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The First Detection of Multiple Honeybee Pathogens in Libyan Bees *Apis Mellifera* L.

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Abstract: *Beekeepers in Libya complain from acute colony population decline and migration after the season, resulting in decrease in honey production. Bee samples were analyzed by reverse transcription-PCR (RT-PCR) for the presence of five viruses DWV, BQCV, SBV, ABPV, and CBPV in addition to Nosema apis and N. ceranae, The results have shown the presence of BQCV and CBPV in all analyzed samples (queen, worker larvae and adult workers), while SBV and ABPV appeared in worker larvae only, our results show that individual queen and worker larvae harbor more than one virus simultaneously, sample of adult workers show the presence of Nosema ceranae.*

Keywords: *RT-PCR, Apis mellifera L., Virus, Nosema Spp.*

1. INTRODUCTION

In the last five decades beekeeping thrived and spreaded for wide regions in Libya accompanied that in the last decade appearance of many problems where beekeeper complains after the season from acute colony population decline and migration, resulting in decrease in honey production. Up to now there is no one in Libya diagnose pathogens like viruses or nosema in the lab, although the appearance of CBPV, SBV, and DWV symptoms.

Most of viruses are symptomless whereas SBV and CBPV produce clinical signs that are clearly identifiable by beekeeper (Anderson and Gibbs 1988, Ball and Bailey 1997).

The role of viruses in honeybee pathogenesis is of increasing concern; recent evidence shows that virus-induced disease can be exacerbated and persistent infections activated by infestation with the parasitic mite *Varroa destructor*, and the incidence of mite infestation is also rising. Furthermore, the consequences of virus infections are becoming more significant (Grabensteiner et al 2001).

Varroa destructor play principal role for transmitting viruses which DWV, SBV, ABPV, and KBV found in varroa samples (Tentcheval et al 2004).

The histological data were confirmed by quantitative RT-PCR of dissected organs showed that DWV infection is not restricted to the digestive tract of the bee but spread in the whole body, including queen ovaries, queen fat body and drone seminal vesicle (Fievet et al 2006).

The queen in bee colonies were identified as positive for BQCV, DWV, CBPV, KBV, and SBV, whereas the same viruses detected in their offspring, including eggs, larvae, and adult workers. That suggests vertical transmission of viruses from queen to offspring (Chen et al 2005).

By developing multiplex RT-PCR assay for the simultaneous detection of multiple bee viruses. They found that individual bees can harbor four viruses simultaneously (Chen et al 2004).

In Austria researcher found Simultaneous infections of DWV and ABPV were most frequently observed in colonies suffering from weakness, depopulation, and sudden collapse. The most prevalent virus was DWV, followed by ABPV, SBV, and BQCV viruses (Berényi et al 2006).

Nosema ceranae is a microsporidian parasite described from the Asian honey bee, *Apis ceranae* the parasite is cross-infective with European honey bee, *Apis mellifera* (Chen et al 2008, Fries 2009).

No specific colony level symptoms of infection have been described and no dysentery is reported to be associated with infections. Several studies from Spain suggest that *Nosema ceranae* is a colony

level virulent parasite and that infections eventually lead to colony collapse unless the infections are controlled (Fries 2009).

Infection by *Nosema ceranae* cause disappearance of adult bees, lack of brood attention, reduced colony strength, and heavy winter mortality without any previous evident pathological disturbances. Treatment with fumagillin avoided the loss of surviving weak colonies (Higes et al 2009).

2. MATERIALS AND METHODS

2.1. Detection of Viruses

Material: Single bee with deformed wings, worker larvae sample 1, worker larvae sample 2, worker larvae sample 3, single queen, about 30 worker bees. All sample material was suspended in RNA later (Qiagen)

Bees from each sample were pulverized in liquid nitrogen. RNA extraction was carried out using Total RNA Mini (A&A Biotechnology) according to manufacturer's protocol.

RNA was reverse-transcribed to cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to protocol enclosed by the manufacturer.

To detect viruses specific primer pairs were used (tab. 1)

Table1. *Primers used to detect specific virus sequences.*

Virus	Primer pairs	Source
BQCV	BQCV76f, BQCV299r	(Bakonyi, 2002)
ABPV	ABPV1f, ABPV2r	(Bakonyi, 2002)
CBPV	CBPV1f, CBPV2r	(Ribière et al., 2002)
SBV	SBV1f, SBV2r	(Ghosh, Ball, Willcocksl, & Carterl, 1997)
DWV	DWV2345f, DWV2779r	(Lanzi et al., 2006)

All PCRs were performed in 50µl reactions, each containing 0,3 µM of each (forward and reverse) primer, 0,2mM of each dNTP, 1,5mM $MgCl_2$, 1,5U of Taq Polymerase and 2µl of query cDNA.

