The EuroBiotech Journal
RESEARCH ARTICLE
Environmental Biotechnology

Determination of spore longevity and viability of *Nosema apis* and *Nosema ceranae* according to storage conditions

Erkay Özgör and Nevin Keskin

Abstract

Honey bee colonies are often infected with *Nosema apis and Nosema ceranae* which cause adult honey bee disease called nosemosis**.** All honey bee colony members can be infected with these species. In addition, it is claimed to be the main cause of honey bee winter losses in many countries. *Nosema* spores are expected to resistant the environmental conditions and their infectivity continues for a long time because of long-term durability of fungal spores. In this study, the viability of *Nosema* spores were investigated in terms of storage situations under laboratory conditions. Honey bee samples that were collected from apiaries in 2011 were investigated to detect the presence of *Nosema* species with real-time PCR amplification studies. After determination of *Nosema* species, each sample was divided in two groups. One of these groups was used to find *Nosema* spore concentration. *Nosema* solutions were divided and stored at both -20°C and +4°C. The spore concentration was measured every year in the period 2011-2015. Other group of honey bee samples was also stored at -20°C and every year was used for *Nosema* spore counting. Furthermore, it was examined the infectivity of *Nosem*a spores with sugar solutions which obtained each sample using cage experiment techniques. According to results, when we compare the solutions annually, there is no change at *Nosema* spore concentration of the solution in -20°C and honeybee samples in -20°C. But reduction was seen at *Nosema* spore concentration of the solution in +4°C. *Nosema* spore infectivity tests revealed that infectivity of *Nosema* spores has not changed significantly between 2011 and 2015. This is the first time mixed *Nosema* spores found more infective than one-type spore after prolonged exposure to different conditions.

Department of Biology, Microbiology and Parasitology Laboratory, Hacettepe University, Ankara, Turkey

Corresponding author: E. Özgör
E-mail: erkay@hacettepe.edu.tr

Published online: 20 July 2017
doi:10.24190/ISSN2564-615X/2017/03.03

Introduction

Nosema apis and *Nosema ceranae* are parasitic organisms that cause adult honey bee disease called nosemosis and honey bee colonies are often infected with *Nosema* species. Honey bee colony members that consisting of the queen bee, worker bees and drones can be infected with these species (1). In addition, some researches claim that nosemosis is the main cause of honey bee losses in many countries and *Nosema ceranae* has contributed to CCD (Colony Collapse Disorder) (2,3,4,5).

N. apis and *N. ceranae* that are included to Microsporidia show their effects in different ways. *Nosema apis*-borne nosemosis is one of the most common and most economically damaging diseases in *Apis mellifera*. *N. apis* identified more than hundred years ago and it was known as the only factor of nosemosis seen in honey bees before the discovery of *N. ceranae* (6). *N. apis* may cause deaths of the weak honey bee colonies, but it does not cause to death by itself generally. Although this panzootic parasite does not have high virulence, it shortens the life of the worker bees and causes weakening in the colonies infected at high quantities. In addition, it causes of the decline or loss of a honey bee midgut function (7,8,9,10).

A new species of *Microsporidia*, *N. ceranae,* was discovered in Beijing, China in 1994 and it was detected to infect *Apis cerana,* eastern honey bee. In 2005, natural infection of *N. ceranae* have been identified for the first time in *A. mellifera* colonies with the SSU rRNA gene sequencing method in Taiwan (11). Today, this parasite is commonly found in *A. mellifera* colonies in Europe, North America, South America, North Africa and Austra-

lia (9,12,13,14,15,16). *N. ceranae* is a dangerous pathogen for *A. mellifera* and it shows more lethal effect than *N. apis*. It is known to cause mass deaths and losses in honey bee colonies especially in strong colonies (17,18).

Although *N. apis* and *N. ceranae* spores have similar morphology under the light microscope, *N. ceranae* spores have slightly smaller and more circular form than *N. apis* spores. The main differences between these two species are determined by electron microscopic structure and the small subunit (16S) rRNA and ITS region gene sequences. *N. apis* has 30 polar filaments under a transmission electron microscope while *N. ceranae* has 20-23 polar filaments (19,20,21).

