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Isolation, identification and pathogenicity of *Steinernema carpocapsae* isolated from Tamil Nadu on cattle tick, *Rhipicephalus microplus*

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Abstract
Entomopathogenic nematodes (EPNs) have been considered as potential candidates for biocontrol of ixodid ticks as chemical acaricides are not environmentally friendly. The present study was carried out to isolate the EPNs from soil using insect-bait method and study their pathogenicity on cattle tick *Rhipicephalus microplus* on the basis of percent mortality under laboratory conditions. A total of 25 soil samples were collected from different places of Tamil Nadu and were baited with ticks. Amongst the soil samples, EPNs were observed from only one soil sample of Kattupakkam, Kanchipuram district, Tamil Nadu baited ticks and were identified as *Steinernema carpocapsae* based on the morphological characteristics. The pathogenicity of *S. carpocapsae* was tested against engorged females of *R. microplus* and there was100 percent tick mortality was observed after fifth day of exposure at the concentration of 1000 infective juveniles of the nematode per Petri dish (6 ticks /Petri dish). The study indicated that the local *S. carpocapsae* was effective for control of engorged females of *R. microplus.*

Keywords: Entomopathogenic nematodes, *Steinernema carpocapsae*, *Rhipicephalus microplus*

Introduction
Ticks (Acari: Ixodidae) are most important for transmission of different viral, bacterial and protozoan disease to human and animals. Various chemical acaricides are currently used to manage tick populations. Ticks are prone to develop resistance to readily available acaricides; these compounds are costly; and some of them are not environmentally acceptable. Because of this, we are looking for alternative biological agent-based control strategies. Traditional biological control methods involve identifying, assessing and importing natural enemy from elsewhere, conserving native natural enemies and increasing the number of biocontrol agents [1]. Biological agents have been used extensively to control pests, frequently as a component of integrated pest management (IPM) programs [2].
Entomopathogenic nematodes (EPNs) are soil-inhabiting, lethal insect parasites that belong to the Phylum Nematoda from the families Steinernematidae and Heterorhabditidae and they have managed to prove to be the most successful biological control. The EPNs are mutually associated with bacteria of the family Enterobacteriaceae; the bacteria carried by Steinernematidae are usually a genus *Xenorhabdus*, and that carried by Heterorhabditidae is a species of *Photorhabdus* [3]. The infective juvenile (IJ) is the only free-living stage of these nematodes and can be found by baiting with live insects in soil and epigeal habitats [4]. When the IJ finds a susceptible insect host, it enters the insect through natural openings (anus, spiracles, or mouth), penetrates the haemocoel and releases the symbiotic bacterium. The bacterial cells multiply in the haemocoel and kill the host within 48 hours by septicemia [5]. The developing nematodes feed on the insect cadaver and digested tissues, and undergo two or more generations, thereby, producing new IJ which emerge into the soil as host resources are depleted [3].
Laboratory studies have shown that EPNs are a promising biological agent for the sustainable control of cattle tick, *Rhipicephalus microplus* [6, 7]. The biological method of using EPNs to control the non-parasitic phase of *R. microplus* can be effective because engorged females during oviposition seek environments with high moisture and protection from solar radiation, which also favors survival of the EPNs [8].

As per the bio-geographic assessment, these nematodes have been isolated from all continents (excluding Antarctica) and nearly all regions of the planet [9]. The number of EPN surveys in tropical and subtropical regions has significantly increased during the last decade and as a result, several new species have been recovered and described [10]. Therefore, the objective of this study was to isolate the entomopathogenic nematode from soil samples of Tamil Nadu and analyze their pathogenicity on cattle tick, *Rhipicephalus microplus.*

Materials and Methods

Collection of soil samples

The soil samples were collected from twelve districts of Tamil Nadu namely Chennai, Kanchipuram, Thiruvallur, Chengalpet, Krishnagiri, Dharmapuri, Salem, Vellore, Ariyalur, Erode, Nammakkal and Coimbatore from June 2013 to March 2014. The soil samples were taken primarily in forest areas and agricultural fields with plantation cover of mango, banana as well as animal fodder. A total of 25 soil samples weighing approximately one kilogram were collected from an area of 10 m^2 and to a depth up to 20 cm. The samples were placed in polyethylene bags to prevent water loss and kept in coolers during transit to the laboratory and stored at 4 °C until used.

