Microscopic and molecular detection of Nosema spp. in honeybees of Turkey

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Original article

Microscopic and molecular detection of *Nosema* spp. in honeybees of Turkey

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Abstract – In this study, we aimed to determine the prevalence of *Nosema* spp. in honeybees of Turkey. For this aim, adult honeybee (*Apis mellifera*) samples were collected from 1621 colonies within 95 apiaries located in 22 provinces of Turkey. Samples were examined microscopically. In case of positivity, spore identification was done by multiplex PCR. At the end of microscopic examination, *Nosema* spp. spores were detected in 7 out of 22 provinces (31.8 %), and 16 out of 95 colonies (16.8 %) that represent 1621 colonies. According to PCR results, 1 out of 16 isolates (6.25 %) was *Nosema apis*, and 15 out of 16 isolates (93.75 %) were *Nosema ceranae*. The result of our study indicated that *N.ceranae* is the dominant species in Turkey.

Nosema apis **/** ***Nosema ceranae*** **/ Multiplex PCR / Turkey**

1. INTRODUCTION

Nosemosis is one of the most prevalent adult honeybee diseases. The etiologic agents of the disease are microsporidian parasites, *Nosema apis* and *Nosema ceranae* (Somerville and Hornitzky 2007). Disease spread by fecal-oral route in honeybee colonies. Adult bees get infection either by contaminated water and foods or when they come into contact with spores while they are cleaning the hives. After the spore ingestion, spores germinate in the midgut and then multiply. In a few weeks period, millions of spores occur and spread by feces (Fries 1997; Somerville and Hornitzky 2007).

Gastrointestinal disorders, shortened life span, flying failures, dead bee accumulation at hive entrance, reduction of colony population and honey production, and even colony collapse are the symptoms of nosemosis (Fries 1997; Somerville and Hornitzky 2007; Higes et al. 2008).

N. apis is one of the first described microsporidian parasite and believed to be the only etiological agent of nosemosis in the European honeybee (*Apis mellifera*). However, nowadays, a new-described species, *N. ceranae*, is seen in Asia, Europe, and North and South America, spreading within the European honeybee colonies and starts to replace *N. apis* (Klee et al. 2007; Williams et al. 2008; Utuk et al. 2010).

Some studies showed that *N. ceranae* may be more pathogenic than *N. apis*, and infected colonies may collapse when they are not treated (Paxton et al. 2007; Somerville and Hornitzky 2007; Mayack and Naug 2009).

The disease is generally diagnosed by microscopical examination of spores. Nevertheless, spores of each species are very similar, and it is hard to make species identification by conventional microscopic examination (Fries et al. 1996;

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Martín-Hernández et al. 2007) Therefore, molecular techniques such as PCR (Polymerase Chain Reaction) (Higes et al. 2006), PCR-RFLP (Restriction Fragment Length Polymorphism) (Klee et al. 2007) and DNA sequencing have gained more prominence in the diagnosis of singular or mix infections (Fries et al. 1996; Martín-Hernández et al. 2007)

The aim of this study was to determine the prevalence of *Nosema* spp. in honeybees of Turkey by microscopic and molecular examination of adult honeybee samples, which were collected in a 7-year period.

2. MATERIAL AND METHODS

2.1. Sample collection

Adult honeybees (*Apis mellifera*) were collected from 1621 colonies within 95 apiaries (17±1 colony from each apiary) located in 22 provinces (4±1 apiaries from each of 22 provinces) of Turkey between the years 2006 and 2011 and the months November and June. Study areas are summarized in Figure 1.

2.2. Spore detection and DNA extraction

95 apiary samples that represent 1621 colonies were examined. The abdomens of 20 adult honeybees (*A. mellifera*) from each sample were macerated in 3 mL of distilled water. Three drops of the suspension were placed on a slide under a cover slip and examined microscopically at ×40 magnification, under bright-field or phase-contrast optics. In case of positivity, 1 mL of suspension was filtered and centrifuged for 5 min at 8000 rpm and supernatants were removed. Spores were stored −20 °C until they were used for DNA extraction (Utuk et al. 2010). Genomic DNA extraction was done from the pellets using DNeasy TM Tissue Kit (Qiagen, Hilden, Germany) by following the manufacturer's instructions. Prior to DNA extraction, pellets were washed for five times with phosphate buffer solution (PBS).

2.3. PCR amplification

To amplify partial 16S rRNA genes of *N. ceranae* and *N. apis*, 218MITOC-FOR (5′-cggcgacgatgtgatatgaaaatattaa-3′), 218MITOC-REV (5′-cccggtcattctcaaacaaaaaaccg-3′) and 321APIS-FOR (5′-gggggcatgtctttgacgtactatgta-3′), 321APIS-REV (5′-gggggcgtttaaaatgtgaaacaactatg-3′) primer pairs were used in multiplex PCR (Martín-Hernández et al. 2007).

