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Antagonistic potential of *Steinernema kraussei* and *Heterorhabditis bacteriophora* against Dengue fever mosquito *Aedes aegypti*

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Abstract
The present investigation was undertaken to control *A. aegypti* procured from three different breeding sites i.e., canal water, tap water and sewage water by utilizing the antagonistic potential of two entomopathogenic nematodes *Steinernema kraussie* and *Heterorhabditis bacteriophora*. The trial was conducted in Vector Ecology Lab, Department of Entomology and Plant Nematology Lab, Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan during Feb-April, 2016. The mortality rate of *A. aegypti* larval population was assessed at two different concentrations 70 and 100 infective juveniles (IJs) of both EPNs at variable temperature of 20, 30 and 40 ºC for 24, 48, 72 and 94 hrs time intervals. *S. kraussei* and *H. bacteriophora* produced best mortality at 20 ºC and 30 ºC being the optimum temperatures for their activity respectively. *S. kraussei* at the concentration of 100 IJs produced 100% mortality of *A. aegypti* bred in canal, tap and sewage water at 20 ºC after 48 and 96 hrs. *H. bacteriophora* at the concentration of 100 IJs produced 100% mortality of *A. aegypti* from all breeding sites at 30 ºC after 96 hrs. Activity of *S. kraussei* was significantly decreased with rise in temperature up to 40 ºC, whereas; activity of *H. bacteriophora* was less at low temperature and increased significantly at higher temperature. Increased concentration of IJs and higher time intervals were also found to be directly proportional with mortality rate.

Keywords: Entomopathogenic nematodes, Biological control, *Galleria mellonella*, Sewage water, Canal water, Bacteria

Introduction
Mosquito *Aedes aegypti* is the carrier of dengue virus in Pakistan [1]. Medical importance of mosquitoes lies with their potential to transmit infectious diseases such as dengue fever, malaria and dengue haemorrhagic fever etc. Dengue virus is reported to be endemic in 120 countries around the globe rendering 2/3rd population of world at potential risk of dengue infection [2]. First outbreak of dengue fever virus in Pakistan was reported in 1994 [3]. In 1995, it was first time recognized as an undifferentiated fever in Hubb region, Baluchistan province of Pakistan [4]. Endemic outbreak of dengue fever in Pakistan is always observed after monsoon season [5].
A. aegypti is the major vector for dengue fever in tropical and sub-tropical regions of the world [6]. Destruction of breeding sites is the best way to decrease mosquito population [7]. On record, annually one million deaths are happening by mosquito bite [8]. Sluggish nature and confined population of *A. aegypti* larvae makes it easy to control at larval stage [9]. Insecticides are ineffective to control *A. aegypti* due to its potential to develop resistance against chemicals [10-11]. Use of insecticides is also hazardous to human health and environment due to persistent nature of chemicals [12]. Therefore, researchers are continuously searching for an effective and safer alternate strategy to control *A. egypti* population. Since last decade, several pests have been successfully targeted and suppressed by biological control agents including entomopathogenic nematodes (EPNs) [13-14]. Recently, plenty of efforts have been made to control dengue mosquitoes by entomopathogenic nematodes [13].
Entomopathogenic nematodes such as *Steinernema* and *Heterorhabditis* spp. represent themselves as potential antagonistic agents for control of insect pests. Nontoxicity of EPNs to human beings, target specificity and compatibility with insecticides fit them accurately in the

spectrum of integrated pest management (IPM) [15-16]. *Photorhabdus* and *Xenorhabdus* sp. are symbiotic bacteria associated with *Heterorhabditis* sp. and *Steinernema* sp. respectively [17]. The rapid antagonistic action of *Steinernema* and *Heterorhabditis* is due to associated symbiotic bacteria. EPNs have the remarkable ability to search and locate their target and to penetrate bark and pupation chambers [18]. Insect pests spend more than 90% of their life span in soil making EPNs perfect candidates for their control. Painful reality of EPNs include their sensitivity to environmental conditions (e.g. Moisture and temperature), poor field conditions, long term packing, viability and high cost [19]. EPNs have been successfully used against a variety of soil and foliar insect pests [20] offering excellent potential for their survival and antagonistic activity.

