Small Ruminant Lentivirus genotype E is widespread in Sarda Goat

Reina Ramsés[1,2], Bertolotti Luigi[1], Dei Giudici Silvia[3], Puggioni Giantonella[3], Ponti Nicoletta[3], Profiti Margherita[1], Patta Cristiana[3], Rosati Sergio[1]*

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

[2] Instituto de Agrobiotecnología, CSIC-UPNA-Gobierno de Navarra, Ctra. Mutilva Baja, 31192 Mutilva Baja, Navarra, Spain

[3] Istituto Zooprofilattico Sperimentale della Sardegna, Sassari, Italy

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

Abstract

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

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

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

41 **goat.**

42

Introduction

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

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

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

Materials and Methods

Blood (serum and DNA) and milk samples

Caprine herds or ovine flocks were selected randomly among the Sardinian population, involving the most populated areas on the island.

Individual whole blood from 20 ovine flocks and from 30 caprine herds were initially collected and serum was stored at -20ºC until ELISA testing. Buffy coats and milk were obtained from 21 samples belonging to three of the caprine herds and DNA was extracted using DNA blood minikit (Qiagen). Following this preliminary serological survey, 186 bulk milk samples were collected from additional caprine or mixed herds and subjected to milk-adapted ELISA (Fig. 1). For both sera and bulk milk, appropriate positive and negative controls were included in each test, including samples from three caprine herds characterized in a previous study and known to be infected with genotype B, genotype E and both genotypes (Reina *et al.*, 2009b).

ELISA comparative assays

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

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

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

PCR, sequencing and phylogenetic analysis

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

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

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

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

Virus isolation and genome sequencing

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

Nucleotide accession numbers

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

Results

P16-25 ELISA and sub-ELISA

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

Following this preliminary screening, we used bulk milk from a total of 186 goat herds to estimate the real prevalence of genotype E within the Sarda goat population. Based on milk adapted P16-25 ELISA, serotyping was not always possible due to highly reactive samples, which reached a saturation level against both B and E derived antigens. Although titration of highly reactive samples may has overcome this drawback, subunit-ELISA was able to serotype most milk samples but sensitivity was obviously lower than that obtained by P16-25 ELISA. When both methods were merged, the estimation of 74% of SRLV seroprevalence was found in the Sardinian goat herds. Among these, 19.41% were infected with genotype B, 44.12% with genotype E and 10.59% were infected by

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

PCR sequencing and phylogenetic analysis

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

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

Complete genome sequencing and in vitro properties

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

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

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

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

Discussion

As hypothesised in the previous study, the lack of a specific serological tool allowed no speculation as to the distribution of genotype E in geographical locations different from the one where it was initially described (Reina *et al.*, 2009b). Serological data from different goat herds, sequence analysis of specific PCR products from three infected flocks and the full length proviral genome sequence of a local strain demonstrate that genotype E infection is associated and widely distributed in the Sarda goat, while Sarda sheep seems to harbour a genotype B (CAEV-like) lentivirus, a common feature in Italian sheep population (Grego *et al*., 2002).

Sarda goat, unlike the Roccaverano breed, represents an important goat population with economic significance at a local and national level. Moreover, since the population size of the Sarda goat is not comparable to the Roccaverano breed, in terms of average number of head per flock, farming system, management and productive levels, the biological significance of genotype E as low pathogenic caprine lentivirus needs to be redefined. The tree topology indicates a clear divergence between Roccaverano and *Seui* strains, showing quite different clade structures and features. These differences in the evolutionary pathway can be justified by epidemiological and historical data. In fact, Roccaverano goats were at risk of extinction in the early sixties, when people abandoned rural areas in favour to towns, following industrial development. This social behaviour forced the goat breed to pass through a bottleneck, and drove viral evolution to take advantage, on one hand of reducing virulence and on the other hand of persisting in a small population, limiting the transmission to lactogenic route. In Sardinia,

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

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

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

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

Acknowledgements

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

References

Ajmone-Marsan, P., Negrini, R., Crepaldi, P., Milanesi, E., Gorni, C., Valentini, A., Cicogna, M., 2001, Assessing genetic diversity in Italian goat populations using AFLP markers. Anim Genet 32, 281-288.

Canon, J., Garcia, D., Garcia-Atance, M.A., Obexer-Ruff, G., Lenstra, J.A., Ajmone-Marsan, P., Dunner, S., 2006, Geographical partitioning of goat diversity in Europe and the Middle East. Anim Genet 37, 327-334.

Gjerset, B., Storset, A.K., Rimstad, E., 2006, Genetic diversity of small-ruminant lentiviruses: characterization of Norwegian isolates of Caprine arthritis encephalitis virus. J Gen Virol 87, 573-580.

