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Detection of *Nosema Cerrana*in Samples of Iraqi Bees Using Traditional and Molecular Methods

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Abstract

Background: Microsporidia Nosema are widely distributed in natural environments and transferred among bees via the faecal-oral route. *Nosema* spp. spores have been detected on flowers and transferred to hives along with the bee pollen,The microscopic technique has been used for years in the diagnosis of Nosema disease,Different methods have been developed to distinguish *Nosemaapis* from *Nosemaceranae*.PolymeraseChain Reaction (PCR)is used to detect pathogen types along with other microbes .

Key words :*N.cerana*, honey bees, Molecular techniques.

Introduction:*Nosema sp.* is an internal parasite of the honey bee, *Apis mellifera*, and one of the most significant factors contributing to colony losses (Goulson*et al.*, 2015). Given the global importance of honey bee pollination to the reproduction of floral species and to agricultural productivity, it is important to understand how *Nosema spp.* parasitism affects honey bee health,*Nosema spp*. is an example of a microsporidian: a group of spore-forming, unicellular parasites classified as fungi (Fries, 2010). Bees typically become infected with *Nosema sp*. through the ingestion of spores found in contaminated food and water, by cleaning contaminated combs(Higes*et al.*, 2010) or during trophallaxis (Smith, 2012). Once infected, spores begin to thrive in the epithelial cells of the midgut. Over time, cell walls rupture and spores are excreted through the fecal matter (Chen *et al*., 2009). At very high levels of infection, the symptoms of *Nosemasp*. infection resemble those of dysentery. When infected, bees, which are naturally hygienic and excrete outside of the hive, defecate in and around the hive, spreading the infection to other workers, drones and the queen (Alaux*et al.*, 2011).

Nosema species parasitizing honey bees are morphologically similar when viewed using light microscropy but can be differentiated by ultrastructural features (number of polar filaments) and using molecular methods such as the (PCR) (Fries *et al.* 1996). Because disease in honey bees was attributed solely to *N. apis*, researchers relied on light microscopy to determine prevalence and intensity of Nosema infection and did not commonly use

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molecular techniques. Consequently, *N. ceranae* went undetected in honey bees for 10 years (Huang *et al.* 2007).

However, due to the great similarity between the *N. apis* and *N. ceranae* spores, it is very difficult to distinguish between these two species under the microscope. Molecular techniques for diagnosis from the parasite's DNA have begun to be used widely for this purpose (Traver and Fell 2011).

The aim of the present study was to determine prevalence of *N. ceranae* in Baghdad province.

Material and Methods:

A field survey was conducted in Baghdad governorate of five sites (Al-Karayat, Al-Shula, Al-Rashidiya, Al-Jadriya and Al-Nahrawan) for the period from November 2019 - June 2020 to detect the fungus *N.cerana* using light microscopy in the insect laboratory / college Agricultural engineering sciences at the University of Baghdad and conducting molecular detection using PCR technology for DNA in Laboratorydepartment / Veterinary directorate /Ministry Of Agriculture.

The number of samples taken was 25 bees / hives, and the climatic condition was recorded during the sample collection period, represented by measuring the temperature and the percentage of humidity.

Preparation of samples for microscopic examination*N.cerana* were obtained from samples taken during the survey that was conducted, and their laboratory diagnosis was confirmed by confirming the presence of spores according to the scientific specifications of the pathogen under a light microscope and (lens strength 40).

DNA extraction. DNA was extracted from the solution obtained by crushing each bee in 1 ml of distilled water after being filtered, and the extraction process was carried out using the QIAGEN-supplied extraction kit. The fungus was diagnosed by means of this PCR reaction using a special primer,F5'-CGGCGACGATGTGATATGAAAATATTAA-3 and R. 5'-CCCGGTCATTCTCAAACAAAAAACCG-3'(Khezri*et al.*2018).

The PCRamplification of 16S rRNA gene fragments, first 50 µL reaction mixture contains 5 ng genomic DNA, 3 mM MgCl2, 200 µM of each deoxyribonucleotide triphosphate, 100 ng of primers, 5 µL of 10X PCR buffer (100 mM Tris/HCl, pH 8.3; 15 mM MgCl2; 500 mM KCl) and 1 U of Taq polymerase. Conditions of amplification consist of an initial denaturation cycle at 94 ˚C for 15 sec followed by 25 cycles of denaturation (94 ˚C, 15 sec), primer annealing (61.80 ˚C, 30 sec), primer extension (72.00 ˚C, 45 sec) followed by additional extension step of 7 minutes at 72.00 ˚C. The PCR products were separated by electrophoresis on 1% agarose gel, stained with Ethidium Bromide and visualized under UV transillumination.