PCR was carried out in a final volume of 50 μL, containing 25.75 μL DNase- and RNase-free steril distilled water (Biobasic, Inc), 5 μL 10X PCR buffer, 5 μL 25 mM $MgCl_2$, 6 μL 1 mM dNTP mix, 1 μL of each primer (20 pmol), 2.5 μL of template DNA (100–200 ng), and 0.25 μL of TaqDNA polymerase (1.25 IU) (MBI Fermentas). The PCR conditions were as follows: 2 min at 95 °C (initial denaturation), 35 cycles of 1 min at 95 °C, 1 min at 50 °C, 1 min at 72 °C, and finally 5 min at 72 °C (final extension). The PCR products were separated on agarose gels (1.5 %), stained with ethidium bromide and visualized and photographed on an UV transilluminator (Utuk et al. 2010).

3. RESULTS

At the end of microscopic examination, *Nosema* spp. spores were detected in 7 out of 22 provinces (31.8 %) and 16 out of 95 colonies (16.8 %) (Figure 1). All positive samples were from adult honeybees collected in the years 2009, 2010, and 2011. According to PCR results, 1 out of 16 isolates (6.25 %) was *N. apis*, which gave a 321 bp amplicon, and 15 out of 16 isolates (93.75 %) were *N. ceranae*, which gave a 218–219 bp amplicon (Figure 2). While *N. ceranae* was detected in Ankara, Bursa, Erzurum, Kayseri, Mugla, and Zonguldak provinces, *N. apis* was only detected in Cankırı province of Turkey.

4. DISCUSSION

Nosemosis is an important widespread disease both in Turkey and in the world and has economic impact on beekeeping. To date, studies on nosemosis in Turkey have focused on detecting the prevalence of the disease with microscopic examination, such as 5–8.77 % in Elazig (Simsek et al. 2001; Simsek 2005), 15.74 % in Kars (Topcu and Arslan 2004), 38.5 % in Bingöl (Kutlu and Ekmen 2003) and 24–26.4 % in Bursa (Aydin et al. 2001; Cakmak et al. 2003) provinces. Kutlu and Kaftanoglu (1990), studied the prevalence of *Nosema* spores on the colonies that were

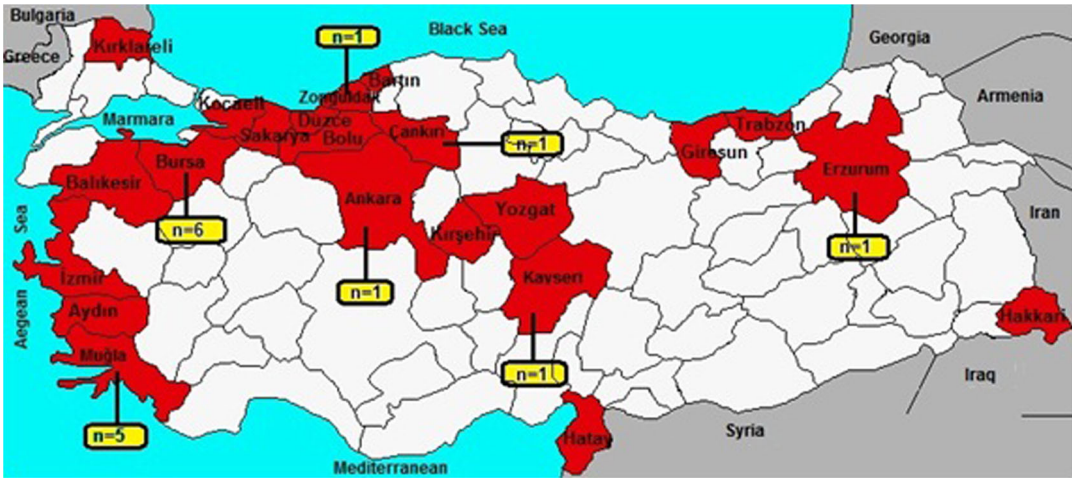

Figure 1. Map of Turkey showing study areas where the adult honeybee (*A. mellifera)* samples were collected and the number of positive colonies against *Nosema* spp. spores.

brought to Adana province for overwintering, Mugla and Aydin provinces for honeydew production. They examined 312 apiaries and sampled 1560 colonies and found that the *Nosema* infection was 31.3 % in Mugla, 29.8 % in Adana, 29.6 % in Dalaman, 28.6 % in Aydin, 25.7 % in Datca, 25 % in Milas, 23.8 % in Fethiye, 23.3 %

