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Diagnosing infection with small ruminant lentiviruses of genotypes A and B by combining synthetic peptides in ELISA

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Highlights

- A synthetic peptide ELISA was developed for detecting SRLV infection.
- Sheep were experimentally infected with genotype A and B SRLV.
- Serological detection was improved, compared with standard methods.
- Combining epitopes from different genotypes improve ELISA sensitivity.

Abstract

The major challenges in diagnosing small ruminant lentivirus (SRLV) infection include early detection and genotyping of strains of epidemiological interest. A longitudinal study was carried out in Rasa Aragonesa sheep experimentally infected with viral strains of genotypes A or B from Spanish neurological and arthritic SRLV outbreaks, respectively. Sera were tested with two commercial ELISAs, three based on specific peptides and a novel combined peptide ELISA. Three different PCR assays were used to further assess infection status.

The kinetics of anti-viral antibody responses were variable, with early diagnosis dependent on the type of ELISA used. Peptide epitopes of SRLV genotypes A and B combined in the same well enhanced the overall detection rate, whereas single peptides were useful for genotyping the infecting strain (A vs. B). The results of the study suggest that a combined peptide ELISA can be used for serological diagnosis of SRLV infection, with single peptide ELISAs useful for subsequent serotyping.

Keywords: Small ruminant lentivirus; Genotype; Serology; Peptide ELISA

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Introduction

Small ruminant lentiviruses (SRLV), including Visna/Maedi virus (VMV) and caprine arthritis-encephalitis virus (CAEV) are widely distributed in sheep and goats. Since no treatment or effective vaccines are available, the control of SRLV infection is based on early diagnosis and culling of infected animals and their progeny. Infected animals often show decreased milk production and quality due to increased somatic cell counts and shortened lactation periods (Turin et al., 2005; Martinez-Navalon et al., 2013).

Use of PCR allows early diagnosis, genotyping and quantification of viral DNA (Gonzalez et al., 2005; Herrmann-Hoesing et al., 2007; Brinkhof et al., 2008; Leginagoikoa et al., 2009), but is mainly employed as a confirmatory test or for research purposes as viral genetic heterogeneity and low viral load limit the use of PCR as a commercial diagnostic assay. Diagnosis of SRLV infection is currently undertaken by serological methods, with agar gel immunodiffusion (AGID) being increasingly replaced by ELISA as a result of its increased sensitivity, objectivity and automation. Indirect and competition ELISAs, based on a monovalent design, have been widely applied for diagnosis of SRLV infection (de Andres et al., 2005; Herrmann-Hoesing, 2010; Muz et al., 2013; Ramirez et al., 2013; Santry et al., 2013; Tolari et al., 2013), and in SRLV eradication programmes (Brinkhof et al., 2010; Herrmann-Hoesing, 2010; Perez et al., 2010; 2013). However, their use has been hindered by unexpected seroconversion (Cardinaux et al., 2013; Ritchie and Hosie, 2014), suggesting that ELISAs need to be designed to cover a greater antigenic range in order to counter SRLV genetic heterogeneity (Grego et al., 2002; de Andres et al., 2013).

Five SRLV genotypes (A–E) have been identified to date. Genotypes A and B include VMV-like and CAEV-like strains, respectively, which are common among SRLV infections across the world and

which have been identified in Spanish neurological and arthritic outbreaks (Glaria et al., 2009; 2012). Genotype C has been reported in sheep and goats from Norway (Gjerset et al., 2006) and genotype E has been found in goats from particular regions of Italy (Grego et al., 2007; Reina et al., 2009).

