Detection of *Nosema cerana* Fries in Egypt and its seasonal fluctuations

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Introduction

The honeybee colony consists of thousands of individual insects living together; such crowded conditions provide an ideal environment for the establishment and spread of diseases and parasites. The brood and adult bees are subjected to infectious diseases (El-Shemy, 1986).The Phylum Protozoa contains several pathogens that attack adult bees. The most important pathogen for honeybee is *Nosema* spp., which attack the epithelium of the mid-gut making a physiological starvation (Malone &Gatchouse, 1998).

The infection does not kill the bee outright, nor does it produce diagnostic symptoms that are readily visible. However, the disease causes severe losses to the beekeeping industry by damaging queen activity, retarding the development of package bees and over-wintered colonies, shortening the lives of worker bees and reducing the quantity of brood reared (Moeller, 1978; Kauko *et al*, 2003 and Webster *et al*, 2004).

Nosema disease is probably the most worldwide disease of the honeybee as its distribution shows from different author works as Nixon, 1982; Fries, 1989; Frazier *et al*, 1994; Malone &Gatehouse,1998; Refaei, 2005 and Butias *et al*, 2011.

The diagnosis of Nosema disease has been traditionally done by detecting spores of *Nosema* spp. through microscopic analyses (Shimanuki and Knox, 2000). However, with the recent finding that both *Nosema ceranae* and *Nosema apis* affect western honey bees (*Apis mellifera*), molecular techniques are required that can reliably differentiate between these different species of microsporidia, because the spores of the two *Nosema* spp cannot be reliably distinguished by their morphology (Fries *et al*., 1996). In addition, microscopic analyses are not as sensitive at detecting low levels of nosema infection as molecular methods, such as PCR, can be. Moreover, microscopic examination of nosema spores is costly, laborious and time-consuming.

Until the end of last century the nosema disease in honeybees is known to be caused by parasitic infection with *Nosema apis* z., whereas the last scientific researches in many countries indicated that the most dominance nosema in the world is *Nosema ceranae* (Martin-Herniandez *et al*, 2011; Stevanovic *et al*, 2011 and Medict *et al*, 2012).

The purpose of this study is to clarify the following points such as:

A- The fluctuation in natural nosema infection levels with respect to season, colony strength, harvesting the honey crop and the impact of oriental hornet in increasing the infection.

B-Diagnose the most dominance *Nosema* spp. infections in honeybees using multiplex PCR assay as a first study in Egypt.

Materials and Methods

This study was carried out at the apiary of Agricultural Experimental Station, Faculty of Agriculture Cairo University during two years 2011; 2012.The bees at this site were established, locally produced hybrids of *Apis mellifera* L. Molecular analysis was conducted in the Faculty of Agriculture Research Bark (FARB)

A-1- Seasonal fluctuations in infection levels:

To investigate seasonal variations in infection levels at Giza region, eight colonies were used in this study. Samples consisting of 15 adult worker bees were taken from the hive entrance of each colony at two weeks intervals throughout one year of experimentation, started in 4-4-2011 until 2-4-2012.

Plastic bags were used to gather the samples of bees which were taken to the fridge to immobilize them and their alimentary systems were removed. Each gut was drawn out of a bee by grasping the posterior abdominal segments with forceps and pulling them away from the bee. The ventricular and small intestines were treated collectively, as one organ sample.

The ventricular is amber and translucent in healthy bees but swollen and milky in nosema - infected bees, later becoming chalky white (Fig.1).