Nosema spores are expected to resistant the environmental conditions and their infectivity continues for a long time because of long-term durability of fungal spores. The aim of this study was to investigate the viability of *Nosema* spores in terms of storage situations under laboratory conditions.

Material and Methods

Collection of honey bee samples

In this study, apiaries that have different geographic and climatic factors were selected and the most appropriate areas were identified. Adult *Apis mellifera anatoliaca* and *Apis mellifera caucasica* from each of 169 apiaries were collected with field studies from 3 different regions (Artvin, Burdur and Isparta) of Turkey in 2011. Especially, an appropriate hive which shows nosemosis symptoms was selected in each apiary and 60 worker bees were taken from each selected hive. After collecting the honey bee samples, it was stored in the dry ice box during the field studies.

Identification of *Nosema spp.* from adult bee samples

Ten apiaries were selected from 169 apiaries and honey bee samples were used from taken these selected apiaries for the *Nosema* spore longevity researches. First of all, these selected adult bee samples were examined in the laboratory for the detection of *Nosema* species. To identify *Nosema* species, Real-time PCR amplification studies were performed. For this purpose, Biospeedy DNA Isolation Kit (Bioeksen, Turkey) was used for DNA isolation. After homogenizing **3** adult honey bee samples, each was transferred to a microfuge tube weighing 200 mg. 400 µl Guanidinium thiocyanate (0.1 M Tris; pH 7.5) was added to the tube and was stirred at 3,000 rpm for 1 minute with vortex. The samples were incubated for 10 min at 95°C. After stirring for 1 min at 3,000 rpm with vortex, the samples were centrifuged for 1 min at 14,000 rpm and supernatant was transferred to a new tube. 200 µl of isopropanol was added in supernatant and mixed well. The mixture was added to a DNA column, waited for 1-2 minutes and after centrifuging at 12,000 rpm for 1 minute the pellet was discarded. 500 µl Wash Buffer (20 mM NaCl, 2 mM Tris-HCl, pH 7.5; 80% v/v Ethanol) was added to the column and pellet was discarded after centrifugation at 12,000 rpm for 1 min. This step was repeated two more times. After the column was centrifuged for 1 min at 12,000 rpm, it was placed in the sterile micro centrifuge tube. 100 µl solubilization buffer was added into the tube, incubated 1 min at room temperature, centrifuged at 14,000 rpm for 1 min and DNA isolates were obtained. These DNA isolates were stored at -20°C.

After DNA isolation, Real-time PCR amplification was performed by modifying the protocol of Bourgeois et al. (22). Biospeedy qPCR 2 × Master Mix (Bioeksen, Turkey) were used for this process. ITS region has been targeted to distinguish *Nosema* species by using *Nosema spp.* specific forward primer ITS-F5'- TGAATGTGTCCCTGTTCTTTGTAC-3', *Nosema apis* specific reverse primer NapisITS-R 5'-TAATTATAATCTCCTTGGTCCGTG-3' and *Nosema ceranae* specific reverse primer NcerITS-R TAAATATAATCTCCTGGTCGGTT. Biorad CFX Connect (Bio-Rad Laboratories, USA) was used in all reactions. The reaction contains 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1 × Reaction Buffer, 0.1U Fast Start Taq DNA Polymerase, 1 × EvaGreen, 4 ng/µl template DNA and 0.5 µM of each primer.

Spore longevity and infectivity studies under laboratory conditions

After determination of Nosema species with molecular studies, each samples was divided in two groups. One of these groups was used for preparing *Nosema* solutions with classical *Nosema* method and it was determined the level of *Nosema* infection microscopically. The spore solutions were obtained by homogenizing the intestinal contents of 10 adult honey bees from each sample. These solutions were centrifuged at 6,000 rpm for 10 minutes. After centrifugation, the supernatant was poured and the pellet was used for counting. It was added 1 ml of distilled water per adult bee on the pellet and was vortexed. These pellet solutions were used for count *N. apis* and *N. ceranae* spores on light microscope with Neubauer slide and infection levels per bee were determined (23) (**Fig. 1**). *Nosema* solutions were divided and stored at both -20°C and +4°C. The spore concentration was measured every year in the period 2011-2015. Other group of honey bee samples was also stored at -20°C and every year was used for *Nosema* spore counting.

