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Molecular detection of *Nosema ceranae* in the apiaries of Kurdistan province, Iran

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Abstract

Nosema disease is one of the most important diseases of adult honey bees worldwide. It is known as silent killer because there are no characteristic symptoms. The aim of the present study was to determine prevalence of *Nosema* species in various towns of Kurdistan province in Iran. A multiplex polymerase chain reaction (multiplex-PCR) was performed for identification of *Nosema* species infecting European honeybee, *Apis mellifera*. A total of 100 samples were collected from apiaries (870 hives) in 10 counties of Kurdistan province, located in the west of Iran. Samples were examined using light microscope and PCR. The light microscope was used to determine the presence of *Nosema* spores in all of the collected samples. Multiplex-PCR based on 16S ribosomal RNA was used to differentiate *N. apis* from *N. ceranae*. Overall prevalence of the microscopic evaluation and PCR method were 29.00% and 32.00%, respectively. The analysis of Nosema isolates from interrogation of DNA databank entries of Kurdistan apiaries (based on rRNA sequence data) indicated that only *N. ceranae* was widespread in these apiaries, and it had already been found in high percentages (50.00%) in Marivan and Kamiaran counties of Kurdistan province. It was shown that only *N. ceranae* was found by PCR assay in the region.

شناسایی مولکولی گونه نوزما سرانه در زنبورستان های استان کردستان، ایران

چکیده

بیماری نوزما از مهم‌ترین بیماری‌های زنبوران بالغ است که اکثر اوقات در زنبورستان ها بدون هیچ‌گونه علائم کلینیکی وجود دارد ازاین‌رو بیماری نوزما به‌عنوان "قاتل خاموش" نامیده می‌شود. هدف از انجام این تحقیق بررسی مولکولی عامل بیماری نوزما در زنبورستان های استان کردستان بود. واکنش زنجیره چندتایی پلیمراز برای شناسایی گونه های نوزما مسبب بیماری در زنبورعسل اروپایی (*آ پیس ملیفرا*) مورد استفاده قرار گرفت. برای انجام این تحقیق تعداد ۱۰۰ زنبورستان (۸۷۰ کندو) در ۱۰ شهرستان استان بر اساس دستورالعمل سازمان دامپزشکی مورد نمونه‌برداری قرار گرفت. نمونه ها با استفاده از میکروسکوپ نوری و PCR مورد بررسی قرار گرفتند. برای تعیین وجود اسپور نوزما ، تمامی نمونه ها با میکرسکوپ نوری مورد بررسی قرار گرفت. روش PCR چندتایی بر اساس ریبوزوم S16 RNA برای تمایز *نوزما آ پیس* و *نوزما سرنا* استفاده شد. شیوع کلی بیماری به روش میکرسکوپی و PCR به ترتیب ۲۹/۰۰ و ۳۲/۰۰ درصد بود. بررسی سویه های نوزمای جدا شده از زنبورستان های استان کردستان بر اساس داده های موجود در بانک اطلاعاتی (بر اساس داد های توالی rRNA) نشان داد که تنها گونه *نوزما سرنا* شیوع گسترده ای در زنبورستان های استان دارد، بطوری که زنبورستان های شهرستان های مریوان و کامیاران دارای شیوع بالایی (۵۰/۰۰ درصد) از این بیماری در استان بودند. تحقیق حاضر نشان داد با روش PCR فقط گونه *نوزما سرنا* در زنبورستان-های استان کردستان شناسایی شد.

واژه های کلیدی: ایران، شناسایی مولکولی، کردستان، نوزما سرانه، نوزموزیس

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Introduction

Nosemosis (Nosema disease) is one of the most serious and prevalent adult honeybee diseases worldwide,[1,2] which it caused by intracellular microsporidian parasites from a genus of *Nosema*. For decades, Nosema disease was particularly ascribed to a single species of *Nosema*, *Nosema apis (N. apis)*, which was first identified in European honeybee, *Apis mellifera*.[3] In 1996, another species of *Nosema* was first recognized in the Asian honey bee, *Apis ceranae*, thus named *Nosema ceranae (N. cerana)*.[4] In 2005, a natural infection of *N. ceranae* was reported in *Apis mellifera* colonies from Taiwan[5] in a short period of time. Infection of *Apis mellifera* with *N. ceranae* was reported in Europe,[6,7] the United States,[8] China,[9] Vietnam and worldwide.[10] *Nosema apis* infection causes a fast acting and short duration syndrome; however, this has not been the case for *N. ceranae,* in which it has been noticed in relation to nonspecific symptoms such as gradual population decline, higher autumn/winter colony deaths or low honey production.[11] Further, it has recently been shown that *N. ceranae* does not show the seasonality that is seen with *N. apis*.[12] The different symptoms presented by these *Nosema* species in honey bees highlight the need to observe two different clinical types: Nosemosis type A caused by *N. apis* and Nosemosis type C caused by *N. ceranae*.[12] Detection of *Nosema* species depends upon microscopic observation, molecular methods or transmission electron microscopy.[13] The spores of the two *Nosema* species are very similar, and microscopic examination cannot differentiate between *N. apis* and *N. ceranae*.[11] Several PCR protocols have been described, including PCR with specific primers,[8] PCR-RFLP,[10,14] Real-time PCR,[15,16] or multiplex PCR.[17] The aim of the present study was to determine prevalence of *Nosema ceranae* in various towns of Kurdistan province in Iran.