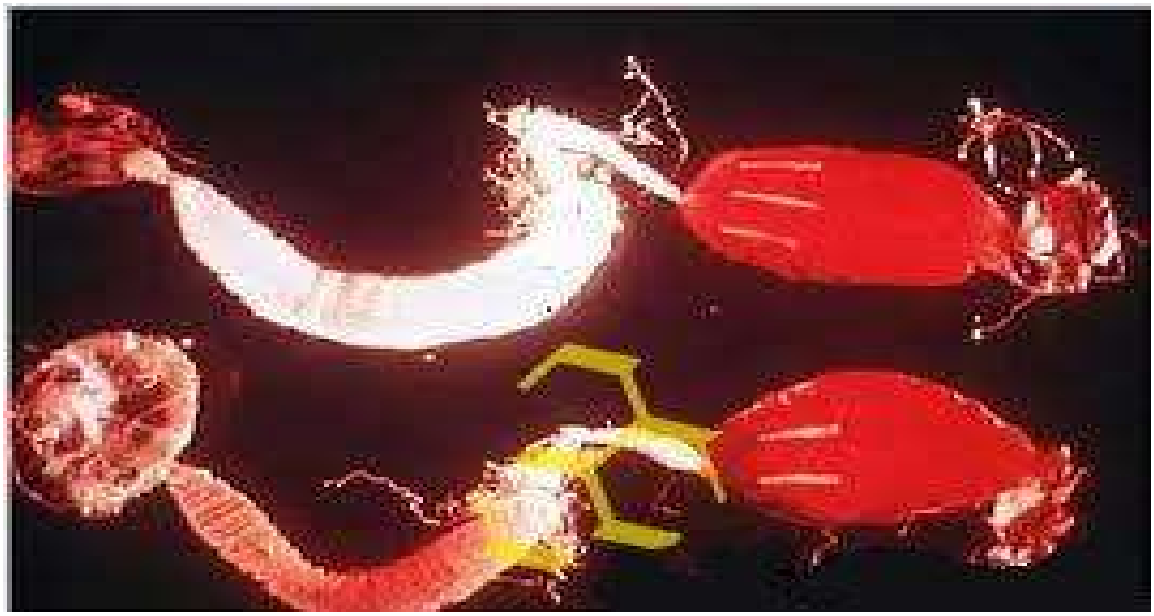

Fig.1. Comparison between the ventricular of healthy (down) and infected bees (up) (White 1918)

The rectum was separated to facilitate the counting of spores (Pickard & El-Shemy, 1989). The mentioned organs (ventricular and small intestine) of each colony sample (15 bees) were treated as a whole sample which were homogenized in 15 ml. of Nigrosine solution (0.5 g strain in 1 liter of distilled water) One drop of this solution was placed in a haemocytometer to give a liquid depth of 0.2 mm under small grid areas of 0.0625 mm2. the volume of liquid under each small grid square was 0.0125 µl. The number of nosema spores which are oval bodies of 5×3 µm (Fig.2) under 10 grid squares (0.125 µl) was manually counted under a microscope at 400 ×magnification (Cantwell, 1970). This figures was converted to No. of spores / one bee (× 8000).

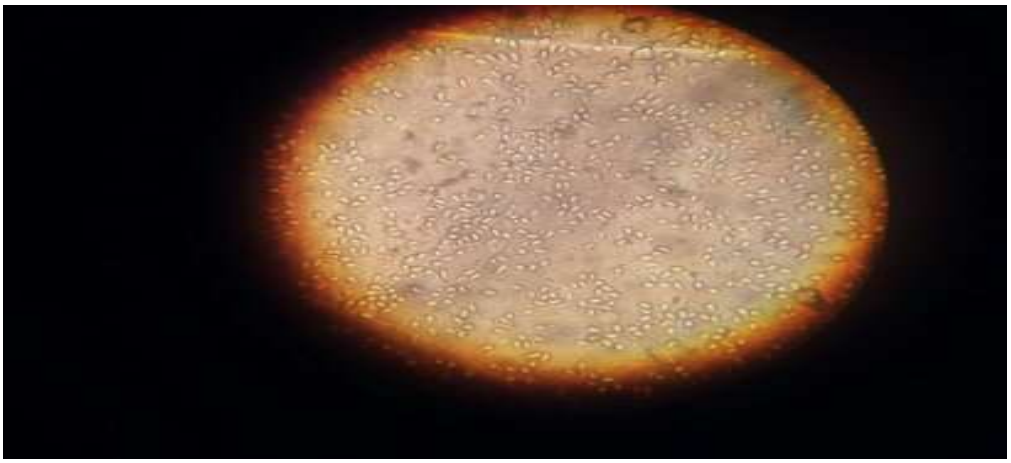

Fig.2. Nosema spores (oval bodies of 5×3 µm)

The seasonal variations in infection level according these figures were recorded throughout the year. The equipments were washed and dried carefully between examinations.

