



Cytokine profile in lambs naturally infected with sheeppox virus

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Abstract

In this study, in order to reveal the immune response against the disease in naturally infected sheep with SPPV, the expressions of various pro- or anti-inflammatory cytokines such as tumour necrosis factor alpha (TNF- α), interferon gamma (IFN- γ), interleukin-1beta (IL-1 β), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10) and interleukin-12 (IL-12) were evaluated immunohistochemically. The material of this study consisted of tissue samples taken from 24 sheep, which were brought as dead for routine histopathological examination to the Department of Pathology. Avidin–biotin–peroxidase method was used for immunohistochemistry. Characteristic pox lesions were observed in the skin, lungs and kidneys. In histopathological examinations, pox cells, which are very characteristic for the diagnosis of the disease, were observed in all three tissues. Capripoxvirus nucleic acid was detected in 8 of the 24 tissues. Samples were sequenced, and a phylogenetic tree was constructed with reference strains from GenBank. Strains from the study clustered with sheeppox virus references. In conclusion, the levels of pro-inflammatory cytokines such as TNF- α , IFN- γ , IL-1 β , IL-2, IL-8 and IL12 (Th1) were much more dominant compared to the levels of anti-inflammatory cytokines: IL-10 and IL-6 (Th2). This supported the fact that the cellular immune response is much more effective than the humoral immune response in sheeppox.

Keywords Cytokine · Immune response · Lamb · Polymerase chain reaction · Sheeppox virus

Introduction

Sheeppox virus (SPPV), goatpox virus (GTPV) and lumpy skin disease virus (LSDV) are three members of the *Capripoxvirus* (CAPV) genus in the Poxviridae family (Ben Chehida et al. 2018). These viruses are double-stranded and enveloped DNA viruses and share a similar nucleotide sequence with up to 99% identity (Aldaiarov et al. 2016). Although *Capripoxvirus* infections are generally species-specific, some species variations in SPPV have been reported to infect both sheep and goats (Chahota et al. 2022). In contrast, LSDV affects cattle and domestic Asian water buffalo but does not naturally infect sheep and goats (Zewdie et al. 2021).

SPPV can survive in the environment for long periods (Oreiby et al. 2022). The virus can be transmitted by inhalation, close contact or mechanically (fly bite) (Beytut 2010). The incubation period of the disease is 2–12 (6 to 8 days in average). In sheep infected by the virus, before the formation of multifocal necrotic lesions on the skin, clinical symptoms such as increased body temperature (40–42 °C), loss of appetite, depression, inflammation of the conjunctiva,

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a viscous or purulent nasal discharge, rapid breathing and increased heart rate can be detected (Zhu et al. 2013). In addition to skin lesions, smallpox lesions are detected in many internal organs, including the lungs and digestive tract (Embury-Hyatt et al. 2012). The disease causes high morbidity and mortality depending on the virus isolate and host type (Roy et al. 2008). Even if the disease affects all age groups, death is mostly seen in young lambs (Beytut 2010).

Pox virus infections, which tend to spread rapidly and cause pandemics, are of great importance for the public health and socio-economic conditions of the countries. These diseases cause great economic damage to the international trade of animals and animal products such as meat, wool and leather (Oğuzoğlu et al. 2006; Oreiby et al. 2022). Sheepox (SP), a highly contagious and systemic viral disease of sheep, has been included in the diseases of interest by the World Organization for Animal Health (WOAH) (WOAH. 2023). The disease is mostly distributed in Africa, the Middle East, Asia, Turkey, Bulgaria and Greece (Oreiby et al. 2022). Sheepox can be confused with diseases such as contagious ecthyma, blue tongue, small ruminant plague, dermatophytosis, sheep scabies, urticaria and parasitic pneumonia (Ben Chehida et al. 2018; Zewdie et al. 2021). The diagnosis of sheepox is performed based on clinical findings with very characteristic features, virus isolation, virus neutralization tests, serological evaluations such as ELISA and molecular methods such as polymerase chain reaction (PCR) (Zro et al. 2014).

Restriction of animal movements and quarantine is insufficient to contain *Capripoxvirus* outbreaks; therefore, vaccination is considered the most important element in controlling this disease. Although humoral immunity against *Capripoxviruses* provides protection, the main defence develops through cellular defence (Chibssa et al. 2021; Oreiby et al. 2022).

In this study, in order to reveal the immune response against the disease in naturally infected sheep with SPPV, the expressions of various pro- or anti-inflammatory cytokines such as tumour necrosis factor alpha (TNF- α), interferon gamma (IFN- γ), interleukin-1beta (IL-1 β), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10) and interleukin-12 (IL-12) were evaluated immunohistochemically.

Materials and methods

Animals

The material of this study consisted of tissue samples taken between years 2012–2022 from 24 sheep, which were brought as dead for routine histopathological examination to the Department of Pathology, Faculty of Veterinary

Medicine of Kafkas University. All sheep were bred only in Kars province. According to the anamnesis information obtained from the owners, it was noted that the animals exhibited various clinical signs such as high fever, loss of appetite, discharge from the mouth, eyes and nose and cough. Additionally skin, lung and kidney tissue samples from 6 healthy sheep were also taken for control purposes.

Molecular methods

Formalin-fixed paraffin-embedded (FFPE) tissue blocks belonging to 24 cases were used for DNA extraction. The method used was described by Pikor et al. (2011). The presence of *Capripoxvirus* nucleic acid was investigated by polymerase chain reaction (PCR). ORF095 (the homologue of A4L gene) was targeted because, according to the publication by Madhavan et al. (2020); after the performance of PCR and sequencing, it allows for distinguishing between SPPV, GTPV and LSDV. The expected amplicon size was 486 bp. The PCR product was visualized in a transilluminator after electrophoresis in a 1% agarose gel containing Safe-Red (Safe View™ Cat No: G108-R, Canada) DNA stain.

For phylogenetic analysis, positive amplicons suitable for sequencing were selected. These amplicons were sent to a commercial company for Sanger sequencing (BM Yazilim Danis. ve Lab. Sis. Ltd. Sti, Ankara).

For constructing a phylogenetic tree, the reference strains of SPPV, GTPV and LSDV were obtained from the GenBank database. The sequence alignment was performed using Bioedit Version 7.0.5.3 (Hall 1999), and the phylogenetic analysis was performed using MEGA7 software as previously described (Tamura et al. 2011). The neighbour-joining and maximum likelihood methods of software were selected. For neighbour-joining, the sequence differences were calculated with the Kimura two-parameter model. The confidence level was assessed by bootstrapping using 1000 replicates. For maximum likelihood Tamura–Nei model was used with the nearest-neighbour-interchange (NNI) method. Similarity/identity matrices were generated using MatGAT with BLOSUM50 scoring (Campanella et al. 2003).

Histopathological examinations

Skin, lung and kidney tissue samples taken from the sheep were fixed in a 10% formaldehyde solution. Serial sections of 5 μ m thickness were taken from the paraffin blocks prepared after the routine tissue follow-up procedures. Haematoxylin and eosin (H&E) staining was performed on the sections in order to evaluate the histopathological changes in the tissues. The sections were evaluated in detail under a light microscope by at least two pathologists, and the observed lesions were photographed.

Immunohistochemical studies

Serial sections taken from paraffin blocks prepared from skin, lung and kidney tissues with a thickness of 4 microns were stained with TNF- α , IFN- γ , IL-1 β , IL-2, IL-6, IL-8, IL-10 and IL-12 commercial antibodies using the avidin–biotin–peroxidase (ABC) technique following the manufacturer's procedures. Information regarding the primary antibodies used in the study is given in Table 1. The Thermo Scientific Histostain IHC Kit (HRP, Broad-spectrum, REF: TP-125-HL) was used to conduct all the immunostainings. Amino ethyl carbazole (AEC, Thermo Scientific, REF: TA-125-HA) was applied as the chromogenic substrate and incubated for 15 min. After staining with Mayer haematoxylin, the slides were rinsed with distilled water for 5 min and coated with AEC mount.

