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Short Communication

Susceptibility of Adult Engorged Ticks, *Rhipicephalus microplus* (Acari: Ixodidae) to a Native Heterorhabditid Isolate (Nematoda: Heterorhabditidae) in Colima, Mexico

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JMO, EGV, LJGM, AMRG, MGR, and RLG performed field work and laboratory bioassays. JMO, SRS, WCC, and MIU did statistical analysis and interpretation. JMO, SRS, JEF, and MIU translated and corrected the language of the manuscript.

Key words
Heterorhabditis*, México, Grasslands, Cattle ticks, *Rhipicephalus (Boophilus).

ABSTRACT

A survey of entomopathogenic nematodes (EPNs) belonging to the families Steinernematidae and Heterorhabditidae was conducted in the municipality of Tecomán, Colima, Mexico in an effort to obtain alternative controls for cattle ticks. Soil cores from grasslands were baited with last instars of the great wax moth (GWM), *Galleria mellonella* L., and an EPN isolate was obtained, it belonged to the genus *Heterorhabditis* and identified as JMO71. The nematodes were cultivated in GWM larvae, and the emerging infective juveniles (IJs) were collected from White traps, and pooled in canted neck vented plastic flasks. The susceptibility of an important livestock arthropod, the cattle tick, *Rhipicephalus microplus* Canestrini to JMO71 was determined. Ticks were manually collected from cross-bred beef cattle, and then the heterorhabditid nematodes assayed against these ticks using the Petri dish assay. Concentrations of 0, 500, 1000, 2000, 4000 and 8000 IJs were applied in 1 mL of sterile distilled water dispensed on the surface of a couple of moistened filter papers. For each treatment concentration, 10 adult engorged ticks were placed in the Petri dishes, and each treatment was replicated four times; the experiment was replicated two times. Tick mortality was recorded daily for 9 days. Our EPNs were able to parasitize engorged ticks, and tick mortality ranged between 70-100% after 9-d post-exposure. The faster and higher tick mortality was obtained with the concentration of 2000 IJs causing 100% mortality after 3 days post-exposure. This is the first report of a native Mexican heterorhabditid nematode causing cattle tick mortality.

Entomopathogenic nematodes (EPNs) belong to the families Steinernematidae and Heterorhabditidae, they are microbial obligate pathogens that infect a wide range of insects and other arthropods in the laboratory. In field conditions, they infect mainly the soil-dwelling forms of insects such as caterpillars, cutworms, crown borers, grubs, corn root worms, cranefly, thrips, fungus gnat, and beetles (Miles *et al*., 2012), and few other soil arthropods. EPNs in the genera *Steinernema* and *Heterorhabditis* are considered

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lethal pathogens of insects due their association with the symbiont bacteria, *Xenorhabdus*, and *Photorhabdus*, respectively (Burnell and Stock, 2000), and the third-stage infective juvenile nematodes (IJs) are proven to be the most effective as biological control organisms (Kaya and Gaugler, 1993). In Mexico, the Universidad de Colima has conducted studies to determine the potential of exotic, and native EPNs to control insect pests, evaluating them in laboratory and field conditions against Mexican fruit flies, *Anastrepha* spp., fall armyworm, *Spodoptera frugiperda* (J. E. Smith), and agave weevil, *Scyphophorus acupunctatus* Gyllenhal. However, few years ago, a native steinernematid strain, *Steinernema diaprepesi*, was isolated in a survey from grasslands in Colima, México, and evaluated against engorged cattle ticks*, Rhipicephalus* ≈*Boophilus microplus* Canestrini, and was found to cause significant adult tick mortality (Molina-Ochoa *et al*., 2009).

Cattle tick is one of the main ectoparasitic pests of bovine in tropical and subtropical regions around the world. Ticks are controlled commonly with chemical acaricides; however, ticks have developed resistance to most of these products (George *et al*., 2004). The EPNs, their infectivity to and susceptibility of female ticks have been previously reported in Israel (Samish and Glazer, 1992), Brazil (de Oliveira-Vasconcelos *et al*., 2004) and México, recently (Molina-Ochoa *et al*., 2009; Rosales-Gutiérrez, 2015). In an effort to obtain alternative controls for cattle ticks, we surveyed grasslands in Tecomán, Colima, Mexico, and a heterorhabditd isolate was recovered, and it was bioassayed against adult engorged ticks under laboratory conditions.

Materials and methods

Soil cores were collected from grasslands cultivated with a native *Panicum* sp. called "Zacatón", in the municipality of Tecomán, Colima, Mexico. Each soil sample, weighing approximately 1 Kg, was a composite of five random subsamples collected at least 100 m apart each site at a depth of 10-20 cm in an area of 20 m^2. Soil samples were placed in polyethylene bags to avoid the loss of moisture and kept in coolers containing refrigerant gel packs during the transit to the laboratory and stored at 15°C in the Entomopathogenic Nematology Laboratory of the Universidad de Colima at Tecomán, Colima, Mexico.

