

Sero-Prevalence Survey of Small Ruminant Lentivirus (SRLV) Infections in Kosovo

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Abstract

The objectives of this cross-sectional study were to detect the presence of small ruminant lentiviral infections in Kosovo and estimate the serological prevalence for the year of 2016. A total of 5.272 sheep and 435 goats were tested using a commercially available indirect enzyme-linked immunosorbent assay (ELISA) for Maedi-Visna/Caprine Arthritis-Encephalitis, giving an overall individual sero-prevalence in sheep of 34.8% (95% confidence interval 31.8% to 38.0%), and a flock prevalence of 85%, and in goats an overall individual sero-prevalence of 15.6% (95% confidence interval 7.2% to 25.6%) and flock prevalence of 35%. Sero-prevalence in sheep was higher in the South and West of Kosovo, whereas in goats was higher in the East and South. There were no statistically significant differences in sero-prevalence between sheep in different age groups <2 year to ≥4 year. There were statistically significant differences between the age groups in goats: chi square 25.74 (3d.f.) with P-value <0.0001. Increasing sero-prevalence observed in goats up to 4 years old with a sharp drop in goats older than four years highlights the need for further investigation based on clinical impact and genotype characterization. While retained sero-positive sheep in the population beyond 4 years old may suggest mild clinical impact of small ruminant lentiviral infections in Kosovo sheep.

Keywords: Small ruminant lentivirus, Maedi-Visna, Caprine Arthritis-Encephalitis, ELISA, Kosovo

Kosova'daki Küçük Ruminant Lentivirus (SRLV) Enfeksiyonlarının Sero-prevalansının Araştırması

Öz

Bu kesitsel çalışmanın amacı, Kosova'da küçük ruminant lentiviral enfeksiyonların varlığını saptamak ve 2016 yılı için serolojik prevalansı tahmin etmektir. Toplam 5.272 koyun ve 435 keçi ticari olarak temin edilebilen dolaylı enzim bağımlı immünosorbent test (ELISA) kullanılarak Maedi-Visna/Keçi Arthritis-Ensefalitis hastalığı yönünden kontrol edildi. Koyunlarda bireysel sero-prevalans %34.8 (%31.8 ila %38.0; %95 güven aralığı), sürü prevalansı % 85 bulunurken keçilerde %15.6'lık bireysel sero- prevalans (%7.2 ila %25.6; %95 güven aralığı) ve %35'lik sürü prevalansı belirlendi. Hastalık sero-prevalansı koyunlarda Kosova'nın güneyinde ve batısında, keçilerde ise doğusu ve güneyinde daha yüksekti. Koyunlarda <2 ila ≥4 yaş aralığındaki farklı yaş gruplarının sero prevalansında istatistiksel olarak anlamlı bir fark yoktu. Keçilerdeki yaş grupları arasında ise istatistiksel olarak anlamlı farklılıklar vardı: ki-kare 25.74 (3d.f.) P değeri <0.0001. Dört yaşına kadar olan keçilerde sero prevalansının artması ancak 4 yaşından büyük keçilerde görülen keskin düşüş, klinik etki ve genotip karakterizasyonuna dayanarak daha fazla araştırma yapılması gerektiğini vurgulamaktadır. Populasyondaki dört yaşından büyük sero-pozitif koyunların sürüde tutulması, küçükbaş hayvan lentiviral enfeksiyonlarının Kosova koyunlarında hafif klinik etkisi olduğu yönünde değerlendirilebilir.

Anahtar sözcükler: Küçük ruminant lentivirüs, Maedi-Visna, Keçi Arthritis-Ensefalitis, ELISA, Kosova



