Isolation and Determination of Morphological Characterization of Heterorhabditid Entomopathogenic Nematodes (EPNs) from Kashere, Nigeria

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Abstract

Entomopathogenic nematodes that harbor symbiotic bacteria in their intestines have attracted great interest for pest control in recent times due to their relative safety to man, farm animals and the environment. The objective of this study is to isolate indigenous entomopathogenic nematode-bacteria complex for potential use in the control of local insect pests. A total of 60 soil samples were collected from Kashere and its environs and tested for presence of these important entomopathogens by baiting with live Galleria mellonella larvae. Infective juveniles of the nematodes were recovered from only two of the samples from one of the locations, (Kashere). Developmental stages of the nematodes were studied and keys were used to identify the nematodes as Heterorhabditis bacteriophora. Further studies will establish their biological control potential and target pests.

Keywords: Entomopathogenic; Biological Control; Heterorhabditis bacteriophora; Pests; Kashere

Introduction

Entomopathogenic nematodes, a group of specialized nematodes that carry symbiotic bacteria in their intestines are lethal parasites of many insects' worldwide [1]. They belong to two families, Steinernematidae and Heterorhabditidae. The Steinernematidae according to the report of Noosidum., et al [2] harbor specific symbiotic bacteria in their intestines such as Xenorhabdus spp. while the Heterorhabditidae harbor Photorhabdus spp. They have been developed and used as important biological control agents for many important crops [3]. The free living forms of these nematodes are the infective juveniles and are usually found in the soil. They penetrate insect hosts through natural openings such as the mouth, the anus, the spiracles and sometimes through the cuticle. Once inside the insect, they release their associated bacterial symbionts which multiply inside the host producing a number of virulence factors such as toxins, hydrolytic enzymes, antimicrobial compounds and hemolysins that kill the host within 24 - 72 hours. The bacteria convert the insect host to nutrients for the developing nematodes which undergo 2 - 3 generations within the insect host. As a result of limiting nutrients the nematodes take in their symbiotic bacteria and exit the host en mass to seek for new hosts in the environment according to Noosidum., et al [2].

Their success as biological control agents is attributed to their high reproducibility, ease of mass production, their harmlessness to microbes, humans, animals and plants, Shapiro-Ilan., et al [3] as well as their inability to confer pest resistance and resurgence [4]. For a successful biological control regime therefore, it is important to isolate native nematodes-bacteria complexes which according to Noosidum., et al [2], are better suited for the control of local insect pests since they are adapted to the local environmental conditions. The

demerits of using exotic entomopathogenic nematode-bacteria complex include the possible displacement of natural organisms as well as their effects on non-target pests. Also exotic nematode-bacteria complex are not adapted to local environmental conditions hence poor infectivity may occur as observed by Grewal, et al [5]. The utilization of an indigenous nematode-bacteria complex as biocontrol agent of insect pests requires the isolation and proper identification of indigenous species in order to achieve better management and control of local pest populations.

**Aim of the Study**

The aim of this study is to isolate and identify indigenous nematode-bacteria complex from Kashere area of Gombe State Nigeria, an area reknown for the cultivation of arable crops.

**Materials and Methods**

**Study site**

The study was conducted in Kashere and its environs, Tumu, Pindiga and Sabon Layi settlements of Gombe State. The area is located between latitude 09°46'01.2''N and longitude 10°57'00.0''E of the equator in North-eastern Nigeria. Most of the inhabitants are farmers who practice subsistent agriculture for local consumption with a few large scale farmers who are into commercial farming.

**Sample collection**

Larvae of *Galleria mellonella* L. used as bait were collected from Kashere, to ensure compliance with local environmental conditions. A total of 60 soil samples were randomly collected from cultivated farms in the study sites. The soil samples were taken from a depth of 5 to 10 cm and held in 250 ml plastic containers before being taken to the Biological Science Laboratory of Federal University of Kashere for analysis.

**Rearing and identification of Galleria mellonella bait**

Honey combs were obtained from an apiary maintained by the Department of Biological Sciences, Federal University, Kashere, Gombe State. The combs were placed in 1 litre transparent jars and kept in a dark cupboard at ambient temperature. The jars were observed every three days for the presence of larvae of greater wax moth, *Galleria mellonella*. The larvae were identified using keys described by Aliyu, et al [6] and subsequently used in all experiments.

