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Nematode-symbiotic bacterial toxin complexes as an alternative larvicidal bioinsecticide agent against *Culex pipiens* (Diptera: Culicidae)

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Abstract: *Culex* species are major vectors for several serious illnesses, where they are responsible for spreading of viral diseases. Gram-negative symbiotic bacteria, *Xenorhabdus indica* and *Photorhabdus luminescens laumondii* are linked with nematode parasites that are very dangerous to insect larvae. In the current investigation, anion exchange chromatography was used to partially purify *X. indica* and *P. luminescens laumondii* protein toxin complexes, crude and fractions of them were tested for their insecticidal efficacy against *Culex pipiens* late third-instar larvae, *in vitro* at varying concentrations. The protein toxin complexes of two bacterial species were analyzed using SDS–Polyacrylamide Gel Electrophoresis. Ten bands with molecular weights from 281 to 27.4 kDa were found in the bound fraction. In comparison, seven bands with molecular weights between 315 and 58 kDa were found in the unbound fraction when analyzing *P. luminescens laumondii* crude toxin complexes. The molecular weight distribution of a crude *X. indica* toxin complex showed 16 bands, from 315 to 17 kDa. All nine bands, with molecular weights ranging from 315 to 30 kDa, were found in the bound fraction, whereas only four bands, with molecular weights ranging from 93 to 17 kDa, were found in the unbound fraction. The LC_{50} values for the crude, unbound, and bound toxin of *P. luminescens laumondii* were 3142.25, 28.97 and 39.63 mg/L, respectively, whereas those values for *X. indica* were 256.52, 294.22 and 443.03 mg/L, respectively. The present results also, revealed that *P. luminescens* (HP88) fractions had a significantly higher larval growth inhibition activity than *X. indica* (Ab) fractions. In conclusion, this work introduces a largely purified toxin from *P. luminescens laumondii* that may be used in the management of *Culex* sp. and, as a result, aid in the integrated pest management (IPM) program for mosquito and subsequently for these viral diseases.

Keywords: Entomopathogenic bacteria; *Xenorhabdus*. *Photorhabdus*; *Culex* sp.; Biocontrol; SDS-PAGE.

1. Introduction

Mosquitoe, *Culex pipiens* is an important vector of a variety of virus-borne infections, they are crucial in the transmission of many infectious diseases, including Rift Valley fever, St. Louis encephalitis, Western equine encephalitis, Japanese encephalitis, and West Nile fever [1-3]. *C. pipiens* is one of the major vectors for West Nile virus (WNV) [4].

Mosquito control is still based essentially on traditional chemical insecticides which have disadvantages, such as toxicity for non-target organisms and environmental pollution. Additionally, the continuous usage of these insecticides over a long time can cause insecticide resistance in mosquito populations [5,6]. So, it has become necessary to investigate alternative methods that are safe for human, environment and non-target organisms. Biological control is one of the promising alternative eco-friendly methods used for mosquito control, where, it has high specificity, and sustainable because it has a slow rate of insect resistance development [7].

Entomopathogenic nematodes with their symbiotic bacteria introduce promising solution as biological agent for pest control strategy [8-11]. In nature, a symbiotic relationship was developed between some species of the bacterial genera *Xenorhabdus* and *Photorhabdus* and class Nematoda, families Steinernematidae and Heterorhabditidae, respectively [5, 12-14]. These bacteria species have been illustrated worldwide to have insecticidal characteristics.

