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DETECTION OF HONEY BEE VIRUSES ON THE TERRITORY OF UKRAINE

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ABSTRACT

A survey of honey bee viruses on the territory of Ukraine has been conducted for the first time in 2016-2017 years. The samples of adult bees, affected combs and mites were collected from apiaries in two regions (Kyiv and Cherkasy). Detailed studying of the bee samples revealed following morphological changes: deformed wings, saccular brood, blackened pistil, changing of the body color. Virus-like particles were observed under TEM after purification of bee samples. Symptomless samples were also analyzed for the presence of bee viruses by RT-PCR. According to results of RT-PCR, we concluded that the following viruses circulate on the territory of Ukraine: Acute Bee Paralysis Virus (ABPV), Kashmir Bee Virus (KBV), Israeli Acute Paralysis Virus (IAPV), Black Queen Cell Virus (BQCV), Sacbrood Bee Virus (SBV) Chronic bee paralysis virus (CBPV). Taken together, these data indicate that bee virus infections occur in bee populations on the territory of Ukraine. Despite the lack of symptoms viruses are often present in colonies and environmental factors might result disease outbreaks that lead to the activation of viral replication in bees.

Key words: *bee virus, bee colony, mites, vector.*

INTRODUCTION

Pollination of flowering plants is an important ecosystem service provided by wild insect pollinators and managed honey bees. Hence, losses and declines of pollinating insect species threaten human food security and are major concerns not only for apiculture or agriculture but for human society in general. Over the last 15 years, dramatic colony losses have been reported regularly from different regions all over the world. The current scientific consensus is that no single factor is causing honey bee colony death. However, frequently virus infections are among the key players in colony losses and therefore viruses are rightly considered a major threat to the health of honey bees, both at individual and at the colony level (Gisder, 2015). The most commonly observed and best known honey bee viruses are 30-nm isometric particles containing a single-stranded positive RNA. These

viruses include Israeli acute paralysis virus, Kashmir bee virus, Acute bee paralysis virus, Black queen cell virus, Deformed wing virus, Sacbrood virus, Slow bee paralysis virus and the recently discovered Lake Sinai viruses, which are a phylogenetically unique, globally distributed group of viruses, including LSV1-7 and other variants (Brutscher, 2016). According to epidemiological data, the distribution of these viruses in honey bee colonies appear to be worldwide (Ahmad, 2016), most likely resulting from intensive exchanges of honey bee stocks throughout the world. RNA viruses are readily detected in bee samples obtained from colonies of varying health and viral presence, and abundance varies by season and geographic location (McMenamin, 2015). Viruses of the honey bee typically infect the larvae, but the symptoms are often most obvious in adult bees. The transmission of the viruses usually occurs horizontally (e.g. through the bee feces, royal jelly, the beekeeper), but the transmission of the main bee viruses occurs vertically (from the queen to the brood). Many viruses are also transmitted by mites Varroa. *Varroa destructor* has greatly contributed to increase the incidence of viral diseases (Moore, 2014). Varroa, in fact, is a passive carrier of bee viruses that are transmitted to the bees through the mite's saliva. Varroa mites feed on hemolymph, which allows viruses transfer directly into the open circulatory system that reaches every cell in the insect body. In addition, mites weaken the immune system of the bees, which can lead to the reactivation of latent viral infections already present in the body of the bees. Honey bee viruses are not limited to honey bees. They also have been found in other non-Apis bee species, other colony inhabitants like small hive beetle, and in pollen and nectar (Bailey and Gibbs, 1964; Genersch et al., 2006; Philip, 2014).

Identification of bee virus is difficult due to the small size of viral particles. Specific laboratory methods are required for accurate diagnosis. However, symptoms of some viral diseases are more visible, especially with overt infection. A lack of symptoms does not guarantee the absence of a virus. Viruses can remain in a latent form within the host, acting as a reservoir of infection, complicating diagnosis and control, and causing outbreaks under particular conditions (Yue, 2006).

Despite the great losses of bee colonies and observation of virus like disasters, specific bee viruses have not been tested on the territory of Ukraine. These viruses are usually undervalued by Ukrainian beekeepers: they can cause serious economic losses alone and if associated with other bee diseases. The aim of present work was to identify honey bee viruses in apiaries of Ukraine.

MATERIAL AND METHODS

Sample collection

This study was conducted during the spring and summer of 2016. The first step included the monitoring of bee colonies for symptoms of viral infection with subsequent sampling and processing. The samples of bees and affected combs were obtained from two regions in Ukraine: Kyiv and Cherkasy. Healthy bees with no clinical signs of disease were collected at the same time. Additional samples of

Varroa destructor mites were collected, as these species serve as vectors for viruses. Each sample consisted of approximately 100 bees or mites, representing one location or apiary. A part of the samples was purified for preliminary identification through electron microscopy. The remaining part was stored at −20 ^{o} C until used for RT-PCR studies.

