemic in India. Various control measures are used for the control of *Aedes* sp. population such as elimination of breeding sites, use of chemical control (organo-chlorides, organo-phosphates, and pyrethroids) genetic and biological control (*Beauveria bassiana* and *Metarhizium anisopliae*). However, repeated uses of inorganic pesticides pave ways to development of insecticide resistant and toxic to other non-targeted organisms. *Aedes* sp. might develop resistance to *B. bassiana* and *M. anisopliae* (Fukruksa *et al.*, 2017) [8]. Since single method of control is ineffective the effective mosquito control strategies including insecticides, biocontrol agents and environmental management are necessary produce the desired results (Poopathi and Tyagi, 2006) [23]. So, the present study was focused on controlling of *A. aegypti* by the bio pesticide *Steinernema – Xenorhabdus* symbiont and its biochemical changes such as protein, carbohydrate and lipid due to *Steinernema – Xenorhabdus* symbiont infection.

Materials and Methods
A total of ten soil samples were collected from different agricultural location and around

Pollachi and Udumalpet areas, Tamilnadu. 250 gram of soil samples were collected at a depth of 15 – 20 cm from the surface. The larvae of greater wax moth *Galleria mellonella* (Lepidoptera: Galleridae) were used for baiting the nematodes (Bedding and Akhurst, 1975) [2]. The *G. mellonella* larvae were cultured and maintained in a large container (30 cm Height and 10 cm Diameter) at room temperature (28 °C – 31 °C) on an artificial diet (contains corn meal, wheat flour, wheat bran, milk powder, glycerin, honey and yeast). Entomopathogenic nematodes were recovered from soil sample using the "insects baiting method" as described by Bedding and Akhurst (1975) [2]. Five number of 4th instar larvae of *G. mellonella* were placed in 100ml plastic containers with 50 grams of moist soil. Soils collected from different areas were placed separately in closed containers and tiny holes were made for respiration throughout the baiting period. Larvae were checked for infection every day and infected larvae were replaced with live larvae in the containers. The dead larvae were isolated and thoroughly rinsed in 0.01% formalin and placed in White's trap (Kaya and Stock, 1997) [15] until the emergence of third-stage infective juveniles of nematodes. To confirm the genus of the symbiotic bacteria associated with the EPNs, a loop of parasitized larval haemolymph were streaked on NBTA media as described by Akhrust, 1980. The plates were incubated at 28^0C for 24 hours. Bacterial colony color and bioluminescence property was noted. For further identification of symbiotic bacteria, morphological studies such as gram's staining and motility test was done.

Plate 1: Isolated EPNs

Mass rearing of *A. aegypti* was carried out in laboratory at room temperature (28 ˚C – 31 ˚C). Larval mortality bioassays were carried out in the laboratory condition. Nematodes in 50 ml of dechlorinated water was added in concentrations of 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 IJs per ten larvae as treatment of T_1, T_2, T_3, T_4, T_5, T_6, T_7, T_8, T_9, and T_{10} respectively. After 10 min, ten *A. aegypti* larvae (1st, 2nd, 3rd, and 4th instar) and pupa were placed in each treatment and maintained in laboratory. Three replications for each treatment from T_1 to T_{10} were maintained along with untreated control. Larval mortality was recorded after 24 and 48 hours. The larval death was confirmed by observing the larva frequently. Parasitized larvae of *A. aegypti* were dissected out to check for the presence of EPNs in their body under light microscope. Protein estimation by Lowry's method (Lowry *et al*., 1951) [16]. Carbohydrates estimation by Roe's method (Roe, 1995) [24]. Lipids estimation by Folch's Method (Folch *et al.*, 1957) [6] was carried out in the laboratory.

Probit analysis was done to find the lethal concentrations (LC_{50} and LC_{90}) of IJs against mosquito larvae (3rd and 4th) and pupae in after 24 and 48 h of application with a reliability interval of 95%. To determine whether there was a statistical significant difference among treatments having different dose of IJs against different life stages of *A. aegypti* was done according to Duncan Multiple Range Test (DMRT) and Regression analysis was done between the mortality percentages of different life stages of *A. aegypti*. All the statistical analysis was done by using SPSS V16.0 software. Results with $p<0.05$ were considered as a statistically significant.

