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Research Article

Monitoring of *Nosema* Infections Levels During Hygienic Honey Bee Breeding Programs in Turkey

Rahsan IVGIN TUNCA [1] Devrim OSKAY [2] Sezai ERGINOGLU [3]

[1] Mugla Sıtkı Koçman University, Ula Ali Koçman Vocational School, Apiculture Program, TR-48640 Ula, Mugla - TURKEY
[2] Namık Kemal Universiy, Faculty of Agriculture, Department of Agricultural Biotechnology, TR-59030 Tekirdag - TURKEY
[3] Mugla Honeybee Breeding Association,Veterinarian, TR-48100, Mugla - TURKEY

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Abstract

The objective of this study was to follow *Nosema* infection levels and species under hygienic bee breeding program for resistance to American foulbrood (*Paenibacillus larvae*). The incidence of *Nosema* parasite infection levels and detection of the species of *Nosema* were evaluated in 5 periods during 2012-2014 for Mugla honey bees known as an ecotype of *Apis mellifera anatoliaca* in the hygienic bee breeding program. During the hygienic breeding program, no organic or synthetic chemical treatments were applied against nosemosis in the colonies. The incidences of *Nosema* spores were followed in 123 colonies at five time periods. Although the correlations were negative for between spores-temperature (r = -0.115; P>0.01) and positive for spores- humidity (r = 0.013; P>0.01) but not significant statistically. Molecular diagnosis showed that only *N. ceranae* spores were detected from samples during 5 seasons. In conclusion, nosema infection levels decreased under hygienic bee breeding programme but further monitoring studies should be performed in order to decide whether the nosema spores decrease due to hygienic behavior. To our knowledge, this is the first long- term and unique study for observation of *Nosema* during breeding program in Turkey so far.

Keywords: *Nosema ceranae, Nosema apis, Breeding program, Mugla ecotype, Turkey*

Türkiye'deki Hijyenik Bal Arısı Islah Çalışması Süresince *Nosema* Enfeksiyon Düzeyinin Takibi

Özet

Bu çalışmada, Amerikan Yavru Çürüklüğü (*Paenibacillus larvae*) hastalığına dirençli olan hijyenik balarısı ıslah programında *Nosema* enfeksiyon düzeyinin takibi amaçlanmıştır. Islah programında *Apis mellifera anatoliaca* ekotipi olarak adlandırılan Muğla balarısında 2012-2014 yılları arasında 5 dönem boyunca *Nosema* türleri ve nosema enfeksiyon düzeyi belirlenmiştir. Islah çalışması süresince kolonilere nosema için her hangi bir ilaç uygulaması yapılmamıştır. Beş dönem boyunca 123 kovanın nosema sporu bulaşıklığı takip edilmiştir. Spor sayısı- sıcaklık arasında negatif korelasyon (r = -0.115; P>0.01) ve spor sayısı nispi nem arasında pozitif korelasyon (r = 0.013; P>0.01) bulunmasına karşın ilişki istatistiksel olarak önemli değildir. Moleküler tanımlamada, beş sezon boyunca alınan örneklerde yalnızca *N. ceranae* sporu tespit edilmiştir. Sonuç olarak, hijyenik arı yetiştirme programı süresince nosema enfeksiyon seviyeleri azaldığı gözlenmiştir. Fakat nosema sporlarının azalmasının nedeninin hijyenik davranışa bağlı olup olmadığına dair karar verebilmek için başka hijyenik çalışmalarda da gözlemler yapılmalıdır. Bu çalışma bugüne kadar Türkiye'deki ıslah program süresince *Nosema* düzeyinin takibini içeren en uzun süreli ve tek çalışmadır.

Anahtar sözcükler: *Nosema ceranae, Nosema apis, Islah programı, Muğla ekotipi, Türkiye*

INTRODUCTION

The Microsporidia have more than thousand species (160 genera and 1300 species) and Nosema species being Microsporodian are parasitic for invertebrates [1-4]. There were two microsporidian species of genus *Nosema*, *Nosema apis* and *Nosema ceranae* in honey bees. It was supposed that *N. ceranae* was specific for *Apis cerana* and *N. apis,* a pathogen specific for the *Apis mellifera,* gave rise to nosemosis previously [5,6].