A-2- Colony strength and its relation to fluctuations in infection levels:

The mentioned experimental colonies (8 colonies) were established as nuclei and nearly were at the same strength in the beginning of experiment (4-4-2011) and headed by young mated queens. The strength of these colonies was recorded (At two weeks intervals throughout the year) by counting the number of combs covered with bees and also the number of sealed brood combs were measured.

The relations between the nosema infection level and other factors such as; colony strength, harvesting the honey crop and the effect of oriental hornet in preventing the cleansing flights of bees were studied.

In all mentioned experiments the infection levels were calculated and statistically analyzed. The means were compared at 5% probability levels by LSD test according to Snedecor and Cochlon (1967).

B - Molecular detection of *Nosema ceranae*

B-1- The honeybee Samples:

Samples of adult honey bees were collected from the entrance of different colonies from three different regions in Egypt (Beni- Suef, Giza and Kafr el-Shekh). 5 bees were collected from each entrance of ten colonies in each region and were microscopically examined for presence of *Nosema* spp. spores (Shimanuki and Knox, 2000).

B-2- DNA extraction:

Abdomens were excised from honey bees, and DNA was obtained by direct DNA extraction method based on modifications of the protocol of Hunt (1997)

B-2-1HBRC method

Abdomens of a sample honey bees were washed with extraction buffer prepared as per Hunt (1997).

PCR amplifications

DNA samples obtained using the previous described method above were used in the co-amplification of the 16S rRNA gene of *N. apis* or *N. ceranae* (Martin-Hernandez *et al*., 2007) and the honey bee ribosomal protein RpS5 gene (Thompson *et al*., 2007) in the same reaction. All PCR reactions were done with a Master Mix (Fermentas International Inc., Canada).

Separation and quantification of PCR amplicons

PCR products were separated by electrophoresis in 1.1% agarose gels and stained with ethidium bromide. Included was a 100 bp DNA ladder. The intensity of the amplified bands captured in pictures with a digital camera, was measured in pixels using the Scion Image computer software (Scion Corporation, Frederick, MD, USA) as per Dean *et al*. (2002).

Results and Discussion

A-1- Seasonal fluctuations in infection levels.

Data presented in Table (1) showed nosema infection levels (spore count / bee / collected at a hive entrance) to be at their highest during April, 2011 (7.17×10^6 spores) then this parameter gradually decreased during the following months, May and June. The corresponding figures during these periods were 3.39 and 1.28×10^6 spores/bee/col. The infection levels reached to 0.26×10^6 spores/bee/col. at the end of June. The differences in infection levels between these periods were significant. The reduction in infection levels during early summer may be due to suitable weather conditions for defecatory frights.

Table (1): Seasonal variation in the infection level (No. of spores*10^6/ bee /colony) of honeybee colonies with nosema disease.

