

1 **Molecular epidemiology of common pathogenic viruses infected *Varroa* mites in Iranian**
2 **apiaries**

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11 **Abstract**

12 **Introduction:** Various agents can infect honey bees, the most valuable pollinator, for example
13 bacteria, fungi, parasites and viruses in apiculture. The *Varroa destructor* (*V. destructor*) mite as
14 one of the most significant pests of honey bees has caused severe losses to population of honey
15 bees in the country in recent years. Considering the role of *Varroa* mites in honeybee's health via
16 transmitting viral diseases to this insect, the aim of this study was to detect common honey bee
17 pathogenic viruses including acute bee paralysis virus (ABPV), chronic bee paralysis virus
18 (CBPV), black queen cell virus (BQCV), deformed wing virus (DWV), sacbrood virus (SBV), and
19 kashmir bee virus (KBV) in *V. destructor* mites obtained from apiaries across Iran. **Materials and**
20 **methods:** Number of 274 apiaries of 31 provinces were selected and honey bee samples were
21 collected, randomly. The collected samples were examined for *V. destructor* contamination, and
22 then *V. destructor* were isolated and counted in each sample. After that, isolated mites from each
23 province were tested for studied viruses, using RNA extraction and RT-PCR. **Results:** The results

24 showed that out of a total of 274 samples, *V. destructor* was detected in 122 apiaries (44.52%)
25 from 26 provinces of the country. From these 26 provinces, 22 (84.61%), 4 (15.38%) and 12
26 (46.15%) samples were positive for DWV, KBV and SBV, respectively, and no infection was
27 observed for ABPV, BQCV and CBPV. **Discussion:** The presence of viral infections in *V.*
28 *destructor* mites indicates the role of this pest in transmitting the infections to honeybees, which
29 should be considered as an important problem in apiculture and so it should be required careful
30 management.

31 **Keywords:** Epidemiology, Honey bee sample, Pathogenic viruses, Phylogenetic study, *Varroa*
32 *destructor*.

33 **1. Introduction**

34 Honey bee (*Apis mellifera*), a vital important factor in the agricultural industry, is
35 pollinator for a wide range of crops [1]. However, over the past few decades, honey bee health has
36 declined globally because of the spread of numerous parasites and pathogens, including bacteria
37 and viruses, a problem facilitated by the international trade in honey bees [2]. Among parasitic
38 agents, *Varroa destructor* (*V. destructor*) is an external parasite of honey bees which effects on
39 the apiculture industry [3].

40 There are six major common pathogenic viruses which are of particular importance,
41 including acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV), black queen cell
42 virus (BQCV), Deformed wing virus (DWV), Sacbrood virus (SBV), and Kashmir virus (KBV)
43 [4]. These honey bee viruses can transmit between bees in a colony both horizontally and
44 vertically. Horizontal transmission occurs through *Varroa* mites, interaction between adult bees,
45 and feeding of pupae by nurse bees, and vertical transmission occurs through drones and queens
46 [5]. In colonies with heavy infested with *Varroa* mites, the detection of high viral loads in sick or

47 dead bees suggests that *Varroa* mite infestation may stimulate the virus to replicate and induce
48 disease from a state of asymptomatic infection to symptomatic infection. *Varroa* mites can activate
49 ABPV and play a role in causing observable inoculation of bees by reducing bee defenses and
50 directly injecting the virus into the hemolymph. Also, virus replication can be caused by other
51 factors. Some studies have shown that ABPV was detected in honeybees in apiaries while ABPV-
52 positive *Varroa* mites were not diagnosed. This proposes that *varroa* mites are not the only factor
53 causative to the spread of ABPV infection [5]. Sacbrood virus infection has been detected in large
54 numbers of adult bees in apiaries infected with *Varroa* mites. It has been reported the high levels
55 of virus in *Varroa* mites, especially in the mouthparts and digestive tract. Also, the positive
56 relationship between the occurrence of SBV in *Varroa* mite samples and SBV in adult bee samples
57 was detected [5].

