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## UNIVERSITÀ DEGLI STUDI DI TORINO

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## **An insight into a combination of ELISA strategies to diagnose small ruminant lentivirus infections**

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### **Abstract**

A single broadly reactive standard ELISA is commonly applied to control small ruminant lentivirus (SRLV) spread, but type specific ELISA strategies are gaining interest in areas with highly prevalent and heterogeneous SRLV infections. Short (15-residue) synthetic peptides ( $n = 60$ ) were designed in this study using deduced amino acid sequence profiles of SRLV circulating in sheep from North Central Spain and SRLV described previously. The corresponding ELISAs and two standard ELISAs were employed to analyze sera from sheep flocks either controlled or infected with different SRLV genotypes. Two outbreaks, showing SRLV-induced arthritis (genotype B2) and encephalitis (genotype A), were represented among the infected flocks. The ELISA results revealed that none of the assays detected all the infected animals in the global population analyzed, the assay performance varying according to the genetic type of the strain circulating in the area and the test antigen.

Five of the six highly reactive (57–62%) single peptide ELISAs were further assessed, revealing that the ELISA based on peptide 98M (type A ENV-SU5, consensus from the neurological outbreak) detected positives in the majority of the type-A specific sera tested (Se: 86%; Sp: 98%) and not in the arthritic type B outbreak. ENV-TM ELISAs based on peptides 126M1 (Se: 82%; Sp: 95%) and 126M2 (Se: 68%; Sp: 88%) detected preferentially caprine arthritis encephalitis (CAEV, type B) and visna/maedi (VMV, type A) virus infections respectively, which may help to perform a preliminary CAEV vs. VMV-like typing of the flock. The use of particular peptide ELISAs and standard tests individually or combined may be useful in the different areas under study, to determine disease progression, diagnose/type infection and prevent its spread.

**Keywords:**

## Introduction

The control of small ruminant lentiviruses (SRLV), which include the visna/maedi virus (VMV) and the caprine arthritis encephalitis virus (CAEV), largely depends on early detection and elimination of infected animals from the flock (Patel et al., 2012). Therefore, the use of the relevant assay and its updating becomes essential (Lacerenza et al., 2006; Reina, 2009 #360). Main antibody detection methods include agar gel immunodiffusion (AGID), linked immunosorbent assay (ELISA), and the confirmatory techniques radioimmunoprecipitation (RIPA) and Western blot (WB) (de Andres et al., 2005; Reina et al., 2009b).

AGID has been progressively substituted by ELISAs (Peterhans et al., 2004) with improved sensitivity and automation to detect serum, milk and semen antibodies (de Andres et al., 2005; Peterhans et al., 2004; Ramirez et al., 2009; Reina et al., 2009a). Although competitive ELISA methods based on monoclonal antibodies to viral envelope protein (ENV-SU, gp135) epitopes have been developed (Herrmann-Hoesing et al., 2010), indirect ELISAs have been the most frequently applied in Europe (Carrozza et al., 2009; Reina et al., 2009a). However, few of these have been compared internationally (Brinkhof and van Maanen, 2007; Ramirez et al., 2009; Toft et al., 2007).

Difficulties in ELISA antigen design strive mainly in the high antigenic and genetic variability of SRLV, being these viruses classified into genotype A which involves classical VMV-like viruses, genotype B assigned to CAEV-like viruses (Shah et al., 2004), genotype C found in goats and sheep from Norway (Gjerset et al., 2009), genotype D observed in ruminants from Switzerland and Spain (Reina et al., 2006; Shah et al., 2004) and genotype E detected in Italian goats (Grego et al., 2007; Reina et al., 2009b). Interestingly, genotype A derived antigens seem more suitable than genotype B antigens to detect heterologous infection (Lacerenza et al., 2006). On the other hand, seroprevalence against genotype E may be underestimated using commercially available ELISAs ("standard" ELISAs henceforth) (Reina et al., 2009b).

Control programs of Central and Southern Europe have employed indirect ELISAs based on whole virus or viral recombinant proteins (Reina et al., 2009a; Saman et al., 1999; Zanoni et al., 1989), differing in performance according to the range of antigenic specificities or the antibody concentration required (Ramirez et al., 2009). Antibody titer may vary along the animal's life (Varea et al., 2001), which may lead to misdiagnosis by ELISA. These findings, together with the high genetic/antigenic variability inherent to these viruses makes no single technique or test sufficient for use as "gold standard" to determine the infection status of the animal (Reina et al., 2009a). In the absence of this standard, the positivity to at least two diagnostic techniques, based on either antibody or virus-related detection, has been used in some studies as criterion for the presence of infection (Saman et al., 1999; Varea et al., 2001).

In Spain, a country with over 30 million of small ruminants (about four times sheep compared to goats), SRLV infection affects from 10% to 90% of animals depending on the geographic area, and outbreaks with neurological (Glaria et al., 2012) and arthritic forms of the disease (Glaria et al., 2009) have been described in the North-Central area (Castilla-León and Aragón, respectively). Together with the limited implementation of control programs, this has led to significant production and animal welfare losses. Like in other European countries, AGID has been replaced by indirect ELISAs (de Andres et al., 2005) in Spain. Comparison studies on different standard ELISAs have been initiated in Spanish goats (Contreras et al., 1998; Sanchez et al., 2001) and sheep (Ramirez et al., 2009). Elitest (Elitest-MVV Hyphen-Biomed, France) (Saman et al., 1999) has been successfully applied alone and in combination with PCR, allowing an efficient detection of SRLV infection (Alvarez et al., 2006; Barquero et al., 2011; Leginagoikoa et al.,

2006, 2010; Reina et al., 2006) and being implemented at present in areas of North-Central Spain (Leginagoikoa et al., 2010; Perez et al., 2010). As a result, some flocks have reached a seronegative status (Juste et al., 2012; Perez et al., 2010). However, like in other areas, seroconversion may reappear sporadically, decreasing the flock value (Brulisauer et al., 2005).

