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First Phylogenetic Perspective on Molecular Epidemiology of *Echinococcus granulosus sensu lato* in Dogs in Sistan and Baluchestan Province, Southeastern Border of Iran

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ABSTRACT

Background: Echinococcosis or Hydatid disease is a zoonotic disease that is caused by *Echinococcus granulosus*. The disease is a high public health concern in Iran, but there is little known about the genetic diversity and epidemiology of Echinococcus spp. in Iranian shepherd dogs.

Materials and Methods: Fifty shepherd dogs were investigated for the adult worm of *E. granulosus* from May 2020 to April 2021 in Sistan and Baluchestan Province, the southeastern border of Iran. DNA extraction of samples and amplifying was done, and sequence analysis of mitochondrial genes (*Cox1* and *Nad1*) was performed.

Results: Out of 50 shepherd dogs, 11 cases (22%) were infected with *E. granulosus*. No significant difference was observed regarding demographic factors (P>0.05). The phylogenetic analyses of *Cox1* and *Nad1* sequences demonstrated G1 genotype (sheep strains) in all isolates. Based on sequence analyses, a low (*Cox1*, Hd [haplotype diversity: 0.200; Hn [number of haplotypes]: 2) to moderate (*Nad1*, Hd: 0.533; Hn: 4) genetic (haplotype) diversity of *E. granulosus* G1 genotype and low nucleotide diversity (π: 0.00052-0.00243) were observed.

Conclusion: The first identification of a sheep strain (G1) in the final host in Sistan and Baluchestan Province indicates that potential intermediate hosts play a secondary role in preserving the biology of the dog-sheep cycle. The present study's findings enrich our knowledge about the prevalence of *E. granulosus*, the classification of strains, and the genetic diversity of the parasite in Iranian herding dogs. This information helps develop strategies and programs for monitoring and controlling infection in stray dogs in the region.

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Introduction

The vulnerability of the unconfined population of the infected dogs to parasitic infections in densely populated human areas is a widely known reality in the transmission of human hydatidosis. Cystic Echinococcosis (CE) is the most crucial zoonotic disease linked with helminths (Echinococcus spp.). It has significant impact on the disability of people in endemic areas across the world, chiefly the Middle East, New Zealand, Russia, Australia, China, South America, and North Africa [1-7].

Annually, the total cost of CE in Iran was estimated at 232.3 million dollars [8]. Vagabond dogs, as the main hosts, harbor the adult stage of parasites, whereas the intermediate hosts, such as herbivores, serve the larval stage of parasites in their bodies, especially the liver and lung [2]. Accordingly, to develop a control and surveillance system for preventing and monitoring the hydatid disease, various features of adult worm *Echinococcus granulosus* isolates must be recognized in separate, non-overlapping geographic areas [9-11]. *E. granulosus* isolates include a variety of intraspecies in terms of morphology, epidemiology, genetics, and host specificity [12, 13]. Out of 10 strains of genus *E. granulosus* (G1–G10), six strains (G1, G2, G3, G5, G6, and G7) have been currently and genotypically reported from different endemic areas in Iran [14, 15].

A high rate of (5%-49%) infection by stray dogs with *E. granulosus* has been reported in different parts of Iran [16]. Problems in the field studies, including contamination with viral infections (e.g., rabies), stray dog trapping, and high risk of hydatidosis in the course of experiments, explain the dearth of knowledge about the molecular-epidemiology characterization and morphometric features of *E. granulosus* adult stage in the Iranian vagabond dogs or even those in worldwide [17-21].

Nevertheless, several researchers were successful in their experiments on the larval stages utilizing morphologic and or genotypic characterizations in the intermediate hosts, such as pigs, buffalo, sheep, camels, goats, and cattle [14, 22-25]. It is of utmost importance to determine the genetic diversity patterns of the adult stage of *E. granulosus* to understand the current parasite cycles in endemic areas of Iran [26, 27].

With this background in mind, this study aimed to evaluate the phylogenetic perspectives on the genetic diversity and epidemiology of *E. granulosus* isolates in dogs. Accordingly, the prevalence of *E. granulosus*, taxonomy of strains, and genetic characterization of the isolated parasite can be determined, which will be suitable for monitor and controlling the infected stray dogs in Sistan and Baluchistan Province, Southeast Iran.

