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Investigation of *Nosema* spp. and Some Important Viral Diseases in Honey Bee (*Apis mellifera L.*) Colonies in The Greater Caucasus Region of Azerbaijan: The First Molecular Detection and Analysis of Viral Diseases of Honey Bee

Rafiga Gazi[1], Rahsan Koc Akpinar[2], Emre Ozan[3], Levent Aydin[4], Barat Ahmadov[1]

1 - Institute of Zoology of the Ministry of Science and Education of the Azerbaijan Republic, Baku, Azerbaijan
2 - Ministry of Agriculture and Forestry, Samsun Veterinary Control Institute, Bee Diseases Laboratory, Samsun, Türkiye
3 - Department of Laboratory Animals, Faculty of Veterinary Medicine, Ondokuz Mayis University, Samsun, Türkiye
4 - Department of Parasitology, Faculty of Veterinary Medicine, Bursa Uludag University Bursa, Türkiye

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Corresponding author
Rafiga Gazi
Institute of Zoology, The Ministry of Science and Education
Republic of Azerbaijan.
E-Mail: r.aliyeva@hotmail.com

Abstract

Parasitic and viral factors seen in beekeeping farms are among the most critical causes of colony losses in honey bees. Within the scope of this study, *Nosema* spp microsporidial disease, one of the bee diseases that is important for bee health in Azerbaijan, and nine bee viruses that are important in colony losses. The presence of acute bee paralysis virus, Black Queen cell virus, Chronic bee paralysis virus, Deformed wing virus, Filamentous virus, Kashmir bee virus, Sacbrood virus, Israel acute bee paralysis virus and *Varroa destructor* 1-virus was investigated. For this purpose, adult bee samples taken from various beekeeping farms in Gabala province in the Greater Caucasus region of Azerbaijan were examined with microscopic and molecular methods, and the presence of *Nosema ceranae*, DWV-A, SBV, AmFV, DWV-B, and BQCV was detected in the farms. DWV-A, SBV, AmFV, DWV-B,, and BQCV viruses were sequenced, and a phylogenetic tree was constructed. Phylogenetic analysis was mainly based on comparison of nucleotide similarities with isolates obtained from Genbank; the highest identity (100%) was between the BQCV isolate and the South African isolates. In addition, 99% identity was recorded with the SBV isolate and the Turkish isolate, VDV-1 with Georgia, and AmFV with the Argentinian isolates. Amino acid similarity with a 100% value was observed between Albania and Turkey with BQCV isolation, and between Turkey and SBV isolation. Additionally, DWV showed similarity with Austria, Belgium, the Netherlands, Italy, France, Lebanon, Iran, and Israel.

Introduction

Beekeeping is one of the significant economic fields in Azerbaijan and worldwide. The honey bee and its products are in high demand due to their significant economic, medical, and food value. Bee productivity should be high to ensure the honey supply for the domestic market and export. Beekeepers need to work with healthy and strong bees to maintain and further increase the productivity of bees, which has increased significantly over the last few years. However, in recent years, mass bee deaths have been observed in Azerbaijan, likely due to various factors (SSCRA, 2025). These factors may include unfavorable climatic conditions, environmental pollution, anthropogenic influences, antibiotics, parasites and diseases, and the use of pesticides (Nazarov & Ahmadov, 2017). Among the diseases causing large-scale bee losses, microsporidian diseases (like Nosemosis) and viral infections are particularly significant. Nevertheless, information concerning the presence or prevalence of these diseases in Azerbaijan is either insufficient or entirely lacking (Khanbekova et al., 2013; Ütük et al., 2019).

Nosemosis is a common and significant disease in beekeeping, found worldwide and affecting the intestines of adult honey bees. Recent research has shown that Nosemosis is a fungal disease caused by the Nosema (Vairimorpha) genus of the Microspora phylum. Two microsporidians, *Nosema ceranae* (*N. ceranae*) and *Nosema apis* (*N. apis*), have been identified in Western honeybee (*Apis mellifera L.*) colonies worldwide (Paxton et al., 2007). *N. neumanni* is the third species, and it is reported only in Uganda (Chemurat et al., 2014). Studies have shown that *N. ceranae* causes more severe infections and higher death rates than *N. apis* (Chemurat et al., 2014; Doğanay & Aydın, 2021; Akpınar et al., 2024). The spread of nosemosis is influenced by poor care and nutrition, dampness, hive congestion, and diseases such as amebiasis. *N. apis* can be affected by climatic conditions (Jabal-Uriel et al., 2022; Mohammadian et al., 2018; Schafer et al., 2022).