All reactions were carried out in Mini Cycler 25 (MJ Research). Thermal protocol for BQCV, ABPV, CBPV and SBV consisted of a single initial activation step in 94^{o}C for 3 minutes followed by 40 PCR cycles (denaturation: 94^{o}C for 1 min, annealing: see Tab. 2, elongation: 72^{o}C for 1 min). The reaction was closed with a final elongation step in 72^{o}C for 2 minutes.

Table2. *Annealing temperatures for specific primer pairs.*

Primer pairs	Annealing temperatures and times
BQCV76f, BQCV299r	50^{o}C for 1 minute
ABPV1f, ABPV2r	55^{o}C for 1 minute
CBPV1f, CBPV2r	50^{o}C for 1 minute
SBV1f, SBV2r	50^{o}C for 1 minute

Thermal protocol for DWV consisted of an initial activation step in 94^{o}C for 3 minutes followed by 35 PCR cycles (denaturation: 94^{o}C for 30 seconds, annealing: 53^{o}C for 1 minute., elongation: 72^{o}C for 30 min). The reaction was closed with a final elongation step in 72^{o}C for 10 minutes.

The size of PCR product for each virus is presented in Table 3.

Table3. *The size of PCR product for each virus.*

Virus	PCR product size (bp)
BQCV	224
ABPV	398
CBPV	455
SBV	646
DWV	435

Products were separated electrophoretically in 1,5% agarose gel. Visualization under UV light and documentation of obtained results was carried out using GelDoc-It Imaging System (UVP)

2.2. Identification of Nosema Species

A sample of about 30 frozen adult bees was crushed in distilled water in extraction bags (BIOREBA) (presence of *Nosema* spores in the suspension was confirmed microscopically using 20µl of the suspension). The resulting filtrate was then centrifuged in 800g for 6 minutes. Supernatant was

discarded and the spore pellet was crushed in liquid nitrogen using sealed pipette tip (to crush spores). The proper DNA extraction was performed using High Pure Template Preparation Kit (Roche Diagnostic) according to manufacturer's protocol for DNA extraction from yeast.

Duplex PCR was performed as described in (Martín-Hernández et al., 2007) using primers to detect *N. ceranae* and *N. apis*, respectively: 218MITOC-FOR, 218MITOC-REV and 321APIS-FOR, 321APIS-REV. PCR products were separated electrophoretically on a 1,5% agarose gel and visualized under UV light using GelDoc-It Imaging System (UVP). Band size for *N. ceranae* is 218 bp, and for *N. apis* 321 bp.

3. RESULTS AND DISCUSSION

3.1. Detection of Viruses

The results of RT-PCR have shown the presence of four viruses ABPV, BQCV, SBV, and CBPV in the analyzed samples. BQCV and CBPV appeared in all analyzed samples (queen, worker larvae and adult workers as in the Fig. 1 and 2). The detection of viruses in queen raises the possibility of a vertical transmission pathway wherein infected queens can pass virus through their eggs to their offspring (Chen et al 2005). SBV detected in larvae is consistent with the symptoms that we observed in the colonies from which the samples were collected (Fig.1).

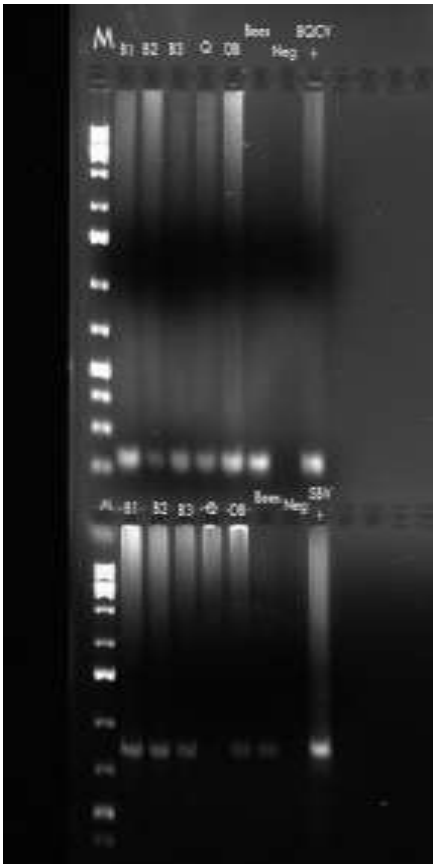

Fig1. *Gel electrophoresis of the product amplified with BQCV and SBV primers. BQCV, Black queen cell virus; SBV, sac brood virus; M, marker; B1,B2,and B3, samples of worker larvae collected from the brood of colonies appear sac brood symptoms; Q, sample of queen; DB, deformed wing bee sample; Bees sample of 30 adult workers collected from the apiary(which crawl on the earth); Neg, negative control; +, positive control.*

Figure 3 shows ABPV in the samples of worker larvae, although bee colonies did not show symptoms of paralysis, as we got negative results for adult bee samples. The result of DWV is not clear then neglected

Our results show that individual larvae can harbor more than one virus, which was also demonstrated by (Chen et al 2004).

