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

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20

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.

43

44 **Introduction**

45   Small ruminant lentiviruses (SRLV), including Visna/Maedi virus (VMV) and caprine arthritis-

46 encephalitis virus (CAEV) are widely distributed in sheep and goats. Since no treatment or effective

47 vaccines are available, the control of SRLV infection is based on early diagnosis and culling of

48 infected animals and their progeny. Infected animals often show decreased milk production and

49 quality due to increased somatic cell counts and shortened lactation periods (Turin et al., 2005;

50 Martinez-Navalon et al., 2013).

51

52   Use of PCR allows early diagnosis, genotyping and quantification of viral DNA (Gonzalez et

53 al., 2005; Herrmann-Hoesing et al., 2007; Brinkhof et al., 2008; Leginagoikoa et al., 2009), but is

54 mainly employed as a confirmatory test or for research purposes as viral genetic heterogeneity and

55 low viral load limit the use of PCR as a commercial diagnostic assay. Diagnosis of SRLV infection is

56 currently undertaken by serological methods, with agar gel immunodiffusion (AGID) being

57 increasingly replaced by ELISA as a result of its increased sensitivity, objectivity and automation.

58 Indirect and competition ELISAs, based on a monovalent design, have been widely applied for

59 diagnosis of SRLV infection (de Andres et al., 2005; Herrmann-Hoesing, 2010; Muz et al., 2013;

60 Ramirez et al., 2013; Santry et al., 2013; Tolari et al., 2013), and in SRLV eradication programmes

61 (Brinkhof et al., 2010; Herrmann-Hoesing, 2010; Perez et al., 2010; 2013). However, their use has

62 been hindered by unexpected seroconversion (Cardinaux et al., 2013; Ritchie and Hosie, 2014),

63 suggesting that ELISAs need to be designed to cover a greater antigenic range in order to counter

64 SRLV genetic heterogeneity (Grego et al., 2002; de Andres et al., 2013).

65

66   Five SRLV genotypes (A–E) have been identified to date. Genotypes A and B include VMV-like

67 and CAEV-like strains, respectively, which are common among SRLV infections across the world and

68   which have been identified in Spanish neurological and arthritic outbreaks (Glaria et al., 2009; 2012).

69   Genotype C has been reported in sheep and goats from Norway (Gjerset et al., 2006) and genotype E

70   has been found in goats from particular regions of Italy (Grego et al., 2007; Reina et al., 2009).

71

72   Serological testing is usually more sensitive and specific when using homologous rather than

73   heterologous antigens (Lacerenza et al., 2006). However, genotype A-derived antigens are more

74   likely to detect cross-reacting antibodies than genotype B- or E-derived antigens (Grego et al., 2005;

75   Carrozza et al., 2009; Rachid et al., 2013; Tolari et al., 2013). The limitations of current monovalent

76   serological tests can be explained by the antigenic variation and mutation rate of SRLV, linked to the

77   appearance of different serotypes (Glaria et al., 2012; Cardinaux et al., 2013). The use of synthetic

78   peptides might allow bespoke diagnostic tests to be developed that are matched to the specific

79   epitopes of the viral serotype under investigation (Lacerenza et al., 2006; Mordasini et al., 2006; de

80   Andres et al., 2013).

81

82   The aim of the present study was to assess a new combined-peptide ELISA (cPE) for

83   serological diagnosis of infection with SRLV genotypes A and B and to compare results with ELISAs

84   using individual and combined peptides and with commercially available immunoassays.

85

86   **Materials and methods**

87   *Viral strains*

88   Two SRLV strains were used, strain 697 (genotype A) from a neurological outbreak (Glaria et

89   al., 2012) and strain 496 (genotype B) from an arthritic outbreak (Glaria et al., 2009) of SRLV. Strains

90   697 and 496 were sub-cultured once in blood derived macrophages (BDM) and ovine skin fibroblasts

91 (SF), respectively. Strain 496 culture supernatants were titrated by limiting dilution (Reed, 1938), and

92 those from strain 697 by measuring reverse transcriptase (RT) activity produced in the supernatant

93 using a commercial kit (HS-Lenti RT activity, Cavidi). Culture supernatants were filtered through a

94 0.44 μm filter and stored in aliquots containing 10^6 $TCID_{50}$/mL at -80 °C until used in experimental

95 infection.

