Phylogenetic Relationships and Acaricidal Effects of Beauveria bassiana Obtained from Cattle Farm Soils Against Rhipicephalus microplus

Authors: Agustín Fernández-Salas, Miguel Ángel Alonso-Díaz, Rogelio Alejandro Alonso Morales, Roberto Lezama-Gutiérrez, and José Antonio Cervantes-Chávez

Source: Journal of Parasitology, 104(3) : 275-282

Published By: American Society of Parasitologists

URL: https://doi.org/10.1645/17-162

BioOne Complete (complete.BioOne.org) is a full-text database of 200 subscribed and open-access titles in the biological, ecological, and environmental sciences published by nonprofit societies, associations, museums, institutions, and presses.

Your use of this PDF, the BioOne Complete website, and all posted and associated content indicates your acceptance of BioOne’s Terms of Use, available at www.bioone.org/terms-of-use.

Usage of BioOne Complete content is strictly limited to personal, educational, and non-commercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

BioOne sees sustainable scholarly publishing as an inherently collaborative enterprise connecting authors, nonprofit publishers, academic institutions, research libraries, and research funders in the common goal of maximizing access to critical research.
PHYLOGENETIC RELATIONSHIPS AND ACARICIDAL EFFECTS OF BEAUVERIA BASSIANA OBTAINED FROM CATTLE FARM SOILS AGAINST RHIPICEPHALUS MICROPLUS

Agustín Fernández-Salas, Miguel Ángel Alonso-Díaz, Rogelio Alejandro Alonso Morales, Roberto Lezama-Gutiérrez, and José Antonio Cervantes-Chávez

1 Facultad de Ingeniería Agronómica y Zootecnia, Complejo Regional Centro, Benemérita Universidad Autónoma de Puebla, Carretera Cañada–Morelos Km. 7.5, El Salado, C.P. 75460, Tecamachalco, Puebla, Mexico.
2 Centro de Enseñanza, Investigación y Extensión en Ganadería Tropical, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, México, Martínez de la Torre, 93600, Veracruz, Mexico.
3 Departamento de Genética y Bioestadística, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, CDMX, 04510, Ciudad de México.
4 Facultad de Ciencias Biológicas y Agropecuarias, Universidad de Colima, Tecoman, 28930, Colima, Mexico.
5 Unidad de Microbiología Básica y Aplicada, Campus Aeropuerto, Universidad Autónoma de Querétaro, Querétaro, 76140, Querétaro, Mexico.
6 Correspondence should be sent to Miguel Ángel Alonso-Díaz at: alonsoadm@unam.mx

ABSTRACT: The objectives of the present study were to isolate Beauveria bassiana strains from cattle farm soils, analyze the phylogenetic relationships among the fungal strains isolated from these soils, and determine the acaricidal effect of B. bassiana isolates on engorged Rhipicephalus microplus tick strains resistant or susceptible to chemical acaricides. Six strains of B. bassiana were obtained and isolated from cattle farm soils in the Mexican tropics using the Galleria bait method, and their acaricidal effect was assessed against 2 populations of R. microplus (“Media Joya” chemical acaricide-resistant strain or “CLAR” chemical acaricide-susceptible strain) using the adult immersion test. The BbV03 strain produced 86.7% and 60% mortality in resistant and susceptible ticks on day 20, respectively, whereas the BbV04 strain produced 66.7% and 53.5% mortality in resistant and susceptible ticks on day 20, respectively. The BbV03 and BbV04 strains reduced egg laying on both R. microplus populations. There was no statistical difference in the acaricidal effect of B. bassiana strains among chemical acaricide-susceptible or -resistant R. microplus populations (P > 0.05). The BbV03 strain was the most virulent against R. microplus with an LC50 of 2 × 10^7 and LC99 of 7 × 10^8 conidia/ml. We found that the 6 B. bassiana isolated clustered in the same clade with other previously reported B. bassiana strains (from GenBank) but were separated into 3 different sub-clades. This study shows that some B. bassiana strains are a promising coadjutant alternative for biological tick control, including tick populations that are resistant to chemical acaricides. Beauveria bassiana is present in the pastures of tropical cattle farms, and there are genetic variations between B. bassiana strains in this ecosystem that might play an important role in the natural control of R. microplus in cattle farm paddocks.

