OPEN ACCESS
Advances in Animal and Veterinary Sciences
Research Article

Molecular Detection of *Peste des Petits Ruminants virus* from Different Sample Materials of Sheep, Northeastern Turkey

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Abstract | *Peste des Petits Ruminants* (PPR) is a highly contagious disease of domestic and wild small ruminants. In this study, a totally 85 materials were used consist of different samples such as lymph nodule, nasal swap, lung, spleen from sheep (n=32), each from different flocks, suspected the PPR infection as clinically and macroscopic pathological lesions in Kars region, Northeastern Turkey. Reverse transcriptase polymerase chain reaction (RT-PCR) was used for the molecular detection of PPR infection. PPRV nucleic acid was detected in 48 of 85 materials by RT-PCR. According to the results of RT-PCR, positivity rates as 62.5% (15/24), 60% (6/10), 50% (13/26), 56% (14/25) were detected in the samples of lymph nodule, nasal swap, lung, spleen, respectively. This study showed that PPR infection is common in the sheep being raised in the region where the study was carried out. The samples of lymph nodule, nasal swap, lung, spleen are more valuable as the diagnostic material from sheep in the detection of PPR infections by RT-PCR. In addition, it is determined that RT-PCR is sensitive and reliable method in the diagnosis of PPR infections.

Keywords | Diagnosis, Northeastern Turkey, PPR, RT-PCR, Sheep

Editor | Kuldeep Dhama, Indian Veterinary Research Institute, Uttar Pradesh, India.
Received | February 03, 2016; **Revised** | February 15, 2016; **Accepted** | March 04, 2016; **Published** | March 18, 2016
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Citation | Yilmaz V (2016). Molecular detection of *Peste des Petits Ruminants virus* from different sample materials of sheep, Northeastern Turkey. Adv. Anim. Vet. Sci. 4(3): 169-173.
DOI | http://dx.doi.org/10.14737/journal.aavs/2016/4.3.169.173
ISSN (Online) | 2307-8316; **ISSN (Print)** | 2309-3331

INTRODUCTION

Ovine rinderpest (Peste des Petits Ruminants, PPR) is an acute or subacute viral disease in domesticated and wild ruminants, especially sheep and goats, that is economically significant, has high mortality and morbidity rates and is characterized by fever, loss of appetite, nasal flow that is serous in the beginning and mucopurulent in advanced stages, hemorrhage and erosions in the mucous of the tongue, lips, and palate, stomatitis, conjunctivitis, diarrhea and bronchopneumonia (Baron et al., 2011; Albina et al., 2013). The PPR virus is part of the *Morbillivirus* genus in the *Paramyxoviridae* family and it has been determined to have antigenic proximity to *Rinderpest* and 5 other viruses [(*Canine distemper virus, Measles virus, Dolphin distemper virusu, Phocine (seal) distemper virusu* and *Porpoise distemper virus*)] in the *Morbillivirus* genus (Singh et al., 2014). The incidence of infection in sheep and goats increases with age. The course of the disease is generally peracute in young animals resulting in death. Although the mortality and morbidity rates may vary, they can be as high as 90-100% (Kumara et al., 2014). The most significant mode of transmission is close contact between viremic animals in the herd with other sensitive animals. The oral, nasal and conjunctival fluids and stool of infected animals contains the virus in abundance (Munir, 2014). There are four genetic lineages of PPRV: the first and second lineages include viruses originating in West Africa, the 3rd lineage is from East Africa, Arabia and Southern India and the 4th lineage is from the Middle East and South Asia (Kerur et al., 2008). PPRV infection was described for the first time in West Africa in 1942 (Gargadennec and Lalanne, 1942). It later spread from there to the Sudan in East Africa and from there to Middle Eastern countries and over a large geographical area as far away as Bangladesh (Saritha et al., 2015). Although existence of the infection in Turkey was officially announced in 1999 (OIE, 1999), previous epidemic reports had noted the existence of the virus in the country (Alcigir et al., 1996; Tatar and Alkan, 1999). Ozkul et al. (2002) reported that the PPR

viruses they isolated in Turkey belonged to the 4th genetic lineage. Later, there were PPR epidemics in the Trakya region of Turkey in 2004.