96

97 *Experimental infection and longitudinal sampling*

98   Two-month old castrated male sheep of the Rasa Aragonesa breed (n = 16) were obtained

99 from accredited SRLV-free flocks (Perez et al., 2010) and housed at the Animal Facilities of the

100 Veterinary Faculty, University of Zaragoza. All animals were tested periodically by ELISA (Elitest;

101 Saman et al., 1999) and PCR for 2 months prior to experimental infection.

102

103   Experimental infection was performed by intra-medullary inoculation into the trochanteric

104 fossa as described previously (Reina et al., 2011). On the day of challenge, sheep were allocated to

105 one of three groups, each housed separately. Sheep from groups A and B (n = 6 each) were injected

106 with 1 mL (10^6 $TCID_{50}$) of virus containing cell culture supernatant, for strains 697 and 496,

107 respectively. The control group (group C; n = 4) received 1 mL of sterile filtered cell culture medium.

108 A second injection was undertaken in the same animals 60 days later, following the same procedure.

109

110   Blood samples in EDTA anticoagulant were collected before inoculation and at weekly

111 intervals until day 308 (week 44 from the first experimental infection) and separated plasma stored

112 at -20 °C prior to testing. Peripheral blood leukocytes (PBL), obtained following red blood cell lysis,

113 were resuspended in PBS and stored at -80 °C until DNA extraction for PCR studies. Sequential

114 culling of selected animals (one control and two from each of the infected groups) was carried out at

115 weeks 21, 35 and 46 post-infection.

116

117 All procedures were carried out under Project License PI09/10 approved by the Ethics

118 Committee for Animal Experiments (University of Zaragoza). The care and use of animals were

119 performed accordingly with the Spanish Policy for Animal Protection RD53/2013, which meets the

120 European Union Directive 2010/63 on the protection of animals used for experimental and other

121 scientific purposes.

122

123 *Commercial ELISAs*

124 Two commercially available ELISAs were used, namely the Elitest-MVV (Hyphen-Biomed),

125 which is based on p25 recombinant protein and a transmembrane synthetic peptide, both derived

126 from genotype A (Saman et al., 1999) and AG-Chekit CAEV/MVV (IDEXX Laboratories), based on the

127 Swiss MVV-OLV whole virus antigen (Zanoni et al., 1994). Both assays were performed according to

128 the manufacturers' recommendations.

129

130 *Peptide ELISAs*

131 Peptide ELISAs were designed using the synthetic peptides 98M (VDMPQSYIEKQKRNK),

132 126M1 (ELDCWHYHQYCVTST) and 126M2 (ELDCWHYQHYCVTST), either individually or in

133 combination (cPE) (de Andres et al., 2013). Peptides were obtained from Thermo Scientific, diluted

134 in carbonate buffer (pH 9.6) at 1 mg/mL and stored at -20 ºC until use. Peptide 98M is based on a

135 SU5 consensus sequence among strains identified from the Spanish neurological outbreak. Peptides

136 126M1 and 126M2, based on the transmembrane region, were identical except for inversion of

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

138 respectively.

139

140   Initially, 96-well microplates (Maxisorp, Nunc) were coated with 100 ng of synthetic peptide,

141 either individually or in equimolar amounts for the cPE and allowed to dry overnight at 37 °C. Plates

142 were washed with phosphate buffered saline (PBS) supplemented with 0.1% Tween 20 (PBS-T) and

143 blocked with 2.5% bovine casein (Sigma–Aldrich) for 1 h at 37 °C. Serum samples were diluted 1 in

144 20 in dilution buffer (PBS containing 1.25% bovine casein) and plates incubated for 1 h at 37 °C.

