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The First Report on the Prevalence of Nosema spp. Disease in Honeybees (Apis mellifera L.) in Sulaymaniyah City, Kurdistan Region, Iraq.

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Abstract:

The microsporidian parasite *Nosema ceranae* and *Nosema apis* are a global problem in honeybee *Apis mellifera* L. populations and it is known to cause winter mortality. The current study was aimed to investigate the prevalence of *Nosema* disease and their geographical distribution in Sulaymaniyah governorate. For this purpose, between April and June, a total of 34 samples have been collected from different apiaries of city center, 18 surrounded localities, and districts of Sulaymaniyah governorate. Light microscopic examination was used to identify *Nosema* spp. spores in both asymptomatic and symptomatic worker bees. Nosema Spore load also measured using hematocytometer method.

Results of microscopical examination exhibited the presence of *Nosema* spp. spores in 91% (31/34) samples collected from geographically varied regions. Apiary from Shorsh region has been detected as a negative sample for Nosema disease, which is situated 67 Km south of the city. Moreover, microscopic enumeration of spore load was revealed that the city center and suburb regions with the maximum spore loads (Log_{10} 7.46±0.09 and 7.10±0.11 / ml, respectively), followed by other localities and district regions ranging (Log_{10} 6.82±0.09 - 6.00±0.05 / ml). However, the lowest spore load concentration has been detected in both Zrguez and Sangaw regions with (Log_{10} 5.63±0.13 and 5.55±0.13 / ml, respectively).

This finding for the first time suggests that *Nosema* spp. is dominant in post-winter *Apis mellifera* worker bees and it can be considered as a real threat of reducing bee colonies and production of honey in Sulaymaniyah governorate. Further molecular studies should be achieved to determine the most prevalence species in the city and to control nosemosis in honeybee populations.

Keywords: Honeybees, *Nosema* spp**,** Microscopic enumeration, Nosema Spore load.

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الملخص:

يعتبر الطفيلي الميكروسبوريدي *Nosema apis* and *Nosema ceranae* مشكلة عالمية في مجتمعات *نحل* العسل *Apis mellifera* L.،من المعروف انة يتسبب في وفيات الشتاء، هدفت الدراسة الحالية إلى معرفة مدى انتشار مرض النوزيما وتوزيعه الجغرافي في محافظة السليمانية. لهذا الغرض، تم جمع 34 عينة من المناحل مختلفة في وسط المدينة، بين شهري نيسان وحزيران ،في 18 منطقة من المناطق المحيطة بالمحافظة. تم استخدام الفحص المجهري لتحديد جراثيم النوزيما في كل من الشغالات المصاحبة بالاعراض والشغالات عديمة بالاعراض، تم قياس وعد سبورات النوزيما أيضًا باستخدام hematocytometer method.

وقد اظهرت نتائج الفحص المجهري انة بنسبة المئوية لجراثيم النوزيما في العينات التي تم جمعها من المناطق الجغرافية المتنوعة بلغت 91٪ (34/31). ان منحل شورش الذي يقع على بعد 67 كم جنوب المدينة السليمانية خاليا من الاصابة بمرض النوزيما، علاوة على ان اقصى عدد لجراثيم مرض النوزيما في عينات وسط المدينة تراوحت (7.46 Log10 ± 0.09 و 7.10 ± 0.11 / مل) على التوالي متبوعا بالمواقع والمناطق الاخرى والتي تراوحت (6.82 Log10 ± 0.09 - 6.00 ± 0.05 / مل). و اوضحت نتائج الدراسة ان ادنى نسبة لجراثيم المرض في المنطقي زركويز و سنكاو بلغت (5.63 Log10 ± 0.13 و 5.55 ± 0.13 / مل)على التوالي.

كشفت نتائج البحث ولأول مرة ان مرض النوزيما يعد سببا رئيسيا لانخفاض عدد شغالات نحل العسل خلال فصل الشتاء، ويمكن اعتباره تهديدا حقيقيا لانهيار مستعمرات النحل وانتاج العسل في محافظة السليمانية. يجب اجراء المزيد من الدراسات الجزئية لتشخيص الانواع الاكثر انتشارا في المدينة والسيطرة على مرض النوزيما في تجمعات نحل العسل.

الكلمات المفتاحية: مرض النوزيما، نحل العسل، العد المجهري، حامل ابواغ النوزيما.

