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Short Communication

Improving molecular discrimination of *Nosema apis* and *Nosema ceranae*

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ABSTRACT

Nosema apis and *Nosema ceranae* are the causative agents of nosemosis, a contagious honeybee disease that weakens bee colonies. The species are discriminated through several PCR-based methods including a multiplex PCR recommended by the World Organization for Animal Health (OIE). In this study, the OIE protocol was compared to two other PCR protocols using different PCR kits with the same primer pairs as described in OIE. The results showed that the three PCR protocols have similar sensitivity but only the kit dedicated to multiplex PCR could detect small quantities of one *Nosema* species when greater quantities of the other were also present. However, singleplex PCR methods are currently the most sensitive methods for discerning each species. These results have important implications for epidemiology and the understanding of the disease.

1. Introduction

Nosemosis, a disease affecting adult honeybees, occurs worldwide and causes a decrease in the colony's population, colony strength, adult bee longevity and winter survival rates (Coineau and Fernandez, 2007; Ritter, 2008). The etiological agent is *Nosema apis,* but recently another microsporidian of the genus *Nosema*—*Nosema ceranae*—has also been implicated in colony population depletion (Higes et al., 2006). *N. apis* was first described in 1909 by E. Zander in European honeybees, *Apis mellifera* (cited in Morse and Flottum 1997). Subsequently, *N. ceranae* was described for the first time in the Asian honeybee *Apis cerana* by Fries et al. (1996). The jump from *A. cerana* to *A. mellifera* was thought to occur in the 1990s, when the first observations were reported (Invernizzi et al. 2009). Today, *N. ceranae* is found more frequently than *N. apis* in some European honeybee colonies (Klee et al., 2007; Paxton et al., 2007).

A reliable distinction between the two pathogens has important implications in the epidemiology and understanding of the disease. Morphological discrimination by optical microscopy is difficult. Therefore, recent molecular techniques were developed to perform a robust diagnosis (Higes et al. 2006; Klee et al. 2007; Martin-Hernandez et al. 2007; Erler et al. 2012). Martin-Hernandez et al. (2007) have proposed a multiplex PCR which detects specific 16SrRNA sequences of both species. This protocol has been recommended by the World Organisation for Animal Health (OIE, Ritter 2008) and used in more than ten studies (Higes et al. 2010; Martin-Hernandez et al. 2007, 2009). However, the disadvantage of a multiplex PCR is its low sensitivity for certain amplicons and cross-reactions in the event of competition (Elnifro et al., 2000; Mothershed and Whitney, 2006).

The aim of this study was to compare the OIE detection protocol with two other PCR protocols: one used a conventional PCR kit (Platinum® Taq DNA polymerase, Invitrogen) and the other used a kit developed for multiplex PCR (Multiplex PCR Master Mix, Qiagen). These two additional protocols used same primer pairs as OIE process but different PCR chemistries. We first assessed the detection limit of the three PCR protocols (DL_{PCR}) by determining the lowest number of nucleic acid targets detected for each *Nosema* species and we assessed the detection limit of the method (DL_{method}) from *Nosema* spores subjected to DNA extraction to PCR analysis, for each PCR protocol. Secondly, we tested if one *Nosema* species present in small quantities was detected when a greater quantity of the other species was also present. Two experiments were performed, one using plasmid of each *Nosema* species and the other using spores of each *Nosema* species. The quantities determined in the first step were therefore mixed with increasing dilutions of the other species. We subsequently determined the dilution at which the small quantities of one species or the other were always amplified at low dilution. The final step entailed comparing the results obtained with the two PCR protocols other than OIE protocol. Ninety-nine honeybee samples infected with *Nosema* spores were analyzed. Results are discussed with regards to the

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choice of PCR protocol, implications for epidemiology and understanding of the disease.

2. Materials and methods

2.1. Experiment design

Three different PCR protocols using different PCR kits and parameters were tested. The first was that used in the OIE protocol (High Fidelity PCR Master Mixture, Roche, Martin-Hernandez et al., 2007; PCR No. 1). The second protocol used the Platinum® Taq DNA Polymerase (Invitrogen, PCR No. 2) and the third protocol used a kit developed for multiplex PCR (Multiplex PCR Master Mix, Qiagen, PCR No. 3). The two *Nosema* species were discriminated with the primers described in Martin-Hernandez et al. (2007).

Firstly, the detection limit of each PCR protocol was evaluated in singleplex PCR (detection of only one *Nosema* species) with serial dilutions of a known quantity of *N. apis* and *N. ceranae* templates of plasmid DNA. These plasmid DNA templates contained one of the two PCR products (*N. apis* or *ceranae*) amplified with primers described in Martin-Hernandez et al. (2007). They were obtained using the procedure described in Blanchard et al. (2007). The purified plasmids were sequenced to verify their specificity, spectrophotometrically quantified and converted into DNA copy number using the molecular weight of plasmid and the *Avogadro* constant. The limit of detection of each PCRs was assessed from 10-fold serial dilution containing 10^8–10^2 copies of plasmid DNA by template volume. The quantity of plasmid DNA copies in which the lowest amplification was observed was thus determined as the DL_{PCR}. The DL_{method} was then assessed in conditions of duplex PCR from 10-fold serial dilution of spores of each *Nosema* species.

