

Original Article

A study on Isolation and Molecular Identification of *Bordetella avium* from Iranian Commercial and Backyard Broiler Turkeys within 2016-2018

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ABSTRACT

Bordetellosis or turkey coryza, caused by *Bordetella avium*, has been an issue for turkey industry since its first description in 1967 when it was reported for the first time. *Bordetella avium* causes a highly contagious upper respiratory disease in turkeys. Therefore, this study aimed to isolate and characterize this species from commercial and backyard turkeys in Tehran, Isfahan, and Northern provinces of Iran. For the purpose of the study, 625 tracheal swabs were taken from 425 commercial poults and 200 backyard poults aged 2-6 weeks from September 2016 to September 2018. The swabs were immediately plated on MacConkey and blood agar plates and then pooled (5 swabs/pool) in tubes, containing 2 mL distilled water, to perform direct polymerase chain reaction (PCR) for the identification of *B. avium*. A total of 17 swab pools were found to be positive for *B. avium*. A subset of seven positive samples were sequenced for the flanking region of *piuA* gene. The analysis of the sequences indicated that the sequences were 98%, 96%, and 98% similar to *B. avium* 197N (AM167904.1), 4142 (AY925058.1), and 4156 (AY925068.1) sequences, respectively. To the best of our knowledge, the current study is the first attempt toward the molecular detection and characterization of *B. avium* in Iran. It is highly recommended to perform further studies to isolate, characterize, and differentiate the regional isolates in order to help the developing turkey industry of Iran meet the increasing demands for protein in the diet of the citizenry.

Keywords: Molecular identification; *Bordetella avium*; Turkey; Iran; Phylogenetic tree

Une Étude sur l'Isolément et l'Identification Moléculaire de *Bordetella avium* Provenant de Dindes Commerciales et de Basse-cour Irlandaises entre 2016-2018

Résumé: La bordetellose ou coryza de la dinde, causée par *Bordetella avium*, représente un problème pour l'industrie de la dinde depuis sa première description en 1967. *Bordetella avium* provoque une maladie des voies respiratoires supérieures très contagieuse chez les dindes. Par conséquent, cette étude visait à isoler et caractériser cette espèce chez les dindes commerciales et de basse-cour à Téhéran, Isfahan et les provinces du nord de l'Iran. A cet effet, 625 écouvillons trachéaux ont été prélevés sur 425 dindonneaux commerciaux et 200 dindonneaux de basse-cour âgés de 2 à 6 semaines entre septembre 2016 et septembre 2018. Les écouvillons ont été immédiatement étalés sur des plaques de MacConkey et de gélose au sang, puis regroupés

(5 écouvillons/pool) dans des tubes, contenant 2 ml d'eau distillée, pour effectuer une réaction en chaîne par polymérase (RCP) directe pour l'identification de *B. avium*. Au total, 17 pools d'écouvillons étaient positifs pour *B. avium*. Un sous-ensemble de sept échantillons positifs a été séquençé pour la région flanquante du gène *piuA*. L'analyse des séquences a indiqué que les séquences étaient 98%, 96% et 98% similaires aux séquences de *B. avium* 197N (AM167904.1), 4142 (AY925058.1) et 4156 (AY925068.1), respectivement. Au meilleur de nos connaissances, cette étude représente la première tentative de détection moléculaire et de caractérisation de *B. avium* en Iran. Nos résultats recommandent fortement la mise en place d'autres études afin d'isoler, caractériser et différencier les isolats régionaux pour l'industrie en développement de la dinde d'Iran à répondre aux demandes croissantes de protéines dans l'alimentation des citoyens.

