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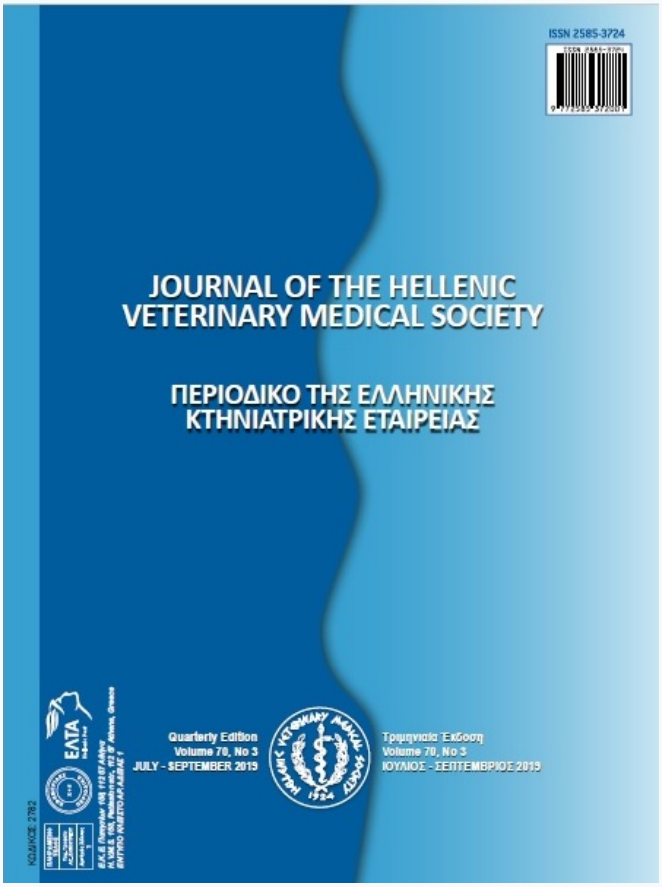

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Research article
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Molecular analysis of Peste des Petits Ruminants Virus from outbreak in Turkey during 2010-2012

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ABSTRACT. The aim of the study is to determine the epizootiology of *Peste des petits ruminants* (PPR) in Turkey during 2010-2012, using molecular genotyping.

Samples of blood (n=193), swab (n=7) and tissue (n=374) were collected from sheep (n=473) and goats (n=101) suspected of having PPRV infection from an outbreak in 50 provinces of Turkey during 2010–2012. These samples (n=574) were tested using reverse transcription polymerase chain reaction (RT-PCR) and real-time reverse transcription polymerase chain reaction (RT-qPCR) targeting selected parts of the fusion (*F)* and the nucleocapsid (*N)* genes. Positivity ratios were 35.5%, 39.3%, and 44.4% with regards to RT-PCR targeting the *F* and the *N* genes, and RT-qPCR targeting the latter gene (*N*), respectively. The overall positivity rate was 45.8%.

For sequence analyses, *F*-gene (n=53) and *N*-gene (n=60) positive samples representing different provinces were selected. After phylogenetic analysis, the circulating PPRV was located in lineage IV according to two gene regions. The *F*-gene partial sequence analysis at the nucleotide level showed 98.2-100% resemblence among 53 for *F*-gene, and 97.9-98.9% and 91.3-92.4% to Turkey2000 and Nigeria75/1 sequences, respectively. The *N*-gene partial sequence analysis at the nucleotide level showed 94.2-100% resemblence among 60 for *N*-gene, and 94.2-98.3% and 89.3-90.9% to Turkey2000 and Nigeria75/1 sequences, respectively.

The result of this study indicates that PPRV infection is enzootic in Turkey, and belongs to the lineage IV, which is present in three haplogroup. The phylogenic analysis indicates the spread of the virus is associated with unauthorized movement of stock.

