HOSTED BY
ELSEVIER

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Original article http://dx.doi.org/10.1016/j.apjtb.2016.11.004

Molecular identification of *Nosema* species in provinces of Fars, Chaharmahal and Bakhtiari and Isfahan (Southwestern Iran)

Fatemeh Aroee[1*], Hamidreza Azizi[1], Behrouz Shiran[2], Khodadad Pirali Kheirabadi[1]

[1]*Department of Pathobiology, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran*

[2]*Department of Plant Breeding and Biotechnology, Faculty of Agriculture, Shahrekord University, Shahrekord, Iran*

ARTICLE INFO

Article history:
Received 4 Feb 2016
Received in revised form 22 Feb, 2nd revised form 24 Mar, 3rd revised form 25 Mar 2016
Accepted 2 May 2016
Available online 10 Nov 2016

Keywords:
Microsporidia
Nosema apis
Nosema ceranae
PCR

ABSTRACT

Objective: To molecularly identify *Nosema* species in provinces of Isfahan, Fars, Chaharmahal and Bakhtiari.

Methods: One hundred and eighty adult honey bees suspected with nosemosis from provinces of Fars (different counties), Isfahan, and Chaharmahal and Bakhtiari were tested. In order to determine the species of *Nosema*, previously developed PCR and primers based on 16S rRNA gene were used. PCR products were purified and sent to the Korean company of Macrogen for sequencing.

Results: Only *Nosema ceranae* was determined in all samples based on their molecular profile. Sequences of the 16S rRNA gene were sent to GenBank/NCBI (samples accession numbers KP318660–KP318663).

Conclusions: This species currently exists in European honeybee apiaries of *Apis mellifera* in the studied provinces.

1. Introduction

Species of Microsporidia are opportunistic, intracellular and spore-producing parasites that infect all types of animals in nature. *Nosema* is one of the microsporidian species and has been increasingly proposed as the most important pathogen factor in insects. Two species, namely, *Nosema apis* (*N. apis*) and *Nosema ceranae* (*N. ceranae*) have been shown to infect the midgut epithelial cells in honeybees [1]. Infection with *Nosema* has significant negative impacts on honeybees, causing dysentery, shortened lifetime of honeybees, delayed fertility of queens, and colony size reduction [1]. Over the last 10 years, an increase in microsporidian parasite infections in the European honeybee [*Apis mellifera* (*A. mellifera*)] has been detected in several European countries [2,3]. Also, beekeepers have reported increasing honeybee colony death and low production in the same areas [4]. *N. ceranae*, originally found in Asian honey bees [*Apis cerana* (*A. cerana*)], is highly pathogenic and is now a common cause of infection in European honey bees [1]. This phenomenon is very surprising for beekeepers because the seasonal phenology of *N. ceranae* is different from that of *N. apis*, causing more effective and significant problems for beekeepers in warm climates and in summer months [1]. The most important difference between two species of *Nosema* is that *N. ceranae* can cause death in colony more quickly. Honey bees can die 8 days after infection with *N. ceranae* which is faster than infection with *N. apis*. It seems that the foraging behavior of bees is mostly affected. Bees leave the colony and are very weak to return, thus dying in the field around the colony [4]. The proportion of non-returning bees has been evaluated, in which a loss of 19% infected bees was observed as compared with 7% of a control group, while the distance was only 30 m, which is very short as compared with the foraging distances of honey bees [mean foraging distance: 1 000 m in May, 5 500 m in August; mean: 1 543 m (range: 62–10 000 m)] [5,6].

Nosema infection has been shown to cause economic and biological damage in colonies, and due to the difficulty in distinguishing between *Nosema* species, molecular and epidemiological studies have been conducted in different geographical areas to identify the species seemed essential [1]. However, it is difficult to morphologically distinguish between *N. ceranae* and *N. apis*

*Corresponding author: Fatemeh Aroee, Department of Pathobiology, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran.
Tel: +98 383 2324401
E-mail: aroee.fatemeh@yahoo.com
Foundation Project: Supported by the Shahrekord University (No. 41).
Peer review under responsibility of Hainan Medical University. The journal implements double-blind peer review practiced by specially invited international editorial board members.

2221-1691/Copyright © 2016 Hainan Medical University. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

(absence of clear morphological characteristics) and as such, molecular methods are best to distinguish between *Nosema* species.