Mots-clés: Identification moléculaire; *Bordetella avium*; Dinde; L'Iran; Arbre phylogénétique

INTRODUCTION

Bordetella is a genus of small Gram-negative bacilli that includes such species as *B. avium* which is an important pathogen affecting avian species (Kerstens et al., 1984). *Bordetella avium*, previously known as *Alcaligenes faecalis*, is the causative agent of bordetellosis or turkey coryza, first described in Canada in 1967. Bordetellosis is a highly contagious upper respiratory tract disease, with a higher economic loss in turkeys, compared to that in other avian species (Venne and Brugere-Picoux, 2015). *Bordetella avium* might also infect some other avian species, such as cockatiels (*Nymphicus hollandicus*), as the causative agent of locked jaw syndrome (Grespan et al., 2012). In turkeys, bordetellosis mostly occurs in 2-6-week-old poults and is characterized by a sudden onset and rapid spread with high morbidity and low mortality. It presents with foamy conjunctivitis, sneezing, and moist tracheal rales as the first clinical signs and then progresses to dyspnea signs (Jackwood and Saif, 2013). This disease might also occur in adult turkeys with dry coughing as the only clinical sign (Kelly et al., 1986), and the signs of the disease subside after 2-4 weeks (Panigrahy et al., 1981). The economic loss of this disease could be so high that it necessitates studying the prevalence of bordetellosis in the US and selection of appropriate control measures (Jackwood and Saif, 2013). This is even more important for countries new to turkey

industry, such as Iran. Iran started the turkey industry approximately 20 years ago and ranked third in turkey meat production in Asia based on the Food and Agriculture Organization reports (Salahi, 2014). Various assays have been introduced to detect *Bordetella avium* as the causative agent of bordetellosis. These tests include culture (Register and Jackwood, 2016), commercial enzyme-linked immunosorbent assay (ELISA; Synbiotics Corporation, Kansas City, USA), microagglutination assay (Jackwood and Saif, 2013), and molecular assays, such as *PvuII* Ribotyping (Sacco et al., 2000), restriction endonuclease analysis using either *HinfI* or *DdeI* (Sacco et al., 2000), and polymerase chain reaction (PCR) (Savelkoul et al., 1993). There are also other assays for the better characterization of *B. avium*, such as biochemical tests to distinguish *B. avium* from other non-fermentative bacteria and *B. hinzii* (Blackall and Farrah, 1986) and hemagglutination of guinea pig red blood cells to evaluate the pathogenicity (Jackwood and Saif, 2013). Despite the increasing demand for the development of more rapid assays with good sensitivity and specificity, there is only one PCR assay to detect *B. avium*, and the diagnosis mostly relies on classic methods, such as culture and biochemical tests using the API 20 NE system (bioMérieux Inc., Durham, USA). Savelkoul et al. (1993) described *B. avium*-specific PCR assay in 1993. They used a DNA fragment from *B. pertussis*, encoding the *fim2* fimbrial

subunit gene with adjacent sequences as a probe to find homologous sequences in *B. avium* DNA and found a 650-bp *EagI* DNA fragment specific to *B. avium*. They claimed that the PCR resulted in the amplification of a 500-bp fragment with only *B. avium*. However, they did not test the specificity and sensitivity of the assay with regard to other pathogens found commonly in turkey respiratory tract and *B. hinzii*, as another *Bordetella* species found in turkeys. In another study, Register and Yersin (2005) evaluated the assay and demonstrated 100% sensitivity and 98.8% specificity in the identification of *B. avium* isolated from different geographic locations over 25 years. Accordingly, they recommended the evaluated version of the PCR as the standard PCR method in peer reviews and references (Register and Yersin, 2005). To the best of our knowledge, *B. avium* has not been detected and molecularly characterized in Iran up to the time of publishing the current study. Accordingly, there is extremely limited knowledge regarding the genetic aspect, clinical manifestation, and role of bordetellosis in the turkey industry of Iran. Meanwhile, it is important to mention that since backyard turkeys are densely reared in the Northern provinces of Iran, namely Gilan and Mazandaran. However, given the lack of any specific regulations for restricting the contact of care takers and people who work in turkey industry, these kinds of turkeys should be also considered in the evaluation of bordetellosis and the effects of the disease on turkey industry. The incidence of bordetellosis in turkey has caused huge economic losses in the US (Jackwood and Saif, 2013). Regarding this and given the recent detection of *B. avium* in the Middle East (Türkyilmaz et al., 2009), the present surveillance study was conducted to evaluate the current status of emerging turkey industry in Iran regarding *B. avium*. This investigation was performed using culture and PCR as a rapid standard molecular assay (Register and Yersin, 2005), followed by the comparison of positive samples with the available sequences from previous studies in GenBank.