After coating, the prepared slides were observed under a light microscope (Olympus Bx53), and the sections were photographed using the Cell ^P program (Olympus Soft Imaging Solutions GmbH, 3,4). Detailed analyses of the captured photographs were made using the program ImageJ (1.51j8).

The immunohistochemical staining results were analysed with a grading system based on the number of positive cells in the areas examined, which presented immune-positive reactions and reflected most strongly the staining character. For the quantification of immune-positive reactions in tissues, the analysis was started based on the areas of high-intensity reaction. In each tissue (skin, lung and kidney), 3 different areas were evaluated with a 50 $\times$ magnification. The numbers of positively stained cells were recorded separately, and the average of these 3 fields was considered the average positive cell number of that animal. Rating was designated as (–) no immunoreactivity; (+) weak, 1–10% positive; (++) moderate, 11–59% positivity; and (+++) severe positivity over 60% (Beytut 2017).

Results

Molecular results

After performing PCR, *Capripoxvirus* nucleic acid was detected in 8 of the 24 cases. Among these 8 cases, 3 amplicons were suitable for sequencing, and it was done successfully by a commercial firm.

Three sequences were submitted to GenBank, and accession numbers were obtained (OQ302232, OQ302233, OQ302234). *Capripoxvirus* genus identification is based on ORF095, which encodes virus core protein. This gene is homologous with vaccinia virus A4L gene. When phylogenetical analyses are performed, *Capripoxvirus* isolates are separated to three clusters, defining three different members of the genus, namely, SPPV, GTPV and LSDV. Our isolates were clustered with SPPV references (Fig. 1). This result confirmed that the causative agent was SPPV.

Nucleotide and amino acid changes of our isolates are compared with the reference SPPV strains using the alignment. Results of the changes in nucleotide and amino acid are shown in the Fig. 2 and Fig. 3 respectively. For isolate OQ302232, nucleotide changes in bases are 153 (C $\rightarrow$ T), 225 (A $\rightarrow$ T), 279 (T $\rightarrow$ A), 288 (T $\rightarrow$ A), 300 (A $\rightarrow$ C), 334 (A $\rightarrow$ T), 339 (A $\rightarrow$ G), 351 (T $\rightarrow$ A), 354 (A $\rightarrow$ C), 373 (T $\rightarrow$ C), 376 (A $\rightarrow$ T), 379 (A $\rightarrow$ T), 418 (G $\rightarrow$ A), 422 (A $\rightarrow$ T), 430 (A $\rightarrow$ G) and 433 (A $\rightarrow$ C). For isolate OQ302233, nucleotide changes in bases are 246 (T $\rightarrow$ A), 286 (G $\rightarrow$ A), 322 (C $\rightarrow$ T) and 387 (C $\rightarrow$ T). These changes resulted in amino acid changes. For OQ302232 isolate, amino acid changes at residues were 93 (D $\rightarrow$ E), 96 (D $\rightarrow$ E), 112 (I $\rightarrow$ F), 117 (D $\rightarrow$ E), 125 (S $\rightarrow$ P), 126 (I $\rightarrow$ F), 127 (M $\rightarrow$ L), 140 (D $\rightarrow$ N), 141 (D $\rightarrow$ V), 144 (S $\rightarrow$ G) and 145 (K $\rightarrow$ Q). OQ302233 isolate amino acid changes at residues were 96 (D $\rightarrow$ N) and 108 (L $\rightarrow$ F).

A second phylogenetic tree was constructed to examine the close related strains (Fig. 4). The closest related SPPV reference strains with our isolates are based on NCBI's BLAST search (KX398477, KX398475, MG458395,

Table 1 Information on primary antibodies used in immunohistochemical studies

Primary antibodies	Pretreatment	Company and catalogue numbers	Dilution	Incubation condition
TNF- α	Microwave open	MyBioSource, MBS2025729, monoclonal	1/100	Overnight, 4 °C
IFN- γ	Microwave open	MyBioSource, MBS2091397, polyclonal	1/100	Overnight, 4 °C
IL-1 β	Microwave open	MyBioSource, MBS2026862, polyclonal	1/50	Overnight, 4 °C
IL-2	Microwave open	Biorbyt, orb657069, polyclonal	1/200	Overnight, 4 °C
IL-6	Microwave open	MyBioSource, MBS2012740, monoclonal	1/100	Overnight, 4 °C
IL-8	Microwave open	MyBioSource, MBS2004056, polyclonal	1/200	Overnight, 4 °C
IL-10	Microwave open	MyBioSource, MBS2026258, polyclonal	1/200	Overnight, 4 °C
IL-12	Microwave open	MyBioSource, MBS1490500, polyclonal	1/50	Overnight, 4 °C

Fig. 1 Phylogenetic tree constructed with the neighbour-joining method using MEGA7 software in reference to sheep-pox virus, goatpox virus and lumpy skin disease virus strains. The sequences of this study are indicated with black dots