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INTRODUCTION

Maedi-Visna (MV) in sheep and Caprine Arthritis-Encephalitis (CAE) in goats are caused by two closely related viruses, commonly referred to as small ruminant lentiviruses (SRLVs). SRLVs share many features, but are genetically heterogeneous as shown by phylogenetic analysis^[1,2]. Cross-infection between small ruminant species is possible^[3,4]. The main routes of transmission are via colostrum/milk and horizontal transmission through aerosol via the natural close contact between the dam and her progeny, especially under intensive housing or grazing conditions. Intrauterine transmission occurs infrequently^[5,6]. Some resistance to lentivirus infection may be related to host genetics^[7]. Once established, infection is lifelong and persistent. Infected animals are a constant reservoir of infection^[8]. Incubation period is long and highly variable. Most infected animals will remain asymptomatic during their productive lifespan. Often it takes years before clinical infection becomes apparent and 30% of infected animals will develop slow progressive multi-systemic chronic disease^[1,5]. MV is clinically manifested as chronic progressive interstitial pneumonia (maedi) and/or progressive neurologic form of the disease (visna) usually in adult sheep, although it has been reported in older lamb and encephalitis in 4-6 months old lambs^[9-13]. The most common manifestation of CAE infection in goats is polysynovitis-arthritis in adult goats and encephalomyelitis, which is generally seen in kids 2-4 months old but has been described in older kids and adult goats^[14,15]. Chronic indurative mastitis is seen in both species^[16,17].

Small ruminant lentivirus infection causes underestimated substantial losses in the small ruminant industry due to reduced animal production and increased replacement rates^[5]. There are no specific treatments for any of the clinical syndromes associated with MV or CAE virus infection and to date, no vaccines are available^[18]. Diagnosis is based on clinical signs, flock history and confirmed by serological tests, PCR, western blot, radio-immunoassay and radioimmuno-precipitation assay. Both agar gel immune-diffusion (AGID) and enzyme-linked immuno-sorbent assay (ELISA) are

considered referent tests, according to the 2008 OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*^[18,19]. However, delayed and intermittent seroconversion, maternal antibodies, high antigenic and genotype variations represent major drawbacks for use of serological tests in control strategies^[1,19-21]. SRLV infections are reported in neighbouring and other countries around Kosovo, including Macedonia^[22-28]. There is record of samples from a goat flock in Kosovo testing positive (ELISA) for CAE in 2009^[29]. Because there was no recent evidence of existing SRLV infection in Kosovo the initial aim of the surveys reported here was to detect evidence of infection and to perform the first structured and country based serological survey for SRLV in Kosovo.

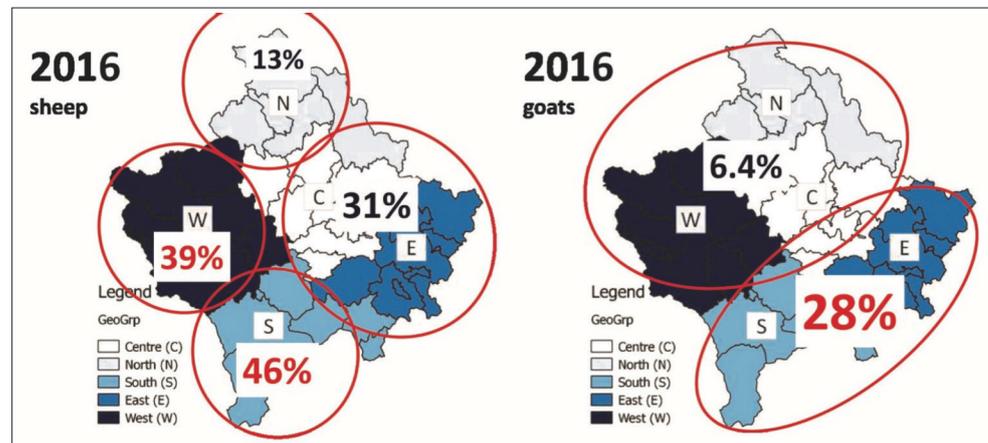
MATERIAL and METHODS

Kosovo is a landlocked country in South-eastern Europe with a humid continental climate. The total area is 10.908 km², divided into 37 municipalities that for convenience can be grouped into five geographical areas: North, east, central, west, and south (Fig. 1).

Survey Design and Implementation of Sampling Activity

Because there was no recent evidence of existing SRLV infection in Kosovo the initial aim of the surveys reported here was detection of evidence of infection. The survey sample was determined for multiple purposes which were to detect the presence of SRLV and to estimate the sero-prevalence of brucellosis in small ruminants in Kosovo. The survey design set out to collect 8.000 samples from sheep and goats on 500 premises. This number of samples, divided between sheep and goats in similar proportion to that in the whole population (slightly above 10% goats), was calculated to provide at least 95% probability of detecting the both infection if present at a minimum individual prevalence of 0.1% in sheep and 0.5% in goats. In the final analysis, having established the presence of both infections, the results obtained were sufficient to provide estimates of sero-prevalence of SRLV infection with 95% confidence intervals of $\pm 3\%$ in sheep and $\pm 10\%$