Virus isolation and purification

Adult bees were crushed in liquid nitrogen and homogenized in 700 µl phosphate buffer (pH 7.0) supplemented with 0.02% diethyldithiocarbamate. The resulting suspension was clarified with carbon tetrachloride and centrifuged for 15′ at 800× g and 4 h at 100,000× g (4 °C). The resulting pellet was resuspended in PBS.

Transmission electron microscopy

To estimate the presence of virus like particles in investigated samples transmission electron microscopy was employed. For this purpose formvar films were placed on the 400-mesh copper grids and were dipped into a sample for 2 min and negatively stained in 2% uranyl acetate. The preparations were dried and viewed under an electron microscope at an instrumental magnification of 90.000.

RNA isolation and PCR

The primers were chosen to target the conservative genome region of seven viruses (ABPV, KBV, IAPV, BQCV, DWV, SBV, CBPV. Five pairs of oligonucleotide primers were selected in accordance with data from the literature (Tab.1) (Miranda 2010, Ahmad M. Mouhanna 2016, Khaliunaa Tsevegmid 2016).

Table 1- Sequence of primers used in the study to target different region of honey bee virus genome

AKI*	F-5’- GGCGAGCCACTATGTGCTAT
	R-5’- ATCTTCAGCCCACTT
DWV	F-5’-CTTACTCTGCCGTCGCCCA
	R-5’-CCGTTAGGAACTCATTATCGCG
BQCV	F-5’-AGTGGCGGAGATGTATGC
	R-5’-GGAGGTGAAGTGGCTATATC
SBV	F-5’- ACCAACCGATTCCTCAGTAG
	R-5’- TCTTCGTCCACTCTCATCAC
CBPV	5’- ACTCCCGTCGTTGTGTTCTC
	5’- GGCGATTGGTATTTGTTTGG

*AKI primer for *Acute Bee Paralysis Virus* (ABPV), *Kashmir Bee Virus* (KBV), *Israeli Acute Paralysis Virus* (IAPV)

RNA Extraction

Individual samples were homogenized in 700 µl sterile tubes with TE reagent according to the manufacturer's instructions in PureLink RNA mini kit, USA. Next step were homogenization in TE buffer and adding 700 µl of lysis buffer and centrifugation at 8000 rpm speed for 2 min. After the samples were mixed with 10 µl mercaptoethanol and 200 µl of lysis buffer. Obtained solution was centrifuged again and the middle fraction was selected. The samples were transferred to a column for RNA isolation and centrifuged at approximately 11,000 rpm. The sediment was drained from the column and washed according to the instructions of sample buffer bottle label. Total RNA was dissolved in 50 µL of sterile water and was stored at −80 °C until further analysis. The quantity and purity of RNA in each sample was measured by agarose gel electrophoresis.

PCR assays

AKI

Synthesis of cDNA was performed with 3 µg of RNA samples from using an oligo-primer and reverse transcriptase. PCR was conducted with forward and reverse primers for IAPV, ABPV, KBV, BQCV, DWV. Amplification viruses (IAPV, ABPV, KBV) were conducted under the following conditions: 50°C 30 min reverse transcription, 2 min denaturation at 94°C, in 35 cycles which included: denaturation at 94°C for 30 sec, 30 sec annealing at 50°C, extension at 68°C for 45 sec.

BQCV

The PCR volume was 25 µl, containing 2µl template cDNA, 5µl of 5X Reaction Buffer, 0.75 µl of dNTPs Mix (10 mM), 0.75µl of each forward and reverse primers (10µM) and 0.5µl of Taq polymerase. The conditions of the reaction were the next: incubation for 2 min at 95°C, 20 sec denaturation at 98°C., annealing at 57°C for 20 sec, 30 sec extension at 72°C and 2 min for a final step at 72°C.

DWV

RT-PCR was completed as follows: 30 min at 50 ° (reverse transcription) and denaturation 15 min at 95°C, followed by 40 cycles of amplification for 30 sec at 94°C, 45 sec at 55°C, and 45 sec at 72°C.

SBV

Temperature profile for SBV RT-PCR: 30 min at 50°C (reverse transcription) and denaturation for 5 min at 95°C, followed by 40 cycles of amplification 20 sec at 95°C, 20 sec at 55 °C, and 1 minute at 72°C.

CBPV

The thermal cycling conditions were next: 5 min at 94°C (denaturation of the template and activation of the enzyme), followed by 35 cycles consisting of denaturation at 94°C for 15 sec, 30 sec annealing at 55°C and 30 sec to 2 min extension at 68°C (depending on the primer pair), completed by a final 10 min extension at 68°C.

RESULTS AND DISCUSSION

In recent years, Ukrainian beekeepers reported several observation cases of virus-like disorders; however, the causative agent has not been identified. The samples of adult honey bees originating from the affected honey bee colonies and symptomless colonies, from different apiaries in Kyiv and Cherkasy regions, were collected and tested for the presence of seven honey bee viruses. Detailed studying of the bee samples revealed following morphological changes: deformed wings, saccular brood, blackened queen bee, changing of the body color. Affected combs with symptoms of chilled brood, spotty brood pattern, dead larvae in cells were also observed (Fig.1).