Present investigation was undertaken for comparative analysis of the effect of *S. kraussei* and *H. bacteriophora* on mortality rate of dengue virus carrier *A. aegypti* at different doses and temperature conditions in canal, sewage and tap water.

Materials and methods

Mosquitoes rearing

A. aegypti was reared under controlled conditions in three different kinds of water sewage, canal and tap water at 27 ± 2 °C temperature, 65-70% of relative humidity (RH), with photoperiod of 12:12 (D:L) in Vector Ecology Laboratory, Department of Entomology, University of Agriculture, Faisalabad during Feb-April, 2016. The adult mosquitoes were provided with 10% sucrose solution and kept in rearing cages (30 x 30 x 30 cm), whereas, adult females were fed on albino rat diet [21].

Galleria mellonella rearing

Larvae and adults *G. mellonella* wax moth were obtained from honey bee infected hives of University of Agriculture, Faisalabad. Late larval instars were collected and stored at 7-8 °C. The adults were placed in plastic jars, closed with muslin cloth to allow passage of fresh air. The freshly laid eggs were collected on paper folds in the plastic jars. These eggs were inoculated on artificial cereal diet prepared by wheat, rice, oat and maize porridge (20g), yeast granules (50g) by dissolving in 80 mL warm honey solution and glycerol solution (100 mL) [22].

Multiplication of EPNs on *G. mellonella*

Cultures of *S. kraussei* and *H. bacteriophora* were obtained from and multiplied Plant Nematology Lab, Department of Plant Pathology, University of Agriculture, Faisalabad. *G. mellonella* larvae were used as a bait for multiplication of entomopathogenic nematodes. Larvae were surface sterilized with 0.1% formalin solution to avoid surface contamination and then placed in plastic petri plates, lined with double ply Whatman No. 1 filter papers. Aqueous solution containing 300 infective juveniles J2 was added in each petri plate.

Extraction of EPNs

A modified white trap [23] comprised of a plastic container (9 × 9 × 6 cm) was filled with sterilized distilled water up to 1 cm depth. A small petri plate (5 × 3.5 cm^2) was placed at the bottom of container in inverted position. Whatman No. 1 filter paper of 9 cm dia. was placed on the petri plate in such a way that its edges were moistened by distilled water. Four to five dead cadavers of *G. mellonella* were placed on filter paper and the container was closed with a lid. The traps were incubated at 28 °C until the nematode progeny emerged out. The infective juveniles started to leave the cadaver 8-20 hours after infection. Infective juveniles moved into the water in the trap observed under stereomicroscope and harvested daily until complete recovery. The container was refilled with distilled water after each harvest. The water containing nematodes was collected in clean glass beakers, diluted by adding more water and allowed to settle down for 2-3 hours. Supernatant was siphoned out followed by counting of nematodes under stereomicroscope in 1mL of aqueous solution thrice. The mean count of three aliquots was multiplied with total volume of water. *S. kraussei* and *H. bacteriophora* were identified based on color changes in infected larvae of *G. mellonella* and morphometric measurements under stereomicroscope. *S. kraussei* and *H. bacteriophora* infected larvae developed red brick and grey color respectively after mortality [24]. Nematode cultures ware diluted up to approximately 5000/mL IJs and stored in a refrigerator at 10-15 °C in clean small plastic cups. The continuous supply of oxygen was maintained by using aerators being used in fish aquariums tanks

Isolation of bacteria

S. kraussie and *H. bacteriophora* infected cadavers of *G. mellonella* were surface sterilized in 70% alcohol for few seconds, transferred to a clean tissue paper to dry for few minutes. NBTA medium was prepared by dissolving Nutrient agar 37 g and Bromothymol Blue 25mg in 1000 mL water followed by autoclaving at 121º C and 15 psi pressure. The medium was cooled to 45 ºC and 4 mL of 1% 2, 3, 5-Tiphenyl tetrazolium chloride was added and distributed in all petri plates. A drop of hemolymph was taken form pierced cadavers of *G. mellonella* using a sterilized needle and streaked on NBTA medium containing petri plate followed by incubation at 28 °C. After 48-hours colonies of *Xenorhabdus* sp. and *Photorhabdus* sp. obtained from hemolymph of infected cadavers were transferred to nutrient agar containing petri plates. The pathogenicity of isolates was confirmed by injecting *Xenorhabdus* sp. and *Photorhabdus* sp. culture into *G. mellonella* larvae.

