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Original Article

Molecular Identification of Six Honeybee Viruses in Iranian Apiaries

Moharrami *, M., Modirrousta, H.

Department of Honeybee, silk worm and wildlife disease, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

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Corresponding Author: mojmoharrami@yahoo.com

ABSTRACT

The identification of honeybee viruses is of serious importance, particularly considering the lack of information on the natural incidence of viral infections in honeybee populations worldwide. Moreover, the global spread of *Varroa destructor* in honeybee colonies has a significant effect on the viral infection. In the present study, 160 samples of adult bee from apparently healthy colonies but with a background of parasitic diseases, tremor, and paralysis, were collected during 2011-2012. The samples belonged to 23 different provinces of Iran. They were sent to Razi Vaccine and Serum Research Institute, Karaj, Iran, for further analysis, and examined for the presence of viruses using reverse transcription polymerase chain reaction assay. According to the results, out of 160 samples, 9 (5.8 %), 40 (25.6 %), 12 (7.8 %), 34 (21.8 %), 7 (4.5 %), and 29 (18.5%) cases were positive for 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). The samples collected from 18 provinces (78 %) were positive for at least one virus. Among all samples, 83 (53.2 %) specimens were infected with at least one virus. The highest prevalent virus was BQCV, followed by DWV, SBV, CBPV, ABPV, and KBV, respectively.

Keywords: Honey bee viruses (ABPV-BQCV-CBPV- DWV- KBV-SBV), RT-PCR, Iran

Identification moléculaire de six virus d'abeilles domestiques dans les ruchers iraniens

Résumé: L'identification des virus d'abeilles est d'une importance capitale, compte tenu en particulier du manque d'informations sur l'incidence naturelle des infections virales chez les populations d'abeilles du monde entier. De plus, la propagation mondiale de *Varroa destructor* dans les colonies d'abeilles a un effet significatif sur l'infection virale. Dans la présente étude, 160 échantillons d'abeilles adultes de colonies apparemment en bonne santé, mais présentant des antécédents de maladies parasitaires, de tremblements et de paralysie, ont été collectés au cours de la période 2011-2012. Les échantillons appartenaient à 23 provinces différentes de l'Iran. Ils ont été envoyés à l'Institut de recherche sur le sérum et les vaccins de Razi, à Karaj, en Iran, pour une analyse plus approfondie, et examinés pour rechercher la présence de virus utilisant l'essai de la transcription inverse de la réaction en chaîne par polymérase. Selon les résultats, sur 160 échantillons, 9 (5,8%), 40 (25,6%), 12 (7,8%), 34 (21,8%), 7 (4,5%) et 29 (18,5%) cas étaient positifs pour le virus de la paralysie aiguë de l'abeille (ABVP), le virus de la cellule noire de reine (BQCV), le virus de la paralysie chronique de l'abeille (CBPV), le virus des ailes déformées (DWV), le virus de l'abeille du Cachemire (KBV) et Le virus du couvain sacciforme (SBV). Les échantillons prélevés dans 18 provinces (78%) étaient positifs pour au moins un virus. Parmi tous les échantillons, 83 échantillons (53,2%) étaient infectés par au moins un virus. Le virus le plus prévalent était le virus BQCV, suivi des virus DWV, SBV, CBPV, ABPV et KBV, respectivement.

Mots-clés: le virus de la paralysie aiguë de l'abeille (ABVP), le virus de la cellule noire de reine (BQCV), le virus de la paralysie chronique de l'abeille (CBPV), le virus des ailes déformées (DWV), le virus de l'abeille du Cachemire (KBV), Le virus du couvain sacciforme (SBV), Transcription inverse - réaction en chaîne de la polymérase (RT-PCR), Iran

