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Original Article

Epidemiological Study and Phylogenetic analysis of Common Pathogenic Viruses of Honeybee in Apiaries of Iran

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ABSTRACT

Several agents such as bacteria, fungi, parasites and viruses can infect honey bees in apiculture. Viruses are one of the most important threats to the health of honeybees. The aim of this research was to diagnose common honeybee pathogenic viruses, including Acute Bee Paralysis Virus (ABPV), Black Queen Cell Virus (BQCV), Chronic Bee Paralysis Virus (CBPV, Deformed Wing Defect (DWV), Kashmir Bee Virus (KBV), and Sacbrood Virus (SBV) in apiaries across Iran. From Autumn to Winter 2022, honey bee samples were randomly collected from 31 provinces across the country. After samples preparation and RNA extraction, the target fragments were amplified using the RT-PCR method. Desired standard viruses and distilled water were used as positive and negative controls, respectively. The PCR products were sequenced and compared with the Genbank database. Results showed that, out of 274 samples collected from apiaries, 21 (7.66%), 21 (7.66%), 11 (4.01%), 247 (90.15%), 31 (11.1%) and 91 (33.21%) were positive for ABPV, BQCV, CBPV, DWV, KBV and SBV, respectively. The highest level of infection was related to DWV and SBV, while the lowest level of infection was related to CBPV. All provinces were infected with at least one virus, and in some, all the studied viruses were detected in apiaries. Sequencing results confirmed the RT-PCR findings. Phylogenetic analysis showed several mutations in the sequences of all studied viruses. The presence of viral infections in the country's apiaries indicates that viral diseases are a significant concern and require targeted management strategies for prevention.

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1. Introduction

Honey bees, as one of the most important insects, play a key role in providing many valuable pollination services for many major agricultural crops (1). They also produce products such as honey, pollen, royal jelly, wax, and other products that contribute to the agricultural economy (2). Many different agents infect honey bees, such as bacteria, fungi, parasites and more than 30 viruses in apiculture (3). Although honey bee viruses typically infect larvae or pupae, the disease symptoms are usually appeared in adult bees (4). Viruses can be transferred to the queen through pollen and the honey, and then passes on from the eggs to the next generations (5). In pollen and honey, honey bee viruses can increase the risk of infection in healthy bee colonies through contact with infected colonies and feeding on contaminated honey and/or pollen. The point is that horizontal transmission of viruses can hide the danger of introducing new viruses (6).

The most frequently detected honeybee viruses worldwide, associated with economic losses, include acute bee paralysis virus (ABPV), black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), deformed wing virus (DWV), Kashmir bee virus (KBV), and sacbrood virus (SBV). ABPV is one of the most important viruses found in *Apis mellifera* (*A. mellifera*). This virus may be transmissible even in the absence of *Varroa destructor* (*V. destructor*) (7).

In addition, BQCV, as a positive-sense single-stranded RNA virus, was first reported in queen larvae and pupae. CBPV is a positive-sense single-stranded RNA virus with an unknown classification, belonging to the realm *riboviria* (8). DWV is one of the most important honey bee pathogens, causing annual colony losses, worldwide (3). It should be kept in mind that the proportion of bees with deformed wings is usually less than one percent in an infected colony. However, a large number of bees can be observed without disease symptoms, despite having relatively high infection with DWV (9). Infected worker honey bees with severe BQCV show symptoms of disorientation similar to those caused by DWV (10). Another serious virus is KBV, which is very prevalent in Australia and the United States of America (USA); however, its presence has also been reported in Europe (11). The last important virus affecting *A. mellifera* larvae is SBV. This single-stranded positive-sense RNA virus belongs to the order *Picornavirales* and the family *Dicistroviridae*. SBV is transmitted from infected bees to young larvae, and the infected larvae die at the pre-pupal stage (12).

The aim of this study was to diagnose prevalent pathogenic viruses in honeybee apiaries across Iran, using molecular techniques and phylogenetic tree mapping.

2. Materials and Methods

2.1. Data collection and sample preparation

Sampling was conducted randomly based on the number of apiaries and the prevalence rate of 40% (reported by the Iran veterinary organization). The population size was estimated using Cochran's formula (13):

$$N = z^2(pq)/d^2$$

Where, d (error) was equal 0.06, p was equal 0.4, q was equal 0.6 and z (normal variable) was equal 1.96 and the confidence factor was 95%. Accordingly, we should have considered at least about 260 apiaries, however, the number of 274 apiaries from 31 provinces of the country were considered. Whereas for each apiary, a number of hives were randomly selected and irrespective of the clinical symptoms of the disease, and a number of bees were collected from each comb in the sterile containers (more than 50 adult bees for each apiary). Subsequently, all adult bee samples collected from each apiary were pooled and transported to the laboratory under a cold chain for viral analysis.

2.2. Sample preparation

For each sample, a total of 100 adult bees were homogenized by adding diethylpyrocarbonate (DEPC)-treated water. The mixture was then centrifuged at 20,000×g for 1 minute, and the supernatant was collected and stored at -20°C until use.

2.3. RNA extraction

An amount of 140 µL from each prepared samples was used for RNA extraction, based on the protocol described by Berényi et al. (11) and utelizing the QIAmp Viral RNA Mini kit (QIAGEN, Germany) according to the manufacturer's instructions.