58 It has been shown that KBV can transmit from KBV-infected mites to healthy pupae and
59 also mites in colonies without any KBV infection [6]. *Varroa* mites are responsible for the spread
60 of DWV to all tissues of the honey bee by feeding on the hemolymph and reactivating viral
61 infections. When DWV is transmitted by *Varroa* mites, this virus can have devastating effects on
62 honey bees by causing morphological deformities and even death [7].

63 Considering the role of *Varroa* mites in transmitting viral diseases to honey bees, it seems
64 it is necessary to investigate the prevalence of viral infections in *Varroa* mites. In the present study,
65 RT-PCR method was used to identify common honey bee pathogenic viruses in *Varroa* mites.

66 **2. Materials and methods**

67 **2.1. Data collection and sample preparation**

68 Sampling was accomplished randomly based on the number of apiaries in Iran by
69 province and prevalence rate of 40% (reported by Iran Veterinary Organization). Using Cochran's
70 formula, population size was estimated [8]:

$$71 N = z^2[pq]/d^2$$

72 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
73 was equal 1.96 with a confidence factor of 95%. Thus, $N \approx 260$. So, the number of 274 apiaries
74 from 31 provinces of Iran were considered, whereas for each apiary, a number of hives were
75 randomly selected regardless of the clinical symptoms of the disease. Then, a number of bees were
76 collected from each comb in the sterile containers (overall more than 50 adult bees for each apiary).
77 All collected adult bee samples from each apiary were pooled and were sent to the lab using a cold
78 chain for further analysis.

79 To count and separate mites in each sample, 200–250 bees were immersed in ethanol and
80 shaken for 10 minutes. Then, using a mesh (2–3 mm pores), the bees and mites were separated,
81 counted, and the percentage determined. For each province, all the isolated mites were mixed
82 together and crushed well in a sterile porcelain mortar. After that, 200 μ L of DEPC water was
83 added and homogenized. Afterward, the homogenized solution was transferred into a sterilized 1.5
84 mL tube and centrifuged at 20,000 g for 1 min. The supernatant was collected for RNA extraction
85 and stored at -20 °C until use [9].

86 **2.2. RNA extraction**

87 RNA extraction for *Varroa* mite samples was performed using QIA amp -Viral RNA
88 Mini kit and cDNA synthesis was performed using kit (Biotech, Germany) according to the
89 manufacturer's instructions in a 0.2 ml tube and a final volume of 20 μ l. Primers introduced by
90 Berenyi et al. [10] were used. PCR reaction was performed using (1.5 mM mgcl₂) Taq 2× Master

91 mix (Ampliqon, Denmark). The molecular weight of the PCR product was examined on a 1.2%
92 agarose gel containing safe stain DNA.

93 **2.3. Purification and DNA sequencing**

94 PCR products were purified using High Pure PCR Product Purification Kit [Roche,
95 Germany] according to the manufacture's instruction. For sequencing, two replicates of purified
96 PCR products of DWV isolated from *Varroa* mite samples were selected and sequenced by
97 Microsynth (Switzerland).

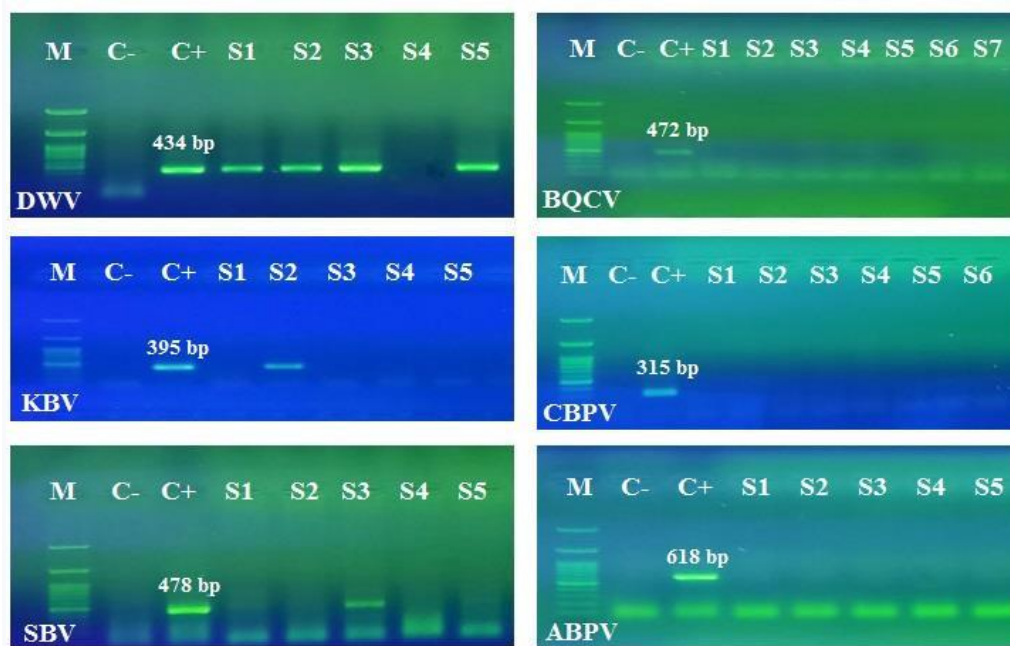
98 **2.4. Statistical analyses and sequence analysis**

