An insight into a combination of ELISA strategies to diagnose Small Ruminant Lentivirus infections

X. de Andrés,[1,a] H. Ramírez,[1,‡,a], L. Bertolotti,[2] B. San Román,[1] I. Glaria,[1] H. Crespo,[1] P. Jáuregui,[1] E. Minguijón,[3] R. Juste,[3] I. Leginagoikoa,[3] M. Pérez,[4] L. Luján,[4] J.J. Badiola,[4] L. Polledo,[5] J.F. García-Marín,[5] J.I. Riezu,[6] F. Borrás-Cuesta,[6] D. de Andrés,[1§] S. Rosati,[2] R. Reina,[1,b] B. Amorena[1,b*]

[1]Institute of Agrobiotechnology (CSIC-UPNA-Gobierno de Navarra) Navarra, Spain; [2]Department of Animal Production, Epidemiology and Ecology, University of Turin, Turin, Italy; [3]NEIKER-Tecnalia, Derio, Bizkaia, Spain; [4]Veterinary Faculty, University of Zaragoza, Zaragoza, Spain; [5]Veterinary Faculty, University of León, León, Spain; [6]Centre for Applied Medical Research (CIMA), University of Navarra, Navarra, Spain; [‡]Present address: Laboratory of Virology, Genetics and Molecular Biology. Facultad de Estudios Superiores Cuautitlán, UNAM. C-4, Cuautitlán Izcalli, State of Mexico, Mexico; [§] Present address: Cátedra de Recursos Cinegéticos y Piscícolas. Universidad de Córdoba, Córdoba, Spain

[a] Both authors have contributed equally to this work

[b] Both authors have contributed equally to this work

* Corresponding author. Tel.: 34 948168006; fax: 34 948232191

E-mail address: bamorena@unavarra.es (B. Amorena)

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 analyse 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 0,65 0.77 (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: Visna/Maedi, sheep, ELISA antibody diagnosis, synthetic peptide, genotyping, Spain

1. 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).

70 Interestingly, genotype A derived antigens seem more suitable than genotype B antigens to
71 detect heterologous infection (Lacerenza et al., 2006). On the other hand, seroprevalence
72 against genotype E may be underestimated using commercially available ELISAs
73 ("standard" ELISAs henceforth) (Reina et al., 2009b).

74 Control programs of Central and Southern Europe have employed indirect ELISAs
75 based on whole virus or viral recombinant proteins (Reina et al., 2009a; Saman et al.,
76 1999; Zanoni et al., 1994), differing in performance according to the range of antigenic
77 specificities or the antibody concentration required (Ramirez et al., 2009). Antibody titer
78 may vary along the animal's life (Varea et al., 2001), which may lead to misdiagnosis by
79 ELISA. These findings, together with the high genetic/antigenic variability inherent to
80 these viruses makes no single technique or test sufficient for use as "gold standard" to
81 determine the infection status of the animal (Reina et al., 2009a). In the absence of this
82 standard, the positivity to at least two diagnostic techniques, based on either antibody or
83 virus-related detection, has been used in some studies as criterion for the presence of
84 infection (Saman et al., 1999; Varea et al., 2001).

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

95 alone and in combination with PCR, allowing an efficient detection of SRLV infection
96 (Alvarez et al., 2006; Barquero et al., 2011; Leginagoikoa et al., 2006; Leginagoikoa et al.,
97 2010; Reina et al., 2006) and being implemented at present in areas of North-Central Spain
98 (Leginagoikoa et al., 2010; Perez et al., 2010). As a result, some flocks have reached a
99 seronegative status (Perez et al., 2010). However, like in other areas, seroconversion may
100 reappear sporadically, decreasing the flock value (Brulisauer et al., 2005).
101 This study, involving known and newly designed ELISA assays, aims to explore
102 the possibilities of a fine tuning of ELISA testing in different areas infected with different
103 SRLV, and proposes diagnostic strategies to prevent viral spread in these areas through
104 flock typing and to detect infection in animals seronegative to standard assays.
105
106 **2. Materials and methods**
107 *2.1. Animals and samples*
108 Two panels of sheep sera from North-Central Spain were used. In a preliminary
109 study, aiming to determine the degree of reactivity of 60 peptides in an ELISA format, the
110 panel of sera employed (n = 128) was obtained from different flocks widely distributed in
111 this area, known to have type A (Glaria et al., 2012) and type B (Glaria et al., 2009)
112 infections (Table 1). Most of these sera, 112 (87.5%) were known positives in one or both
113 standard ELISAs, specifically 85 (66.4%) in Elitest, 108 (84.3%) in Chekit and 81 (63.2%)
114 in both standard tests. At a second stage, a serum panel of increased size (involving 496
115 additional animals) was used for a detailed study on reactivity patterns and specificities.
116 Accordingly, animal Groups 1 to 6 were established from different flocks, with the
117 following characteristics (Table 2 shows further details on groups' description). Group 1
118 was negative to VMV infection according to standard tests, without having been submitted
119 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).