Mass production of *S. kraussei* and *H. bacteriophora*

For mass rearing of IJs of *S. kraussie* and *H. bacteriophora* chicken offal method was used [25]. Chicken offal consists of a porous foam (Polyether polyurethane) providing large surface volume ratio and adequate interstitial space. Fresh chicken offal (without gizzards and gall bladders) was homogenized by adding 20% w/v water in a blender. The homogenate was evenly distributed on the foam by kneading 10 parts of the homogenate and 1 part of the foam. Kneaded material 80 g was taken and placed in 500 mL flasks. The flasks were cleaned, plugged with cotton and wrapped with aluminum foil followed by sterilization at 121 °C and 15 psi pressure for 30 minutes in an autoclave. It was left to cool up to 45 °C and shaken well to separate coated foam particles. The material in each flask was inoculated in a laminar flow chamber with 10 mL of 1-day-old yeast extract broth cultures of symbiotic bacteria followed by incubation for four days at 23 °C**.**

Inoculation and harvesting of nematodes

Inoculum of each nematode was obtained from 6-weeks-old culture flasks containing approximately 99 % IJs [26]. The monoxenic suspension of infective juveniles was added to flasks at the rate of 100 IJs through pipettes in laminar flow cabinet. Sterilized water 10 mL was added to flask. The flasks were kept at 23-30 °C. After 15 days of inoculation IJs were harvested by following method. The sponge culture was taken

and placed on a 20-mesh sieve kept in a pan of tap water with adequate water to submerge the foam and incubated at 15-20 °C in a growth chamber for 24-48-hours. Nematode extraction was accomplished by using active migration and sedimentation in baermann funnel apparatus. Harvesting trays were lined with aluminum foil, a cloth fabric was placed over the foil and water was added to cover the fabric. The flasks were emptied over the cloth. The nematodes migrated from the cloth into the water reservoir.

***In vitro* Bioassay**

Water was collected from three breeding sites i.e., Sewerage water, Tap water and Canal water. The water collected from different sources was used for rearing of mosquitoes. *In vitro* effect of 2-weeks-old culture of *S. kraussei* and *H. bacteriophora* at different concentrations on mortality of *A. aegypti* from each water source was assessed. Mosquito larvae bred in sewage, canal and tap water were taken and 10 larvae from each breeding site were placed in petri plates for each treatment and inoculated with IJs of nematodes at different concentrations 70 and 100 IJs followed by incubation at variable three different temperatures 20, 30 and 40 °C. The experiment was conducted with 3rd to 4th instar larvae of *A. aegypti*. One treatment was maintained as control. Mortality rate of *A. aegypti* was calculated at 24, 48, 72 and 96-hours.

Statistical analysis

The data collected on mortality rate was subject to statistical analysis using a statistical software M-Stat (ver.2.3. Faisalabad, Pakistan). Means were separated from each other using least significant difference test. Interactions were analysed by ANOVA. Significant interactions were used to explain the data.

