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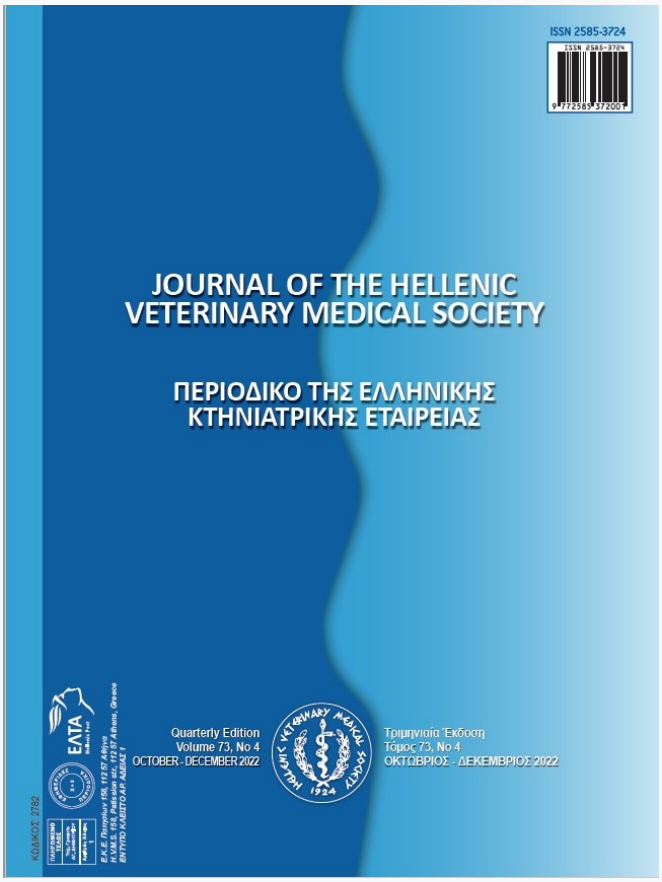

Investigation of the Epidemiology of Small Ruminant Lentivirus Infections southeast part of the Marmara region of Turkey

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Research article
Ερευνητικό άρθρο

Investigation of the Epidemiology of Small Ruminant Lentivirus Infections southeast part of the Marmara region of Turkey

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ABSTRACT: Small ruminant lentiviruses (SRLVs) are viral pathogens that are common in goats and sheep, affect production, and cause significant economic losses in small ruminant breeding. Caprine arthritis encephalitis virus (CAEV) and Maedi-Visna virus (MVV) are prototypes of SRLVs. Both of them affect animal health and welfare in sheep and goats and cause progressive and persistent infections in the small ruminant industry. The present study aimed to reveal the epidemiological status of lentivirus infection in the sheep region in Yalova province located in the southeast part of the Marmara region and determine the circulating genotypes by conducting the sequence analysis of the samples detected positive by a molecular method and molecular characterization of the detected field strains. To that end, 231 sheep blood samples were used between May 2016 and April 2018. Based on sampling results of the PCR test and ELISA tests, 5.62% (13/231) and 5.19% (12/231) positivity rates were found in sheep, respectively. According to the ELISA test results, a significant difference was found in terms of age groups (6 months -1 age, 1-3 age, >3 age) ($\chi 2$: 6.01; p=0.04). Furthermore, the sequence analysis of the gag gene region detected the existence of the A genotype of small ruminant lentiviruses in sheep. The data obtained from the study revealed a low seroprevalence course of SRLV infection in the study area in the absence of a systematic disease control program.

Keywords: ELISA, PCR, sheep, lentivirus, Turkey

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INTRODUCTION

Small ruminant lentiviruses (SRLVs) were identified as caprine arthritis encephalitis virus (CAEV) in goats and Maedi-Visna virus (MVV) in sheep (Gomez-Lucia et al., 2018). Maedi ("breathlessness") and visna ("wasting") are diseases that were brought to Iceland in 1933 with imported sheep (Andrésdóttir 2018). MVV infection or ovine progressive pneumonia (OPP), as it is known, is a slowacting disease of sheep caused by persistent infection (Heaton et al., 2012). SRLVs belong to in the Lentivirus genus of the Retroviridae family. SRLVs are a positive polarity, single-stranded, enveloped RNA virus. Infectious viruses have three structural genes: gag, pol, and env genes. The gag gene encodes the viral capsid proteins, the pol gene encodes the viral enzymes protease, reverse transcriptase, and the env gene encodes the envelope glycoproteins (Stonos et al., 2014; Gomez-Lucia et al., 2018). Upon comparing the nucleotide sequence of various isolates of Maedi-Visna virus and CAEV, it was revealed that these viruses were close relatives. Molecular epidemiology studies have demonstrated that both viruses represent a wide genetic diversity that can infect sheep and goats (Reina et al., 2006).

