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Investigation of deformed wing virus, black queen cell virus, and acute bee paralysis virus infections in honey bees using reverse transcriptase-polymerase chain reaction (RT-PCR) method

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Abstract: Viruses are one of the most potential risk factors that negatively affect the different life stages of honey bees. This study was conducted to determine the presence of infections caused by the deformed wing virus (DWV), black queen cell virus (BQCV), and acute bee paralysis virus (ABPV) in honey bees in the beekeeping regions of Burdur, along with obtaining information about their prevalence in this particular region. In our study, the adult bees were taken from 31 different beehives and comb samples that had different honey bee breeds and were sampled randomly from 15 beekeeping areas within the region. The collected samples were analyzed using the reverse transcriptase-polymerase chain reaction (RT-PCR), and the prevalence of DWV, BQCV, and ABPV infections were determined to be 74.19% (23/31), 25.81%, (8/31) and 74.19% (23/31), respectively. In this study, the distribution of positive samples and the rates of multiple infections were determined in the colonies. Of the positive honey bee samples, 12 (%38.71) were detected only for one virus, 9 (%29.03) were positive for two viruses (DWV-ABPV) and 8 (%25.81) were positive for all three viruses. In the present study, the presence of the three bee viruses that caused significant damage to the colonies by multiple infections in the Burdur region was determined with RT-PCR. To our knowledge, this is the first report of three mentioned bee infections in honey bees in the Burdur region. The revealed epidemiological conditions lead to the conclusion that serious measures are needed to control these infections in this region.
Keywords: Acute bee paralysis virus, black queen cell virus, deformed wing virus, honey bee, RT-PCR.

Bal arılarında deforme kanat virus, siyah kraliçe hücre virus ve akut arı felci virus enfeksiyonlarının reverz transkriptaz-polymerase chain reaction (RT-PCR) metodu kullanılarak araştırılması

Özet: Viruslar bal arılarının farklı yaşam evrelerini olumsuz yönde etkileyen potansiyel risk faktörlerinden birisidir. Bu çalışma, Burdur yöresinde arıcılık işletmelerinde bulunan bal arılarında deforme kanat virus (DKV), siyah kraliçe hücre virus (BKHV) ve akut arı felci virus (AAFV) enfeksiyonlarının varlığının virolojik olarak saptanması ve bu yöredeki yaygınlıkları hakkında bilgi edinilmesi amacıyla yapılmıştır. Araştırmada söz konusu yörede bulunan 15 arıcılık işletmesinden tesadüfi örnekleme ile farklı arı ırklarından 31 farklı kovandan ergin arı ve yavrulu petek örneği usulüne uygun olarak alındı**.** Toplanan numuneler reverse transcriptase-polymerase chain reaction (RT-PCR) metodu ile analiz edildi ve DKV, BKHV, AAFV enfeksiyonlarının prevalansı sırasıyla %74,19 (23/31), %25,81 (8/31), %74,19 (23/31) oranlarında belirlendi. Çalışmanın yürütüldüğü kolonilerde tespit edilen pozitifliğin ırklara göre dağılımı ve çoklu enfeksiyon oranları tespit edildi. Pozitif örneklerin 12'si (%38,71) sadece bir virusa, 9'u (%29,03) iki virusa (DKV-AAFV) ve 8'ide (%25,81) üç virusa karşı pozitif bulundu. Bu çalışmada Burdur yöresinde çoklu enfeksiyonlarla kolonilercde önemli hasara neden olan üç arı virusunun varlığı RT-PCR ile belirlendi. Bu çalışma, Burdur bölgesindeki bal arılarında adı geçen üç virusun varlığına ilişkin bilinen ilk raporu sunmaktadır. Tespit edilen epidemiyolojik sonuçlara göre yörede bu enfeksiyonların kontrolü amacıyla ciddi önlemlerin alınması gerektiği kanaatine varıldı.
Anahtar sözcükler: Akut arı felci virus, bal arısı, deforme kanat virus, siyah kraliçe hücre virus, RT-PCR.