INTRODUCTION

Apis mellifera L. (European honeybee) is one of the most important economical insects due to its various products and contribution to the pollination of agricultural plants. However, many pathogens, including viruses, threaten beehives and cause severe losses to apiculture (Chen and Siede, 2007). The diagnosis of the bee viral infections is difficult because the honeybee viruses usually persist as inapparent infections and cause no obvious signs (Fievet et al., 2006). The most commonly detected viruses worldwide associated with 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). The majority of these infections occur concurrently with varroasis, a disease caused by an external parasitic mite that attacks the *Apis cerana* (Asiatic honeybee) and *Apis mellifera*. The ABPV is one of the important viruses infecting *A. mellifera*. This virus is a positive-sense single-stranded ribonucleic acid (RNA) virus of the order of *Picornavirales*, family of *Dicistroviridae*, and genus of *Aparavirus*, with a genome length of 9.491 kb. The virus persists as an inapparent infection in nature. It is probably transmitted in the absence of *Varroa destructor* (*V. destructor*) via the salivary gland secretions of the adult bees and the food contaminated with these secretions (Anderson, 1991). Mites can also act as vectors in the spread of the virus from bee to bee. Honeybee larvae can become infected with the virus by ingesting food contaminated with viral particles and secreted by the infected nurse bees. It is presumed that this virus plays a role in cases of sudden collapse of honeybee colonies (Nordström et al., 1999). The CBPV is the causal agent of chronic paralysis known to induce the significant losses in honeybee colonies (Bailey and Woods, 1977). This virus is an unclassified single-stranded positive-sense RNA virus, and the genome is 5.979 kb in length. The CBPV is transmitted by the ingestion of infected food or through the contact of infected materials with open wounds. This infection entails two groups of manifestations, one of which include inability to fly, trembling, crawling, and asymmetrical outspread of wings, followed by dysentery and death within a few days. The other group of manifestations consists of black discoloration due to hair loss, rendering the affected individuals unrecognizable to the guard bees, leading to their dismissal in the colony. The DWV is a virus most commonly found in *A. mellifera*, although it has also been detected in (*A. cerana*) from China. This virus is a single-stranded positive-sense RNA virus from the order of *Picornavirales*, family of *Dicistroviridae*, genus of *Iflavirus*, with a genome length of 10.14 kb. The appearance of wing deformity due to DWV apparently depends on the stage of infection. The introduction of the virus and establishment of overt infection in developing honeybee brood are closely linked to the feeding activities of *V. destructor* mites. The pupae infected with the virus at the white-eye stage of development survive to emerge; however, they may have poorly developed wings and die prematurely. Most of the young bees emerging from the mite-infected cells in an infected colony appear normal, although they can contain as much virus as deformed individuals, and their productivity, and lifespan are similarly reduced. The virus has been associated with bee mortalities,

particularly in the presence of *V. destructor*. Reports from several countries indicate that there is a significant association between DWV, *V. destructor,* and the honeybee colony collapse (Nordström et al., 1999). The KBV was first detected in *A. cerana* from India (Bailey and Woods, 1977). This virus is a single-stranded positive-sense RNA virus of the order of *Picornavirales*, family of *Dicistroviridae*, genus of *Aparavirus*, and the genome length of 9.524 kb. It is thought to be the most virulent of the all known honeybee viruses. Very low titers are required for the infection, and the virus can be multiplied rapidly when it is introduced into the haemolymph of both adult bees and pupae, causing death within three days (Allen and Ball, 1995). The KBV is very closely related to ABPV; in this regard, both of them are approximately 70% identical throughout the whole genome (de Miranda et al., 2004). According to the reports, bees might be infected by both viruses simultaneously. Larvae can survive after they ingest KBV, and some of them become inapparently infected adults (Anderson, 1991). The BQCV causes a disease in *A. mellifera* queen larvae (Anderson, 1993). This virus is a single-stranded positive-sense RNA virus of the order of *Picornavirales*, family of *Dicistroviridae*, genus of *Triatovirus*, and genome length of 8.55 kb. The infected queen dies in the prepupal or pupal stage, and her cell wall color turns to dark brown or black. The dead larva contains many virus particles. The BQCV is a common infection of field bees that are infected with *Nosema apis* (*N. apis*), a small, unicellular parasitic fungus which causes nosemosis or nosema, the most common and widespread disease of adult honeybees. Over-wintering colonies show significantly higher losses when infected with the virus and *N. apis*, compared to the time they are infected with *N. apis* alone (Bailey and Woods, 1977). Sacbrood is a disease of *A. mellifera* larvae caused by SBV. Sacbrood is a single-stranded positive-sense RNA virus of the order of *Picornavirales*, family of *Dicistroviridae*, and genus of *Iflavirus*, with a genome length of 8.832 kb. The virus might be present and multiplied in young adult bees without causing obvious symptoms or disease, except for the change in behavior, which can manifest as the loss of appetite for pollen. However, such bees may have a short life span (Bailey and Woods, 1977). Infected bees pass SBV in the food to the young larvae, which then become infected and die in the prepupal stage. The disease has a seasonal occurrence, with common outbreaks in the spring and spontaneous disappearing in the summer (Bailey and Woods, 1977). With this background in mind, the present study aimed to investigate the presence of honeybee viruses in diseased honeybee colonies in 23 provinces of Iran using RT-PCR.