Keywords: Fusion gene, molecular epidemiology, Nucleocapsid gene, Peste des petits ruminants virus, Sequence analysis,

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INTRODUCTION

Peste des petits ruminants (PPR) is a highly contagious and economically important viral disease affecting small ruminants, caused by *Peste des petits ruminants virus* (PPRV) classified within the genus *Morbillivirus* in the family *Paramyxoviridae* (Banyard *et al.*, 2010). PPRV is a nonsegmented, negative sense, single-stranded RNA virus that encodes six structural proteins; nucleocapsid protein (*N*), phosphoprotein (*P*), matrix protein (*M*), fusion protein (*F*), hemagglutinin (*H*), RNA-dependent RNA polymerase (*L*), and two nonstructural proteins (*V* and *C*). According to phylogenetic analysis, PPRV can be classified into four genetic lineages based on the fusion (*F*) and/or nucleocapsid (*N*) gene (Couacy-Hymann *et al*., 2002; Ozkul *et al*., 2002; Kwiatek *et al.,* 2007; Munir *et al*., 2012a; Munir, 2012b; Mahajan *et al*., 2014). These lineages are generally correlated with the geographical distribution of the virus (Shaila *et al*., 1996). Lineage IV is prevalent in Asian countries although all four lineages have been found in Africa (Libeau *et al*., 2014).

The presence of PPR in Turkey was first reported in lambs in 1993 based on postmortem and immunohistochemical findings (Alcigir *et al*., 1996). Then it was detected serologically and virologically in 1998 (Tatar and Alkan, 1999). The presence of the disease in Turkey was declared officially by the World Organisation for Animal Health (OIE) in 1999 (Food and Agriculture Organization, 2012). PPR has been a notifiable disease in Turkey since 1997. The complete genome of PPRV isolated from infected sheep in 2000 was sequenced in 2004 (isolate Turkey2000, GenBank acc. No. AJ849636). Phylogenetic analysis revealed that Turkey2000 is closely related to lineage IV, originating from the Middle East, the Arabian peninsula and Asia (Bailey *et al*., 2005). Since 1993, PPR constitutes a significant health threat for sheep and goat farmers in Turkey despite to the unnual vaccination program. Together with PPRV, Rinderpest virus, the etiological agent of Rinderpest or cattle plague, is grouped in the same family *Paramyxoviridae* (Banyard *et al.*, 2010). Eradication status of Rinderpest in Turkey was declared by OIE (Food and Agriculture Organization, 2011), so experiences gained during in this eradication process had become very importand for designing of control and eradication program of PPR.

In the light of information mentioned above, the aim of the study was to determine the epizootiology of PPR in Turkey during 2010-2012, using molecular genotyping.

MATERIALS AND METHODS

Clinical Specimens

The samples (193 blood, 7 nasal swab, 162 lung, 116 spleen, 4 rectum, 3 small intestine and 89 lymph node) were taken from sheep (n=473) and goats (n=101) raised in 50 provinces in Turkey during

Figure 1. Sampled provinces (marked with yellow) and the positivity rates per provinces: **Province's number:** *The positivity rate per province*; **1:***59.25%*; **2:***57.14%*; **3:***50%*; **4:***66.66%*; **5:***28.57%*; **6:***100%*; **7:***50%*; **8:***100%*; **9:***38.46%*; **10:***33.33%*; **11:***100%*; **12:***0%*; **13:***100%*; **14:***44.44%*; **15:***0%*; **16:***30.95%*; **17:***33.33%*; **18:***100%*; **19:***16.66%*; **20:***25%*; **21:***100%*; **22:***50%*; **23:***50%*; **24:***36%*; **25:***100%*; **26:***40.42%*; **27:***53.33%*; **28:***100%*; **29:***0%*; **30:***52.63%*; **31:***60%*; **32:***36.84%*; **33:***55.55%*; **34:***50%*; **35:***75%*; **36:***33.33%*; **37:***47.61%*; **38:***50%*; **39:***100%*; **40:***66.66%*; **41:***46.66%*; **42:***100%*; **43:***25%*; **44:***100%*; **45:***100%*; **46:***100%*; **47:***61.53%*; **48:***80%*; **49:***100%*; **50:***0%.*

2010–2012 (Figure 1). Suspecion of PPRV infection was established based on clincal findings. Most of the animals had PPR symptoms such as fever, discharges from the eyes and nose, mouth lesions, respiratory distress, sometimes diarrhea, death and abortion. All the collected samples were kept at -80ºC for the nucleic acid extraction. All procedures performed in this study involving animals were in accordance with the ethical standards of the Animal Experiments Local Ethics Committee of Ankara University (Approval date: 24/03/2010, Approval number: 2010-59-298).