Stored soil samples were processed within a 1 week of collection. To bait the soils for the recovery of EPNs each sample of 1 Kg was thoroughly mixed, and a ca. 240 cc was placed into a 250 CC plastic container. Then, five last instar larvae of the great wax moth (GWM), *Galleria mellonella* L. were placed in the soil sample, and the container was covered with a lid and inverted (Kaya and Stock, 1997). The container was held at room temperature (20±3°C) for a period of 7-8 days.

GWM larvae were infected and killed by EPNs during the 15-day baiting period. Cadavers of GWM were recovered from the baited traps, disinfected with a solution of Sodium hypochlorite 1% during 3 min, rinsed with distilled water for 3 times. They were collected at 3-day intervals over 15 days after set-up and transferred to White traps to collect the emerging IJs (Kaya and Stock, 1997). IJs that emerged were pooled from each sample and were used to infect fresh GWM last instars; IJs were collected in 200 mL canted neck tissue culture flasks, and were stored in an aqueous suspension at low temperature (7-10°C) and used within 1 month of collection from the White traps to verify their pathogenicity and allow for progeny production for identification at the genera level, considering the characteristic color and other signs of the GWM cadavers infected with heterorhabditids (Woodring and Kaya, 1988; Kaya and Stock, 1997), as well as to carry out the bioassays. Soil samples that were negative for EPN during the first round were baited again with GWM last instars; then, all the soil samples were maintained at room temperature. An isolate of heterorhabditid nematode was recovered from a sandy loam soil and designated as *Heterorhabditis* sp. JMO71.

Adult engorged ticks were manually collected from naturally infested cross bred cattle in the municipality of Tecomán, Colima. Ticks were deposited in plastic containers of 500 ml and then covered with a perforated lid. They were transported to the laboratory, disinfected with 0.1% sodium hypochlorite for 10 seconds and then rinsed three times with distilled water to remove the hypochlorite. Ticks were then placed in Petri plates 90 x 100 mm containing a double layer of filter paper (Whatman No. 1; Whatman International Ltd., England) moistened with distilled water and incubated at 25 ± 1°C and a 12 h light-12 h dark photoperiod, and 90% RH. Adult engorged ticks were not sexed.

We used the Petri dish bioassay procedure (Freitas-Rivero *et al*., 2005; Reis-Menini *et al*., 2008) to evaluate the susceptibility of adult engorged ticks to concentrations of 0, 500, 1000, 2000, 4000, a 8000 IJs of *Heterorhabditis* sp. JMO71. EPN concentrations were applied in 1 mL of sterile distilled water dispensed with a pipet on the surface of a couple of moistened filter papers (Whatman No. 1) in Petri dishes, only sterile distilled water (1 mL) was added to the controls. For each treatment concentration, 10 adult engorged ticks were placed in the Petri dishes (60 x 15 mm) and each treatment was replicated 4 times; and the experiment was replicated two times. The Petri dishes of each replication were subsequently placed in double plastic bags and incubated in the dark at room temperature (23 ± 2° C) to avoid the desiccation. Tick mortality was recorded every 24 h for 9 d (de Oliveira-Vasconcelos *et al*., 2004). Ticks were considered as dead by the absence of movement after stimulation, showing gummy body and reddish cuticle (Woodring and Kaya, 1988). Cadavers

were incubated and later examined to verify the presence of EPN.

Analysis of variance was used for determining differences between treatments on tick mortality, and among different nematode concentrations and times post-exposure. Angular transformation was performed on the percent of mortality by square-root transformation before the analysis. A bifactorial arrangement (6 X 9; 54 treatments) of a completely random design was used; factor A was the nematode concentrations and factor B the exposure times. Means were separated by the Tukey test (P=0.05). Mean lethal concentration values (LC_{50}), and mean survival time (ST_{50}) were calculated by Probit analysis (Finney, 1971) using a software SAS version 7.12 (SAS, 1998).