This study, involving known and newly designed ELISA assays, aims to explore the possibilities of a fine tuning of ELISA testing in different areas infected with different SRLV, and proposes diagnostic strategies to prevent viral spread in these areas through flock typing and to detect infection in animals seronegative to standard assays.

## **Materials and methods**

### *Animals and samples*

Two panels of sheep sera from North-Central Spain were used. In a preliminary study, aiming to determine the degree of reactivity of 60 peptides in an ELISA format, the panel of sera employed ( $n = 128$ ) was obtained from different flocks widely distributed in this area, known to have type A (Glaria et al., 2012) and type B (Glaria et al., 2009) infections (Table 1). Most of these sera, 112 (87.5%) were known positives in one or both standard ELISAs, specifically 85 (66.4%) in Elitest, 108 (84.3%) in Chekit and 81 (63.2%) in both standard tests. At a second stage, a serum panel of increased size (involving 496 additional animals) was used for a detailed study on reactivity patterns and specificities. Accordingly, animal Groups 1–6 were established from different flocks, with the following characteristics (Table 2 shows further details on groups' description). Group 1 was negative to VMV infection according to standard tests, without having been submitted to any control measure. Groups 2 and 6, both seronegative to Elitest, were from flocks originally infected with SRLV but had been included in the last decade in an eradication program using Elitest until becoming seronegative to this test; Group 3 animals were from a flock infected with different SRLV types but free of diseased animals; and Groups 4 and 5 were from SRLV infected flocks belonging to a neurological and an arthritic outbreak, respectively. Animals of the infected groups were further divided into positive (P) and negative (N) to Elitest in order to further analyze peptide ELISA reactivities compared to standard tests (Table 3). Also, for the study on disease status, sera from the arthritic and the neurological outbreaks were distributed into the clinically affected and asymptomatic categories (Table 4).

In addition, sera from 130 sheep and goats from UK, Italy and Iceland, belonging to flocks consistently SRLV-seronegative upon retesting, were used as negative controls to determine peptide ELISA cut-off values. The study on peptide ELISA strain specificity was done with sera from 71 animals infected with a known SRLV genotype (Figs. 1 and 2): 37 sheep from North-Central Spain naturally infected with genotypes A ( $n = 28$ ) and B ( $n = 9$ ), 10 sheep from the United Kingdom experimentally infected with strain Ev1 of genotype A (Niesalla et al., 2009); 1 sheep experimentally infected with the Icelandic clone Kv1772 from genotype A, kindly provided by Dr. V. Andrésdóttir; 15 Mexican goats naturally infected with genotype B1 (Ramirez et al., 2011); and 8 Italian goats experimentally infected with genotype E Roccaverano strain (Reina et al., 2011).

### *Peptide design*

Peptides were designed on the basis of newly described sequences from two outbreaks of arthritis and neurological disease from Spain as well as sequences available from databases. The gag and env nucleotide sequences of SRLV present in Spanish animals were obtained using primers (Fig. 1S, Annex I in Supplementary material) and the PCR procedures described below. Deduced amino acid sequences were aligned with those available from GenBank database and analyzed (<http://tools.immuneepitope.org/>

tools/bcell/iedb input) regarding hydrophobicity, flexibility, accessibility, turns, exposed surface, polarity and antigenic propensity of polypeptide chains that have been correlated with the location of antibody epitopes (Kolaskar and Tongaonkar, 1990). After discarding non-antigenic regions, 60 peptides of 15 amino acids each were designed (Fig. 1S, Annex II in Supplementary material), corresponding to the viral nucleocapsid GAG p14 NC (n = 7), GAG p17 matrix MA (n = 11), capsid GAG p25 CA (n = 13), surface envelope ENV gp135 SU (n = 8) and transmembrane ENV gp46 TM (n = 21) viral proteins.

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Peptides were chemically synthesized (Thermo scientific), diluted upon arrival in carbonate buffer (pH 9.6) at 1 mg/ml and stored at  $-20^{\circ}\text{C}$  until tested in the corresponding screening ELISA.

#### *Peptide ELISA procedure*

Each peptide ELISA was carried out as previously described (Reina et al., 2009b), with slight modifications. Briefly, 96-well microplates (Maxisorp Nunc<sup>TM</sup>) were coated with 300ng of the selected peptide in carbonate buffer (pH 9.6) or carbonate buffer alone as negative control. Plates were allowed to dry overnight at  $37^{\circ}\text{C}$  and then blocked with 2.5% bovine casein for 1 h at  $37^{\circ}\text{C}$ . After washing in PBS with 0.1% Tween 20, serum samples diluted

1/20 in PBS containing 1.25% bovine casein were added and plates incubated for 1 h at  $37^{\circ}\text{C}$ .

Subsequently, protein G (0.2  $\mu\text{g/ml}$ , Pierce) diluted in the same buffer was added and plates were incubated for 1 h at  $37^{\circ}\text{C}$ . After a final washing step, the reaction was developed with ABTS (Millipore) and absorbance read at 405 nm. Net absorbance was obtained by subtracting the absorbance in the well without antigen from the absorbance of the well with antigen.