Viral diseases in honey bee colonies vary in severity, from asymptomatic to mass mortality. In general, the occurrence of symptomatic and overt infections in different life stages of honey bees may vary depending not only on the presence of viruses but also on the co-existence of other pathogens such as *Varroa* spp. and *Nosema* spp., and environmental stress factors such as malnutrition, cold, and humidity (Beaurepaire et al., 2020; Gedremedhn et al., 2020). While many viruses known to exist in honey bees have RNA, a small number of them have DNA. For this reason, research on viral diseases in honey bees generally focuses on RNA viruses, although studies on DNA viruses have also been conducted (Aubert et al., 2008). Some of these include: Acute bee paralysis virus (ABPV, species *Aparavirus apisacutum*), Black queen cell virus (BQCV, species *Triatovirus nigereginacellulae*), Chronic bee paralysis virus (CBPV), Deformed wing virus (DWV-A, species *Iflavirus aladeformis*) Kashmir bee virus (KBV, species *Aparavirus kashmirense*), Sacbrood virus (SBV, species *Iflavirus sacbroodi*), Israeli acute paralysis virus (IAPV, species *Aparavirus israelense*), Varroa destructor virus-1 (DWV-B, species *Iflavirus vadestruente*), and *Apis mellifera* filamentous virus (AmFV) (Bailey, 1982; Beaurepaire et al., 2020; ICTV, 2023). Most studies have focused on RNA viruses belonging to the *Picornavirales, a group of* viral agents. Within this order, ABPV, KBV, and IAPV are classified in the genus *Aparavirus* in the *Dicistroviridae* family, while BQCV is classified in the genus *Triatovirus*. DWV-A, DWV-B, and SBV are also classified in the genus *Iflavirus* in the *Iflaviridae* family (ICTV 2023). CBPV is categorized as an unclassified ssRNA(+) virus within the *Ribovira* realm. The DNA virus AmFV is part of the *Baculoviridae* family (Beaurepaire et al., 2020).

ABPV, KBV, and IAPV, also known as Acute Bee Paralysis Virus Complex (ABPV complex), are widespread worldwide (Beaurepaire et al., 2020). Although these viruses are closely related genetically and highly virulent at high titers, they generally show a subclinical infection and widespread prevalence at low titers. In particular, ABPV and IAPV may lead to symptoms such as tremors, inability to fly, hair loss or darkening, progressive paralysis, and even death. However, these individual instances of paralysis rarely progress to colony paralysis. In addition, larvae and pupae infected with ABPV and KBV can cause a sharp decline in the adult bee population within the colony. The ABPV complex agents are known to be associated with *Varroa destructor* in their transmission, and the diseases are more commonly observed in the summer and autumn. (Beaurepaire et al.2020;de Miranda et al.2010).

BQCV and SBV infections typically cause fatal diseases in the larvae and pupae of honey bees. BQCV infections are more commonly observed in the spring and summer when *N. apis* activity is intense, as BQCV is closely associated with this parasite. While BQCV is predominantly seen in queen bee larvae and pupae, the disease can also affect worker bee larvae. In SBV, this is not the case, and the most susceptible age group is 2- to 4-day-old larvae. In SBV, dying larvae resemble a fluid-filled jumpsuit. In BQCV, the dark color of the honeycomb eyes in which the dead larvae are found is striking (Aubert et al., 2008; Beaurepaire et al., 2020; Tuncer & Yeşilbağ, 2009).

CBPV, one of the oldest known bee viruses, can cause diseases that range from subclinical infections to fatal outcomes. CBPV is primarily transmitted through direct contact or feeding; however, vertical transmission is suspected since it has also been detected in eggs, larvae, pupae, and adult bees (Yeşilbağ, 2021). The disease is more commonly observed in the spring and summer months. In affected bees, the disease can progress in two different forms. In Syndrome 1, bees exhibit swollen abdomens, an inability to fly, and trembling. In Syndrome 2, also known as “black robbers,” the bees are smaller, hairless, and darker in color. Mortality occurs in both cases (Beaurepaire et al., 2020; Yeşilbağ, 2021).

It is known that one of the most important factors in the transmission of DWV-A and DWV-B is *V. destructor*. The nucleic acids of these viruses, which differ in their replication within *V. destructor* and in the virulence of the diseases they cause in bees, are known to be 84% identical (Beaurepaire et al., 2020; Ongus et al., 2004). DWV-A typically causes a disease characterized by wing deformities in adult bees, and infections are particularly observed during the winter months (Beaurepaire et al., 2020).

AmFV infections, which are typically transmitted via the fecal-oral route, are often observed in conjunction with *N. apis*, and the mite is believed to play an important role in transmission. AmFV, more commonly seen in the spring, has milky-white hemolymph, one of its most notable symptoms. In addition, symptoms such as abdominal swelling and a reduced ability to fly may also be observed (Bailey & Ball, 1991; Beaurepaire et al., 2020).

This study aims to investigate the presence of the microsporidian disease *Nosema* spp. and nine different virus species in honey bee samples collected from various beekeeping farms in the Greater Caucasus region of the Republic of Azerbaijan. Additionally, this study aims to conduct the first molecular analysis of viral agents and examine the status of multiple infections.

Materials and Methods

Collection of the bee samples and providing positive controls

The bee samples were collected from the beekeeping farms of the Gabala region of the Great Caucasus of Azerbaijan to detect nine bee viruses and Nosemosis. Samples were collected from 25 hives, with five hives sampled from each of the five beekeepers. From each hive, at least 100-200 adult bee samples were collected, and the samples were stored at -20°C until microscopic and PCR analyses were performed. All positive controls used in the tests for the diagnosis of Nosemosis and viral bee diseases were obtained from the Samsun Veterinary Control Institute, which operates under the Ministry of Agriculture and Forestry of the Republic of Türkiye and serves as the national reference laboratory for viral bee diseases and Nosemosis.