In Italy, the first national investigation of honey bee viruses was conducted in 2009, where dead adult bee samples were collected from hives with symptoms of severe losses and lack of productivity where analyzed by RT-PCR, they detect IAPV, ABPV, and DWV (Formato et al. 2011). In 2010 the further

viral species detected in the eight Kashmir Bee Virus positive samples where: ABPV, DWV, CBPV, SBV, and BQCV (Cersini et al 2013).

Bee samples collected from Giza Governorate in Egypt which detect deformed wing virus (DWV) in all body parts of crippled bees while Kakugo Virus (KV) was detected specifically in the head of aggressive worker honeybees but not in the thorax or abdomen(Abd-El-Samie et al 2014)

Results of the first molecular report in Algeria show that the 40 apiaries examined, 40% were infected with DWV (Loucif-Ayad et al 2013)

The spread of viruses which may result from global trade of *A. mellifera* queens, nucleus and package bees among many countries, many shipments of queens and bees have been imported to Libya for many years especially from Italy and other countries, may result to introduce viruses.

The results obtained via PCR show, that those four viruses (thought to be common in most parts of the world) are also present in Libya, however more research is needed for proper screening for those (and other) viruses since the sample in this study was only preliminary, thus very small.

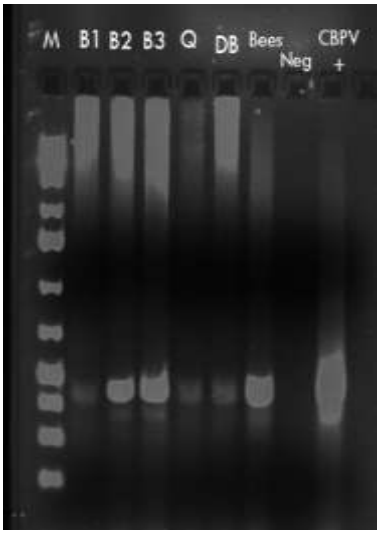

Fig2. *Gel electrophoresis of the product amplified with CBPV primers. CBPV, Chronic bee paralysis virus*

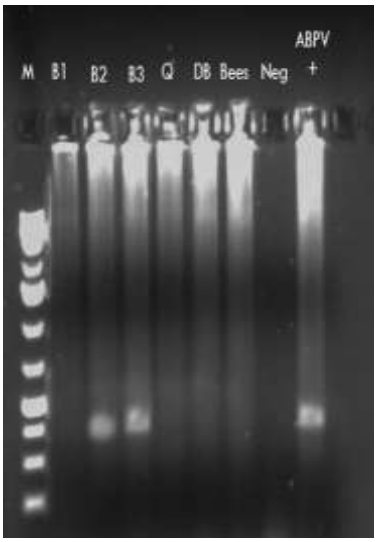

Fig3. *Gel electrophoresis of the product amplified with ABPV primers. ABPV, Acute bee paralysis virus; M, marker; B1,B2,and B3, samples of larvae collected from the brood of colonies appear sac brood symptoms; Q, sample of queen; DB, deformed wing bee sample; Bees sample of 30 bees collected from the apiary(which crawl on the earth); Neg, negative control; +, positive control.*

3.2. Detection of Nosema Spp.

The multiplex PCR results show only the presence of *Nosema ceranae* in the sample, so we can conclude, that it is present in Libya, however we cannot exclude the possibility for *Nosema apis* to be present there too, since a wide screening is needed.

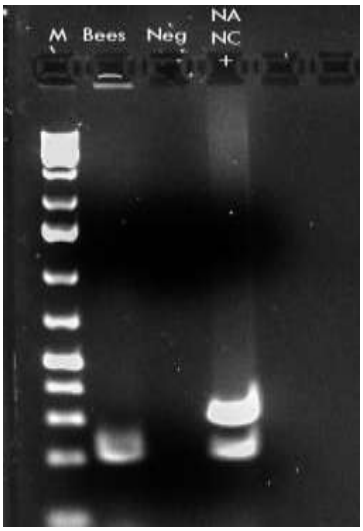

Fig4. *RT-PCR result for N. a. and N.c . bp. M, marker; Bees, sample of 30 adult bees; Neg, negative control; +, positive control N.a., Nosema apis; N.c., Nosema ceranae.*

In this study we record five pathogens in two apiaries, ABPV, BQCV, SBV, CBPV and *Nosema ceranae*. It is the first time they were detected in Libya, and we think it is a strong reason to run a detailed and wide survey in different areas of the country. Given the world-wide colony losses it is necessary to detect the pathogens and their prevalence, especially in such a big country. Only then it will be possible to undertake proper steps to fight colony decline.

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