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Research Article

The Role of Viral and Parasitic Pathogens Affected By Colony Losses in Turkish Apiaries

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Abstract

Parasites and viruses are considered major agents of bee colony loss. The aim of this study is to detect the presence of seven viruses and three parasites - namely, Israeli acute bee paralysis virus (IAPV), deformed wing virus (DWV), sacbrood virus (SBV), acute bee paralysis virus (ABPV), black queen cell virus (BQCV), Kashmir bee virus (KBV), chronic bee paralysis virus (CBPV), *Nosema, Varroa* and *Tropilaelaps* spp. - in Turkish apiaries affected by colony loss. For this purpose, nine apiaries were examined in 2016, 38 in 2017 and 29 in 2018. The results show that DWV was the most prevalent virus in Turkish apiaries, identified in 44.7% of the samples. The second-most widespread virus was ABPV, in 35.5% of the samples, while the prevalence of BQCV, SBV, CBPV and IAPV was 28.9%, 22.3%, 18.4% and 6.5%, respectively. However, KBV was not identified in any of the apiaries. With regard to microsporidian and parasitological investigations, *Nosema* was found in 10, *Varroa* was detected in 21 and both *Varroa* and *Nosema* were simultaneously identified in seven apiaries. *Tropilaelaps* spp. was not detected. In some examples, two, three, four and even five viral agents were detected simultaneously. Mixed viral infections were common in some colonies, which had between two and five different viruses. In conclusion, viruses and mixed infections in particular play a major role in declining colonies.

Keywords: *Apis mellifera, Bee viruses, Colony losses, Nosemosis, Turkey, Varroasis*

Koloni Kaybından Etkilenen Türk Arılıklarında Viral ve Paraziter Patojenlerin Rolü

Öz

Parazitler ve viruslar koloni kayıplarına sebep olan önemli etkenlerdir. Bu çalışmada Türkiye arılıklarından koloni kaybı şikayeti ile gelen örnekler yedi viral etken; İsrail akut arı felci virusu (IAPV), deforme kanat virusu (DWV), sacbrood virusu (SBV), akut arı felci virusu (ABPV), siyah kraliçe hücre virusu (BQCV), kaşmir arı virusu (KBV), kronik arı felci virusu (CBPV) ve paraziter etkenler olan *Nosema, Varroa* ve *Tropilaelaps* spp. yönünden incelenmiştir. Bu amaçla 2016 yılından dokuz, 2017 yılından 38 ve 2018 yılından ise 29 örnek teste tabi tutulmuştur. Koloni kaybı yaşanan arılıklarda en fazla tespit edilen virus %44.7 ile DWV olmuştur. İkinci en yaygın virus ise %35.5' le ABPV'dir. BQCV, SBV, CBPV ve IAPV prevalansı ise sırasıyla %28.9, %22.3, %18.4 ve %6.5 olarak bulunmuştur. KBV ise örneklerin hiçbirinde tespit edilmemiştir. Parazitolojik incelemeler sonucunda *Nosema* 10 arılıkta belirlenirken, *Varroa* 21 arılıkta tespit edilmiştir. Yedi arılıkta ise hem *Nosema* hem de *Varroa* belirlenmiştir. *Tropilaelaps* spp. ise örneklerin hiçbirinde bulunmamıştır. Bazı örneklerde ikili, üçlü, dörtlü ve hatta beşli viral etkenler aynı anda tespit edilmiştir. Sonuç olarak, koloni kayıplarına yol açabilecek birçok faktör vardır, özellikle virüsleın ve miks enfeksiyonların azalan kolonilerde önemli bir rol oynadığı düşünülmektedir.

Anahtar sözcükler: *Apis mellifera, Arı virusları, Koloni kaybı, Nosemosis, Turkey, Varroasis*

INTRODUCTION

Honeybees *(Apis mellifera L.)* are economically and biologically indispensable due to their balancing and unique pollinator roles in the ecosystem [1]. The total global value of crops pollinated for food production by honeybees was estimated at nearly €153 billion [2]. Unfortunately, significant colony losses have recently been

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observed in honeybee populations [3]. There was a loss of approximately 10 million bees in the United States in the winter of 2006-2007, when an agent causing the sudden disappearance of all adult bees in a hive led to colony collapse disorder (CCD) [4]. It is believed that the reduction in honeybee populations is caused by parasitic diseases, viruses, microsporidians, genetic factors, severe seasonal weather conditions, malnutrition and exposure to pesticides [4-9].