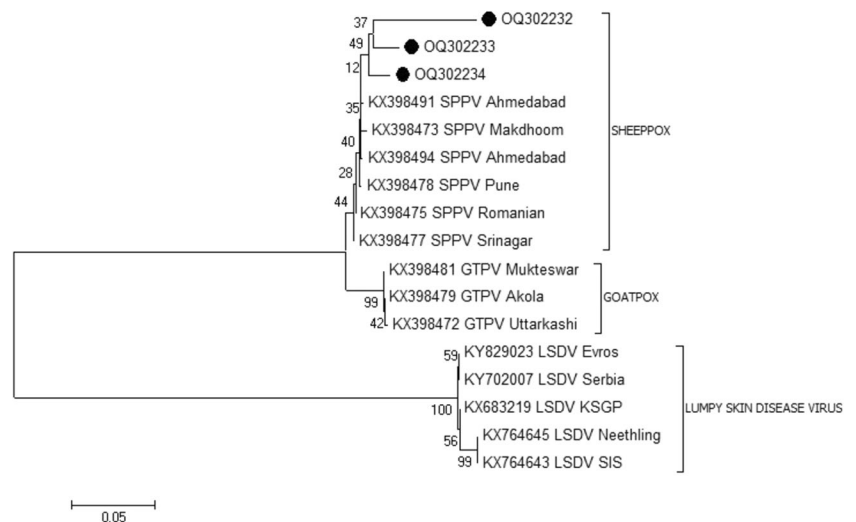
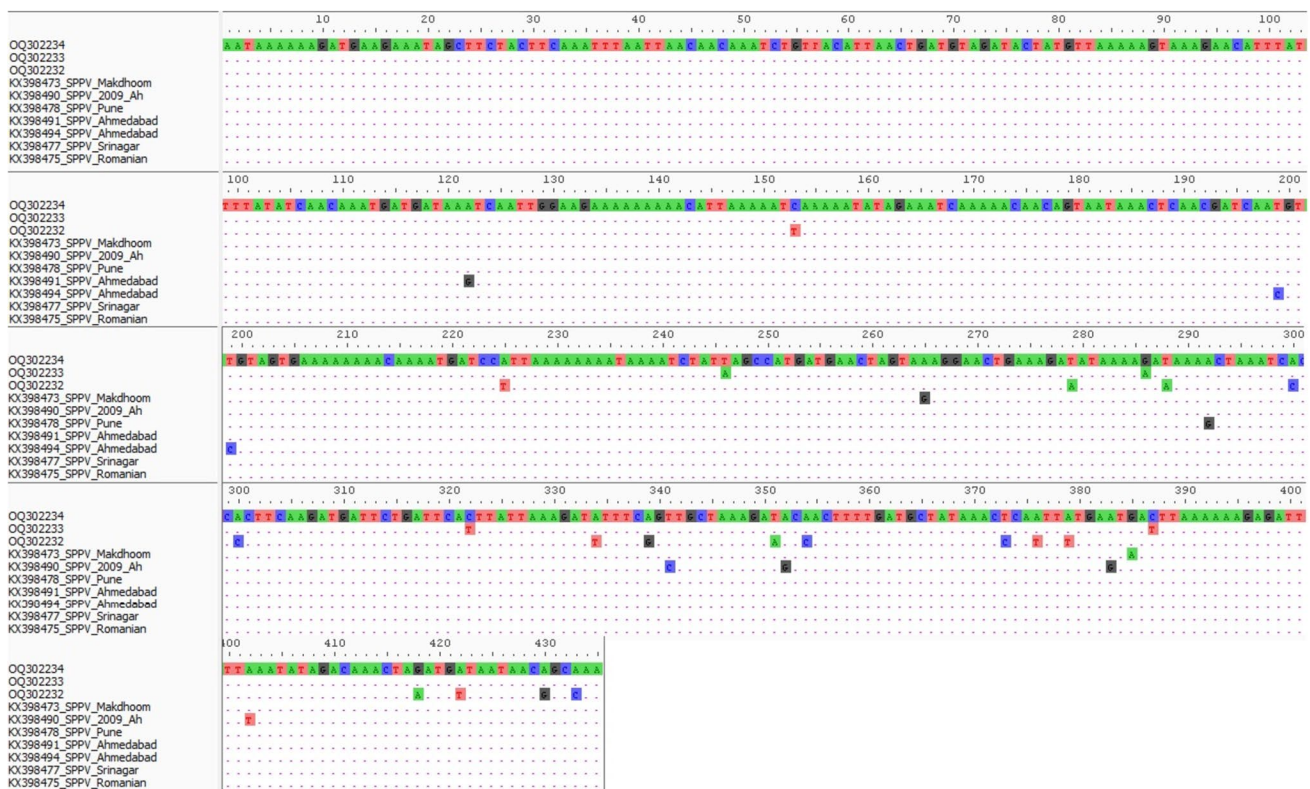


Fig. 2 The comparison of ORF095 gene nucleotides of sheep-pox virus and our isolates with references

MN072631, MN072630, MN072629, MN072628, MN072626, ON961656) which include two Turkish strains one GTPV and one LSDV strain. While OQ302234 was closer to other references, OQ302232 was found to be more closely related to GTPV strain.

Two similarity/identity matrices with the study isolates were prepared, one including closely related SPPV

references (Fig. 5A) and the other one just including study isolates and two other Turkish isolates (Fig 5B). Similarity index of the strains showed between 95.3 and 100% identity.

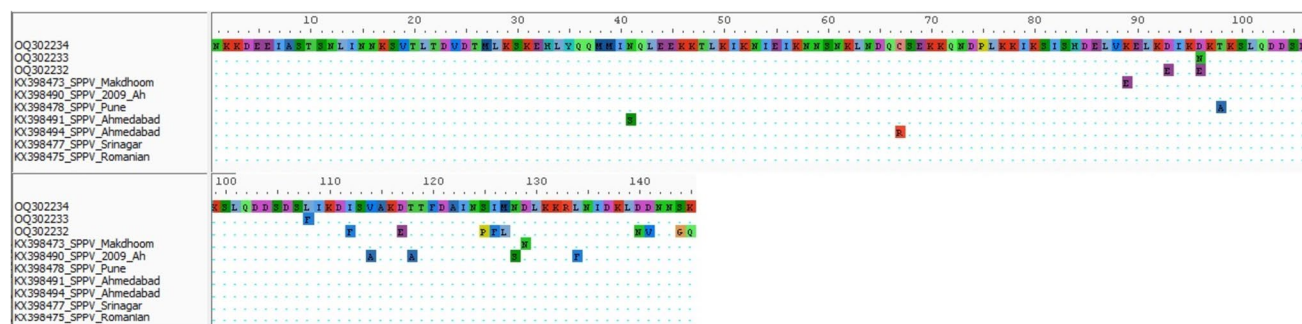
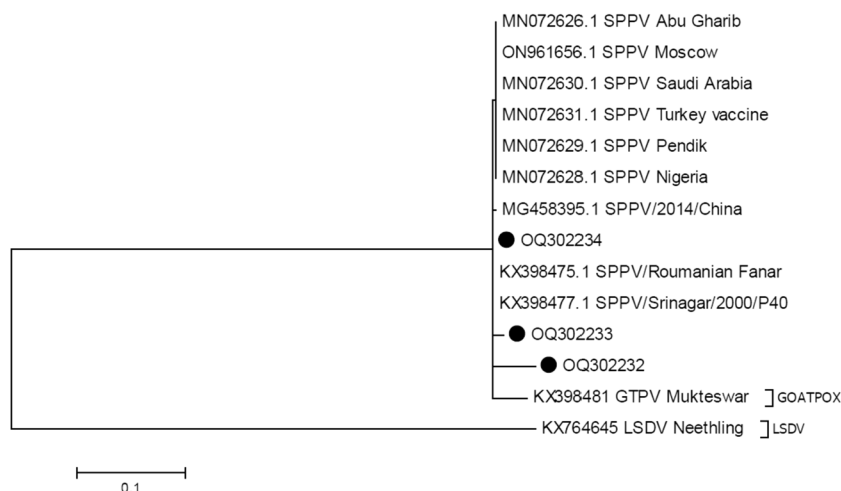


Fig. 3 Amino acid comparison of ORF095 gene of sheeppox virus and our isolates with references

Fig. 4 Phylogenetic analysis of close related strains using maximum likelihood method. The sequences of this study are indicated with black dots



	1	2	3	4	5	6	7	8	9	10	11	12
1. OQ302232		95.3	96.2	96.0	96.0	96.0	96.0	96.0	96.0	96.0	96.2	96.2
2. OQ302233	95.3		99.1	98.8	98.8	98.8	98.8	98.8	98.8	98.8	99.1	99.1
3. OQ302234	96.2	99.1		99.8	99.8	99.8	99.8	99.8	99.8	99.8	100.0	100.0
4. MN072629.1 SPPV Pendik,	96.0	98.8	99.8		100.0	100.0	100.0	100.0	100.0	99.5	99.8	99.8
5. MN072631.1 SPPV Turkey vaccine,	96.0	98.8	99.8	100.0		100.0	100.0	100.0	100.0	99.5	99.8	99.8
6. MN072630.1 SPPV Saudi Arabia,	96.0	98.8	99.8	100.0	100.0		100.0	100.0	100.0	99.5	99.8	99.8
7. MN072626.1 SPPV Abu Gharib,	96.0	98.8	99.8	100.0	100.0	100.0		100.0	100.0	99.5	99.8	99.8
8. ON961656.1 SPPV Moscow,	96.0	98.8	99.8	100.0	100.0	100.0	100.0		100.0	99.5	99.8	99.8
9. MN072628.1 SPPV Nigeria,	96.0	98.8	99.8	100.0	100.0	100.0	100.0	100.0		99.5	99.8	99.8
10. MG458395.1 SPPV/2014/China,	96.0	98.8	99.8	99.5	99.5	99.5	99.5	99.5	99.5		99.8	99.8
11. KX398475.1 SPPV/Roumanian Fanar,	96.2	99.1	100.0	99.8	99.8	99.8	99.8	99.8	99.8	99.8		100.0
12. KX398477.1 SPPV/Srinagar (2000/P40),	96.2	99.1	100.0	99.8	99.8	99.8	99.8	99.8	99.8	99.8	100.0	

A

	1	2	3	4
1. OQ302232		95.3	96.2	96.0
2. OQ302233	95.3		99.1	98.8
3. OQ302234	96.2	99.1		99.8
4. MN072629.1 SPPV Pendik,	96.0	98.8	99.8	
5. MN072631.1 SPPV Turkey vaccine,	96.0	98.8	99.8	100.0

B

Fig. 5 **A** The per cent similarity of ORF095 gene nucleotides of our isolates with reference strains. **B** The per cent similarity of Turkish only strains

Macroscopic findings

Characteristic pox lesions were observed in animals, especially in the areas with reduced wool. Papular lesions were mostly concentrated on the eyelids, lips, nostrils, under the tail, in the inner part of the legs and on the skin of the breasts (Fig. 6A).

