sciendo

J Vet Res 68, 2024
DOI:10.2478/jvetres-2024-0025

Comparison of serological and molecular methods for differentiation between genotype A and genotype B strains of small ruminant lentiviruses

Monika Olech[1]✉, Jacek Kuźmak[2]

[1]Department of Pathology, [2]Department of Biochemistry, National Veterinary Research Institute, 24-100 Puławy, Poland
monika.olech@piwet.pulawy.pl

Received: November 10, 2023 *Accepted: April 24, 2024*

Abstract

Introduction: Small ruminant lentiviruses (SRLV) cause multisystemic, degenerative and chronic disease in sheep and goats. There are five genotypes (A, B, C, D and E), of which A and B are the most widespread. The purpose of this study was to evaluate the serotyping efficiency of the Eradikit SRLV Genotyping ELISA and the molecular typing efficiency of a newly developed nested real-time PCR targeting the long terminal repeat–*gag* (LTR-gag) region using samples from animals infected with subtypes of SRLV known to circulate in Poland. **Material and Methods:** A total of 97 sera samples taken from 34 sheep and 63 goats were immunoassayed, and 86 DNA samples from 31 sheep and 55 goats were tested with the PCR. All ruminants were infected with known SRLV strains of the A1, A5, A12, A13, A16, A17, A18, A23, A24, A27, B1 and B2 subtypes. **Results:** A total of 69 (80.2%, 95% confidence interval 71.6%–88.8%) out of 86 tested samples gave positive results in the PCR. In 17 out of the 86 (19.8%) samples, no proviral DNA of SRLV was detected. The differentiation between MVV (genotype A) and CAEV (genotype B) by PCR matched the predating phylogenetic analysis invariably. No cross-reactivity was observed. On the other hand, the proportion of samples genotyped the same by the older phylogenetic analysis and the Eradikit SRLV Genotyping ELISA was 42.3%. The test was unable to classify 40.2% of samples, and 17.5% of sera were incorrectly classified. **Conclusion:** Our results showed that the Eradikit SRLV genotyping kit is not a reliable method for predicting SRLV genotype, while the nested real-time PCR based on the LTR-*gag* region did prove to be, at least for genotypes A and B.

Keywords: SRLV, genotypes, nested real-time PCR, ELISA genotyping, serotyping.

Introduction

Small ruminant lentiviruses (SRLV) are a group of viruses of the *Retroviridae* family causing multisystemic, degenerative and chronic disease in sheep and goats. The viruses are divided into five genotypes (A, B, C, D and E) and at least 34 subtypes (A1–A27, B1–B5 and E1 and E2) (31). This group of viruses includes maedi-visna virus (MVV), typically infecting sheep, and caprine arthritis encephalitis virus (CAEV). Respectively these two viruses define genotype A (MVV-like strains) and B (CAEV-like strains). The A and B genotypes are the most widespread in goat and sheep populations worldwide, whereas the other three genotypes are restricted to limited geographical areas (12, 16). Strains belonging to genotype A were detected in goats and genotype B strains in sheep, confirming the possibility of interspecies transmission of SRLV (23, 28–31). Genotype B is considered the most virulent genotype for goats, while genotype A seems to be more pathogenic in sheep (2, 6, 24). In Poland, SRLV occur quite commonly, with herd-level seroprevalence of 61% and 33.3% in goats and sheep, respectively (19, 33). Genetic studies identified two genotypes (A and B) and 12 subtypes (A1, A5, A12, A13, A16, A17, A18, A23, A24, A27, B1 and B2) circulating in Poland. Updated knowledge of the different circulating genotypes could raise the effectiveness of control programmes, it being difficult at present to completely eliminate the viruses.

The main target cells for SRLV are monocytes and macrophages. Following the transformation of its RNA to DNA by the viral reverse transcriptase, the SRLV genome integrates as a provirus into the monocyte and macrophage genomes. The SRLV genome is comprised of three structural genes and three accessory genes. The structure is formed by *gag* (encoding capsid, matrix and nucleocapsid proteins), *pol* (encoding reverse transcriptase, protease and integrase) and *env* (encoding the surface

and transmembrane protein), and information for the synthesis of proteins that regulate viral replication is carried by *vif*, *rev* and *vpr*-like. Additionally, non-coding long terminal repeats (LTRs) are located at both ends of the integrated (proviral) linear DNA (24).