Glaria I, Reina R, Crespo H, de Andrés X, Ramírez H, Biescas E, Pérez MM, Badiola J, Luján L, Amorena B, de Andrés D., 2009, Phylogenetic analysis of SRLV sequences from an arthritic sheep outbreak demonstrates the introduction of CAEV-like viruses among Spanish sheep. Vet Microbiol Jul 2;138(1-2):156-62. Epub 2009 Mar 13.

Grego E, Profiti M, Giammarioli M, Giannino L, Rutili D, Woodall C, Rosati S., 2002, Genetic heterogeneity of small ruminant lentiviruses involves immunodominant epitope of capsid antigen and affects sensitivity of single-strain-based immunoassay. Clin Diagn Lab Immunol Jul;9(4):828-32.

Grego, E., Bertolotti, L., Quasso, A., Profiti, M., Lacerenza, D., Muz, D., Rosati, S., 2007, Genetic characterization of small ruminant lentivirus in Italian mixed flocks: evidence for a novel genotype circulating in a local goat population. J Gen Virol 88, 3423-3427.

Harmache, A., Vitu, C., Guiguen, F., Russo, P., Bertoni, G., Pepin, M., Vigne, R., Suzan, M., 1998, Priming with tat-deleted caprine arthritis encephalitis virus (CAEV) proviral DNA or live virus protects goats from challenge with pathogenic CAEV. J Virol 72, 6796-6804.

Hotzel, I., Kumpula-McWhirter, N., Cheevers, W.P., 2002, Rapid evolution of two discrete regions of the caprine arthritis-encephalitis virus envelope surface glycoprotein during persistent infection. Virus Res 84, 17-25.

Nei, M., 1987, Molecular Evolutionary Genetics. Columbia University Press, New York, NY.

Posada, D., Crandall, K.A., 1998, MODELTEST: testing the model of DNA substitution. Bioinformatics 14, 817-818.

Reina, R., Grego, E., Bertolotti, L., De Meneghi, D., Rosati, S., 2009a, Genome analysis of small ruminant lentivirus genotype E: a caprine lentivirus naturally deleted for dUTPase subunit, vpr-like accessory gene and the 70 bp repeat of U3 region. J Virol 83, 1152-1555.

Reina, R., Grego, E., Profiti, M., Glaria, I., Robino, P., Quasso, A., Amorena, B., Rosati, S., 2009b, Development of specific diagnostic test for small ruminant lentivirus genotype E. Vet Microbiol.

Robertson, D.L., Anderson, J.P., Bradac, J.A., Carr, J.K., Foley, B., Funkhouser, R.K., Gao, F., Hahn, B.H., Kalish, M.L., Kuiken, C., Learn, G.H., Leitner, T., McCutchan, F., Osmanov, S., Peeters, M., Pieniazek, D., Salminen, M., Sharp,

P.M., Wolinsky, S., Korber, B., 2000, HIV-1 nomenclature proposal. Science 288, 55-56.

Ronquist, F., Huelsenbeck, J.P., 2003, MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572-1574.

Saltarelli, M., Querat, G., Konings, D.A., Vigne, R., Clements, J.E., 1990, Nucleotide sequence and transcriptional analysis of molecular clones of CAEV which generate infectious virus. Virology 179, 347-364.

Sechi, T., Usai, M.G., Miari, S., Mura, L., Casu, S., Carta, A., 2007, Identifying native animals in crossbred populations: the case of the Sardinian goat population. Anim Genet 38, 614-620.

Shah, C., Boni, J., Huder, J.B., Vogt, H.R., Muhlherr, J., Zanoni, R., Miserez, R., Lutz, H., Schupbach, J., 2004, Phylogenetic analysis and reclassification of caprine and ovine lentiviruses based on 104 new isolates: evidence for regular sheep-to-goat transmission and worldwide propagation through livestock trade. Virology 319, 12-26.

Staskus KA, Retzel EF, Lewis ED, Silsby JL, St Cyr S, Rank JM, Wietgrefe SW, Haase AT, Cook R, Fast D, et al., 1991, Isolation of replication-competent molecular clones of visna virus, Virology Mar;181(1):228-40.

