Original Article

Molecular Investigation of *Brucella* Species Belongs to Sheep and Goats in Seropositive Samples from an Endemic Area of Hamedan Province; Famenin Brucellosis Cohort Study

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ABSTRACT

Brucellosis is a zoonotic infectious disease in western regions of Iran, especially in Hamedan province. Following the Famenin brucellosis cohort study, the main aim of the current study was the molecular detection of *Brucella* species (spp.) in sheep and goats from Famenin, Hamedan, Iran. A total of 23 *Brucella*-seropositive samples (sheep=21 and goats=2), which had been screened from 1,660 animals in the Famenin cohort study, were used to detect *Brucella*-DNA using the *BCSP31* target gene and IS711 locus. In total, 20 of 23 samples were positive for *Brucella* infection by using specific primers. Additionally, *Brucella melitensis* (*B. melitensis*) and *Brucella abortus* (*B. abortus*) were confirmed in 90% (n=18) and 10% (n=2) of positive samples, respectively. There was no sample with the co-infection of *B. abortus* and *B. melitensis*. In this study, *B. abortus* was isolated from one of the goat samples. This is the first report on *Brucella* spp. in animals in the region. It was found that *B. melitensis* is the dominant spp. responsible for brucellosis in animals from Famenin. Molecular techniques are reliable tools to detect *Brucella* infection, especially in cases without serology findings and conclusive results.

Keywords: Animal, *Brucella abortus*, *Brucella melitensis*, Famenin, Molecular detection

1. Introduction

Brucellosis, which is caused by various species (spp.) of *Brucella* (an intracellular gram-negative coccobacilli bacteria), is one of the endemic diseases in Iran (1). It also goes by other native names, such as Mediterranean fever, undulant fever, gastric remittent fever, and Malta fever (2). There are more than 10 spp. in the genus of *Brucella*, but only *Brucella melitensis* (*B. melitensis*), *Brucella abortus* (*B. abortus*), *Brucella suis*, and *Brucella canis* (in rare cases) are characterized as human pathogens (3). The incidence of human brucellosis is directly related to the prevalence of *Brucella* infection in animals (4). The major cause of brucellosis in bovines is *B. abortus* in some countries, while the most pathogenic spp. in humans is *B. melitensis*, the common spp. in sheep and goats (5). In animals, brucellosis has severe effects on reproductive organs, which causes abortion and reproductive defects. After the abortion, the shedding of the pathogen is continued by female animals (6). Brucellosis is also one of the abortion causes in Iranian sheep herds (1, 7).

Brucellosis is spread through direct and indirect contact with the tissues and secretions of infected animals, as well as through the consumption of unpasteurized dairy products. It can show significant clinical signs in humans, including undulating fever, anorexia, sweating, arthritis, splenomegaly, hepatomegaly, and lymphadenopathy (4). The disease has been controlled successfully in some countries; however, the infection is distributed in areas with poor sanitation or without a launch control program (2). In endemic areas, such as Iran, brucellosis control is costly and requires a supportable budget. In previous decades, Iran has faced dire challenges in controlling brucellosis, which is the reason for significant financial and public health problems throughout the country (4). The importance of identifying and distributing brucellosis reservoirs is well-established for the execution of complete control and prevention programs. Additionally, specifying the risk factors contributing to the brucellosis outbreak in animals is of excessive significance (3). The prevalence of brucellosis is high in some regions of Iran, including Lorestan, Kermanshah, and Hamedan. Famenin is one of the regions with an increased incidence of brucellosis in Hamedan province (8). The incidence rate in Hamedan is estimated at 31-41 cases per 100,000 humans (8). Brucellosis is an occupational disease whose prevalence is higher in farmers than in others (9). Moreover, the consumption of non-pasteurized dairy products, especially cheese and milk, plays a vital role in the prevalence of human brucellosis (10).

