Molecular Characterization and Phylogenetic analysis of Deformed Wing Virus in Honey Bees (*Apis Mellifera*), North of Iran

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Abstract

Deformed wing virus (DWV) is one of the major bee viruses which can be transmitted by the *Varroa destructor* mite and is a key contributor to honey bee colony collapse. To date, three main variants of the Deformed wing virus (DWV-A, DWV-B, DWV-C) have been described. In the present study, bee samples showing signs of DWV infection and *Varroa* infestation were collected from two different farms in Alborz province, northern Iran. The samples were screened for the presence of DWV genome and also other bee viral pathogens including Chronic bee paralysis virus, Acute bee paralysis virus, Kashmir bee virus and sac brood virus by using PCR assay. PCR results indicated that among collected samples, two samples were positive for DWV and all samples were negative for other viral disease. Phylogenetic analysis revealed that the detected DWVs clustered within the DWV-A genotype. Homology analysis of isolate UT-SHN07

showed approximately 99% similarity to isolate PA and DWV-A-OR-OCT2018-EVR23 from USA, as well as to DWV-Iraq-2023 and the reference sequence NC004830 from Iraq and Italy respectively. For the other isolate, UT-SHN62, homology analysis showed 100% similarity with isolate PA isolated from USA and 99% similarity with DWV-A-OR-OCT2018-EVR23, DWV-Iraq-2023, NC004830 and Gotland-2 sequences from the USA, Iraq, Italy and Sweden respectively. Iran is ranked among top 10 honey-producing countries, and continuous monitoring of circulating viruses and *Varroa* mites (key contributors to colony losses) is essential. Further molecular and phylogenetic studies are recommended to better understand the current status of honey bee viral disease in Iran.

Keywords

Honeybee, Deformed wing virus, Colony loss, Phylogeny, Homology

1. Introduction

Since the beginning of 21st century, substantial honey bee losses have been reported across the northern hemisphere (1). It's estimated that nearly one-third of the typical western diet depends on bee pollination. The honey bee, *Apis mellifera*, is recognized as the primary insect responsible for pollinating a wide range of crops, including fruits, nuts, vegetables and oilseeds, with an estimated annual added market value exceeding 15 billion dollars (2, 3). In addition to pollination, honey bees contribute to the economy by producing various hive products such as honey, beeswax, propolis, royal jelly and others (4). Over the past 15 years, sever winter colony losses have been reported from different regions worldwide raising growing concern about the health status of bee colonies. Nevertheless, over the past 60 years, the number of managed bee colonies has increased all over the world (5). Like many other species, honey bees are susceptible to a variety of pathogens that can cause considerable colony. Among these, viruses are considered one of major threats to honey bee health and survival (4).

Viruses that infect honey bees are primarily classified within the order *Picornavirales,* and are positive sense single-stranded RNA viruses (+ssRNA). Common bee viruses include members of the Dicistroviruses such as Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), Acute bee paralysis virus (ABPV) and Black queen cell virus (BQCV); the Iflaviruses such as Deformed wing virus (DWV), Kakugo virus, Varroa destructor virus-1 (also known as DWV-B), Sacbrood virus (SBV), and Slow bee paralysis virus (SBPV) and a taxonomically unclassified viruses Chronic bee paralysis virus (CBPV) and Lake Sinai viruses (LSVs) (6).

Deformed wing virus (DWV), transmitted by the ectoparasite mite *Varroa destructor*, causes wing deformities in honeybees and is considered one of the main viruses associated with the colony collapse, premature death and reduced performance in asymptomatic bees (7, 8). Transmission of DWV by *V.destructor* to pupae can result in clinical symptoms such as pupal death, the emergence of adult bees with deformed wings, bloated and shortened abdomen, and discoloration. However, in the absence of

V.destructor mites, DWV infection appears to cause no visible symptoms or detectable impact on host fitness (7). Although infection with DWV in honey bees is strongly associated with V.destructor mite infestation, it can also exist as a covert (symptomless) infection in colonies where the mite is absent (9). DWV is a picorna-like virus classified in Iflaviridae family. Its genome encodes a poly protein that is subsequently cleaved by viral and/or cellular enzyme into structural and non-structural proteins required for the viral replication cycle (10). Mature Virions of Iflaviridae family, are spherical without envelope and measuring 22-30 nm in diameter. They contain three major structural proteins (VP1, VP2 and VP3) and a smaller capsid protein VP4 which found in some species (11).

