

**A SURVEY OF DEFORMED WING VIRUS AND ACUTE BEE PARALYSIS VIRUS IN HONEY BEE COLONIES FROM SERBIA USING REAL-TIME RT-PCR**

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In this study 55 honey bee colonies from different Serbian regions were monitored for the presence of Deformed Wing Virus (DWV) and Acute Bee Paralysis Virus (ABPV) using TaqMan-based real-time RT-PCR assay. The results revealed the presence of DWV in each sampling location, and ABPV in 10 out of 11 apiaries. High frequency of DWV (76.4%) and ABPV (61.8%) positive samples in asymptomatic colonies can be the consequence of inefficient and postponed *Varroa* treatment concerning the role of this mite in the transmission and activation of honey bee viruses. The real-time RT-PCR technique described in this paper is proved to be the most reliable method for this kind of investigation.

**Key words:** DWV, ABPV, *Varroa destructor*, Serbian apiaries

## INTRODUCTION

The honey bee *Apis mellifera* L. can be affected by various pathogens including numerous viruses, bacteria (*Paenibacillus larvae*, *Melissococcus plutonius*), fungi (*Nosema spp.*, *Ascosphaera apis*), and parasitic mites (*Varroa destructor*, *Acarapis woodi*, *Tropilaelaps spp.*). Bee viruses affect bee morphology, physiology, and behavior and have been often linked with weakening and dying colonies [1]. So far, more than 18 different viruses have been identified and described in honey bee colonies [2,3]. Most of known bee viruses exist and co-exist in individuals or colonies causing unapparent and persistent infections [4-6]. However, due to widespread of *V. destructor* as a virus-transmitting ectoparasite, two viruses are of growing interest: Deformed wing virus (DWV) and Acute bee paralysis virus (ABPV).

DWV is one of the viruses mostly related to colony losses [3,7]. DWV is a 30 nm

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icosahedral particle consisting of a single, positive strand RNA genome [8], typical for iflaviruses, a genus of the family *Iflaviridae*, within the order *Picornavirales* [9,10]. As widely and globally distributed, DWV is the most studied of all honeybee viruses [4,11-18]. DWV has been detected in adult bees, their pupae and larvae [19], as well as in *Varroa* mites [12] and *Tropilaelaps mercedesae* [20]. Transmitted vertically by queens and drones [21-23] or horizontally through larval food [24] DWV causes benign, symptomless infections. However, transmission of DWV to pupae via *V. destructor* that acts as both a mechanical and biological vector is considered to be crucial in the manifestation of the morphological changes in adult bees [25]. Bees with typical signs of the overt DWV infection - deformed wings, shortened and bloated abdomen and miscolouring [26], are not viable and their lifespan is less than 67 h after emergence [25]. Furthermore, the viral replication and subsequently high enough virus titer in the mites prior to transmission are substantial for the induction of an apparent DWV infection [27]. Hence, the higher the number of DWV-transmitting mites and consequently the number of deformed bees, the greater the chances for fatal DWV infection and colony collapse [28].

ABPV has a geographical distribution similar to that of *A. mellifera* and usually exist in a low concentration as a covert infection [29,30]. Together with Israeli acute paralysis virus (IAPV) and Kashmir bee virus (KBV), ABPV is a putative member of the genus *Apavirus*, family *Dicistroviridae*, within the order *Picornavirales* [9,10]. Common feature of viruses belonging to *Dicistroviridae* is a bicistronic, single-stranded RNA genome, consisting of two open reading frames (ORFs). In the wake of *V. destructor* in Europe and its establishment as a virus-transmitting parasite, the number of overt ABPV infections in severely *Varroa*-infested colonies has rapidly increased [11,13,31,32]. This phenomenon is related with the finding of ABPV extreme virulence when directly injected into the bee haemolymph causes bee mortality within a few days [16]. Nevertheless, the exact role of the *Varroa* mite in the increase in ABPV virulence still remains elusive. Due to rapid progression from paralysis to death in individual bees, effects of covert ABPV infections, including trembling and inability to fly, are rarely visible at colony level [16]. Two surveys conducted in Germany [3,33] revealed a significant relation between ABPV infection in autumn and colony mortality in the following winter season. Since ABPV can frequently be detected in bees from both asymptomatic and collapsing colonies [12,13,18], it is difficult to define the influence of ABPV infection on colony mortality. Different techniques have been used in the detection of bee viruses, including indirect fluorescent-antibody analysis, agarose gel immunodiffusion (AGID), enzyme-linked immunosorbent assay (ELISA), reverse transcription-PCR (RT-PCR) and real-time RT-PCR [34-39]. Development of real-time RT-PCR assays has significantly increased the sensitivity and specificity in the detection of honey bee viruses [40].

