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Article

Molecular detection of Nosema ceranae and determining the percentage of colony infection and workers in some provinces of Iraq

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ABSTRACT

Nosema ceranae is one of the most serious diseases affecting adult honey bees worldwide. It is referred to as a silent killer because there are no distinguishing symptoms. So, Polymerase chain reaction(multiplex PCR) was used to identify the rRNA marker for Nosema ceranae in 2700 Apis mellifera samples collected from three Governorates (AL-Najaf ، Wasit ، Diyala) in Iraq over six months in 2021-2022. DNA sequencing analysis of the N. ceranae samples revealed no intra-specific variation in the 219 bp of the 16s SSU of N. ceranae from Iraq, and the strain was recorded in NCBI with number OM985045.1 ، OM985047.1 and OM9790821.1 respectively. Before using PCR, the samples were examined using an optical microscope. It was discovered that the high percentage of infection in colonies in Autumn 2021 was 70.3 % in Diyala in October, the low percentage was 47.5% in Wasit in December, and The high percentage in workers was 92.7 % in October in Diyala. The low percentage is 52.0% in Wasit in December. The highest number of spores per bee was recorded in Diyala in November it was $86.9x10^5$ spores ، the lowest number was $49.5x10^5$ spores recorded in Wasit in October. In Spring 2022, the high percentage in colonies was 54.4% in April in Al-Najaf in May. The low percentage was 32.3% ، the high percentage in workers was 84.0% in March in Diyala, and the low percentage was 38.7% in May in AL-Najaf ، The high number of spores was recorded in November in Diyala was $86.9x10^5$spores. The lowest number was $48x10^4$spores in May in Al-Najaf.

Keywords: Nosema ceranae, colonies infection, Honey bees

INTRODUCTION

Honey bees are important pollinators for a variety of crops [1]. The growing global demand for this pollination service [2, 3]. Honey bee health has thus received considerable attention, particularly in light of reports of dramatic and unexpected colonies losses between (2000-2010) [4]. Several pathogens infecting honey bees have been identified as high-risk stressors, reducing individual host lifespan and having a

negative impact on the colony [5]. Troubles bees' health has contributed to a reduction in colony supply [6]. Nosemosis is one of these troubles caused by two distinct microsporidia that harm honey bees (Apis mellifera L.): Nosema apis, which is responsible for nosemosis type A, and Nosema ceranae, which is responsible for nosemosis type C [6]. This disease affects adult honey bees world‹ wide It's caused by a Microsporidia group Nosema spore-forming unicellular parasite [7]. Nosema apis [8] ‹ Nosema ceranae [9], and another species, Nosema neumanni, have been discovered in Uganda, honey bees [10]. Nosema ceranae is a species of Nosema spp originally discovered in the Asian honey bee Apis ceranae [9]. The first infection of Nosema ceranae in Apis mellifera colonies from Taiwan after a short period ‹ was reported in 2005 [11]. N.ceranae reported in Iraq for the first time in 2018 with the number Mk072950.1 in NCBI [12]. The spores of Nosema spp. have an ovoid shape. Nosema ceranae spores are around 2.7- 4.7 µm long, which makes them smaller than Nosema apis spores, which are about 3-6 µm long [13].

Infection with Nosema apis generates a fast-acting, short-term disease; however, this has not been the case with Nosema ceranae, which has been linked to nonspecific symptoms such as slow population loss, increased autumn and winter colony mortality, and low honey product [6]. The effects of Nosema ceranae on Apis mellifera colonies vary depending on the study and geographic location [14]. In the United States, colonies affected by colony collapse disorder (CCD) had a slightly higher prevalence and load of N. ceranae than control colonies [15]. Infection with N. ceranae can cause trouble. Chemical communication in infected colonies (based on pheromonal signals) is disrupted between workers and between workers and infected queens [16]. One of the most used approaches for detecting and quantifying Nosema spp. is microscopy [13]. The advantages of this method include the ability to examine it immediately and the ability to estimate infection strength by counting spores using a hemocytometer (Malassez counting chamber) and a light microscope at 400 magnification [17, 18]. However, because different Nosema species have comparable spore diameters [19], this method could be more effective for distinguishing species.