Three main variants of DWV have been described: DWV-A, DWV-B and DWV-C, with DWV-A and DWV-B being the most prevalent globally in honey bee colonies (12). However, more recently a newly identified virus named Egypt bee virus (EBV) has been proposed as a potential fourth variant of DWV. EBV appears to be more closely related to DWV-C than to either DWV-A and DWV-B (13). The DWV-A variant is associated with colony loss. However, the effects of DWV-B on colony health remain a subject of debate. While DWV-B has been detected in healthy colonies, it has also been suggested that this variant may offer protective benefits to colonies through a mechanism known as superinfection exclusion (14).

According to FAOSTAT 2023 report, Iran ranked third among the top ten natural honey-producing countries, with an annual production of approximately 100000 tons of honey (15). It has been reported that in Iran, pesticides and diseases, particularly *Varroa* mite infestations, are among the primary causes of colony losses and reduction in honey production. Consequently, viruses transmitted by this mite are believed to contribute significantly to these losses. However, limited information is available regarding viral infections and their relationship with Varroa mites and colony losses in Iran (16). DWV was first reported in Iran by Ghorani et al in 2017 and since then, several studies have confirmed the presence and circulation of this virus among bee colonies and mites in the country (16-20).

Since Iran ranks among the top ten honey-producing countries, monitoring the presence and circulation of viruses and mites in bee colonies is of great importance, as these factors can significantly affect colony health and, consequently performance. In this study, we investigated the simultaneous presence of Deformed Wing Virus (DWV) and Varroa mites in honey bee colonies located in Alborz Province, northern Iran.

2. Material and methods

2.1 Sample collection

Samples were collected from honey bee colonies showing signs of wing deformities at two separate farms located in Alborz Province, northern Iran. A total of 24 colonies were sampled from first farm (farm A) and 15 colonies from the second farm (farm B). The sampled colonies showed darkened brood cells, deformed and atrophied wings, and, in some cases, symptoms suggestive of wing paralysis. Samples from each farm were first screened for *Varroa destructor* infestation, then pooled and homogenized. All specimens were

submitted to the Virology Laboratory of the Faculty of Veterinary Medicine, University of Tehran, and stored at –20 °C until further processing.

2.2 RNA extraction and RT-PCR

Samples from each colony were pooled to generate a single representative sample per farm. The pooled samples were then crushed and homogenized using a ceramic mortar with diethylpyrocarbonate (DEPC) treated water. The homogenates were centrifuged in 20,000 × g for 1 minute, and 200 µl of the supernatant was used for total RNA extraction. RNA extraction was performed using Sinapure™ RNA extraction kit according to the manufacturer's instructions. The extracted RNA was eluted into 50 µl of RNAase free water and stored at -20 °C until synthesis of cDNA. For cDNA synthesis, 1µl of random hexamer primer was added to 10µl of total RNA. The mixture was incubated at 70 °C for 5 minutes, followed by immediate cooling at 5°C for 1 minute. In the next step, 9µl of cDNA master mix containing 1µl of MMLV reverse transcriptase enzyme and 4µl of 5X RT-buffer (Yekta Tajhiz Azma, Iran), 1µl of dNTPs (SinaClone, Iran) and 2µl of DEPC-treated water were added resulting in a final reaction volume of 20µl. For the second step, the mixture kept in 25 °C for 5 minutes, after than incubated in 42 °C for 1 hour and then heated to 85 °C for 5 minutes and then cooled to 5 °C for 1 minute.

