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Research Paper

Molecular Epidemiology of Common Pathogenic Viruses Infected *Varroa* Mites in Iranian Apiaries

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ABSTRACT

Introduction: Various agents can infect honey bees, the most valuable pollinator, for example bacteria, fungi, parasites and viruses in apiculture. The *Varroa destructor* mite as one of the most significant pests of honey bees has caused severe losses to population of honey bees in the country in recent years. Considering the role of *Varroa* mites in honeybee's health via transmitting viral diseases to this insect, the aim of this study was to detect common honey bee pathogenic viruses including acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV), black queen cell virus (BQCV), deformed wing virus (DWV), Sacbrood virus (SBV), and Kashmir bee virus (KBV) in *V. destructor* mites obtained from apiaries across Iran.

Materials & Methods: Number of 274 apiaries of 31 provinces were selected and honey bee samples were collected, randomly. The collected samples were examined for *V. destructor* contamination, and then *V. destructor* were isolated and counted in each sample. After that, isolated mites from each province were tested for studied viruses, using RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR).

Results: The results showed that out of a total of 274 samples, *V. destructor* was detected in 122 apiaries (44.52%) from 26 provinces of the country. From these 26 provinces, 22(84.61%), 4(15.38%) and 12(46.15%) samples were positive for DWV, KBV and SBV, respectively, and no infection was observed for ABPV, BQCV and CBPV.

Conclusion: The presence of viral infections in *V. destructor* mites indicates the role of this pest in transmitting the infections to honeybees, which should be considered as an important problem in apiculture and so it should be required careful management.

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1. Introduction

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

There are six major common pathogenic viruses which are of particular importance, including acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV), black queen cell virus (BQCV), deformed wing virus (DWV), Sacbrood virus (SBV), and Kashmir virus (KBV) [4]. These honey bee viruses can transmit between bees in a colony both horizontally and vertically. Horizontal transmission occurs through *Varroa* mites, interaction between adult bees, and feeding of pupae by nurse bees, and vertical transmission occurs through drones and queens [5]. In colonies with heavy infested with *Varroa* mites, the detection of high viral loads in sick or dead bees suggests that *Varroa* mite infestation may stimulate the virus to replicate and induce disease from a state of asymptomatic infection to symptomatic infection. *Varroa* mites can activate ABPV and play a role in causing observable inoculation of bees by reducing bee defenses and directly injecting the virus into the hemolymph. Also, virus replication can be caused by other factors. Some studies have shown that ABPV was detected in honeybees in apiaries while ABPV-positive *Varroa* mites were not diagnosed. This proposes that *varroa* mites are not the only factor causative to the spread of ABPV infection [5]. SBV infection has been detected in large numbers of adult bees in apiaries infected with *Varroa* mites. It has been reported the high levels of virus in *Varroa* mites, especially in the mouthparts and digestive tract. Also, the positive relationship between the occurrence of SBV in *Varroa* mite samples and SBV in adult bee samples was detected [5].

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

Considering the role of *Varroa* mites in transmitting viral diseases to honey bees, it seems it is necessary to investigate the prevalence of viral infections in *Varroa* mites. In the present study, reverse transcription-polymerase chain reaction (RT-PCR) method was used to identify common honey bee pathogenic viruses in *Varroa* mites.

2. Materials and Methods

2.1. Data collection and sample preparation

Sampling was accomplished randomly based on the number of apiaries in Iran by province and prevalence rate of 40% (reported by Iran Veterinary Organization). Using Cochran's formula (Equation 1), population size was estimated [8]:

1. $N = z^2[pq]/d^2$

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%. Thus, N≈260. So, the number of 274 apiaries from 31 provinces of Iran were considered, whereas for each apiary, a number of hives were randomly selected regardless of the clinical symptoms of the disease. Then, a number of bees were collected from each comb in the sterile containers (overall more than 50 adult bees for each apiary). All collected adult bee samples from each apiary were pooled and were sent to the lab using a cold chain for further analysis.