Infection with SRLV persists throughout life and there is no treatment for it or vaccine against it. Therefore, the use of control programmes is the only way to avoid the spread of SRLV infection. These programmes rely on diagnostic tools to identify positive animals so that they may be eliminated. Therefore, the use of an appropriate test is crucial for the effective prevention and control of SRLV infections. Detection of SRLV infections is most commonly achieved using serological methods that detect antibodies to SRLV, while detection of the integrated provirus in infected monocytes and macrophages can be accomplished using PCR strategies. The two most commonly used tests for detecting specific antibodies against SRLV are agar gel immunodiffusion and enzyme-linked immunosorbent assays (ELISA). Agar gel immunodiffusion is highly specific, but its sensitivity is low, so it is increasingly being replaced by ELISA because of the immunosorbent assay's good sensitivity, objectivity and ability to be automated. In addition, it is easy to perform and results are obtained quickly. Most diagnostic ELISAs use recombinant capsid and/or transmembrane proteins as antigens (27). More recently, real-time PCR assays have been developed for quantitative, sensitive, rapid and large-scale detection of SRLV (3, 5, 9, 10, 18, 20). However, despite advances in diagnostic techniques for detecting SRLV, there is no test capable of detecting all strains. This is mainly due to the high genetic and antigenic heterogeneity of these viruses. Many of the currently available diagnostic tests are still based on a monostrain format (genotype A or B), as it was assumed that the antigens of either strain can detect antibodies against both MVV and CAEV in infected animals' sera (14, 38). However, it has been observed that ELISAs are more sensitive and specific when homologous antigens are used rather than heterologous ones (21, 37). The low cross-reactivity between genotype-mismatched SRLV antigen and antibody pairs starkly limits the diagnostic performance of monostrain ELISAs in a population where animals are infected with an SRLV genotype different from the one used in the test (6, 21, 37, 39). The high heterogeneity of the SRLV genome also hinders the usefulness of PCR to detect all SRLV strains, so it is suggested that PCRs should rather be developed based on strains circulating in a given area. Therefore, information on circulating genotypes would be helpful in selecting appropriate tests, especially in areas where genetic testing has not been conducted.

Current tools for SRLV characterisation include partial region sequencing and heteroduplex mobility assays, but these methods are quite complicated, take a long time and are not suitable for routine diagnosis (11, 28). Recently, the initial classification of A and B SRLV genotypes has become achievable by ELISA. To date, two such ELISAs have been developed, one based on the matrix and the other on the capsid epitope (17, 25). In addition, ELISAs based on the variable SU5 protein can be used as serotyping tools to provide information on SRLV subtypes, because the SU5 epitope is considered subtype specific (7, 25). Unfortunately, almost all of these ELISA tests have been developed in-house by non-commercial organisations and are not available worldwide. There is only one commercial test (the Eradikit SRLV Genotyping ELISA offered by In3 Diagnostic, Turin, Italy) that can distinguish between the A, B and E SRLV genotypes. However, its serotyping efficiency has not been well defined. In addition to serological tests, PCR protocols have been recently developed to detect the A and B genotypes and distinguish between them. This method uses genotype-specific primers and probes to detect genotype-specific nucleotide sequences (1, 10, 20, 40).

With the intention of simplifying the serotyping of SRLV field isolates and thereby improving the effectiveness of control programmes, this study investigated the serotyping efficiency of the Eradikit SRLV Genotyping ELISA test and the molecular typing efficiency of the newly developed real-time PCR targeting the LTR-*gag* region using samples originating from animals infected with known SRLV subtypes circulating in Poland.

Material and Methods

Samples. A panel of 183 fully typed samples was included in this study. This panel comprised 86 peripheral blood leukocyte (PBL) pellet samples originating from 31 sheep and 55 goats infected with SRLV, and 97 SRLV-positive serum samples originating from 34 sheep and 63 goats. All 183 samples were retrieved from a frozen collection at the National Veterinary Research Institute (Puławy, Poland). The genotype of each of the 183 samples was assigned by genetic analysis based on *gag* gene amplification and sequencing (29–32, 34, 35). The samples analysed in this study originated from animals infected with SRLV subtypes A1, A5, A12, A13, A16, A17, A18, A23, A24, A27, B1 and B2 (29–32, 34, 35). Genomic DNA was extracted from PBL pellets using a NucleoSpin Blood Quick Pure kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's recommendation. The quality and quantity of DNA were assessed in a nanophotometer (Implen, Munich, Germany). The extracted DNA was tested sample by sample with a nested real-time PCR while serum samples were tested with an Eradikit SRLV Genotyping ELISA (In3 Diagnostic, Turin, Italy) against three antigens. Both PBL and serum samples were available from 78 animals (26 sheep and 52 goats), which made a comparison possible for this group of samples between the results of the nested real-time PCR and those of the ELISA.

Nested real-time PCR assay. The DNA extracted from the 86 PBL samples was tested using a nested real-time PCR. The reaction was performed as previously described by Schaer *et al.* (40) with slight modifications. The first step, consisting of a conventional PCR, was performed using a Whatman Biometra thermocycler (Göttingen, Germany). The reaction included 2U of OptiTaq DNA Polymerase (EURx, Gdańsk, Poland), 1× PCR buffer with 1.5 mM of $MgCl_2$, 300 nM of each primer, 0.2 mM of deoxynucleotide triphosphate mix and 1 µg of extracted DNA. Amplification was performed in a total volume of 25 µL according to the following cycling conditions: initial denaturation at 95°C for 5 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and elongation at 72°C for 1 min; and final elongation at 72°C for 10 min. All products of the first PCR were then tested in the second step with genotype-specific real-time PCRs using primers and probes specific for detection and discrimination of genotypes A and B of SRLV. The qPCR was performed in a 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA). The reaction mixture for each PCR test contained 10 µL of 2× QuantiTect Probe PCR Master Mix (Qiagen, Hilden, Germany), 900 nM of each primer, 200 nM of the specific probe and 5 µL of the product of the first PCR step. Amplification profiles consisted of a hold stage of 15 min at 95°C and a PCR stage of 40 cycles at 94°C for 15 s and 60°C for 60 s. A no-template control consisting of deionised H_2O was prepared as a negative control and included in each run. All samples were tested with primers and probes designed for detection of MVV-like and CAEV-like viruses.