To detect DWV, a pair of primers targeting the polyprotein coding region were used: DWVF: 5′-CTTACTCTGCCGTCGCCCA-3′ and DWVR: 5′-CCGTTAGGAACTCATTATCG-3′). These primers amplify a 160 bp fragment of DWV genome. The PCR conditions were as follows: initial denaturation at 94 °C for 1 minute; 35 cycles of denaturation at 94 °C for 1 minute, annealing at 56 °C for 1 minute, and extension at 72 °C for 1 minute; followed by a final extension at 72 °C for 10 minutes. To evaluate PCR products, 5 µL of each reaction was loaded onto a 1.5% agarose gel, stained with ethidium bromide, and visualized under ultraviolet (UV) light using a transilluminator.

2.3 Sequencing and phylogenetic analysis

Positive samples were submitted for Sanger sequencing by Codon Genetic Group (Tehran, Iran). The quality of the obtained sequences was evaluated using NCBI BLAST and FinchTV software (version 1.4.0). Phylogenetic analysis was performed using MEGA 7 software with the Maximum Likelihood method based on the General Time Reversible (GTR) model with 1000 bootstrap replicates. For this analysis, the sequences obtained in the present study were compared with previously submitted DWV sequences available in the NCBI database. The sequences of the current isolates were submitted to GenBank and available under accession numbers: PV975901 (isolate UT-SHN07) and PV975902 (isolate UT-SHN62).

3. Results

Phylogenetic analysis of the detected DWV isolates revealed that they belong to Deformed wing virus variant A (DWV-A) (Figure 1). Homology analysis showed that the sequence UT-SHN07 from the present study share approximately 99% nucleotide identity with sequences AY292384 and OR361536 from the

USA OR130297 and NC004830 from Iraq and Italy respectively. Results of homology analysis for other isolate in this study, UT-SHN62, indicated 100% similarity between the sequence of this isolate and sequence AY292384 isolated from USA. The sequence of UT-SHN62 has also showed almost 99% similarity with sequences OR361536 (USA), OR130297 (Iraq), NC004830 (Italy) and, MZ867711 (Sweden) (table 1). Investigation of Varroa infestation demonstrated that, 13 out of 24 colonies (54%) from farm A and 3 out of 15 (20%) from farm B, were infested with mites.

4. Discussion

The global population of honeybees (*Apis mellifera*) is under pressure due to habitat loss, environmental stress, and specially pathogens such as viruses that can cause lethal epidemics (21). Honeybees play a vital role in pollination and contribute billions of dollars in added value to agriculture (22). Although many viral infections can be asymptomatic, they are of great concern as they can harm honeybees at various developmental stages, including egg, larva, pupa, adult, worker, drone, and queen (22). Among these viruses, Deformed Wing Virus (DWV), along with its vector, the Varroa destructor mite, appears to be a major global threat to honeybee populations (21).

The RT-PCR method developed to amplify a specific nucleotide sequence located in virus genome, present in sample is considered as valuable tool for diagnosis of viral disease in honey bees as it is fast, reliable and specific (especially when target-specific primers are used) (17). In addition to molecular detection, Phylogenetic analysis serves as a useful approach for defining the diversity and differences between nucleotide sequences of various virus strains. These differences are affected through different ways including the replication cycles, the accuracy of replication and the phenotypic effects of mutations. The quantity of these variation is evaluated by phylogenetic algorithms in order to define the probable genetic relationships between isolates (23).

Colony losses have been associated with infestation by an exotic mite, *Varroa destructor*, which feeds on honey bee hemolymph and can harbor different honeybee viral pathogens specially deformed wing virus. Severe DWV infection can result in pupal death, wing deformities, shortened abdomen and cuticle discoloration in adult bees, which often die shortly after pupation, causing colony, collapse (21). In colonies that don't show the symptoms of DWV disease, *Varroa destructor* mites are either absent or present in low levels. The severity of DWV infection has been shown to be directly correlated with the prevalence and intensity of *Varroa destructor* infestation within the colony (16).