However, other studies illustrated that *N. ceranae* could infect *A. mellifera.* In the last decade, *N. ceranae* has expanded its distribution in the world and the replacement of *N. apis* by *N.ceranae* was reported by many researchers [7-10]. The serious colony losses referred to colony collapse disorders (CCD) were observed in the last decade and attracted great

İletişim (Correspondence)
+90 252 2115647
rivgin@gmail.com

attention of scientists, but the reasons are still unclear [11]. The studies indicated that *Nosema* was considered the possible suspect for colony losses. Furthermore, the researchers thought that many factors such as beekeeping practices, host susceptibility and various combinations of pathogens resulted in colony losses [12,13]. Also researchers indicated that synergistic effects of various pesticides and *N. ceranae* combination increased honey bee mortality [14-16]. Recent studies illustrated that *N. ceranae* is the most prevalent bee pathogen and it does not show any prior clinical symptoms [7,8,17]. The one possible explanation for the higher pathogeny of *N. ceranae* is that it has better adaptation than *N. apis* to different temperature conditions [18,19]. Another finding is that *N. ceranae* infection exerts a higher immune suppression than *N. apis* [20] and *N. ceranae* infection X imidacloprid affect the immune response [14]. On the other side, if honey bee colonies have enough protein and energy reserves, honey bees can tolerate *N. ceranae* infection as the host energy intake is increased by the parasite. Otherwise, it experiences energy stress if the host does not have enough energy reserve [21,22]. *Nosema* is still considered as the possible suspect for colony losses causing economic loss. Many survey studies indicated that *Nosema* infections led to colony losses worldwide [9,10,13,23]. Universities and several governmental institutions in many countries have performed breeding and selection programs supporting to increase honey, pollen production and gentleness. Recently, breeding programs for increased disease resistance including hygienic behavior of colonies to American foulbrood [24,25], and *Varroa destructor* were performed in many countries [26-30]. In Turkey, breeding programs have generally been applied for conservation of subspecies or native populations and used widely for enhancing desirable traits *(www.tagem.gov.tr)*. In contrast, the breeding programs for disease resistance have not been widely applied before. The negative effects of diseases could often be compensated by pharmaceuticals, and other management techniques.

This study was a part of a hygienic behavior breeding program against American foulbrood which has been coordinated by universities and Mugla Beekeepers Association. The incidence and levels of *Nosema* infections and the type of *Nosema* were determined in 5 periods during 2012-2014 for Mugla ecotype in the program which recorded the hygienic test values in colonies. In here, *Nosema* spore monitoring results and types of *Nosema* are given. More detailed results on breeding program will be prepared for publications. The reason why *Nosema* spores have been observed during this breeding program is that selected populations are very important, and so *Nosema* spores were observed during five periods in order to see the effect of *Nosema* spores in any colony loss of selected population considering the pathogen effect of nosema.

The objective of this study was to follow *Nosema* infection levels and species under hygienic selection program for resistance to American foulbrood. To our knowledge, this is the first long- term study for observation of *Nosema* during breeding program in Turkey so far.

MATERIAL and METHODS

The colonies used in the present study were screened in order to detect the presence of *Nosema* spores using microscopic method. During the hygienic breeding programme, no organic or synthetic chemical treatments have been applied against nosemosis. The honey bee samples were taken from each colony in both spring and autumn in 2012-2014 and kept in alcohol for spore counts in 123 colonies.

Determination of Nosema Infection Levels

Twenty older foragers from each colony were sampled from in order to determine the number of spores per bee in a pooled sample. Homogenates were prepared according to the OIE terrestrial manual [31]. *Nosema* spp. spores were microscopically determined from each homogenate at 400x magnification. The spores were counted on the haemocytometer [31] and the average number of spores per bee was calculated [32].

Molecular Detection of Nosema spp.

Total DNA was extracted from each homogenate using DNA isolation kit (Fermentas K512). Isolated DNA was analyzed by multiplex PCR in order to confirm the *Nosema* species of the spores as previously described using 321APIS FOR/321APIS REV and 218MITOC FOR/218MITOC REV primers specific for *N. apis* or *N. ceranae* respectively [33]. PCR amplification was detected in agarose gel (1.5%) electrophoresis and visualized under UV after ethidium bromide staining [33]. Molecular detection of *Nosema* spp. was performed in all samples for each season. The positive PCR products were compared with the controls for *N. ceranae* and *N. apis* provided by Etlik Veterinary Control Central Research Institute (Ankara, Turkey).