The PCR technique is very sensitive for detecting microsporidian infection because it enables detection of the parasite at very low levels of infection and can identify all stages of the microsporidian life cycle [4].

An increase in microsporidian infections in the European honey bee (*A. mellifera*) has been detected in Iran in the last 3 years [4]. There is a relationship between *N. ceranae* and colony collapse disorder, as reported by several researchers [7,8]. As pointed out in a study, the different causes of colony collapse disorder should not be ignored [4].

Most investigators in America found that *N. apis* is more related to colony collapse disorder than *N. ceranae*. There are indications that *N. ceranae* may play a key role in colony collapse disorder and it most likely collaborates with other pathogenic agents or conditions [9].

Further investigation showed that the disease caused by *N. ceranae* is far more common than that caused by *N. apis* in its new host, and *N. ceranae* has been shown to be highly pathogenic to European honey bees [10].

It has been reported that the only agent of nosemosis in Iran is *N. apis* [11,12]. The prevalence of nosemosis in European honey bees in Mazandaran Province was studied by Nabian *et al.* They observed that *N. ceranae* is the only species of *Nosema* in Mazandaran Province [4]. In a study by Razmaraii *et al.* on *Nosema*, the prevalence of nosemosis in East Azerbaijan was investigated [1]. They observed *N. ceranae* as the only species of *Nosema* in the province of East Azerbaijan [1].

While *N. ceranae* has successfully invaded a new host, there is a dearth of information regarding microsporidian infection in *A. cerana*, and the original host of *N. ceranae* [11].

Nosema is transmitted horizontally. Although it is not generally considered as highly virulent, *N. apis* is thought to be epidemic, and cause significant economic losses [13]. *Nosema* infection significantly increased worker bee mortality, but had no influence on food consumption. Spore intensity and infection mortality caused by *N. ceranae* is considerably greater than that caused by *N. apis* [14]. This supports claims that *N. ceranae* could be one stressor responsible for elevated colony losses that have been observed recently [15–17]. The production of high spores could be a mechanism by which apparent rapid extension of this horizontally-introduced *Nosema* occurred [14].

Hence, nosemosis is often referred to as “the silent killer”, because there are no obvious signs of disease and as such, diagnosis is difficult [18]. The provinces of Isfahan, Fars, Chaharmahal and Bakhtiari are important in the beekeeping industry of the country. Therefore, this study was carried out to detect the microsporidians (*N. apis* and *N. ceranae*) from honey bee in the aforementioned provinces using the PCR method.

2. Materials and methods

2.1. Sample collection

In this study, 60 honey bees collected in the Parasitology Laboratory of General Veterinary Office of Fars Province and suspected with nosemosis were sent to the laboratory of counties of Kazeroon, Lar, Mamasani and Meymand. Also, in collaboration with the Veterinary Organization of Chaharmahal and Bakhtiari and Isfahan provinces, 120 suspicious honey bees of these two provinces were prepared and transferred to the laboratory.

2.2. Spore detection

In total, 180 adult honey bees suspected with nosemosis were macerated in 100 mL of distilled water. The suspension was filtered and centrifuged at 1 000 r/min for 20 min, and the supernatants were removed, then the sediment was transferred into 1.5 mL of distilled water, so that the presence of spores of *Nosema* was examined using light microscopy (magnification, 400×). The obtained spores were stored at −20 °C [1,4].

2.3. DNA extraction

The total genomic DNA was extracted from 100 mg spores material using a DNA extraction kit (BioFlux, China) following the manufacturer's instructions. The purified total DNA was quantified by gel electrophoresis. Negative control samples, including healthy bees, were tested as well.

2.4. PCR design methodology

The primers designed for this study were based on the 16S rRNA gene because of given gene sequence of *N. ceranae* (samples accession numbers KP318660–KP318663) available in the GenBank/NCBI database. Specific primers were selected for both *N. apis* and *N. ceranae* taking into account that primer sequences are specific to each of the two *Nosema* species.

