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 all over the country. From Autumn to Winter 2022, honey bee samples were randomly collected from 31 provinces of the country. After samples preparation and RNA extraction, the target fragments were amplified and accomplished using 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 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 viruses and the lowest level of infection was related to CBPV virus. All provinces were infected with at least one virus, and in some provinces, all studied viruses were observed in apiaries. The sequencing results confirmed the RT-PCR results. Phylogenetic analysis showed the occurrence of several mutations in the total sequences in all studied viruses. The presence of viral infections in the country's apiaries shows that viral diseases should be noticed as significant problem and special management should be considered to solve it.

Key words: Honey bee, Phylogenetic analysis, Epidemiological study, RT-PCR, Pathogenic viruses

1. Introduction

Honey bees as one of the most important insects, play a key role in supplying many valuable pollination services for the most agricultural crops (1). They also produce many 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 through pollen and the honey transfer to the queen and then viruses transfer from the eggs to the next generations (5). In pollen and honey, viruses of honey bee can increase the risk of infection in healthy bee colonies via infected colonies and also feeding with honey and/or pollen. The point is that horizontal way of spreading of viruses can hide the danger of introducing new viruses (6).

The most frequently detected viruses worldwide related to economic loss 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*. This virus probably can transmit in the absence of *Varroa 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 virus with an unknown classification that belongs to the realm riboviria (8). DWV is one of the most important pathogens of honey bees which causes loss honey bee colony, annually (3). It should be kept in mind that the ratio of bees with wings deformed is usually less than one percentage in an infected colony, but, a large number of bees can be observed without disease symptoms with relatively high amounts of infection with DWV (9). Infected worker honey bees with severe BQCV show symptoms of disorientation like those of DWV (10). Another serious virus is KBV which is a very prevalent in Australia and United States of America (USA), however, its presence has been reported in Europe, too (11). The last virus that is important in *A. mellifera* larvae is SBV. This virus as a single-stranded positive-sense RNA virus is belonging to Picornavirales order and Dicistroviridae family. SBV is transmitted through infected bees to young larvae and then the infected larvae die in the prepupal stage (12).

The aim of this project was to diagnose prevalent honeybee pathogenic viruses in apiaries all over Iran, using molecular technique as well as phylogenetic tree mapping.

2. Materials and methods

2.1. Data collection and sample preparation

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

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

Where, d or error was equal 0.06, p was equal 0.4 and q was equal 0.6 and z or the normal variable was equal 1.96 with a confidence factor of 95%. So, N ≃ 260. So, 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 (overall more than 50 adult bees for each apiary). Subsequently, all collected adult bee samples from each apiary were pooled and were sent to the lab using a cold chain to check the viruses under study.

2.2. Sample preparation

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

2.3. RNA extraction

Amount of 140 µL of each prepared samples used for RNA extraction based on Berényi et al. (11) using 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 were synthesized using cDNA synthesis kit (Biotechrabbit, Germany), following the manufacturer's recommendations. Positive controls were prepared from Feredrich-Loeffler-Institute (Germany). Table 1 shows size and the primer pairs sequences of all studied viruses. All PCR reactions were accomplished using 50 ng genomic DNA, 20 pmol of each specific forward and reverse primers, 12.5 µL Taq DNA Polymerase Master Mix RED 2x (Ampliqon, Denmark) in a final volume of 25 µL.

For all studied viruses PCRs were set as an initial denaturing at 95°C for 15 min, followed by 40 cycles of 94°C for 30 secs, annealing temperatures of 55°C for 50 secs, and extension at 72°C for 1 min. The final extension was set at 72°C for 7 min. Then, PCR products were checked by 1.2% agarose gel electrophoresis.

2.5. Purification and DNA sequencing

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

2.6. Sequence analysis and statistical analyses

To determine the number of apiaries for sampling, population size was evaluated using Cochran's formula (13). And, results of molecular process were evaluated by descriptive statistics. Using the neighbor-joining method, phylogenetic trees of all sequences were assembled by MEGAX 4.0 program (14) and bootstrap support was estimated based on 1000 replicates.

