1 **Small Ruminant Lentivirus genotype E is widespread in Sarda Goat**

2

3 Reina Ramsés^{1,2}, Bertolotti Luigi¹, Dei Giudici Silvia³, Puggioni Giantonella³,
4 Ponti Nicoletta³, Profiti Margherita¹, Patta Cristiana³, Rosati Sergio^{1*}

5

6 ¹ Dipartimento di Produzioni Animali, Epidemiologia, Ecologia, Facoltà di
7 Medicina Veterinaria, Università degli Studi di Torino, Via Leonardo da Vinci 44,
8 10095 Grugliasco (TO), Italy

9 ² Instituto de Agrobiotecnología, CSIC-UPNA-Gobierno de Navarra, Ctra.

10 Mutilva Baja, 31192 Mutilva Baja, Navarra, Spain

11 ³ Istituto Zooprofilattico Sperimentale della Sardegna, Sassari, Italy

12 *Corresponding author:

13 Rosati Sergio, Dipartimento di Produzioni Animali, Epidemiologia ed Ecologia,
14 via Loenardo da Vinci, 44, 10095, Grugliasco (TO), Italy. Phone 0039 011
15 6709187, fax 00390116709196.

16

17 **Abstract**

18 The highly divergent SRLV genotype E has recently been characterized in Italy as
 19 a low pathogenic caprine lentivirus in the Roccaverano breed. The availability of
 20 a genotype specific diagnostic test based on a comparative assay, using a
 21 combination of genotype specific recombinant antigens allows a wide serosurvey
 22 in other goat populations. The island of Sardinia still has the highest small
 23 ruminant population of any Italian region and crossbreeding has been limited to
 24 goats, mainly with the Maltese breed.

25 A serological survey was carried out on sheep flocks and goat herds, using
 26 individual sera as well as a bulk milk-adapted procedure. Genotype E was
 27 identified in more than 50% of goat herds and none of the sheep flocks thus
 28 supporting the idea that this genotype is specifically associated with the goat
 29 species. The full length proviral sequence of a Sardinian isolate revealed and
 30 confirmed the deletion of dUTPase subunit and the absence of both *vpr* gene and
 31 the 71 bp repeat of the LTR. Genetic similarity of this isolate with the prototype
 32 strain Roccaverano was no more than 84%, supporting the designation of two
 33 subtypes within genotype E. Nevertheless, in vitro properties of the Sardinian
 34 strain were different from those of the Roccaverano strain in terms of ability to
 35 infect synovial membrane and produce syncytia. Remarkable differences in the
 36 HV1 and HV2 of the *env* gene were recorded, with the Sardinian isolate
 37 displaying sequence motif more similar to arthritic strains. Data presented suggest
 38 diffusion of genotype E is wider than previously thought.

39

40 Keywords: **small ruminant lentivirus / genotype e / pathogenic subtype / sarda**

41 **goat.**

42

Accepted Manuscript

43 Introduction

44 Small ruminant lentiviruses (SRLV) are a group of viruses displaying different
 45 genetic, antigenic and biological properties in their natural hosts. These viruses
 46 cause slow progressive multi systemic diseases involving joints, mammary
 47 glands, brain and lungs. Beside the Maedi Visna Virus (MVV) and Caprine
 48 Arthritis Encephalitis Virus (CAEV), prototypes of genotype A and B
 49 respectively, additional genotypes C, D and E have been described (Reina *et al.*,
 50 2009a; Shah *et al.*, 2004). The latter has so far been identified in the Roccaverano
 51 goat, an endangered Italian breed. Full length genome analysis of the prototype
 52 strain *Roccaverano* revealed unusual genetic organization with natural deletions
 53 of the dUTPase subunit of the *pol* gene and the absence of *vpr* gene (previously
 54 characterized as *tat* gene) (Reina *et al.*, 2009a). We proposed the designation of
 55 low pathogenic caprine lentivirus to characterize this viral cluster for two main
 56 reasons: i) reduced viral load and disease progression have been observed using
 57 CAEV molecular clones artificially deleted for the same gene or gene subunit; ii)
 58 the arthritic clinical index in a Roccaverano flock infected with genotype B is
 59 significantly higher than that found in a flock infected with genotype E (personal
 60 observations). A recent study has indicated that, due to antigenic diversity of *gag*
 61 encoded proteins among genotypes A, B or E, distribution of E-like infection in
 62 other small ruminant population would require a specific antigen design. To
 63 address this problem a comparative assay was proposed using the recombinant
 64 P16 (matrix) and P25 (capsid antigen) fusion protein from both B and E
 65 genotypes. This test was able to selectively detect genotype E infected animals,