2.2. 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.

Peptides were chemically synthesized (Thermo scientific) and diluted upon arrival in carbonate buffer (pH 9.6) at 1 mg/ml and stored at -20ºC until tested in the corresponding screening ELISA.

2.3. Peptide ELISA procedure

Each peptide ELISA was carried out as previously described (Reina et al., 2009b), with slight modifications. Briefly, 96-well microplates (Maxisorp_NuncTM) were coated with 300 ng of the selected peptide in carbonate buffer (pH 9.6) or carbonate buffer alone as negative control. Plates were allowed to dry overnight at 37ºC and then blocked with 2.5% bovine casein for 1 h at 37ºC. 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 37ºC. Subsequently, protein G (0.2 µg/ml, Pierce) diluted in the same buffer was added and plates were incubated for 1 h at 37ºC. 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.

170 *2.5. Polymerase chain reaction (PCR) and sequencing*

171 PCR was used to determine the nucleotide sequences for peptide design and, when
172 needed, to confirm the infection status. Briefly, genomic DNA was extracted from
173 peripheral blood mononuclear cells (PBMC) or tissue samples (central nervous system or
174 carpal joint from clinically affected animals of the neurological and the arthritic outbreak,
175 respectively) using QIAamp® DNA Blood Mini Kit (Qiagen) as described elsewhere
176 (Crespo et al 2012). Tissue samples were previously lysed (1 h at 56ºC) in buffer (100 mM
177 Tris-HCl pH 8.5; 5 mM EDTA; 400 mM NaCl; 0.2% SDS) with Proteinase K (50 µg per
178 10^7 cells; Sigma).

179 *Gag* and *env* regions were amplified with previously described primers (Glaria et
180 al., 2009) or those designed in this work (Supplementary material). The reaction mix of the
181 PCRs consisted of: 1× Reaction Buffer (Biotools), 2 mM $MgCl_2$ (Biotools), 225 µM of
182 each dNTP (Biotools), 600 nM of each primer, 0.04 U/µl of Pfu DNA polymerase
183 (Biotools) and 0.5 to 1 µg of sample DNA to a final volume of 30 µl. PCR conditions
184 were: initial denaturation step for 5 min at 94ºC followed by 45 cycles of 94ºC for 40 s,
185 annealing at 50 to 58 ºC (depending on the primers used) for 50 s and extension at 72ºC for
186 a variable period (depending on the size of the amplicon); and a final extension step (10
187 min at 72ºC). Amplicons were cloned into pGEMT-easy® vector (Promega) following the
188 manufacturer's instructions and then sequenced using BigDye® Terminator v3.1 chemistry
189 on a 3730 DNA Analyzer (Applied-Biosystems).

190 *2.5. Standard ELISAs*

191 Two standard ELISAs were used following the corresponding manufacturer's
192 instructions: Elitest based on a GAG p25 recombinant protein and a TM synthetic peptide
193 as antigens derived from genotype A (Saman et al., 1999); and Chekit (AG-CHEKIT

194 CAEV / MVV kit, IDEXX Switzerland) based on whole virus antigen (Zanoni et al.,
195 1994).

196 *2.6. Data analysis*

197 In the screening study of the 60 peptide ELISAs (first stage), the cut-off value was
198 fixed to 0.35 according to reactivity of negative sera (mean value plus three times standard
199 deviation), a value close to 0.3 applied previously in similar studies (Mordasini et al.,
200 2006). At a second stage, when 5 of these ELISAs were further investigated, the cut-off
201 value was calculated individually for each peptide ELISA, using ROC curve analysis.
202 Sensitivity and specificity of each ELISA test were determined using 2 by 2 contingency
203 tables. The associated 95% confidence intervals (CI) were estimated using Exact Binomial
204 test (RCoreTeam, 2012).