The selected primers were 321APIS (F5′-GGGGGCATGT CTTTGACGTACTATGTA-3′), 321APIS (R5′-GGGGGGCGT TTAAAATGTGAAACAACTATG-3′) and NAF (5′-CCATTG CCGGATAAGAGAGT-3′), NAR (5′-CCACCAAAAACTCC CAAGAG-3′) for *N. apis* and 218CERANAE (R5′-CGGCGA CGATGTGATATGAAAATATTAA-3′), 218CERANAE (F5′-CGGCGACGATGTGATATGAAAATATTAA-3′) and NCF (5′-CGGATAAAAGAGTCCGTTACC-3′), NCR (5′-TGAG-CAGGGTTCTAGGGAT-3′) for *N. ceranae* [1,4]. The 20 μL of PCR volume contained 0.8 μmol/L of each primer, 0.5 μmol/L each of deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate and deoxythymidine triphosphate, 1.5 mmol/L $MgCl_2$, 1× buffer, 0.2 IU *Taq* DNA polymerase and 4 ng of *Nosema* template DNA. Amplification was performed in an Eppendorf Mastercycler gradient thermalcycler under following conditions: predenaturation stage at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min, and then followed by an extension stage of 5 min at 72 °C. Amplification products were separated by electrophoresis on 1.4% agarose gels. The gels were stained with ethidium bromide and photographed using an UV gel documentation system (UVI technology). A 100 bp DNA ladder was used as a size standard.

DNA was purified using a DNA purification kit (BioFlux, China) [4]. The purified DNA products were used for sequencing insert with both forward and reverse primers M13. Sequencing was performed by a company of Macrogen from Korea. The *N. ceranae* and *N. apis* sequences were aligned and compared using data from GenBank. Sequence analyses were performed using data available in NCBI.

3. Results

The honey bee samples were investigated using PCR for 16S rRNA gene of *Nosema* species. The amplified DNA fragment showed that the extraction of DNA from the samples was

performed successfully. *N. ceranae* was detected in samples from Isfahan, Meymand, Mamasani, Kazeroon and Shahre Kord counties (Table 1). *N. apis* was not detected in any of the samples. In this study, the 16S rRNA gene was successfully applied to detect *Nosema*. Also, the differentiation of *N. apis* and *N. ceranae* was easy through the application of the mentioned primer pairs (NC, 218CERANAE and NA, 321APIS primers). The sizes of amplified products ranged from approximately 189 to 200 bp and the expected amplicon sizes (218 bp for *N. ceranae*) and sequences were yielded. In agarose gels, neither undesirable amplicons nor primer interaction were detected. The sequences of 16S rRNA gene were used to investigate diagnosis, and the PCR products were sequenced and submitted to GenBank/NCBI (accession numbers of samples of Isfahan Province, Kazeroon, Mamasani and Shahre Kord were KP318662, KP318660, KP318663 and KP318661, respectively).

In Canada, the reproduction of *Nosema* species, the effects of *N. apis* and *N. ceranae* and the co-infection of both species in European honeybee were investigated. *N. ceranae* had significantly the greatest infection intensity and host mortality; mortality due to *N. apis* was similar to that caused by co-infection, and spore intensity was reduced probably due to inter-specific competition [14].

Studies have investigated the exclusive competition of intracellular gut parasites (microsporidian) and the spread of infections emanating from European honey bees in Germany. They provided a clear evidence of competition between microsporidian species, *N. apis* and *N. ceranae*, in host of *A. mellifera*. Infection is an important determinant of the result of competition between microsporidian species; the first microsporidian significantly inhibits the growth of the second microsporidian, regardless of species [22].

Table 1

Analysis of PCR for DNA extraction based on 16S rRNA using NA, 321APIS primers for *N. apis* and NC, 218CERANAE primers for *N. ceranae*.

Species	Primers	Meymand	Kazeroon	Lar	Shahre Kord	Isfahan	Mamasani	Control samples
N. apis	NA	−	−	−	−	−	−	−
	321APIS	−	−	−	−	−	−	−
N. ceranae	NC	−	+	−	+	+	+	−
	218CERANAE	+	+	−	+	+	+	−

−: Absence; +: Presence.

The sequences were compared with those of related species obtained from GenBank and the sequences obtained were confirmed by *N. ceranae* diagnosis.

The BLAST search against GenBank observed the highest similarity (99% or 93%) with *N. ceranae* 16S rRNA sequence. The presence of *N. ceranae* was detected in all samples from Meymand, Mamasani, Kazeroon and Shahre Kord counties and Isfahan Province.

The results of this study showed that *Nosema* in provinces of Fars, Isfahan and Chaharmahal and Bakhtiari is exclusively *N. ceranae*.