Although, *Xenorhabdus* and *Photorhabdus* species have different evolutionary origins, they have similar life-cycle [15]. The entomopathogenic nematodes (EPNs) larvae live and searching for insect larvae in the soil [16]. When the EPNs larvae found the insect larvae, it penetrates the insect's larvae body through natural openings, such as the mouth, spiracles or anus [17], or they bore a hole into the insect's cuticle and directly reach the hemocoel, where symbiotic bacteria will be released by defecation (e.g. Steinernematidae) and regurgitation (e.g. Heterorhabditidae) [18, 19]. The symbiotic bacteria replicate, and release compounds in the hemocoel which could suppress the host immune response, as a protection strategy for symbiosis with the nematode [20, 21]. Bacteria reproduce in the hemocoel, beginning a lethal septicemia for the host insect [9], causing its death in about 24 to 48 h. Toxin complexes from *Xenorhabdus* and *Photorhabdus* spp. bacteria represent novel insecticidal proteins. Three distinct proteins make up the toxin complex: XptA2, XptB1, and XptC1, which stand for products of the class A, B, and C toxin complex genes, respectively. Sheets et al. [22] showed that recombinant XptA2 and co-produced recombinant XptB1 and XptC1 combine with a 4:1:1 stoichiometry. XptA2 forms a tetramer of ~1,120 kDa that bound to solubilized insect brush border membranes and induced pore formation in black lipid membranes. The high molecular weight insecticidal complexes' toxins were initially identified in *Photorhabdus luminescenens* strain W14 supernatant [23]. Using various chromatography steps four different complexes were separated and labelled Tca, Tcb, Tcc, and Tcd [24]. Purified Tca disrupted the insect mid gut epithelium in a manner similarly to the δ-endotoxins from "*B.t.*" [25]. However, supernatants of some *Xenorhabdus* strains like *Photorhabdus*, also demonstrate oral toxicity against insects [26].

Our study was conducted to investigate and highlight the use of *Xenorhabdus* and *Photorhabdus* for *Culex pipiens* biological control as promising future techniques for their biotechnological applicability. Thus, this study was carried out for isolation and purification of Toxin Complexes-Cell and cell free of symbiotic bacteria *Xenorhabdus indica* (*X. indica*) mutual with *Steinernema abbasi* (Ab) (native isolate from Sultanate of Oman) in comparison with *Photorhabdus luminescens lamondii* (*P.luminescens lamondii*) mutual with *Heterorhabditis bacteriophora* (HP88). Also, characterization of TCs of two bacteria species by SDS PAGE was done.

2. Materials and Methods

2.1. Source of Bacteria

The nematodes *Steinernema abbasi* and *Heterorhabditis bacteriophora* HP88 were in the Sultanate of Oman and Egyptian soils, respectively. The Nematodes culture is mass propagated on wax moth, *Galleria mellonella* according to Mohamed and Coppel [27] in Parasitology and Animal Diseases Department, National Research Center, Egypt. The symbiotic bacteria *X. indica* and *P. luminescens laumondii* have been isolated from the nematodes *Steinernema abbasi* and *Heterorhabditis bacteriophora* HP88.

2.2. Bacterial Strains and Growth Conditions

Bacteria *X. indica* (Ab) and *P. luminescens laumondii* (HP88) had been isolated from their symbiotic nematodes using the method of Akhurst [28]. For each subculture, the phase status was culturing on NBTA agar (2.3% nutrient agar [Difco], 0.0025% bromothymol blue [Merck], 0.004% 2,3,5- tri-phenyl-tetrazolium [Merck]). On NBTA agar, phase I colonies are blue while phase II are red. According to Cabral *et al*. [29], twenty infective juveniles (IJs) were surface sterilized for 10 minutes in sodium hypochlorite (1%), then washed in sterilized distilled water (DW), placed in a Petri dish containing 5 ml of TSBYE (3% tryptic soy broth [Difco], 0.5% yeast extract [Difco]), and grind using grinder pistol. The dishes were incubated for 24 hrs at 30°C and streaked on NBTA plates (2.3% nutrient agar [Difco], 0.0025% bromothymol blue [Merck], 0.004% 2,3,5-triphenyltetrazolium [Merck]). The presence of *Xenorhabdus* or *Photorhabdus* colonies was proved by coloring adsorption on NBTA plates, manufacture of luminescence, and antibiotic activity. The isolated bacteria were kept on NBTA plates at 10°C and subcultured once a week.