401 **Figure 1**. Map of the Sardinia island, divided in municipalities. A) goat herds
402 density. Grey level indicates goat herd density within the municipality: white =
403 less than 1st quartile (3 herds); light grey = between 1st and 2nd quartile (11 herds);
404 grey = between 2nd and 3rd quartile (15 herds); dark grey = more than the 3rd
405 quartile. B) Municipalities including flocks tested with bulk milk analysis (grey).
406 Circles = E positive flocks; squares = B positive flocks; triangles = coinfected (or
407 uncharacterised) flocks.
408
409 **Figure 2**. Net absorbance against E (x axis) and B (y axis) antigens. Dashed
410 diagonal line represents equal reactivity versus both antigens. Vertical and
411 horizontal dotted lines represent ELISA E and B cut-offs respectively.
412 A) Data from 19 ovine flocks and from 19 caprine herds. White circles: median
413 absorbance of samples belonging to goat herds. White triangles: median
414 absorbance of samples belonging to sheep flocks. Black squares: reactivity
415 detected in herds previously characterized (Reina *et al.*, 2009b) infected with B
416 (*a*), E (*c*) or both strains (*b*). Vertical and horizontal bars represent the variation
417 (interquartile range) in the distribution of absorbances within flock against B and
418 E antigens respectively.
419 B) Data from 186 goat herds tested using bulk milk sub-ELISA. Dots represent
420 tested flocks.
421
422 **Figure 3**. Phylogenetic tree constructed by Bayesian analysis of 33 partial *gag*
423 gene sequences (consensus alignment length: 525 bp). New sequences are

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

425 Posterior probabilities of clades are indicated above branches.

1 **Table 1**. Nucleotide sequence of primer pairs.