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

2.2. RNA extraction

RNA extraction for *Varroa* mite samples was performed using QIA amp -Viral RNA Mini kit and cDNA synthesis was performed using kit (Biotech, Germany) according to the manufacturer's instructions in a 0.2 mL-tube and a final volume of 20 µL. Primers introduced by Berényi et al. [10] were used. PCR reaction was per-

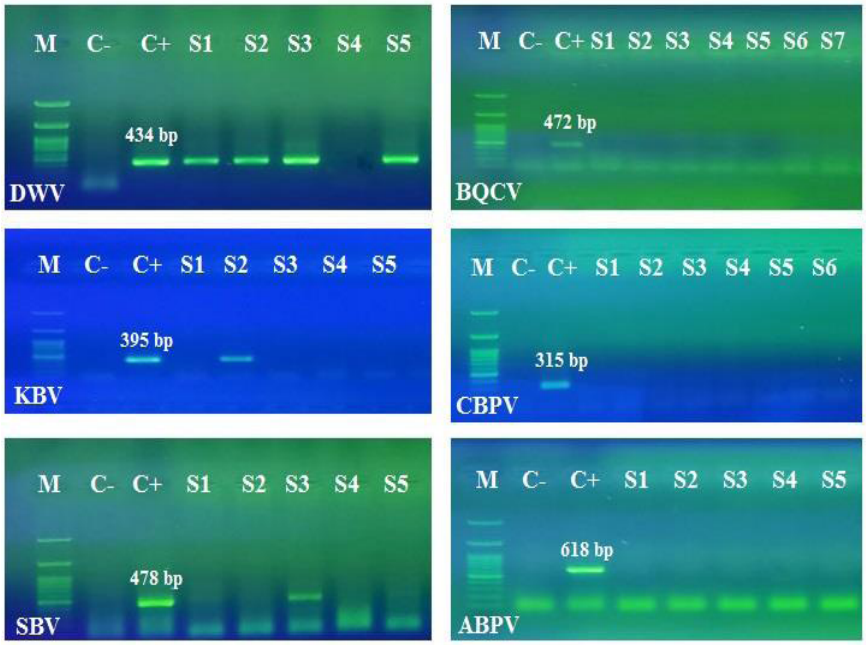

Figure 1. Results of PCR products examples on 1.2 % agarose gel electrophoresis for the studied viruses isolated from *Varroa* mite

Note: Lane M: Ladder; Lane C-: Negative control; Lane C+: Positive control; S1-S7: Samples.

formed using (1.5 mM $mgcl_2$) Taq 2× Master mix (Ampliqon, Denmark). The molecular weight of the PCR product was examined on a 1.2% agarose gel containing safe stain DNA.

2.3. Purification and DNA sequencing

PCR products were purified using High Pure PCR Product Purification Kit [Roche, Germany] according to the manufacture´s instruction [11]. For sequencing, two replicates of purified PCR products of DWV isolated from *Varroa* mite samples were selected and sequenced by Microsynth (Switzerland).

2.4. Statistical analyses and sequence analysis

To estimate the number of apiaries for sampling, population size was calculated using Cochran's formula [8]. Then, results of molecular process were evaluated by descriptive statistics. Phylogenetic tree of sequences of DWV isolated from *Varroa* mite samples were assembled by MEGAX 4.0 program using the neighbor-joining method [12] and bootstrap support was estimated based on 1000 replicates.