Formulation of DNA standards. A reference plasmid encompassing the target LTR-gag region was used to generate a standard curve based on 10-fold serial dilutions of plasmid DNA from 10^9 to 10^1. The templates were obtained after amplification of samples originating from animals naturally infected with subtype A5 of genotype A or subtype B1 of genotype B of SRLV using primers designed for the real-time PCR as described above. After amplification, PCR products were analysed by electrophoresis on 2% agarose gel, and after purification were cloned into the pDRIVE vector with a TA cloning kit (Qiagen). Following transformation, the plasmid was isolated and linearised with *Hind*III. Finally the product was gel purified using a NucleoSpin Plasmid kit (Macherey-Nagel, Hamburg, Germany). The DNA copy number of recombinant plasmids was calculated using a DNA Copy Number and Dilution Calculator (ThermoFisher Scientific, Waltham, MA, USA). A new standard curve was generated for every run along with unknown samples.

Serological testing. The 97 serum samples were tested using the Eradikit SRLV Genotyping kit. In this ELISA, separate strips of wells in the plates are coated with immunodominant epitopes of capsid antigens specific for genotypes A, B or E. Briefly, samples were diluted 1:20 in sample diluent and incubated for 60 min at 37°C. Following three washes, a peroxidase-labelled anti sheep/goat IgG antibody was added and the plate was incubated for 60 min at 37°C. After washing, the substrate was added, the mixture was incubated for 15 min and the colorimetric reaction was read at 405 nm. The results were calculated using an Excel file for automatic calculations downloadable from the manufacturer's website. The results were given as inconclusive, positive for one (A, B or E) or indeterminate.

Results

Differentiation between MVV and CAEV using real-time PCR. Of the PBL samples, 73 were from animals infected with known SRLV strains representing subtypes A1, A5, A12, A13, A16, A17, A18, A23, A24 and A27 of genotype A, and 13 were from animals infected with subtypes B1 and B2 of genotype B. All samples were tested separately with the primers and probe specific for MVV (MVV assay) and separately with the primers and probe specific for CAEV (CAEV assay). Of the 86 samples tested, 69 gave positive results by nested real-time PCR; however, in the remaining 17 (19.8%) proviral DNA of SRLV was not detected. Therefore, the diagnostic sensitivity of this PCR was 80.2% (95% confidence interval (CI) 71.6%–88.8%). The samples that yielded negative results represented subtypes A1 (n = 3), A12 (n = 3), A13 (n = 1), A17 (n = 3), A27 (n = 1), B1 (n = 2) and B2 (n = 4). Among the 73 genotype A (MVV) samples, 62 (84.9%) gave positive results with primers and a probe specific for MVV. In the other 11 (15.1%), neither MVV nor CAEV was detected. None of the 73 genotype A samples tested positive using the primers and probe specific for CAEV. Subtypes A5, A16, A18, A23 and A24 were detected without fail, while 86.0%, 81.2%, 77.0% and 75.0% of the instances of subtypes A13, A12, A17 and A27 were detected, respectively. Only 25% of the occurrences of subtype A1 were detected. Regarding samples representing subtype B, 7 out of 13 (53.8%) were positive with the primers and probe specific for CAEV, but in the remaining 6 (46.2%), neither CAEV nor MVV was detected. None of the samples tested positive using the primers and probe specific for MVV (Table 1). The detection rate of subtype B1 was 60.0% and that of subtype B2 was 50.0%. Agreement between the nested real-time PCR and the prior phylogenetic analysis was assessed by calculating the kappa coefficient. When all 86 samples were analysed, the kappa coefficient was estimated as 0.47 (95% CI 0.31–0.63), indicating moderate concordance. When only PCR-positive samples (69) were analysed, the kappa was 1.00 (95% CI 1.00–1.00), indicating that the differentiation between MVV (genotype A) and CAEV (genotype B) by real-time PCR was 100% concordant with the phylogenetic analysis.

The analytical sensitivity of the MVV and CAEV assays was evaluated using plasmid DNA carrying

MVV-like (subtype A5) and CAEV-like (subtype B1) DNA, respectively. Both assays were able to detect fewer than five copies per reaction. The reaction efficiencies of the MVV assay ranged from 88.0% to 100% and its R^2 was 0.986–0.996. The CAEV assay showed reaction efficiency in a 73.5%–95.5% range and an R^2 of 0.981–0.994.

Serological differentiation between MVV and CAEV infections. The total number of serum samples with a concordant genotype result in the phylogenetic analysis and the present serological test was 41 (out of 97 – 42.3%). As many as 17 out of 97 (17.5%) samples were incorrectly classified. The kappa value of the agreement between the previous phylogenetic analysis and the Eradikit test results was 0.15 (95% CI 0.05–0.28), indicating poor agreement. Samples totaling 30 out of the 82 (36.6%) of genotype A were correctly classified as MVV infected, but 14 (17.1%) and 1 out of 82 (1.2%) of the genotype A-infected sera were misclassified as genotype B-infected and genotype E-infected sera, respectively. The sera of genotype B in 11 out of 15 (73.3%) instances were correctly classified as CAEV infected, but 2 out of 13 (13.3%) genotype B-infected sera were misclassified as MVV infected (Table 1). The test was unable to classify 39 out of 97 (40.2%) of samples, which were categorised as inconclusive or indeterminate. The inconclusive results were those for sera of which the optical density (OD) values did not exceed 0.4 for at least one antigen. The indeterminate results were those for samples that showed reactivity to more than one antigen and yielded differences between OD values of <40%. Most of the indeterminate samples (60%) showed high reactivity to all antigens of genotypes A, B and E. Detailed information on inconclusive and indeterminate results is shown in Table 2.

