



RESEARCH ARTICLE

Molecular Epidemiology of Peste Des Petits Ruminants Cases Associated with Abortion in Sheep and Goat in Marmara Region of Turkey, 2018

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ABSTRACT

The aim of this study was to investigate the molecular epidemiology of peste des petits ruminants (PPR) infections associated with abortion in sheep and goat samples from the Marmara region of Turkey during 2018. The study was carried out from 116 sheep and 26 goat abortion cases. PPR virus (PPRV) detection in these samples was performed using real-time RT-PCR (Q-RT-PCR). Then, sequence analysis was performed from PPRV positive samples. Q-RT-PCR results demonstrated that 12 (10.34%) out of 116 sheep abortion samples and 3 (11.53%) out of 26 goat abortion samples were positive for PPRV genome. The sequence results of RT-PCR positive products revealed that the viruses causing the cases belong to lineage IV. Furthermore, molecular analysis showed that present cases were not related to PPRV vaccine strains or its mutants. Marmara region, where this study was conducted, is a neighbour of European countries such as Bulgaria and Greece. The first PPR cases in Europe were reported from Bulgaria at the beginning of 2018 and subsequently, other cases also reported before are mentioned in the present study. This study provides valuable information to understand the epidemiology of recently emerged PPRV cases in Europe and Turkey. Furthermore, because of the prevalence of PPRV in abortion samples in this study, these results suggest that PPRV may be one of the possible etiologic agents of abortions in sheep and goat. However, for clarification of the relationship between abortion and PPRV, there is need more robust epidemiological data and experimental infection studies.

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INTRODUCTION

Peste des petits ruminants (PPR) is a highly contagious and economically important viral disease affecting small ruminants, caused by PPR virus (PPRV) classified within the genus *Morbillivirus* in the family *Paramyxoviridae* (Ozkul *et al.*, 2002). PPRV is a non-segmented, 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 (Kerur *et al.*, 2008;

Parida *et al.*, 2015). PPRV causes acute or subacute infections in small ruminants, especially sheep and goats. This virus may cause widespread and severe lesions in the digestive and respiratory systems and results in deaths in severely affected animals (Parida *et al.*, 2015). Sheep herds constitute the largest livestock population among farm animals in Turkey. Turkey has around 36 million sheep according to 2018 reports (FAO, 2019). Therefore, viral diseases such as PPR cause important economic losses in sheep are carefully monitored by official authorities in Turkey. In addition, PPR is on the notifiable disease list of Turkey. The virus outbreaks were reported for the first time officially in 1999 in Turkey. Since then, several virus outbreaks have been reported in spite of restrictions such as animal movements, notice, and

vaccination in large areas (Ozkul *et al.*, 2002; Yesilbag *et al.*, 2005; Şevik *et al.*, 2015; Altan *et al.*, 2019; Sait *et al.*, 2019). The last outbreaks of PPRV were reported in the Marmara region together with many other reports in Turkey (Altan *et al.*, 2019). Thrace is the part of Marmara region that has a border with Greece and Bulgaria. PPR vaccination policy is mandatory in sheep and goat in the Thrace part of Marmara region. PPR vaccination is performed when disease occurs in the rest of the Marmara. Along with the successful vaccination policy, there are no reported PPR cases in Thrace since 2015. The first PPR outbreak in the area was reported in Bolyarovo municipality in Bulgaria, 2018. Bulgaria has a border with the Thrace part of Turkey (Altan *et al.*, 2019). PPR outbreaks in Marmara region, especially in Thrace, have potential affects to European countries. Because of such a potential impact, epidemiological investigation and genetic characterization of the viruses causing these outbreaks from present study are highly important. Investigation and genetic characterization of the viruses from outbreaks, especially in Thrace part of Marmara region, will help us to understand the epidemiological role of PPR cases and potential effect on the area especially on European countries.