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

3. Results

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

Table 1. The number of *Varroa* mite positive samples and positive studied viruses in infected *Varro* mite samples in each province

Province	Apiary	Varroa Positive	Virus					
			ABPV	BQCV	CBPV	DWV	KBV	SBV
Alborz	6	4	-	-	-	+	+	+
Ardebil	4	2	-	-	-	+	-	-
Bushehr	3	0	-	-	-	-	-	-
Tehran	12	6	-	-	-	+	-	+
Chaharmahal & Bakhtiari	7	3	-	-	-	+	-	-
East Azarbayijan	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

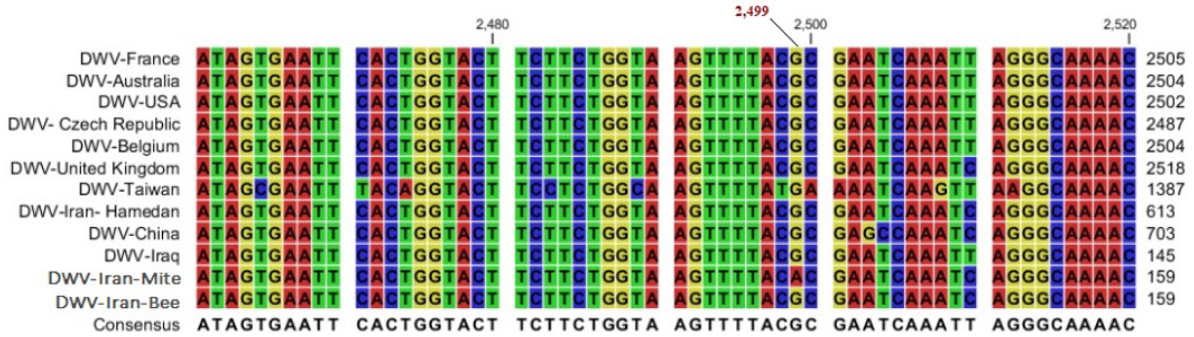

Figure 2. Phylogenetic analysis of DWV sequences isolated in present study and other reports of DWV sequences

Note: Detected nucleotide difference at position 2499 has been shown.

As mentioned earlier, we only sequenced DWV isolated from *V. destructor* mites isolated from bee samples. The identified nucleotide differences between strains and phylogenetic tree for this virus are shown in Figures 2 and 3. The phylogenetic tree shows three clades. DWV isolate obtained from the *V. destructor* mite in our study had a minimal genetic distance with the isolate obtained from the bee sample reported by Torkaman et al. [13] in the same study, as well as to the Iranian isolate registered in the NCBI database (Hamedan-MW566438) with an isolate from China and Iraq, indicating the existence of a common ancestor among these strains. The isolates from Belgium and France are in the same clade, and the sequence from Australia is an out-group. Phylogenetic analysis of DWV in *Varroa* mites showed a substitution mutation (G → A) at position 2499 in comparison with bee samples in the same study [13].

4. Discussion

Recent findings support the key role of *Varroa* mites in the spread of infected viruses of honeybees. Honey bee viral infestation occurs consistently in honey bee populations in spite of the absence of clinical signs suggesting the prevalence of the colony diseases may be caused by environmental factors which cause the activation of virus replication in honey bees [5]. The *V. destructor* mite is broadly recognized as the most destructive risk to the colonies of honey bee on a global scale. *Varroa* mite infestation in honey bee colonies is linked to viral infections, and then, leads to a decline in bee populations and accelerated colony losses [14]. Research has demonstrated the association between *Varroa* mites and viruses, emphasizing the efficiency of *Varroa* mites in transmitting viruses among honey bees and colonies. Therefore, *Varroa* mites control can reduce viral infections in honey bee colonies [14]. This pest is an external parasite of honey bees, which can be important in the transmission of viral diseases. Given the vital role of *V. destructor* in the transmission of honey bee viruses, it is important to identify the viruses that replicate within the mites [15].