پوختە:

مشەخۆری مایکرۆسپۆریدی *Nosema ceranae* و *Nosema apis* بە کێشەیەکی جیهانی هەنگ دادەنرێت، *Apis mellifera* L.، ناسراوە بە هۆکاری مردنی زستانە، ئامانجی توێژینەوەکەمان زانینی ڕادەی دابەشبوونی جوگرافی نەخۆشی نۆزیما بووە لە پارێزگای سلێمانی. بۆ ئەم مەبەستەش ٣٤ نمونە لە هەنگەڵانە جیاوازەکان لە نێوان مانگی نیسان تا حوزەیران لە ناوەندی شارو لە ١٨ ناوچەی دەوروبەری پارێزگاکە وەرگیران. پشکنینی وردبینی بەکارهێنرا بۆ دیاریکردنی سپۆرەکانی نۆزیما لە هەردوو پاڵە ي هەلگري نیشانە و بێ نیشانە، هەروەها ژماردنی سپۆرەکانی نۆزیما بە بەکارهێنانی ڕێگەی هیماتۆسایتۆمێتەر پێوانە کرا.

ئەنجامی پشکنینی وردبینی دەریخست کە ڕێژەی سپۆرەکانی نۆزیما لە نمونەکانی کۆکراوە لە ناوچە جوگرافییە جیاوازەکان گەیشتە 91% (34/31). هەنگەڵاني شۆرش کە دەکەوێتە ٦٧ کم باشوری شاری سلێمانی، دوور بوو لە نەخۆشی نۆسیما، جگە لەوەش زۆرترین ژمارەی سپۆري نۆزیما لە نمونەکان لە ناوەندی شار لە نێوان (7.46 Log10 ± 0.09 و 7.10 ± 0.11 / مل) دوابە دواي یە ك ,دواتر شوێن و ناوچەکانی تر دێن، کە لە نێوان (6.82 Log10 ± 0.09 - 6.00 ± 0.05 / مل). ئەنجامەکانی توێژینەوەکە دەریانخست کە کەمترین ڕێژەی سپۆرەکانی نەخۆشی لە قەزاکانی زرگویز و سە نگاو(5.63 Log10 ± 0.13 و 5.55 ± 0.13 / مل)، دوابە دواي یە ك.

ئەنجامی توێژینەوەکە بۆ یەکەمجار دەرکەوت کە نەخۆشی نۆزیما هۆکارێکی سەرەکییە بۆ کەمبوونەوەی ژمارەی پاڵە کانی هەنگ لە وەرزی زستاندا، هەروەها دەتوانرێت بە مەترسییەکی ڕاستەقینە هەژمار بکرێت بۆ ڕووخانی کۆلۆنی هەنگەکان و بەرهەمهێنانی هەنگوین لە پارێزگای سلێمانی. پێویستە توێژینەوەی زیاتر ئەنجام بدرێت بۆ دەستنیشانکردنی باوترین جۆرەکان لە شارەکەدا و بۆ کۆنترۆڵکردنی نەخۆشی نۆزیما لە کۆلۆنی هەنگ.

کلیلە وشە: نەخۆشی نوزیما، هەنگي هەنگوین، ژماردن بە وردبین، هەلگري سپۆرەکانی نوزیما.

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1. Introduction:

The honey bee (*Apis mellifera* L.) is the insect species act as one of main pollinators in the natural environment [1, 2] , and with many valuable products for human food and treatment[3, 4]. A variety of biotic and abiotic pressures have been exposed to honey bee larvae and adults in recent years such as, pesticides, infection by viruses, bacteria, fungi, and parasites[5]. The combination of existing pressures that can weaken bees' defense mechanisms and destroy their social structure, and increasing susceptibility of colonies to the diseases and collapse[6] . Nosemosis is one of widespread fungal disease affecting honey bees, two main species of microsporidian including *Nosema apis* [7]and *Nosema ceranae* [8] have been recognized with the potential ability to kill individual honey bees (adult worker bees, drones, and queens) and make them more susceptible to infections by other pathogens[9] Furthermore, nosemosis due to several consequences such as shortening the lifespan of worker's honey bees, reducing colony productivity [10], changing the pheromone production by honey bee workers and queens that could lead to queen supersedure[11].

During Nosema infection generally happens when spores are consumed by honey bee colonies with water or food. In a bee's midgut, 30–50 million spores was determined within 2 weeks of the initial infection[12] Eventually, the spores leave the bee in its excrement and spread to new bee colonies through feeding and cleaning operations[13, 14]. A close relationship between spore count and the degree of infection has been established for this disease and it has been used as a parameter to evaluate a colony's need for treatment[15] Nosema pathogens cannot be cultured, and seasonably available[12] .Therefore, several studies considered that spore counting under light microscopic is crucial for identification and differentiation between of that *N. apis* and *N. ceranae* spores[16] .