Abortion is one of the most important risks for sustainable profitability in livestock and causes major economic losses in livestock management. Abortion cases are frequently reported in all ruminants, especially sheep and goats. Although the etiology of abortion in small ruminants has not been fully elucidated, the epidemiological relationship between some microbial agents and abortion was found (Givens and Marley, 2008). Pestiviruses, bluetongue virus, Cache Valley virus, Schmallenberg virus, caprine herpesvirus, and Akabane virus are accepted as the most important virus-induced abortion agents in gestating sheep and goats (Stram *et al.*, 2004; Givens and Marley, 2008; Herder *et al.*, 2012). Due to the abortion cases recorded in goats especially in regions where PPR cases are endemic, it is thought that PPRV may be one of the viral agents causing abortions (Abubakar *et al.*, 2008). However, there are not enough studies or data about the relationship between the virus and abortion of small ruminants in field conditions (Abubakar *et al.*, 2008; Kumar *et al.*, 2018; Yang *et al.*, 2018).

The purpose of this study is to investigate molecular epidemiology of PPR infection associated with abortion in sheep and goat samples from the Marmara region of Turkey within the year 2018.

MATERIALS AND METHODS

Samples: 116 sheep and 26 goats with abortion were obtained in the Marmara region of Turkey, 2018. Province distribution of collected samples and number of selected cases are listed in Table 1. Thrace part of Marmara region is constituted of Kırklareli, Edirne, Tekirdağ, and some portions of Çanakkale and İstanbul provinces (black painted part). The east side of the Marmara region except Thrace is called Anatolian side (Fig. 1).

This study was approved (Decision no: 062019-214) by the Republic of Turkey, Ministry of Agriculture and Forestry, Pendik Veterinary Control Institute and performed in strict accordance with the recommendations of the Animal Experiments Ethics Committee.

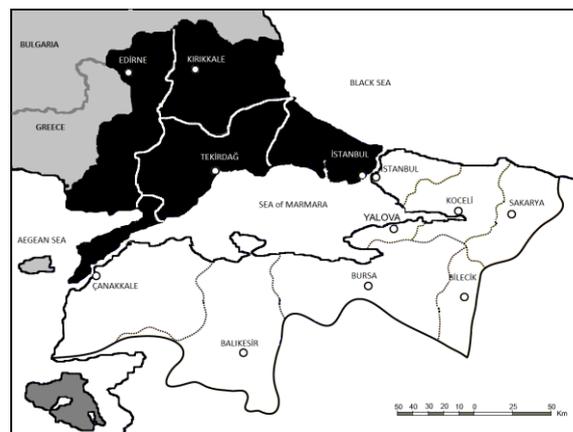


Fig. 1: Marmara region of Turkey and the provinces where samples were collected in this study. Black painted area shows Thrace part of the Marmara region.

RNA extraction: RNA extractions from tissue samples were carried out using a Qiagen RNeasy Mini Kit (Qiagen, Germany). For this aim, initially, the tissue pieces from 3 different regions of the foetal tissues (lymph node, spleen, lung, liver and internal organ specimens) were transferred to 2 ml lysis tubes including Magna Lyser Green Beads (Roche Molecular Systems Inc., Branchburg, New Jersey, U.S.). One ml of distilled water was added on the samples and the tubes were placed in Magna Lyser device. The tissues were homogenized for 60 seconds at 6000 rpm. RNAs from homogenates were obtained according to the manufacturer's recommendations (Qiagen RNeasy Mini Kit, Cat No: 74106, Qiagen Co., Germany).

Detection of common microbial abortion pathogens: The RNAs extracted from clinical samples were tested for detections of pestiviruses (La Rosca and Sandvik, 2009), bluetongue virus (Aytekin *et al.*, 2015), Akabane virus (Stram *et al.*, 2004) and Schmallenberg virus (Tonbak *et al.*, 2013) before PPRV detection.

In addition, the detection of bacterial abortion agents including *Brucella*, *Campylobacter sp.*, *Chlamydomphila abortus*, *Coxiella burnetii* were performed on the clinical samples according to standard test procedures described by Ministry (Tarım Bakanlığı, 2019).