Detection of RNA Using RT-qPCR Assay

Detection of RNA belonging to the *N* gene segment of PPRV using RT-qPCR was performed as previously described (Kwiatek *et al*. 2010). According to this, the RT-qPCR amplification conditions for *N* gene were as follows: an initial reverse transcription for 30 mins at 50ºC and RT-inactivation/Taq-activation for 15 mins at 95ºC, followed by 40 cycles of amplification (95ºC for 1 min, 60ºC for 1 min). All samples were tested further using *F* and *N* genes conventional RT-PCR.

Detection of RNA Using Conventional RT-PCR Assays

PPRV RNA was extracted from tissue samples using Qiagen RNeasy Mini Kit (Qiagen, Germany) and from blood samples using High Pure Viral Nucleic Acid Kit (Roche, Germany) according to the manufacturers' instructions. Extracted PPRV RNA was stored at -80ºC until further use.

The One-step RT-PCR kit (Qiagen, Germany) was used for PPRV RNA detection. The assay was carried out in two seperate reaction mixtures for *F* and *N* genes. Each 20µl reaction mixture contained 10pmol primers, 4µl of 1x One-Step RT-PCR buffer, 0.8µl of 10mmol dNTPs, 0.8µl of One-Step RT-PCR enzyme mix and 3µl of extracted (Ozkul *et al*., 2002; Kerur *et al*., 2008). The RT-PCR amplification conditions for *F* and *N* genes were as follows: a reverse transcription step of 30 mins at 50ºC and 15 mins at 95ºC, followed by 40 cycles at 94ºC for 1 min, 50ºC for 1 min and 72ºC for 2 mins, with a final extension step at 72ºC for 10 mins. RT-PCR products then were electrophoresed in 1.5% agarose gel and visualized by ethidium bromide staining. A 448bp fragment for *PPRV-F* primers and a 463bp fragment for *PPRV-N* primers were amplified in positive reactions.

Lyophilized freeze-dried live PPR vaccine (Nigeria75/1 isolate) obtained from commercial PPR vaccine (Vetal Pestvac K, Vetal Inc., www.vetal.com.tr) was used as a reference virus for this study.

Sequencing and Phylogenetic Analysis

At least one positive sample from each province was selected for sequence analysis and purified from gel using a purification kit (High Pure PCR Product Purification Kit, Roche, Germany) and sequenced using the BigDye Terminator kit (v3.1, Applied Biosystems, USA) with an ABI 3130xl DNA Analyzer (Applied Biosystems, USA). Sequence reads were assembled and edited with DNASTar software package (DNAStar Inc., Madison WI; USA, https://www.dnastar.com/). Assembled sequences were also compared with reference publicly available sequences using the MegAlign tool available in the same software. Tables showing sequence similarity were produced (Data not show). Phylogenetic trees were constructed for the 290 and the 238 base pair (bp) fragments of *PPRV-F* and *PPRV-N*, respectively, using the same publicly availabe sequences used for sequence comparison. Neighbor-joining (NJ) trees were constructed using Molecular Evolutionary Genetics Analysis version 5 (MEGA5) software (https://www.megasoftware.net/), based on the evolutionary distances between different sequences calculated by the Kimura two-parameter model (Tamura *et al*., 2011). The confidence level of the NJ tree was assessed with bootstrapping, using 1,000 replicates.

A phylogenetic network was drawn for *PPRV-F* and *PPRV-N* to determine nucleotide substitutions and molecular evolution at *F* and *N* gene sites targeted RT-PCR. The data were processed using DnaSP v.5 software and the star contraction algorithm and median-joining (MJ) network algorithm (Bandelt *et al*., 1999; Forster *et al*., 2001; Librado and Rozas, 2009).