Rhipicephalus microplus (Canestrini 1888) is among the most important livestock ticks due to the transmission of pathogenic agents, the costs involved in treatment, and the growing resistance to acaricides (Fernández-Salas et al., 2011). The resistance phenomenon observed in R. microplus, coupled with the environmental contamination by secondary chemical metabolites, has motivated an investigation on tick control methods (Samish et al., 2004), such as the use of entomopathogenic fungi (EF).

The acaricidal effect of EF against R. microplus is high when it is evaluated in vitro (Païño et al., 2001; Perinotto et al., 2012; Sun et al., 2013); however, under tropical field conditions this effect is usually reduced (Leemon et al., 2008). The evolutionary climatic adaptation of isolated EF in tropical areas might be an important factor in their efficacy against ticks in those environments as non-native organisms might be more susceptible than native fungi to extreme climatic factors, particularly temperature and incident UV radiation (Rangel et al., 2005). Thus native EF strains might play an important role in the natural control of tick populations in tropical cattle farm paddocks. Beauveria bassiana (Balsamo) Vuillemin, 1912, is an important natural tick pathogen (Fernandes and Bittencourt, 2008) and one of the most studied biological control agents, primarily because it has an extensive arthropod host range with high pathogenicity to target pests, it is easy and inexpensive to produce, it grows rapidly and sporulates abundantly, and it is non-pathogenic to mammals and plants (Feng et al., 1994).

Considering the high and complex chemical resistance that is observed in ticks, a desirable EF agent must be effective against both chemical acaricide-susceptible and -resistant populations. Tick populations may also exhibit differential susceptibility to biological control using EF agents. Thus, when a tick population is resistant to chemical acaricides, it is appropriate to consider the possible interference of chemical acaricide resistance mechanisms with EF agent performance (Perinotto et al., 2012).

When potential EF agents are isolated for use as biological controls, identification often depends only on morphological and culture traits; however, this might not be well enough for a correct differentiation between species of the same genera (Pérez-Gonzales et al., 2014). Previous studies have shown that some habitats can host different species of fungi as well as different genetic groups within these species (Meyling et al., 2009). Thus more reliable and sensitive molecular techniques can ensure the correct differentiation of the EF candidate strains and could help to characterize potential EF strains that provide effective biological control of R. microplus.

It is necessary to find alternative methods to control ticks in areas where chemical acaricides are ineffective. In these areas, ranchers apply high doses of chemicals, contaminating soils and aquifers and poisoning cattle. Unfortunately, available EF strains...
are susceptible to the high temperature and incident UV light of the tropics. Using native microorganisms adapted to these regions might improve the levels of mortality against ticks. The search for new tick control methods using EF is important due to the widespread resistance of ticks to the chemical families used for their control (Murigu et al., 2016) and feasible because studies have demonstrated EF success in the control of ticks (Stafford and Allan, 2010; González et al., 2016).

Therefore, the objectives of the present study were to isolate B. bassiana strains from cattle farm soils, analyze the phylogenetic relationships among the fungal strains isolated from these soils, and determine the acaricidal effect of B. bassiana isolates on engorged R. microplus tick strains resistant or susceptible to chemical acaricides.

MATERIALS AND METHODS

Soil sampling

Twelve different regional municipalities from the center-north zone of the State of Veracruz, Mexico, with heavy livestock activity and high populations of R. microplus and Amblyomma cajennense (Fabricius, 1787) ticks were selected for soil sampling. Six cattle farms were selected for study in each municipality using following criteria: distance between cattle farms equal or greater than 20 kilometers, high tick incidence, and owner consent for sampling.

Soil sampling was carried out from January through November 2013. In each cattle farm, soil samples were obtained from 3 habitats paddocks, hedgerows, and pens or cowsheds. In each habitat 5 soil subsamples of 200–300 g each were collected, homogenized, and deposited in properly identified polyethylene bags (INTA, 2000). Samples for each habitat were collected as follows: for paddocks, subsamples were taken from the ends and center of an “X,” with 50 m between subsample points (none of the paddocks exceeded 10 ha in area) (Rendón, 1994); for hedgerows, subsamples were taken along a transect with subsamples taken at 25 m intervals (see Dalgliesh and Foale, 1998); for pens, subsamples were taken every 30 steps along a zigzag transect spread across the pen (see Pleysier, 1995). Samples were taken with a blast-hole soil sampler (Lord 0225™).