MATERIAL AND METHODS

This study was conducted on 17 farms and 200 backyard 2-6-week-old turkey poults from four provinces (Figure 1). Tracheal swabs (n=625) were taken from the farms (25 swabs each) and backyard turkey poults from September 2016 to September 2018 (Table 1). It is necessary to mention that all backyard poults showed respiratory signs. However, regarding the commercial turkeys, although they were all sick with general clinical signs, only 5 cases out of the 17 commercial turkey farms showed respiratory signs. All 625 swabs were immediately plated on MacConkey and blood agar with a quadrant streak method. Subsequently, every five swabs were pooled in tubes, containing 2 mL distilled water, and kept in -20 °C for direct DNA extraction. After discs, containing 30 µg aztreonam and 10 µg ampicillin (Padtan Teb Co., Iran), were placed on the initial inoculation on both MacConkey agar and blood agar plates, all plates were incubated for 48 h hours at 37 °C. Clear or brownish raised center colonies growing in the zone of inhibition were selected and passed on to both MacConkey and blood agar plates to purify the isolates. Fifty-five Gram-negative bacillus isolates were glycerolized and frozen at -80 °C for further characterization by PCR. As the next step, DNAs from the glycerolized isolates and pooled swabs were extracted using the standard boiling method. To detect *B. avium*, PCR was performed on the extracts using the MM2001 PCR Master Mix (Sinaclon, Iran), via the primers derived from the region flanking of *piuA* gene (Register and Yersin, 2005) (Table 2). The PCR was carried out in a total volume of 25 µl per sample, consisting of 7.5 µl water, 12.5 µl 2x buffer, 1 µM of each primer, and 3 µl template sample DNA. The cycling conditions included 3 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 50 °C, and 45 sec at 72 °C, followed by a final extension step of 5 min at 72 °C. The positive control for *B. avium* (197N) was kindly provided by the Faculty of Veterinary Medicine, Adnan Menderes University, Turkey. In addition, nuclease-free distilled water was

used as the negative control for PCR. It is necessary to note that in addition to the controls mentioned, a 16S rRNA-specific PCR was also performed on all samples detected as negative, with the *B. avium* set of primers as a positive control (Register and Yersin, 2005). Electrophoresis was performed on PCR products on a 2% agarose gel with 0.5 µg/ml ethidium bromide (100V, 197 mA, 40 min). The bands were visualized by ultraviolet illumination and compared with a 100-bp ladder. Finally, *B. avium*-specific PCR products with a length of 520 bp were sequenced partially using the Sanger method for the *piuA* gene region of *B. avium*. The sequences were compared with other *B. avium* *piuA* gene sequences available in the GenBank by Blast (<http://ncbi.nlm.gov/bla>). The phylogenetic tree was then drawn with the p-distance-based neighbor-joining method (1000 Bootstrap) using the Mega software, version 7.

RESULTS

Polymerase chain reaction identification from swabs. Out of 125 pools, 17 samples were positive using *B. avium*-specific primer PCR (Figure 2). Out of

these samples, 10 specimens belonged to the turkey farms of Mazandaran, and 4 and 3 samples had been collected from the backyard turkey swabs of Gilan and Mazandaran, respectively. Seven positive samples were sequenced at the Avian Coronavirus Lab, University of Tehran. Comparison of the sequences obtained in this study with 22 sequences from other studies in the phylogenetic tree revealed that basically, the sequences fell into two groups, one having two closely related subgroups (i.e., 197-N and the sequences of the current study) and the other with the isolates from different parts of the US, Germany, UK, and Australia. The analysis of the sequences indicated that the sequences were 98% similar to the *B. avium* 197N (AM167904.1) isolated from turkey flocks in Ohio, the US, in the 1980s. The sequences were also respectively 96% and 98% similar to *B. avium* strain 4142 (AY925058.1) and *B. avium* strain 4156 (AY925068.1), confirming these isolates as *B. avium*.