The clinical picture is generally not observed in SRLV infections, and since animals are persistently infected, they shed the virus with colostrum, milk, and respiratory secretions. Infection occurs through the consumption of colostrum and milk from infected sheep or respiratory touch with contaminated animals (Bolea et al., 2006; OIE, 2017). SRLV infections cause chronic, weak, and fatal lymphoproliferative disease with lesions observed most commonly in the mammary gland, lungs, lymph nodes, and brain (Alvarez et al., 2005; Heaton et al., 2012).

It is possible to ensure the control and prevention of SRLV infections with the removal/culling of infected animals from the flock. Serological tests are used at this stage. On the other hand, there are various practices, such as replacing infected animals with the offspring of seronegative mothers or rearing separately newborn animals separated from infected mothers immediately after birth. Such approaches may be effective when they are applied continuously to eradicate the progression of the infection (Scheer-Czechowski et al., 2000; Tavella et al., 2018; Kalogianni et al., 2020). Nevertheless, such initiatives can usually be costly. The high genetic variability of SRLV may present challenges reducing the effective implementation of eradication programs (Gomez-Lucia et al., 2018; Olech et al., 2021).

Since there is no effective vaccine despite numerous studies on strategies to control infection, the general view is based on detecting seropositive animals (Kalogianni et al., 2020). Furthermore, the presence of new genotypes increasing antigenic heterogeneity in the field may be in a wider range than the scope of the current ELISA tests. Thus, the serological reply may not always be detected. Consequently, the status of new outbreaks of infection in flocks screened by the ELISA test puts the ELISA performance at risk, leading to diagnostic failures (Echeverría et al., 2020; Ramírez et al., 2021). Due to the low antibody production in seronegative animals, polymerase chain reaction (PCR) and molecular diagnosis can add diagnostic value to serodiagnosis (Brinkhof et al., 2010; Ramírez et al., 2021). Serum samples obtained from infected animals may give negative results in serological tests if seropositivity occurs late after infection. PCR has frequently been used to complement serological methods since some seronegative animals give positive results in PCR. Moreover, ELISA positive results due to colostral antibodies can be obtained in PCR-negative samples in lactating lambs from infected mothers (De Andrés et al., 2005; Reina et al., 2009).

Serology represents the most appropriate way to diagnose SRLV infections. Agar gel immunodiffusion (AGID), radioimmunoassay (RIA), ELISA, radioimmunoprecipitation (RIPA), and western blot are among diagnostic methods (De Andrés et al., 2005; OIE, 2017). PCR and diagnostic methods may facilitate the detection of the virus in seronegative animals, indicating a benefit for combining serological and molecular methods (Gayo et al., 2019).

MVV, among small ruminant lentiviruses, causes infection in adult sheep. Many European countries organize joint eradication programs for MVV infection (OIE, 2017; Gomez-Lucia et al., 2018). The presence of MVV infection in Turkey dates back to 1975 (Alibaşoğlu and Arda, 1975). Studies on the presence of infection in Turkey are mostly based on serological methods. In the studies conducted, seropositivity in sheep varies from region to region and according to the number of samples collected, and seroprevalence has been reported to vary between 2.9% and 28.1% (Gürçay and Parmaksız, 2013; Un et al., 2018). Turkish MVV sequences were reported for the first time in 2013 with the molecular characterization of LTR and gag genes (Muz et al., 2013).

Considering the studies carried out in Turkey, MVV infection, one of the SRLV infections in question, has been investigated more serologically (Burgu et al., 1990; Çelik et al., 2018; Un et al., 2018). Although the infections mentioned above have been investigated at the geographical region and provincial level, limited studies have been performed in the study region due to the significant share of sheep breeding (Karaoğlu et al., 2003). This epidemiological study aimed to investigate the presence of SRLV infection in the Yalova region from serological and molecular aspects.