Results and Discussion

Out of ten samples, Entomopathogenic nematodes were recovered from only one sample from Pongaliyur, Pollachi soil sample. Both biotic (vegetation and host availability) and abiotic (temperature, soil type, depth, moisture) factors are responsible for the presence of EPN in soil (Molyneux, 1985) [18]. The nematodes were harvested from the cadaver of infected larvae of *G. mellonella* by White's trap method (Kaya and Stock, 1997) [15] for further bioassay study against *A. aegypti*. The infected *G. mellonella* was black in colour, the results were in par with the Kaya and Nelson (1985) [14] this shows that the isolated EPN, in this study belongs to *Steinernema* Genus. Parasitized larvae turned black color because of the symbiotic bacteria which produce Mcf toxins that induce apoptosis in haemocytes and epithelial tissues, resulting in blood infection and abundance of tissue damage (Castagnola and Stock, 2014) [4]. According to Akhrust (1980) [1], *Xenorhabdus* sp. in NBTA media appeared as maroon color colonies. Similar observation was recorded in the present study. The bacteria were confirmed to the *Xenorhabdus* sp. as it was gram negative, motile and showed no bioluminescence. These bacteria live in symbiosis with nematodes and both share a complex life cycle which involves both symbiotic and pathogenic stages. The symbiotic bacteria will be released to haemocoel of host body by nematodes, although in most cases the bacteria alone are highly virulent while they circulate in the insect haemocoel and cause the death of the host due to septicemia by *Xenorhabdus* sp. (Forst and Nealson, 1996) [7].

Pathogenicity studies showed that no mortality was observed in 1st and 2nd instar larvae of *A. aegypti* after 24 and 48 h of application. The reason may be the whole nematodes were rarely ingested by 1st and 2nd instar larvae. This shows the penetration of nematode into the mosquito larvae was deterred because of the size. Similar observation was made by Dadd, (1971) [5] in *Culex pipiens* were the 1stand 2nd instar larvae were not infected by the nematode. Zohdy *et al*., (2013) [31] also studied that EPN's were not able to enter into 1st and 2nd instar larvae of *Culex quinquefasciatus*.

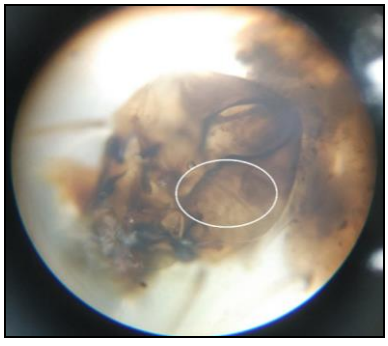

Plate 2: IJs identified in head region of *A. aegypti.*

As per the present study, increasing nematode numbers

(concentration) (df = 10, F=158.27, p < 0.01) in the treatments has significantly influenced the mortality rate of *A. aegypti* on all the tested life stages (df = 2, F = 405.15, p < 0.01) at both 24 and 48 h after application (df = 1, F = 141.84, p < 0.01). Similarly, interaction between treatment and life stage, treatment with time and vice-versa reached significant mortality (p < 0.01) while interaction of all the factors is not significant at p >0.05. Present study shows that mosquito larval mortality has increased when IJs has increased. Highest host mortality percentage was achieved with a highest numbers of IJs and nil or very low mortality was observed in treatments having low number of IJs. Cagnolo and Almiron, (2010) has reported that the IJs of *Steinernema rarum* were exposed against larvae of *Culexapicinus Philippi* and highest mosquito larval mortality (75%) was achieved with the highdose of 400:1 and no mortality was observed in lower doses tested. Infective rate of *S. rarum* was high at 23 ± 2°C. Similarly, Molta and Hominick, (1989) [17] observed the rate of melanization of the nematodes (= mortality %) and dosage are positively correlated with each other. Previous studies have shown that infection rate of *Steinernema carpocapsae* was higher than the *Heterorhabditis indica* against *Culex gelidus. Xenorhabdus ehlersii* effectively gave 100% mortality at 96 h, in both fed and unfed conditions (Wongdyan *et al*, 2008) [30]. This may be due to the bioactive compounds which were produced by *Xenorhabdus ehlersii* that are effective in killing mosquito larvae within a short period (Fukruksa *et al.*, 2017) [8].

