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This is a pre print version of the following article:

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1693810 since 2019-02-20T18:53:46Z

Published version:

DOI:10.1016/j.rvsc.2018.01.008

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09 November 2026

Viral load, tissue distribution and histopathological lesions in goats naturally and experimentally infected with the Small Ruminant Lentivirus Genotype E (subtype E1 *Roccaverano* strain).

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***Abstract**:*

Small Ruminant Lentivirus (SRLV) subtype E1, also known as *Roccaverano* strain, is considered a low pathogenic virus on the basis of natural genetic deletions, *in vitro* properties and on-farm observations. In order to gain more knowledge on this atypical lentivirus we investigated the *in vivo* tropism of *Roccaverano* strain in both, experimentally and naturally infected goats. Antibody responses were monitored as well as tissue distribution and viral load, evaluated by real time PCR on single spliced (*gag/env)* and multiple spliced (*rev)* RNA targets respectively, that were compared to histopathological lesions. Lymph nodes, spleen, alveolar macrophages and mammary gland turned out to be the main tissue reservoirs of genotype E1-provirus. Moreover, mammary gland and/or mammary lymph nodes acted as active replication sites in dairy goats, supporting the lactogenic transmission of this virus. Notably, a direct association between viral load and concomitant infection or inflammatory processes was evident within organs such as spleen, lung and testis.

Our results validate the low pathogenicity designation of SRLV genotype E1 *in vivo,* and confirm the monocyte-macrophage cell lineage as the main virus reservoir of this genotype. Accordingly, SRLV genotype E displays a tropism towards all tissues characterised by an abundant presence of these cells, either for their own anatomical structure or for an occasional infectious/inflammatory status.

Introduction:

Small ruminant lentiviruses (SRLV) cause a slow progressive multi-systemic disease that may involve joints, lungs, brain and mammary glands. SRLVs display different genetic and antigenic properties highly depending on host genetic background (Stonos et al., 2014). Five different SRLV-genotypes (A-E), characterized by peculiar biological and pathological properties, have been described so far (Juganaru et al., 2011; Valas et al., 2000). While genotypes A (including Visna/Maedi prototypes) and B (including Caprine Arthritis Encephalitis Virus, CAEV) are widely distributed, genotypes C and E are geographically restricted to Norway and Italy respectively (Gjerset et al., 2009; Grego et al., 2007a). Moreover, genotypes A, B and C may infect both goats and sheep, yet genotype E remains specific to the caprine species.

Genetic characterization of genotype E from different geographic areas supported the designation of two subtypes, namely E1 (*Roccaverano* strain) and E2 (*Seui* strain). Both genotypes display the same genomic organization, including deletions in the *dUTPase* subunit of the *pol* gene, the *Vpr-like* gene (referred as *tat* in previous studies) and the 70-base pair repeat of the U3 region (Grego et al., 2007a; Reina et al., 2009). However, *Seui* strain retained some cytopathogenicity *in vitro*, evidenced by the development of syncitia, most likely due to the *env* hypervariable regions HV1 and HV2 that are more similar to the arthritic prototype strains (Reina et al., 2010).

The low pathogenic potential of *Roccaverano* strain is further supported by the lack of clinical signs in *Roccaverano* breed herds naturally infected with genotype E strains. Naturally double infected animals (genotype E1 and genotype B) present milder disease compared to animals infected exclusively by genotype B, leading to hypothesize a certain degree of sheltering function of *Roccaverano* strain (Bertolotti et al., 2013; Grego et al., 2009, 2007b; Reina et al., 2009). Indeed, immunization studies employing *Roccaverano* as immunogen and genotype B as challenge strains demonstrated a cytotoxic T lymphocytes (CTL)-mediated protective role towards heterologous

73 SRLV infection (Bertolotti et al., 2013).

74 Given the differences in the genomic organization and *in vitro* properties of *Roccaverano* strain as
75 compared to other known SRLV genotypes (Grego et al., 2007c; Juganaru et al., 2011) and its
76 prophylactic effect, we deemed it interesting to explore natural and experimental *in vivo* infection.

