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RESEARCH ARTICLE

Pathology and Phylogenetic Analysis of Capripoxvirus in Naturally Infected Sheep Sheeppox Virus

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ABSTRACT

Sheeppox and goatpox is a contagious viral disease of sheep and goats characterized by fever, generalized papules or nodules in skin and mucosal surface. DNA virus belonging to the genus capripoxvirus. The disease with high mortality and morbidity causes significant economic losses in small ruminants. In this study, 20 tissues from 8 sheep which were considered having natural sheeppox based on the macroscopic and histopathologic evaluation were further investigated by immunohistochemical (iNOS, SP-A, HSP-70), and Polymerase Chain Reaction (PCR) method. Papules, typically pox lesions, noticeable on the skin surfaces were examined macroscopically. Immunohistochemically; iNOS and SP-A were most intensely stained whereas HSP-70 was low stained. PCR method was used for the detection of A29L gene of capripoxvirus. Positive samples obtained from sheep were used for molecular characterization. A phylogenetic analysis was performed using sequence of the partial A29L gene and by comparing with reference sheeppox viruses isolates obtained from Gene Bank. The results of the sequence analysis were similar among themselves, they were found different (99-100% identity) from the other sheeppox viruses around the world. This study provides firstly phylogenetic analysis of sheeppoxviruses from Van province in Turkey.

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INTRODUCTION

Capripox virus is a member of the subfamily Chordopoxvirinae, which are enveloped, double-stranded DNA viruses, comprising 8 genus within the poxviridae family (Fenner, 1996). The Capripoxvirus genus includes sheeppoxvirus (SPPV), goatpoxvirus (GTPV) and lumpy skin disease virus (LSDV) causing disease in sheep, goat or cattle, respectively (Tuppurainen *et al.,* 2014). These diseases are characterized by fever, generalized papules or nodules, vesicles, internal lesions and tend to spread rapidly in animals and cause systemic disease in all ages of animals. The young animals are affected by this virus mostly (Babiuk *et al.*, 2008; Zhu *et al*., 2013; Kirmizigul *et al*., 2016).

Sheeppox is the most severe and highly contagious disease in sheep. The mortality and morbidity rates of the disease are about 80-100% (Yeruham *et al.*, 2007). It is an important problem in animal husbandry since it leads to death in a short time due to advanced pneumonia and severe infections accompanied by reduced milk production, live weight loss, increased abortion rates, losses and damages of wool and leather. Countries experience important economic losses depending on their socioeconomic situations and eradication methods, since this rapidly transmitted virus causes large pandemics (Oguzoglu *et al.*, 2006). Sheeppox virus infection requires rapidly and correct diagnosis to prevent any outbreaks. More effective preventive precaution in addition to the early detection of infected animals should be performed to control programs (Zro *et al.*, 2014). The virus has been eradicated from European countries; however, it still causes pandemics particularly in Asian and African countries (Bhanuprakash *et al.*, 2006). The infection is classified as a highly infectious disease with a priority to fight against by the World Animal Health Organization (OIE, 2016).

Along with the increase in the body temperature, the consecutive development of papules, vesicles and pustules

in different regions of the body has been accepted as the typical symptom of the disease and the clinical findings comprise an important place in the diagnosis of sheeppox. Polymerase Chain Reaction (PCR) are the leading technique used for this purpose (Babiuk *et al.,* 2008; Bhanuprakash *et al.,* 2006). Electron microscopy is capable of identifying the capripoxvirus virions in the skin lesions; however, no discrimination can be performed for the SPPV, GTPV or the LSDV. Specific staining techniques are needed for this discrimination (Babiuk *et al.,* 2008). PCR is a sensitive, specific, rapid diagnostic method used frequently for many diseases including sheep and goat pox (Balinsky *et al.,* 2008). A29L gene is RNA polymerase subunits (35-kDa) and it has DNA-directed RNA polymerase activity and the high level of conservation of capripoxvirus genomes (Zheng *et al.,* 2007).

