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Published version:

DOI:10.1016/j.vetmic.2012.12.004

Small ruminant lentivirus genotype B and E interaction: Evidences on the role of Roccaverano strain on reducing proviral load of the challenging CAEV strain

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Abstract
Live attenuated vaccines provide the most consistent protective immunity in experimental models of lentivirus infections. In this study we tested the hypothesis that animals infected with a naturally attenuated small ruminant lentivirus field strain of genotype E may control a challenge infection with a virulent strain of the caprine arthritis encephalitis virus (CAEV-CO). Within genotype E, Roccaverano strain has been described as attenuated since decreased arthritic pathological indexes were recorded in Roccaverano-infected animals compared to animals of the same breed infected with genotype B strains. Moreover, under natural conditions, animals double-infected with genotypes B and E appear less prone to develop SRLV-related disease, leading to a putative protective role of Roccaverano strain. Here we present evidence that goats experimentally infected with the avirulent genotype E SRLV-Roccaverano strain control the proviral load of a pathogenic challenge virus (CAEV-CO strain) more efficiently than naive animals and appear to limit the spread of histological lesions to the contralateral joints.

Introduction
Small ruminant lentiviruses (SRLV) are distributed around the world causing a multisystemic disease in sheep and goats leading to production losses as well as to consequences in animal trade and welfare. Target organs typically, the lungs, udder, carpal joints and the central nervous system, are affected by inflammatory process characterized by a dense cellular infiltrate of mononuclear leukocytes, mainly lymphocytes and, to a lesser extent, B cells, plasma cells and macrophages the latter being the SRLV target cell in vivo (Lyall et al., 2000; von Bodungen et al., 1998; Wilkerson et al., 1995). Control is mainly based on serological diagnosis and culling of the seropositive animals. However, detection of antibodies is affected by antigenic heterogeneity among SRLV genotypes, which makes serological testing an inaccurate control method if heterologous diagnostic antigen is employed (Ramirez et al., 2009; Reina et al., 2009c). SRLV are genetically divided into five genotypes (Bertolotti et al., 2011; Grego et al., 2007; Shah et al., 2004). Among them, it is well known the association between the genotype B and the caprine arthritis encephalitis disease, causing a threat on animal welfare and in milk production of small ruminants. In contrast, the lastly described genotype E was first isolated in few local Roccaverano breed goats in Piedmont, Italy, where no SRLV related clinical signs were reported by breeders or practitioners. Histopathological evidence of pathology was also reduced when evaluated under natural and experimental

conditions (Grego, unpublished observations). These findings together with natural deletions of the dUTPase subunit of the pol gene and the vpr-like gene, within viral genome, led us to tentatively term Roccaverano strain, as low pathogenic caprine lentivirus (Juganaru et al., 2011; Reina et al., 2011). In addition, the Roccaverano strain was identified in a flock in which animals were found co-infected with the pathogenic CAEV-like genotype B, with no apparent development of SRLV-related disease. Current opinion among local breeders and practitioners is that the Roccaverano goat breed is resistant to CAEV infection, compared to Alpine and Saanen breeds. How- ever, Roccaverano breed goats infected by field genotype B strains did develop arthritis (Reina et al., 2009c). In the light of the discovery of genotype E infection, preexisting to the introduction of CAEV-like strains in this local population, a new approach should be taken into account to explain breed resistance: could genotype E infection act as live attenuated vaccine, able to induce resistance to superinfection versus heterologous strains?

We recently evaluated immunological parameters in goats experimentally infected with the Roccaverano strain using homologous and heterologous antigens. Results clearly indicated that humoral and T cell proliferation responses were strictly detected against recombinant antigens from the homologous genotype, while cytotoxic-T-lymphocyte (CTL) activity was not strain specific, being surprisingly higher against genotype B infected antigen presenting cells (Reina et al., 2011). Thus CTL activity is the sole adaptive immune response which could be associated with protection against the heterologous strain. Following these experiments, in this study we have explored the potential protective role of genotype E in vivo under strictly controlled conditions by infecting goats with Roccaverano strain followed by a challenge with pathogenic CAEV-CO strain. Results indicate that previous infection with genotype E results in a decreased proviral load of the challenge strain, acting itself as a potential natural prevention strategy to control SRLV infection.

Materials and methods

Cells and viruses

Caprine peripheral blood mononuclear cells (PBMCs) were obtained from SRLV-free animals and derived macrophages (BDMs) were allowed to differentiate for 9–15 days as described (Juganaru et al., 2011). BDMs were used to propagate and titrate Roccaverano strain. Goat synovial membrane primary cell line was used to propagate and titrate CAEV Cork strain.

