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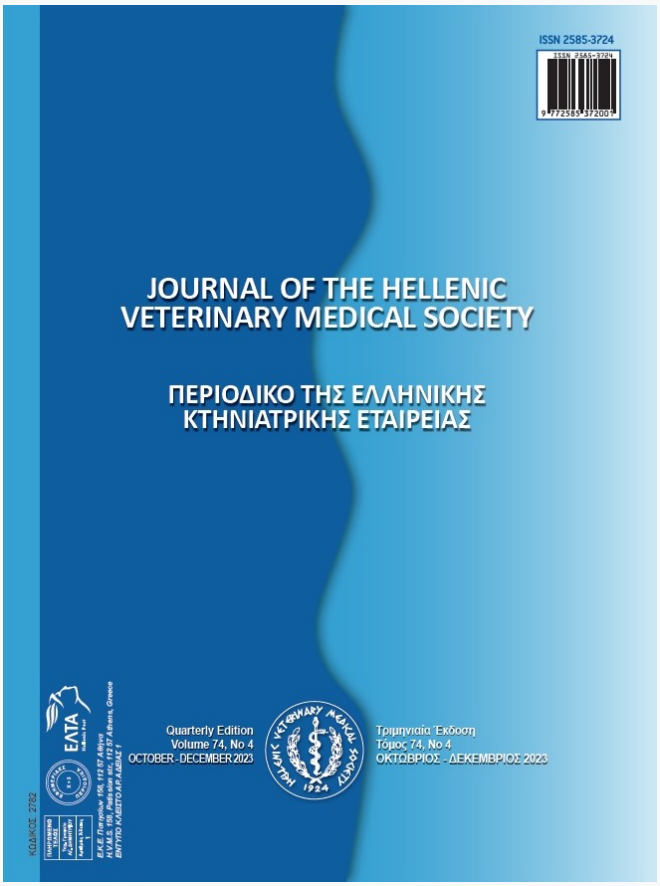

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Research article
Ερευνητικό άρθρο

Molecular characterization of peste des petits ruminants virus strains circulating in sheep and goats in Iran

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ABSTRACT: Peste des petits ruminants virus (PPRV) infection is an acute to sub-acute viral disease in both domestic and wild small ruminants. Recent outbreaks of PPRV in Turkey's Marmara region and in Europe (Georgia and Bulgaria) highlight the potential risk of PPR spreading to a larger geographic area. In order to achieve a successful control and eradication program, evaluating etiyological data prior to developing disease control strategies is an essential criterion. The aim of this study was to perform molecular characterization of PPRVs found in sheep and goats in Iran. For this purpose, a total of 341 animal specimens were collected from sheep (n = 271) and goats (n = 70) with clinical signs of PPRV infection from twelve different provinces. RT-real time-qPCR assay based on nucleoprotein (N) with a plasmid standard reference, which is rapid and sensitive for the diagnosis of infection, was used for the detection of PPRV nucleic acid. In the RT-real time-qPCR assay, a positivity rate of 29,91% (102/341) was detected for PPRV nucleic acid. At the nucleotide level, the N-gene partial sequence analysis of sixteen viral sequences obtained from four provinces of Iran showed 96.8%-100% similarity and 97.6%-100% and 88.2%-89% similarity to the Turkey2000 reference isolate and Nigeria 75/1 vaccine strain, respectively. Except for two viral sequences, the secondary protein structure of the approximately 80 amino acid long nucleoprotein region in the sixteen viral sequences revealed structural similarity in the alpha-helix and beta-leaf structures for all PPRVs of Iranian origin. In the phylogenetic tree, PPRVs circulating in Iran are homologous, belong to genetic lineage IV, and are closely related to the Turkey2000 isolate. According to the results of this work, it is emphasized that PPRV circulates in Iran, causes outbreaks and deaths, and should be controlled. In addition, further studies on the molecular analyses of the N protein of the Iranian isolates will help clarify the origin of the disease and determine the genetic diversity of the virus.

***Keywords*:** Iran, PPRV, Phylogenetic analysis, Real time-qPCR

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INTRODUCTION

Peste des petits ruminants virus (PPRV) infection is a highly contagious viral disease of small domestic and wild ruminants that causes economic losses due to high morbidity and mortality (Dhar et al., 2002; Ebrahimzadeh et al., 2016). This infection is characterized by fever, anorexia, nasal discharge, hemorrhage, erosions in the oral mucosa (tongue, lip, palate), stomatitis, conjunctivitis, diarrhea, and bronchopneumonia in small ruminants (Kwiattek et al., 2007).

The etiological agent known as peste-des-petits ruminants virus (PPRV) was renamed small ruminant morbillivirus (SRMV) by the international committee on taxonomy of viruses (ICTV) in 2016. PPRV belongs to the *Morbillivirus* genus of the *Paramyxoviridae* family, as it has biological and physicochemical properties closely related to the other morbilliviruses, which include measles virus (MV), rinderpest virus (RPV), canine distemper virus (CDV), phocine morbillivirus (PMV), porpoise distemper virus (PDV), and dolphin morbillivirus (DMV) (Liu et al., 2018). Morbilliviruses are linear, non-segmented, single-stranded, negative sense RNA viruses with a genome of approximately 15-16 kb and a diameter of 200 nm (Norrby and Oxman, 1990). The viral genome consists of two nonstructural proteins (V and C) and six transcriptional units (3'-N-P/C/V-M-F-H-L-5') encoding six structural proteins, respectively (Diallo, 1990). The nucleoprotein (N) that surrounds the viral nucleic acid is the most abundant and has a 52-56 base long leader sequence. Various functions are reported for this sequence, such as beginning replication, generating positive-strand RNA, and reducing host cell transcription of abundant viral protein in both virions and infected cells. The N gene has a single transcription promoter containing a non-coding region at the 3' end upstream of the first codon (Norrby and Oxman, 1990).

