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Phylogenetic analysis of SRLV sequences from an arthritic sheep outbreak demonstrates the introduction of CAEV-like viruses among Spanish sheep

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Abstract

Small ruminant lentiviruses (SRLV) cause different clinical forms of disease in sheep and goats. So far in Spain, Maedi visna virus-like (MVV-like) sequences have been found in both species, and the arthritic SRLV disease has never been found in sheep until a recent outbreak. Knowing that arthritis is common in goats, it was of interest to determine if the genetic type of the virus involved in the sheep arthritis outbreak was caprine arthritis encephalitis virus-like (CAEV-like) rather than MVV-like. Alignment and phylogenetic analyses on nucleotide and deduced amino acid sequences from SRLV of this outbreak, allowed a B2 genetic subgroup assignment of these SRLV, compatible with a correspondence between the virus genetic type and the disease form. Furthermore, an isolate was obtained from the arthritic outbreak, its full genome was CAEV-like but the pol integrase region was MVV-like. Although its LTR lacked a U3 repeat sequence and had a deletion in the R region, which has been proposed to reduce viral replication rate, its phenotype in sheep skin fibroblast cultures was rapid/high, thus it appeared to have adapted to sheep cells. This outbreak study represents the first report on CAEV-like genetic findings and complete genome analysis among Spanish small ruminants.

Keywords: small ruminant lentiviruses, ovine, caprine, arthritis, outbreak
1. Introduction

Spanish SRLV partial genetic sequences have been described recently (Grego et al., 2002; Reina et al., 2006), even though SRLV disease had been detected in Spanish sheep long ago, mainly causing pneumonia and mastitis in sheep and arthritis in goats. Recently, a disease characterized by carpal joint synovitis, together with interstitial mastitis and/or pneumonia has been detected among sheep from a farm in the Aragonese region of Spain (Biescas, 2006).

In this study, SRLV sequences from a unique Spanish SRLV ovine arthritis outbreak were obtained and analyzed genetically and a complete viral genome sequenced, revealing a phylogenetic proximity to CAEV-like prototypes B2. The results suggest a link between viral genetic type and disease form, independently of the in vitro phenotype of the isolate.

2. Material and Methods

2.1 Animals and samples

SRLV-seropositive (Elitest, Hyphen Biomed) arthritic adult sheep of the Rasa Aragonesa breed were used. Animals (Nos. 44, 84, 258, 449, 496 and 497) belonged to flocks with high infection prevalence. EDTA-blood mononuclear cells (PBMC) and bronchoalveolar lavage (BAL) were obtained. Tissues from mammary gland, brain, lung and articular joint were collected and embedded in RNAlater (Qiagen).

2.2 Virus isolation and phenotyping

Synovial fluid (SF) from both carpal joints (left and right) of the arthritic sheep No. 496 was incubated with sheep choroid plexus (SCP) and foetal ovine synovial membrane (FOS) cells. Culture supernatants were used to further infect skin fibroblast cells (SFC) and then titrate the virus (isolate 496) according to RT-activity of supernatants (Reverse Transcriptase Assay
chemiluminescent, Roche). The correlation between known (EV1 strain) TCID\textsubscript{50} and RT activity was determined. The phenotype of isolate 496 was identified as low, medium or high (titres of less than 10\textsuperscript{6}, from 10\textsuperscript{6} to 10\textsuperscript{7} and higher than 10\textsuperscript{7}, respectively); and as rapid, intermediate or slow (when syncytia appeared within a week, a few weeks or over a month, respectively). RT activity was determined at days 0, 1, 2, 3, 5, 6, 7, 8, 9, 21 and 42 post-inoculation (p.i.).