Table 1: LC_{50} and LC_{90} of different stages of *A. aegypti* at both 24 and 48 h of after application under laboratory condition.

Time	Stages	*LC_{50}	95% Confidential Limit		*LC_{90}	95% Confidential Limit		CHI Square Value
			LCL	UCL		LCL	UCL	
After 24 Hours	3rd Instar	1263.71	1022.15	5411.56	1695.71	1264.37	9834.29	0.5558
	4th Instar	1084.39	829.24	2399.49	2010.22	1401.79	5752.47	0.192
	Pupae	1060.33	836.41	1941.39	1859.57	1351.933	4239.06	0.566
After 48 Hours	3rdInstar	1184.06	975.58	2204.58	1671.02	1280.47	3815.57	0.95
	4thInstar	768.06	609.67	1104.68	1542.79	1171.79	2847.91	0.284
	Pupae	831.74	678.27	1176.53	1538.54	1188.08	2671.78	0.323

*LC50=Lethal Concentration brings out 50% mortality. *LC90 = Lethal Concentration brings out 90% mortality. LCL = Lower Confidence Limit; UCL = Upper Confidence Limit.

Probit analysis was done to determine LC_{50} and LC_{90} for all tested stages (3rd, 4th and Pupae). The results are presented in Table-1. Wongdyan *et al.*, (2008) [30] has reported that *S. carpocapsae* required at 2000 and 4000 IJs for killing more than 50% of *C. gelidus* larvae under laboratory condition and isolated EPNs in this study, required around 1500 to 1700 IJs to achieve 90% host mortality in all the tested stages. Muhammad *et al* (2017) [19], has reported that 100 IJs of *S. kraussei* produced 100% mortality in*A. Aegypti* bred in canal, tap and sewage water at an optimum temperature of 20°Cafter 48 and 96 h of application.

The regression analysis indicates that increasing the IJs numbers from 100 to 1000 IJs / 4th instar (R^2 = 0.99, p <0.001) and pupae (R^2 = 0.97, p <0.001) resulted a highly significant increase in host mortality at 24 h of after application. Similarly host mortality after 48 h of after application was found to be highly significant in both 4th instar (R^2 = 0.99, p<0.001) and pupae (R^2 = 0.97, p<0.001).