77

78 **Materials and methods:**

79 *Experimental animals and sample collection:*

80 Two groups of *Roccaverano* breed goats were used for this research: Group A composed by 9
81 newborn kids [5 females (ID: 1-5) and 4 males (ID: 6-9)] coming from a single flock naturally
82 infected by *Roccaverano* strain.

83 Eight kids were fed with colostrum and milk pool obtained from positive (naturally infected by
84 *Roccaverano* strain) goats of the same flock. One female kid (5) was kept in a separate box as
85 negative control and received virus-free commercial colostrum and milk.

86 Group B was composed of 6 SRLV-free animals of the same age of group A, coming from a long
87 term negative flock. Four of them were experimentally infected: 2 goats via the intra-tracheal
88 route (VIT) and 2 by intra-bone marrow injection via the *trochanteric fossa* (VTF). Two different
89 doses of virus in the inoculum were used: 2ml of 10^5 $TCID_{50}$/ml (VIT-L andVTF-L) and 10^6 $TCID_{50}$/ml
90 (VIT-H andVTF-H) of genotype E1 strain *Roccaverano*. The use of both routes of infection allowed
91 us to compare direct infection of alveolar macrophages with the infection of promonocytes in the
92 bone marrow. Since pulmonary lesions are not described in the infection by this genotype, we
93 wondered whether infection of precursor cells in the bone marrow would establish a persistent
94 infection more efficiently. The strain used for the inoculation was produced and titrated according
95 to the procedure previously reported by Reina et al. (2011). The remaining two goats were mock
96 infected and kept as negative controls.

97 The two experimental groups and the control animals were kept in separated boxes and rooms at
98 the experimental farm (CISRA) of the Department of Veterinary Sciences, Grugliasco campus,
99 University of Turin. Experiments were carried out in compliance with the relevant National
100 legislation on experimental animals and animal welfare, upon authorization by the competent
101 authority (Italian Ministry of Health-Directorate General Animal Health-Office VI; permit no.
102 07/2009B).
103 Serum and buffy coats were collected at monthly interval during 6 months from all goats included
104 in the experiment. Subsequently, animals were euthanized according to the procedure set by
105 Ethical Review Committee, with the authorization by the relevant Office, Italian Ministry of Health,
106 Veterinary Services (prot. N. 7/2009-B), and submitted to necropsy.
107 During post mortem, pieces of approximately 0.5cm^3 of different tissues (Table 1) were cut and
108 stored in RNA-later (RNA Stabilization Reagent, Qiagen GmbH, Hilden, Germany) at -80°C until
109 DNA and RNA extraction were performed.
110 Moreover, lymph nodes (mesenteric, mediastinal, prescapular, inguinal/supra-mammary), small
111 and large intestine, liver, kidney, lung, spleen, thymus, choroid plexus, cerebral cortex, carpal
112 synovial membrane and mammary gland were aseptically sampled, formalin-fixed and embedded
113 in paraffin wax for light microscopy.
114
115 *Infection monitoring:*
116 Sera collected at monthly interval throughout the study period (6 months) were used to monitor
117 the presence of SRLV antibodies by home-made indirect ELISA test as described by Reina et al.
118 (2009). Briefly, homologous recombinant p16-25 antigen was produced and purified. ELISA plates
119 were coated with 100ng/ml per well and sera tested at 1/20 dilution. Net absorbances were
120 obtained by subtracting antigen absorbance from the one obtained without antigen. Results were