Congestion, oedema and consolidation were detected in the lungs. Some cases were mild, however, in some cases, severe and coalescent. Multifocal typical pox nodules, 2–3 cm in diameter, with a bright-white appearance separated by

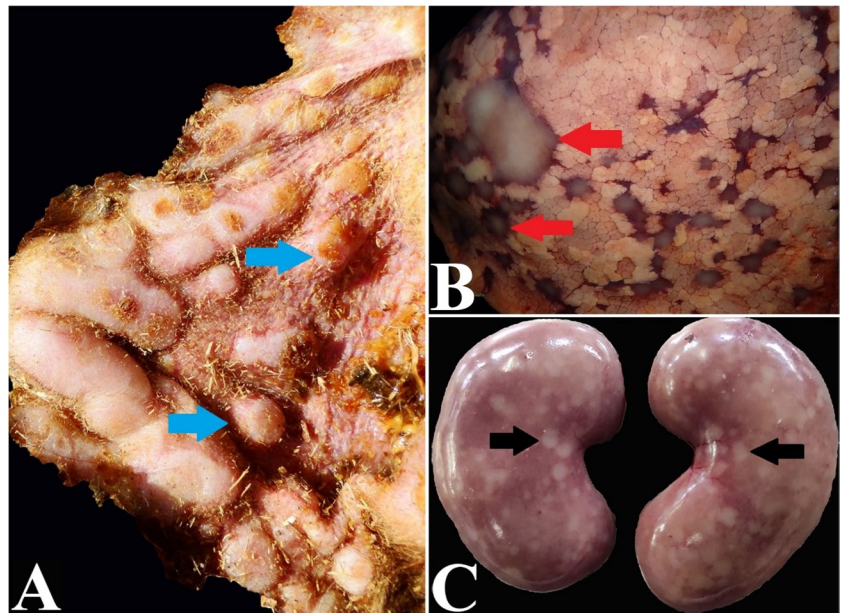
sharp hyperemic borders were detected. These pox nodules were particularly prominent in the caudal lobes (Fig. 6B).

In the kidneys, multifocal large and small white round nodules were detected in the cortex. These lesions were also present on the cut surface of the organ (Fig. 6C).

Microscopic findings

The histopathological examination of the skin revealed significant hydropic degeneration and microvesicles in the keratinocytes in the stratum spinosum layer. Eosinophilic

Fig. 6 **A** Papillary lesions on the ventral surface of the tail (blue arrows). **B** Multifocal or fused pox lesions in the lung surrounded by a hyperemic ring (red arrows). **C** Multiple whitish nodular lesions along the cortex in the kidney (black arrows)



inclusion bodies (Guarneri) were found in the cytoplasm of the degenerated keratinocytes. In addition to these changes, acanthosis and hyperkeratosis were detected in the epidermis around the lesioned areas. The lesions were concentrated in both the surface epithelium and the hair follicle epithelium. In the dermis layer, vasculitis was observed in addition to an inflammatory cell infiltration of mostly mononuclear cells. Histiocyte-like large pox cells, which are very characteristic in the diagnosis of the disease, presented vacuoles, and the chromatin was concentrated in the nuclear membrane. In the vacuolated cytoplasm of these cells, eosinophilic inclusion bodies were found, mostly single, sometimes two (Fig. 7A–D).

In the lung, interstitial pneumonia, characterized by proliferative alveolitis, bronchitis, bronchiolitis and, occasionally, foci of necrosis, was observed. Thickening was observed in the alveolar walls due to mononuclear cell infiltration and the hyperplasia of type 2 pneumocytes. Eosinophilic material and neutrophil leukocytes were also present in many alveoli. In the nodular foci, the presence of necrosis, haemorrhage and mononuclear cells such as alveolar macrophages, histiocytes, lymphocytes and pox cells was remarkable. Pox cells had vacuolated nuclei and exhibited marginal hyperchromasia. Intracytoplasmic inclusion bodies were observed both in the interalveolar septa and in the cytoplasm of the pox cells in the proliferating bronchi and bronchioles (Fig. 8A–D).

In the kidney, focal nonpurulent interstitial nephritis was detected. Large foci of necrosis were observed in some areas. There was degeneration of the tubular epithelium and hyaline cylinders in the lumina of the tubule. Unlike those in the skin and lung, the presence of smallpox

cells was detected, even though a small number, in the kidney tissue (Fig. 9A–D).

Immunohistochemical findings

The cytokines in the organs of healthy sheep were insignificant. The immunopositivity scores of sheeppox cases are given in Table 2. According to the immunohistochemical examinations of the skin, in sheeppox cases, the predominant cytokines were TNF- α and IFN- γ . Positive staining was predominantly detected in sheeppox cells in the dermis layer. In addition, it was noted that mononuclear cells gave a positive reaction regarding these cytokines. The expressions of IL-1 β , IL-2, IL-6, IL-8, IL-10 and IL-12 were, on a staining pattern and cell basis, also similar to TNF- α and IFN- γ . Immunopositive reactions were mostly detected in sheeppox cells. However, the intensity of the staining was much lighter (Fig. 10A–H). In the lung, similarly to the results of skin staining, the most severe reactions belonged to TNF- α and IFN- γ . There were positive stainings for all cytokines in the cytoplasm of proliferating bronchial and bronchiolar epithelial cells. Considerable cytokine expression was also observed in alveolar macrophages. In addition to these cells, positive immune reactions were detected in sheeppox cells in the alveolar wall and proliferating bronchi and bronchioles. In addition, positive staining was also detected in the inflammatory infiltration, which consisted mostly of mononuclear cells (Fig. 11A–H). According to the immunohistochemical staining results in the kidney tissue, among other cytokines, the strongest staining belonged to TNF- α and IFN- γ . Expressions of both TNF- α and IFN- γ and other interleukins were