**Isolation of nematode-bacteria complex from soil: Insect baiting technique**

Infective juveniles of the nematodes (nematode-bacteria complex) were recovered from the soil samples by baiting with live larvae of *G. mellonella*. Thereafter, five (5) fifth instar larvae of *G. mellonella* were placed on the surface of each of the 60 soil samples earlier kept in their respective plastic containers. To maximize level of recovery of nematode-bacteria complex, second and third baiting of the soil samples were done using fresh larvae of *G. mellonella* according to the method of Shapiro-Ilan and Gaugler [7]. The containers were inverted upside down and kept at room temperature in a cupboard to facilitate infection of the larvae by the nematode-bacteria complexes. They were subsequently monitored and checked daily for the presence of dead larvae of *G. mellonella* which usually occurred within 24 - 72 hrs.

**Recovery of nematode-bacteria complex from infected G. mellonella larvae by use of White trap method**

White traps consisting of the lid of a small plastic Petri dish (50 mm) lined with moistened filter paper were designed and used. The small Petri dish was in turn placed in a large Petri dish plate of 90 mm size that contained distilled water. Any dead larva or bait was properly rinsed in distilled water to remove soil particles and sterilized with 70% hypochlorite solution before being placed on the White
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trap. The white traps were incubated at room temperature and observed for emergence of the nematodes-bacteria complexes. Nematode-bacteria complexes emerging from the *G. mellonella* cadavers migrated into the larger Petri dish and were trapped in the water contained in it according to the method of Grewal [8].

**Morphological characterization using light microscopy**

For morphological observation, first generation hermaphrodites were collected after 3 days post infection and second generation males and females of the nematodes were collected from 4 - 5 days post infection. The cadavers were rinsed in water and disinfected in 70% hypochlorite solution. They were then dissected out in distilled water. Infective juveniles of the nematodes were collected directly from the white traps after their first emergence from the cadavers. Wet mount was prepared by placing the nematodes on clean slides. The slides were gently covered with cover slips and viewed under the research microscope connected to a camera for capturing the images. Characterization was based on various important morphological characters in the keys developed by Nguyen and Smart [9], Nikdel, *et al.* [10] and Seddiqi, *et al.* [11].

**Results**

Larvae obtained from honey combs were successfully reared in the laboratory on artificial medium. The larvae were also used successfully to trap infective juveniles of entomopathogenic nematodes from 2 out of 60 soil samples collected from all the study sites shown in table 1. Infected cadavers were recognized by a change in colour. Preliminary identification was based on colour change of infected *G. mellonella* cadavers from milky white of healthy larvae to dark red colour of infected cadavers 48 - 72 hours after their death according to the description of Kaya and Stock [12]. The sticky nature of infected cadavers characteristic of *H. bacteriophora* was also used in the identification. Infective juveniles of the nematodes were observed to exit the dead cadavers and move into the white trap successfully.

<table>
<thead>
<tr>
<th>Location</th>
<th>Samples containing dead baits</th>
<th>Samples from which infective juveniles emerged</th>
<th>Samples from which infective juveniles did not emerge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pindiga</td>
<td>7</td>
<td>None</td>
<td>7</td>
</tr>
<tr>
<td>Tumu</td>
<td>4</td>
<td>None</td>
<td>4</td>
</tr>
<tr>
<td>Kashere</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Sabon lal (Billiri)</td>
<td>3</td>
<td>None</td>
<td>3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>18</strong></td>
<td><strong>2</strong></td>
<td><strong>16</strong></td>
</tr>
</tbody>
</table>

*Table 1: Infective juveniles of nematodes isolated from the study sites (N=15).*

SD = 0.1810, SE = 0.0234 at 0.05% confidence level.

**Identification of the nematodes**

**Hermaphroditic females- first generation adults**

Upon entry into an insect host, the infective juveniles develop into hermaphroditic females (Plate 1). They are robust with the head being slightly rounded and continued with the body. The esophagus is long and muscular with a swollen basal bulb. The vulva is situated near the middle of the body. Vulval opening is a transverse slit and the upper lip is hypertrophied. The cuticle anterior to the vulva is slightly protruding. The tail is relatively short and blunt. The hermaphrodites are self fertilizing. They laid eggs which were retained in their body. Initially, they were oviparous and later they become ovoviviparous.
Amphimictic females- second generation adults

The amphimictic females are second generation adults (Plate 2). They are similar to the hermaphroditic females but the shape of the head is different. They have a long and muscular esophagus which ends with a distinct basal disc and the isthmus is covered by the upper part of the intestine. The vulva is located in the middle of body with the vulval lips protruding outward and covered with a hardened deposit. The tail is conical and pointed. After mating the females lay eggs which are retained in their body. After a few days the eggs develop into juveniles within the females. The juveniles fed on the contents of the mother and the mother usually bursts to release the juveniles leaving an empty cuticle, a process referred to as endotokia matricide.