66 based on different reactivity against homologous antigen (Reina *et al.*, 2009b).
 67 Since only few infected flocks have been recorded to date in the Piedmont region
 68 (North-West Italy), it is difficult both to speculate on the distribution of genotype
 69 E infection in other countries and to assess if genotype E might be present in other
 70 goat populations displaying different biological behaviour (i.e. virulence). In Italy,
 71 several local goat populations have been subjected to unplanned crossbreeding
 72 with imported breeds to increase milk production. The introduction of B1 subtype
 73 (CAEV-like strains), especially from France, is commonly believed to have
 74 occurred through importation of French Alpine and Saanen breeds in the early
 75 eighties (Grego *et al.*, 2007). Pathogenic strains such as those belonging to
 76 subtype B1, tend to spread horizontally among adult animals. For this reason,
 77 even if more than 50 goat breeds are currently farmed, B1 strains appeared to be
 78 widespread in many regions. Local breeds with limited crossbreeding or
 79 introduction represent a good starting point to investigate the presence of
 80 genotype E. The Sarda goat lives only in Sardinia and represents more than 20%
 81 of the Italian goat population (about 300,000 heads). Crossbreeding has mainly
 82 occurred in the past with the Maltese breed, while introduction of B1 infected
 83 goats has been limited (Ajmone-Marsan *et al.*, 2001; Sechi *et al.*, 2007). In the
 84 present study, a large number of small ruminant flocks were tested using genotype
 85 E and B comparative ELISA assay. While sheep flocks were negative to genotype
 86 E, surprisingly, more than 50% of the goat herds resulted positive, suggesting that
 87 genotype E infection is widespread in the Sarda goat. Genome analysis of a
 88 Sardinian viral isolate revealed a similar genome organization within genotype

89 and moderate pathogenic behaviour in vitro. Different viral evolutionary strategies
 90 in the two different sized populations and potential genotype E reservoir in other
 91 countries are also discussed.

92

93 **Materials and Methods**

94 *Blood (serum and DNA) and milk samples*

95 Caprine herds or ovine flocks were selected randomly among the Sardinian
 96 population, involving the most populated areas on the island.
 97 Individual whole blood from 20 ovine flocks and from 30 caprine herds were
 98 initially collected and serum was stored at -20°C until ELISA testing. Buffy coats
 99 and milk were obtained from 21 samples belonging to three of the caprine herds
 100 and DNA was extracted using DNA blood minikit (Qiagen). Following this
 101 preliminary serological survey, 186 bulk milk samples were collected from
 102 additional caprine or mixed herds and subjected to milk-adapted ELISA (Fig. 1).
 103 For both sera and bulk milk, appropriate positive and negative controls were
 104 included in each test, including samples from three caprine herds characterized in
 105 a previous study and known to be infected with genotype B, genotype E and both
 106 genotypes (Reina *et al.*, 2009b).

107

108 *ELISA comparative assays*

109 A previously described ELISA test was used to serotype samples, consisting of
 110 P16-25 recombinant antigen derived from genotypes B (strain IT-Pi1) and E
 111 (strain Roccaverano) (Reina *et al.*, 2009a). Briefly, ELISA microplates

112 (Immunomaxi TPP) were coated with 100 ng of each recombinant antigen and
 113 water as negative control. After drying and blocking steps, serum samples were
 114 applied at 1/20 dilution and plates incubated at 37° for 1h. Following the washing
 115 step, peroxidase labelled Mab anti-sheep/goat IgG was applied and plates
 116 incubated as above. After the final wash, development was carried out using
 117 ABTS and plates were read at 405 nm. Net absorbances were obtained by
 118 subtracting the absorbance of negative antigen from the absorbance of each
 119 recombinant antigen. Cut-off value was defined as percentage of reactivity $\geq 20\%$
 120 of the absorbance of positive control included in each plate.