Furthermore, the infectivity of *Nosema* spores with solutions which obtained each sample was examined using cage experiment techniques. For this purpose, infectivity test were carried out with 30 *Nosema*-free worker bees per cage using each solution (-20°C and +4°C) in specific proportions. 30 cages were used for all samples and spore storage conditions, and the bees were infected with doses of 100,000 spores per bee. It was investigated whether there is any increase at *Nosema* spore concentrations. To confirm that the spores were infective, bees were sacrificed a week post-infection, and their abdomens were macerated in a mortar in 1 ml of water using a pestle. The presence and number of spores in each group was confirmed and determined by light microscopy in a Neubauer slide. Negative (uninfected) and positive controls infected with fresh *N. ceranae* spores were analysed in parallel. The evaluations were performed in terms of the amount of spores in bees with the results obtained from infectivity studies.

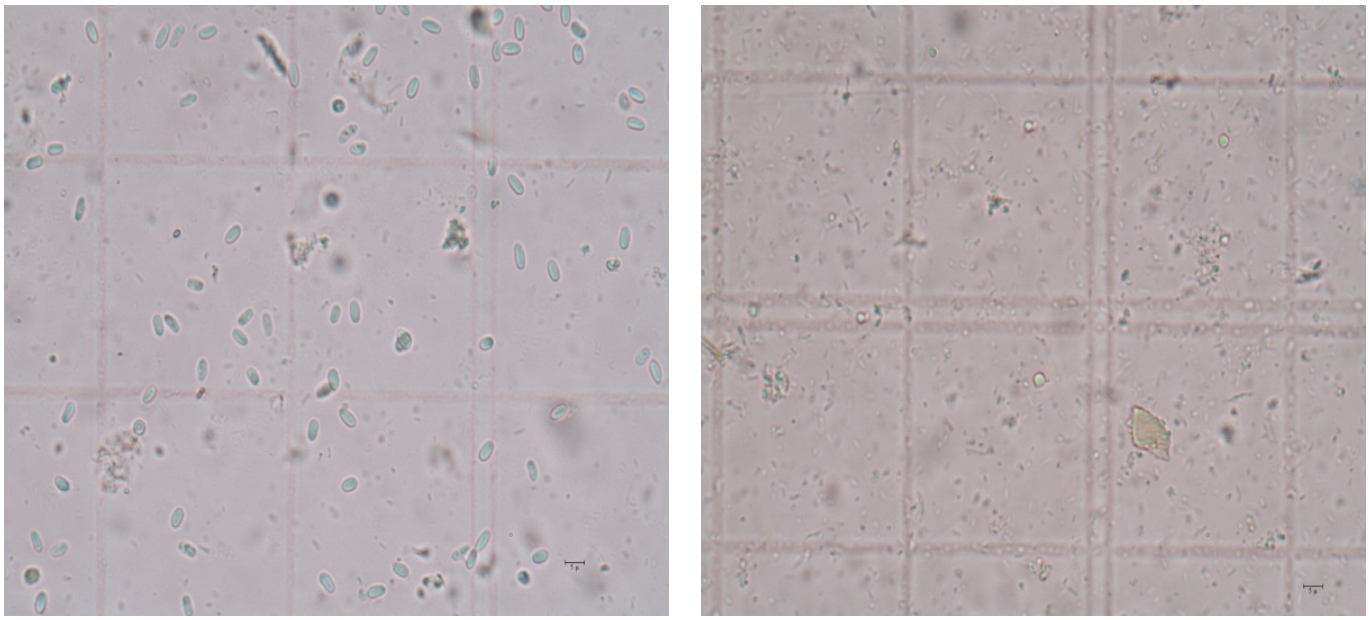

Figure 1. Microscopic views of *Nosema apis* (left) and *Nosema ceranae* (right) spores (Figure scales are 5 µm).

Statistical analysis

After biological analysis, SPSS 14 software was used for statistical evaluation of data. *Nosema* spore concentrations were compared in terms of storage conditions in all solutions using one-way ANOVA. The differences between the storage conditions are shown by the LSD test and *Nosema* concentration and storage conditions were evaluated with dual comparisons due to the statistical results.