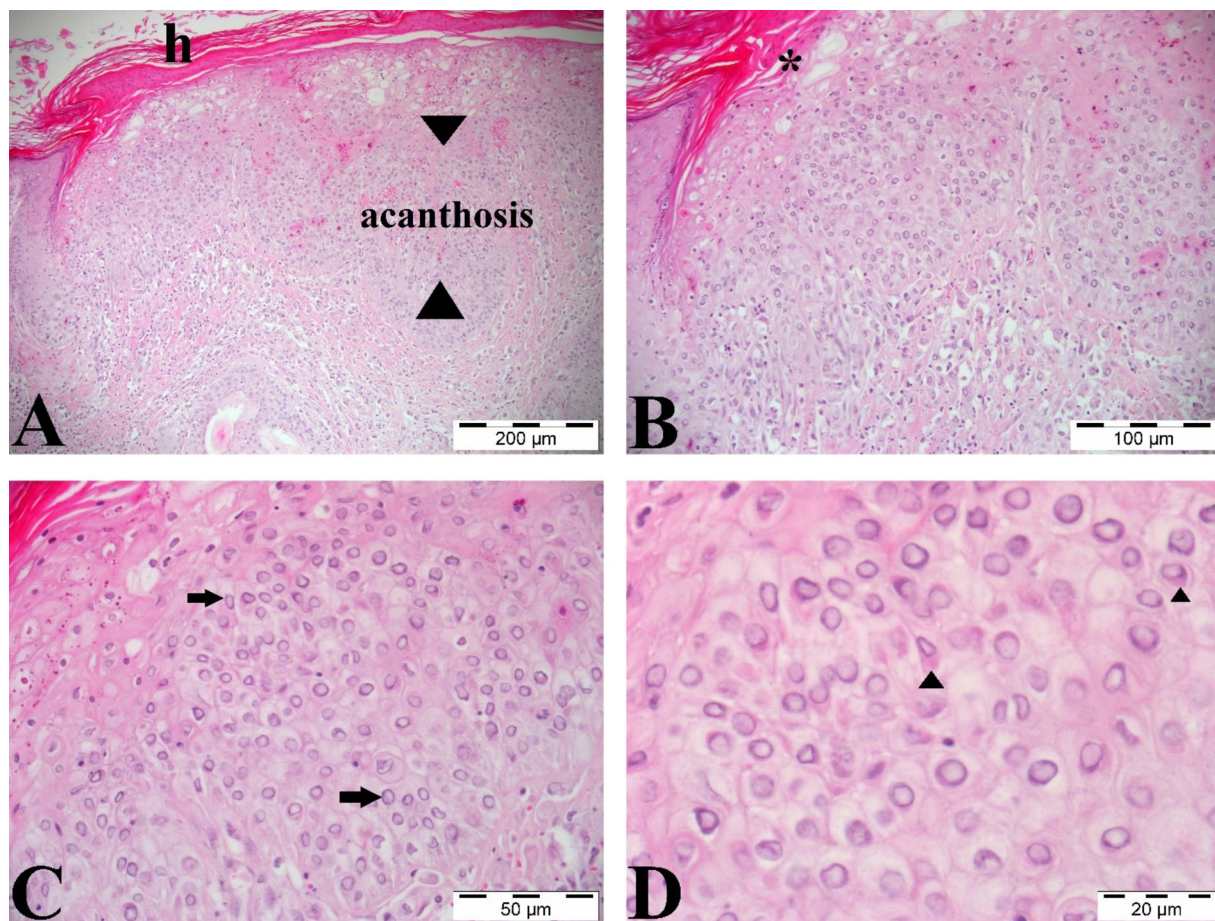


Fig. 7 **A** Skin, hyperkeratosis (h) and acanthosis in the epidermis, bar = 200 µm. **B** Microvesicles (star), bar = 100 µm. **C** Sheeppox cells (arrows), bar = 50 µm. **D** Sheeppox eosinophilic inclusion bodies (arrowheads) in the cytoplasm of cells, bar = 20 µm

mostly detected in degenerated tubular epithelial cells and nonpurulent interstitial nephritis foci (Fig. 12A–H).

Discussion

PCR is a very sensitive and reliable technique in the diagnosis of this disease (Zhu et al. 2013). In the current study, the systematic necropsy of 24 lambs with characteristic sheeppox clinical findings was performed. In these animals, both macroscopic and microscopic examinations revealed pox lesions scattered to various organs. These findings in the skin (Gulbahar et al. 2006; Ozmen et al. 2009), lung (Beytut 2010; Oreiby et al. 2022) and kidney (Chahota et al. 2022; Zro et al. 2014) were consistent with the literature data. Although all 24 cases presented signs of poxvirus when examined macro- and microscopically, only 8 could be confirmed by PCR. This is an expected outcome when working with FFPE tissues as the genetic material deteriorates with the embedding procedures and over time (Pikor et al. 2011). As the selected FFPE extraction procedure may

cause chemical contamination of the nucleic acid, not all the amplicons were suitable for sequencing. For this reason, non-positive cases are deemed false-negative cases.

When nucleotides of our isolates are compared with the same references, differences of many changes are found especially in isolate OQ302232. These changes resulted in 11 different amino acid variations. Isolate OQ302233 had minor nucleotide changes which resulted in 2 amino acids. OQ302234 isolate had no change when compared to references. OQ302232 seems the most divergent among the isolates as can be seen in Fig. 4. However, it is not certain this situation has any role in immune response when our results are taken into consideration.

When compared with the closest references, per cent similarities are between 95.3 and 100%. The major difference in this similarity percentage comes from OQ302232 as other isolates have 99.1% similarity. When OQ302232 is excluded from comparison, isolates of the study and references have 98.8–100% similarity. This shows the ORF095 has high degree of conservation among isolates which have been reported from close vicinities.