The clinical signs of brucellosis are nonspecific in both humans and animals. Therefore, it is necessary to use an efficient and precise method to diagnose the disease quickly. Its early diagnosis is also helpful in initiating the therapeutic process in humans and health services in animals (11). There are three main laboratory methods to diagnose brucellosis in animals and humans, including bacteria isolation using culture, serology and immunology, as well as molecular and DNA identification. Despite the development of new methods with high sensitivity and specificity in recent years, culture is still introduced as the “gold standard” in the laboratory detection of brucellosis due to its clinical and epidemiological connection, low cost, and availability (12). Many investigations have been conducted to identify various spp. of *Brucella* in humans and animals using molecular techniques (11). Different techniques based on serology are used to identify brucellosis cases, including the Rose Bengal plate test (RBPT), Wright serum agglutination test (SAT), 2-Mercapto-Ethanol (2-ME), Enzyme-linked immunosorbent assay, and Coombs test (antihuman globulin) (13, 14). In Iran, RBPT, SAT, and 2-ME tests are commonly applied to detect brucellosis in animals (7).

Regarding previous reports in Iran, there is no updated data on *Brucella* spp. in animals from Hamedan province (4, 12, 15). Following the Famenin brucellosis cohort study, the current project sought to detect *Brucella* spp. in sheep and goats from Famenin, Hamedan, Iran, using molecular methods.

2. Materials and Methods

2.1. Study Design

The present study is part of a huge project on the cohort of brucellosis in the Famenin area, Hamedan, Iran (8, 13). In the first stage (13), the seroprevalence of brucellosis in animals was evaluated in the region. This study was conducted to confirm the seropositive samples and determine *Brucella* spp. in animals.

2.2. Study Location

Hamedan province (34.77°N and 48.58°E, an area of 19,546 km2) is located in the vicinity of the Alvand Mountain, West of Iran, with dry summers and a cold, semi-arid climate in winter. The average temperature is 11.3°C, which may reach -30°C in the winter. It is the exchange focal point of a ripe ranch district where grain is developed; therefore, agriculture and animal breeding are the popular jobs and the primary sources of income in the area. Famenin (a city in the Northeast of Hamedan province) is considered one of the significant centers for agriculture and animal husbandry (Figure 1). There are 2,200,000 sheep and goats in Hamedan province, including 141,000 sheep and 15,000 goats from the Famenin area. Mehraban (a native race in the region) is the dominant breed of sheep in Hamedan province.

2.3. Sampling

All 1,660 sera samples (sheep=1470 and goats=190) obtained in the previous investigation were re-evaluated using RBPT, SAT, and 2-ME (16). Twenty-three positive samples (sheep=21 and goats=2) were confirmed using the Iranian Veterinary Organization (IVO) protocol (Table 1) parallel to the previous study (7, 13). The sera samples of 23 seropositive animals were used for DNA extraction and molecular detection.