121 Bulk milk samples were tested using the same P16-25 ELISA and a previously
 122 described subunit ELISA (sub-ELISA) (Reina *et al.*, 2009b) in which microplates
 123 were coated with 200 ng/well of the immunodominant epitope of capsid antigen
 124 derived from genotype B (sequence KLNEEAERWRRNNPPPP) and E (sequence
 125 KLNKEAETWMRQNPQPP). Since both peptides were expressed as GST fusion
 126 protein, an equimolar amount of GST was used as negative control. Net
 127 absorbances were obtained by subtracting the absorbance against GST antigen
 128 from that of each recombinant subunit. Milk samples were used at 1/2 dilution in
 129 both assays and procedures were carried out as above. For P16-25 ELISA, a
 130 standard curve was generated using two fold dilutions of bulk milk sample into
 131 negative bulk milk, the former obtained from a caprine herd with known
 132 seroprevalence and known to be infected only with E genotype. Cut off was
 133 defined as the absorbance level of the dilution corresponding to 20% prevalence
 134 and included in each plate.

135

136 *PCR, sequencing and phylogenetic analysis*

137 DNA was extracted from individual blood and milk samples and used to amplify a
 138 partial region of the *gag* gene (Grego *et al.*, 2007). Briefly, DNA was analyzed by
 139 a nested PCR designed to amplify a 1.3 kb fragment in the first round and a 0.8 kb
 140 fragment in the second one. The result of the nested amplification was sequenced
 141 directly using an ABI PRISM 310 Genetic Analyzer (Applied Biosystem, Monza,
 142 Italy). Nucleotide sequences were aligned using Clustal X algorithm, in respect of
 143 the amino acidic coding frame and were compared to SRLV homologous
 144 sequences available on GenBank.

145 Genetic similarity was expressed as nucleotide and aminoacid diversity (Nei,
 146 1987), or mean proportion of differences among sequences. Taken into account
 147 the peculiar genomic organization of isolates within genotype E, the amount of G
 148 to A transitions was analyzed to investigate the possible role of genome deletions
 149 on the viral mutation rate. The evaluation of the amount of G-to-A substitutions
 150 was carried out using hand made functions in R computer software (R
 151 Development Core Team 2007), available upon request.

152 Selective pressure was evaluated calculating the ratio (ω) of non synonymous
 153 substitutions per non synonymous sites (d_N) and the number of synonymous
 154 substitutions per synonymous sites (d_S); evaluation of selective pressure was
 155 performed considering the overall number of substitutions and analyzing
 156 mutations at site-specific level (SNAP www.hiv.lanl.gov).

157 In order to describe the phylogenetic relationships among new and SRLV
158 reference sequences, we created a dataset including samples belonging to the
159 genotype E published previously (Grego *et al.*, 2007) and sequences from A, B
160 and C genotypes as outgroups. The phylogenetic tree was created evaluating the
161 best model of molecular evolution (ModelTest software, (Posada and Crandall,
162 1998)) and using Bayesian heuristic approaches (MrBayes software, (Ronquist
163 and Huelsenbeck, 2003)).

164

165 *Virus isolation and genome sequencing*

166 An uncharacterized viral isolate, which had been previously obtained by co-
167 culture of the buffy coat with a primary culture of choroid plexus cells, was traced
168 back in our laboratory as a frozen supernatant. It had been isolated in a Sardinian
169 caprine herd, found reactive against genotype E antigen in the present study, from
170 an adult animal suffering unspecific arthritis. DNA extracted from the buffy coat
171 of the same animal was also available. Viral isolate (hereafter named *Seui*) was
172 cultured on caprine foetal synovial membrane (CFSM) and analyzed for syncytia
173 formation, immunocytochemistry, RT activity (Cavidi) and PCR. DNA from
174 infected cells was used to obtain the complete genome sequence of the *Seui*
175 isolate using primers described in Table 1. Rev transcripts analysis was carried
176 out by RT-PCR with primers already described (Gjerset *et al.*, 2006).