Materials and Methods

Study area, sampling, and preparation

The samples were obtained from different areas in Southeast Iran, including Zabol, Zahedan, Iranshahr, Saravan, and Chabahar cities (Figure 1), which are all suburb regions that include livestock farming and shepherd dogs. Fifty shepherd dogs were fed 5 mg/kg praziquantel, and after 24 to 48 hours, their feces were evaluated to find any adult worms of *E. granulosus*. In total, 50 shepherd dogs (29 males and 21 females) were investigated from May 2020 to April 2021. After dog feces examination, *E. granulosus* worms were isolated and normal saline was used to wash them three times. Worms are stored in 70% ethanol until molecular tests.

DNA extraction and PCR amplification

A commercial kit (WizPrep™ gDNA Mini Kit [Cell/Tissue], Wizbiosolutions, South Korea) was used to extract the total genomic DNA from worms according to the manufacturers' protocol and kept at -20°C up to PCR amplification. For the amplification of the cytochrome oxidase subunit 1 gene fragment, we used JB3 (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and JB4.5 (5′-TAAAGAAAGAACATAATGAAAATG-3′) primers, and for the amplification of the NADH dehydrogenase subunit 1 gene, we employed MS1 (5′-CGTAGGTATGTTGGTTTGTTTGGT-3′) and MS2 (5′-CCATAATCAAATGGCGTACGAT-3′) primers [28, 29].

The single round-PCR amplification was conducted in 25 µL reaction volumes containing 2.5 µL of 10×PCR buffer, 0.7 µL (1.5 mM) MgCl2, 0.5 µL (10 mM) of dNTP Mix, 0.3 µL (5u/µL) of Taq DNA polymerase, 1.5 µL of each forward and reverse primers (10 pmol), 5 µL of DNA template (50 ng) and 13 µL deionized distilled water. The details of thermal cycling conditions for amplifying *Cox1* and *Nad1* genes were described in other studies [28, 29]. PCR products were then electrophoresed on 1.5% agarose gel, and then fragments of 444 bp for the *Cox1* gene and 400 bp for the *Nad1* gene were investigated under ultraviolet light utilizing a transilluminator.

Sequencing, phylogenetic analysis, and genetic diversity

Twenty PCR products of *Cox1* (n: 10) and *Nad1* (n: 10) genes were sequenced by Bioneer Corporation (South Korea). Sequences were aligned and edited with the reference sequence of *E. granulosus* genotype G1 (RefSeq: KC660075) retrieved from GenBank utilizing Sequencer Tmv.4.1.4 software. To confirm the taxonomic status genotypes of *E. granulosus*, a phylogenetic tree based on a maximum likelihood algorithm with the Kimura 2-parameter model was constructed using MEGA software. To calculate the degree of genetic diversity of *Cox1-Nad1* genes among sequences of *E. granulosus*, diversity indices (Haplotype diversity: Hd, haplotype number: Hn, and nucleotide diversity: π) were estimated by DnaSP software v. 5.1.

Results

Out of 50 shepherd dogs, 11 (9 males and 2 females) cases (22%) were infected with *E. granulosus*. Table 1 tabulates the number of non-infected and infected dogs regarding gender and age groups.

Considering the demographic factors, there is no difference between male and female adults in terms of the frequency of parasitism (P>0.05). Moreover, no significant difference was observed between the contamination rate and age groups (P>0.05) (Table 1).

Nucleotide sequence analysis, phylogenetic tree, and diversity indices

Amplification of the partial-length *Nad1* and *Cox1* genes yielded PCR products of approximately 444 bp and 400 bp, respectively. In 20 edited sequences, the majority of genotypes were identified as G1. No mixed infection of *E. granulosus* genotypes was found during the analysis of overlapped chromatograms. Distance-based maximum likelihood cladistic trees generated by *Cox1* and *Nad1* sequences demonstrated that the *E. granulosus* G1 genotype was assigned to its specific clade. In other words, sequence analysis of *Cox1* gene (n: 10) (Accession numbers: MZ823583 to MZ823592) and *Nad1* gene (n:10) (Accession numbers: MZ855265 to MZ855274) indicated that all isolates belong to G1 genotype) (Figures 2 and 3).