145 Peroxidase-conjugated anti-ruminant IgG (EG5; Ingenasa) was added (1/100 in dilution buffer) and

146 plates incubated for 1 h at 37 °C. One hundred microlitres per well of 2,2'-azino-bis(3-

147 ethylbenzothiazoline-6-sulphonic acid) (ABTS, Millipore) were added as the substrate and the optical

148 density measured at 405 nm (OD_{405}) after 15–40 min.

149

150   Serum samples were tested alongside positive and negative control sera, included on each

151 plate. Antibody positive serum was obtained from a naturally infected sheep, whereas negative

152 control sera came from animals belonging to SRLV-free certified flocks (Pérez et al., 2010).

153 Absorbance values were calculated by subtracting the negative control OD_{405} (diluent only, no

154 peptide) from each sample OD_{405}. Samples with absorbance values >0.3 were considered positive,

155 based on a threshold value determined previously for sera from uninfected sheep (de Andres et al.,

156 2013).

157

158 *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

183   697 by 21 and 23 bp in C/O and LTR PCRs, respectively, and by direct sequencing of GAG amplicons,

184   performed with samples from each infected group, taken 44 weeks post infection (see Appendix:

185   Supplementary material).

186

187   *Gag* sequences from strain 496-infected animals, obtained at the end of the study period,

188   showed nucleotide differences of 1.27% (i.e. 10 base changes in 787 nucleotides). These mutations

189   were all synonymous changes except one, G381E in the *gag* p55 polyprotein. Sequences from strain

190   697-infected animals showed nucleotide differences in *gag* of 2.85% (22 base changes in 772

191   nucleotides) with non-synonymous mutations located at A310T, _421InsK, T428N and K429G, all of

192   which were downstream of the reported p25 immunogenic regions and upstream immunogenic

193   epitopes described in p14 (Lacerenza et al., 2008).

194

195   Evidence of infection was seen within the first month of exposure, although PCR reactivity

196   subsequently fluctuated during the study period (Table 1). Use of GAG primers in PCR showed the

197   greatest number of positives, with all of the 496-infected animals and all but one of the 697-infected

198   animals PCR positive by week 28. Nine weeks after the first experimental infection, PCR was most

199   likely to detect infection by strain 496 (Table 1). In contrast, the LTR PCR detected the least number

200   of positives, particularly in animals exposed to the 697 strain. After the second challenge, these

201   differences in performance of the PCRs were maintained, with the exception of results in weeks 9

202   and 16 (Table 1).

203

204   *Serological analysis*

205   None of the samples from the control group (*n* = 4) were found to be positive by ELISA

206   during the study period (Table 2). In contrast, all the SRLV-inoculated animals (*n* = 12) showed

207   positive results in at least one of the ELISAs and/or PCRs. As expected from the peptide sequence

208   (based on genotype A), the monovalent 98M peptide ELISA only detected those animals exposed to

209   the 697 strain (Table 2). The ELISA using peptide 126M1 (based on genotype B) identified those

210   animals exposed to the 496 strain, although not exclusively, as one of the animals exposed to the

211   697 strain also tested positive on two occasions (Table 2). Peptide 126M2 was designed on the SRLV

212   genotype A sequence, but the ELISA detected exposure to genotype B (strain 496) in more animals

213   than those exposed to genotype A (strain 697), although in decreased proportions compared to

214   using the 98M or 126M1 ELISAs for detecting exposure to genotype A and B, respectively. Thus, in

215   monovalent ELISAs, peptides 98M and 126M1 could detect exposure to a particular genotype (A vs.

216   B, respectively), but were unable (98M) or inefficient (126M1) for detecting both types of infection.

217

218   A relatively low proportion of animals showed evidence of seroconversion by cPE, Elitest or

219   Chekit up to 7 weeks post-infection with the two SRLV strains (Fig. 1). Genotype-A specific antibodies

220   were detected by cPE after this period in more animals and at more time points compared to the

221   commercial ELISAs. Chekit, Elitest and cPE preferentially detected infection with strain 496

222   (genotype B) compared with strain 697 (genotype A). In most cases, cPE results were the same as

223   the combined results of the monovalent peptide ELISAs, with one exception at week 11, where one

224   animal exposed to the 697 strain was positive in the 98M peptide ELISA but negative in the cPE