RESULTS

Detection of RNA in the field samples using RT-PCR assays

PPRV RNA was detected using the RT-qPCR assay targeting the *N* gene in 44.4% of the samples (255/574) and using RT-PCR assays targeting the *F* nad *N* genes in 204 (204/574, 35.5%) and 226 samples (226/574, 39.3%), respectively. RT-PCR results for specific test materials were shown in Table 1. Depending on year, positivity rates in the targeted population were 46.0-47.1% and 42.8-44.0% with regards to sheep and goats, respectively (Table 2).

Table 1. Positivity rates according to test materials

PCR	Organs	Blood	Swab	Total Positivity
N gene RT-qPCR	150/374 (40.1%)	100/193 (51.8%)	5/7 (71.4%)	255/574 (44.4%)
F gene RT-PCR	119/374 (31.8%)	81/193 (41.9%)	4/7 (57.1%)	204/574 (35.5%)
N gene RT-PCR	130/374 (34.7%)	92/193 (47.6%)	4/7 (57.1%)	226/574 (39.3%)

Table 2. Positivity rates according to year and species

Year	**Total test material**	**Sheep**	**Goat**	**Total positivity**
2012	74	25/53 (47.1%)	9/21 (42.8%)	34/74 (45.9%)
2011	389	156/339 (46.0%)	22/50 (44.0%)	178/389 (45.7%)
2010	111	38/81 (46.9%)	13/30 (43.3%)	51/111 (45.9%)
Total	574	219/473 (46.3%)	44/101 (43.5%)	263/574 (45.8%)

Table 3. Nucleotide and amino acid substitutions in *PPRV-N* sequences compared to TU00

Position in whole genome	Nucleotide substitutions	Amino acid substitutions	**Position in whole genome**	Nucleotide substitutions	Amino acid substitutions	**Position in whole genome**	Nucleotide substitutions	Amino acide substitutions
1415.nt	G→A	R→K	**1473.nt**	T→C		**1583.nt**	C→T	P→L
1452.nt	A→C	K→N	**1482.nt**	C→T		**1615.nt**	A→G	R→G
1460.nt	G→A	G→E	**1529.nt**	G→A	R→K			

Sequence and phylogenetic analysis

The *F* (n=53) and *N* genes (n=60) sequence corresponding to the strains of the PPRV that were detected, were deposited in GenBank under three groups with the following accession numbers: from JQ388615 to JQ388664; from JQ519907 to JQ519965, and from JX117877 to JX117880. Analysis of the *F* gene nucleotide sequences revealed that the nucleotide sequence identity among *PPRV-F* samples ranged from 98.2% to 100% whereas similarity with previously characterized Turkish isolates (Tu96 and Tu00) ranged from 97.9% to 99.3%. Amino acid sequence identity for *PPRV-F*, Tu96 and Tu00 was 97.8-100%. No amino acid substitutions were detected in *PPRV-F* compared to Tu96 and Tu00. Compared to the Nigeria75/1 vaccine strain, *PPRV-F* had 28 nucleotide substitutions with a similarity of 91.3-92.4%. A phylogenetic tree for *PPRV-F* gene was drawn using sequences analysed in this study, and sequences of strains from Iran, Iraq, Syria, Egypt, India and China (Tibet), and the reference sequences of the four lineages. As shown in figure 2, all *PPRV-F* studied here and other Asian sequences grouped together in lineage IV. The oligonucleotide sequences of strains from Egypt, India and Iraq were more similar than those of other countries (Iran, China (Tibet), Morocco, Kuwait, Pakistan) compared to those isolated in Turkey.

Analysis of the *N* gene nucleotide sequences revealed that the nucleotide sequence identity among *PPRV-N* samples ranged from 97.2% to 100% whereas similarity with previously characterized Turkish isolate (Tu00) was 97.5%-98.9%. Amino acid sequence identity was 94.2-100% among *PPRV-N* samples and 94.2-98.3% for *PPRV-N* and TU00. Nucleotide sequence identity for *PPRV-N* with the Nigeria75/1 vaccine strain were 89.3-90.3%. The phylogenetic tree of *PPRV-N* and other sequences from GenBank was shown in figure 3. All *PPRV-N* samples were clustered into lineage IV, which is exclusive to Asian and Middle East countries. All *PPRV-N* isolates sequenced here and reported earlier were similar to the PPRV isolates obtained from Iran and Iraq. But Turkey96 formed a separate branch within lineage IV with PPRV isolates from Iran, Israel, Nigeria, and

Morocco. Seven-12 nucleotide substitutions were detected between Turkey96 sequence and other *PPRV-N* sequences analysed here (Table 3).