The diversity indices were calculated for the *E. granulosus* G1 genotype in southeast Iran based on the *Nad1* and *Cox1* sequences. Based on sequence analyses, a low (*Cox1*, Hd: 0.200; Hn: 2) to moderate (*Nad1*, Hd: 0.533; Hn: 4) genetic (haplotype) diversity of *E. granulosus* G1 genotype and low nucleotide diversity (π: 0.00052-0.00243) were observed in *E. granulosus* isolated from a definitive host (Table 2).

Discussion

Hydatid cyst infection has always been considered one of the important health and economic problems in human societies, and its occurrence in domestic animals leads to irreparable economic losses. Because stray dogs usually live around cities and rural areas, they have a critical role in transmitting this parasite to humans. In addition, shepherd and guard dogs are in very close contact with humans, and a lack of attention to food hygiene and their environment may also have a significant impact on the transmission and establishment of the hydatidosis infection cycle [30].

There are several reports of dogs infected with *E. granulosus* in Iran. According to a study carried out by Shariatzadeh et al. in East Azerbaijan Province, Iran, the prevalence of *E. granulosus* was reported to be 20%. This prevalence caused concern among health experts because a study conducted by Garedaghi et al. reported a prevalence rate of 12.5% in the same area, and the results of the study by Shariatzadeh et al. could be a severe warning for the environmental protection agency and health experts to take the necessary decisions to control and prevent the spread of this parasitic infection [25, 31].

Eslami and Hosseini conducted a study on dogs in 13 provinces of Iran; the rate of infection of dogs with the *E. granulosus* parasite varied between 3.3% in Sistan Baluchestan Province and 63.3% in Isfahan Province [32]. Also, in Kerman Province, adjacent to Sistan and Baluchestan Province, Sharifi and Zia-Ali reported a 7.4% prevalence rate of E granulosus in stray dogs [33].

There is no other study to determine the prevalence of *E. granulosus* in dogs in Sistan and Baluchestan Province. In the present study, the prevalence of *E. granulosus* infection in shepherd dogs in Sistan and Baluchestan Province was estimated to be 22%, different from the Eslami and Hosseini study in 1998. This huge difference can be attributed to the lack of control over the stray dog population, the increase in the unsanitary slaughter of livestock around the city and even the city itself, or the unprincipled disposal of viscera infected with hydatid cysts.

Table 1 tabulates the frequency of E granulosus infection among the two age groups of dogs (2-4 years and 4-6 years). The lowest frequency was related to the age group

