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## Antibody response in sheep experimentally infected with different small ruminant lentivirus genotypes

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Received 21 December 2005; received in revised form 28 March 2006; accepted 28 March 2006

### Abstract

Two groups of sheep were experimentally infected by intratracheal route with two small ruminant lentivirus (SRLV) isolates belonging to different genotypes (It-561 genotype A3 and It-Pi1 genotype B2). Seroconversion was evaluated using recombinant homologous and heterologous matrix protein/capsid antigen fusion protein. Results clearly indicate that seroconversion against homologous antigen was detected well in advance as regards heterologous antigen in both groups, although the advantage of using homologous antigen was less evident in detecting seroconversion against the caprine arthritis encephalitis virus (CAEV)-like strain, compared with the maedi-visna virus (MVV)-like infection. Commercially available ELISAs detect CAEV-like seroconversion earlier than MVV-like infection suggesting a closer relationship between CAEV-like isolate and the antigen used in the latter ELISA tests. Seven recombinant subunits developed from matrix protein and capsid antigen of strain K1514 (prototype A1) were used to better define the antibody response in sheep infected with It-561 isolate. Two animals clearly reacted against type specific epitopes in the early stage of infection.

This study highlights the relative insensitivity of gag encoded cross-reacting epitopes during the early stage of infection and suggests the development of novel diagnostic tests based on both genotype specific antigens.

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**Keywords:** Small ruminant lentiviruses (SRLV); Type specific seroconversion; A and B genotypes

### 1. Introduction

Small ruminant lentiviruses (SRLV), namely maedi-visna virus (MVV) and caprine arthritis encephalitis

virus (CAEV), are a heterogeneous group of infectious agents affecting sheep and goat which cause chronic debilitating diseases known as ovine maedi-visna, ovine progressive pneumonia, caprine arthritis encephalitis. Infections, which are widespread, cause severe economic loss due to chronic mastitis, pneumonia, arthritis and early culling (Bulgin, 1990). In several countries, eradication programs have been implemented which

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are mainly based on a test and slaughter policy until the whole flock remains negative for at least 3 years (OIE *Terrestrial Animal Health Code*, 2005). Although sheep and goat lentivirus infections have been considered specie-specific for a long time, several reports now indicate that natural cross species infection may occur and the eradication of infection in one animal species (i.e. the goat) cannot rule out the presence of infection in the other (Peterhans et al., 2004). A new phylogenetic classification of small ruminant lentiviruses 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 (Shah et al., 2004a; Pisoni et al., 2005). Two additional strains, isolated from a Norwegian goat and Swiss sheep showed high divergence from other groups and are tentatively classified into the novel groups C and D (Shah et al., 2004a). Antigenic heterogeneity of small ruminant lentiviruses further complicates the field situation. Immunodominant epitopes of capsid antigen and matrix protein have been shown to be variable between CAEV-like (B1 and B2) MVV-like (A1-7) strains regardless of the animal they have been isolated from, suggesting that both sheep and goats may be serologically reactive against small ruminant lentivirus infection in a type specific manner (Grego et al., 2002, 2005). Despite the highly genetic heterogeneity of SRLV, they can be antigenically divided into two main groups based on the available amino acid sequence of capsid antigen major linear epitopes: group A, which includes subtype A1–A5 and group B (subtype B1 and B2). A third group might be related to Norwegian isolate which shows some divergence from group A and B. Other structural proteins such as matrix protein was found to be variable between some A and B subtypes but sequence information are to date still limited to few isolates. Currently available diagnostic tests are based on a single strain ELISA format, using whole virus, recombinant and synthetic antigens (de Andres et al., 2005). These tests are usually employed in both species since cross-reacting epitopes have been found in all structural proteins (Gogolewski et al., 1985). However, the aforementioned antigenic variability has not been yet taken into account. Two Italian ovine lentivirus field

isolates have recently been characterised and genetically classified into MVV-like (isolate It-561) and CAEV-like (It-Pi1) genotypes which belong to subtypes A3 and B2, respectively based on the novel classification. In order to evaluate if the antigenic differences between isolates could represent a diagnostic drawback, two groups of sheep were experimentally infected with such isolates and seroconversion was detected using homologous and heterologous recombinant matrix protein/capsid antigen fusion protein (named P16-P25 thereafter) as well as commercially available ELISA. This study highlights the antigenic heterogeneity of SRLV and the relative insensitivity of gag encoded cross-reacting epitopes during the early stage of infection.

## 2. Materials and methods

### 2.1. Viral strains and animals

SRLV strain It-561 (subtype A3) and It-Pi1 (subtype B2) were used. They were originally isolated from sheep showing typical maedi gross and histopathological lesions and severe arthritis, respectively. Genetic and antigenic characterisation of capsid antigen and matrix protein of the viruses have been published (Grego et al., 2002; Grego et al., 2005). Original isolates were passed once, titrated on ovine foetal choroid plexus cells at low number of passages and stored in aliquots at  $-80^{\circ}\text{C}$  until used for experimental infection. Since *in vitro* adaptation was kept to a minimum, different titre per volume of inoculum was obtained for the two strains, reflecting their ability to grow or adapt in cell culture system.