Results

S. kraussei produced maximum mortality of *A. aegypti* bred in tap water up to 100% after 96 hrs at 20 ºC. While, mortality by *S. kraussei* was significantly reduced at 40 ºC. Mortality rate was more at higher concentration of IJs. *H. bacteriophora* also produced 100% mortality of tap water bred larvae at 30 ºC after 96 hours. The activity of *H. bacteriophora* was quite low at 20 ºC (Table 1). Similar trends were observed on mortality of *A. aegypti* larvae bred in canal and sewage water (Table 2 and 3). *S. kraussei* and *H. bacteriophora* consistently exhibited higher mortality of *A. aegypti* larvae from different breeding sites at 20 ºC and 30 ºC respectively (Table 1, 2 and 3). The activity of *S. kraussei* was remarkably reduced with increase in temperature with least at 40ºC whereas, *H. bacteriophora* had least antagonistic activity at 20 ºC that significantly increased with rise in temperature with optimum activity at 30 ºC. The optimum temperature for activity of *S. kraussei* and *H. bacteriophora* was found to be 20 ºC and 30 ºC respectively (Table 1, 2 and 3). Higher concentration of IJs and increased exposure was directly proportional with larval mortality. *S. kraussei* and *H. bacteriophora* exhibited remarkable antagonistic activity up to 100% at variable temperature conditions. It was found that temperature had a highly significant effect on antagonistic activity of nematodes. Fluctuations in temperature drastically affected the activity of both nematodes. The means were separated from each other using LSD test.

Table 1: Effect of *S. kraussei* and *H. bacteriophora* on mortality of *A. aegypti* larvae bred in tap water[a]

Treatment	Concentration No. of IJs	Temperature ºC	Mortality (%)			
			After 24 hrs	After 48 hrs	After 72 hrs	After 96 hrs
S. kraussei	70	20	80.0bcd	93.3ab	96.7a	100.0a
		30	26.7kl	43.3hij	80.0bcd	93.3ab
		40	0.0m	3.3m	26.7kl	43.3hij
	100	20	100.0a	100.0a	100.0a	100.0a
		30	36.7ijk	63.33ef	86.7abc	96.7a
		40	33.3ijk	56.7fgh	80.0bcd	96.7a
H. bacteriophora	70	20	0.0m	3.3m	13.3lm	33.3ijk
		30	56.7fgh	66.7def	86.7abc	90.0abc
		40	13.3lm	23.3kl	43.3hij	66.7def
	100	20	30.0jk	46.7ghi	60.0fg	86.7abc
		30	70.0def	86.7abc	100.0a	100.0a
		40	26.7kl	43.3ghi	76.7cde	93.3ab

[a]Mean values in the columns with same letter are not significantly different from each other at P < 0.05, analysed and separated using LSD test, values represent average of 3 replicates.

Table 2: Effect of *S. kraussei* and *H. bacteriophora* on mortality of *A. aegypti* bred in canal water[a]

Treatment	Concentration No. of IJs	Temperature ºC	Mortality (%)			
			After 24 hrs	After 48 hrs	After 72 hrs	After 96 hrs
S. kraussei	70	20	40.0lmno	46.7klmn	70.0efgh	100.0a
		30	6.7tuv	20.0rst	36.7mnopq	53.3ijkl
		40	3.3tuv	16.7stu	33.3nopqr	43.3klmno
	100	20	100.0a	100.0a	100.0a	100.0a
		30	43.3klmno	70.0efgh	93.3abc	96.7ab
		40	36.7mnopq	66.7fghi	90.0abcd	93.3abc
H. bacteriophora	70	20	0.0v	3.3uv	26.7pqrs	50.0jklm
		30	80.0cdef	90.0abcd	96.7ab	100.0a
		40	23.3qrs	36.7mnopq	63.3ghij	83.3bcde
	100	20	26.7pqrs	43.3klmno	70.0efgh	93.3abc
		30	76.7defg	93.3abc	96.7ab	100.0a
		30	30.0opqrs	56.7hijk	76.7defg	93.3abc

[a]Mean values in the columns with same letter are not significantly different from each other at P < 0.05, analysed and separated using LSD test, values represent average of 3 replicates.

Table 3: Effect of *S. kraussei* and *H. bacteriophora* on mortality of *A. aegypti* bred in sewage water[a]