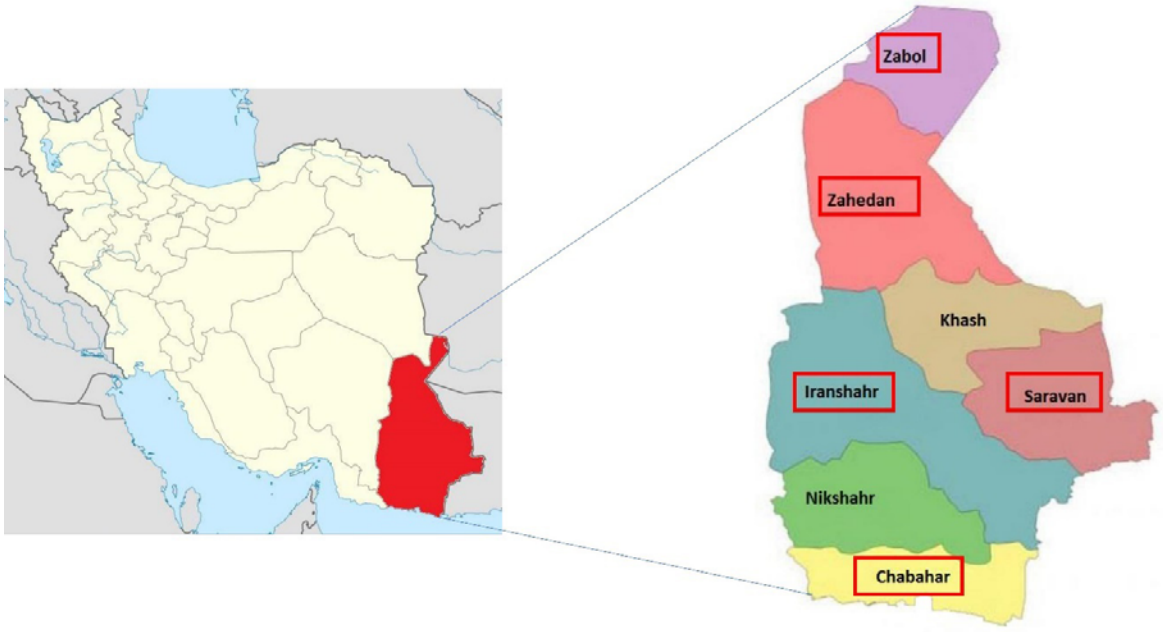

Figure 1. Geographical location of Sistan and Baluchestan Province, Iran

of 2-4 years. However, the two age groups of dogs showed no significant difference in the distribution of infection (P=0.977). There is no information on the association of *E. granulosus* infection with different age groups. However, in studies conducted by Eslami and Hosseini and Maleky and Moradkhan, no significant differences were observed between dog age groups and *E. granulosus* infections [32, 34].

The frequency distribution of *E. granulosus* infection by sex of dogs in Table 1 also confirmed that male dogs

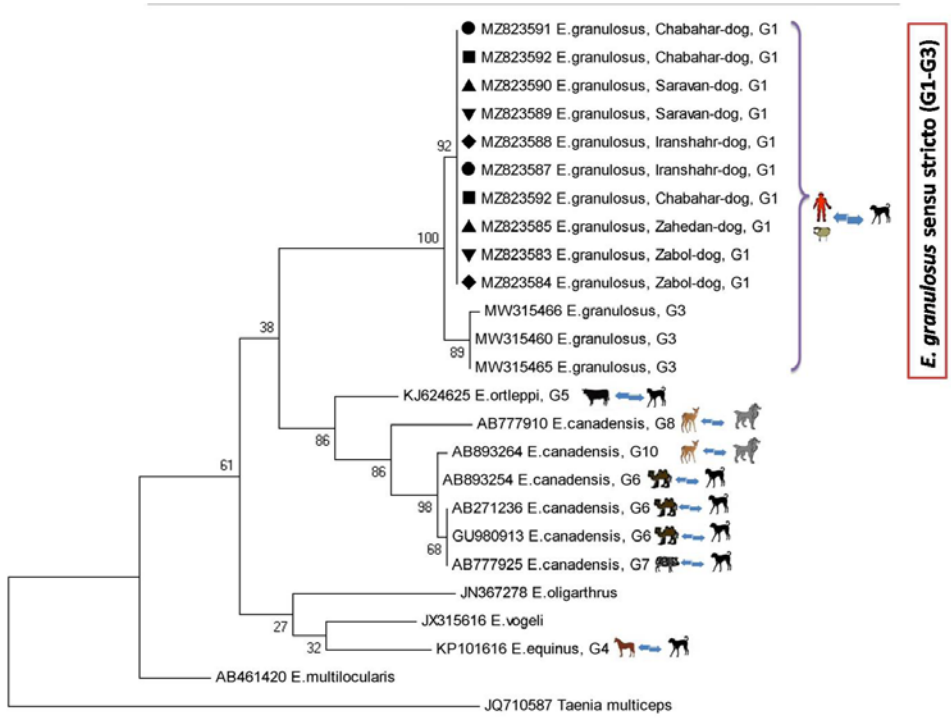

Figure 2. A distance-based maximum likelihood cladistic tree of *Echinococcus granulosus* G1 genotype based on the *Cox1* gene

Only bootstrap values higher than 70% are indicated on each branch. The collected location characterized genotype (G1) and registered accession numbers marked by geometric shapes. *Taenia multiceps* was considered an out-group branch (Accession No: JQ710587)