MATERIAL AND METHODS

Sampling. Sampling was performed during July-September 2011 and 2012 from 23 provinces of Iran in different geographic regions (Caspian weather, mountains, desert or semi-desert) (Table 2) with the collaboration of National Veterinary Organization, Iran. In total, the samples were taken from 160 apiaries. Each sample consisted of approximately 100-500 honeybees from colonies suffering from symptoms of depopulation, sudden collapse, paralysis, and *V. destructor* infestation. All samples were submitted to the Honeybee Department of Razi Vaccine and Serum Research Institute, Karaj, Iran, under a cold chain condition, and then stored at -20 °C until use.

RNA extraction. Sixty adult bees from each apiary were selected and homogenized in a ceramic mortar with sterile diethylpyrocarbonate-(DEPC) treated water. The homogenates were centrifuged at 20,000 g for 1 min, and 140 µl of the supernatant was used for RNA extraction (Berenyi et al., 2006). RNA was extracted by the QIAmp Viral RNA Mini kit (QIAGEN, Germany) according to the manufacturer's instructions.

Primers. Primers specific for the genomes of ABPV, BQCV, CBPV, DWV, KBV, and SBV viruses were used as described by Berenyi et al. (2006) (Table 1).

Reverse transcription polymerase chain reaction. Reverse transcription and amplifications were performed

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

Primer name	Primer sequence (5′-3′)	Primer position on the genome	Length of amplified product
ABPV f	GTG CTA TCT TGG AAT ACT AC	7928-7947	618
ABPV r	AAG GYT TAG GTT CTA CTA CT	8527-8546	
CBPV f	TGT CGA ACT GAG GAT CTT AC	111-130	315
CBPV r	GAC CTG ATT AAC GAC GTT AG	407-426	
DWV f	ATT GTG CCA GAT TGG ACT AC	2345-2364	434
DWV r	AGA TGC AAT GGA GGA TAC AG	2760-2779	
KBV f	GAT GAA CGT CGA CCT ATT GA	5406-5425	394
KBV r	TGT GGG TTG GCT ATG AGT CA	5781-5800	
BQCV f	AGT AGT TGC GAT GTA CTT CC	252-271	477
BQCV r	CTT AGT CTT ACT CGC CAC TT	710-729	
SBV f	ACC AAC CGA TTC CTC AGT AG	221-240	487
SBV r	CCT TGG AAC TCT GCT GTG TA	689-708	

Table 2. Samples collected for the detection of six honeybee viruses from apiaries located in 23 provinces in different geographic areas of Iran during summer 2011-2012

