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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.
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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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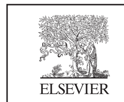

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were thought to be species-specific. However, *N. ceranae* was shortly reported also in *A. mellifera* in many countries throughout the world (Fries, 2010; Higes et al., 2010; Klee et al., 2007; Paxton, 2010). Some studies have shown that the geographic 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 ($\geqslant$5.0–<10.0 million spores per bee), and high ($\geqslant$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

(Ferroglio et al., 2013). The high prevalence (63%) of *N. ceranae* together with the absence of *N. apis* infection and *N. apis*/*N. ceranae* co-infection in the present survey corroborates the findings of other authors that *N. ceranae* is definitely spread in Italy and has basically replaced *N. apis* (Klee et al., 2007). High thermotolerance at 60 and 35 °C, resistance to desiccation, significant decrease in viability after freezing, and rapid degeneration of *N. ceranae* spores maintained at 4 °C were observed under experimental conditions (Fenoy et al., 2009). Therefore, it has been proposed that *N. ceranae* may be more prevalent in warmer climates (Fries, 2010; Higes et al., 2010) such as the typical Mediterranean climate that occurs in the study area. The present prevalence is close to values as high as 70% and 75% observed in China (Yan et al., 2013) and United States (Runckel et al., 2011) but considerably higher than 1.3–14.9% and 33.7% previously reported in Germany (Gisder et al., 2010) and Australia (Giersch et al., 2009), respectively. Different prevalence values reported in literature may be due to differences in number of apiaries examined, sampling methods, geographical areas, characteristics of honeybee population, diagnostic techniques, and other biotic and abiotic factors.

Based on our findings, microscopy is still a valuable, relatively cheap and simple method to screen for the presence of *Nosema* infection in apiaries since a perfect agreement between microscopy and M-PCR was observed in this cross-sectional study. Similar results were also reported by Michalczyk1 et al. (2011). Unfortunately, very strong morphological similarities occur between *N. apis* and *N. ceranae* spores, resulting in a high risk of misdiagnosis. Both *Nosema* species spores are smooth and darkly outlined with elongated-elliptical shape and bright centre. *N. apis* spores end rounded and measure 6.0 μm in length and 3.0 μm in width. *N. ceranae* spores end sharper and measure 4.4 μm in length and 2.2 μm in width (Huang, 2012; Michalczyk1 et al., 2011). The main differences are noted with respect to the length of the polar filament, and they can be detected only under an electron microscope (Fries, 2010; Higes et al., 2010; Paxton, 2010).

In this scenario, molecular techniques such as M-PCR are needed for a reliable identification of *Nosema* to species level (Michalczyk1 et al., 2011). Indeed, the advent of new highly sensitive and specific molecular tools has played a key role for detection of *N. ceranae* in *A. mellifera* and for retrospective analyse of samples, showing that *N. ceranae* is not a new microsporidian agent in *A. mellifera* but it has infected this host during the last twenty years (Ferroglio et al., 2013; Higes et al., 2010). It is likely that the delay in a correct identification of *N. ceranae* in *A. mellifera* is attributable to the routine use of microscopy as a diagnostic technique for the identification of *Nosema* spores (Higes et al., 2010). Moreover, in accordance with the Italian laws and regulations on veterinary hygiene, *N. apis* infected apiaries with clinical evidence of disease must be subjected to strict sanitary measures. However, the same measures are not needed in cases of *N. ceranae* infected apiaries (see http://www.sivempveneto.it/leggi-tutte-le-notizie/3664-parere-ministero-misure-polizia-veterinaria-solo-nei-casi-di-nosema-apis-clinicamente-manifesta). Therefore, accurate identification of *Nosema* spores to species level by molecular tools should be especially useful for Italian beekeepers.

Nosema spores are primarily spread to neighbouring bees through faecal matter contaminating the environment (faecal-oral pathway) or, alternatively, they can also reach the crop and be regurgitated to other colony members during food exchange (oral-oral pathway) (Smith, 2012). Therefore, infections by both *Nosema* species can be transmitted among bees via ingestion of environmentally resistant mature spores from contaminated wax, combs, other hive interior surfaces, and water (OIE, 2013). Other potential routes of transmission include contamination of pollen, beekeeping material, and honey as well as cleaning activities and trophallaxis (Higes et al., 2010). Auto-infections can also occur (Higes et al., 2007). In our survey, we did not attempt to identify any source of infection. However, all these routes of spread and transmission of infective spores may have played a role in the presence of *N. ceranae* in the *A. mellifera* colonies investigated. After ingestion, the spores pass through the digestive tract until they reach the host midgut and germinate; this implies that the polar filament is extruded and mechanically penetrates through the cell membrane into the epithelial cells of the ventriculus (Fries, 1988; Higes et al., 2007). Then, the infective sporoplasm is entered through the polar filament into the host cell cytoplasm where *Nosema* replication with spore production starts (Fries, 1988; Higes et al., 2007). After being completely filled with spores, infected ventricular cells undergo degeneration and lysis (Maiolino et al., 2014). *N. ceranae* infection can occur all year round and may show low seasonality (Huang, 2012). Foraging bees are more likely to be heavily infected (Fries et al., 2013).

In contrast to nosemiasis caused by *N. apis*, *N. ceranae* affected bees do not exhibit defecation near or inside the hive with evident dysentery but the main clinical symptom is dwindling, i.e. the progressive reduction in the number of bees in a colony with no apparent cause, until the point of collapse (Huang, 2012; Paxton, 2010). Sometimes dwindling may affect the whole apiary and other times only specific colonies may show symptoms (van Engelsdorp et al., 2009). The disease sometimes occurs rapidly but may also occur over several months (van Engelsdorp et al., 2009).

The pathological mechanisms causing these differences remain unknown since both microsporidia affect the same tissue and cause similar lesions (Huang, 2012). It has also been reported that *N. ceranae* is more virulent than *N. apis*, affects learning and homing behaviour, causes higher energy costs and immune suppression, and affects queen health (Huang, 2012). Despite this, no hives of the 24 *N. ceranae*-positive apiaries in this survey showed any clinical sign at the time of sampling and had history of signs referable to nosemiasis caused by *N. ceranae*. In addition, low infection levels (up to 4,100,000 spores per ml per bee) were detected in pooled samples. This is in agreement with the results of Mulholland et al. (2012) and Smart and Sheppard (2012) who reported similar (up to 3,962,500 spores) or much more higher (up to 87,000,000) spore counts in single bees from colonies infected with *N. ceranae* without any clinical sign of nosemiasis. These findings suggest that *N. ceranae* may be less virulent than it was previously thought, or that the Italian *N. ceranae* strain spread in Central Italy apiaries may be less pathogenic than others, or that the cohort of Italian *A. mellifera* populations investigated may have developed some degree of resistance to *N. ceranae*, or finally that a combination of these factors may occur.

5. Conclusions

Overall, our results provide evidence that *N. ceranae* infection occurs frequently in the cohort of apiaries examined despite the lack of clinical signs. This suggests that colony disease outbreaks might probably be caused by other factors (Mutinelli et al., 2010), both known and unknown, that singly or in combination may lead to higher susceptibility of honeybees to *N. ceranae*. Results of this study provide basic knowledge about the impact of *Nosema* infection on honey bee breeding in different geographic areas of Central Italy. Further research focusing on virulence variation of different *N. ceranae* strains and genetic variation in resistance of different honeybee lineages is urgently required.

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Conflict of interest

The Authors declare no competing interests.

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