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Full Article

Molecular Identification of *Nosema* species in East-Azerbaijan province, Iran

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ABSTRACT

Nosema is a genus of microsporidia, which have significant negative impacts on honeybees. The aim of this study is the epidemiological evaluation and molecular characterization of *Nosema* spices in various counties of East-Azerbaijan province (Northwest of Iran). 387 samples were collected from colonies maintained in various counties of East-Azerbaijan province. Samples after preparation were examined by a light microscope for presence of *Nosema* spores. PCR method (SSUrRNA gene) was used to differentiate between *Nosema apis (N. apis)* and *N. ceranae*. Descriptive statistics were used for data analysis. Total infection prevalence of the microscopic evaluation and PCR tests were 225 (58.1%) and 260 (67.1%) respectively, total validity of PCR test against the microscopic test was computed equal to 1.1 in this case. Disease distribution in various counties of study area was variable and *N. ceranae* was the only *Nosema* species found to infect honeybees. The one species presence and different distribution of *Nosema* positive samples in various counties of East-Azerbaijan province may be due to multiple reasons. Furthermore, epidemiological information helps us to improve disease management practices in the studied area, apply new hygiene policy and reduce extra costs of production.

Keywords: Epidemiology, Molecular Characterization, Nosema, East-Azerbaijan, Iran

INTRODUCTION

Microsporidia are opportunistic intracellular spore-forming parasites in nature infecting all animal phyla (Chen *et al* 2009, Mayack & Naug 2009). *Nosema* is a genus of microsporidia and increasingly has been recognized as an important insect pathogen (Alaux *et al* 2011, Higes *et al* 2008). *Nosema apis* and *Nosema ceranae* are parasites infecting the midgut epithelial cells of adult honey bees (Forsgren & Fries 2010). These infections have significant negative impacts on honeybees, causing dysentery, shortened life spans of

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honey bees, supersedure of infected queens, and decrease in colony size (Chen *et al* 2009). Severe honeybee colony losses have occurred in the past several years in the United States, Asia, and Europe. Most of these losses have been attributed to *Nosema* spices infection (Cornman *et al* 2009, Gajger *et al* 2010, Lotfi *et al* 2009, Nabian *et al* 2010).

For years, Nosemosis of the European honeybee was exclusively attributed to *Nosema apis (N. apis)*. *N. ceranae*, a species originally found in the Asian honeybee (*Apis cerana*) and it is now a common infection of European honeybees and is highly pathogenic to its new host (Chen *et al* 2009b). This is problematic for beekeepers because *N. ceranae* has a different seasonal phenology than *N. apis*, causing more significant problems for beekeepers in summer months and in warm climates (Bourgeois *et al* 2010). Thus, investigation of *Nosema* spices seems important. Routine optical microscopy assessment can confirm infection with both *Nosema* species, but it is impossible to distinguish between the species because of the absence of clear morphological characteristics for species recognition. Thus, it is necessary to use molecular diagnostic tools and identification methods (Gajger *et al* 2010).

According to the economic and biologic damages caused by *Nosema* in colonies and difficulty to distinguish between the species, molecular and epidemiological studies in various geographical regions sound essential.

The aim of the present study was to determine the molecular prevalence and epidemiology of *Nosema* spices in various counties of East-Azerbaijan province of Iran.

MATERIALS AND METHODS

Study area. East-Azerbaijan province is located in North-West of Iran and has an area about 47,830 km² (2.8 % of Iran's area) and is located between 36.45 °C and 39.26 °C north latitude and 45.5 °C and 48.22 °C east longitude. According to the latest divisions of the country, this province has 19 counties. East Azerbaijan enjoys a cool, dry climate, being in the main a mountainous region. However, the gentle breezes off the Caspian Sea have some influence on the climate of the low-lying areas. Temperatures run up to 8.9 °C in Tabriz, in the winter dropping to -15 °C at least.

Samples collection. Honey bees were collected from colonies maintained in various counties of East-Azerbaijan province (Table 1) during April – June, 2011. Sample size calculated in α=5%, $Z_{1-a/2}$=1.96, P=50% and d=5% condition and stratified random sampling method was used for the sample collection. All the bee samples were transferred to the parasitology laboratory of Razi research institute immediately and stored at –20 °C prior to examination.