2.3. Extraction and FPLC Purification of Protein Toxin Complexes of X. indica and P. luminescent laumondii

According to Sheets et al. [22] cell pellets were produced from fermentation of 2-liter culture of *X. indica* and *P. luminescent laumondii* for 24hrs. The pellets were suspended in 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1mM DTT, 10% glycerol and lysozyme (0.6 mg/ml). A small amount of glass beads (0.5 mm dia.) had been added and cells were disturbed by sonication. Then broken cells were centrifuged at 10,000 rpm for 60 minutes. at 4°C., supernatant was then collected, and bacterial protease inhibitor cocktail was added (Sigma, St. Louis), and stored at -80°C until used.

2.4. Purification of Photorhabdus and Xenorhabdus Toxin Complexes Using Anion Exchange Chromatography

The extraction was dialyzed against 25 mM Tris-HCl, pH 8.0 overnight. The protein was then loaded onto a Q Sepharose XL (1.6 x 10 cm) anion exchange column, (Pharmacia). Bound proteins were eluted using a linear 0 to 1 M NaCl gradient in 5 column volumes. In the early fractions, the toxin complexes with high molecular weight were eluted. Bound and unbound fractions were collected. Protein contents for crude and fractions were determined according to Lowry *et al.* [30]. The crude and fractions were stored at -80°C until used for bioassay application and electrophoresis characterization.

2.5. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Polyacrylamide gels (SDS-PAGE) was used as described by Laemmli [31]. Before loading to the gel, both crude protein toxin complexes, bound and unbound fractions were individually mixed with sample buffer containing 5% 2-mercaptoethanol. After separation, the gel has been stained with Commassie brilliant blue. Relative molecular weights of bands were calculated using marker supplied by Fermentas International INC. Canada

2.6. Oral Bioassay Application of Toxin Complex Crude and Fractions on Culex sp. Larvae

In this study, *Culex* sp. late third instar larvae were treated with partially purified toxin complex fractions of protein cells from *P. laumondii* and *X. indica* bacteria. five concentrations of *P. Laumoundii* and *X. indica* (Crude protein, bound and unbound fractions) (25, 50, 100, 200 & 500 mg/L) were prepared dechlorinated water in white, clear plastic cups (100 ml) supplied with food. Ten third-stage, well-fed larvae are in each cup, replicated five times. Dechlorinated water was used in the control replications. The tests ran seven days at normal room temperature.

2.7. Statistical Analysis

For the toxicity tests, the LC_{25} and LC_{50} values were calculated using the software for probit analysis Biostat ver. 2.1 [32]. For combaring data, one-way analysis of variance (ANOVA) was used

followed by Tukey's studentized test when significant differences were found at $P < 0.05$ using SAS software, 2002 [33].

3. Results

3.1. Electrophoretic Profile of Bacteria Toxin Complexes (TCs)

Several protein bands of varying molecular weights were resolved by SDS-PAGE of *P. luminescens lamondii* and *X. indica* protein toxin complexes (crude, bound, and unbound fractions), as shown in **Figure 1**. *P. luminescens laumondii* crude toxin complexes resolved to 16 bands with molecular weights spanning 309 to 17 kDa, with 10 bands at 281, 246, 195, 161, 123, 72, 62, 41, 38, and 27.4 kDa found in the bound fraction and 7 bands at 315, 288, 197, 135, 99, 78, and 58 kDa found in the unbound fraction. Sixteen bands with molecular weights between 315 and 17 kDa were seen in *X. indica* crude toxin complexes; nine of these bands (315, 237, 183, 71, 63, 60, 56, 34, and 30 kDa) were detected in the bound fraction, while only four bands (93, 41, 25 and 17 kDa) were seen in the unbound fraction.