Attenuated SRLV strain Roccaverano (genotype E, subtype E1) was isolated and characterized in previous studies (Reina et al., 2009b). The pUC9kb-CAEV (Hess et al., 1986) and pCAEVLTR-cat (Pyper et al., 1986) plasmids containing two fragments that allow reconstitution of replication-competent CAEV-CO virus upon co-transfection of primary goat synovial membrane cells (GSM) were kindly supplied by Dr. R. Vigne (Marseille, France).

Animals

Animals from a certified SRLV-free herd formed by Roccaverano breed goats as previously described (Reina et al., 2009c), were used in this study. A total of 22 female goats were purchased as weaned kids and introduced into the experimental facilities at the Faculty of Veterinary Medicine, University of Turin, Grugliasco, Italy (CISRA FMV UNITO) 18 months before the experimental infection. Animals were tested monthly and found consistently negative for SRLV antibodies using genotype A-, B- and E- derived antigens for a one-year period. Animals included in experimental and control groups had similar age distribution, ranging from 9 to 24 months. During the experiment, a total of 22 goat kids were born between 243 and 355 days post challenge. In detail, 7 kids born from animals infected with Roccaverano strain, 11 from animals infected with CAEV-CO and 4 from uninfected animals.

Experiments were carried out in compliance with the relevant National legislation on experimental animals and animal welfare, upon authorization by the competent authority (Italian Ministry of Health-Directorate General Animal Health-Office VI; permit no. 07/2009B).

Experimental design

Animals were divided into three groups. Group A included 8 animals that were subjected to experimental infection with Roccaverano strain inoculated intra-tracheally with 2 ml of 2.5 x 10^5 TCID50/ml. After 4 months, animals were challenged with CAEV-CO through direct inoculation of 0.5 ml of 106 TCID50/ml per animal in the right carpal joint, after removal of an equal volume of synovial fluid. Group B included 8 animals infected with the CAEV-CO strain at the same time, dose and challenge method as group A. Group C included 6 healthy animals, as control group.

EDTA blood samples were obtained in all groups 15 days prior to infection (-15), at weekly interval until day 49, and bi-weekly until day 132, corresponding to the day of the challenge. After the CAEV-CO infection, EDTA blood samples were collected from all animals at weekly interval until day 169, bi-weekly until day 205, and then every 4–6 weeks until the end of experiment which lasted over one year after challenge with a total of 28 time points. From each blood sample plasma was collected after centrifugation and stored at -20°C for serological analysis and buffy coats were recovered, resuspended in PBS and stored at -20°C until DNA extraction for PCR studies. After parturition, colostrum was sampled and analyzed for proviral load as well as antibodies and blood samples were collected from all kids bi-weekly until the end of the experiment.

Serological analysis

In order to evaluate the serological response against each infecting strain, genotype specific and strain specific ELISA tests were applied. The first employs a recombinant p16-25 fusion protein derived from genotype B or E, which has been proven in previous study to discriminate between SRLV genotype B or E infection (Reina et al., 2009c). The second ELISA test is based on strain specific SU5 synthetic peptides which detect specific and precocious antibodies being successfully described as markers of infection (Bertoni et al., 2000; Mordasini et al., 2006). Synthetic peptide sequences of Roccaverano and CAEV-CO strains used in the second ELISA test (strain specific) were QVRAYTYGVIEMPTGYETPTIRRR and KVRAYTYGVIEMPE- NYAKTRIINRK respectively. All animals were tested at each time point and seroconversion against the two variants of the same antigen was independently monitored.

DNA extraction and proviral load quantification

DNA was isolated from buffy coats with DNeasy Blood and Tissue kit (Qiagen, Germany) and quantified by fluorimetry with Picogreen dsDNA Quantitation kit (Invitrogen, Carlsbad, CA). Two sets of strain-specific primers and probes were designed with Beacon Designer 7 (Premier Biosoft International) to set up a duplex q-PCR targeting pol sequences. Primer and probe sequences are reported in Table 1. PCR reactions were carried out in triplicate with Quantitech Multiplex PCR kit (Qiagen) in a volume of 25 ml with 200 nM each primer, 300 nM probe and 500 ng of DNA. After an initial activation at 95 8C for 15 min, reactions were subjected to 50 cycles of 94 8C for 15 s and 60 8C for 1 min. Negative controls were included in each assay, as well as serially diluted positive controls allowing quantification through estimations on a standard curve. In more details, two different plasmids carrying Roccaverano and CAEV-CO pol gene fragments spanning the real-time amplicons were