A number of 13 sheep of the Appenninica breed were selected from a long term negative flock and housed in the experimental station of Istituto Zooprofilattico Sperimentale of Perugia at 8 month of age. They were kept there for another 6 months before experimental infection and tested twice with negative results for small ruminant lentivirus antibodies. On the day of infection, the sheep were randomly divided into three groups, each housed in different independent units. Group A of five sheep were infected by intratracheal route with 1 ml of virus containing cell culture supernatant (strain It-561,  $4.6 \times 10^3$  TCID<sub>50</sub>/ml); Group B of four sheep

were infected by the same route with 1 ml of viral containing cell culture supernatant (strain It-Pi1,  $4.6 \times 10^4$  TCID<sub>50</sub>/ml); the third group (C) received 1 ml of mock consisting of uninfected cell culture medium. All sheep were sampled before inoculation on day of infection, and at regular intervals until Day 300. Serum samples were obtained from each blood sample and stored in aliquots at  $-20^\circ\text{C}$ .

## 2.2. Recombinant antigens

The *gag* gene of strains It-561 and It-Pi1 have been previously cloned, sequences and deposited in the GenBank database under accession number AY265455–AY265456 (Grego et al., 2005). The *gag* fragment encoding the P16-P25 fusion protein was amplified and cloned in frame with Glutathione S-transferase (GST) into pGex6his, a modified pGex6P expression vector (Amersham) that carries a 6xhis tail at C' terminus. In order to facilitate directional cloning, a site directed PCR mediated mutagenesis was carried out to suppress two BamHI restriction sites within *gag* fragment of strain It-561. Positive clones were selected from SDS-PAGE analysis of IPTG induced cultures for expression of GST/P16-P25 fusion protein. Sequence analysis of positive clones confirmed the identity of gene fragment compared with previously published data for each viral isolate. Fusion protein was purified using glutathione sepharose 4B affinity matrix using an FPLC system (Smith and Johnson, 1988). On column cleavage of GST was carried out with PreScission<sup>TM</sup> protease (Amersham Biosciences) and the resulting P16-P25 was further purified by immobilised metal ion affinity chromatography. Purity and yield of recombinant antigens were evaluated by SDS-PAGE and modified Lowry method (DC protein assay, BioRad). They were designated as It-561/P16-P25 and It-Pi1/P16-P25.

Recombinant capsid antigen-transmembrane epitope fusion protein (P25/TM) derived from Icelandic strain K1514 has been developed earlier (Rosati et al., 2004).

Seven subunits of matrix ( $n = 3$  this study) and capsid antigen ( $n = 4$  previous study: Rosati et al., 1999), were generated from the Icelandic strain K1514 (prototype A1), encompassing the hydrophilic domains as detected by Kite and Doolittle analysis (Kyte and Doolittle, 1982). All subunits were expressed and

purified as GST fusion proteins and used in an ELISA format to evaluate the reactivity of sera which were reactive in a type specific manner against homologous P16-P25. Recombinant subunits were designated P16a, P16b, P16c, P25a, P25b, P25c, P25d, according to previous studies (Rosati et al., 1999; Grego et al., 2005). In addition P25b subunit, previously generated from the CAEV-like It-128 (Grego et al., 2002), and having amino acid sequence identical to It-Pi1, was used alongside group A P25b antigen in some experiments.

## 2.3. ELISAs

Several ELISAs were used in the study. For recombinant P16-P25 ELISA, microplates were coated overnight uncovered at  $37^\circ\text{C}$  with 100 ng/well of It-561/P16-P25 (lanes 1, 4, 7, 10), It-Pi1/P16-P25 (lanes 2, 5, 8, 11), and water (lanes 3, 6, 9, 12). After two washes, plates were blocked with 2.5% bovine casein for 1 h and washed as before. Sera were diluted 1/10 in PBS containing 1.25% bovine casein and incubated (200  $\mu\text{l}$ /well) for 1.5 h at  $37^\circ\text{C}$ . After four washes, plates were incubated with peroxidase labelled anti-sheep IgG monoclonal antibody (Sigma) (200  $\mu\text{l}$ /well) for 1 h at  $37^\circ\text{C}$ . After the final washes, reaction was developed with TMB and reaction stopped after 10 min with 0.2 M sulphuric acid. Reactivity of each serum sample against the recombinant antigens was calculated subtracting the absorbance of the same serum against negative control antigen (water) and expressed as percentage of reactivity respect to the positive reference serum enclosed in each plate. The latter consisted in a pool of positive sera which was equally reactive against It-561 and It-Pi1 antigens. Preliminary evaluation was carried out using a panel of 167 negative and 49 positive samples obtained from long term negative and infected flocks, respectively. Cut off was defined as having a reactivity of  $>40\%$  of that of the positive serum.