Statistical Analysis

ANOVA tests were used to determine the variation in *Nosema* prevalence and the degree of infections over the seasons. Multiple comparison tests were applied to spore counts by Tukey B and correlation test were done using SPSS for Windows Version 16.0 (SPSSInc., Chicago, IL, USA). Selected three positive PCR products from positive samples for *N. ceranae* were sequenced and the sequence similarity analyses were performed using BLAST database search.

RESULTS

The Prevalence of Nosema Spores

During the hygienic behavior breeding program, prevalence of *Nosema* spores was followed in 123 colonies.

During the 2012-2014 period of breeding program, we observed seasonality in spore densities in colonies. The first year (2012) of the breeding program, *Nosema* sporecounts were very high in May and November *(Fig. 1)*. The highest percentage of infected colonies was observed in November 2012 (76%). In the second year, *Nosema* spores were decreased dramatically and the lowest percentage of infected colonies was observed in November 2013 (18%) and nearly similar percentage was observed in May 2014 (19%). Descriptive statistics results of spore counts for sampling seasons were given in *Table 1*. Spore numbers from sampled seasons were decreased during years in breeding programs. The lowest mean numbers of spores were observed in November 2013. Analysis of variance was performed to determine of significance among variables (spore numbers in sampled seasons). Variance analysis illustrated that the differences among spores variables were highly significant (P<0.001). Multiple comparison; Tukey B test was conducted to determine differences which arise from variables. According to multiple comparisons test, spore numbers in May 2012 and November 2012 were different from each other and May 2013-November 2013-May 2014 spore numbers *(Table 1)*.

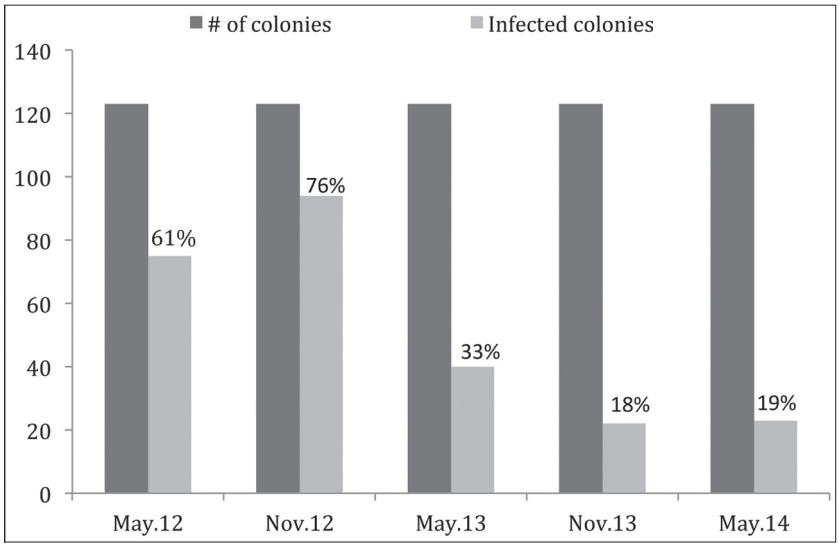

Fig 1. Studied and infected colonies in years 2012-2013-2014

Table 1. *Descriptive statistics results of spores for five seasons*

Group	Mean	N	St. Dev.
May 2012	52. 10^4b	123	12.10^5
November 2012	106.10^4a	123	13.10^5
May 2013	7. 10^4c	123	15. 10^4
November 2013	5.10^4c	123	16. 10^4
May 2014	6. 10^4c	123	18. 10^4

The group means having the different letter (a, b and c) in the same column were different from each other, P≤0.05

Average temperature and relative humidity values were obtained from Turkish State Meterological Service (on electronic data base: *http://tumas.mgm.gov.tr/wps/portal/*) for months during selection periods. Correlation tests were performed between spores- temperature and humidity spores. Negative correlation was detected between spores temperature (r = -0.115; P>0.01) and positive correlation spores- humidity (r = 0.013; P>0.01). But the correlations were not statistically significant. During the selection program, mean spore numbers, mean temperature (Temp.), and relative humidity (RH%) were given in *Fig. 2*.

