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Determination of Mixed Virus Infection in Honey Bees from Erzurum, Türkiye

Zeynep SELVİTOPİ, Gözde Büşra EROĞLU[1*]

[1] Erzurum Technical University, Faculty of Science, Department of Molecular Biology and Genetics, Erzurum, Türkiye
Zeynep SELVİTOPİ ORCID No: 0000-0001-5297-1579
Gözde Büşra EROĞLU ORCID No: 0000-0001-8988-1315

**Corresponding author: gozdebusra.eroglu@erzurum.edu.tr*

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Abstract: Honeybees are one of the most important pollinators of agricultural products Especially the worker bees, which make up the majority of the honey bee population, produce products with high economic value such as honey, pollen, propolis, royal jelly, bee venom, and beeswax. Mass deaths are observed in honey bees grown in Erzurum province, where beekeeping activities are carried out intensively in the Eastern Anatolia region of Türkiye. Epidemic diseases seen in honey bees in Erzurum have a very negative effect on the development and progress of beekeeping activities. Most of the diseases that cause epidemics in bees and cause sudden death and colony loss are of viral origin. In this study, viral pathogens that cause death in honey bees in Erzurum province were investigated and it was determined that two honeybee viruses, the black queen cell virus (BQCV), and the deformed wing virus, caused intense epidemics. In addition, phylogenetic analyzes revealed that all BQCV isolates found in this study clustered quite far from BQCV isolates previously isolated from Türkiye, while DWV isolates clustered close to Hakkari and Lithuania isolates.

Erzurum, Türkiye Bal Arılarında Karışık Virüs Enfeksiyonunun Belirlenmesi

Anahtar Kelimeler
Bal arısı virüsleri, Siyah kraliçe hücre virüsü, Deforme kanat virüsü, Koloni çöküşü

Öz: Bal arıları tarımsal ürünlerin en önemli tozlaştırıcılarıdır. Özellikle bal arısı popülasyonunun büyük bir çoğunluğunu oluşturan işçi arılar bal, polen, propolis, arı sütü, arı zehri ve bal mumu gibi oldukça çeşitli ve ekonomik değeri yüksek ürünler üretmektedir. Türkiye'nin Doğu Anadolu bölgesindeki arıcılık faaliyetlerinin yoğun olarak gerçekleştirildiği Erzurum ilinde yetiştirilen bal arılarında toplu ölümler görülmektedir. Erzurum ili bal arılarında görülen salgın hastalıklar arıcılık faaliyetlerinin gelişimini ve ilerlemesini oldukça olumsuz etkilemektedir. Arılarda salgın oluşturarak ani ölüm ve koloni kayıplarına yol açan hastalıkların büyük bir çoğunluğu viral kaynaklıdır. Bu çalışmada Erzurum ili bal arılarında ölüme neden olan viral patojenler araştırılmış ve siyah kraliçe hücre virüsü ve deforme kanat virüsü olmak üzere iki adet bal arısı virüsünün yoğun salgına neden olduğu belirlenmiştir. Ayrıca filogenetik analizler bu çalışmada bulunan tüm BQCV izolatlarının daha önce Türkiye'den izole edilen BQCV izolatlarına oldukça uzak kümelendiğini, DWV izolatlarının ise Hakkari ve Lithuania izolatlarına yakın kümelendiğini ortaya koymuştur.

1. INTRODUCTION

Türkiye is known as one of the 12 bee gene centers in the world due to its geographical location and rich flora diversity. It also contains 20% of the honey bee species found in the world [1]. Since the products obtained from bees have a high commercial value, beekeeping has been an important source of income in Anatolia since ancient times. However, sudden bee deaths and colony losses occur due to diseases frequently seen in colonies. Beekeeping producers often use broad-spectrum chemical drugs without knowing the origin or the cause of the disease. Toxic drugs used leave residues in bee products and cause resistance development in vectors (*Varroa destructor*) [2]. The most common factors causing colony collapse and disease in bees are viral pathogens [3, 4]. While some of the viral pathogens seen in honey bees continue to exist silently without showing any specific symptoms, some of them cause important