177

178 *Nucleotide accession numbers*

179 Nucleotide sequences of partial *gag* fragments and the complete genome of strain
 180 *Seui* were submitted to the GenBank database and given accession numbers
 181 GQ428519-36 and GQ381130 respectively.

182

183 **Results**

184 *P16-25 ELISA and sub-ELISA*

185 Serological test was conducted on a total of 504 animals from 19 goat herds
 186 (n=309) and 19 sheep flocks (n=195) and only goats showed the presence of
 187 genotype E infection in Sardinia, reaching absorbance values comparable to those
 188 of the positive controls used. Serum P16-25 ELISA was able to serotype the
 189 infection and although some animals reacted against both antigens (genotype B
 190 and E), most reacted in a type specific manner against genotype E antigen (Fig
 191 2A).

192 Following this preliminary screening, we used bulk milk from a total of 186 goat
 193 herds to estimate the real prevalence of genotype E within the Sarda goat
 194 population. Based on milk adapted P16-25 ELISA, serotyping was not always
 195 possible due to highly reactive samples, which reached a saturation level against
 196 both B and E derived antigens. Although titration of highly reactive samples may
 197 has overcome this drawback, subunit-ELISA was able to serotype most milk
 198 samples but sensitivity was obviously lower than that obtained by P16-25 ELISA.

199 When both methods were merged, the estimation of 74% of SRLV
 200 seroprevalence was found in the Sardinian goat herds. Among these, 19.41% were
 201 infected with genotype B, 44.12% with genotype E and 10.59% were infected by

both genotypes or not characterised. Finally, 18.24% of the flocks were found negative by both assays. Results clearly indicate that genotype E is widely distributed on the island of Sardinia, reaching a prevalence twice the levels found for genotype B.

206

207 *PCR sequencing and phylogenetic analysis*

A total of 18 partial sequences (0.8 Kb) of the *gag* gene were obtained and analyzed. The mean nucleotide diversity among Sardinian samples was 9.915% (standard error of the mean 1.105%). Analyses on G to A transitions showed that the amount of this specific mutation was 24.75% (standard error of the mean = 0.60%) of the total number of substitutions and it is similar to that of Roccaverano cluster in Piedmont (Reina *et al.*, 2009a). The evaluation of selective pressure showed the presence of purifying selection ($\omega = 0.032$).

Phylogenetic relationships among new Sardinian samples, sequences from Piedmont belonging to genotype E and reference sequences are described in the phylogenetic tree reported in Figure 3. Tree topology clearly indicates the divergence between Roccaverano and Sardinian clusters.

219

220 *Complete genome sequencing and in vitro properties*

Sardinian genotype E (strain Seui) was able to infect synovial membrane as assessed by the presence of characteristic CPE, immunocytochemistry, RT activity and PCR.

224 DNA extracted from infected CFMS with *Seui* strain was used to amplify the
 225 complete proviral genome in six steps (LTR, LTR-*gag*, *gag*, *gag-pol*, *pol*, and
 226 *env*). Rev transcripts were successfully generated by RT-PCR. Since the complete
 227 sequence was obtained by overlapping PCR fragments, it may not reflect the
 228 sequence of a single provirus. However *env* sequences obtained from PBMC,
 229 coculture and milk from the isolation's animal presented a divergence less than
 230 1%. Furthermore, *Rev* sequences presented a divergence of 0.14% compared with
 231 *env* sequence obtained from DNA indicating that the provirus sequence is
 232 representative of a replication competent virus.

233 The mean nucleotide diversity between *Seui* and Roccaverano strain was 14.643%
 234 (SEM: 1.104%). This result supports the definition of two different subtypes
 235 within the genotype E, according to the previously proposed criteria (Shah *et al.*,
 236 2004).