Males- second generation adults

The males are also adults found in the second generation. They are similar to the females but appear smaller in size. Their cuticle is smooth under light microscope. The head is rounded and continues with the body. The bucal cavity is funnel shaped and is shorter than that of the females (Plate 3 and 4). The isthmus is distinct. The spicules (Plate 4) are paired and separate. The manubrium (head) of the spicule is rounded and the lamina is ventrally curved. Gubernaculum is thinner and about half the size of the spicules. The Gubernaculum is also ventrally curved. They assist in guiding the spicules during mating. The bursa peloderan is open and supported by nine pairs of papillae. The papillae are arranged according to the formular 1,2,3,3 (Plate 4) that is: 1 pair consisting of one each, followed by another pair consisting of two each then 2 pairs consisting of three each. The terminal pair has the median one (8th pair) shorter than the others. The genital papillae are sensory appendages. The tail is conical and ventrally curved with a pointed tip.

Plate 2: Amphimictic female of Heterorhabditis bacteriophora (Mgx800).
Arrow showing the protruding vulva at middle of the body.

Plate 3: Infective juveniles and adult male of *H. bacteriophora* (Mgx800).

A= Head of adult male (*♂*) with short oesophagus.

B= Head of an infective juvenile worm (*IJ*).

C= Tail of an infective juvenile worm (*IJ*).

D= Ejected spicule and gubernaculum on the tail of the male. The bursa pelloderan is open and supported with 9 pairs of papillae.

E= The 8th pair of the papillae is shorter than the rest hence it is not touching the rim of the bursa.

Plate 4: Lateral view of tail of male *H. bacteriophora* (Mgx800).

A= The ventrally curved gubernaculum

B= Ventrally curved spicule.

1-9= Arrangement of 9 pairs of caudal papillae according to the formular 1,2,3,3.
Infective juveniles

The body is slim, elongated and usually taper at both ends. They are enclosed by the sheath of the second stage juvenile and this is present immediately after harvesting. The sheath is usually lost during storage in water. Exsheated infective juveniles of *Heterorhabditis* spp. have two prominent ridges along the middle and form four ridges near the anterior part when heat fixed and treated with preservatives. The tail is elongated with a pointed terminus. Symbiotic bacterial cells are found in the intestine.

Discussion and Conclusion

Larvae of *G. mellonella* were successfully obtained from honey combs, reared and maintained on artificial medium in the laboratory and subsequently used for all experiments. They were identified as *G. mellonella* by studying their developmental stages using the descriptive keys of Aliyu., *et al* [6]. The occurrence of infective juveniles of the nematodes was relatively low in this study. Low recovery of infective juveniles of the nematodes from Kashere (Table 1) could be due to the following. Firstly, *G. mellonella* has been widely used as bait to trap entomopathogenic nematodes from soil. However, some species like *Steinernema scapterisci* do not reproduce well in the larvae of *G. mellonella*, hence limiting the isolation of such species. This finding is consistent with that of Phan., *et al* [13]. Shapiro-Ilan and Gaugler [7] reported that wax moth were the most susceptible insect bait for isolating nematodes from soil samples. However, Spiridonov and Moens [14] observed that the nematode *Steinernema scapterisci* does not invade and infect wax moth hence an alternative bait is used. Secondly, only room temperature was used in baiting of the soil samples for the isolation of infective juveniles. Consequently, temperature may have affected the rate of recovery of infective juveniles of the nematodes as previously observed by Hazir., *et al* [15]. The third reason for low recovery of the nematodes-bacteria complexes may be attributed to the large dependence on chemical pesticides by farmers in the study area to control and manage pest insects in agricultural produce which could have affected the natural pathogens of these crops including infective juveniles of the nematodes. These observation are consistent with the earlier report of Laznik., *et al* [16] who isolated entomopathogenic nematode-bacteria complexes from only 2.5% of soil samples in Slovenian soils as a result of heavy use of chemical insecticides in that area. Low recovery of nematodes-bacteria complexes in this study is also consistent with the report of Hominick [17].