Real-time RT-PCR for PPRV detection: Real-time RT-PCR (Q-RT-PCR) for PPRV detection was performed using Real-time Ready RNA Virus Master kit (Lot No. 44199300, Roche Diagnostic, Germany) for all samples (Kwiatk *et al.*, 2010).

The detection limit analysis of the Q-RT-PCR was carried out using vaccine virus strain (Nijerya 75/1) of PPRV ($10^{4.75}$ TCID₅₀/ml). For detection of the melting curve, the dissociation temperature range study was performed with temperature increase at the rate of 1.2°C per sec. from 68°C to 95°C. The results were detected with continuous reading of fluorescence.

The amplification of the N region and sequence analysis: A partial region of N gene of PPRV was amplified using RT-PCR from Q-RT-PCR positive samples (OIE, 1999). The amplifications were performed

using a Qiagen one-step virus RT-PCR kit (Lot No.151024842, Diagnostic, Germany) according to the manufacturer's protocols. The specific RT-PCR products were visualized using 1% agarose gel electrophoresis.

The amplified RT-PCR products were purified using a High Pure PCR Product Purification Kit (Lot No. 11732668001, Roche, Germany) and sequenced using an ABI 3100 automatic sequencer. The sequences from both directions were assembled together for each strain to obtain consensus sequences, which were then used in multiple alignments of strains from different isolates using ClustalW with BioEdit v.7.0.9.0. Phylogenetic tree of PPRV based on the PPRV-N gene constructed using the neighbour-joining method in the Kimura two-parameter model in Mega X v.10.0.4 software program. In addition to the nucleotide sequence analysis, the amino acid sequences of the partial N gene were obtained from nucleotide sequences using BioEdit v.7.0.9.0. The amino acid sequences of the partial N gene of the viruses were compared with vaccine strain (SMR Nigeria/75/1, Gene Bank accession number: KY628761) and Turkish strain (PPR Turkey 2000, Gene Bank accession number: AJ849636.2).

RESULTS

Analysis of common microbial abortion pathogens:

The results of detection of common microbial abortion pathogens were evaluated according to the references cited in material-method. These results were revealed that all abortion cases were negative for these microbial agents.

The detection with real-time RT-PCR of PPRV: In the Q-RT-PCR, the mean cycle threshold (Ct) values of the virus control and PPRV positive samples were 24 and 26, respectively. The detection limit of Q-RT-PCR was found as 5 virion/ μ l. According to the result of the melting curve analysis, the specific amplification was identified by the generation of a melt peak at 82.6°C.

The results of Q-RT-PCR analysis of 116 sheep and 26 goat abortion cases revealed the presence of the PPRV genome in 12 sheep and 3 goat samples (Table 1). According to the results, 10.34% (12/116) of sheep samples and 11.53% (3/26) of goat samples were found to be PPRV positive. In this study, no PPR cases were detected in Thrace part of the Marmara region.

RT-PCR and Sequence analysis: Amplified partial N gene products of 463 bp length were determined on agarose after conventional RT-PCR (Fig. 2). Then, the amplified products were sequenced. According to the sequence analysis, all samples showed 99% nucleotide sequence similarity to each other. The sequences of four samples obtained from this study were deposited in GenBank (Accession numbers; from MK732927 to MK732930). It was also found that the virus causing abortion classified in lineage IV as a result of the phylogenetic analysis performed from this study and previous PPRV strains (Fig. 3).

Table 1: The provinces in which samples were collected and the distribution of RT-PCR positive samples.

Provinces	Numbers of sheep samples	PPRV positive (%) in sheep	Number of goat samples	PPRV positive (%) in goat
Balikesir	10	2	-	-
Bilecik	3	1	2	-
Bursa	12	2	2	-
Çanakkale*	22	3	7	1
Edirne	27	-	2	-
İstanbul*	4	2	3	1
Kırklareli	21	-	9	-
Kocaeli	3	-	-	-
Sakarya	5	1	1	1
Tekirdağ	7	-	-	-
Yalova	2	1	-	-
Total	116	12 (10.34%)	26	3 (11.53%)

*; PPRV positive samples were determined in the Anatolian part of these provinces.