99 To estimate the number of apiaries for sampling, population size was calculated using
100 Cochran's formula [8]. Then, results of molecular process were evaluated by descriptive statistics.
101 Phylogenetic tree of sequences of DWV isolated from *Varroa* mite samples were assembled by
102 MEGAX 4.0 program using the neighbor-joining method [11] and bootstrap support was estimated
103 based on 1000 replicates.

104 To ensure the results, given the high prevalence of DWV among mite samples, 2 samples
105 of PCR products from DWV positive cases that had strong bands were selected and sent to
106 Microscience (Switzerland) for sequencing. Sequencing was performed using the forward primer
107 of each sample. The obtained sequences were blasted to determine the similarity with other
108 sequences available in the NCBI database and the homology was examined. Sequence comparison
109 and information on genetic parameters such as the number of mutations, nucleotide diversity, and
110 the number of sites where similar substitutions occurred, as well as construction of a phylogenetic
111 tree, were conducted using CLC Sequence Viewer software (v. 6.8.2.)

112 **3. Results**

113 Figure 1 shows the results of PCR products of DWV, KBV and SBV infection which
 114 were isolated from *Varroa* mites in different provinces. As presented, out of 274 samples in all
 115 provinces, 122 (44.52%) apiaries were positive for *Varroa* mite which belonged to 26 provinces
 116 of the country. In these 26 provinces, viral infection was observed in 24 provinces which 22
 117 (84.61%), 4 (15.38%) and 12 (46.15) provinces were positive for DWV, KBV and SBV,
 118 respectively, and no infection was observed for ABPV, BQCV and CBPV. In four provinces, viral
 119 infection for DWV, KBV and SBV was observed, simultaneously. In six provinces, DWV and
 120 SBV was observed, simultaneously. Table 1 shows the number of *varroa* mite positive samples
 121 and positive studied viruses in infected *varroa* mite samples in each province.



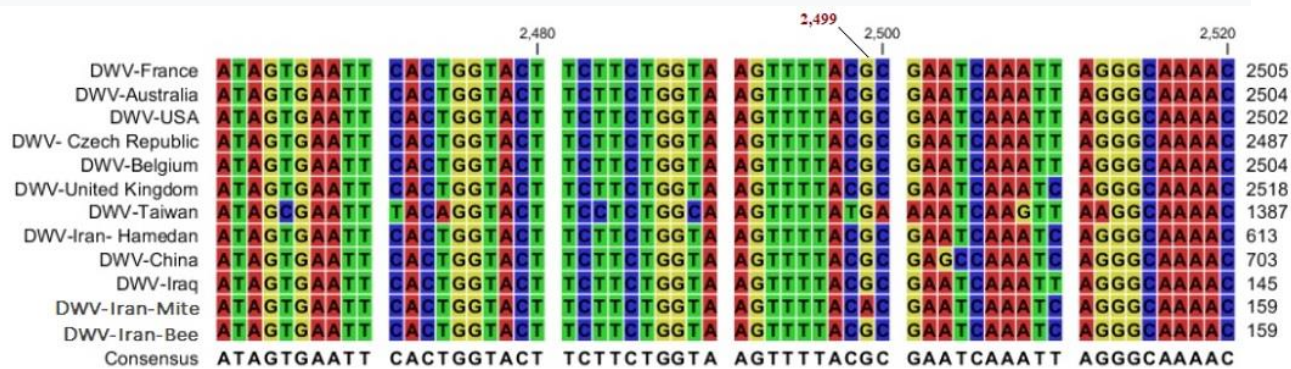
122
 123 **Figure 1** Results of PCR products examples on 1.2 % agarose gel electrophoresis for the studied
 124 viruses isolated from *Varroa* mite. M: Ladder; C-: Negative control; C+: Positive control; S1-S7:
 125 Samples.