3. Results

The results of molecular detection showed that out of 274 samples, 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. Table 2 shows the number of positive samples by province for all studied viruses in all over the country. Due to the large number of PCR photos, some selected examples are shown in Figure 1.

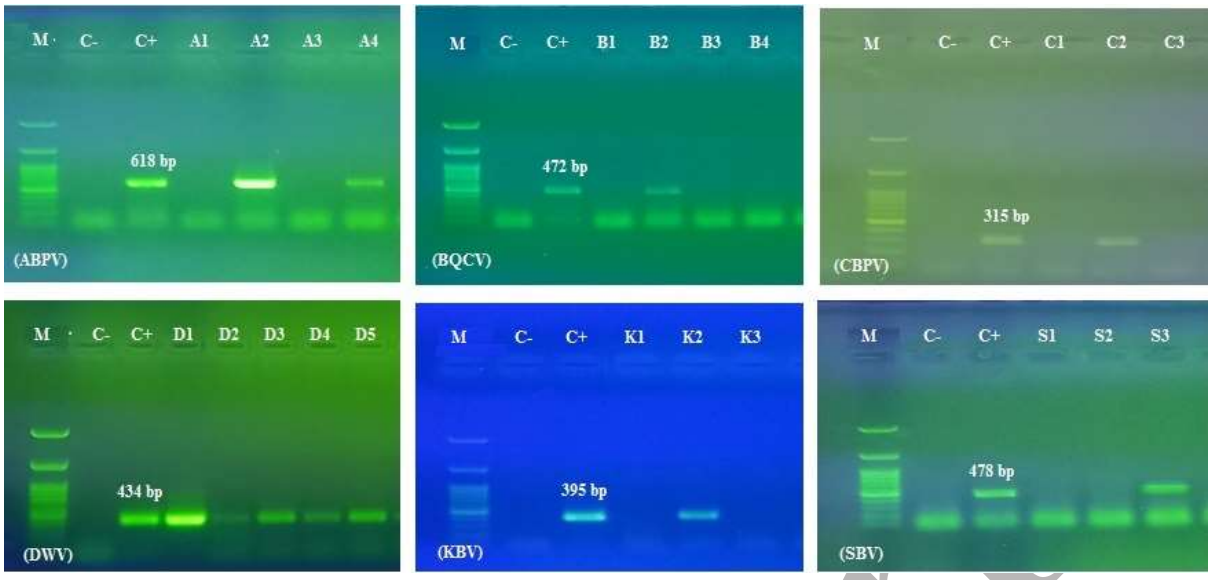
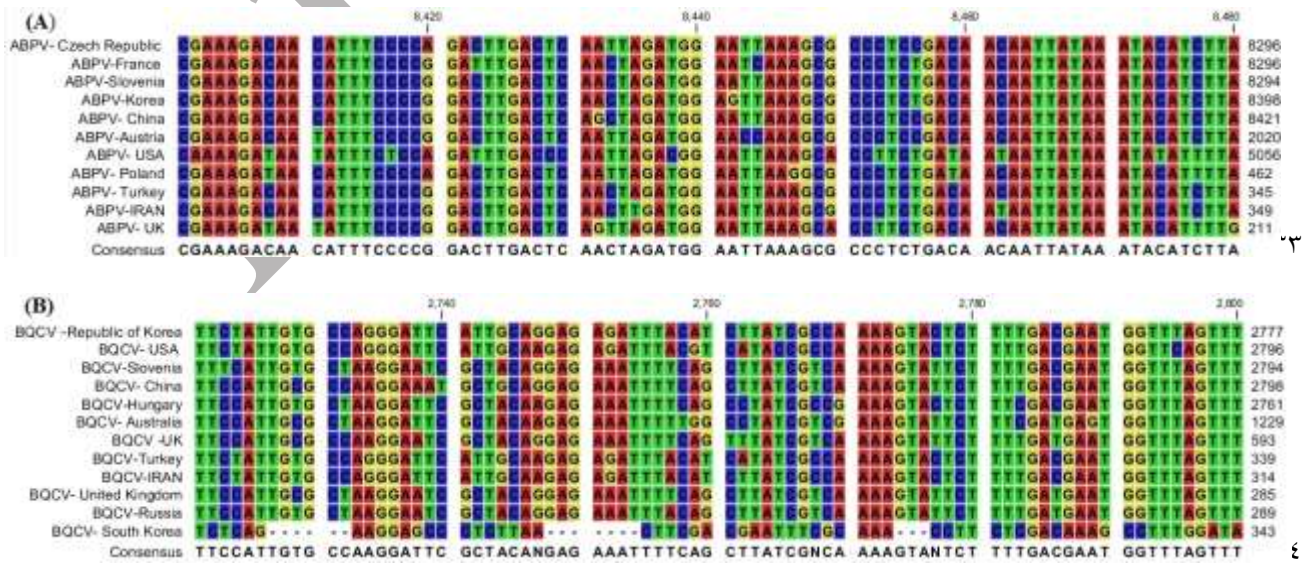