Honeybee colony losses are a multifactorial phenomenon that include biotic and abiotic causes [10]. Pathogens such as viruses and parasites are biotic factors. Most important honey bee pathogens known to affect colony health have also been defined by ANSES (French Agency for Food, Environmental and Occupational Health And Safety). These are; a predatory beetle *(Aethina tumida)*, two ectoparasitic mites *(Varroa destructor* and *Tropilaelaps* spp.), two fungi *(Nosema apis, N. cerenae)*, two bacteria which cause American foulbrood and European foulbrood *(Paenibacillus larvae and Melissococcus plutonius)* and three viruses (CBV, DWV, and ABPV).

Varroosis is a disease of honeybees caused by mites in the genus Varroa. It is the most important honeybee disease to affect honeybees and cause destructive losses in colonies. The Varroa mite can be observed on both adult bees and brood. This mite acts as a vector for viruses (particularly DWV) [11,12]. *Tropilaelaps* spp. causes similar damage as *Varroa* infestations; it is a parasite of the brood of large honeybees, such as *Apis dorsata, A. laboriosa, and A. breviligula*. As in *Varroa*, it has been reported that it causes colony losses especially when heavy infestation in *A. mellifera* [13]. In addition, this mite can also act as a vector for viruses of the honeybees [14,15]. *Nosema* is an extremely important and common honeybee disease caused by the microsporidia *Nosema apis* and *N. ceranae*. It is transmitted through ingestion of spores by adult bees [16]. The epidemiology of BQCV is generally associated with *Nosema* infection [17,18].

More than 20 distinct viruses have been identified that affect the genus *Apis* [19]; this number changes continuously with the identification of new viruses, such as apis rhabdovirus-1, apis rhabdovirus-2 [20] and varroa orthomyxo-virus-1 [21]. Bee viruses in general belong to one of two families: Iflaviridae or Dicistroviridae. They are non-enveloped, single-stranded and positive-sense RNA viruses [1]. With the exception of the *Apis mellifera* filamentous virus and the *Apis* iridescent virus, which have DNA genomes, all of these viruses are single-stranded RNA viruses. Apart from chronic bee paralysis virus, which has an isometric shape, most of these viruses cannot be distinguished using particle morphology [22]. Israeli acute bee paralysis virus (IAPV), deformed wing virus (DWV), sacbrood virus (SBV), acute bee paralysis virus (ABPV), black queen cell virus (BQCV), Kashmir bee virus (KBV) and chronic bee paralysis virus (CBPV) are the most common viruses that cause infections in honeybee colonies [1,23].

Turkey has significant beekeeping potential, with 7.4 million colonies, and ranks 3rd in the world after China and India, which is proof of the importance of Turkey in the global trade of beekeeping [24]. Scientists and beekeepers are intensely preoccupied honey bee colony health problems for more than a decade in Turkey [5,25]. So it is important to investigate colony losses. The objective of this study was to determine the presence of pathogens affecting colony losses in Turkish apiaries.

MATERIAL and METHODS

Sample Preparation

Alive or dead honey bee samples were sent by complaints with colony losses. A total of 2280 honey bee samples from 76 different apiaries *(Fig. 1)* that occurred in seven provinces (Adana, Aydın, Bursa, Izmir, Kütahya, Muğla and Manisa) of Turkey between 2016 and 2018 were tested. For this purpose, nine apiaries were examined in 2016, 38 in 2017 and 29 in 2018.

Fig 1. The geographical location of the apiaries affected by colony losses in seven provinces

Spore and Parasite Detection by Microscopic and Macroscopic Investigation

To determine infestations of both *Varroa* and *Tropilaelaps spp*. mites, a pool was created from approximately 100-300 honey bees after adding 70% ethyl alcohol to remove mites from the honeybee. Then the jar was sealed and shaken for several min. Floating mites were then observed on a surface with 70% ethyl alcohol.

To identify *Nosema spp*. spores, at least 60 adult bee abdomens were masserated in 20-30 mL sterile distilled water, then the suspension was centrifuged for 10 min at 3500 rpm and after that 2-3 drops of the suspension were placed on a slide under a coverslip and examined with the light microscope at 400x magnification (Olympus BX53).

RNA Extraction

Thirty adult bees were homogenized with 9 mL Eagle Minimum Essential Medium (EMEM) (Sigma, U.K.) followed by centrifugation for 30 min at 3500 rpm and 4°C. The supernatant was stored at -80°C until analyzed. For RNA extraction 200 µL of supernatant was used. Total RNA extraction was carried out using High Pure Viral RNA Kit (Roche, Germany) following the manufacturer's instructions.