Samples preparation. The abdomens of 20 honeybees from each colony were ground up in 4 ml of sterile distilled water. The suspensions were filtered to remove coarse bee parts, centrifuged at 1000 g for 20 min and the supernatants removed. Pellets of isolated spores were resuspended in 1.5 ml of distilled water and transferred to a fresh tube.

Microscopic evaluation. One drop of sample was examined by a light microscope for presence of Nosema spores. Wet mount slides were photographed using IDS imaging development systems GmbH. Then spore sizes were analyzed with Axiovision LE software.

Molecular identification. Identification was done with SSUrRNA gene amplification with PCR method. Briefly, samples total genomic DNA was extracted according to the AccuPrep kit protocol provided by the manufacturer (BioNeer, Korea). The SSUrRNA gene was amplified by using the PCR method with Taq DNA polymerase and primers NAF (5' - CCATTGCCGGATAAGAGAGT 3') and NAF (5' CCACCAAAAACTCCCAAGAG 3') for *N. apis* (with 269 bp product) and NCF (5' CGGATAAAAGAGTCCGTTACC 3') and NCF (5' TGAGCAGGGTTCTAGGGAT 3') (with 250 bp product) for *N. ceranae* (Chen *et al* 2009a). The conditions for thermal cycling were as follows: denaturation of the target DNA at 96 °C for 2 minutes

followed by 30 cycles at 94°C for 15 second, primer annealing at 57°C for 30 second and primer extension at 72°C for 30 second. At the end of the cycling, the reaction mixture was held at 72°C for 7 minutes and then cooled to 4°C. PCR amplification was detected by agarose gel (1.5%) electrophoresis and was visualized by ultraviolet (UV) after ethidium bromide staining. Documentation was done with Kodak M12 software.

Statistics analysis. Data were examined using a commercially available statistical package (IBM SPSS version 19 for Windows), and comparisons were made using the descriptive statistics.

RESULTS

Results of the PCR and microscopic examinations of all 387 samples are presented in Table 1. All of 387 samples were investigated by PCR for SSUrRNA gene of *N. apis* and *N. ceranae*. The highest percentages (>75%) of PCR positive samples were from eight counties of East-Azerbaijan: Ahar, Ajabshir, Heris, Kaleybar, Marand, Shabestar and Varzeghan. The intermediate percentages (25% to 75%) of infection were detected in Hashtrood, Jolfa, Maraghe, Miyane and Osku. The lowest percentages (<25%) of nosemosis were detected in Azarshahr, Bonab, Bostanabad, Charoymagh and Malekan. Total infection prevalence in the province with the microscopic evaluation was 58.1 % while in PCR test was 67.1% and the total validity of PCR test against the microscopic test was computed equal to 1.1 in this case. Our results showed that *N. ceranae* was the only *Nosema* species found to infect honeybees from our widespread geographic collection in East-Azerbaijan (Figure 1). Light microscopy revealed that fresh *N. ceranae* spores were oval or rod shaped, varied in size with a mean length 4.9 ± 0.28 μm and a mean width 2.09 ± 0.06 μm (N = 100).

DISCUSSION

According to results of this study in microscopic and PCR assays, the percentage of total *Nosema* positive samples was 58.1% and 67.1% respectively. In the previous work (2010), the highest infection rate was reported in June (59.8 %) in East-Azerbaijan province (Razmaraii & Karimi, 2010). According to lotfi et al (2009) study maximum infection was observed in May (83.3%) and in Arasbaran region of mentioned province. Furthermore, Tavassoli et al (2009) reported low Nosemosis infection rate in the early months of the year in Urmia (Northwest of Iran). In Stevanovic et al (2010) study the percentages of *Nosema* positive samples were 78.6% in Bosnia and Herzegovina and 94.6% in Montenegro. According to Gisder et al (2010) the proportions of Nosema positive samples were variable from 22.4% to 35.4% in Germany.