Figure 1. Results of some selected examples of PCR products on 1.2 % agarose gel electrophoresis for all studied viruses. M: Ladder; C-: Negative control; C+: Positive control; A-S: Samples of studied viruses.

The results of sequence mutations in studied viruses 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 the occurrence of mutations in the total sequences in all studied viruses.



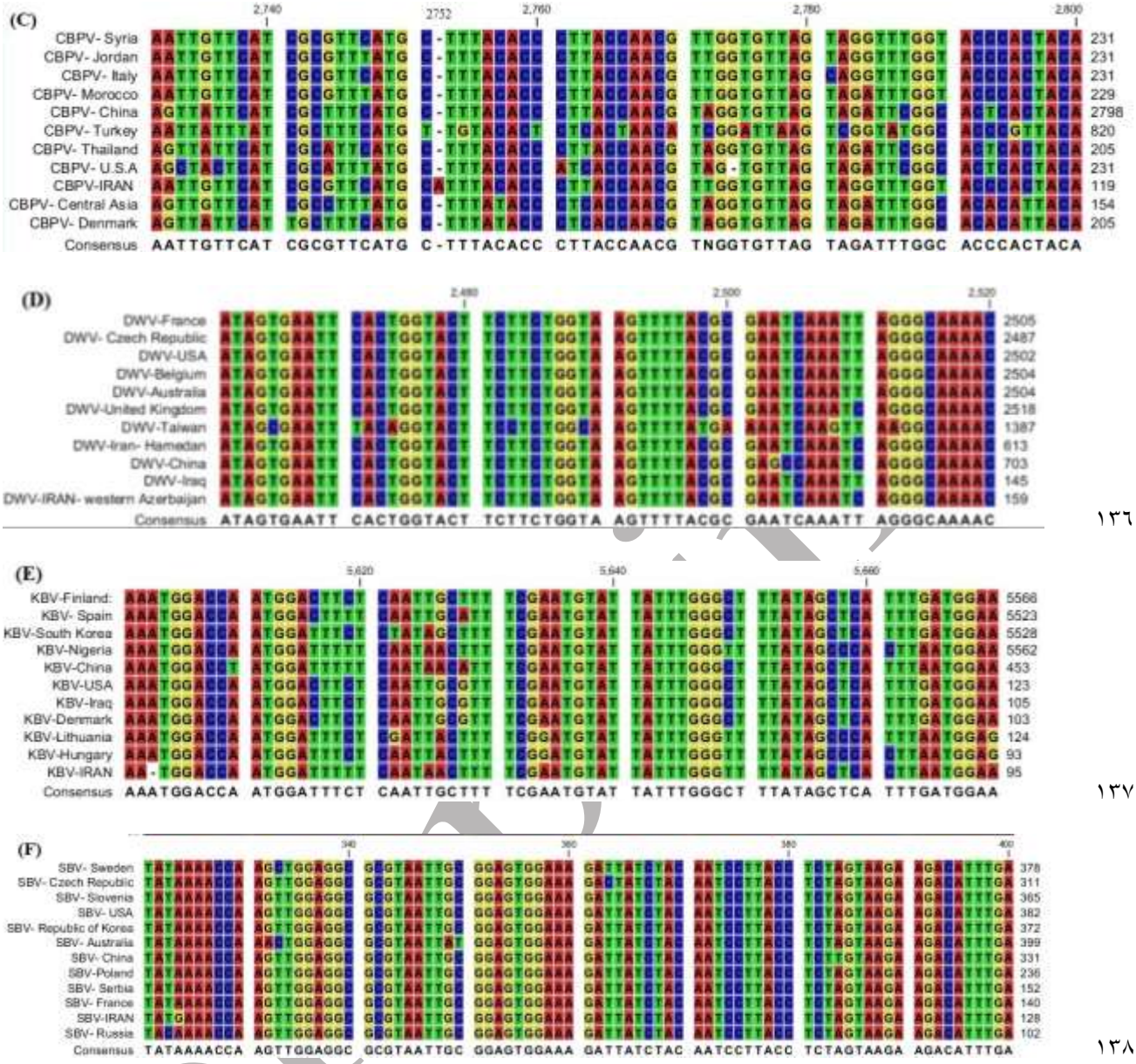
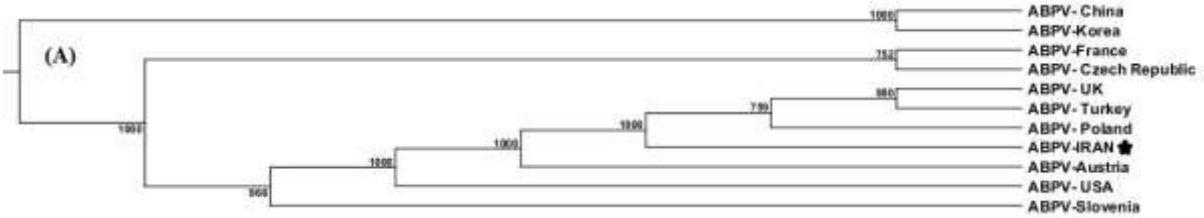


