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Molecular detection of *Nosema* spp. and black queen-cell virus in honeybees in Van Province, Turkey

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Abstract: This study was planned to determine the prevalences of the *Nosema* spp. and the black queen-cell virus (BQCV) among honeybees (*Apis mellifera*) raised in the province of Van by PCR and to determine the molecular characteristics of the determined isolates. A total of 260 adult worker bees from 26 colonies at 5 apiary locations belonging to the province of Van in April and May 2015 were collected for this reason. Samples were examined microscopically. In the case of positivity, spore identification was done by multiplex PCR. Reverse transcription/PCR analysis (RT/PCR) was carried out for the BQCV analysis. At the end of the microscopic examination, *Nosema* spp. spores were detected in 8 out of 26 colonies (32.5%). The result of multiplex-PCR revealed *Nosema ceranae* positivity in all of the samples, but no *Nosema apis* was determined. As a result of the RT/PCR tests of the samples BQCV was detected in 23 (88.5%) of the total 26 colonies. This study is the first to investigate *Nosema* spp. and BQCV with the PCR technique in bees raised in the province of Van.

Key words: *Nosema ceranae*, honeybee disease, black queen-cell virus, Turkey

1. Introduction

Beekeeping has become an industry that has demonstrated progress in the recent years in Turkey, as in the rest of the world. Every year, there is an increase in the number of beekeepers, the number of hives, and the honey production per hive (1,2). Turkey is an important region for beekeeping in terms of its surface area, topographic character, climate, and vegetation. Compared to developed countries, the honey yield per hive in Turkey is at low levels. This situation suggests that proper colony management and the struggle with diseases are insufficient in beekeeping (1,2). In bees, many diseases are observed caused by bacteria, viruses, parasites, and fungi. One of the bee parasites, *Nosema*, is an extremely important and common honeybee disease caused by microsporidia *Nosema apis* and *Nosema ceranae*, which are quite effective in adult honeybees (3,4). Although the life cycles of these species are similar, in the studies carried out, *N. ceranae* has been reported to be more pathogenic, or in other words to have more damaging features (3,5).

The disease is transmitted via the fecal-oral route in honeybee colonies (6). The adult bees contract the disease through water and food contaminated with spores, or when moving away from contaminated feces while cleaning the hives. In infected colonies, in particular, digestive system disorders, shortening of the life span, flying failure, dull white and pale coloring of the intestines, collection of dead bees in the hive entrance, reduced colony population and honey production, and even collapse of the colony may be observed (6,7).

The microscopic technique has been used for years in the diagnosis of *Nosema* disease. However, due to the great similarity between the *Nosema apis* and *Nosema ceranae* spores, it is very difficult to distinguish between these two species under the microscope. Molecular techniques for diagnosis from the parasite's DNA have begun to be used widely for this purpose (8,9).

The bee viruses that were described in the early 20th century have become one of the biggest threats to the health of honeybees. Since then, at least 18 viruses have been reported to infect honeybees worldwide (10). Among these, black queen-cell virus (BQCV) has been reported to cause significant losses in bees. The virus is transmitted to the bees by food and reproduces in the cytoplasm of the intestinal epithelial cells. It infects developing queen bee larvae and their pupae. Furthermore, it has been

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determined that BQCV can be seen in adult bees at higher rates than in pupae (11). The epidemiology of BQCV is generally associated with *Nosema* infection, which is a protozoan disease of bees (12,13). BQCV density has been reported to increase in spring and summer, at which times *Nosema* spp. infection is intense. In addition, the virus reproduces faster in adults that are infected with *Nosema* spp. (10). Recently, especially in international studies (14,15), the bee viruses have been reported to play a role in the majority of colony collapse events. In studies performed, the possibility of simultaneous infection of viral and parasitic pathogens of honeybee colonies in which collapse has been observed has been found to be higher (14–17). This study aimed to investigate the *Nosema* species (*N. apis* and *N. ceranae*) and BQCV positivity and their prevalences using the PCR technique in honeybees collected from the province of Van.

2. Materials and methods

2.1. Sample collection

This study was conducted with 260 adult worker bees from 26 colonies collected from 5 apiary locations belonging to the center of the province of Van in April and May 2015. The bee colonies provided from the fieldwork samples were determined to be distant from each other and dead bee samples were provided at specified times, which were determined with the colony owners during the study. At least 10 adult worker bees from each colony were collected. The main clinical feature in all the colonies was the presence of signs of diarrhea and bee death. All the bee samples were stored at –20 °C prior to examination.