Culture analysis. A total of 57 colonies were suspected of *B. avium* infection in the culture procedures; however, none of the isolates were positive in *B. avium* *piuA* gene PCR. This result did not occur

Table 1. Distribution of the farms (25 tracheal swabs from each farm)

Province	Commercial/backyard	Age of the flocks	No. of samples	Respiratory signs	General clinical signs	Humid/ dry area
1. Gilan	Commercial, Four farms	Three at 4 WOA and one at 5 WOA	100	No	Yes	Humid
2. Isfahan	Commercial, Four farms	Two at 5 WOA and two at 6 WOA	100	Yes (3 farms)	Yes	Dry
3. Mazandaran	Commercial, Four farms	One at 2 WOA and three at 3 WOA	100	Yes (2 farms)	Yes	Humid
4. Tehran	Commercial, Five farms	Two at 4 WOA and three at 3 WOA	125	No	Yes	Dry
5. Gilan	Backyard	Various ages between 2 and 6 WOA	50	Yes	Yes	Humid
6. Isfahan	Backyard	Various ages between 2 and 6 WOA	50	Yes	Yes	Dry
7. Mazandaran	Backyard	Various ages between 2 and 6 WOA	50	Yes	Yes	Humid
8. Tehran	Backyard	Various ages between 2 and 6 WOA	50	No	Yes	Dry

Table 2. Sequence of primers used to detect *Bordetella avium*

Primer	Bacterial species	Sequence (5'→3')	Amplicon size	Reference
univ16S-3	Veterinary clinical bacteria	5'-AGAGTTTGATCCTGGCTCAG-3'	~520 bp	(Cai et al., 2003)
Univ16S-4	Veterinary clinical bacteria	5'-GCGGCTGCTGGCACG-3'		
N- <i>avium</i>	<i>B. avium</i>	5'-GCGGCGTCAACACATACTCTTGAT-3'	~520 bp	(Register and Yersin, 2005)
C- <i>avium</i>	<i>B. avium</i>	5'-AGGGAGGTCAGATAGCTCTAGAAT-3'		

due to the lack of DNA as shown by a positive PCR for the 16sR-DNA gene (data not shown).

Phylogenetic analysis. The tree constructed as described above revealed a close grouping of the isolates from Iran that is more related to the largest group than the other four isolates (Figure 3). All other isolates were from Europe and the US obtained over a period of nearly 40 years.

DISCUSSION

Since the 1970s, bordetellosis has been considered a major health problem in young turkeys (Register and Jackwood, 2016). Although the infection causes low mortality, its high morbidity could be problematic for weight gain in turkeys as this happens in the early weeks of life. This might have a higher impact in countries with emerging turkey industries as higher and more cost-effective production is expected. The aim of this study was to investigate the presence of *B. avium* in turkeys exhibiting coryza symptoms. Four provinces were selected for the study, two of which (i.e., Tehran and Isfahan) had the densest turkey production. However, in the two other provinces (i.e., Sari and Mazandaran) with humid weather turkey production was less dense. *Bordetella avium* is susceptible to very dry environmental conditions (Van Alstine, 1987). As mentioned, all samples were plated on both MacConkey and blood agar with aztreonam and ampicillin discs being dispensed in the first step. There is evidence regarding the resistance of *B. avium* to aztreonam and ampicillin (Beach et al., 2012). This resistance may be due to the lack of penicillin-binding protein 3 (*PBP3*) gene. The mentioned antibiotics were used to inhibit the growth of other bacteria, such as *Escherichia coli*, without affecting *B. avium* that may have been present. However, if the reported resistance to ampicillin and aztreonam is not present universally, the potential *B. avium* bacteria could have been inhibited resulting in the nonappearance of *B. avium* in the 57 colonies tested. In a study performed in Hungary, the minimum inhibitory concentration (MIC)