Molecular Diagnosis of Nosema spp.

DNA isolation was done from *Nosema* spores positive samples using commercial isolation kit. Multiplex PCR were performed to detect of *Nosema* types, *N. apis* and *N. ceranae (Fig. 3)*. Molecular diagnosis illustrated that only *N. ceranae* spores were detected from samples during 5 seasons. Selected positive samples for *N. ceranae* were sequenced. BLAST database search illustrated that the nucleotide sequences of amplification products from the *Nosema* infested honeybees were 99% identical with *N. ceranae* sequence from many countries deposited in GenBank database in this study.

DISCUSSION

The results in here were interpreted with hygienic behavior values which increased in colonies during 3 years of selection. Selection on queens with an artificial insemination showed a steady increase in hygienic bees from 43% in 2012 to 63% in 2013 and 91.7% in 2014. The percentage of bees with hygienic behavior was significantly different among the years (P<0.001) [34]. The results revealed that hygienic behavior increased, and on contrary, infected colony numbers and spore loads significantly decreased in the 5 periods. During breeding program, 3-4% over wintering colony losses were observed through the years, and no suddenly bee death was observed. Honey production was not measured. During these periods, no *Nosema* symptoms were observed in colonies.

The significant differences have been detected in prevalence of *Nosema* spp. from 1990s until now by many studies in the world. The first period examination of *Nosema* spore distribution was carried

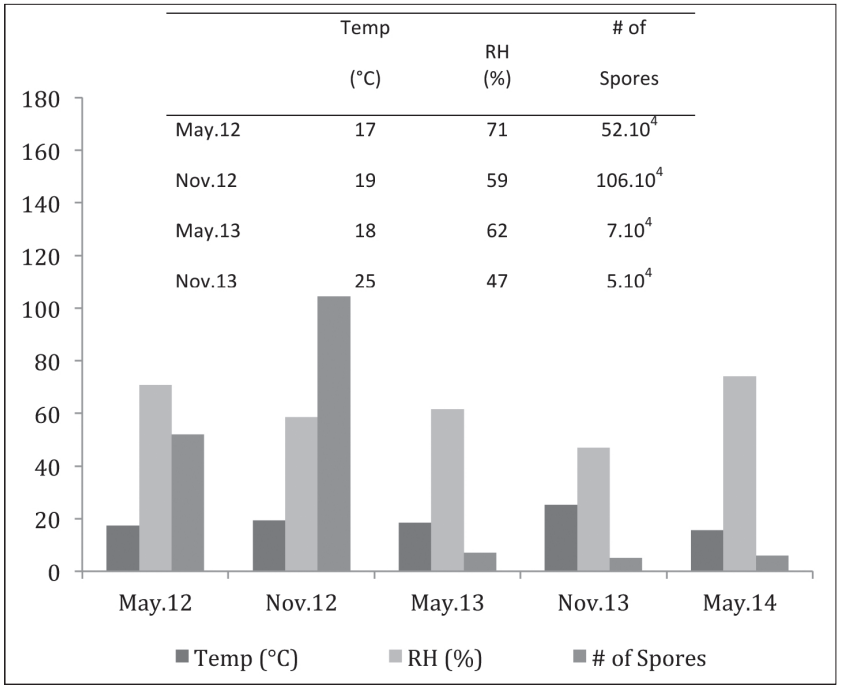

Fig 2. Average temperature (Temp.), relative humidity (RH%), and mean spores numbers under selection

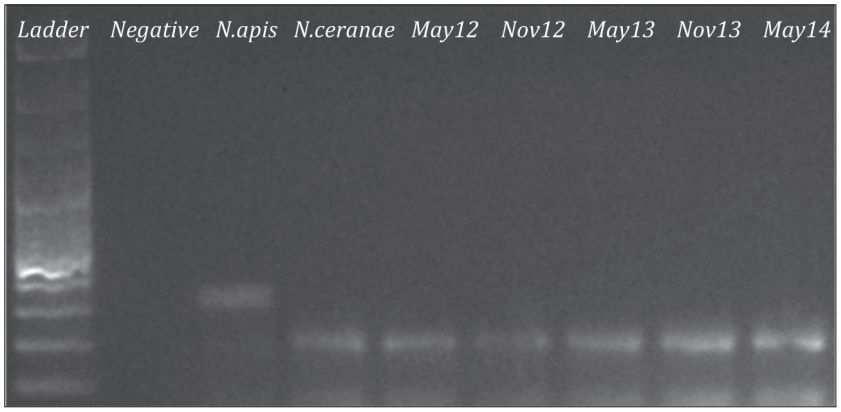

Fig 3. PCR results of positive samples on gel: Line 1: 100bp DNA ladder, line 2: negative control, line 3: *N. apis*, line 4: *N. ceranae*, line 5- 9: Positive samples