2.2. Spore detection by microscopic investigation and DNA extraction

The bees obtained by sampling were dissected without delay and examined under a light microscope. Five apiary samples that represent 26 colonies were examined. The abdomens of 10 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 the light microscope. In the case of positivity, 1 mL of suspension was filtered and centrifuged for 5 min at 8000 rpm and supernatants were removed. Spores were kept below –20 °C until nucleic acid extraction (18). After removal of the supernatant, DNA extraction was performed from the sediment at the bottom using a tissue kit (Thermo, GeneJET Genomic DNA Purification Kit). The extracts were kept below –20 °C until the PCR.

2.3. PCR amplification

The specific primer pairs of the 16S rRNA gene of *N. ceranae* and *N. apis* were used in PCR, which were designed for multiplex PCR by Martín-Hernández et al. (18), and the characteristics of the primers are presented in Table 1. PCR was carried out in a final volume of 50 µL, containing 29.25 µL of DNase- and RNase-free sterile distilled water (BioBasic, Inc.), 5 µL of 10X PCR buffer, 5 µL of 25 mM $MgCl_2$, 6 µL of 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 Taq DNA 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, and 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 (3).

2.4. Virus extraction

Every 10 bees collected from the same colony were accepted as one sample. The wings and the heads of the bees that were collected in a sterile tube were plucked. The remaining chests and abdomens were homogenized with 10 mL of phosphate-buffered saline at 4 °C. The homogenized preparation was centrifuged at 4500 rpm for 15 min, and the obtained supernatant was used for viral RNA extraction. RNA extraction was performed using a viral nucleic acid isolation kit (Thermo Scientific GeneJET Viral DNA and RNA Purification Kit).

Reverse-transcriptase PCR (RT-PCR) assays were performed using a cDNA synthesis kit (Thermo Scientific RevertAid First Strand K1622, USA). Initially the content of mixture 1 (containing 3 µL of sterile distilled water, 0.5 µL of random hexamer primer (0.2 µg/ µL), and 3 µL of RNA) was prepared. The tubes were then placed into a thermal cycler and incubated for 5 min at 70 °C. The tubes were immediately placed on an ice box for cooling. In the second step, mixture 2 was prepared (containing 2.0 µL of

Table 1. Sequences of the primers used in multiplex PCR for detecting *Nosema* spp.

Primers	Primer sequence (5' - 3')	Amplification target	Length of amplicon
218MITOC 218MITOC	CGGCGACGATGTGATATGAAAATATTAA CCCGGTCATTCTCAAACAAAAAACCG	16S rRNA (*N. ceranae*)	218–219
321APIS 321APIS	GGGGGCATGTCTTTGACGTACTATGTA GGGGGGCGTTTAAAATGTGAAACAACTATG	16S rRNA (*N. apis*)	321

5X reaction buffer, 1.0 µL of 10 mM dNTP mix, and 0.5 µL of M-MuLV reverse transcriptase) and was distributed in amounts of 3.5 µL for each tube. These tubes were incubated for 45 min at 48 °C in a thermal cycler (19).

Finally the PCR mix was prepared with a total volume of 30 µL that contained 3 µL of cDNA, 3 µL of Taq buffer (10X,750 mM Tris-HCl, 200 mM $(NH_4)_2SO4$, 0.1% (v/v) Tween 20), 2 µL of $MgCl_2$ (25 mM), 1 µL of each primer (10 mM), 1 µL of the solution of four deoxynucleoside triphosphates (10 mM), 0.25 µL of Taq DNA polymerase (5 U/µL), and 18.75 µL of molecular biology-grade water (MBI Fermentas, Lithuania).

For general BQCV detection, universal primers (P1, 5'–3' TGG TCA GCT CCC ACT ACC TTA AAC; and P2, 5'–3' GCA ACA AGA AGA AAC GTA AAC CAC) were used according to Benjeddou et al. (19) to amplify specifically a 700-bp product.

The PCR reactions were performed using a thermal cycler (Prime, Techne, Bibby Scientific Ltd., UK) and using an initial denaturation at 95 °C for 2 min followed by 40 cycles consisting of denaturation (95 °C for 30 s), annealing (55 °C for 60 s), and elongation (68 °C for 120 s) as action conditions and a final extension step at 68 °C for 7 min. The PCR products were analyzed by 1.5 % agarose gel electrophoresis.

2.5. DNA sequence analysis

After purification of the samples selected from the obtained positive products with a commercial purification kit (High Pure PCR Cleanup Micro Kit, Roche, Germany), prior to sequence analysis, they were subjected to capillary electrophoretic separation in a specialized laboratory (Refgen, Ankara, Turkey) and sequence analyses of the products were performed. The sequence results of the samples were obtained through examination of the sequencing results with the BioEdit sequence alignment program. The BLAST analyses of the obtained sequences were performed on the Internet in GenBank, and the percentage of their similarity to the same strains around the world was identified. The samples were recorded in GenBank-NCBI and accession numbers were obtained (KU521775, KU521776, KU521777, and KU521778).