121 expressed as the % relative to a positive control included in each plate. Buffy coat samples were
122 obtained by gradient centrifugation and the SRLV provirus quantified by *gag* and *env* qPCRs.
123
124 *Histology*
125 Histology was performed on mediastinal lymph nodes and on the following lung lobes (left cranial,
126 right cranial, right medial and the right caudal) and blind scored for pathological lesions by two
127 independent pathologists, as described by Reina et al. (2008). Sections of 3-µm thickness were
128 stained with haematoxylin-eosin (H&E). Pathological changes typical of SRLV infection were
129 screened and scored. In the lung, lymphoid follicle hyperplasia and interstitial pneumonia as well
130 as BALT (Bronchus-associated lymphoid tissue) hyperplasia, perivascular infiltrates, congestion and
131 oedema were scored on a scale of 0–4; in the mediastinal lymph node, lymphoid follicle reactivity
132 and cortical hyperplasia were scored on a scale of 0–2.
133
134 *Immunohistochemistry*
135 Frozen sections (3-µm thick) were collected on poly-L-lysine-coated slides and processed for
136 immunohistochemistry by using a mouse serum raised against recombinant genotype E capsid
137 antigen (Juganaru et al., 2010). All tissues collected were covered with cryo-embedding media
138 (OCT Kaltek, Padova, Italy) and processed by routine methods to provide 5-6 µm thick cryosections
139 for immunohistochemical labelling by the Envision Plus TM peroxidase method (Dako Cytomation,
140 Glostrup, Denmark). The frozen sections were thawed at room temperature for 30 to 60 seconds
141 without drying and immersed immediately in ice-cold methanol (5 min.). After fixation, the slides
142 were rinsed briefly in 1X phosphate-buffered saline (PBS), pH 7.4, and subjected to
143 immunostaining. Specific primary polyclonal anti-*gag* antibody was diluted 1:1000 and applied to
144 sections 3 hours at RT. Following a washing step, a HRP conjugate anti mouse IgG was added and

145 slides incubated as above. After a final washing step, diaminobenzidine (DakoCytomation,
146 Glostrup, Denmark) served as the chromogen and Mayer's haematoxylin as the counterstaining.
147 Controls omitting primary antibody or replaced with non-immune homologous serum were also
148 analysed. Samples from an uninfected, seronegative goat were also used as negative control.
149 Cases with cytoplasmic immunoreactivity, represented by brown discoloration at the level of
150 lymphocytes, were considered positive.
151
152 *Nucleic acid extraction and cDNA:*
153 DNA and RNA were extracted with QiaZol Lysis Reagent (Qiagen, Hilden, Germany) according to
154 the manufacturer's instructions from 50 mg of tissue disrupted using the Tissue Lyser
155 (Homogenizer, Qiagen GmbH, Hilden, Germany). Afterwards, nucleic acids were quantified and
156 checked for quality by NanoDrop 2000 (Spectrophotometer, Thermo Fisher Scientific Inc.,
157 Waltham, MA) and stored at -80°C until use. RNA was retro-transcribed using QuantiTect Reverse
158 Transcription Kit (Qiagen, Hilden, Germany). To check for possible cross-contamination, tissues
159 from lentivirus negative animals were processed in parallel with the tissue samples (1/10) and
160 included in the PCRs. Each sample was processed in duplicate.
161
162 *GAPDH and ATPase PCR:*
163 In order to assess DNA and RNA quality as well as the absence of PCR inhibitors we amplified the
164 extracted nucleic acids using primers specific for glyceraldehyde-3-phosphate dehydrogenase
165 (GAPDH) gene and ATPase α subunit mRNAs (Homan et al., 1997; Woodall et al., 1997).
166
167 *The qPCR assay:*
168 Specific primers and probes for each target gene were designed on the basis of the same

