Genetic characterization of small ruminant lentivirus in Italian mixed flocks: evidence for a novel genotype circulating in a local goat population

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In this study, characterization of the gag gene of small ruminant lentiviruses was carried out in Italian mixed flocks. The nearly complete gag gene was amplified and sequenced. Within genotype A, subtype A1 and a novel subtype, A8, were found in goats, and another novel subtype, A9, was found in both sheep and goats. Subtype B1 was found in both host species and subtype B2 was identified only in sheep. A novel, highly divergent sequence was obtained from goats in two epidemiologically related flocks and is proposed to represent a novel genotype, E. Major epitopes of matrix and capsid antigen were highly divergent, suggesting that serological identification of animals infected with genotype E may have been missed by using currently available diagnostic tests. A recombinant subunit ELISA, based on genotype E-specific epitopes, was developed and a third independent flock carrying this genotype was identified, based on serology.

Infections by ovine and caprine lentiviruses, referred to as small ruminant lentiviruses (SRLV), cause slow-progressive, insidious diseases that greatly affect flock productivity and limit international animal trade. Two genetically and antigenically related viruses, members of the genus Lentivirus of the family Retroviridae, are responsible for lifelong persisting infection: maedi visna virus (MVV) and caprine arthritis encephalitis virus (CAEV). Although sheep and goat lentivirus infections have, for a long time, been considered species-specific, several reports now indicate that natural cross-species infection may occur (Rolland et al., 2002; Zanoni, 1998), and the eradication of infection in one animal species (e.g. goat) cannot rule out the presence of infection in the other (Peterhans et al., 2004). A new phylogenetic classification of SRLV was recently proposed: MVV prototypes originally isolated from sheep are now referred to as group A, further divided into several subtypes isolated from sheep (A1, A2), goats (A5, A7) or both species (A3, A4, A6) (Shah et al., 2004a, b). CAEV prototypes originally isolated from goats are referred to as group B, divided into two subtypes isolated from both species (Pisoni et al., 2005; Shah et al., 2004a). Two additional strains, isolated from a Norwegian goat and a Swiss goat, showed high divergence from other groups and are tentatively classified into the novel groups C and D (Shah et al., 2004a). In Italy, genotype A and subtype B2 have been described in sheep, and subtype B1 has been found in sheep and goats. These studies, however, refer to a limited number of viral strains that were isolated more than 15 years ago (Grego et al., 2002) or were obtained in restricted populations or by using genotype-specific genetic markers (Pisoni et al., 2006). Immunodominant epitopes of capsid antigen (CA) and matrix protein are variable between CAEV-like (B1 and B2) and MVV-like (A1–A7) strains, regardless of the host animal, suggesting that both sheep and goats may be serologically reactive against SRLV infection in a type-specific manner (Grego et al., 2002, 2005). Mixed flocks, in which sheep and goats live in close contact, represent a suitable environment to evaluate the degree of interspecies transmission of different SRLV genotypes and to detect possible recombination events. Further, mixed flocks represent a challenge for established and newly developed diagnostic tests, as the potential pool of heterogeneous SRLV genotypes may affect their sensitivity.

In this study, both serological and genetic characterizations of SRLV infection were performed in mixed flocks. Phylogenetic analyses were carried out to investigate the heterogeneity of SRLV. Genetic and serological analyses
were compared, to gain more understanding of naturally circulating viral strains.

The geographical area under study is located in the north-west part of Italy in the Piedmont region, province of Asti, which has a small ruminant population, mainly goats, of about 3200 heads. Since the early 1980s, the local goat breed Roccaverano has no longer been predominant, as imported breeds such as Alpine and Saanen were introduced to improve milk production. The Langhe sheep breed is sometimes associated in mixed flocks. Eight small-size mixed flocks were selected, based on a preliminary serological evaluation, and divided into groups of high (33–80 %) and low (5–12 %) prevalence of SRLV infection. Serum samples and Buffy coats were obtained from six to 12 sheep and an equal number of goats in each flock. Bulk milk samples were obtained from 12 additional goat flocks in the same province and 38 sheep flocks from the neighbouring province of Cuneo.

DNA was obtained from Buffy-coat samples by using a Qiagen DNeasy blood kit. DNA was first analysed by a hemi-nested PCR designed to amplify 1.3 kb, and samples with a negative result were tested by a nested PCR designed to amplify 0.8 kb. Two forward degenerate primers, GAG F1 (5'-TGTTGARKCTAGMTAGACATGG-3' and GAG F2 (5'-GCGGACGGCACSCACCA-CG-3'), were designed, based on conserved regions. The two reverse primers, POL R1 (5'-CATAGGRGGHGCAGGAC-GGCASCA-3') and POL R2 (5'-GCCAGCGGASCACA-CG-3'), were modified slightly compared with those described by Shah et al. (2004a) as reverse primers for a short gag fragment.