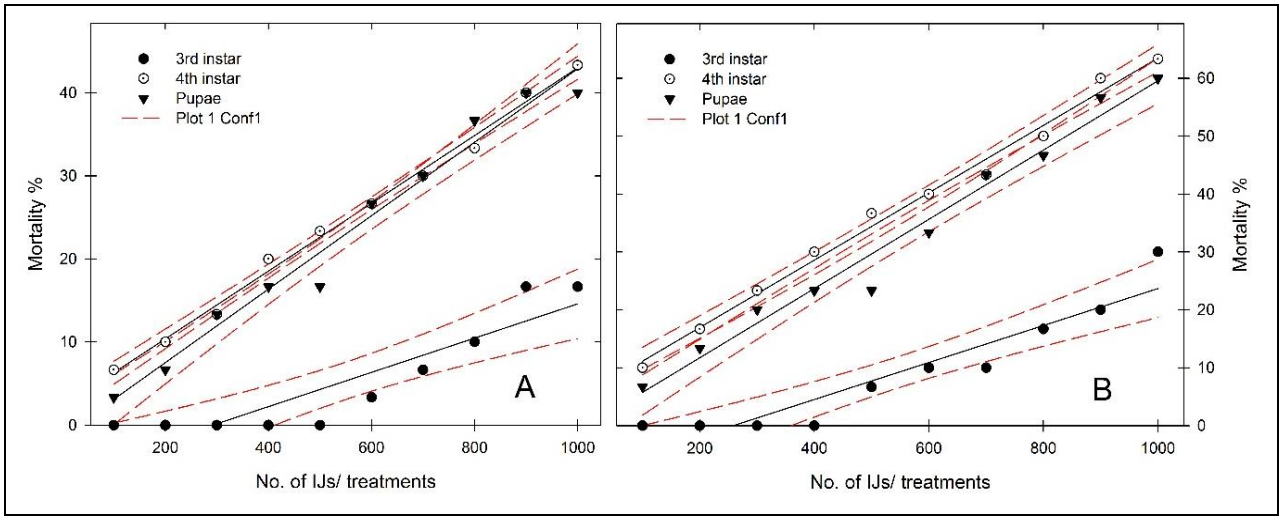

Fig 1: Regression analysis between the number of IJs/Treatments and host mortality percentage of different stages of *A. aegypti* after 24 h (Fig. A) and 48 h (Fig. B) of application.

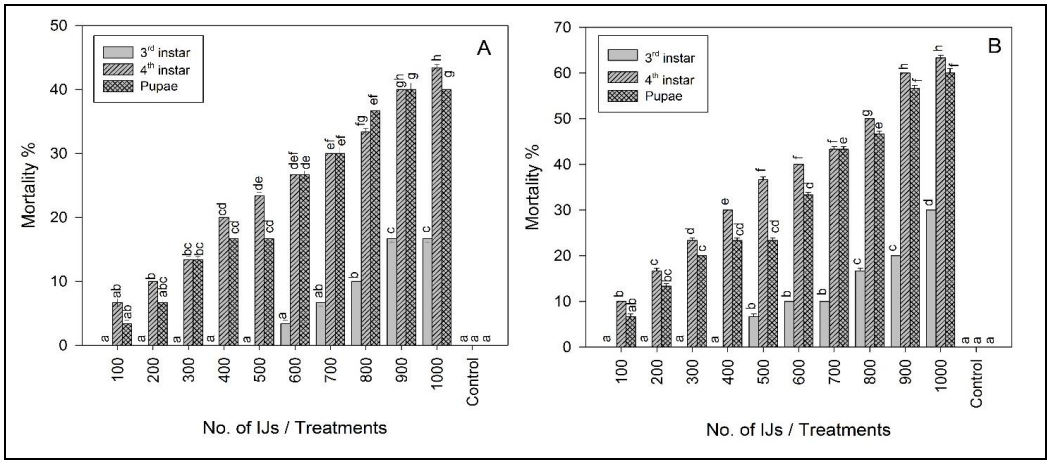

Fig 2: Mortality percentage of different stages of *Aedes aegypti*after24 h (Fig. A) and 48 h (Fig. B) of application. Bar diagram with error bar indicates mean ± SD. Within a group means followed by the same letter (s) are not significantly different at 5% level by DMRT.

Host mortality of 3rd instar larva was significant at 24 h (R^2 = 0.82, p<0.01) and highly significant at 48 h (R^2 = 0.88, p<0.001) of after application. The result which undoubtedly indicates that 3rd, 4th instars and pupae were highly susceptible to isolated EPNs (Figure 1).

Multiple comparison of mortality mean difference between the stages of host larva on the host mortality indicated that 4th instar larvae (2.818 ± 0.058) shows maximum mortality followed by the pupae (2.530 ± 0.058) and 3rd instar larva (0.667 ± 0.058) and between the exposure time, 48 h showed highest mean mortality (2.404 ± 0.47) followed by 24 h (1.606 ± 0.47). Mosquito mortality was higher in 4th instar larvae compared to other tested stages; this may be due to the decrease in physical activities for upcoming molting stage. This might be a reason for higher mortality in 4th instar larvae than others. Wongdyan *et al.*, (2008) [30] has noted that *S. carpocapsae* shared high mortality at 48 h of after application. According to Onilda *et al.*, (2013) [20] when enterobacteria of *X. nematophila* ingested orally by third late or fourth early instars larvae of *A. aegypti*, could cause 52% mortality in fed and 42% inunfed larvae up to 96 h of after application and *X. nematophila* appeared to cause a higher mortality rate in fed larvae, when compared to unfed larvae of *A. aegypti*, so bacterial virulent factor differs among the insects based on their feeding habit.