Materials and Methods

Study area. Kurdistan is located in the west of Iran with an area of 28,000 Km^2. This province lies between the eastern longitude 45° 33′ 11″ and 51° 13′ 7″ and northern longitude 34° 24′ 16″ and 37° 52′ 12″. The weather conditions are similar to the Mediterranean area in which rainfall occurs in winter, moderate rain occurs in autumn and spring and no rainfall occurs during the summer season. With respect to the climate, the region is defined as having cold winters, hot summers, neutral springs and autumns with a wide range of temperature changes. According to the latest divisions of the country, this province has 10 counties.

Samples collection. A total of 100 apiaries were randomly sampled from November 2014 to September 2015 in 10 counties of Kurdistan province. The apiaries were selected randomly and according to the instructions of the Iranian Veterinary Organization (IVO). The required samples were taken from 5.00% of the colonies in each apiary[18] and the samples were immediately transferred to the honey bee research department of Razi Vaccine and Serum Research Institute, Karaj, Iran.

Preparation of samples for microscopic examination. The abdomens of 20 adult dead honey bees were macerated in 10 mL distilled water and were crushed in a mortar. Then, the suspension was passed through a 100 µm mesh sieve to remove the debris and was centrifuged for 6 min at 800 g. Finally, the supernatant was discarded and the pellet was examined under the common light microscope at 400× magnification. This methodology was used to determine the presence of *Nosema* spores in all of the collected samples.[13]

Preparation of samples for PCR. The abdomens of 20 adult dead honey bees from each apiary were macerated in 10 mL distilled water (PCR grade), and the suspension was then filtered and centrifuged at 800 *g* for 6 min. Spore germination was induced with 200 µL freshly prepared germination buffer (0.50 M sodium chloride, 0.50 M sodium hydrogen carbonate, pH to 6.0 with ortho-phosphoric acid), and the mixture was incubated at 37 ˚C for 15 min.[13, 17]

DNA extraction. DNA was extracted using DNA extraction kit (Takapozist, Tehran, Iran) according to the manufacturer's instructions.

Polymerase chain reaction. PCR amplification of 16S rRNA was performed using PCR kit (Sinaclon, Tehran, Iran) in an Eppendorf Mastercycler® gradient thermal cycler (Eppendorf, Hamburg, Germany) according to OIE terrestrial manual 2008 for *N. ceranae*, *N. apis* and *N. bombi*. For multiplex PCR amplification of partial 16S rRNA (= SSU rRNA) gene fragments, first 50 µL reaction mixture contains 5 ng genomic DNA, 3 mM $MgCl_2$, 200 µM of each deoxyribonucleotide triphosphate, 100 ng of primers, 5 µL of 10X PCR buffer (100 mM Tris/HCl, pH 8.3; 15 mM $MgCl_2$; 500 mM KCl) and 1 U of Taq polymerase. Conditions of amplification consist of an initial denaturation cycle at 94 ˚C for 15 sec followed by 25 cycles of denaturation (94 ˚C, 15 sec), primer annealing (61.80 ˚C, 30 sec), primer extension (72.00 ˚C, 45 sec) followed by additional extension step of 7 minutes at 72.00 ˚C.[19] The PCR products were separated by electrophoresis on 1.00% agarose gel, stained with safe stain (Baiometra, Berlin, Germany) and visualized by UV transillumination. In this study, primers targeting small subunit 16S rRNA gene of *N. apis*, *N. ceranae* and *N. bombi* were used (Table 1). Positive controls for *N. ceranae* and *N. apis* were prepared from Department of Honeybee-Silkworm and Wildlife Diseases, Razi Vaccine and Serum Research Institute, Karaj, Iran. The PCR products were sent for both forward and reverse sequencing using Sanger method (Bioneer, Daejeon, South Korea), and revealed sequences were verified by Bioedit software (version 7.0.5; Ibis Therapeutics,Carlsbad, USA).[20]