2.4. cDNA synthesis and polymerase chain reaction (PCR)

cDNA for all studied viruses was synthesized using a cDNA synthesis kit (Biotechrabbit, Germany), following the manufacturer's recommendations. Positive controls were obtained from the Feredrich-Loeffler-Institute (Germany). Table 1 presents the sizes and primer pair sequences of all studied viruses. Each PCR reaction was carried out using 50 ng of genomic DNA, 20 pmol of each specific forward and reverse primers,

Table 1. Oligonucleotide primer pairs employed in RT-PCR assays.

Viruses	Sequence	Size	GenBank accession number
ABPV	F: 5'- GTG CTA TCT TGG AAT ACT AC-3' R: 5'- AAG GYT TAG GTT CTA CTA CT-3'	618 bp	AF150629
BQCV	F: 5'- AGT AGT TGC GAT GTA CTT CC-3' R: 5'- CTT AGT CTT ACT CGC CAC TT-3'	472 bp	AF125252
CBPV	F: 5'- TGT CGA ACT GAG GAT CTT AC-3' R: 5'- GAC CTG ATT AAC GAC GTT AG-3'	315 bp	AF375659
DWV	F: 5'- ATT GTG CCA GAT TGG ACT AC-3' R: 5'- AGA TGC AAT GGA GGA TAC AG-3'	434 bp	AJ489744
KBV	F: 5'- GAT GAA CGT CGA CCA ATT GA-3' R: 5'- TGT GGG TTG GCT ATG AGT CA-3'	395 bp	AY275710
SBV	F: 5'- ACC AAC CGA TTC CTC AGT AG-3' R: 5'- CCT TGG AAC TCT GCT GTG TA-3'	478 bp	AF092924

and 12.5 µL Taq DNA Polymerase Master Mix RED 2x (Ampliqon, Denmark), in a final volume of 25 µL.

For all studied viruses, PCR protocols included an initial denaturing at 95°C for 15 minutes, followed by 40 cycles of 94°C for 30 seconds, annealing at55°C for 50 seconds, and extension at 72°C for 1 minute. A final extension step was performed at 72°C for 7 minutes. PCR products were subsequently analyzed by 1.2% agarose gel electrophoresis.

2.5. Purification and DNA sequencing

PCR products were purified using the High Pure PCR Product Purification Kit (Roche, Germany), according to the manufacture´s instruction. For each fragment, two replicates of the purified PCR product were sequenced by Microsynth (Switzerland).

2.6. Sequence analysis and statistical analyses

To determine the number of apiaries for sampling, the population size was determined using Cochran's formula (13). Molecular results were analyzed using descriptive statistics. Phylogenetic trees of all sequences were constructed using the neighbor-joining method, in MEGAX 4.0 (14) and bootstrap support was assessed based on 1000 replicates.

3. Results

The results of molecular detection showed that out of 274 samples, 21 (7.66%) were positive for ABPV, 21 (7.66%) for BQCV, 11 (4.01%) for CBPV, 247 (90.15%) for DWV, 31 (11.1%) for KBV, and 91 (33.21%) for SBV. Table 2 presents the number of positive samples by province for all the viruses studied across the country. Due to the large number of PCR images obtained, some selected images are shown in Figure 1.

The results of sequence mutations in viruses studied in Iran compared with reference sequences in NCBI are presented in Figure 2. Since polymorphic differences between similar sequences can be observed in terms of sequence composition and length, the results indicated mutations in the total sequences of all studied viruses. Nucleotide differences detected between strains are shown in some columns. The phylogenetic trees for studied viruses are shown in Figure 3.

The results of sequence identity analysis for our ABPV isolate, compared with other ABPV sequences in the NCBI database, showed a homology range of 89.34%-98.57%, indicating high similarity with previously reported strains. The highest sequence identity was observed with a strain from Turkey (Accession No. KY465554.1). For CBPV, the homology results were in the range of 81.12%-97.30%. The highest homology (97.30%) was detected in a sample from Italy (Accession No. LR797924.1). For other studied viruses, the highest homologies were detected in samples from Turkey (Accession No. FJ588532.1) for BQCV, Belgium (Accession No. KX783225.1) for DWV, Nigeria (Accession No. MN296283.1) for KBV, and Poland (Accession No. OR513789.1) for SBV.

4. Discussion

Honey bees are recognized for their positive effects on many different areas. They produce several products such as honey, beeswax, royal jelly, and propolis. They also play an important role in plant pollination (15). Some biotic and abiotic factors impact bees' welfare and survival. Among these, changes in land use and management intensity, climate change, beekeeper's management practices, lack of forage (nectar and pollen),

Table 2. The number of positive samples by province for all studied viruses in all over the country.