In the previous study, it was believed that N. apis pathogen can cause disease in European honey bee, A. mellifera, and N. ceranae only affect the Asian honey bee, A. cerana[8] .Several studies confirmed that N. ceranae has the ability to infect A. mellifera over the world[17] other studies emphasized that N. ceranae causes nosemosis in European honey bees more than that in warmer climates caused by N. apis [17]Currently, the distribution of N. ceranae and N. apis is practically identical, and the two species can co-infect honey bees[18].

The prevalence of the disease is seasonal dependent, the N. apis infection normally peaks in the spring and declines in both summer and fall in most places[19] . The variations in climatic conditions between different geographic regions may be one of these factors. From 2015 till to now the entire world suffers from this Epidemic disease. The first detection of N. ceranae is variable among the countries such Turkey in 2010[20], Iran in 2011[21], Jordan in 2014 [22] Saudi Arabia in 2016[23], Azerbaijan in 2016[24] Recently in 2020, the first detection of N. ceranae was reported in the northern region of Baghdad (Capital of Iraq)[25]. Sulaimaniyah city is consider one of the leading cities in Iraq for honey production, however, the consequences of several honey bee diseases decreased the level of honey production and economically affected the apiculture[7, 8]. Therefore, the evaluation of infected apicultures with symptoms of Nosema disease is fundamental to determine the prevalence of the disease in the area of study. In this perspective, the main goal of this study was to investigate the prevalence of Nosema spp. in honeybee of different localities and districts of Sulaymaniyah city. Moreover, to find the correlation between spore load and severity of illness per each selected honey colony.

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2. Materials and methods

According to our knowledge this is the first investigation on *Nosema* spp. existence in Sulaymaniyah governorate honey bees. For this purpose, from April and June 2022, thirty-four 34 apiaries have been selected from 18 regions of Sulaymaniyah governorate, Kurdistan region, north of Iraq. Special precautions were taken prior sample collection such as none of the honey bee colonies were treated against Nosema infection for at least six months and the adult bees were mainly targeted for diagnosis of diseases with no sign of fly [26] . Four colonies per each apiary (two strong hive and two weak hive), and in each hive fifty adult worker honey bees were collected from peripheral combs in the brood area, without hatching bees, or in a super above a queen excluder to avoid sampling newly emerged, uninfected bees[27]. The honey bees were placed in the small box with cribriform cover in order to permit the bee for breathing until arrive to the lab for analysis.

2.1. Microscopic Identification

Samples are generally prepared from crushed abdomen of infected or suspected bees with Nosema disease and examined under microscope (figure 1). Despite the presence of minor differences in the structure and spore size of *N. ceranae* and *N. apis*. However, it still difficult to differentiate between them when routinely diagnosed under light microscope.

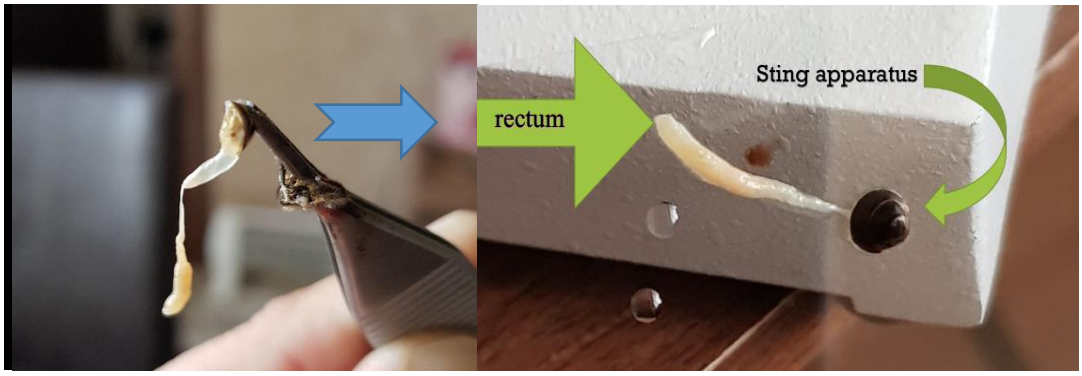

Figure 1: honey bee rectum infected with Nosema disease.