126 **Table 1.** The number of *varroa* mite positive samples and positive studied viruses in infected *varro*
 127 mite samples in each province.

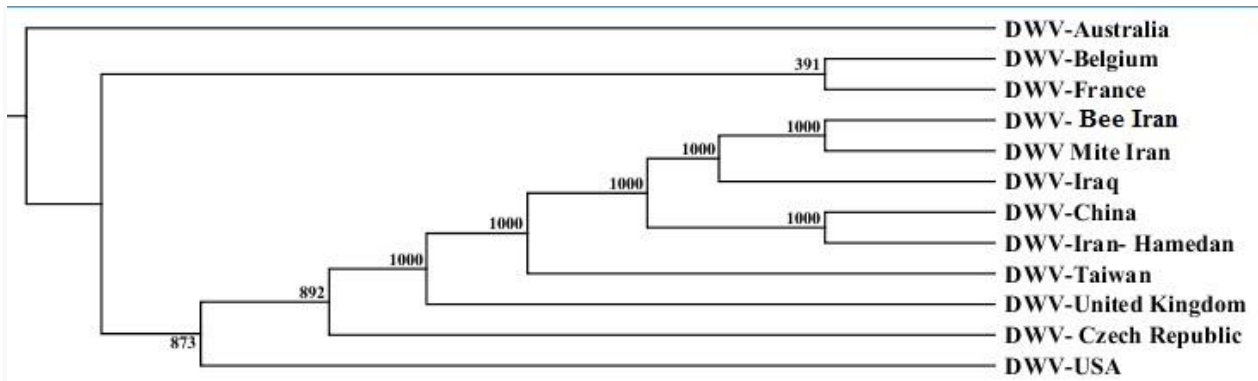
Province	Apiary	<i>Varroa</i> positive	ABPV	BQCV	CBPV	DWV	KBV	SBV
Alborz	6	4	-	-	-	+	+	+
Ardebil	4	2	-	-	-	+	-	-
Bushehr	3	0	-	-	-	-	-	-
Tehran	12	6	-	-	-	+	-	+
Chaharmahal & Bakhtiari	7	3	-	-	-	+	-	-
East Azarbaijan	15	7	-	-	-	+	-	-
Esfahan	9	4	-	-	-	-	-	+
Fars	21	6	-	-	-	+	-	+
Ghazvin	5	3	-	-	-	+	+	+
Ghom	2	2	-	-	-	+	-	+
Gilan	14	5	-	-	-	+	-	-
Golestan	19	11	-	-	-	+	+	+
Hamedan	10	3	-	-	-	+	-	-
Hormozgan	1	0	-	-	-	+	-	-
Ilam	6	0	-	-	-	+	-	-

Kerman	7	3	-	-	-	-	-	-
Kermanshah	13	5	-	-	-	+	-	-
Khuzestan	5	1	-	-	-	-	-	-
Kohkilouyeh & Buyer Ahmad	8	4	-	-	-	+	+	+
Kordestan	8	3	-	-	-	+	-	+
Lorestan	10	5	-	-	-	+	-	-
Markazi	5	5	-	-	-	+	-	-
Mazandaran	22	13	-	-	-	+	-	+
North Khorasan	7	4	-	-	-	-	-	-
Razavi Khorasan	18	8	-	-	-	+	-	+
Semnan	2	2	-	-	-	+	-	-
Sistan&Baluchestan	7	4	-	-	-	+	-	-
South Khorasan	3	0	-	-	-	+	-	-
West Azarbayijan	17	9	-	-	-	+	-	+
Yazd	2	2	-	-	-	+	-	-
Zanjan	6	2	-	-	-	+	-	-
Total	274	122	0	0	0	22	4	12