Microscopic detection of Nosema

The preliminary diagnosis of Nosemosis was made using a microscopic method. For this purpose, the abdomen of 30 adult bee samples taken from each hive was removed. These samples were placed in a sterile container, and 15 mL of distilled water (0.5 mL per bee) was added. It was then crumbled in a sterile container. A 0.1 mL sample of the obtained suspension was transferred onto a hemocytometric slide. After placing a cover glass, the sample was examined under a light microscope with a 40x magnification (Human et al., 2013).

Homogenization, nucleic acid extraction, and Multiplex-PCR of Nosema

After the microscopic diagnosis of Nosemosis, samples taken from all hives were subjected to Multiplex PCR. For this purpose, the abdomens of 30 adult bees taken from the same hive were transferred to 7 mL tubes, and 3 mL of PBS (SIGMA, 806544-500ML, USA) was added. The tubes were homogenized using an automatic homogenizer (Bead Ruptor Elite, Bead Mill Homogenizer, Sku 19-042e, Omni International, USA) and then centrifuged at 4000 rpm for 10 minutes at +4 °C. DNA extractions were performed from the obtained supernatants using a DNA extraction kit (Pure Link Genomic DNA mini kit, Invitrogen), following the manufacturer's instructions. The extracted DNAs were stored at -20 °C until testing. The primers used in the Multiplex PCR are listed in Table 1 (Martin-Hernandez et al., 2007; Ütük et al., 2010).

PCR was performed in a final volume of 50 ml, consisting of 23.5 µL DNase and RNase-free sterile distilled water, 5 µL 10X Taq Buffer ($(NH4)_2SO_4$), 6 µL $MgCl_2$ (25 mM), 2 µL dNTPs mix (10 mM), 2 µL of each primer (*N. ceranae* and *N. apis*-10 pmol), 0.5 ml of TaqDNA polymerase (1.25 IU) (Thermo Scientific), and 5 µL of template DNA. The reaction was carried out as follows: The reaction was performed as follows: 95 °C (2 min) for the initial denaturation, followed by 35 cycles of 95 °C (1 min) for denaturation, 50 °C (1 min) for annealing, 72 °C (1 min) for extension, and a final extension of 72 °C (5 min). The products were visualized in a 1.5% agarose gel containing ethidium bromide, with a 218-219 bp band for *N. ceranae* and a 321 bp band for *N. apis* (Ütük et al., 2010).

Homogenization, total nucleic acid extraction, and One-step RT-PCR of viruses

To investigate viral bee diseases, 15 samples collected from the same hive were transferred into 7 mL tubes, and 3 mL of PBS (SIGMA, 806544-500ML, USA) was added. The samples were homogenized using an automatic homogenizer (Bead Ruptor Elite, Bead Mill Homogenizer, SKU 19-042E, OMNI International, USA) and then centrifuged at +4 °C at 4000 rpm for 15 minutes. Total nucleic acid extraction was performed using the supernatants with a commercial kit (High Pure Viral Nucleic Acid Kit, REF: 11858874001, Roche, Germany), following the manufacturer's instructions (Akpinar et al., 2024). The obtained total nucleic acids were stored at -20 °C until analysis.

The extracted total nucleic acids were used in PCR tests to diagnose nine viral diseases. Although AmFV is a DNA virus, and the other viral agents (ABPV, BQCV, CBPV, DWV-A, DWV-B, IAPV, SBV, and KBV) are RNA viruses, one-step RT-PCR tests were performed under the same thermal conditions using the same mix components (excluding primers) in separate tubes. A commercially available one-step RT-PCR kit (RT-PCR Kit, QIAGEN, Germany) was used for the analyses. Although the kit was designed for RNA-based tests, it was considered that processing the samples obtained from the same hive in a single run would minimize potential nucleic acid losses caused by freeze-thaw-related thermal stress. This approach was possible because the DNA integrity remained intact during the reverse transcription (RT) step, and subsequent stages allowed amplification from either cDNA or DNA (Bozdeveci et al., 2024). Information on the primers used for each disease is provided in Table 1. For the one-step RT-PCR, the reaction mixture was prepared with 12.5 µL of RT-PCR Master Mix, 0.8 µL of each 10 pmol primer, 0.25 µL of RT Mix, and 5.65 µL of DNase- and RNase-free sterile distilled water. Then, 5 µL of the total nucleic acid sample was added to the mixture, bringing the final reaction volume to 25 µL. The prepared reaction mixtures were transferred to a thermal cycler (T100 Thermal Cycler, BIO-RAD, Singapore), and the thermal conditions were applied as follows: RT at 50 °C for 30 minutes (one cycle), followed by an initial denaturation at 95 °C for 15 minutes (one cycle). This was followed by 40 amplification cycles consisting of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 60 seconds, and extension at 72 °C for 60 seconds. Finally, a final extension step was performed at 72 °C for 10 minutes (Chen et al., 2006; Gauthier et al., 2015; Cox-Foster et al., 2007; Sguazza et al., 2013; Stoltz et al., 1995).