Province	Apiary	ABPV	BQCV	CBPV	DWV	KBV	SBV
Alborz	6	0	0	1	1	0	**6**
Ardebil	4	0	0	0	4	0	**0**
Bushehr	3	0	0	0	3	0	**0**
Tehran	12	1	0	0	12	0	**4**
Chaharmahal & Bakhtiari	7	0	0	0	7	0	**0**
East Azerbayijan	15	0	1	0	11	0	**0**
Esfahan	9	0	0	0	9	0	**5**
Fars	21	0	2	0	19	1	**8**
Ghazvin	5	0	0	0	5	0	**2**
Ghom	2	0	0	1	2	0	**2**
Gilan	14	2	0	3	13	4	**3**
Golestan	19	3	4	1	13	3	**1**
Hamedan	10	0	0	2	9	0	**1**
Hormozgan	1	1	1	0	1	0	**1**
Ilam	6	0	0	0	5	1	**0**
Kerman	7	0	0	0	7	2	**1**
Kermanshah	13	0	0	0	13	4	**4**
Khuzestan	5	0	0	0	5	0	**1**
Kohkilouyeh & Buyer Ahmad	8	1	1	0	8	3	**0**
Kordestan	8	0	1	0	8	0	**0**
Lorestan	10	1	1	0	10	1	**1**
Markazi	5	1	1	0	5	2	**3**
Mazandaran	22	5	5	2	16	0	**5**
North Khorasan	7	2	0	0	7	0	**3**
Razavi Khorasan	18	2	4	0	18	1	**7**
Semnan	2	0	0	0	2	0	**2**
Sistan&Baluchestan	7	1	0	0	6	0	**4**
South Khorasan	3	0	0	0	3	1	**3**
West Azerbayijan	17	1	1	0	17	7	**16**
Yazd	2	0	0	0	2	0	**1**
Zanjan	6	0	0	0	6	1	**3**
Total	274	21	21	11	247	31	**91**

