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Original Article

FIRST DETECTION OF *NOSEMA CERANAE* AND *NOSEMA APIS* IN GREATER WAX MOTH *GALLERIA MELLONELLA*

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Abstract

The greater wax moth *Galleria mellonella* L. (Lepidoptera: Pyralidae), one of the pests of honey bee (*Apis mellifera* L.) colonies, has spread almost all over the world. Although the *G. mellonella* is often reported to infest weak honey bee colonies that are exposed to pesticides and diseases, it is also a threat for healthy colonies. Therefore, there is a fairly high probability of transmission of both microflora-specific bacteria and pathogen micro-organisms, especially *Nosema* species, between these organisms (Moth and bees). The aim of this study was to investigate the presence of *Nosema* species in greater wax moth *G. mellonella* collected from apiaries as well as grown in laboratory conditions. Adults and late instar larva of wax moth were used for detecting *Nosema apis* and *Nosema ceranae*. Real-time PCR amplification studies were performed and specific ITS regions were targeted to distinguish *Nosema* species. Real-time PCR results showed that *N. apis* and *N. ceranae* were found in both phases of *G. mellonella*. This is the first study to confirm that *N. apis* and *N. ceranae* are present in greater wax moth collected from apiaries and grown at laboratories in Turkey.

Keywords: *Galleria mellonella*, honey bee, *Nosema apis, Nosema ceranae*, real-time PCR

INTRODUCTION

The greater wax moth *Galleria mellonella* L. (Lepidoptera:Pyralidae) is an economic pest of honey bee (*Apis mellifera*) colonies and nowadays has spread almost all over the world, including Europe, Asia, North America and Australia (Ellis, Graham & Mortensen, 2013), especially spread to low altitude regions with a temperate climate (Akyol & Korkmaz, 2008). The waxworms of the greater wax moth have been shown to be an excellent model organism for in vivo toxicology and pathogenicity testing, replacing the use of small mammals (Hu & Webster, 2010; Coleman et al., 2011; Harding et al., 2013). In addition to the use of the wax moth as a model organism for research on insect physiology, genomics and proteomics, they have an important role in the apicultural industry (Ellis, Graham, & Mortensen, 2013). The larval phase but not the adult phase causes severe damage to the combs and leads to significant economic loss in the beekeeping sector.

Female wax moths lay almost 0.5 mm eggs in crevices and cracks of the bee hive. Larvae emerge from the eggs within 5-8 days under normal conditions (24-26°C) which varies depending on the temperature. Following development, the larvae weave a cocoon, transform into adults and complete their life cycle (Spangler, 1985; Williams, 1997; Ellis, Graham & Mortensen, 2013. Besides their natural habitat, wax moths can be reared in laboratory conditions with the appropriate diet (Ellis, Graham, & Mortensen, 2013).

The wax moth is often reported in honey bee colonies that are weakened by exposure to pesticides and diseases but is also a threat for healthy colonies. Its constantly interacts with honey bees because its essential nutrient is

honeycomb which is inside bee nests (Ellis, Graham, & Mortensen, 2013). Therefore, both microflora-specific bacteria and pathogen microorganisms, especially *Nosema* species, very likely are transferred between the moths and bees, which increases the probability of contact with honey bee pathogens and parasites. Wax moths mostly prefer weak and diseased honey bee colonies because of the easier access to nutrients. This situation increases the probability of contact with honey bee pathogens and parasites.

Besides reports on *Nosema galleriae* infection and lytic activity through the injection of *Nosema algerae* and *Nosema plodiae* (Kučera & Weiser, 1975; 1985; Lipa, 1977), there has not been any other related with other *Nosema* species in *G. mellonella*. *Nosema apis* and *Nosema ceranae* are parasitic microsporidia that cause Nosemosis, an adult honey bee disease and infects all colony members i.e. the queen bee, worker bees and drones (Chen et al., 2009). Nosema infections are thought to be the main cause of honey bee winter losses in many countries (Higes et al., 2005; Higes et al., 2008; vanEngelsdorp & Meixner, 2010). Although having a similar morphology under the light microscope, *N. ceranae* spores are slightly smaller and more circular than that of *N. apis*. The main differences between these two species was determined by electron microscopic structure, the small subunit (16S) rRNA gene sequences and ITS regions. Under an electron microscope, *N. apis* spores were observed to have 30 polar filaments while *N. ceranae* spores 20-23 polar filaments (Fries et al., 1996; 2006; Fries, 2010).