205 Fisher's exact test was used for comparisons of frequencies of binomial data
206 (positive/negative). Between-group differences of absorbance and index means were
207 submitted to analysis of variance and means comparison using the PROC GLM of the SAS
208 statistical package (SAS institute, Cary, NC. USA), with the Tukey-Kramer adjustment for
209 multiple comparisons in the LSMEANS statement. Differences were considered
210 statistically significant if $p < 0.05$. Estimates of agreement between tests using kappa and
211 confidence intervals (CI 95 %) were obtained as described previously (Landis and Koch,
212 1977). Analysis of correlation was applied to assess the relationships between the different
213 tests using the CORR procedure of the SAS statistical package.

214

215 **3. Results**

216 *3.1. Preliminary peptide screening*

217 The 60 synthesized peptides were used individually as coating antigens in ELISA
218 assays. For a preliminary peptide screening using field samples, the panel of 128 sera (112

219 positive to Elitest and/or Chekit) from sheep of North Central Spain was tested in the
220 peptide ELISAs. The results obtained on the degree of reactivity presented by each of the
221 60 peptide ELISAs (Table 1) indicate that few of the peptide ELISAs (10%) were highly
222 reactive, with positive results at a frequency close to that observed of positive reactions to
223 two commercial tests, Elitest and Chekit (around 60%). This preliminary screening
224 allowed us to select for further studies five of these peptides [91 and 98M (envelope
225 surface, SU region), 126M1, 126M2 and 139 (envelope transmembrane, TM region)].
226 Peptide 91 ELISA, was used as a low-reactivity ELISA control assay and the other four
227 ELISAs were highly reactive (Table 1).
228
229 *3.2. Reactivity of sera in the ELISAs according to the genotype of the infecting virus*
230 In order to determine the type of antibody specificity detected by the five selected
231 peptide ELISAs and to assess the sensitivity and specificity of these ELISAs, a group of
232 infected animals (n = 71) was used to obtain PCR and sequencing data so that the virus
233 was genotyped in each of these animals. Also, a cut-off value was established in each of
234 the five ELISAs, using for this the negative control serum panel (n = 130). None of the
235 sera of this control panel reacted beyond the positivity threshold value in the peptide
236 ELISAs. To assess the antibody specificity detected by each of the peptide ELISAs (91,
237 98M, 126M1, 126M2 and 139), the provirus from 71 animals from different sources (as
238 specified in the Material and Methods section) was genotyped by gag PCR-sequencing,
239 alignment and phylogenetic analysis (Fig 1).
240 Sera from these animals were distributed into three groups according to the genotypes (A,
241 B and E) of the SRLV infecting the animals, and then tested in the peptide ELISAs (Fig.
242 2). The results indicated that genotype E was very poorly detected by these peptides.
243 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 Scottish 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., 2011). 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, were 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.

3.3. 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.

3.4. 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, 4 and 5; 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

294 with the reduced serum panel (n = 128; Table 1), with the lack of reaction observed with
295 the uninfected control sera under study (n = 130), and with the correspondence existing
296 between the antigen type of both the circulating strain and the ELISA peptide.

297 Analysis of results per group revealed that Groups 1 and 2, both negative to Elitest,
298 showed limited positive results to Chekit and most of the peptides, but in Group 1 a
299 substantial proportion (40%) of the animals were seropositive to 98M ELISA. Among the
300 infected Groups (3, 4 and 5), Group 3 with mixed (types A and B) SRLV infections,
301 presented a higher reactivity in Chekit compared to Elitest and 126M2 was the most
302 reactive peptide (Table 2). In Group 4 (neurological outbreak, type A) both standard tests
303 performed similarly, but with a slightly decreased frequency compared to 98M ELISA. In
304 Group 5 (arthritic outbreak, type B), Chekit detected more positives than Elitest and
305 126M1 was the most sensitive ELISA. Finally, Group 6 positives were only detected by
306 peptide ELISAs, 126M1 and 126M2 being the most reactive.