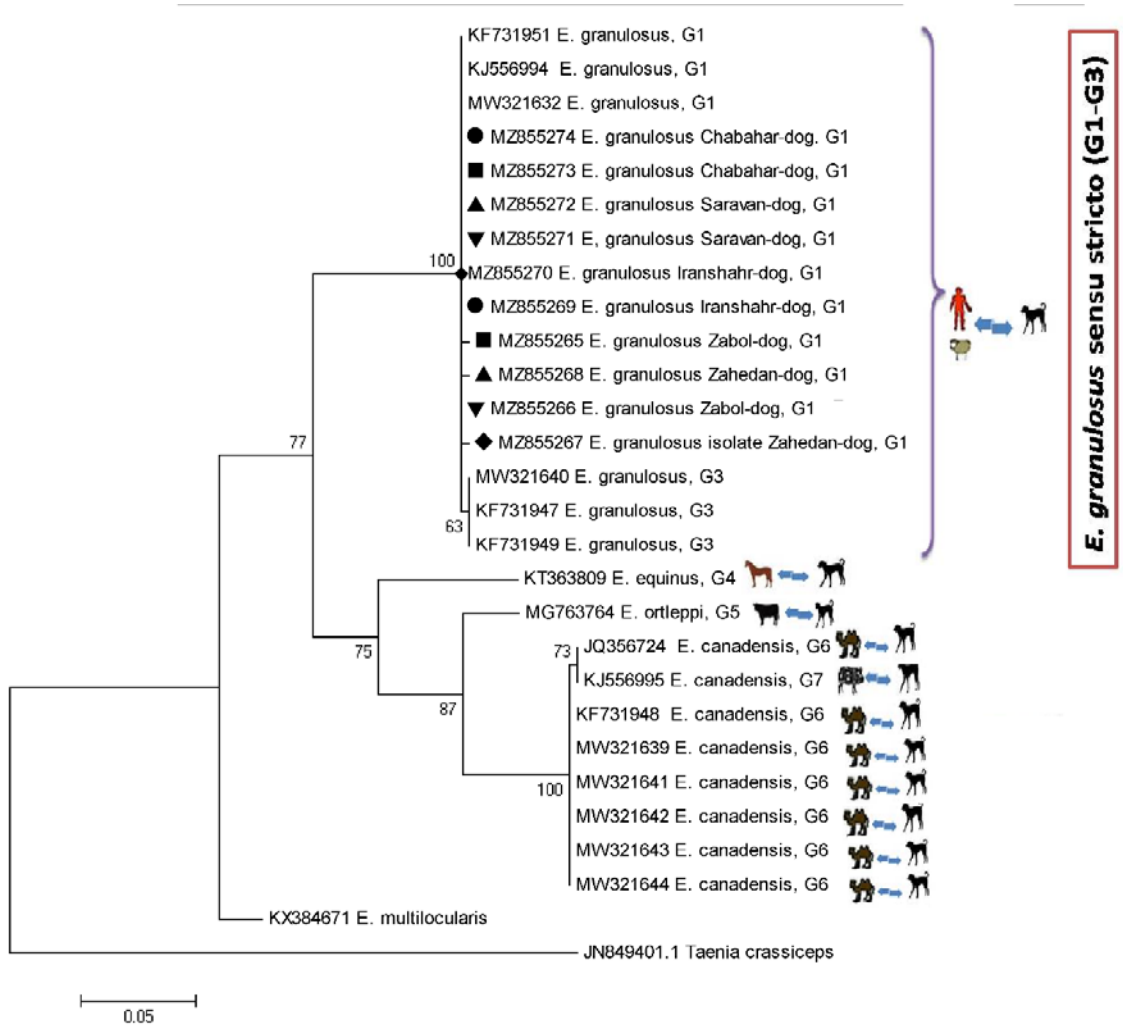

Figure 3. A distance-based maximum likelihood cladistic tree of *Echinococcus granulosus* G1 genotype based on the *Nad1* gene
Only bootstrap values higher than 70% are indicated on each branch. The collected location characterized genotype (G1) and registered accession numbers marked by geometric shapes. *Taenia crassiceps* was considered an out-group branch (Accession No: JN849401).

were more infected with *E. granulosus* than female ones, but this difference was not significant. Emampour et al. and Shariatzadeh et al. reported that *E. granulosus* infection in female dogs was slightly higher than in male dogs, but this difference was not significant [25, 35]. In general, there is limited information on the relationship between *E. granulosus* infection and the sex of the dog. Therefore, this finding requires more detailed studies.