#### *2.4. Polymerase chain reaction (PCR) and sequencing*

PCR was used to determine the nucleotide sequences for peptide design and, when needed, to confirm the infection status. Briefly, genomic DNA was extracted from peripheral blood mononuclear cells (PBMC) or tissue samples (central nervous system or carpal joint from clinically affected animals of the neurological and the arthritic outbreak, respectively) using QIAamp<sup>®</sup> DNA Blood Mini Kit (Qiagen) as described elsewhere (Crespo et al., 2012). Tissue samples were previously lysed (1 h at  $56^{\circ}\text{C}$ ) in buffer (100 mM Tris-HCl pH 8.5; 5 mM EDTA; 400 mM NaCl; 0.2% SDS) with Proteinase K (50  $\mu\text{g}/10^7$  cells; Sigma). Gag and env regions were amplified with previously described primers (Glaria et al., 2009) or those designed in this work (Supplementary material). The reaction mix of the PCRs consisted of:  $1\times$  reaction buffer (Biotools), 2 mM  $\text{MgCl}_2$  (Biotools), 225  $\mu\text{M}$  of each dNTP (Biotools), 600 nM of each primer, 0.04 U/ $\mu\text{l}$  of Pfu DNA polymerase (Biotools) and 0.5–1  $\mu\text{g}$  of sample DNA to a final volume of 30  $\mu\text{l}$ . PCR conditions were: initial denaturation step for 5min at  $94^{\circ}\text{C}$  followed by 45 cycles of  $94^{\circ}\text{C}$  for 40 s, annealing at  $50\text{--}58^{\circ}\text{C}$  (depending on the primers used) for 50 s and extension at  $72^{\circ}\text{C}$  for a variable period (depending on the size of the amplicon); and a final extension step (10 min at  $72^{\circ}\text{C}$ ). Amplicons were cloned into pGEMT-easy<sup>®</sup> vector (Promega) following the manufacturer's instructions and then sequenced using BigDye<sup>®</sup> Terminator v3.1 chemistry on a 3730 DNA Analyzer (Applied-Biosystems).

#### *Standard ELISAs*

Two standard ELISAs were used following the corresponding manufacturer's instructions: Elitest based on a GAG p25 recombinant protein and a TM synthetic peptide as antigens derived from genotype A (Saman et al., 1999); and Chekit (AG-CHEKIT CAEV/MVV kit, IDEXX Switzerland) based on whole virus antigen (Zanoni et al., 1989).

### *Data analysis*

In the screening study of the 60 peptide ELISAs (first stage), the cut-off value was fixed to 0.35 according to reactivity of negative sera (mean value plus three times standard deviation), a value close to 0.3 applied previously in similar studies (Mordasini et al., 2006). At a second stage, when 5 of these ELISAs were further investigated, the cut-off value was calculated individually for each peptide ELISA, using ROC curve analysis. Sensitivity and specificity of each ELISA test were determined using 2 by 2 contingency tables. The associated 95% confidence intervals (CI) were estimated using Exact Binomial test (RCoreTeam, 2012).

Fisher's exact test was used for comparisons of frequencies of binomial data (positive/negative). Between-group differences of absorbance and index means were submitted to analysis of variance and means comparison using the PROC GLM of the SAS statistical package (SAS institute, Cary, NC, USA), with the Tukey–Kramer adjustment for multiple comparisons in the LSMEANS statement. Differences were considered statistically significant if  $p < 0.05$ . Estimates of agreement between tests using kappa and confidence intervals (CI 95%) were obtained as described previously (Landis and Koch, 1977). Analysis of correlation was applied to assess the relationships between the different tests using the CORR procedure of the SAS statistical package.

## **Results**

### *Preliminary peptide screening*

The 60 synthesized peptides were used individually as coating antigens in ELISA assays. For a preliminary peptide screening using field samples, the panel of 128 sera (112 positive to Elitest and/or Chekit) from sheep of North Central Spain was tested in the peptide ELISAs. The results obtained on the degree of reactivity presented by each of the 60 peptide ELISAs (Table 1) indicate that few of the peptide ELISAs (10%) were highly reactive, with positive results at a frequency close to that observed of positive reactions to two commercial tests, Elitest and Chekit (around 60%). This preliminary screening allowed us to select for further studies five of these peptides [91 and 98M (envelope surface, SU region), 126M1, 126M2 and 139 (envelope transmembrane, TM region)]. Peptide 91 ELISA, was used as a low-reactivity ELISA control assay and the other four ELISAs were highly reactive (Table 1).

### *Reactivity of sera in the ELISAs according to the genotype of the infecting virus*

In order to determine the type of antibody specificity detected by the five selected peptide ELISAs and to assess the sensitivity and specificity of these ELISAs, a group of infected animals ( $n = 71$ ) was used to obtain PCR and sequencing data so that the virus was genotyped in each of these animals. Also, a cut-off value was established in each of the five ELISAs, using for this the negative control serum panel ( $n = 130$ ). None of the sera of this control panel reacted beyond the positivity threshold value in the peptide ELISAs. Sera from these animals were distributed into three groups according to the genotypes A, B and E of the SRLV infecting the animals (Fig. 1), and then tested in the peptide ELISAs (Fig. 2). The results indicated that genotype E was very poorly detected by the peptides under study. Peptide 98M (consensus sequence from the neurological outbreak; Fig. 2S of the Supplementary material) detected almost exclusively genotype A infections (including those by Spanish 697, Icelandic Kv1772 and British Ev1 strains), distinguishing it from infections with other genotypes ( $p < 0.0001$  for ELISA mean absorbance comparisons on genotype A vs. genotypes B or E). This is in line with a previous study in which this peptide was able to discriminate between animals affected with neurological vs. arthritic disease (Glaria et al., 2012). In contrast with 98M, peptide 126M1 reacted especially with genotype B infected sera ( $p <$

0.001) from Spanish (B2) and Mexican (B1) origins, as observed in mean comparisons vs. genotype A or genotype B vs. E ( $p < 0.005$ ). Similarly, mean comparisons of peptide 126M2 ELISA results allowed the distinction between A and B infections although cross-reacting antibodies were detected at a higher proportion compared to 126M1 ELISA. Finally, peptide 139 detected low percentages of infected animals either with genotype A or B. A detail on the sensitivity and specificity values of each peptide ELISA is provided in Table 5 and the corresponding ROC curves in Fig. 3. The group of negative sera obtained from the different European countries, was not reactive in the peptide ELISAs, confirming the very low proportion of false positive reactions in these assays. Overall, these results reveal the utility of peptide ELISAs to detect and distinguish in a group of animals, genotype A vs. B infections and the existence of an association between the antibody specificity detected by peptide ELISAs and the type-specific antigen design.

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vetimm.2012.12.017>.