Results

Nosema species were initially identified in honey bee samples which are collected from 169 apiaries (**Fig 2**). These were detected by amplification with real-time PCR species in honey bee samples. Determination of presence of *N. apis* and *N. ceranae* in 10 selected samples using proliferation threshold and melting curves obtained from qPCR results, cycle number (Ct) and melting temperature (Tm) data are given in **Table 1**. It was evaluated that qPCR was performed with Ct <35 and positive results were obtained for *N. ceranae* at Tm 82°C and *N. apis* at Tm 85°C.

The changes of *Nosema* spores in terms of storage situations under laboratory conditions were investigated and pre-prepared solution or freshly crushed honeybee solution were counted from all samples every year. When compared samples in terms of storage conditions, it was observed that there is a significant difference in *Nosema* spore concentration (**Table 2**). The differences between the storage conditions were evaluated one by one and this difference was appeared to be due to the reduction of the amount of *Nosema* in the + 4°C storage condition (**Table 3**). Temperature is an effective factor for decreasing *Nosema* spore quantities although environmental conditions are not affect the concentration of spores.

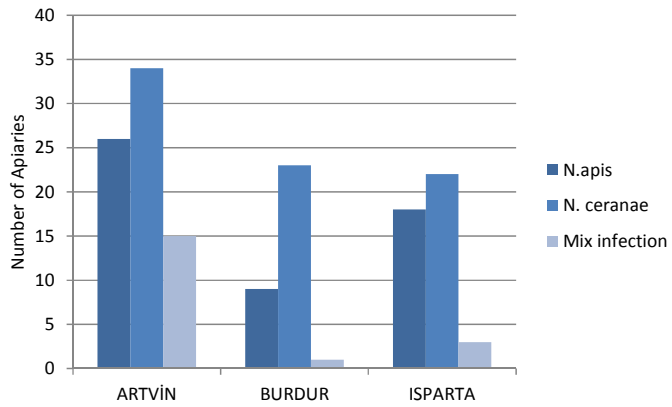

Figure 2. Distribution of *Nosema* species between 169 apiaries by province.

Table 1. *Nosema* species determination results in ten selected samples depending on the melting temperature (Tm) and threshold cycle number (Ct) obtained from the Q-PCR results

Sample No	Ct	Tm	Results
1	32.24	85	*N. apis*
1	34.18	82	*N. ceranae*
2	27.96	85	*N. apis*
3	34.22	82	*N. ceranae*
4	29.45	85	*N. apis*
5	30.75	82	*N. ceranae*
6	21.99	85	*N. apis*
6	31.22	82	*N. ceranae*
7	33.84	82	*N. ceranae*
8	31.49	82	*N. ceranae*
9	24.47	85	*N. apis*
10	21.31	85	*N. apis*

Table 2. The comparison of *Nosema* spore count in terms of storage conditions (One-way ANOVA, $p < 0.05$)

Nosema	Sum of Squares	df	Mean Squares	F	Sig.
Between groups	868.926	2	434.463	5.181	.007
Within groups	12327.496	147	83.861		
Total	13196.421	149			

The viability of *Nosema* spp. spores at each of the storage conditions assessed was expressed as their percentage mortality, assessed and Infection rates were varied based on deaths of honey bees. İt was evident that storage duration had no adverse effects on spore viability while differences of storage temperature had adverse effect on concentration of spores. The increase in spore infectivity assessed as the percentage of dead was observed in *N. ceranae* and *N. apis* spores stored at -20°C. Mix *Nosema* samples showed the highest infectivity in all conditions (**Table 4**).

Discussion

N. apis and *N. ceranae* are known to spread worldwide and this situation has become one of major problems for the beekeeping sector. In this study, Real-time PCR studies showed that *Nosema* species are widespread at honey bee colonies in Turkey. It is noteworthy that both species were found in some samples.

According to storage conditions, when we compare the solutions annually, there is no significant change at *Nosema* spore concentration of the solution in -20°C and honeybee samples in -20°C. But reduction was seen at *Nosema* spore concentration of the solution in +4°C. Indicating that *Nosema* spore intensity can be maintained more in low temperatures whether in solution or in the intestines of honeybees.