237 Proviral sequence revealed that the hallmarks of genotype E were confirmed in
 238 the Sardinian isolate. Residual dUTPase subunit presented additional four amino
 239 acids respect to Roccaverano strain. Differences were also observed in the hyper
 240 variable regions of *env* gene (HV1 and HV2), the Sardinian isolate displaying
 241 sequence motifs more similar to arthritic strains (Table 2). Long terminal repeats
 242 included all the described enhancer elements already present in the Roccaverano
 243 strain, except for the AP-4 site tandem repeat, a common feature of CAEV
 244 isolates.

245

246 **Discussion**

247 As hypothesised in the previous study, the lack of a specific serological tool
 248 allowed no speculation as to the distribution of genotype E in geographical
 249 locations different from the one where it was initially described (Reina *et al.*,
 250 2009b). Serological data from different goat herds, sequence analysis of specific
 251 PCR products from three infected flocks and the full length proviral genome
 252 sequence of a local strain demonstrate that genotype E infection is associated and
 253 widely distributed in the Sarda goat, while Sarda sheep seems to harbour a
 254 genotype B (CAEV-like) lentivirus, a common feature in Italian sheep population
 255 (Grego *et al.*, 2002).
 256 Sarda goat, unlike the Roccaverano breed, represents an important goat population
 257 with economic significance at a local and national level. Moreover, since the
 258 population size of the Sarda goat is not comparable to the Roccaverano breed, in
 259 terms of average number of head per flock, farming system, management and
 260 productive levels, the biological significance of genotype E as low pathogenic
 261 caprine lentivirus needs to be redefined. The tree topology indicates a clear
 262 divergence between Roccaverano and *Seui* strains, showing quite different clade
 263 structures and features. These differences in the evolutionary pathway can be
 264 justified by epidemiological and historical data. In fact, Roccaverano goats were
 265 at risk of extinction in the early sixties, when people abandoned rural areas in
 266 favour to towns, following industrial development. This social behaviour forced
 267 the goat breed to pass through a bottleneck, and drove viral evolution to take
 268 advantage, on one hand of reducing virulence and on the other hand of persisting
 269 in a small population, limiting the transmission to lactogenic route. In Sardinia,

270 viral evolution might have displayed a different behaviour, increasing or
 271 maintaining a certain degree of both virulence and horizontal transmission. To
 272 date it is difficult to speculate about the pathogenic role of genotype E for several
 273 reasons. First of all, the genomic organization of the Sardinian isolate is similar to
 274 the Roccaverano strain, lacking both dUTPase subunit and *vpr* gene. In other
 275 SRLV models, dUTPase and *vpr* were specifically associated with an increased
 276 viral load, tissue distribution and lesion severity, compared to the deleted
 277 counterpart (Harmache *et al.*, 1998). In addition, the presence of other pathogens,
 278 such as *Mycoplasma* spp, *Fusobacterium necrophorum*, *Bacteroides nodosus*,
 279 which had consistently been reported in the Sarda goat, could lead to lentivirus-
 280 induced overlapping clinical signs. Finally, the viral isolate used in this study had
 281 originally been obtained from co-cultivation of peripheral blood mononuclear
 282 cells with choroid plexus or synovial membrane cells, while viral isolates from
 283 direct explantation of synovial membrane of arthritic goats are still unavailable for
 284 genotype E. In vitro study, however, seems to attribute to the Seui strain a certain
 285 degree of cytopathogenicity at least in terms of ability to infect synovial
 286 membrane and syncytia formation, while replication of the Roccaverano isolate
 287 in the same cell system is greatly reduced (personal observation; manuscript in
 288 preparation). Cell tropism has been attributed to sequence variation in U3 region
 289 of LTR, related to specific transcription factor binding sites, as well as variation in
 290 the hyper variable (HV) regions of the *env* gene. We first analysed the structure of
 291 viral enhancer elements and significant similarity was found between the two
 292 strains, except for a genuine AP4 tandem repeat which is present in *Seui* as well as