2.2. Microscopic enumeration of Nosema spores

Using a normal hemocytometer is the simplest method for counting *Nosema* spp. spores. According to the protocol, which was previously described by [28] .The entire digestive tract of 1-2 honeybees were pooled, squashed, mixed with 1 ml sterilized distilled water (SDW) and thoroughly homogenized with SDW using mortar and pestle. Subsequently, a drop of homogenized digestive tract of bees was transferred to the hemocytometer chamber. After few second of distribution throughout the chamber a cover slip was placed and examined under light microscope using magnification power (400X). Finally, spore counting has been done through counting spores in each five squares of the hemocytometer. The spore load per bee in the sample, the obtained average number must be multiplied by 50000 [29] (figure 2).

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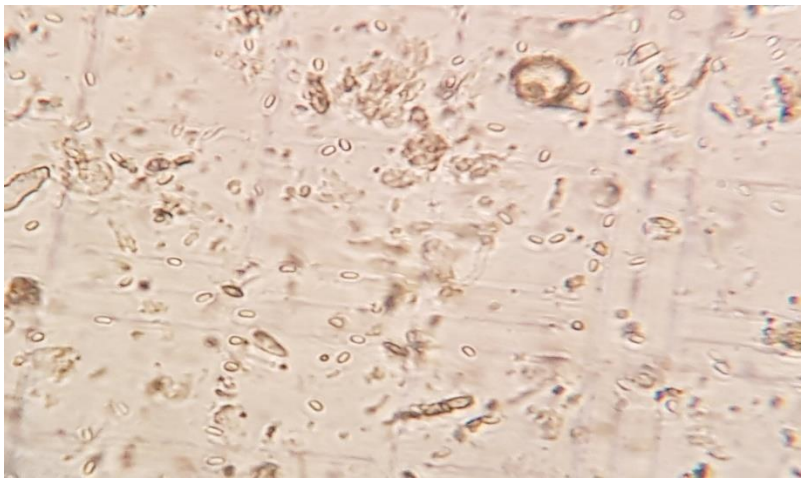

Figure 2: Spores observed for counting in five of the 16 observation fields of a hemocytometer. The observations were performed at 400× magnification under a light microscope. The real concentration of the spore solution was calculated based on the spores counted using each concentration of diluted spore solution, from total count per 5 large squares (80 small squares) x 50,000.

2.3.Statistical analysis:

GraphPad prism version 9 (Graphpad, California, USA) was used to analyse all data. The experimental results were expressed as mean ± standard deviation of the mean (SD). Groups were compared by analysis of variance using One-way, and descriptive statistical analysis.

3. Results:

For the purpose of this survey, a total of 34 samples were collected from apiaries *Apis mellifera* distributed in the central part and surrounded areas of Sulaymaniyah governorate. Results of light microscopic examination exhibited that majority of apiaries 91% (31 / 34) under the study was infected by *Nosema* spp. (Figure 3). Moreover, microscopic enumeration of serial spore dilution revealed that the Sulaymaniyah center and suburb with the highest spore load (Log10 7.46±0.09 and 7.10±0.11 / ml, respectively), followed by all other regions around Sulaymaniyah province, which was exhibited the greatest variation ranging between (Log10 6.82±0.09 - 6.00±0.05 / ml) (table 1). However, lowest spore load concentration has been detected in both Zrguez and Sangaw with (Log10 5.63±0.13 and 5.55±0.13 / ml, respectively) (table-1) (Figure 4). Surprisingly, Nosema spores has not been detected in Shorsh the north part of the Sulaymaniyah city, which is about 67 km away from the city center. It can be concluded that almost all the cases included in this survey approached the hazard zone of Nosema infection.