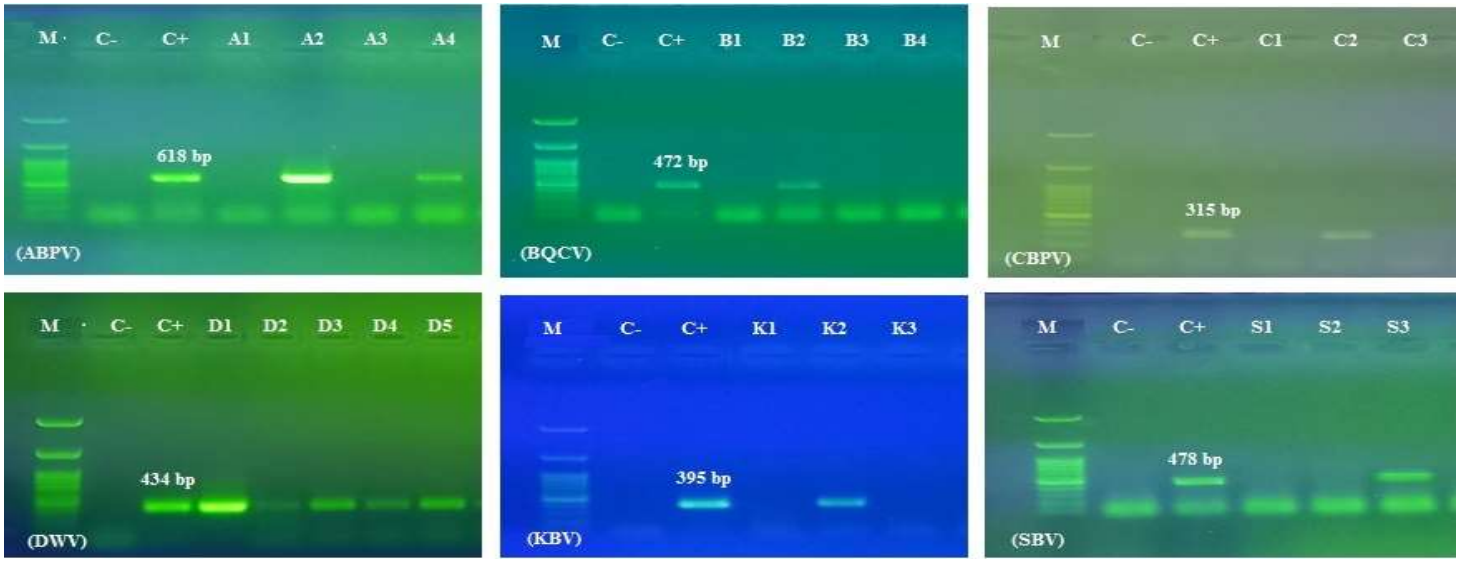
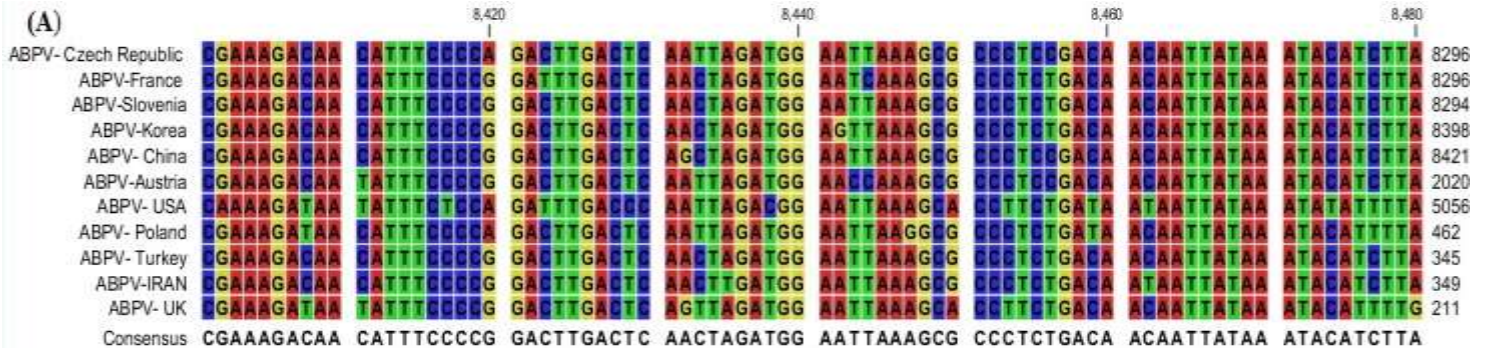
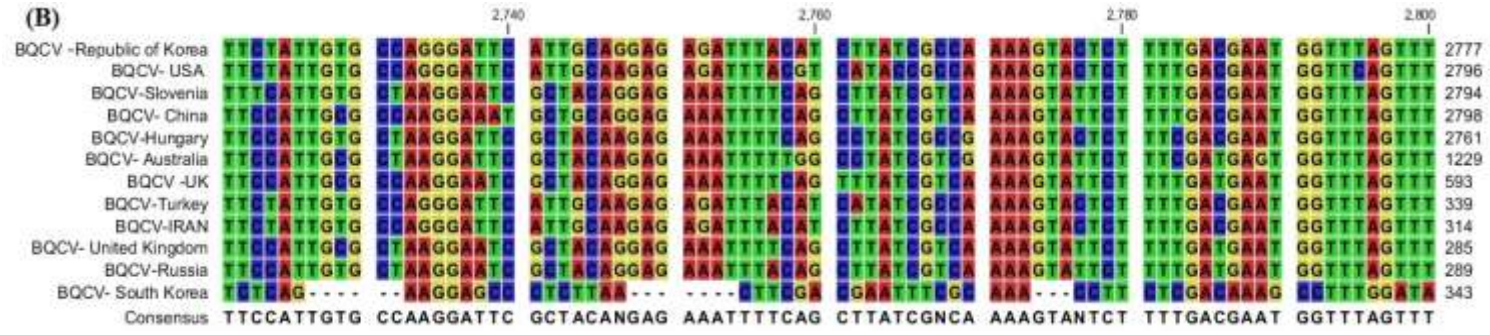
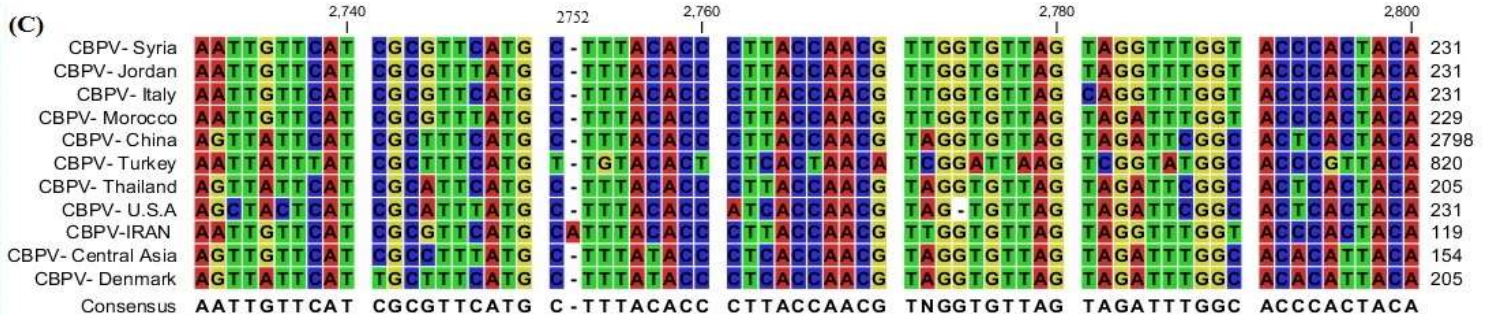
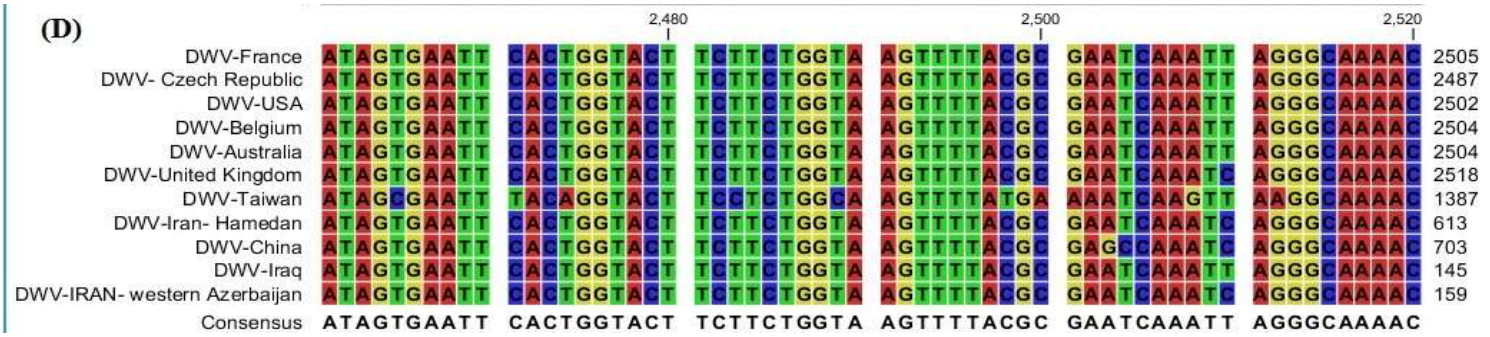