In Iran different studies have reported the presence and circulation of bee viruses among colonies. In 2017, Ghorani et al examined 89 Iranian honey bee samples showing signs of depopulation, sudden collapse, paralysis or dark coloring. Using RT-PCR method with DWV specific primers targeting the structural protein encoding gene, they found that 16 samples (17.97%) were positive for DWV making it the most prevalent virus detected in this study (17). In 2018, Moharrami et al screened 156 apiaries for DWV infection using RT-PCR method with specific primers targeting a 435 bp fragment of the polyprotein

encoding gene. DWV was detected 34 apiaries (21.8%) with 21.8% of adult bees and 20.51% of pupae testing positive (24). In another study by Moharrami et al in 2018, analyzed 160 adult bee samples from 23 provinces, detecting DWV in 34 samples (21.8%) ranking it as the second most prevalent virus (20). In a more recent study by same group (2023), 45 samples were tested for six major bee viruses with emerging as the most prevalent, present in 37.7% of samples (19). Additionally, Shojaei et al, screened 30 apiaries using RT-PCR with primers targeting partial VP2, VP4 and VP1 and partial RNA helicase gene. Their findings showed DWV infection in 36.6% of apiaries were infected with DWV including 8, 0, and 3 positive samples on capped larvae and workers, and Varroa mites respectively (25). All together, these studies suggest that DWV is one of the most prevalent honey bee viruses, currently circulating among honeybees in Iran.

In addition to Iran, neighboring and nearby countries have also reported the presence of DWV in their honey bee colonies. In a study conducted by ÇAĞIRGAN et al in 2021, a total of 111 samples were collected from seven provinces in Turkey to screen for seven major honey bee viruses. Among the detected viruses, 22 out of 111 samples tested positive for DWV making it the most prevalent virus in this study (19.8%) (26). In another study by Karapinar et al, samples were collected from 26 hives, of which 18 hives (69.23%) were positive for DWV infection (27). Haddad et al, investigated the distribution of DWV in Middle Eastern and North African (MENA) countries. For this propose, 111 adult worker bee samples were collected from 12 countries including Lebanon, Syria, Iraq, Palestine, Jordan, Egypt, Libya, Tunisia, Algeria, Morocco, Yemen and Sudan. According to the results, the highest prevalence was observed in Syria and Lebanon with lower rates reported in Palestine, Jordan and Iraq (28).

In the present study, homology analysis revealed 100% and 99 % sequence similarity of isolates UT-SHN62 and UT-SHN07, respectively, with isolate PA/USA isolate reported from the United States. The genome of this isolate (PA/USA) was studied completely and phylogenetic analysis revealed that it clustered in genotype A of DWV (29).

Besides their vital role in ecosystem, honey bees contribute significantly to the economy through products like honey, beeswax, propolis and royal jelly. Viruses, particularly DWV, pose a serious threat to bee health and colony sustainability. Present study is performed for detection and phylogeny of DWV isolated from 2 different farms in Alborz province in the north of Iran. According to this study, both isolates were clustered in genotype A of deformed wing viruses. Monitoring of common circulating viruses is necessary for finding new ways in order to prevent and control viral bee disease and subsequently prevent colony losses and decrease of production. Constant monitoring can help to improve the quality of production and increasing the exportation potential of honey bee products. Further studies are recommended for genotyping of circulating deformed wing viruses in Iran.

Conflict of Interest

The authors declare no conflict of interest.

Author's contribution

Conceptualization, Methodology and Supervision: A. G.

Sample collection: S. J.

Formal Analysis and Software: Z. Z K, S. S.

Original Draft and Writing: F. J, Z. ZK, S. S, A. B, N. S.

Review and Editing: A. G, S. J.

Ethics committee Approval

Our study did not involve any invasive procedures on animals therefore, an ethics committee approval is unnecessary.

Data Availability

The data that supporting the findings of this study are available upon request from the corresponding author.

