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Original article

Prevalence of the microsporidian *Nosema ceranae* in honeybee (*Apis mellifera*) apiaries in Central Italy

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ABSTRACT

Nosema ceranae and *Nosema apis* are microsporidia which play an important role in the epidemiology of honeybee microsporidiosis worldwide. Nosemiasis reduces honeybee population size and causes significant losses in honey production. To the best of our knowledge, limited information is available about the prevalence of nosemiasis in Italy. In this research, we determined the occurrence of *Nosema* infection in Central Italy. Thirty-eight seemingly healthy apiaries (2 to 4 hives each) were randomly selected and screened from April to September 2014 ($n = 11$) or from May to September 2015 ($n = 27$). The apiaries were located in six areas of Central Italy, including Lucca ($n = 11$), Massa Carrara ($n = 9$), Pisa ($n = 9$), Leghorn ($n = 7$), Florence ($n = 1$), and Prato ($n = 1$) provinces. Light microscopy was carried out according to current OIE recommendations to screen the presence of microsporidiosis in adult worker honeybees. Since the morphological characteristics of *N. ceranae* and *N. apis* spores are similar and can hardly be distinguished by optical microscopy, all samples were also screened by multiplex polymerase chain reaction (M-PCR) assay based on 16S rRNA-gene-targeted species-specific primers to differentiate *N. ceranae* from *N. apis*. Furthermore, PCR-positive samples were also sequenced to confirm the species of amplified *Nosema* DNA. Notably, *Nosema* spores were detected in samples from 24 out of 38 (63.2%, 95% CI: 47.8–78.5%) apiaries. Positivity rates in single provinces were 10/11, 8/9, 3/9, 1/7, or 1/1 ($n = 2$). A full agreement (Cohen's Kappa = 1) was assessed between microscopy and M-PCR. Based on M-PCR and DNA sequencing results, only *N. ceranae* was found. Overall, our results highlighted that *N. ceranae* infection occurs frequently in the cohort of honeybee populations that was examined despite the lack of clinical signs. These findings suggest that colony disease outbreaks might result from environmental factors that lead to higher susceptibility of honeybees to this microsporidian.

1. Introduction

Microsporidia are unicellular spore-forming microorganisms. They are classified as highly evolved fungi, are intracellular obligate parasites of eukaryotes, and include species that parasitize insects (e.g. bumblebees, silkworms, and others), fishes, or mammals (Keeling, 2009). The genus *Nosema* belongs to this group of fungi (Keeling, 2009). *Nosema apis* and *Nosema ceranae* are the two best known *Nosema* species because they invade the midgut epithelial cells of adult honeybees (*Apis mellifera*), i.e. worker bees, drones, and queens, and they are the aetiological agents of the honey bee disease known as nosemiasis or nosemosis (Fries, 1988, 2010; Higes et al., 2007, 2010). This disease negatively affects productivity and survival of honeybee colonies, adult bee longevity, queen bees, brood rearing, bee biochemistry, pollen collection and other bee behaviours (Botias et al., 2013; Huang, 2012). In addition, *N. ceranae* was associated with a particular form of nosemiasis that may lead to colony depopulation and collapse (Huang, 2012; Paxton, 2010), although the specific causes of most losses remain undetermined (van Engelsdorp et al., 2009). Originally, *N. apis* and *N. ceranae* were reported in European honey bees (*A. mellifera*) and Asian honey bees (*Apis cerana*), respectively, and

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were thought to be species-specific. However, *N. ceranae* was shortly reported also in *A. mellifera* in many countries throughout the world (Fries, 2010; Higes et al., 2010; Klee et al., 2007; Paxton, 2010). Some studies have shown that the geographic distribution of *N. apis* and *N. ceranae* may overlap and co-infections may occur (Milbrath et al., 2015). It also appears that *N. ceranae* has displaced *N. apis* becoming more and more prevalent in many countries, including Italy (Klee et al., 2007). Studies on the impact of *Nosema* on honeybee colonies need data concerning its prevalence, particularly in asymptomatic colonies. To the best of our knowledge, little is reported about the prevalence of *Nosema* in Italian apiaries (Ferroglio et al., 2013). Therefore, in this research we evaluated the prevalence and distribution of *Nosema* infection in 38 apiaries located in different provinces of Central Italy. First, light microscopy was carried out according to current OIE recommendations to screen the presence of microsporidiosis in adult worker honeybees. Second, since the morphological characteristics of *N. ceranae* and *N. apis* spores are similar and can hardly be distinguished by optical microscopy, all samples were also screened by multiplex polymerase chain reaction (M-PCR) assay based on 16S rRNA-gene-targeted species-specific primers to differentiate *N. ceranae* from *N. apis*. Furthermore, PCR-positive samples were also sequenced to confirm the species of amplified *Nosema* DNA.