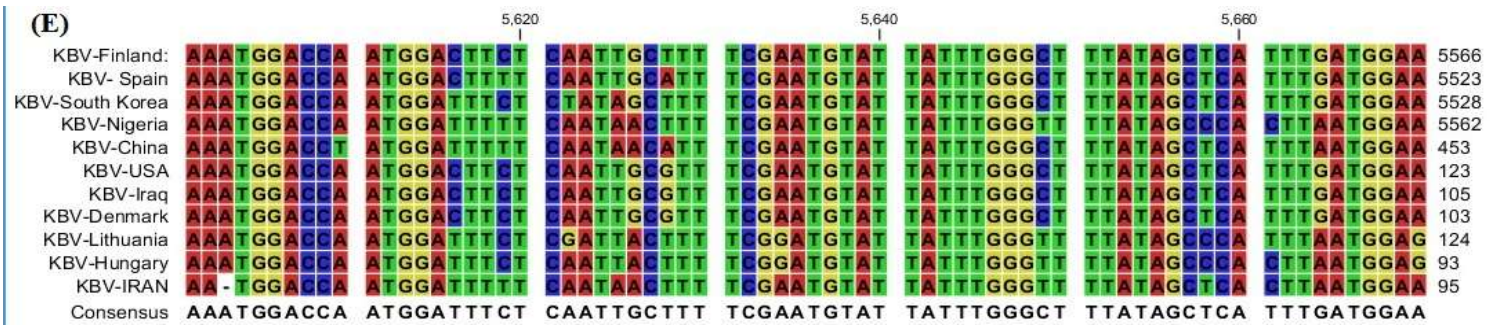
Figure 1. Results of some selected examples of PCR products on 1.2 % agarose gel electrophoresis for all studied viruses. M: Ladder; C-: Negative control; C+: Positive control; A-S: Samples of studied viruses.


(A)
8,420
8,440
8,460
8,480
ABPV- Czech Republic 8296
ABPV-France 8296
ABPV-Slovenia 8294
ABPV-Korea 8398
ABPV- China 8421
ABPV-Austria 2020
ABPV- USA 5056
ABPV- Poland 462
ABPV- Turkey 345
ABPV-IRAN 349
ABPV- UK 211
Consensus CGAAAGACAA CATTTCCCCG GACTTGACTC AACTAGATGG AATTAAAGCG CCCTCTGACA ACAATTATAA ATACATCTTA


(B)
2,740
2,760
2,780
2,800
BQCV -Republic of Korea 2777
BQCV- USA 2796
BQCV-Slovenia 2794
BQCV- China 2798
BQCV-Hungary 2761
BQCV- Australia 1229
BQCV -UK 593
BQCV-Turkey 339
BQCV-IRAN 314
BQCV- United Kingdom 285
BQCV-Russia 289
BQCV- South Korea 343
Consensus TTCCATTGTG CCAAGGATTC GCTACANGAG AAATTTTCAG CTTATCGNCA AAAGTANTCT TTTGACGAAT GGTTTAGTTT


(C)
2,740
2752
2,760
2,780
2,800
CBPV- Syria 231
CBPV- Jordan 231
CBPV- Italy 231
CBPV- Morocco 229
CBPV- China 2798
CBPV- Turkey 820
CBPV- Thailand 205
CBPV- U.S.A 231
CBPV-IRAN 119
CBPV- Central Asia 154
CBPV- Denmark 205
Consensus AATTGTTCAT CGCGTTCATG C-TTTACACC CTTACCAACG TNGGTGTTAG TAGATTTGGC ACCCACTACA


(D)
2,480
2,500
2,520
DWV-France 2505
DWV- Czech Republic 2487
DWV-USA 2502
DWV-Belgium 2504
DWV-Australia 2504
DWV-United Kingdom 2518
DWV-Taiwan 1387
DWV-Iran- Hamedan 613
DWV-China 703
DWV-Iraq 145
DWV-IRAN- western Azerbaijan 159
Consensus ATAGTGAATT CACTGGTACT TCTTCTGGTA AGTTTTACGC GAATCAAATT AGGGCAAAAC


(E)
5,620
5,640
5,660
KBV-Finland: 5566
KBV- Spain 5523
KBV-South Korea 5528
KBV-Nigeria 5562
KBV-China 453
KBV-USA 123
KBV-Iraq 105
KBV-Denmark 103
KBV-Lithuania 124
KBV-Hungary 93
KBV-IRAN 95
Consensus AAATGGACCA ATGGATTTCT CAATTGCTTT TCGAATGTAT TATTTGGGCT TTATAGCTCA TTTGATGGAA