prepared. The former included a 3309 bp insert (nucleotides 3782–7090 along Roccaverano genome) while the latter carried a 453bp fragment, corresponding to nucleotides 1589–2131 in CAEV-CO genome. Serial dilutions from106 to 5copies/reaction of both plasmids were included in each assay to generate standard curves.
The coefficient of variation (COV) of the proviral copy number per reaction of the three replicas for each DNA template was calculated. A threshold value of 0.28 was established for DNAs having more than 10 copies per reaction and outliers were excluded from further analysis. No COV analysis was applied to DNA samples carrying less than 10 copies per reaction. Proviral load values were expressed as the average copy number per microgram of template DNA.

T cell proliferation
Measurement of T cell proliferation against homologous and heterologous antigens was carried out as described elsewhere (Reina et al., 2011). Briefly, PBMCs prepared by buffy coat centrifugation on Ficoll gradient were plated in 96-well plates at a concentration of 105 cells/well in RPMI-1640 medium (Sigma–Aldrich Company Ltd.) supplemented with 2 mM L-glutamine, 50 mM beta-mercaptoethanol, 100 U penicillin and 100 mg streptomycin/ml, 10% FBS (RPMI 10). PBMCs were incubated in quadruplicate with recombinant heterologous (genotype B) or autologous (genotype E) P25 antigen, or GST (as negative control) at equimolar amounts. Antigens were plated at 25, 12 and 6mg/ml in 200ml and after a five-day incubation, cells were labeled with 1 mCi of [3H] thymidine (Amersham) for 5 h. Incorporated radioactivity was determined using a Filter Cell Harvester 1540 (Wallac) and a Beta counter. Proliferation was measured as a stimulation index (SI) normalizing incorporated radioactivity in P25 wells with that obtained in the GST wells. The SI was calculated for each antigen using the formula: SI = cpm with antigen/cpm with GST protein.
An individual animal was considered to show positive T cell reactivity if the SI was greater than 3 in at least two antigen dilutions.

Clinical and histopathological examination
In order to determine the development of clinical arthritis, the circumference ratio between carpus and the contralateral metacarpus (c/m) was monitored. As commonly known, ratios above 1.8 indicated the presence of clinical arthritis (Ravazzolo et al., 2006).
Following euthanasia of experimental goats, samples from the left and the right carpal joints were collected and fixed in buffered formalin for 48h. Two synovial tissue samples from each sample were embedded in paraffin for histopathology. Four-micron sections were stained with hematoxylin and eosin staining (H&E) according to routine procedures. The slides were observed blindly by two independent pathologists monthly on three occasions. Each time, five pictures at 20x were evaluated from each sample. On the basis of the enlargement of the synovial lining cell layer, the density of the resident cells and the inflammatory infiltrate, histological lesions were blindly recorded as no lesion, mild, moderate or severe lesions. The mean value of the three lesion scores was considered as the final score. Final lesion score of the left and the right synovial samples were compared. Difference between scores (Delta Score, DS) recorded at the left and the right carpal joints was calculated for each animal, in order to quantify the worsening in clinical lesions in the contralateral joint compared to the inoculation site.

Statistical analysis
Real time PCR sensitivity and specificity were analyzed with Rotor-Gene software version 1.7 (Qiagen). Blood proviral load, clinical and histopathological lesion scores from groups A and B

were analyzed and compared using Student's t test or Wilcoxon rank-sum test at each time point (considering the normality of the distributions, tested using Shapiro–Wilk normality test), considering $p = 0.05$ as the level of significance. Fisher's exact test was applied for T cell proliferation frequencies of positive animals and to quantify the difference between groups considering qualitative proviral load estimation. All statistical tests were performed using R statistical software (R Core Team, 2012).

Results

Serological response

Seroconversion against Roccaverano and CAEV-CO strains was evaluated using indirect ELISA containing p16-25 recombinant protein and SU5 synthetic peptide derived from each of the two genotypes (Fig. 1).

In animals belonging to the group A, serological response was mounted exclusively against homologous antigens p16-15 and SU5 from 14 days post infection till the day of challenge on day 133. Peaks were reached at 44, 72, 105 and 142 days p.i. and afterwards decreased transiently to become consistently positive upon 233 days drawing the typical two-phase curve. Interestingly, the maximal antibody reactivity was reached before challenge. After CAEV-CO challenge, group A developed antibodies against genotype B antigen, thus becoming positive versus both genotypes with reactivity values close to the threshold until day 491 in which average reactivity reached more than 20% compared with positive controls (Fig. 1).