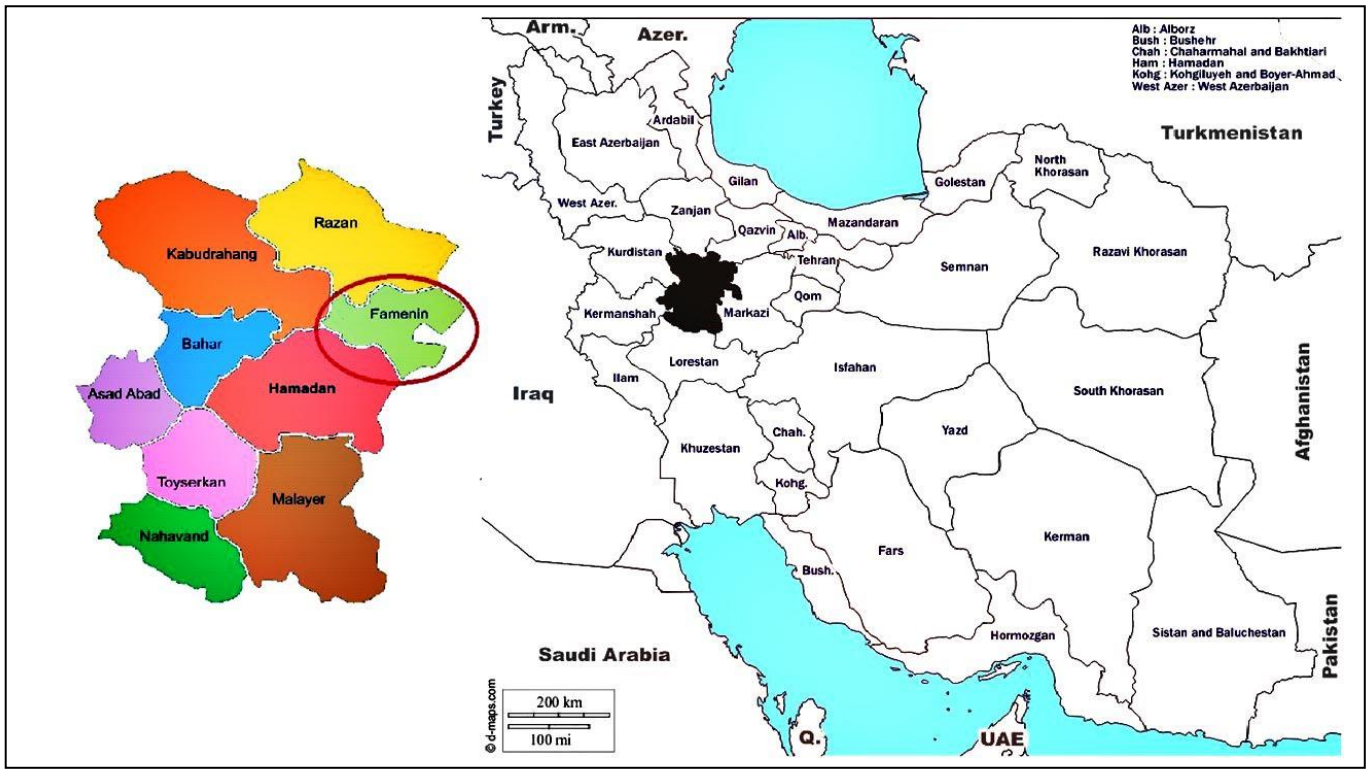

Figure 1. Geographic location of Hamedan province and Famenin, studied area

Table 1. The Iranian Veterinary Organization manual to detect brucellosis in sheep and goats based on serology tests

RBPT	SAT	2-ME	Brucellosis result
Positive	≥ +4/40	Each titer of antibody	Positive
	≤ +3/40	≥ +1/20	Positive
		≤ +4/10	Negative

2.4. Molecular Identification

2.4.1. DNA Extraction

Genomic materials were extracted from serum-positive samples using a DNA purification commercial kit (Sinaclon, Iran, Cat. No. PR881613) based on the supplier's instructions. The extracted materials were analyzed qualitatively and quantitatively using electrophoresis and NanoDrop (Eppendorf, Germany) by reading A260 and A280.

2.4.2. DNA Amplification

The extracted DNA was subjected to a polymerase chain reaction (PCR) using the BCSP31-B4 and BCSP31-B5 primers (Table 2) to detect the genus of *Brucella* (17). The total volume of BCSP31-PCR reactions was 12.5 µl, including 6.25 µl 2X PCR master mixes (Amplicon, Denmark), 0.5 µl of each primer, 4 µl DNA template, and distilled water (D/W) up to 12.5 µl. *Brucella* spp. were determined using specific-spp. primers with amplicons of 498 and 731 bp for *B. abortus* and *B. melitensis*, respectively (12) (Table 2). The forward primer of IS711 is unique for detecting *Brucella* spp., but reverse primers are diverse and are derived from specific loci on chromosomal DNA for *B. abortus* and *B. melitensis* (Table 2). IS711-PCR was performed in a total volume of 12.5 µl using a mixture similar to BCSP31-PCR. The PCR profile was accomplished on a thermocycler (T100 PCR thermal cycler, USA) by the following steps: primary denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 90 sec, primer annealing at 64°C for 1 min, and primer extension at 72°C for 1 min with a final extension cycle at 72°C for 5 min. The amplified products (10 µl) were evaluated using electrophoresis on 1.5% agarose gel (Figures 2-4). We used strains of *B. abortus* (ATCC 23455) and *B. melitensis* (ATCC 23457) as positive controls and D/W as the negative control in the reactions.

