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Characterization and pathogenicity of symbiotic bacteria associated with entomopathogenic nematode: *Steinernema* species KALRO

[1]*NGUGI C N., [2]WACHIRA P M., [3]MBAKA J N., [2]OKOTH S., [2]MANGUA P W

[1]Kenya Agricultural and Livestock Research Organization (KALRO), Horticulture Research Institute (HRI) P.O. Box 220-01000, Thika
[2]University of Nairobi, Kenya P.O. Box 30197-0100, Nairobi
[3]Kenya Agricultural and Livestock Research Organization (KALRO), Horticulture Research Institute (HRI) P.O Box 220-01000, Thika

***Corresponding author**: ceciliahngugi20@gmail.com

Abstract

The soil inhibiting entomopathogenic nematodes (EPNs), in the family Steinernematidae and Heterorhabditidae, are useful insect biological control agents. They have been used in the management of economically important crop pests. The EPNs are mutually associated with symbiotic bacteria genus *Xenorhabdus* and *Photoharbdu*s respectively. The study aimed to isolate, characterize and evaluate the pathogenicity of symbiotic bacteria associated with EPN *Steinernema* sp. Kalro (Accession MW151701). The EPN *Steinernema* sp. Karlo was multiplied using the insect baiting technique. Its bacteria symbiont was isolated and characterized based on microscopic, biochemical, and physiological features like Gram staining, urease, motility test, and glucose fermentation test). Molecular identification and phylogenetic analysis were performed on 16S rDNA nucleotide sequence. Pathogenicity of the bacteria isolate was evaluated against *Tuta absoluta* larvae with mortality data recorded after 24 and 48hours of exposure to the bacterial cell suspension. The bacteria were found to be motile and glucose fermentation positive. Sequence analysis of 16S rDNA region resulted in 1500bp sequence with maximum similarity of between 97 and 98.93%, with *Xenorhabdus* spp Accessions from Genbank. It closely matched to *Xenorhabdus* sp. My8NJ with 98.93% similarity (Accession AB507811.1). Mean percent larval mortality of 68±4.9 and 88±8.0 in the lowest cell suspension was observed in 24 and 48h of exposure. It's concluded that, the symbiotic bacteria associated with *Steinernema* sp. Kalro is *Xenorhabdus* sp. strain Kalro Genbank Accession MW245845. The bacteria is a potential biological control agent against *Tuta absoluta* larvae. Further classification of the bacteria to species level and pathogenicity trials in the screen house and field are recommended.

Keywords: *Entomopathogenic nematodes; Steinernema sp.; Kalro; Xenorhabdus sp.; strain Kalro, Pathogenicity; Tuta absoluta*

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Introduction

Entomopathogenic nematodes (EPNs) in Steinernematidae and Heterorhabditidae families are soil-inhabiting soft non-segmented roundworms, are lethal against economically important crop pests (Aiswarya *et al*., 2017; Cruz-Martinez *et al*., 2017). In the free-living non-

feeding stage of EPNs, the infective juveniles (IJ) infect insects by penetrating through body orifices and cuticles. The EPNs *Steinernema* and *Heterorhabditis* species are symbiotically associated with pathogenic bacteria species *Xenorhabdus* and *Photorhabdus* respectively (Ferreira & Malan, 2014; Kalia *et al*., 2014; Abdolmaleki *et al*., 2016; Salgado-Morales *et al*., 2019). These bacteria belong to the family Enterobacteriaceae in the gamma subdivision of proteobacteria (Sangeetha *et al*., 2016; Salgado-Morales *et al*., 2019). The *Xenorhabdus* bacteria inhabit the bacterial vesicle of *Steinernema* while *Photorhabdus* are found in the gut of *Heterorhabditis* EPNs. Once the IJs invade an insect, the pathogenic bacteria are released from the intestines into the hemocoel of the host insect (Dillman & Sternberg, 2012; Ferreira & Malan, 2014; Abdolmaleki *et al*., 2016; Aiswarya *et al*., 2017). The bacteria multiply as the EPNs nourish on them and insect tissue and reproduce killing the insect host within 24-48 h (Shapiro-Ilan *et al*., 2015; Ulug *et al*., 2015). The symbiont bacteria provide pathogenicity, degrade and break down host tissues and suppress the immunity of the host during which the EPN reproduce and complete the growth cycle. The bacteria also produce antibiotics and enzymes in addition to toxins. The mutualism of bacteria and EPNs is vital as it inhibits the development of resistance in the host pest (Proschak *et al*., 2011; Poinar and Grewal, 2012; Dillman & Sternberg, 2012; Kalia *et al*., 2014). Different EPNs harbor specific bacterial symbiont which is believed to dictate the virulence of the vector nematode. Biochemical, phenotypic, and DNA analysis have been used in the identification of bacteria. Recently, 16S rDNA sequencing and phenotypic evaluation have also been used in the characterization and identification of new bacteria species (Salvadori *et al*., 2012; Sangeetha *et al*., 2016; Ahmed *et al*., 2018). This study aimed at determining the identity of symbiotic bacteria associated with indigenous EPN *Steinernema* sp. Karlo, through biochemical and molecular methods.

Materials and Methods

Isolation of bacteria from infective juveniles of entomopathogenic nematode Steinernema sp. Karlo and hemolymph of Galleria mellonella larvae

Symbiotic bacteria from entomopathogenic nematode (EPN) *Steinernema* sp. Karlo (Accession MW151701), was isolated from infective juveniles (IJs) according to Akhurst, (1980). The IJs were picked, crushed and a loopful of the macerated material was inoculated in Luria-Bertani (LB) broth (10 g Bacto-Tryptone, 5 g Bacto-Yeast extract, and 10 g NaCl in a litre of distilled water) and plates with NBTA media. Ten replications were made for each media and incubated for 48 h at 35°C.

Galleria mellonella larvae were infected with EPN *Steinernema* sp. Karlo and bacteria associate isolated according to Woodring & Kaya, (1988). A third segment from the head of *G. mellonella* was cut and the hemolymph streaked onto nutrient agar and NBTA plates using a sterile wire loop. The plates were incubated for 48 h at 35°C for bacteria growth. Sub culturing was done until colonies of the same size and morphology were obtained. For short-term storage (three-four months) the bacterium was maintained on nutrient agar plates at 28°C and liquid broth at 4°C.