Serological testing is usually more sensitive and specific when using homologous rather than heterologous antigens (Lacerenza et al., 2006). However, genotype A-derived antigens are more likely to detect cross-reacting antibodies than genotype B- or E-derived antigens (Grego et al., 2005; Carrozza et al., 2009; Rachid et al., 2013; Tolari et al., 2013). The limitations of current monovalent serological tests can be explained by the antigenic variation and mutation rate of SRLV, linked to the appearance of different serotypes (Glaria et al., 2012; Cardinaux et al., 2013). The use of synthetic peptides might allow bespoke diagnostic tests to be developed that are matched to the specific epitopes of the viral serotype under investigation (Lacerenza et al., 2006; Mordasini et al., 2006; de Andres et al., 2013).

The aim of the present study was to assess a new combined-peptide ELISA (cPE) for serological diagnosis of infection with SRLV genotypes A and B and to compare results with ELISAs using individual and combined peptides and with commercially available immunoassays.

Materials and methods

Viral strains

Two SRLV strains were used, strain 697 (genotype A) from a neurological outbreak (Glaria et al., 2012) and strain 496 (genotype B) from an arthritic outbreak (Glaria et al., 2009) of SRLV. Strains 697 and 496 were sub-cultured once in blood derived macrophages (BDM) and ovine skin fibroblasts

(SF), respectively. Strain 496 culture supernatants were titrated by limiting dilution (Reed, 1938), and those from strain 697 by measuring reverse transcriptase (RT) activity produced in the supernatant using a commercial kit (HS-Lenti RT activity, Cavidi). Culture supernatants were filtered through a 0.44 µm filter and stored in aliquots containing 10^6 $TCID_{50}$/mL at -80 °C until used in experimental infection.

Experimental infection and longitudinal sampling

Two-month old castrated male sheep of the Rasa Aragonesa breed (*n* = 16) were obtained from accredited SRLV-free flocks (Perez et al., 2010) and housed at the Animal Facilities of the Veterinary Faculty, University of Zaragoza. All animals were tested periodically by ELISA (Elitest; Saman et al., 1999) and PCR for 2 months prior to experimental infection.

Experimental infection was performed by intra-medullary inoculation into the trochanteric fossa as described previously (Reina et al., 2011). On the day of challenge, sheep were allocated to one of three groups, each housed separately. Sheep from groups A and B (*n* = 6 each) were injected with 1 mL (10^6 $TCID_{50}$) of virus containing cell culture supernatant, for strains 697 and 496, respectively. The control group (group C; *n* = 4) received 1 mL of sterile filtered cell culture medium. A second injection was undertaken in the same animals 60 days later, following the same procedure.

Blood samples in EDTA anticoagulant were collected before inoculation and at weekly intervals until day 308 (week 44 from the first experimental infection) and separated plasma stored at -20 °C prior to testing. Peripheral blood leukocytes (PBL), obtained following red blood cell lysis, were resuspended in PBS and stored at -80 °C until DNA extraction for PCR studies. Sequential

culling of selected animals (one control and two from each of the infected groups) was carried out at weeks 21, 35 and 46 post-infection.

All procedures were carried out under Project License PI09/10 approved by the Ethics Committee for Animal Experiments (University of Zaragoza). The care and use of animals were performed accordingly with the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

Commercial ELISAs

Two commercially available ELISAs were used, namely the Elitest-MVV (Hyphen-Biomed), which is based on p25 recombinant protein and a transmembrane synthetic peptide, both derived from genotype A (Saman et al., 1999) and AG-Chekit CAEV/MVV (IDEXX Laboratories), based on the Swiss MVV-OLV whole virus antigen (Zanoni et al., 1994). Both assays were performed according to the manufacturers' recommendations.

Peptide ELISAs

Peptide ELISAs were designed using the synthetic peptides 98M (VDMPQSYIEKQKRNK), 126M1 (ELDCWHYHQYCVTST) and 126M2 (ELDCWHYQHYCVTST), either individually or in combination (cPE) (de Andres et al., 2013). Peptides were obtained from Thermo Scientific, diluted in carbonate buffer (pH 9.6) at 1 mg/mL and stored at -20 ºC until use. Peptide 98M is based on a SU5 consensus sequence among strains identified from the Spanish neurological outbreak. Peptides 126M1 and 126M2, based on the transmembrane region, were identical except for inversion of

amino acid residues HQ (126M1) or QH (126M2) present in CAEV-like and VMV-like sequences, respectively.