225   (Table 2). The study results indicate that the cPE was able to detect exposure to genotype A and B of

226   SRLV early after challenge and efficiently throughout the study period.

227

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

252   All but one of the animals that were experimentally challenged with SRLV strains 496 and

253   697 became PCR positive on at least one occasion during the study period. PCR identified infected

254   animals, albeit in small proportions within the first month post-challenge in the strain 697-infected

255   group, but in increased proportions during the experimental period, confirming the utility of PCR as

256   a diagnostic tool. However, PCR results fluctuated over time, representing a drawback for field

257   diagnosis of SRLV infections where often a single sample is assessed. Variability in the PCR assay to

258   detect virus in infected animals might have been related to mutations affecting the primer binding

259   sites. Although, *gag* sequencing at the end of the study revealed some mutations, particularly in the

260   animals exposed to the 697 strain, none of these were located at primer binding sites.

261

262   Our study shows the benefits of the cPE for detecting SRLV infections and the use of

263   individual peptides to genotype the infecting virus. These observations are consistent with our

264   previous field studies on the diagnosis of subclinical and clinical infections using a combination of

265   single peptide ELISAs (Glaria et al., 2012; de Andres et al., 2013), where use of the 98M peptide was

266   genotype A specific, whereas the capacity of peptides 126M1 and 126M2 differed in their specificity

267   (de Andres et al., 2013). As expected, strain 496 (genotype B) infections were preferably detected by

268   peptide 126M1, cPE and Chekit ELISAs. The relatively low detection rate by Chekit in genotype A-

269   infected animals was unexpected, as this assay is based on use of whole virus antigen that would be

270   expected to recognise cross-reacting antibodies. The single amino acid inversion in the TM peptides

271   employed led to a change in antibody profile, confirming previous observations (de Andres et al.,

272   2013). This difference became most evident by week 20 and indicates the diagnostic value of these

273   peptides in detecting VMV-like or CAEV-like infections, as reported in clinical outbreaks (Glaria et al.,

274   2009).

275

276   Elitest, based on genotype-A derived antigens, detected seropositive animals infected with

277   the 496 strain by week 28, most likely because some of the epitopes were binding cross-reactive

278   antibodies. This is in agreement with the general trend of genotype A-derived antigens to better

279   detect cross-reacting antibodies compared with genotype B-derived antigens (Gogolewski et al.,

280   1985; Lacerenza et al., 2006; Carrozza et al., 2009). However, the low proportion of positive animals

281   detected by Elitest was unexpected (only 1/6 animals at week 20 in the experimental group infected

282   with genotype A) particularly in the light of the greater number in this group with positive test

283   results using the other ELISAs containing type A antigens (cPE and 98M). This low Elitest detection

284   rate in the animals infected with strain 697 was not due to failures in experimental procedure, since

285   PCR confirmed infection with each genotype in both groups.

286

287   The lower proportion of seropositive animals in the 697 strain-infected group, as compared

288   with those infected with the 496 strain, might be related to differences in viral loads. Quantitative

289   PCR, performed in target tissues has revealed increased proviral loads in animals infected with the

290   496 strain (unpublished data). Consistent with this observation, replication dynamics, according to

291   the proportion of PCR-positive results, indicated an advantage for strain 496 relative to strain 697 on

292   the different test dates (except at the beginning of the study). Viral load differences may have been

293   caused by strain differences in replication dynamics in vivo, in agreement with strain differences

294   previously found in vitro, with phenotypes rapid/high vs. slow/low for strains 496 and 697,

295   respectively (Glaria et al., 2009; 2012). Alternatively, a breed effect may be involved, such that strain

296   697 infection might have increased replication and pathogenicity in the Assaf breed (Benavides et

297   al., 2006) but not in the Rasa Aragonesa breed used in the present study.

298

299   The introduced mutations found in both SRLV genomes during the course of the infection

300   period, although mainly synonymous, may have affected the host-pathogen interaction or infection

301   dynamics and might have been responsible for an attenuated phenotype and decreased antibody

302   response in 697 strain-infected sheep. Alternatively, the differences in viral titration methods used

303   for both strains may have led to an overestimation of the 697 virus titre and thus injection of

304   relatively less virus for experimental infection, explaining the decreased serological reactivity found

305   in group A sheep.