During the past several years, beekeepers in Serbia frequently report colony depopulation, morphological changes of adult bees (dark color and deformed wings), symptoms of central nervous system disorder (trembling and inability to fly), or sudden collapse of entire colonies. When it comes to bee pathogens related with colony decline in Serbia, there are recent investigations of *Nosema* sp. [41,42] and *V. destructor* [43,44]. The presence of bee viruses in Serbia was first reported by Kulinčević et al. [45] when four honey bee viruses were identified: ABPV, Egypt bee virus, J strain (EBV), Cloudy wing

virus (CWV) and Black queen cell virus (BQCV). However, these viruses were detected using only serological methods and in bees sampled from one location (Belgrade). Also, the results from a recent study [46] showed the presence of five viruses in honey bee samples from Serbia. The mentioned investigation encompassed only apiaries from northern Serbia and the detection of bee viruses was based upon the conventional RT-PCR assay. Surveys conducted in France, Austria, Hungary and Slovenia revealed that ABPV and DWV viruses are prevalent in European honey bees, present in 37-70% and 70-97% of bee samples, respectively [12,13,15,47]. Thus, survey of DWV and ABPV in Serbia is important for the assessment of their impacts on beekeeping in this country. The goal of this study was to investigate the occurrence and prevalence of DWV and ABPV in asymptomatic honey bee colonies from different regions of Serbia. TaqMan one-step real-time RT-PCR is used because of its capability to detect a small amount of viral copies in samples from apparently healthy colonies. This helps to increase the throughput, reduces the chance of carry-over contamination and eliminates post-PCR processing as a potential source of error [48].

## MATERIAL AND METHODS

### ***Honey bee samples***

Bee samples were collected from 55 seemingly healthy colonies located at 11 apiaries (5 colonies per apiary) distributed in northern, southern, eastern, western and central Serbia (Figure 1). Colonies considered being valid for honey production were randomly selected within each apiary and checked for the absence of clinical signs of viral infections. Samples consisting of approximately 100 worker bees were taken from each colony during the second half of September. Samplings were done by licensed veterinarians. Live bees were collected with sterile single-use vessels, placed into sterile test tubes, immediately stored in dry ice, transported to the laboratory and stored at -20°C until processed.