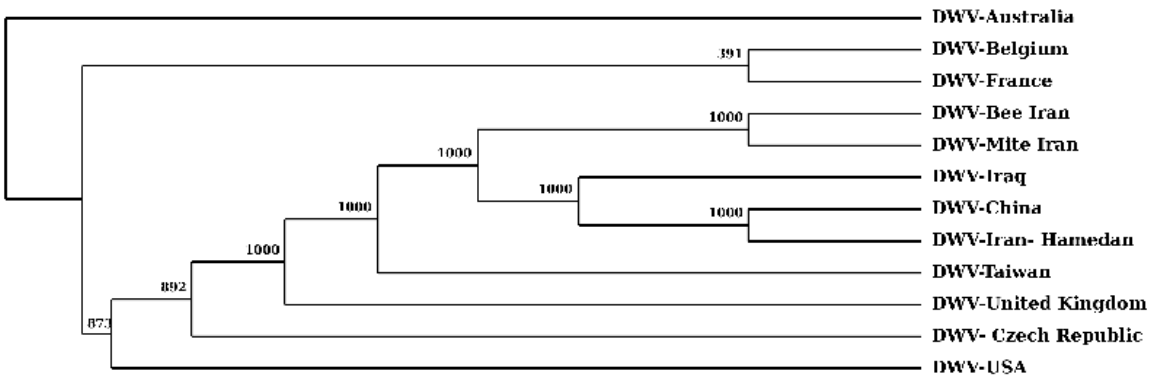

Figure 3. Phylogenetic tree and evolutionary relationship of nucleotide sequence of studied DWV isolated from *Varroa* mite and other reports of DWV sequences

Note: High bootstrap rates at the branches can be seen.

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

Our results showed the prevalence of *V. destructor* mites in Iranian apiaries which was about 44.52%. Of the collected samples from all provinces, *V. destructor* mites were found in 26 provinces, whereas viral infection was found in isolated mites only in 24 provinces. Of these, 84.61%, 15.38% and 46.15% were positive for DWV, KBV, and SBV, respectively, and no infection was found for ABPV, BQCV, and CBPV. Torkaman et al. [13] also examined the prevalence of viral infection in honey bee samples collected as the same study in terms of infection with the six mentioned viruses. Their results showed that out of 274 honey bee samples, 7.66% ABPV, 7.66% BQCV, 4.01% CBPV, 90.15% DWV, 11.13% KBV and 33.21% SBV samples were positive. While, DWV, SBV and KBV viruses showed a higher prevalence than other viruses [13]. They also showed ABPV, BQCV, and CBPV were less prevalent than other studied viruses, which further highlights the role of *Varroa* mites in the transmission of viral diseases. The results of our study were consistent with those of Torkaman et al. [13], where both showing the highest infection rates for DWV and SBV. *Varroa* mites from 22 out of 26 provinces (84.61%) were infected with DWV, which, given the high infection rate of the studied apiaries with this virus (90.15%) [13]. It has been reported that the most common and virulent viruses affecting the health of *A. mellifera* in the world are DWV and BQCV [16]. Findings of present study also signifies DWV has a high prevalence in the *V. destructor* mite population as well as adult bees. Although viruses can be detected in mites, yet viral infection is also observed in mite-free colonies [17]. Given that free *Varroa* mites colonies also showed infection with some viruses, other transmission routes should be noted such as contaminated food (pollen, honey, etc.) and queens and male honey bees [13].

In previous study Torkaman et al. [13] showed that the most infestation was related to DWV and SBV, respectively, which is consistent with the high prevalence of infection of the *V. destructor* studied with these viruses. Shen et al. [18] showed that under natural and artificial infestations with *Varroa* mites, honey bee pupae contained significantly higher levels of KBV and DWV and also KBV structural proteins than pupae free from *Varroa* mites. Linear regression analysis showed a positive correlation between the number of *Varroa* mites introduced and the levels of viral RNAs. Their research suggests that infection by *Varroa* mites suppresses the immunity of honey bees and leads to the activation of a persistent and latent viral infection [18].

In Brazil, by examining six pathogenic viruses (DWV-BQCV-SBV-IAPV-ABPV-CBPV), Brzoskowski Chagas et al. [19] showed that cases of simultaneous infection with ABPV, BQCV, and DWV in both states. While, in all beekeeping with positive virus samples, the mite *V. destructor* was also reported, indicating the transmission of these viruses through *Varroa* mites [19]. Tentcheva et al. [5], examined six pathogenic viruses DWV-BQCV-SBV-KBV-ABPV-CBPV in adult bees, pupae and *V. destructor* samples from apparently healthy honey bee colonies in France using PCR technique. In their studied *Varroa* samples, four viruses were identified; DWV (100%), SBV (45%), ABPV (36%) and KBV (5%) of apiaries [5].