Figure 2. Phylogenetic analysis of studied viruses and evolutionary relationships based on ABPV(A), BQCV(B), CBPV(C), DWV(D), KBV(E) and SBV (F) sequences. Detected nucleotide differences between strains are shown in some columns.

The phylogenetic trees for studied viruses are shown in Figure 3.



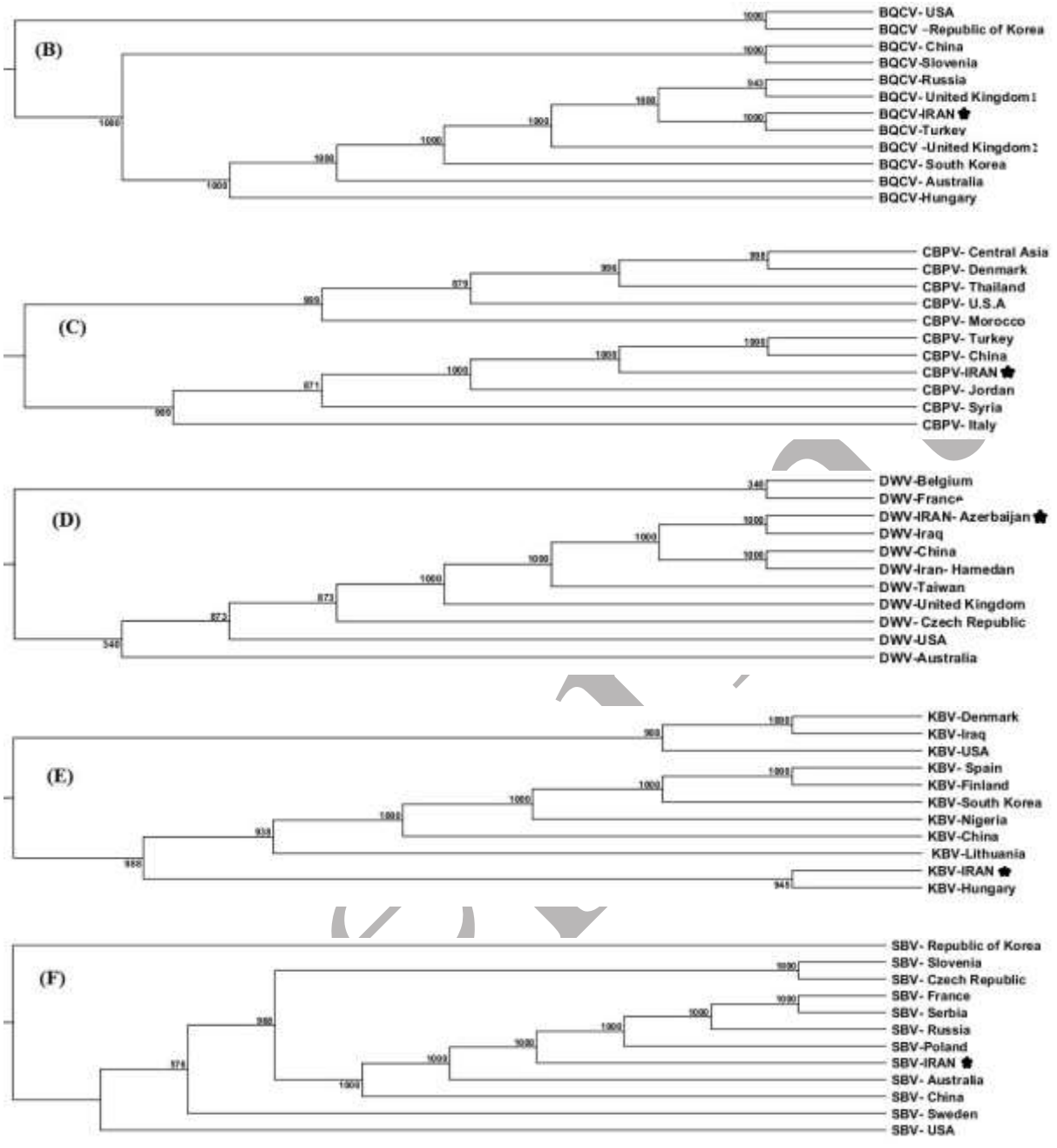


Figure 3. Phylogenetic trees and evolutionary relationships of nucleotide sequences of studied viruses. High bootstrap rates at the branches can be observed. A: ABPV; B: BQCV; C: CBPV; D: DWV; E: KBV and F: SBV.

The sequence identity results of our ABPV sequence in comparison with other ABPV sequences in database showed that the homology is in the range of 89.34%-98.57%, which indicates the high similarity of our ABPV with other strains in NCBI and the highest homology was belong to strain of 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 with a sample from Italy (Accession No. LR797924.1). For other studied viruses, the highest homologies were detected with samples from Turkey (Accession No. FJ588532.1) for BQCV, from Belgium (Accession No. KX783225.1) for DWV, from Nigeria (Accession No. MN296283.1) for KBV and from 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. Also, they play an important role in plants 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), the use of pesticides in agriculture, parasites and pathogen infections have been more considered (16, 17).

