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Short Communication

Evaluation of Entomopathogenic Fungi Isolated from Adult Dog Fleas, *Ctenocephalides canis* (Siphonaptera: Pulicidae)

C.A. Angel-Sahagún[1], J.E. Ortega Palomares[1], A.A. Hernández-Rangel[1], C. Cruz-Vázquez[2], R. Montesinos-Matias[3], M. Valencia-Posadas[1] and A.M. Cruz-Avalos[4]*

[1]*Departamento de Veterinaria y Zootecnia. División de Ciencias de la Vida, Campus Irapuato-Salamanca, Universidad de Guanajuato, México.*
[2]*División de Estudios de Posgrado e Investigación, Instituto Tecnológico El Llano Aguascalientes, México.*
[3]*Centro Nacional de Referencia de Control Biológico, SENASICA-DGSV, México.*
[4]*Universidad Tecnológica del Suroeste de Guanajuato. Guanajuato, México.*

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Key words
***Beauveria bassiana, Metarhizium anisopliae, Ctenocephalides canis*, Dogs, Biological control**

ABSTRACT

The objective of this study was to evaluate the pathogenicity of *Metarhizium anisopliae* (*Ma*) and *Beauveria bassiana* (*Bb*) isolates, obtained from adult *Ctenocephalides canis*, the dog flea, under laboratory conditions. Nine, monosporic cultures were isolated. Each were pathogenic when exposed by immersion at a concentration of $1x10^8$ conidia/ml, causing between 10-100% mycosis at ten days post-inoculation. Four isolates identified as *Bb9*, *Bb6*, *Ma9* and *Ma10*, were the most pathogenic as mycosis reached 100%, which places them as potential candidates to be used as biological control agents.

The dog flea, *Ctenocephalides canis* (Curtis) (Siphonaptera: Pulicidae) is a hematophagous ectoparasite with cosmopolitan distribution that primarily affects dogs. Flea infestation in dogs is common and it is responsible for discomfort in both dogs and their owners due to their eating habits such as blood feeding and inoculation of allergenic substances that cause itching, skin irritation, and eventually flea allergic dermatitis. In addition, they are intermediate hosts of internal parasites and vectors of some microorganisms responsible for diseases of importance in public and veterinary health (Blagburn and Dryden, 2009).

The control of flea infestations mainly includes the use of insecticides of different chemical families. However, the indiscriminate use of them has prompted the development of resistance in different populations around the world (Coles and Dryden, 2014). Entomopathogenic fungi (EPF) represent a biocontrol alternative that has been documented in various ectoparasites of veterinary importance both *in vitro* and in the field conditions; the presence of EPF in the insects under natural conditions has also been demonstrated (Fernandes *et al*., 2012; Galindo-Velasco *et al*., 2015; Cruz-Vázquez *et al*., 2015). In the case of fleas, there are antecedents in which they have been shown to be susceptible to infection by *Beauveria bassiana* (Hypocreales: Cordycipitaceae) and *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae), under laboratory conditions (De Melo *et al*., 2007, 2008) and their presence within insect under natural conditions (Ortega-Palomares *et al*., 2014). The fungi isolated from an insect may be specifically adapted as biological control agents of the same insect (Fernandes and Bittencourt, 2008), to our knowledge, there is only one report of the occurrence of EPF in *C. canis* (Ortega-Palomares *et al*., 2014). Therefore, the objective of this study was to evaluate the pathogenicity of *Metarhizium anisopliae* (*Ma*) and *Beauveria bassiana* (*Bb*) isolates, obtained from adult *Ctenocephalides canis*, the dog flea, under laboratory conditions.

Materials and methods

Flea specimens from dogs maintained in the Irapuato Canine Control Center (ICCC) were collected. At the time

* Corresponding author: cruz20_5@hotmail.com
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of the study, the ICCC kept dogs that came from urban and suburban areas of the municipality of Irapuato, all of them considered street dogs. The ICCC is located in the city of Irapuato in the state of Guanajuato, Mexico. The site is located in the north-central region of Mexico at an altitude of 1739 meters above sea level, between 20°40' North latitude and 101°21'L. The climate in the area is sub-humid with a rainfall ranging from 600-900 mm which occurs mainly in the summer and a temperature ranging from 16-22 °C.

The ICCC were visited weekly over the period from May 2012 to January 2013, during which 20 dogs were selected to undergo a thorough physical examination of the head, neck, body, flanks, tail and the ventral region to establish the presence of fleas. From each dog that tested positive for fleas all possible flea specimens were collected using a comb and placed in a plastic container to be transported to the Laboratory. The specimens were transferred to Petri dishes (90 × 10 mm) using an adhesive tape to hold them motionless. The genus and species identification was performed on the selected specimens of *C. canis* under microscope using descriptions and the appropriate taxonomic keys (Linardi and Santos, 2012). Fleas were incubated at 25 ± 1°C, 16/8 hours (light/dark), and 80% relative humidity in less than four hours after being collected. After ten days and mycosis on the flea surface was identified, it was cultured on Sabouraud dextrose agar with yeast extract and 500 ppm chloramphenicol. This procedure was repeated until a pure culture was obtained. Subsequently, each isolate was incubated for 21 days at 25 ± 1°C with a photoperiod of 12:12 h light/dark. Conidia from each isolate were extracted from the culture medium by scraping and suspending them in sterile distilled water with 0.1% (v/v) Tween 80 (Ángel-Sahagún *et al*., 2010).