3. Results

In this study, a total of 260 adult worker bees from 26 colonies in 5 apiary locations (Erek, Kasımoğlu, Özalp Yolu, Bostaniçi, Kale Yolu) that belonged to the center of the province of Van were investigated. In the dissection study, spores that belonged to *Nosema* spp. were found in the intestines and in the body cavity (Figure 1). *Nosema* spp. spores were detected in 8 out of 26 colonies (32.5%). All of the bees that were found to be positive by microscopic examination were also found to be positive by the PCR technique. Following agarose gel electrophoresis

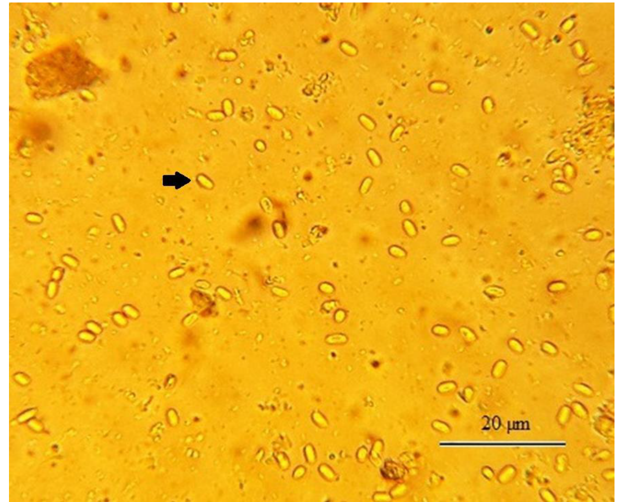

Figure 1. The presence of a *Nosema* spore (macerated abdomen suspension of adult honeybees).

and staining of the obtained products with ethidium bromide, 8 amplicons exhibited a length of 218–219 bp, which corresponds to *Nosema ceranae* (Figure 2). No coinfections (mixed *N. apis*/*N. ceranae*) were found. The nucleotide sequences of amplification products from the *Nosema*-infected honeybee samples were 99% identical to the *N. ceranae* sequence deposited in the GenBank database. The detected sequence analysis data belonging to *N. ceranae* are listed in Table 2.

The results of RT-PCR tests of the *Nosema*-positive colonies demonstrated BQCV positivity in 23 (88.5%) of 26 colonies. As a result of the gel electrophoresis of the obtained PCR products, specific bands were detected at a molecular weight of 700 bp (Figure 3). Sequence analysis of 4 BQCV-positive samples was performed and the results were evaluated and recorded in GenBank (KU521775, KU521776, KU521777, and KU521778).

4. Discussion

Nosemosis is an extremely important and common honeybee disease caused by *Nosema apis* and *Nosema ceranae* (3,4). As in the rest of the world, *Nosema* disease has a wide range of distribution in Turkey, and it is speculated to cause major losses from time to time. The diagnosis of the disease is made by traditional microscopic methods and some molecular techniques (20,21). In previous studies from Turkey, the prevalence of *Nosema* disease was found to be between 4% and 54.16% by microscopic techniques (1,22). However, the spores of these species are very similar to each other morphologically, and hence such distinctions between them are quite difficult with traditional microscopic methods. The diagnosis of the disease is made by traditional microscopic methods and