Nitric oxide (NO) is synthesized from L-arginine by nitric oxide synthetase (NOS). It has antimicrobial, anti-inflammatory a bacterial, viral, parasitic infections. Inducible nitric oxide synthetase (iNOS) increase throughout immun responses to viral replication. Because of NO production became maximum in the virus infected lung, NO correlate with the pathogenesis of viral pneumonia (Akaike and Maeda, 2000). Heat shock proteins (HSPs- stress proteins), overexpressed in bacterial and viral infections (Hacker *et al.,* 2009). HSP70 proteins have been found associated with viral proteins and that is determined that it promotes viral replication (Sedger and Ruby, 1994). Pulmonary surfactant proteins play an important role in pulmonary innate immune defense against viral, bacterial pathogens and prevent alveolar collapse by reducing surface tension. SP-A is determined as intense in the cytoplasm of hyperplastic type II pneumocytes and Clara cells in the lungs of sheep with natural sheeppox and retrovirus (Beytut *et al*., 2009; Beytut, 2010; Bratcher and Gaggar, 2013).

In this study, tissue samples obtained from sheep with clinically and macroscopically suspicious sheeppox disease were used to confirm the diagnosis with PCR, phylogenetic analysis was performed on the obtained PCR products by sequencing. Additionally, iNOS, SP-A and HSP-70 were immunohistochemically investigated in the lung lesions of the animals with diagnosed sheeppox.

MATERIALS AND METHODS

The samples were obtained from 20 tissues of 8 sheep with macroscopic findings of sheeppox, which were brought from the farms of 5 different regions of the Van to Yuzuncu Yil University Veterinary Medicine Faculty for necropsy.

Pathological examination: All tissue samples were fixed in 10% neutral buffered formalin, processed routinely, embedded in paraffin, sections stained with hematoxylin and eosin (HE) for histologic examination. Immunohistochemical staining was performed using the avidin–biotin immunoperoxidase complex method. The lung sections on adhesive slides were deparaffinized and dehydrated. Peroxidase activity was blocked in 3% hydrogen peroxide-methanol solution. The sections were washed with phosphate-buffered solution (PBS) and non-specific proteins were blocked by incubating protein blocking sera for 5 min. Sections were incubated with primary antibodies for 60 min at room temperature (RT) (Table 1). After washing with PBS, sections were incubated for 20 min with biotinylated goat anti-rabbit (HSP-70, SPA) or biotinylated rabbit anti-mouse (iNOS) antibodies at RT. After another PBS rinse, sections were treated with streptavidin–horseradish-peroxidase complex for 20 min. After washing in PBS, amino-ethyl carbazole (AEC) was used as chromogen and Mayer's hematoxylin was used for counterstaining. All sections were treated with PBS after each step. As controls substituting PBS for the primary antibody were included for each slide run. The rest of procedure was identical. Ten different fields on each slide were examined at high magnification. The intensity of staining was scored as no staining (-), low (+), moderate (++), and severe (+++). The extent of staining was scored as - (0%-), + (1-10 %), ++ (1-50%), and +++ (51% and higher) according to the percentage of positively stained cells. Each field was graded according to the score and then the total score was divided by ten. This way the average score was calculated for each slide.

DNA extraction and PCR: For PCR, a total of 20 different tissue samples were used from 8 of 12 sheep with macroscopic findings of sheeppox (Table 2). Extraction of Viral DNA was performed according to the GeneJET Viral DNA and RNA purification kit procedure (Thermo Scientific, catalog number: K0821). A29L gene specific CPVS (Forward) 5'-AACTGCCGTCAATGAA GAATGG-3' primers and CPVA (reverse) 5'-TTTCAAAGCTTGTTTTTAACGTRGG-3' primers and 35 cycles of PCR procedures were performed for the Nucleic Acid Amplification (Zheng *et al.,* 2007). 413 base-pair sized DNA products amplified with 1.5% strength agarose gel stained with ethidium bromide gel imager identified under UV light.