2. Materials and methods

2.1. Bee sample collection

Thirty-eight seemingly healthy apiaries (2 to 4 hives each) were randomly selected and screened from April to September 2014 ($n = 11$) or from May to September 2015 ($n = 27$). The apiaries were located in six provinces of Central Italy, including Lucca ($n = 11$) (43°51′N 10°31′E), Massa Carrara ($n = 9$) (44°02′00″N 10°08′00″E), Pisa ($n = 9$) (43°43′N 10°24′E), Leghorn ($n = 7$) (43°33′N 10°19′E), Florence ($n = 1$) (43°46′17″N 11°15′15″E), and Prato ($n = 1$) (43°52′50.93″N 11°05′47.62″E). The apiaries were visited only once. In each sampling, fifty forager bees were collected at the entrance of each sampled hive (Fries et al., 2013). No hives of the apiaries sampled had history of signs referable to nosemiasis and no signs of the disease were present at the time of sampling.

2.2. Microscopic analysis

Light microscopy was used to screen the presence of *Nosema* spores in adult worker honeybees according to OIE recommendations (2013). Spore counting was estimated in some randomly selected positive samples ($n = 5$) by haemocytometer as described by Fries et al. (2013). Infection levels were classified as low (<5.0 million spores per bee), medium (⩾5.0–<10.0 million spores per bee), and high (⩾10.0 million spores per bee) according to Yücel and Goğaroğlu (2005). As morphological characteristics of *N. ceranae* and *N. apis* spores are similar and can hardly be distinguished by optical microscopy, all samples were also screened by multiplex polymerase chain reaction (M-PCR) assay based on 16S rRNA-gene-targeted species-specific primers to distinguish between *N. ceranae* and *N. apis*.

2.3. Molecular analysis

DNA was extracted by DNeasy Blood & Tissue Kit (Qiagen S.p.a., Milan, Italy) following to the manufacturer's instructions. According to OIE manual recommendations (2013), the primers used for *N. apis* were 321 APIS FOR 5′-GGGGGCATGTCTTTGACGTAC TATGTA-3′ and 321 APIS REV 5′-GGGGGGCGTTTAAAATGTGAAA CAACTATG-3′. Those used for *N. ceranae* were 218 MITOC FOR 5′-CGGCGACGATGTGATATGAAA-ATATTAA-3′ and 218 MITOC REV 5′-CCCGGTCATTCTCAAACAAAA-AACCG-3′. The parameters for DNA amplification were the following: an initial PCR activation step of 2 minutes at 94 °C, followed by 10 cycles of 15 seconds at 94 °C, 30 s at 61.8 °C, and 45 s at 72 °C, and 20 cycles of 15 s at 94 °C, 30 s at 61.8 °C, and 50 seconds at 72 °C plus a final extension step at 72 °C for 7 min. The M-PCR products were visualized in a 2% agarose TAE gel with a band at 321 bp for *N. apis* and at 218–219 for *N. ceranae*.

2.4. Sequence analysis

DNA samples from all M-PCR-positive samples were also sequenced to confirm the species of amplified *Nosema* DNA The nucleotide sequences obtained were compared with all *N. apis* and *N. ceranae* sequences available in the GenBank™ database using BLAST tool (Altschul et al., 1997).

2.5. Data analysis

Prevalence of positivity rates were calculated as follows:

$$\text{Prevalence } (\%) = [(\text{number of positive apiaries})/(\text{number of examined apiaries})] * 100$$

The corresponding 95% confidence intervals (95% CI) were calculated and differences among prevalence values were compared by Fisher's exact test. P values <0.05 were considered significant. Range, mean and standard deviation (SD) of spore counts were determined. The Cohen's Kappa coefficient was used as a measure of agreement between microscopy and M-PCR. The following ranges were considered for interpretation of the Cohen's Kappa coefficient: poor agreement = less than 0.00, slight agreement = 0.00–0.20, fair agreement = 0.21–040, moderate agreement = 0.41–0.60, substantial agreement = 0.61–0.80, and almost perfect agreement = 0.80–1.00.

3. Results

Nosema infections were detected in apiaries from all the six provinces examined, with single prevalence rates of 10/11, 8/9, 3/9, 1/7, or 1/1 ($n = 2$). Overall, a total of 24 out of 38 (63.2%, 95% CI: 47.8–78.5%) apiaries tested positive for *Nosema* infection by light microscopy and M-PCR. Therefore, the Cohen's Kappa coefficient for the association between results of microscopy and results of M-PCR was 1, indicating that there was a perfect level of agreement between the two diagnostic methods in all the bee samples.

Results of the M-PCR assay revealed that all the 24 positive bee samples were infected only by *N. ceranae*. Counts ranged from 125,000 to 4,100,000 (mean ± SD = 2,070,000 ± 1,521,052) spores per ml per bee. With respect to the year of sample collection, prevalence was higher in 2014 (9/11, 81.8%, 59–100%) than in 2015 (15/27, 55.6%, 36.8–74.3%), However, this difference was not statistically significant ($P = 0.1596$). The comparison of DNA sequences from all the 24 M-PCR-positive samples with the *Nosema* sequences available in the GenBank™ database showed 100% identity with *N. ceranae*.

4. Discussion

The identification of *N. ceranae* in the six Tuscan provinces surveyed in Central Italy was expected, given that this *Nosema* species has previously been reported in different Italian regions (Ferroglio et al., 2013; Klee et al., 2007; Maiolino et al., 2014). *N. ceranae* is not a recent fungal pathogen for Italian honeybees, since it has been detected in honeybee samples collected in Northern Italy in 1993