Soilmoisture, CDMX, Mexico) at a depth of 200 mm (INTA, 2000) and 30 mm diameter, without considering organic matter. Samples were transported in plastic coolers for treatment and analysis at the Animal Health Laboratory of the Centro de Enseñanza, Investigación y Extensión en Ganadería Tropical.

Isolation of entomopathogenic fungi from soil samples

The Galleria mellonella bait method was used to isolate EF from soil samples (Zimmerman, 1986). For each soil sample, the sample was moistened with distilled water and passed through a 2 mm sieve to remove rocks, garbage remains, and plant roots; 300 g of sieved soil was deposited in containers; and 5 third instar G. mellonella larvae were added to each bait. Containers were incubated at 27 ± 2 °C for 10 days and inverted every 2 days to promote contact between larva and soil. After incubation, Galleria trap larvae were removed from the soil, disinfected with sodium hypochlorite 0.5% for 3 min, washed with distilled water 3 times, and dried with absorbent paper. Larvae were deposited individually in Petri dishes (60 × 10 mm) with Whatman no. 1 filter papers (Neocitec, CDMX, Mexico), and incubated for 10 days at 27 ± 2 °C and 85–95% relative humidity (RH). Larvae were inspected daily for signs of mycosis, to check the moisture conditions, and to discard pupae and larvae infected with bacteria.

Taxonomic identification, reproduction, and conservation of fungi isolates

When mycosis appeared on Galleria larvae and conidia covered their bodies, a conidial sample was taken with a sterile bacteriological loop, sowed on potato dextrose agar (PDA) amended with 0.1 g/L of streptomycin and 0.5 g/L of oxytetracycline, and incubated at 27 ± 2 °C for 21 days. Larvae of Galleria infected with opportunistic fungi were discarded. Taxonomic identification was conducted based on the morphological characteristics of the reproductive structures, form and size of the conidia, and growth characteristics according to taxonomical keys (Humbert, 1996). Monosporic cultures were prepared on PDA plates, using 21-day-old conidia of putative EF. After obtaining the monosporic cultures, EF were identified again and sowed on inclined PDA tubes for purification. Finally, after 21 days of growth, the conidia were collected by scraping in distilled water with 15% of glycerol, where 1 ml was deposited in cryotubes and stored at −80 °C.

Molecular identification of entomopathogenic fungi

Each strain from the collection was sowed on PDA at 28 °C until the mycelium appeared. DNA from the mycelium was obtained as described by Hoffman and Winston (1987). The DNA concentration was estimated using a NanoDrop (Thermo Fisher Scientific, Inc., Waltham, Massachusetts), and stored at 4 °C for subsequent analysis.

The internal transcription sequences (ITSs) from ribosome genes were amplified by PCR using a T 100 JB Lab thermocycler (Bio-Rad, CDMX, Mexico), with the universal primers ITS1 (5′ TCC GTA GGT GAA CCT GCG G 3′) and ITS4 (5′ TCC GCT TAT TGA TAT GC 3′) (White et al., 1990), which amplified a 550 bp fragment, including the partial sequence of the 18S subunit, ITS1, 5.8S subunit, ITS2, and the partial sequence of the 28S subunit from ribosomal DNA.

The mixtures for the PCR reactions were prepared in 0.2 ml Eppendorf tubes in a final volume of 12.5 µl. Each reaction contained 0.25 µl of each primer (Sigma-Aldrich, St. Louis, Missouri), 0.625 µl of MgCl2, 0.2 µl of dNTPs (Invitrogen, Carlsbad, California), 0.15 µl of Taq polymerase (Amplificasa®, BioTecmol, CDMX, Mexico), 1.25 µl of 10× PCR reaction buffer, 1 µl of DNA, and 8.77 µl of sterile distilled water. Thermal cycling was performed according to the following scheme: 1 initial denaturalization cycle of 94 °C for 5 min, then 38 cycles (denaturation: 94 °C for 30 sec; annealing: 56 °C for 30 sec; extension: 72 °C for 1 min), and 1 final extension cycle at 72 °C for 5 min. PCR products were visualized on 1% agarose gels in 1× TAE. A GelPilot® 100 bp size marker was used; gels were stained with ethidium bromide (0.1 µg ml−1) and visualized under UV light using a photo-documenter machine. All PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. The 5.8S DNA region amplified was sequenced using an ABI3730 X1 at the Laboratorio Nacional de Genómica para la Biodiversidad.
(CINVESTAV, Irapuato, Guanajuato, Mexico). Sequences obtained from fungi strains were aligned using BLAST (GenBank) with the goal of determining percentages of homology with species reported.