Fig 1. Representation of the spatial patterns of MVV/CAEV antibodies sero-prevalence in sheep and goat across Kosovo in 2016



in goats. The sampling frame of premises was derived from the official identification and registration (I&R) database maintained by Kosovo Food and Veterinary Agency (KFVA). A list of 2.666 premises with recently registered sheep or goats was extracted. Premises with fewer than 10 registered sheep and goats recorded were omitted from the sampling frame because these flocks would not have sufficient numbers of eligible animals to make sampling viable. These smaller flocks were 22% of all flocks but contained less than 1% of the registered population so their exclusion will have had little impact on the representative nature of the sample. Based on the registrations in the I&R database, the resulting sampling frame included about 270.000 sheep and 28.000 goats held on 2.078 premises (297 goats only, 1.202 sheep only and 579 mixed species). Distribution of samples according to geographic area are shown in *Table 1*.

Sampling was conducted in two stages. At the first stage, a random selection of premises was made from the sampling frame. The selection of premises from the sampling frame was stratified by flock size and intentionally biased towards larger flocks to ensure that the resulting sample of individual animals matched the whole population in terms of the flock sizes in which individuals exist.

At the second stage a pre-defined number of animals was set for sampling by the field staff during the visit to each selected premises. The target animals for sampling were adult animals, 12 months old or over (almost all were female). The number of animals to be sampled per premises was defined according to the total number of small ruminants registered in the premises (5 samples in flocks of 10-39, 10 samples in flocks of 40-99 and 20 samples in flocks of 100 or more). The sampling instructions for field teams included a breakdown of samples to take by species, based approximately on the proportions of sheep

and goats registered in each selected premises.

Field sampling was organised by a single contracted private veterinary company. Sample collection for the survey was carried out between May and September 2016. Bleeding of animals in the field was carried out by locally sub-contracted licensed private veterinary practitioners (PVPs). Blood samples were taken into plain vacutainer tubes. Each sample was given a unique ID and the species, sex and estimated age in years of each sampled animal was also recorded. Instructions were issued that samples were kept cool in transit. Samples were gathered by the contracted veterinary company in Pristina, checked and recorded and then forwarded to the Kosovo Food and Veterinary Laboratory (KVFL) for storage and testing.

Laboratory Diagnostic Testing

On receipt at KVFL the serum was separated and stored at -20°C. Samples were tested using an indirect ELISA based on the use of an immunogenic peptide of transmembrane protein (TM, ENV gene) and of the recombinant p28 protein which enters into the composition of the viral capsid (GaG gene) (Maedi-Visna/CAEV Antibody Test Kit REF: P00303-10 IDEXX MVV/CAEV p28 Ab Screening). Anti-p28 antibodies can appear slightly later than the antiviral envelope protein antibodies. The use of this stable protein allows the serological detection of a wide spectrum of serological variants. The cut-off point was calculated according to the manufacturer's instructions. Samples with sp% ≥ 120 of the control positive were classified positive and samples with sp% < 120 negative. Samples with sp% > 110 but < 120 could be classified as 'inconclusive'.

Statistical Analysis

Asymmetric Wilson score confidence intervals taking into

Geographic Area	Total Samples Tested (MV/CAE ELISA)	Registered Population	% of Registered Population Sampled
SHEEP SAMPLES			
Centre	918	46660	2.0%
East	1137	54198	2.1%
North	503	32195	1.6%
South	1339	72196	1.9%
West	1375	68452	2.0%
Overall	5272	273701	1.9%
GOAT SAMPLES			
Centre	104	7091	1.5%
East	124	8661	1.4%
North	47	2820	1.7%
South	61	3987	1.5%
West	99	7087	1.4%
Overall	435	29646	1.5%

account the sample size and the total population (sampling fraction) were calculated for prevalence estimates using the online statistical toolbox at *OpenEpi.com*^[30]. This method provides exact, non-symmetrical confidence intervals for estimates based on simple random samples that are robust even when sample size is small and/or the prevalence is close to 0% or 100%^[31-33]. The data were analysed using the method described by Bennett et al.^[34] so that the effect of two-stage sampling could be taken into account, using premises as the unit of sample clustering. This analysis included a calculation of the overall sampling design effect. To take account of the two-stage sampling design, the lower and upper bounds of the calculated intervals were inflated by a factor of the square root of the design effect.