Seroconversion in animals from group B was readily observed after 2–3 weeks after challenge at day 133 against homologous antigen and remained well above detection limit till the end of the experiment. As expected, seroconversion against genotype E antigens was not generally observed with few time points close to the threshold limit (Fig. 1a and c).

Seroconversion against SU5 antigen reflected that to p16-25. Group A showed early seroconversion against both genotypes post-challenge while group B showed seroconversion exclusively against CAEV-CO SU5-derived antigen.

Seroconversion in goat kids was evaluated against genotypes B and E using both p16-25 and SU5 ELISA tests employing either plasma or colostrum as sample source. Both ELISA tests showed a slow decrease of passive immunity without apparent seroconversion in both groups up to 16 weeks after birth (Fig. 2).

3.2. Proviral load quantification

The linear range of amplification of the duplex q-PCR was determined from 106 to 5 copies per reaction using Roccaverano and CAEV-CO plasmid dilutions. The amplification of the standard dilutions showed linearity over six orders of magnitude ($1x10^6$ to 10 copies/reaction) with a target specificity of 100%. The assay was able to amplify at least one replica of the standard dilution containing 5 copies. The mean amplification efficiency of both targets was 97.0%.

The proviral load of both strains showed considerable variation during the time course of the experiment. Considering the CAEV strain, according to Fig. 3, differences between animal groups showed a trend toward significance at 317 days post challenge (Wilcoxon sum rank test $p < 0.10$) and progressively CAEV proviral load became significantly higher in animals belonging to the group B than ones belonging to the group A during the three following time points (day 364 $p < 0.05$, day 407 $p < 0.05$, day 462 $p < 0.05$). At the end of the experiment an increasing CAEV proviral load in group A animals resulted in non-significant differences between groups.

Roccaverano proviral load was analyzed in animals from group A showing detectable copies along all the post- challenge period reaching maximum peak at day 407 (mean number of

copies = 101.73/mg DNA). Genotype E proviral copy numbers were below the limit of detection in animals belonging to the group B as expected since they were not infected with Roccaverano strain (not shown).
Four group A and seven group B goats gave birth to seven and eleven kids respectively. Colostrum and blood were sampled from the mothers and blood was collected from the kids during four months. Reactions containing proviral load values within the limit of detection (4 copies/ 100ng DNA) were considered as positive (Table 2). Mothers from group A showed positive reactions to genotype E PCR in blood as expected since real time PCR was carried out between 373 and 485 days after infection with Roccaverano strain. Interestingly, only half of the mothers presented positive proviral loads in the colostrum. After challenge, although the majority of the adult animals from group A showed positive reactions for CAEV- CO provirus, statistical differences in the proportion of positive animals between groups A and B were found at days 317 and 407 (one-tailed Fisher's exact test $p < 0.05$). Group B animals showed as expected CAEV-CO positive proviral loads in some of the time points analyzed (Table 2). None of the kids born from group A goats carried the proviral CAEV genome, which was detected in three of the eleven kids born to group B goats. This result could suggest a possible difference in CAEV transmission to the progeny caused by the genotype E interference but, considering the small sample size, no statistical analyses can be carried out (Table 2).

T cell proliferation
As previously shown, infection with genotype E resulted in a genotype specific T cell proliferation (Reina et al., 2011). Challenge with CAEV-CO induced a broader response showing positive reactions versus both genotype derived antigens (data not shown).
Infection with CAEV-CO (group B) induced similarly a strain specific response with no detectable reaction against genotype E derived antigen.

Clinical and histopathological examination
Arthritic pathological score, expressed as the ratio between circumferences of the right carpal joint and the contralateral metacarpal one, was calculated for each animal at each time point. All animals showed ratio levels below 1.8 throughout the whole experiment and no differences were recorded among groups (Wilcoxon sum rank test $p > 0.05$ at each time point).
Right carpal joints (inoculation site) were generally more affected than left ones as expected. Precisely, lesions in the right synovial membrane samples were more severe than in the left in 5 goats belonging to the group A, while in 2 animals no difference was observed. In the group B, CAEV-CO infection worsened prognosis in the contralateral carpal joint since only 2 goats had right synovial membrane lesions more severe than those present in the left synovial membrane, while in 4 animals no difference was observed. No synovitis was observed in both right and left carpal joints of the group C animals (Table 3). Two out of three animals from group A showing severe lesions in the right carpal joint displayed no lesion in the left and a total of 5 animals did not show any worsening in the contralateral joint compared to the inoculated one. In group B this tendency was observed only from severe to low degree of lesion and only two animals presented better condition in the contralateral carpal joint (Table 2). In two cases (animals 2631 and 2634) the comparison between the left and the right synovial membrane samples was not possible because the synovial lining cell layer was not adequately present in two out of the four samples collected from each animal. In order to quantify the effect of CAEV infection in carpal joint lesions, we evaluated the difference between the scores obtained from left and the right carpal joint clinical evaluation. Negative DSs were observed in both groups, but given the low number of individuals, DSs distributions