diseases such as deformation in the wings of bees, paralysis in their legs, death, and even colony destruction [5, 6]. These viruses are pathogenic for all life stages of bees and are frequently seen in mixed infections in which an individual is infected with more than one virus at the same time [6]. Although conventional PCR methods using specific primers were used commonly for the determination of honeybee viruses, new-generation sequencing technologies developed in recent years have increased the number of known honeybee viruses. Thanks to this method, which is particularly suitable for the detection of asymptomatic viruses, the number of known honeybee viruses exceeded 30 [7-9]. The majority of these viruses have an RNA genome and are found in the Dicistroviridae and Iflaviridae families [10, 11]. The viral diseases that cause the most important problems in honey bee breeding, are DWV and BQCV. The viral pathogens in question can be transmitted to bees by the varroa mite, as well as the bee trade [12]. These two viruses are also very common in Turkish honey bees. DWV and BQCV viruses were detected for the first time in the Black Sea region in studies conducted in 2009-2010 in Türkiye [13, 14]. Although viral diseases of honey bees grown in many localities of Türkiye were detected, there are no studies on diseases and pathogens of honey bees in Erzurum, which has honey forests in the Eastern Anatolia region and is one of the attraction centers of beekeeping. This study, it was aimed to determine viral diseases to be obtained from apiaries located at different points in Erzurum province.

2. MATERIAL AND METHOD

2.1. Sample Source and RNA Isolation

A total of 37 queens and 279 worker bee samples were collected from 48 apiaries in Erzurum city center and its districts (Table 1).

The collected samples were brought to the laboratory on ice. It was stored at -80 °C until RNA isolation. Indispin/Cador pathogen kit was used to isolate RNA from samples (Indical Bioscience Cat. No: SP54104). Before proceeding to the manufacturer's instructions, every bee, 1 ml of PBS, and sterile steel balls were placed in each microcentrifuge tube and waited in the tissue lyser (Qiagen) device until the bees were completely disintegrated. Afterward, the tissue pieces were precipitated by centrifugation at 6000 rcf at 4°C for 3 minutes and the supernatant was taken into a clean tube. After this stage, the kit was used. Nanodrop was used to determine the quality and density of RNA. Samples with good quality and density were stored at -80 ℃ until used in the next step.

Table 1. Sample numbers collected from localities

Locality	Apiaries	Sample (Queen/worker)
Aşkale	1	0/10
Aziziye	2	1/14
Çat	2	4/19
Hınıs	2	0/10
Horasan	2	1/10
İspir	3	2/14
Köprüköy	3	2/16
Narman	1	0/10
Palandöken	4	3/21
Pasinler	5	4/17
Pazaryolu	5	3/15
Olur	1	1/10
Oltu	2	1/20
Şenkaya	4	6/12
Tekman	1	0/13
Tortum	2	2/20
Uzundere	4	4/26
Yakutiye	4	3/22
Total	**48**	**37/279**

2.2. One-step Reaction and Phylogenetic Analysis

One-step reaction kit (EURx Cat. No: E0803-02) was used to synthesize the isolated RNAs into complementary DNA (cDNA) and to perform the polymerase chain reaction. The reaction prepared according to the kit procedure is as follows: in 25 µL of reaction volume with 60 ng of RNA, 1 µL of 10 µM sense and reverse primers, 12.5 µL of 2x master buffer mix, 1 µL of Master Enzyme mix, and up to 25 µL of nuclease-free water. The PCR condition was as follows: 94 °C for 5 min. for pre-denaturation, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. The information of the primers used in the reaction is given in Table 2. After the reaction was finished, the banded samples were loaded onto a 1% agarose gel treated with ethidium bromide and run at 75 V for 45 min. Afterward, the gel was visualized in the imaging system under ultraviolet light.