Table 2. Information on primers in regard to Gene type and product size that used to detect of *Brucella* in animals from Famenin

Gene	Primer	Product size	Reference
BCSP31-B4	Forward: 5´ tgg ctc ggt tgc caa tat caa 3´	224 bp	(12)
BCSP31-B5	Reverse: 5´cgc gct tgc ctt tca ggt ctg 3´		
IS711 *B.abortus*	Forward: 5´ tgc cga tca ctt aag ggc ctt cat 3´	498 bp	(14)
IS711 *B.abortus*	Reverse: 5´ gac gaa cgg aat ttt tcc aat ccc 3´		
IS711 *B. melitensis*	Forward: 5´ tgc cga tca ctt aag ggc ctt cat 3´	731 bp	(14)
IS711 *B. melitensis*	Reverse: 5´ aaa tcg cgt ctt tgc tgg tct ga 3´		

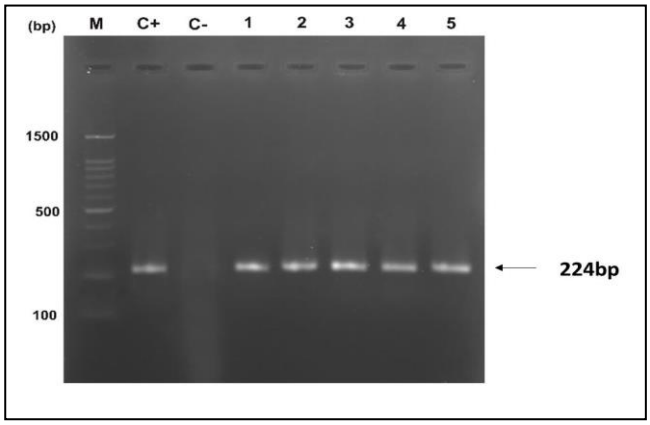

Figure 2. The PCR products electrophoresis to detect of *Brucella* genus. Lane M: 100 bp marker; lane C+: *B. melitensis* standard strain (Razi vaccine and serum research institute) as positive control; lane C-: physiological serum as negative control. Lanes 1 to 12: positive samples

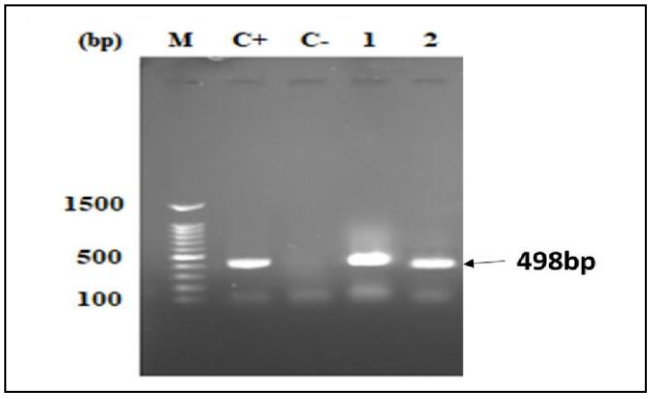

Figure 3. The PCR products electrophoresis to detect of *Brucella abortus*. Lane M: 100 bp marker; lane C+: *B. abortus* standard strain (Razi vaccine and serum research institute) as positive control; lane C-: physiological serum as negative control. Lanes 1 to 5: positive samples

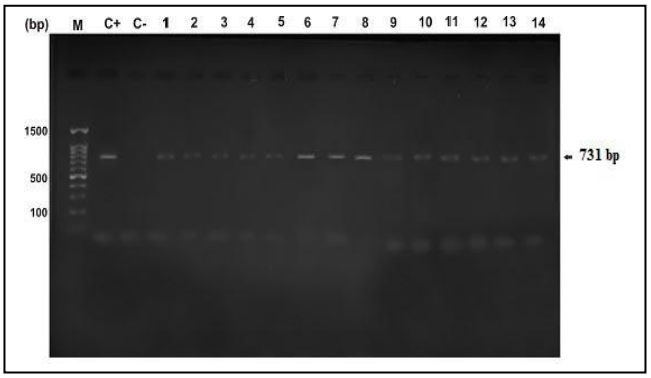

Figure 4. The PCR products electrophoresis to detect of *Brucella melitensis*. Lane M: 100 bp marker; lane C+: *B. melitensis* standard strain (Razi vaccine and serum research institute) as positive control; lane C-: physiological serum as negative control. Lanes 1 to 14: positive samples

3. Results

After the molecular evaluation of seropositive samples (n=23), 20 (86.95%) were positive for *Brucella* spp. (Figure 2). In the second stage, 2 (10%) and 18 (90%) cases were confirmed for *B. abortus* and *B. melitensis*, respectively (Figures 3 and 4). There was no sample with co-infection of *B. abortus* and *B. melitensis*, and *B. abortus* was isolated from one of the goat samples. In the molecular evaluation, three negative samples were detected with a lower serology titer than other samples (Table 3).