The first PCR was carried out in a 50 μl reaction volume, using 1 μg DNA sample, 1 × PCR buffer (Qiagen), 300 nM GAG F1 and POL R1 primers, 200 μM each dNTP and 1 U HotStar Taq DNA polymerase (Qiagen). The amplification profile was 95°C for 15 min, 35 cycles of 94°C for 30 s, 55°C for 1 min and 72°C for 2 min, and a final extension of 72°C for 10 min. The second PCR was carried out using almost the same conditions as described above, using 4 μl of the first PCR product, but adding 1 × Q buffer (Qiagen) and using 45 amplification cycles and annealing at 60°C for 1 min. GAG F2 and POL R2 were used for the nested PCR, or GAG F1 and POL R2 for the hemi-nested PCR. A subset of PCR-positive DNA samples also underwent a second round of PCR to amplify the pol gene fragment, as described by Shah et al. (2004a).

Nested PCR fragments (0.8 kb) were purified and sequenced directly. Hemi-nested PCR fragments (1.3 kb) were gel-purified and cloned into pCR4-TOPO (Invitrogen) and at least two independent clones were sequenced. Sequencing was carried out on an ABI PRISM 310 Genetic Analyzer.

Sequences were aligned by using CLUSTAL W (Thompson et al., 1997) with a set of reference sequences available in GenBank. Phylogenetic trees were created by using MrBayes ver. 3.1.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003), considering a model of molecular evolution estimated by MODELTEST ver. 3.7 (Posada & Crandall, 1998, 2001). Tree statistics and phylogenetic manipulations were performed with the computer program PAUP* ver. 4.0b10 (Swofford, 2003). Genetic diversity was expressed as nucleotide diversity (Nei, 1987). Sequence alignments were analysed by using SimPlot software ver. 3.2 (Ray, 1999) to calculate percentage identity, and bootscan analysis (Salminen et al., 1995) was used to evaluate recombination break points.

An indirect ELISA based on recombinant CA and transmembrane-domain fusion protein has been developed and described previously (Rosati et al., 2004). This test, based on the Icelandic strain K1514, was used as a screening test to estimate the seroprevalence in each flock. Additionally, a multi-epitope ELISA was developed based on genotype A- and B-derived matrix protein (P16mvv and P16caev) and the immunodominant subunit of CA (B3mvv and B3caev). Following the identification of a cluster of highly divergent sequences in two flocks, a third version of the latter immunodominant subunit of CA was generated and used in some experiments. All antigens were expressed as glutathione S-transferase (GST) fusion proteins, as described previously (Rosati et al., 1999); the subunit ELISA was carried out as described in a previous study (Lacerenza et al., 2006), with the exception that serum or bulk milk samples were diluted 1/20 or 1/2, respectively. ELISA net absorbances (measured at 405 nm) were obtained by subtracting the A405 against GST from the A405 against each antigen. Net A405 readings >0.4 were scored as positive; a genotype-specific response was recorded when A405 against putative homologous antigen was >0.4 compared with the heterologous counterpart.

In total, 137 blood samples were obtained from eight mixed flocks. Thirty-eight out of 55 gag PCR-positive samples were sequenced directly (0.8 kb) or cloned and sequenced (1.3 kb). Distribution of genotypes/subtypes in sheep or goats of the flocks is summarized in Table 1. The

### Table 1. Distribution of 38 sequences in the mixed flocks analysed, based on viral genotype/subtype and animal species

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Abbreviations: g, goat; s, sheep.
Phylogenetic tree topology was comparable to that reported by Shah et al. (2004a) (Fig. 1). For instance, heterogeneity within each group was high, with high values of nucleotide diversity in group A (mean nucleotide diversity, 17.0%; range, 1.0–22.0%) and group B (mean, 14.2%; range, 0.4–19.0%). Within group A, two novel subgroups, A8 and A9, were described based on the nucleotide difference classification reported by Shah et al. (2004a). To assign the novel subtypes, the pol gene fragment encoding integrase was sequenced in a subset of samples and compared with the homologous pol sequence of subgroup A7, for which gag sequences were not available. The nucleotide diversity between the novel sequences classified as A8 and A9 and the A7 subgroup was >22%.

A new cluster of ten sequences formed a separate clade. The difference between this new group and genotype A (mean, 31.1%; range, 28.5–32.9%), B (mean, 29.7%; range, 27.6–31.5%) and C (mean, 31.2%; range, 31.0–31.5%), as well as the nucleotide diversity with the integrase pol fragment of group D (>26%), support our proposal that these isolates represent a novel genotype, E.

