Iranian J Parasitol: Vol. 6, No.3, 2011, pp.89 -95

Tehran University of Medical Sciences Publication
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Iranian J Parasitol

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Iranian Society of Parasitology
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Short Communication

First Detection of *Nosema ceranae*, a Microsporidian Protozoa of European Honeybees (*Apis mellifera*) In Iran

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(Received 17 Feb 2011; accepted 21 July 2011)

ABSTRACT

Background: Nosemosis of European honey bee (Apis *mellifera*) is present in bee colonies worldwide. Until recently, *Nosema apis* had been regarded as the causative agent of the disease, that causes heavy economic losses in apicultures. *Nosema ceranae* is an emerging microsporidian parasite of European honeybees, *A. mellifera*, but its distribution is not well known. Previously, nosemosis in honeybees in Iran was attributed exclusively to *N. apis*.
Methods: Six *Nosema* positive samples (determined from light microscopy of spores) of adult worker bees from one province of Iran (Savadkouh- Mazandaran, northern Iran) were tested to determine *Nosema* species using previously- developed PCR primers of the 16 S rRNA gene. As it is difficult to distinguish *N. ceranae* and *N. apis* morphologically, a PCR assay based on 16 S ribosomal RNA has been used to differentiate *N. apis* and *N. ceranae*.
Results: Only *N. ceranae* was found in all samples, indicating that this species present in Iran apiaries.
Conclusion: This is the first report of *N. ceranae* in colonies of *A. mellifera* in Iran. It seems that intensive surveys are needed to determine the distribution and prevalence of *N. ceranae* in different regions of Iran.
Keywords: Honeybee, *Nosema apis*, *Nosema ceranae*, PCR

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Introduction

For several decades, the only known causal agent of Nosemosis in European honeybees (*A. mellifera*) was the unicellular microsporidium *N. apis* (1).

During the last 10 years, an increase in infections by microsporidian parasites in the honey bee (*A. mellifera*) has been detected in several European countries (2- 4), while increasing numbers of honey bee colony death and low production in the same areas have been reported by beekeepers. In 2006, Higes et al. reported a new microsporidium *N. ceranae*, as the main etiological agent of Nosemosis in Spain (5). Shortly after, the presence of *N. ceranae* was confirmed in Europe, America, and Asia (6-14). *N. ceranae* is highly pathogenic in European honeybees and is associated with reduced honey production and increased winter mortality (5, 15).

The spores of the two *Nosema* species are very similar and can hardly be distinguished by light microscopy, so that in the absence of clear morphological characteristics for species recognition, other techniques using molecular markers may greatly assist in the diagnosis and identification of honeybee microsporidians (5, 16, 17). The PCR technique provides a very sensitive test for detecting microsporidian infection because it enables detection of the parasite at very low levels of infection and can reveal all the stages of its life cycle (18, 19).

During the last 3 years, an increase in infections by microsporidian parasites in the honeybee (A. *mellifera*) has been detected in Iran. In addition, increasing number of honeybee colony death and low production in the same areas has been reported by beekeepers. In this study the aim was to detect of honeybee microsporidians (*N. apis* and *N. ceranae*) using PCR.

Former reports of the distribution of Nosemosis in Iran (20, 21), assumed that only one *Nosema* species, *N. apis*, infected honey bees. The prevalence of Nosemosis in honeybee colonies in Arasbaran region (Northwestern Iran) was studied by Lotfi et al. (2009). They showed the highest-level infection in the spring (20). The presence of the reports based on the incidence of colony depopulation and high prevalence of nosemosis, with different clinical and epidemiological pattern in Iran, noticed us that its cause may potentially be this addition species.

Materials and Methods

Samples

Based on the retrospective study of the prevalence of hive depopulation in Mazandaran Province, Northern Iran, six adult bee samples from Savadkouh of Mazandaran were collected and tested for diagnosis of *Nosema* spp.

Spore detection

The abdomens of 10 adult honeybees from each sample were macerated in 10 ml of distilled water and the suspension was filtered and centrifuged for 6 min at 800 *g*. Pellets were analyzed by phase-contrast microscopy (magnification, _400) to verify the presence of spores(22). This methodology was employed for determination of the presence of *Nosema* spores in all the samples used in this study.

DNA extraction

For DNA extraction, spore germination was induced with 200 µl of freshly prepared germination buffer (23), and preparations were incubated at 37°C for 15min (24). DNA extraction was conducted using a High Pure PCR template preparation kit (catalog no. 1796828; Roche Diagnostic) as previ-

ously described (15). Negative controls including uninfected honeybees were processed in parallel to detect possible contamination.