Table 2: Alignments of nucleotide and the amino acid sequences of N region of the PPR virus (TR Çanakkale-2018, MK732930) detected from the outbreaks the vaccine strain (Nigeria/75/1, KY628761) and Turkish strain (Turkey 2000, AJ849636.2).

NGR→TR 2000	Nigeria / 75/1 a.a*	NGR→TR 2018	NGR→TR 2000	Nigeria / 75/1 Nt*	NGR→TR 2018	NGR→TR 2000	Nigeria / 75/1 a.a*	NGR→TR 2018	NGR→TR 2000
C→T	416	S→L	S→L	1459.	C→G	C→G	489.	E→K	E→K
T→C	420	L→S	L→S	1465.	G→A	G→A	490.	H→R	H→.
T→C	421	V→A	V→A	1469.	A→G	A→.	491.	A→.	A→.
A→G	435	H→R	H→R	1473.	T→C	T→.	492.	Q→R	Q→.
A→G	436	R→.	R→.	1475.	A→G	A→.	494.	S→G	S→G
T→C	439	M→T	M→T	1480.	A→G	A→G	498.	K→R	K→R
C→A	440	N→K	N→K	1493.	A→G	A→G	499.	L→P	L→P
T→C	443	L→.	L→.	1496.	T→C	T→C	500.	P→.	P→.
G→.	450	R→K	R→.	1500.	G→A	G→A	504.	F→S	F→S
T→C	458	I→T	I→T	1510.	T→C	T→C	505.	R→W	R→W
A→G	461.	M→E	M→E	1513.	C→T	C→T	506.	R→K	R→K
T→A	465.	R→.	R→.	1517.	G→A	G→A	509.	K→Q	K→Q
C→T	466.	L→P	L→P	1525.	A→C	A→C	512.	N→R	N→R
T→C	466.	L→P	L→P	1535.	A→G	A→G	512.	N→R	N→R
A→G	469.	R→Q	R→Q	1536.	C→G	C→G	517.	L→.	L→.
G→A	473.	K→G	K→G	1551.	A→G	A→G	528.	L→P	L→P
A→G	473.	K→G	K→G	1583.	T→C	T→C	536.	P→L	P→.
A→G	476.	L→.	L→.	1607.	C→T	C→.	546.	R→K	R→K
G→.	480.	M→T	M→T	1637.	G→A	G→.	552.	R→.	R→.
T→C	484.	E→K	E→K	1656.	A→G	A→G			
G→A	487.	Q→E	Q→E						

*; Nucleotide (nt) and amino acid (a.a.) positions are named according to the Nigerian strain (Nigeria/75/1). NGR; Nigeria/75/1 strain, TR 2018; TR Çanakkale-2018 strain, TR 2000; Turkey 2000 strain. Bold letters are different sequences in TR 2018 and Turkey 2000 strains.

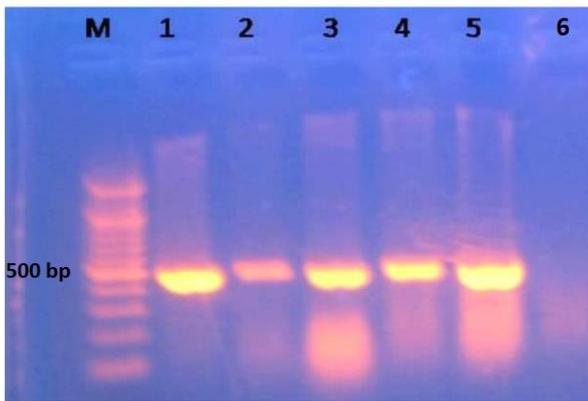


Fig. 2: Agarose gel electrophoresis of the partial N gene regions (463 bp length) of PPRV amplified with RT-PCR from the abortion samples. M; 100 bp DNA ladder, Line 1; Positive control (PPRV vaccine strain), Lane 2- 5; RT-PCR positive clinical samples, Lane 6; RT-PCR negative clinical sample.