PPRV has been isolated in many countries, such as Nigeria (Taylor and Abegunde, 1979), Sudan (El Hag Ali and Taylor, 1984), Saudi Arabia (Abu Elzein et al., 1990), India (Nanda et al., 1996), Turkey (Ozkul et al., 2002), and Iran (Marashi et al., 2017). This virus was also detected for the first time in the European Union, namely in Bulgaria, in 2018 (Altan et al., 2019). Previous studies has identified four genetic lineages in sequence analyses of the F and N protein gene regions in PPRV isolates from around the world (Dhar et al., 2002; Sait and Bilge Dagalp 2019).

According to Food and Agriculture Organization of the United Nations (FAO) data, the goat and sheep population in Iran was estimated to be 63.24 million heads (goats 16.66 head and sheep 46.58 head) in 2020 (FAO, 2022). Small ruminants are widely bred animal species that play a very important role in the rural economy and are fed in an intensive or extensive production system (Peacock, 2008). Due to this virus, outbreaks and death cases have been reported in different areas of Iran at different times, and it causes large economic losses each year. Abdoullahpour et al. (2006) reported a high rate of mortality in a flock of sheep (430 deaths) in the 2004 outbreaks in Tehran province, Iran. In December 2012, an outbreak of PPR in a sheep flock in Karaj and Alborz provinces with 50% morbidity and 60% mortality rates was reported pathologically, clinically, and virologically (Mohebbie et al., 2018). In a study conducted in the south of Iran, the seropositivity rates among the herds of sheep and goats were 13.7% and 8.3%, respectively (Shadmanesh, 2014). In another study performed between 2009 - 2012, the PPRV seropositivity rate was found to be 53.38% (268/502) in small ruminants in nine different provinces of Iran (Babaoglu et al., 2021). In winter 2019, in two unvaccinated goat flocks with clinical signs of PPR infection and a mortality rate of 10%, viral nucleic acid was detected in all goat samples (oral swabs and blood samples) in the province of Alborz in the north of Iran (Alidadi et al., 2021). In previous studies, phylogenetic analysis based on F and N proteins showed that the PPRVs circulating in Iran are involved in the IV_{th} genetic lineage (Esmailzadeh et al., 2011; Shahriyari et al., 2019; Alidadi et al., 2021). Based on this data, the present study was designed to investigate the circulation and molecular characterization of PPRV strains in small ruminants in Iran.

MATERIALS AND METHODS

Ethical statement

The study was approved by the Ankara University Animal Experiments Local Ethics Committee (Decision No. 2010-56-281) and Ministry of Agriculture and the Forestry of Republic of Turkey (Approval No. 18.01.2010-001632).

Study area and sample collection

In this study, samples were taken from sheep and goat flocks suspected of having PPRV infection and reported to private veterinary clinics during 2009-2012. They were housed in publicly owned farms in different regions of Iran, especially in provinces bordering other countries (Figure 1). At the time of sampling, the

mentioned areas were not vaccinated against PPRV infection. A total of 341 animal specimens were collected from 271 sheep and 70 goats with clinical signs (pneumonia, ocular discharge, diarrhea, stomatitis) of PPRV infection and from slaughtered or dead small ruminants suspected of having PPRV infection. These samples, including anticoagulant blood samples (n = 296), nasal swabs (n = 5), and tissue samples (n = 5), were collected from 12 different provinces in Iran (Table 1). The collected samples were transported under cold-chain conditions to the virology department lab at the Ankara University Faculty of Veterinary Medicine.

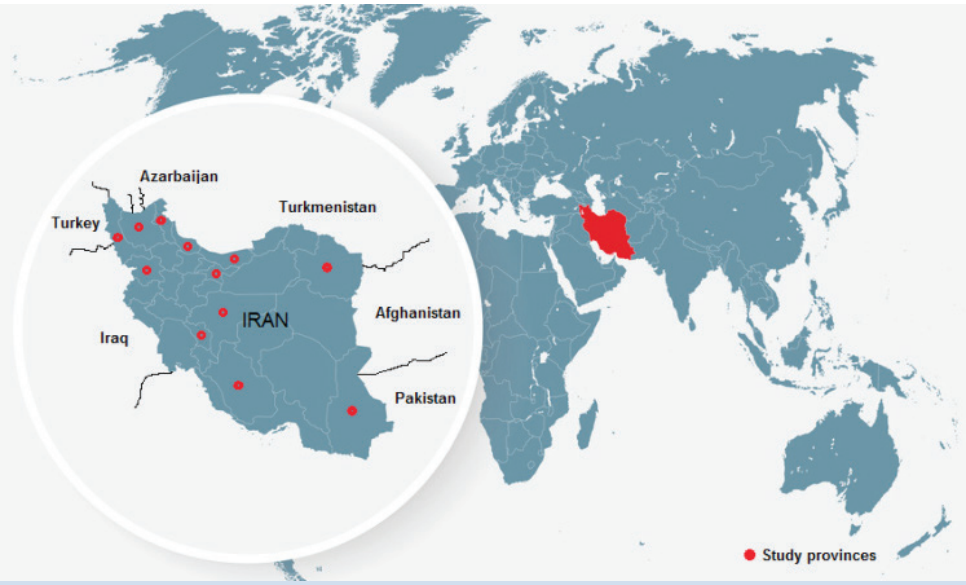

Figure 1. The geographical distribution of the present study samples, taken from 12 provinces on the map of Iran (red circles), and the location of neighboring countries

Table 1. Distribution of the samples collected in this study by province, animal species, sample types, and the number of samples found positive in RT-real time qPCR and conventional RT-PCR assays