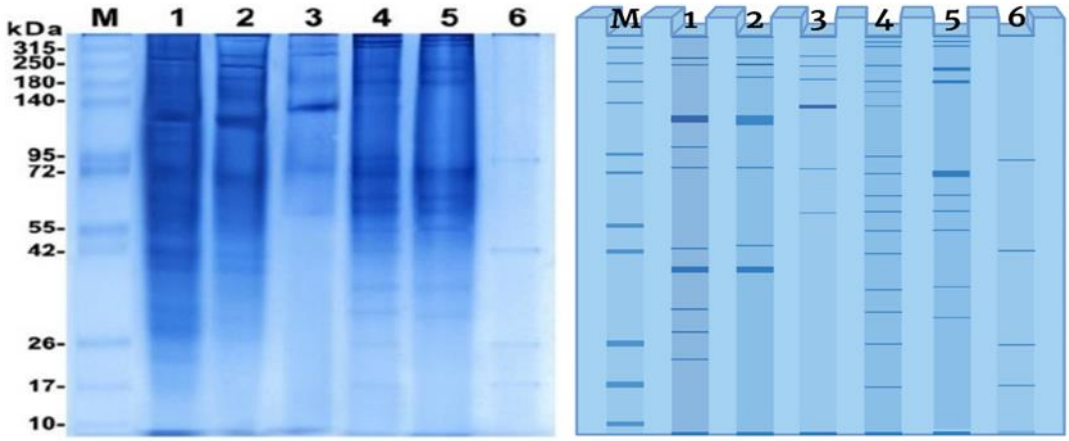

Figure 1. Electrophoretic profile of *Photorhabdus luminescens laumondii* (HP88) and *Xenorhabdus indica* (Ab) toxins complexes. Lane 1: Crude extract of HP88, lane 2: Bound fraction of HP88, lane 3: Unbound fraction of HP88, lane 4: Crude extract of Ab, lane 5: Bound fraction of Ab, lane 6: Unbound fraction of Ab. Lane M: Molecular weight standards.

By SDS–PAGE of *P. luminescens lamondii* and *X. indica* protein toxin complexes (crude, bound and unbound) were resolved into several protein bands with different molecular weights as shown in **Figure 1**. A crude toxin complexes of *P. luminescens laumondii* resolved to 16 bands of molecular weights ranged from 309 to 17 kDa, 10 bands of molecular weights 281, 246, 195, 161, 123, 72, 62, 41, 38, and 27.4 kDa were detected in bound fraction, while 7 bands of molecular weights 315, 288, 197, 135, 99, 78 and 58 kDa were detected in unbound fraction. A crude toxin complexes of *X. indica* exhibited 16 bands of molecular weights ranged from 315 to 17 kDa, 9 of them of molecular weights 315, 237, 183, 71, 63, 60, 56, 34, and 30 kDa were detected in bound fraction, while only four bands of molecular weights 93, 41, 25 and 17 kDa were detected in unbound fraction.

In current study, concerning *electrophoretic* profile of the two eluted fractions, bound fraction of P. *luminescens laumondii* resolved to 10 bands of molecular weights ranged from 281 to 27.4 kDa. The same number of bands was detected in bound fraction of *X. indica* but with higher molecular weights ranged from 315 to 30 kDa. Although, *P. luminescens laumondii* fraction exhibited less molecular weight mass, it proved the higher toxic effect on *Culex* larvae.

3.2. Biological Activity of Toxin Complexes (Crude and Fractions)

Partial purification of large mass proteins of *Photorhabdus luminescens lamondii* (HP88) and *Xenorhabdus indica* (Ab) bacterial cells using anion exchange chromatography resulted into two fractions (bound and unbound). Bound and unbound fractions and crude extract were used to evaluate their insecticidal activity on late third instar larvae of *Culex* sp. for seven days

post-treatment. Presented results revealed high oral toxicity of *P. luminescens* (HP88) TCs than *X.indica* (Ab) in bound and unbound fractions **(Table 1).**

Table 1. Toxicity of *Photorhabdus luminescens laumondii* (HP88) and *Xenorhabdus indica* (Ab) toxin complexes protein cells (TCs) fractions against late third instar larvae of *Culex pipiens* after 7 days post-treatment.