Morphological and biochemical analysis of bacteria isolated on different media

The bacterial phenotypic and biochemical assessments were done according to the Kumar *et al*., (2011) method.

Gram Staining

A small drop of distilled water was placed on a sterile slide. A loop full of the inoculum of bacteria isolate was scooped from colonies on NBTA media and spread thinly on the slide. The smear was heat fixed by passing the slide over a flame 3 times. The smear was flooded with Crystal violet stain for 1 min, washed in distilled water, Gram's iodine was added, let to stand for 1 min, and washed off. The smear was decolorized using acetone counterstained with Safranin for 3 min, rinsed with distilled water, air-dried, and observed under the microscope (Leica DM 500), at x100 objective with oil immersion.

Symbiotic bacteria growth on differential media

A loopful of pure bacteria isolate culture was

streaked on Nutrient agar (NA), NBTA, and MacConkey media. Uninoculated plates were used as negative controls (-ve). All the media plates were incubated for 3 days at 35 °C and observed for bacterial growth and colony characteristics (shape, color, margins).

Glucose fermentation (Acid gas production) test
Acid and gas production tests were done using glucose broth (0.015 g Bromocresol Purple, 10 g glucose, 5 g Sodium chloride, and 1lt distilled water). Sterile glucose broth (10 ml) was dispensed in test tubes with Durham's tubes. The broth was inoculated with a loopful of bacteria isolate. Gas production and medium color change observed

Motility test
Semi-solid NA media which had been prepared at half strength was inoculated with bacteria isolate culture by a single stab at the center half depth of the tube using a sterile straight wire after which it was observed for motility (cloudiness).

Catalase test (Slide test)
Symbiotic bacteria isolate (0.5 ml), 24 hr old inoculum was placed on a clean sterile dry glass slide using a sterile loop. A drop of freshly prepared 3% Hydrogen peroxide (H_20_2) was added and mixed with the inoculum. The setup was observed for a catalase (fizzling) reaction. To avoid false-positive catalase results, a wooden stick was used instead of a metal loop.

Urease test
Urease broth and slants containing Phenol red indicator were inoculated with a loopful of 36 hr old bacterial culture. The setup was incubated for 48 h to 7 days at 35°C and medium color change was observed.

Bacterial DNA extraction
Total genomic DNA was extracted from fresh bacterial cultures (24 h) grown in Nutrient broth and Nutrient agar (NA plates). The bacteria suspension (1 ml) was centrifuged for 5 min at 14000 rpm, the supernatant was decanted and TE buffer (Tris 1.0m and EDTA 0.5m), 500 µl was added. Equal amounts of 500 µl of lysis buffer were added and sample mixtures were incubated in a water bath at 37°C for 30 min. Phenol Chloroform Isoamyl (25:24:1 w/v) alcohol 500 µl and centrifuged for 10 min at 14000 rpm after which Absolute ethanol, of 500 µl was added and the samples were incubated for 30min at -20 °C. The samples were then centrifuged for 10 min at 14000 rpm to pellet the DNA, the supernatant discarded, 1000 µl of wash buffer (95% alcohol) added, and the sample mixture centrifuged for 5 min at 14000 rpm. The samples were air-dried for 40min. The DNA was resuspended in 10 µl TAE buffer (Sambrook *et al*., 1989; Weisburg *et al*., 1991; Chen & Kuo, 1993). The presence of genomic DNA was determined by gel electrophoresis. The genomic DNA was sent to Biotech Inqabar South Africa for PCR using forward (27f-AGAGTTTGATCATGGCTCAG) and reverse (1492r-ACGGGCGGTGTGTGC), (Weisburg *et al*., 1991). The PCR conditions were 94°C for 5 min, 94°C for 3 min, 50°C for 1 min, 68°C for 1 min and 68°C for 10 min holding at hold at 4°C.

Bacteria isolate sequence analysis
The sequence editing and alignment were done using BioEdit Sequence Alignment Editor and MUSCLE on SeaView version 4 Alignment and Analysis program. A similarity search of the Genbank database was then done using the Basic Local Alignment Search Tool (BLAST) at https://blast.ncbi.nlm.nih.gov. to identify the bacteria isolate. The best BLAST hits obtained were retrieved and their sequences aligned using the SeaView alignment tool (Hall, 1999; Edgar, 2004; Gouy et al., 2010). In addition, BLAST Pairwise sequence alignment of the isolate bacteria sequence and the closest accession at NCBI (https://www.ncbi.nlm.nih.gov/) was done.

Phylogenetic analysis of symbiotic bacteria isolate
The retrieved 16S rDNA sequences were used in phylogenetic tree reconstructed using the Neighbour-Joining method on SeaView program version 4 (Edgar, 2004; Gouy et al., 2010). Bootstrap analyses were performed with 1000 replications at a 70% threshold for relatedness. Bacteria Escherichia coli (J01859) was used as an outgroup.

Score	Expect	Identities	Gaps	Strand
2678 bits(1450)	0.0	1484/1500(99%)	3/1500(0%)	Plus/Plus

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Query  1    AGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGCAGCGGGGGAAAGCTT  60
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Sbjct  1    AGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGCAGCGGGGGAAAGCTT  60

Query  61   GCTTTCCTGCCGGCGAGCGGCGGACGGGTGAGTAATGTCTGGGGATCTGCCCGATGGAGG  120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GCTTTCCTGCCGGCGAGCGGCGGACGGGTGAGTAATGTCTGGGGATCTGCCCGATGGAGG  120

Query  121  GGGATAACCACTGGAAACGGTGGCTAATACCGCATAACCTCTTTGGAGCAAAGTGGGGGA  180
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  GGGATAACCACTGGAAACGGTGGCTAATACCGCATAACCTCTTTGGAGCAAAGTGGGGGA  180