Calculation of the overall sero-prevalence estimates also took into account the distribution of samples by geographic area. The proportions of the registered sheep or goat population contained within each geographic area are used as weighting factors to adjust the overall prevalence estimate for Kosovo according to the relative population in each geographic area. Sero-prevalences for different age groups (by year cohort) where information on estimated age was provided

were calculated using Wilson score 95% CI as for simple random sample (no accounting for possible design effect).

Differences in sero-prevalence between groups were assessed for statistical significance using the chi-square statistic where more than two groups were involved and the Fisher exact test for 2-way comparisons only (Table 2).

RESULTS

Of the planned 8.000 samples from 500 premises, 6.013 were collected from 356 premises. This difference in sample size reflects the degree of uncertainty in the I&R database which was expected. The total planned sample size had been increased to take this problem into account. Out of 6013 samples collected, 306 samples were classified as haemolysed or did not fulfil other criteria to be tested. The total number of sheep samples tested was 5.272 from 318 premises (average just over 16 per premises) and the total number of goat samples tested was 435 from 54 premises (average just under 8 per premises). These samples represent an overall 1.9% of sheep population and 1.5% of goat population.

Table 2. Maedi-Visna sero-prevalence (ELISA) in sheep and Caprine Arthritis-Encephalitis sero-prevalence (ELISA) in goats across Kosovo in 2016, by age

SHEEP SAMPLES			
Estimated Age (years)	n Samples Tested	n MV ELISA Positive	%MV Positive With 95%CI
1 to <2	899	332	36.93% (33.84% to 40.13%)
2 to <3	1718	567	33.00% (30.82% to 35.26%)
3 to <4	1311	467	35.62% (33.08% to 38.25%)
≥4	1052	352	33.46% (30.67% to 36.37%)
not specified	292	137	46.92% (41.27% to 52.64%)
GOAT SAMPLES			
Estimated Age (years)	n Samples Tested	n CAE ELISA Positive	%CAE Positive With 95%CI
1 to <2	116	12	10.34% (6.02% to 17.21%)
2 to <3	86	17	19.77% (12.72% to 29.40%)
3 to <4	105	30	28.57% (20.81% to 37.85%)
≥4	128	8	6.25% (3.20% to 11.85%)

Table 3. Maedi-Visna sero-prevalence (ELISA) in sheep across Kosovo in 2016, by geographic area

Geographic Area	Number of Premises Sampled (and Number of Samples Tested)	Sero-prevalence (%) with 95% CI
Centre	53 (918)	30.83% (24.87% to 37.13%)
East	68 (1.137)	31.05% (24.96% to 37.43%)
North	33 (503)	12.92% (7.95% to 18.95%)
South	84 (1.339)	45.93% (40.89% to 51.01%)
West	80 (1.375)	39.20% (32.10% to 46.47%)
Overall	318 (5.272)	35.19% (32.13% to 38.29%)
Kosovo (Weighted for geographic area)		34.84% (31.79% to 37.94%)

Table 4. Caprine Arthritis-Encephalitis sero-prevalence (ELISA) in goats across Kosovo in 2016, by geographic area

Geographic Area	Number of Premises Sampled (and Number of Samples Tested)	Sero-prevalence (%) with 95% CI
Centre	12 (104)	4.81% (2.10% to 10.73%)
East	20 (124)	30.65% (8.05% to 56.79%)
North	4 (47)	4.26% (0.58% to 16.30%)
South	7 (61)	21.31% (8.00% to 40.06%)
West	11 (99)	9.09% (4.12% to 17.67%)
Overall	54 (435)	15.40% (7.05% to 25.40%)
Kosovo (Weighted for geographic area)		15.55% (7.20% to 25.55%)