Initially, 96-well microplates (Maxisorp, Nunc) were coated with 100 ng of synthetic peptide, either individually or in equimolar amounts for the cPE and allowed to dry overnight at 37 °C. Plates were washed with phosphate buffered saline (PBS) supplemented with 0.1% Tween 20 (PBS-T) and blocked with 2.5% bovine casein (Sigma–Aldrich) for 1 h at 37 °C. Serum samples were diluted 1 in 20 in dilution buffer (PBS containing 1.25% bovine casein) and plates incubated for 1 h at 37 °C. Peroxidase-conjugated anti-ruminant IgG (EG5; Ingenasa) was added (1/100 in dilution buffer) and plates incubated for 1 h at 37 °C. One hundred microlitres per well of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, Millipore) were added as the substrate and the optical density measured at 405 nm (OD_{405}) after 15–40 min.

Serum samples were tested alongside positive and negative control sera, included on each plate. Antibody positive serum was obtained from a naturally infected sheep, whereas negative control sera came from animals belonging to SRLV-free certified flocks (Pérez et al., 2010). Absorbance values were calculated by subtracting the negative control OD_{405} (diluent only, no peptide) from each sample OD_{405}. Samples with absorbance values >0.3 were considered positive, based on a threshold value determined previously for sera from uninfected sheep (de Andres et al., 2013).

Polymerase chain reaction

159 Genomic DNA was extracted from PBL using the DNA Blood Mini Kit (Qiagen). PCR was used
160 to amplify selected regions of the *gag* gene, yielding amplicons of 510 base pairs (bp) (PCR named
161 C/O) (Glaria et al., 2009), or 800 bp (PCR named GAG) (Grego et al., 2007) and long terminal repeats
162 (LTR) of approximately 300 bp (depending on the strain) (Glaria et al., 2009). Each reaction mix
163 consisted of Reaction Buffer 1× (Biotools), 2 mM $MgCl_2$ (Biotools), 240 µM of each dNTP (Applied
164 Biosystems), 20 pmol of each primer, 0.02 U/µL of Taq DNA polymerase (Biotools) and 1 µg of
165 sample DNA to a final volume of 25–50 µL. PCR consisted of 45 cycles of denaturation at 95 ºC for 1
166 min, annealing (55–60 ºC) for 1 min and elongation at 72 ºC for 1 min/Kb of amplified DNA, followed
167 by a final elongation step at 72 ºC for 10 min.

168

169 Since SRLV strains 697 and 496 differed by 21 and 23 bp in C/O and LTR amplicons,
170 respectively, PCR products from test samples were subjected to agarose gel electrophoresis
171 alongside DNA amplified from cells infected in vitro with strains 697 or 496. Using this internal
172 control, the viral strain, infecting each animal, could be identified. To identify viral mutations, four
173 GAG amplicons of each strain, obtained at the end of the study, were purified with a PCR extraction
174 kit (ATP Biotech), cloned into the pGEM-T easy vector (Promega) and submitted for sequencing
175 (StabVida).

176

177 **Results**

178 *PCR analysis*

179 PCR results confirmed that samples from group C, the mock-inoculated sheep, were negative
180 for virus (data not shown) and that the experimental procedure was successful, in terms of the
181 infection status of animals in groups A and B. The identity of the viral strain present in infected
182 animals was verified by analysing the PCR amplicon length, that differed between strains 496 and

697 by 21 and 23 bp in C/O and LTR PCRs, respectively, and by direct sequencing of GAG amplicons, performed with samples from each infected group, taken 44 weeks post infection (see Appendix: Supplementary material).