Nosema spore infectivity tests revealed that infectivity of *Nosema* spores has not changed in all comparative samples between 2011 and 2015. Thus, there were no changes in the spore longevity in case of long term storage of *Nosema*. Although it has been many years, *Nosema* spores can become infective.

As a result of infectivity tests, although spores protected their vitality in all storage conditions, there was a decrease in the infectivity of the samples at +4°C (80%). Sample 1 and 6 which have Mix *Nosema* spores shows 100% infectivity in all conditions. It has been observed that mixed *Nosema* spores can protect their infectivity for a longer period of time. Thus, it was found that mixed *Nosema* may be more infective than one-type spore for the first time after prolonged exposure to different conditions.

A maximum of twenty percent of the inoculated bees escaped infection, all of which dies within 17 to 29 days. After the examination it was shown that all dead bees from the inoculated cages showed 100 % infection. It means that some of *Nosema* spores can be alive at all storage condition and these spores retain their virulence.

Table 3. Multiple comparisons of storage conditions of *Nosema* samples by LSD test

(I)Storage	(J)Storage	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
-20 Solution	+4 Solution	5.1352*	1.8315	.006	1.5157	8.7547
	-20 Honeybee	5.960E-02	1.8315	.974	-3.5599	3.6791
+4 Solution	-20 Solution	-5.1352*	1.8315	.006	-8.7547	-1.5157
	-20 Honeybee	-5.0756*	1.8315	.006	-8.6951	-1.4561
-20 Honeybee	+4 Solution	-5.9600E-02	1.8315	.974	-3.6791	3.5599
	-20 Solution	5.0756*	1.8315	.006	1.4561	8.6951

* The mean difference is significant at the .05 level.

Table 4. Percentage of mean infectivity of *Nosema*-treated bees in cage experiment

Sample	1	2	3	4	5	6	7	8	9	10
-20 Honeybee	100	96.6	96.6	93.3	96.6	100	96.6	93.3	90	96.6
-20 Solution	100	90	96.6	96.6	93.3	100	96.6	90	96.6	93.3
+4 Solution	100	86.6	83.3	86.6	90	100	86.6	90	80	86.6
Positive control	100									
Negative control	0									

From this study, it is seen that the *N. apis* and *N. ceranae* spores can be very hardy microsporidians. When kept different storage conditions, these spores were found to be viable for 5 years. Revell (24) shows that refrigerated *N. apis* spores keeps its vitality almost 7 years. Our results are consistent with his results. But study of Collado et al. (25) gave opposite results compared to ours. They found that the best storage temperatures of both *Nosema* species were 25 and 4 °C. It is thought that *Nosema* species used in these studies may be varied by regional and genetic differences in based of resistance mechanisms. For this reason, it is necessary to carry out further studies related to storage conditions of *Nosema* spores using samples collected from different regions or countries.

In conclusion, we showed that *Nosema* spore longevity can vary due to storage conditions. Although the storage conditions effective on *Nosema* spore concentration, it has not led any change on *Nosema* infectivity with both *Nosema* species. It has been shown that *Nosema* spores may be infective after many years even if viable spores are in very small quantities.

Conflict of Interest Statement

The authors confirm that this article content has no conflict of interest.