Viruses as one of the most important threats to the health of honeybees were identified at the beginning of the 20th century. The viruses of honeybee typically remain as unapparent infections without any signs of disease. They affect honeybee health, dramatically and so, the lives of infected honey bees under these conditions will be shorted (3). Also, viruses in infected cases without clinical symptoms may cause serious or lethal diseases in individual honey bees or even 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 showed that each colony involved at least with more than one pathogenic virus (11). The results of our study demonstrated that the most infections were belong to DWV and SBV, respectively, which was consistent with mentioned reports (11).

In France, it is reported that in 4% of adult bees, CBPV was detected during the summer, but, pupae collected samples were free of CBPV in apparently healthy colonies from

360 hives during spring, summer and autumn in 2002 (18). Our results indicated that only 11 samples were found positive for CBPV, which most of them were belong to Northern provinces. The sequence of ABPV in our samples showed polymorphic sites including deletion and translocation with other strains in NCBI, as well as BQCV. The studied ABPV isolate was the most similar to ABPV isolates from Turkey, Korea and Slovenia, whereas, our BQCV isolate was close to the isolates from Turkey and Slovenia. As can be seen in phylogenetic tree, due to the short length of the branch between our sequence and that of Turkey, it can be concluded that this sequence may has a common ancestor with the stated strains.