of *B. avium* isolates for ampicillin was reported to range from ≤ 0.03 $\mu\text{g/ml}$ to 1 $\mu\text{g/ml}$ (Szabo et al., 2015). There are also some reports on the variable (Malik et al., 2003) or very low (Blackall et al., 1995) resistance to ampicillin for *B. avium*. On the other hand, there are many reports regarding the coinfection of turkeys with *B. avium* and other bacteria, such as *Escherichia coli* (Pumford et al., 2008), *Chlamydia psittaci*, *Klebsiella pneumoniae*, and *Pseudomonas fluorescens* (Hinz et al., 1992). This might also explain the inability to isolate *B. avium* colonies since all the above-mentioned species are fast growing and might overwhelm on MacConkey and blood agar. Regarding this, it is recommended to perform further studies to isolate *B. avium*, check the MICs of different antibiotics, and work on different concentrations of antibiotics in order to develop a new culture media suitable for the strains detected in Iran. All the positive samples in *B. avium* specific PCR belonged to the humid area. The location of the positive samples found in this study shows the importance of humidity for *B. avium*. Accordingly, this issue highlights the fundamental importance of screening for *B. avium* in the Northern provinces of Iran, located next to the Caspian Sea. This finding, together with the detection of *B. avium* in the Middle East and neighboring countries (El-Sukhon et al., 2002; Ozbey and Muz, 2006; Türkyilmaz et al., 2009; Smialek et al., 2015; Abo-State et al., 2018), necessitates the development of a surveillance program for this emerging industry in Iran, especially in Northern provinces. Regarding the studies conducted on *B. avium* in the Middle East and neighboring countries, Türkyilmaz et al. (2009) studied the lung, tracheal, and serum samples obtained from turkeys with respiratory clinical signs in Turkey using culture, ELISA, and PCR. Although *B. avium* was successfully detected in PCR assay, and the antibody response to *B. avium* was shown in ELISA, the bacterium was not isolated from both tracheal and lung samples. They suggested the administration of antibiotics before sampling as a possible reason for the

failure of bacterial isolation. The lack of any veterinary feed directive regulations in Iran might be another reason explaining our findings. Smialek et al. (2015) also investigated the day-old and +23-week-old turkey flocks using ELISA between 2012 and 2014. They found that the anti-*B. avium* IgY antibodies and mean titers increased with bird aging, indicating bordetellosis as a very common infection in Polish turkey flocks. They recommended the vaccination of turkey flocks with a live *B. avium* vaccine. This should be considered after studying the epidemiology of bordetellosis and careful evaluation of the results in Iran. On the other hand, El-Sukhon et al. (2002) investigated 100 broiler chicken farms in the northern and middle parts of Jordan and found the complication of *Escherichia coli*,

Ornithobacterium rhinotracheale, and *B. avium* as the causative agents of airsacculitis. In the current study, *B. avium* isolates were partially sensitive to ampicillin as opposed to the isolates studied in the US (Beach et al., 2012). Hence, it is required to investigate airsacculitis cases in broiler chicken flocks and role of *B. avium* in future studies. The phylogenetic tree constructed based on 29 sequences of the *piuA* obtained from GenBank showed that the isolates from Iran grouped together tightly. Most of the 22 comparison sequences were isolated in the US. The implications of this result cannot be appreciated until the full genome sequencing of the isolates is completed. To the best of our knowledge, the current study is the first attempt toward identifying *B. avium* in sick turkeys in Iran.



Figure 1. Location of sampled provinces in Iran (These provinces are the leaders of the growing turkey industry in Iran.)