169 *Roccaverano* strain that was used as inoculums, and circulating in the naturally infected flock using
170 Beacon designer (Premier Biosoft). In detail, two real time PCR techniques targeting single spliced
171 targets (*gag* and *env* genes) and one on a multiple spliced target (*rev* gene) were designed (Table
172 2). Negative and positive controls were included every 10 samples analysed. Negative controls
173 included RNase and DNase-free water and nucleic acid extracts from negative samples to
174 determine any possible cross-reactivity or contamination (false positive results). Positive controls
175 were nucleic acid extracts of macrophages cultures infected with *Roccaverano* strain. All
176 experiments were run in triplicate and were replicated twice.
177 The qPCR assays were performed with 100ng of DNA extracted from each sample. The 25µl
178 reactions contained 5µl of DNA extracts in RNase and DNase free water, 2XQuantiTect SYBR Green
179 or PROBE PCR Master Mix (QIAGEN) and primers and probe as reported in Table 2.
180 *Gag* cycling conditions included denaturation at 95°C for 5 min; 45 cycles of 95°C for 15s, 63°C for
181 30s; and a final extension at 72°C for 7 min (Applied Biosystems 7300 Real Time PCR System).
182 *Env* cycling conditions included denaturation at 95°C for 5 min; 50 cycles of 95°C for 30s, 60°C for
183 30s; and a final extension at 72°C for 7 min (Applied Biosystems 7300 Real Time PCR System).
184 *Rev* cycling conditions included denaturation at 95°C for 5 min; 50 cycles of 95°C for 15s, 60°C for
185 30s; and a final extension at 72°C for 7 min (Applied Biosystems 7300 Real Time PCR System).
186 Post amplification melting temperature (Tm) analysis from 50 to 95°C at 0.5°C increments was
187 conducted to determine specific real time PCR products (*Gag* Tm ~84°C). (*Rev* Tm ~ 83.6 °C). SRLV
188 genotype A and B strain, previously collected in our laboratory, were used to evaluate the
189 *Roccaverano* strain specificity of these qPCRs. None of these reaction gave a positive signal.
190
191 *Standard curve:*
192 *Gag*, *env* and *rev* amplicons were cloned in TOPO TA cloning system (Invitrogen,Carlsbad, CA, USA)

193 and plasmids used serially diluted from 10^7 to 10^1 copies to obtain the standard curve (cycle
194 threshold versus quantity) in real time PCR. Moreover, a standard curve based on different RT-
195 qPCR reactions with known copy numbers of *rev* transcripts from the plasmid containing the *rev*
196 fragment obtained by PCR was generated. The Riboprobe kit (Promega, Madison, WI, USA) was
197 used to produce the standard transcripts following the manufacturer's instructions.
198
199 *Sequencing (not shown)*:
200 Some PCR (*gag, env*) positive samples were directly sequenced (BMR genomics, Padova, Italy) to
201 confirm the infection by genotype E1 and to exclude potential laboratory contamination. The
202 chromatograms were edited and the sequences aligned against known reference sequences using
203 multiple-alignment software provided in the BioEdit package, version 7.0.5.2.12. (Hall, 2011) and
204 were submitted for BLAST analysis.
205
206 **Results**:
207 *ELISA genotype E*:
208 Naturally infected kids from group A showed a serological pattern consistent with maternal
209 antibody transfer (IgG antibodies) in the bloodstream of the new born kids that was not observed
210 in the control animal that received commercial colostrum and milk. Maternal antibodies declined
211 after 120 days after infection (dpi) and three goats showed an increased antibody titre at 150-180
212 dpi (Fig. 1).
213 Experimentally infected animals (group B), showed a rapid antibody response in the two goats
214 which received the highest viral dose (at 60 dpi for VTF-H, and at 90 dpi for VIT-H), while amongst
215 the two animals which received the lower dose, only the goat inoculated VIT-L seroconverted at
216 120 dpi, and VTF-L showed an increased signal at the last collection timepoint, although still below

217 the cut-off value (Fig. 2).

218

219 *Pathological and histopathological examinations*:

220 In the group A animals, a severe lymphoid hyperplasia, particularly in the mesenteric lymph node,
221 and a severe lymphoplasmacytic enteritis were observed. There were no significant lesions in
222 other organs except in goat 9, which suffered from enlargement and inflammation of the left
223 testicle showing severe multifocal granulomatous orchitis at the histological examination; and
224 goats 4 and 8 that showed severe multifocal pulmonary lesions, with moderate to severe
225 eosinophilic pneumonia secondary to parasitic infestation.

226 In the animals of group B, the main histological findings were severe reactive lymphoid
227 hyperplasia, particularly evident in the mesenteric lymph node associated with severe
228 lymphoplasmacytic enteritis in the infected goats. Regarding the histopathological blind-score
229 evaluation, significant lesions were represented by mild lymphoplasmacytic infiltrate of the carpal
230 synovial membrane and mild lymphoplasmacytic infiltrate of choroid plexus in one case (VIT-L)
231 and moderate splenic lymphoid hyperplasia in all cases (Table 3).