Query  181  CCTTCGGGCCTCACGCCATCGGATGAACCCAGATGGGATTAGCTAGTAGGTGGGGTAAAG  240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  CCTTCGGGCCTCACGCCATCGGATGAACCCAGATGGGATTAGCTAGTAGGTGGGGTAAAG  240

Query  241  GCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGGACTGA  300
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Sbjct  241  GCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGGACTGA  300

Query  301  GACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGAGCGCAAGC  360
           ||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  301  GACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGC  360

Query  361  CTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGG  420
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Query  421  GGAGGAAGGCACAAGGTCGAATACACTGTGCGATTGACGTTACCCACAGAAGAAGCACCG  480
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Query  481  GCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACT  540
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Sbjct  481  GCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACT  540

Query  541  GGGCGTAAAGCGCACGCAGGCGGTCAATTAAGTTAGATGTGAAATCCCCGGGCTTAACCT  600
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Query  601  GGGAATGGCATCTAAGACTGGTTGGCTAGAGTCTCGTAGAGGGGGGTAGAATTCCACGTG  660
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Query  661  TAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACG  720
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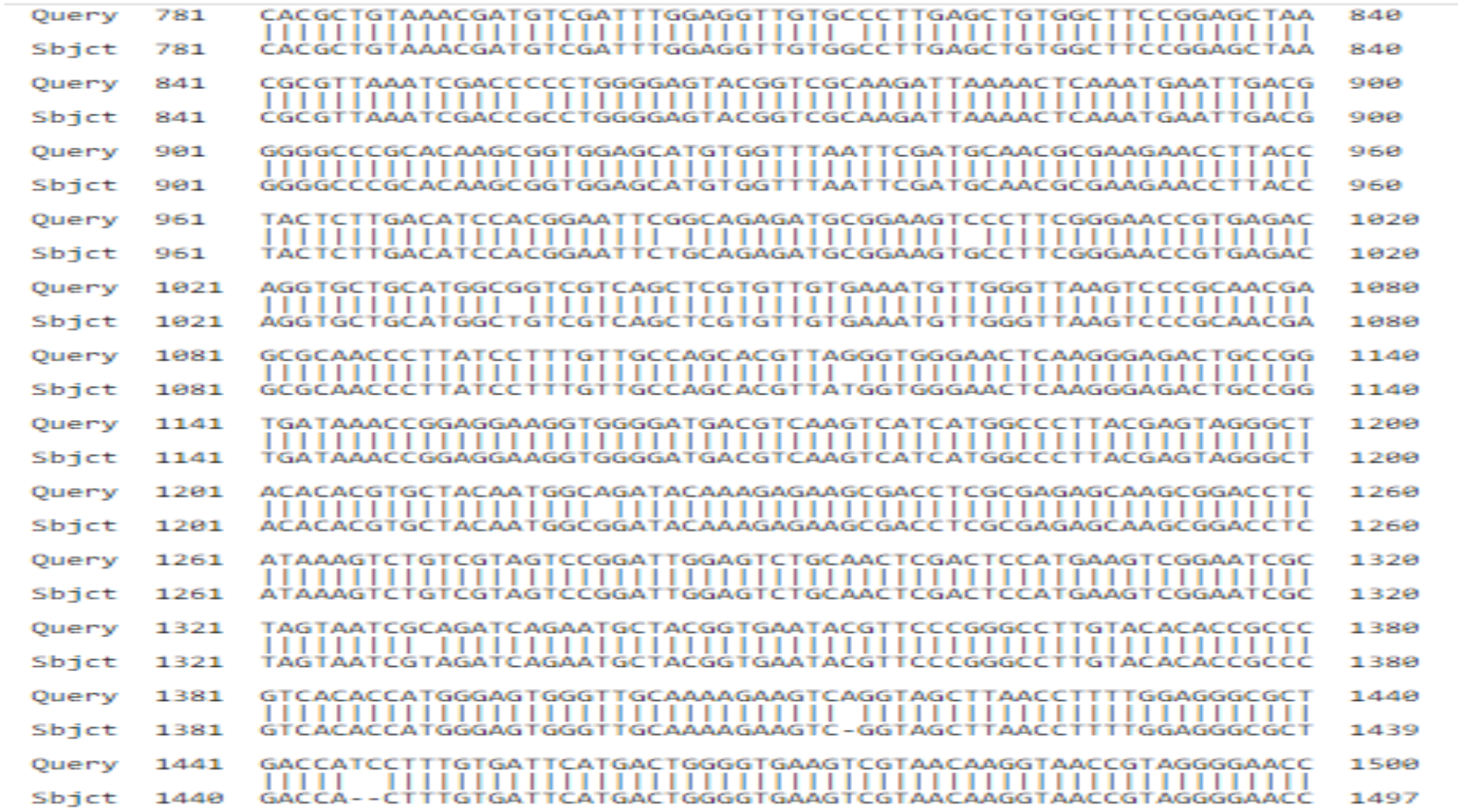


Figure 1. Pairwise sequence alignment of ITS1 region of present Xenorhabdus sp. strain Kalro (MW245845) symbiont bacteria with the most closely related species Xenorhabdus sp. My8NJ. Query= *Xenorhabdus* sp.strain Kalro (MW245845); Sbjct= *Xenorhabdus* sp. My8NJ

Pathogenicity of symbiotic bacteria isolate against Tomato leafminer Tuta absoluta 2nd stage larvae

Pathogenicity of bacteria isolates against 2nd instar larvae of *T. absoluta* was carried out. The *T. absoluta* larvae were obtained from a culture maintained on a tomato crop established in a screen house at KALRO-Kandara. Pathogenicity evaluation was according to Yooyangket *et al*., (2018) method. A single colony of bacteria isolate was inoculated in 3 ml of LB broth and incubated at 30°C for 48 h. The bacteria broth culture was centrifuged at 150 rpm and the bacterial pellet was suspended in 1 ml sterile distilled water. Serial dilution of bacterial cell suspension was done resulting in four concentrations; $0.9x10^9$, $1,7x10^9$, $2.6 x10^9$, and $3.6 x10^9$, used to evaluate the pathogenicity of the bacteria isolate against the 2nd stage larvae of *T. absoluta*. The experiment was comprised of five treatments in 5 replicates; control (distilled water), $0.9x10^9$, $1,7x10^9$, $2.6 x10^9$, and $3.4 x10^9$ /ml distilled water of bacterial concentrations. Each treatment comprised of 5 larvae individually placed in a petri dish lined with a filter paper thus a total of 25 larvae were used. In each treatment, 1 ml of bacterial cell suspension was dispensed in each Petri dish. Data on *T. absoluta* mortality was recorded at 24 and 48 h exposure time.