4. Discussion

The prevalence of *N. ceranae* and *N. apis* was 61% and 28%, respectively in China, but in Taiwan, the prevalence was 73% and 33% for *N. ceranae* and *N. apis* [10]. The prevalence rates of *N. apis* and *N. ceranae* in Slovakia were 90.9% and 9.1%, respectively. In 2009 and 2010, a gradual increase in the prevalence of *N. ceranae* and decrease in prevalence of *N. apis* were recorded in Slovakia [19]. The only species found in different climatic regions of Croatia was *N. ceranae* [18]. Bourgeois *et al.* suggested that the only sample prepared from the midgut is suitable for molecular analysis and detection of *Nosema* species [20]. They observed a prevalence rate of *N. apis* and *N. ceranae* and co-infection of both species during different seasons of the year. *N. ceranae* was identified in the months of June and July. In April, *N. apis* was identified. In the months of May, July and August, there was co-infection of both species [21]. The prevalence of *Nosema* species detected by light microscopy method was 58.1%, and by PCR method it was 67.1% in East Azerbaijan Province. The only species detected was *N. ceranae* [1]. The only reported microsporidian species removed from honey bee samples in Mazandaran was *N. ceranae* [4].

The results of previous studies on *Nosema* species in Iran and other countries are consistent with those of this research.

The results of this study indicated that *N. ceranae* was transferred from *A. cerana* to *A. mellifera* and was not limited to its original host, but distributed in the study region. These results have been reported in various studies in different regions of the world [1,14,23,24].

As previously reported, the only agent of nosemosis in Iran was *N. apis* [11,12]. It was assumed that the only agent of honey bees infection was *N. apis*. According to the reports, the incidence of colony depopulation and high prevalence of nosemosis, with different clinical and epidemiological patterns in Iran may potentially be due to this addition species [4].

N. ceranae spores are oval and rather uniform in shape. Its size is revealed by light microscopy. Since species of Microsporidia are often difficult to distinguish using morphological criteria, a quick and accurate molecular genetics method for the identification of *Nosema* is important [1].

In different geographical regions, infections with *N. ceranae* have different colony level effects. The seasonal variations and gross colony level symptoms described for *N. apis* seem not to be present in *N. ceranae*. There are differences between the two parasites, but virulence differences remain conclusively verified. The spores of *N. ceranae* appear to be much vulnerable than those of *N. apis*, in particular when freezing, and the apparent replacement of *N. apis* for *N. ceranae* remains enigmatic [25].

The results of this study showed that *N. ceranae* was the only *Nosema* species found to infect European honey bees in provinces of Isfahan, Fars and Chaharmahal and Bakhtiari.

Therefore, further studies are required to determine the prevalence and distribution of *N. ceranae* in other provinces [4].

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

The authors are grateful to the Biotechnology Laboratory of Agriculture Faculty in University of Shahre Kord. Thanks to the Parasitology Laboratory of Veterinary Faculty in University of Shahre Kord and Veterinary Organization of Fars for preparing the samples.