References

1. vanEngelsdorp 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 Patho 2010; 103: 80-95.
2. Higes M, Martín R, Sanz A, Alvarez N, Sanz A, Garcia MP, Meana A. El síndrome de despoblamiento de las colmenas en España. Consideraciones sobre su origen. Vida Apícola 2005; 133: 15-21.
3. Higes M, Martin-Hernandez H, Botias C, Bailon EG, Gonzalez-Porto AV, Barrios L, Nozal MJD, Bernal JL, Jimenez JJ, Palencia PG, Meana A. How natural infection by *Nosema ceranae* causes honeybee colony collapse. Environ Microbiol 2008; 10(10): 2659-2669.
4. Chen PY, Evans JD, Murphy C, Gutell R, Zuker M, Gundensen-Rindal D, Pettis JS. Morphological, molecular and phylogenetic characterization of *Nosema ceranae*, a microsporidian parasite isolated from the European honey bee, Apis mellifera. J Eukaryot Microbiol 2009; 56(2): 142-147.
5. Oldroyd BP. What's Killing American Honey Bees? PLoS Biol 2007; 5(6): e168.
6. Zander E. Tierische Parasiten als Krankenheitserreger bei der Biene Münchener Bienenzeitung 1909; 31: 196-204.
7. Fries I, Ekbohm G, Villumstad E. *Nosema apis* sampling techniques and honey yield. J Apicult Res 1984; 23: 102-105.
8. Ritter W. Enfermedades de las abejas. In: Acribia S.A. (Ed) 2001; Zaragoza, Spain.
9. Klee J, Besana AM, Genersch E, Gisder S, Nanetti A, Tam DQ, Chinh TX, Puerta F, Ruzi JM, Kryger P, Message D, Hadjina F, Korpela S, Fries I, Paxton RJ. Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*. J Invertebr Patho 2007; 96: 1-10.
10. Ptaszyńska AA, Borsuk G, Mułenko W, Demetraki-Paleolog J. Differentiation of *Nosema apis* and *Nosema ceranae* spores under Scanning Electron Microscopy (SEM). J Apicult Res 2014; 53: 537-554.
11. Huang WF, Jiang JH, Chen YW, Wang CH. A *Nosema ceranae* isolate from the honeybee *Apis mellifera*. Apidologie 2007; 38: 1-8.
12. Higes M, Martin R, Meana A. *Nosema ceranae*, a new microsporidian parasite in honeybees in Europe. J Invertebr Patho 2006; 92: 93-95.
13. Paxton RJ, Klee J, Korpela S, Fries I. *Nosema ceranae* has infected *Apis mellifera* in Europe since at least 1998 and may be more virulent than *Nosema apis*. Apidologie 2007; 38: 558-565.
14. Invernizzi C, Abud C, Tomasco I, Harriet J, Ramallo G, Campá J, Katz H, Gardiol G, Mendoza Y. Presence pf *Nosema ceranae* in honey bees (*Apis mellifera*) in Uruguay. J Invertebr Patho 2009; 101: 150-153.
15. Paxton RJ. Does infection by *Nosema ceranae* cause 'Colony Collapse Disorder' in honey bees (*Apis mellifera*)?. J Apicult Res 2010; 49: 80-84.
16. Ütük AE, Pişkin FÇ, Kurt M. Türkiye'de *Nosema ceranae*'nın ilk moleküler tanısı. Ankara Univ Vet Fak 2010; 57: 275-278.
17. Martín-Hernández R, Meana A, Prieto L, Martínez-Salvador A, Garrido-Bailón E, Higes M. Outcome of colonization of *Apis mellifera* by *Nosema Ceranae*. Appl Environ Microb 2007; 73: 6331-6338.
18. Higes M, Martín-Hernández R, Meana A. *Nosema ceranae* in Europe: an emergent type C Nosemosis. Apidologie 2010; 41: 375-392.
19. Fries I. *Nosema ceranae* in European honey bees (*Apis mellifera*). J Invertebr Patho 2010; 103: 573-579.
20. Fries I, Martin R, Meana A, García-Palencia P, Higes M. Natural infections of *Nosema ceranae* in European honey bees. J Apicult Res 2006; 45: 230-233.
21. Fries I, Feng F, DaSilva A, Slemenda SB, Pieniazek NJ. *Nosema ceranae* n. sp. (Microspora, Nosematidae), morphological and molecular characterization of a microsporidian parasite of the Asian honey bee *Apis cerana* (Hymenoptera: Apidae). Eur J Protistol 1996; 32: 356-365.
22. Bourgeois AL, Rinderer TE, Beaman LD, Danka RG. Genetic detection and quantification *Nosema apis* and *N. ceranae* in the honey bee J Invertebr Pathol 2010; 103: 53-58.
23. Cantwell GE. Standart methods for counting *Nosema* spores. Am Bee J 1970; 110: 222-223.
24. Revell IL. Longevity of refrigerated *Nosema* spores – *Nosema apis*, a parasite of honey bees. J. Econ Entomol 1960; 53(6): 1132-1133.
25. Collado JGS, Higes M, Barrio L, Martín-Hernández R. Flow cytometry analysis of *Nosema* species to assess spore viability and longevity. Parasitol Res 2014; 113(5): 1695-1701.