232

233 *Immunohistochemistry*:

234 Natural infected goats showed positivity in mesenteric lymph nodes and in spleen while the
235 mammary glands were negative. On the contrary, in experimentally infected animals the spleen
236 was negative and the signal in the mammary gland was dependent on the viral dose administered
237 (Table 4). Notably, positivity in the lymph nodes was confined to the intracytoplasmic district of
238 the perifollicular cells.

239 The negative control, analysed by histological and immunohistochemical investigations, did not
240 show significant lesions or positive reactions in the target tissues (data not shown).

241

242 *Provirus quantification in blood leukocytes*

243 Both assays (*gag* and *env* qPCR) displayed a dynamic detection pattern comprised from 10^7 to 10^1
244 copies, with a Pearson's linear correlation ranging between 0.98 and 0.99 respectively. Provirus
245 was detected in all animals and the sequencing of amplified target genes (*gag* and *env*) confirmed
246 the SRLV genotype E1 infection (data not shown).
247 At the beginning of the experiment, all tested animals in the PCR-*env* time-course analysis were
248 negative (data not shown). The proviral load in blood samples of group A was highly variable with
249 three goats above 10^4 copies/100 ng of DNA and a peak at 120 dpi in five goats. In VTF animals
250 from group B*,* infected cells were present at 30 and 60 dpi and decreased in the subsequent
251 experimental time points. In the remaining two animals (VIT) the viral load was negative or weakly
252 positive throughout all the experiment (Table 5).

253

254 *Tissue PCRs*

255 The copy numbers obtained by *gag* and *env* real time PCR in all animal tissues are reported in
256 Figure 3 for group A and in Figure 4 for group B. In both groups the *gag* gene qPCR was more
257 sensitive than the *env* gene qPCR. Indeed the positivity in group A was 15/112 tested tissues to
258 *gag* qPCR and 5/112 tested tissues to *env* qPCR, and in group B 30/60 tested tissues to *gag* qPCR,
259 5/60 tested tissues to *env* qPCR (copy number higher than 10).
260 In group A there were differences among both, individuals and tissues. In detail, goats 1, 3 and 8
261 showed higher and more dispersed proviral load in the various tissues tested. Spleen and
262 bronchoalveolar lavage resulted positive in 4 animals, and the highest viral load of this group was
263 detected in bronchoalveolar lavage of goat number 5. Notably, Peyer's patches, mammary lymph
264 node and mediastinal lymph nodes were positive in 2 out of 6 naturally infected animals. The

mesenteric lymph node, mammary gland, synovial membrane, choroid plexus and thymus resulted negative in all animals. The animals 2 and 6, and the control (animal 5) resulted negative to all the qPCRs (data not shown).

Interestingly, DNA from testicle of male 9, which suffered from orchitis, gave positive results (Figure 3).

The qPCR (*gag* and *env*) analysis of group B showed the presence of provirus in almost all the tissues analysed, although proviral load was highly variable with remarkable differences depending on the route and dose. The highest number of infected tissues was detected in goat VIT-H, and the goat inoculated VTF-H presented the lower number of tissues with a detectable proviral load (Figure 4). Infected cells were found in mediastinal lymph nodes, mammary gland, mammary lymph node and spleen from all the 4 experimentally infected goats independent from the route and dose. The highest copy numbers were found in Peyer's patches from goat VIT-L (192798/100ng genomic DNA). The VIT infected animals showed positive provirus amplification in Peyer's patches as well as the choroid plexus in contrast to VTF inoculation. In VIT goats a slight positivity was detected in lungs and a marked positivity in the bronchoalveolar lavage of goat VIT-H was also observed. Instead, the mesenteric and mammary lymph nodes and, to a lesser extent, the bone marrow samples were found positive in the two VTF animals.

The sequences of some positive samples were analysed and contamination was excluded since *gag* and *env* specific sequences were identified as *Roccaverano* strain (data not shown).