293 several CAEV isolates, while a point mutation is present in one of the two
 294 repetitions in the Roccaverano strain. In the *env* gene, amino acid motif in the
 295 HV1 and HV2 regions were clearly different between strains, the Seui being more
 296 similar to arthritic isolates. It should be noted that compartmentalization studies of
 297 viral quasispecies revealed that different motifs in the HV1-2 regions of CAEV
 298 are a normal finding in the same animal (Hotzel *et al.*, 2002) and arthritic related
 299 sequences might have resulted from an in vitro adaptation of *Seui* isolate to
 300 synovial membrane cells. For this reason we sequenced a PCR fragment
 301 encompassing the HV region from PBMC of the same animal from which the
 302 *Seui* strain was isolated and identical amino acid sequence was obtained.
 303 Therefore, we suppose that higher, if any, in vivo virulence of the *Seui* strain
 304 could be attributable to different cell tropism related to U3 and/or HV sequences.
 305 Sequence analysis of the Sardinian strain *Seui* revealed 84% similarity with the
 306 Roccaverano strain supporting the definition of the genotype E and, possibly two
 307 subtypes, following the criteria recommended in the HIV field, where at least two
 308 epidemiologically unlinked isolates should be sequenced in their entirety
 309 (Robertson *et al.*, 2000). Divergence between genomes was not clearly
 310 attributable to specific gene or gene fragment. Interestingly, a certain degree of
 311 variability was found in the *pol* gene corresponding to residual dUTPase subunits.
 312 This seems to confirm that dUTPase was lost during evolution and residual
 313 sequence is not subjected to functional constrain except for frame conservation
 314 and spacer function between RNaseH and Integrase subunits. On the contrary, the

entire ORF of *vpr* was absent in both strains and it is difficult to speculate if *vpr* gene has ever been present in genotype E.

If we assume that the Sarda goat is an ancient breed which came to Sardinia during the Mediterranean colonization of navigators from Middle East and, to the best of our knowledge, there has been limited introduction of improved breeds, we may also assume that SRLV genotype E has strictly been associated with the Sarda goat population, representing an excellent model to study a long lasting host-pathogen interaction and co-evolution. Moreover, phylogeographical partitioning of goat breeds suggests that the Sarda goat belongs to a West Mediterranean cluster, including French (Corse, Rove, Pyreneene) and Spanish (Brava, Verata, Payoya, Florida, Malagueña, Guadarrama) breeds (Canon *et al.*, 2006). Since serological tools adapted to bulk milk in this study proved to be very sensitive and dependable for the detection of the genotype E, a wider serological survey including these populations would be essential, in order to identify additional infection foci and evaluate more accurately the biological significance and impact of genotype E in SRLV control programs.