Bacterial Species	Protein fraction	LC_{25}[a] (mg/L)	LC_{50}[b] (mg/L)	Lower limit[c]	Upper limit[c]	Slope ± SE[d]	(χ^2)[e]
P. l. laumondii	Crude protein	95.31	3142.25	832.99	5973.24	0.444 ± 0.135	0.025
	Bound fraction	7.22	28.97	18.21	39.67	1.118 ± 0.147	0.081
	Unbound fraction	10.04	39.63	27.23	52.08	1.131 ± 0.143	0.21
X. indica	Crude protein	43.06	256.52	183.43	419.36	0.87 ± 0.132	0.029
	Bound fraction	67.03	294.22	218.02	449.45	1.05 ± 0.137	0.201
	Unbound fraction	91.89	443.03	305.07	793.32	0.987 ± 0.140	0.254

[a] The concentration causing 25% mortality.
[b] The concentration causing 50% mortality.
[c] Confidence limits.
[d] Slope of the concentration-mortality regression line ± standard error.
[e] Chi square value.

The LC_{50} values of (HP88) TCs were 3142.25, 28.97 and 39.63 mg/L for crude protein cells, bound and unbound fractions, respectively. Also, the LC_{50} of (Ab) TCs protein cells was 256.52, 294.22 and 443.03 mg/L for crude, bound, and unbound fractions, respectively. These results indicated that the purification of multiple proteins of *P. luminescens* (HP88) and *X.indica* (Ab) increased the toxic activity of TCs protein cells towards *Culex* sp larvae. The percentage of larvae growth inhibition was also recorded and revealing oral toxicity of (HP88) and (Ab) TCs protein cells **(Figure 2)**.

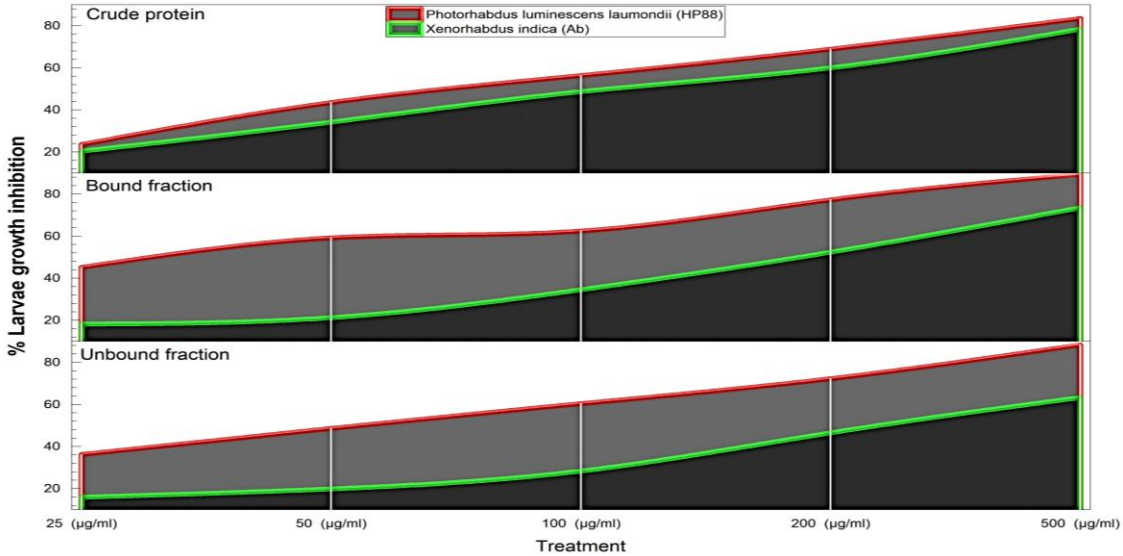

Figure 2. larval growth inhibition activity of *Photorhabdus luminescens laumondii* (HP88) and *Xenorhabdus indica* (Ab) toxin complexes protein cells (TCs) fractions against larvae of *Culex pipiens.*

Generally, *P. luminescens* (HP88) fractions had a significantly higher larval growth inhibition activity than *X. indica* (Ab) fractions. Results also, showed that growth inhibition effect increased with increasing concentration. The highest inhibition percentage was recorded by bound fraction of 500mg/L *P. luminescens* (HP88) with 89.33 ± 2.8%.