Result and Discussion:

The survey results showedthat the fungus *N. cerana* was found in all the study sites in the governorate of Baghdad, and the infection rate increased when the temperature was moderately cold (28-32 degrees Celsius) and the humidity rate (40-50%), where

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the highest infection was recorded in the Shula in November 2019, as it reached 71%, followed by Al-Rashidiya, Al-Jadiriya, , Al-Kureyatand Al-Nahrawanas it reached 66%, 48%, 40% and 20%, respectively(Figure 1).

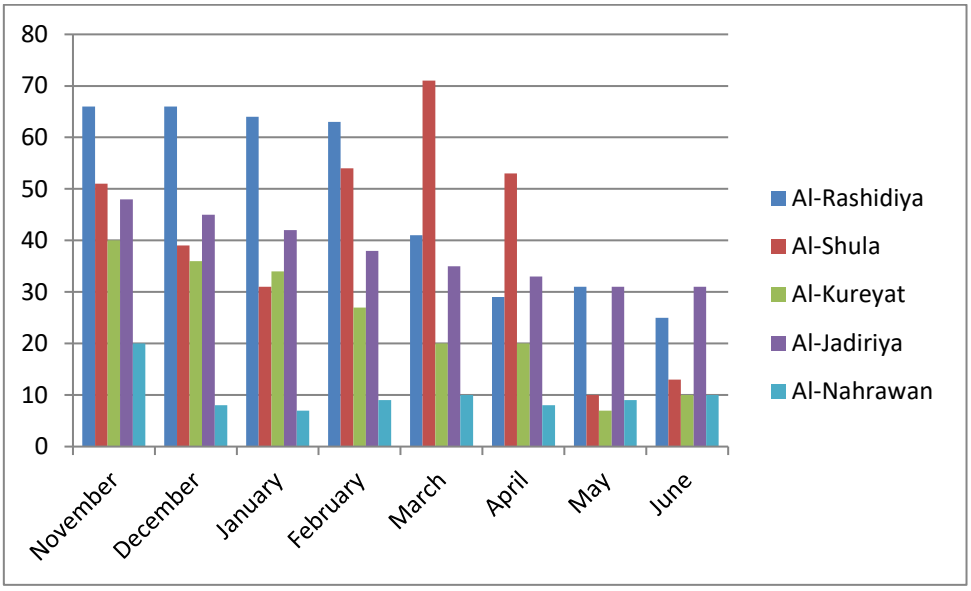

Figure (1) Morbidity rates in the regions where the survey was conducted during the months of samples collection.

The incidence of the disease increases in the wet months and with moderate temperature tilted to cold, which is usually the flowering period for most plants and is considered the period of the main activity of honey bees, as the results of the survey showed an increase in the severity of the disease in Months with a moderate climate inclined to cold and humid, which is the period of the spring season, and the results showed that the lowest incidence of infection was in the hot dry months.This results were consistent with that of Higes*et al.* (2007) who reported thatNosemosis infection significantly is affected by temperature change. Infection is directly proportional to temperature around the beehives and both agent of nosemosis disease shows 99% vitality at 33 ºC, and also between 25 ºC and 37 ºC temperatures.

Environmental conditions also strongly influence many parasitic relationships and, regardless of the effects of altitude, flora and colony management, in warm countries like Spain the influence of temperature on the consequences of *N. ceranae* has been observed (Martín-Hernández *et al.*, 2012).

The results of light microscopy of the samples taken from the surveyrevealed the presence of large numbers of *Nosema* spp. spores. *Nosema* spores were oval shapedthat belong to the fungus *Nosem spp.* (Figure2).

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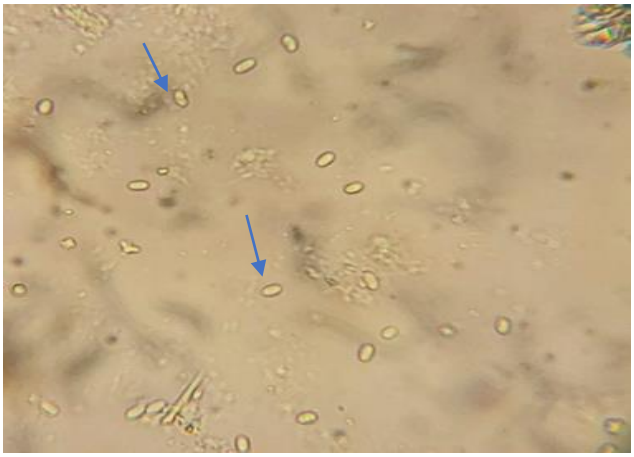