Introduction

Viruses are one of the most potential risk factors that negatively affect the different life stages of honey bees. About 26 honey bee viruses have been reported so far in the literature (16, 27, 40). Many studies conducted in beekeeping have shown that the deformed wing virus (DWV), black queen cell virus (BQCV), and acute bee paralysis virus (ABPV) are the main viruses causing

heavy losses in bees (41, 43, 44). The viral nucleic acid of DWV is a single-stranded positive polarity RNA (25, 28), and the structure is approximately 10.1 kb long. The DWV virus belongs to the *Iflavirus* genus of the *Iflaviridae* family. Iflaviruses differ from the dicistroviruses through a single ORF region present in their genome (45). The deformed wing virus of honeybees is closely associated with characteristic wing deformities, paralysis, abdominal bloating, and rapid mortality of emerging adult bees (25).

The *Varroa destructor* is a major vector for DWV. This ectoparasitic mite receives the virus through the hemolymph of the infected bee and transmits the virus while feeding on another bee. DWV can be transmitted across bee colonies through both vertical and horizontal transmission. Vertical transmission takes place through drone sperm and queen bee eggs while horizontal transmission takes place through larval foods (9, 33, 48, 49). DWV causes colony effects such as bee deformity, malformed appendages (crumpled/vestigial wings), shortened abdomen, weight loss (11, 38), a probable decline in lifespan (23), and consequently irregular and decreased bees (40).

BQCV is the most common (approximately 80%) honey bee virus, followed by the deformed wing and sacbrood viruses (43). It is an RNA virus that belongs to the *Triatovirus* genus of the *Dicistroviridae* family (42). This virus has a non-enveloped structure with cubic symmetry and carries a single-stranded positive polarity nucleic acid (ssRNA) (26). Being an etiological agent of a fatal disease in honeybee queen larvae and pupae, the BQCV infection is observed on the sealed cell wall of the queen's pupa along with the presence of dead pupae (5). The *Nosema apis* and *Nosema ceranae* microorganisms act as a vector in the epidemiology of the BQCV infection (1, 4, 7, 44). Also, the transmission may occur through feeding or contaminated foods (2). BQCV is one of the most common but least known honey bee pathogens, with the most common clinical symptoms consisting of blackened cells and the death of queen larvae or pupae (42). It is observed that the dead pupae in the cell take up a dark color and appear brownish to black. Simultaneously, the blackening of the cell wall occurs on the larvae and pupae as well. This virus causes diarrhea in adult bees (28), where the infected queen bee becomes weak and contaminated (14).

Acute bee paralysis is frequently seen in honey bees and is one of the causes of collapsing colonies. Acute bee paralysis virus (ABPV) is an RNA virus that belongs to the *Aparavirus* genus of the *Dicistroviridae* family (12). It has a cubic symmetry and non-enveloped structure that carries single-stranded viral nucleic acid (ssRNA) (46).

The disease caused by ABPV can be seen across all the biological phases of honey bees. In its natural condition, the virus spreads through the oral secretions (royal jelly) of infected adult bees, which are transmitted to young pupal bees during the feeding process. Bees display the symptoms of the viral disease through their feces. The parasitic mite *V. destructor* is the vector of this virus (30). Depending on the ABPV infection, acute or subclinical diseases are formed in bees. Symptoms and deaths connected to acute bee paralysis occur mostly in colonies infested with *Varroa* mites. Adult bees show symptoms of paralysis as trembling after 5–6 days of incubation. Progressively, some of the bees may appear dark and hairless, while flightless honey bees die within 1–2 days (28). The incidence of the ABPV infection varies in different countries based on their colony capacities and *Varroa* control programs.

Although there is a considerable amount of honey bee population in our country and the world, the data on bee viruses is a limited reference. This study aimed to determine the presence, prevalence, and distribution rates of the bee colonies infected with the DWV, BQCV, and ABPV, causing significant colony losses in the Burdur region using the RT-PCR method.

Materials and Methods

Samples and isolation procedure: In this study, sampling was carried out between June and September 2019 within the Burdur region. The sample size was calculated at a 90-95% confidence interval to determine the number of samples in this research (13). Thirty-one (from 15 apiaries) adult bees and pupa samples were taken from beehives and brought to the laboratory in the cold chain. The collected honey bee samples consisted of different races, including ten Anatolian races (five Muğla ecotypes and five Ege ecotypes), three Syrian, two Belfast, seven Italian, two Carpathian, and seven Carniolan races**.**

The presence of a clinically deformed wing in some honey bees was observed during our field studies (Figure 5). Also, all honey bees were checked during the laboratory stage of the research for *Varroa* mites under a stereomicroscope.