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Table 1- Nucleotide sequence variation for deformed wing virus of isolates in present study compared with previous sequences retrieved from NCBI GeneBanck.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	UT-SHN07 (PV975901)																					
2	UT-SHN62 (PV975902)	99.3																				
3	PA/USA (AY292384.1)	99.3	100																			
4	DWV-A-OR-OCT2018-EVR23/USA (OR361536.1)	99.3	98.6	98.6																		
5	DWV-Iraq-202/Iraq (OR130297.1)	98.6	99.3	99.3	98																	
6	Deformed wing virus Reference sequence/Italy (NC_004830.2)	98.6	99.3	99.3	97.9	98.6																
7	Gotland-2/Sweden (MZ867711.1)	98	98.6	98.6	97.2	99.3	98.6															
8	85-DWV/France (KX373899.2)	97.9	97.2	97.2	97.2	96.5	97.2	97.2														
9	Chilensis_A1/Chilie (JQ413340.1)	97.2	96.5	96.5	96.5	95.7	96.4	96.5	97.2													
10	Warwick-2009/United Kingdom (GU109335.1)	96.5	97.2	97.2	95.8	97.9	97.2	98.6	96.5	96.5												
11	Austria 1414/ Austria (KU847397.1)	96.5	97.2	97.2	95.8	96.5	97.2	97.3	95.8	95	95.8											
12	AmE711/USA (KT004425.1)	95.7	95	95	96.5	94.3	94.2	93.5	93.4	94.2	91.8	93.4										
13	Kakugo virus/ japan (AB070959.1)	94.3	95.1	95.1	93.6	94.3	95	95.1	93.6	92.7	93.5	96.5	89.4									
14	Korea1/ South Korea (JX878304.1)	94.1	94.9	94.9	93.4	95.7	94.9	96.4	93.4	94.2	94.9	94.9	91	92.6								
15	Korea2/ South_Korea (JX878305.1)	93.4	94.2	94.2	92.6	95	94.1	95.7	92.6	93.5	94.1	95.7	90.3	93.5	94.9							
16	DWV-B-MI-OCT2018-EVR22/ USA (OR361558.1)	70.7	71.8	71.8	71.9	70.7	73.4	70.7	70.7	66	70.3	72.9	68.9	66.8	65.3	67.3						
17	Leuven-dw1/ Belgium (KX783225.1)	69.8	71	71	71.1	69.8	72.6	69.8	69.8	65.2	69.5	72.1	68.1	68.7	64.5	66.4	97.9					
18	DWV-B-OR-OCT2018-EVR24/ USA (OR361560.1)	69.4	70.6	70.6	70.7	69.4	72.2	69.4	69.4	64.6	69	71.6	67.5	68.2	63.9	65.9	99.3	98.6				
19	DWV_B_Netherlands/ Netherlands (MN538209.1)	69.4	70.6	70.6	70.7	69.4	72.2	69.4	69.4	64.6	69	71.6	67.5	68.2	63.9	65.9	99.3	98.6	100			
20	VDV-1_Ox/ United Kingdom (KC786222.1)	69.4	70.6	70.6	70.7	69.4	72.2	69.4	69.4	64.6	69	71.6	67.5	68.2	63.9	65.9	99.3	98.6	100	100		
21	DWV-B-ND-JUN2018-EVR03/ USA (OR361540.1)	68.2	69.4	69.4	69.5	68.2	71	68.2	68.2	63.3	67.8	70.5	66.3	64.2	62.6	64.6	98.6	96.5	97.9	97.9	97.9	
22	Varroa destructor virus-1 Reference sequence/ Netherland (NC_006494.1)	67.1	68.3	68.3	68.4	69.5	70	69.5	67.1	62.1	69.1	69.4	65.2	63.1	64	66	98	97.2	97.3	97.3	97.3	98