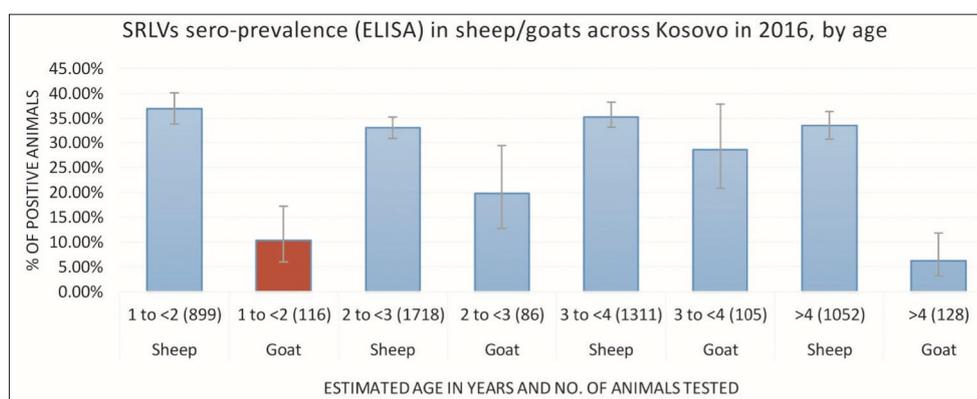


Fig 2. MVV/CAEV antibodies sero-prevalence (ELISA) in sheep and goats across Kosovo in 2016, by age. Estimated age in years and no. of animal tested (in brackets) are shown. The error bars indicate the Wilson score 95% CI calculated as for a simple random sample

The percentage sampling was similar in all geographic areas except the North, where a slightly lower percentage of sheep and higher percentage of goats were sampled. Any possible bias in the overall sero-prevalence estimate as a result of this was accounted for by using a weighted estimate.

Overall individual prevalence in sheep was 35% and flock prevalence was 85%. Average within flock prevalence for sheep was 40%. In total, 67 goat samples were positive giving an overall individual prevalence of 15% and flock prevalence of 35%. Average within flock prevalence for goats was 29%. *Table 3* and *Table 4* show the sero-prevalences calculated for each geographic area and the overall sero-prevalence both unweighted and weighted to account for differences between geographic distribution of the sample and the target population.

Sero-prevalence for SRLV antibodies in sheep is highest in the South and West geographic areas. Sero-prevalence for SRLV antibodies in goats is highest in the East and South geographic areas. There are statistically significant differences in SRLV sero-prevalence in sheep between geographic areas as indicated by an overall chi-square value of 203 (4d.f.) with a P-value of <0.00001. In pairwise comparisons, all geographic areas are significantly different from one another except the Centre and East (*Fig. 1*). There are statistically significant differences in SRLV sero-prevalence in goats between geographic areas as indicated by an overall chi-square value of 40.2 (4d.f.) with a P-value of <0.00001. Not all geographic areas are significantly different from one another. The distinct spatial groupings

are the South and East together, with an aggregated prevalence of 27.6% and the Centre, North and West together, with an aggregated prevalence of 6.4%. The difference between these two aggregated prevalences is statistically significant (chi-square value of 34.95 (1d.f.) with a P-value of <0.00001), while differences within these two groupings are not significant (Centre vs North vs West: chi-square p-value 0.3683; South vs East: chi-square P-value 0.2465) (*Fig. 1*).

The sero-prevalences for different age groups, where information on estimated age was provided with the sample are shown in *Table 2* and *Fig. 2*, with Wilson score 95% CI calculated as for a simple random sample (not accounting for possible design effect).

There are no statistically significant differences between the four age groups in sheep (from <2 year to ≥ 4 year): chi square 5.285 (3d.f.) with P-value =0.1521. There are statistically significant differences between the age groups in goats: chi square 25.74 (3d.f.) with P-value =0.00001082. The sero-prevalence of the age group 1 to <2 years is significantly lower than the sero-prevalence of the age group 3 to <4 years. Also, the sero-prevalence of the age group ≥ 4 years is significantly lower than the sero-prevalence of the age groups 2 to <3 years and 3 to <4 years.

DISCUSSION

Although KFVL has previously tested samples from small ruminants for SRLV and found positive results, this had

not been as part of a formal survey and these results had not been reported internationally. The results reported in this paper are resulting from the first structured and country-based survey assessing the SRLV infection across Kosovo. Sero positive sheep and goats were found in all five geographic areas, which means that the headline result of the survey is that there is clear evidence that SRLV infection is present in sheep and goats throughout Kosovo. It is difficult to compare and elaborate the results with neighboring countries due to the extended period of time in reports and differences in study design. Nevertheless, the present data as well as those from neighboring countries suggest that SRLV infection at least in sheep must be endemic in the region. Although, the survey was not designed to estimate flock prevalence with any specified accuracy or precision, flock-level statistics were calculated and reported in the results but only succinctly even though the sample sizes are small. In particular, when analysing the sheep and goat samples separately the number of goat samples available per flock tended to be much fewer than the number of sheep samples. The probability of detecting goat flocks affected by SRLV would therefore be quite low. The flock-level estimates should be viewed cautiously, with the possibility in mind that the true percentage of flocks affected, particularly goat flocks affected by SRLV, could be higher than the percentage apparent from the survey results.

