1 **Viral load, tissue distribution and histopathological lesions in goats naturally and experimentally**

2 **infected with the Small Ruminant Lentivirus Genotype E (subtype E1 *Roccaverano* strain).**

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25   ***Abstract***:

26   Small Ruminant Lentivirus (SRLV) subtype E1, also known as *Roccaverano* strain, is considered a

27   low pathogenic virus on the basis of natural genetic deletions, *in vitro* properties and on-farm

28   observations. In order to gain more knowledge on this atypical lentivirus we investigated the *in*

29   *vivo* tropism of *Roccaverano* strain in both, experimentally and naturally infected goats. Antibody

30   responses were monitored as well as tissue distribution and viral load, evaluated by real time PCR

31   on single spliced (*gag/env)* and multiple spliced (*rev)* RNA targets respectively, that were

32   compared to histopathological lesions. Lymph nodes, spleen, alveolar macrophages and mammary

33   gland turned out to be the main tissue reservoirs of genotype E1-provirus. Moreover, mammary

34   gland and/or mammary lymph nodes acted as active replication sites in dairy goats, supporting the

35   lactogenic transmission of this virus. Notably, a direct association between viral load and

36   concomitant infection or inflammatory processes was evident within organs such as spleen, lung

37   and testis.

38   Our results validate the low pathogenicity designation of SRLV genotype E1 *in vivo,* and confirm

39   the monocyte-macrophage cell lineage as the main virus reservoir of this genotype. Accordingly,

40   SRLV genotype E displays a tropism towards all tissues characterised by an abundant presence of

41   these cells, either for their own anatomical structure or for an occasional infectious/inflammatory

42   status.

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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

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

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

265   mesenteric lymph node, mammary gland, synovial membrane, choroid plexus and thymus

266   resulted negative in all animals. The animals 2 and 6, and the control (animal 5) resulted negative

267   to all the qPCRs (data not shown).

268   Interestingly, DNA from testicle of male 9, which suffered from orchitis, gave positive results

269   (Figure 3).

270   The qPCR (*gag* and *env*) analysis of group B showed the presence of provirus in almost all the

271   tissues analysed, although proviral load was highly variable with remarkable differences

272   depending on the route and dose. The highest number of infected tissues was detected in goat

273   VIT-H, and the goat inoculated VTF-H presented the lower number of tissues with a detectable

274   proviral load (Figure 4). Infected cells were found in mediastinal lymph nodes, mammary gland,

275   mammary lymph node and spleen from all the 4 experimentally infected goats independent from

276   the route and dose. The highest copy numbers were found in Peyer's patches from goat VIT-L

277   (192798/100ng genomic DNA). The VIT infected animals showed positive provirus amplification in

278   Peyer's patches as well as the choroid plexus in contrast to VTF inoculation. In VIT goats a slight

279   positivity was detected in lungs and a marked positivity in the bronchoalveolar lavage of goat VIT-

280   H was also observed. Instead, the mesenteric and mammary lymph nodes and, to a lesser extent,

281   the bone marrow samples were found positive in the two VTF animals.

282   The sequences of some positive samples were analysed and contamination was excluded since

283   *gag* and *env* specific sequences were identified as *Roccaverano* strain (data not shown).

284

285   *Multiple spliced (MS) mRNA expression patterns*

286   To assess viral active replication sites, MS *mRNA* expression was analysed in all tissues by *rev* rt-

287   qPCR. The *rev* assay displayed a dynamic detection range comprised from 10^7 to 10^1 target copies,

288   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

336   supporting its designation as the viral reservoir tissue of genotype E1, as previously suggested for

337   other lentiviruses (Embretson et al., 1993). Tissue positivity correlated with the presence of

338   macrophages in spleen (sinusoid lining cells), lungs (dust-alveolar cells) or testis (testicular

339   macrophages). The genotype E1 was also present in the mammary gland or in associated tissues;

340   this may ensure the SRLV transmission to offspring since the main transmission route in goats is

341   lactogenic (Blacklaws et al., 2004; Gjerset et al., 2009). The data obtained from the mammary

342   gland supported the results obtained by Deubelbeiss et al. (2014), in which the low pathogenic

343   virus genotype A4 was abundantly represented in the mammary gland. In contrast to the

344   genotype A4, the increased genotype E1 proviral load was not involved in the development of

345   histopathological lesions, thus confirming genotype E1 as a highly attenuated SRLV.

346   Although this experiment is not sufficient to document the long term progression of the lesions,

347   the poor ability of E1 subtype to infect cell types other than macrophages (Juganaru et al., 2011)

348   may reduce tissue antigen expression thus interfering with the immunopathological process

349   characteristic in SRLV-induced disease (Blacklaws, 2012).

350   There was no evidence of viral replication in group A animals and a low viral replication rate was

351   detected in group B. We can speculate that natural infection with *Roccaverano* strain, although

352   established, proceeded with a viral replication rate below the detection limit of the qPCR

353   technique used. The analysis of RNA MS further confirmed that the optimal route of inoculation

354   was intratracheally since the highest rate of viral replication was detected. Mammary gland and/or

355   mammary lymph nodes in three animals of group B showed the highest RNA MS viral load. This

356   result further supports that lactogenic transmission is an efficient natural route of this strain as

357   previously demonstrated for subgroups B1 and A10 (Pisoni et al., 2010). The bone marrow seems

358   not to be a relevant replication site of this virus as reported for genotype B of SRLV (Ravazzolo et

359   al., 2006).

360　Interestingly, we found a high genotype E1 viral load in tissues and in blood with a ongoing

361　inflammatory condition such as orchitis (goat 9), lung parasitism (goat 4) and bronchitis (goat 8). A

362　possible explanation is that the recruitment of macrophages at the inflammation sites, secondary

363　to a infective status, increases the chances of infection as it has been previously described for

364　*Brucella ovis* and CAEV (de la Concha-Bermejillo et al., 2013; Preziuso et al., 2013).

365　In conclusion, we suggest that: i) the most probable and efficient reservoirs of genotype E1 SRLV

366　are the monocyte-macrophage cells, and consequently all organs/tissues characterised by an

367　abundant presence of these cells, either for their own anatomical organization (e.g. mammary

368　glands) or for occasional recruitment induced by an infection/inflammatory status; ii) proviral load

369　is a more constant and reliable marker than viral genomic RNA to assess the virus presence in

370　infected animals (Ravazzolo et al., 2006); iii) the absence of pathological manifestations in

371　*Roccaverano* infected goats, together with accumulated deletions and common ancestor

372　estimations, may reflect a step further in the attenuation of SRLV, likely consequence of the long

373　co-evolution between this lentivirus and goats.

374

375

376　**Conflict of interest**

377　No competing financial interests exist.

378

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