PCR design methodology

The 16 SrRNA locus was selected to perform *N. apis* and *N. ceranae* PCR. Published sequences in the GenBank database from *N. apis*(accession number U26534, DQ235446) and *N.ceranae* (accession number U26533, DQ078785) were compiled. Specific primers for both *N. apis* and *N. ceranae* were visually selected taking into account that primer sequences were specific to each of the two species. The selected primers were 321 Apis for *N. apis* (FOR5'-GGG GGC ATG TCT TTG ACG TAC TAT GTA-3',
321APIS REV 5'-GGG GGG CGT TTA AAA TGT GAA ACA ACT ATG-3') and 218 MITOC for *N. ceranae* (FOR 5'-CGG CGA CGA TGT GAT ATG AAA ATA TTA A-3')
218MITOC: (REV 5'-CCC GGT CAT TCT CAA ACA AAA AAC CG-3').
In the single- gradient PCR test of both primers, the annealing temperature was 61.8. PCR was performed using a Mastercycler gradient (Eppendorf) in 50µl volumes containing 25 µl of High Fidelity PCR Master Mixture(catalog no.12140314001;Roche Diagnostic), 0.4 µM of each primer, 0.4 mMof each deoxynucleoside triphosphate, 3mMCl_2mg, 0.2mg/ml bovine serum, 0.1%TritonX-100, and 5 µl of *Nosema* template DNA.
The thermocycler program consisted of 94◦C for 15 second followed by 25 cycles of 15s at 94◦C, 30s at 61.8◦C and 45s at 72◦C and a final extension step at 72◦C for 7 min. Negative controls (from DNA extraction) were included in all PCR experiments. Amplified PCR products were electrophoresed through 1.5% agarose TAE gel in standard TAE buffer, stained with ethidium bromide, and visualized using UV illumination. All six samples and controls were checked some times.
The PCR products were purified with a QIA quick PCR purification Kit (catalog no. 28104;QIAGEN) as previously described (5) and fully sequenced in both directions. These sequences were aligned and compared to the *N. ceranae* and *N. apis* consensus using Sequencher;GeneCodes, AnnArbor,MI).Sequence similarity analyses were performed using BLAST database search.

Results

Adult honey bee samples from 3 apiaries in Savadkouh (Mazandaran Province) were collected. All of the analyzed samples were positive for presence of *Nosema* spore (Fig.1). DNA was extracted from spores and amplified with common primer pair derived from 16SrRNA of *Nosema* spores. The amplified DNA fragment revealed that the isolation of DNA from spores was successfully performed (Fig.2, Lane1-6). The 16SrRNA gene has seen successfully applied to identify *Nosema*. Also the application of the mentioned primer pairs (MITOC and APIS) could easily differentiate Two different species (*N. apis* and *N. ceranae*).
PCR and sequencing analyses of both strands using the designed primers (321 APIS and 218MITOC primers) yielded the expected amplicon sizes (218 bp for *N. ceranae*) and sequences. Neither primer interaction nor undesirable amplicons were detected in agarose gels. None of the samples was identical to *N. apis* (U26534, DQ235446). To verify the species diagnosis three PCR products were sequenced and the partial 16SrRNA sequences were submitted to GenBank (accession numbers U26533 and DQ078785). The generated consensus sequences created from both forward and re-

verse sequences were compared with those of related species obtained from Gen Bank. Diagnosis was confirmed with the sequences obtained, which corresponded to *N .ceranae*. All the samples analyzed were positive for *N. ceranae*, clearly showing its current presence in this region.

Blast search against Gen Bank revealed the highest similarity (99.8 or 100%) with *N.ceranae* 16SrRNA partial sequence.

In all of the samples analyzed, the presence of *N. ceranae* was detected (Fig. 2). All six positive and three negative controls, tested 3 times showed the same results which demonstrated the reliability of the method.

The expected PCR products 218bp for *N. ceranae* are observed. Our results showed that *N. ceranae* was detected in all six macerated abdomens of adult honeybees. The presence of *N. ceranae* in Iran was verified.

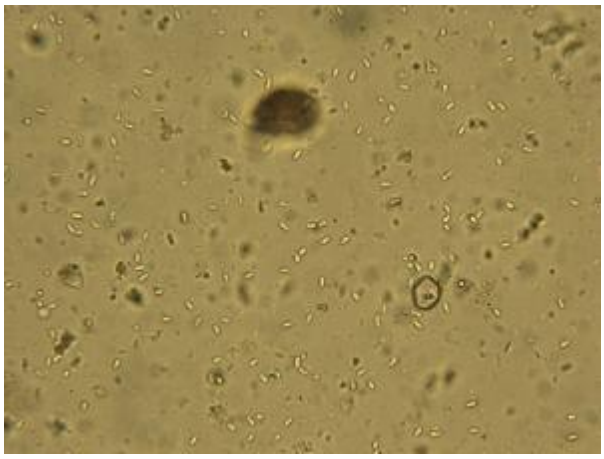

Fig.1: The presence of *Nosema* spore, (wet mount, 400X) in macerated abdomens suspension of adult honeybees