Gag sequences from strain 496-infected animals, obtained at the end of the study period, showed nucleotide differences of 1.27% (i.e. 10 base changes in 787 nucleotides). These mutations were all synonymous changes except one, G381E in the *gag* p55 polyprotein. Sequences from strain 697-infected animals showed nucleotide differences in *gag* of 2.85% (22 base changes in 772 nucleotides) with non-synonymous mutations located at A310T, _421InsK, T428N and K429G, all of which were downstream of the reported p25 immunogenic regions and upstream immunogenic epitopes described in p14 (Lacerenza et al., 2008).

Evidence of infection was seen within the first month of exposure, although PCR reactivity subsequently fluctuated during the study period (Table 1). Use of GAG primers in PCR showed the greatest number of positives, with all of the 496-infected animals and all but one of the 697-infected animals PCR positive by week 28. Nine weeks after the first experimental infection, PCR was most likely to detect infection by strain 496 (Table 1). In contrast, the LTR PCR detected the least number of positives, particularly in animals exposed to the 697 strain. After the second challenge, these differences in performance of the PCRs were maintained, with the exception of results in weeks 9 and 16 (Table 1).

Serological analysis

None of the samples from the control group (n = 4) were found to be positive by ELISA during the study period (Table 2). In contrast, all the SRLV-inoculated animals (n = 12) showed positive results in at least one of the ELISAs and/or PCRs. As expected from the peptide sequence (based on genotype A), the monovalent 98M peptide ELISA only detected those animals exposed to the 697 strain (Table 2). The ELISA using peptide 126M1 (based on genotype B) identified those animals exposed to the 496 strain, although not exclusively, as one of the animals exposed to the 697 strain also tested positive on two occasions (Table 2). Peptide 126M2 was designed on the SRLV genotype A sequence, but the ELISA detected exposure to genotype B (strain 496) in more animals than those exposed to genotype A (strain 697), although in decreased proportions compared to using the 98M or 126M1 ELISAs for detecting exposure to genotype A and B, respectively. Thus, in monovalent ELISAs, peptides 98M and 126M1 could detect exposure to a particular genotype (A vs. B, respectively), but were unable (98M) or inefficient (126M1) for detecting both types of infection.

A relatively low proportion of animals showed evidence of seroconversion by cPE, Elitest or Chekit up to 7 weeks post-infection with the two SRLV strains (Fig. 1). Genotype-A specific antibodies were detected by cPE after this period in more animals and at more time points compared to the commercial ELISAs. Chekit, Elitest and cPE preferentially detected infection with strain 496 (genotype B) compared with strain 697 (genotype A). In most cases, cPE results were the same as the combined results of the monovalent peptide ELISAs, with one exception at week 11, where one animal exposed to the 697 strain was positive in the 98M peptide ELISA but negative in the cPE (Table 2). The study results indicate that the cPE was able to detect exposure to genotype A and B of SRLV early after challenge and efficiently throughout the study period.

228 The kinetics of the antibody response, based on use of cPE, Elitest or Chekit are summarized
229 in Fig. 1. ELISA absorbance values of uninfected animals remained below the threshold in the three
230 ELISA tested. Seroconversion in animals exposed to the 496 strain was detected early after
231 inoculation and at sampling times thereafter in cPE and Chekit (Fig. 1D, F). In contrast, exposure to
232 the 697 strain was only successfully detected serologically using the cPE, likely due to incorporation
233 of peptide 98M (Fig. 1A). Elitest detected relatively few animals exposed to either SRLV genotype
234 (Fig. 1B, E).

235

236 **Discussion**

237 Decreased prevalence and improved control of SRLV infection has been achieved using
238 serological testing based on the use of ELISAs (Scheer-Czechowski et al., 2000; Christodoulopoulos,
239 2006; Brinkhof et al., 2010; Perez et al., 2013). In addition, conventional and real time PCRs have
240 been designed for broad and specific detection of SRLV, even in seronegative animals (Brajon et al.,
241 2012; Kuhar et al., 2013). Recently, alternative methods, based on identifying the TMEM154 gene,
242 have been proposed to classify susceptible/resistant SRLV sheep populations (Leymaster et al., 2013;
243 Sider et al., 2013).