2.3. PCR procedures
Genomic DNA was extracted from PBMC, tissue samples and infected SFC with QIAamp\textsuperscript{®} DNA Blood Mini Kit (QIAGEN). PCRs were performed using Pfu DNA polymerase (Biotools) and 1 µg DNA. Partial amplification of LTR (300 nt) and \textit{gag} (490 nt) regions from outbreak sequences was performed with previously described primers (Reina et al., 2006); \textit{pol} (1.2 kb) and \textit{gag-pol} (1.8 kb) PCR were done as previously described (Shah et al., 2004). Table 1 shows the set of primers used for complete sequencing of the arthritic isolate (Table 1). RNA from FOS, GSM (goat synovial membrane) cells and SFC infected in vitro with isolate 496 was extracted with RNeasy\textsuperscript{®} Mini Kit (QIAGEN) for \textit{rev} transcripts. Reverse transcription was done with SuperScript\textsuperscript{TM} II (Invitrogen) using oligo-dT. Amplification of \textit{rev}-encoding regions was performed using adapted primers originally described by Gjerset et al. (2006).

2.4. Cloning, sequencing, alignment and phylogenetic analysis
Amplicons were cloned by triplicate into pGEMT-easy\textsuperscript{®} vector (Promega) and then sequenced using BigDye\textsuperscript{®} Terminator v3.1 chemistry on a 3730 DNA Analyzer (Applied-Biosystems). Multiple alignments were made with ClustalX and PHYLIP (Phylogeny Inference Package version 3.5c) programs and trees produced by the Neighbour Joining
method with Kimura’s correction, using 1000 bootstrap confidence limits. Results with over 500 bootstraps were considered highly likely.

GenBank MVV and CAEV prototype sequences were included: EV1 (S51392), SAOMVV (M31646), KV1514 (M10608), P1OLV (AF479638), 85/34 USA (AY101611), Swiss Goat Isolate (AY445885), CAEVCo (M33677), CAEV Gansu (AY900630) and CAEV1GA (AF322109). The phylogenetic tree on the 1.2 kb pol segment included previously described Spanish sequences (Reina et al., 2006), one from sheep (Ov1) and two from goats (C1 and C3). This tree and the tree based on the 1.8 Kb gag-pol segment include sequences with a four-digit numeric assignment (Shah et al., 2004). The gag-pol tree also includes [B2] sequences from Leroux et al. (1995) and Grego et al. (2002) (GenBank accession numbers: U35808, U35679 and U35680, and AY265456, respectively).

Partial sequences from the six animals under study and the whole genome sequence of isolate 496 were given GenBank accession numbers: FJ187802-FJ187812 for gag amplicons; FJ187813-FJ187820 for LTR amplicons; and FJ195346 for complete genome.

3. Results

3.1. Sequence similarity and phylogenetic analysis

Sequences from short LTR (300 nt) and gag (490 nt) amplicons (Reina et al., 2006) from the tissues of the arthritic sheep involved in the study (brain was not found infected) were CAEV-like and highly similar to each other. An isolate, 496, was obtained from sheep No. 496 and its genome (9136 nt) fully sequenced, being CAEV-like according to sequence similarity analysis (Table 2) and belonging to the B2 genetic sub-type, using current phylogenetic classification (Fig. 1).

3.2. Biological properties of the isolate
Productive infection (RT-positive supernatants and presence of CPE) was first observed in FOS and SCP cells after 3 days p.i. with SF from carpal joint of animal No. 496. Supernatants of these cultures were used to infect and titrate the virus (496) in SFC cultures. These cells formed syncytia and yielded RT-positive supernatants within 2-4 days p.i. (Fig. 2). The virus titre reached a value close to $10^8$ TCID$_{50}$/ml at 5 to 9 days p.i. Thus, the phenotype of isolate 496 was rapid in FOS and SCP cells, and rapid/high in SFC.