Even though clinical findings may suggest PPR infection, a definitive diagnosis requires laboratory testing (OIE, 2013). Diagnosis of PPR infection is based on four fundamental principles, namely virus isolation, antigen detection, nucleic acid detection and antibody identification from serum with sequencing. The ELISA test is widely used in field studies (Balamurugan et al., 2012; Kihu et al., 2015). Virus isolation and neutralization tests in cell cultures are time-consuming, costly and not very productive. The diagnostic methods frequently used today to diagnose PPR infection are antigen-ELISA, RT-PCR and subsequent nucleotide sequencing (Forsyth and Baarrett, 1995; Balamurugan et al., 2012; Kumara et al., 2014; Saritha et al., 2015). Organ materials such as lymph nodes, spleen, liver and lung as well as oral and nasal swab samples can be used in PCR tests to identify PPRV nucleic acid (Forsyth and Baarrett, 1995; Albayrak and Alkan, 2009; Baydar et al., 2013; Sevik, 2014).

The purpose of this study was to investigate the incidence of the PPR virus in sheep raised in the Kars region in Northeastern Turkey using the RT-PCR method and to identify the diagnostic value of the RT-PCR test in diagnosing PPR infections.

MATERIAL AND METHODS

Study Area and Sample Collection

This study was conducted in Kars region in Northeast part of Turkey. The Kars region, located in Northeastern Turkey (43.05° E and 40.36° N), which is the most important livestock production area in Turkey, is mountainous and has a cold climate (Figure 1). A totally 85 materials (10 nasal swap, 26 lung, 25 spleen and 24 lymph node) collected from lambs (n=32), aged between 1 and 24 months, in the 12 flocks suspected to have PPR infection as clinically were tested. Definite vaccination records of the sampled animals were not obtained in this study. Lambs with clinical signs of PPRV infection were develope fever, anorexia, dehydration, dullness, mucoprulent oculonasal discharge, lacrimation, conjuctivitis, dyspnea and diarrhea. For serological diagnosis, the blood samples were collected before necropsy. The blood samples was collected from jugular vein in nonheparinised vacotainer tubes for analysis C-ELISA. The samples were centrifuged at 1.500 g for 10 min to separate the serum and then stored at -20°C until they are tested. Nasal swaps centrifuged at 3.000 xg for 5 min remove the suspended solids. The supernatants were stored at –80°C until used. Necropsy was performed on short times the after death and fresh samples of the lung, spleen, and lymph nodes were placed in 2 ml of PBS diluent with MagNA Lyser Green Beads (Roche, Mannheim, Germany) and were homogenized at 3.000×g for 3 min by MagNa Lyser (Roche, Mannheim, Germany). Homogenates were centrifuged in Eppendorf tubes at 12.000×g for 3 min to remove the suspended solids, without removing the beads. The supernatants were stored at -80°C until testing with RT-PCR.

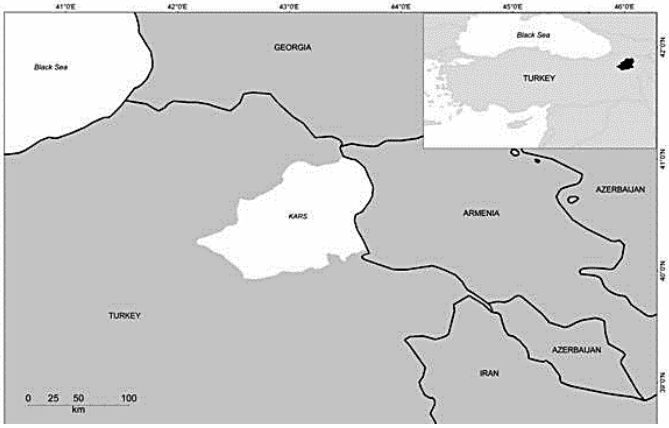

Figure 1: Geographical positioning of the Kars region in which the study was performed

Competitive Enzyme Linked Immunosorbent Assay (C-ELISA)

Commercial ELISA (IDEXX, France) used for the detection of antibodies against PPRV in serum samples was carried out according to the manufacturer's instructions.