Nucleotide sequence and phylogenetic analysis: The positive products of PCR were selected; 3 were purified using a commercial purification kit prior to the sequence analysis, undergoing capillary electrophoretic separation, and sequence analysis was performed. Phylogenetic analysis (Neighbor-Joining) with bootstrap analysis (1,000 replicates) and Kimura 2-parameter correction was conducted using the MEGA software package v5.0. (Kumar *et al.,* 2004).

RESULTS

Macroscopically, typical sheeppox papules were observed in the skin (6 cases), the tongue (5 cases), abomasum (1 case). Grey-white, pale foci, approximately 1-2 cm were detected in the lung (7 cases) and in the kidney (1 case). Histopathologicaly, epidermal hyperplasia, vacuolar and necrotic keratinocytes, eosinophilic intracyto-plasmic inclusion bodies were observed in epidermis of the skin. Mononuclear cell infiltration, characteristic sheeppox cells, inclusion bodies and vasculitis were observed in the dermis (Fig. 1a). Pustules and necrotic foci were observed on the tongue. Interstitial pneumonia, mononuclear cell infiltration, proliferative alveolitis and bronchiolitis, sheeppox cells, intracytoplasmic inclusion bodies were observed in the lungs (Fig. 1b); epithelial hyperplasia, spongiosis, sheep pox cells were observed in the abomasums sheeppox cells were detected in kidneys.

Table 1: Characteristics of antibodies used for immunohistochemistry

Antibodies	*Origin*	*Dilutions*	*Source*
iNOS*	Mouse monoclonal	1:100	Thermo Fisher Scientific
HSP-70*	Rabbit monoclonal	1:100	Thermo Fisher Scientific
SPA*	Rabbit polyclonal	1:500	Chemicon

*Heat-induced antigen retrieval was performed using citrate buffer.

Table 2: The results of PCR amplification of A29L gene of necropsy samples

Case no		Sample no	Material	PCR
1	Gürpınar	1	Skin	+
		2	Tongue	+
		3	Stomach	+
2	Muradiye	4	Skin	+
		5	Lung	+
		6	Tongue	+
3	Gürpınar	7	Skin	+
		8	Tongue	+
		9	Lung	+
4	Özalp	10	Lung	+
		11	Kidney	+
		12	Skin	+
5	Erciş	13	Skin	+
		14	Tongue	+
		15	Lung	+
6	Başkale	16	Lung	+
7	Özalp	17	Lung	+
8	Muradiye	18	Skin	+
		19	Tongue	+
		20	Lung	+

Table 3: İmmunostaining. Score; no staining (-), low (+), moderate (++), and marked (+++).

Case	SP-A	HSP-70	iNOS
1	+++	+	++
2	+++	+	+++
3	+++	+	+++
4	+++	+	++
5	+++	+	+++
6	+++	+	++
7	+++	+	++

The detailed immunohistochemical findings was shown in Table 3. In all cases, SP-A was moderate or intensely stained in the cytoplasm of the type II pneumonitis and Clara cells (Fig. 2a). In all positive cases, positive staining was identified by diffuse labeling in the cytoplasm and the hyperplastic cell membrane. iNOS protein expression was intense or moderate in hyperplastic epithelial cells of lungs (Fig. 2b). HSP-70 immunostaining were weak in alveolar septa (Fig. 2c). SP-A staining was also immuno-positive in the normal sheep lungs, but was much less intense than in lung with sheeppox. iNOS and HSP-70 expression was also not detected in the lung tissues of normal sheep.

All tissues were tested by PCR and determined to be positive (Fig. 3). Three samples were selected that were strongly positive with PCR (V1-skin, V5-lung, V11-kidney) and sequence analysis was performed (Refgen, Ankara). BLAST searches in GenBank revealed these sequences to be 98-100% identical to various poxvirus strains. The phylogenetic map prepared comparing the A29L gene sequences obtained from the study between each other and the reference sequences obtained from the gene bank have been demonstrated in Fig. 4. (GenBank Accession No: V1-KU521783, V5-KU521784, V11-KU521785). The accession numbers used in phylogenetic analysis were: KJ546376, KJ546375, KJ546377, KJ546378, KC951854, AY077832, AY077833, AY077834, AY077835 and AY077836, respectively.