The PCR amplicons were run in TAE buffer on a 1% agarose gel containing ethidium bromide, and the gel was

visualized using a UV transilluminator. The results were evaluated based on the expected amplicon sizes for each virus (Table 1). Samples that tested positive for viral diseases were marked and stored at -20 °C for subsequent sequence analysis.

Sequencing and phylogenetic analysis

BM Labosis sequenced PCR products from positive samples in Ankara, Türkiye. Bidirectional sequencing using the Sanger method was conducted. Nucleotide sequences were assembled using BioEdit version 7.2.5 (Hall, 1999) and submitted to the GenBank database. Consensus nucleotide sequences were compared with existing GenBank data. For each virus, separate datasets were created by comparing sequence data with appropriate gene regions in GenBank, using parameters such as amino acid/nucleotide similarities and samples from different countries (Supplementary Tables S1-5). Phylogenetic tree construction was performed using MEGA 11 version 11.0.13 (Tamura et al., 2021). Maximum likelihood methods were employed for tree construction, and assessments were made with 1000 bootstrap replicates.

Results

This study revealed the presence of different bee viruses in adult bees in the Gabala region of the Great Caucasus of Azerbaijan. As a result of the tests performed on adult bee sampling from a total of 25 hives across five different beekeepers, BQCV, DWV-A, DWV-B, SBV, and AmFV were detected in Azerbaijan. BQCV and AmFV were detected in all hive samples, while SBV was found in 20 out of 25 hives. DWV-A and DWV-B were detected in fewer hives, 9 out of 25 and 5 out of 25, respectively. However, the sampled beekeepers did not find ABPV, CBPV, KBV, and IAPV. In the microscopic diagnosis of Nosemosis, the pathogen was detected in five hives from only one beekeeper. The multiplex-PCR results from all samples were consistent with those of the microscopic method. However, while all samples were identified as *N. ceranae*, no *N. apis* was detected in any of the samples. The results according to beekeepers and hives are presented in Supplementary Table S6.

In this study, which investigated the presence of nine different bee viruses and two Nosemosis pathogens, no samples were found to be infection-free. Although BQCV and AmFV were detected in all pooled samples according to the hives from which the samples were taken, at least triple infections were detected in the pooled samples. Nine different infection combinations were identified, with the most common infection pattern being the triple infection of BQCV-AmFV-SBV (10/25-40%). Triple infections (14/25-56%) were the most frequently detected in the hives, followed by quadruple infections (8/25-32%) and quintuple infections (3/25-12%) (Table 2).

Table 1. Primers used for PCR of ABPV, BQCV, CBPV, DWV-A, DWV-B, KBV, SBV, IAPV, AmFV, *N. ceranae,* and *N. apis.*

Viruses/Parasites	Primers(5'→3')	Gene Region	Amplicon size (bp)	Reference
ABPV	F: TTATGTGTCCAGAGACTGTATCCA R: GCTCCTATTGCTCGGTTTTTCGGT	ABPVgp2	900	Benjeddou et al., 2001
BQCV	F: CTTTATCGAGGAGGAGTTCGAGT R: GCAATAGATAAAGTGAGCCCTCC	ORF2	536	Sguazza et al., 2013
CBPV	F: TCAGACACCGAATCTGATTATTG R: ACTACTAGAAACTCGTCGCTTCG	ORF3	570	Blanchard et al., 2008
DWV-A	F: TGGTCAATTACAAGCTACTTGG R: TAGTTGGACCAGTAGCACTCAT	DWVgp1	269	Sguazza et al., 2013
DWV-B	F: GCCCTGTTCAAGAACATG R: CTTTTCTAATTCAACTTCACC	VDV1_gp1	430	Yanez et al., 2014
KBV	F: GATGAACGTCGACCTATTGA R: TGTGGGTTGGCTATGAGTCA	KBVgp1	415	Stoltz et al., 1995
SBV	F: CGTAATTGCGGAGTGGAAAAGATT R: AGATTCCTTCGAGGGGTACCCTCATC	SBVgp1	342	Sguazza et al., 2013
IAPV	F: CGATGAACAACGGAAGGTTT R: ATCGGCTAAGGGGTTTGTTT	IAPVOB_gp1 IAPVOB_gp2	767	Cox-Foster et al., 2007
AmFV	F: CAGAGAATTCGGTTTTTGTGAGTG R: CATGGTGGCCAAGTCTTGCT	APL35_gp110	551	Gauthier et al., 2015
N. ceranae	F: CGGCGACGATGTGATATGAAAATATTAA R: CCCGGTCATTCTCAAACAAAAAACCG	16S RNA	218-219	Martín-Hernández et al., 2007
N.apis	F: GGGGGCATGTCTTTGACGTACTATGTA R: GGGGGGCGTTTAAAATGTGAAACAACTATG	16S RNA	321	

The sequence analysis of a PCR product selected from BQCV-positive study samples was performed, yielding a partial sequence of the structural polyprotein encoded by the ORF2 (BQCVgp2) gene region of BQCV. The sequence data, which is 498 nucleotides long and encodes 166 amino acids, was named Az_BQCV_3 and submitted to GenBank with the accession number OQ819160. Az_BQCV_3 showed the highest nucleotide (nt) similarity of 99.35% with the Turkish isolate KX273094, followed by the Albanian isolate MK212108 (99.13%) and the Iraqi isolate LC713311 (98.91%) (Muz & Muz, 2018). The lowest nt similarity was observed with the Japanese isolate LC635471 (93.04%) (Supplementary Table S7). These nt similarities were also consistent with the clustering of the samples in the phylogenetic tree (Figure 1). The highest amino acid (aa) similarity was 100% with the Turkish isolate KX273094, the Albanian isolate MK212108, and the South African isolate NC_003784 (Leat et al., 2000; Muz & Muz, 2018). Notably, despite a 95% nt similarity with the South African isolate, the aa similarity was 100% (Supplementary Table S7) (Leat et al., 2000). No previously unreported amino acid residues were detected in the aa sequence of Az_BQCV_3 (Supplementary Table S12).