Province	Animal Species	Samples			RT-real time PCR	RT-PCR
		Leucocyte	Swab	Tissue	Positive	Positive
Ardabil	Sheep	24	--	--	--	--
	Goat	4	--	--	--	--
Khorasan	Sheep	--	--	1	--	--
	Goat	--	--	2	2	2
East Azarbaijan	Sheep	120	--	--	38	14
	Goat	--	--	--	--	--
Esfahan	Sheep	16	--	--	5	3
	Goat	4	--	4	4	--
Fars	Sheep	1	--	2	1	--
	Goat	10	--	6	10	--
Gilan	Sheep	21	--	--	--	--
	Goat	3	--	--	--	--
Kordestan	Sheep	--	--	--	7	--
	Goat	8	--	--	--	--
Mazandaran	Sheep	--	--	--	--	--
	Goat	7	--	--	--	--
Shahrekord	Sheep	46	--	--	11	--
	Goat	8	--	--	--	--
Sistan-Baluchestan	Sheep	--	--	15	4	--
	Goat	--	1	8	1	1
Tahran	Sheep	--	--	--	--	--
	Goat	5	--	--	2	1
West Azarbaijan	Sheep	19	4	2	13	5
	Goat	--	--	--	--	--
Total	**341**	**296**	**5**	**40**	**102**	**26**

Construction of the standard reference plasmid for PPRV quantitation

In order to construct the standard reference plasmid, a conventional RT-PCR assay was performed on the control virus (PPRV Nigeria 75/1 vaccine strain) according to procedures previously described (Kerur et al., 2008). The PCR product (464 bp) after purification (Roche, Germany) was inserted into the pJET 1.2/ blunt vector and used to transform *Escherichia coli* (JM 109 competent) (Fermentas, Lithuania). Plasmid DNA obtained after cloning was photographed with a 1% agarose gel and checked by double cutting with restriction endonuclease enzymes (BglII). Then, plasmid DNA was measured, and concentrations were converted into copies per microliter for real time-qPCR purposes (Salvi et al., 2008). To calculate the copy number of the standard reference DNA molecules, the formula "plasmid copies/µl = (C×NA)/ (MW×109)" was used. For the plasmid copy number to construct a standard curve for a quantitative, real time-qPCR assay performed in 7-step dilutions, calculations were made according to log ten bases and with a negative control (Rotor-Gene Q6000, Qiagen, Germany).

Viral RNA extraction, reverse transcription (RT), real time-qPCR, and RT-PCR

Viral RNA extraction was performed on 341 samples by the method described by Chomczynski and Sacchi (1987). Viral RNAs were translated into cDNA to be used for real time-qPCR, conventional RT-PCR, and sequence analysis. For this purpose, the RevertAid first strand cDNA synthesis kit was used according to the manufacturer's instructions (ThermoScientific, USA).

To detect PPRV nucleic acid in real time-qPCR, synthesized primers, and a probe that encoded the nucleoprotein (N) gene region, as described previously (Bao et al., 2008). The threshold line constructed was used in parallel with the plasmid standards for the assessment of positive and negative samples. Samples that did not intersect with this line were considered negative, and quantitation was not performed within the scope of the study. As a result of optimization processes and RT-real time-qPCR assay, the desired positivity graph was obtained. Conventional RT-PCR was applied to the samples that were found positive in RT-real time-qPCR for sequence analysis according to the protocol described by Kerur et al. (2008).

Sequencing, secondry protein structure and phylogenetic analysis

Partial N gene sequences of sixteen animal samples were obtained on a CEQ 8000 genetic analyzer using the Dye Terminator Loop Sequencing Kit. (DTCS Beckman Coulter, USA). Viral sequences were aligned with regional, reference, and global viruses obtained from GenBank. The protein analysis section of the CLC bio program (CLC Main Workbench v5.0) was used to perform multiple alignments of the 80 amino acid long region of the gene encoding the N protein in order to predict its secondary structure. Thus, we will have an idea about the secondary structure of the amino acid sequences.

Phylogenetic analysis was constructed based on nucleotid sequences to reveal the genetic relationship between vaccine strains, RPV-Kabete O(Uo2679) sequence as an outgroup and other circulating PPRV country strains and the all sixteen sequences obtained in this study. For this, bootstrap analysis (500 replications and 111 random matches) was performed according to the maximum likelihood (ML) method using MEGA 5. Kitamura's double parameter was used in the analysis, and the bootstrap values were shown on the phylogenetic map.

RESULTS

The partial N protein was found to be positive, with the threshold cycle values (CT) ranging from 12 to 32 in parallel with the copy number of the quantitative standard in real time-qPCR. In this assay, the PPRV nucleic acid positivity rate was 29.91% (102/341) for all tested samples, including 31.08% (92/296) of blood samples, 40% (2/5) of nasal swab samples, and 20% (8/40) of tissue samples. The N-gene coding primer pairs and probe sequences used in real time-qPCR are located within the 464-bp long amplicon sequence produced by the N-gene coding primer pairs used in conventional RT-PCR.

A total of 102 field materials (blood: 92, nasal swab: 2, and tissue: 8) defined as positive in real time-qPCR were subjected to RT-PCR specific primers based on the N gene to amplify a 464 bp length product. Positivity rates of 21.73% (20/92) in blood samples, 100% (2/2) in swab samples, and 50% (4/8) were detected in tissue samples. The PPRV positivity rate was determined as 25.50% (26/102) in all samples on the RT-PCR.

As a result of conventional RT-PCR based on the N gene region, sequence analysis was performed for 16 animal samples (blood = 12, nasal swab = 1, and tissue = 3). From 26 samples found positive in RT-

PCR, 16 samples were selected from strong PCR amplicons, not more than one sample from the same animal, and included in sequence analysis. The accession number (JX89847-JX89869) was obtained by submitting each sample to the National Center for Biotechnology Information (NCBI). Information on these samples based on sequence code, province, animal species, and sample type is presented in Table 2. All the partial N-gene sequences of these samples showed 96.8-100% nucleotide identity with each other; they were 95.2-95.6% identical to the PPRV strain (DQ840185) detected in Iran in 1998. The genetic similarity of the viral sequences detected in this study was determined to be 96.4-100% with the PPR viruses detected in Turkey from 2000-2011. As a result of the comparison with the sequence of the Nigeria 75/1 vaccine strain (HQ197753) that was used for the control and prevention of PPRV infection in Iran, nucleotide identity was detected at 88.2-89% (Table 3).