Table 1. *Primers used in this study*

Agent	Primer Pairs	Amplicon Length	Ref.
IAPV-F IAPV-R	*CGAACTTGGTGACTTGAAG* *GCATCAGTCGTCTTCCAGG*	110 bp	[4]
DWV-F DWV-R	*TGGTCAATTACAAGCTACTTGG* *TAGTTGGACCAGTAGCACTCAT*	269 bp	[26]
SBV-F SBV-R	*CGTAATTGCGGAGTGGAAAGATT* *AGATTCCTTCGAGGGTACCTCATC*	342 bp	[26]
AIV-F ABPV-R	*GGTGCCCTATTTAGGGTGAGGA* *ACTACAGAAGGCAATGTCCAAGA*	460 bp	[26]
BQCV-F BQCV-R	*CTTTATCGAGGAGGAGTTCGAGT* *GCAATAGATAAAGTGAGCCCTCC*	536 bp	[26]
AIV-F AIV-R	*GGTGCCCTATTTAGGGTGAGGA* *TGCACGGGAAGTATAAATAATTCT*	641 bp	[27]
CBPV-F CBPV-R	*AACCTGCCTCAACACAGGCAAC* *ACATCTCTTCTTCGGTGTCAGCC*	774 bp	[26]

PCR Amplification

The target fragments of IAPV, DWV, SBV, ABPV, BQCV, KBV, CBPV were amplified using specified primers for each virus as detailed in *Table 1*. The total volume of each PCR reaction was 50 µL, consisting of 30 µL of water, 10 µL of reaction buffer (One-step transcriptor, Roche), 2 µL of forward primer (10 p/mol), 2 µL of reverse primer (10 p/mol), 1 µL of Taq polymerase (Roche) and 5 µL of RNA. Cycling was a multi-step process that included one cycle at 50°C for 30 min for reverse transcription followed by initial denaturation at 95°C for 7 min, then denaturation at 95°C for 20 sec, followed by annealing at 56°C for 20 sec, extension at 72°C for 30 sec for 45 cycles, before the final extension at 72°C for 10 min. At the end of the reaction, a 10-µL PCR product was used for 1% agarose gel electrophoresis.

RESULTS

In this study, the most common colony losses occurred in March, April, and June at rates of 38.1%, 21%, and 13.1%, respectively. A single *Varroa destructor* infection was detected in 21 samples. *Nosema* was detected in 10 samples and co-infection with both *Nosema* and *Varroa* was observed in eight samples *(Table 2, Table 3)*. *Tropilaelaps spp.* were not identified.

The results of this study also showed that DWV was the most prevalent honey bee virus affecting colony losses in Turkish apiaries as it was found in 44.7% (34/76) of the samples, followed in descending order of prevalence by ABPV, BQCV, SBV, CBPV, and IAPV with prevalence rates of 35.5% (27/76), 28.9% (22/76), 22.3% (17/76), 18.4% 14/76), and 6.5% (5/76), respectively *(Table 2, Table 3)*. KBV was not found in any of the samples collected from apiaries.

Mixed viral infections were also detected in our study *(Fig. 2)*, and most of the Turkish colonies were positively diagnosed with two viruses (19/76). Furthermore, three-

Table 2. *The provinces distributions of samples and the numbers of pathogens*

Provinces	*SMP	Varroa	Nosema spp.	IAPV	DWV	SBV	ABPV	BQCV	CBPV
Adana	9	4	1	5	1	0	2	2	1
Aydın	12	3	4	0	4	2	2	3	2
Bursa	3	1	0	0	2	0	0	0	1
İzmir	33	14	6	0	19	7	14	11	6
Kütahya	2	1	0	0	0	0	0	1	1
Manisa	2	0	1	0	0	0	0	0	0
Muğla	15	7	6	0	8	8	9	5	3
TOTAL	76	29	18	5	34	17	27	22	14

** **SMP:** Samples*

Table 3. *The monthly distributions of samples sent with the complaint of colony losses and the numbers of pathogens*

Months	*CL	Varroa	Nosema spp	IAPV	DWV	SBV	ABPV	BQCV	CBPV
March	29	6	6	5	10	0	4	6	4
April	16	6	7	0	8	6	7	3	2
June	10	5	3	0	3	6	7	6	5
October	9	6	1	0	6	0	6	4	2
November	5	3	0	0	4	1	1	1	0
September	4	2	1	0	2	2	0	2	1
May	2	1	0	0	0	2	2	0	0
February	1	0	0	0	1	0	0	0	0
TOTAL	76	29	18	5	34	17	27	22	14