Results and discussion

Current study effort was focused on isolating EPNs that may ubiquitous to grasslands in the municipality of Tecomán, Colima, Mexico. Ten soil samples were collected from grasslands. A heterorhabditid nematode isolate was recovered from 1 of the 10 soil samples with a prevalence of 10%. *Heterorhabditis* sp. JMO71 was highly pathogenic to engorged ticks, *R. microplus* in laboratory. Mortality rates increased with nematode concentration (r =0.478, P< 0.001) and time post-exposure (r = 0.501, P < 0.001), similar results were reported by Freitas-Rivero *et al*. (2005) with *Steinernema carpocapsae*, Molina-Ochoa *et al*. (2009) with *S. diaprepesi,* Monteiro *et al*. (2010) and (2012) with *H. bacteriophora* HP88 and Rodrigues *et al*. (2012) with *H. indica* against *B. microplus*. According to the authors mentioned above, longer exposure times allow greater number of nematodes to locate and penetrate the host, which apparently increase the effects on mortality of engorged ticks, *B. microplus* measured in this study.

Highest mortalities (80.6 % ± 34, 79.9% ± 28, and 75.4% ± 34) were obtained with the concentrations 2000, 8000 and 4000 IJ, respectively, after 9-d post-exposure (Supplementary Table SI), but concentration of 2000 IJ caused 100% mortality after 5 day post-exposure; the LC_{50} for the 5th day had 801.8, and 1364.4 IJs as lower and upper 95% confidence intervals (CI) (P < 0.001, Y = -0.55 + 0.0004 X) (Supplementary Table SII). The survival time for percent mortality of engorged ticks by the application of different concentrations of EPNs showed also that 2000 IJs had the lowest mean survival time (2.3 ± 0.768; 2.1, 2.4 lower, and upper CI; X^2 = 404.2; P < 0.001; Y = 2.94 – 1.30 X) (Supplementary Table SIII), and needed less than three days for causing 100% mortality (Supplementary Table SI).

Molina-Ochoa *et al*. (2009) reported that cattle ticks are not specific hosts of *S. diaprepesi*, speculating that this is one reason for their low cumulative mortalities (40%) obtained; they mentioned also that the nematodes body length and width (1002 µm, and 34 µm, respectively) could reduce the opportunities to penetrate the natural openings of the engorged ticks. Comparing the Molina-Ochoa *et al*. (2009) results for *S. diaprepesi* (Colimense strain) with ours reported here, *Heterorhabditis* sp. isolate JMO71 caused almost 200% higher mortality with similar nematode concentrations. We speculate that *Heterorhabditis* sp. isolate JMO71 caused higher mortalities after 9-d because: 1) *Heterorhabditis* species have smaller range of body lengths and body widths in the IJ (<800 µm, and about 29-20 µm, respectively) (Nguyen 2010); 2) when in contact with an insect host, the IJs exsheath the previously retained J2 cuticle and enter the hemocoel through the insect mouth, anus or spiracles or by penetrating the exoskeleton using a buccal 'tooth'-like structure (Bedding and Molyneux, 1981), Monteiro *et al*. (2010) reported the rupture of the tick cuticle due to the penetration of the IJs using this keratinous tooth; even when Samish *et al*. (2008) reported that penetration of ENs in ticks occurs primarily through the anal or genital pore, and 3) the cruiser foraging behavior could be another reason (Lewis *et al*., 1995).

The present study demonstrates that under laboratory conditions, *B. microplus* engorged ticks had high susceptibility to *Heterorhabditis* sp. JMO71, with mortality rates above those found with the use of other nematodes, such as *H. bacteriophora* CCA, *Steinernema glaseri* Santa Rosa, and *S. carpocapsae* Santa Rosa and All strains (Vasconcelos *et al*., 2004; Freitas-Rivero *et al*., 2005), but similar to those from Monteiro *et al*. (2010) and (2012) with *H. bacteriophora* HP88, and Rodrigues *et al*. (2012) with *H. indica* with 100% of control.

We speculate that the EPNs could be applied as a strategy of the Integrated Pest Management (IPM) in controlling adult cattle engorged ticks in the host animal interfering with the biological parameters of the non-parasite phase of engorged females as discussed by Vasconcelos *et al*. (2004), Freitas-Rivero *et al*. (2005) and Reis-Menini *et al*. (2008).

Morphometrical and molecular characterizations are the next steps for identifying this *Heterorhabditis* sp. (isolate JM071) to the level of species. We will also investigate the potential of *Heterorhabditis* sp. isolate JMO71 for control of other pests of veterinary importance such as the dog ticks, *Rhipicephalus sanguineus* (Acari: Ixodidae).

Conclusions

A native heterorhabditid nematode was recovered from a survey conducted in grasslands in Tecomán, Colima, Mexico. Adult engorged tick mortality rates were associated with nematode concentration and time exposure, and 100% mortality rate of engorged ticks was obtained with the concentration of 2000 IJs of *Heterorhabditis* sp. JMO71 after 5 day post-exposure.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: http://dx.doi.org/10.17582/journal.pjz/2018.50.2.sc2

Statement of conflict of interest

Authors have declared no conflict of interest.

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