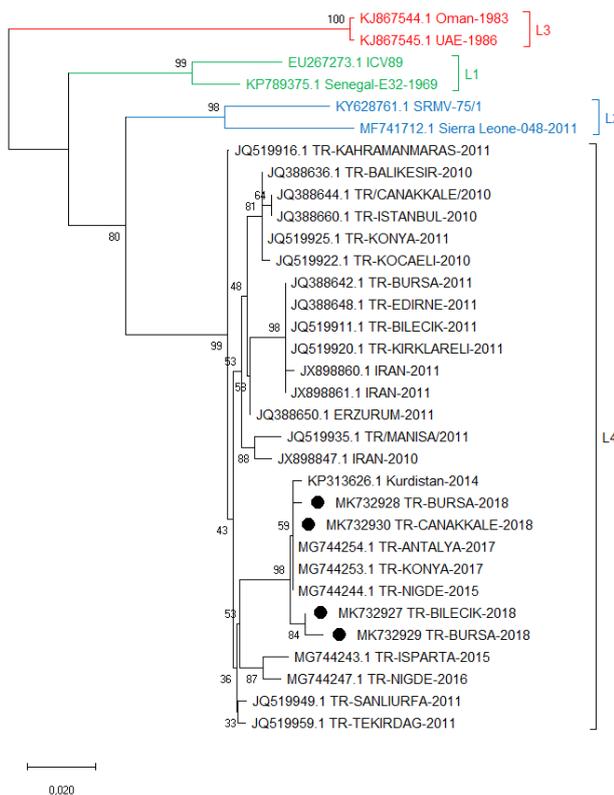


Fig. 3: Phylogenetic tree of PPRV based on the PPRV-N gene (382 bp), constructed using the neighbour-joining method in the Kimura two-parameter model in Mega X v.10.0.4 software. Numbers indicate bootstrap values (1,000 replicates). The scale bar at the bottom represents genetic distances in nucleotide substitutions per site. Horizontal distances are proportional to sequence distances. The phylogenetic tree indicates clear division of PPRV strains into four lineages. Black Dot: Sequences studied here.

The partial N gene of PPRV (TR Çanakkale-2018) nucleotide and amino acid sequence identities were compared with the vaccine strain (SMR Nigeria/75/1, KY628761) and Turkish strain (PPR Turkey 2000, AJ849636.2). Although many nucleotide and amino acid changes were determined once compared to the Nigerian strain, seven nucleotide changes and four amino acid changes (Bold letters) were detected in comparison with the Turkey 2000 strain (Table 3).

DISCUSSION

Pestivirus, bluetongue virus, Schmallenberg virus, Akabane virus, *Brucella*, *Chlamydomphila abortus*, *Campylobacter fetus* and *Coxiella burnetii* are accepted as common abortion agents in Turkey as in many countries (Stram *et al.*, 2004; La Rosca and Sandvik, 2009; Tonbak *et al.*, 2013; Aytekin *et al.*, 2015). All samples included in this study were negative for the microbial agents mentioned above. Furthermore, according to the real-time RT-PCR results performed with these samples, 15 of 142 (10.56%) samples were found as positive for PPRV. These results indicate that PPRV may be one of the possible etiologic agents of abortion in endemic areas of the PPR.

For genotypic identification, amplification and sequence studies for the partial N gene were performed with the real-time RT-PCR positive samples. The sequences results revealed that all clinical samples had >99% nucleotide sequence identity among themselves. These results point out that identical virus circulates from the last outbreak to date. In addition to these results, the phylogenetic tree analysis showed that strains are classified under the lineage IV along with previously reported PPRV strains from Turkey. Furthermore, the viruses detected in the present study have a high proportion of nucleotide and amino acid similarity with PPRV strain (GenBank accession number: AJ849636.2) from the 1999 Turkey epidemic (Table 2). These data suggest that the circulating viruses in Turkey are in the same lineage since 1999 when PPRV was firstly reported in Turkey.