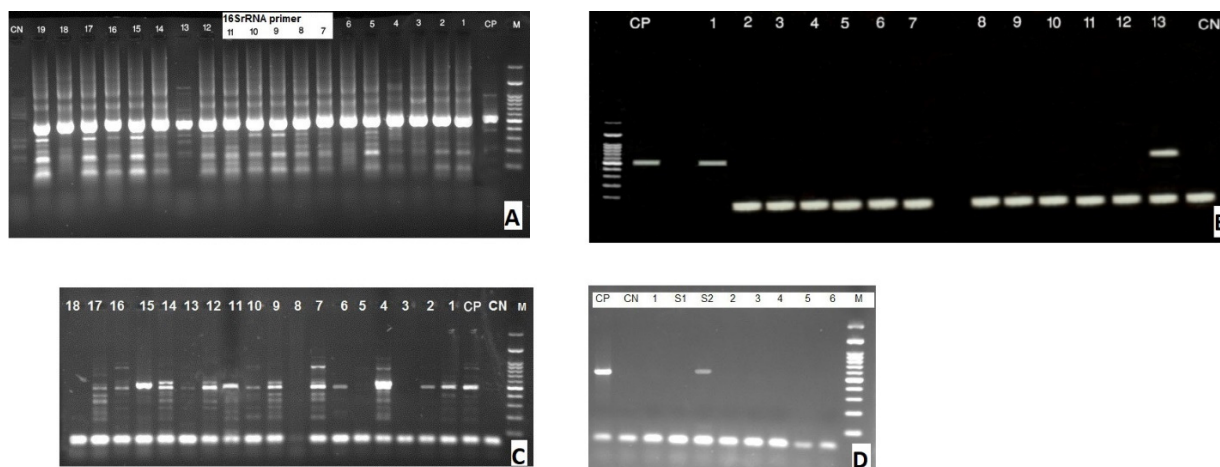


Figure 2. Representative results obtained from polymerase chain reaction with 16S r-RNA primer set (A) and Bordetella avium-specific primer set (B, C, and D) [(A) Lanes: M, 100 bp ladder; CN, Negative control; CP, *B. avium* Positive Control; 1-19, suspected colonies (B) Lanes: M, 100 bp ladder; CN, Negative control; CP, *B. avium* Positive Control; 1-13, pooled swabs (C) Lanes: M, 100 bp ladder; CN, Negative Control; CP, *B. avium* positive control; 1-18, pooled swabs; (D) Lanes: M, 100 bp ladder; CN, Negative Control; CP, *B. avium* positive control; 1-6 and S1-S2, pooled]

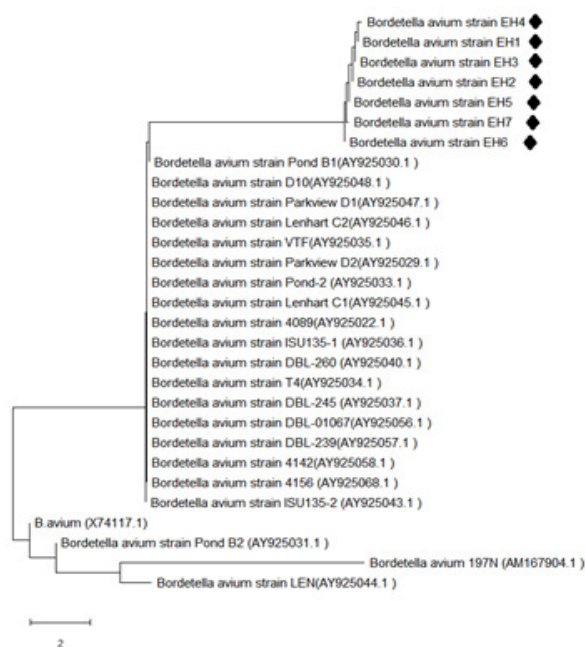


Figure 3. Phylogenetic analysis of *Bordetella avium*-positive polymerase chain reaction products (shown with black diamonds) (It was drawn with p-distance based on neighbor-joining method (1000 Bootstrap) using Mega software, version 7. The optimal tree with the sum branch length of 24.73008862 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The differences in the composition bias among sequences were considered in evolutionary comparisons. This analysis involved 29 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 479 positions in the final dataset. Evolutionary analyses were conducted in MEGA X software. The analysis of the sequences indicated that the sequences were 98% similar to *B. avium* 197N (AM167904.1) and 96% and 98% similar to *B. avium* strain 4142 (AY925058.1) and *B. avium* strain 4156 (AY925068.1) sequences, respectively, indicating all three were evolved from a common ancestor.

It is recommended to perform further studies to isolate, characterize, and differentiate the regional isolates of *B. avium* in order to help the developing turkey industry of Iran meet the increasing demands for protein in the diet of the populace. That means to help the turkey industry of Iran, which increased from 5,916 turkeys in 2003 to 1,703,882 turkeys in 2016 (Iran Agriculture Statistics II published by the Ministry of Agriculture Jihad, last updated on March 2018),

provide the protein requirement of the increasing population of Iran with a population growth rate of 1.24% (Statistical Center of Iran, 2019).

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Authors' Contribution

Study concept and design: Hassanzadeh M., Barrin A.
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 Analysis and interpretation of data: Hassanzadeh, M., Ehsan M., Barrin A., Ghalyanchi Langeroudi A., Temple L.
 Drafting of the manuscript: Ehsan M.
 Critical revision of the manuscript for important intellectual content: Temple L., Hassanzadeh M., Bozorgmehri Fard M. H.
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 Administrative, technical, and material support: University of Tehran., Hassanzadeh M., Barrin A., Temple L., Turkyilmaz S.

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