were not statistically different (Wilcoxon test p = 0.1277). On the other hand, only DSs calculated on animals belonging to group A were statistically different from zero, which means ‘‘no changes’’ (Shapiro–Wilk normality test W = 0.88, p = 0.2153; Student’s t test t = 2.97, $p < 0.05$), showing that lesions recorded in the contralateral carpal joint were less severe than ones recorded at the inoculation site. In contrast, DSs in group B were not statistically different from zero (Wilcoxon test p = 0.1729), suggesting a stronger ability of the infection to reach the contralateral carpal joint.

Discussion

Attenuated virus vaccines are currently used for the prevention of a wide range of viruses such as Influenza virus (Sylte and Suarez, 2012), SIV (Sparger et al., 2008), EIAV (Ma et al., 2009), chicken pox or yellow fever (Craigo et al., 2011) conferring different degrees of protection. Among the strategies applied to immunize against HIV or SIV lentiviral infections, those based on live attenuated viruses have reached the highest levels of protection (Daniel et al., 1992). There are also several examples of effectiveness within lentiviral animal infections such as Feline immunodeficiency virus (FIV) and Equine infectious anemia virus (EIAV). FIV vaccines based on modified attenuated live viruses have been compared with subunit vaccines delivered as plasmid DNA or as protein, inducing moderate levels of cellular immunity and significant antibody responses conferring increased protection rates (Uhl et al., 2008). Regarding EIAV, among the various strategies explored, including immunizations based on attenuated viruses, inactivated virus particles, protein subunits, DNA vaccines, and live vectors, the highest level of protection was achieved when using attenuated viruses, likely due to the continuous antigen exposure and optimized maturation of the immune response. However, there is an inverse relationship between the level of protection and the level of attenuation, indicating that a minimal replication rate is needed for eliciting protective immune responses (Craigo et al., 2011). In the early 70s an attenuated strain (DLV120) obtained by in vitro passages in donkeys, conferred protection against EIAV challenge (Shen, 1983), and was extensively used in China with promising results (Ma et al., 2009).

Immunization against SRLV has been explored deeply in the last decades with occasionally disappointing results. Various strategies have been applied including artificially attenuated whole virus (Cutlip et al., 1987; Harmache et al., 1996; Zhang et al., 2003), subunit vaccines supplied as protein or as expression plasmids or recombinant viruses (de Andres et al., 2009; Gonzalez et al., 2005; Niesalla et al., 2009; Reina et al., 2009a). CAEV deletions of vif, essential for viral replication (Harmache et al., 1995a), induced weak responses with no protection whereas CAEV tat-, replication competent (Harmache et al., 1995b) conferred some degree of protection but still induced inflammatory lesions, further confirming the inverse relationship between attenuation and protection (Harmache et al., 1998).

Early approaches within DNA immunization were carried out with env and tat encoding plasmids and conferred some degree or protection related to Th1-biased response (Beyer et al., 2001; Cheevers et al., 2001, 2003). The newest approaches employed, in a series of vaccination experiments, DNA plasmids encoding gag and env viral genes and recombinant modified Vaccinia Ankara as inocula together with the employment of immunologic adjuvants such as IFN-gamma or B7 costimulatory molecules (de Andres et al., 2009). Immunity was stimulated enough to control viremia and proviral load in tissues but not in terms of reduced lesion development, with an increased inflammation in target tissues, probably masking a competent immune response (de Andres et al., 2009; Niesalla et al., 2009; Reina et al., 2009a). These SRLV vaccine experiments confirmed that virus-specific immune responses are a double edged sword that can contribute to either control or enhance virus replication or disease. In all previous vaccination studies, a common experimental design was the antigenic