#### *Detection of clinical vs. asymptomatic infections by peptide ELISAs*

Knowing that TM peptides may detect preferentially clinical SRLV infections (Bertoni et al., 1994), we determined if any of the four ENV highly reactive peptides (98M, 126M1, 126M2 or 139) reacted preferentially with SRLV affected animals from the arthritic or neurological outbreaks. SRLV-infected animals analyzed in each outbreak were distributed into diseased vs. asymptomatic categories and the results obtained with the peptide ELISAs compared (Table 4). Statistical differences between reaction frequencies showed that clinical stages were detected preferentially with peptide 139 ELISA in both outbreaks. Furthermore, when considering jointly both outbreaks, clinical stages were preferentially detected with the peptide 139 ELISA and tended to be preferentially detected with peptide 126M1 also from the TM region. Independently of the clinical vs. asymptomatic status, sera reacted more frequently in the 98M (genotype A) and 126M1 (genotype B) ELISAs when they were obtained from the neurological and the arthritic outbreaks, respectively, in agreement with the genotype-related results shown in Fig. 1. None of the standard tests yielded significant differences in any of the comparisons of this table.

#### *Serological studies in animal groups of North Central Spain*

Five of the eight most reactive peptides ELISAs were selected for further studies, and these as well as both standard tests (Elitest and Chekit) were tested against a large panel of sera ( $n = 496$ ) collected in North Central Spain. In the three groups infected according to standard tests (3–5 and Table 2), the main genetic types of circulating SRLVs were firstly established by PCR-sequencing of a representative sample of animals within each group. Accordingly, these were of types A (Group 4); B (Group 5); and either A or B (Group 3).

Overall (global population, Table 2), sera showed an intermediate degree of reactivity (frequency of positive reactions close to 50%) in both standard tests and in the joint result of the five peptide ELISAs (not shown), but the reactivity decreased to 26–34.7% when the four most reactive peptide ELISAs were individually considered and down to 6.6% in the case of peptide 91 ELISA. This is in line with the results obtained with the reduced serum panel ( $n = 128$ ; Table 1), with the lack of reaction observed with the uninfected control sera under study ( $n = 130$ ), and with the correspondence existing between the antigen type of both the circulating strain and the ELISA peptide.

Analysis of results per group revealed that Groups 1 and 2, both negative to Elitest, showed limited positive results to Chekit and most of the peptides, but in Group 1 a substantial proportion (40%) of the animals were seropositive to 98M ELISA. Among the infected Groups (3–5), Group 3 with mixed (types



A and B) SRLV infections, presented a higher reactivity in Chekit compared to Elitest and 126M2 was the most reactive peptide (Table 2). In Group 4 (neurological outbreak, type A) both standard tests performed similarly, but with a slightly decreased frequency compared to 98M ELISA. In Group 5 (arthritic outbreak, type B), Chekit detected more positives than Elitest and 126M1 was the most sensitive ELISA. Finally, Group 6 positives were only detected by peptide ELISAs, 126M1 and 126M2 being the most reactive. The proportion of discordant reactions between both standard tests varied between animal groups and the proportion of the animals negative to one or both standard tests but positive to a peptide ELISA varied between animal groups and between peptide ELISAs.

The four most reactive peptide ELISAs differed from each other in reactivity patterns, according to the main circulating SRLV type of the animal group. In all the cases, peptides detected infections according to their antigenic design, thus genotype A infections were better detected with peptide 98M (Group 4) and genotype B infections were detected mainly with 126M1 (Group 5).

Among the animals from groups 3, 4 and 5 positive to Elitest (Table 3), the highest proportion of positives to peptide ELISAs was found in Group 4P with type A infections (91.6% in 98M ELISA), followed by Group 5 with type B infections (72.5% in 126M1 ELISA) and Group 3 with mixed (A and B) infections (50% in 126M1 ELISA).

Analysis of sera from the Elitest negative groups revealed that Groups 2 and 6 (both certified by Elitest) did not reach 30 percent reactivity in any of the peptide ELISAs and did not react in Chekit. However, sera from Group 1 (naturally negative to Elitest) had a reactivity similar to that found in group 4N (Table 3) from A-infected flocks, reaching reactivity levels well above 30 percent in peptide 98M ELISA and of 14–23 percent in Chekit.

To further assess if Elitest-negative animals with a substantial reactivity to 98M ELISA were truly infected, a follow up study was done one to two years later on Group 4Elitest-N, involving 48 animals which were analyzed by PCR, standard tests and peptide ELISAs. The results corroborated that the group was infected, since 6 out of 8 of the animals became Elitest positive and 20 of the animals were found infected according to PCR. PCR analysis revealed that most of these animals were infected (16 of the amplicons were sequenced) by genotype A and of these, the majority (75%) were seropositive in peptide 98M ELISA (genotype A antigen) and in the Chekit assay. Taken together, these results indicate that the peptide ELISAs enable general and especially type specific serotyping of infected animal groups according to the reactivity pattern differences between them.

## **Discussion**

A single diagnostic assay is commonly applied to detect SRLV infections in each particular area under study. However, 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. In this work, we explored comparatively different ELISA-based strategies in animals infected with different genotypes of SRLV in areas of North-Central Spain. The assays involved in the work were two standard tests, Elitest and Chekit, and ELISAs based on different synthetic peptides derived from SRLV proteins of genotypes A and B.