No	Province	Apiary	ABPV	CBPV	DWV	KBV	QBCV	SBV	Type of virus
1	Alborz	2	2	0	1	2	0	0	**3**
2	Ardebil	6	0	0	4	0	0	3	**2**
3	Chaharmahal&Bakhtiari	6	1	0	1	0	3	1	**4**
4	East Azarbayijan	24	0	1	11	1	9	10	**5**
5	Esfahan	3	0	0	0	0	0	0	**0**
6	Fars	12	0	0	0	0	0	0	**0**
7	Ghazvin	2	2	1	1	1	2	0	**5**
8	Gilan	6	1	0	3	0	3	0	**3**
9	Golestan	2	0	0	2	0	0	0	**1**
10	Hamedan	6	0	0	0	0	1	0	**1**
11	Ilam	7	0	0	0	0	0	0	**0**
12	Kerman	6	0	0	0	1	2	0	**2**
13	Kermanshah	5	0	0	0	0	4	0	**1**
14	Kohkilouyeh& Buyer Ahmad	4	1	1	0	0	0	0	**2**
15	Markazi	4	0	0	0	1	1	1	**3**
16	Mazandaran	1	1	0	0	0	0	0	**1**
17	North Khorasan	10	1	2	4	0	3	6	**5**
18	RazaviKhorasan	35	0	6	4	1	10	3	**5**
19	Semnan	4	0	1	0	0	1	3	**2**
20	South Khorasan	4	0	0	0	0	1	0	**1**
21	West Azarbayijan	2	0	0	0	0	0	0	**0**
22	Yazd	2	0	0	0	0	0	0	**0**
23	Zanjan	8	0	0	3	0	0	2	**2**
	Total	160	9	12	34	7	40	29	

with a one-step RT-PCR kit (QIAGEN, Germany), following the manufacturer's recommendations. Amplifications were employed in an Eppendorf Master Cycler Gradient. The reverse transcription was performed at 50 °C for 30 min, followed by a denaturation step at 95 °C for 15 min, and 40 cycles consisting of 30 sec at 94 °C, 50 sec at 55 °C, and 1 min at 72 °C. The reactions were completed by a final elongation step for 7 min at 72 °C (Berenyi et al., 2006). The PCR products were electrophoresed in a 1.2% agarose gel and stained with safe stain. Bands were photographed under ultraviolet light by means of a digital camera (Panasonic, Japan). Positive controls (RNA of six viruses) were kindly provided by Feredrich-Loeffler-Institute, Germany. Healthy bees from healthy apiaries were used for negative control.

RESULTS

In this study, adult honeybees were collected from apparently healthy apiaries (n=160) with the histories of parasitic diseases, tremors, paralysis, and unprecedented deaths. The apiaries were located in 23 provinces with

different geographical profiles (Table 2). The RT-PCR successfully identified the samples containing six honeybee viruses, and no PCR fragments were amplified from the total RNA of healthy bees (Figure 1, Table 1).

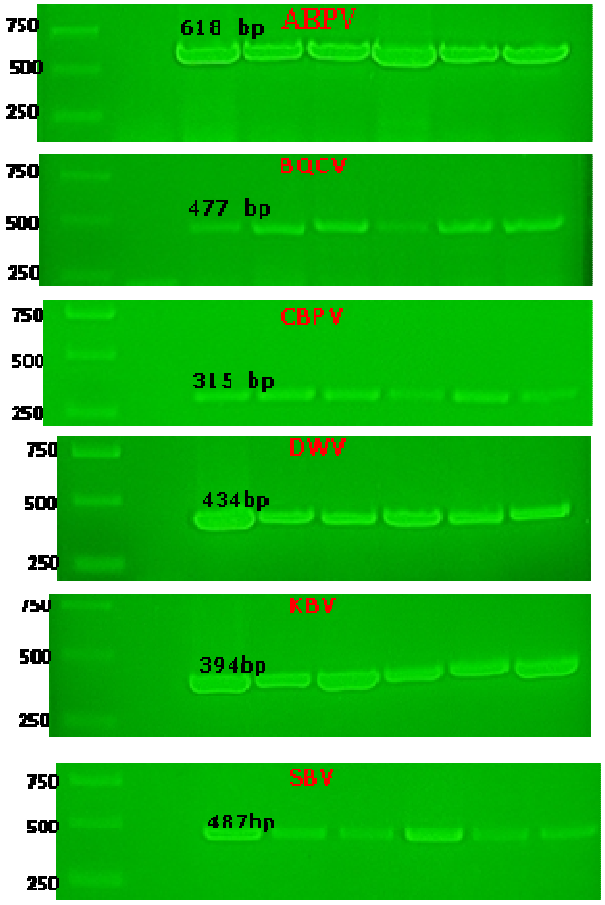

Figure1. Visualization of some positive reverse transcription polymerase chain reaction products obtained from amplification process (L: ladder 1kb, C-: negative control, C+: positive control, others: positive samples)