Table 2. The samples were identified in the sequence, secondary protein structure, and phylogenetic analyses in the present study

No	Sample ID	Location (province)	Animal	Sample type	Accession No
1	IR/TBZ1/10	East Azarbaijan	Sheep	Blood	JX898847
2	IR/MSHD339/11	Central Khorasan	Goat	Lymph node	JX898848
3	IR/TBZ2/10	East Azarbaijan	Sheep	Blood	JX898849
4	IR/TBZ51/10	East Azarbaijan	Sheep	Blood	JX898850
5	IR/TBZ54/10	East Azarbaijan	Sheep	Blood	JX898851
6	IR/TBZ58/10	East Azarbaijan	Sheep	Blood	JX898852
7	IR/TBZ59/10	East Azarbaijan	Sheep	Blood	JX898853
8	IR/TBZ64/10	East Azarbaijan	Sheep	Blood	JX898854
9	IR/TBZ65/10	East Azarbaijan	Sheep	Blood	JX898855
10	IR/TBZ66/10	East Azarbaijan	Sheep	Blood	JX898856
11	IR/TBZ83/10	East Azarbaijan	Sheep	Blood	JX898857
12	IR/TBZ89/10	East Azarbaijan	Sheep	Blood	JX898858
13	IR/URM329/11	West Azarbaijan	Sheep	Blood	JX898859
14	IR/URM332/11	West Azarbaijan	Sheep	Nasal Swab	JX898860
15	IR/URM333/11	West Azarbaijan	Sheep	Lung	JX898861
16	IR/ZHN336/11	Sistan-Baluchestan	Goat	Lymph node	JX898862

Table 3. Nucleotide percent identity-divergency ratio of the PPRV partial sequence of the N gene: Samples written in red are PPRV sequences from the current study; black are sequences obtained from the GenBank; and green is an outgroup sequence (RPV) obtained from the GenBank

Percent Identity

Seq	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	
1		96,8	88,2	93,3	53,3	96	94,9	95,2	96	95,6	95,6	95,6	95,6	95,6	95,6	95,6	96	95,6	95,2	95,6	95,2	95,6	95,2	95,2	95,6	1 Iran98(DQ840185)
2	3,2		89	94,5	55,6	96,8	98	98,4	99	98,8	98,8	98,8	98,8	98,8	98,8	98,8	99	98,8	98,4	98,8	98,4	98,8	98,4	98,4	98,8	2 Iraq-11(JF969755)
3	11,8	11		87	52,1	87,8	87,8	88,2	89	88,6	88,6	88,6	88,6	88,6	88,6	88,6	89	88,6	88,2	88,6	0,89	88,6	88,2	88,2	88,6	3 Nigeria75-1(HQ197753)
4	6,7	5,5	13		53,3	96	93,3	93,7	94	93,3	93,3	93,3	93,3	93,3	93,3	93,3	93	93,3	93,3	93,3	92,9	93,3	93,7	93,7	93,3	4 Pakistan-10(JN009674)
5	46,7	44,4	47,9	47		54,9	56,4	56,4	55,2	55,2	55,2	55,2	55,2	55,2	55,2	55,2	55	55,2	54,9	55,2	54,9	55,2	56,4	56,4	55,2	5 RPV-Kabete O(U02679)
6	4	3,2	12,2	4	45,1		94,9	95,2	96	95,6	95,6	95,6	95,6	95,6	95,6	95,6	96	95,6	95,2	95,6	95,2	95,6	95,2	95,2	95,6	6 Tajikestan-04(DQ840198)
7	5,1	2	12,2	6,7	43,6	4,8		99,6	97	96,8	96,8	96,8	96,8	96,8	96,8	96,8	97	96,8	96,4	96,8	96,4	96,8	99,6	99,6	96,8	7 TR-AGRI-11-16(JQ519909)
8	4,8	1,6	11,8	6,3	43,6	4,8	0,4		98	97,2	97,2	97,2	97,2	97,2	97,2	97,2	97	97,2	96,8	97,2	96,8	97,2	100	100	97,2	8 TR-EDIRNE(JX117878)
9	4	0,8	11	6,3	44,8	4	2,8	2,4		99,6	99,6	99,6	99,6	99,6	99,6	99,6	100	99,6	99,2	99,6	99,2	99,6	97,6	97,6	99,6	9 Turkey00(AJ563705)
10	4,4	1,2	11,4	6,7	44,8	4,4	3,2	2,8	0		100	100	100	100	100	100	100	100	99,6	100	98,8	100	97,2	97,2	100	10 IR-TBZ1-10(JX898847)
11	4,4	1,2	11,4	6,7	44,8	4,4	3,2	2,8	0	0		100	100	100	100	100	100	100	99,6	100	98,8	100	97,2	97,2	100	11 IR-MSHD339-11(JX898848)
12	4,4	1,2	11,4	6,7	44,8	4,4	3,2	2,8	0	0	0		100	100	100	100	100	100	99,6	100	98,8	100	97,2	97,2	100	12 IR-TBZ2-10(JX898849)
13	4,4	1,2	11,4	6,7	44,8	4,4	3,2	2,8	0	0	0	0		100	100	100	100	100	99,6	100	98,8	100	97,2	97,2	100	13 IR-TBZ51-10(JX898850)
14	4,4	1,2	11,4	6,7	44,8	4,4	3,2	2,8	0	0	0	0	0		100	100	100	100	99,6	100	98,8	100	97,2	97,2	100	14 IR-TBZ54-10(JX898851)
15	4,4	1,2	11,4	6,7	44,8	4,4	3,2	2,8	0	0	0	0	0	0		100	100	100	99,6	100	98,8	100	97,2	97,2	100	15 IR-TBZ58-10(JX898852)
16	4,4	1,2	11,4	6,7	44,8	4,4	3,2	2,8	0	0	0	0	0	0	0		100	100	99,6	100	98,8	100	97,2	97,2	100	16 IR-TBZ59-10(JX898853)
17	4,4	1,2	11,4	6,7	44,8	4,4	3,2	2,8	0	0	0	0	0	0	0	0		100	99,6	100	98,8	100	97,2	97,2	100	17 IR-TBZ64-10(JX898854)
18	4,4	1,2	11,4	6,7	44,8	4,4	3,2	2,8	0	0	0	0	0	0	0	0	0		99,6	100	98,8	100	97,2	97,2	100	18 IR-TBZ65-10(JX898855)
19	4,8	1,6	11,8	6,7	45,1	4,8	3,6	3,2	0,8	0,4	0,4	0,4	0,4	0,4	0,4	0,4	0,4	0,4		99,6	98,4	99,6	96,8	96,8	99,6	19 IR-TBZ66-10(JX898856)
20	4,4	1,2	11,4	6,7	44,8	4,4	3,2	2,8	0	0	0	0	0	0	0	0	0	0	0,4		98,8	100	97,2	97,2	100	20 IR-TBZ83-10(JX898857)
21	4,8	1,6	11	7,1	45,1	4,8	3,6	3,2	0,8	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,6	1,2		98,8	96,8	96,8	98,8	21 IR-TBZ89-10(JX898858)
22	4,4	1,2	11,4	6,7	44,8	4,4	3,2	2,8	0	0	0	0	0	0	0	0	0	0	0,4	0	1,2		97,2	97,2	100	22 IR-URM329-11(JX898859)
23	4,8	1,6	11,8	6,3	43,6	4,8	0,4	0	2,4	2,8	2,8	2,8	2,8	2,8	2,8	2,8	2,8	2,8	3,2	2,8	3,2	2,8		100	97,2	23 IR-URM332-11(JX898860)
24	4,8	1,6	11,8	6,3	43,6	4,8	0,4	0	2,4	2,8	2,8	2,8	2,8	2,8	2,8	2,8	2,8	2,8	3,2	2,8	3,2	2,8	0		97,2	24 IR-URM333-11(JX898861)
25	4,4	1,2	11,4	6,7	44,8	4,4	3,2	2,8	0	0	0	0	0	0	0	0	0	0	0,4	2,8	1,2	0	2,8	2,8		25 IR-ZHN336-11(JX898862)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	