The tendency toward an increased ability of type-A strain antigens to detect cross-reacting antibodies in type B infections has been recognized previously (Lacerenza et al., 2006). Consequently, type A derived antigens become less discriminatory at the time of distinguishing infections of the types A and B. This occurred here when comparing the ELISAs of peptide 126M2 (type A-derived) vs. 126 M1 (type B-derived) or Elitest (derived from type A strain) vs. Chekit, whose reactivity appeared to be directed mainly toward genotype B infection even though this assay involves the MVV OLV whole virus antigen (Zanoni

et al., 1989). On the other hand, the Chekit assay, based on whole virus protein, may have covered a broader antigen spectrum compared to Elitest, which is based on a two component monostrain design (TM synthetic peptide and p25 recombinant protein). This may partially explain the Elitest-negative Chekit-positive results obtained (54 of the 496 sera; 10.88%). The Chekit assay may have detected also antibodies unrelated to SRLV, as those cross-reacting with bluetongue virus (BTV) according to recent findings (Valas et al., 2011), because in the area of study vaccination against BTV serotypes 1 and 8 was in course at the time of sampling (Sánchez Matamoros et al., 2009). In agreement with previous reports (Reina et al., 2010), both assays, Elitest and Chekit, may have underestimated genotype E seroprevalence as they were not fully detecting animals experimentally infected with a strain of this genotype. Comparatively in the global population, each of the standard tests detected infection more efficiently than single peptide ELISAs (with exceptions like 98M ELISA in the Castilla-León region), likely because both standard tests displayed at least two viral proteins as coating antigens. Thus, peptide combinations may be required to increase the antigenic spectrum. However, standard ELISAs failed to detect a proportion of animals seropositive to peptide ELISAs and were less informative than peptide ELISAs regarding the genotyping of the infection present in a particular area, so relevant in epidemiological studies, control of virus spread and animal exchange programs.

Only eight of the 60 peptides screened were highly reactive, suggesting that these eight peptides corresponded to well conserved antigenic regions along the viral genome. Peptide 77/GAG p14 (Table 1) ELISA had apparently a high reactivity but positivity could not be confirmed in a high proportion of animals by PCR or standard ELISAs. Therefore, many positive reactions against peptide 77 may have been non-specific. Lack of reactivity to the remaining peptides tested was unexpected, although the probability of any epitope prediction algorithm to identify a real epitope is usually less than 60%. Several reports using WB based on both matrix and capsid proteins have shown that these proteins are highly reactive with sera from naturally and experimentally infected animals, suggesting that linear epitopes are located in these antigenic regions (Houwens and Nauta, 1989; Rosati et al., 1994; Zanoni et al., 1989). This could be due to the algorithm chosen for epitope prediction, length of the selected region and efficiency of passive immobilization on solid phase which is strictly sequence dependent. In line with this, the highest performance among the four highly reactive peptide ELISAs studied in depth corresponded to the peptide ELISA 98M, but only when applied to detect type-A specific antibodies (as expected since 98M was in a type-specific amino acid region), elicited in experimental infections with Ev1 strain (Reina et al., 2008), the Kv1772 clone (Andersson et al., 1993) or natural infections such as those of the Castilla-León region (Group 4). This peptide corresponds to an immunodominant epitope of a variable region (Fig. 2S of Supplementary material) homologous to that of the SU5 peptide 1163 detecting antibodies with high avidity (Mordasini et al., 2006). In contrast, peptide 91 ELISA had by far the lowest performance in the screening study (n = 128), as well as in the second-phase study (n = 496). Likely, the immunodominance of this epitope was hindered, since its sequence included a conserved cysteine among the available SRLV sequences downstream a potential N-linked glycosylation site (Valas et al., 2000), but lacked a precedent glycosylation site. In line with previous findings (Glaria et al., 2009), a single inversion within the amino acid sequence (HQ in 126M1 and QH in 126M2; Table 1) biased the peptide reactivity toward a CAEVlike (B-type) and a VMV-like (A-type) profile, respectively. Type-specific reactivity of the three ENV-TM peptides was less marked than that of the ENV-SU 98M peptide corresponding to the hypervariable domain SU5, in agreement with previous studies (Mordasini et al., 2006). Compared to the Elitest ENV-TM peptide (Saman et al., 1999), 126M1 and 126M2 were shorter, lacking residues GGQ at the aminoterminal and KS at the carboxy-terminal, which may affect the antigen coating.

Amongst the three animal groups (1, 2 and 6) apparently uninfected (Elitest-negative), Group 1 was found positive mainly according to peptide 98M ELISA, strongly suggesting genotype A infections may trigger anti-ENV-SU (98M) antibodies undetectable by the p25 or the ENV-TM peptide used in Elitest. In addition, Groups 2 and 6 were similarly reactive in peptide 126M1 and 126M2 ELISAs, suggesting the presence of two circulating genotypes (A and B) in these flocks. Thus, the selection of peptides for complementary tools may differ between areas in this scenario of known virus genotype. Particularly in Elitest-negative animals, genotype A infections may be detected by 98M and 126M2 ELISAs (in Groups 3 and 4), whereas genotype B infections may be detected by 126M1 ELISA (in Group 5). Therefore, peptide testing of animals assumed to be free of infection may help to detect and classify the infection avoiding its persistence. However, since specificity of peptide ELISAs did not reach 100%, non-specific reactions should not be discarded when testing properly controlled flocks by standard ELISAs. Commercial tests have shown that it is possible to eradicate or at least to keep under control SRLV infections in commercial flocks in the UK, Italy and Spain. The results presented here indicate that these tests might miss a few cases that are picked out with the new peptide ELISAs. These new tests might detect earlier phases or lower humoral responses helping to accelerate SRLV infection eradication.

Sera from clinically affected animals tended to be more reactive with the peptide 139 ELISA, which appeared to detect high affinity antibodies produced along with viral replication burst, disease onset and pathogenesis. CAEVlike TM peptides such as TM3, analogous to 126M1, also detected preferentially clinical (type B) infections (Bertoni et al., 1994). TM3, 126M1 and 126M2 peptides have two cystein residues that may trigger the production of enhancing antibodies to favor viral entry, as it occurs in HIV infections (Robinson et al., 1991), undermining the role of early cellular responses (de Andres et al., 2009; Juganaru et al., 2011). However detection of these antibodies is less informative from the point of view of early diagnosis since frequency of non-clinical infections detected by ELISAs based on peptide 139 may be underestimated.