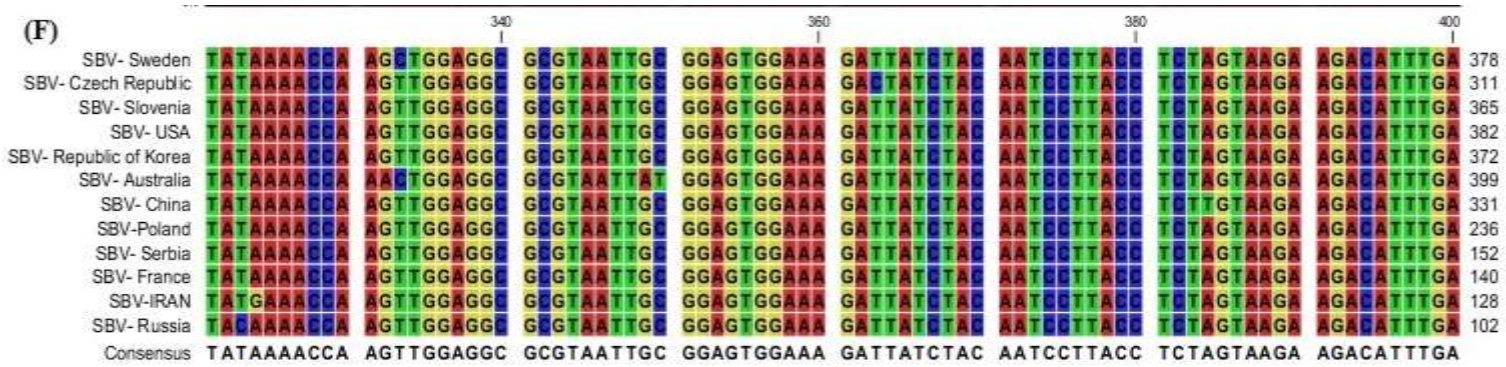
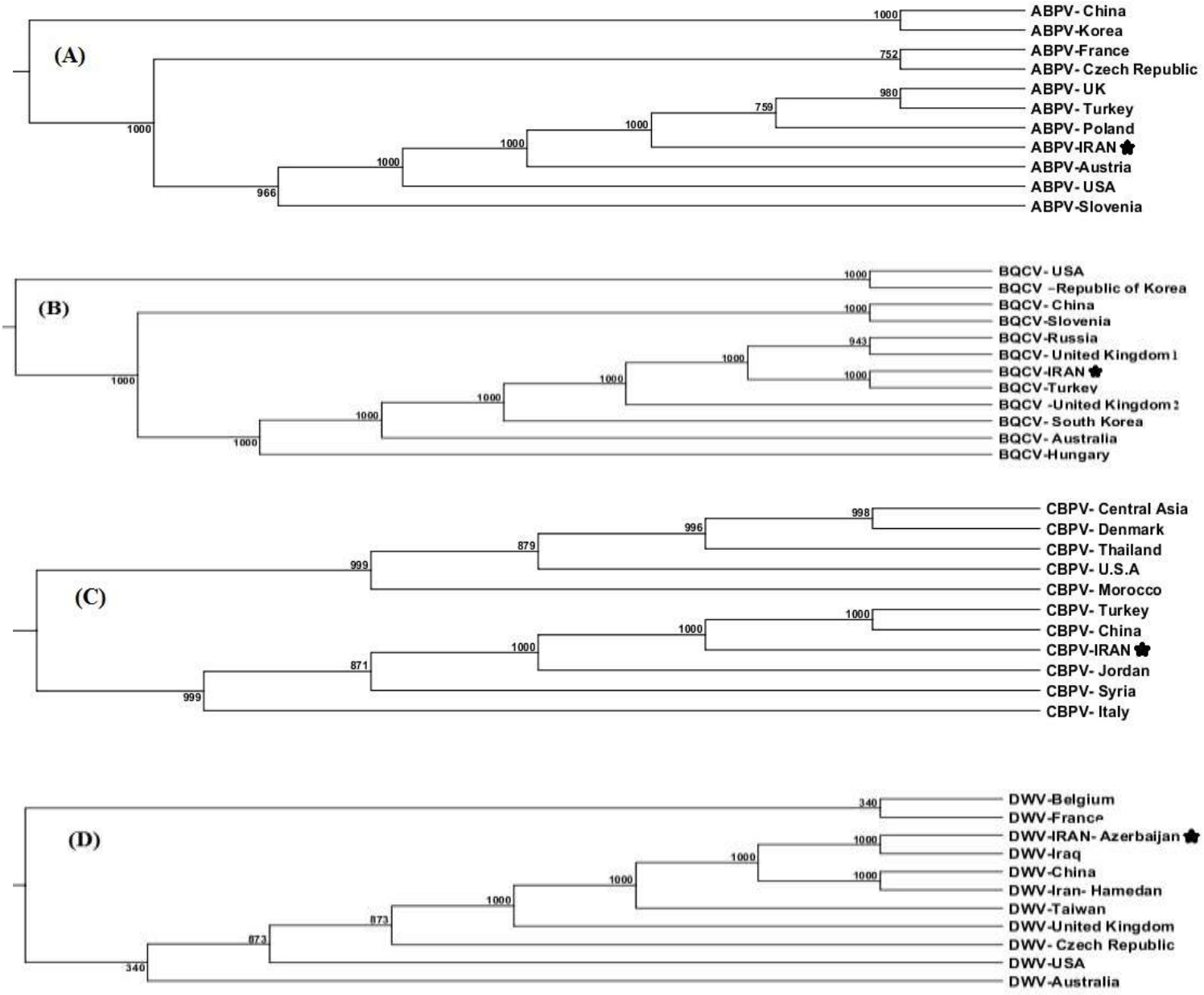