4. Discussion

Culex sp. serves as vectors of one or more important diseases of human, animals and birds. These species transmit arboviral diseases, which affects millions of people every year [34]. There no available vaccine or effective drugs against these diseases [35]. Synthetic insecticides have been the prime control method for many years to suppress *Culex Pipiens* populations. As illustrated in our results *X. indica* and *P. luminescens laumondii* toxin complexes proved insecticidal effect on late third instar larvae of *Cx. Pipiens*. Crude and fractions of *P. luminescens laumondii* toxin were the most potent than *X. indica*, LC_{50} were 3142.25, 28.97 and 39.63 mg/L for crude extract, bound and unbound fractions, respectively. While, LC_{50} of *X indica* toxin were 256.52, 294.22 and 443.03 mg/L for crude, bound and unbound, respectively. Collectively, the bound fractions of the two bacteria spp. proved the highest toxic effect compared with crude extract and unbound fraction. These results demonstrated the success of current toxins purification. To our knowledge, there is no available literature concerned the effect of X. *indica* and *P. luminescens laumondii* toxins on *Culex* sp. The toxicity of *X. indica* and *P. luminescens laumondii* metabolites which illustrated in current research against *Culex sp* was previously confirmed by different authors against other arthropods by different tools. For example, *P. luminescens laumondii* toxin was injected in locust nymph, *Schistocerca gregaria* [36] and larvae of *Manduca sexta* [23]. By orally fed of *X. indica* to fire ant [37] and beet army worm (*Spodoptera exigua*) [38]. Our findings were in line with da Silva et al. [7] who used those two species of bacteria and concluded that the bacteria, *P. luminescens laumondii* was more infective than *X. nematophila* against *Aedes aegypti.* This pathogenicity is based on their ability of multiplication and production of toxic substances inside the insect haemocoel and causing septicaemia [39]. Both produce toxin complexes, lipases, proteases, lipopolysaccharides, and other active components, may be responsible for the insecticidal activity of the bacteria, *Xenorhabdus* sp. and *Photorhabdus* sp. [40, 41]. In addition, the bacterial proteinases produced in the insect hemocoel cause histological lesions leading to insect death. Furthermore, on exposing larvae of *Aedes Aegypti* to *X. indica* and *P. luminescens laumondii* they showed abnormal behavior so called cannibalism [7]. Also, both bacteria species were toxic to locust adults and nymphs [36]. Based on the electrophoretic profile of the two eluted fractions, the present investigation found that the bound fraction of *P. luminescens laumondii* resolved to 10 bands with molecular weights between 281 and 27.4 kDa. With molecular weights ranging from 315 to 30 kDa, the same number of bands was seen in the bound fraction of *X. indica*. Although the laumondii fraction of *P. luminescens* had a lower molecular weight than the rest of the species, it was the most poisonous to *Culex* larvae. None of this was respected.

5. Conclusions

This study corroborates the use of nematode-symbiotic bacterial toxin complexes as an alternative larvicidal bioinsecticides agent against *Culex pipiens* larvae. Therefore, the present observations open opportunities for more specific compounds for insect and biological control agents used against mosquitoes. Entomopathogenic bacteria have to consider in integrated pest management (IPM) programs for mosquito control.

6. Patents

N/A

Supplementary Materials: N/A

Author Contributions: HAE, FHG, AMS, HSH, EEH, and SEE designed the experiment; HAE, and EEH conducted the experiment; HAE, and EEH analyzed the results; HAE prepared a manuscript draft; HSH, and SEE edited the manuscript; HAE, FHG, AMS and EEH revised the manuscript for technical and scientific accuracy. All authors have read and agreed to the published version of the manuscript.

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