The samples were found to be similar between each other. When it is considered that the mutation rates in DNA viruses are lower, the virus within the city was observed to be of the same strain. When the samples were compared to the sheep and goat poxviruses in the gene bank, varying similarities were observed. The resulting of sheeppox virus strains were determined to be 100% homology to the strains obtained from America (AY077832, AY077833, AY077834) and were determined 1-2% diversity from Egypt (KJ546376, KJ546375, KJ546377, KJ546378) and China (KC951854) strains.

The sheep poxvirus strains obtained in the phylogenetic analysis were found to be similar to the other sheeppox viruses compared (Fig. 5), and the goat poxvirus was observed to include differences in the 66th base (G→A), 81st base (A→C), 120nd base (T→C), 252nd base (G → C) and the 301st base (A→G). However, these differences were determined not to cause a difference in the amino acids.

DISCUSSION

Although electron microscopy has been clinically used for a long time in the detection of sheeppox virus, different strains of viruses within the family Poxviridae sheeppox, goatpox, Lumpy Skin Disease virus cannot be morphologically discriminated. Capripoxviruses are serologically identical, but certain identification is performed by molecular techniques (Venkatesan *et al.,* 2014). Molecular techniques should be used in order to determine the antigenic character of the virus and the described viral strain should be phylogenetically analyzed (Bhanuprakash *et al.,* 2006; Tuppurainen *et al.,* 2014). It is obvious that the PCR technique is time-advantageous. Furthermore, some studies have determined that the specificity and sensitivity of this test more sensitive compared to the virus isolation technique in the detection of sheeppox virus (Balinsky *et al.,* 2008). In this study the primers are selected spesific A29L gene. This gene has DNA-directed RNA polymerase activity and the high level of conservation of capripoxvirus genomes (Zheng *et al.,* 2007). All necropsy materials used in our study were tested via PCR and determined to be positive.

It was observed that the sensitivity depends on the race among sheep. The Merinos race with a thinner wool were more sensitive. Sheeppox disease may be observed at any age of the sheep (Mahmoud *et al*., 2016). However, the mortality rate may be as high as 80-100% among lamb. In this study, infection was observed in lamb younger than 1 month as well (Bhanuprakash *et al.,* 2006; Zhu *et al*., 2013).

The papules generally were reported on the skin, in abomasa mucosa, tongue, rumen, palate, trachea and esophagus. The grayish-white pale foci, 1-2 cm in diameter round, were reported in especially the diaphragmatic lobe of lung, the kidney and liver. Typical papules were observed in the skin (6 cases), the tongue (5 cases) and the abomasum (1 case). Grey-white, faint foci were detected in the lungs (7 cases) and the kidney (1 case).

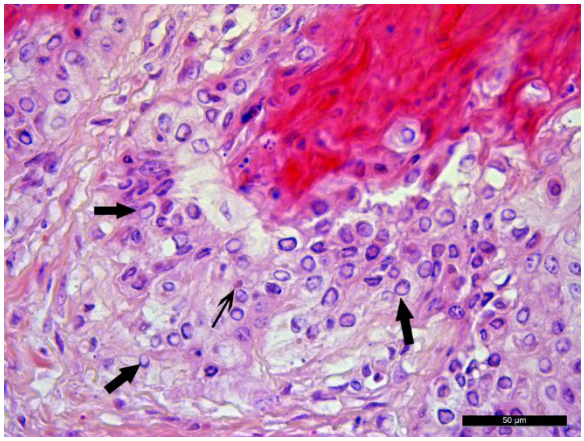

Fig. 1A: Sheeppox virus infected cells (bold arrows) and inclusion bodies (thin arrows), Dermis, HE. Bar = 50 µm.