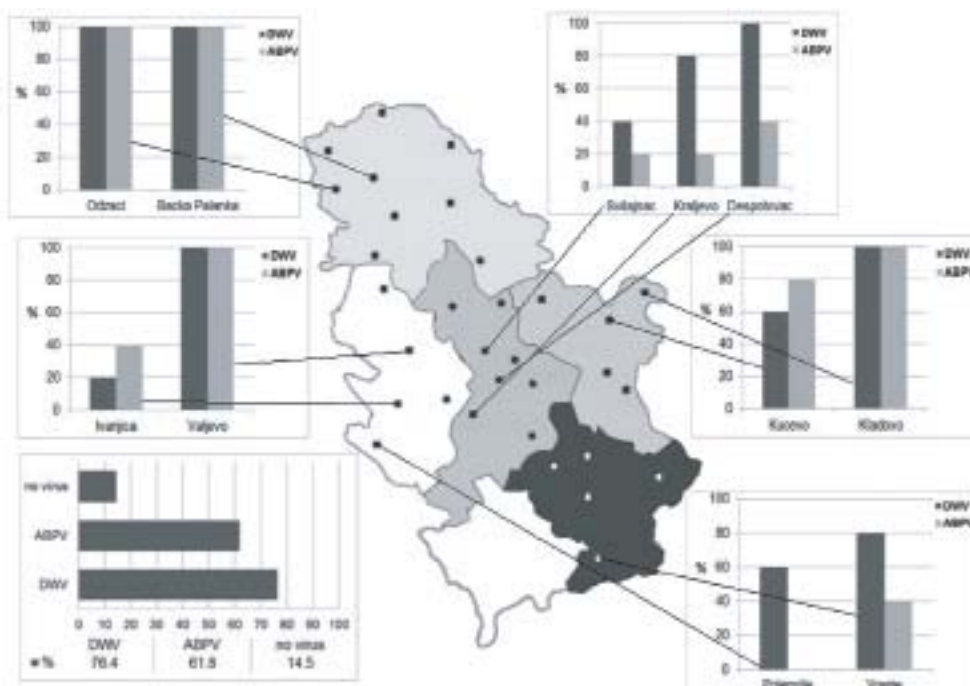
### ***Sample preparation and RNA extraction***

From each sample, 30 randomly selected bees were crushed and homogenized in a sterile mortar in the presence of 5 mL PBS solution. After homogenization, purification of samples was conducted by centrifugation for 15 min at  $5,000 \times g$  and 140  $\mu$ L of supernatant was collected and used for RNA extraction. Total RNA was extracted using ZR Viral RNA Kit™ (Zymo Research, Orange, CA) according to manufacturer's recommendations. Extracted RNA was quantified by spectrophotometry and an average of 2  $\mu$ g was used for single real-time RT-PCR reaction. Isolates were stored at -80 °C until use.

### ***Real-time RT-PCR assay***

To achieve the best optimization of the reaction, the real-time RT-PCR assay was conducted with positive controls for DWV and ABPV, kindly provided by the Veterinary Institute "Kraljevo" (Serbia). The positive controls were obtained after conventional

RT-PCR and proved to be DWV and ABPV by sequencing. The primer pairs and PCR program used for RT-PCR were those recommended by the “French Agency for Food, Environmental and Occupational Health & Safety” (ANSES) [49].



**Figure 1.** Occurrence and distribution pattern of DWV and ABPV among sampling location in Serbia.

All real-time RT-PCR reactions were performed on Rotor-Gene Q 5plex (Qiagen, Germany) and the presence of DWV and ABPV in bee samples were tested in a separate single step reaction using the Rotor-Gene Probe RT-PCR Kit (Qiagen, Germany). The primer pairs and probes (Table 1) were selected based upon the previous report of Chantawannakul et al. [14]. Both primers and TaqMan probes were obtained by Eurofins MWG Operon (Germany). The 5'-terminal reporter dye for each probe was 6-carboxyfluorescein (FAM) and the 3'- quencher was tetra-methylcarboxyrhodamine (TAMRA). After several trials, the final concentration of 800 nM for primers and 400 nM for probes in the reaction were affirmed as most appropriate. All analyses were carried out in a 25 µL reaction volume containing: 12.5 µL of 2x Rotor-Gene Probe RT-PCR Master Mix (Qiagen, Germany), 0.8 µM of each primer (one pair of primers per reaction), 0.4 µM of each probe, 0.25 µL Rotor-Gene RT Mix (Qiagen, Germany), RNase-free water and 2 ng of RNA template. The thermal cycling profile included a reverse transcription step of 50°C for 10 min, followed by an initial activation step of 95°C for 5 min and 45 cycles of denaturation (95°C for 15 sec), primer annealing (50°C for 30 sec) and DNA extension (72°C for 30 sec). Fluorescence was acquired during the elongation step. Negative controls (no template) were included in each reaction run.