Dates	Colonies								Mean
	1	2	3	4	5	6	7	8	
04/04/2011	6.4	8.76	3.08	8.8	5.2	8.96	9.64	6.52	**7.17^{a}**
02/05/2011	2.56	4.4	3.64	2.8	3.5	3.52	5.6	1.12	**3.39^{c}**
30/05/2011	1.6	1	1.68	1.24	1.6	2.16	0.24	0.72	**1.28^{de}**
27/06/2011	0.06	0.52	0.36	0.04	0.3	0.2	0.08	0.52	**0.26^{e}**
25/07/2011	0.27	2.2	0.36	0.16	0.06	0.66	0	0	**0.46^{de}**
22/08/2011	1.12	1.76	0.44	0.2	0.96	0.68	0.44	0	**0.70^{de}**
19/09/2011	2.96	1.2	1.8	0.36	1.8	0	0.32	0.8	**1.16^{de}**
17/10/2011	4	1.2	0	0	0	0.04	0.28	0	**0.69^{de}**
14/11/2011	2.8	1.2	0	0	0	0.16	0.6	8	**1.60^{d}**
12/12/2011	0.28	0.06	0	0	0	0	0.12	0.6	**0.13^{e}**
09/01/2012	0	0	0	0.48	0	1.8	0.12	2	**0.55^{de}**
06/02/2012	0.32	0	1.36	1.6	1.88	2.16	1.28	3.76	**1.55^{d}**
05/03/2012	1.84	4.76	3.44	7.12	7.4	2	3.61	6.64	**4.60^{b}**
02/04/2012	2.11	4.83	2.67	4.43	6.03	1.43	4.23	2.23	**3.49^{bc}**
Mean	**1.88^{ab}**	**2.28^{a}**	**1.34^{b}**	**1.94^{ab}**	**2.05^{ab}**	**1.70^{ab}**	**1.90^{ab}**	**2.35^{a}**	

SD value at 0.05
Treatments: 0.89 Dates: 1.19 Interaction: 3.36

The lowest infection level induced during December (0.13 × 10^6 spores/bee/col). Also, the mean no. of sealed brood combs during this period reached to the lowest value (0.34 and 1.19 combs / col.) the decreasing in brood rearing resulted reduction in infection levels. These data confirmed by Kruber (1968) who stated that brood rearing has been considered to be a stimulus to nosema reinfection.

Results in Table (1) indicated that the incidence of nosema infection levels tend to increase at the end of winter and early spring during the second year, 2012 as a result of starting rearing of brood and reached to the greatest value at the beginning of April, 2012.

These results are in agreement with those of Pickard & El-Shemy (1989). They reported that the peak of nosema infection levels induced during spring.

As clear in Table (1) and Fig. (3) a slightly increase (0.46×10^6 spores / bee / col.) in infection level induced during July (one month after harvesting clover honey crop). Also, the infection level tend to raise again (1.16×10^6 spores/bee/col.) during September (one month after harvesting the second boney crop). The increasing of infection levels during these periods may be due to the cleaning of contaminated combs after honey extraction and reinfection with nosema spores. Similar results were obtained by Bailey (1953) who stated that the primary means of spore transmission was workers cleaning combs soiled by excreta. Also, the colony disturbance alone can increase nosema infection and this usually happen during honey extraction.

The attacking of oriental hornet reached to its peak during October and November (Ibrahim, 2009). So, the mean no. of spores / ventricular and small intestine tend to increase during November (1.6×10^6 spores / bee / col.). As a result of inhibiting bees from defecatory flights and eliminate the contaminated feces. So the reinfection start to build up again.

Generally, the variations in colony infection levels between the periods throughout the year are significantly differed and can be considered in a generalized annual cycle of summer / winter / autumn / spring to allow the collation of data from different latitudes. When this done, Bailey (1955, England), Doull & Cellier (1961, Australia), Clinch (1974, New Zealand), Dyess& Wilson (1978, Mississippi), Morse (1978, Canoda), Pickard & El-Shemy (1989, Wales), El-Shemy (1991, Manzala north of Egypt) and the current study find no absolute annual cycle for nosema infection levels but a cycle of relative infection levels. In a year where the infection is almost near zero in summer, the autumn period will show a slow increase in infection. At the onset of spring period, the greatest rate of increase in infection is expected and a maximum annual level is reached.

In the second spring 2012, the cycle may be moved out of phase according to climatic conditions and colony stress.