### 3.3. Comparative analysis of the genetic regions and proteins of the isolate

#### LTR region

The isolate lacked a LTR repeat present in some MVV-like isolates (37-53 nt) or CAEV-like isolates (70 nt) (Fig. 3). Although the AP-4 site and the TATA-box were conserved, the AP-1 site distribution was similar to that of CAEVCo. However, distal to TATA box, 496 had one AP-1 site, whereas CAEVCo had two sites (one of them corresponding to the repeat), and the MVV-like sequence KV1514 had 4 sites (two belonging to the repeat). Moreover, 496 LTR presented only one highly conserved AML (vis) site, lacking the AML sequence close to the TATA box present in MVV-like sequences. The distribution of CAAAT, GAS and TAS sites was similar to that of CAEVCo, except that 496 lacked the 70 nt repeat that also contains these sites. In addition, the R region of 496 presented a deletion, similar to that found in CAEVCo, other highly pathogenic strains such as SAOMVV and some strains from sub-clinically infected sheep (Angelopoulou et al., 2008) (Fig. 3).

#### Gag region

The 496 gag-p17 sequence presented two conserved motifs within the inmunodominant epitope and a seven amino acid deletion (Grego et al., 2005; Pisoni et al., 2005) found in other CAEV-like and not in MVV-like sequences. In the 496 gag-p25 protein, two major epitopes
and a major homology region were quite conserved (Fluri et al., 2006; Rosati et al., 1999). In the gag-p14 protein, a CAEV-like one-amino acid deletion at the N-terminal, and a deletion at the carboxy-terminal sequence, absent from CAEVCo, but present in some MVV-like sequences, was observed (not shown).

**Pol region**

Significantly, a stretch of 17 amino acids in the *pol* integrase DNA binding domain of isolate 496 was MVV-like (PGDWEGPTQVLWKGEGA), although 496 had a CAEV-like sequence in other *pol* sub-regions (not shown).

**Env region**

The sequence of the 496 predicted signal peptide was similar in length to other CAEV-like sequences and 9-10 amino acids shorter than MVV-like sequences. The predicted mature 496-SU protein included 22 cysteine residues, conserved among all SRLV, as well as 19 potential N-glycosylation sites. Like in CAEVCo, V1-V4 domains (Mordasini et al., 2006; Valas et al., 2000) were conserved, unlike the variable region of SU5. The TM subunit presented 4 potential N-glycosylation sites conserved in all SRLV. One of the immunodominant epitopes, widely used in ELISA diagnosis, was also detected in isolate 496 and had a CAEV-like (HQ) sequence.

**Rev, vif and tat regions**

Three *rev* transcripts (562 nt, 483 nt and 437 nt) were obtained from SFC and two (562 nt and 437 nt) from FOS and GSM cells. Amplicon sequencing revealed the presence of four *rev* exons: 1) in the LTR region; 2) at the 3’ end of *pol*; 3) at the 5’ end of *env*; and 4) at the 3’ end of *env*. The longest amplicon was integrated by exons 1, 3 and 4 and its putative open
reading frame (ORF) corresponded to a deduced protein sequence of 134 amino acids, analogous to that described in the CAEV Gansu strain (Gazit et al., 1996). Alignment of this sequence with other SRLV-Rev protein sequences revealed a low similarity with known SRLV isolates (Table 2), although the functional N-terminal, the basic (arginine-rich tract), and the Nuclear Export Signal domains (Abelson and Schoborg, 2003) were present in the 496 Rev sequence. The 496 Rev responsive element (RRE) was located in the TM region like in previously described sequences (Saltarelli et al., 1994). The RNA secondary structure of the putative RRE domain was predicted to form a stable stem-loop similar to that of CAEV-like sequences (Kalinski et al., 1991) (not shown).

Tat and Vif proteins (CAEV-like; Table 2) of 496 isolate presented an 8 nt overlap between tat and vif ORFs, like most of the CAEV-like isolates (Gjerset et al., 2006).