Twenty adult bees and honeycomb samples were grouped in each sample. The samples were homogenized in a sterile mortar with 4-5 mL of phosphate buffer saline (PBS) along with 1000 µg of streptomycin and 1000 IU of penicillin per mL. Following this process, the samples were transferred to 15 mL sterile tubes and centrifuged for 30 min at 4000 rpm. Supernatants were stored at –80 °C until further extraction of RNA.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR): Total RNA extraction was performed by placing the supernatants obtained from homogenate samples into the automatic extraction appliance (Roche, Magna Pure, Germany). The

obtained 31 supernatants were further used for the experiment, out of which 200 µL was used for each bee sample. RNA samples, thus, obtained were stored at –80 °C until required for reuse. As per the procedure, a separate primary concentrate was prepared for all three viruses using a high pure viral nucleic acid kit elution buffer (no: 11858874001). Further, OneStep RT-PCR Kit (Grisp, Xpert OneStep RT-PCR Kit GK64.0100 Portugal) was used for reverse transcription, where the RT-PCR mix for each sample was prepared in a sterile Eppendorf tube as follows: 12.5 µL of Fast PCR Master mix, 1 µL each of forward GSP (10 p/mol) and reverse GSP (10 p/mol), 5 µL of extracted RNA sample, 1.25 µL of RTase Mix, and 4.25 µL of RNase-free water.

RT-PCR was performed using specific primers for the VP1-VP2 gene of DWV, the VP3 gene of BQCV, and the VP2 gene for ABPV. The specific primers are shown in Table 1. The thermal cycling conditions were as follows: one cycle for reverse transcription at 45 °C for 10-15 min followed by initial denaturation at 95 °C for 3 min. Then, 35 cycles of denaturation step at 95 °C for 10 sec, annealing at 56 °C for 10 sec, and finally an extension step at 72 °C for 15 sec with a final extension of one cycle at 72 °C for 1 min. The annealing temperature used to amplify all the viruses was the same. Viral RNA was amplified using the Techne TC-412 device.

We used the positive RNA controls from the Izmir Bornova Veterinary Control Institute to achieve optimization in PCR. The products were electrophoresed on a 1.5% agarose gel containing ethidium bromide and then visualized under a UV transilluminator.

***Varroa destructor* determination:** The honey bees brought to the laboratory were analyzed for the presence of *Varroa* mite. The average size of the mature *Varroa* mites is 1.2 mm while the width is 1.6 mm, and the male mite's size is smaller than the size of the female mite. Mature female *Varroa* mites are reddish brown, but male mites are tan in color (21, 30). The sedimentation method was used to separate the *Varroa* from the wastes of the hive wood floor. One part of waste was mixed with 10 parts of oil. *Varroa* mites that accumulated on the surface of the oil were collected after the waste had precipitated. The bees were placed in jars of warm water at 8-12°C to gain a clear result and one drop of detergent was dropped on them. The honey bees were then examined for *Varroa* mite approximately 5-10 min. after being removed from the jars (21).

Results

The prevalence of all three viruses among the 31 samples collected from 15 apiaries is shown in Table 2. Based on the results of the viral-specific PCRs, out of 31 samples, twenty-three (74.19%) were found to be coinfected with DWV and ABPV, while eight (25.81%) were found to be positive for BCQV nucleic acid. As predicted, the DWV primers amplified a 269 bp fragment from the VP1-VP2 gene of DWV, while a 460 bp amplicon was amplified from the VP2 gene of ABPV and 536 bp amplicon was obtained from the VP3 gene of BQCV. The gel images obtained from the RT-PCR results are presented in Figures 1, 2, and 3.

Table 1. Primers used for all three viruses and the size of their amplicons.

Primer	Nucleotide sequences	Target gene	Product length (bp)	Reference
DWV-F	TGGTCAATTACAAGCTACTTGG	VP1-VP2	269 bp	39
DWV-R	TAGTTGGACCAGTAGCACTCAT			
BQCV-F	CTTTATCGAGGAGGAGTTCGAGT	VP3	536 bp	39
BQCV-R	GCAATAGATAAAGTGAGCCCTCC			
AIV-F	GGTGCCCTATTTAGGGTGAGGA	VP2	460 bp	39
ABPV-R	ACTACAGAAGGCAATGTCCAAGA			

Table 2. Prevalence distribution of all three viruses based on the bee races.