**Table 1.** Sequence of the TaqMan primers and probes used for the detection of DWV and ABPV

Primer/Probe	Target	Sequence (5'-3')	Reference
DWV958F DWV9711R DWV9627T	<i>Deformed wing virus</i>	CCTGGACAAGGTCTCGGTAGAA ATTTCAGGACCCACCCAAAT CATGCTCGAGGATTGGGTCGTCGT	Chantawannakul et al. (2006)
APV95F APV159R APV121T	<i>Acute bee paralysis virus</i>	TCCTATATCGACGACGAAAAGACAA GCGCTTTAAATTCATCCAATTGA TTTCCCGGACTTGAC	Chantawannakul et al. (2006)

F- forward primer; R - reverse primer; T - probe. Probes consist of oligonucleotides with a 5'-reporter dye (FAM, 6-carboxy-Xuorescein) and a 3'-quencher (TAMRA, tetra-methylcarboxyrhodamine)

## RESULTS

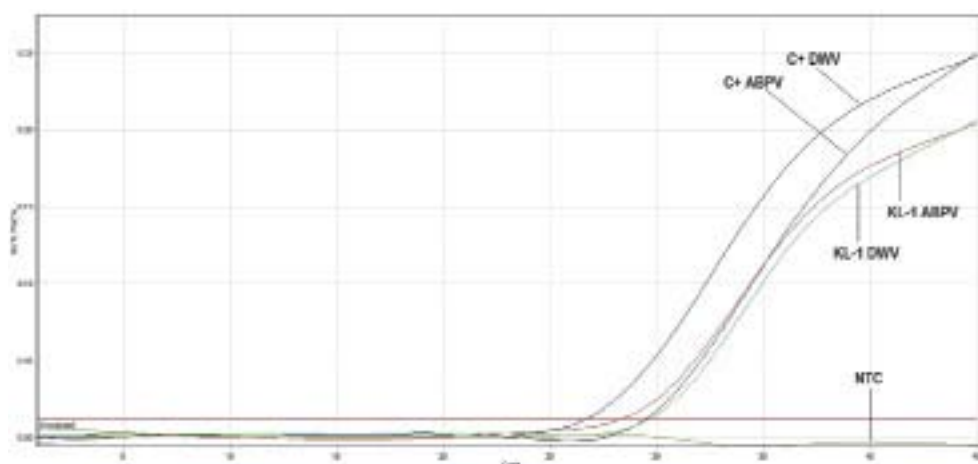
DWV was found to be more prevalent than ABPV. Among all analysed bee samples, 76.4% was positive for DWV and 61.8% for ABPV. Although both viruses were affirmed in all monitored Serbian regions, only DWV was detected in every sampled apiary (Figure 1, Table 2). Figure 1 shows the distribution and prevalence of DWV and ABPV in the sampled locations. The northern region of Serbia, represented by sampling locations Odzaci and Backa Palanka, showed the highest frequency of DWV and ABPV, as both were found in 100% of samples. Also, both viruses were found in all samples from locations Valjevo (Western Serbia) and Kladovo (Eastern Serbia). ABPV nucleic acid was not detected in any of the samples from the westernmost sampled location (Prijepolje).

Altogether, at least one of the investigated viruses was detected in 85.5% of samples (in 47 out of 55 colonies). Simultaneous infections with DWV and ABPV were present in 50.9% of samples (28/55 colonies). Single infections with either DWV or ABPV were detected in 23.6% (13/55 colonies) and 9.1% (5/55 colonies) of samples, respectively. Only 14.5% (8/55) of sampled colonies were negative for the presence of DWV and ABPV by real-time RT-PCR assay (Table 2).