MATERIAL AND METHODS

Sampled animals and the preparation of samples

The present study was conducted on sheep blood samples sent to the laboratory between May 2016 and April 2018. The sample size was calculated based on a 90% confidence interval and a desired relative precision of 5% by considering the prevalence rate determined in the study previously conducted in Turkey (Dean et al., 2013). The sampled animals were selected randomly, and the sampled animals belonged to different enterprises. To obtain serum, separated sera from the blood samples taken into coagulant tubes after centrifugation at 2500 rpm for 10 minutes were transferred to sterile tubes. They were stored at -20 ºC until further analysis. To obtain viral nucleic acid, the blood samples taken into vacuum tubes containing anticoagulant were centrifuged at 1800 rpm and +4 ºC for 10 min, and 300 µl were transferred into sterile 1.5 ml Eppendorf tubes for the detection of the virus nucleic acid from the leukocyte layer. The samples were stored at -80 ºC until testing.

Antibody ELISA

A commercially available ELISA (MVV/CAEV p28 Ab Screening Test, IDEXX, USA) kit was used to detect MVV-specific antibodies. The ELISA test was applied and the results were evaluated according to the protocol specified by the manufacturer. The measurement was performed at 450 nm to obtain the Optical Density (OD) data, and the OD data were calculated.

Viral nucleic acid isolation

To investigate the presence of SRLV viral nucleic acid in a total of 231 samples (sheep leukocytes), viral nucleic acid isolation was performed according to the protocol specified by the manufacturer using a Roche MagNa Pure LC automatic extraction device and commercial extraction kit (MagNA Pure LC Total Nucleic Acid Kit, REF:03264793001, Germany). The nucleic acid obtained was stored at -80 °C until its use in molecular studies.

Polmerase Chain Reaction analysis

To investigate the presence of SRLV viral nucleic acid in the leukocyte samples collected, Polmerase Chain Reaction (PCR) was carried out using the primers specific to the GAG gene region and specified by Daltabuit-Test et al., (2008). cDNA synthesis was performed using a commercial High-Capacity cDNA Reverse Transcription Kit (Catalog number: 4368814, Thermo Fisher Scientific, USA) in line with the kit procedure. The Solis BioDyne 5X HOT FIREPOL Blend Master Mix (Cat: 04-25-00S25) was used for PCR. The mix components used in PCR were prepared as (20 µl including 5X HOT FIREPOL Blend Master Mix 5 µl, primer F (10 pmol/µl) 0.5 µl, Primer R (10 pmol/µl) 0.5 µl, Nuclease Free Water 13 µl, and cDNA 2 µl). The PCR conditions were set as follows: 40 cycles consisting of initial denaturation at 95 ºC for 15 min, denaturation at 95 ºC for 1 min, annealing at 56 ºC for 1 min, and extension at 72 ºC for 1 min, and final extension at 72 ºC for 10 min.

Agarose gel electrophoresis

A 1% agarose gel (Prona, EU) containing Safe-Red (Safe ViewTM Cat No: G108-R, Canada) was prepared to visualize the products obtained as a result of PCR. Tris Acetate Ethylene Diamine Tetra Acetic acid (TAE) solution was used as a buffer solution to prepare and run the agarose gel. PCR samples were loaded into the frozen agarose wells. Then, the products were subjected to electric current (8 volt/cm), and after approximately 30 min, the 748 bp long DNA bands formed as a result of PCR were visualized by the gel imaging system (Figure 1).

Sequencing and phylogenetic analysis

The purification and sequence analysis of PCR products were performed in the form of service procurement. The raw data obtained after the analysis were identified using the (BankIt: GenBank Submissions) service available from the Basic Local Alignment Search Tool (BLAST) web page of the National Center Biotechnology Information (NCBI) service. After selecting one of the positive samples for sequence analysis, it was separated from the gel and sequenced using a purification kit. The sequences were compared among themselves using Aliview