Divergency

In the sequence analysis of the partial N gene based on the alignment of the amino acids, no divergence was detected in any amino acid. In comparison with the Turkey2000 (AJ563705) full gene sequence, except for three samples (IR/TBZ89/10, IR/URM332/11, and IR/URM333/11), it was determined that A→G change in the 1363rd nucleotide caused a K→R amino acid change. As a result of the alignment of the amino acids with the Nigeria 75/1 vaccine strain sequence, it was determined that there were 19 amino acid changes (Figure 2).

Using the CLC bio program (CLC Main Workbench v5.0) with multiple alignments, structural similarity was detected between the viruses isolated from Iran and Turkey in the analysis of the secondary protein structure of the approximately 80 amino acid long region of the gene encoding the N protein of the 16 sequences originated from this study. These similarities in alpha-helix and beta-leaf structures were shown to be in similar patterns for all PPRV strains of Iranian origin, except for two viruses (IR-URM332-11 and IR-URM333-11) detected in West Azarbaijan province. It was identified that 20 of the 80 amino acids in the N protein of Iranian strains differed from the vaccine strain, while the viruses detected in Turkey showed identity with the Nigeria75/1 vaccine strain (Figure 3).

All sixteen viral sequences originating from this study with accession numbers were included in the phylogenetic analysis. The RPV-Kabete O (Uo2679) sequence was used as an outgroup, and other PPRV sequences (n = 38) with data in the GenBank were