306

307   Use of synthetic peptides from different SRLV genotypes, combined in the same ELISA well,

308   improved detection of early and late stages of infection, compared with the use of commercially

309   available ELISAs. Those same peptides used individually, were able to identify the specific SRLV

310   genotype involved in the infection. It is important that the antigens used in serological testing are

311   reviewed periodically and revised to ensure that they match the antigenic profile of the target virus.

312   This is particularly important for those viruses that undergo antigenic shift and drift. Updated ELISAs

313   may be required to diagnose infections by emerging SRLV strains in order to achieve early diagnosis

314   and improved sensitivity for SRLV control programs. Use of synthetic peptides is particularly suited

315   to this strategy as additional antigenic epitopes can be added to the ELISA relatively quickly and cost-

316   effectively.

317

318   **Conclusions**

319   ELISAs can be used for serological testing of animals exposed to different SRLV genotypes.

320   Phylogenetic and epidemiological data should be employed to select peptide epitopes for use in

321   ELISAs that are capable of detecting antibodies generated following exposure to SRLV. Genotype-

322   specific diagnosis of SRLV infection is possible with specific synthetic peptides in ELISA testing. In

323   addition, a combination of peptides in the same ELISA well broadens the specificity and facilitates

324   detection of divergent SRLV strains.

325

326   **Conflict of interest statement**

327   Individual and combined peptide ELISAs (98M, 126M1, 126M2 and cPE) are patented

328   (Spanish patent ES 2387243) and are being commercialised by Ingenasa. None of the authors has

329   any financial or personal relationships that could inappropriately influence or bias the content of the

330   paper.

331

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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			Combined PCR result
		LTR	C/O	GAG	
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

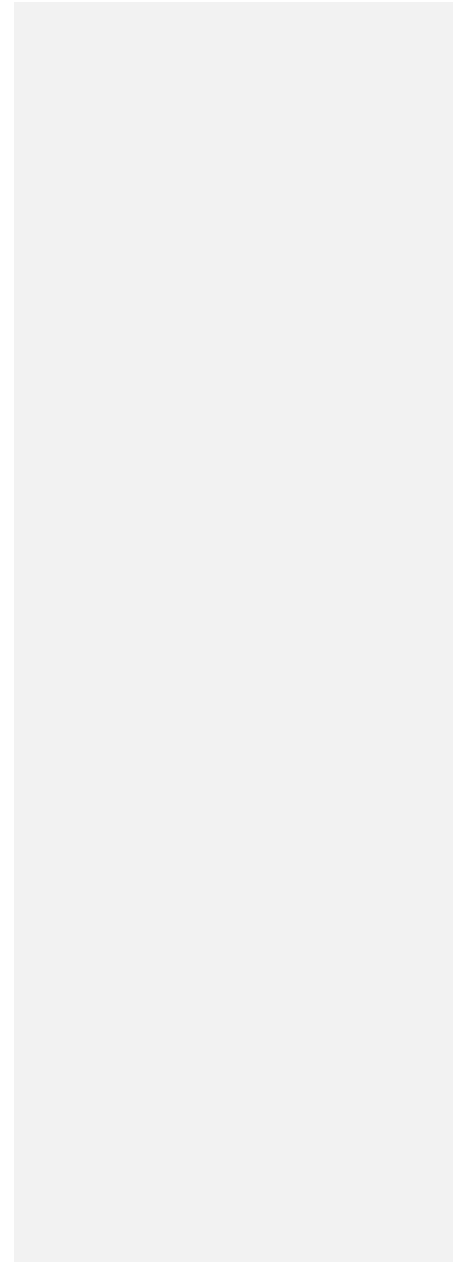| 16 | 697 | 0/6 | 2/6 | 3/6 | 4/6 |
	496	3/6	0/6	5/6	5/6
28	697	0/4	1/4	2/4	2/4
	496	2/4	3/4	4/4	4/4

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.