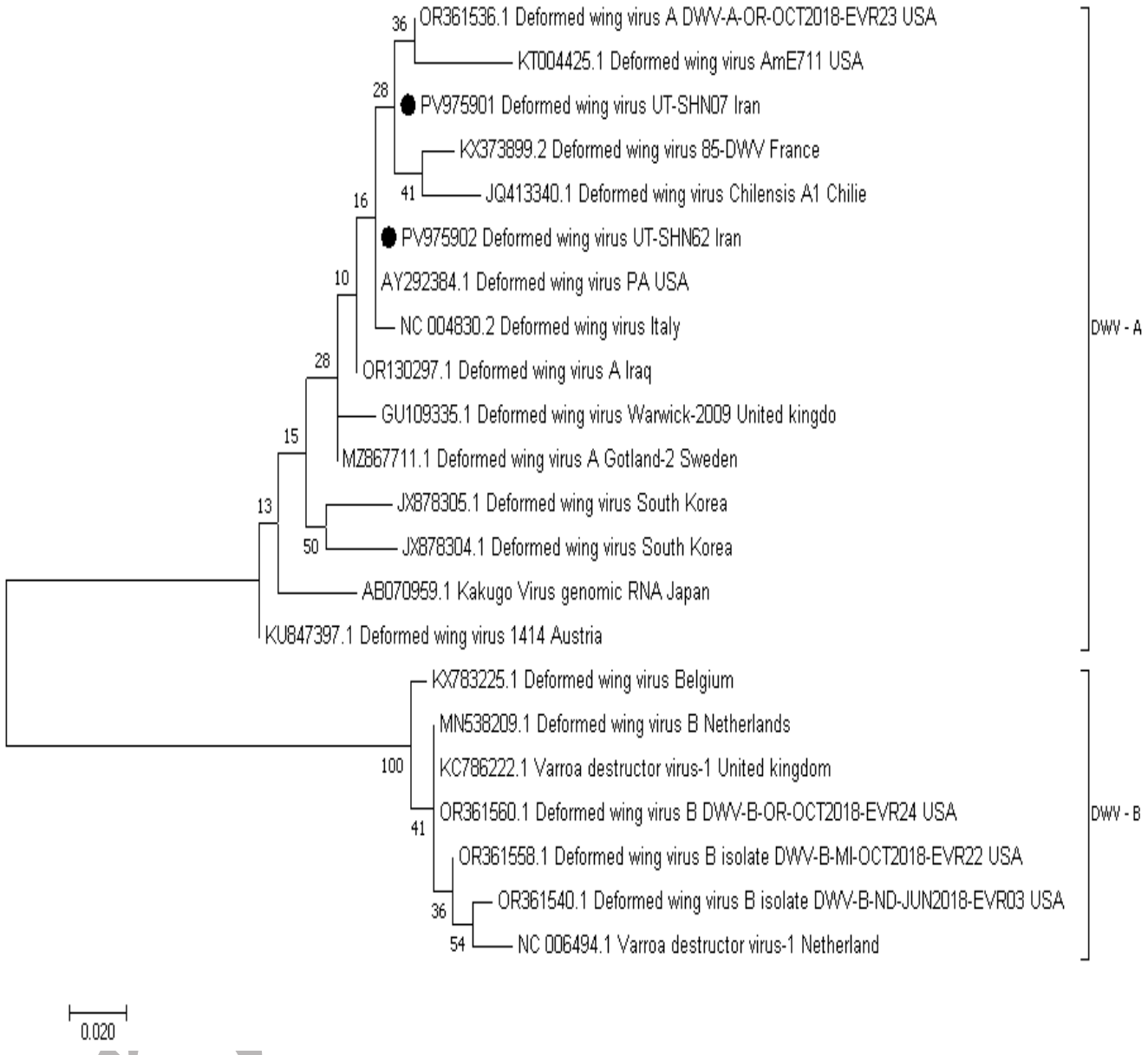

Figure 1- Phylogenetic analysis of deformed wing virus. Phylogenetic analysis was performed with MEGA 7 software, using Maximum Likelihood method based on General Time Reversible model with 1000 bootstrap. Black circles represent for isolates in this study. According to this phylogenetic tree, current isolates are clustered with isolates in genotype A of deformed wing virus.