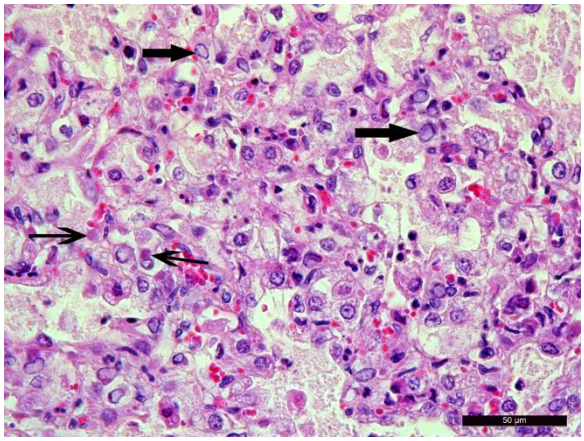

Fig. 1B: Sheeppox virus infected cells (bold arrows) and inclusion bodies (thin arrows), Lung, HE. Bar = 50 µm.

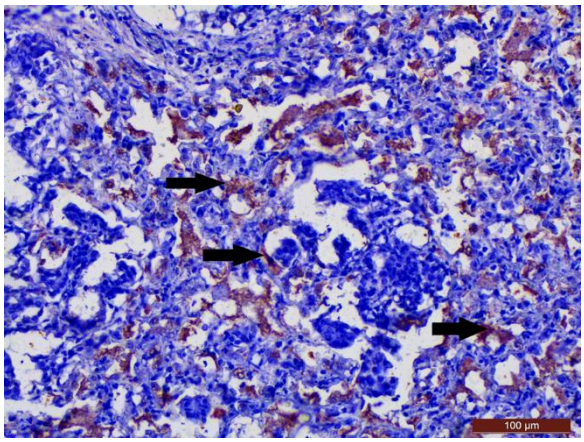

Fig. 2A: Sheep; lung. Immunohistochemistry (IHC) of ovine sheeppox, Surfactant protein (SP-A) intracytoplasmic positive immunostaining in sheeppox virus infected alveolar type II cells (arrows). IHC. Bar = 50 µm.

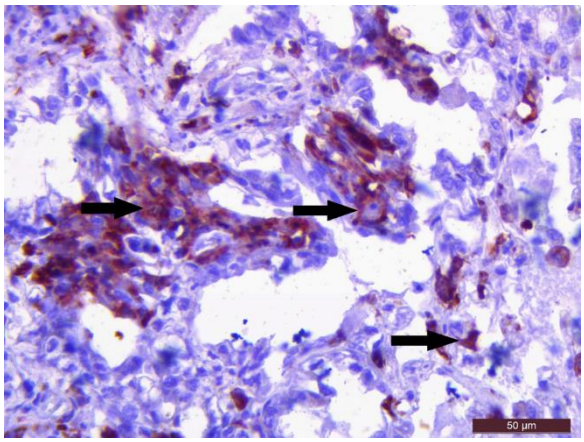

Fig. 2B: Sheep; lung. Immunohistochemistry (IHC) of ovine sheeppox. iNOS expression in cytoplasm of tumor cells which cells are infected by sheeppox virus (arrows). IHC. Bar = 50 µm.

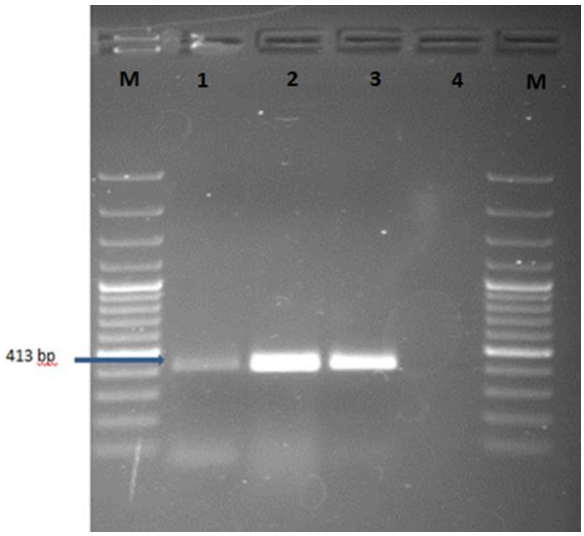

Fig. 3: PCR product of amplified A29L gene of sheeppox viruses. [M: Marker (100 bp DNA ladder) 1,2,3: spesific product for Sheeppoxvirus, 4: negative control (Distile water)].