Multiple spliced (MS) mRNA expression patterns

To assess viral active replication sites, MS *mRNA* expression was analysed in all tissues by *rev* rt-qPCR. The *rev* assay displayed a dynamic detection range comprised from 10^7 to 10^1 target copies, with a correlation between 0.98 and 0.99. MS *mRNA* was absent in all tissues of group A animals.

289 *Rev* expression patterns (MS mRNA) differed amongst the animals from group B (Fig. 4). The goat
290 inoculated VIT-H had the highest rate of virus replication in the following tissues: mediastinal
291 lymph nodes (2904 copies/100 ng DNA), mammary gland (1774 copies/100ng DNA), spleen (661
292 copies/100ng DNA), choroid plexus (296 copies/100ng DNA) and bronchoalveolar lavage (145
293 copies/100ng DNA); goat VIT-L: mammary lymph nodes (288 copies/100ng DNA) and mammary
294 gland (1827 copies/100ng DNA); goat VTF-L: mammary lymph nodes (567 copies/100ng DNA) and
295 spleen (661copies/100ng DNA); goat VTF H did not show detectable viral load in tissues.
296
297 **Discussion:**
298 *Roccaverano* strain of SRLV is naturally characterized by the presence of three distinctive genetic
299 deletions which hypothetically may confer a unique low pathogenicity *in vivo* accompanied by the
300 absence of any classical lentiviral cytopathic effect *in vitro* (Juganaru et al., 2011). Immunization
301 with *Roccaverano* strain elicited humoral and T-cell responses, accompanied by a broadly-reactive
302 cytotoxic T lymphocytes induction, which lead to protection against the highly pathogenic SRLV
303 subtype B. (Bertolotti et al., 2013) These features, together with its sheltering capacity towards
304 pathogenic lentiviral infections, prompted us to test some biological features of SRLV *Roccaverano*
305 strain *in vivo*. For this, goats naturally (group A) and experimentally (group B) infected were
306 evaluated for seroconversion, pathological lesions and distribution of virus in tissues. Moreover,
307 we differentiated the anatomical districts in which the virus was actively replicating from those
308 which were simply colonized, by monitoring MS RNA and proviral load respectively.
309 Almost half of the naturally infected animals showed seroconversion, likely reflecting the
310 circulation of infective virus in the herd. Animals experimentally infected, which received a higher
311 viral dose, presented a rapid seroconversion, as expected.

312 The severity of the pathological lesions has been linked to the ranks of viral replication (Turelli et
313 al., 1997). Thus, low levels of viral replication may account for a slow progression of the disease
314 (Blacklaws, 2012). In addition, it has been shown that virus with deletions of the dUTPase and vpr-
315 like genes display reduced viral loads and low pathogenicity *in vivo* (Hizi and Herzig, 2015; Lerner
316 et al., 1995; Lichtenstein et al., 1995; Sato et al., 2013). The histopathological analysis, together
317 with the analysis of viral replication conducted in this study, confirm the above literature data,
318 thus supporting the *in vitro* previous designation of genotype E1 as a low pathogenic SRLV (Grego
319 et al., 2007a).
320 In naturally infected animals, the proviral load in blood mimicked its presence in tissues.
321 Conversely, in experimentally infected animals the proviral load in blood varied significantly
322 among goats even considering the same route of infection. This suggests an important role of the
323 individual genetic background, as previously demonstrated for other viral genotypes (da Cruz et
324 al., 2013). However, definitive conclusions on this regard were hampered by ethical and technical
325 issues since the experimental protocol authorization restricted the use of only one animal per
326 route and dose of virus infection.
327 The tissue PCR analysis demonstrated that the intra-tracheal inoculation route was more efficient
328 for the establishment of the infection. The viral load and the number of infected tissues was
329 higher in animals of group B than in group A, most likely reflecting that experimentally infected
330 goats (group B) have received a higher viral dose compared to the naturally infected animals. In
331 agreement with this theory it has been hypothesised that the establishment of a SRLV infection
332 may require two -or more- subsequent contacts with the virus (Shah et al., 2004).
333 The tissue distribution of genotype E1 observed in our study is quite similar to the one reported in
334 animals infected with SRLV genotype B (Ravazzolo et al. 2006). The most commonly infected tissue
335 by SRLV genotype E1 seems to be the lymphoid compartment, in both experimental groups