4. Discussion

The appearance of a focus with a new form of SRLV disease (arthritis) in the history of Spanish sheep prompted us to isolate and investigate the genetic characteristics of the virus involved. SRLV amplicon sequences from the outbreak including those of isolate 496, were close to each other and to the CAEV-like prototype CAEVCo, although a small genetic variability was found within the host, like in previous SRLV studies (Pisoni et al., 2007). Interestingly, isolate 496 showed a high replication rate in vitro regardless of the ovine or caprine origin of the cells used, a phenotype commonly found in sheep and not in goat isolates (Barros et al., 2005). The original goat virus may thus have adapted to the new ovine host, acquiring genetic properties that confer the rapid/fast phenotype.

SRLV from the outbreak belonged to the B2 CAEV-like subgroup, in contrast with the previously described sequences in Spain from sheep and goats, which belonged to A and D
MVV-like groups (Reina et al., 2006). Thus, there appears to be an agreement between the phylogenetic group to which strain 496 was assigned, and the clinical form observed. The presence of LTR repeats, of 71 nt in CAEV-like and 37-57 nt in MVV-like isolates, and an increased number of sites proposed to regulate viral activity, appears to affect the strain phenotype and tropism (Barros et al., 2004; Murphy et al., 2007; Oskarsson et al., 2007), leading to enhanced promoter activity and a rapid/high lytic phenotype. Alternatively, deletion of these repeats has been reported to abolish the activity of the promoter in SCP cells in some SRLV infections (Oskarsson et al., 2007). The absence of both repeats from isolate 496 LTR indicates that these duplications are not essential for a fast/high phenotype.

Conservation of GAS and TAS motifs, as well as AP-4 site, TATA-Box and PolyA-signal confirmed previous findings (Barros et al., 2004), strongly suggesting that maintenance of these sites is essential to the virus. Deletion of one of the two CAAAT sites of LTR may result in viruses which are non-replicative in SCP cells (Oskarsson et al., 2007). Here we describe sequences from a virus fully replicative in SCP cells lacking the CAAAT in the U3 repeat region of LTR. However, in the 496 sequence there were two extra CAAAAT sites, both of them downstream the CAAAT site, which may help to explain the high replication capacity of this isolate.

A 13-14 nt deletion has been found previously in the R region of the SRLV-LTR from asymptomatic sheep and not in SRLV of pneumonia-affected sheep (Angelopoulou et al., 2008). Interestingly, in our study this deletion was found in sequences from the clinically affected sheep involved, regardless of the sample source, suggesting that factors besides this deletion are involved in the appearance of clinical symptoms.

In contrast with the remaining regions of the 496 genome, the integrase DNA binding domain of this isolate was MVV-like. Whether this is related to the MVV-like phenotype (rapid/high) of isolate 496 observed in vitro is unknown. This phenotype is also compatible with the
appearance of a high number of affected animals in this outbreak, considering that a high viral load may enhance the development of clinical signs (Zhang et al., 2000).

In the SU5 immunodominant epitope, the 496 sequence presented a two amino acid deletion compared with CAEVCo and three amino acid substitutions compared with a SRLV consensus sequence (Mordasini et al., 2006). In the immunodominant TM epitope (Bertoni et al., 1994) the two amino acid inversion (HQ being CAEV-like) could be of diagnostic value in this outbreak.

The features of the CAEV-like highly replicative isolate from the arthritic sheep outbreak under study highlights a possible correspondence between genotype and phenotype and provides further evidence of controversy in this research area within the field of SRLV.

Acknowledgements

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Conflict of interest

There is no conflict of interest.
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Duplicated sequence motif in the long terminal repeat of maedi-visna virus extends cell

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cross-reacting epitope of maedi visna virus and caprine arthritis-encephalitis virus capsid
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lentiviruses based on 104 new isolates: evidence for regular sheep-to-goat transmission


Table 1. Primers used for sequencing the complete genome of isolate 496.