Nitric oxide is known to block the rate-limiting enzyme of DNA synthesis, ribonucleotide reductase, show an antiviral effect by inhibiting the viral replication in DNA and RNA viruses via deamination of the cellular DNA (Türköz and Özerol, 1997). Neuronal-nNOS, Endothelial-eNOS and Inducible-iNOS are three forms of nitric oxide synthetase. nNOS, eNOS are in their inactive form within the tissues in the physiological period, and the enzyme inducible nitric oxide synthetase (iNOS) is not present under normal circumstances. iNOS is synthesized from macrophages, neutrophils and vascular endothelial cells in pathological conditions and in the presence of inflammatory mediators such as tumor necrosis factor (TNF), interleukin 1 (IL-1), IFN and endotoxins. Stimulation of the L-Arginine-NO pathway leads to nitric oxide synthesis for hours or even days. NO is essential for maintaining the normal physiological events; however, in high concentrations, it has harmful effects and causes damage. Therefore, iNOS can cause both protective and cytotoxic effects in acute inflammatory events (Türköz and Özerol, 1997). The increased amount of iNOS in the acute period of the infection will lead to the reduction of the causative factor after a time. In chronic infection, the amount of iNOS will decrease with the reduction in the causative amount of the factor. Influenza A viruses (H5N1) cause severe pulmonary diseases in animals. The amount of iNOS in the lungs increase in proportion with the increase in the number of macrophages and neutrophils and the amount of free oxygen radicals (Perrone *et al.*, 2008). In our study, iNOS immunopositive staining was mostly observed in epithelium of bronchi and bronchioles.

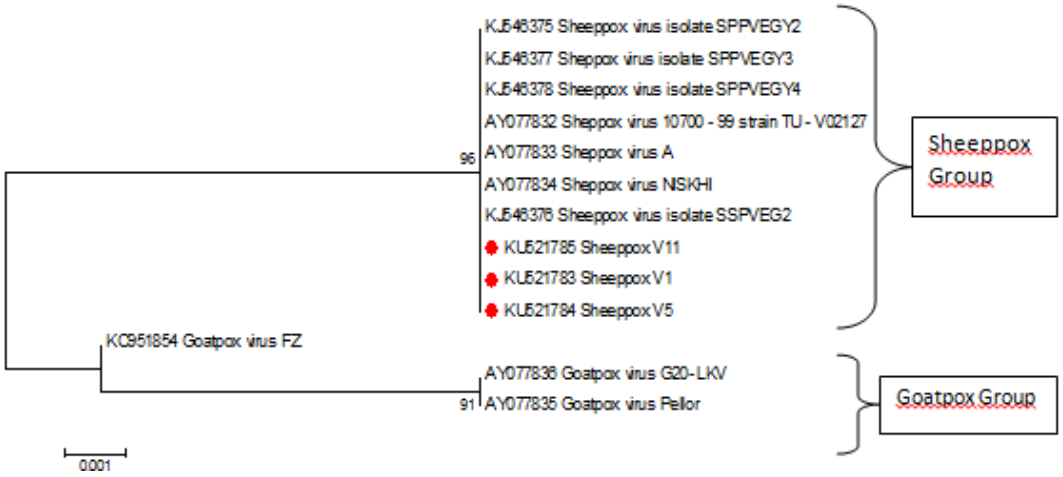

Fig. 4: Phylogenetic tree of sequences from this study and reference sequences.