Table 2. Primers set data used in virus screening of honey bees

Primers and target genes	Sequences	tm	References
CBPV (RdRP)	**F:** GCAAACTGCCCACCAATAGT **R:** TGGTACGGAAGGTGTGTCAA	55°C	[15]
SBV (cp gene)	**F:** TATTCAGGGGGACGCTACAC **R:** AGTGCTGCTTGAAACCCTGT		
IAPV (cp gene)	**F:** TTGGCGTGCAACTATGTGTT **R:** TCTTCTGCCCACTTCCAAAC		
BQCV (cp gene)	**F:** GACAGCGTGCCAAAGAGAG **R:** GCGAACCCGTCCAATACTTA		
KBV (cp gene)	**F:** CACATTCCGAACAATAA **R:** GCGATAGGAATTTTGCGGTA		
DWV (Non-structural protein)	**F:** TTGGTATGCTCCGTTGACTG **R:** ATTCCTCAGAAGTTGGTTTCG		
ABPV (cp gene)	**F:** GTATGGAAGTGGGCTGAGGA **R:** CGCGGTACTAAAAAGCTACGA		[16]

The samples obtained from the band were sent to Sentebiolab (Ankara, Türkiye) for sequence analysis. The name of the isolates was confirmed using the nucleotide blast program after trimming the head and end parts of the sequence results with low quality. In addition, access numbers were obtained for each isolate and recorded in the National Center for Biotechnology Information (NCBI) database. For phylogenetic analysis, the samples in the database and detailed information in Table 3 were used. In addition, Israel acute bee paralysis virus (IAPV) in the same family (Dicistroviridae) was used as the outgroup for BQCV, and Sacbrood virus (SBV) in the same family (Iflaviridae) with it was used as the outgroup for DWV. The nucleotide sequences of polyprotein for DWV and BQCV isolates were aligned using the program BioEdit (7.1.3.0). For phylogenetic relationship analysis, the Maximum Composite Likelihood model with 1000 bootstrap in the Neighbor-joining method was used to generate a phylogenetic tree using the MEGA 11 program.

Table 3. Information of the viruses used in phylogenetic analyzes

Virus	Origin	Accession number	References
DWV	Lithuania	KF840794	[17]
	Hakkari, Türkiye	KP835214	[15]
	Erzincan, Türkiye	MW962981	[18]
		MW962982	
	Van, Türkiye	KU521779	[19]
		KU521782	
	Erzurum, Türkiye	OQ475006	This study
		OQ475007	
		OQ475005	
		OQ475004	
		OQ475003	
		OQ475002	
		OQ475011	
		OQ475012	
		OQ475013	
		OQ475014	
	Italy	KF311109	[20]
	Switzerland	JF346617	[21]
	Sweden	JF346611	
	Yugoslavia	JF346630	
	China	JF346640	
SBV (outgroup)	France	AH012541	unpublished
BQCV	Tasmania	MF004373	[22]
	Belgium	HG764797	[23]
	Italy	MK238795	[24]
	Syria	LT844588	[25]
	Japan	KT717337	[26]
	China	MF092814	[27]
	USA	KY627847	[28]
	France	MH133351	unpublished
	Isparta, Türkiye	MW433904	[29]
	Karaman, Türkiye	MW433911	
	Konya, Türkiye	MW433906	
	Niğde, Türkiye	MW433915	
	Aksaray, Türkiye	MW433916	
	Bingöl, Türkiye	MZ357974	[30]
	Van, Türkiye	KU521775	[19]
	Erzurum, Türkiye	OQ475008	This study
		OQ475009	
		OQ475010	
		OQ475015	
		OQ475016	
IAPV (outgroup)	Russia	OL314256	unpublished

3. RESULTS

3.1. PCR Reactions

The samples in which BQCV and DWV diseases were determined were found in 7 apiaries in 4 different localities of Erzurum (Cat, Pasinler, Pazaryolu, Uzundere). As a result of the PCR reactions performed for virus screening, DWV was detected in 10 samples (3%) and BQCV was detected in 5 samples (1.6%) (Figure 1). Besides, samples collected from the Çat yolu locality were found to have mixed infections with both DWV and BQCV (Table 3). However, the presence of other bee viruses (CBPV, SBV, KBV, ABPV, and IAPV) was not detected in Erzurum.