**Growth of B. bassiana strains for in vitro bioassays**

The isolated *B. bassiana* strains were grown on 1% of a PDA yeast extract in test tubes that had 500 ppm of chloramphenicol (Sneh, 1991). Cultures were incubated at 25 ± 1 °C and 80% RH for 3 wk. Conidia were harvested by scraping and suspended in sterile distilled water containing 0.1% (v/v) of Tween 80. Then the suspension was poured into a sterile glass test tube and homogenized using a vortex mixer. Conidial concentrations were determined by direct counts using an improved Neubauer hemocytometer and adjusted to $1 \times 10^8$ conidia/ml by dilution, with 0.1% (v/v) of Tween 80 in distilled water. Conidia viability was determined by placing 100 μl of the conidial suspensions on PDA and counting colonies after 24-48 hr of incubation (Alves, 1998). For each strain, conidia viability of at least 98% was required for further testing.

**Tick collection**

Four free-tick male calves (3/4 Holstein × 1/4 Zebu) were maintained in individual pens for artificial infestation. Two calves were infested with 5,000 larvae of *R. microplus* susceptible to acaricides (Media Joya strain), whereas the other two were infested with the same number of larvae of *R. microplus*, but resistant to acaricides (CLAR strain). Tick infestations were applied with a brush on the dorsum of the calf every 15 days (3 times). The susceptible tick strain was obtained from the Centro Nacional de Servicios de Constatación en Salud Animal, which is recommended by the Food and Agriculture Organization as a reference strain for Latin America. The acaricide-resistant tick strain was obtained from a cattle farm in the municipality of Martínez de la Torre, Veracruz, Mexico. The CLAR strain was determined to be multiresistant to commercial acaricides (organophosphates, pyrethroids, and amides) and endectocides (ivermectin) (Fernández-Salas et al., 2012a, 2012b). Engorged female ticks were collected from the holding pen floors 19 to 21 days post-infestation and transported to the laboratory, disinfected with 1% of sodium hypochlorite, washed 3 times with distilled water, dried with sterile absorbent paper, and used immediately in the adult bioassays.

The management and care of the calves were approved by the Subcomité Institucional para el Cuidado y Uso de Animales Experimentales, a FMVZ-UNAM program, protocol number DC-2013-14.

**Acaricidal evaluation of B. bassiana**

The acaricidal effect of *B. bassiana* strains on engorged female ticks was evaluated using the adult immersion test (Drummond et al., 1967). Conidia suspensions for each *B. bassiana* strain were prepared ($1 \times 10^8$ conidia/ml), and 10 engorged female ticks ranging from 0.2 to 0.3 g were treated with the conidia. The control group (10 ticks) was exposed only to distilled water and Tween 80. Three replications of each treatment were performed. Treated groups were immersed in the conidial concentration or control group for 1 min and then dried by placing them on paper towels. Ticks from each group were adhered to masking tape strips in Petri dishes and incubated at 27 ± 1.5 °C and 70–80% RH to allow tick oviposition and to allow the fungi to produce its acaricidal effect. Ticks were individually examined under a stereoscopic microscope for fungal infections, and mortality was recorded every 2 post-treatment days (PT), during a total period of 20 days. Ticks were considered dead if there was an absence of movement after stimulation, cessation of Malpighian tube movement, and observation of mycelia emerging from the cuticle. Mortality was calculated using the corrected formula (Abott, 1925) recommended by the Food and Agriculture Organization.