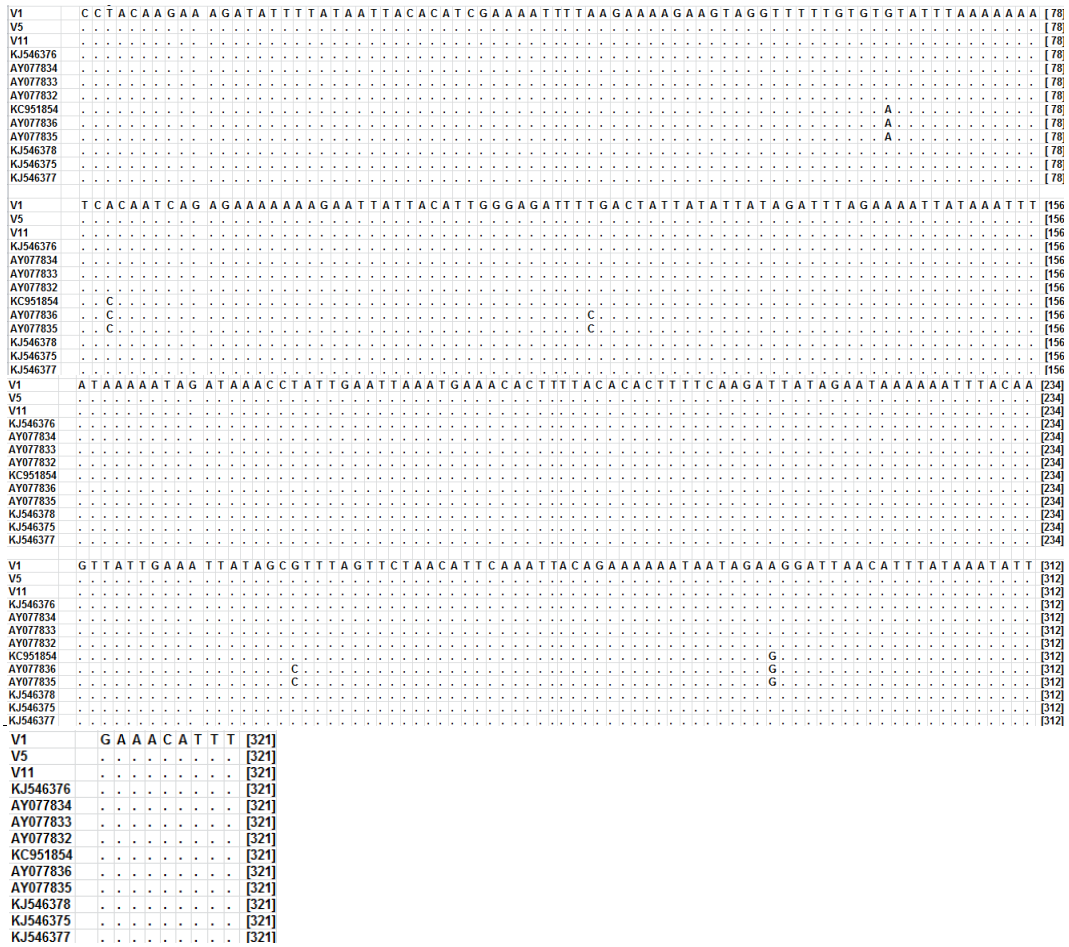

Fig. 5: Comparison of sequences of Sheeppox virus reference field isolate from genbank

The pulmonary collections surfactant protein SP-A and SP-D play important roles in innate lung defense, limiting lung inflammatory responses and they are synthesized from alveolar type II pneumocytes. With various stimulating agents, SP-A and SP-D bind to the viral, bacterial, fungal pathogens and inactivate the pathogen. SP-A, SP-B, SP-D are also synthesized from respiratory tract cells (Clara cells, submucosal cells). It has also been reported that SP-A is present in small amount in healthy lungs, but is more intense in the cytoplasm of hyperplastic type II pneumocytes and Clara cells in the lungs of sheep with natural sheeppox and retrovirus (Beytut *et al.*, 2009; Beytut, 2010). In the current study, SP-A was detected in type II pneumocytes.

HSP-70 has a role in many physiological and pathological processes including regulation of the cell cycle, apoptosis, viral infections, neurodegenerative disorders and oncogenesis. The cell-protective and anti-inflammatory effects of HSP- 70 have been reported in the animal models of various diseases such as pulmonary fibrosis, gastric ulcer, inflammatory intestinal disease and Alzheimer (Tanaka *et al.*, 2010; Namba *et al.*, 2011; Wang *et al.*, 2012).