Bee race	Number of samples	DWV (+)/%	BQCV (+)/%	ABPV (+)/%
Muğla	5	5 / 100	3 / 60	5 / 100
Syria	3	3 / 100	-	1 / 33.33
Belfast	2	2 / 100	-	1 / 50
Italian	7	5 / 71.43	3 / 42.86	7 / 100
Carpathian	2	2 / 100	-	2 / 100
Ege	5	4 / 80	2 / 40	2 / 40
Carniole	7	2 / 28.57	-	5 / 71.43
Total	**31 Samples**	**23 / 74.19**	**8 / 25.81**	**23 / 74.19**

Figure 1. DWV RT-PCR gel image [M: Marker, 1–31: Samples, NK: Negative Control, PK: Positive Control, PK1: Positive control] (269 bp).

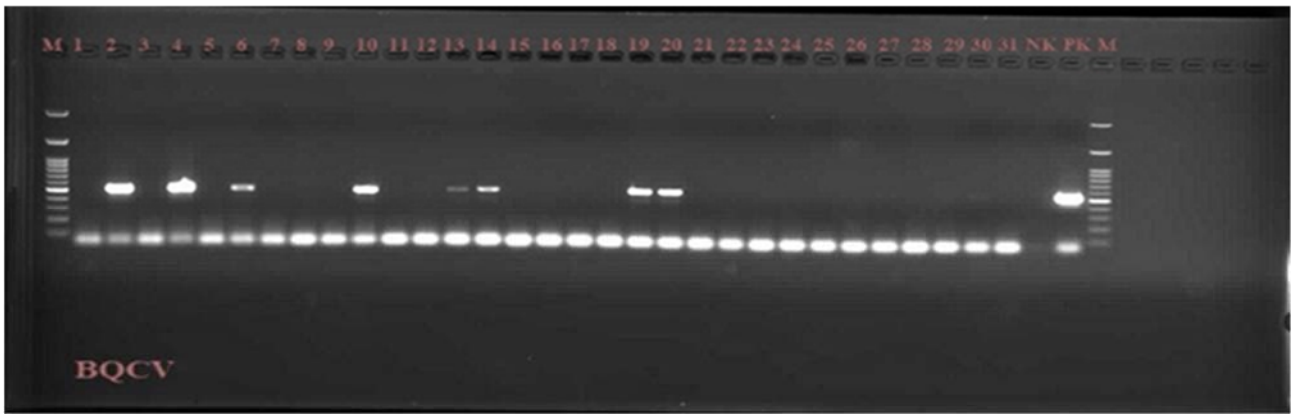

Figure 2. BQCV RT-PCR gel image [M: Marker, 1–31: Samples, NK: Negative Control, PK: Positive Control] (536 bp).

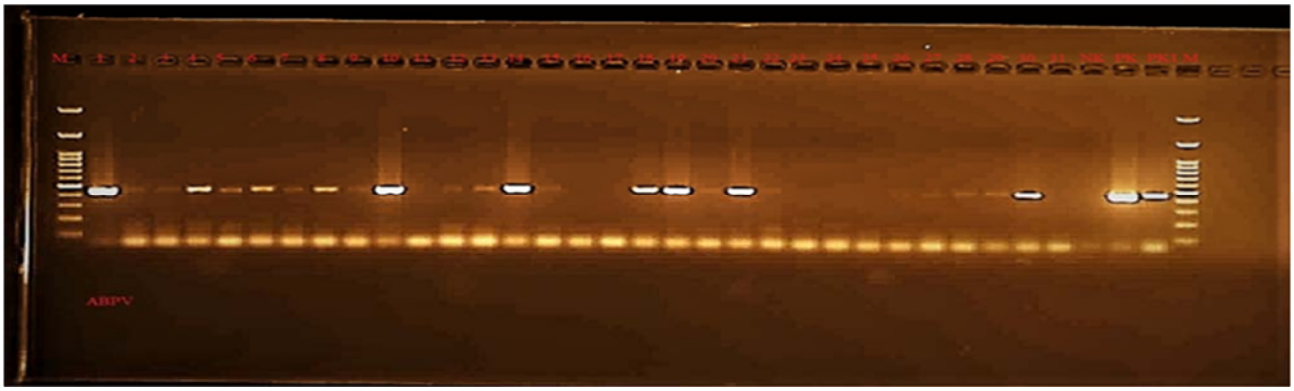

Figure 3. ABPV RT-PCR gel image [M: Marker, 1–31: Samples, NK: Negative Control, PK: Positive Control, PK1 Positive control] (460 bp).