Table 1. Nested real-time PCR and ELISA SRLV genotyping results for classification of peripheral blood lymphocyte and serum samples of sheep and goats

Test type	Samples (n)	Sample genotype (n)		Positive for genotype A (MVV) (n)		Positive for genotype B (CAEV) (n)		Positive for genotype E (n)		Inconclusive or indeterminate (n)	
		A	B	Genotyped previously as A	Genotyped previously as B	Genotyped previously as A	Genotyped previously as B	Genotyped previously as A	Genotyped previously as B	Genotyped previously as A	Genotyped previously as B
Nested real-time PCR	86	73	—	62 (84.9%)	—	0	—	n/a	—	11 (15.1%)	—
		—	13	—	0	—	7 (53.8%)	—	n/a	—	6 (46.2%)
Eradikit SRLV Genotyping ELISA	97	82	—	30 (36.6%)	—	14 (17.1%)	—	1 (1.2%)	—	37 (45.1%)	—
		—	15	—	2 (13.3%)	—	11 (73.3%)	—	0	—	2 (13.3%)

Table 2. Inconclusive and indeterminate results obtained using the Eradikit SRLV Genotyping kit for classification of peripheral blood lymphocyte and serum samples of sheep and goats

	Inconclusive OD					Indeterminate OD				
No.	Subtypes	Host	Genotype A	Genotype B	Genotype E	Subtypes	Host	Genotype A	Genotype B	Genotype E
1.	A1	sheep	0.163	0.33	0.26	A1	goat	1.111	0.859	0.716
2.	A5	goat	0.251	0.202	0.172	A5	goat	0.589	0.777	0.634
3.	A5	goat	0.291	0.359	0.255	A5	goat	2.325	2.471	1.023
4.	A5	goat	0.323	0.262	0.21	A5	goat	1.806	1.726	0.265
5.	A5	goat	0.144	0.246	0.142	A12	goat	1.028	0.296	0.836
6.	A12	sheep	0.169	0.384	0.206	A12	goat	1.017	1.334	0.83
7.	A12	sheep	0.193	0.347	0.152	A12	goat	1.012	0.981	0.952
8.	A12	sheep	0.178	0.236	0.29	A12	goat	2.561	2.494	1.142
9.	A13	sheep	0.191	0.162	0.297	A13	sheep	1.256	1.026	1.159
10.	A17	goat	0.393	0.344	0.297	A13	sheep	0.775	0.82	0.701
11.	A17	goat	0.336	0.341	0.18	A13	sheep	0.151	0.547	0.687
12.	A18	sheep	0.349	0.325	0.374	A16	goat	0.944	0.763	0.314
13.	A18	sheep	0.206	0.255	0.355	A17	goat	2.559	2.433	2.797
14.	A18	sheep	0.395	0.209	0.348	A17	goat	2.456	2.438	0.434
15.	A23	sheep	0.183	0.262	0.298	A17	goat	1.181	1.378	0.736
16.	A23	sheep	0.17	0.216	0.241	A17	goat	0.912	0.921	0.344
17.	A27	goat	0.251	0.228	0.317	A17	goat	0.468	0.457	0.158
18.	A27	goat	0.211	0.168	0.119	A23	sheep	0.478	0.267	0.355
19.	B2	sheep	0.17	0.272	0.177	A27	goat	0.795	1.045	0.233
20.						B2	sheep	0.553	0.606	0.473

OD – optical density

Correlation between serological and molecular typing of SRLV. To evaluate the agreement between the Eradikit SRLV Genotyping kit and the real-time PCR, the results were compared for the samples of 78 animals which were a PBL and serum sample pair and therefore possible to test with both methods. We found that the results for 36 (46.2%) out 78 sample pairs concurred. Agreement based on kappa was poor (0.16; 95% CI 0.02–0.30). The classification of 28 out of the 36 samples was MVV or CAEV infected, but the remaining 8 samples gave inconclusive results in both the real-time PCR and ELISA. Divergent results were obtained for 42 out of 78 (53.8%) samples. Of these 42 samples, 25 were classified as MVV by real-time PCR and as inconclusive or indeterminate by the ELISA. Seven samples classified as MVV infected by real-time PCR were classified as CAEV infected using the ELISA test, and one sample was classified as genotype E infected. Six and three samples which were negative by real-time PCR were respectively classified as CAEV and MVV infected by the ELISA.

Discussion

It has been shown that the amino acid sequences of one immunodominant epitope of the capsid antigen are not conserved and have a variable region specific for each SRLV genotype. Therefore, ELISAs based on this region can be tools for classifying SRLV genotypes circulating in the field (13, 17). In this study, we evaluated the serotyping efficiency of the Eradikit SRLV Genotyping ELISA kit, the only commercial test based on capsid antigens able to distinguish between the A, B and E SRLV genotypes. For this purpose, a panel of 97 serum samples from animals infected in Poland with known SRLV isolates representing subtypes A1, A5, A12, A13, A16, A17, A18, A23, A24, A27, B1 and B2 was tested.

