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Short Communication

FIRST IDENTIFICATION OF *NOSEMA CERANAE* (MICROSPORIDIA) INFECTING *APIS MELLIFERA* IN VENEZUELA

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Abstract

***Nosema ceranae* is a pathogen of *Apis mellifera*, which seems to have jumped from its original host Asiatic honey bee *Apis ceranae*. Nosemosis which affects the honey bee *Apis mellifera* is caused by two parasitic fungi described as etiologic agents of the disease. *Nosema apis* was the only microsporidian infection identified in *A. mellifera* until *N. ceranae* in Taiwan and Europe. Nosema spp. positive samples of adult worker bees from the Venezuelean state of Lara were determined through light microscopy of spores. Samples were then tested to determine Nosema species (*N.apis/N.ceranae*) using previously reported PCR primers for the 16S rRNA gene. A multiplex PCR assay was used to differentiate both *N. apis* and *N. ceranae* species. Only *N. ceranae* was found in the analyzed samples and the percentage of infected foragers fluctuated between 18% and 60%.**

Keywords: honeybee, microsporidian, Multiplex PCR, *Nosema ceranae, Nosema apis*, Venezuela.

INTRODUCTION

Nosemosis which affects the honey bee *Apis mellifera* is caused by two parasitic fungi described as etiologic agents of the disease. *Nosema apis was* the only microsporidian infection identified in *A. mellifera* until *Nosema ceranae* in Taiwan (Huang et al., 2005) and Europe (Higes, et al., 2006). This emerging pathogen has now spread worldwide (Chauzat et al., 2007; Klee et al., 2007; Chen et al., 2008) and has been associated with bee disappearance or colony depopulation (Higes et al., 2006; Martin-Hernandez et al., 2007). The highly prevalent disease spreads throughout resistance spores, causing a reduced honey production and pollen collection, queen supersedure and decreased colony population and ultimately great economic damages (Mayack & Naug, 2009; Higes et al., 2010; Martin-Hernandez et al., 2011). In addition, experimental data suggest that *N. ceranae* exhibits a competitive advantage over *N. apis*, which possibly explains its wide dispersion. Environmental conditions may influence disease epidemiology (Fries, 2010) and increase the probabilities of it occupying new ecosystems. *N. ceranae* parasitizing African hybrids has been progressively identified in the South American countries of Brazil, Uruguay, Argentina and Chile (Klee et al., 2007; Invernizzi et al., 2009;

Medici et al., 2012; Rodriguez et al., 2012) as well as Mexico and Costa Rica (Calderón et al., 2008; Guzmán-Novoa et al., 2011). In Venezuela, the development of the beekeeping industry has been affected by the presence of Africanized honeybees, as a result, honey production declined in the early 1980s (Piccirillo & González, 1995) but then slowly recovered in 2012 according to Beekeeping Development Foundation (FUNDAPI) data. Preliminary studies were carried out in the state of Zulia to analyze the health status of *A. mellifera* colonies in Venezuela (Piccirillo & González, 1995), but were only focused on the detection of Nosema spp. spores without specifying molecular characterization. The aim of this study was to detect the presence of *Nosema ceranae* in Venezuela.

MATERIAL AND METHODS

For the preliminary detection of Nosema spp. spores, samples of five beehives were collected in September 2014. Returning flight bees stored in ethanol 96° were received from an experimental apiary of Lisandro Alvarado Centrooccidental University (U.C.L.A.), Lara , Venezuela (Fig. 1). The abdomens of thirty worker honeybees were individually crushed in 1ml sterile water and analyzed under light microscope (400X) to quantify infective propagules using a hemocytometer (Cantwell, 1970). Twenty bees from each sample were used for spore collection. The abdomens were manually macerated with glass rods in 5ml of sterile water in 15ml sterile glass tubes for five minutes. Another 5ml were added, filtered and centrifuged at 2000 rpm for six minutes. The supernatant was discarded, the pellet was suspended in 1 ml of sterile water and the solution was vortexed with glass beads for 1 min at maximum speed. All samples were stored at 4°C until DNA extraction which was performed with a High Pure PCR template preparation kit (Cat. No. 11796828001; Roche Diagnostic). The 16S rRNA locus was selected to perform *N. apis - N. ceranae* Multiplex PCR according to Martín-Hernández et al. (2007). Amplifications were carried out using 25µl reaction cocktail containing 12.5 µl of Fast Start PCR Master Mixture (Cat. No. 04710444001; Roche Diagnostic), 0.4 µM of each primer, 0.4 mM of each deoxynucleoside triphosphate, 0.1% Triton X-100, and 2.5 µl of *N. apis* or *N. ceranae* DNA template. The thermocycler program consisted of 94°C for 2 min, followed by ten cycles of 15 s at 94°C, 30 s at 61.8°C, and 45 s at 72°C, 20 cycles of 15 s at 94°C, 30 s at 61.8°C, and 50 s at 72°C plus an additional 5 s of elongation for each successive cycle, and a final extension step at 72°C for 7 min. Positive and negative controls were included in all PCR experiments. Amplification products were visualized by electrophoresis in 1.5 % agarose gel in TBE (Tris 10.8g, boric acid 5.5g, EDTA 0.5 M pH 8 4ml/100ml), using size ladder (100pb) and GelRed pre-staining. In addition, one PCR product was purified and sequenced. The resulting sequence was BLAST searched to examine their similarity with sequences deposited in GenBank. Multiple alignments of sequences of the 16S rRNA gene from *Nosema ceranae* and *Nosema apis* in other

Fig. 1 Study area

countries was performed with ClustalX 2.0 (Larkin et al., 2007).

RESULTS AND DISCUSSION

Spore count intensity values showed in most honeybee samples a spore number s near zero, with some rather high values exceeding $5.0x10^6$ spores/ bee. Although the percentage of infected foragers fluctuated between 18% and 60%, the average value for 150 abdomens reached 45.2% infection. This value exceeds the 34.3% obtained in the previous prevalence analysis of 1219 abdomens of twenty-four colonies in the neighboring state of Zulia (Piccirillo & González, 1995). Amplification products revealed the only presence of *Nosema ceranae* in four samples and the absence of both *Nosema apis* as well as a co-infection in the remaining one. These results suggest that *N. ceranae* has a competitive advantage over *N.apis*, such as a higher degree of infection or virulence. Nevertheless, the number of analyzed samples is not enough to discard the presence of *N. apis.* Our work represents the first report of *N. ceranae* in Venezuela and serves as a basis for future studies. The obtained sequence of 168 bp was compared with isolates from other geographical regions with high probability match (100% identity) to *N. ceranae* sequences to confirm identification. Intensive surveys of colonies established in different biogeographic regions are needed to determine the distribution and prevalence of this new agent in Venezuela. It will provide knowedge on how local production is affected by the disease under the influence of Central America's particular climate. Our results highlight the need to incorporate the disease into a sanitary management strategy adapted to the productive conditions of the country.

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