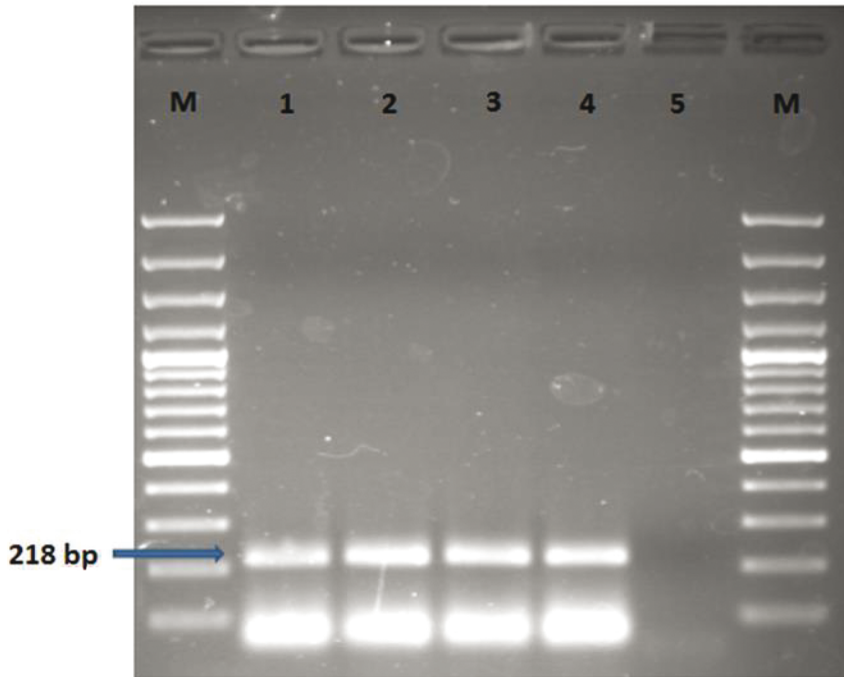

Figure 2. PCR analysis of extracted DNA from *Nosema* spore using primers (218MITOC) specific for *Nosema ceranae* derived from 16 S rRNA of the protozoa (lanes 1–4) and the negative control (lane 5).

Table 2. Sequence of *N. ceranae* detected in Van Province.

Sequencing results
GTATTACaTAaTTGAATTTGAGTTTTTTGGCTCTGGGGATAGTATGATCA
GCAAGATTGAAAATTAAAGAAATTGACGGAAGAATACCACAAGGAGTG
GATTGTGCGGCTTAATTTGACTCAACGCGAGGTAACTTACCAATATTTT
TTATTTTGAGAGAACGGTTTTTTGTttgAGAATGACCGGGA

some molecular techniques (20–22). Therefore, molecular biological techniques such as PCR, multiplex-PCR, DNA sequencing, and PCR-RFLP are used today in the diagnosis of infections or mixed infections caused by the two pathogens (3).

Having used multiplex-PCR diagnosis for *Nosema* spores and different primer pairs targeting the rRNA gene for discrimination of the species, Michalczyk et al. (23) reported that *Nosema ceranae* was the dominant species in European honeybees. Traver and Fell (24) studied 586 managed *A. mellifera* colonies from Virginia in 2009 using real-time PCR to detect *Nosema apis* and *Nosema cerenea*. Nabian et al. (25) determined *N. ceranae* for the first time in European honeybee colonies in Iran by sequencing the 16S rRNA gene. In Hungary, differentiation between *N. ceranae* and *N. apis* spores by PCR analysis reported a low incidence of *N. apis* in Hungarian apiaries (26). Webster et al. (27) noted that PCR analysis was more sensitive than examination for spores by light microscopy in detecting *N. apis* infection. Different researchers who used the same technique determined *N. ceranae* for the first time in European honeybee colonies in Canada and the central USA (28), France (20), Spain (29), Jordan (30), and the United States (31). On the other hand, Klee et al. (21) and Szalanski et al. (32) reported that the PCR-RFLP method was quite fast and reliable in diagnosing the species and discriminating the pathogen species.

Along with extensive studies on the determination of the species of *Nosema* spores at the molecular level in the world (23,32,33), the number of studies related to the agent for the disease seems to be high in Turkey; however, these studies have been limited to the general presence