***CL:** Number of colony losses*

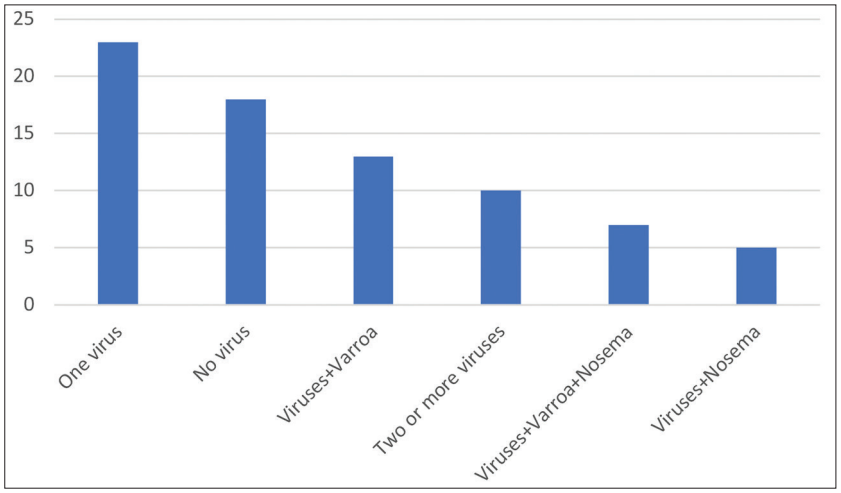

Fig 2. Mixed infections identified in Turkish apiaries affecting colony losses

virus infections were detected in 8 out of 76 samples, four-virus infections in 7 out of 76, while a five-virus infection was detected in one colony. In some apiaries, mixed viral infection was detected simultaneously with other parasitic infestations. A single virus was detected in 22 apiaries and no virus was detected in 19 apiaries.

Varroa, Nosema and viruses were co-occuring in some colonies. Parasitic infestation was found in apiaries that were positive for IAPV, DWV, SBV, ABPV, BQCV and CBPV at rates of 80%, 59.4%, 70.5%, 70.3%, 86.3%, 57.1% respectively. *Nosema* was detected in three, *Varroa* in four, and both *Nosema* and *Varroa* simultaneously in one of specimens without any detected virus.

DISCUSSION

In past years, bacteria, viruses, microsporidia, and parasites have all been reported to play an important role in colony losses [4,5,28,29]. Honey bee losses can be caused by either biotic or abiotic agents [10]. In this study, the presence of pathogens affecting colony losses were investigated in Turkish apiaries.

Most of the viruses identified in this study were RNA viruses; they were DWV, ABPV, BQCV, SBV, CBPV, IAPV. The general pattern of high DWV prevalence in our study is consistent with other published data concerning this matter [30,31]. The presence of DWV has been reported in apiaries with collapsed colonies in the Hatay province of Turkey [25]. ABPV was the second-most widespread bee virus in apiaries with colony losses in our study. In the previous studies from randomly sampled colonies in Turkey, ABPV was not found in the Black Sea Region [32] and it was determined at low incidence in the Aegean Region [33,34]. ABPV is one of three viruses, along with DWV and CBPV, that ANSES emphasizes in colony losses. The presence of DWV and ABPV in the current study are consistent with the ANSES report and the German bee-monitoring project findings [29]. BQCV is one of the most commonly detected bee viruses in Turkish colonies [35], and BQCV prevalence in the current study is consistent with previous studies in Turkey [32,34]. In contrast to this study, BQCV was the most frequently detected virus in Belgium Colony Collapse Disorders (CCD) [36]. CBPV is one of the most important viral agents causing colony losses and overt clinical signs. CBPV was detected in seven of 28 (25%) samples from the Black Sea region in Turkey [32] but other studies showed CBPV to be relatively rare in most provinces [34,37]. Cox-Foster et al.[4] showed a positive correlation between CCD and IAPV in their results. In this study, we detected IAPV in five different apiaries in just