Figure 2. Phylogenetic analysis of studied viruses and evolutionary relationships based on ABPV(A), BQCV(B), CBPV(C), DWV(D), KBV(E) and SBV (F) sequences.



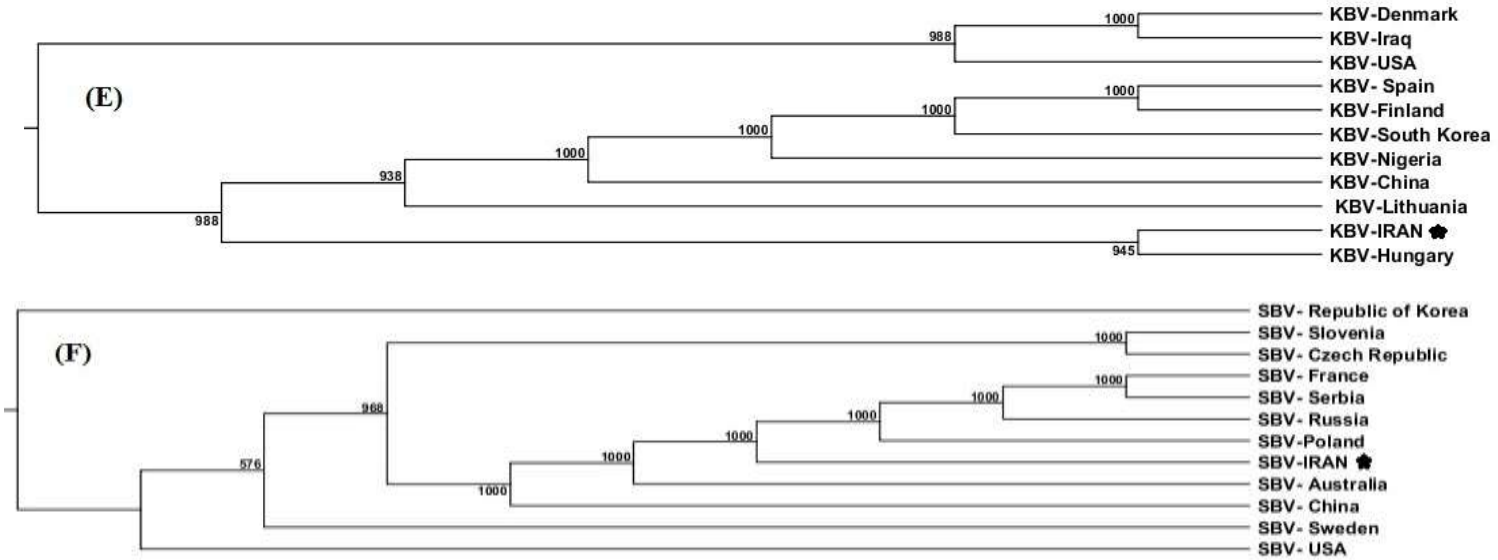


Figure 3. Phylogenetic trees and evolutionary relationships of nucleotide sequences of studied viruses. High bootstrap rates at the branches can be observed. A: ABPV; B: BQCV; C: CBPV; D: DWV; E: KBV and F: SBV.

the use of pesticides in agriculture, and parasites and pathogen infections have received more attention (16, 17).

Viruses, as one of the most important threats to the health of honeybees, were identified at the beginning of the 20th century. Honeybee viruses typically remain as unapparent infections without any signs of disease. They dramatically affect honeybee health, and as a result, the lives of infected honey bees under these conditions will be shortened (3). Also, viruses in infected cases without clinical symptoms may cause serious or lethal diseases in individual honey bees or even lead to the collapse of entire colonies (11).

In Austria, the prevalence of ABPV (68%), BQCV (30%), CBPV (10%), DWV (91%), and SBV (49%) indicated heavy infections in honeybee colonies. It is shown that each colony was involved with at least one pathogenic virus (11). The results of this study demonstrated that the most infections belonged to DWV and SBV, respectively, which was consistent with the mentioned reports (11).

In France, it was reported that CBPV was detected in 4% of adult bees during the summer, while pupae sample collected from apparently healthy colonies across 360 hives during spring, summer, and autumn in 2002 were free of CBPV (18). Findings of the current study indicated that only 11 samples tested positive for CBPV, which most of them were belonged to Northern provinces.