Ticks

Engorged female ticks of *Rhipicephalus microplus* were collected from natural field infestations in cattle farm at different places of Tamil Nadu. Ticks were identified according to Hoogstraal *et al*. [11]. The collected ticks were incubated at 25 °C and 75% Relative Humidity for laboratory experiments.

Isolation of entomopathogenic nematode (EPNs)

The isolation of entomopathogenic nematodes from soil sample was followed as per the method described in insect baiting technique (*Galleria* trap) [4] with minor modifications. The soil samples were mixed with small quantity of water to moisten the soil and facilitate the movement of nematodes. Approximately 200 to 250 g of moist soil were placed in plastic boxes. Ten engorged female ticks were added on the soil surface in each box. Cover the container with a lid and turn upside down and kept in dark at room temperature 22- 25 °C. Check containers every 2 - 3 days for up to seven to 10 days. The dead ticks were collected, washed in sterile water and placed in a Petri dish covered with lid and kept in BOD incubator at 25 °C. The IJs of nematodes were recovered from dead ticks after two days of incubation [5]. The IJs isolated were stored in sterile distilled water in tissue culture flask at 16°C until used for bioassay. The IJs were further propagated by repeating the process described above [4, 12]. The morphological characteristics of IJs, female and male nematodes were identified using a light microscope, as described by Nguyen and Smart [13].

EPN bioassay with ticks

The pathogenicity of *Steinernema carpocapsae* was tested against engorged female tick, *Rhipicephalus microplus* under laboratory conditions. Six ticks were placed on What man filter paper in each Petri dish and exposed with infective juveniles (IJs) of *S. carpocapsae* at different concentrations *viz*., 250, 500, 750 and 1000 IJs in 1 ml of distilled water. Water only was applied to the control Petri dishes. The Petri dishes were placed in BOD incubator at 25ºC and 75% RH. Each treatment was replicated three times. The mortality of ticks was recorded every day and percentage mortality was recorded. The dead ticks infected with IJs of *S. carpocapsae* were surface sterilized in 70% ethanol for 10 min, flamed and allowed to dry in a laminar airflow for 2 min. The ticks were opened with sterile needle and scissor; care being taken not to damage the gut and a drop of the haemolymph was examined under microscope for the presence of symbiotic bacteria [14].

Results and Discussion

Of the collected soil samples, the EPNs were isolated from dead ticks collected from only one fodder soil sample from Kattupakkam, Kanchipuram district, Tamil Nadu. The IJs of EPNs emerged from dead tick by insect bait method showing whitish thread and clumpy in nature on gross examination (Fig.1). The nematode was identified as *Steinernema carpocapsae* based on the morphological characteristics of male, female and IJs. Microscopy revealed that the female nematode having an esophagus with a slightly swollen meta corpus, a narrow isthmus and an enlarged posterior bulb (Fig. 2). In males, the tail end was rounded, showing paired spicules curved, a long gubernaculum and no bursa (Fig. 3). The IJ showed a slender body with tapering ends, reduced oesophagus and intestine with a conical tail having a pointed terminus (Fig. 4). Bioassay studies, there was no mortality of *R. microplus* ticks was recorded on first 2 days after treatment in all the concentrations of IJs. On 3rd, 4th and 5th day, the mortality percentage of 72.2, 88.8 and 100%, respectively was recorded only at a concentration of 1000 IJs of *S. carpocapsae* per Petri dish (6 ticks per dish) in the present study. The dead ticks treated with *S. carpocapsae* appeared swollen and dark brown or black in color (Fig.5). The dead ticks were examined under microscope and found that the IJs multiplied inside the haemocoel of tick (Fig. 6). Current study, the entomopathogenic *Xenorhabdus* bacteria associated with *S. carpocapsae* nematode was isolated from dead ticks infected with IJs. The bacterium appeared motile and peritricously flagellated rods under the microscope (Fig.7). The brown or black coloration of the tick body could be an indicative of the pigments produced by the monoculture of mutualistic bacteria *Xenorhabdus* growing in the host ticks and this colour usually occur in insect hosts which were parasitized by steinernematids [3].