Our results revealed that the percentage of samples with their genotype assignment by sequencing in agreement with the assignment by serology was 42.3. This value is higher than the one obtained by Acevedo Jiménez *et al.* (1), who obtained only 26% of results consistent between sequencing and serological typing using the Eradikit SRLV Genotyping kit. However, the present value was much lower than that achieved by Nogarol *et al.* (26), who showed that 98.23% of genotyped sera were correctly classified using capsid antigens derived from genotypes A, B, C and E. The research performed by Acevedo Jiménez *et al.* (1) and Nogarol *et al.* (26) revealed that ELISAs based on the capsid antigens were unable to serotype 40.2% and 30.0% of analysed seropositive samples, respectively. The unserotypeable samples gave OD values lower than 0.4 for all antigens. We found that 19.6% of tested sera had low (OD < 0.4) reactivity with the antigens used in the Eradikit SRLV Genotyping kit, and their results were considered inconclusive. This can be explained by the basis of the kit on a single epitope representing one specific subtype; therefore, the weaker reactivity of sera from animals infected with different subtypes is not surprising. Even single amino acid substitutions can significantly affect the antigenicity and thus the genotypic specificity of the serologic response. The low reactivity of positive sera against immunodominant capsid epitopes may also suggest a late stage of infection, when antibodies to the p25 SRLV core protein are not always detectable. It is known that antibodies against capsid antigens are detectable early after infection and tend to decrease later, while antibodies against the envelope predominate at later stages of infection (20–33 weeks after infection) (4, 8, 21, 22, 39). Therefore, in many ELISAs antigens from the capsid and the envelope are used together to identify seropositive animals at all stages of infection. Furthermore, it should also be noted that the tested samples were archival and had been thawed and refrozen several times, which could have affected the stability of the antibodies.

It was observed that 20.6% of the tested sera gave indeterminate genotype results because they contained antibodies which reacted highly with all test antigens, and the reaction strengths with individual antigens differed too little to permit discrimination. This result was similar to that obtained by Nogarol *et al.* (26), who showed that the genotype of 17.07% of analysed positive sera was indeterminate using capsid antigens. This outcome may indicate the carriage of more than one SRLV subtype in a given animal; however, the real-time PCR did not confirm any co-infection. Higher reactivity of sera with both genotype A and B antigens may indicate co-infection with MVV and CAEV, and such a phenomenon was observed in sheep and goats in Poland earlier (28–31). However, it is difficult to explain the high reactivity of sera to all three antigens (A, B and E) observed in 60% of the indeterminately genotyped samples from this study, especially since genotype E has never been detected in Poland. Highly heterogeneous genotype E was identified only in Roccaverano goats in mainland Italy and Sarda goats on Sardinia (36). Therefore, these positive reactions are more likely to indicate the presence of nonspecific reactions or the occurrence of cross reactions. Despite the C-terminal epitopes of the P25–B3 subunit of genotype B (RRNNPPPP), genotype A (VRQNPPGP) and genotype E (MRQNPQPP) being different, all three genotypes' N-terminal epitopes are similar (LNE/KEAER/TW); therefore, cross-reactions are possible (17, 26). Such cross-reactivity was also observed when matrix and SU5 antigens were used (6, 15, 40). We also observed that 75% of indeterminate sera in this study originated from goats, which is in line with previous reports indicating that the spectrum of antibody reactivity is wider in goats than in sheep (17).

Nogarol *et al.* (26) found that only 1.5% of samples were incorrectly classified using capsid antigens derived from genotypes A, B, C and E. Acevedo Jiménez *et al.* (1) noted 7.6% of samples to be misclassified using the Eradikit

SRLV Genotyping kit. Our results included 17.5% of sera incorrectly classified by the Eradikit ELISA. These differences may be due to the sera sample panels having been different in these studies. Nogarol *et al.* (26) tested samples from animals infected with the A1, A8, A9, B1 and B2 subtypes. Acevedo Jimenez *et al.* (1) tested sera from animals infected with the A1, A2 and B1 subtypes. In our study, sera from animals infected with SRLV subtypes A1, A5, A12, A13, A16, A17, A18, A23, A24, A27, B1 and B2 were analysed, and most of these subtypes (A12, A13, A17, A18, A23, A24 and A27) are found only in Poland. We noted that most of the misclassified samples (82.3%) were genotype A-infected sera which reacted most strongly with an antigen derived from a genotype B strain. These observations differ from those of Lacerenza *et al.* (21), who suggested that genotype B strains may induce an antibody response against more conserved epitopes of the Gag protein. This phenomenon is not fully explained and requires further research. A further impediment to the specificity of the Eradikit ELISA worth mentioning is that the kit's recombinant proteins are fused to glutathione S-transferase, and some animals have antibodies that react with the GST portion of the fusion protein, which can lead to false-positive reactions (8).