Parasitized larvae were dissected under light microscope and IJs were recorded at the head region of *A. aegypti* (Plate 2). IJs failed to complete its life cycle within the host body which indicates that some factors arrest the IJs survival rate in host body. According to Peschiutta *et al,* (2014) [21] the *A. aegypti* infected with *Heterorhabditis bacteriophora* showed greater infection in the head region compared to other parts of the body. Wongdyan *et al,* (2008) [30] found that the nematodes infected were seen mostly in thorax.

The biochemical analysis showed that protein content in 4th instar larvae (df=1, F=600.25, p<0.01) and Pupae (df=1, F=1.98, p<0.01); carbohydrate content in 4th instar larvae (df=1, F=411.11, p<0.01) and Pupae (df=1, F=152.17, p<0.01); lipid content in 4th instar larvae (df=1, F=139.68, p<0.01) and Pupae (df=1, F=52.72, p<0.01) decreased significantly when compared to control groups. The treatments resulted in percentage of reduction of 19%, 32% and 11% for protein, carbohydrate and lipid respectively in 4th instar larvae. Likewise in pupae, percentage of reduction of 53%, 12% and 11% for protein, carbohydrate and lipid respectively. This significant decrease in protein, carbohydrate and lipids content of parasitized larvae may be due to energy loss through immune reaction against infection and also due to the utilization of protein, carbohydrate and lipids resources by nematode bacterial complex for their growth and reproduction (Santhana Bharathi *et al.*, 2016) [26].

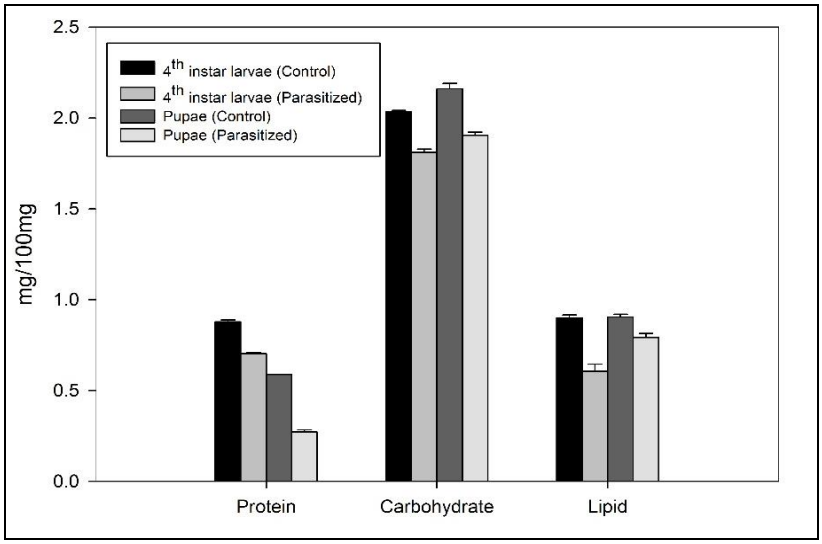

Fig 3: Estimation of protein, carbohydrate and lipid content in 4th instar and pupae of *Aedes aegypti* infected by *Steinernema* sp. after 48h of application. (*Mean ± SD, n=3).*significant at p<0.001 with control groups.