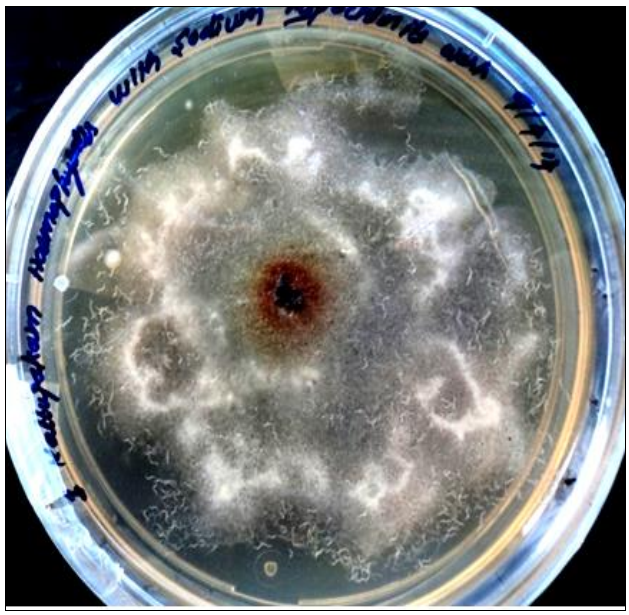

Fig 1: Infective juvenile stage of *Steinernema carpocapsae* emerged from tick cadaver showing whitish thread and clumpy in nature

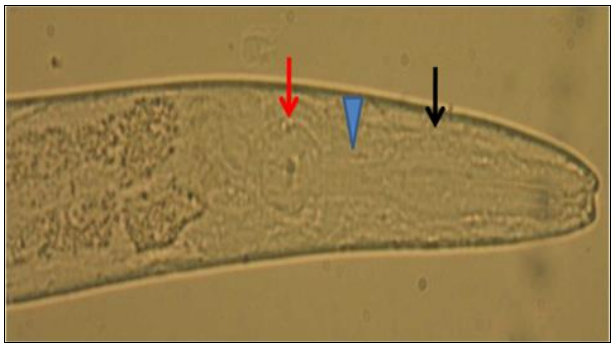

Fig 2: Female head end showing oesophagus with meta corpus (black arrow), isthmus (blue arrowhead) and posterior bulb (red arrow) (40X)

Fig 3: Male tail end showing equal paired curved spicules (black arrow) and gubernaculum (triangle) (40X)

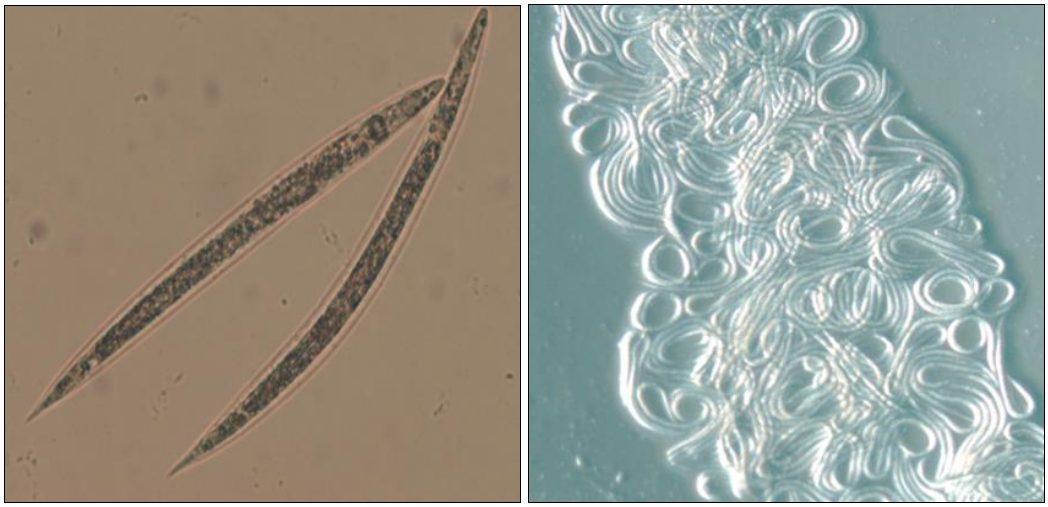

Fig 4: Infective juvenile (3rd stage larva) of *Steinernema carpocapsae* (10X)