The sequence of ABPV in our samples showed polymorphic sites including deletions and translocations, when compared with other strains in NCBI- similar patterns were also observed with BQCV. The studied ABPV isolate was most similar to ABPV isolates from Turkey, Korea and Slovenia, whereas our BQCV isolate was similar to the isolates from Turkey and Slovenia. As presented in phylogenetic tree, the short length of the branch between our sequence and that of Turkey suggests that this sequence may have a common ancestor with the stated strains.

The sequence analysis of studied CBPV indicated an insertion in locus 2752 of the virus's genome. The ABPV phylogenetic tree revealed high bootstrap values at the branches (between 75.2% and 100%), indicating high accuracy in constructing the tree. In the following, several branches and sub-branches were formed, and the Iran ABPV sequence was separated into a distinct node from the UK, Turkey and Poland strains. As seen in the tree, sequences related to strains from China and Korea were separated from the other strains. In the first branch of this group, the sequence from Slovenia was classified as an out-group. For other viruses, the phylogenetic tree showed high bootstrap values similar to the ABPV tree. The phylogenetic tree for BQCV indicated that sequences related to strains from the USA and the Republic of Korea were separated from the other strains.

For DWV, sequences belonging to Belgium and France were separated from the other strains. In cases of KBV, sequences from Iraq, Denmark, and the USA were distinct from the other strains, and the ancestral origins of sequences from Iran and Hungary were the same. Also, the SBV sequence from the Republic of Korea was excluded as an out-group. For most of the viruses studied, the findings showed a shared ancestor between the sequences analyzed in this study and those from Turkey, with mutations contributing to genetic divergence over time.

It has been demonstrated that pollen can be used as a reliable source to diagnose viral diseases in honeybees. In contrast, consumption of infected pollen as honeybee feed can spread the virus horizontally (19). It has been shown that stresses affecting the honeybee immune system can activate latent viral infections in colonies (20). In Italy, Power et al. (21) showed that DWV was the most widespread, followed by ABPV, BQCV, KBV, and SBV. However, there were no positive samples for CBPV. In a significant number of samples, co-infection with several viruses was observed. The highest frequency belonged to DWV-ABPV infection, which was often associated with BQCV infection (21). In China, Ding et al. (22) reported that the prevalence of DWV ranged from 41% to100% (22). In Iran, Ghorani et al. (23) reported a frequency of about 21.73% in Kurdistan province, whereas in this research, a high proportion of samples (about 90%) were infected by DWV across the country. Other reports have shown a high level of DWV infections in honeybees in countries such as, Russia, Serbia and France (45%, 76.4%, and 97%, respectively) (17), which may be due to significant *V. destructo*r infestation (19). Results of this study showed considerable DWV infection in 90% of apiaries across the country, which should be taken into account in diseases management of honeybee colonies.

KBV infection can be transmitted via contaminated food resources within the colony such as brood food, honey, pollen, and royal jelly (24). The results of this study demonstrated that only about 11% of the samples were infected by KBV, and its phylogenetic analysis showed the greatest similarity to isolates from Nigeria, Lithuania, and Hungary.

It is reported that SBV infection is much higher during brood seasons, especially during spring, when large numbers of susceptible larvae and young adults can be affected (11, 17). Although our sampling time was during autumn and wither, our results showed that about 30% of the samples were infected with SBV, showing the greatest similarity to isolates from Poland and Australia.

It has been suggested that beekeepers should take preventive measures to reduce the spread of honeybee diseases, Since even a small population of sick bees can quickly transmit infections throughout the colony (25).

Therefore, preventive measures such as, establishing colonies in areas rich in nectar and pollen, replacing old and contaminated frames, changing the queen every two years, avoiding purchase of hives and related equipment from unidentified sources, conducting epidemiological monitoring of diseases using molecular diagnostic methods, and identifying contamination in bee export products- especially honey- can be vital (25). Results of this study showed that out of 31 provinces across the country, three provinces (9.68%) showed infection by at least one virus, eight provinces (25.8%) showed infection by two viruses, 10 provinces (32.26%) showed infection by three viruses, and only Golestan province (3.22%) showed infection by all six viruses.

Hence, due to the lack of available treatments for viral diseases, management strategies remain the primary means of controlling such infections in apiaries (18). Additionally, greater attention should be paid to the control of *V. destructo*r (19), which plays a critical role in the spread of these viruses. As a result, direct inspection of bee colonies by informed personnel is essential for controlling/reducing viral diseases. This goal can be achieved through active management and by preventing colonies from exposure to infection sources and other stressors. In conclusion, the findings of this study demonstrated considerable infections with studied pathogenic viruses in most apiaries across country, and these results indicated that viral diseases should be recognized as a significant problem. Therefore, an effective strategy using preventive measures should be considered to prevent the spread of viral honeybee diseases.

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Authors' Contribution

Study concept and design: M.T, M.M, H.P, G.N.B, and M.B.
Acquisition of data: M.T, M.M and M.B.
Analysis and interpretation of data: M.T and M.B.
Drafting of the manuscript: M.T and M.B.
Critical revision of the manuscript for important intellectual content: M.T, M.M, H.P, G.N.B, and M.B.
Statistical analysis: M.T and M.B.
Administrative, technical, and material support: M.T, M.M, H.P, G.N.B, and M.B.

Ethics

Not applicable.

Conflict of Interest

The authors declare that there is no conflict of interest.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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