homology between vaccine antigen and challenge strain, leading to conclude that humoral response, with different degree of stimulation recorded in the different vaccine strategies, may play a deleterious role in protection.
In this study, we infected goats with the naturally attenuated strain Roccaverano from the genotype E of SRLV, in order to induce protection against both the proviral load and the development of SRLV-related pathology. Goats were double infected with Roccaverano and CAEV-CO strains with an interval of 4 months (group A) or single-infected with CAEV-CO (group B). Serological analysis showed that, although a normal antibody response was elicited in both groups according to the strains used, a clear type specific reactivity was obtained, being the pre-challenge of group A reactive exclusively against genotype E antigen, becoming reactive in the post- challenge period against both antigens. Group B as expected showed reactivity against genotype B antigen. Similar results were obtained in lymphoproliferation assay, leading to suppose that, if any protection occurs, this may not be related to a Th2 response, which is thought to be non-effective to control the infection (Gonzalez et al., 2005; Perry et al., 1995).
Goats infected with genotypes E and B indeed showed lower proviral loads than goats single infected with genotype B strongly indicating that the Roccaverano infection may have induced a sustained immune response able to control genotype B proviral load and possibly, target tissues colonization. Proviral load and lesion development have been associated in a wide number of SRLV infection studies (Crespo et al., 2012; Herrmann- Hoesing et al., 2009; Ravazzolo et al., 2006; Zhang et al., 2000). Assuming this relationship, histopathological results were in line with proviral load ones, since dissemination of lesions was lower in Roccaverano infected animals. Even so, macroscopic lesions (arthritic pathological score) evaluation did not show evident differences. Explanations for this lack of contralateral lesions in group B, may include differences between reference strain CAEV-CO and field strains causing arthritis in Roccaverano goats.
Infection with Roccaverano and CAEV-CO strains induces exclusively homologous antibody and T proliferative responses, but infection with Roccaverano conferred protection against heterologous infection which is probably due to cross-reactive CTL activity from our previously published results (Reina et al., 2011).
Finally, we provided clear evidence that both viruses and antibodies against both SRLV strains passed to the progeny, although genotype B was not detected in kids raised by group A mothers (Fig. 2 and Table 2). This decreased viral flow to the progeny could reflect the lower proviral load found in the mothers, thus reducing viral transmission. Probably due to the short period of observation of the kids, we had not chance to evaluate the seroconversion against the viruses, but we only detected the decreasing of maternal antibody response.
The main goal of a vaccine is to achieve protection not only toward homologous strains but also against heterologous infection, this goal would be even more important in controlling lentiviral infections including SRLV, that are probably the most widely distributed lentivirus and one of the most heterogeneous. Genotype E Roccaverano strain infection could be of extensive application not only in naive goats but also in goats already infected with pathogenic genotype B. Roccaverano infection may open new approaches to naturally immunize against SRLV being the first naturally attenuated vaccine conferring protection against increased viral loads and lesion development.

Acknowledgements
This work was funded by the Italian Ministry of Instruction, University and Research PRIN 2008 and Piedmont Region "Ricerca Sanitaria Finalizzata" 2008; and from projects from CICYT (no. AGL2010-22341-C04- 01) and the Government of Navarra (no. IIQ14064.RI1). The

authors acknowledge dr. R. Maritano for his contribution in animal management and dr. D. Arnulfo, dr. R. Vanni and dr. L. Gay for the assistance during animal autopsies.

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Table 1
Primers and probes.

Primer or probe	Sequence (5′–3′)	Nucleotide position	Amplicon length (bp)
Roccaverano_Fw	AACACAGGAAGAGAAATAGTGAGG	3863–3886	114
Roccaverano_Rev	GAGCGATCTTGCATCTTGGTG	3976–3956	
Roccaverano_Probe	*FAM*-CCAGTGTTCTTCTTCCGCCTCCGG-*BHQ-1*	3949–3926	
CAEV-Co_Fw	AAAGAATGCAGAGGAAAGAGAGAC	1763–1786	106
CAEV-Co_Rev	GGTGCTGAAGTTATTCCATAGGAG	1868–1845	
CAEV-Co_Probe	*HEX*-CGGACGGCACCACACGTATCCC-*BHQ-1*	1841–1820	

Table 2
Proviral load of Roccaverano (RV) and CAEV-CO viral strains in mothers, colostrum and kids.