The effect on the reproductive efficiency index (REI) was evaluated 10 days PT. The egg mass from each tick was collected and weighed using an analytical scale. After weighing, eggs were deposited into glass vials (10 ml) with cotton plugs and incubated to allow egg hatching and to evaluate potential residual effects of EF during egg maturation. After 21 days, hatching rates for the different treatments were estimated by counting eggshells. Each bioassay was repeated, and the REI average of both repetitions is reported.

**Treatment of R. microplus with the selected B. bassiana strains**

Based on the mortality and REI of *R. microplus*, 2 *B. bassiana* strains (BbV03 and BbV04) were selected to assess their LC$_{50}$ and LC$_{99}$. Another series of adult immersion tests with engorged females using the CLAR strain (the Media Joya strain was no longer used because there was no difference in mortality among tick populations) was performed at concentrations of $1 \times 10^8$, $1 \times 10^7$, $1 \times 10^6$, and $1 \times 10^5$ conidia/ml. Bioassays were performed under the same conditions described above. A mortality analysis between the 4 concentrations was performed on day 20. The mortality of the control group was lower than 10%.

**Statistical analysis**

Treatment effects on adult mortality and egg hatching inhibition were analyzed using a Kruskal–Wallis test. The reproductive efficiency index (REI = egg mass weight/initial weight of the engorged female) was determined for each engorged female tick. A single ANOVA factor was used to determine the statistical differences of susceptibility among tick populations and REI. Calculation of LC$_{50}$ and LC$_{99}$ was performed using a probit analysis. A $P$ value $< 0.05$ was considered significant.

The sequences obtained from fungi DNA were edited and assembled using the BioEdit program (Hall, 1999). Multiple sequence alignments were made using the Clustal W program (Thompson et al., 1994). Maximum parsimony analyses and the phylogenetic tree of the strains were performed with the Molecular Evolutionary Genetic Analysis software 5.0 (Tamura et al., 2013), using the close-neighbor-interchange algorithm. Branch robustness was estimated by bootstrap analysis with 1,000 sampling repetitions of the data. Sequences were aligned using the GenBank BLAST program to determine percent homology with previously reported species and to confirm their identity.

**RESULTS**

The entomopathogenic fungi isolated were identified as *B. bassiana*. Our 6 *B. bassiana* strains clustered into the same clade...
with other previously reported *B. bassiana* strains; however, they were separated into 3 different sub-clades in the phylogenetic tree (2 isolates each) (Fig. 1). Percentage mortality effects of the 6 *B. bassiana* isolates on resistant or susceptible strains of *R. microplus* engorged females are presented in Table I. BbV03 and BbV04 showed the highest acaricidal effect against *R. microplus*. In reproductive parameters, the BbV03 strain reduced the egg laying significantly followed by BbV04 against resistant and susceptible ticks (Table II). None of the fungal strains had any significant residual effects on the egg-hatching inhibition of *R. microplus* (P > 0.05). There was no difference in the effect of *B. bassiana* strains among the *R. microplus* populations tested (P > 0.05; Table III). The BbV03 strain showed the strongest dose-effect relationship against *R. microplus* (Table IV).

**DISCUSSION**

In this study, 6 different strains of *B. bassiana* were isolated from soils (BbV01, BbV02, BbV03, BbV04, BbV05, and BbV06) in cattle farms. Few prior studies have reported EF isolations...
from paddocks (Yip et al., 1992). In most studies, EF have been isolated from tick or insect corpses that were found in paddocks (da Costa et al., 2001). The isolation of EF from cattle farm soils documents the composition of the fungal community in this ecosystem. The successful use of the EF as biocontrol agents in tropical environments requires a better understanding of the ecology of indigenous EF populations (Pérez-Gonzáles et al., 2014). The presence of EF in paddocks might highlight their ecological importance given their ability to colonize and control tick eggs, larvae, nymphs, and adults (Fernandes and Bittencourt, 2008). The results presented herein contribute to the knowledge of EF in cattle farm soils, which is important for EF conservation measures and bio-control agent isolation from these habitats.