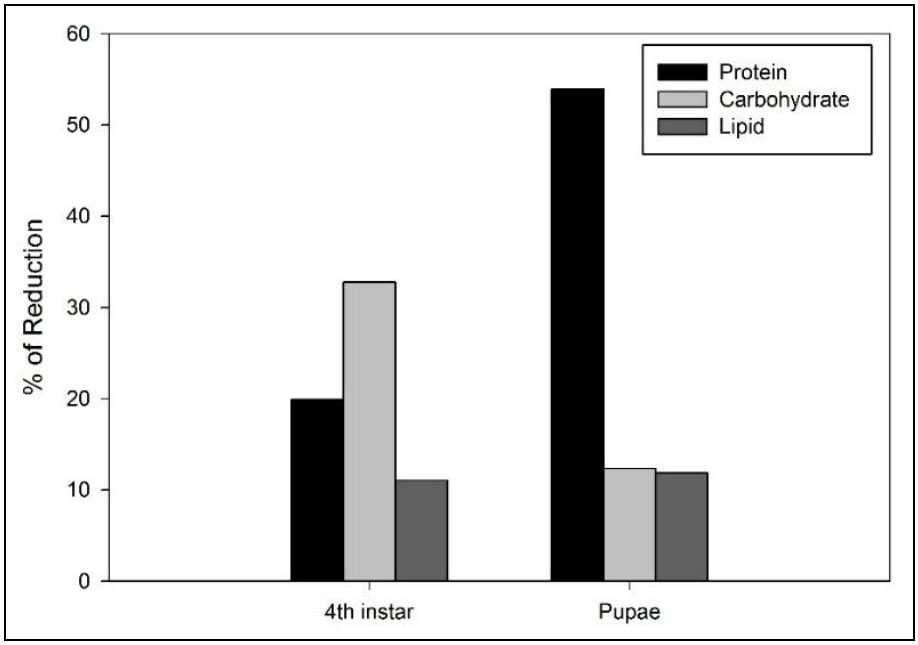

Fig 4: Reduction percentage of biochemicals in 4th instar larvae and pupae of *A. aegypti* after 48 h of application.

Xenorhabdus sp. produce a large number of insecticidal toxins to help them harvest nutrients from the insect hosts (Castagnola and Stock, 2014) [4]. The proteinaceous toxins produced by their symbionts (Vinay *et al.*, 2014) [29] in host body may be the reason for larval mortality and bio chemical changes. According to Hananand El-Sadawy (2009) [10], The EPNs secreting proteolytic enzymes into haemocoel of host body for hydrolysis of the host protein leads to hormone regulated disturbance in haemolymph of host. The decline of lipid content in parasitized larvae is due to endoparasite action on host chemical composition. The bacterium *X. nematophila* can produce bio active compounds with insecticidal properties such as toxin complexes or lipopolysaccharides, may be true in the study also. When *Xenorhabdus* sp. infected with *Helicoverpa armigera* (Santhana Bharathi *et al.*, 2016) [26], *Spodoptera litura* (Sindhu, 2016) [27], *Leucinodes orbonalis* (Sujatha, 2017) [28] showed decreased biochemical content in parasitized larvae when compared to non-parasitized (control) larvae. The Txp40 protein has been identified in both *Photorhabdus* sp. and *Xenorhabdus* sp. This has caused damage to the insect midgut and the fat body in dipterans and lepidopteran insects (Castagnola and Stock, 2014) [4], this is in line with the present study as the mosquito *A. aegypti* is also a dipteran. But further studies should be carried out for knowing their insecticidal properties for better bio control approaches against various insects.

Conclusion

The present work has highlighted the efficiency of the *Steinernema – Xenorhabdus* symbionts against dengue and chikungunya viral vector *A. aegypti*. Isolated EPNs has showed mosquito larvicidal activity in all instars and pupae except 1st and 2nd instars because of the host size. Soil nematodes have an insecticidal activity against aquatic pests like *A. aegypti* as aquatic habitat provides an outstanding environment for EPNs survival. Study paves way to identify novel bacterial toxins against Dengue viral vector *A. aegypti* and minimize the risk of insect resistant development. So, isolated EPNs can be exploited as efficient bio pesticide in near future. However, these laboratory findings cannot assurance the role of EPNs to control mosquito larvae at large scale in natural habitat. Hence, their antagonistic potential should be studied at large scale for practical applications.

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