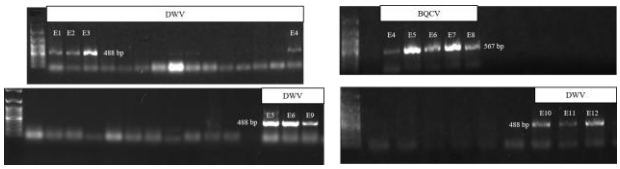

Figure 1. Samples with virus-positive band detected in PCR analysis

The nucleotide sequencing samples were deposited in the database with the accession numbers indicated in Table 3. In addition, it was determined that the sequences showed 98% BQCV and 99% for DWV similarity with the samples in the database, respectively (Table 4).

Table 4. Informations about samples with virus detected

District	Sample code	Virus	Accession number	Similarity rate
Catyolu	E1	DWV	OQ475002	99%
	E2		OQ475003	99%
	E3		OQ475004	99%
	E4	DWV	OQ475005	99%
		BQCV	OQ475008	98%
	E5	DWV	OQ475006	99%
		BQCV	OQ475009	98%
	E6	DWV	OQ475007	99%
		BQCV	OQ475010	98%
Uzundere	E7	BQCV	OQ475015	98%
	E8		OQ475016	98%
Pasinler	E9	DWV	OQ475011	99%
	E10		OQ475012	99%
	E11		OQ475013	99%
Pazaryolu	E12		OQ475014	99%

*Samples with mixed infections are indicated in bold.

3.2. Phylogenetic analyses

Phylogenetic similarity tree results Erzurum DWV isolates clustered close to Hakkari, Türkiye and Lithuania isolates (Figure 2a). Erzurum BQCV isolates clustered quite far from all BQCV isolates previously isolated from Türkiye (Figure 2b).

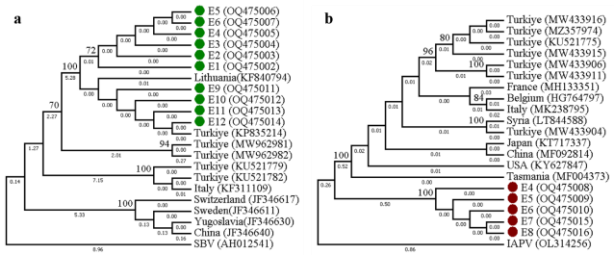

Figure 2. Phylogenetic analysis of honey bee viruses in Erzurum **a.** DWV isolates, **b.** BQCV isolates

4. DISCUSSION AND CONCLUSION

BQCV, which is in the family of Dicistroviridae, has taken this name because it was first detected in queen bees in Australia [31]. However, later studies in France and Austria reported that this virus was seen in the larval stages [6, 32]. DWV in the Iflaviridae family was first isolated from *Apis cerena*, a honey bee species found in the fareast [33]. Subsequently, this virus has spread to regions of Europe, North America, South America, Africa, Asia, and the Middle East [34, 35]. The most important findings of the disease are shrinkage and wrinkling of the wings caused by wear and tear. In the advanced stages of infection, deterioration in the body size of the bee and color change is also observed. This virus causes infection not only in the honey bee, but also in *Bombus* bees, which are known as important pollinators of tomatoes [36-38].

It is known that BQCV and DWV have a high prevalence worldwide. Choe et al. (2012) reported that BQCV virus is the most common pathogen in Korean honey bees [39]. Wang et al. (2016) declared that the prevalence of BQCV is high in the Yunnan region of China [40]. Ghorani et al. (2017) found that DWV is the most common honeybee virus in Iran (Mazandaran, Hormozgan, Kurdistan and Khorasan Razavi) [41]. Truong et al. (2023) reported that the three most common viruses were DWV (52.63%), BQCV (55.26%), and SBV (52.63%) as a result of their study on the prevalence of honeybee pathogens and parasites in South Korea between 2017-2021 [42].