Nosema spp. can be vertically and horizontally transmitted in high quantities among honey bees (Higes et al., 2008). Although there are many factors in the spreading of *Nosema*, that *Nosema spp*. is known to mostly spread by transfer between organisms because of its host-switching property. It is necessary to investigate the probability of *Nosema* transmission between honey bees and other species which interact with them. The aim of this study was to investigate the presence of *Nosema* species in greater wax moth *G. mellonella* both collected from apiaries in the Isparta and Artvin provinces of Turkey and grown away from its natural habitat in laboratory conditions.

MATERIAL AND METHODS

Microscopic examination of intestinal contents of *Galleria mellonella* larvae and adults

In this study, the classical *Nosema* detection method applied to honey bees was used for *G. mellonella* samples (Fig. 1). Twenty homogeneous colonies of *Apis mellifera anatoliaca* and

Fig. 1. Late instar larvae (left) and adult (right) phases of *Galleria mellonella.*

Apis mellifera caucasica in the Isparta and Artvin provinces of Turkey were visited from January 2012 to April 2012 in order to find wax moth samples and adults and late instar larvae of *G. mellonella* samples were collected from wax moth-positive apiaries. All samples were stored at -20°C until they were used for experiments. Adult and late instar larval samples of wax moth which were reared in the laboratory conditions were also selected in the same quantities for the study. All samples were washed first with 1% sodium hypochlorite solution and then with sterile distilled water to avoid any surface contamination and to determine whether they had Nosema spores in their gastrointestinal tracts. All materials used in this study were sterilized to prevent external contamination and new sterile materials were used before work with each sample. The solutions were prepared through homogenization of the intestinal content of fifteen greater wax moths per sample. Larva solutions were prepared by using the clean gut tissues of the larvae. The adult forms were homogenized to make adult solutions. Larva and adult samples were homogenized separately in order for these two stages of the wax moth to be compared. These solutions were centrifuged at 6,000 rpm for 10 minutes, and afterwards the supernatant was poured and the pellet was used for examination. 1 mL of distilled water was added per sample on the pellet and vortexed. These pellet solutions were used for the morphological detection of *N. apis* and *N. ceranae* spores with light microscope using an improved Neubauer slide (Cantwell, 1970).

DNA isolation

The Biospeedy DNA Isolation Kit (Bioeksen, Turkey) was used for DNA isolation. After homogenization of the moth samples, each was transferred to a microfuge tube weighing 200 mg. 400 µL Guanidinium thiocyanate (0.1 M Tris; pH 7.5) was added to the tube and vortexed at 3,000 rpm for one minute. The samples were incubated for 10 min at 95°C. After stirring for 1 min at 3,000 rpm with vortex, the supernatant was centrifuged for 1 min at 14,000 rpm and then transferred to a new tube. 200 µL of isopropanol was added to the supernatant and mixed thoroughly. The mixture was added to a DNA column followed by a 1-2 minute pause; after centrifugation at 12,000 rpm for 1 minute the pellet was discarded. 500 µL Wash Buffer (20 mM NaCl, 2 mM Tris-HCl, pH 7.5; 80% v/v Ethanol) was added to the column and pellet was discarded after centrifugation at 12,000 rpm for 1 min. This step was repeated two more times. After the column was centrifuged for 1 min at 12,000 rpm, it was placed in the sterile micro centrifuge tube. 100 µL solubilization buffer was added to the tube, incubated 1 min at room temperature and centrifuged at 14,000 rpm for 1 min, after which DNA isolates were obtained and stored at -20°C.

Real-time PCR amplification (qPCR)

After DNA isolation, real-time PCR amplification was performed through the modification of the protocol of Bourgeois et al. (2010). Biospeedy qPCR 2 × Master Mix (Bioeksen, Turkey) were used for this process. The ITS region has been targeted to distinguish *Nosema* species with the use of *Nosema spp.* specific forward primer ITS-F5'- TGAATGTGTCCCTGTTCTTTG-TAC-3', *N. apis* specific reverse primer N.apisITS-R 5'-TAATTATAATCTCCTTGGTCCGTG-3' and *N.ceranae* specific reverse primer NcerITS-R TAAATATAATCTCCTGGTCGGTT. Biorad CFX Connect (Bio-Rad Laboratories, USA) was used in all reactions. The reaction contained 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1 × Reaction Buffer, 0.1U Fast Start Taq DNA Polymerase, 1 × EvaGreen, 4 ng/µL template DNA and 0.5 µM of each primer. The optimization of specific primer pairs in the device was provided and thermocycling program given in Tab. 1 was applied. To determine whether there was a production of the expected product during qPCR, a melting curve analysis between 65°C - 98°C was performed. qPCR data were analyzed with CFX Manager Software 3.0.