The differences in the sequence of the *gag* gene also allowed the development of PCR tests differentiating genotypes A and B. Acevedo Jiménez *et al.* (1) developed a nested PCR based on the *gag* gene–encoded capsid protein which attained 55.0% sensitivity. Kuhar *et al.* (20) developed a real-time PCR based on the *gag* gene–encoded matrix protein which showed 79.0% sensitivity, while Michiels *et al.* (2018) developed a real-time PCR also based on the *gag* gene which achieved 83.3% sensitivity in goats and 88.0% in sheep. The primers used in the nested real-time PCR performed in this study allow amplification of the LTR-*gag* fragment in order to detect either genotype A or genotype B SRLV. This real-time PCR was developed by Schaer *et al.* (40), who estimated that its sensitivity was 75.5%. Our study resolved the sensitivity of this test to be 80.2%. The less than 100% sensitivity may be due to the storage of the archival samples tested in this study for a quite a long time in the freezer, which may have reduced the amount of proviral DNA. However, the MVV and CAEV assays were highly sensitive, with a detection limit of five plasmid copies per reaction, being more sensitive than the real-time PCR designed by Kuhar *et al.* (20), which detected 100 copies per reaction. In addition, the samples used in the study were from a different pool of samples than those used for sequencing, so the negative results may be due to SRLV compartmentalisation. It can be assumed that the reason is not SRLV diversity, since the negative samples came from animals infected with subtypes A12, A13, A17 and A27, of which more than 75.0% were detected. Only subtype A1 was poorly detected, 25.0% of such samples having been identified, which may indicate the weaker ability of this method to detect this subtype.

In addition, Schaer *et al.* (40) indicated that all real-time PCR–positive samples were correctly classified as MVV or CAEV. Our results confirmed these findings. The MVV assay was able to detect various SRLV strains belonging to genotype A (subtypes A1, A5, A12, A13, A16, A17, A18, A23, A24 and A27), and the CAEV assay was able to detect subtypes B1 and B2. When genotype A strains were tested with CAEV-specific primers and probe, and genotype B strains were tested with MVV-specific primers and probe, no amplification was observed. Thus, the nested real-time PCR used in this study showed 100% specificity. When Kuhar *et al.* (20) used the real-time PCR developed by themselves, similarly no cross-reactivity was observed when samples from animals infected with the B1, B2, A1, A3, A4, A5, A14 and A15 subtypes were tested. These results indicate that these real-time PCR methods are reliable tools for accurately differentiating genotype A (MVV) from genotype B (CAEV). However, in order to validate the PCR applied here for international use, samples representing all other subtypes should be tested.

Conclusion

Our results showed that the Eradikit SRLV genotyping kit is not a reliable method for predicting SRLV genotype. Cross-reactivity was noted, many samples could not be serotyped, and some were misclassified. Unlike this ELISA, the nested real-time PCR based on the LTR-*gag* region proved to be a very good tool for error-free differentiation of the A and B genotypes. This preliminary classification of SRLV genotypes can help in the selection of ELISAs or the design of new PCR assays to be used in a given area for detecting SRLV.

Conflict of Interests Statement: The authors declare there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: This research received no external funding.

Animal Rights Statement: None required.

References

1. Acevedo Jiménez G.E., Tórtora Pérez J.L., Rodríguez Murillo C., Arellano Reynoso B., Ramírez Álvarez H.: Serotyping versus genotyping in infected sheep and goats with small ruminant lentiviruses. Vet Microbiol 2021, 252, 10893, doi: 10.1016/j.vetmic.2020.108931.
2. Blatti-Cardinaux L., Pisoni G., Stoffel M.H., Zanoni R., Zahno M.L., Bertoni G.: Generation of a molecular clone of an attenuated lentivirus, a first step in understanding cytopathogenicity and virulence. Virology 2016, 487, 50–58, doi: 10.1016/j.virol.2015.09.027.
3. Brajon G., Mandas D., Liciardi M., Taccori F., Meloni M., Corrias F., Montaldo C., Coghe F., Casciari C., Giammarioli M.,