Table 1. Primers used for identification of *Nosema* species by multiplex PCR

Species	Primer sequence (5`-3`)	Fragment size	References
N. ceranae	5'-CGGCGACGATGTGATATGAAAATATTAA-3' 5'-CCCGGTCATTCTCAAACAAAAAACCG-3'	218-219	17
N. apis	5'-GGGGGCATGTCTTTGACGTACTATGTA-3' 5'-GGGGGGCGTTTAAAATGTGAAACAACTATG-3'	321	17
N. bombi	5'-TTTATTTTATGTRYACMGCAG-3` 5`-GACTTAGTAGCCGTCTCTC-3`	171	19

Results

The results of microscopic examination and PCR of all 100 samples are presented in Table 2. Microscopic examination showed 29.00% samples of apiaries were infected by *Nosema* spores (Fig. 1).

Table 2. Distribution of *erm* genes in isolates

Counties	Apiary numbers	Microscopic positive (%)	PCR positive (%)
Bane	10	10	10
Bijar	10	30	30
Dehgolan	10	30	30
Divandarreh	10	20	20
Kamyaran	10	50	60
Marivan	10	50	60
Qorveh	10	40	40
Sanandaj	10	40	50
Saqqez	10	20	20
Sarvabad	10	0	0
Total (%)	100	29	32

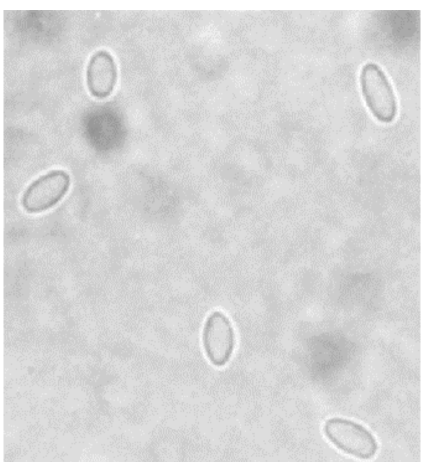

Fig. 1. Spores of *Nosema* spices in light microscopy (400×).

In this study, PCR results showed that 32 samples (32.00%) were positive for *N. ceranae,* which showed a specific amplicon at 218bp (Fig. 2).

In all of the positive samples, the presence of *N. ceranae* was detected. The analysis of *Nosema* isolates from interrogation of DNA databank entries of Kurdistan apiaries (based on rRNA sequence data) indicated that only *N. ceranae* was widespread in these apiaries, and it had already been found in high percentages (50%) in Marivan and Kamiaran counties of Kurdistan province.

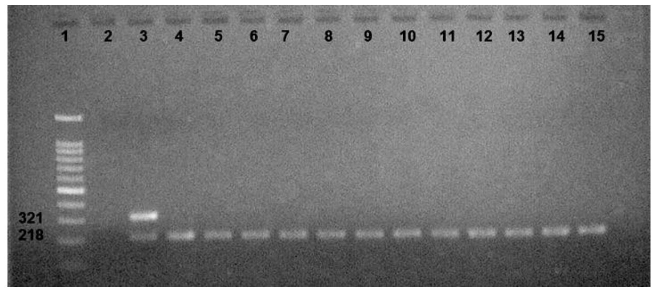

Fig 2. Lane 1: Ladder 100 bp (Smobio, Hsinchu, Taiwan), Lane 2: Negative control, Lane 3: Positive control, Lanes 4 -15: *N. ceranae*.