129 As mentioned earlier, we only sequenced DWV isolated from *V. destructor* mites
 130 isolated from bee samples. The identified nucleotide differences between strains and phylogenetic
 131 tree for this virus are shown in Figures 2 and 3. The phylogenetic tree shows three clades. DWV
 132 isolate obtained from the *V. destructor* mite in our study had a minimal genetic distance with the
 133 isolate obtained from the bee sample reported by Torkaman et al. [12] in the same study, as well
 134 as to the Iranian isolate registered in the NCBI database (Hamedan-MW566438) with an isolate
 135 from China and Iraq, indicating the existence of a common ancestor among these strains. The
 136 isolates from Belgium and France are in the same clade, and the sequence from Australia is an out-
 137 group. Phylogenetic analysis of DWV in *Varroa* mites showed a substitution mutation (G → A)
 138 at position 2499 in comparison with bee samples in the same study [12].



139
 140 **Figure 2** Phylogenetic analysis of DWV sequences isolated in present study and other reports of
 141 DWV sequences. Detected nucleotide difference at position 2499 has been shown.
 142



143

144 **Figure 3** Phylogenetic tree and evolutionary relationship of nucleotide sequence of studied DWV
 145 isolated from *Varroa* mite and other reports of DWV sequences. High bootstrap rates at the
 146 branches can be seen.

147 **4. Discussion**

148 Recent findings support the key role of *Varroa* mites in the spread of infected viruses of
 149 honeybees. Honey bee viral infestation occurs consistently in honey bee populations in spite of the
 150 absence of clinical signs suggesting the prevalence of the colony diseases may be caused by
 151 environmental factors which cause the activation of virus replication in honey bees [5]. The *V.*
 152 *destructor* mite is broadly recognized as the most destructive risk to the colonies of honey bee on
 153 a global scale. *Varroa* mite infestation in honey bee colonies is linked to viral infections, and then,
 154 leads to a decline in bee populations and accelerated colony losses [13]. Research has demonstrated
 155 the association between *Varroa* mites and viruses, emphasizing the efficiency of *Varroa* mites in
 156 transmitting viruses among honey bees and colonies. Therefore, *Varroa* mites control can reduce
 157 viral infections in honey bee colonies [13]. This pest is an external parasite of honey bees, which
 158 can be important in the transmission of viral diseases. Given the vital role of *V. destructor* in the
 159 transmission of honey bee viruses, it is important to identify the viruses that replicate within the
 160 mites [14].

161 When a honey bee colony is infected by *Varroa* mites, the risk of colony collapse
162 increases due to the weakening caused by the *Varroa* mites' and continuous feeding on the
163 hemolymph of developing larvae, pupae and adult bees. In fact, it has been shown that *Varroa* can
164 be an active carrier of about 19 honey bee viruses. Therefore, this parasite can increase viral titers
165 and, by overcoming the mechanical and immune defenses of the honey bee, by reducing nutrients
166 and blood cells in the hemolymph, can effectively transmit viruses by direct injection into the
167 hemolymph [3]. Regarding the growth of the *Varroa* mite population which is influenced by
168 condition of the colony, the number of mites can increase between 12 and 800 times. Hence,
169 *Varroa* mite can be a huge problem in apiculture industry [3].