Furthermore, because of the changes in size and shape among the internal life stages of Nosema spp, identifying all life stages using a typical light microscope is difficult [20]. As a result, microscopy is not an effective tool for detecting types of Nosema spp. At low infection levels, [21]. The Polymerase Chain Reaction (PCR) detection method has been widely utilized to diagnose this pathogen infection [22]. Based on variations in the size of conventional PCR amplicons, PCR detection methods for Nosema spp. have been developed for N. ceranae and N. apis identification [23]. Later, a semi-quantitative triplex PCR assay was developed to estimate spore counts [24], and quantitative real-time PCR was developed to detect N. apis and N. ceranae simultaneously using multiple primers in one reaction [25]. Despite long research, the mechanisms underlying N. ceranae infection's high prevalence and pathological effects in A. mellifera populations remain ununderstandable [26, 27]. Due to the fast dispersion and high losses in bee colonies in Iraq, the study was aimed to diagnose N.ceranae by microscopic and PCR to prove the disease and determine the percentage infection of colonies and workers, in addition to calculating the spores of N.ceranae in some provinces in Iraq.

MATERIALS AND METHODS

Samples were taken from honey bees from three Provinces in Iraq and from three locations in every Province between Autumn 2021 and Spring 2022 in the middle of every month to determine the percentage of infection and the abundance of N. ceranae in samples of bees in apiaries of this regions. Microscopic examination of the digestive system of the worker, which preparation suspension drop of spores to be examined under the optical microscope ‹ It used samples of N. ceranae spores

as a standard, which was determined using optical microscopy at 400x -1000x [28, 29] ، the microscopic technique is based on a procedure created by Cantwell 18; OIE 2018). In a nutshell, the technique is crushing honey bee abdomens in ultrapure water with a mortar and pestle at a rate of one milliliter (1 ml/bee).To remove big debris, the solution is filtered through two layers of muslin (thin, loosely woven cotton cloth) and centrifuged for six minutes at 800 xg for six minutes to remove heavy debris and clean up the spores. To restore the initial dilution of 1 ml/bee, the pellets are re-suspended in a homogenous suspension. Finally, the sample is examined under a microscope in a calibrated hemocytometer to detect and count N. ceranae. spores. The quantitative (number of N. ceranae. spores per ml, i.e., per bee, based on dilution) and qualitative analytical It should be emphasized that the visual counting approach has a significant level of measurement uncertainty. It varies depending on the number of N. ceranae spores and particles (such as pollen and yeast) present in the bee's digestive tract, which can make N. ceranae spore detection and identification difficult.

Samples preparation for PCR

Each apiary's 25 adult, dead honey bees' abdomens were macerated in 10 mL of distilled water (PCR grade), and the resulting suspension was filtered before being centrifuged at 800g for six minutes. 200 µL of newly made germination buffer (containing 0.50 M sodium chloride, 0.50 M sodium hydrogen carbonate, and ortho-phosphoric acid to adjust the pH to 6.0) was used to promote spore germination, which was then incubated at 37 °C for 15 min [30].

DNA extraction

Genomic DNA was isolated from plant root samples according to the protocol of ABIOpure Extraction

Polymerase chain reaction (PCR)

According to the OIE Terrestrial Manual 2008 for N.ceranae, 16S rRNA was amplified using a PCR kit (Macrogen Korea) in a Thermal cycler (Thermo Fisher Scientific, USA). N. ceranae. The first 50 µL reaction mixture for multiplex PCR amplification of incomplete 16S rRNA gene fragments 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; 500Mm KCL) and 1 Unit of Taq polymerase. The conditions of amplification are an initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 56 °C for 30 extension at 72°C for 30 sec, and a final extension step of 7 minutes at 72 °C. 1.5 % agarose gel electrophoresis was used to separate the PCR products. Safe stain (Bromega, USA) was used to stain the products, and UV transillumination was used to see what was left behind. PCR products were separated by electrophoresis on a 1.00% agarose gel stained with a safe stain (Bromega, USA). It was visualized by ultraviolet radiation. In this study, primers targeting the 16S rRNA gene of the small subunit of N. ceranae were used [13].