In the lungs and plasma of rats with experimental pulmonary fibrosis induced by silicon dioxide (SiO2), HSP-70 was investigated (Wang *et al.*, 2012). In the lungs of Silicon dioxide applied study groups; on 7th day HSP-70 expression was increased, on 14th day HSP-70 expression was increased to the highest level and was

reduced again after that day. In the plasma HSP-70 was found at the highest level after 21st day. In this study HSP-70 expression in lung tissue also detected in light intensity although it was higher than the control tissue. This situation is explained that infection wasn't during the acute phase. In this study, iNOS, SP-A and HSP-70 were found as playing a role in sheeppox which is viral infection.

In a research conducted in Eastern Turkey aimed to identify the viruses obtained from the seasonal cases and to attempt to comment on the seasonal variations in occurrence of sheeppox cases (Eroksuz *et al.,* 2008). They reported that the cases occurring in the March and April and it could be concluded that being in the pasture of time for mother ewes plays role in the epidemiology of the seasonal cases. In this study the necropsy materials were obtained in October-December. Sheeppox virus endemics occur frequently in the periods of October- November and espesially in lambs in March- April in Turkey (Oguzoglu *et al.,* 2006).

Sheeppoxvirus is highly transmissive and notification of the disease is obligatory in many countries. The capripoxvirus genomes were determined very similar to (no less than 96% nucleotide identity) sequences of sheeppoxvirus and goatpoxvirus strains (Tulman *et al.,* 2002; Venkatesan *et al.,* 2014). Also in another study based on P32 gene of capripoxvirus obtained from goat, sheep and cattle indicated that goatpoxvirus and lumpy skin disease virus are more closely related than sheeppoxvirus is to lumpy skin disease virus (Hosamani *et al.,* 2004). In some countries, the sheep and goats are kept in the same barns, and since transmission is observed between these species, it is more difficult to obtain information on the diagnosis and epidemiology of the infection. For this purpose, our study aimed to make a comparison with some goat strains during the phylogenetic analysis. Among our three sequences, V1-KU521783, V5-KU521784, V11- KU521785, shared 100% nucleotide identities. The sequences obtained from the samples of our study for our phylogenetic map constituting a branch with the sheep pox sequences obtained from Egypt (KJ546376, KJ546375, KJ546377, KJ546378) and United States (AY077832, AY077833, AY077834), whereas the goat pox sequences (AY077835, AY077836, KC951854) composed a separate branch.

There is limited number of study about molecular characterization of sheeppox virus in Turkey. In a study presented in different necropsy spescimens of a sheep, samples were used to sequencing analysing which were determined by PCR as positive (Yazıcı *et al.*, 2008). This sequence of virus strain was determined 98-100% homology between strains of Kazakhstan and India.

The resulting of sheeppox virus strains was determined to be 100% homology to the strains obtained from America (AY077832, AY077833, AY077834) and was determined % 1-2 diversity from Egypt (KJ546376, KJ546375, KJ546377, KJ546378). Van Turkish poxviruses sequences obtained from sheep were found to be 98% identity with AY077835, AY077836 (United States) and 99% identity KC951854 (China) strains which from obtained goats.

Conclusions: In this study, presence of sheeppox virus into the necropsy materials obtained from the sheep was confirmed by PCR along with the specific pathological and immunohistochemical changes for the disease and phylogenetic analysis was performed. Because of this disease causes epidemics in our country, it is important to determine in a short time and to use control methods in sheep. We believe that this study including the current molecular characterization data supported by immunohistochemical data belonging to the regional strains of sheeppox virus would be useful in further studies and in strategies to be developed in the battle against the disease.

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Authors' contributions: FI and SY performed the pathological experiments, ZK and ED performed PCR, sequence analysis and phylogenetic analysis. All authors critically revised the manuscript and approved the final version.

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