Group	Goat no.	Virus	Proviral load (days)											Colos-trum		Kids	
			133	149	162	205	233	247	317*	347	407*	462	491				
A	2588	RV	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+
		CAEV-CO	–	+	+	–	+	+	–	–	–	–	+	–	–	–	–
	2633	RV	+	+	+	–	–	+	–	+	+	+	+	–	+	–	
		CAEV-CO	+	+	–	–	–	+	–	+	–	+	–	–	–	–	
	50010	RV	+	+	+	+	+	+	+	+	+	+	+	–	–		
		CAEV-CO	–	–	–	+	+	+	–	+	–	–	–	–	–		
	50011	RV	+	–	+	+	+	+	+	+	+	+	+	+	+		
		CAEV-CO	–	–	–	+	–	–	–	+	–	–	+	+	–		
B	2587	CAEV-CO	nd	+	+	+	+	+	+	+	+	+	+	–	–	+	
	2631	CAEV-CO	nd	+	+	–	+	+	+	+	+	+	+	–	–	–	
	2635	CAEV-CO	nd	+	+	+	+	+	+	+	+	+	+	–	–		
	2637	CAEV-CO	nd	+	–	–	–	–	–	–	+	–	+	–	–		
	50012	CAEV-CO	–	–	+	+	+	+	+	+	+	+	+	+	+	+	
	50014	CAEV-CO	–	+	–	–	–	+	–	+	–	–	–	–	–		
	50015	CAEV-CO	–	+	+	–	+	+	+	+	+	+	+	–	–		

nd: not detectable
* Significant difference between proportion of CAEV-CO positive animals belonging to groups A and B (Fisher's exact test $p < 0.05$).

Table 3
Synovitis observed in right (R) and left (L) carpal joints.

Group	Animal no.	Right (R)	Left (L)	Comparison (R versus L)	Delta Score[a] (L–R)
A	50011	Low	No	>	Neg
A	2638	Moderate	Low	>	Neg
A	2629	Severe	Low	>	Neg
A	2633	Severe	No	>	Neg
A	2634	Unclassified	Moderate	[b]	[b]
A	50013	Low	Low	=	0
A	2588	Low	Low	=	0
A	50010	Severe	No	>	Neg
B	2637	Low	Low	=	0
B	2635	Severe	Low	>	Neg
B	50015	Low	Low	=	0
B	50012	Severe	Low	>	Neg
B	50014	Low	Low	=	0
B	2631	Moderate	Unclassified	[b]	[b]
B	2587	Low	Low	=	0
C	7686	No	No	=	0
C	7837	No	No	=	0
C	7841	No	No	=	0

[a] See text for details.
[b] Comparison not possible.

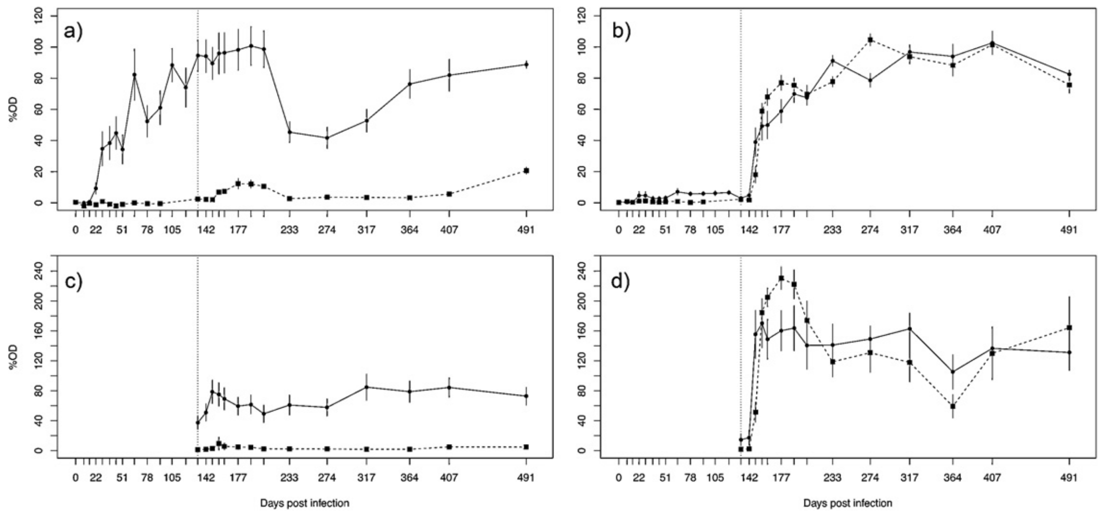

Fig. 1. Seroconversion to type specific antigens. Seroconversion against SRLV genotype E Roccaverano strain (a: p16-25 antigen; c: SU5 antigen) and against SRLV genotype B CAEV-Co strain (b: p16-25; d: SU5) in animals belonging to the group A (circles and solid line) and to the group B (squares and dashed line). Seroconversion is indicated as the average of reactivity among animals and it is expressed as the percentage against the homologous positive control. Vertical solid lines represent the standard error of the mean, calculated among animals belonging to the same group and within the same collection time. Vertical dotted line represents the time of challenge (day = 133).