244

245 Despite advances in diagnostic techniques, genetic variability and antigenic variation of SRLV
246 can lead to failure to detect all infections (Cardinaux et al., 2013; de Andres et al., 2013). In the
247 present study, we performed a serological survey of animals experimentally infected with Spanish
248 SRLV strains 697 and 496, assessing the performance of a new multivalent ELISA (cPE), based on the
249 use of a combination of synthetic peptides, comparing its performance alongside monovalent
250 peptide ELISAs and two commercially available ELISAs.

251

All but one of the animals that were experimentally challenged with SRLV strains 496 and 697 became PCR positive on at least one occasion during the study period. PCR identified infected animals, albeit in small proportions within the first month post-challenge in the strain 697-infected group, but in increased proportions during the experimental period, confirming the utility of PCR as a diagnostic tool. However, PCR results fluctuated over time, representing a drawback for field diagnosis of SRLV infections where often a single sample is assessed. Variability in the PCR assay to detect virus in infected animals might have been related to mutations affecting the primer binding sites. Although, *gag* sequencing at the end of the study revealed some mutations, particularly in the animals exposed to the 697 strain, none of these were located at primer binding sites.

Our study shows the benefits of the cPE for detecting SRLV infections and the use of individual peptides to genotype the infecting virus. These observations are consistent with our previous field studies on the diagnosis of subclinical and clinical infections using a combination of single peptide ELISAs (Glaria et al., 2012; de Andres et al., 2013), where use of the 98M peptide was genotype A specific, whereas the capacity of peptides 126M1 and 126M2 differed in their specificity (de Andres et al., 2013). As expected, strain 496 (genotype B) infections were preferably detected by peptide 126M1, cPE and Chekit ELISAs. The relatively low detection rate by Chekit in genotype A-infected animals was unexpected, as this assay is based on use of whole virus antigen that would be expected to recognise cross-reacting antibodies. The single amino acid inversion in the TM peptides employed led to a change in antibody profile, confirming previous observations (de Andres et al., 2013). This difference became most evident by week 20 and indicates the diagnostic value of these peptides in detecting VMV-like or CAEV-like infections, as reported in clinical outbreaks (Glaria et al., 2009).

Elitest, based on genotype-A derived antigens, detected seropositive animals infected with the 496 strain by week 28, most likely because some of the epitopes were binding cross-reactive antibodies. This is in agreement with the general trend of genotype A-derived antigens to better detect cross-reacting antibodies compared with genotype B-derived antigens (Gogolewski et al., 1985; Lacerenza et al., 2006; Carrozza et al., 2009). However, the low proportion of positive animals detected by Elitest was unexpected (only 1/6 animals at week 20 in the experimental group infected with genotype A) particularly in the light of the greater number in this group with positive test results using the other ELISAs containing type A antigens (cPE and 98M). This low Elitest detection rate in the animals infected with strain 697 was not due to failures in experimental procedure, since PCR confirmed infection with each genotype in both groups.

The lower proportion of seropositive animals in the 697 strain-infected group, as compared with those infected with the 496 strain, might be related to differences in viral loads. Quantitative PCR, performed in target tissues has revealed increased proviral loads in animals infected with the 496 strain (unpublished data). Consistent with this observation, replication dynamics, according to the proportion of PCR-positive results, indicated an advantage for strain 496 relative to strain 697 on the different test dates (except at the beginning of the study). Viral load differences may have been caused by strain differences in replication dynamics in vivo, in agreement with strain differences previously found in vitro, with phenotypes rapid/high vs. slow/low for strains 496 and 697, respectively (Glaria et al., 2009; 2012). Alternatively, a breed effect may be involved, such that strain 697 infection might have increased replication and pathogenicity in the Assaf breed (Benavides et al., 2006) but not in the Rasa Aragonesa breed used in the present study.