RESULTS

Microscopic examination showed that both *N. apis* and *N. ceranae* spores were found microscopically in all homogenates of *G. mellonella*

Table 1

Q-PCR thermocycling program designed for the molecular determination of *Nosema apis* and *Nosema ceranae*

Detection Format		Reaction Volume		
SYBR Green		20 µl		
Programs				
The Program Name	Number of cycles	Analysis Mode		
Pre-incubation	1			
Proliferation	45	Counting		
Melting Curve	1	Melting Curve		
Cooling	1			
Temperature Targets				
Target (°C)	Reading Mode	Retention (hh:mm:ss)	Speed (°C/s)	Reading (Per °C)
Pre-incubation				
95		00:10:00	4,8	-
Proliferation				
95		00:00:15	4,8	-
53		00:00:15	2,5	-
72	Single	00:00:30	4,8	-
Melting Curve				
95		00:00:05	-	-
65		00:01:00	-	-
98	Continuous	-	0.5	10
Cooling				
40	Single	00:00:30	2,5	-

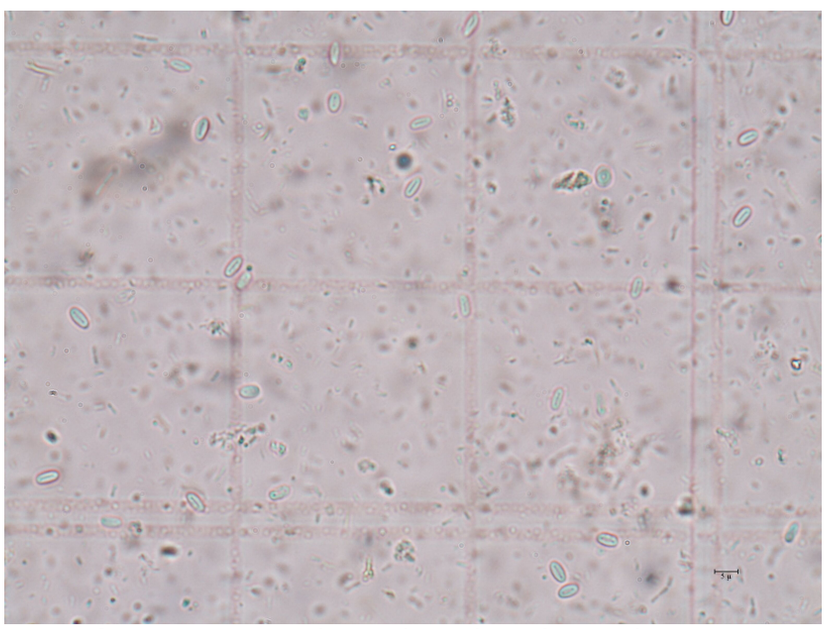

Fig. 2. Microscopic image of Nosema spores in the intestine of *Galleria mellonella* larvae collected from apiaries.

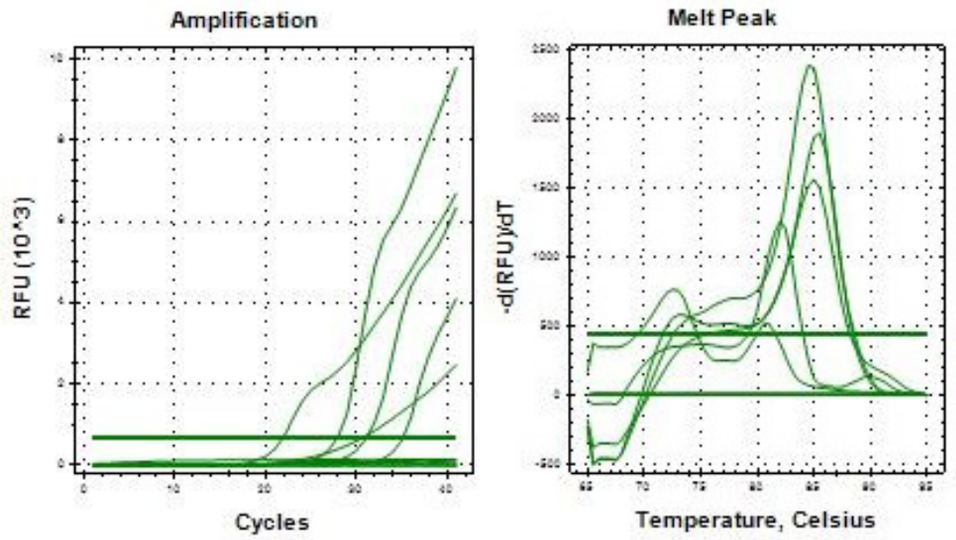

Fig. 3. Real-Time PCR proliferation threshold and melting curves obtained for *Nosema ceranae* and *Nosema apis* detection. Curves indicate positive results in terms of the presence of Nosema specie.