As a result of the sequence analysis performed for DWV-A, 267 nt and 89 aa partial sequence data of the DWVgp1 gene region encoding the polyprotein were obtained and then named as Az_DWV_3 and uploaded to GenBank with accession number OQ819161. Az_DWV_3 shows the highest nt similarity with the Iranian isolate MW566435, at 97%, and the lowest nt similarity with the Chinese isolate MZ821821 and the Chilean isolate JQ413340, at 83.90%. Additionally, Az_DWV_3 exhibited high nt similarity, ranging from 95.88% to 96.63%, with the Austrian isolate (OL803828), the French isolates (MW980113 and MZ014649), and the Israeli isolate (MW397639) (Supplementary Table S8) **(**Daughenbaugh et al., 2021). Following these nt similarities, the phylogenetic tree showed that the isolates clustered together (Figure 1). In terms of aa similarity, Az_DWV_3 showed 100% similarity with eight isolates, which was consistent with the high nt similarities (Benaets et al., 2017; Daughenbaugh et al., 2021). These eight isolates and Az_DWV_3 were found to differ in six aa residues (S360Q, S361T, Q370H, Y390F, I401V, and A414T) compared to the NCBI reference isolate (NC_004830) (Supplementary Table S13) (Lanzi et al., 2006).

A partial sequence of 402 nt and 134 aa of the VDV1_gp1 gene region encoding the polyprotein of DWV-B was obtained. It was named Az_VDV1_1 and submitted to GenBank under accession number OQ819163. Az-VDV1_1 shows the highest nt similarity (98.26-99%) to the Georgian isolates (KX911801, KX911803, KX911804, KX911805, and KX911808) (Radzeviciute et al., 2017). It exhibits 98.24% nt similarity with the NCBI reference isolate NC_006494, which was identified in the Netherlands (Supplementary Table S9) (Ongus et al., 2004). The nt similarity between Az_VDV1_1 and these isolates also leads to their clustering together in the phylogenetic analysis (Figure 1). The highest aa similarity with Az_VDV1_1 is shown by the Georgian isolate KX911805 with 99.25% (Supplementary Table S9). There are two differences in aa residues (S103G and K118Q) between AZ_VDV1_1 and the NCBI reference isolate. The K118Q aa mutation is present in both Az_VDV1_1 and isolate KX911805, whereas the S103G aa mutation is found only in Az_VDV1_1 but not in KX911805 (Supplementary Table S14) (Ongus et al., 2004; Radzeviciute et al., 2017).

A partial sequence of 333 nt and 111 aa of the SBVgp1 (Pol) gene region encoding the polyprotein of SBV was obtained. It was named Az_SBV_3 and submitted to GenBank under accession number OQ819162. Az_SBV_3 exhibits the highest nucleotide (nt) similarity with Turkish isolates (MZ357971 and MH251274), at approximately 99% (Güller & Kurt, 2022; Kalaycı et al., 2019). The lowest nt similarity is observed at 88.68% with the Chinese isolate KF960044 and the Vietnamese isolate KJ959614 **(**Supplementary Table S10). The high nt similarity between Az_SBV_3 and the Turkish isolates is also consistent with their clustering in the phylogenetic tree (Figure 1). In terms of aa similarity, Az_SBV_3 shows the highest similarity with the Turkish isolates MZ357971 (100%) and MH251274 (99.05%) (Güller & Kurt, 2022; Kalaycı et al., 2019). The lowest aa similarity is also with the Chinese isolate KF960044 (89.62%) (Supplementary Table S10). There are seven aa residue differences between the study isolate Az_SBV_3 and the NCBI reference isolate NC_002066 (T58A, D68G, F82Y, T113A, I117L, T124S, and L144T). These seven aa residues are also present in the two Turkish isolates, and MH251274 has an additional S60C aa mutation (Supplementary Table S15) (Ghosh et al., 1999; Güller & Kurt, 2022; Kalaycı et al., 2019).

Table 2. Status of infection combinations in pooled adult bee samples within hives.