Orrù G.: Development and Field Testing of a Real-Time PCR Assay for Caprine Arthritis-Encephalitis-Virus (CAEV). Open Virol J 2012, 6, 82–90, doi: 10.2174/1874357901206010082.
4. Brinkhof J., van Maanen C.: Evaluation of five enzyme-linked immunosorbent assays and an agar gel immunodiffusion test for detection of antibodies to small ruminant lentiviruses. Clin Vaccine Immunol 2007, 14, 1210–1214, doi: 10.1128/CVI.00282-06.
5. Brinkhof J.M., van Maanen C., Wigger R., Peterson K., Houwers D.J.: Specific detection of small ruminant lentiviral nucleic acid sequences located in the proviral long terminal repeat and leader-gag regions using real-time polymerase chain reaction. J Virol Methods 2008, 147, 338–344, doi: 10.1016/j.jviromet.2007.10.013.
6. Cardinaux L., Zahno M.L., Deubelbeiss M., Zanoni R., Vogt H.R., Bertoni G.: Virological and phylogenetic characterization of attenuated small ruminant lentivirus isolates eluding efficient serological detection. Vet Microbiol 2013, 162, 572–581, doi: 10.1016/j.vetmic.2012.11.017.
7. Carrozza M.L., Mazzei M., Lacerenza D., Del Chiaro L., Giammarioli M., Marini C., Rutili D., Rosati S., Tolari F.: Sero-conversion against SU5 derived synthetic peptides in sheep experimentally infected with different SRLV genotypes. Vet Microbiol 2009, 137, 369–374, doi: 10.1016/j.vetmic.2009.01.032.
8. Celer V. Jr., Celer V.: Detection of antibodies to ovine lentivirus using recombinant capsid and transmembrane proteins. J Vet Med B Infect Dis Vet Public Health 2001, 48, 89–95, doi: 10.1046/j.1439-0450.2001.00430.x.
9. Chassalevris T., Chaintoutis S.C., Apostolidi E.D., Giadinis N.D., Vlemmas I., Brellou G.D., Dovas C.I.: A highly sensitive semi-nested real-time PCR utilizing oligospermine-conjugated degenerate primers for the detection of diverse strains of small ruminant lentiviruses. Mol Cell Probes 2020, 51, 101528, doi: 10.1016/j.mcp.2020.101528.
10. De Regge N., Cay B.: Development, validation and evaluation of added diagnostic value of a q(RT)-PCR for the detection of genotype A strains of small ruminant lentiviruses. J Virol Methods 2013, 194, 250–257, doi: 10.1016/j.jviromet.2013.09.001.
11. Germain K., Valas S.: Distribution and heterogeneity of small ruminant lentivirus envelope subtypes in naturally infected French sheep. Virus Res 2006, 120, 156–162, doi: 10.1016/j.virusres.2006.03.002.
12. Gjerset B., Storset A.K., Rimstad E.: Genetic diversity of small-ruminant lentiviruses: characterization of Norwegian isolates of Caprine arthritis encephalitis virus. J Gen Virol 2006, 87, 573–580, doi: 10.1099/vir.0.81201-0.
13. Glaria I., Reina R., Ramírez H., de Andrés X., Crespo H., Jauregui P., Salazar E., Luján L., Pérez M.M., Benavides J., Pérez V., Polledo L., García-Marín J.F., Riezu J.I., Borrás F., Amorena B., de Andrés D.: Visna/Maedi virus genetic characterization and serological diagnosis of infection in sheep from a neurological outbreak. Vet Microbiol 2012, 155, 137–146, doi: 10.1016/j.vetmic.2011.08.027.
14. Gogolewski R.P., Adams D.S., McGuire T.C., Banks K.L., Cheevers W.P.: Antigenic cross-reactivity between caprine arthritis-encephalitis, visna and progressive pneumonia viruses involves all virion-associated proteins and glycoproteins. J Gen Virol 1985, 66, 1233–1240, doi: 10.1099/0022-1317-66-6-1233.
15. Grego E., Bertolotti L., Carrozza M.L., Profiti M., Mazzei M., Tolari F., Rosati S.: Genetic and antigenic characterization of the matrix protein of two genetically distinct ovine lentiviruses. Vet Microbiol 2005, 106, 179–185, doi: 10.1016/j.vetmic.2004.12.007.
16. Grego E., Bertolotti L., Quasso A., Profiti M., Lacerenza D., Muz D., Rosati S.: Genetic characterization of small ruminant lentivirus in Italian mixed flocks: evidence for a novel genotype circulating in a local goat population. J Gen Virol 2007, 88, 3423–3427, doi: 10.1099/vir.0.83292-0.
17. Grego E., Profiti M., Giammarioli M., Giannino L., Rutili D., Woodall C., Rosati S.: Genetic heterogeneity of small ruminant lentiviruses involves immunodominant epitope of capsid antigen and affects sensitivity of single-strain-based immunoassay. Clin Diagn Lab Immunol 2002, 9, 828–832, doi: 10.1128/cdli.9.4.828-832.2002.
18. Herrmann-Hoesing L.M., White S.N., Lewis G.S., Mousel M.R., Knowles D.P.: Development and validation of an ovine progressive pneumonia virus quantitative PCR. Clin Vaccine Immunol 2007, 14, 1274–1278, doi: 10.1128/CVI.00095-07.
19. Kaba J., Czopowicz M., Kuźmak J., Olech M., Witkowski L., Moroz-Fik A., Mickiewicz M., Biernacka K., Nalbert T., Bereznowski A., Szaluś-Jordanow O., Potârniche A.V., Spinu M., Markowska-Daniel I., Bagnicka E.: A large-scale study on the seroprevalence of small ruminant lentiviral infection in the Polish goat population. Prev Vet Med 2023, 213, 105885, doi: 10.1016/j.prevetmed.2023.105885.
20. Kuhar U., Barlič-Maganja D., Grom J.: Development and validation of TaqMan probe based real time PCR assays for the specific detection of genotype A and B small ruminant lentivirus strains. BMC Vet Res 2013, 9, 172, doi: 10.1186/1746-6148-9-172.
21. Lacerenza D., Giammarioli M., Grego E., Marini C., Profiti M., Rutili D., Rosati S.: Antibody response in sheep experimentally infected with different small ruminant lentivirus genotypes. Vet Immunol Immunopathol 2006, 112, 264–271, doi: 10.1016/j.vetimm.2006.03.016.
22. Michiels R., Van Mael E., Quinet C., Adjadj N.R., Cay A.B., De Regge N.: Comparative Analysis of Different Serological and Molecular Tests for the Detection of Small Ruminant Lentiviruses (SRLVs) in Belgian Sheep and Goats. Viruses 2018, 10, 696, doi: 10.3390/v10120696.
23. Minardi da Cruz J.C., Singh D.K., Lamara A., Chebloune Y.: Small ruminant lentiviruses (SRLVs) break the species barrier to acquire new host range. Viruses 2013, 5, 1867–1884, doi: 10.3390/v5071867.
24. Minguijón E., Reina R., Pérez M., Polledo L., Villoria M., Ramírez H., Leginagoikoa I., Badiola J.J., García-Marín J.F., de Andrés D., Luján L., Amorena B., Juste R.A.: Small ruminant lentivirus infections and diseases. Vet Microbiol 2015, 181, 75–89, doi: 10.1016/j.vetmic.2015.08.007.
25. Mordasini F., Vogt H.R., Zahno M.L., Maeschli A., Nenci C., Zanoni R., Peterhans E., Bertoni G.: Analysis of the antibody response to an immunodominant epitope of the envelope glycoprotein of a lentivirus and its diagnostic potential. J Clin Microbiol 2006, 44, 981–991, doi: 10.1128/JCM.44.3.981-991.2006.
26. Nogarol C., Bertolotti L., Klevar S., Profiti M., Gjerset B., Rosati S.: Serological characterization of small ruminant lentivirus: a complete tool for serotyping lentivirus infection in goat. Small Rumin Res 2019, 176, 42–46, doi: 10.1016/j.smallrumres.2019.05.010.
27. Olech M.: The genetic variability of small-ruminant lentiviruses and its impact on tropism, the development of diagnostic tests and vaccines and the effectiveness of control programmes. J Vet Res 2023, 67, 479–502, doi: 10.2478/jvetres-2023-0064.
28. Olech M., Croisé B., Kuźmak J., Valas S.: Evidence for interspecies transmission of small ruminant lentiviruses in sheep and goats in Poland. Bull Vet Inst Pulawy 2009, 53, 165–168.
29. Olech M., Kuźmak J.: Molecular Characterization of Small Ruminant Lentiviruses of Subtype A5 Detected in Naturally Infected but Clinically Healthy Goats of Carpathian Breed. Pathogens 2020, 9, 992, doi: 10.3390/pathogens9120992.
30. Olech M., Kuźmak J.: Molecular Characterization of Small Ruminant Lentiviruses in Polish Mixed Flocks Supports Evidence of Cross Species Transmission, Dual Infection, a Recombination Event, and Reveals the Existence of New Subtypes within Group A. Viruses 2021, 13, 2529, doi: 10.3390/v13122529.
31. Olech M., Kycko A., Kuźmak J.: Molecular Characterization of Small Ruminant Lentiviruses Isolated from Polish Goats with Arthritis. Viruses 2022, 14, 735, doi: 10.3390/v14040735.
32. Olech M., Murawski M., Kuźmak J.: Molecular analysis of small-ruminant lentiviruses in Polish flocks reveals the existence of a novel subtype in sheep. Arch Virol 2019, 164, 1193–1198, doi: 10.1007/s00705-019-04161-9.
33. Olech M., Osiński Z., Kuźmak J.: Bayesian estimation of seroprevalence of small ruminant lentiviruses in sheep from