Our observation with light microscopy showed that spores of *N. ceranae* are oval shaped and rather uniform in shape and size. Thus, a quick and accurate molecular genetics method of detection of *Nosema* is important for identification because species of microsporidia are often difficult to distinguish using morphological criteria (Klee *et al* 2007). We used of PCR method for *Nosema* species identification. This methodology detected infection agent in 35 samples, which were found to be negative using microscopy. Furthermore, in the present study *N. ceranae* was the only *Nosema* species found to infect honeybees. These results are similar to Stevanovic et al (2010) study. 28% of bees collected in China, were positive for *N. apis* and 61% for *N. ceranae* and this ratio in Taiwan were 33% and 73% respectively (Chen *et al* 2009a). Martin-Hernandez, et al (2007) reported 53.8% prevalence for *N. ceranae* and 9.3% for *N. apis. N. ceranae* was observed in some regions of Europe . First Detection of Nosema ceranae were reported by Whitaker et al (2010) In turkey. Valera et al (2011) reported high prevalence of viable spores of *N. ceranae* in central Europe and central Asia. Mentioned literature shows the various distributions of *Nosema* species in the world.

Colonization is a transmission process involving the spread of a parasite into new geographical areas and

Table 1. prevalence of *N. ceranae* in various counties of East-Azerbaijan province of Iran.

Province	Apiary numbers	Sample size	Microscopic positive (%)	PCR positive (%)	PCR/Microscopic rate (test validity)
Ahar	98	13	8 (61.5)	11 (84.6)	1.1
Ajabshir	43	7	5 (71.4)	6 (85.7)	1.2
Azarshahr	58	8	0 (0)	0 (0)	1
Bonab	22	3	0 (0)	0 (0)	1
Bostanabad	69	9	2 (22.2)	2 (22.2)	1
Charoymagh	31	5	0 (0)	0 (0)	1
Hashtrood	74	10	3 (30)	4 (40)	1.3
Heris	26	4	3 (75)	3 (75)	1
Jolfa	33	5	3 (60)	3 (60)	1
Kaleybar	225	32	24 (75)	28 (87.5)	1.1
Malekan	21	3	0 (0)	0 (0)	1
Maraghe	587	78	47 (60.2)	54 (69.2)	1.1
Marand	240	34	24 (70.5)	29 (85.2)	1.2
Miyane	151	19	13 (68.4)	13 (68.4)	1
Osku	348	48	20 (41.6)	22 (45.8)	1.1
Sarab	172	25	18 (72)	20 (80)	1.1
Shabestar	167	24	15 (62.5)	21 (87.5)	1.4
Tabriz	329	41	26 (63.4)	29 (70.7)	1.1
Varzeghan	156	20	14 (70)	15 (75)	1
Total	2850	387	225 (58.1)	260 (67.1)	1.1

leads to establishment of that species in a host population in which it was not previously presented (Martin-Hernandez *et al* 2007). Our results suggest that *N. ceranae* was not restricted to its original host and jumped from *Apis cerana* to *Apis mellifera* and become distributed in study area. These results were frequently reported in various studies in different reagents of the world (Klee *et al* 2007, Chen *et al* 2008, Yoshiyama & Kimura 2011).

The presence and various distributions of *N. ceranae* positive samples in the counties of East-Azerbaijan province may be due to five reasons; 1) Infection agent may have been introduced into this area, 2) migratory condition of commercial beekeepers in East-Azerbaijan, 3) contaminated equipment, 4) climate changes, 5) parasite host changing.

The data presented in this study indicate a strong need for epidemiological and pathogenetic studies to identify distribution, species of infection, host-parasite relations and infection kinetics. This information provides awareness and obliges us to apply new hygiene policy for honeybee keepers. Furthermore, help us to improve disease management practices in the studied area and reduce extra costs of production.

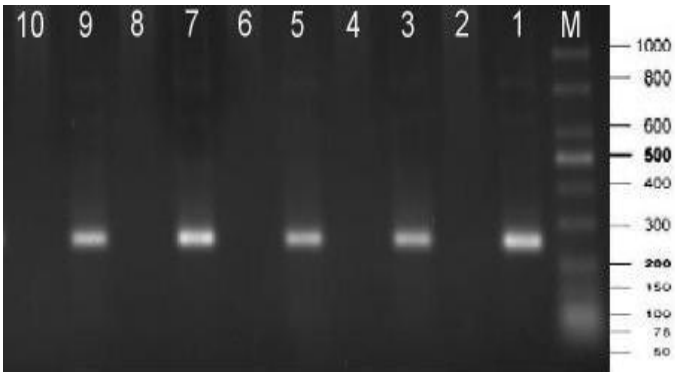

Figure 1. Electrophoresis results of SSUrRNA gene with special primers for *N. apis* (2, 4, 6, 8 and 10) and *N. ceranae* (250 bp) (1, 3, 5, 7 and 9), Lane M related to marker.

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