	Triple Infections		**Quadruple Infections**				**Quintuple Infections**		
	BQCV AmFV DWV-A	BQCV AmFV SBV	BQCV AmFV SBV DWV-A	BQCV AmFV SBV DWV-B	BQCV AmFV SBV *N.ceranae*	BQCV AmFV DWV-A *N.ceranae*	BQCV AmFV SBV DWV-A DWV-B	BQCV AmFV SBV DWV-A *N.ceranae*	BQCV AmFV SBV DWV-B *N.ceranae*
Number of Hives	4	10	2	3	2	1	1	1	1
Rate of Hives	16%	40%	8%	12%	8%	4%	4%	4%	4%
Total Rate	56%		32%				12%		

A partial sequence of 531 nt and 177 aa of the APL35_gp110 gene region encoding the BRO-B protein of AmFV was obtained. It was named Az_AmFV_5 and submitted to GenBank under accession number OQ819164. Az_AmFV_5 shows the highest nt similarity of 99.27% with the Argentinian isolate MN025284, while the lowest nt similarity is 96.59% with the Hungarian isolate MH431927 (Supplementary Table S11) (Zana et al., 2019). These differences in nt similarity are also consistent with the clustering of Az_AmFV_5 and the isolates in the phylogenetic tree (Figure 1). Az_AmFV_5 also exhibits the highest aa similarity (99.26%) with the Argentinian isolate. In comparison, the lowest aa similarity (94.85%) is observed with the Hungarian isolate and Syrian isolate (LT844586) (Supplementary Table S11) (Kubaa et al., 2018; Zana et al., 2019). Compared to the NCBI reference isolate NC_027925, which was obtained from Switzerland,

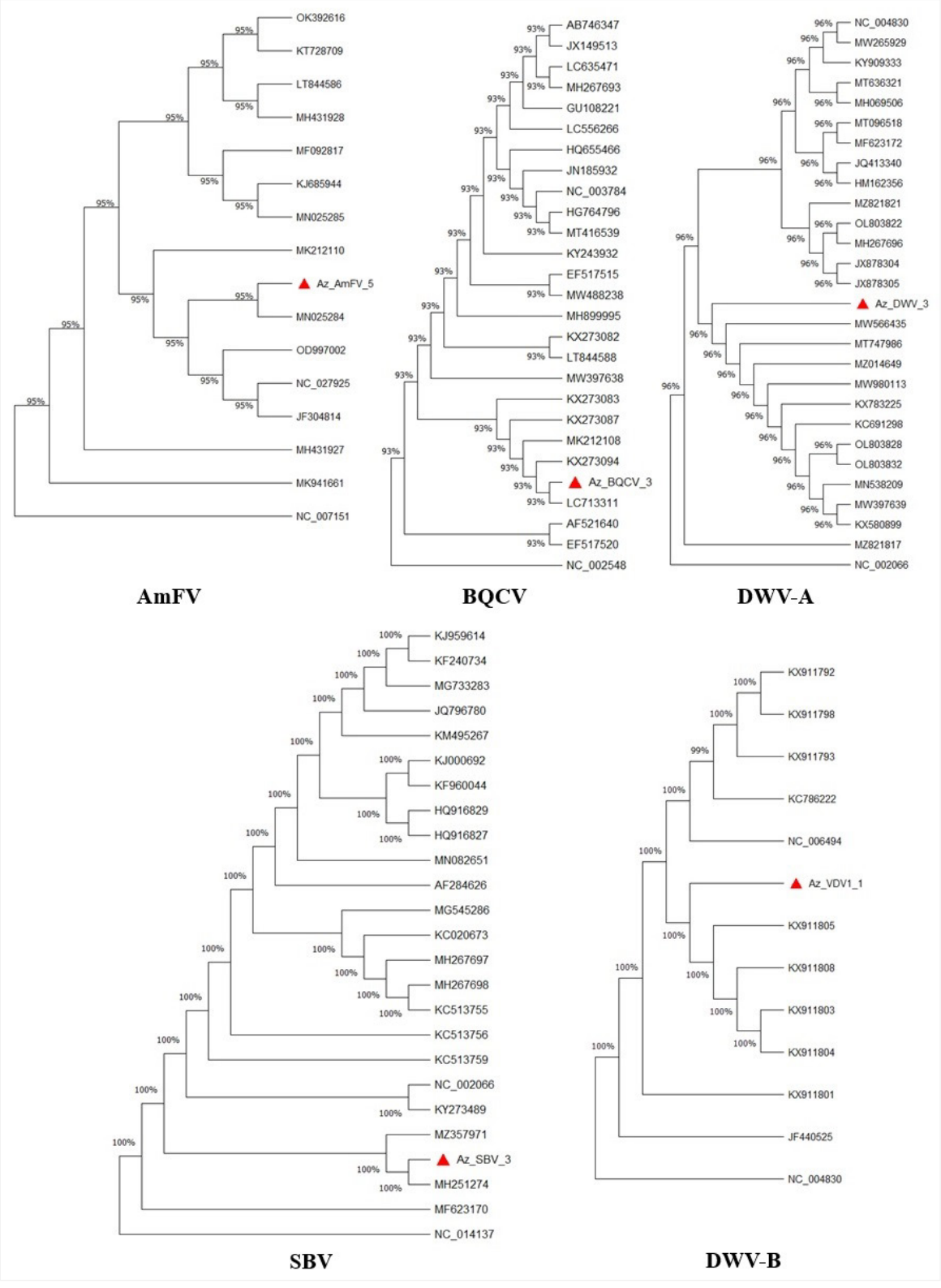

Fig 1. The phylogenetic trees of five viral disease isolates from Azerbaijan and other countries. Sequences from this study are indicated by a ▲.

the study isolate Az_AmFV_5 contains three different aa residues (K196H, N198K, and D231N). The Argentinian isolate MN025284, which has the highest aa similarity to Az_AmFV_5, shares the K196H and D231N aa mutations, whereas the N198K aa residue is unique to the study isolate (Supplementary Table S16) (Gauthier et al., 2015).