RNA Extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from the nasal swap and tissue homogenate using RNeasy Mini Kit 36 (Qiagen, Germany), according to manufacturer's instruction. The obtained RNA extracts were further amplified by RT-PCR using primers: PPRVF1b: AGTACAAAAGAT TGCTGATCACAGT and PPRVF2d: GGGTCT CGAAGG CTAGGC CCGAATA that target F protein-coding gene described by Forsyth and Barrett (1995). One-step RT-PCR amplification was done using commercial kit Qiagen One Step RT-PCR kit chemistry (Qiagen, Germany), according to manufacturer's instruction. Briefly, the amplification reaction was carried out in a volume of 20 µL containing 7 µL Molecular Grade Water, 0.8 µL 10 pmol of forward and reverse primers, 4.0 µL buffer, 0.8 µL dNTP mix (containing 10 mM of each dNTP), 0.8 µL enzyme mix, 4.0 µL 5x Q-Solution, 2.6 µL template RNA. The amplification conditions (Thermocycler Gradient, Eppendorf, Germany) were as follows: reverse transcription stage at 50°C for 30 min, followed by an initial PCR activation step at 94°C for 15 min, 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 7 min. Specific PCR product of 448 bp was detected and visualised by electrophoresis on 1.5 agarose gel (Prona, Spain) stained with ethidium bromide.

RESULTS AND DISCUSSION

PPRV antibodies were detected in 23 of 32 serum samples by C-ELISA (71.8%). PPRV nucleic acid was detected in 48 of 85 materials by RT-PCR. According to the results of RT-PCR, positivity rates as 62.5% (15/24), 60% (6/10), 50% (13/26), 56% (14/25) were detected in the samples of lymph nodule, nasal swap, lung, spleen, respectively (Table 1). This study concluded that lymph node samples have the highest diagnostic value in detection PPRV with RT-PCR. The specific 448 bp band was obtained from the DNA amplification of F protein-coding gene using the primers PPRVF1b and PPRVF2d (Figure 2). Because there is a financial problem, PCR positive products from different clinical samples were not sequenced in this study. Also, PCR positives samples will use further more molecular investigation in the future.

Table 1: PPRV RNA positivity rate according to various specimens of sheep

Sample	Tested	Positive (%)
Lymph nodule	24	15 (62.5%)
Nasal Swap	10	6 (60.0%)
Lung	26	13 (50.0%)
Spleen	25	14 (56.0%)
Total	85	48 (56.4%)

Studies on the prevalence of PPR antibodies in sheep (Taylor et al., 1990; Lefevre et al., 1991; Tatar and Alkan, 1999; Yener et al., 2004; Yesilbag et al., 2005; Banyard et al., 2010; Singh, 2011) have established seroprevalence over large areas. Taylor et al. (1990) found that PPRV antibody prevalence in healthy sheep sampled in a seroprevalence study conducted in Oman was 23.7%. Tatar and Alkan (1999) tested 206 serum samples from sheep and goats older than six months with clinical symptoms using the C-ELISA test and found that the PRR seropositivity rate in sheep serums was 87.95% and 90% in goat serums. Similarly, serum samples taken prior to necropsy in this study from 32 lambs with clinical symptoms suspected of being PPR infection were tested with C-ELISA, and the presence of antibodies specific to PPRV were detected in 23 of them. The seropositivity rate was 71.8%, which can be viewed as an indicator of the existence of the PPR infection in the Kars region. Although clinical and necropsy findings may be sufficient for diagnosis of PPR infection in endemic regions, laboratory testing is required for a definitive diagnosis. In this study, all of the sheep suspected of having PPR had the clinical and virological findings.

It is recommended that conjunctival fluid, nasal fluid, oral and rectal mucosa and anticoagulant blood samples be taken from live animals to diagnose PPR infection while samples of mesenteric and bronchial lymph nodes, lung, spleen and intestinal mucosa should be taken from deceased animals (OIE, 2000). Although samples taken from dead animals are not generally suitable for virus isolation, they should be taken within two hours, depending on ambient temperature, in cases where isolation is mandatory (Munir, 2013; Nizamani et al., 2014). PCR is a superior virus isolation technique because it allows one to use isolation materials with small amounts of the virus that have begun to decompose in order to make a diagnosis. PCR's increased effectiveness depends on many factors, such as number of cycles, the quality of the starting material, the length of the target DNA and variability of annealing and elongation temperatures.