one province (Adana), and it was identified simultaneously with other pathogens like *Varroa* and/or *Nosema*. These additional pathogens were detected in four apiaries, while IAPV alone was detected in one apiary, which may indicate that honey bee losses in the province of Adana may be due to IAPV. KBV is thought to be exotic for Europe [17] and has not been detected in previous studies by Cagirgan [34] in the Aegean Region of Turkey. SBV is one of the most widely distributed honey bee viruses. This virus can infect both larvae and adult stages of honey bees, but larvae are far more susceptible than adult bees. Worker bees play an important role in the transmission of this disease to the colonies [22]. In our study, SBV was only examined in adult bees. The Turkish genotype of SBV has been reported in colonies of *Apis mellifera* in Turkey [38]. It was previously reported in 2.7% of 111 colonies from different apiaries in seven provinces of the Aegean Region [34]. In this study, the prevalence of SBV in apiaries with colony losses was found to be higher than in the study conducted using the random sampling method by Cagirgan [34].

Honeybee colonies are commonly infected simultaneously by many viruses, often without exhibiting overt signs [39]. Mixed viral infections were detected in honeybee samples, but these simultaneous infections consisted of various pathogens as detailed in *Fig. 2*. These results are consistent with those obtained by Van Engelsdorp et al.[40], where a higher percentage of CCD colonies were co-infected with a greater number of disease agents than control colonies. Our mixed viral infection ratio, which was 46.1% (35/76), may indicate the presence of CCD in this study, but in most cases, mixed viral infections were observed simultaneously with *Varroa* and *Nosema* (25/35). There are multiple factors that can lead to colony losses, depending on the pathogen, pathogen titers and quality of nutrition. We were unable to obtain data about the quality of nutrition.

Varroa and *Nosema* were found in samples with rates of prevalence of 38.1% and 23.6%, respectively. *Varroa* is a major problem in beekeeping not only in Turkey, but across the world [41]. It was previously reported in 55% of 394 apiaries from seven provinces of the Aegean Region in Turkey [33]. *Varroa* is considered the main factor in winter colony losses according to Topalska et al.[42] and Van Engelsdorp et al.[43]. However, the honeybee losses in our study frequently occurred in spring. In Turkey, the presence of *Nosema* was confirmed earlier [44,45]. It has been reported that colony losses are caused by *Nosema* in the Hatay overwintering region and Southeastern Marmara [45]. Francis et al.[27] reported that viral titers will rise in honeybee populations from spring to autumn. *Nosema* peaked in the period between March to June and was detected in 18 colonies. On the other hand, the lower levels of *Nosema* detected in autumn could be due to the possible application of antifungal treatments [46]. When we compared our results with other Turkish colonies without colony losses [20,47], we found that their *Nosema spp.* levels were approximately equal to levels determined to affect colony losses in our study. Although *Nosema spp.* has been associated with CCD [4], according to a 5-year German study, there is no correlation between *Nosema* and honeybee losses [48]. Lack of correlation between *Nosema* and honeybee losses demonstrates the importance of mixed viral infections in our study. Therefore, mixed viral infections may be significant for honeybee losses in Turkish apiaries.

Many studies indicate that there is no single pathogen associated with colony losses [40,49]. However, in our study, viral agents like CBPV, BQCV, DWV, ABPV, SBV, parasites like *Varroa spp.* and microsporidia like *Nosema spp.* have been identified individually in some apiaries. Virus titers and excessive parasitic infestation may lead to individual colony losses in apiaries [50]. Moreover, many of the colony losses in early spring may be explained by the fact that colonies are weak and vulnerable at the end of winter. As use of pesticides is another cause for loss, colony losses could also be due to pesticides in apiaries where no viral or parasitic agent was detected. There is no up-to-date proof regarding the effect that simultaneous viral infections could have on the health of honeybees. Therefore, new research is required since the contribution of mixed viral infections to honeybee losses without any stress factors such as poor nutrition or parasites should be investigated further.

In conclusion, there are multiple factors that can lead to honeybee losses. Viruses in particular play a major role in declining colonies, and are a likely contributor to honeybee deaths in Turkey.

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Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Statement of Author Contributions

Methodology: A. A. Çağırgan, K. Pekmez, M. Kaplan, B. Ozkan, H. Yeşilöz, F. Arslan, H. Yeşilöz; Sample preparation: A. A. Çağırgan, K. Pekmez, M. Kaplan, B. Ozkan, H. Yeşilöz, F. Arslan; Investigation: A. A. Çağırgan, K. Pekmez, M. Kaplan, H. Yeşilöz, A. Beyazıt; Data evalution: G. Kalaycı, A. A. Çağırgan, A. Beyazıt; Writing: A. A. Çağırgan

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