Discussion

Although Azerbaijan ranks below the world average in terms of bee colony numbers and honey production, it still has a significant production capacity to meet domestic demand (TEPGE 2022). Moreover, there has been a significant increase in both colony numbers and honey production each year (TEPGE, 2022; SSCRA, 2025). Between 2016 and 2022, the number of honey bee colonies increased by 39%, while honey production increased at a much higher rate of 175.74% (TEPGE 2022; SSCRA 2025). To increase the total number of bees and the honey yield per colony, the presence of healthy bee colonies and their protection from disease are crucial. However, while there are only a limited number of studies on nosemosis, a disease that causes significant infections in bees, there is only one study on viral diseases in Azerbaijani honeybees (Girisgin et al., 2022; Khanbekova et al., 2013; Ütük et al., 2019). With such limited data on the health of Azerbaijan's bee population, it is almost impossible to develop and implement strategies to support the beekeeping sector or identify bottlenecks. For this reason, our study investigates the presence of nine different viral pathogens and *Nosema* spp. pathogens in a limited sample, analysing 25 hives from five beekeepers in the Gabala region of the Greater Caucasus in Azerbaijan. Despite the small sample size and regional limitation, molecular analyses of the viruses detected were performed. The primary objective of our study is not to determine the prevalence of diseases in Azerbaijan's bee population but rather to establish the presence of viruses in the country, provide insights into virus circulation in Azerbaijan, and lay the groundwork for more comprehensive studies. In addition, this research marks the first molecular analysis of bee viruses in Azerbaijan, allowing comparisons with isolates from other countries and providing valuable insights into potential combinations of multiple infections that may contribute to Colony Collapse Disorder (CCD).

Although limited in number, studies have demonstrated the presence of nosemosis in Azerbaijani bee populations. However, while *N. apis* could not be detected in many studies, the prevalence of *N. ceranae* has been reported to reach up to 62%. Only one study has reported the presence of *N. apis*, with a prevalence of up to 87% in the Nakhchivan Autonomous Region (Girisgin et al., 2022; Khanbekova et al., 2013; Ütük et al., 2019). In our study, *N. ceranae* was detected in only five out of 25 hive samples, while *N. apis* was not found. All five positive samples originated from the same beekeeper. Although it is not appropriate to compare the prevalence due to the small number of samples, it is similar to previous studies in terms of the type of nosemosis detected in our study (Girisgin et al., 2022; Khanbekova et al., 2013; Ütük et al., 2019).

In the only study on bee viruses in Azerbaijan, the presence of BQCV, DWV-A, and viruses from the Iflaviridae family (KBV and/or IAPV) was detected. At the same time, CBPV and SBV were not found (Khanbekova et al., 2013). In our study, BQCV, DWV-A, DWV-B, SBV, and AmFV were detected using molecular methods, whereas ABPV, CBPV, KBV, and IAPV were not found. Similar to the previous study, BQCV and DWV-A were also identified in our research; additionally, the presence of DWV-B, SBV, and AmFV has been demonstrated for the first time in Azerbaijan.

Interactions with neighboring countries influence the health of a country's bee population due to factors such as the natural movement of bees, commercial beekeeping activities, and contamination of flowers by various agents (Beaurepaire et al., 2020; Chen et al., 2006; Radzeviciute et al., 2017). Azerbaijan shares borders with Armenia, Iran, Georgia, and Russia. It also shares a border with Türkiye via the Nakhchivan Autonomous Republic. In our study, BQCV and AmFV were detected in all hive samples. Similar to our study, BQCV was detected at high rates in Russia (100%) and Türkiye (95.8%). However, despite sampling from 89 apiaries, BQCV was not detected in Iran (Akpınar et al., 2024; Ghorani et al., 2017; Zinatullina et al., 2018). The reason for this difference between Iran and Azerbaijan concerning BQCV may be that the study samples carried out in Iran were mainly collected in the south of the country. The situation is similar for AmFV. While AmFV was detected in all samples in our study, its prevalence was reported to be 50% in Armenia and 60% in Türkiye (Arzumanyan et al., 2023; Bozdeveci et al., 2024). The reason for this difference in AmFV prevalence may be that our study sampled from only one region of Azerbaijan with a relatively small sample size. In support of this view, a study conducted in Türkiye reported an overall AmFV prevalence of 60%, but in some sampled sites, AmFV was detected in all samples (Bozdeveci et al., 2024). In our study, SBV was detected in 20 out of 25 hive samples. Similarly, SBV was detected at a high prevalence in Russia and Türkiye, whereas it was not detected in Iran (Akpınar et al., 2024; Ghorani et al., 2017; Zinatullina et al., 2018). DWV-A is among the bee viruses detected in Armenia, Russia, Iran, and Türkiye, and was identified in nine out of 25 hive samples in our study. While DWV-A has been reported with a prevalence of up to 90% in Türkiye, Russia, and Armenia, it was found at a much lower rate in our study (Akpınar et al., 2024; Arzumanyan et al., 2023; Ghorani et al., 2017; Zinatullina et al., 2018). DWV-B, on the other hand, has only been reported in Türkiye (40%) and was found with a lower prevalence in our study (5/25, 20%) (Bozdeveci et al., 2024).