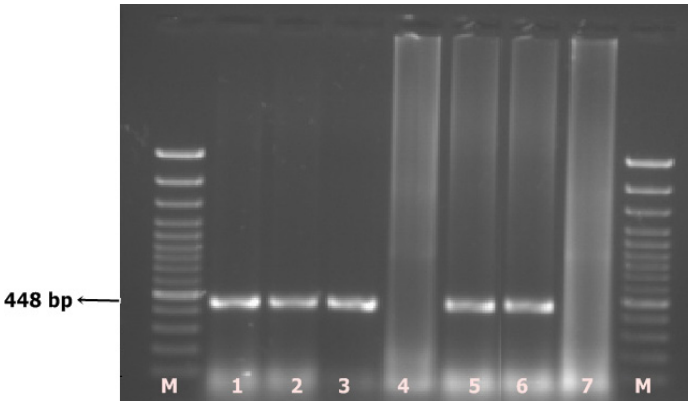

Figure 2: Agarose Gel Electrophoresis of PPRV RT-PCR Positive Specimens and Controls. **Lines M)** 100 bp DNA Ladder; **1)** Positive Control; **2)** Positive Lymph Node Sample, **3)** Positive Nasal Swap Sample; **4)** Negative Control; **5)** Positive Lung Sample; **6)** Positive Spleen Sample; **7)** Negative Sample

Forsyth and Barret (1995) were unable to detect viral nucleic acid in lung, oral and nasal swab samples even though they did detect PPRV nucleic acid in lymph node, spleen, conjunctival swab and blood samples using the RT-PCR method. Researchers have reported that lymph node samples are more valuable than other necropsy samples and should be preferred. In a study conducted by Albayrak and Alkan (2009), they found PPRV nucleic acid in nasal and conjunctival swab samples with RT-PCR but could not detect it in oral swab and blood samples. Researchers have reported that nasal and conjunctival swab samples taken from animals with clinical symptoms are more valuable as diagnostic material. Baydar et al. (2013) stated that the most valuable necropsy material for diagnostic purposes was different materials such as lymph nodes, spleen, lung, oral-nasal swab and blood, and they found that positivity rates for lymph nodes, nasal and oral swabs, blood, lung and spleen were 54.2%, 66.6%, 45%, 46.2% and 46.2% respectively. In the current study, materials such as nasal swab, lung, spleen and lymph nodes were used to diagnose PPR infection with RT-PCR. The high diagnostic value of the materials that were used has been established. Positivity rates for lymph nodes, nasal swab, lung and spleen

were 62.5% (15/24), 60% (6/10), 50% (13/26), and 56% (14/25) respectively. The results of the research show that lymph node samples have the highest diagnostic value in detection PPR infection with RT-PCR. In addition, this study concluded that the RT-PCR method is a sensitive and reliable method for diagnosing PPR infection. The results of this study are similar to the results of other studies.

The location for the study was chosen because Kars region is located in the middle of an animal transit route between North-East Anatolia and Caucasus functioning as a bridge between Asia and Europe. In addition, Kars region is one of the regions made intensive of livestock. Region has 27.3% about of small ruminants in the Turkey. The Kars Region shares a border with Armenia and Georgia, it is possible that the virus entered Turkey via uncontrolled animal movement from neighboring countries where PPRV is endemic.

These results demonstrate that PPR infections previously unreported in sheep raised in the Kars region of Northeast Anatolia have rapidly spread in the area where animal movements and sheep operations are more intense than they are in other geographical regions. Factors that play a significant role in the rapid spread of the disease in both this region and other regions are uncontrolled animal movements, illegal smuggling of animals from neighboring countries and transportation of infected subclinical animals between regions. The fact that treatment for the PPRV infection generally begins on the assumption that it is a respiratory and/or digestive infection caused by bacteria results in higher morbidity and mortality rates for the infection. Important building blocks for control of infections include taking measures related to transmission of the virus, the use of rapid and sensitive diagnostic techniques and certain specific practices (such as administering hyperimmune serum and quarantine). Precautions such as quarantine are very important in countries like Turkey which have extensive land borders with neighbors where the potential for infection is high. This study also concluded that the RT-PCR method used to diagnose PPRV infection is very sensitive and has diagnostic value while necropsy samples, such as nasal swab, lung, spleen and lymph nodes, are valuable as diagnostic material and lymph node samples have the highest diagnostic value in detection PPRV with RT-PCR. Furthermore, phylogenetic analysis of the PCR positive products obtained from this study will play an important role in future studies on protection and control strategies to combat and prevent the spread of PPRV infection.

ACKNOWLEDGEMENT

There exists no any Acknowledgements.

CONFLICT OF INTEREST

There exists no any conflict of interest.

AUTHOR'S CONTRIUTION

No Author contributed in creating the article.

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