331

332 **Acknowledgements**

333 This work has been partially funded by the Italian Ministry of Health, “Ricerca
334 Corrente” program id IZSSA/03.

335

References

- 336
337
338 Ajmone-Marsan, P., Negrini, R., Crepaldi, P., Milanese, E., Gorni, C., Valentini,
339 A., Cicogna, M., 2001, Assessing genetic diversity in Italian goat populations
340 using AFLP markers. *Anim Genet* 32, 281-288.
- 341 Canon, J., Garcia, D., Garcia-Atance, M.A., Obexer-Ruff, G., Lenstra, J.A.,
342 Ajmone-Marsan, P., Dunner, S., 2006, Geographical partitioning of goat
343 diversity in Europe and the Middle East. *Anim Genet* 37, 327-334.
- 344 Gjerset, B., Storset, A.K., Rimstad, E., 2006, Genetic diversity of small-ruminant
345 lentiviruses: characterization of Norwegian isolates of Caprine arthritis
346 encephalitis virus. *J Gen Virol* 87, 573-580.
- 347 Glaria I, Reina R, Crespo H, de Andrés X, Ramírez H, Biescas E, Pérez MM,
348 Badiola J, Luján L, Amorena B, de Andrés D., 2009, Phylogenetic analysis of
349 SRLV sequences from an arthritic sheep outbreak demonstrates the
350 introduction of CAEV-like viruses among Spanish sheep. *Vet Microbiol* Jul
351 2;138(1-2):156-62. Epub 2009 Mar 13.
- 352 Grego E, Profiti M, Giammarioli M, Giannino L, Rutili D, Woodall C, Rosati S.,
353 2002, Genetic heterogeneity of small ruminant lentiviruses involves
354 immunodominant epitope of capsid antigen and affects sensitivity of single-
355 strain-based immunoassay. *Clin Diagn Lab Immunol* Jul;9(4):828-32.
- 356 Grego, E., Bertolotti, L., Quasso, A., Profiti, M., Lacerenza, D., Muz, D., Rosati,
357 S., 2007, Genetic characterization of small ruminant lentivirus in Italian mixed
358 flocks: evidence for a novel genotype circulating in a local goat population. *J*
359 *Gen Virol* 88, 3423-3427.
- 360 Harmache, A., Vitu, C., Guiguen, F., Russo, P., Bertoni, G., Pepin, M., Vigne, R.,
361 Suzan, M., 1998, Priming with tat-deleted caprine arthritis encephalitis virus
362 (CAEV) proviral DNA or live virus protects goats from challenge with
363 pathogenic CAEV. *J Virol* 72, 6796-6804.
- 364 Hotzel, I., Kumpula-McWhirter, N., Cheevers, W.P., 2002, Rapid evolution of
365 two discrete regions of the caprine arthritis-encephalitis virus envelope surface
366 glycoprotein during persistent infection. *Virus Res* 84, 17-25.
- 367 Nei, M., 1987, *Molecular Evolutionary Genetics*. Columbia University Press,
368 New York, NY.
- 369 Posada, D., Crandall, K.A., 1998, MODELTEST: testing the model of DNA
370 substitution. *Bioinformatics* 14, 817-818.
- 371 Reina, R., Grego, E., Bertolotti, L., De Meneghi, D., Rosati, S., 2009a, Genome
372 analysis of small ruminant lentivirus genotype E: a caprine lentivirus naturally
373 deleted for dUTPase subunit, vpr-like accessory gene and the 70 bp repeat of
374 U3 region. *J Virol* 83, 1152-1555.
- 375 Reina, R., Grego, E., Profiti, M., Glaria, I., Robino, P., Quasso, A., Amorena, B.,
376 Rosati, S., 2009b, Development of specific diagnostic test for small ruminant
377 lentivirus genotype E. *Vet Microbiol*.
- 378 Robertson, D.L., Anderson, J.P., Bradac, J.A., Carr, J.K., Foley, B., Funkhouser,
379 R.K., Gao, F., Hahn, B.H., Kalish, M.L., Kuiken, C., Learn, G.H., Leitner, T.,
380 McCutchan, F., Osmanov, S., Peeters, M., Pieniazek, D., Salminen, M., Sharp,

- 381 P.M., Wolinsky, S., Korber, B., 2000, HIV-1 nomenclature proposal. *Science*
382 288, 55-56.
- 383 Ronquist, F., Huelsenbeck, J.P., 2003, MrBayes 3: Bayesian phylogenetic
384 inference under mixed models. *Bioinformatics* 19, 1572-1574.
- 385 Saltarelli, M., Quérat, G., Konings, D.A., Vigne, R., Clements, J.E., 1990,
386 Nucleotide sequence and transcriptional analysis of molecular clones of CAEV
387 which generate infectious virus. *Virology* 179, 347-364.
- 388 Sechi, T., Usai, M.G., Miari, S., Mura, L., Casu, S., Carta, A., 2007, Identifying
389 native animals in crossbred populations: the case of the Sardinian goat
390 population. *Anim Genet* 38, 614-620.
- 391 Shah, C., Boni, J., Huder, J.B., Vogt, H.R., Muhlherr, J., Zanoni, R., Miserez, R.,
392 Lutz, H., Schupbach, J., 2004, Phylogenetic analysis and reclassification of
393 caprine and ovine lentiviruses based on 104 new isolates: evidence for regular
394 sheep-to-goat transmission and worldwide propagation through livestock trade.
395 *Virology* 319, 12-26.
- 396 Staskus KA, Retzel EF, Lewis ED, Silsby JL, St Cyr S, Rank JM, Wietgreffe SW,
397 Haase AT, Cook R, Fast D, et al., 1991, Isolation of replication-competent
398 molecular clones of visna virus, *Virology* Mar;181(1):228-40.
- 399

400

Figure 1. Map of the Sardinia island, divided in municipalities. A) goat herds density. Grey level indicates goat herd density within the municipality: white = less than 1st quartile (3 herds); light grey = between 1st and 2nd quartile (11 herds); grey = between 2nd and 3rd quartile (15 herds); dark grey = more than the 3rd quartile. B) Municipalities including flocks tested with bulk milk analysis (grey). Circles = E positive flocks; squares = B positive flocks; triangles = coinfectd (or uncharacterised) flocks.