307 The proportion of discordant reactions between both standard tests varied between
308 animal groups and the proportion of the animals negative to one or both standard tests but
309 positive to a peptide ELISA varied between animal groups and between peptide ELISAs.

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

315 Among the animals from groups 3, 4 and 5 positive to Elitest (Table 3), the highest
316 proportion of positives to peptide ELISAs was found in Group 4P with type A infections
317 (91.6% in 98M ELISA), followed by Group 5 with type B infections (72.5% in 126M1
318 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 year later on Group 4-Elitest-N, involving 48 animals which were analyzed by PCR, standard tests and peptide ELISAs. The results corroborated that the group was infected, since 6 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.

4. 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

344 Chekit, and ELISAs based on different synthetic peptides derived from SRLV proteins of
345 genotypes A and B.

346 The tendency towards an increased ability of type-A strain antigens to detect cross-
347 reacting antibodies in type B infections has been recognized previously (Lacerenza et al.,
348 2006). Consequently, type A derived antigens become less discriminatory at the time of
349 distinguishing infections of the types A and B. This occurred here when comparing the
350 ELISAs of peptide 126M2 (type A-derived) vs. 126 M1 (type B-derived) or Elitest
351 (derived from type A strain) vs. Chekit, whose reactivity appeared to be directed mainly
352 towards genotype B infection even though this assay involves the MVV OLV whole virus
353 antigen (Zanoni et al 2004). On the other hand, the Chekit assay, based on whole virus
354 protein, may have covered a broader antigen spectrum compared to Elitest, which is based
355 on a two component monostrain design (TM synthetic peptide and p25 recombinant
356 protein). This may partially explain the Elitest-negative Chekit-positive results obtained
357 (54 of the 496 sera; 10.88%). In the area of study vaccination against BTV serotypes 1 and
358 8 had been performed since 2008 (Sánchez Matamoros et al., 2009). Whether the Chekit
359 assay detected also antibodies BTV-related but unrelated to SRLV (Valas et al., 2011) is
360 unknown, since paired serial samples were unavailable. In agreement with previous reports
361 (Reina et al., 2010), both assays, Elitest and Chekit, may have underestimated genotype E
362 seroprevalence as they were not fully detecting animals experimentally infected with a
363 strain of this genotype. Comparatively in the global population, each of the standard tests
364 detected infection more efficiently than single peptide ELISAs (with exceptions like 98M
365 ELISA in the Castilla-León region), likely because both standard tests displayed at least
366 two viral proteins as coating antigens. Thus, peptide combinations may be required to
367 increase the antigenic spectrum. However, standard ELISAs failed to detect a proportion of
368 animals seropositive to peptide ELISAs and were less informative than peptide ELISAs

369 regarding the genotyping of the infection present in a particular area, so relevant in
370 epidemiological studies, control of virus spread and animal exchange programs.
371 Only eight of the 60 peptides screened were highly reactive, suggesting that these
372 eight peptides corresponded to well conserved antigenic regions along the viral genome.
373 Peptide 77 (77/GAG p14; Table 2) ELISA had apparently a high reactivity but positivity
374 could not be confirmed in a high proportion of animals by PCR or standard ELISAs (or
375 peptide ELISAs presented here). Therefore, many positive reactions against peptide 77
376 may have been non-specific. Lack of reactivity to the remaining peptides tested was
377 unexpected, although the probability of any epitope prediction algorithm to identify a real
378 epitope is usually less than 60%. Several reports using WB based on both matrix and
379 capsid proteins have shown that these proteins are highly reactive with sera from naturally
380 and experimentally infected animals, suggesting that linear epitopes are located in these
381 antigenic regions (Houwers and Nauta, 1989; Rosati et al., 1994; Zanoni et al., 1989). This
382 could be due to the algorithm chosen for epitope prediction, length of the selected region
383 and efficiency of passive immobilization on solid phase which is strictly sequence
384 dependent. In line with this, the highest performance among the four highly reactive
385 peptide ELISAs studied in depth corresponded to the peptide ELISA 98M, but only when
386 applied to detect type-A specific antibodies (as expected since 98M was in a type-specific
387 amino acid region), elicited in experimental infections with Ev1 strain (Reina et al., 2008),
388 the Kv1772 clone (Andresson et al., 1993) or natural infections such as those of the
389 Castilla-León region (Group 4). This peptide corresponds to an immunodominant epitope
390 of a variable region (Fig 2S of Supplementary material) homologous to that of the SU5
391 peptide 1163 detecting antibodies with high avidity (Mordasini et al., 2006). In contrast,
392 peptide 91 ELISA had by far the lowest performance in the screening study (n = 128), as
393 well as in the second-phase study (n = 496). Likely, the immunodominance of this epitope