ABPV, CBPV, KBV, and IAPV, which were not detected by molecular methods in our study, are reported

bee viruses in Russia and Türkiye. Studies have reported the prevalence of KBV at 37.5% in Russia, ABPV at 70% in Armenia, IAPV at 54% in Iran, and CBPV at 8% in Türkiye (Akpınar et al., 2024; Arzumanyan et al., 2023; Khezri et al., 2020; Zinatullina et al., 2018). Although the absence of these four viruses in our study is promising for Azerbaijani beekeeping, the small sample size highlights the need for larger-scale studies. The fact that the study samples were collected in Gabala, in the northern part of Azerbaijan, near the Russian border, may explain the similarity of the results with those from Russia. In addition, the fact that the studies conducted in Iran and Turkey were conducted in sites far from the Azerbaijani border may explain the differences observed.

When multiple infection combinations were analyzed in our study, single infections, dual infections, and disease-free hives were not detected in pooled hive samples. The presence of nine different infection combinations in our study indicates significant risks to the country's beekeeping industry. The fact that triple infections were the most common at 56% is similar to the data from Türkiye. However, while in Türkiye the BQCV/DWV-A/SBV triple infection was the most common (22.1%), in our study, the BQCV/AmFV/SBV triple infection was detected in 10 out of 25 hive samples (40%). This difference could be attributed to the fact that AmFV infections were not investigated in the Turkish study and that DWV-A infections were detected at a lower rate in our study compared to Türkiye. In addition, the presence and diversity of quadruple and quintuple infections in our study are similar to those reported in Türkiye (Akpınar et al., 2024). However, although *N. ceranae*, considered an important factor in BQCV transmission, was detected in only five hive samples from a single beekeeper, BQCV was found in all study samples. For Azerbaijan, this suggests that there may be other important sources of BQCV transmission among bees besides *Nosema* spp.

This study was the first phylogenetic analysis of bee viruses in Azerbaijan. It was found that the BQCV isolate Az_BQCV_3 had 99.35% nt and 100% aa similarity with the Turkish isolate KX273094, and a similar situation was found between the SBV isolate Az_SBV_3 and the Turkish isolate MZ357971, and the isolates had 99.06% nt and 100% aa similarity (Güller & Kurt, 2022; Muz & Muz, 2018). For DWV-B, the isolate Az_VDV1_1 showed the highest similarity to the Georgian isolate KX911805, with 99% nt and 99.25% aa similarity (Radzeviciute et al., 2017). Regarding DWV-A, the isolate Az_DWV_3 exhibited 97% nt and 100% aa similarity to the Iranian isolate MW566435. However, Az_DWV_3 also showed a similar level of similarity to the Austrian isolate OL803828 (96.25% nt and 100% aa). The situation is slightly different for AmFV, as isolate Az_AmFV_5 showed the highest similarity to the Argentine isolate MN025284, with 99.27% nt and 99.26% aa similarity. The sequence data obtained from the phylogenetic analyses of BQCV, DWV-A, DWV-B, SBV, and AmFV provide significant evidence that these viral agents in Azerbaijan not only interact with viruses in neighbouring countries such as Iran, Georgia, and Türkiye but may also interact with those in distant countries as far away as Argentina and Austria.

In conclusion, this study represents the most comprehensive investigation conducted in Azerbaijan regarding disease agents, as it examines Nosema spp. and nine honey bee viruses. Additionally, it is the first study in Azerbaijan to detect the presence of DWV-B, SBV, and AmFV. BQCV and DWV-A were also detected in the study. It is also the first study in the country to perform phylogenetic analyses and to investigate multiple infection combinations. Despite being conducted in a limited location with a relatively small sample size, the phylogenetic analyses provide significant insights. The similarities between the detected viral isolates and those from neighboring countries, as well as with distant countries such as Argentina and Austria, along with identifying nine different infection combinations, are valuable findings. To ensure the sustainability of the increasing bee population and honey production, this study emphasizes the need for more extensive research on honey bees in Azerbaijan. Additionally, it highlights the necessity of taking serious precautions to prevent situations such as CCD, which could lead to the sudden decline of bee colonies.

Supplementary information

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Ethics approval: There is no animal or human study that requires ethics permission.

Availability of data and material: The data that support the findings of this study are openly available in the GenBank database at https://www.ncbi.nlm.nih.gov/nucleotide/ with accession numbers OQ819160-OQ819164.

Authors' Contribution

R.G.: Conceptualization, methodology, investigation, formal analysis, writing-original draft, writing-review and editing.
B.A.: Conceptualization, methodology, investigation, formal analysis, writing-original draft, writing-review and editing.
R.K.A.: Formal analysis.
E.O.: Data curation.
L.A.: Writing-review & editing.

All authors reviewed and approved the final version of the article.

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