In this study, we isolated *B. bassiana*. In prior studies, *B. bassiana* was one of the principal EF isolated from soils (Asencio et al., 2003) and has been reported as one of the most important biological controls for ticks (Jonsson, 2004). Precise species and strain identification is needed due to the similarity that exists between pathogen and non-pathogen microorganisms for mammals. It is also important to establish the reproductive characteristics, temperature and humidity requirements, and virulence profiles of EF strains for use against ticks (Bishoff et al., 2009; Rehner et al., 2011). Meyling et al. (2009) demonstrated that a particular habitat can host different EF species and distinct genetic groups within a single EF species. The boundaries between species in the EF genera are based on morphological studies, but genetic analyses are necessary to establish clear relationships between species. Phylogenetic analysis revealed genetic distance between *B. bassiana* isolates and the presence of 3 probable phylogenetic lineages (Fig. 1). This suggests that cattle farm soils harbor a genetic complex of *B. bassiana* fungi. Neelapu et al. (2009) demonstrated that the *B. bassiana* complex varies narrowly in different lineages and shows a clear phylogenetic separation. Thus, we probably recovered 3 different sub-species of *B. bassiana*, but differentiation at the sub-species level was not possible based on the sequences obtained.

In terms of mortality, similar results (82.29–86.54%) were reported for *R. microplus* engorged females using 4 *B. bassiana* isolates at a higher dose of conidia (1 × 10⁸ conidia/ml) (Campos et al., 2010). Paia˜o et al. (2001) reported a mortality mean of 88.7% when using 5 *B. bassiana* strains at 1 × 10⁸ conidia/ml, and Sun et al. (2013) obtained a 100% of mortality against *R. microplus* after treatment with *B. bassiana* (B.BAT17 strain) on the 10th day. Other studies reported non-significant mortality effects when using a *B. bassiana* (Bb986) strain on *R. microplus* (Camargo et al., 2012). Differences among studies could be attributed to the virulence and pathogenicity of each *B. bassiana* strain, or this variation could be due to the interaction of several factors inherent to ticks, such as their immunological defense (Anderson et al., 2011). Moreover, fungi might lose their virulence or pathogenicity because of the constant propagation or management on artificial media. It has been reported that *B. bassiana* has different metabolic responses depending on whether it is grown on pupal extracts or root exudates (Luo et al., 2005). Indeed, it has been hypothesized that an isolate may focus its energy into the vegetative growth instead of producing a large amount of toxin in response to nutrient availability (Kershaw et al., 1994). The fungi evaluated in the present study were recently

### Table I. Mortality index (mean mortality, %) for 6 *Beauveria bassiana* isolates on CLAR-resistant or Media Joya–susceptible strains of *Rhipicephalus microplus*. R: resistant; S: susceptible. Mortality rates were adjusted according to control group.

<table>
<thead>
<tr>
<th>Strain</th>
<th>R</th>
<th>S</th>
<th>R</th>
<th>S</th>
<th>R</th>
<th>S</th>
<th>R</th>
<th>S</th>
<th>R</th>
<th>S</th>
<th>R</th>
<th>S</th>
<th>R</th>
<th>S</th>
<th>R</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BbV01</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.3</td>
<td>3.3</td>
<td>10</td>
<td>6.7</td>
<td>13.3</td>
<td>6.7</td>
<td>16.7</td>
<td>6.7</td>
<td>23.3</td>
<td>13.3</td>
<td>23.3</td>
<td>13.3</td>
</tr>
<tr>
<td>BbV02</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.3</td>
<td>10</td>
<td>6.7</td>
<td>13.3</td>
<td>6.7</td>
<td>16.7</td>
<td>6.7</td>
<td>23.3</td>
<td>13.3</td>
<td>23.3</td>
<td>13.3</td>
</tr>
<tr>
<td>BbV03</td>
<td>3.3</td>
<td>3.3</td>
<td>10</td>
<td>6.7</td>
<td>16.7</td>
<td>23.3</td>
<td>36.7</td>
<td>30</td>
<td>43.3</td>
<td>40</td>
<td>60</td>
<td>46.7</td>
<td>83.3</td>
<td>56.7</td>
<td>86.7</td>
<td>60</td>
</tr>
<tr>
<td>BbV04</td>
<td>6.7</td>
<td>6.7</td>
<td>10</td>
<td>23.3</td>
<td>16.7</td>
<td>26.7</td>
<td>30</td>
<td>40</td>
<td>36.7</td>
<td>63.3</td>
<td>40</td>
<td>63.3</td>
<td>46.7</td>
<td>66.7</td>
<td>53.3</td>
<td></td>
</tr>
<tr>
<td>BbV05</td>
<td>6.7</td>
<td>6.7</td>
<td>10</td>
<td>6.7</td>
<td>13.3</td>
<td>13.3</td>
<td>26.7</td>
<td>16.7</td>
<td>30</td>
<td>16.7</td>
<td>30</td>
<td>26.7</td>
<td>33.3</td>
<td>30</td>
<td>33.3</td>
<td>36.7</td>
</tr>
<tr>
<td>BbV06</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.3</td>
<td>6.7</td>
<td>3.3</td>
<td>10</td>
<td>10</td>
<td>16.7</td>
<td>13.3</td>
<td>16.7</td>
<td>16.7</td>
<td>16.7</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