The second part of the experiment was designed to test the DL_{PCR} for a given species with the dilution range of the other species. Each different PCR was performed three times to test result reproducibility. Negative controls (with no DNA template) were run in all PCRs to detect possible contamination. DNA fragments were separated by electrophoresis in a 2% agarose gel at 100 V for 60 min in TAE 1X and revealed after ethidium bromide (0.5 μg/ml) staining under ultraviolet light. The same experiment was conducted to test the DL_{method} for a given species with the dilution range of the other species.

Moreover, to detect possible PCR inhibition and competition, a complementary experiment was conducted to test the DL_{PCR} as described above using DNA extraction from healthy honey bee as diluent for 10-fold serial dilution of each plasmid.

2.2. Multiplex PCR conditions

The first multiplex PCR was run under the conditions described in Martin-Hernandez et al. (2007) with the High Fidelity PCR Master Mixture (Roche) (PCR No. 1). The second multiplex PCR used the Platinum Taq DNA Polymerase (Invitrogen, PCR No. 2) according to the manufacturer's recommendations with 0.4 μM of each primer, 0.4 mM of dNTP and H_2O to complete to 20 μl and 5 μl of DNA plasmid preparations or extracted DNA from *Nosema* spores. The PCR was run on an Eppendorf thermocycler with an initial denaturation at 94 °C for 2 min followed by 35 cycles of 30 s at 94 °C, 30 s at 62 °C and 30 s at 72 °C and a final extension of 7 min at 72 °C. The third multiplex PCR (PCR No. 3) using the Multiplex PCR Master Mix (Qiagen) was performed in a final volume of 20 μl containing 1X of reaction buffer Qiagen Multiplex PCR Master Mix, 0.1 μM of each primer, DNA plasmid preparations or extracted DNA from *Nosema* spores, and H_2O. The cycling conditions were: initial denaturation at 95 °C for 15 min followed by 35 cycles of 15 s at 94 °C, 45 s at 62 °C and 45 s at 72 °C and a final extension for 10 min at 72 °C.

2.3. Application to honeybee samples and DNA extraction

The *Nosema* species were determined by multiplex PCR on a total of 99 honeybee samples positive for *Nosema* by microscopic observation of spores. DNA extraction was performed from homogenates of bee abdomens, prepared as described in OIE protocol. Briefly, ten bee abdomens were crushed, filtered and washed twice with distilled water. After counting of the spores, the solution is concentrated in 1.5 ml of distilled water and 80 μl (corresponding to 0.5 bee abdomen) are subjected to DNA extraction using the High Pure PCR Template Preparation Kit (Roche Diagnostic). Elutions are performed with 200 μl of elution buffer, according to the manufacturer's recommendations. Performance of different extraction DNA kits was previously tested, without significant difference (data not published). These samples were collected between 2009 and 2010 and analyzed with both protocols No. 2 and No. 3 to compare the results of *Nosema* species discrimination.

3. Results

No amplification occurred for negative controls (nontemplate controls) and replicates of each protocol generated similar results.

In experiments conducted with PCR protocol No. 1, *N. ceranae* was detected up to 10^3 plasmid DNA copies per reaction and *N. apis* up to 10^4 plasmid DNA copies per reaction, corresponding to the PCR detection limit of *N. ceranae* and *N. apis* with this protocol, respectively at 10^3 and 10^4 copies per reaction. When 10^4 plasmid DNA copies of *N. ceranae* (corresponding to the DL_{PCR} multiplied by 10 allowing to give a more accurate result) was mixed with a quantity of *N. apis* from 10^8 to 10^3 plasmid DNA copies, *N. ceranae* was detected up to 10^6 plasmid DNA copies of *N. apis* (low amplification was observed with 10^7 copies of *N. apis*). Similarly, 10^5 plasmid DNA copies of *N. apis* (corresponding to the DL_{PCR} multiplied by 10) was detected up to 10^6 plasmid DNA copies of *N. ceranae* (Fig. 1). With PCR protocol No. 2, the PCR detection limit was substantially lower (10^2 and 10^3 plasmid DNA copies of *N. ceranae* and *N. apis* respectively) than the detection limit of PCR protocol No. 1. *N. ceranae* prepared at the limit of detection multiplied by ten (10^3 copies) was detected when mixed with all quantities of plasmid DNA copies of *N. apis*. However, *N. apis* was not amplified when the quantity of *N. ceranae* was above 10 times the plasmid copies: 10^4, 10^5 and 10^6 plasmid DNA copies of *N. apis* were detected up to 10^5, 10^6 and 10^7 plasmid DNA copies of *N. ceranae* respectively (Fig. 1). With PCR protocol No. 3, the PCR detection limit was similar to that of PCR protocol No. 2 (10^2 and 10^3 plasmid DNA copies of *N. ceranae* and *N. apis* respectively). Using this third protocol, *N. apis* and *N. ceranae* were detected whatever the quantity of plasmid DNA copies tested.

For these three protocols, similar results were observed when DNA from healthy honey bee was used as diluent for the 10-fold serial dilution of plasmid. These results suggested that there is no PCR inhibition or DNA competition.

The detection limits of the method were then assessed for each protocol and each *Nosema* species. DL_{method} of protocols Nos. 2 and No. 3 were similar and determined at 46 and 376 spores of *N. ceranae* and *N. apis* respectively per reaction, while the DL_{method} of the first protocol was one hundred fold higher (4600 and 37600 spores of *N. ceranae* and *N. apis* respectively). The experiments conducted to test the DL_{method} for a given species with the dilution range of the other species confirm the results obtained with plasmids. With the protocol No. 3, *N. apis* and *N. ceranae* were detected whatever the quantity of spores tested, while with the others protocols,