To look for variation among the sequences, a phylogenetic network analysis was drawn for the *PPRV-F* and *PPRV-N* nucleotide sequences. According to this, all *PPRV-F* sequences were clustered under three haplogroups (H1, H2, H3) in lineage IV (Figure 4). As shown in the phylogram, sequences of strains from India (India03-FJ750562) and Iraq (Iraq2009-AY948429) were closest to the Turkish sequences, with one nucleotide substitution. Similarly, all *PPRV-N* sequences were mainly clustered under three haplogroups (H1, H2, H3) in lineage IV (Figure 5). In general, sequences of strains from Iraq (Iraq2011-JF969755) and Iran (JX898860 and JX898861) were closest to the *PPRV-N* sequences analysed here. Especially, sequences of strains from Iran were identical with H1.

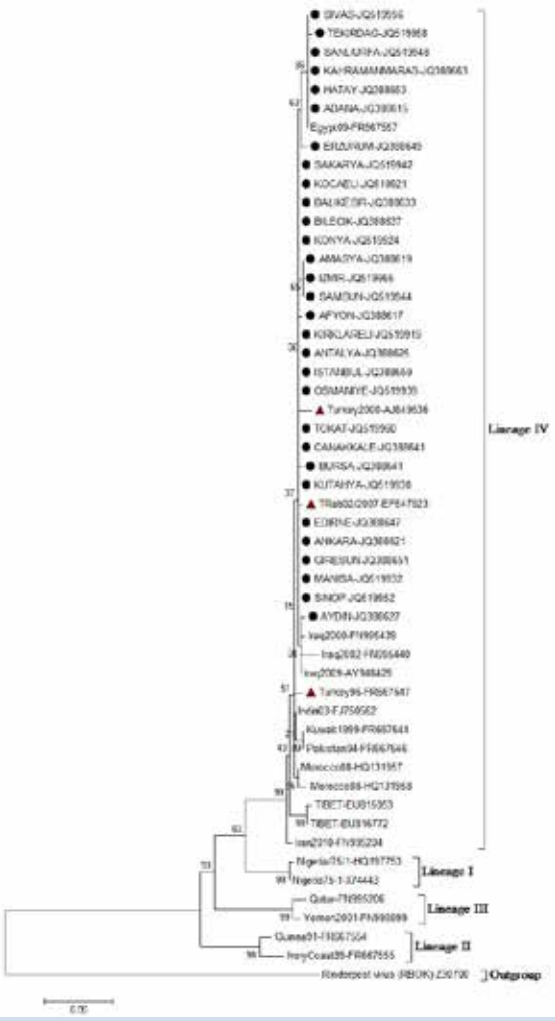

Figure 2. Phylogenetic tree of PPRV based on the PPRV-F gene (290 bp), constructed using the neighbour-joining method in the Kimura two-parameter model in Mega5 v.5. Numbers indicate bootstrap values (1,000 replicates). The scale bar at the bottom represents genetic distances in nucleotide substitutions per site. Horizontal distances are proportional to sequence distances. The phylogenic tree indicates clear division of PPRV strains into four lineages. ▲: Previously characterized sequences.