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<tr>
<th>Designation</th>
<th>Sequence (5'-3')</th>
<th>Orientation</th>
<th>Location</th>
<th>Region</th>
<th>Size (nt)</th>
<th>Source</th>
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</thead>
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<td>env-CAEV1</td>
<td>GGA GCT TTG GCA GAA CTC AC</td>
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<td>8979-8998</td>
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<tr>
<td>LTR</td>
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<td>Rv</td>
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<td>LTR</td>
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<td>Zanoni et al., 1992</td>
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<tr>
<td>LTR</td>
<td>TGA CAC AGC AAA TGT AAC CGC AGA</td>
<td>Fw</td>
<td>152-176</td>
<td>LTR</td>
<td>1260</td>
<td>Zanoni et al., 1992</td>
</tr>
<tr>
<td>CAEV-Oslo</td>
<td>GGC ATC ATG GCT AAT ACT TCT AA</td>
<td>Rv</td>
<td>1412-1390</td>
<td>gag</td>
<td></td>
<td>Rimstad et al., 1993</td>
</tr>
<tr>
<td>Wie5</td>
<td>CTT GTG TCC GAG GAT TTT GA</td>
<td>Fw</td>
<td>1324-1343</td>
<td>gag</td>
<td>596</td>
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<td>Wie3</td>
<td>CGC TTG TGC TAA TAA CTG CAT</td>
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<td>1920-1900</td>
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<td>env</td>
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<td>Rv</td>
<td>9227-9206</td>
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</table>

p/c: Personal communication.
Table 2. Similarity (%) between the amino acid sequences of isolate 496 and those of VMV-like and CAEV-like prototypes.

<table>
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<tr>
<th>Protein compared</th>
<th>VMV-like prototypes</th>
<th>CAEV-like prototypes</th>
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<tr>
<td>Gag</td>
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<td>91.0-92.1</td>
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<tr>
<td>Pol</td>
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<td>Env</td>
<td>55.9-59.3</td>
<td>75.8-76.8</td>
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<td>24.6-26.8</td>
<td>56.0-58.0</td>
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<td>TM</td>
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<td>Vif</td>
<td>54.1-57.0</td>
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<td></td>
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</table>
Figure captions

Fig. 1. Nucleotide phylograms involving isolate 496 nucleotide sequences. (a) complete sequence phylogram assigning isolate 496 to a CAEV-like phylogenetic group; the phylogram involves complete GenBank SRLV sequences, VMV-like (EV1, SAOMVV, P1OLV, KV1514 and 85/34 USA and a Swiss Goat isolate) and CAEV-like (CAEV1GA, CAEV Gansu and CAEVCo). (b) 1.2 kb $gag-pol$ segment (Shah et al., 2004) and (c) 1.8 kb $pol$ segment (Shah et al., 2004) phylograms indicating that 496 belongs to the phylogenetic sub-type B2. 496 and previous study (Reina et al., 2006) isolates are encircled. Bootstrap values are indicated. Phylogenetic subtypes are within brackets.

Fig. 2. Viral production of isolate 496 measured by reverse transcriptase (RT) activity in culture supernatants of different cell types: Sheep choroid plexus (SCP), foetal ovine synovial membrane (FOS) cells and heterologous (het) and autologous (aut) sheep skin fibroblast cells (SFC).

Fig. 3. Alignment of U3-R sequences of the LTR region from SRLV isolates. Sequences are aligned against KV1514 prototype. Dots indicate identity with KV1514 and dashes represent gaps. The boundaries between U3, R and U5 are indicated by straight arrows. A bent arrow indicates the start site of transcription. AP-1, AP-4 and AML(vis) motifs, the TATA box and the polyadenylation signal are marked by boxes. TAS and GAS sites are underlined. The polypurine tract region is marked with a horizontal line above the sequence. The grey boxes represent the CAAAT sequences. Repeat regions are within brackets. The sequence of isolate 496 is in boldface.
Fig. 1a. Glaria et al.
Fig. 1b. Glaria et al.
Figure 2. Glaria et al.

![Graph showing RT activity over days post-inoculation](image-url)
Fig. 3. Glaria et al.
Fig. 1c. Glaria et al.