Table 3. The results of *Brucella*-infection in Famenin in regard to serology and molecular methods and also type of animals.

sample no.	Type of animals	Serology results			PCR results		
		RBPT	SAT	2-ME	*Brucella* sp.	*B. melitensis*	*B. abortus*
1	goats	+	+3/80	+2/20	+	+	-
2		+	+2/20	+2/20	+	-	+
3		+	+4/80	+4/80	+	+	-
4		+	+4/80	+3/80	+	+	-
5		+	+4/80	+4/80	+	+	-
6		+	+4/80	+4/80	+	+	-
7		+	+4/20	+4/20	+	+	-
8		+	+4/160	+4/160	+	+	-
9		+	+4/40	+4/40	+	+	-
10		+	+3/40	+3/40	+	+	-
11		+	+3/40	+3/40	+	+	-
12		+	+1/20	+1/20	+	+	-
13	sheep	+	+2/20	+2/20	+	+	-
14		+	+2/20	+2/20	+	+	-
15		+	+3/80	+4/40	+	+	-
16		+	+4/80	+4/80	+	+	-
17		+	+2/40	+2/40	+	-	+
18		+	+3/20	+3/20	+	+	-
19		+	+2/40	+2/40	+	+	-
20		+	+3/40	+2/20	+	+	-
21		+	+4/20	+2/20	-	-	-
22		+	+4/20	+4/20	-	-	-
23		+	+2/20	+1/20	-	-	-

4. Discussion

Brucellosis is a neglected zoonotic disease in developing countries (2). The disease is endemic in Iran, and animals have a significant role in transmitting the infection to humans (1). The implementation of appropriate control strategies is critical in reducing human brucellosis (3). The identification of risk options for the disease is the first step toward designing an effective plan to limit the infection. The main risk factors for brucellosis in the herds are the type of animal spp., common grazing locations, abortion management, sharing of male animals for mating, and keeping animals together (16). Brucellosis is of particular concern due to its importance for health and the significant economic losses it causes in the livestock industry (1). The most prevalent spp. in Iran are *B. melitensis* and *B. abortus*, and there are reports on *B. melitensis* and *B. abortus* co-infection (4). The determination of co-infection between *B. abortus* and *B. melitensis* is related to keeping various animals together, which is a risk factor that needs to be appropriately managed (16).

In serology evaluation, different levels of antibodies show evidence of infection (8, 13). *Brucella*-DNA could not be identified in three of the seropositive samples. It can be due to infection with other strains, such as vaccines, cross-reaction with other pathogens, the low quality of extracted DNA, and a previous disease without bacteremia. Similar outputs are reported by other researchers (5, 6). They believe some animals may be silent carriers of *Brucella* infection, and more positive cases are reported in molecular techniques, compared to serology (18). Therefore, they are a potential source of infection for other animals and humans.

In the previous evaluation of brucellosis in the Famenin area, 2.73% and 1.30% of animals had antibodies to *Brucella* infection in rapid RBPT and complementary tests, such as SAT and 2-ME, respectively. The overall brucellosis rate in animals was 1.3% (zero in cattle, 1.43% in sheep, and 1.05% in goats) (13). In a report by Dadar, Shahali (1) from Iran, the prevalence of *Brucella* spp. in livestock was 10.18%, with 3.79% in Hamedan province. In addition, 4% of raw milk, 2.3% of cheese, and 9.5% of cream samples were diagnosed as positive for *Brucella* contamination in Hamedan using bacteriology methods (19). On the other hand, in a molecular study by Gharekhani, Yakhchali (20), the bulk milk samples of 149 dairy farms in Hamedan were not *Brucella*-positive. In Mamani, Majzoobi (9) report, 17%, 15%, and 8.1% of butchers, slaughterhouse workers, and veterinary staff, respectively, were positive for brucellosis in Hamedan. Additionally, *Brucella* infection in animals was reported to be zero in horses, 3.3% in dogs, 4.6% in goats, and 3% in sheep (7, 21, 22).