Because the PPRV vaccinations have been applied in most of the provinces in Marmara region, the cases may suggest that outbreaks can be caused by vaccine or mutants of the vaccine strain (Fosyth and Barrett, 1995). Therefore, in the present study, the genomic comparison of the detected viruses with the PPRV vaccine strain (Nigeria 75/1) was performed. Both phylogenetic analysis (Fig 3) and nucleotide sequence identity between 89.1-89.1% showed that these outbreaks were not caused by vaccination or vaccine virus.

Turkey has applied PPR regional vaccination for many years. As a result of these vaccinations, especially in many regions where the vaccination program was meticulously implemented, serious decreases in the frequency of the cases were reported. However, the illegal animal trade and smuggling from Iraq, Syria, Iran, and Armenia that they are Turkey's eastern and southern neighbours, cause weaknesses in control against many viral infections such as PPR, foot and mouth disease (FMD), sheep-goat pox. Multiple alignment of the N gene indicated that the Turkish strains detected in this study were closely related to the Kurdistan isolate (GenBank number: KP313626) (Hoffmann *et al.*, 2012) with 98.7-99.7% nucleotide identity. There was high nucleotide similarity (between 97.8-98.2%) with Iran 2010 strain (GenBank number: JX898860). Our PPRV sequences have high nucleotide similarity with previously reported PPRV isolates of Turkey's neighbouring countries (Hoffmann *et al.*, 2012). According to these results, it is thought that PPRVs outbreaks in Turkey may be related

with illegal animal or wild animals' transportations and vectors movements from the neighbouring countries.

PPR outbreak was officially reported in Turkey for first time in 1999, PPR outbreaks have been reported often in Turkey (OIE, 1999; Yesilbağ *et al.*, 2005; Şevik and Sait, 2015; Altan *et al.*, 2019; Sait and Dagalp, 2019). No PPR cases were reported in the Thrace since 2015 (Altan *et al.*, 2019). There have been a great importance of identification and investigation of the epidemiology of the virus causing outbreaks from present study area due to first PPRV cases reported in Bulgaria in 2018 (Altan *et al.*, 2019; OIE, 2019). Although the information about the same outbreaks in Marmara region has been reported by other study (Altan *et al.*, 2019), the study was carried out on PPRVs samples causing 2010-2012 epidemics in the Marmara region. Besides, this study demonstrated the presence of PPRV in Marmara region during the year 2018. In addition, PPR positivity in the Thrace region could not be determined during routine field surveys performed by Pendik Research Institute. The results suggested that there was not a probable relationship between outbreaks of Turkey and Bulgaria. Unfortunately, chance didn't come to compare PPRV causing 2018 outbreaks in Bulgaria and Turkey due to the absence of PPRV gene submission from Bulgaria outbreaks. Although this study didn't provide any data on the relationship between the PPR outbreaks reported in Bulgaria and Turkey, the identification of the viruses causing the outbreak in this study may be valuable for future epidemiological studies.

In the present study, the samples from abortion materials were sent to Pendik Veterinary Institute with official reports and anamnesis obtained from the animal owners. Although mild respiratory and digestive symptoms were reported, PPR-specific findings and deaths following these symptoms were not reported on the farms from which PPRV positive sheep and goat samples were collected. PPR can cause acute, severe disease, or subclinical disease in sheep (Parida *et al.*, 2015), compatible with our findings. In addition, PPR vaccination in many provinces, where samples are collected, may be one of the reasons for the secret course of the clinical course.

Conclusions: According to the present study data, there may be a possible relationship between PPR outbreaks and abortion cases. Therefore, we recommend PPRV detection in the abortion diagnosis in the PPR outbreaks reported in sheep and goat. However, for clarification of the relationship between abortion and PPRV in the animals, there is the need of new epidemiological studies and experimental infections.

Authors contributions: HB and ZP designed the study and manuscript preparation. The sampling, data collection

and laboratory analysis were made by AS, OS, ZP and FTO. All the authors read the manuscript.

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