In order to avoid interference of maternal antibodies, only adult >1 year old sheep and goats were included in this study. It is recommended that serologic or molecular testing of lambs and kids occur at least 4-6 months following weaning [18]. Previous studies have shown that if lambs are allowed to suckle naturally from positive dams and weaned at 8 months, maternal SRLV antibody is detectable starting the first day after suckling and up to 52 weeks of age in some lambs [29]. In addition, under the same conditions, the SRLV provirus may be detectable in the peripheral blood mononuclear cell of lambs up to 24 weeks old [29]. Present data suggest that different epidemiological scenarios might apply for sheep and goats. There are no statistically significant differences in SRLV sero-prevalence between sheep in different age groups from <2year to ≥4 year. The fact that sero-prevalent sheep appear to be retained in the population beyond 4 years old may suggest that the clinical impact of SRLV infection is mild in Kosovo sheep. This is in accordance with the fact that mortality due to SRLV infection in sheep may be low in enzootic areas [5]. In contrast, for SRLV sero-prevalence in goats, there are statistically significant differences between the age groups. There is a steady increase in sero-prevalence from age group 1 to <2 years to age group 3 to <4 years, followed by a sharp decrease, with sero-prevalence in the age group ≥4 years being significantly lower than the sero-prevalence of the age groups 2 to <3 years and 3 to <4 years. This highlights the need for further investigation based on clinical impact of infection and genotype

characterization. However, this could also be explained as an artefact of the lower sample size per flock. This should be further explored through investigations with farmers and PVPs which can be focussed on those flocks where antibodies have been detected.

Kosovo has a very long tradition of sheep production. Sheep production is one of the sectors within Kosovo agriculture that suffered the most severe decline in the post-war period (after 1999). By November 2001, sheep populations were at 56% of their pre-war levels [35]. Contrary, in former Yugoslavia, the number of goats dramatically dropped after the law of banning goats from fields had been passed in 1954 and lasted till 1989. Since then goat production was never important in Kosovo. After post-war (1999) small ruminant population bottleneck, there was a steady increase of sheep and goat population with a replacement from imported animals as a donation, and from other different sources (frequently uncontrolled). Therefore, multiple-source infections should be expected.

SRLV are heterogeneous, the strains circulating in different areas may differ from each other and thus the performance of diagnostic tests in these areas might vary accordingly. Clear differences have been found among the different ELISA tests in analytical and diagnostic sensitivity and overall diagnostic performance, whereas no significant differences in specificity were found [19,36,37]. Interestingly, genotype A derived antigens seem more suitable than genotype B antigens to detect heterologous infection [19,38]. On the other hand, sero-prevalence against genotype E may be underestimated using commercially available ELISAs [39]. Although, IDEXX MV/CAE p28 Ab screening Kit used in this study is designed to detect a wide range of serological variants, some sensitivity issues might be expected [37]. Therefore, before any strategy planning, further genotyping of the SRLVs is pivotal. Parturition in small ruminants in Kosovo is exclusively during winter months. Bleeding of animals was done during summer. Therefore, false negative results due to the fluctuation of antibodies during periparturient period [20] are ruled out. In the absence of vaccination, test and slaughter is the only currently available strategy for control and elimination of these infections. However, this is not financially feasible under current conditions in Kosovo.

Moreover, any attempts of control strategies on a large scale will be hampered by uncontrolled and illegal movements of animals. Small ruminants are confined for long periods due to the harsh Balkan winter and that increases the risk of horizontal transmission. Some control of infection is possible with MV and CAE by separating lambs/kids from infected dams at the time of birth and running separate herds/flocks of uninfected and infected animals but this is complicated and probably not practical for many livestock keepers under conditions in Kosovo. However, at present, it is the only applicable measure to control the prevalence of SRLV infections in Kosovo. Owners are usually unaware

of the role of SRLV infections in animal welfare and economics of sheep and goat farming. Existing authorities should pursue and encourage an active information policy through pre-existing animal health information channels and private veterinarians. Regional cooperation is a must in order to ensure a successful control program.

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