394 was hindered, since its sequence included a conserved cysteine among the available SRLV
395 sequences downstream a potential N-linked glycosylation site (Valas et al., 2000), but
396 lacked a precedent glycosylation site. In line with previous findings (Glaria et al., 2009), a
397 single inversion within the amino acid sequence (HQ in 126M1 and QH in 126M2; Table
398 1) biased the peptide reactivity towards a CAEV-like (B-type) and a VMV-like (A-type)
399 profile, respectively. Type-specific reactivity of the three ENV-TM peptides was less
400 marked than that of the ENV-SU 98M peptide corresponding to the hypervariable domain
401 SU5, in agreement with previous studies (Mordasini et al., 2006). Compared to the Elitest
402 ENV-TM peptide (Saman et al., 1999), 126M1 and 126M2 were shorter, lacking residues
403 GGQ at the amino-terminal and KS at the carboxy-terminal, which may affect the antigen
404 coating.

405 Amongst the three animal groups (1, 2 and 6) apparently uninfected (Elitest-
406 negative), Group 1 was found positive mainly according to peptide 98M ELISA, strongly
407 suggesting genotype A infections may trigger anti-ENV-SU (98M) antibodies undetectable
408 by the p25 or the ENV-TM peptide used in Elitest. In addition, Groups 2 and 6 were
409 similarly reactive in peptide 126M1 and 126M2 ELISAs, suggesting the presence of two
410 circulating genotypes (A and B) in these flocks. Thus, the selection of peptides for
411 complementary tools may differ between areas in this scenario of known virus genotype.
412 Particularly in Elitest-negative animals, genotype A infections may be detected by 98M
413 and 126M2 ELISAs (in Groups 3 and 4), whereas genotype B infections may be detected
414 by 126M1 ELISA (in Group 5). Therefore, peptide testing of animals assumed to be free of
415 infection may help to detect and classify the infection avoiding its persistence.

416 Sera from clinically affected animals tended to be more reactive with the peptide
417 139 ELISA, which appeared to detect high affinity antibodies produced along with viral
418 replication burst, disease onset and pathogenesis. CAEV-like TM peptides such as TM3,

419 analogous to 126M1, also detected preferentially clinical (type B) infections (Bertoni et al.,
420 1994). Thus, the frequency of non clinical infections as detected by ELISAs based on
421 peptides 139 and 126M1 may be underestimated. TM3, 126M1 and 126M2 peptides have
422 two cystein residues that may trigger the production of enhancing antibodies to favor viral
423 entry, as it occurs in HIV infections (Robinson et al., 1991), undermining the role of early
424 cellular responses (de Andres et al., 2009; Juganaru et al., 2011). However detection of
425 these antibodies is less informative from the point of view of early diagnosis.

426 Often the information on genetic types of circulating strains is not available and a
427 unique serological diagnostic technique is applied blindly. In this scenario of unknown
428 virus genotype, PCR-based techniques (Barquero et al., 2011; Brinkhof et al., 2010), WB
429 (Ramirez et al., 2009) or the heteroduplex mobility assay may be helpful for initial
430 classification of SRLV circulating genotypes (Germain et al., 2008; Germain and Valas,
431 2006) enabling proper antigen design for new type specific ELISA. These ELISAs may be
432 useful to diagnose infection in populations of limited size, animals of high value apparently
433 seronegative or flocks close to an eradication stage and to trace back infections of
434 epidemiological interest. The appearance of mixed infections, new variants by mutation,
435 recombination or introduction of new viral types into flocks and herds, point out the need
436 of constant surveillance on the technique implemented in order to improve diagnosis and
437 control the virus spread.