Discussion

There is a little information about the epidemiological factors and clinical symptoms of this disease in different areas in Iran which have distinct beekeeping management and climatic conditions. The present study showed that *N. ceranae* was the only *Nosema* species found to infect honey bees from apiaries in Kurdistan province in the west of Iran. Lotfi *et al.* reported *N. apis* is widely distributed in East Azerbaijan province in northwest of Iran, being diagnosed in 85.00% of apiaries.[21] Modirrousta *et al.* by multiplex PCR assay showed the samples were collected from five provinces of Iran (Alborz, East-Azerbaijan, Qazvin, Gilan and Tehran) in 2004-2013, were positive for *N. ceranae*.[22] In Mazandaran province, north of Iran, only *N. ceranae* was found by PCR assay.[23] Tavassoli *et al.* reported apiaries of Urmia, northwest of Iran, infected by *N. apis*.[24] The prevalence of *N. ceranae* in East-Azerbaijan by microscopic and PCR methods was 58.10% and 67.10%, respectively.[25] Aroee *et al.* reported that honey bee colony of Esfahan, Chaharmahal and Bakhtiari, and Fars was infected with *N. ceranae*.[26] In Iran, there are reports showing that the frequency of *Nosema* infection has increased.[25,27] During the last 10 years, an increase in infections by microsporidian parasite in honey bee (*A. mellifera*) has been detected in several European countries.[28] Both in the North American continent[29] and in Europe,[17] the proportion of *N. ceranae* infections appears to dominate in warmer climates compared to more temperate regions, whereas *N. apis* may be presently more prevalent in cooler climates.[1,7] Two types of *Nosema* that cause this disease are different from each other in terms of pathogenicity, symptoms and epidemiology, by which it is possible to diagnose the disease. Differences have been documented in Europe with regard to the epidemiological pattern of *Nosema* caused by *N. ceranae,*[30] thus this disease has a prolonged incubation period without obvious clinical symptoms, which can lead to the death of colonies.[6] The symptoms of *N. ceranae* include longer breeding period in the cold months, disproportion between the nurse bees and the larvae population in the hive in the warm months of the year, reduced honey production in the hive, wakened hive, reduced population of adult bees and destruction of hives over 1.5 to 2 years.[31] There are two mechanisms involved in the loss of such contaminated hives: first, in the cold season more than 50.00% of the hive population is lost at one side of the hive, the mean number of spores per bee is over 10 million, and contamination is evident if the queen is found; second, the hive death occurs in early spring, the mean number of spores per bee is less, and the queen is not contaminated with disease. Further, in these two mechanisms the ratio of old/young bees is different in various seasons. In early spring, the ratio of contaminated/non-contaminated young bees is reduced, thereby postponing the queen contamination.[12] A disease occurs due to a complex relationship between the triple sides of epidemiology, including disease cause, host, and environmental conditions. In domestic animals, the host is largely influenced by the maintenance and management conditions.[32] The host is one of the triple sides involved in the occurrence of every disease. The hosting differences lead to differences in severity of the disease; for example, laboratory research using bees with different ages has been reported to yield different results indicating the effect of factors associated with the host on the results obtained from the research.[33] Environmental conditions such as altitude effects, type of plants in the region, and management of apiary highly affect the parasitic relationships, as *N. ceranae* has been reported in Spain under the influence of temperature on the spread of *N. ceranae*. Currently, contamination of honey bee colonies with different species of Nosema around the world does not follow a different weather pattern.[34] The studies conducted in regions where both types of *Nosema* have been prevalent have shown that *N. ceranae* is more prevalent in warmer areas, areas with Mediterranean climate, and prevalence of *N. apis* is higher in regions with moderate climate. This subject should be considered while the migratory bees enter such regions.[1] Environmental factors have dramatically affected the competition between *N. apis* and *N. ceranae*, as in many areas around the world *N. ceranae* is the only species identified in honey bees.[8-10,22,35] However, in some areas around the world, due to special weather conditions, *N. apis* is more prevalent.[36,37] Recent studies at different levels (i.e. individual, colony, apiary, country, different races of honey bee) as well as analysis of the reasons involved in the prevalence of *Nosema* species around the world have indicated that no substitute has been found for *N. apis* and *N. ceranae* worldwide.[34] Only *N. ceranae* is the dominant species during the year, and prevalence of *N. apis* depends on a specific epidemiological pattern (occurrence in spring and fall or more generally in colder seasons). This theory has been also proposed in the past,[38,39] and recent studies in the U.S. have confirmed it.[40] Many studies have been carried out on Nosema and its prevalence in spring, when the disease is severe.[41,42] This is exactly when only *N. apis* is found as the cause of Nosemosis in the hives, an agent that is not active in tropical and subtropical areas;[42] whereas, *N. ceranae* can be diagnosed all year in different geographical latitudes in colonies.[17,34] There are a few studies on *Nosema* species in Iran, some of which have used microscopic observation technique. Given the many similarities of the spores of these two species, their differential diagnosis by microscopic observation is very difficult and at times impossible.[11] Hence, molecular techniques must be used in this regard. Analysis of studies published in Iran demonstrates that studies that have used molecular techniques to investigate the causative agent of *Nosema* have reported microsporidian *N. ceranae* as the causative agent of this disease in Iran. On the other hand, studies using microscopic observation technique have introduced *N. apis* as the cause of this disease, and no report has ever been presented regarding the molecular diagnosis of *N. apis* in Iranian apiaries. Also, considering numerous reports from around the world about the replacement of *N. ceranae* with *N. apis* and changes in epidemiologic symptoms of the disease,[6,7,10,17] *N. ceranae* is probably the cause of *Nosema* infection in Iran; however, further studies are required to confirm this issue.

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Conflict of interest

The authors have no conflict of interest.

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