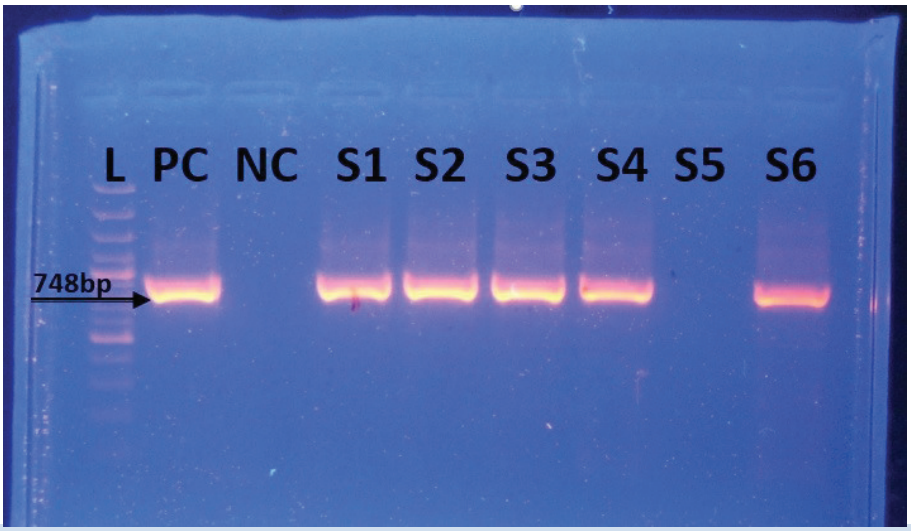

Figure 1. Image of PCR products

and Multiple Sequence Comparison by Log-Expectation (MUSCLE) software. The sequences were aligned with the other data obtained from GenBank using the multiple alignment feature of the same program. MEGA version X (https://www.megasoftware.net/) program was used for phylogenetic analysis. To that end, bootstrap analysis (1000 replicates) was performed on all data converted to the FASTA format according to the neighbor-joining method. The p-distance parameter was used in the analysis.

Statistical Analysis

Analysis of all data obtained in the study and to determine the statistically significant differences among the groups, were processed using R programme (R Core Team, 2018).

RESULTS

The seropositivity rate was determined as 5.19% (12/231) as a result of the ELISA test conducted to detect SRLV-specific antibodies. Furthermore, seropositivity rates were found as 3.70%, 2.40%, and 10.13% in the age range of (6 months-1 age, 1-3 age, >3 age, respectively) as a result of the ELISA test. It was statistically significant (χ^2: 6.01; p:0.04) upon comparing seropositivity rates in sheep on the age basis (Table 1). According to the results of the PCR test conducted in the gag gene region of SRLV for specific viral nucleic acid detection, the existence of viral nucleic acid was detected in 5.62% (13/231).

The partial sequence analysis of the samples detected as positive by PCR was performed for the gag gene, and a well-read sample as a result of the analysis was entered into the Genbank data system, and the accession number was obtained (MN186565 TR Yalova 2016). It was determined that the obtained sequences were included in the A genotype in the phylogenetic tree made by comparing the obtained sequences with the previously detected SRLVs in the Genbank data system (Figure 2).

DISCUSSION

SRLV infections are still one of the biggest threats in sheep and goat species despite the control programs carried out by serological screening using the existing tools in different countries of the world for almost a quarter of a century (Ramirez et al., 2021). SRLV infections can be detected by PCR in seronegative infected individuals, and the application of PCR together with serology naturally increases the detection sensitivity (Brinkhof et al., 2010). The procedure containing ELISA testing together with molecular methods should be considered not only for seroepidemiological studies but also when measuring produc-

Table 1. ELISA test results of different age groups in serum samples

Factor	Factor Levels	Number	Positive	Negative	%	χ2	p -value
Age	6 month-1 age	27	1	26	3.70	6.01	0.04
	1-3 age	125	3	122	2.40		
	>3 age	79	8	71	10.13		
	total	231	12	219	5.19		