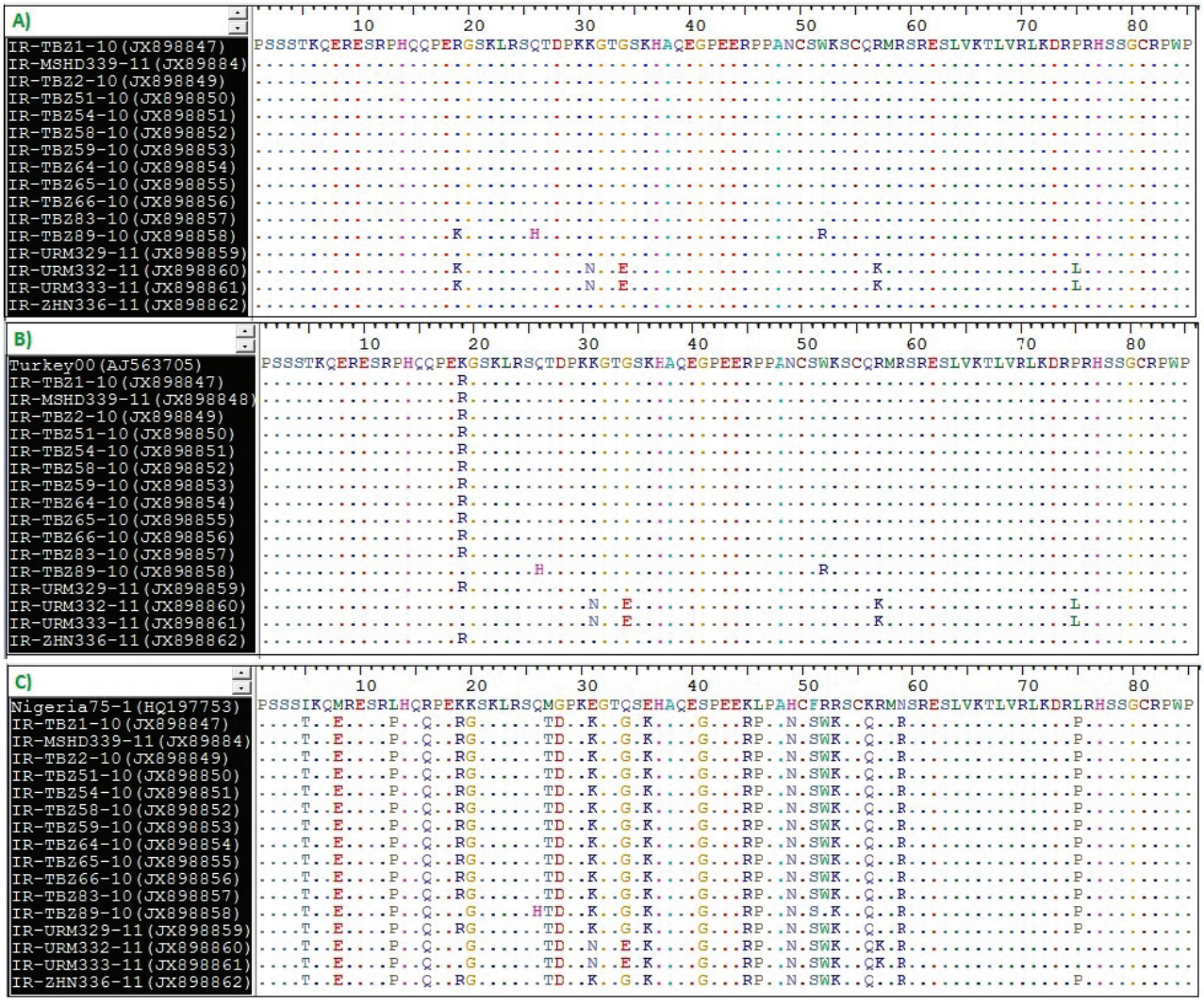

Figure 2. The alignment of PPRV partial N protein sequences obtained in this study at the amino acid level; (A) with among them, (B) with the Turkey2000 reference isolate, and (C) with the Nigeria 75/1 vaccine strain

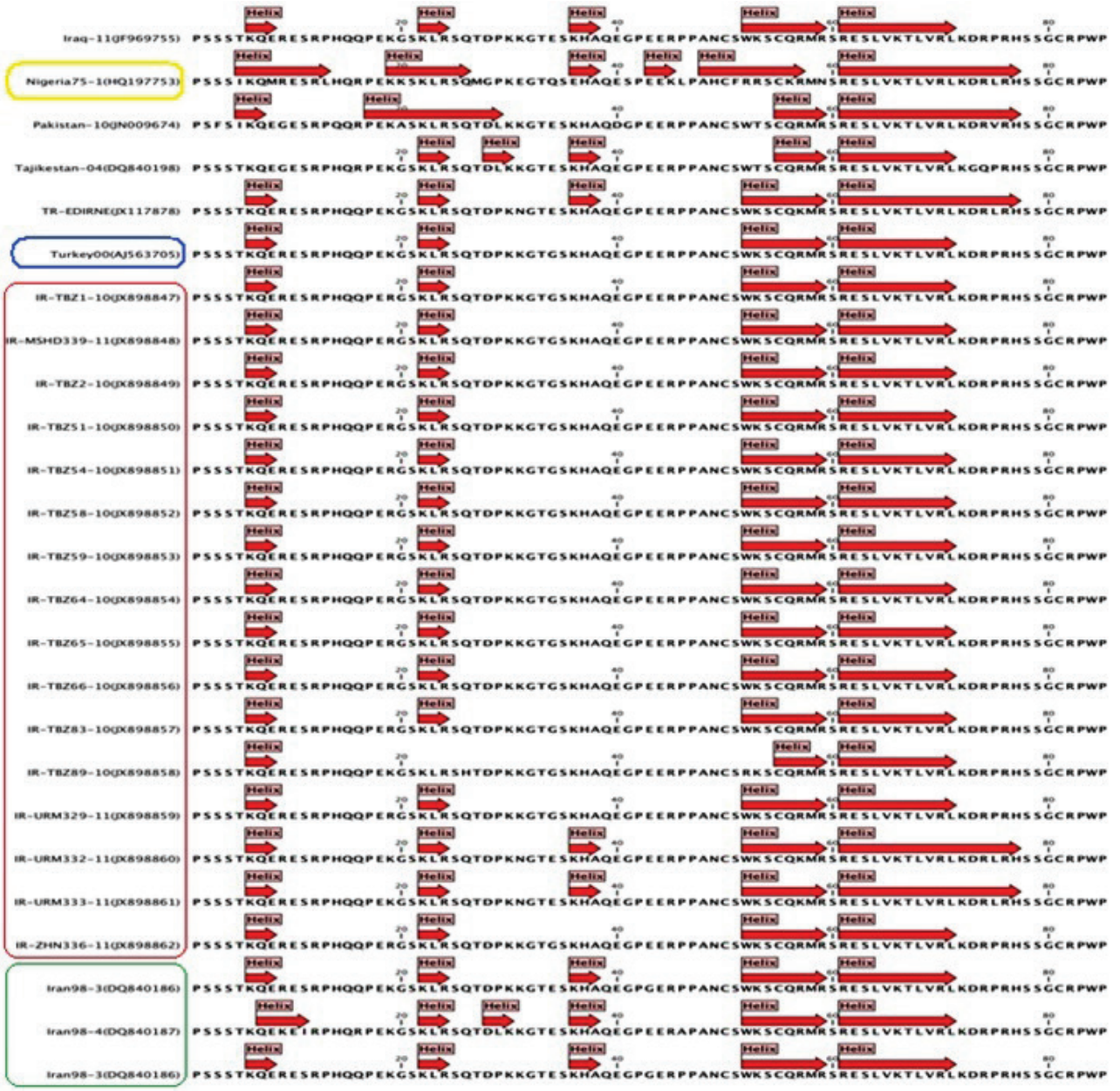

Figure 3. Analysis of the secondary structure of the 80 amino acid long region of the gene encoding the PPRV partial N protein by using multiple alignments metoth of CLC bio program. Samples in the present study are located in the red rectangle

used in a phylogenetic tree drawn with bootstrap analysis (500 replications and 111 random matches) by the maximum likelihood (ML) method using the partial sequences of the N gene that originated from this study. According to the phylogenetic tree, the sequences of the viruses in question shared the same branch, are identical with each other, and are located in the same lineage group (lineage IV). Although two viral sequences (IR-URM332-11, IR-URM333-1) were obtained from West Azerbaijan province and located in the same branch, it was observed that they were closer to the sequences of viruses detected in Turkey after 2010. In addition, it was determined that sequences (n = 14) obtained from four provinces (Central Khorasan, East Azarbaijan, Sistan-Baluchestan, West Azerbaijan) of Iran showed relationships with the sequences of viruses detected in Iran in 1998 (DQ840185), Turkey in 2000 (AJ563705), and Iraq in 2011 (JF969755). Figure 4 shows the phylogenetic tree constructed from all samples, the outgroup sample, and viral sequences (n = 38) with data in GenBank.