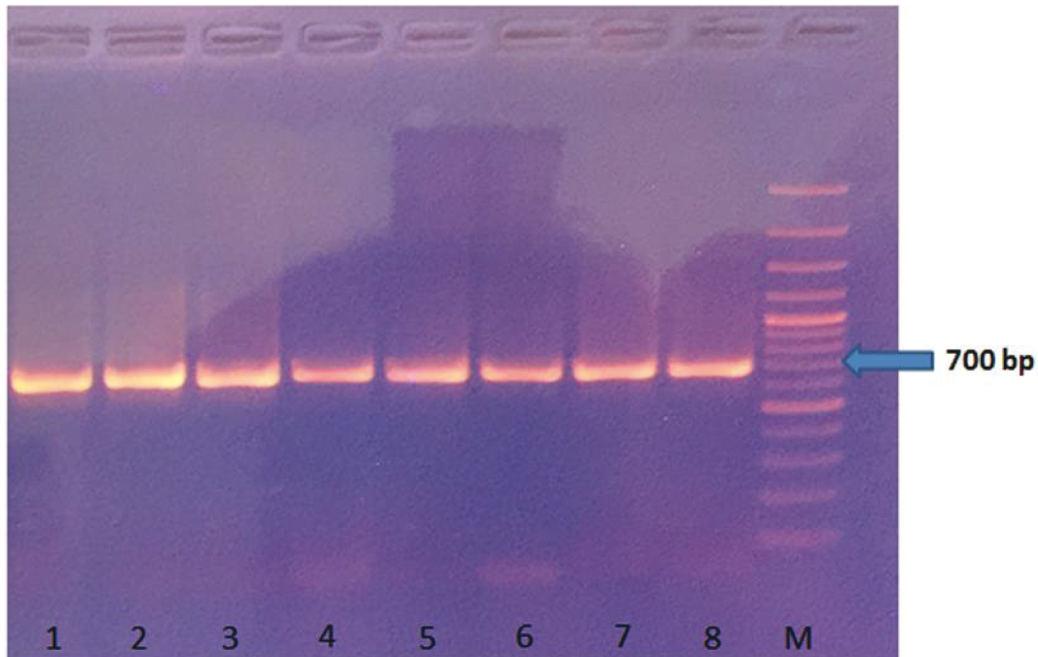

Figure 3. RT-PCR from a 700-bp section of black queen-cell virus RNA. M: Molecular weight marker (MBI Fermentas Vilnius, Lithuania). Lanes 1–8: positive samples.

of the disease in content. In the last few years, however, determination of the disease at species level and species-specific molecular biological studies have been carried out (3,4,34,35). Ütük et al. (3) detected *N. ceranae* for the first time in two bee samples that were sent to their laboratory from the Samsun and Giresun provinces of Turkey by using multiplex PCR. In the same year, in the samples they studied by PCR technique in the Eastern Black Sea Region, Whitaker et al. (4) detected *N. ceranae* and *N. apis*. Similarly, in the samples collected from Hatay and the South Marmara regions, Muz et al. (34) determined *Nosema* by detecting spores and their species at the molecular level. As a result of PCR performed with specific primers that replicated the 16S rRNA gene region, Tosun (35) reported only the presence of *N. ceranae* in the province of Ordu. Utuk et al. (36) reported *Nosema ceranae* as the dominant species in Turkey using the PCR method. In this study, while *Nosema ceranae* was detected in adult honeybees as a result of PCR in 5 apiaries in the province of Van, no *N. apis* or mixed *N. apis/N. ceranae* infections were detected in the samples. Our results show that *N. ceranae* is the only *Nosema* species found to infect honeybees in the geographic territory of Van. The prevalence of the disease was identified to be 32.5%, and the species was confirmed by sequence analysis. In addition, this study is the first microscopic and molecular examination for detection of *Nosema* spp. in the province of Van. The benefits of molecular methods include increased sensitivity, specificity, and, perhaps more significantly, ability to identify all developmental stages of *Nosema* sp. (18).

BQCV is a common virus that affects honeybees; however, its implication in honeybee mortality is currently poorly understood (37). The virus was detected in 86% of adult samples and 23% of pupae in a survey of healthy French bee colonies (11), and recently in Austrian apiaries (13). Limited data are available regarding the distribution of BQCV in Turkey. In studies determining the presence of the infection in Turkey, the rate was determined as 21.42% in colonies in the Black Sea region and 32.2% in colonies in the province of Hakkari (38,39). In the current study, the presence of BQCV was determined as 88.5%. This determined prevalence rate has attracted attention, as it is higher than that of the Black Sea region and the province of Hakkari.

In studies conducted in previous years, a positive correlation was reported between BQCV epidemiology and *Nosema* parasites (6,11). In a study by Berényi et al. (13), which was conducted in Austria, *Nosema apis* infection was detected as 78%, while in the same bees, the density of BQCV was determined as 75%. Similarly, in cases in which the viruses and parasites are seen together, the bee mortality has been reported to increase (12,40,41). In the present study, *N. ceranae* and BQCV infections were determined to have been seen simultaneously in the bee colonies. Concurrent detection of BQCV and *Nosema* parasites is a common finding obtained by different researchers (14,40,41). In contrast with the above-

mentioned articles, in different studies conducted in Spain and France, no clinical signs were detected in *N. apis* infections, which have a high number of spores and it is not seen simultaneously with BQCV (22). Again, in cases in which BQCV and *N. apis* were seen simultaneously, there is no precise information on the presence of clinical signs. This situation seems to support other studies that indicated that *N. apis* is less pathogenic compared to *N. ceranae* (8,36).

As a result of this study, the presence of *Nosema ceranae* and BQCV species and their spread were determined for the first time in honeybees in the province of Van using the PCR method. The possibility that a coinfection between *Nosema* spp. and BQCV could weigh down the clinical course should be taken into account. In the future, it will be necessary to establish the relationships between all the factors implicated in bee mortality.

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