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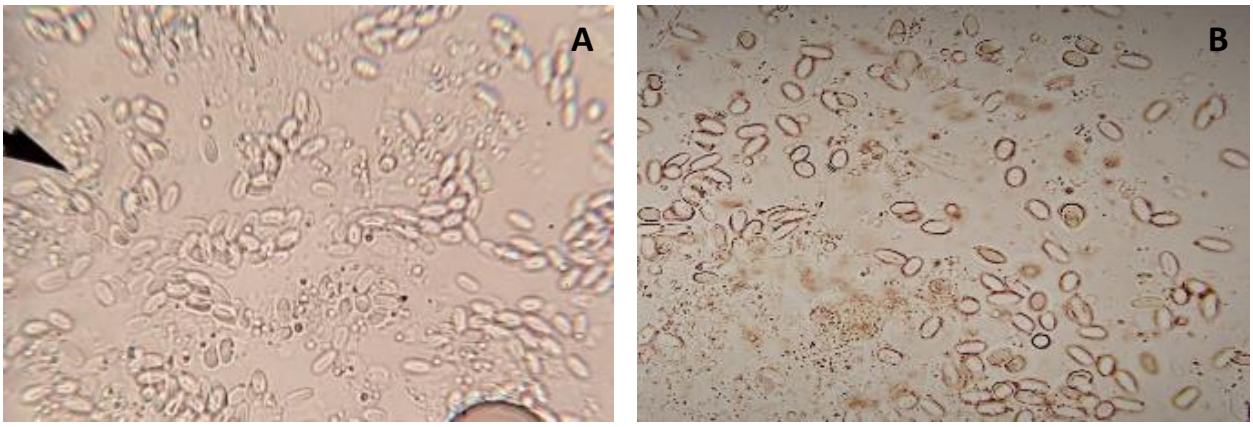

Figure 3: Microscopic examination of *Apis mellifera* infection **(A)** Sever infection by spores of *Nosema* spp. **(B)** Moderate infection by spores of *Nosema* spp. The observations were performed at 1000× magnification under a light microscope.

Table 1: The result of spore load of *Nosema* spp. of the honeybee colonies in Sulaymaniyah governorate. The values are the mean±Standard deviation of variable numbers of inspected apiaries during April and June, 2022.

Place name	**Number of apiaries**	**Mean of Spore number/bee**
Sulaymaniyah center	3	7.46±0.09
Sulaymaniyah suburb	3	7.10±0.11
Qalladze	2	6.82±0.09
Raniah	2	6.73±0.13
Qaradagh	2	6.64±0.15
Penjween	2	6.61±0.02
Saburawa	1	6.57±0.21
Darbandikhan	2	6.56±0.04
Sharazwr	2	6.51±0.10
Chwarta	2	6.33±0.07
Mawat	2	6.22±0.03
Saidsadq	2	6.20±0.06
Chamchamal	2	6.19±0.13
Bazyan	1	6.14±0.09
Dukan	1	6.05±0.05
Halabja	2	6.00±0.05
Zrguez	1	5.63±0.13
Sangaw	1	5.55±0.13
Shorsh	1	0.00±0.00

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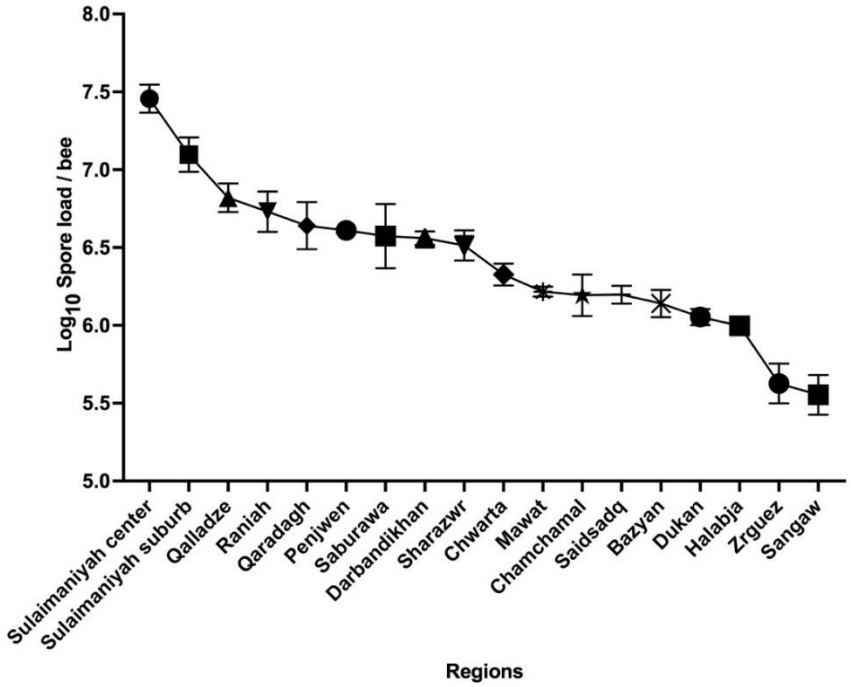

Figure 4: Analysis of microscopic enumeration of purified Nosema spores. The real concentration of the spore solution was calculated based on the spores counted using each concentration of diluted spore solution. Values are derived from one to three apiaries; pooled sample of each apiary represent 40-45 honeybee individuals. Data (mean ± standard deviation) were analyzed using one-way ANOVA. Spores observed for counting in four of the 16 observation fields of a hemocytometer are shown. The white bar in the bottom right corner represents 20 μm, and the arrows indicate individual spores.