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

2

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

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

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

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

6

Figure1

Click here to download high resolution image

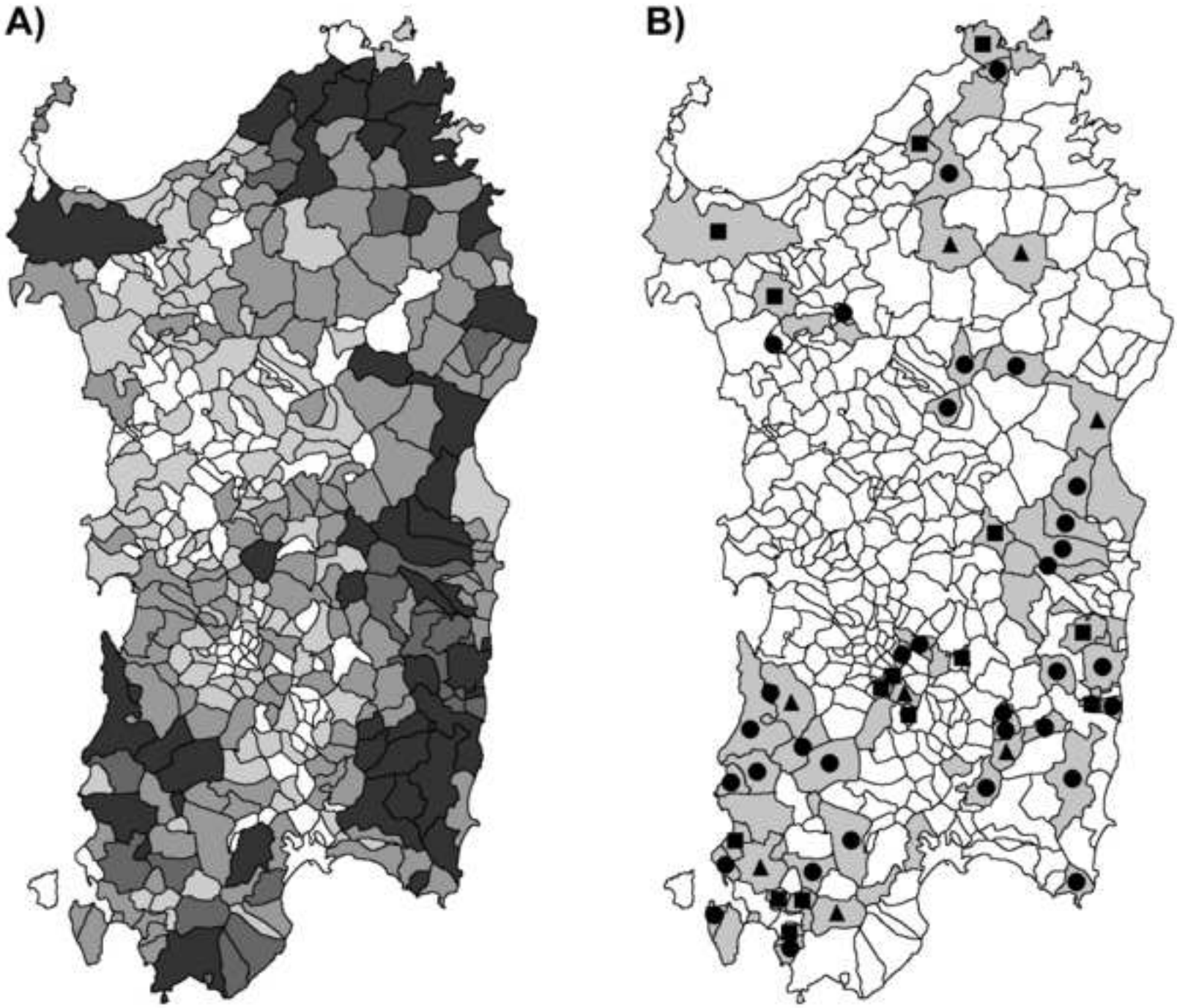

Figure2

Click here to download high resolution image

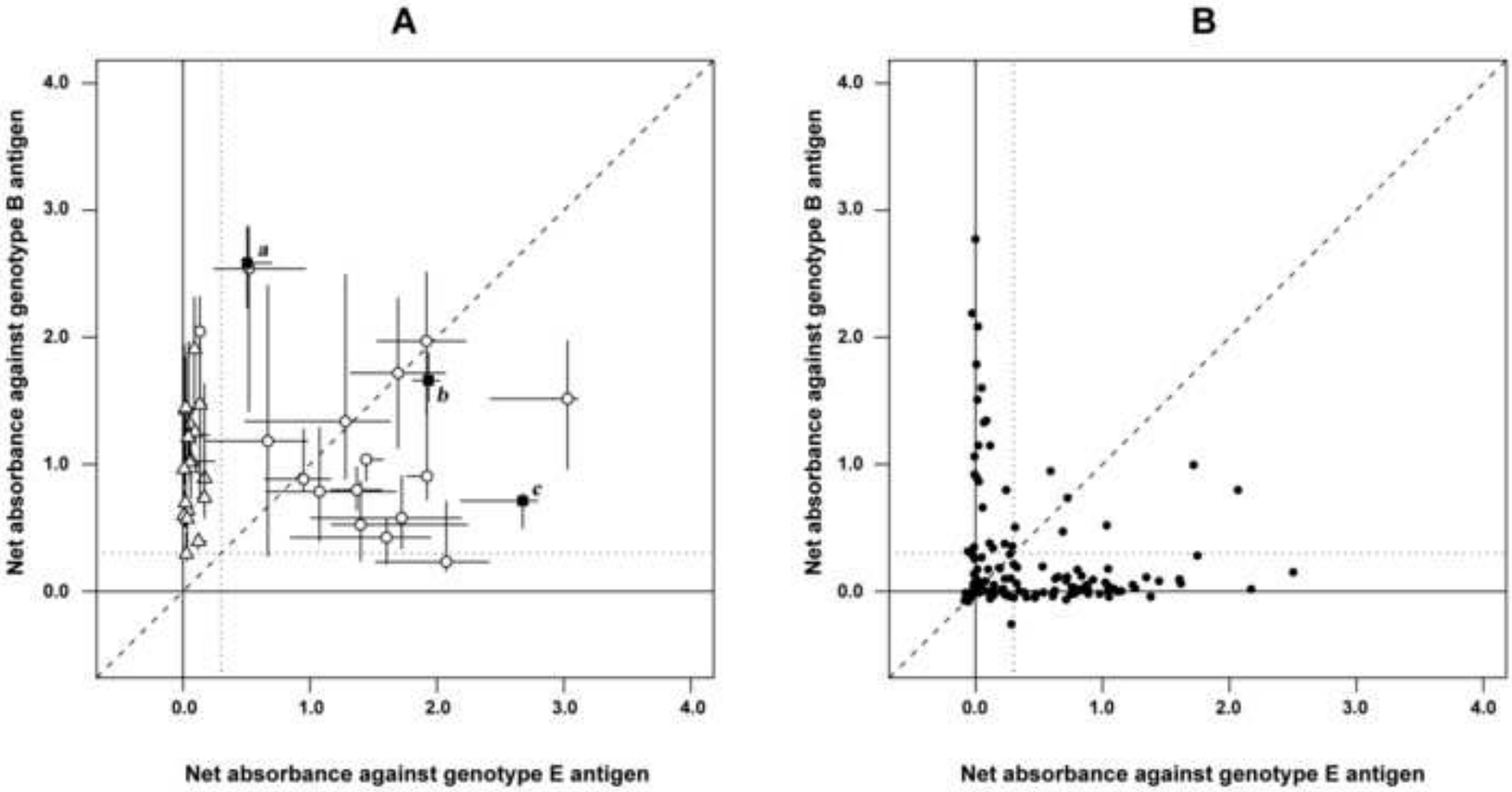

Figure 3

Click here to download high resolution image