Poland. Prev Vet Med 2017, 147, 66–78, doi: 10.1016/j.prevetmed.2017.09.001.

34. Olech M., Rachid A., Croisé B., Kuźmak J., Valas S.: Genetic and antigenic characterization of small ruminant lentiviruses circulating in Poland. Virus Res 2012, 163, 528–536, doi: 10.1016/j.virusres.2011.11.019.
35. Olech M., Valas S., Kuźmak J.: Epidemiological survey in single-species flocks from Poland reveals expanded genetic and antigenic diversity of small ruminant lentiviruses. PLoS One 2018, 13, e0193892, doi: 10.1371/journal.pone.0193892.
36. Reina R., Bertolotti L., Dei Giudici S., Puggioni G., Ponti N., Profiti M., Patta C., Rosati S.: Small ruminant lentivirus genotype E is widespread in Sarda goat. Vet Microbiol 2010, 144, 24–31, doi: 10.1016/j.vetmic.2009.12.020.
37. Reina R., Grego E., Profiti M., Glaria I., Robino P., Quasso A., Amorena B., Rosati S.: Development of specific diagnostic test for small ruminant lentivirus genotype E. Vet Microbiol 2009, 138, 251–257, doi: 10.1016/j.vetmic.2009.04.005.
38. Rosati S., Mannelli A., Merlo T., Ponti N.: Characterization of the immunodominant cross-reacting epitope of visna maedi virus and caprine arthritis-encephalitis virus capsid antigen. Virus Res 1999, 61, 177–183, doi: 10.1016/s0168-1702(99)00031-3.
39. Rosati S., Profiti M., Lorenzetti R., Bandecchi P., Mannelli A., Ortoffi M., Tolari F., Ciabatti I.M.: Development of recombinant capsid antigen/transmembrane epitope fusion proteins for serological diagnosis of animal lentivirus infections. J Virol Methods 2004, 121, 73–78, doi: 10.1016/j.jviromet.2004.06.001.
40. Schaer J., Cvetnic Z., Sukalic T., Dörig S., Grisiger M., Iscaro C., Feliziani F., Pfeifer F., Origgi F., Zanoni R.G., Abril C.E.: Evaluation of Serological Methods and a New Real-Time Nested PCR for Small Ruminant Lentiviruses. Pathogens 2022, 11,129, doi: 10.3390/pathogens11020129.