Figure 2. Net absorbance against E (x axis) and B (y axis) antigens. Dashed diagonal line represents equal reactivity versus both antigens. Vertical and horizontal dotted lines represent ELISA E and B cut-offs respectively.

A) Data from 19 ovine flocks and from 19 caprine herds. White circles: median absorbance of samples belonging to goat herds. White triangles: median absorbance of samples belonging to sheep flocks. Black squares: reactivity detected in herds previously characterized (Reina *et al.*, 2009b) infected with B (a), E (c) or both strains (b). Vertical and horizontal bars represent the variation (interquartile range) in the distribution of absorbances within flock against B and E antigens respectively.

B) Data from 186 goat herds tested using bulk milk sub-ELISA. Dots represent tested flocks.

Figure 3. Phylogenetic tree constructed by Bayesian analysis of 33 partial *gag* gene sequences (consensus alignment length: 525 bp). New sequences are

424 reported in bold. Genbank accession numbers are reported within brackets.

425 Posterior probabilities of clades are indicated above branches.

Accepted Manuscript

Table 1. Nucleotide sequence of primer pairs.

Amplicon	Length (Kb)	Primer forward (5'→3')	Primer reverse (3'→5')
LTR	0.3	TGACACAGCAAATGTAACCGCAAG	CCACGTTGGGCGCCAGCTGCGAGA
LTR-GAG	0.8	TGACACAGCAAATGTAACCGCAAG	CCCTGGGGGCTGTGGATTCTG
GAG	1.3	TGGTGARKCTAGMTAGAGACATGG	CATAGGRGGHGCAGCGGCASCA
GAG-POL	2.6	AACCGGGTCATCTAGCAAGAC	CTATCCAGAGAATTTGCACGTCTTG
POL	0.8	GGTGCCTGGACATAAAGGGATTG	GCCACTCTCCTGRATGTCCTCT
ENV	3.0	ATGGACAAGAAGGACGGG	GTGGTTACATTTGCTATGTC
REV	0.5	TGCGGTCCTCGCAGGTGGC	TGAGGCGATCTCCACTCCATC

2

Table 2. Comparison of HV1 and HV2 *env* protein aminoacidic motifs between

Seui strain, different SRLV genotypes and the Roccaverano strain. Dots indicate

identity. HV1 and HV2 regions are highlighted in grey (Hotzel *et al.*, 2002).

Strain	Genotype	Ref	HV1	HV2
Seui strain	E2	This work	I-GNNTVIGNCSAQK	GHWTCKPRTKEGKTDSLVI-GGKK
PBMC colture	E2	This work		
SM colture	E2	This work		
CAEV Cork	B1	(Saltarelli <i>et al.</i> , 1990)	V...G.IT...TTN	NK...A..QRD...A....
CAEV-63	B1	(Hotzel <i>et al.</i> , 2002)	VDR.Q.IT...VTN	NK...A..QR...A....
Ov496	B2	(Glaria <i>et al.</i> , 2009)	V...G.IT...VTN	NK...A..WRG.MS...A...Q
K1514	A	(Staskus <i>et al.</i> 1991)	V...G.IT...VTN	NK...AA.R.GSRR...A.-RD
1GA	C	(Gjerset <i>et al.</i> , 2006)	I...S.LQ.Q.NRSN	R.YVN-----D
Roccaverano	E1	(Reina <i>et al.</i> , 2009a)	L.DAQG.....KEN	NQ.....QRGNR...V..GA-RR

6

Figure1

[Click here to download high resolution image](#)

Figure2

[Click here to download high resolution image](#)

Figure 3
[Click here to download high resolution image](#)