Fig 5: Bioassay with *S. carpocapsae* treated ticks appeared as swollen and black in color

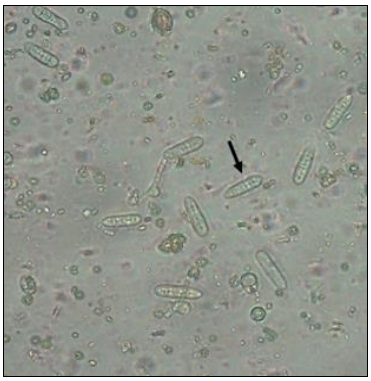

Fig 7: *Xenorhabdus* symbiotic bacteria collected from haemolymph of dead tick 40X (Black arrow)

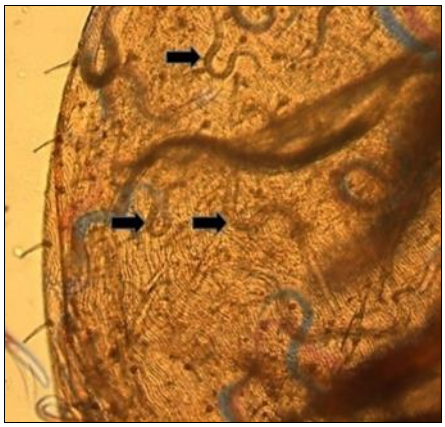

Fig 6: Tick haemocoel filled with nematode *S. carpocapsae* (10X) (Black arrows)

The insect baiting technique with larvae of the greater wax moth, *Galleria mellonella*, is the most commonly used method for recovering IJs of entomopathogenic nematodes from soil [4]. However, in this study the ticks were used for the first time for isolation of EPNs from soil samples from Tamil Nadu. Inside the insect host, the bacteria multiply and release a variety of virulence factors, including toxin complexes, hydrolytic enzymes, hemolysins and antimicrobial compounds [15], thus providing nutrients for the nematode development and reproduction within the insect cadaver.The nematode cannot establish within its insect host without the bacteria. The three-way *Xenorhabdus-steinernema*-insect interaction serves as a model system for mutualism and pathogenicity. Tick mortality caused by EPNs seems to be due to the rapid proliferation of the nematode symbiotic bacteria within the ticks [16].

Similar studies were conducted by various authors *viz*., Zhiou *et al*. [17] who reported mortality of engorged *Ixodes scapularis* females infected with *S. carpocapsae* to be higher than that of uninfected controls and that the LC_{50} was 347.8 infective juveniles per Petri dish (5 ticks per dish). The IJs of various EPNs strains (*S. glaseri, S. riobravus, S. carpocapsae, S. feltiae and Heterorhabditis bacteriophora*) appear to be the most effective in killing ticks which invaded and killed 30 to 100% of replete females [18]. The mortality in *R. bursa* and *R. sanguineus* adult ticks was recorded after 0.3 to 8.0 days of their exposure in Petri dishes to five entomopathogenic nematode strains [6]. Maru *et al*. [19]. also recorded a cent percent mortality of *R. microplus* at 500 *S. carpocapsae* IJs/Petri plate after the fourth day of inoculation.

The susceptibility of ticks to EPNs infection has been varied according to tick species and stage of ticks. The fully engorged female ticks were most susceptible, whereas the preimaginal stages were least sensitive, and the eggs were completely resistant [20]. Many factors influence the efficacy of EPN in a given system, including host species, host life stage, nematode species & strain [21] and nematode dose or concentration [22]. Many researchers found that EPNs were successful to control different insect pests [8] and different species of ticks [6]. Thirteen ixodid tick species and two Argasid species were susceptible to nematodes when adult stages were targeted [6]. Laboratory studies revealed that these EPNs are effective for controlling a wide range of tick species and significant effort is required to demonstrate their potential in the field.

Conclusion

In this study, the entomopathogenic nematode *S. carpocapsae* was isolated from soil baited ticks from Tamil Nadu and this could be the first report from this region. This study indicated that this nematode was highly effective for control of engorged female tick *R. microplus* and could be used as a viable alternative approach for sustainable control of ticks.

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