The results in Table (1) show that the levels of nosema infection in experimental colonies (8 colonies) were variable. The colony number (8) showed the highest mean level of infection (2.35×10^6 spores / bee / col.) throughout the year, whilst the colony number (3) showed the lowest value (1.34×10^6 spores/bee/col.).

The infection level in some colonies reached to zero during some periods of study, especially during autumn and winter. The variation in infection levels between the experimental colonies may be due to colony conditions and rearing of brood (Morse, 1978).

A-2- Colony strength and its relation to variation in infection level:

The results given in Tables (2 & 3) indicate that the colony no.8 was the strongest colony between all the experimental colonies (The number of combs covered with bees and number of sealed brood combs). The variations between this colony and other ones in these parameters were significant. As clear in Table (1) this colony (no.8) showed the highest mean level of infection throughout the year (2.35×10^6 spores/bee/col.). The corresponding figures of mean no. of combs covered with bees and no. of sealed brood combs were 5.93 and 3.93, respectively.

Table (2): Number of combs covered with bees in honeybee colonies throughout the year.

Dates	Colonies								Mean
	1	2	3	4	5	6	7	8	
04/04/2011	5.5	4.5	5	3.75	4	4	4	4.5	**4.41^{e}**
02/05/2011	7.5	5	5	4	4.5	6	4.5	5.5	**5.25^{d}**
30/05/2011	7.5	6.5	4.5	4.5	4.5	6.5	5	6	**5.63^{d}**
27/06/2011	9.5	9.5	7.5	5.5	7	7.5	6.5	7.5	**7.56^{b}**
25/07/2011	11	11	8.5	5.5	7	8.5	7.5	8.5	**8.44^{a}**
22/08/2011	9.5	9.5	8.5	6	8	8.5	7	7	**8.00^{ab}**
19/09/2011	7.5	6.5	7	5.5	6	7	6.5	6.5	**6.56^{c}**
17/10/2011	4	4	4	3	3.5	4	3.5	5	**3.88^{f}**
14/11/2011	1.75	1.75	1.75	1.25	1.75	2.5	3	4	**2.22^{h}**
12/12/2011	1.75	1.75	1.5	1.5	1.5	2.25	4	4	**2.28^{gh}**
09/01/2012	2.25	2.25	2.5	2	2	2.5	4	4.5	**2.75^{g}**
06/02/2012	1.5	2	3	2	2.25	2	4.5	4.5	**2.72^{gh}**
05/03/2012	2.25	2.5	3.5	2.75	2.5	3	6.5	6.5	**3.69^{f}**
02/04/2012	5	5	5	4	4	5	9	9	**5.75^{d}**
Mean	**5.46^{b}**	**5.13^{bc}**	**4.80^{c}**	**3.66^{e}**	**4.18^{d}**	**4.95^{c}**	**5.39^{b}**	**5.93^{a}**	