Fig.1. Larvae with characteristic lesions in honeycombs

Electron microscopy revealed the presence of spherical virus-like particles about 30 nm in diameter in mite sample (Fig. 2). No significant differences in virion size and morphology were observed among the virus particles. Observed virions had a diameter and morphology compatible with that of the genus *Dicistroviridae:* ABPV, KBV, IAPV, BQCV, and *Iflaviridae*: DWV, SBV, CBPV.

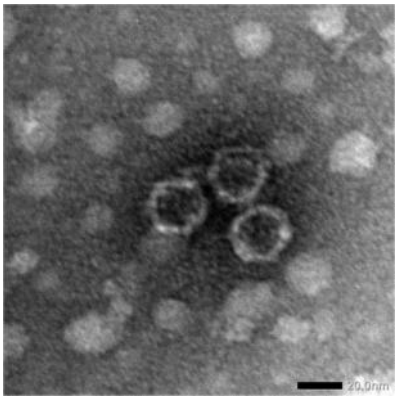

Fig.2. Electron micrograph of virus-like particles, observed in purified mites sample.

For exact diagnosis collected samples were analyzed for the presence of viruses with RT-PCR using specific primers. The following viruses were identified in samples of mites: BQCV, SBV (Fig. 3.). ABPV, KBV, IAPV, BQCV were detected in adult bees (Fig. 4).

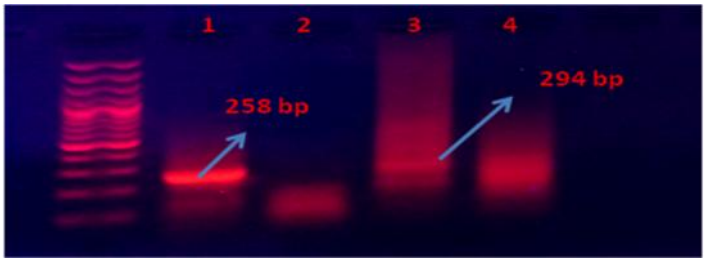

Fig. 3. RT-PCR results of bee and mites samples for SBV and BQCV: 1, 2- SBV; 3,4-BQCV; 1,3- samples bees; 2,4- sample bee

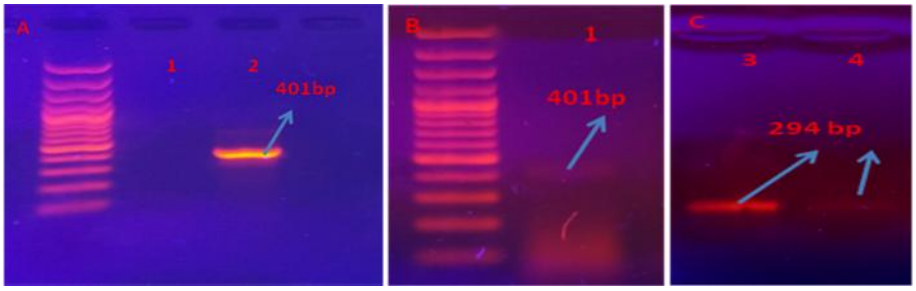

Fig. 4. RT-PCR samples results mites and bees: A, B- with primers for ABPV, KBV, IAPV; C- with primers for BQCV; 2- mites; 1, 3, 4-samples bees

RT-PCR analysis revealed the presence of two viruses: *Black Queen Cell Virus (BQCV)* and *Sacbrood bee virus (SBV)* in samples from affected combs.
Our investigations finally confirmed the circulation of specific honey bee viruses in Ukrainian apiaries. These results can be regarded as a base for establishing epidemiological surveillance of honey bee diseases in other regions of the country. The next step of our research will include sequencing of the samples for further comparison with foreign isolates and determination of phylogenetic relationships of Ukrainian isolates. Identified *Acute Bee Paralysis Virus*, *Kashmir Bee Virus*, *Israeli Acute Paralysis Virus*, *Black Queen Cell Virus*, *Sacbrood Bee Virus* are the most significant viruses as they can cause great economic losses and reduction in biodiversity in natural ecosystems. Thus, diagnosis of virus infections is the key component for surveillance, control and monitoring of honey bee diseases.

CONCLUSIONS

Beekeeping in Ukraine is a major economic activity. Ukraine is the top honey-producing country in Europe and has the largest number of managed honey bee colonies, however, the presence of honey bee viruses has never been identified in

Ukrainian apiaries. We report here the first survey of bee viruses distribution in Ukrainian apiaries. As a result of our work we estimated the presence of BQCV, CBPV, DWV, IAPV and SBV, ABPV and KBV. ABPV, KBV, IAPV, BQCV, SBV were the most prevalent in the tested samples. Obtained data is in accordance with results reported after identification of bee viruses in other countries. Honey bee colonies can suffer from multiple virus infections without showing obvious pathological symptoms, thereby confounding diagnoses. Therefore, rapid and accurate diagnosis of virus infection is a critical component of honey bee disease surveillance and control programs.

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