out between 1999 and 2002 [10]. During this period, the smallest numbers of *Nosema* positive samples were detected during the summer and the spores looked like *N. apis*. In the other years among 2003-2005, *Nosema* positive samples in all months showed tendency to increase of spores [10]. In Spain, the high number of colony losses had been related with *N. ceranae* more common than *N. apis* [9,13]. The coexistence of both *N. ceranae* and *N. apis* in colonies with the higher prevalence of *N. ceranae,* a sign of a developmental advantage over *N. apis,* and different developmental preferences according to prevalence under the different climatic regions in Spain has been revealed through the other studies [35]. The majority of colonies were found to be infected with mixed *Nosema* spp. in another survey study performed in Sweden in 2007 [17]. With intend of determining the prevalence of *Nosema* spp. in Germany, the long term studies has been made during the five years [36]. Each spring and autumn periods, samples were collected from these colonies and there were no relation between colony losses and detectable levels of infection with *N. apis* or *N. ceranae* in this report [36]. Although, the samples collected in Finland from the 1990s infected with *N. apis*, collected samples from colonies after 2000s infected with either *N. ceranae* or association with *N. apis* [8]. Although the climatic conditions are quite similar, the prevalence of *N. ceranae* has been determined to be higher in Finland than in Sweden or Norway [7]. The *Nosema* spores were detected during four years and the infection level altered both winter and summer periods during this four year period as a decrease in winter 2009-2010 and 2011-2012 in Poland [37]. *N. ceranae* has been reported in France since 2002 and a high variability in spore was observed in studied colonies [38]. The study completed in order to determine the prevalence of *Nosema* spores in Slovakia during the period of two years (2009-2010) reported that the prevalence of *N. ceranae* gradually increased whereas the prevalence of *N. apis* decreased [39]. In Iran, the percentage of *Nosema* positive samples changed according to regions and seasons and *N. ceranae* was the only *Nosema* species in studied colonies [40-43]. The study completed in Eastern part of world illustrated that the percentage of *N. apis* and *N. ceranae* varied from 28% to 61% in China and 33% and 73% in Taiwan, respectively [2]. All these studies above contained seasonal variation of *Nosema* spore loads but not during breeding projects in long term.

In Turkey, the survey studies for colony losses and diseases have shown that the presence of *Nosema* spores in different regions. The percentage of *Nosema* changed from 2% to 9% during years and regions according to these studies but the species of *Nosema* spores were not distinguished [44,45]. Up to now, many studies have reported the presence of *N. ceranae* and *N. apis* from different regions of Turkey [46-48]. Also the replacement of *N. apis* by *N. ceranae* was indicated [49-51].

Nosema ceranae is the only species in sampled colonies in our study. The sequence results for present study also showed high level identity of *N. ceranae* sequences from many European countries and also Australia, Iran, Lebanon, and Thailand according to BLAST database search.

This study is the unique in that the levels of *Nosema* infections were followed in colonies under selection for resistance to another pathogen. In the world, bee breeders in Denmark informed that they have aimed to improve resistance to *Nosema* spp. in their breeding stocks [52].

The infected colony numbers and spore loads were observed in three years. During the five seasons, the number of infected colonies and spores were decreased. In studied colonies, there were negative correlation observed between spores-temperature and positive correlation spores-relative humidity. But these correlations were not significant statistically. In conclusion the *Nosema* infection levels decreased under hygienic bee breeding programme for American Foulbrood disease but further monitoring studies should be performed in order to decide whether the *Nosema* spores decrease due to hygienic behavior. The level of *Nosema* spores observed in colonies under breeding programme for resistance to another pathogen is the unique and long term study in Turkey

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