Data analysis

The symbiotic bacteria isolate sequence and phylogeny analysis were done using BioEdit v.7.5. and SeaView v.4. Bioinformatics programs. Larval mortality data were subjected to Statistical analysis, Analysis of variance (ANOVA) using Genstat, 15th edition, Statistical software. The means were separated using Fisher's protected least significant difference test at a 5% significance level.

Results

Physiological and Biochemical

Microscopy, biochemical and physiological analysis of isolate bacteria is summarized in Table 1

Table 1. Microscopy, biochemical and physiological analysis of isolate bacteria

Microscopy and Biochemical analysis	Result
Gram stain	-
	Rod-shaped
Colony shape and color	Convex with irregular edges
NA	Cream White
NBTA	Dark Blue
MacConkey	Red
Glucose fermentation	+
Catalase	-
Urease	+

(-)= Negative; (+)= Positive; NA=Nutrient agar; NBTA=Nutrient BromoThymol Agar

Sequence and phylogenetic analysis

The length of the 16S sequence of rDNA obtained from symbiotic bacteria was 1500 bp. The BLASTn results of 16S rDNA of the symbiotic bacteria had sequence maximum identity/similarity of between 97 and 98.93%, with *Xenorhabdus* spp retrieved from Genbank. The isolate was closely matched to *Xenorhabdus* sp. My8NJ with 98.93% similarity (Accession AB507811.1) isolated from EPN *Steinernema* sp. MY8, from Japan. Other relatives with a close similarity index to the isolate were; *Xenorhabdus ishibishii* (AB243427) 98.93%, *X. ishibishii* strain GDh7 (NR117216.1) 98.79%, *X. eopokensis* DI20 (NR156925.1) 98.06%, *Xenorhabdus* sp. GD328 (GQ149085) 98.94%, *X. griffinae* ID10 (NR043643.1) 98.04%, *Xenorhabdus* sp. VN13

(FJ51800.1) 97.46%, *X. ehlersii* strain DSM (NR042327.1) 97.34%, *X. poinarii* strain NC (FJ515806) 97.27%, and *X. thuongxuanensis* (NR156924.1) with 97% identity (Table 2). Pairwise sequence alignment revealed bacteria isolate close matches in nucleotide bases with symbiotic bacteria *Xenorhabdus* sp. My8NJ (Figure 1).

Table 2. Symbiotic bacteria species used in phylogenetic analysis of 16S rDNA of present isolate (Xenorhabdus species)

Bacteria isolate relatives	Sequence length	Max Score	Query Cover	E value	% Identity	Accession No.	Isolate source
Xenorhabdus sp. MY8NJ	1497	2678	100	0.0	98.93	AB507811.1	*Steinernema* sp. my8
Xenorhabdus ishibashii	1537	2678	100	0.0	98.93	AB243427.1	*Steinernema aciari*
X. ishibashii Gdh7	1480	2636	98	0.0	98.79	NR117216.1	*Steinernema aciari*
X. eopokensis DL20	1496	2591	99	0.0	98.06	NR156925.1	*Steinernema* sp.
Xenorhabdus sp. GDC328	1480	2575	98	0.0	98.04	Gq149085.1	*Steinernema leizhouense*
Xenorhabdus griffinae IDIO	1473	2562	98	0.0	98.04	NR043643.1	*Steinernema hermaphroditum*
Xenorhabdus sp.VN13	2015	2555	99	0.0	97.46	FJ515800.1	*Steinernema* sp.
X. ehlersii strain Dsm	1524	2545	100	0.0	97.34	NR042327.1	*Steinernema serratum*
Xenorhabdus poinarii NC	2014	2540	100	0.0	97.27	FJ515806.1	*Steinernema glasieri*
Xenorhabdus thuongxuasensis	1526	2534	100	0.0	97.00	NR156924.1	*Steinernema* sp.
Escherichia coli	1541					J01859.1	-

The phylogenetic tree constructed using retrieved *Xenorhabdus* sp. from Genbank is shown in Figure 2. Two major clades of bacteria *Xenorhabdus* sp. were obtained from the tree. The bacteria isolate clustered in a clade sub-branch with *Xenorhabdus sp*. My8NJ (AB507811.1), *X. ishibishii* (NR117216), and *X. graffinae* (NR043643). These were the most convergent *Xenorhabdus* species to bacteria isolate while *E. coli* (J01859) was most unrelated among the selected bacteria species.