Our results showed that 51.87% (83/160) of the samples were infected with at least one virus (Figure 2). Out of the 160 apiaries, 7 (4.37%), 9 (5.62%), 12 (7.5 %), 29 (18.12%), 34 (21.26%), and 40 (25 %) cases were infected with KBV, ABPV, CBPV, SBV, DWV, and BQCV, respectively. The most frequent infection was caused by BQCV, followed by DWV, SBV, CBPV, ABPV, and KBV (Figure 3). As depicted in Figure 4, 62.5% of the samples were infected with only one virus, whereas 21.69%, 10.85%, and 4.81% of the specimens were simultaneously infected with two, three, and four viruses, respectively.

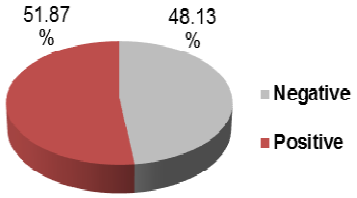

Figure 2. Percentage of infected samples at least with 1 virus

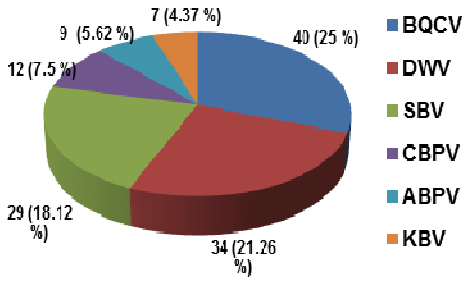

Figure 3. The frequency of viruses in infected samples

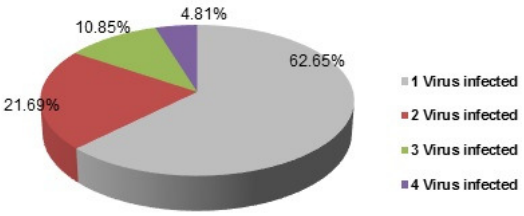

Figure 4. Percentage of simultaneously infected samples with 1, 2, 3, 4 viruses

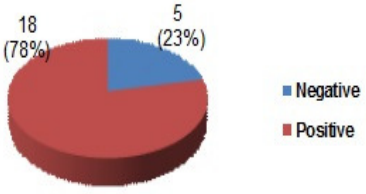

Figure 5. The frequency of contaminated and uncontaminated provinces

DISCUSSION

Based on these results, DWV and BQCV were the most frequent viruses detected by RT-PCR assay. Among the 23 provinces, the samples obtained from 18 (78%) provinces were positive for at least one virus, whereas five provinces appeared devoid of the mentioned viruses (Figure 5). More than 70% (13 out of the 18 provinces) of the positive provinces were infected with two or more viruses. Table 2 demonstrates the distribution profile of the viruses in different regions of the country. Furthermore, one, two, three, four, and five viruses were detected in five, three, three, one, and four provinces among 18 infected provinces, respectively. This study is the first molecular investigation on the dispersion of six viruses of economic importance among the Iranian apiaries, providing hints for the identification of the causes of the abnormal reduction or loss of adult bees in bee colonies. Our results are consistent with those reported in other studies performed across the world. Berenyi et al. (2006) conducted a study in Austria on the occurrence of six pathogenic virus patterns (i.e., ABPV, BQCV, CBPV, DWV, KBV, and SBV) in 90 colonies. In the mentioned study, the most common viruses were reported as DWV (91%), ABPV (68%), SBV (49%), BQCV (30%), and CBPV (10%). Furthermore, most of the samples were positive with more than one virus (Berenyi et al., 2006). Investigations of bee colonies in France demonstrated DWV (97%), SBV (86%), CBPV (28%), ABPV (58%), BQCV (86%), and KBV (17%) infections in adult bee samples (Tentcheva et al., 2004).