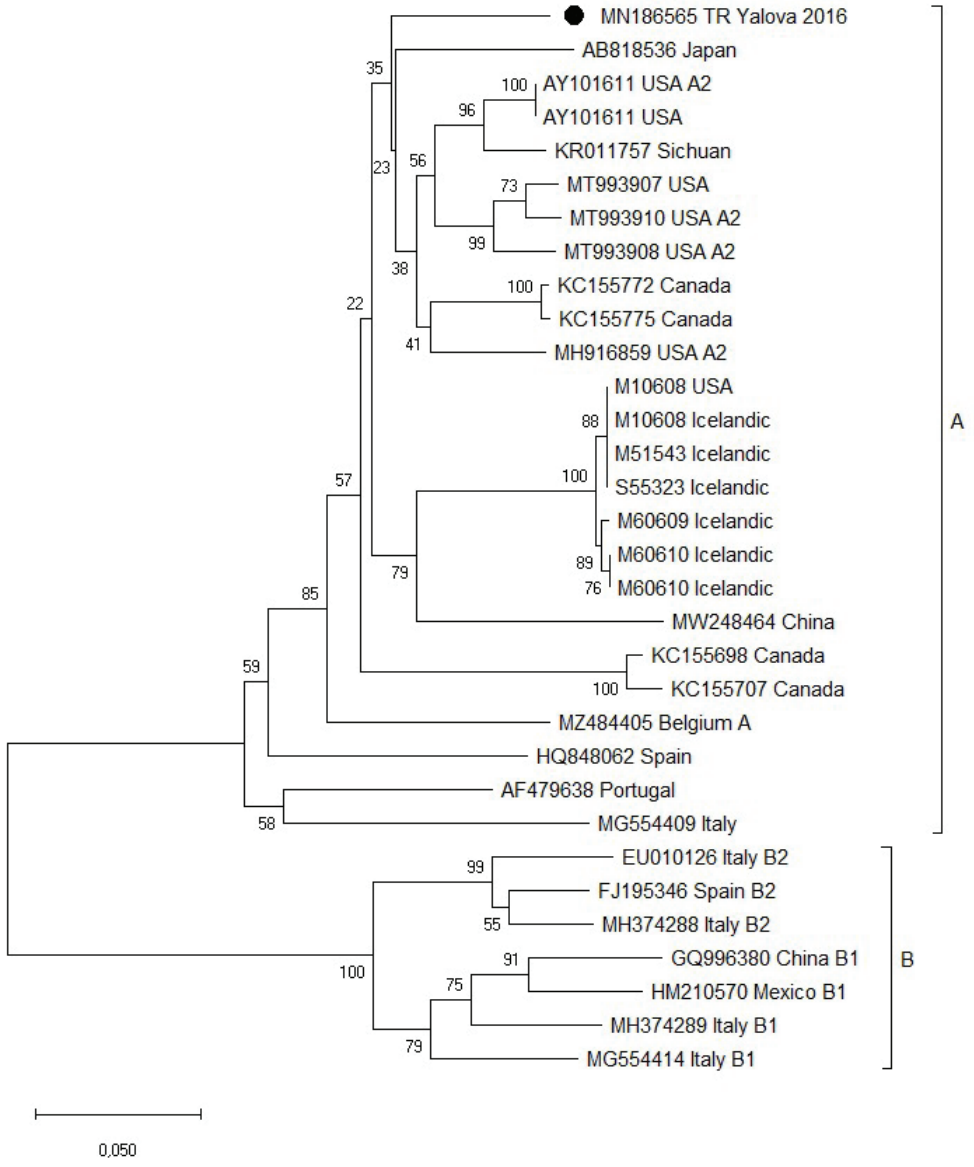

Figure 2. Phylogenetic tree for the gag gene region of SRLV

tion losses. Thus, the detection of infected animals will decrease the risk of continued infection in flocks (Ramirez et al., 2021).

MVV, identified for the first time in Iceland in 1939, was reported in many sheep-rearing countries worldwide, apart from Australia and New Zealand (Shuaib et al., 2010). Although MVV data are scarce in the Mediterranean countries such as Spain and Italy, where the dairy sheep sector has advanced, field observations indicate increased seroprevalence. The European countries, which realized this situation (Germany, France, the Netherlands, Switzerland, Italy, and Spain) have implemented control and eradication programs. However, the efficiency of these control programs in controlling the disease is controversial (Peterhans et al., 2004; Kalogianni et al., 2020). The studies conducted worldwide reported seroprevalence as 18.64% in Italy (Tavella et al., 2018), 6-9% in Belgium (Michiels et al., 2018), 10.2% in Poland (Central Eastern) (Junkuszewa et al. 2016), 2.5% in Canada (Manitoba) (Shuaib et al., 2010), and 28.8% in Germany (Mecklenburg Western-Pomerania), (Hüttner et al., 2010). These heterogeneities among studies can generally be attributed to country-specific production systems, biosecurity measures when introducing new animals to farms, and the analysis method used.