### Table II. Reproductive efficiency index (in grams) of *Rhipicephalus microplus* engorged females treated with *Beauveria bassiana*.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Control group*</th>
<th>Resistant ticks*</th>
<th>Difference from control group</th>
<th>Reduction %</th>
<th>Susceptible ticks*</th>
<th>Difference from control group</th>
<th>Reduction %</th>
</tr>
</thead>
<tbody>
<tr>
<td>BbV01</td>
<td>0.58783A</td>
<td>0.57303A</td>
<td>0.015</td>
<td>2.55</td>
<td>0.58231A</td>
<td>0.005</td>
<td>0.85</td>
</tr>
<tr>
<td>BbV02</td>
<td>0.58783A</td>
<td>0.59034A</td>
<td>0.0</td>
<td>0.0</td>
<td>0.57993A</td>
<td>0.007</td>
<td>1.19</td>
</tr>
<tr>
<td>BbV03</td>
<td>0.52074A</td>
<td>0.32177B</td>
<td>0.199</td>
<td>38.2</td>
<td>0.34455B</td>
<td>0.176</td>
<td>33.8</td>
</tr>
<tr>
<td>BbV04</td>
<td>0.52074A</td>
<td>0.39039B</td>
<td>0.130</td>
<td>25.0</td>
<td>0.38923B</td>
<td>0.131</td>
<td>25.16</td>
</tr>
<tr>
<td>BbV05</td>
<td>0.52074A</td>
<td>0.50790A</td>
<td>0.013</td>
<td>2.5</td>
<td>0.49871A</td>
<td>0.022</td>
<td>4.22</td>
</tr>
<tr>
<td>BbV06</td>
<td>0.52074A</td>
<td>0.52675A</td>
<td>0.0</td>
<td>0.0</td>
<td>0.51003A</td>
<td>0.010</td>
<td>1.9</td>
</tr>
</tbody>
</table>

* Different letters after numbers indicate a significant statistical difference (*P* < 0.05).
isolated from cattle farms and their reproduction on artificial media was low. Although the evaluated strains did not reach 100% mortality, our results are encouraging because B. bassiana strains are generally less pathogenic against ticks than other EF (Gindin et al., 2002). Moreover, we observed that nearly 90% of the infected ticks were covered with mycelia and allowed B. bassiana sporulation (data not shown). In the field, tick bodies showing sporulation may serve as reservoirs or vectors of fungal conidia, permitting the fungi to be transmitted from an infected tick to susceptible ones.

The effect of B. bassiana strains on engorged female reproduction (egg laying and egg hatching) could help to decrease tick populations in pastures. Perinotto et al. (2012) and Sun et al. (2013) reported similar fungal effects. Other studies have shown a higher egg-laying reduction (Campos et al., 2010; Sun et al., 2013) but provided LC50 values (Campos et al., 2010; Sun et al., 2013) but not LC99 values. The determination of LC50 as a unique parameter of infectivity could result in a high variability of the acaricidal effect (Fernández-Ruvalcaba et al., 2005), while an LC99 estimate could be an important indicator of the real virulence of the EF strains. Our LC50 and LC99 data showed dose-dependent acaricidal effects of BbV03 and BbV04 within acceptable virulence degrees. These results allow correct dosing of the BbV03 and BbV04 strains during field acaricidal evaluations, but additional research is required to develop effective formulations for tick control in the field.