The monosporic culture of each isolate was obtained following the technique previously reported by Cortez-Madrigal *et al*. (2003). The taxonomic identification was made using the keys of Humber (1997), based on macro-aspects, such as color, diameter and mycelial texture, while a light microscope was used for identification of mycelium and micromorphological conidial characteristics.

The collected specimens of *C. canis* were from dogs naturally infested which were maintained in the ICCC as previously described. Groups of ten fleas were formed and were placed on 5 cm of adhesive tape; each group was immersed for 5s in a 1×10^8 conidia/ml concentration of the isolate to be studied, that were prepared in sterile distilled water containing 0.1% (v:v) Tween 80. The control groups were immersed in a sterile water suspension with Tween 80 (0.1%). Each treatment was replicated four times. Fleas of the different groups were placed in Petri dishes (90 x 10mm) on a double layer of filter paper (Whatman No.1) moistened with sterile distilled water and incubated at 25 ± 1°C under conditions of 12:12h light/dark. The presence of growth of fungal mycelium was recorded every 12h for 10 days.

Statistical assessment of pathogenicity was carried out under a completely random design including the nine fungi isolates and a control treatment with four repetitions each. Mortality percentages and growth of fungal mycelium were estimated and analyzed using ANOVA having previously made an angular transformation of proportions (Y= arc sen $\sqrt{p}$) and Tukey´s means comparison test at $P \leq 0.05$. All analyses were performed with the SAS statistical software (SAS Institute, 1997).

Results and discussion

A total of 1,222 fleas were collected during the study period and identified as *C. canis*. In addition, five isolates of *M. anisopliae* (*Ma*) and four of *B. bassiana* (*Bb*) were obtained (Table I). This is the first report of an isolation of *M. anisopliae* in *C. canis*, whereas *B. bassiana* has been previously reported in this flea in Mexico (Ortega-Palomares *et al*., 2014).

Table I. Percentage of mycosis 10 days post treatment with entomopathogenic fungi isolated from *C. canis*.

Species of entomo-pathogenic fungus	Collection date	Key	Mycosis (%) *
M. anisopliae	10-05-12	*Ma*9	100.0 ± 0a
M. anisopliae	10-05-12	*Ma*10	100.0 ± 0a
B. bassiana	25-07-12	*Bb*6	100.0 ±0a
B. bassiana	26-07-12	*Bb*9	100.0 ± 0a
M. anisopliae	10-05-12	*Ma*11	95.8 ± 7.2ab
M. anisopliae	10-05-12	*Ma*12	41.7 ± 7.2b
M. anisopliae	10-05-12	*Ma*13	35.9 ± 13.1c
B. bassiana	26-07-12	*Bb*8	40.0 ±0c
B. bassiana	25-07-12	*Bb*7	10. 4 ± 10.0d
Control	---	---	0.0 ± 0d

* Averages within the same column with different letters indicate significant differences (p <0.05). ±, Standard deviation.

The nine isolates tested were pathogenic to *C. canis* causing 10-100% of growth of fungal mycelium, at ten days post-inoculation. Isolates of *B. bassiana* caused growth of fungal mycelium between 10 and 100%, while *M. anisopliae* ranged from 35-100% (Table I). All fungi were re-confirmed from the infected cadavers post experiment. Analysis of variance showed significant differences among treatments (F= 94.9; df= 9; P <0.0001) while the Tukey test determined that the isolates *Ma*9, *Ma*10, *Bb*6, *Bb*9 were the pathogens that caused 100% of the growth of fungal mycelium and formed part of the most virulent group.

All species could infect the dog fleas, demonstrating

the susceptibility of the adult stage *C. canis* to these pathogens; previous studies have demonstrated the ability to infect adult cat fleas, *C. felis,* with strains of *M. anisopliae* and *B. bassiana* (de Melo *et al*., 2008). Samish *et al*. (2020) found that *M. brunneum* and *M. robertsii* cause mortalities of up to 100% of the flea *C. felis* under conditions favorable for entomopathogens. In the present study, favorable conditions of temperature and relative humidity were provided for the EPF used, however, some strains did not exceed 40% mortality, probably because the flea species presents differences in susceptibility. Pathogenicity in our isolates was variable, but *Bb*6, *Bb*9, and *Ma*9 were the most outstanding with 100% growth of fungal mycelium.

Few reports of the biological control of fleas exist. It is limited to entomopathogenic nematodes and fungi. Evaluations with entomopathogenic nematodes have shown results of up to 100% mortality under laboratory conditions in the different development phases of *C. felis* (Samish *et al*., 2020).

The use of EPF is another alternative for controlling pet ectoparasites, not only for dogs and cats but for any domestic or wild animal. Currently, control depends primarily on the use of chemicals, and thus there is always the possibility of fleas develop resistance, but by the multifactorial mechanism of action of EPF it is unlikely that insects generate resistance. In conclusion, this study identified different isolates of EPF obtained from adult. *C. canis*. Four isolates (Bb9, Bb6, Ma9 and Ma10) showed outstanding pathogenicity, which places them as potential candidates to be used as biological control agents.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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