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4. Discussion:

Nosema spore-forming parasites, which attack the epithelial lining of the middle intestine of worker bees, queens, and drones, are the source of the bee disease known as nosemosis[30]. In many European countries during the past ten years, there has been a rise in microsporidian parasite infections in honeybees (*A. mellifera*)[31] The foraging force appears to be the most affected, despite beekeepers reporting an increase in colony deaths and low output in the same regions. They depart the colony but are too feeble to make it back, dying in the outside[21].

Although use of light microscope does not accurately differentiate between species of Nosema disease, however, it still the most standard practical methods to determine *Nosema* spp.

Obtained results were indicated that most of collected samples (91%) with Nosema infection and appeared as asymptomatic cases. In parallel to the positive cases of *Nosema* spp. in this survey the prevalence of *N. ceranae* has been detected in many other countries including 80.6% in Poland[32], 95%-97% in Hungary[33],15%- 100% in Turkey[34] ,41%-91% in Canada[35],63% in Italy56[36]% in Saudi Arabia[37], and 77% in Bulgaria[38].

This study also demonstrated variable spore loads among the sampling regions included in this survey. A study was confirmed that spore load during Nosema disease can reach 50000000 spores (Log_{10} 7.69 Spores / ml)[12] ,and one of infected individual bee is enough to infect the entire hive apiary if it die in the bee feeder or in the source of water. High or low spore loads can be present in infected asymptomatic bees[27]. Large numbers of dead bees inside the colony and stains of diarrhea at hive entrances are two obvious signs of infection by *N. apis*[39]. In contrast, *N. ceranae* nosemosis symptoms are less apparent (asymptomatic) and characterized by weak growth of colonies, large reduction in colony size, and the infection may be detected all year long[39].

The fluctuation of spore load among the regions used in this survey might be directly attributed to the variable climate factors of each region. Generally, inspection of this disease is usually performed on dead bee samples collected from the bottom of the hive in spring and winter times[40]**.** The survey was done in April and June, which is the temperature degree between 19 and 32℃. The most significant environmental factors that contributed to the infection in honeybees spreading fast are temperature and humidity in the area around the beehives[41]. It has been postulated that *N. ceranae* is more predominant in warmer countries[33] and more resistant to temperature fluctuations than infection caused by *N. apis*[42] Many studies confirmed that the maximum infection rate was recorded between 21 ºC and 35 ºC temperatures for *N. ceranae*, which is optimum temperature for survival of *N. ceranae* and *N. apis*[42] . *N. ceranae* has been actively isolated in summer and throughout the year, which makes infection by *N. ceranae* much more severe than *N. apis* infection[43]. Furthermore, It was emphasized that humidity of rainfall has a significant impact on Nosema infection [44], Malon[45] stated that in dry weather between (40-49 ℃), *N. apis* lost the viability of infection in 3 to 45 days. Humidity not only with the direct impact on spore survival of Nosemosis, however, it also increase the ability of spores to travel to other bees and cause infection[43] .

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The presence of huge numbers of Nosema spore load in relatively all inspected apiaries probably attributed to that the most of beekeepers during winter time are returning their apiaries from mountain to Sulaymaniyah center or suburb. While, the city is filled with more than 3300 beekeepers, which most of them dose not known about disease. Consequently, it facilitated the spread and increased the rate of reinfection of Nosema disease. As well as, most of the beekeepers are feed their hives outside the colony and treat the hives with fumagillin and other unknown drugs to prevent them from diseases, which may cause immune compromising in the honeybee's colony. Researchers described that fumagillin is a chemical that causes cancer, and fumagillin residue in honey poses a threat to people's health[46]. Surprisingly, one of the beekeepers prevented their apiaries from Nosema disease along the season through feeding the honeybees with a mixture of sugar and lemon juice. In support to this finding, previous study was approved that mixture of vitamins and amino acids in a dietary protects the honeybees from the severity of Nosema disease[47] .

It can be concluded that this study provides a basic information about the prevalence of Nosema disease in relatively all apiaries distributed in Sulaymaniyah governorate center and all other regions included in this study. Nosemosis makes a real threat on production of honey and reduction of bee colonies, which may cause high losses for apiculture and consequently in agriculture. Further molecular studies should be performed to determine the most distributed species and to control of nosemosis in honeybee populations.

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