LSD value at 0.05

Treatments: 0.39 Dates: 0.52 Interaction: 1.47

Table (3): Number of sealed brood combs in honeybee colonies throughout the year

Dates	Colonies								Mean
	1	2	3	4	5	6	7	8	
4-4-2011	4.50	2.50	4.00	2.25	2.50	3.25	3.00	3.00	**3.13^{cd}**
2-5-2011	6.00	3.00	3.50	3.00	2.50	4.00	3.00	4.00	**3.63^{bc}**
30-5-2011	5.00	4.50	3.50	2.50	3.50	3.50	3.00	5.00	**3.81^{b}**
27-6-2011	7.50	7.00	6.00	4.50	4.00	6.00	5.00	6.00	**5.75^{a}**
25-7-2011	6.50	8.00	7.00	4.00	5.50	5.50	5.50	5.50	**5.94^{a}**
22-8-2011	5.50	6.50	5.50	4.00	5.50	6.00	5.00	5.00	**5.38^{a}**
19-9-2011	4.50	4.50	4.00	3.50	4.00	4.00	4.00	4.00	**4.06^{b}**
17-10-2011	1.00	1.00	2.50	0.50	0.00	0.50	1.50	2.00	**1.13^{e}**
14-11-2011	0.25	0.00	0.00	0.00	0.00	0.50	1.00	1.00	**0.34^{f}**
12-12-2011	1.00	0.50	0.50	0.75	0.75	1.50	2.00	2.50	**1.19^{e}**
9-1-2012	1.25	1.25	1.50	1.00	1.25	1.75	2.50	3.00	**1.69^{e}**
6-2-2012	0.75	1.25	2.00	1.00	1.25	1.00	3.50	3.00	**1.72^{e}**
5-3-2012	1.50	1.75	2.50	1.75	1.00	2.00	5.50	5.00	**2.63^{d}**
2-4-2012	3.00	3.00	3.00	3.00	2.00	3.00	7.00	6.00	**3.75^{b}**
Mean	**3.45^{bc}**	**3.20^{c}**	**3.25^{bc}**	**2.27^{d}**	**2.41^{d}**	**3.04^{c}**	**3.68^{ab}**	**3.93^{a}**	

LSD value at 0.05

Treatments: 0.46 Dates: 0.60 Interaction: 1.71

The experimental colonies showed variations in infection level. There was a positive correlation between level of infection and activity in rearing brood as clear in colony no.8. These results confirmed the results of Oertel (1964). Who reported that the peak infections appeared to follow periods of brood – rearing. Also, Pickard & El-Shemy (1989) found significant differences in infection level between hives in the same apiary.

B - Molecular detection of *Nosema ceranae*

From the 3 region samples subjected to PCR analysis, *Nosema ceranae* was detected at two regions (Giza and Kafr el-Shekh) these positive samples were from beekeepers selected randomly from the previous regions. The two positive *N. ceranae* samples showed, no intraspecific DNA sequence variation among the 208 bp 16Ssequences. (Fig.3&4)

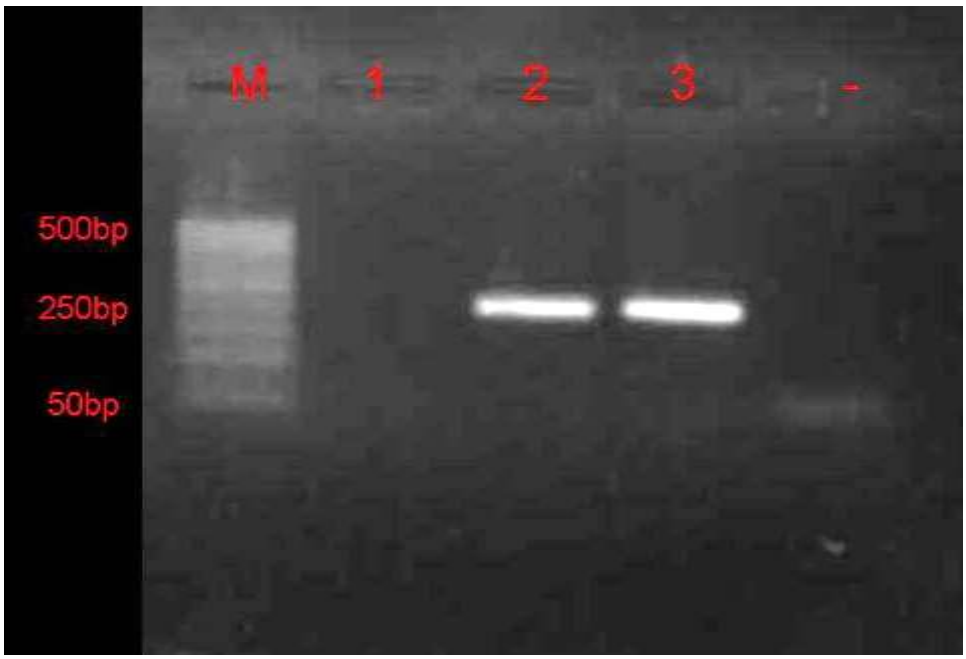

Fig.3 Detection of the 16S rRNA PCR product specific for *Nosema ceranae* (218 bp).