In a study conducted by Nielsen et al. (2007) in Denmark on winter colony loss, KBV, BQCV CBPV, ABPV, DWV, and SBV infections were observed in 1, 1, 4, 11, 55, and 78 apiaries, respectively. In another study, the co-infection of one to three viruses was observed in a large number of samples (Nielsen et al., 2008). In an investigation carried out by Chen et al. on 56 bee colonies in the USA, it was revealed that 75% of these colonies were infected with at least one or more viruses. In this regard, 38% of the colonies were infected with BQCV, DWV, KBV or SBV. Additionally, 50%, 7%, and 5% of the colonies were reported to be infected with two, three, and four viruses, respectively. In the mentioned study, co-infection (43%) with BQCV and DWV was the most frequent finding (Chen et al., 2006). In another study performed by Haddad et al. (2008) in Jordan on 13 bee colonies, DWV, SBV, and ABPV were present in 92%, 8%, and 16% of colonies, respectively. However, they found no CBPV, BQCV, and KBV (Haddad et al., 2008). Based on the evidence, KBV infection is closely related to ABPV. It has been shown that this virus can be associated with varroasis. The KBV was identified in the African honeybee colonies, which were infected with V. destructor in Costa Rica (Calderón et al., 2003). Yasar et al. showed that the DWV is present in varroasis-infected colonies in Turkey (Gülmez et al., 2009). As a conclusion, the results of those studies may confirm the importance of viral infections alone and in combination with *V. destructor*, Nosema, and other parasitic factors. The interference may have a significant impact on the health, productivity, longevity, and survival of the honeybee colonies (Tentcheva et al., 2004). The synergistic effect between N. apis and viruses, such as filamentous, Y, and Queen viruses, was previously reported (Bailey and Woods, 1977). Diarrhea in adult bees is usually caused by *N. apis* and CBPV infection; as a result, the virus is excreted via feces in the diarrhea stools and contaminate the environment. Toplak et al. revealed that the results of studies confirm the importance of CBPV infection in combination with Nosema (Toplak et al., 2013). In addition, Bailey and Woods demonstrated a close relationship between BQCV and *N. apis*, and the pathogenicity of the BQCV was exacerbated by *N. apis* infection (Bailey and Woods, 1977). The researchers concluded that the combination of *N. apis* and BQCV infection was more severe than *N. apis* infection alone. The DWV is one of the most common bee viruses in the world, which has been observed in most of the Varroa-infected colonies; this indicates the close association of this virus with Varroa infestation (Paxton et al., 2007; Higes et al., 2009). Another common way of SBV transmission can

be the infestation of colonies with *V. destructor*. The ability of mites to transmit SBV has been experimentally shown in healthy pupae. The evidence suggests that highly pathogenic viral diseases have been exacerbated by Varroa mites and remain as persistent infections in bees (Grabensteiner et al., 2001). Studies conducted in France show similarities between KBV and ABPV infections. However, they are slightly different in terms of seasonal outbreaks (Tentcheva et al., 2004). Both viruses have a low prevalence; however, the occurrence of ABPV in summer is associated with Varroa mites and is more common than KBV. On the other hand, KBV is more prevalent in the spring and less prevalent in the fall. The KBV is widely spread in the world; nonetheless, it has a low prevalence in the North America and Australia. There is little information about the prevention of KBV infection in colonies and its role in the death of colonies infested with V. destructor (Anderson, 1991). To gather data on the relationship between viral and parasitic infections, environmental factors and clinical symptoms in bee colonies or in the apiary, it is necessary to track the dispersion and circulation of the viruses in infected colonies (Evans and Hung, 2000; Higes et al., 2009). The most important aspect of the present research is to identify six pathogenic honeybee viruses for the first time in the apiaries obtained from 23 provinces in Iran. This study presents six viruses in the bee colonies with their remarkable differences in the distribution pattern. The spread of viruses in different regions may be influenced by geographical conditions and viral concentrations in their apiaries. The population density of colonies in different areas provides a great potential for close contact between colonies, which creates important scenarios for the transmission of infectious agents. The RT-PCR as a quantitative method could provide more detailed information, regarding the relationship between the viruses and symptoms and may be used as a great tool for future studies.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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