The introduced mutations found in both SRLV genomes during the course of the infection period, although mainly synonymous, may have affected the host-pathogen interaction or infection dynamics and might have been responsible for an attenuated phenotype and decreased antibody response in 697 strain-infected sheep. Alternatively, the differences in viral titration methods used for both strains may have led to an overestimation of the 697 virus titre and thus injection of relatively less virus for experimental infection, explaining the decreased serological reactivity found in group A sheep.

Use of synthetic peptides from different SRLV genotypes, combined in the same ELISA well, improved detection of early and late stages of infection, compared with the use of commercially available ELISAs. Those same peptides used individually, were able to identify the specific SRLV genotype involved in the infection. It is important that the antigens used in serological testing are reviewed periodically and revised to ensure that they match the antigenic profile of the target virus. This is particularly important for those viruses that undergo antigenic shift and drift. Updated ELISAs may be required to diagnose infections by emerging SRLV strains in order to achieve early diagnosis and improved sensitivity for SRLV control programs. Use of synthetic peptides is particularly suited to this strategy as additional antigenic epitopes can be added to the ELISA relatively quickly and cost-effectively.

Conclusions

ELISAs can be used for serological testing of animals exposed to different SRLV genotypes. Phylogenetic and epidemiological data should be employed to select peptide epitopes for use in ELISAs that are capable of detecting antibodies generated following exposure to SRLV. Genotype-specific diagnosis of SRLV infection is possible with specific synthetic peptides in ELISA testing. In

addition, a combination of peptides in the same ELISA well broadens the specificity and facilitates detection of divergent SRLV strains.

Conflict of interest statement

Individual and combined peptide ELISAs (98M, 126M1, 126M2 and cPE) are patented (Spanish patent ES 2387243) and are being commercialised by Ingenasa. None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Figure Legend

Fig. 1. Kinetics of antibody responses in sheep after experimental infection, based on ELISA testing. Top panels (A, B and C): ELISA absorbance values (minus background) obtained with the combined peptide ELISA (cPE), Elitest or Chekit, respectively, in animals infected with strain 697 (genotype A) (continuous lines). Bottom panels (D, E and F): ELISA absorbance values (minus background) obtained with cPE, Elitest or Chekit ELISAs in animals infected with strain 496 (genotype B) (continuous lines). Animals were injected with virus on weeks 0 and 8. Results from the mock inoculated control animals (C1-C4) are also included in the graphs (dashed lines). Date of sequential culling is indicated by interruption of the line.

Table 1
Proportion of positive sheep in LTR, C/O and GAG PCR assays, considering results individually and in combination, at different time points, following challenge with small ruminant lentivirus (SRLV) strains 697 or 496.

Week post challenge	SRLV strain	Individual PCRs			
		LTR	C/O	GAG	Combined PCR result
4	697	0/6	1/6	0/6	1/6
	496	0/6	0/6	0/6	0/6
9	697	1/6	1/6	1/6	2/6
	496	4/6	4/6	2/6	4/6

16	697	0/6	2/6	3/6	4/6
	496	3/6	0/6	5/6	5/6
28	697	0/4	1/4	2/4	2/4
	496	2/4	3/4	4/4	4/4

Table 2

Serological response of control (*n = 4*) and infected (*n* = 6 per group) sheep at different time points following challenge with SRLV strains 697 or 496, assessed by the monovalent (98M, 126M1 or 126M2) and the combined peptide ELISAs (cPE). Symbols correspond to Fig. 1. Blank spaces indicate culled animals.

Comment [A1]: PRE-EDITOR: Table 2 for rekey.

Appendix

Supplementary material

Alignment of deduced protein sequences obtained at 44 weeks post-infection with SRLV strain 496 (A) or 697 (B). Reference sequence is Ov496 (GenBank: FJ195346.1). An example of three independent clones from each experimental infection is shown.