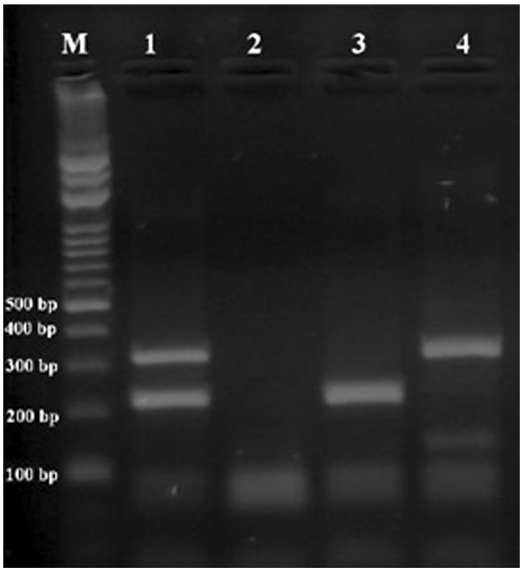

Figure 2. M: Marker **1:** Positive controls of *N. apis* and *N. ceranae* **2:** Negative control, **3:** 218 bp amplicon of Ankara, Bursa, Erzurum, Kayseri, Mugla, and Zonguldak isolates **4:** 321 bp amplicon of Cankırı isolate.

in Koycegiz, and 20.5 % in Marmaris. In a wide range research, Aydin et al. (2005) have studied on 230 colonies within 37 apiaries from 26 provinces and seven districts of Turkey, and they have found a prevalence of 60 % at apiaries level and 14 % at colony level. Utuk et al. (2011) evaluated the samples sent to the Veterinary Control Central Research Institute, Bee Diseases Laboratory between the years 2006 and 2010. In this period, they received 140 applications from 36 different provinces of Turkey and found the prevalence of nosemosis as 4.28 %. In this study, *Nosema* spp. spores were detected in 7 out of 22 provinces (31.8 %) and 16 out of 95 colonies (16.8 %).

According to the latest molecular studies, both *N. ceranae* and *N. apis* are present in Turkey. Utuk et al. (2010) detected *N. ceranae* for the first time in two bee samples that were sent to their laboratory from Samsun and Giresun provinces of Turkey by using multiplex PCR. Whitaker et al. (2011) have studied on 84 honeybee samples from 20 provinces of Turkey and detected *N. ceranae* in three samples from Artvin, Hatay, and Muğla and *N.apis* in four samples from Sivas, Izmir, Bitlis, and Gaziantep provinces by using conventional PCR and DNA sequencing. Muz et al. (2010) have reported *N. ceranae* and *N. apis* from Hatay and Southern Marmara by using conventional PCR. In this study, we reported *N. ceranae* in 15 samples from Ankara, Bursa,

Erzurum, Kayseri, Mugla, and Zonguldak provinces and *N. apis* in 1 sample from Cankırı province of Turkey. According to our results, *N. ceranae* is the dominant species in Turkey.

Most studies showed that *N. ceranae* is more pathogenic and causes more energetic stress on honeybees than *N. apis* (Paxton et al. 2007; Mayack and Naug 2009; Martín-Hernández et al. 2011). As a result of the coevolution period of European honeybee and *N. apis*, they are well adapted to each other. But in the case of *N. ceranae*, this period is shorter than *N. apis* for the adaptation to European honeybee, and physiological adaptation mechanism in the host-parasite relationship may be less efficient. This causes increased appetite, more food consumption, shortened life span, decrease in energy levels, reduced colony population, and even colony collapse (Paxton et al. 2007; Mayack and Naug 2009). In addition to its effect on energetic stress, *N. ceranae* causes immunosuppression and provokes the secondary infections in honeybees (Antúnez et al. 2009). If the expansion of *N. ceranae* continues at this pace, there may be heavier colony losses in the future than in the past. Molecular studies that contain all regions in Turkey have to be performed and the current situation of the diseases has to be determined. In the light of the new data, awareness should be raised among beekeepers against the possible threat of *N. ceranae*.

Détection microscopique et moléculaire de *Nosema* spp. chez les abeilles de Turquie

Nosema apis **/** ***Nosema ceranae*** **/ PCR multiplexe /** ***Apis mellifera***

Die Identifizierung von *Nosema* spp. in der Türkei mitttels Mikroskopie und molekularer Methoden

Nosema apis / Nosema ceranae **/ Multiplex-PCR / Türkei /** ***Apis mellifera***

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