The distribution of the resultant data was evaluated according to the races and is as follows: twenty-three DWV positive samples were observed in the Anatolian race of Muğla ecotype, four in the Anatolian race of Ege ecotype, three in Syria, two in Belfast, five in Italian, two in Carpathian and two in Carniole bee races, respectively. Out of the eight BQCV positive samples, three were determined to be of the Anatolian race of Muğla ecotype, two were from the Anatolian race of Ege ecotype, and three were from the Italian bee race. Out of twenty-three ABPV positive samples, five were found to be of the Anatolian race of Muğla ecotype, two were of the Anatolian race of Ege ecotypes, one of Syrian and Belfast races, seven of the Italian race, five of Carniole, and two were of the Carpathian bee race (Table 2).

Positive results were evaluated in the colonies in terms of DWV, BQCV, and ABPV, with multiple infection rates also being determined. When the distribution of study data by race is evaluated; 23 DWV positive samples; have been observed in Muğla, 4 in Ege

ecotype, 3 in Syria, 2 in Belfast, 5 in Italian, 2 in Carpathian and 2 in Carniole, respectively. 8 BQCV positive for example; 3 of them were determined in Muğla, 2 of them in Ege ecotype and 3 of them in Italian bee race. 23 ABPV positive samples were found to be 5 in Muğla, 2 in Ege ecotypes, 1 in Syria and Belfast, 7 in Italian, 5 in Carniole and 2 in the Carpathian bee race (Table 2).

In addition, positive results in terms of DWV, BQCV and ABPV were evaluated and multiple infection rates in colonies were determined. When these results were evaluated, 2 (6.45%) honey bee samples were found negative for all the viruses controlled. Of the positive honey bee samples, 12 (38.71%) were detected only for one virus, 9 (29.03%) were positive for two viruses (DWV-ABPV) and 8 (25.81%) were positive for all three viruses (Figure 6).

The obtained samples were identified as *Varroa destructor* by morphological analysis (Figure 4).

Figure 4. *Varroa* spp.-infested bee samples encountered during the research are marked with red signs (Original).

Figure 5. Images with deformed wings detected in bees at some sampled apiaries (Original).

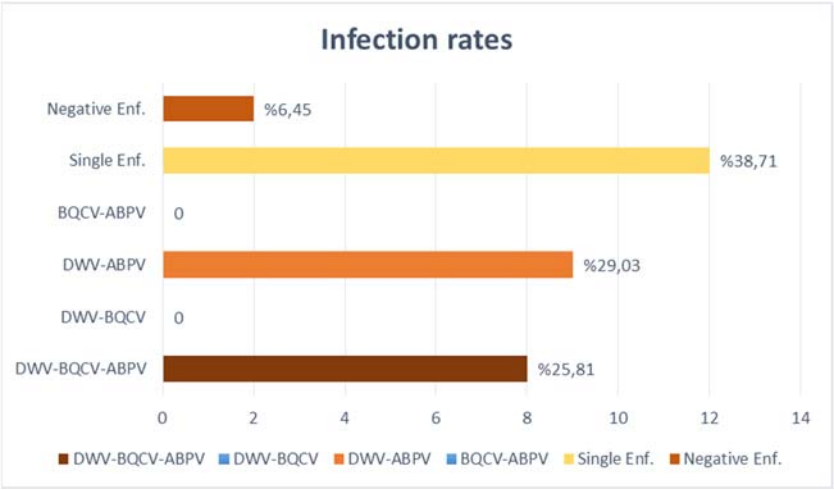

Figure 6. Multiple infections were observed in the controlled colonies.

Discussion and Conclusion

Honey bee products such as royal jelly, honey, and pollen are very valuable in terms of nutrition and health and also in the industries. Additionally, bees are critical for the continuity of the ecosystem. Therefore, it is very important to protect the health of honey bees and protect them against diseases. Especially, it is essential to prevent the unexplained colony losses in beehives or minimize the losses for the future and continuation of our world (3, 7, 18, 29).