In Hungary, Bakonyi et al. [20] detected ABPV in *Varroa* mites in apiaries suffering from colony collapse disorder (CCD), while more than 87% of the collapsed apiaries showed ABPV infection in bee samples. In our study, ABPV infection was not observed in mite samples and infection of the total apiary rate with this virus was less than 8%, indicating *Varroa* mite infection along with viral diseases plays an effective role in viral infection of honey bee colonies [20].

Studies by Chen et al. showed that KBV can be transmitted from KBV-infected mites to healthy pupae and mites in a KBV-free colony [6]. Shen et al. identified KBV and SBV in honey bee samples and also, for the first time, in *V. destructor* mites. The simultaneous identification of viruses in *V. destructor* and honey bees emphasizes the importance of mites in the transmission of various honey bee viruses [18]. Furthermore, it was shown that DWV is not able to replicate in all mites. Interestingly, viral replication in mites is associated with DWV infection in honeybees, which suggests that symptoms are more likely to occur if the virus is transmitted by mites [21]. In another study conducted by Minoo et al. [22] rates of

DWV and ABPV detection in *Varroa* mites were 100% and 77%, respectively, while, in *Tropilaelaps* mites, they detected 100% DWV, 57% ABPV, 30% CBPV and 23% VDV-1. Arzumanyan et al. reported infection of DWV in all *Varroa* mites [23]. The presence of DWV, SBV, KBV, IAPV, BQCV, and CBPV in *Varroa* mites collected from Korean apiaries has been reported by Moon et al. (2024) [24]. They showed the highest prevalence of viral infection belonged to DWV (87%), followed by IAPV (65.2%), SBV (17.4%), and less than 10% belonged to other viruses. In the present study, the prevalence of DWV was higher than SBV and KBV in *Varroa* mite samples, which was consistent with the above mentioned study. These results indicate that the *V. destructor* plays an effective role in the propagation and transmission of viral diseases in honey bees. This means that one of the reasons for the low prevalence of the pathogenic viruses including ABPV, BQCV and CBPV in honey bee samples [13] can be due to the non-infectiousness of the *Varroa* mite samples. Of course, it has been showed that there is no relationship between the spread of CBPV and the *V. destructor* mite [25].

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

In the last decade, KBV has been recognized as the most important virus among several viruses related with CCD in apiaries infected with the *V. destructor* mite [10]. Reports show that in the beekeeping industry, control of *Varroa* mites and viruses is a vital factor in preserving the health of honey bees. So, beekeepers should take preventive methods to prevent the spread of bee diseases. Given the lack of treatment for viral diseases, management strategies may be the only way to control viral diseases [5]. Also, given the importance of the *V. destructor* mite in transmitting viral diseases, more attention should be paid to controlling this pest. Consequently, regular inspection of colonies by trained personal is essential to control or reduce viral infections. Recent research has suggested the use of thermal interventions, particularly hyperthermia, as a suitable method for controlling *Varroa* mites. These studies have also explained the effect of heat stress in decreasing viral infections among infected bees. Hyperthermia treatment may be effective in treating viral infections by decreasing *Varroa* mite infestation or by inducing the production of heat shock proteins, which have potential antiviral properties [26].

5. Conclusion

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

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Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

Conceptualization, study design, administrative, technical, and material support, review and editing: all authors; Data acquisition: Maryam Torkaman, Mojtaba Moharrami, and Masoumeh Bagheri; Analysis and data interpretation: Maryam Torkaman and Masoumeh Bagheri; Statistical analysis: Maryam Torkaman and Masoumeh Bagheri. Writing the original draft: Maryam Torkaman and Masoumeh Bagheri.

Conflict of interest

The authors declared 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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