Table 1. Age groups and gender frequency of *Echinococcus granulosus* in 50 Shepherd dogs from Southeast Iran

Dogs	No. (%)				
	Age Groups (Years)		Gender		Total
	2-4	4-6	Female	Male	
Infected	4(22.2)	7(21.9)	2(9.5)	9(31.0)	11
Non-infected	14(77.8)	25(78.1)	19(90.5)	20(69.0)	39
Total	18	32	21	29	50

Table 2. Diversity indices of *E. granulosus* G1 genotype based on nucleotide sequences of *Cox1*/*Nad1* Genes

Country (Province)/ Genotype	Host (N; Genotype)	Gene	Diversity Indices				
			N	Hn	Hd±SD	Number of Nd (π)	Segregating Sites
Iran (Sistan and Baluchestan)/*E. granulosus sensu* stricto	Dog (10; G1)	*Cox1*	10	2	0.200±0.154	1	0.00052
	Dog (10; G1)	*Nad1*	10	4	0.533±0.180	3	0.00243

N: number of isolates; Hn: number of haplotypes; Hd: haplotype diversity; Nd: nucleotide diversity

In the present study, the G1 genotype was identified in all *E. granulosus* samples for both genes (*Cox1* and *Nad1*). Haplotype diversity analysis showed low haplotype diversity based on the *Cox1* gene, and only two new haplotypes were identified, but based on the *Nad1* gene, moderate haplotype diversity and four new haplotypes were identified. Therefore, the *Nad1* gene showed genetic diversity better than the *Cox1* gene. So far, a few molecular studies have been performed on genotyping of the adult worm of *E. granulosus* in Iran. In Parsa et al.'s study on 71 stray dogs, genotypes G1, G2, and G3 were detected. G2 genotype was reported for the first time in the final host in Iran [21]. In their study in East Azerbaijan Province (2015) to identify *E. granulosus* genotypes in dogs, Shariatzadeh et al. reported the most prevalent and common genotype, G1 [25]. Gholami et al. in Mazandaran Province in their study reported that the highest genotype of *E. granulosus* in dogs was the G1 genotype [36]. Ghabdian et al. examined 100 stray dogs to determine the genotypes of *E. granulosus*. After sequencing, it was found that all worms isolated from infected dogs had a G1 genotype [37]. A study conducted by Arbabi et al. on Isfahan dogs showed that the predominant genotype was sheep strain (G1) [38]. Heidari et al. conducted a study in Khorasan Province for molecular identification and genotyping of *E. granulosus* strains isolated from canines, G1 genotype was identified in all isolates [39]. Mirbadie et al. studied *E. granulosus* genotypes in domestic and stray dogs in Iran and identified G1, G3, and G7 genotypes. This research was the first report of pig strain (G7) in the final hosts in the Middle East [40]. In addition, Keyhani et al. conducted a study for molecular identification and genotyping of *E. granulosus* strains isolated from dogs in Kerman Province, the neighbor of Sistan and Baluchestan Province. The results showed that all isolates had a G1 genotype [41].

A possible reason that only the G1 genotype was identified in the present study may be because, in the current study, only sheepdogs were investigated for *E. granulosus* strains, as the first study on the final host in Sistan and Baluchestan Province. In other studies, primarily stray dogs have been investigated for *E. granulosus* genotypes, and more genetic diversity has been identified. Therefore, further studies on stray dogs and other final hosts (wild cycle) seem necessary to better identify circulating genotypes in Sistan and Baluchestan Province.

Conclusion

From the studies conducted in Iran, the common genotype in Iranian dogs, as well as in the adjacent areas of Sistan and Baluchestan Province, is the G1 genotype. The predominance of G1 genotype in the dogs studied in the present study showed that sheep play a greater role as intermediate hosts in the *E. granulosus* cycle in Sistan and Baluchestan Province. The study results reveal the need for further studies on the knowledge of dogs infected with *E. granulosus*. Because of the high frequency of infection obtained in this study and the urban population at risk, monitoring and attention of veterinary organizations and competent health authorities regarding the control of this parasite seem to be necessary.

Ethical Considerations

Compliance with ethical guidelines

The current study protocol was approved by the Ethics Committee of Mazandaran University of Medical Sciences, Sari, Iran (Code: IR.MAZUMS..REC.1398.4712).

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Authors contribution's

Conceptualization and supervision: Shirzad Gholami, Ahmad Daryani, Shahabbedin Sarvi, and Adel Spotin; Methodology: Davood Anvari, and Seyed Abdollah Hosseini; Investigation, writing-original draft, and writing-review & editing: All authors; Sample collection:

Davood Anvari; Data analysis: Adel Spotin, and Seyed Abdollah Hosseini; Funding acquisition and Resources: Shirzad Gholami.

Conflict of interest

The authors declared no conflict of interests.

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