Often the information on genetic types of circulating strains is not available and a unique serological diagnostic technique is applied blindly. In this scenario of unknown virus genotype, PCR-based techniques (Barquero et al., 2011; Brinkhof et al., 2010), WB (Ramirez et al., 2009) or the heteroduplex mobility assay may be helpful for initial classification of SRLV circulating genotypes (Germain et al., 2008; Germain and Valas, 2006) enabling proper antigen design for new type specific ELISA. These ELISAs may be useful to diagnose infection in populations of limited size, animals of high value apparently seronegative or flocks close to an eradication stage and to trace back infections of epidemiological interest. The appearance of mixed infections, new variants by mutation, recombination or introduction of new viral types into flocks and herds, point out the need of constant surveillance on the technique implemented in order to improve diagnosis and control the virus spread.

## **Conclusion**

This study, showing the results obtained with different diagnostic strategies in particular geographic areas involving heterogeneous SRLV infections, outlines the interest of peptide assays to type the infections and control viral spread and encourages the development of highly sensitive ELISA tests involving multiple strain designs.

## **Conflict of interest statement**

None of the authors has personal or financial relationship with people or organization that could influence or bias the results presented in this paper. Experimental

results and test designs and procedures produced in this paper, including ELISAs and newly characterized sequences are patent pending.

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**Table 1**

Percentage of sera reactive to the 60 synthetic single peptide<sup>a</sup> ELISAs (1 type of peptide/ELISA) analyzed in this study using a panel of 128 sera from sheep flocks of North Central Spain.

No. of ELISAs	Reactivity (%)		Peptide name/viral protein–peptide amino acid sequence used in this study
33	Very low	(<9%)	75/GAGp14-PERKGRNQGMGQKCY 76/GAGp14-PEKGKRNQPQRCY 91/ENVgp135-CSLPHKNESNKWTCA 157/ENVgp46-KNKKERVDCQDREQR 48/GAGp25-KLNEEAERWVRQNPP 81/GAGp14-GNNRRGPRVVPASAPP 77/GAGp14-MQKDCRQKKQQGNRR 98M/ENVgp135-VDMPQSYIEKQKRNK 126M1/ENVgp46-ELDCWHYHQCVTST 126M2/ENVgp46-ELDCWHYHQCVTST 139/ENVgp46-HIAQRDASRIPDVWT 140/ENVgp46-QLAQEQARRIPDVWE
15	Low	(9–20%)	
4	Medium	(23–31%)	
2	High	(46–47%)	
6	Very high	(57–62%)	

<sup>a</sup> See Supplementary material for sequences of the 60 peptides and the protein from which they were designed.

**Table 2**

Percentage of sera reactive in different areas of North Central Spain using five synthetic single peptide ELISAs (peptides 91, 98M, 126M1, 126M2 and 139), jointly considered (global population) and the standard ELISAs Elitest and Chekit.

Animal group (number of animals): SRLV type	Province (region)	Breed/outbreak	Standard ELISA		Peptide ELISA				
			Elitest	Chekit	91	98M	126M1	126M2	139
1(50)	Guipúzcoa (País Vasco)	Latxa	0	14	6	40	16	12	8
2 (50)	Vizcaya (País Vasco)	Latxa	0	0	10	12	20	22	0
3 (62): A, B	Navarra (Navarra)	Mixed	45.2	71	16.1	22.5	32.2	29	8
4 (157): A	León, Zamora (Castilla-León)	Assaf/neurologic	61.1	60.5	9	69.4	17.8	38.2	45.2
5 (117): B2	Zaragoza (Aragón)	Rasa Aragonesa/arthritis	87.2	100	5.1	6.8	66.6	41.8	41.8
6(60)	Teruel (Aragón)	Rasa Aragonesa	0	0	10	0	26.6	26.6	0
Global population tested(496): A, B	All the tested provinces		45.5	51.2	6.6	31.6	32.2	26	34.7

<sup>a</sup> Inferred according to previous studies in the pertinent areas and flocks (Glaria et al., 2009, 2012; Ramirez et al., 2009).

**Table 3**

Percentage of sera reactive in each of the five synthetic peptide ELISAs (91, 98M, 126M1, 126M2 and 139) and in the standard ELISA Chekit using infected groups (3–5) and the global population tested, each distributed into positive (P) and negative (N) categories according to the standard Elitest results.

Animal group, Elitest reaction (no. of sera): SRLV type	Chekit	Peptide ELISA				
		91	98M	126M1	126M2	139
3ElitestP (28): A, B	93	25	39.3	50	39.3	17.9
ElitestN(34)	53	8.8	8.8	17.6	20.6	0
4ElitestP (96): A	84.4	9.3	91.6	21.8	53.1	63.5
ElitestN(61)	23	8.2	34.4	11.5	14.7	16.4
5ElitestP (102): B2	100	5.9	7.8	72.5	45	46.1
Elitest N(15)	100	0	0	26.6	20	13.3
Global population tested ( $n = 496$ ) <sup>a</sup> : A, B						
ElitestP (213)	100	8.4	45.5	50.2	49.3	49.3
ElitestP (13)	0	7.7	77	0	30.8	23
Elitest N (54)	100	13	27.8	16.7	26	14.8
Elitest N (216)	0	7.4	14.8	17.1	17.6	3.7

<sup>a</sup> Elitest P and N categories in the global population have each been further classified into two subcategories in the global population analyzed on the basis of reactivity to Chekit.



**Table 4**

Reactivity (percentage of positive reactions) in 4 peptide ELISAs (98 M, 126M1, 126M2, 139), using sera from SRLV diseased and asymptomatic seropositive animals (by Chekit or Elitest standard tests) from flocks located in Castilla-León and Aragón and belonging to the neurological (type A) and arthritic (type B) outbreaks, respectively.