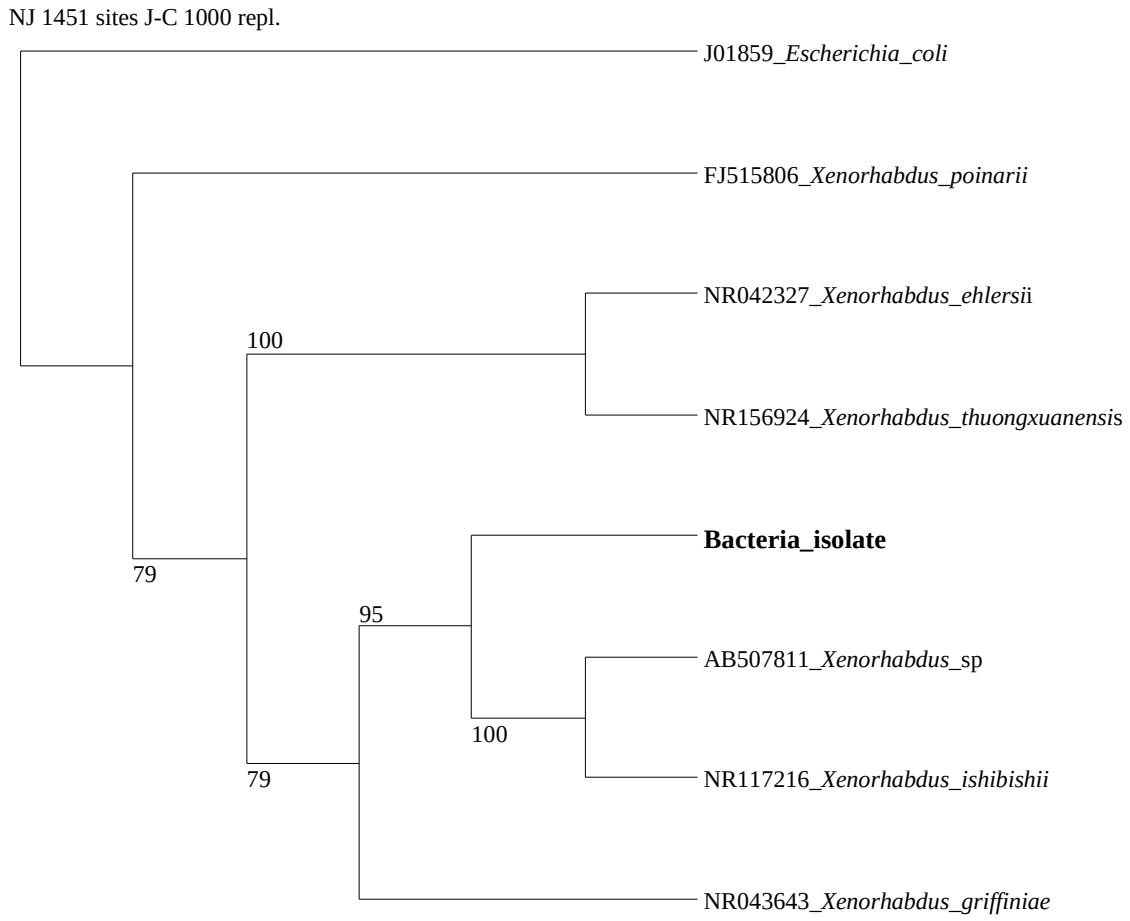


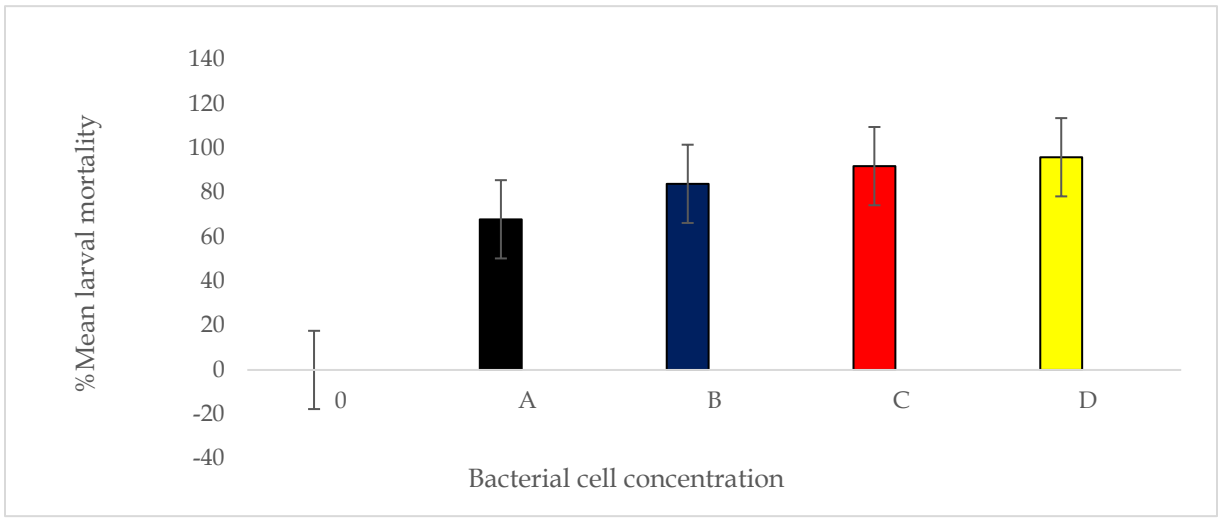
Figure 2. Phylogenetic relationship of bacteria isolate based on 16S rDNA sequence analysis through Distance Method, Neighbor-Joining

Pathogenicity of bacteria isolate against the 2nd stage larvae of Tuta absoluta under laboratory condition

Larval mortality of *Tuta absoluta* was observed only in the bacteria treatments over 48 h of exposure. At 24 h of exposure, there was no larval mortality recorded in the control experiment. The larval mean mortality of 68±4.9, 84±4.0, 92±4.9, and 96±4.0 %, respectively, was observed in bacteria concentrations $0.9x10^9$, $1,7x10^9$, $2.6\ x10^9$, and $3.6\ x10^9$ (Figure 3 A). There was a significant difference ($P<0.05$) between the control and all the other treatments. A significant difference ($P<0.05$) between bacteria concentration $0.9x10^9$, $1.7x10^9$, $2.6\ x10^9$, and $3.6\ x10^9$ was observed.

At 48 h exposure time mean mortality of 0±0.0, 88±8.0, 96±4.0, 96 ±4.0, and 100±0.0% was recorded in the control and bacteria concentrations $0.9x10^9$, $1.7x10^9$, $2.6\ x10^9$, and $3.6\ x10^9$ respectively (Figure 3B). There was a significant difference ($P<0.05$) between the control and all the treatments but there was no significant difference among the treatments with bacteria.