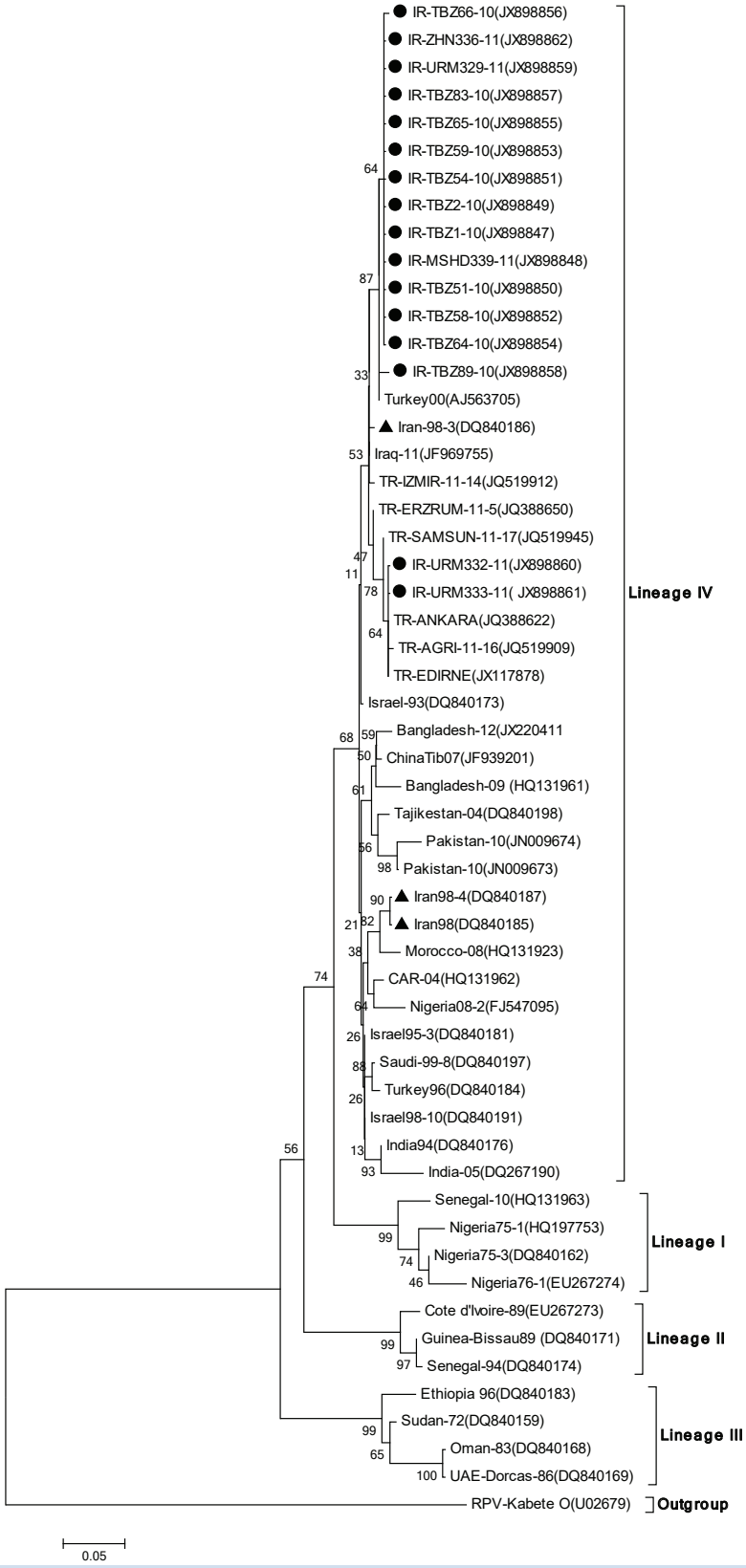

Figure 4. Phylogenetic tree constructed by the maximum likelihood (ML) method using the PPRV partial sequences of the N gene and other PPRV sequences with data in the GenBank. Sixteen viral sequences with accession numbers (JX89847 to JX89869) were included in the phylogenetic analysis. In addition, the RPV-Kabete O(Uo2679) sequence was used as an outgroup in this analysis. The black circle sign (●) indicates the sequences from the present study, and the black triangel sign (▲) indicates the samples previously isolated in Iran

DISCUSSION

For many years, PPRV infection was considered an African-specific disease, localized in West and Middle Africa. Later, the infection was identified as endemic in the Middle East, Arabian Peninsula, Turkey, Iran, Iraq, Pakistan, India, Bangladesh, China, and Kazakhstan outside of Africa, partly because of improved detection systems (Losos, 1994; Taylor et al., 2002; Wang et al., 2009). The disease has recently been reported in Europe in Georgia (Donduashvili et al., 2018) and Bulgaria (Altan et al., 2019). PPRV infection is one of the most important animal diseases in Iran, causing a total loss of 1.5 million US dollars in sheep and goats. After the first detection of PPRV infection in Iran in 1995, the disease spread throughout the country and caused great economic losses (Alidadi et al., 2021). In the following years, despite the vaccination of susceptible animals with the RPV and PPRV vaccines and some control measures, varying rates of PPR infection were detected in different provinces of Iran (Abdollahpour et al., 2006; Bazarghani et al., 2006; Esmailzadeh et al., 2011; Shadmanesh, 2014; Mohebbi et al., 2018; Shahriyari et al., 2019; Alidadi et al., 2021).