Group	Status	Standard test		Peptide ELISA			
		Chekit	Elitest	98M	126M1	126M2	139*
4	Neurological disease (n = 24)	87.5	100	100	16.7	62.5	87.5
	Asymptomatic (n = 96)	88.5	100	91.7	21.9	53.1	63.5
5	Arthritic disease (n = 12)	100	100	8.3	91.7	58.3	83.3
	Asymptomatic (n = 117)	100	87.2	6.8	66.7	42.7	40.1

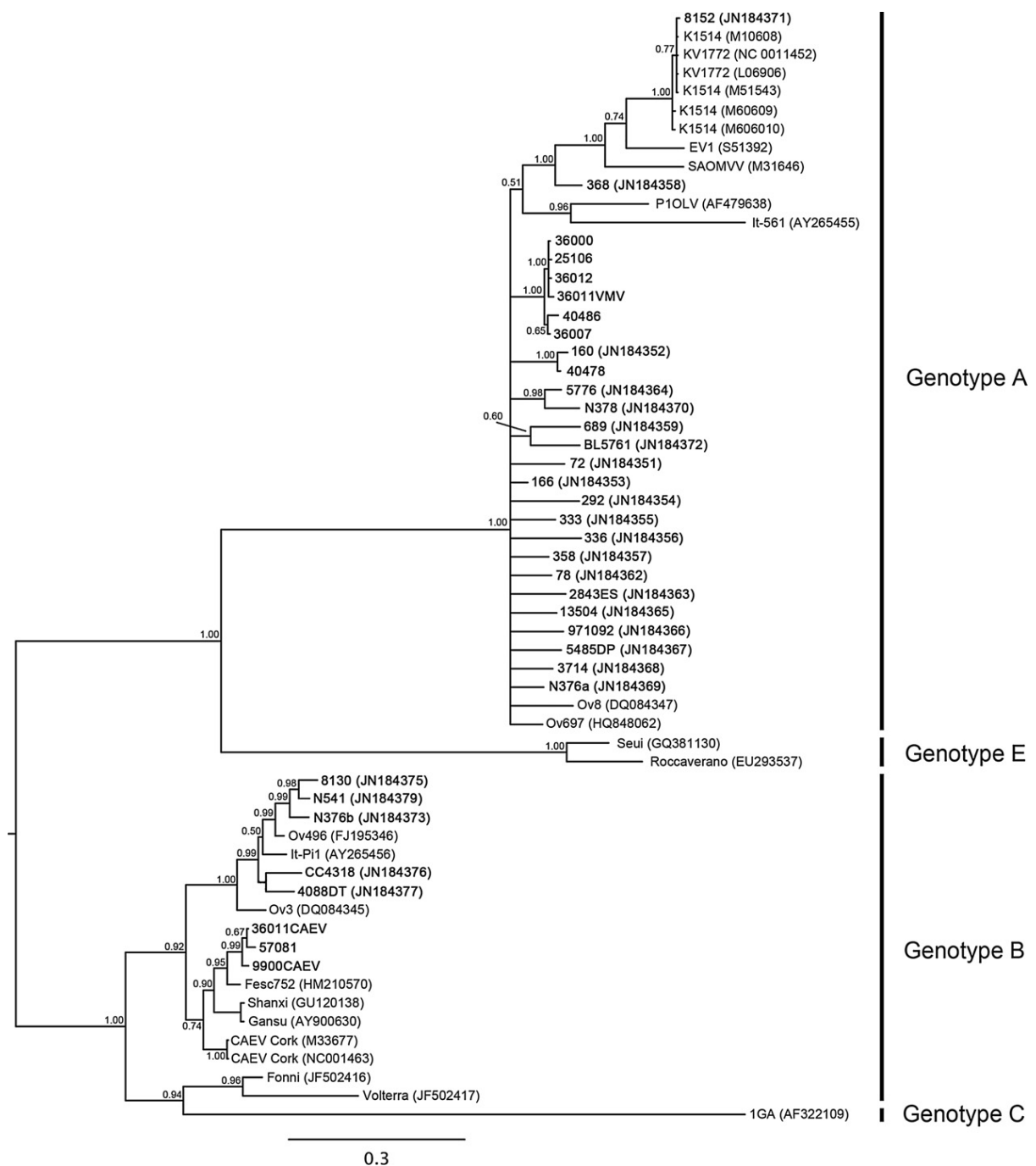
\*  $p < 0.0001$ : Statistical differences using Fisher's exact test were found between diseased vs. asymptomatic animals from the same outbreak only in the peptide 139 ELISA.

**Table 5**

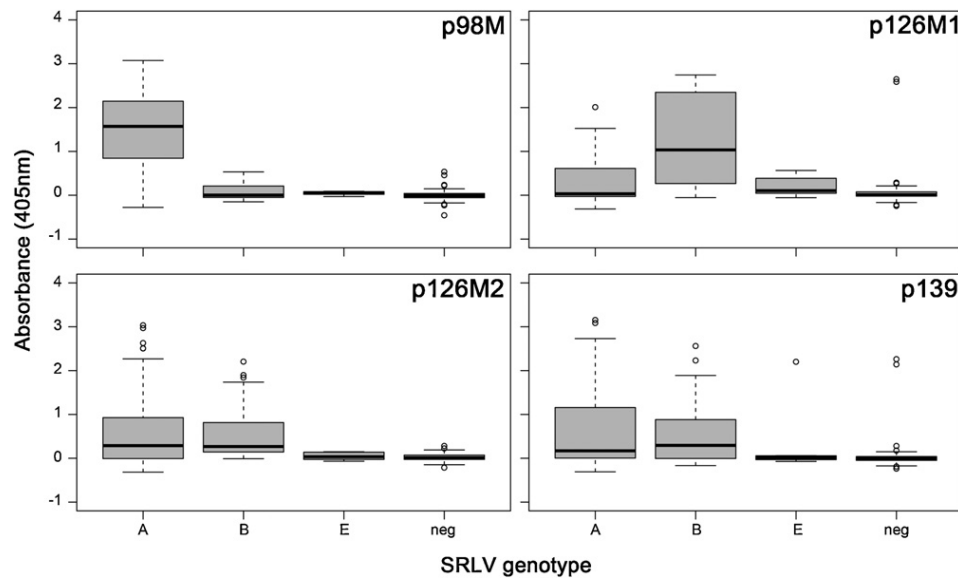
Sensitivity and specificity of peptide ELISA and standard test (Elitest) according to the SRLV genotype involved. Best performance peptide ELISA is reported in bold. Standard Elitest data are highlighted in italics.