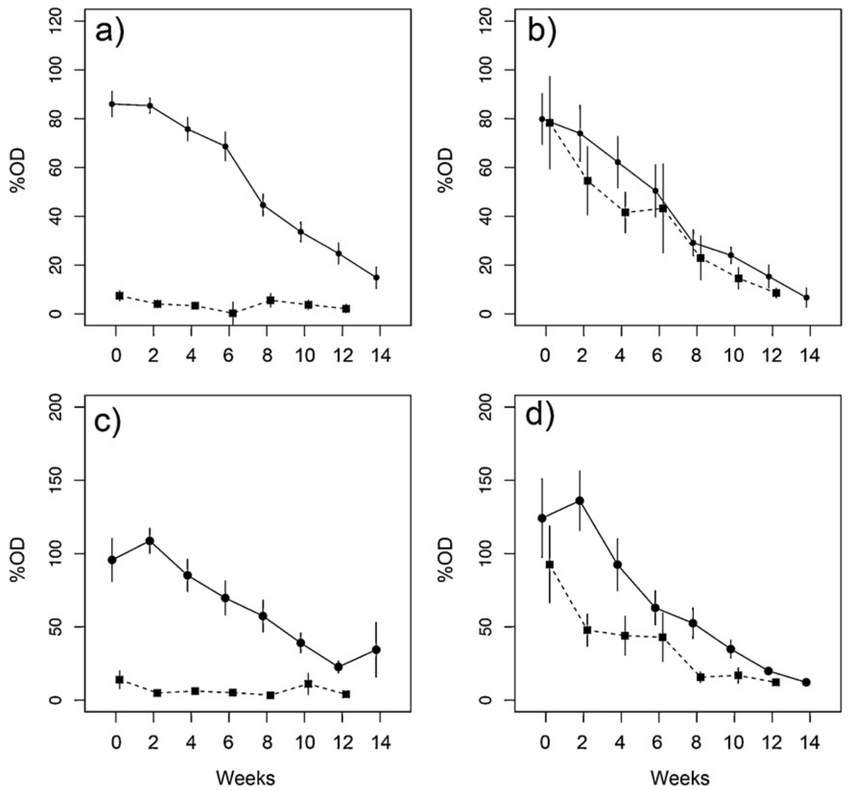

Fig. 2. Seroconversion in the progeny. Seroconversion against SRLV genotype E Roccaverano strain (a: p16-25 antigen; c: SU5 antigen) and against SRLV genotype B CAEV-Co strain (b: p16-25; d: SU5) in kids born from animals belonging to the group A (circles and solid line) and to the group B (squares and dashed line). Seroconversion is indicated as the average of reactivity among animals and it is expressed as the percentage against the homologous positive control. Vertical solid lines represent the standard error of the mean, calculated among animals belonging to the same group and within the same collection time.

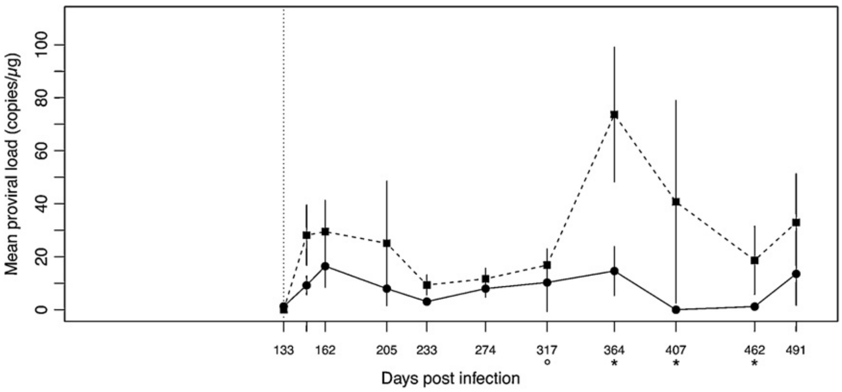

Fig. 3. CAEV-CO proviral load. Mean number of CAEV-CO provirus copies in blood of animals belonging to the group A (circles and solid line) and B (squares and dashed line). Vertical solid lines represent the standard error of the mean, calculated among animals belonging to the same group and within the same collection time. Vertical dotted line represents the time of challenge (day = 133). Statistical differences are reported on the x axis ('8': Wilcoxon rank sum test $p < 0.10$; '*': Wilcoxon rank sum test $p < 0.05$).