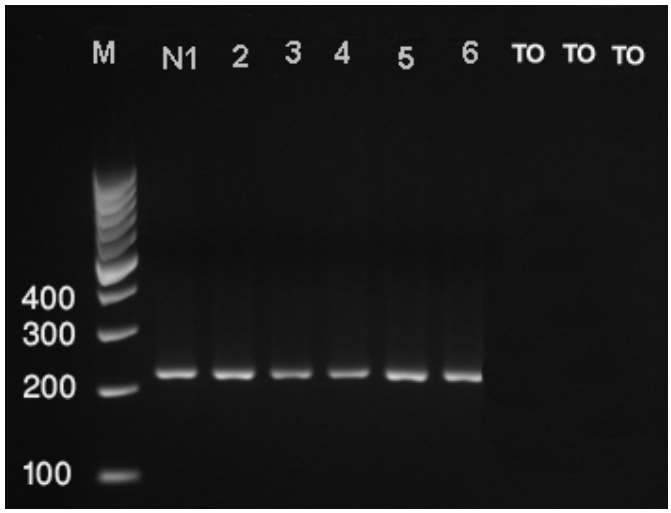

Fig. 2: PCR analysis of extracted DNA from *Nosema* spore using primers (218 MITOC) specific for *Nosema ceranae* derived from 16 SrRNA of the protozoa(Lane 1-6), and the negative controls (TO1-3)

Discussion

During the last 10 years, an increase in infections by microsporidian parasites in the honeybee (*A. mellifera*) has been detected in several European countries (2-4), while increasing numbers of honeybee colony death and low production in the same areas have been reported by beekeepers. One of the principal hypotheses that might explain these problems is the recent entry and rapid dissemination of *N. ceranae* in Europe (16). The most significant difference between the two types of *Nosema* (*N. ceranae* and *N. apis*) is how quickly *N. ceranae* can cause a colony to die. Bees can die within 8 days after exposure to *N. ceranae*, which is faster than bees exposed to *N. apis*. The foraging force seems to be affected the most. They leave the colony and are too weak to return, thus dying in the field.

Former reports of the distribution of *Nosema* disease in Iran (20, 21), assumed that only

one *Nosema* species, *N. apis*, infected honey bees. The presence of the reports based on the incidence of colony depopulation and high prevalence of nosemosis, with different clinical and epidemiological pattern in Iran, noticed us that its cause may potentially be this addition species. The most significant difference between the two microsporidian, is how quickly *N. ceranae* can cause a colony to die. The foraging force to be affected the most. They leave the colony and are too weak to return, thus dying in the field.

Regarding to the similarity of spores of the two *Nosema* species and it is hardly distinguishing by optical microscopy, so that we use PCR technique in the diagnosis and identification of honeybee microsporidians.

The aim of performing this study was to show the probability of presence of new species of *Nosema* in Iran using small- sub unit rRNA (16SrRNA). Based on molecular data, our studies confirm that the microsporidia isolated from Savadkouh samples of honeybees are *N. ceranae*.

Polymerase Chain Reaction with the *N. ceranae* specific primers, 218MITOC were positive in all six samples sent to our department for *Nosema* analysis, Subsequent PCR assays confirmed the suitability of our design. The *N. ceranae* SSUrRNA gene-specific primers used here successfully amplified a 218-bp fragment from *N.ceranae* spores. Conversely, these primers did not amplify DNA preparations from uninfected honeybees.

This is the first molecular survey on *N. ceranae* in the country attempting to identify and characterize the species of this genus.

However, this is the first study that provides evidence concerning the species of *N. ceranae*, which causes severe disease among honeybees in Iran.

It seems that the lack of seasonality detected and the increasing number of pathological samples sent to our laboratory in last years without any compatible *Nosema* signs of infection can be related to the *N. ceranae* prevalence in other regions in Iran that needs to investigate. Some differences in the epidemiology and pathology caused by this agent may explain this situation.

According to earned results, we can not state the exact time of the beginning of infection, but we suggest its occurrence in this country. Over the past years, many beekeepers in the country have noticed a change in the course of nosemosis in their colonies, especially loss of honeybees the whole year around and quick die off colonies in the wintertime, *N. apis*, on the other hand, generally affects colonies during spring (25). It seems that these differences may be due to the emergent parasite, *N. ceranae*, as the relationship between recent Iran honeybee colony losses and nosemosis.

Analyses of more colonies with and without apparent *Nosema* disease are needed to determine the prevalence of this new agent in Iran. According to announcement of the many of the investigators, there is a relationship between *N. ceranae* and colony collapse disorder. It should be noticed that the other variety of reasons of the occurrence of disease should not be ignored. Recent survey and experimental work suggest that *N. ceranae* is a serious threat to the global beekeeping industry (3, 5, 15).

Intensive surveys are needed to determine the distribution and prevalence of *N. ceranae* in Iran.

Acknowledgments

This study was supported by Veterinary Organization of Iran. We thank beekeepers in mention province for preparing the bees samples. The authors declare that there is no conflict of interests.

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