In the present study, *B. abortus* was rarely detected in sheep and goat samples, similar to other studies (1, 6, 12, 15). This result is due to keeping different livestock spp. together, which is common in Iran. The top reported spp. of *Brucella* in humans and animals are *B. melitensis* and *B. abortus* in the neighboring countries of Iran and the Middle East. The seroprevalence rate of brucellosis in animals is higher in these regions than in other locations worldwide (2). In Abnaroodheleh, Emadi (23) study, *B. melitensis* biovar 1 has been introduced as the main pathogen for abortion in Iranian sheep and goats. Following genotypic analyses on Iranian isolates (from animals and humans), *B. melitensis* biovar 1 and *B. abortus* biovar 3 were found to be the most common biovars. While *B. abortus* appeared to be closed mainly to cattle, *B. melitensis* is common in sheep, goats, and cattle (12). Alamian and Dadar (24) isolated *B. melitensis* biovar 1 and 2 from Iranian farm dogs for the first time. *Brucella* transmission from various animals to dogs may occur through the consumption of infected reproductive materials or aborted fetuses (22). Therefore, the presence of free-roaming dogs in the herd is a potential risk for transmitting the infection to farmers and farms.

In a similar study conducted in Iranian dairy farms (5), *Brucella* was detected in 28% of seropositive animals using PCR, with all isolates being *B.*

abortus. In another study in Eastern Iran, 23 animals were seropositive with titers of ≥1/40 and ≥1/20 in SAT and 2-ME, respectively (6). In the molecular evaluation, the frequency of *B. abortus* was higher than *B. melitensis*. Moreover, *B. abortus* was detected in 35% and 15% of blood and milk samples, respectively, and 2% of the samples were co-infected with *B. melitensis* and *B. abortus*, as well as two biovars of *B. abortus*. They reported the vaccinal strains (*B. abortus* S19 and *B. melitensis* Rev.1) in the positive samples. In a study conducted in Brazil by Miyashiro, Scarcelli (25), *Brucella* was not isolated in cheese from any samples using bacteriology. However, 19.3% were positive for *B. abortus* in PCR. Interestingly, 80% of them were positive for the vaccinal strain. Regarding the high sensitivity of molecular methods, vaccinal strains should be considered when designing the primers. Mirkalantari, Masjedian (15) detected the same genotype of *B. melitensis* isolates from sheep and humans, which confirmed a possible epidemiological connection between them. Dadar, Alamian (4) reported both *B. melitensis* and *B. abortus* contribute to the human brucellosis burden in Iran, but *B. melitensis* has a higher role than *B. abortus*. This pattern is confirmed in previous studies (2, 4, 12).

A 'One Health' policy, such as the development of veterinary services and the extension of health training, has proven strikingly viable in controlling brucellosis (3). It is recommended to keep different livestock spp. and genders separate to prevent transmitting non-specific *Brucella* spp. Additionally, specific pastures should be considered for each herd to block the cross-movement of animals between herds. We suggest combining different laboratory methods, such as serology, microbiology, and molecular biology, to minimize the detection limits of infection in the herds.

This study is the first report on *Brucella* spp. diversity in animals using sera samples and molecular techniques from Hamedan province. *B. melitensis* is the principal spp. responsible for brucellosis in sheep and goats in Famenin. Molecular analyses, such as phylogenetic studies and fingerprinting techniques of *Brucella* isolates from humans and animals in the endemic regions, are needed for identifying the genetic diversity and dominant spp., as well as the mode of transmission and distribution of the infection. Molecular evaluation is considered a reliable tool to detect *Brucella* infection, especially in cases with no serology findings and conclusive results. Regarding the structure of the health system in Iran, coordination between the Ministry of Health and the IVO to design the health policy on the One Health approach is highly recommended.

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Authors' Contribution

Study concept and design: M. A.
Acquisition of data: M. A.
Analysis and interpretation of data: H. G. and F. S.
Drafting of the manuscript: J. G.
Critical revision of the manuscript for important intellectual content: M. A. and J. G.
Administrative, technical, and material support: S. A. and M. V.
Study supervision: M. A.

Ethics

The study was confirmed by the Ethical Committee of Hamedan University of Medical Sciences, Iran (IR.UMSHA.REC.1399.7). Additionally, ethical standards have been carefully observed during the preparation of results and submission the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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