170 Our results showed the prevalence of *V. destructor* mites in Iranian apiaries which was
171 about 44.52%. Of the collected samples from all provinces, *V. destructor* mites were found in 26
172 provinces, whereas viral infection was found in isolated mites only in 24 provinces. Of these,
173 84.61%, 15.38% and 46.15% were positive for DWV, KBV, and SBV, respectively, and no
174 infection was found for ABPV, BQCV, and CBPV. Torkaman et al. [12] also examined the
175 prevalence of viral infection in honey bee samples collected as the same study in terms of infection
176 with the six mentioned viruses. Their results showed that out of 274 honey bee samples, 7.66%
177 ABPV, 7.66% BQCV, 4.01% CBPV, 90.15% DWV, 11.13% KBV and 33.21% SBV samples
178 were positive. While, DWV, SBV and KBV viruses showed a higher prevalence than other viruses
179 [12]. They also showed ABPV, BQCV, and CBPV were less prevalent than other studied viruses,
180 which further highlights the role of *Varroa* mites in the transmission of viral diseases. The results
181 of our study were consistent with those of Torkaman et al. [12], where both showing the highest
182 infection rates for DWV and SBV. *Varroa* mites from 22 out of 26 provinces (84.61%) were
183 infected with DWV, which, given the high infection rate of the studied apiaries with this virus

184 (90.15%) [12]. It has been reported that the most common and virulent viruses affecting the health
185 of *Apis mellifera* in the world are DWV and BQCV [15]. Findings of present study also signifies
186 DWV has a high prevalence in the *V. destructor* mite population as well as adult bees. Although
187 viruses can be detected in mites, yet viral infection is also observed in mite-free colonies [16].
188 Given that free *Varroa* mites colonies also showed infection with some viruses, other transmission
189 routes should be noted such as contaminated food (pollen, honey, etc.) and queens and male honey
190 bees [12].

191 In previous study Torkaman et al. [12] showed that the most infestation was related to
192 DWV and SBV, respectively, which is consistent with the high prevalence of infection of the *V.*
193 *destructor* studied with these viruses. Shen et al. [17] showed that under natural and artificial
194 infestations with *Varroa* mites, honey bee pupae contained significantly higher levels of KBV and
195 DWV and also KBV structural proteins than pupae free from *Varroa* mites. Linear regression
196 analysis showed a positive correlation between the number of *Varroa* mites introduced and the
197 levels of viral RNAs. Their research suggests that infection by *Varroa* mites suppresses the
198 immunity of honey bees and leads to the activation of a persistent and latent viral infection [17].

199 In Brazil, by examining six pathogenic viruses) DWV-BQCV-SBV-IAPV-ABPV-
200 CBPV) , Chagas et al. [18] showed that cases of simultaneous infection with ABPV, BQCV, and
201 DWV in both states. While, in all beekeeping with positive virus samples, the mite *V. destructor*
202 was also reported, indicating the transmission of these viruses through *Varroa* mites [18].
203 Tentcheva et al. [5], examined six pathogenic viruses DWV-BQCV-SBV-KBV-ABPV-CBPV in
204 adult bees, pupae and *V. destructor* samples from apparently healthy honey bee colonies in France
205 using PCR technique. In their studied *Varroa* samples, four viruses were identified; DWV (100%),
206 SBV (45%), ABPV (36%) and KBV (5%) of apiaries [5].

207 In Hungary, Bakonyi et al. [19] detected ABPV in *Varroa* mites in apiaries suffering
208 from colony collapse disorder (CCD), while more than 87% of the collapsed apiaries showed
209 ABPV infection in bee samples. In our study, ABPV infection was not observed in mite samples
210 and infection of the total apiary rate with this virus was less than 8%, indicating *Varroa* mite
211 infection along with viral diseases plays an effective role in viral infection of honey bee colonies
212 [19].

213 Studies by Chen et al. [6] showed that KBV can be transmitted from KBV-infected mites
214 to healthy pupae and mites in a KBV-free colony [6]. Shen et al. [17] identified KBV and SBV in
215 honey bee samples and also, for the first time, in *V. destructor* mites. The simultaneous
216 identification of viruses in *V. destructor* and honey bees emphasizes the importance of mites in the
217 transmission of various honey bee viruses [17]. Furthermore, it was shown that DWV is not able
218 to replicate in all mites. Interestingly, viral replication in mites is associated with DWV infection
219 in honeybees, which suggests that symptoms are more likely to occur if the virus is transmitted by
220 mites [20]. In another study conducted by Minoos et al. [21] rates of DWV and ABPV detection in
221 *Varroa* mites were 100% and 77%, respectively, while, in *Tropilaelaps* mites, they detected 100%
222 DWV, 57% ABPV, 30% CBPV and 23% VDV-1 [21]. Arzumanyan et al. [22], reported infection
223 of DWV in all *Varroa* mites [22]. The presence of DWV, SBV, KBV, IAPV, BQCV, and CBPV
224 in *Varroa* mites collected from Korean apiaries has been reported by Moon et al. (2024) [23]. They
225 showed the highest prevalence of viral infection belonged to DWV (87%), followed by IAPV
226 (65.2%), SBV (17.4%), and less than 10% belonged to other viruses. In the present study, the
227 prevalence of DWV was higher than SBV and KBV in *Varroa* mite samples, which was consistent
228 with the above mentioned study. These results indicate that the *V. destructor* plays an effective
229 role in the propagation and transmission of viral diseases in honey bees. This means that one of