The sequence analysis of studied CBPV indicated an insertion in locus of 2752 of the sequence of this virus. The ABPV phylogenetic tree revealed a high bootstrap rate at the branches of the tree (between 75.2% and 100%), which indicates the high accuracy in drawing the phylogenetic tree. In the following, several branches and sub-branches were formed and Iran ABPV sequence was separated in one nodes from the UK, Turkey and Poland strains. As seen in this tree, sequences related to strains of China and Korea were separated from other strains. In the first branch of this category, the sequence from Slovenia was removed as an out-group. For other viruses the phylogenetic tree showed a high bootstrap rates as well as ABPV tree. The phylogenetic tree for BQCV indicated that sequences related to strains of USA and republic of Korea were separated from other strains. For DWV, sequences belong to Belgium and France were separated from other strains, while for KBV, sequences of Iraq, Denmark and USA were separated from other strains and the ancestor of sequences from Iran and Hungary were the same. Also, the sequence of SBV from republic of Korea was removed as an out-group. For most of studied viruses, findings showed that there is a same ancestor for our studied sequences and Turkey sequences, and the mutations have led to genetic distances.

It is demonstrated that pollen can be used as a reliable source to diagnose viral diseases in honeybee. In contrast, consumption of infected pollen as honeybee feed can spread the virus

horizontally (19). It has shown that stresses which affect the honeybee immune system can activate latent viral infections in colonies (20). In Italy, Power et al. (21) have shown that DWV was the most widespread followed by ABPV, BQCV, KBV and SBV. However, there was not positive samples for CBPV. In a significant number of samples, the co-infection of several viruses was observed, whereas, the highest frequency was belonging 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%-100% (22). In Iran, Ghorani et al. (23) reported that frequency of was about 21.73% in Kurdistan province whereas in our research, high range of samples (about 90%) were infected by DWV in all over the country. Other reported have shown high level of DWV infection in honeybee in other countries such as Russia, Serbia and France (45%, 76.4% and 97%, respectively) (17), which can be because of significant *Varroa destructo*r infection (19). Results of our study showed considerable infection by DWV in 90% of apiaries of the country that should be noted in diseases management of honeybee colonies.

The KBV infection can transmit via contaminated food resources in the colony such as brood food, honey and pollen royal jelly, orally (24). Our results demonstrated that only about 11% of our samples were infected by KBV and its phylogenetic analysis showed most similarity to the isolates from Nigeria, Lithuania and Hungary.

It is reported that SBV infection is much higher in 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 the Autumn and Wither, our results showed that about 30% of our samples were infected with SBV which had most similarity to isolates from Poland and Australia.

It has been suggested that beekeepers should take preventive measures to prevent the spread of honeybee diseases. Since, even a small population of sick bees can quickly cause spread the diseases to the colony (25), so, preventive measures such as the creation of a colony

in areas rich in nectar and pollen, changing old and polluted frames, changing the queen every two years, avoid doing from buying hives and its requirements from unidentified sources, monitoring epidemiological examination of diseases using the molecular diagnostic method, identifying the contamination of bee export products especially honey, can be vital (25). Results of our study showed that out of 31 provinces of 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 three viruses and only Golestan province (3.22%) showed infection by all 6 viruses.

Hence, due to the lack of treatments for viral diseases, the management strategies can be as the only way to control of viral diseases in apiaries (18), and also it should be more pay attention to control of *Varroa destructo*r (19). As a result, using direct inspection of the colonies by informed persons for controlling/reducing the viral diseases is necessary. This important can be accomplished by an active management and preventing the colony from being exposed to sources of infections and other stressors. In conclusion, our finding demonstrated that considerable infections for studied pathogenic viruses in most apiaries of the country, and these results indicated that viral diseases should be noticed as significant problem. So, an effective strategy using preventive measures should be considered to prevent the spread of viral honeybee diseases.

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Authors' contributions

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.

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Table 1. Oligonucleotide primer pairs employed in RT-PCR assays (13).

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

	R: 5'- TGT GGG TTG GCT ATG AGT CA-3'		
SBV	F: 5'- ACC AAC CGA TTC CTC AGT AG-3'	478 bp	AF092924
	R: 5'- CCT TGG AAC TCT GCT GTG TA-3'		

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 Azarbayijan	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 Azarbayijan	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

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