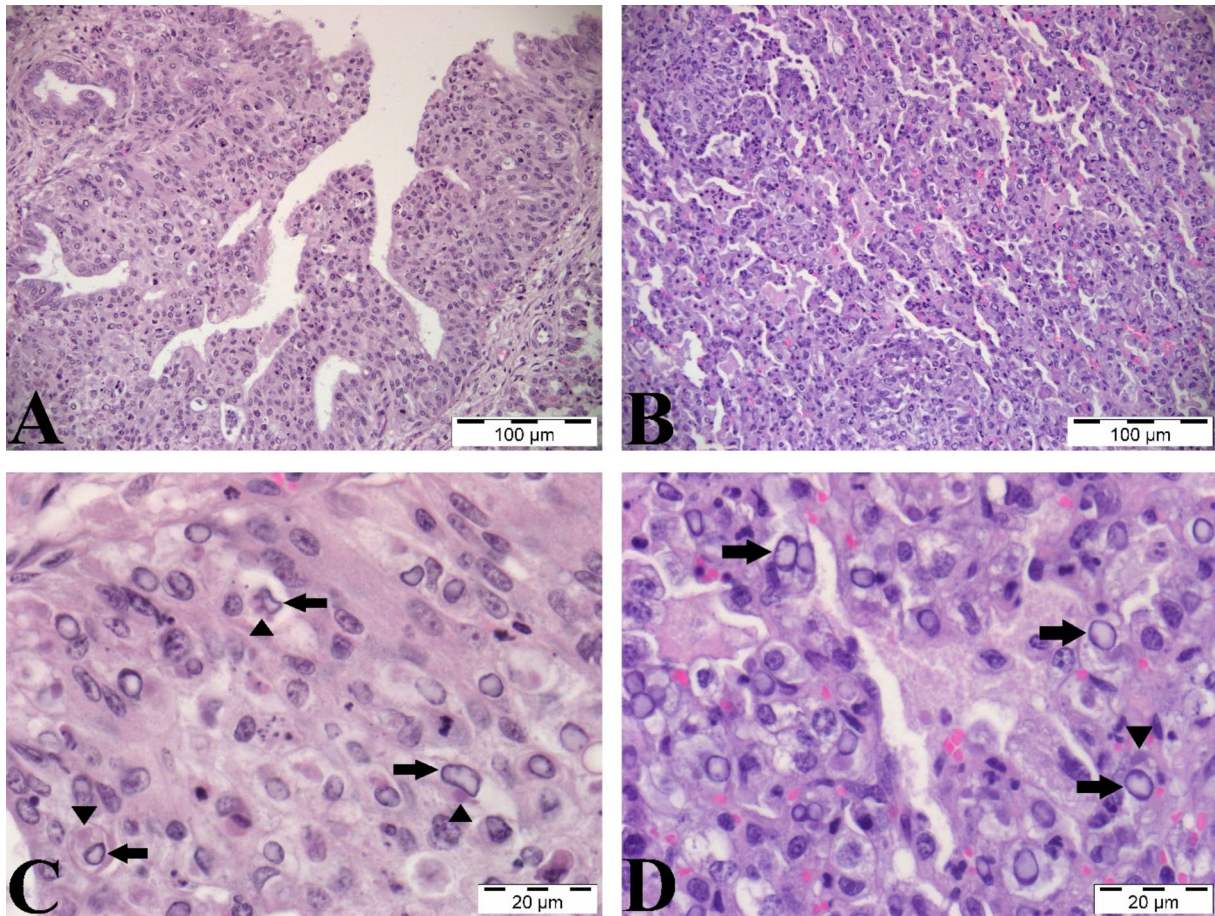


Fig. 8 **A** Proliferation in bronchiolar epithelial cells, bar = 100 µm. **B** Proliferative alveolitis, bar = 100 µm. **C** Sheeppox cells (arrows) and inclusion bodies (arrowheads) in bronchioles, bar = 20 µm. **D** Sheeppox

cells displaying marginal hyperchromasia in the alveolar wall (arrows) and few inclusion bodies (arrowhead), bar = 20 µm

Virus transmission and spread are usually limited by various host defence mechanisms; however, viruses have also developed various variations to reproduce even in hosts with sufficient immunity despite these defence mechanisms. Innate immune mechanisms such as the complement system, IFNs and the inflammatory response, as they are rapidly induced and lead to the activation of specific immune responses mediated by cytotoxic T cells or antibodies, are highly effective in the defence against pathogens (Alcamí et al. 1998). Viral infections cause the release of different types of interferons (such as types 1–3) and pro-inflammatory cytokines (Zeng et al. 2016). Sheeppox is transmitted to animals by inhalation or ingestion (Maxie 2016). Afterwards, viral agents settle in the mucous membrane of the oronasal pharynx, especially in the tonsils. Here, it infects and replicates into epithelial cells, mucosal lymphocytes, macrophages and dendritic cells. Both cellular and humoral defences are effective against the disease (Zachary 2017).

Cytokines are soluble mediators of the immune system that play critical roles in anti-viral defence (Alcamí and

Smith 1996). In sheeppox, pro-inflammatory chemokines and cytokines are released from macrophages infected by the virus, activating infected but inactive lymphocytes and macrophages (Zachary 2017). In this study, a detailed evaluation of cytokine expressions was made in the organs of lambs that died from a natural sheeppox infection.

TNF- α is an important cytokine in the regulation of early-stage defence against viral infections (Alcamí et al. 1998). TNF- α is closely associated with the emergence of inflammation and plays a key role in the coordination and activation of the adaptive immune responses in diseases (Zhang et al. 2021). Zeng et al. (2016) has reported GTPV infection has increased inflammatory cytokines like TNF- α , (IL)-1 β , IL-6 and IL-18. IFN- γ is an important multifunctional cytokine involved in the defence against infections caused by viral and microbial pathogens in the host and activates various pathways to inhibit viruses directly (Varshovi et al. 2017). In addition, it triggers the activation of monocytes and ensures the transformation of these cells into macrophages and multinucleated giant cells (Badr et al. 2022).

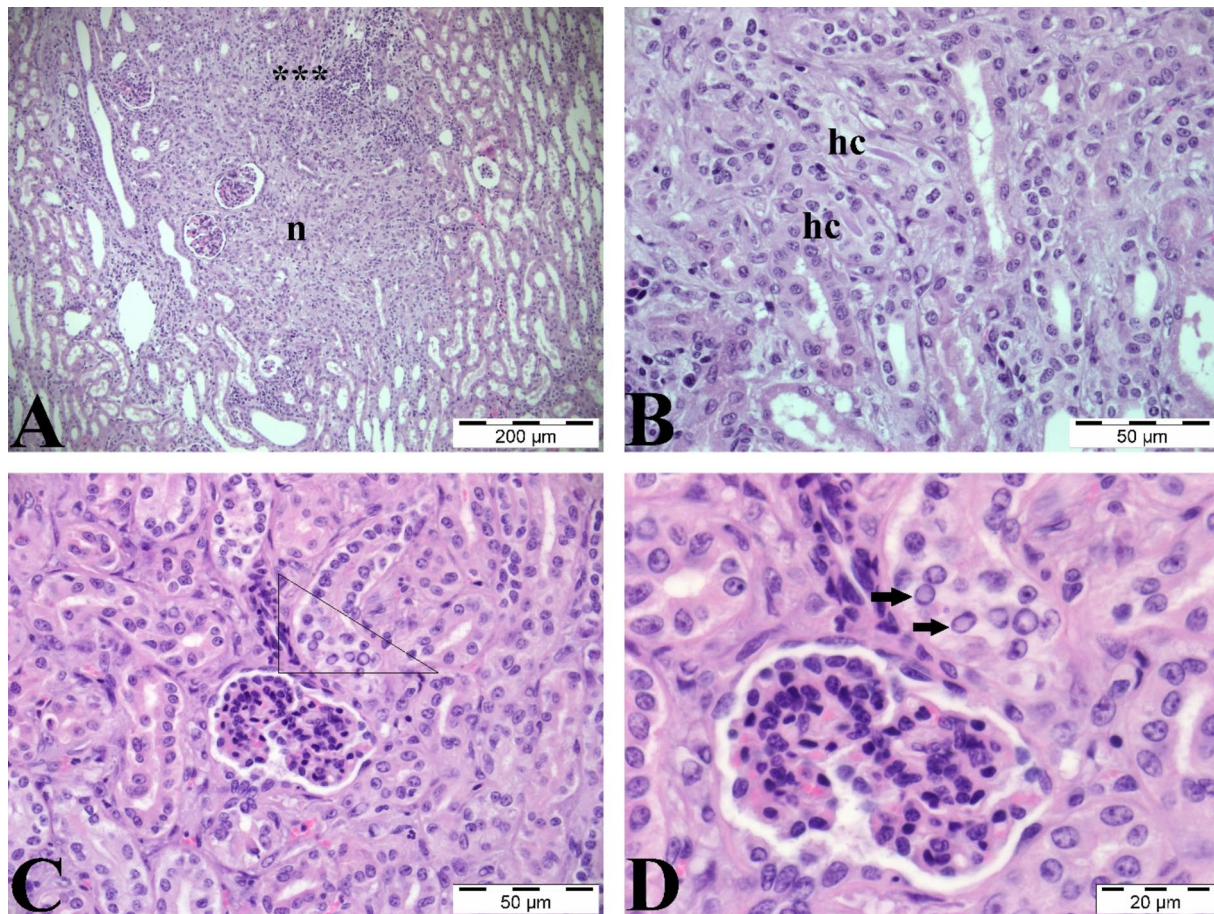


Fig. 9 **A** Large area of necrosis in the kidney (*n*) and inflammatory cell infiltration (stars), bar = 200 µm. **B** Eosinophilic hyaline cylinders (*hc*) in the lumen of degenerated or necrotized tubules, bar = 50

microns. **C** Many pox cells around the glomerulus (triangle area), bar = 50 µm. **D** Pox cells exhibiting marginal hyperchromasia (arrows), bar = 20 µm

IFN- γ plays a vital role in controlling poxvirus infections by participating in both acquired and innate immunity (Kara et al. 2018). IFN- γ is mostly released from activated macrophages and natural killer cells (NK) (Rasool et al. 2006). Varshovi et al. (2017) compared two different vaccines one SPPV origin and GTPV origin. Although humoral response time was similar, GTPV induced more neutralizing antibodies. They also examined cellular response in vitro, and lymphocyte proliferation index and IFN- γ and IL-4 production levels were higher in favour of GTPV vaccine. They concluded GTPV vaccine produced better humoral and cellular response which will provide enough protection for LSDV. In this study, TNF- α and IFN- γ staining was quite intense compared to other cytokines. This shows the importance of TNF- α and IFN- γ in anti-viral defence. Positive staining was mostly observed in sheeppox cells. These findings supported that sheeppox cells were virus-infected monocytes, macrophages and fibroblasts.