Table 2

Nosema species determination results depending on the melting temperature (Tm) and threshold cycle number (Ct) obtained from the qPCR results

Sample No	qPCR Target	Ct	Tm	Results
1 (Larvae)	*N.apis*	31,14	85	*N.apis* positive
1 (Larvae)	*N.ceranae*	35,16	82	*N.ceranae* positive
2 (Adult)	*N.apis*	27,96	85	*N.apis* positive
2 (Adult)	*N.ceranae*	-	-	*N.ceranae* negative
3 (Larvae)	*N.apis*	-	-	*N.apis* negative
3 (Larvae)	*N.ceranae*	30,75	82 and 73	*N.ceranae* positive
4 (Adult)	*N.apis*	21,99	85	*N.apis* positive
4 (Adult)	*N.ceranae*	-	-	*N.ceranae* negative

samples (Fig. 2). Although one *Nosema* species was detected in all sample groups, the two *Nosema* species were observed in only one sample group. Besides the *Nosema* spores, some protozoan forms were seen in *G. mellonella* larvae and adult intestines.

Sensitivity of the detection assay was determined by amplification with real-time PCR, and these results' proliferation threshold and melting curves shown in Fig. 3. Determination of the presence of *N. apis* and *N. ceranae* using these results, cycle number (Ct) and melting temperature (Tm) data are given in Tab. 2. It was evaluated that qPCR was performed with Ct <35 and positive results were obtained for *N. ceranae* at Tm 82°C and *N. apis* at Tm 85°C.

The real-time PCR results of the *Nosema* species were compared and *N. apis* was shown to be observed in wax moth adults which were both collected from apiary and reared in laboratory. The wax moth larvae reared in the laboratory only had *N. ceranae* while both *Nosema* species were found in larvae collected from the natural habitat (Tab. 2).

Comparison of the molecular and microscopic results revealed that consistent results were obtained in the two working process. *Nosema*

species which were determined by the spore morphology were identical with the results obtained from real-time PCR studies.

DISCUSSION

N. ceranae, a significant pathogen for honey bees, shows a more lethal effect than *N. apis*. It is known to cause mass deaths and losses in honey bee colonies especially in strong colonies (Martín-Hernández et al., 2007; Higes et al., 2010). The spread and transfer of *Nosema* species to other organisms, especially *N. ceranae*, increases transmission to honey bees and wild bees. Therefore, the presence of *Nosema* species must be investigated in organisms which interact with honey bees. Real-time PCR studies showed that *N. apis* and *N. ceranae* were found in both the larvae and adult phases of *G. mellonella,* and thus *Nosema* species may be find in all stages. .

We began to obtain from the molecular samples results of between 20-35 cycles as a minimum relative fluorescence unit (RFU) peak-height level during Real-time amplification. Obtaining the results in lesser cycles showed that specific Nosema primers which targeted ITS regions were selected correctly for this study. The RFU peaks were also separated easily as a result of the sufficient amount of DNA being analyzed. RFU peak heights allowed us to achieve the true results more rapidly.

The results obtained from the Real-time PCR were directly proportional to the microscopic identification results of *Nosema* species. Samples identified as *N. ceranae* and *N. apis* during microscopic examination were also found to be identical with the results obtained from Real-time PCR. These results showed us that microscopic detection of *Nosema* species also provided reliable results in addition to molecular detection studies. However, studies only with light microscopy are not adequate for *Nosema* spore differentiation.

Many *Nosema* species are known to infect many species of invertebrates. There were discussions on each *Nosema* species being host-specific in various studies, but it has been shown in experiments that a *Nosema* species isolated from some Tribolium species infected many species including *G. mellonella* (Fisher & Sanborn, 1962). *N. ceranae* originally found on *Apis ceranae* is now the parasite in *A. mellifera* and replacing *N. apis* which is known to infect honey bees for a long time. This situation shows that there is host-switching property in *Nosema* species and they can be transferred to the *G. mellonella*.

According to these results, *Nosema* species can be found in honey bee pests which may play a role in their transmission among honeybee colonies in addition to other transmission routes. These parasitic species may transfer from one honey bee hive to another with adult wax moths increasing the spread of parasitic *Nosema* species in honey bees. Furthermore, it is believed that *Nosema spp.* live a long time in *G. mellonella* without showing any symptoms because there was no change in the life quality of *G. mellonella*. The presence of *Nosema* species in wax moths grown in laboratory conditions suggests to us that these organisms can be found in the normal microflora of wax moths. This situation will become clear after further research.

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