The existence of healthy bees and bee colonies in our country and the world is very important in terms of both natural life and human health. One of the most important criteria is to raise the bees and their colonies free from the viral agents. Hence, it is necessary to conduct accurate and rapid diagnoses along with combating these diseases by proper protection measures in place. In this way, we can protect the health of honey bees and contribute to future generations and the ecosystem (17, 20, 31, 36, 44). Difficulties are encountered while assessing the epidemiological status of countries because it is not compulsory to report the DWV, ABPV, and BQCV infections in disease reporting systems such as Animal Disease Notification System (ADNS) and World Organization for Animal Health (OIE). However, when the literature related to these viruses was examined, the diseases were observed to be spread worldwide. Depending on the honey bee colony capacities of various countries and the *Varroa* struggle programs, the incidence of these infections varies between 2% and 91% (3, 30, 34, 44, 47).

DWV causes unusually high winter deaths and disease symptoms and is detected at high prevalence in adults and pupae in many countries using molecular diagnostic techniques such as RT-PCR methods (3, 6, 15, 24, 31). The prevalence of DWV in the world is as described below: the most common honey bee virus is found in Thailand (37) and is closely related to the infestation of *Varroa* (8) and *Tropilaelaps* mites (22). A study conducted in Germany showed that all German bees infested by the endemic *Varroa destructor* parasite species were compared with the Swedish bees brought from Sweden. However, this *Varroa* mite has not been reported to date. The German bees were 100% positive for the DWV nucleic acid, while only 40% of Swedish bees were positive for DWV (50). A study was carried out using the RT-PCR method in the Aclun region of Jordan, where there were losses observed in the bee colonies, and it was determined that there was a high rate of DWV in these colonies (19). Shumkova et al. (41) reported the prevalence of DWV in the bee colonies of Bulgaria at a rate lower than that found in other countries. In the study conducted on honey bees located in different climatic regions of Argentina, 35% of the colonies were found to be DWV positive. However, in the same study, approximately 25% of the bees showed binary and triplet viral infections (30).

Many studies have been conducted in our country related to DWV. One study was conducted in the queen bee colonies, where approximately 50% of the hives were reported to be infected with this virus (17). Karapınar et al. (21) detected a high rate of DWV disease, i.e., 69.23% in the province of Van, which was also similar to the prevalence of *Varroa* parasite in these colonies. A large-scale study was conducted in apiaries in the Aegean region which used the multiplex RT-PCR method and determined the DWV rate to be 25.2%. Especially in some apiaries, where there were DWV disease symptoms or high colony loss, the prevalence of viral agents was reported to be higher in the bee population (10). Rüstemoğlu also reported the prevalence of DWV at a rate of 23.3% in a total of 90 apiaries collected from Hakkari province in 2015, which was conducted on the bee samples using the RT-PCR method (36). Kalaycı et al. reported that DWV was the most prevalent virus in the Turkish apiaries with a rate of 44.7% (20).

In this study, DWV was detected in twenty-three (74.19%) of 31 samples collected from 15 apiaries. Our results were parallel with those obtained by Karapınar et al. (21). But, our percentage was found to be higher than the percentages found by Çağırgan (10), Kalaycı et al. (20), and Rüstemoğlu (36). The reason for high DWV prevalence may be attributed to this province being a transit/accommodation area for migratory beekeeping. Considering that the transmission of the virus happens through the *Varroa* mite, the evaluation that the *Varroa destructor* is seen on the bees in many sampled apiaries makes the fight against it not completely realized.

BQCV is one of the most common but least known honey bee pathogens, which causes a disease that leads to blackness and death of the queen, larvae, and pupae in high titers (42). The prevalence of BQCV in Uruguay was found to be 91% using the RT-PCR method in the bee colonies, while the research was being reported as the first case of BQCV in South America (3). Tentcheva et al. (44) screened 36 adult bees and pupa samples collected from apparently healthy colonies during the spring, summer, and autumn seasons using the RT-PCR method and found 86% positive BQCV colonies. A study conducted in Denmark (31) revealed the presence of BQCV in the colony where winter deaths were unusually high. In Australia, 65% of the bee colonies were reported to be BQCV positive (33). Also, the BQCV infection was found at a high prevalence (81%) in Chile province in South America (35). However, another research was conducted by the same researchers in Chile in the following years,

which showed the prevalence of infection to be decreased by 10% (34). However, in the studies conducted in England, Croatia, and Syria, the prevalence of BQCV was reported to be of a lower rate (6, 15, 24).