Genotype	ELISA	Sensitivity (95% CI)	Specificity (95% CI)
A	p91	0.750 (0.616–0.856)	0.168 ( <b>0.108–0.243</b> )
	<b>p98M</b>	<b>0.857 (0.738–0.936)</b>	<b>0.985 (0.946–0.998)</b>
	p126M1	0.393 (0.265–0.532)	0.864 (0.793–0.917)
	p126M2	0.679 (0.540–0.797)	0.879 (0.811–0.929)
	p139	0.607 (0.468–0.735)	0.846 (0.772–0.903)
	<i>Elitest</i>	<i>0.911 (0.804–0.970)</i>	<i>0.898 (0.833–0.945)</i>
B	p91	0.636 (0.407–0.828)	0.237 (0.167–0.319)
	p98M	0.364 (0.172–0.593)	0.947 (0.894–0.978)
	<b>p126M1</b>	<b>0.818 (0.597–0.948)</b>	<b>0.955 (0.904–0.983)</b>
	p126M2	0.773 (0.546–0.922)	0.924 (0.865–0.963)
	p139	0.591 (0.364–0.793)	0.900 (0.835–0.946)
	<i>Elitest</i>	<i>0.727 (0.498–0.893)</i>	<i>0.898 (0.833–0.945)</i>
E	p91	0.750 (0.349–0.968)	0.496 (0.408–0.585)
	p98M	0.750 (0.349–0.968)	0.636 (0.548–0.718)
	<b>p126M1</b>	<b>0.750 (0.349–0.968)</b>	<b>0.667 (0.579–0.746)</b>
	p126M2	0.625 (0.245–0.915)	0.583 (0.494–0.668)
	p139	0.625 (0.245–0.915)	0.523 (0.434–0.611)
	<i>Elitest</i>	<i>0.875 (0.473–0.997)</i>	<i>0.805 (0.725–0.869)</i>

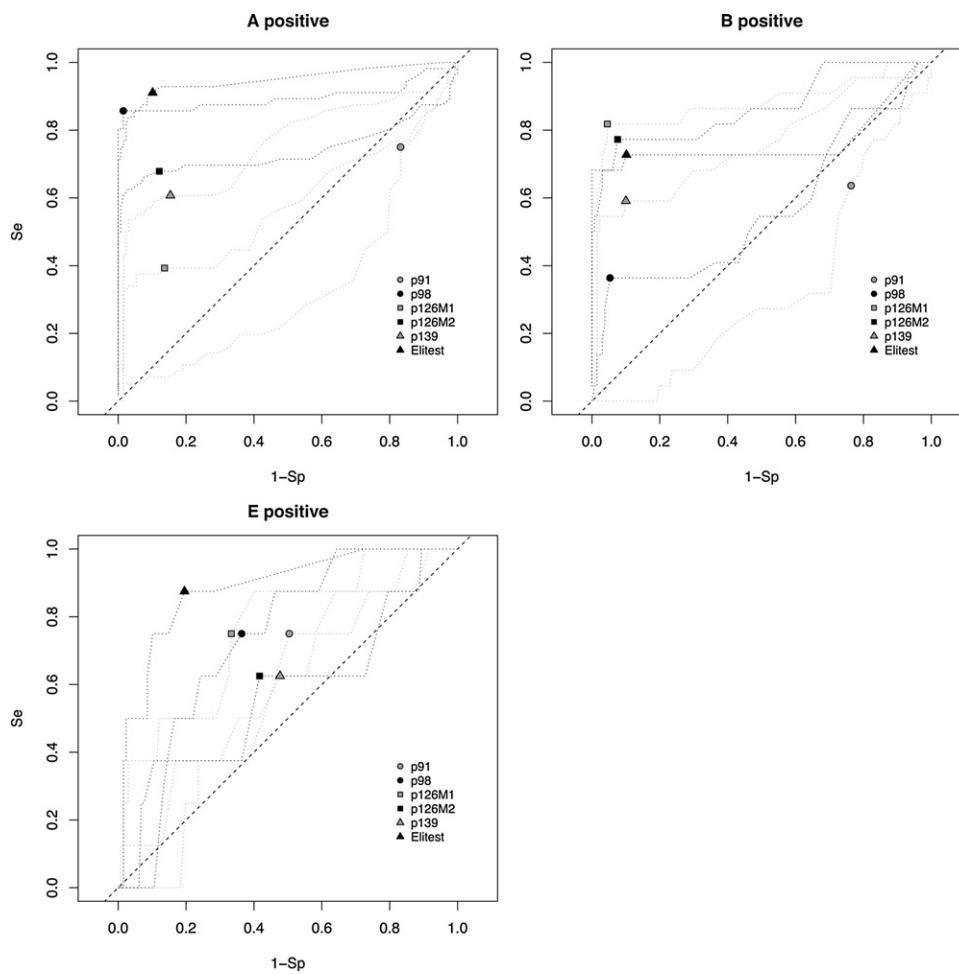
CI: confidence interval; p: peptide.



**Fig. 1.** Phylogenetic tree involving 60 SRLV gag sequences. These include the 37 sequences genotyped used in this study (reported in bold) and reference sequences obtained from GenBank. The SRLV genotypes and subtypes are indicated. GenBank accession numbers are indicated within brackets. Posterior probability for each node is indicated above branches.



**Fig. 2.** Distribution of optical density values obtained in the four synthetic peptide ELISAs. Peptides used were: 98M (top left panel), 126M1 (top right panel), 126M2 (bottom left panel) and 139 (bottom right panel). Sera tested in these ELISAs were from 71 animals infected with A (48), B (23) and E (8) SRLV genetic types and 130 negative animals.



**Fig. 3.** ROC curves illustrating sensitivity and specificity values of five ELISAs (corresponding to peptides 91, 98M, 126M1, 126M2 and 139) and the Elitest standard ELISA found in groups of sera distributed according to genotypes A (top panel) B (middle panel) and E (bottom panel).