3A



3B

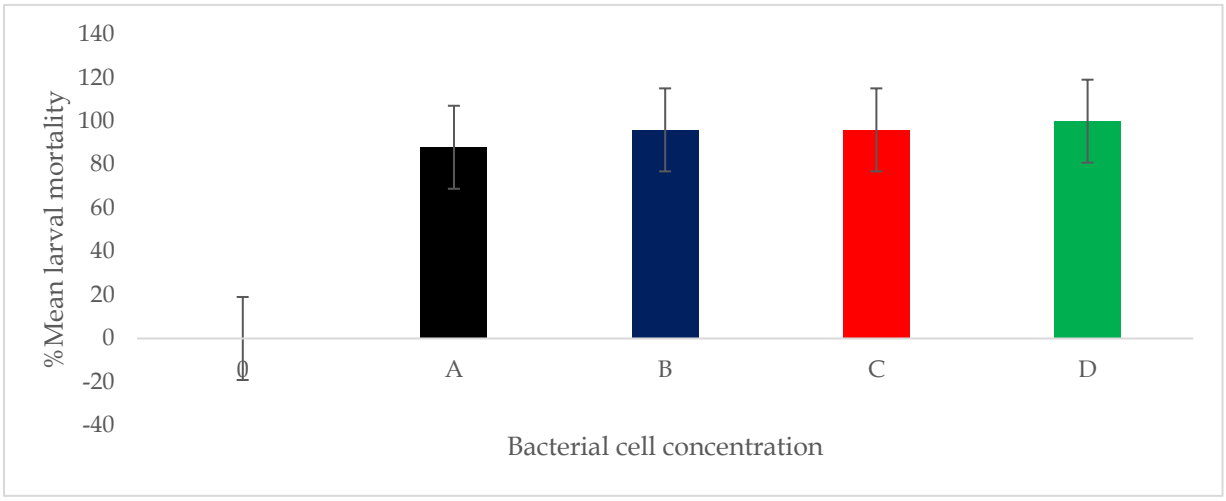


Figure 3. Pathogenicity of bacteria isolate on Tuta absoluta (3A) at 24 h and (3B) 48 h exposure (A)=0.9x10^9; (B) = 1,7x10^9; (C) = 2.6 x10^9 and (D) = 3.6 x10^9

Discussion

Phenotypically isolate symbiont bacterial colonies were creamy white in an appearance on nutrient agar (NA) medium, raised, circular with smooth to irregular edges, shiny, and opaque in appearance. The bacterial cells were also rod-shaped and the results conformed to Shields and Cathcart, (2011); Sugar *et al*., (2012), and Kampfer *et al*., (2017). The bacteria was Gram-stain-negative as pink coloration was observed as it was unable to retain crystal violet stain (dark blue) suggesting they have a thin cell wall. It is reported that Steinernematidae EPNs harbor mutualistic bacteria, *Xenorhabdus* spp. that are Gram-negative (Kumar *et al*., 2011; Kampfer *et al*., 2017). The blue bacteria colonies on a clear zone on NBTA indicated bacteria ability to absorb Bromothymol blue dye and reduce Triphenyltrizolium chloride (TTC) in the medium. The growth of red-colored bacterial colonies on the MacConkey medium was an indication the study bacteria were Gram-negative. This is because only Gram-negative bacteria flourish in bile salt in the MacConkey medium absorbing neutral red dye hence red colony appearance (Kumar *et al*., 2011; Kampfer *et al*., 2017). Positive glucose fermentation and acid gas production were observed from isolated symbiotic bacteria. This was indicated by gas

accumulation in Durham tubes and a change in media color from violet/purple to yellow (Kumar *et al*., 2011). The finding disagrees with Thomas & Poinar, (1979), who reported glucose and other carbohydrates fermentation without acid gas production from entomopathogenic *Xenorhabdus nematophilic* bacteria.

The symbiont bacteria was motile confirmed by turbidity observed extending away from the stab line of inoculation. Bacteria motility is a key in the classification and host-pathogen relationship in causing infections (Easom & Clarke, 2008; Shields & Cathcart, 2011). The negative catalase results suggested the inability of the bacteria isolate to breaks down H_2O_2 into water and Oxygen. A characteristic feature of EPNs symbiotic bacteria *Xenorhabdus* and *Photorhabdus* spp. (Kumar *et al*., 2011; Kampfer *et al*., 2017). Positive urease reaction was due to the bacteria isolate ability to break down urea into ammonia and carbon dioxide to form ammonium carbonate (alkaline). Ureases in EPNs symbiont bacteria suggest contributing to pathogenic properties of bacteria (Kumar *et al*., 2011; Salvadori *et al*., 2012; Lechowicz *et al*., 2016). The results contradict with Kampfer *et al*., (2017), who reported negative urease activity EPN symbiotic bacteria *Xenorhabdus thuongxuanensis* sp.Nov. and *Xenorhabdus eapokensis* sp.Nov. 6.7.

Molecular identification revealed bacteria isolate was *Xenorhabdus* species of bacteria which was supported by sequence and phylogeny analysis. The closest relative *Xenorhabdus* sp. My8NJ (Accession AB507811.1), with 98.93% similarity had been isolated from EPN *Steinernema* sp. MY8, from Japan. In addition, the bacteria accessions from Genbank showed geographical relatedness in that most of them had been isolated from EPNs *Steinernema* sp. from Asia (China, Japan, Indonesia) in origin.

The bacteria isolate was pathogenic against *Tuta absoluta* as it was able to infect and kill the larvae. This suggested that bacteria isolate overcome *T. absoluta* immune resistance leading to the death of the larvae. It was observed that even outside the EPN vector, bacteria isolate was still lethal to *T. absoluta* larvae. There is a possibility that the bacterial cells entered the larvae hemocoel through the same natural openings (anus, mouth, spiracles), which are entry points for EPN as reported by Shan *et al*. (2019). This is the first scientific research report on EPNs symbiotic bacteria pathogenic to *T. absoluta* larvae in Kenya. However, the larvicidal activity of the EPNs symbiotic bacterial cell suspensions against insect larvae of Diamondback moth (*Plutella xylostella*), Greater wax moth (*Galleria mellonella*), and mosquito has been reported (*Aedes aegypti* and *Aedes albopictus*) (Vanitha *et al*., 2010; Vitta *et al*., 2018; Yooyangket *et al*., 2018; Salgado-Morales *et al*., 2019).