References

[1] Razmaraii N, Sadegh-Eteghad S, Babaei H, Paykari H, Esmaeilnia K, Froghy L. Molecular identification of *Nosema* species in East-Azerbaijan Province, Iran. *Arch Razi Inst* 2013; **68**(1): 23-7.
[2] Higes M, Martín-Hernández R, Meana A. *Nosema ceranae* in Europe: an emergent type C nosemosis. *Apidologie* 2010; **41**(3): 375-92.
[3] Martín-Hernández R, Meana A, Prieto L, Salvador AM, Garrido-Bailón E, Higes M. Outcome of colonization of *Apis mellifera* by *Nosema ceranae*. *Appl Environ Microbiol* 2007; **73**(20): 6331-8.
[4] Nabian S, Ahmadi K, Nazem Shirazi M, Gerami Sadeghian A. First detection of *Nosema ceranae*, a microsporidian protozoa of European honeybees (*Apis mellifera*) in Iran. *Iran J Parasitol* 2011; **6**(3): 89-95.
[5] Higes M, Martin R, Meana A. *Nosema ceranae*, a new microsporidian parasite in honeybees in Europe. *J Invertebr Pathol* 2006; **92**(2): 93-5.
[6] Higes M, Garcia-Palencia P, Martin-Hernández R, Meana A. Experimental infection of *Apis mellifera* honeybees with *Nosema ceranae* (Microsporidia). *J Invertebr Pathol* 2007; **94**(3): 211-7.
[7] Kralj J, Fuchs S. *Nosema* sp. influences flight behavior of infected honey bee (*Apis mellifera*) foragers. *Apidologie* 2010; **41**(1): 21-8.
[8] Wolf S, McMahon DP, Lim KS, Pull CD, Clark SJ, Paxton RJ, et al. So near and yet so far: harmonic radar reveals reduced homing ability of *Nosema* infected honeybees. *PLoS One* 2014; **9**(8): e103989.
[9] Budge GE, Barrett B, Jones B, Pietravalle S, Marris G, Chantawannakul P, et al. The occurrence of *Melissococcus plutonius* in healthy colonies of *Apis mellifera* and the efficacy of European foulbrood control measures. *J Invertebr Pathol* 2010; **105**(2): 164-70.
[10] Chen Y, Evans JD, Zhou L, Boncristiani H, Kimura K, Xiao T, et al. Asymmetrical coexistence of *Nosema ceranae* and *Nosema apis* in honey bees. *J Invertebr Pathol* 2009; **101**(3): 204-9.
[11] Lotfi A, Jamshidi R, Aghdam Shahryar H, Yousefkhani M. The prevalence of nosemosis in honey bee colonies in Arasbaran region (Northwestern Iran). *Am Eurasian J Agric Environ Sci* 2009; **5**(2): 255-7.
[12] Razmaraii N, Karimi H. A survey of *Nosema* of honeybees (*Apis mellifera*) in East Azarbaijan Province of Iran. *J Anim Vet Adv* 2010; **9**(5): 879-82.
[13] Klee J, Besana AM, Genersch E, Gisder S, Nanetti A, Tam DQ, et al. Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee. *Apis Mellifera J Invertebr Pathol* 2007; **96**(1): 1-10.
[14] Williams GR, Shutler D, Burgher-MacLellan KL, Rogers RE. Infra-population and -community dynamics of the parasites *Nosema apis* and *Nosema ceranae*, and consequences for honey bee (*Apis mellifera*) hosts. *PLoS One* 2014; **9**(7): e99465.
[15] Neumann P, Carreck NL. Honey bee colony losses. *J Apic Res* 2010; **49**(1): 1-6.
[16] van Engelsdorp D, Meixner MD. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. *J Invertebr Pathol* 2010; **103**: S80-95.
[17] Villa JD, Bourgeois AL, Danka RG. Negative evidence for effects of genetic origin of bees on *Nosema ceranae*, positive evidence for effects of *Nosema ceranae* on bees. *Apidologie* 2013; **44**(5): 511-8.
[18] Tlak Gajger I, Vugrek O, Grilec D, Petrinec Z. Prevalence and distribution of *Nosema ceranae* in Croatian honeybee colonies. *Vet Med* 2010; **55**(9): 457-62.
[19] Staroň M, Jurovčíková J, Čermáková T, Staroňová D. A scientific note on incidence of *Nosema apis* and *Nosema ceranae* in Slovakia during the years 2009 and 2010. *Slovak J Anim Sci* 2012; **45**(1): 36-8.
[20] Bourgeois L, Beaman L, Holloway B, Rinderer TE. External and internal detection of *Nosema ceranae* on honey bees using real-time PCR. *J Invertebr Pathol* 2012; **109**(3): 323-5.
[21] Sokół R, Michalczyk M. Detection of *Nosema* spp. in worker bees of different ages during the flow season. *J Apic Sci* 2012; **56**(2): 19-25.
[22] Natsopoulou ME, McMahon DP, Doublet V, Bryden J, Paxton RJ. Interspecific competition in honeybee intracellular gut parasites is asymmetric and favours the spread of an emerging infectious disease. *Proc R Soc B* 2015; http://dx.doi.org/10.1098/rspb.2014.1896.
[23] Yoshiyama M, Kimura K. Distribution of *Nosema ceranae* in the European honeybee, *Apis mellifera* in Japan. *J Invertebr Pathol* 2011; **106**(2): 263-7.
[24] Chen Y, Evans JD, Smith IB, Pettis JS. *Nosema ceranae* is a long-present and wide-spread microsporidian infection of the European honey bee (*Apis mellifera*) in the United States. *J Invertebr Pathol* 2008; **97**(2): 186-8.
[25] Fries I. *Nosema ceranae* in European honey bees (*Apis mellifera*). *J Invertebr Pathol* 2010; **103**(Suppl 1): S73-9.