230 the reasons for the low prevalence of the pathogenic viruses including ABPV, BQCV and CBPV
231 in honey bee samples [12] can be due to the non-infectiousness of the *Varroa* mite samples. Of
232 course, it has been showed that there is no relationship between the spread of CBPV and the *V.*
233 *destructor* mite [24].

234 Phylogenetic tree of the studied DWV showed the low genetic distance between the
235 sequence obtained from the *V. destructor* and honeybee samples, which indicates the importance
236 of the *Varroa* mite in the transmission of this virus among honeybees. The high viral replication
237 in mites increases the probability of the mutation. Assuming that only some DWV mutations are
238 capable of causing wing paralysis in developing honeybees, the possibility of such a mutation is
239 possible with a high transcription rate in *Varroa* mites [7]. Our results showed a substitution
240 mutation (G → A) at position 2499 in phylogenetic analysis of DWV in *Varroa* mites.

241 In the last decade, KBV has been recognized as the most important virus among several
242 viruses related with CCD in apiaries infected with the *V. destructor* mite [10]. Reports show that
243 in the beekeeping industry, control of *Varroa* mites and viruses is a vital factor in preserving the
244 health of honey bees. So, beekeepers should take preventive methods to prevent the spread of bee
245 diseases. Given the lack of treatment for viral diseases, management strategies may be the only
246 way to control viral diseases [5]. Also, given the importance of the *V. destructor* mite in
247 transmitting viral diseases, more attention should be paid to controlling this pest. Consequently,
248 regular inspection of colonies by trained personal is essential to control or reduce viral infections.
249 Recent research has suggested the use of thermal interventions, particularly hyperthermia, as a
250 suitable method for controlling *Varroa* mites. These studies have also explained the effect of heat
251 stress in decreasing viral infections among infected bees. Hyperthermia treatment may be effective

252 in treating viral infections by decreasing *Varroa* mite infestation or by inducing the production of
253 heat shock proteins, which have potential antiviral properties [25].

254 **5. Conclusion**

255 Our results showed the importance of the *V. destructor* mite in transmitting viral diseases
256 in honey bee. The findings propose serious control of *V. destructor*, which is an active vector of
257 honey bee viruses (especially DWV). should be placed on the program of regulatory organizations.
258 These measures should be incorporated into the regulatory and apiculture management programs.

259 **Acknowledgments**

260 The authors would like to show their gratitude to Dr. Hossein Modirrousta for his help.
261 Also, authors thank Iran veterinary organization for helping samples collection. This work was
262 supported by Razi vaccine and serum research institute [grant number: 01-18-18-019-01019].

263 **Authors' contributions**

264 Study concept and design: M.T, M.M, H.P, G.N.B, and M.B.

265 Acquisition of data: M.T, M.M and M.B.

266 Analysis and interpretation of data: M.T and M.B.

267 Drafting of the manuscript: M.T and M.B.

268 Critical revision of the manuscript for important intellectual content: M.T, M.M, H.P, G.N.B, and
269 M.B.

270 Statistical analysis: M.T and M.B.

271 Administrative, technical, and material support: M.T, M.M, H.P, G.N.B, and M.B.

272 **Ethics approval**

273 Not applicable.

274 **Conflict of Interest**

275 The authors declare that there is no conflict of interest.

276 **Data Availability**

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

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Preprint