There was a varied percentage of larval mortality of *T. absoluta* larvae on exposure to different bacterial cell concentrations over time. There was positive a correlation between bacterial cell concentration and larval mortality hence increased pest knockdown. The results agree with Kalia *et al*., (2018), who reported mortality of Tobacco cutworm (*Spodoptera litura*), neonates after 96 h of exposure to *Photorhabdus luminescens* bacteria following feeding bioassay.

Conclusion and recommendation

From the study results, it's concluded that bacteria are mutually associated with EPN *Steinernema* sp. Kalro (Accession MW151701), is a *Xenorhabdus* species deposited in Genbank as *Xenorhabdus* sp. strain Kalro (Accession MW245845). The bacteria are a potential biological control agent against *Tuta absoluta* larvae. Further classification of the bacteria to species level and pathogenicity trials against *T. absoluta* in the screen house and field are recommended.

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References

Abdolmaleki, A., Maafi, Z.T., Dastjerdi, H.R., Naseri, B., & Ghasemi, A. (2016). Isolation and identification of entomopathogenic nematodes and their symbiotic bacteria from Kurdistan province in Iran. *Journal of Crop Protection*, 5 (2): 259 - 271. DOI:10.18869/modares.jcp.5.2.259

Ahmed, J.M., Azazy, A.M., Abdelall, M.F., Saleh, W.D., & Ali, M.A. (2018). *Phortohubdus* and *Xenorhabdus* for biocontrol of the leafminer, *Tuta absoluta*. *Bioscience Research*, 15 (3): 2185 - 2193. http://www.isisn.org/BR15(3)2018/2185-2193-15(3)2018BR18-323.pdf

Aiswarya, D., Raja, K.R., Gowthaman, G., Deepak, P., Balasubramani, G., & Perumal, P. (2017). Antibacterial activities of extracellular metabolites of symbiotic bacteria, *Xenorhabdus* and *Photorhabdus* isolated from entomopathogenic nematodes. *Spring*, 3 (2): 80 - 88.

Akhurst, R. (1980). Morphological and functional dimorphism in *Xenorhabdus* spp. Bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. *Journal of General Microbiology*, 121: 303-309 https://doi.org/10.1099/00221287-121-2-303

Chen, W.P., & Kuo, T.T. (1993). A simple and rapid method for the preparation of Gram-negative bacteria genomic DNA. *Nucleic acid Research*, 21 (9): 2260. doi: 10.1093/nar/21.9.2260.

Cruz-Martinez, H., Ruiz-Vega, J., Matadamas-Ortiz, P.T., Cortes- Martinez, C.I., & Rosas-Diaz, J. (2017). Formulation of entomopathogenic nematodes for crop pest control. A review. *Plant Protection Science*, 53: 15 - 24. https://doi.org/10.17221/35/2016-PPS

Dillman, A.R., & Sternberg, P.W. (2012). Entomopathogenic nematodes. *Current Biology*, 22 (11): 430-431. doi: 10.1016/j.cub.2012.03.047

Easom, C.A., & Clarke, D.J. (2008). Motility is required for the competitive fitness of entomopathogenic *Photorhabdus luminescens* during insect infection. *BMC Microbiology*, 8: 168. https://doi.org/10.1186/1471-2180-8-168

Edgar, R.C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and throughput. *Nucleic Acids Research*, 32 (5): 1792 - 1797. https://doi.org/10.1093/nar/gkh340

Ferreira, T., & Malan, A.P. (2014). *Xenorhabdus* and *Photorhabdus*, bacterial symbionts of entomopathogenic nematodes *Steinernema* and *Heterorhabditis* and their *in vitro* liquid mass culture: A Review. *African Entomology*, 22 (1): 1 - 14. https://doi.org/10.4001/003.022.0115

Gouy, M., Guindon, S., & Gascuel, O. (2010). SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Molecular Biology and Evolution*, 27 (2): 221 - 224. https://doi.org/10.1093/molbev/msp259

Hall, T.A. (1999). BioEdit. A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic acids symposium series, 41: 95 - 98. DOI: 10.14601/Phytopathol_Mediterr-14998u1.29

Kalia, V., Sharma, G., & Ganguly, S. (2018). Virulence of native EPN strains and their symbionts alone to polyphagous Lepidopteran pests, *visa vis* model insect *Galleria mellonella* along with *in vivo* production. *Entomology, Orthonology Herpetology*, 7 (2): 210 - 218. DOI: 10.4172/2161-0983.1000210

Kalia, V., Sharma, G., Shapiro-Ilan, D.I., & Ganguly, S. (2014). Biocontrol potential of *Steinernema thermophilum* and its symbiont *Xenorhabdus indica* against Lepidopteran pests: virulence to eggs and larval stages. *Journal of Nematology*, 46 (1): 18 - 26. PMID: 24643472

Kampfer, P., Tobias, N.J., Ke, L.P., Bode, H.B., & Glaeser, S.P. (2017). *Xenorhabdus thungxuanenseis* sp. Nov. and *Xenorhabdus eapokensis* sp. Nov, isolated from *Steinernema* spp. *International*

Journal of Systemic and Evolutionary Microbiology, 67: 1107 - 1114. DOI: 10.1099/ijsem.0.001770

Kumar, S.V., Mulla, S.R., & Suresh, C.K. (2011). Isolation and molecular characterization of symbiotic bacterial isolates associated with entomopathogenic nematode in agro climate zone 5 of Karnataka. *Journal of Phytology*, 3 (11): 25 - 29

Lechowicz, L., Chrapek, M., Czerwonka, G., Korzeniowska-Kowal, A., Tobiasz, A., Urbaniak, M., Matuska-Lyzwa, J., & Kaca, W. (2016). Detection of ureolytic activity of bacteria strains isolated from entomopathogenic nematodes using infrared spectroscopy. *Journal of Basic Microbiology*, 56 (8): 922 - 928. https://doi.org/10.1002/jobm.201500538

Poinar, G.O., & Grewal, P.S. (2012). History of entomopathogenic nematology. *Journal of Nematology*, 44 (2): 153 - 161. PMCID: PMC3578475