In tested samples .

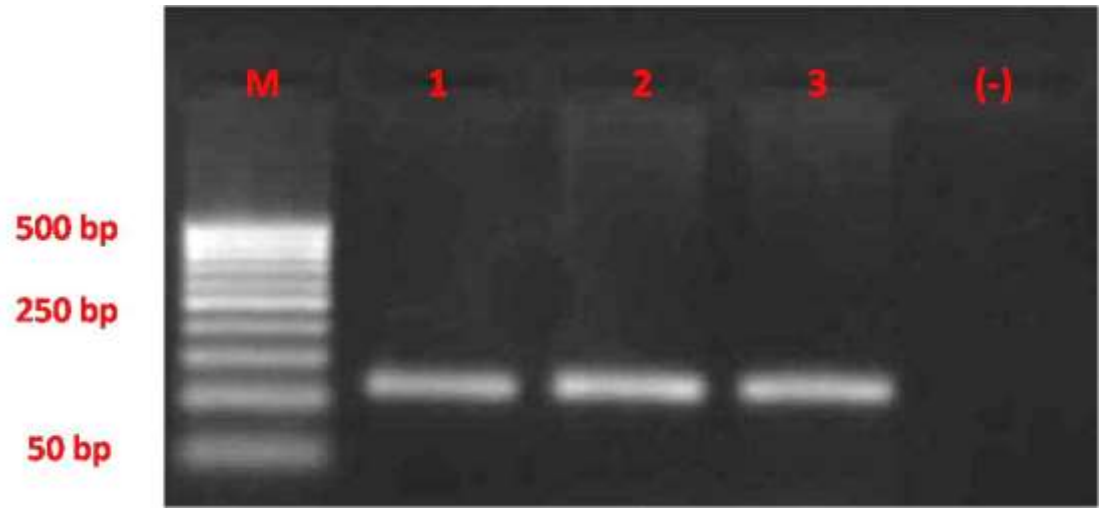

Fig.4 Co-amplification of PCR product specific for the honey bee house-keeping gene, RpS5(115 bp). In tested samples

Summary

This study was carried out at the apiary of Agricultural Experimental Station, Faculty of Agriculture, Cairo University during two years 2011, 2012. To study the seasonal fluctuation of nosema disease, also to diagnose the specie of *Nosema* which more dominance in Egypt.

Eight honeybee colonies were used in the first study. These colonies were nearly at the same strength in the beginning of experiment and headed by young mated queens. Samples of 15 bees were collected from the entrance of each colony at 2 weeks internals throughout the year and examined collectively for the presence of nosema spores.

The obtained results showed that the nosema levels to be at their highest during April, 2011($7.17*10^6$ spores/bee/col.) Then this parameter gradually decreased during the following months and reached to $1.28*10^6$ spores/ bee/col. at the end of June. The infection level increased again during autumn months as a result of severe attacking of oriental hornet and prevents the bees from defecating flights. On the other hand the nosema infection reached to the lowest level during December ($0.13*10^6$spores/bee/col.) as a result of decreasing of brood rearing. Generally, the variations in colony infection level between the periods throughout the year are significantly differed. In the second spring, 2012 the cycle may be moved out of phase according to climatic conditions and colony stress.

In the second experiment, samples of 50 bees were randomly collected from the entrances of ten colonies .From three different regions in Egypt. DNA Samples were obtained from the abdomen of infected bees were used in the co amplifaction of 165r RNA gene of *N. apis* & *N. cerana* and the honeybee ribosomal protein RpS5 gene the obtained results showed that the *Nosema* spp. which is dominance in Egypt is *Nosema ceranae* not *Nosema apis*.

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