Statistical analyses

Statistical analyses were performed using a randomized complete block design with the Genstat 12.1 program to determine the differences between the three provinces in the percentage of colony infection, the percentage of infection of workers and the numbers of N.ceranae spores in different apiaries. Significant variations exist in colonies infection percentage, workers infection and number of spores between the provinces.

RESULTS AND DISCUSSIONS

The result of microscopic and PCR diagnostics showed that the pathogen was Nosema ceranae recorded in all provinces of Iraq studied. We recorded three isolates of N.ceranae in Al-Najaf, Wasit and Diyala, which of N. ceranae were recorded in NCBI with numbers OM985045.1 ، OM985047.1 and OM9790821.1. The results of the study showed that honey bee colonies of infection in N.ceranae were recorded in all Provinces (Najaf, Wasit, and Diayala), and the percentage of infection was high in autumn but less than in the spring season. The highest percentage of infection in Al- Najaf Provinces was 60% and 40 %, in Wasit Provinces 53% and 40 % and in Diayala reached 73.3% and 54.4% in autumn 2021 and spring 2022, respectively [Table 2).

Months		Provinces			Mean
		Al-Najaf	Wasit	Diyala	
October	2021	72.00	62.70	96.00	76.90
November	2021	76.00	58.70	89.30	74.67
December	2021	76.00	52.00	84.00	70.67
March	2022	73.30	52.00	80.00	68.43
April	2022	65.30	54.70	57.30	59.10
May	2022	38.70	37.30	45.30	40.43
Mean		66.88	52.90	75.32	
Factors		Provinces	Months	Interaction	
LSD 0.05		5.06	7.16	12.40	

Table 2. Percentage infection of Colonies with the pathogen N. caranae in different apiaries in three provinces in different periods

Also, the percentage of infection of workers in infected hives was different between all provinces. The study results showed that the highest percentage of infection workers in Al-Najef Province was 76% and 73.3%. In Wasit Province, 61.3% and 54.7%; in Diyala Province, 96% and 80% in autumn 2021 and spring 2022, respectively (Table 3).

Months		**Provinces**			**Mean**
		Al-Najaf	Wasit	Diyala	
October	2021	60.00	53.30	73.30	62.20
November	2021	60.00	46.70	66.70	57.80
December	2021	53.30	46.70	60.00	53.33
March	2022	40.00	33.30	53.30	42.20
April	2022	40.00	40.00	53.30	44.43
May	2022	20.00	26.70	40.00	28.90
Mean		45.55	41.12	57.77	
Factors		Provinces	Months	Interaction	
LSD 0.05		10.73	15.17	26.28	

Table 3. Percentage infection of workers with the pathogen N. caranae in different apiaries in three Provinces during different periods (25 workers)

On the other hand, the results showed that the number of spores in infected workers differed between the Provinces studied. The highest number of spores in infected workers in Al-Najaf Province reached 6200000 and 2580000 spores/ml; in Wasit Province, 5870000 and 2260000 spores/ml; in Diayala Province, 896000 and 3230000 spores /ml in autumn 2021 and spring 2022, respectively (Table 4)

Months		Provinces			Mean
		Al-Najaf	Wasit	Diyala	
October	2021	5717000	4950000	8117000	6261333
November	2021	6200000	5567000	8683000	6816666
December	2021	5050000	5867000	6383000	5766666
March	2022	2583000	2267000	3233000	2694333
April	2022	1000000	933000	1550000	1161000
May	2022	483000	550000	750000	594333
Mean		3505500	3355666	4786000	
Factors		Provinces	Months	Interaction	
LSD 0.05		270000	381900	661400	

Table 4. Number of the spores of the pathogen N.ceranae in workers in different apiaries for three Provinces in Iraq during different periods (number of spores/ ml).