Proschak, G.O., Schultz, K., Herrmann, J., Doowlinng, A.J., Brachmann, A.O., Ffrench-Constant, R., Muller, R., & Bode, H.B. (2011). Cytotoxic fatty acid amides from *Xenorhabdus*. *Chem Bio Chem*, 12: 2011 - 2015. https://doi.org/10.1002/cbic.201100223

Razia, M., Padmanaban, K., Karthik, R.R., Chellapandi, P., & Sivaramakrishnan, S. (2011). PCR RFLP pattern analysis of entomopathogenic nematodes isolated from agroecosystem for implicating their genetic diversity. *Journal of Entomological Zoology*, 6 (1): 404 - 411

Salgado-Morales, R., Martinez-Ocampo, F., Obregon-Barboza, V., Vilchis-Martinez, K., Jimenez-Perez, A., & Dantan-Gonzalez, E. (2019). Assessing the pathogenicity of two bacteria isolated from the entomopathogenic nematode *Heterorhabditis indica* against *Galleria mellonella* and some pest insects. *Insects*, 10 (3): 83. DOI: 10.3390/insects10030083.

Salvadori, J., Defferiari, M., Ligabue-Braun, R., Lau, E., Salvadori, J., & Carlini, C. (2012). Characterisation of entomopathogenic nematodes and symbiotic bacteria active against *Spodoptera frugiperda* (Lepidoptera; Noctuidae) and contribution of bacteria urease to insecticidal effect, *Biological Control*, 63 (3): 253 - 263. https://doi.org/10.1016/j.biocontrol.2012.08.002

Sambrook, J., Fristch, E.F., & Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual (2nd ed.). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sangeetha, B.G., Jayaprakas, C.A., Siji, J.V., Rajitha, M., Shyni, B., & Mohandas, C. (2016). Molecular characterization and amplified ribosomal DNA restriction analysis of entomopathogenic bacteria associated with Rhabditis (*Oscheius*) spp. *Biotechnology*, 6 (1): 32. doi: 10.1007/s13205-015-0326-1

Shan, S., Wang, W., Song, C., Wang, M., Sun, B., Li, Y., Fu, Y., Gu, X., Ruan, W., & Rasmann, S. (2019). The symbiotic bacteria *Alcaligenes faecalis* of the entomopathogenic nematodes *Oscheius* spp. exhibit potential biocontrol of plant and entomopathogenic fungi. *Microbial Biotechnology*, 12(3): 459 - 471. doi: 10.1111/1751-7915.13365

Shapiro-Ilan, D.I., Cottrel, T.E., Mizel, R.F., Horton, D.L., & Zaid, A. (2015). Field suppression of the Peach borer, *Synathedon exitiosa*, using *Steinernema carporcapsae*: Effect of irrigation, sprayable gel and application method. *Biological Control*, 82 (2015): 7 - 12. https://doi.org/10.1016/j.biocontrol.2014.12.003

Shields, P., & Cathcart, L.A. (2011). Motility test medium protocol. Washington, DC: *American Society of Microbiology*, pp. 1 - 10.

Sugar, D.R., Murfin, K.E., Chaston, J.M., Andersen, AW., Richards, G.R., De-Leon, L., Baum, J.A., Clinton, W.P., Forst, S., Goldman, B.D., Krasomil-Osterfeld, K.C., Slater, S., Stock, P., & Goodrich-Blair, H. (2012). Phenotypic variation and host variation of *Xenorhabdus bovienii* SS-2004, the entomopathogenic symbiont of *Steinernema jollieti* nematode. *Environmental Microbiology*, 14 (4): 924 - 939. doi: 10.1111/j.1462-2920.2011.02663.x

Thomas, G.M., & Poinar Jr. G.O. (1979). *Xenorhabdus* gen. Nov., a genus of entomopathogenic nematophilic bacteria of family Enterobacteriaceae. *International Journal of Systematic Bacteriology*, 29 (4): 352 - 360. https://doi.org/10.1099/00207713-29-4-352

Ulug, D., Hazir, C., & Hazir, S. (2015). A new and simple technique for the isolation of symbiotic bacteria associated with entomopathogenic nematodes (Heterorhabditidae and Steinernematidae). *Turkish Journal of Zoology*, 39: 365 - 367. DOI: 103906/zoo-1404-63

Weisburg, W.G., Barns, S.M., Pelletier, D.A., & Lane, D.J. (1991). 16S Ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*, 173 (2): 697 - 703. DOI: 10.1128/jb.173.2.697-703.1991

Yooyangket, T., Muangpat, P., Polseela, R., Tandhavanant, S., Thanwisal, A., & Vitta, A. (2018). Identification of entomopathogenic nematodes and symbiotic bacteria from Nam Nao National Park in Thailand and larvicidal activity of symbiotic bacteria against *Aedes aegypti* and *Aedes albopictus*. PLOS ONE, 13 (4): p. e019568. DOI: 10.1371/journal.pone.0195681

Zolfagharian, M., Saeedizadeh, A., & Abbasipour, H. (2016). Efficacy of two entomopathogenic nematodes as potential biocontrol agents against Diamondback moth, *Plutella xylostella* (L.). *Journal of Biological Control,* 30 (2): 78 - 83. DOI:10.18311/jbc/30/2/14919

Vanitha, L.S., Kumari, M., Jayappa, J., & Chandrashekar, S.C. (2010). Symbiotic bacteria, *Xenorhabdus* spp. of entomopathogenic nematodes: source of antifungal compounds against four plant pathogens. *Asian Journal of Bioscience*, 5 (2): 174 -177

Vitta, A., Thimpoo, P., Meesil, W., Yimthin, T., Fukruksa, C., Polseela, R., Mangkit, B., Tandhavanant, S., & Thanwisal, A. (2018). Larvicidal activity of *Xenorhabdus* and *Photorhabdus* bacteria against *Aedes aegypti* and *Aedes albopictus*. *Asian Pacific Journal of Tropical Medicine,* 8 (1): 31 - 36. DOI: 10.4103/2221-1691.221134