supporting its designation as the viral reservoir tissue of genotype E1, as previously suggested for other lentiviruses (Embretson et al., 1993). Tissue positivity correlated with the presence of macrophages in spleen (sinusoid lining cells), lungs (dust-alveolar cells) or testis (testicular macrophages). The genotype E1 was also present in the mammary gland or in associated tissues; this may ensure the SRLV transmission to offspring since the main transmission route in goats is lactogenic (Blacklaws et al., 2004; Gjerset et al., 2009). The data obtained from the mammary gland supported the results obtained by Deubelbeiss et al. (2014), in which the low pathogenic virus genotype A4 was abundantly represented in the mammary gland. In contrast to the genotype A4, the increased genotype E1 proviral load was not involved in the development of histopathological lesions, thus confirming genotype E1 as a highly attenuated SRLV.

Although this experiment is not sufficient to document the long term progression of the lesions, the poor ability of E1 subtype to infect cell types other than macrophages (Juganaru et al., 2011) may reduce tissue antigen expression thus interfering with the immunopathological process characteristic in SRLV-induced disease (Blacklaws, 2012).

There was no evidence of viral replication in group A animals and a low viral replication rate was detected in group B. We can speculate that natural infection with *Roccaverano* strain, although established, proceeded with a viral replication rate below the detection limit of the qPCR technique used. The analysis of RNA MS further confirmed that the optimal route of inoculation was intratracheally since the highest rate of viral replication was detected. Mammary gland and/or mammary lymph nodes in three animals of group B showed the highest RNA MS viral load. This result further supports that lactogenic transmission is an efficient natural route of this strain as previously demonstrated for subgroups B1 and A10 (Pisoni et al., 2010). The bone marrow seems not to be a relevant replication site of this virus as reported for genotype B of SRLV (Ravazzolo et al., 2006).

Interestingly, we found a high genotype E1 viral load in tissues and in blood with a ongoing inflammatory condition such as orchitis (goat 9), lung parasitism (goat 4) and bronchitis (goat 8). A possible explanation is that the recruitment of macrophages at the inflammation sites, secondary to a infective status, increases the chances of infection as it has been previously described for *Brucella ovis* and CAEV (de la Concha-Bermejillo et al., 2013; Preziuso et al., 2013).

In conclusion, we suggest that: i) the most probable and efficient reservoirs of genotype E1 SRLV are the monocyte-macrophage cells, and consequently all organs/tissues characterised by an abundant presence of these cells, either for their own anatomical organization (e.g. mammary glands) or for occasional recruitment induced by an infection/inflammatory status; ii) proviral load is a more constant and reliable marker than viral genomic RNA to assess the virus presence in infected animals (Ravazzolo et al., 2006); iii) the absence of pathological manifestations in *Roccaverano* infected goats, together with accumulated deletions and common ancestor estimations, may reflect a step further in the attenuation of SRLV, likely consequence of the long co-evolution between this lentivirus and goats.

Conflict of interest

No competing financial interests exist.

Acknowledgements

This work was co-funded by the Italian Ministry of Instruction, University and Research PRIN 2008, by Piedmont Region, “Ricerca Sanitaria Finalizzata’’ 2008 and 2009, and by University of Turin, “Fondi ricerca locale (ex-60%)” 2009. The Authors acknowledge Mr. R. Maritano, CISRA for his valuable contribution in animal management, and Mr. D. Arnulfo and R. Vanni for their competent

384 work and assistance during animal autopsies. We thank Dr. A. Quasso, veterinary officer, Local
385 Health Unit ASLAT, and the Roccaverano goat breeders for their invaluable support and
386 collaboration during field activities.
387 Prof. P. Accornero is acknowledged for his contribution and useful discussion on the postnatal
388 mammary gland development and theory of recruitment.
389 R. Reina was supported by Spanish Ministry of Science and Innovation 'Ramón y Cajal' contract.
390

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