Many researchers reported that the real time RT-PCR (rtRT-PCR) technique based on the N protein-encoding gene is high throughput, sensitive, and provides a rapid diagnosis of PPRV infection. Bao et al. (2008) have shown that the detection limit of the rtRT-PCR assay is 8.1 copies per reaction, increasing the detection rate from 46.7% to 73.3%. This assay is capable of detecting 20% more positive field samples and is faster to perform, making this assay an ideal tool for monitoring large numbers of at risk or diseased animals compared to the conventional PCR method. It was also reported that viral RNA could be detected up to a minimum of 32 copies (Ct = 39) per reaction, especially in samples with low viral RNA loads (Kwiatek et al., 2010). In rtRT-PCR, four different genetic lineages of the PPRV were detected by targeting the N gene. Using reference strains and field materials, they showed that rtRT-PCR was much more sensitive (10 genome copies per reaction) than conventional RT-PCR (Batten et al., 2011).

Multiple molecular analyses are needed to evaluate the evolution of RNA viruses and the resulting mutants in the replication process, as well as the genetic similarity and difference between strains. In this study, the RT-real time-qPCR method targeting the gene region encoding the N protein was used to detect the viral nucleic acid in field materials taken from 341 animals with clinical signs of PPRV infection. As a result of the assay, the presence of viral RNA was quantitatively determined by the minimum detection limit, CT values, and threshold line of all samples, and PPRV nucleic acid was detected at a rate of 29.91% (102/341).

PPRVs have been identified in four different lineages and have been reported to be effective in the investigation of virus spread in the world. Genetic identity and divergences are based on the partial sequence of the fusion protein (F) gene (Ozkul et al., 2002) and the nucleoprotein (N) gene region (Kwiatek et al., 2007). Studies based on N gene sequence analysis show that the clustering of PPR viruses in different lineages is better and thus more accurate epidemiologically (Kwiatek et al., 2010). In the present study, to evaluate the molecular identity and divergence among the viral sequences, multiple molecular analyses were performed. All sixteen samples (Table 2) from four different provinces of Iran were included in the sequence, secondary N protein structure, and phylogenetic analyses. At the nucleotide level, the sixteen PPRV N partial gene sequences in this study showed 96.8-100% identity among them, 97.6-100% identity with the Turkey2000 reference strain, 95.2-99.6% identity with the sequences determined as of 2010 in Turkey, and 88.2-89% identity with the Nigeria 75/1 sequence used as a vaccine strain in Iran. The percent identity of nucleotides revealed that viruses that have been circulating in Iran since 1998 are similar (95.2-96%) and show genetic identity with viruses circulating in neighboring countries obtained from Genbank (Table 3). On the basis of neighboring countries, the nucleotide similarity for Iraq, Pakistan, and Turkey2000 reference isolate is 98.4-99%, 92.9-93.7%, and 97.6-100%, respectively. However, they were in the same genetic line.

In the comparison based on amino acids, no amino acid divergence was detected among the N gene sequences obtained in this study. As a result of alignment with the Turkey00 reference strain, it shows that there was an amino acid (K→R) change in only thirteen samples out of sixteen sequences. In alignment with the Nigeria 75/1 sequence, differences were detected in nineteen amino acids. In the analysis of 80 amino acid length, at least 25% of this protein has changed compared to the past. The main reason for this change is that there will be different PPRV strains belonging to the close geography to which it may be

exposed.

According to the current study's phylogenetic analysis, all sixteen animal sequences have been located in lineage IV, as previously revealed by other molecular studies in Iran (Shahriyari et al., 2019; Alidadi et al., 2019). In a phylogenetic tree drawn by using the partial sequences of the N gene and other PPRV sequences with data in the GenBank, it was found that the fourteen animal sequences of the viruses in question are similar to each other and originated from the same genetic lineage (lineage IV). Two (JX898860 and JX898861) out of sixteen sequences located in lineage IV and showing structural differences in secondary protein structure analysis were found in a different branch in phylogenetic analysis. They were detected in the province of West Azerbaijan, located in the northwest of Iran (on the Turkey and Iraq border), showing great identity with the strains detected in Turkey and Iraq as of 2010. It was found that the viruses obtained from other provinces showed identity with the isolates detected in Iran in 1998, Turkey in 2000, and Iraq in 2011. Close contact is essential for the transmission of this infection. For this, there must be infected animals from neighboring countries. The identity observed between Iran, Turkey, and Iraq sequences indicates that the same strains cause disease outbreaks, and the phylogenetic tree reveals that there is animal movement from eastern to western Iran and in the triangular border region of Iran, Iraq, and Turkey. Thus, the classification of PPRV into lineages based on the N gene sequences appeared to yield a better picture of genetic characterization for the virus strains once again.

The FAO and the World Organization for Animal Health (OIE) have targeted the eradication of PPRV infection by 2030. The PPR global eradication programme (PPR GEP) lays the foundation for eradicating PPR by first reducing its prevalence in the countries currently infected. Due to its location, Iran borders many countries with economically important infectious diseases. Some control strategies, such as vaccination and restriction of animal movements, have been successful in eradicating RPV infection. A control measure like this will reduce the prevalence of PPRV infected animals and prevent the spread of the virus in a country; however, the structure of the small ruminant population is substantially different from that of cattle, and the same strategies can be applied to PPR infection (Baron et al., 2011).

CONCLUSION

The origin of the PPRV outbreaks is not known exactly, but the results of this study have revealed that they developed in Iran with viruses from the same group and that the animal movement and legal or illegal animal trade played a key role. Also, it should not be forgotten that wild animals are carriers and are one of the critical factors in the origin of the PPRV infection. For the implementation of control eradication programs, animal movements and trade should also be considered in the PPR GEP and by country authorities, and multiple molecular analyses based on the N gene are recommended to obtain more data on the genetic characteristics of isolates circulating in the country. As a result, performing multiple molecular analyses of PPRV would be useful for elucidating viral pathogenesis, revealing epidemiological dynamics, and developing vaccines for local strains.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTION

AR Babaoglu and S Bilge Dagalp contributed to the study conception and design. Samples were collected by AR Babaoglu and M Mahzounieh. Material preparation, laboratory tests and analyses were performed by all authors. The first draft of the manuscript was written by AR Babaoglu and S Bilge Dagalp, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscrip.

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