**Table 2.** Overall frequencies of DWV and ABPV infections found in examined honey bee samples from Serbia

Single or simultaneous infections	Type of infection	No. of samples	%
Single	DWV	13	23.6
	ABPV	5	9.1
Simultaneous	DWV, ABPV	28	50.9
Total	DWV	42	76.4
	ABPV	34	61.8
	No viruses	8	14.5

The real-time RT-PCR assay with the use of TaqMan probe based chemistry was initially carried out with positive sequenced DWV and ABPV controls that were not included in the final results (Figure 2). In addition, before the evaluation for the presence of DWV and ABPV in selected samples started, concentrations of primers and probes were set at 0.8  $\mu$ M and 0.4  $\mu$ M, respectively. The sensitivity of the method was investigated by testing 10-fold dilutions of two positive field samples for DWV and ABPV and comparing them to the results of the conventional RT-PCR. The detection limit was determined by the lowest dilution that showed specific amplification for the strong positive field samples selected as templates. Using the same positive samples, conventional RT-PCR had a dilution detection limit of  $10^{-6}$  for both viruses, while the dilution detection limit for real-time RT-PCR was  $10^{-8}$ . TaqMan Ct values for DWV and ABPV in samples from locations Odzaci, Backa Palanka, Valjevo and Kladovo were mainly between Ct 25 and 30 (Figure 2). In the remaining locations where DWV and ABPV were recorded samples reached the threshold between 35 and 40 cycles.



**Figure 2** Amplification plot of a probe-based real-time RT-PCR assay showing DWV and ABPV positive bee samples. C+DWV and C+ABPV are representing positive controls for DWV and ABPV, respectively. KL1-DWV and KL1-ABPV are illustrating the presence of DWV and ABPV in samples from Kladovo. NTC stands for a no template control. Single replicates are shown for clarity, but each sample was tested in duplicate

## DISCUSSION

This study reveals the presence and distribution of DWV and ABPV in Serbian apiaries. There are only two reports concerning the presence of bee viruses in samples originated from Serbia. The first one [45] is showing the existence of ABPV in Serbian honey bees. However, this investigation was conducted 23 years ago and in samples from a restricted area near Belgrade. In addition, methods used in that study were based on electron microscopy followed by immunodiffusion with no implication of molecular techniques. In the second one, Petrovic et al. [46] recorded the presence of both DWV and ABPV, but using conventional RT-PCR and only in apiaries from the northern part

of Serbia. It is noteworthy that the applied methodology in both reports could not provide detection of low virus levels typical for persistent inapparent infections.

A total of 55 honey bee colonies distributed in different regions of Serbian territory were investigated by TaqMan probe-based real-time RT-PCR for the presence of DWV and ABPV, two important honey bee viruses implicated in colony mortality. In most of the apiaries where samples were collected, clinical signs of diseases concerning viral infections were lacking. In this study both monitored viruses were detected using real-time RT-PCR technique. The prevalence of positive DWV and ABPV samples in this survey is consistent with the results of Petrovic *et al.* [46] and reports from other countries. The finding of DWV in 76.4% of samples is within the range of 67-97% DWV-positive samples reported in other surveys [12,13,15,18,46,47,50]. The high prevalence of DWV in all sample locations is expected as an outcome of its close association with *V. destructor*, being the biological and mechanical vector for this virus. The sampling period also contributes to the obtained results, as climate conditions in Serbia during the sampling time still allow bees to forage forcing many beekeepers to postpone the autumn acaricide treatment. The results reported by Bailey *et al.* [4], Tentcheva *et al.* [12], Gauthier *et al.* [6] and Runckel *et al.* [51] show that ABPV is known to persist in apparently healthy colonies. The proportion of 61.8% samples positive to ABPV found in this study is in accordance with the estimates obtained in France (58%) [12], Austria (68%) [13], Hungary (37%) [15], Germany (73%) [33] and Slovenia (40%) [47], but disagrees with findings of researchers in Denmark (10.56%) [38], China (6%) [18], South Korea (0%) [52] and Uruguay (9%) [50]. High prevalence of ABPV in Serbian samples could also be explained by the mite-virus relationship, sampling period and apitechnical measures mentioned earlier.