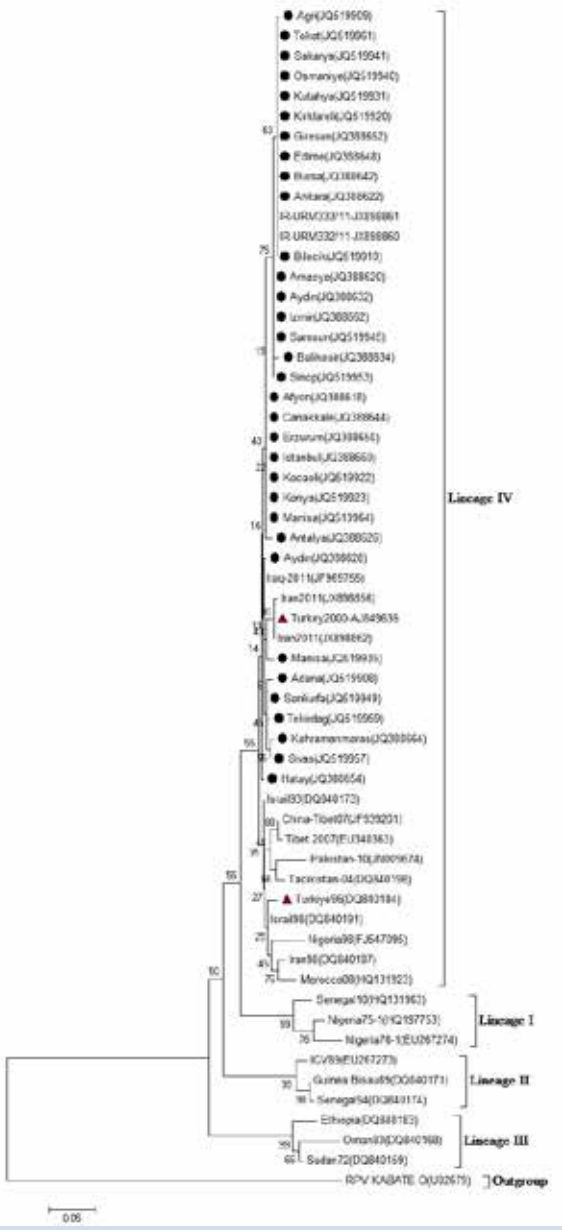

Figure 3. Phylogenetic tree of PPRV based on the *PPRV-N* gene (238 bp), constructed using the neighbour-joining method in the Kimura two-parameter model in Mega 5 v.5. Numbers indicate bootstrap values (1,000 replicates). The scale bar at the bottom represents genetic distances in nucleotide substitutions per site. Horizontal distances are proportional to sequence distances. The phylogenic tree indicates clear division of PPRV strains into four lineages. ▲: Previously characterized sequences.

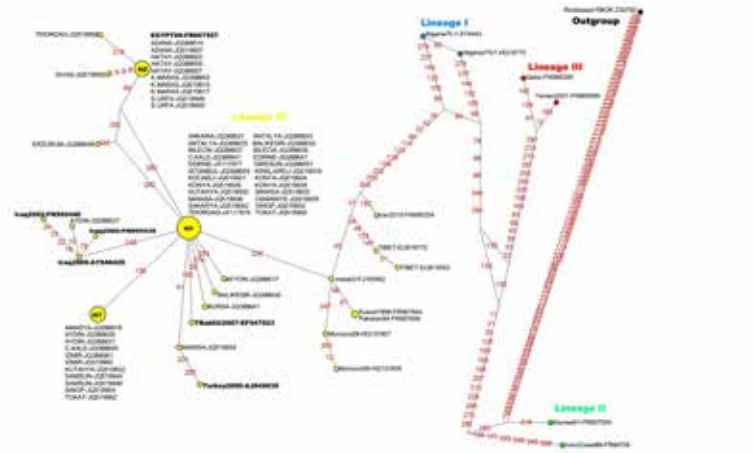

Figure 4. Phylogenetic network analysis based on the *PPRV-F* gene (290 bp). The numbers along the branches represent nucleotide changes. Sequences obtained from this study were usually clustered in three haplogroups (H1, H2 and H3) within lineage IV, marked in yellow.