The temperature and Relative humidity were different between Provinces of Iraq. The decreasing temperature and increasing relative humidity in the autumn may be responsible for increasing the infection N.ceranae Compared to increasing temperature and decreasing Relative humidity in the spring (Table 5).

Date	Province						Mean	
	Al-Najaf		Wasit		Diyala			
	Temp	humidity	Temp	humidity	Temp	humidity	Temp	humidity
October	21.5	37.2	18.6	36.2	17.5	42.3	19.2	38.6
November	19.2	41.0	13.1	56.9	13.8	57.6	15.4	51.8
December	14.9	45.6	12.0	57.2	11.6	59.3	12.8	54.0
March	20.5	38.3	17.8	35.1	16.0	41.2	18.1	38.2
April	21.8	33.7	19.9	30.8	18.2	35.0	20.0	33.2
May	28.0	23.9	26.5	28.6	24.8	25.8	26.4	26.1
Mean	21.0	36.6	18.0	40.8	17.0	43.5	18.7	40.3

Table 5. Average Temperature and Relative humidity during the study in some Iraqi provinces

DISCUSSION

The differences in infection in Colonies, Workers, and several spores depend on the management of the beekeeper, the source of Primary infection, Environmental conditions, especially temperature and Relative humidity, the Source of feeding and sugar solution, and the Source of Pollen. All these factors may be significantly aggregated for dispersion and increased infection in Pathogen N.ceranae. This study agreed with some studies done in Iraq. Studies have shown the presence of pathogens in the colonies of honey bees has been Iraq's infection rate in some areas of Baghdad, ranging from (7-66%) [31]. This agrees with [12] when recoding the disease for the first time in Iraq, where the infection rate ranges from 50-100%.

Infection with N. ceranae has now been confirmed on every continent. Recent reports have confirmed changes in Nosemosis clinical and epidemiological patterns, indicating that N. ceranae is one of the most prevalent pathogens in honeybees around the world [32] and is extremely virulent for its new host, Apis mellifera [20]. According to a study conducted in Van province, Turkey, the prevalence of Nosemosis is 32.50 %. The only Nosema species found to infect honeybees was N. ceranae, and no N. apis or mixed infections were found in the samples [33]. The prevalence of N. ceranae in different climatic zones was significantly different in this study, with the semi-humid climate 71.00% having the highest prevalence, followed by very humid 68.10% and humid 53.80% climates. The high prevalence of infection in humid places is consistent with a study conducted in Turkey, which found that moisture has a greater influence on Nosema ceranae in areas with higher humidity, and humidity has been recognized as a critical component for Nosema species [34]. Temperature affects the multiplication rates of both parasites (N. ceranae and N. apis). However, N. ceranae grows quicker in slightly higher temperatures than N. apis [35]. According to the results of this study, a correlation was observed between the increase in the number of spores ، and the increase in the percentage of infection and between increased Relative humidity and moderate temperatures ، which agrees with the study [31]. The isolates recorded in NCBI with number OM985045.1 ، OM985047.1 and OM9790821.1 (Figure 1)