Circulation of more than one subtype was evident in three flocks (genotypes B1/E, A8/B1/E and A1/A8/B1/B2). The most frequently detected subtype was B1, identified in seven of eight flocks and in 17 out of 38 sequences. The whole gag gene sequence, representative of five of the six genotypes/subtypes, was obtained and the amino acid sequence is shown in Fig. 2. Major linear epitopes of matrix and CA were clearly distinguishable for the A, B and E genotypes.

Among 137 serum samples, 54 were positive for viral antibodies by using the screening test. Agreement between serology and PCR was rather low ($k=0.56$) and seemed to be independent of genotype and/or flock prevalence. Matrix and CA subunits, corresponding to major linear epitopes, were used for serotyping. In flocks with only the B1 subtype, a type-specific (CAEV-like) immune response was evident against both homologous subunits, whereas in flocks with different genotypes, the antibody response was spread over different antigens. This was particularly evident in flocks with B and E genotypes (not shown).

Additionally, a recombinant subunit of type E SRLV major CA was used in serum and bulk milk ELISA to screen a wider range of sheep and goat flocks in the neighbouring provinces of Asti and Cuneo. A third goat flock harbouring the novel genotype was detected, based on type E-specific antibody reactivity (data not shown). The three flocks in which type E SRLV was detected, either directly or indirectly, were of the same Roccaverano breed, the local early goat population.

This study demonstrates that recombinant subunits derived from known genotypes and corresponding to immunodominant epitopes of gag-encoded structural
proteins may represent a cost-effective tool to detect antigenic variability in the field and to improve the sensitivity of indirect diagnosis. One drawback of serology is, however, the limiting diagnostic capability toward known genotypes, whose antigens are readily developed and produced. Thus, type A- or B-derived antigens may fail to identify novel or different viruses in a population.

SRLV sequence availability was, for a long time, limited by the lack of primers able to amplify long fragments in the majority of viral strains. Recently, a new set of degenerate primers has been proposed (Shah et al., 2004a) and improved further in the present study to amplify long and more informative gene fragments of a wide range of viral subtypes. Despite the antigenic diversity of some strains, PCR was able to amplify highly heterogeneous viral subtypes, suggesting that the low sensitivity of PCR is generally more related to viral load in infected animals, rather than genetic divergence among viral isolates. The newly identified cluster, different from any known genotype in both \textit{gag} and \textit{pol} subunit sequences, was classified as type E and was detected exclusively in the local Roccaverano goat breed. Subunit ELISA, based on the type E immunodominant epitope of CA, strongly supports the hypothesis that, even in mixed flocks, the origin of this virus is related epidemiologically to this goat breed. In fact, the bulk milk test was consistently negative for all 38 sheep flocks, and a third genotype E-positive goat flock was detected in which the same goat breed was prevalent. Interestingly, the flock owners were not aware of any of the clinical signs attributable to goat lentivirus before the French breeds entered the population in the early 1980s, supporting the hypothesis that this genotype may represent a low-pathogenic SRLV.

Despite the number of viral subtypes proposed to date in different countries, from the antigenic point of view, the viral strains detected in the present study can be grouped into three main prototypes based on two major CA epitopes, named B and D subunits in a previous study (Rosati et al., 1999). Subunit B shows serological differentiation between genotypes A and B (12/17 identities), genotypes A and E (11/17 identities) and genotypes B and E (13/17 identities), whereas subunit D allows discrimination between genotypes A/B and E (12/19 identities). This remarkable antigenic variation may be
responsible for misdiagnosis of genotype E infections using heterologous antigens. As a voluntary eradication programme was implemented over 15 years ago in most goat flocks in the area and a number of flocks are now considered CAEV-free, it would be important to develop a type E-specific serological test to evaluate the degree of ‘diagnostic-escape’ mutants in flocks in which traditional serological tests have been adopted.

As to the interspecies transmission of SRLV between sheep and goats, we can extend the knowledge of the subtypes found in both species. Subtype A1, previously identified in sheep worldwide, was detected in one goat, and subtype A9 was detected in both species. Genotype E was identified only in goats; however, we still have limited information on the circulation of this novel genotype.

Finally, a similarity plot of all partial gag gene sequences did not reveal any evidence of recombination. Although double infection was serologically evident in several animals, direct and clonal sequences never revealed highly divergent double sequences. As all examined flocks were established a long time ago, no selective advantage of potential recombinant virus, i.e. in terms of viral load or transmission efficiency, would have prevailed over the parental viruses. This finding supports the hypothesis that, even in presence of a double infection, the most-adapted virus sustains the infection better and probably segregates in tissues that allow more efficient transmission.

In conclusion, our data indicate clearly that there is no species barrier in SRLV infections, as well as the fact that co-existence of different SRLV genotypes is a normal feature in mixed flock. Additional sequencing data revealed a novel genotype, highly divergent from any known isolates, that has been missed by diagnosis for a long time; this may influence the success of an eradication programme based on currently available diagnostic tests.

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References