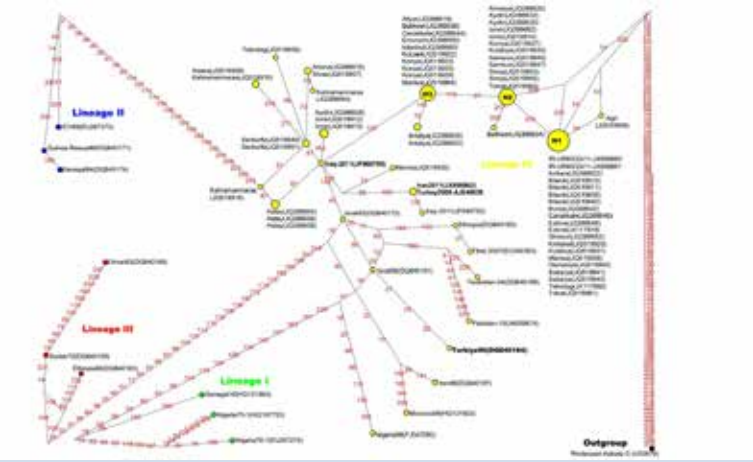

Figure 5. Phylogenetic network analysis based on the *PPRV-N* gene (238 bp). The numbers along the branches represent nucleotide changes. Sequences obtained from this study were clustered in lineage IV, marked in yellow.

DISCUSSION

The infection of small ruminants with PPRV causes significant economic losses across a wide geographical area, including Turkey (Ozkul *et al*., 2002; Kul *et al*., 2007; Banyard *et al*., 2010). After the successful Global Rinderpest Eradication Program in cattle, national and international organisations have undertaken initiatives to control and eradicate PPR. The determining of the seriousness and variability of PPRV infection in susceptible populations is impossible without any effective diagnostic method. Because of that, the accurate and reliable diagnosis constitute the first step of these initiatives (Banyard *et al.,* 2010). Especially in early stage of the disease, the differential diagnosis of PPRV infection is difficult from other diseases with similar symptoms. Thus, rapid alleviation programs, supported by rapid, specific and sensitive diagnostic methods are critical.

For the reasons mentioned above, the existing situation regarding disease prevalence was recorded for the first time, within the context of this study. The mean level of positivity for years 2010 to 2012 using RT-PCR was 45.8% (263/574), which corresponds to 45.9% for year 2010, 45.7% for 2011 and 45.9% for 2012. At same time, the level of positivity for sheep and goats was 46.7-47.1% and 42.8-44%, respectively. The Ministry of Agriculture and Forestry has an annual vaccination program since the first detection of the disease. According to this, all sheep and goats of all ages are vaccinated with PPR vaccine every year in the autumn. In 2010, PPR vaccination campaigns for individually identified lambs and kids were implemented in Turkey as part of a three-year European Union Project. For this, 30 million doses of PPRV vaccine were produced and consigned to the field. Between 2010-2012, in parallel with vaccination, approximately 27 million sheep and goat were ear-tagged and registered in all provinces. Despite all these vaccination campaigns, similar positivity

rates were determined between 2010-2012. In addition to problems mantioned previous studies (Ozkul *et al*., 2002; Banyard *et al*., 2010), the political situation on the south east borderline of Turkey may have hampered the efforts to control of disease and animal movement during this study.

In view of the results of detection limit and field samples, the *N* gene RT-qPCR was more valuable than *N* gene RT-PCR and *F* gene RT-PCR for detecting PPRV infection in this study. These results, which are compatible with those of Kwiatek *et al*. (2007) and Bao *et al.* (2008), indicate that the RT-qPCR assay used in this study was more sensitive than the *F* and *N* genes RT-PCR assays for the diagnosis of PPRV in field samples (Bao *et al.,* 2008; Kwiatek *et al*., 2010; Batten *et al*., 2011). Because the *N* gene is located at the 3' end of the PPRV genome, it is the most expressed gene due to a transcriptional gradient from the 3' to the 5' end of the genome (Ghosh *et al.,* 1995). Therefore, it is probably one of the best targets for maximising sensitivity with regards to RT-qPCR (Kwiatek *et al*., 2010).