Figure (2)*N.spp*.spores under light microscope (40x)

Although the differences in spore size between *N. ceranae* and *N. apis*are not immediately apparent in light microscopy, there is a consistent difference. Spores of *N. ceranae* are clearly smaller compared to spores of *N. apis*. Fresh, unfixed spores of *N. apis* measure approximately 6 x 3 µm (Zander and Böttcher, 1984).whereas, fresh spores of *N. ceranae* measure approximately 4.7 x 2.7 µm,although there is a slight overlap, with the smallest *N. apis*spores being smaller than the largest N. ceranae spores, the average spore size of *N. apis* is approximately 1 µm larger in length. (Fries *et al*., 1996).

Also Chen *et al.,* 2009 declared that the difference in the size of spores between these species is clear, it may still be difficult to detect the difference in routine diagnosis of infected bees using light microscopy,this is particularly true because mixed infections of both species can occur even in individual bees (Burgher-MacLellan *et al*., 2010).

All of the bees that were found to be positive by microscopic examination were also found to be positive by the PCR technique. Following agarose gel electrophoresis(Fig. 3).

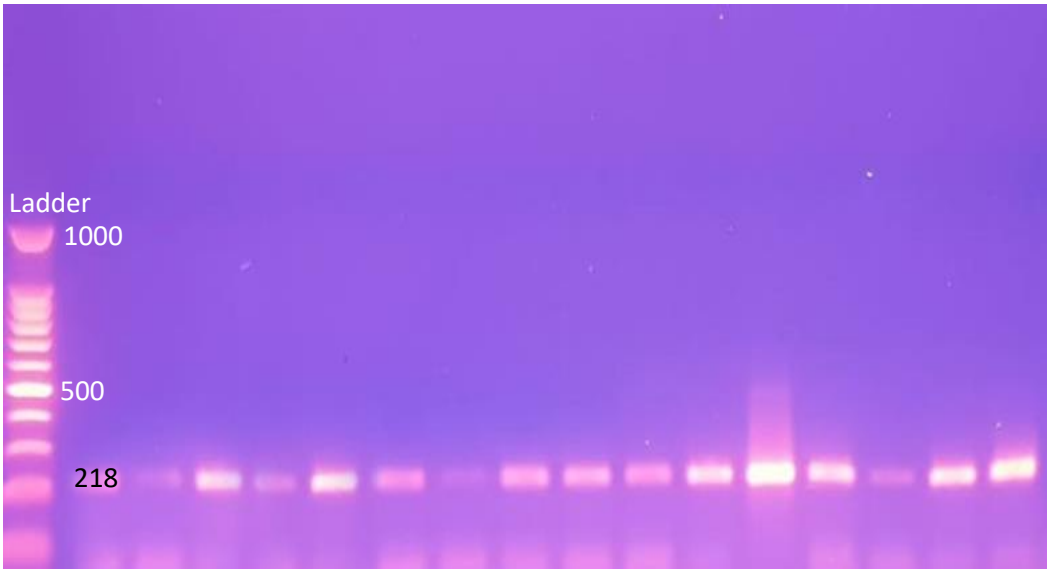

Figure (3) : Gel Electrophoresis of PCR Product for Detection of *16S rRNA*gene fragments of *N. cerana*

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The results of this study showed that *N. ceranae* was the only Nosema species found to infect honey bees, However, the spores of *Nosema spp*. are very similar to each other morphologically, and hence such distinctions between them are quite difficult with traditional microscopic methods.

The diagnosis of the disease is made by traditional microscopic methods and some molecular techniques (Chauzat*et.al*., 2007). Therefore, molecular biological techniques such as PCR, multiplex-PCR, DNA sequencing, and PCR-RFLP are used in the diagnosis of infections or mixed infections caused by the two pathogens (Ütük*et al*., 2010).

Webster *et al*., 2008noted that PCR analysis was more sensitive than examination for spores by light microscopy in detecting Nosema infection,different researchers who used the same technique determined *N. ceranae* for the first time in European honeybee colonies in Canada and the central USA (Williams *et al*., 2008), France (Chauzat*etal.,* 2007), Spain (Higes*et al.*, 2006), and the United States (Chen *et al.*, 2008).

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