In Turkey, the disease was reported for the first time by Alibaşoğlu and Arda (1975) at a rate of 0.02% in animals slaughtered in slaughterhouses in various provinces, and the researchers found seropositivity at

a rate of 0.94% in the samples obtained from slaughterhouses in Istanbul (Yılmaz et al., 2002), 5.29% in Şanlıurfa and its surroundings (Ün et al., 2018), and 10.5% in Van and its surroundings (Ameen and Karapınar, 2018). The researchers detected the presence of Maedi-Visna specific antibodies at a rate of 2.9% in the sheep population sampled in Konya and its surroundings (Yavru et al., 2012), at a rate of 1.5% in Erzurum province and its surroundings (Schreuder et al., 1988), at a rate of 5.7% in Afyonkarahisar (Arık et al., 2015), at a rate of 23.5% in the Black Sea region (Albayrak et al., 2012), at a rate of 7.69% in the Eastern Mediterranean region (Doğan et al., 2021), and at a rate of 16% in Kars and its surroundings (Gezer et al., 2021). Changes in seropositivity rates can be attributed to many factors such as different regions, the number of animals, time period, and animal movements. Factors such as animal density, the occurrence of reinfection, the number of infected animals in the initial flock may explain these findings. The data obtained from this study (5.19%) are higher than the previous findings (1-4%) obtained by Karaoğlu et al. in the Marmara Region (Kocaeli-Sakarya provinces) in 2003, and they are similar to the previous reports in Şanlıurfa and Afyonkarahisar.

In this study, in the distribution of the seroprevalence of MVV infection by age groups (6 months-1 age, 1-3 age, >3 age), of the 27 sheep in the 6 month-1 age group, 1 sheep (3.70%) was determined as seropositive, and 26 sheep (96.30%) were determined as seronegative; of the 125 sheep in the 1-3 age group, 3 sheep (2.40%) were determined as seropositive and 122 sheep (97.60%) as seronegative; of the 79 sheep in the age group of 3 age and above, 8 sheep (10.13%) were found seropositive and 71 sheep (89.87%) seronegative. It was revealed that the lowest percentage of seropositivity among the age groups was 3.70% in the 6 month-1 age group, and the highest seropositivity was 10.13% in the age group above 3 age. The possible reasons for these differences can be attributed to the sampling design, sampling time and number, animals' age, care and feeding conditions. Although infection rates are not very high, it is worrying that the presence of infection is reported in all regions. Hence, it would be beneficial to initiate the voluntary application of control programs. It is important for the sheep breeding industry to encourage enterprises in this regard by taking into account the country's breeding practices. It may be observed more frequently in elderly animals due to the increase in the rate of infection in animals with the progressing age, due to the immunological status, and the continuous encounter with the virus as a result of horizontal transmission as the life span of sheep in the infected flocks increases. The study results are consistent with the high incidence of infection in adult animals, as reported in other studies (Yavru et al., 2012; Norouzi et al., 2015; Tefera and Mulate, 2016).

On the other hand, this study detected 5.19% (12/231) positivity in sheep as a result of the ELISA test and 5.62% (13/231) positivity by the PCR test. Molecular studies carried out in Turkey demonstrate the presence of A and B genotypes of SRLV in the field (Muz et al. 2013; Doğan et al. 2021). In this study, after the molecular analysis of the relevant region, the existence of A genotype of SRLV was determined in sheep. The possible reasons for the difference between these two tests (ELISA (-) / PCR (+)) can be attributed to the early sampling of the infection, low levels of antibodies, or antibodies against different subtypes.

CONCLUSIONS

Consequently, this study is important for revealing the current status of SRLV in Yalova province, where small ruminant breeding is carried out. It is thought that the results obtained may contribute to the control strategy scenarios of pilot region-based SRLV infections in the future. Since seropositive animals in the flock play an essential role in shedding the virus, the most effective way to control SRLV infections is flock management, which prevents viral transmission. Moreover, combined serological and molecular diagnosis will provide a perspective for the control strategy of SRLV infections by identifying the circulating subtypes in the region. Additionally, the early detection of SRLVs will significantly improve the sheep breeding industry and animal health.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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