Arun and Lalramliana [18] and Aliyu., *et al* [6] who separately isolated infective juveniles from 2%- 45% of soil samples analysed in their various study areas. It was also observed that greater sample size increased chances of the sites for isolation of infective juveniles. An extensive study which was conducted in Turkey for isolation of entomopathogenic nematodes involved a sample size of 1080 and out of those, only 22 were positive for nematodes. In South Africa, the most abundant species encountered from soil samples were those of the genus *Heterorhabditis* with only a few *Steinernema* spp. Malan., *et al* [19] collected 202 soil samples in South Africa of which 35 yielded infective juveniles of the nematodes with 89% *Heterorhabditiids*. Hattings., *et al* [20] analysed 1506 soil samples collected from oak woodlands in mountain ranges of Coronado national forests in Southern Arizona and recovered infective juveniles from 28 of the samples recording 78.5% steinernematids and 21.5% *Heterorhabditiids*. In Nigeria, Aliyu., *et al* [6] reported 6% recovery out of 50 soil samples collected from maize and cowpea fields in Yola, Adamawa State of Nigeria.

Cadavers of recovered from soil samples collected from Pindiga, Tumu and Sabon Layi in Billiri Local Government Areas of Gombe State did not produce infective juveniles of the nematodes. The absence of infective juveniles of the nematodes in these samples could be attributed to excessive use of chemical fertilizers and chemical pesticides used in the area. A similar observation has been made by Koppenhofer and Fuzy [4], Shapiro-Ilan., *et al* [3]. Absence of nematodes could also be attributed to the small sample size collected in the area as similarly observed by de Brida., *et al* [21]. The white trap method was used successfully to recover infective juveniles of nematodes from infected *G. mellonella* cadavers. This result is consistent with that of Seddiqi., *et al* [11].

Preliminary identification of the nematodes was based on symptoms of infected *G. mellonella* cadavers based on colour change from milky white to dark red based on the report of Stock., *et al* [22]. Also, the cadavers had a gummy consistency, a characteristic of *Heterorhabditis bacteriophora* reported by Kaya and Gaugler (1993). Other important evidence included the presence of hemaphroditic females as exclusive members of the first generation adults 3 - 4 days post infection. The presence of amphimictic females, males and a

few hermaphroditic females as members of second generation adults 6-7 days post infection which is also consistent with the findings of Dix., et al. [23] who described members of the first generation adults as exclusively hermaphroditic females while in the second generation males, amphimictic females as well as hermaphroditic females were observed. Some researchers like Seddiqi., et al. [11] and Nikdel., et al. [10] described hermaphroditic females with pointed tail but Nguyen and Smart [9] described hermaphroditic females with blunt tails. In the current study hermaphroditic females were observed with blunt tails. This observation is consistent with the description of the original material.

Another valid criterion is the number and arrangement of the genital papillae in males according to the formular 1,2,3,3 (Plate 4) which is consistent with those of described Heterorhabditis bacteriophora isolates previously observed by Seddiqi., et al. [11] and Nikdel., et al. [10]. Poinar [24] described genital papillae of males in H. bacteriophora with 9 pairs according to the formular 1,2,3,3 with the 9th pair being variable. Nikdel., et al. [10] observed males of H. bacteriophora isolated from Iran with 9 pairs also with the formular 1,2,3,3. However, variations occurred in the two terminal groups of three papillae, with the median one shorter than others as also observed in H. baujardi. They attributed these morphological variations to geographical locations and habitats from where the isolates were collected. They also observed that these differences were also due to intraspecific variations among species. Another valid morphological character that was also used for the identification of the isolate was the shape of the spicules and gubernaculums as reported by Stock., et al. [25], Phan., et al. [13], Nguyen., et al. [26], Nikdel., et al. [10] and Seddiqi., et al [11]. In the current study, the spicules were observed to be ventrally curved with pointed tip. The gubernaculums were observed to be ventrally curved as well and shorter than the spicules, findings similar to those described for H. bacteriophora isolates previously studied by Karini., et al. [27], Nikdel., et al. [10], Seddiqi., et al [11]. It was also reported that most entomopathogenic nematodes and associated bacteria recovered from Asia and other developing countries have not yet being properly characterized due to lack of facilities and expertise as well as adequate literature.

In Nigeria, the diversity of these important agents of biological control as well as their use have not yet been properly established as most studies are still at the experimental stage. Due to the advantage of using indigenous isolates of entomopathogenic nematodes to enhance ecological compatibility and reduce significant impact on non-target hosts, it is therefore important to carry out further research to recover more species of the nematodes in the environment. There is also the need to collect large number of soil samples from different depths to increase the chances of isolation of the nematodes. Also, it is important to evaluate the control potential of the native isolates against local pests under local conditions in order to properly manage and control local pests in the Nigerian environment.

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