IL-1 β is an important pro-inflammatory cytokine (Abu-EL-Saad and Abdel-Moneim 2005). IL-2, also known as

the T cell growth factor, is produced from Th1 cells. The most important function of this cytokine is the procurement of variation and development of helper and cytotoxic T lymphocytes, B and NK cells. The stimulation of Th1 cells indirectly causes the activation of macrophages. It leads to the stimulation of Th2 cells and, thus, antibody synthesis (Diker 2005). In addition, it strengthens the cytotoxic effects of NK cells (Arda et al. 1998). IL-8 has chemotactic properties on neutrophils, basophils and some T cell types. It also leads to the activation of neutrophils (Diker 2005). IL-12 is mainly synthesized from macrophages and dendritic cells. By influencing NK and CD8+ T cells, it causes an increase in IFN- γ production and cytotoxic activity (Abbas et al. 2014). In the current study, it was revealed that IL-1 β , IL-2, IL-8 and IL-12 expressions are quite pronounced in cases with severe inflammatory infiltration. Similar to TNF- α and IFN- γ findings, cytokine immunoreactivity was detected in the cytoplasm of sheeppox cells.

IL-6 is an inflammatory cytokine (Zeng et al. 2016; Zhang et al. 2021). By affecting the liver, it induces the production of

Table 2 Immunoreactivity scores of sheepox cases

Case No.	TNF- α	IFN- γ	IL-1 β	IL-2	IL-6	IL-8	IL-10	IL-12
1	+++	+++	++	+	+	++	+	+
2	+++	+++	++	+	+	++	+	+
3	+++	+++	+	++	++	++	++	+
4	++	+++	++	+	++	++	+	++
5	+++	+++	+	+	+	++	+	+
6	+++	++	++	++	++	+	+	+
7	++	++	+	+	+	+	+	++
8	+++	++	+	+	+	+	++	+
9	+++	+++	+++	++	+	++	+	+
10	++	+++	++	+	+	++	++	+
11 (PCR positive)	++	++	++	++	+	++	+++	+
12 (PCR positive)	+++	++	+++	++	+	++	+	+
13	+++	+++	+	+	++	++	+	+
14	+++	+++	+++	++	+	+	++	+
15	+++	+++	++	+	+	+	+	+
16 (PCR positive)	+++	+++	++	+	+	+	+	+
17	++	+++	+++	+	+	+	+++	+
18	+++	++	++	++	+	+	+	+
19	+++	+++	++	+	++	+	+	+
20 (OQ302232)	++	++	+	+	+	+	++	++
21 (PCR positive)	++	++	+	+	+	++	++	++
22 (OQ302234)	+++	++	+	+	+	++	+	+
23 (PCR positive)	+++	+++	++	++	+	++	+	+
24 (OQ302233)	++	+++	++	+	+	++	++	+

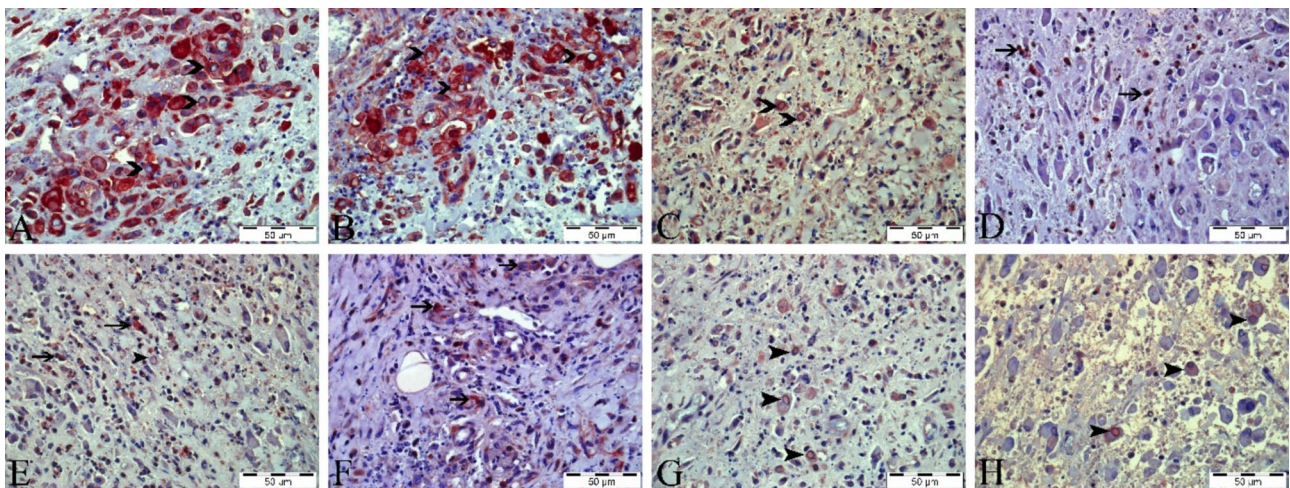


Fig. 10 Skin, IHC, bar = 50 μ m. **A** Severe intracytoplasmic TNF- α staining in sheepox cells (arrowheads). **B** Severe IFN- γ immunoreactivity in the dermis layer, especially in the cytoplasm of sheepox cells (arrowheads). **C** Moderate IL- β expressions in sheepox cells (arrowheads). **D** Weak IL-2 positive staining in mononuclear cells (arrows). **E** Weak IL-6 immunoreactivity in sheepox (arrow-

head) and mononuclear cells (arrows). **F** Moderate IL-8 expression (arrows) in inflammatory cells located around and outside the vessel in the dermis. **G** Mild IL-10 staining in sheepox cells (arrowheads). **H** Mild IL-12 expression in a small number of sheepox cells (arrowheads)

acute phase proteins, as well as the proliferation of antibody-producing cells such as B cells (Abbas et al. 2014). In this study, IL-6 levels in infected lambs were considerably weak.