438

439 **5. Conclusion**

440 This study, showing the results obtained with different diagnostic strategies in
441 particular geographic areas involving heterogeneous SRLV infections, outlines the interest
442 of peptide assays to type the infections and control viral spread and encourages the
443 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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615

Table 1

Percentage of sera reactive to the 60 synthetic single peptide[a] 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%)	
15	Low	(9-20%)	
4	Medium	(23-31%)	75/GAGp14-PERKGRNQGMGQKCY 76/GAGp14-PEKGKRNGPQQRCY 91/ENVgp135-CSLPHKNESNKWTCA 157/ENVgp46-KNKKERVDCQDREQR
2	High	(46-47%)	48/GAGp25-KLNEEAERWVRQNPP 81/GAGp14-GNNRRGPRVVPSAPP
6	Very high	(57-62%)	77/GAGp14-MQKDCRQKKQQGNNR 98M/ENVgp135-VDMPQSYIEKQKRNK 126M1/ENVgp46-ELDCWHYHQYCVTST 126M2/ENVgp46-ELDCWHYQHYCVTST 139/ENVgp46-HIAQRDASRIPDVWT 140/ENVgp46-QLAQEQARRIPDVWE

616 [a] See Supplementary material for sequences of the 60 peptides and the protein from which they were designed.

617

618

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 [a]	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/ arthritic	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

619 [a] Inferred according to previous studies in the pertinent areas and flocks (Glaria et al., 2009, 2012; Ramírez et al., 2009).

620

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, 4 and 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
3 Elitest P (28): A,B	93	25	39.3	50	39.3	17.9
Elitest N (34)	53	8.8	8.8	17.6	20.6	0
4 Elitest P (96): A	84.4	9.3	91.6	21.8	53.1	63.5
Elitest N (61)	23	8.2	34.4	11.5	14.7	16.4
5 Elitest P (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)[a]: A,B						
Elitest P (213)	100	8.4	45.5	50.2	49.3	49.3
Elitest P (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

[a] 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 (98M, 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.

634 **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	(0.108 - 0.243)
	p98M	**0.857**	**(0.738 - 0.936)**	**0.985**	**(0.946 - 0.998)**
	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)
	Elitest	*0.911*	*(0.804 - 0.970)*	*0.898*	*(0.833 - 0.945)*
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)
	p126M1	**0.818**	**(0.597 - 0.948)**	**0.955**	**(0.904 - 0.983)**
	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)
	Elitest	*0.727*	*(0.498 - 0.893)*	*0.898*	*(0.833 - 0.945)*
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)
	p126M1	**0.750**	**(0.349 - 0.968)**	**0.667**	**(0.579 - 0.746)**
	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)
	Elitest	*0.875*	*(0.473 - 0.997)*	*0.805*	*(0.725 - 0.869)*

CI, confidence interval; p, peptide.

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636

637 **FIGURE CAPTIONS**

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

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

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

652

653 Fig. 1S. ANNEX 1: Oligonucleotides used in different PCRs to obtain the gag and env
654 nucleotide sequences of SRLV from natural infections in North Central Spain. ANNEX II.
655 Synthetic peptides used in this study for small ruminant lentivirus specific antibody
656 detection by indirect ELISA.

657 Fig. 2S. Alignment of partial SU sequences obtained in this work and GeneBank
658 sequences. The SU5 region corresponds to base pairs 7720 to 7907 and amino acids 597 to
659 632 of the CAEV-CO molecular clone (accession number **M33677**). Accession numbers

660 are indicated for each sequence. The first eight sequences were obtained in this work from
661 the Castilla-León neurological outbreak (accession numbers **JN184379** to **JN184394**). The
662 98M peptide sequence (consensus) underlined (top), starts at the end of the constant SU5
663 region of the ENV SU protein. The hydrophilic-antigenic region is outlined within a
664 square. The beginning of the SU5 domain of the ENV protein (Mordasini et al., 2006) is
665 indicated by an arrow. See also Glaria et al. (2012) on a section of this figure lacking
666 several of the Spanish sequences her illustrated and other known sequences.

667