In this study, a total of 473 sheep and 101 goats from various flocks were sampled. There was also a difference between sheep and goats in terms of positivity (Table 2). It was found that this finding is in accordace with the studies (Ozkul *et al*., 2002; Kul *et al*., 2007) that suggest that PPRV infection is more prevalent in sheep than goats in Turkey. However it is not compatible with the other reports (Anderson and McKay, 1994; Zhiliang *et a*l., 2009). Possible explanations for these results may be sample size relative the general population, the age of the animals and difference in species-specific animal trade. According to years, the rate of positivity was similar in 2010, 2011 and 2012 (Table 2). The absence of a reduction in rates despite vaccination can be attributed to uncontrolled animal movement on the east and southeast border of the country, which seems to be consistent with the outcome of the relevant network phylogenetic analyses that was conducted (provinces' numbers: 1, 24, 30,40), (Figure 1).

Although several RT-PCR methods have been developed since 1995 for rapid and specific detection of PPRV, genome sequencing has remained the gold standard for confirming the virus (Zhao *et al.,* 2009). This method has been also found useful for analyzing the genetic relationships between PPRV isolates and supported epidemiological investigations on the origin and spread of the virus. In light of this information, phylogenetic analyses of PPRV were conducted by using partial sequences of the *PPRV-F* and *N* genes (Munir et al., 2012a; Munir, 2012b). As in previous studies (Ozkul *et al.,* 2002; Bailey *et al.,* 2005; Yesilbag *et al.,* 2005; Kul *et al.,* 2007), this study showed that prevailing PPRV in Turkey belongs to lineage IV consisting of three main haplogroups. Detection of haplogroups indicates a number of introductions into Turkey. However, these haplogroups also show that PPRV strains in Turkey may have followed different evolutionary courses. It is plausible that multiple introductions from diverse sources were combined, resulting to mixed virus population in Turkey. The belief that border crossing of the virus (Ozkul *et al*., 2002; Banyard *et al*., 2010) is supported by the fact that sequences obtained in this study were clustered with sequences obtained in Iran, Iraq, Egypt and India. Specifically, the greater nucleotide sequence identity in the *N* gene network phylogenetic analysis suggests that there has been a close contact between sequences of strains from Iran and Turkey. To determine this possibility, sequencing should be added to regular virologic surveillance to characterise PPRV in the country.

Through *F* gene partial sequence analysis, it was found out that the PPRV strains showed a level of nucleotide sequence identity that was determined in Turkey to a minimum of 98.2%. Several studies (Kwiatek *et al*., 2007; Kerur *et al*., 2008; Banyard *et al*., 2010; Anees *et al*., 2013) indicated that substitution in the *N* gene is more probable than in the *F* gene, which was confirmed by our results indicating lower level of nucleotide sequence identity with regards to the *N* gene (94.2%). In general, the topology of the phylogenetic and network phylogenetic tree indicated that PPRV samples from different provinces differ mainly in their *PPRV-F* and *PPRV-N* gene sequences. While the PPRV strains in this study are more closely related strains from Egypt and Iraq in the *F* gene phylogenetic tree, they are more closely related to strains from Iran and Iraq in the *N* gene phylogenetic tree. When evaluated together with geographical proximity, the *N* gene-based phylogenetic tree becomes more meaningful. Because the probability of livestock transition from neighboring countries is higher than non-border countries. The possible relationship could be due to the fact that Turkish breeders share the same pastures with their Iraqi and Iranian counterparts. Another possible cause is uncontrolled animal movement, which especially increases before religious ceremonies, such as the festival of the sacrifice.. Like similar studies (Kwiatek *et al.,* 2007; Kerur *et al.,* 2008; Banyard *et al.,* 2010) our data indicate that a molecular genotyping survey targeting the *N* gene of PPRV would be more reliable than the *F* gene based.

CONCLUSIONS

It was concluded that PPR is enzootic in Turkey according to the PCR results of this study. By conducting molecular epidemiological analyses, it was understood that the main cause of this situation is animal movement from different sources according to three haplogroups. So PPR continues to be a major economic burden for sustainable animal production in Turkey. In this respect, an appropriate control and eradication campaign for PPRV infection should be considered similar to the successfully completed RPV eradication program.

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CONFLICT OF INTEREST STATEMENT

The authors declared no conflict of interest. The funders had no role in study design, datacollection and analysis, decision to publish or preparation of the manuscript.

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