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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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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 ELISA 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.

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, 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 since 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).

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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 0.45 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 according to 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 °C 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 (Perez 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

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

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

Results

PCR analysis

PCR results confirmed that samples from group C, the mock-inoculated sheep, were negative for virus (data not shown) and that the experimental procedure was successful, in terms of the infection status of animals in groups A and B. The identity of the viral strain present in infected 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