Although both DWV and ABPV were detected in Serbian apiaries, their occurrence and prevalence differ among sampling regions (Figure 1). Single DWV and ABPV infections in Serbia were present in 23.6% and 9.1% of samples, respectively; however, simultaneous DWV-ABPV infections were recorded in 50.9% of samples (Table 2). Simultaneous infections have been proven in asymptomatic colonies worldwide [53, 13, 6, 38, 18]. Infection with both viruses was recorded in each apiary from sampling locations in northern Serbia (Odzaci and Backa Palanka). High frequency of DWV-ABPV infection in northern Serbia could be connected with increased mite infection due to different control strategies directed against *V. destructor* among Serbian beekeepers. Namely, late forage in this region postpones *V. destructor* treatment with soft acaricides (e.g. thymol, formic and oxalic acids), raising the level of surviving mites and the possibility of virus spreading. In contrast, colonies from higher altitudes, such as Prijepolje and Ivanjica, where the hard acaricide treatment usually finishes by the end of July, had lower prevalence of DWV (60% and 20%, respectively) and ABPV (0% and 40%, respectively) compared to other locations. Percentage of samples where neither of the monitored viruses was detected (Table 2) is higher than in reports of Tentcheva *et al.* [12], Berenyi *et al.* [13], Antúnez *et al.* [50], Toplak *et al.* [47] and Ai *et al.* [18], although several times lower in comparison with 64.4% virus-free samples from Spain [54]. This discrepancy could be explained by the number of viruses searched in each survey.

Several RT-PCR assays [6,12,13,55] or non-specific SybrGreen real-time RT-PCR [33,37,56] have been designed for the purpose of the detection of DWV and ABPV. However, only few probe-based TaqMan real-time RT-PCR protocols have been published for DWV and ABPV [14,39]. Non-specific detection system is characterized by fluorescence enhancement upon binding the intercalating dye (SYBR Green) to dsDNA, while a specific probe is designed to bind within the amplified PCR fragment. Therefore, due to a non-specific binding to all dsDNA products, including primer dimers, SYBR Green detection system is usually less specific. In this study several TaqMan real-time RT-PCR reactions were conducted with positive and sequenced DWV and ABPV controls with the purpose of assay optimization (Figure 2). Unlike manufacturer's recommendations and protocol published by Chantawannakul et al. [14], we had to raise concentrations of primers and probes to 0.8  $\mu$ M and 0.4  $\mu$ M, respectively, to reach the maximum sensitivity of the assay used in this study. In each sampling location where high prevalence of DWV and ABPV was recorded Ct values were mainly between Ct 25 and 30, indicating high levels of these viruses in bee samples. Nevertheless, when sampling was conducted no signs of disease or colony collapse were observed. After serial 10-fold dilutions of strong positive DWV and ABPV field samples used to compare conventional and real-time RT-PCR assay, dilution detection limit of real-time RT-PCR was 100 times lower. This proved the higher sensitivity of real-time RT-PCR as reported by Jamnikar Ciglinečki et al. [39] and Blanchard et al. [57].

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## **ISPITIVANJE PRISUSTVA VIRUSA DEFORMACIJE KRILA I VIRUSA AKUTNE PARALIZE PČELA U PČELINJIM ZAJEDNICAMA IZ SRBIJE UPOTREBOM “REAL-TIME RT-PCR” METODE**

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Ovo istraživanje je obuhvatilo utvrđivanje prisustva virusa deformacije krila (DWV) i virusa akutne paralize pčela (ABPV) u 55 pčelinjih društava poreklom iz različitih delova teritorije Srbije, upotrebom “real-time RT-PCR” metode bazirane na “TaqMan” probama. Rezultati pokazuju prisustvo DWV na svakom ispitivanom lokalitetu, dok je ABPV bio prisutan u 10 od 11 ispitivanih pčelinjaka. Imajući u vidu ulogu ektoparazita *Varroa destructor* u prenošenju i aktivaciji pčelinjih virusa, visoka učestalost uzoraka pozitivnih na DWV (76,4%) i ABPV (61,8%) u asimptomatskim pčelinjim društvima može biti posledica nedovoljno efikasnog i zakasnelog tretmana protiv ove grinje. “Real-time RT-PCR” metoda, opisana u ovom radu se pokazala kao najpogodnija metoda za ovakvu vrstu istraživanja.