Studies on the detection of viral pathogens that cause disease in honey bees have been carried out in our country for the last 15 years. Within the scope of these studies, Gülmez et al. (2009) detected DWV for the first time in Türkiye as a result of their study on honey bees in Ordu province [13]. Muz and Muz (2009) identified DWV in Hatay province [43]. Gümüşova et al. (2010) reported the presence of BQCV and CBPV in honey bees for the first time in Türkiye in their study in the Black Sea regions [14]. Rüstemoğlu and Sipahioğlu (2016) defined ABPV from honey bees in Hakkari province [16]. Muz and Muz (2018) detected BQCV in honey bees collected from different cities in Türkiye [44]. Oguz et al. (2017) determined the prevalence of Nosema and BQCV in honey bees reared in Van [45]. Karapınar et al. (2018) detected four viruses (ABPC, CBPV, DWV, BQCV) in Van honey bees [19]. Kalaycı et al. (2019) detected SBV in honey bees from Muğla province [46]. Rüstemoglu and Sipahioglu (2019) defined 6 viruses (BQCV, DWV, SBV, CBPV, KBV, IAPV) in honey bees in Hakkari province [15]. Çağırgan et al. (2020) detected BQCV, DWV and ABPV disease in Burdur honey bees [47]. Kalaycı et al. (2020) reported that the DWV pathogen was the most common in honey bee samples from Adana, Aydın, Bursa, Izmir, Kütahya, Muğla, and Manisa, while the CBPV pathogen was less common [48]. Aydın (2020) determined that the honey bees of Malatya and Elazığ provinces were infected with DWV and BQCV [45]. Avcı et al. (2022) determined BQCV, DWV, and ABPV in honey bees in Konya, Karaman, Aksaray, Niğde, and Isparta [29]. Güller et al. (2022) detected SBV and BQCV pathogens in their study with honey bees in Bingöl province [30]. Utkan and Eroğlu (2023) declared that 2 different viruses (DWV, CBPV) in honey bees in Amasya province [50]. In addition, Eroglu (2023) determined that honey bee viruses (BQCV and KBV) were found in some wasps (*Vespula germanica*) found collectively dead in Erzurum [51].

Considering the literature studies, it was seen that viral pathogens are common in many beekeeping provinces in our country, and mainly DWV and BQCV pathogens were determined in these studies. In this study conducted in Erzurum, 7 bee viruses common worldwide were scanned by PCR in 316 healthy bees and dead/sick in front of the hive, and two viruses (DWV and BQCV) were detected. When the phylogenetic relationship of these viruses with other isolates in the NCBI database was examined, it was determined that BQCV isolates clustered quite far from BQCV isolates isolated from Türkiye. In addition, it was determined that DWV isolates clustered close to Hakkari and Lithuania isolates. Mixed infection of honeybee viruses has been known since the 1990s and is a frequent occurrence both in the world and in our country [52]. In mixed infection, more than one virus can be present in the same bee individual at the same time. Chen et al. (2004) reported for the first time that four bee viruses (BQCV, DWV, KBV, and SBV) were found in the same sample [53]. In our country, it has been declared in various studies that double, triple, quadruple and quintuple virus infections are seen [15, 48]. In this study, it was determined that both DWV and BQCV were found in the samples taken from Çat locality. The mixed infection will undoubtedly cause bees to die and diseases to spread faster. For this reason, in order to protect bees from viral infections, there is a need for careful hive cleaning and transported beekeeping, as well as vaccine production studies.

The results obtained showed that two viruses (BQCV and DWV) caused an epidemic in Erzurum honey bees and even mixed infections were observed in some samples at the same time. Thus, this study will shed light on the studies planned to prevent viral bee diseases that are common in the region and to protect bee health.

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