Considering the relationship between antibody synthesis and IL-6, it is obvious that the humoral defence that develops against the disease is just as weak. IL-10 is a prototypical

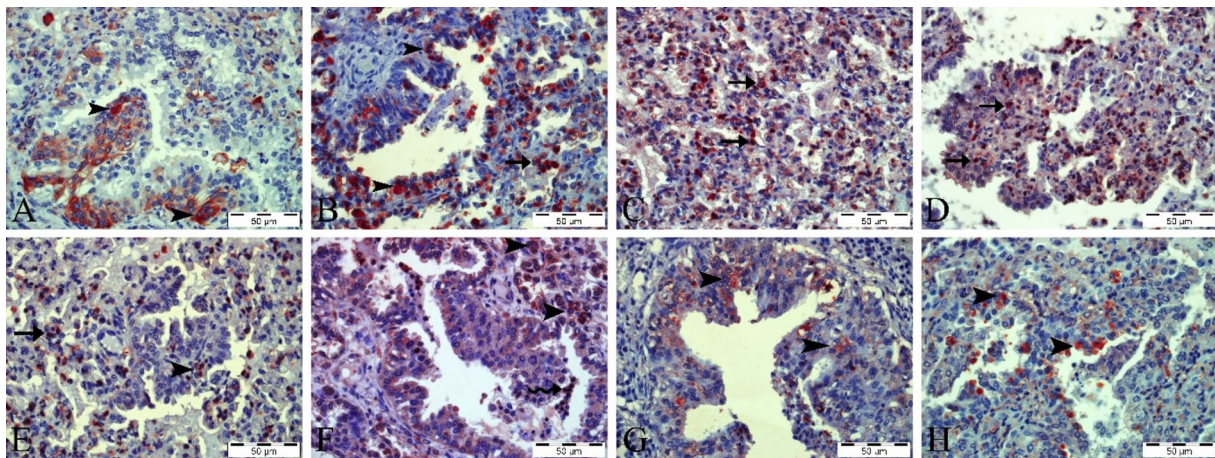


Fig. 11 Lung, IHC, bar = 50 μ m. **A** Severe TNF- α staining in the cytoplasm of proliferating bronchiole epithelial cells (arrowheads). **B** Intracytoplasmic IFN- γ expressions in proliferating bronchioles (arrowheads) and alveolar macrophages (arrow). **C** IL- β immunopositive reactions in mononuclear cells (arrows) in the interalveolar septa. **D** IL-2 expression in inflammatory cells in hyperplastic bronchioles (arrows). **E** IL-6 positive staining in proliferating bronchioles (arrow-

head) and the lymphocytes surrounding them (arrow). **F** IL-8 immunoreactivity in macrophages (arrowheads) and neutrophils in the bronchiolar lumen (folded arrow). **G** Intracytoplasmic IL-10 immunopositive staining in the hyperplastic bronchioles (arrowheads). **H** IL-12 expression in the cytoplasm of bronchiole epithelial cells (arrowheads)

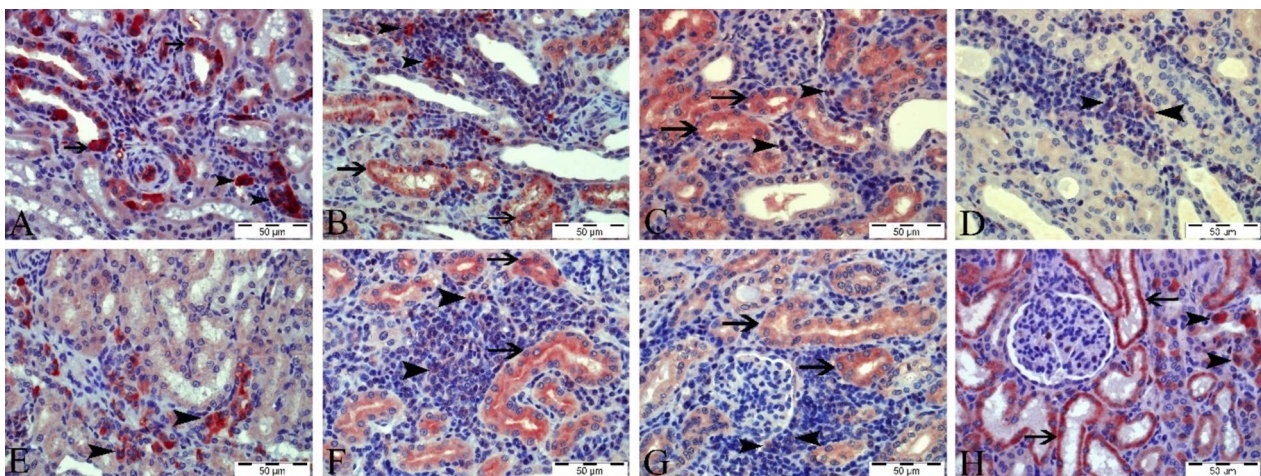


Fig. 12 Kidney, IHC, bar = 50 μ m. **A** Severe TNF- α positive staining in the tubular epithelium (arrows) and the inflammatory cell infiltration (arrowheads). **B** Severe intracytoplasmic IFN- γ immunopositive expression in the degenerated tubular epithelium (arrows) and mononuclear cells (arrowheads). **C** Moderate IL- β immunopositive reactions in inflammatory cells (arrowheads) and the cytoplasm of epithelial cells of tubules (arrows). **D** Weak IL-2 positive staining in inflammatory focus localized between tubular epitheliums (arrow-

heads). **E** Moderate IL-6 expression in the cytoplasm of mononuclear cells (arrowheads). **F** Moderate IL-8 immunoreactivity in tubules (arrows) and the surrounding inflammatory cell infiltration (arrowheads). **G** Mild IL-10 expression in inflammatory foci (arrowheads) and the tubular epithelial cells (arrows). **H** Moderate IL-12 positive staining in the tubular epithelium (arrows) and inflammatory cell infiltration (arrowheads)

anti-inflammatory cytokine (Sareyyüpoğlu et al. 2022). The overexpression of this cytokine leads to immunosuppression and regression of immunity (Zhang et al. 2021). It inhibits the function of antigen-presenting cells and ultimately impairs the induction of anti-viral defence (Chibssa et al. 2021). In addition, it decreases the production of cytokines such as IL-2, IFN- γ and TNF- α from Th1 cells and IL-12 production from

antigen-presenting cells (Abu-EL-Saad and Abdel-Moneim 2005; Ayari-Fakhfakh et al. 2018; Boshra et al. 2015). Zhang et al. (2021) reported GTPV strains caused increased expression of IL-1 β , TNF- α , IL-6 and IL-10 mRNAs. In the current study, a negative correlation was found between IL-10 and IL-2, IFN- γ and TNF- α . In addition, the pro-inflammatory

cytokine profile, rather than the anti-inflammatory, has been shown to be much more effective in sheeppox disease.

Conclusions

The data obtained from the current study show that both pro-inflammatory and anti-inflammatory cytokines play a vital role against natural infection with the sheeppox virus. Compared to the levels of anti-inflammatory IL-10 and IL-6 (Th2) cytokines, the levels of pro-inflammatory cytokines such as TNF- α , IFN- γ , IL-1 β , IL-2, IL-8 and IL12 (Th1) were much more dominant. This supported the fact that the cellular immune response is much more effective than the humoral immune response in sheeppox. When isolates OQ302232, OQ302233 and OQ302234 were compared in terms of cytokine profiles, no dramatic difference could be detected by immunohistochemical analysis. In the literature review, no study was found in which cytokine levels were evaluated by immunohistochemical methods in SPPV and other *Capripoxvirus* diseases such as GTPV and LSDV. In this respect, it is thought that the data obtained from the study may be useful in understanding the immune response to the disease and the role of cytokines in this response and contribute to the literature.

Author contributions E.K.: idea, concept and writing. N.C.: molecular studies and interpretation of data. E.B.: immunohistochemical and histopathological analysis. S.D.: immunohistochemical and histopathological analysis. V.Y.: molecular studies and interpretation of data. H.N.: immunohistochemical and histopathological staining. A.Y.: immunohistochemical and histopathological staining. E.K.: immunohistochemical and histopathological staining.

Data availability Data are available within the article or its supplementary materials.

Declarations

Ethics approval The study was approved by Kafkas University Animal Experiments Local Ethics Committee (decision number: KAU-HADYEK-2021/065) and the General Directorate of Food and Control (E-71037622-325.01-7907977) of the Republic of Türkiye Ministry of Agriculture and Forestry.

Consent to participate All participants gave their consent before participating.

Consent for publication All authors revised and approved the final manuscript.

Conflict of interest The authors declare no competing interests.

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