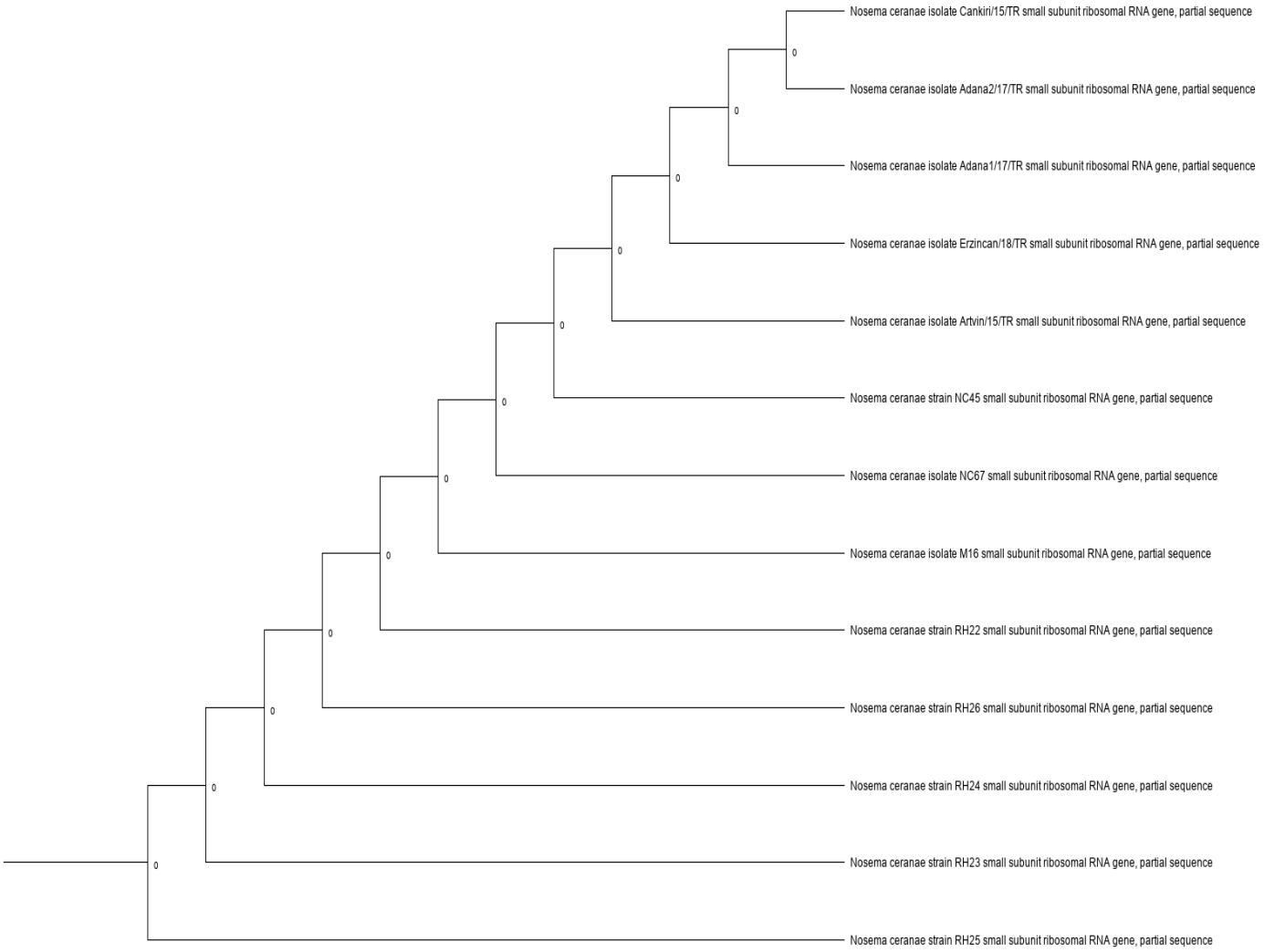

Figure 1. Trees of Nosema ceranae and relationship with other isolates recorder in NCBI.

CONCLUSION

The pathogen N.ceranae was recorded in three provinces (Najaf, Wasit and Diyala) with a high spread and serious damage, and it is expected to spread in most of the provinces of Iraq where bee hives are located, so it is necessary to pay attention to follow-up and control the disease to preserve the honeybee colonies from mortality.

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