In conclusion, this study demonstrates that B. bassiana is present in the pastures of tropical cattle farms and that genetic variations among strains of B. bassiana increase the potential to find an organism highly pathogenic to ticks. Strains BbV03 and BbV04 have acaricidal effects against chemical acaricide-resistant or susceptible strains of R. microplus and should be considered promising co-adjuvants for controlling R. microplus field populations.

Table III. Mortality effect of Beauveria bassiana on Rhipicephalus microplus ticks susceptible (Media Joya) or resistant (CLAR) on day 20 post-exposure.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Mortality of resistant tick strain (%)</th>
<th>Mortality of susceptible tick strain (%)</th>
<th>P value*</th>
<th>Most susceptible tick strain to R. bassiana</th>
</tr>
</thead>
<tbody>
<tr>
<td>BbV01</td>
<td>23.3</td>
<td>13.3</td>
<td>0.0806</td>
<td>No difference</td>
</tr>
<tr>
<td>BbV02</td>
<td>3.3</td>
<td>3.3</td>
<td>-</td>
<td>No difference</td>
</tr>
<tr>
<td>BbV03</td>
<td>86.7</td>
<td>60.0</td>
<td>0.0081</td>
<td>Resistant ticks</td>
</tr>
<tr>
<td>BbV04</td>
<td>66.7</td>
<td>53.3</td>
<td>0.0711</td>
<td>No difference</td>
</tr>
<tr>
<td>BbV05</td>
<td>33.3</td>
<td>36.7</td>
<td>0.3739</td>
<td>No difference</td>
</tr>
<tr>
<td>BbV06</td>
<td>20.0</td>
<td>20.0</td>
<td>-</td>
<td>No difference</td>
</tr>
</tbody>
</table>

* P < 0.05 was considered significant. - = not calculated.

Table IV. Lethal concentration 50% and 99% of 2 Beauveria bassiana strains on Rhipicephalus microplus ticks resistant (CLAR strain) to acaricides. PT: post-treatment; LC: lethal concentration; CI: confidence Interval; = not calculated.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Doses</th>
<th>0</th>
<th>3.3</th>
<th>10</th>
<th>26.7</th>
<th>26.7</th>
<th>43.3</th>
<th>60.0</th>
<th>83.3</th>
<th>86.7</th>
<th>1.51</th>
<th>2 × 10^5 (5 × 10^6 to 4 × 10^6)</th>
<th>7 × 10^4 (2 × 10^4 to =)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BbV03</td>
<td>1 × 10^6</td>
<td>0</td>
<td>3.3</td>
<td>10</td>
<td>26.7</td>
<td>26.7</td>
<td>43.3</td>
<td>60.0</td>
<td>83.3</td>
<td>86.7</td>
<td>1.51</td>
<td>2 × 10^5 (5 × 10^6 to 4 × 10^6)</td>
<td>7 × 10^4 (2 × 10^4 to =)</td>
</tr>
<tr>
<td></td>
<td>1 × 10^7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6.7</td>
<td>13.3</td>
<td>13.3</td>
<td>16.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 × 10^6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.3</td>
<td>3.3</td>
<td>3.3</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 × 10^5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.3</td>
<td>6.7</td>
<td>6.7</td>
<td>6.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.3</td>
<td>6.7</td>
<td>6.7</td>
<td>6.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BbV04</td>
<td>1 × 10^6</td>
<td>0</td>
<td>6.7</td>
<td>6.7</td>
<td>23.3</td>
<td>26.7</td>
<td>40</td>
<td>63.3</td>
<td>63.3</td>
<td>66.7</td>
<td>0.67</td>
<td>4 × 10^5 (1 × 10^5 to 2 × 10^5)</td>
<td>9 × 10^5 (7 × 10^6 to =)</td>
</tr>
<tr>
<td></td>
<td>1 × 10^5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.3</td>
<td>3.3</td>
<td>3.3</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 × 10^4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.3</td>
<td>6.7</td>
<td>6.7</td>
<td>6.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.3</td>
<td>6.7</td>
<td>6.7</td>
<td>6.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENTS
We are very grateful to Consejo Nacional de Ciencia y Tecnología-México (CONACyT) for supporting Agustín Fernández-Salas during his Ph.D. program at FMVZ-UNAM.

LITERATURE CITED