Treatment	Concentration No. of IJs	Temperature ºC	Mortality (%)			
			After 24 hrs	After 48 hrs	After 72 hrs	After 96 hrs
S. kraussei	70	20	53.3hij	73.3defg	90.0abcd	93.3abc
		30	30.0klm	43.3jkl	53.3hij	73.3defg
		40	3.3n	16.7mn	26.7lm	50.0ij
	100	20	60.0ghij	73.3defg	96.7ab	100.0a
		30	30.0klm	53.3hij	76.6cdefg	90.0abcd
		40	13.3mn	26.7lm	43.3jkl	66.7fghi
H. bacteriophora	70	20	6.7n	30.0klm	43.3jkl	60.0ghij
		30	50.0ij	66.7fghi	86.7abcde	100.0a
		40	0.0n	13.3mn	30.0klm	53.3hij
	100	20	26.7lm	53.3hij	76.7cdefg	93.33abc
		30	80.0bcdef	83.3abcdef	90.0abcd	100.0a
		40	26.7lm	46.7jk	66.7fghi	86.7abcde

[a]Mean values in the columns with same letter are not significantly different from each other at $P < 0.05$, analysed and separated using LSD test, values represent average of 3 replicates.

Discussion

S. kraussei and *H. bacteriophora* were highly effective to induce mortality of *A. aegypti* larvae with optimum activity at 20 ºC and 30 ºC. Mortality rate of 100% was recorded by both EPNs. Highest activity of dengue virus carrying mosquitoes is reported in a temperature range of 20-30 ºC [27]. Therefore, the activity spectrum of *H. bacteriophora* and *S. kraussei* in a specific temperature range in this study presents both EPNs as potential candidates and a safer alternate to chemicals to control carrier mosquito *A. aegypti*. *S. kraussei* was more effective at a relatively low temperature than *H. bacteriophora* that was more effective at high temperatures. The variation in efficacy of different species of EPNs has been reported earlier [28]. *H. bacteriophora* was reported to cause 84% mortality of *A. aegypti* at 100:1 concentration. *H. bacteriophora* can infect larvae of *A. aegypti* and the larval mortality increases with the increasing dose [28]. The antagonistic activity of EPNs is due to symbiotic bacteria associated with them. *Photorhabdus* and *Xenorhabdus* bacteria are naturally associated with both *Heterorhabditis* spp. and *Steinernema* spp. respectively [17]. The mortality of insects by EPNs is due to the damage caused by these bacteria to haemocytes of Lepidopteran insects and disintegration of fat bodies, the main reservoir of antimicrobial peptides [29].

Many researchers have reported the antagonistic effect of EPNs on mortality of insect larvae obtained from different breeding sites such as sewage or canal water [30]. The present finding suggested that mortality of mosquito larvae by *H. bacteriophora* was higher at high temperature and doses. The growth conditions of IJs of EPNs had a significant impact on their infectivity and performance [31]. The mortality by *H. bacteriophora* at all doses can be higher because it survives at relatively higher density of nematodes than other EPNs [32]. The biological cycle of *H. bacteriophora* and host mortality provides the possibility of using this EPN in mosquito larvae control [28]. In the present study, the effect of increasing time and dose of IJs on *A. aegypti* mortality was positive with higher mortality rate. The effect of dose had a positive correlation with the mortality of mosquito larvae [33] increasing dose increased the mortality of mosquito larvae. The time of exposure to *Heterorhabditis* and *Steinernema* spp. also affected the larval mortality of *A. aegypti* in a previous finding [34]. The rate of melanisation of mosquito larvae is affected by the dose, while, it remained unaffected by duration of exposure [33]. The temperature sensitive nature of antagonistic activity of *Steinernema* is also reported earlier [35]. The promising potential of *Sterinernema* to parasitize *Culex gelidus* was also reported previously [36]. The present study signifies the importance of the use of *H. bacteriophora* and S. *kraussei* for the antagonistic control of *A. aegyptii*. However, the *in vitro* findings cannot guarantee the role of EPNs to control dengue mosquito larvae at large scale. Hence, their potential should be studied at large scale for practical application of EPN to reduce the population of dengue mosquito larvae.

Conclusion

Based on present investigations it can be concluded that the antagonistic potential of *S. kraussei* and *H. bacteriophora* due to associated symbiotic bacteria has presented them as potential alternate to chemical pesticides. The temperature requirement for antagonistic activity of both EPNs and *A. aegypti* is same making them more suitable to be adopted as biological control of the dengue vector. These EPNs should be formulated as bio pesticides to check the efficacy bio formulations commercially against *A. aegypti* in future.

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