In terms of the studies related to BQCV infection in our country, Gümüşova et al. observed higher BQCV positive rates in adult bees (53%) compared to the larvae (33%) using the RT-PCR method in 2010 (18). Oğuz et al. reported the presence of BQCV nucleic acid in the bees of the Van province at a rate of 88.5% using the RT-PCR method, which was sampled during April and May 2017 (32). In another study conducted in the same province, BQCV was found to be positive in 88.46% (23/26) of the sampled hives. In a study conducted using the multiplex RT-PCR method in apiaries in the Aegean region, the BQCV infection rate was determined to be 25.2% in the bees (10). Rüstemoğlu determined the rate of positive BQCV as 32.2% in the bee samples collected from Hakkari province in 2015 (36).

In our study, the presence of BQCV nucleic acid was detected in eight (25.81%) samples out of 31 bee samples collected from the Burdur region. This rate is lower than the positive rates found in the black sea and eastern provinces of our country (18, 32, 36). However, the BQCV positive rates in our results were corresponding exactly with the rate determined in the Aegean region (10), which is also closer to our area of study. The reason for the lower rate of BQCV-positive bee colonies in our study compared to the DWV and ABPV infections was attributed to the low rate of *Nosema* infection in bee colonies in our area.

Although ABPV presence was not detected in eastern bee colonies of China's Yunnan province, its presence was reported as 2% in Chile and 9% in Uruguay (3, 34, 47). Also, the studies conducted in Europe determined ABPV positive rates to be higher (6, 15, 31, 44) and were accepted as one of the important causes of bee losses.

The presence of ABPV infections in Turkey was reported at different rates since the studies were conducted in different provinces. For the first time in our country, the presence of ABPV (2.2%) was demonstrated using the RT-PCR method in bee samples collected from Hakkari (36). In the following years, Çağırgan (10) reported the prevalence of ABPV as 3.6% in the samples collected from apiaries in the Aegean region while Karapınar et al. (21) did not observe any ABPV infection in their screening of bee colonies in Van province.

In this study, we determined the prevalence of ABPV in the Burdur region to be at the rate of 74.19%. Compared to the other studies conducted in Turkey, our work in the beehive indicated the reason for a high rate of ABPV infection in the bee colonies, commonly consisting of *V. destructor* infestations. An increase in the spread of infectious agents was attributed to the fact that the Burdur region is an accommodation/transit area for migratory beekeeping. Additionally, in our study, varroasis was observed in all of the sampled bees. This is an important finding in terms of the epidemiology of viral pathogens transmitted by the *Varroa* vector.

Honeybee colonies are commonly infected by many viruses simultaneously, often without exhibiting overt signs (15, 28). Mixed viral infections were detected in honeybee samples, which were shown in Figure 6. These results were consistent with those obtained by Kalaycı et al. (20) and Çağırgan (10). Our mixed viral infection ratios were 25.81% and 29.03%, which might indicate the presence of colony losses in this study. But in all cases, multiple viral infections were observed simultaneously along *Varroa*. Also, multiple factors may have led to colony collapses depending on the quality of nutrition and pathogens/pathogen titers.

In conclusion, the data of this research revealed a very high prevalence of pathogenic viruses in the bee population of the Burdur province, which was thought to be the probable reason for the loss of colonies in the hives of Burdur. It is required to detect and use bee breeds to protect the health of the bees that are resistant to viral disease. Also, feeding/hosting conditions, compliance with sanitation, and hygienic measures should not be ignored. In addition to these, it may be beneficial to carry out screening/struggle programs for viral and parasitic factors to protect bees' health. We conclude that conducting regional and national major studies along with serious measures to protect the bees against viral diseases is very important for animal health and our country's economy and ecosystem.

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Ethical Statement

This study was approved by the Burdur Mehmet Akif Ersoy University Animal Experiments Local Ethics Committee (2019-61-493).

Conflict of Interest

The authors declared that there is no conflict of interest.

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