Two commercially available ELISAs were used. One whole virus ELISA (thereafter named WVcomELISA) was from Bommeli Diagnostics (Checkit MVV/CAEV) and a recombinant based ELISA (thereafter named RcomELISA) was from the Institute Pourquier, employing the recombinant capsid antigen and transmembrane protein. Both tests were carried out following the manufacturer's instructions.

A subset of the serum panel was also tested against P25/TM ELISA according to the previously described method (Rosati et al., 2004).

Subunit ELISA was carried out with a subset of sera from group A (all animals sampled at longer intervals) which were mainly reactive against homologous antigen (see below). Plates were coated with 200 ng of the seven GST/subunits (rows A–G) or an equimolar amount of GST (row H). ELISA was performed as for recombinant P16–P25 ELISA, except for cut off calculation. Net absorbance was obtained from each serum subtracting the absorbance against GST as negative antigen. Since no positive control sera were available for each subunit, cut off was arbitrarily defined for each animal as having an absorbance >0.1 of that obtained at time 0.

Subunit ELISA using peptide P25b derived from A and B phylogenetic group was carried out as described (Grego et al., 2002).

### 3. Results

Both P16–P25 recombinant antigens were successfully expressed and purified in soluble form. Cleavage of GST moiety and IMAC purification yield 4 and 9 mg of purified antigens per litre of bacterial culture, respectively (Fig. 1). Preliminary evaluation of specificity and sensitivity of P16–P25 ELISAs, using a subset of well characterised sera, was 99 and 76%, respectively for the two antigens. Sensitivity greater than 90% was obtained combining results of both ELISAs.

Results of experimentally infected sheep are summarised in Figs. 2 and 3 where the percentage of reactivity versus each positive serum in the four ELISA tests (homologous and heterologous P16–P25 ELISA and the two commercially available ELISAs) is shown at different time points p.i. for each animal. In group A (five sheep infected with A3 genotype It-561) seroconversion against homologous P16–P25 ELISA was detected in all animals between 15 and 58 days p.i. Four out of five animals remained clearly positive during the whole study, while one sheep was positive only at Days 58, 99 and 113 p.i., then becoming doubtful or negative. Heterologous P16–P25 ELISA detected the first positive reaction at Day 58 p.i. while commercial ELISAs detected the first

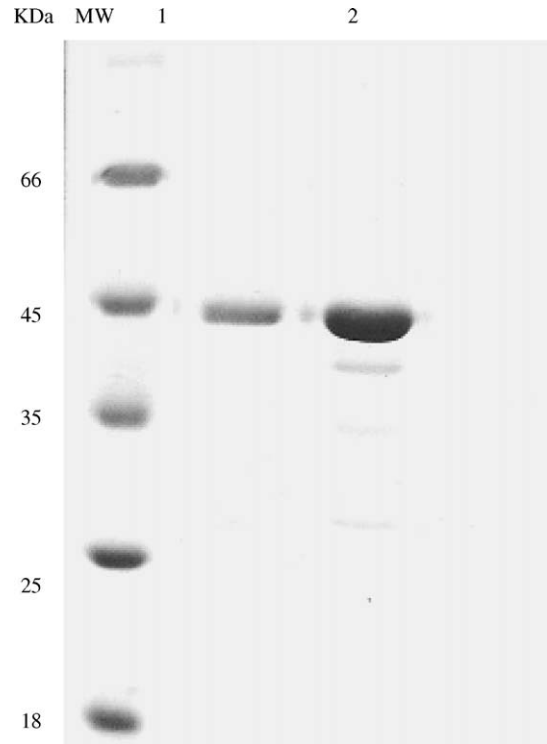


Fig. 1. SDS-PAGE showing purified recombinant P16–P25 fusion proteins after GST cleavage and IMAC. MW, molecular weights standard; lane 1, It-Pi1/P16–P25; lane 2, It-561/P16–P25.

seroconversion at Day 84 (Rcom ELISA) and 128 (WVcom ELISA). Performance of RcomELISA was better than homologous P16–P25 only at 210 days p.i. (four positives versus three) while heterologous P16–P25 and WVcom ELISA were always less sensitive than homologous ELISA. In group B (four sheep infected with B2 genotype It-Pi1) seroconversion against homologous P16–P25 ELISA was detected in all sheep between 23 and 99 days p.i. Animals remained positive throughout the whole study except for one sheep which turned negative from 182 days p.i. Both the heterologous ELISA and the commercial ELISAs reacted in a similar way, although homologous ELISA was more sensitive during the early stage of seroconversion (between 28 and 42 days p.i. and between 99 and 140 days p.i.).

In group C (four sheep mock infected) all animals remained negative throughout the study with all ELISA tests. Results are not reported or discussed thereafter.

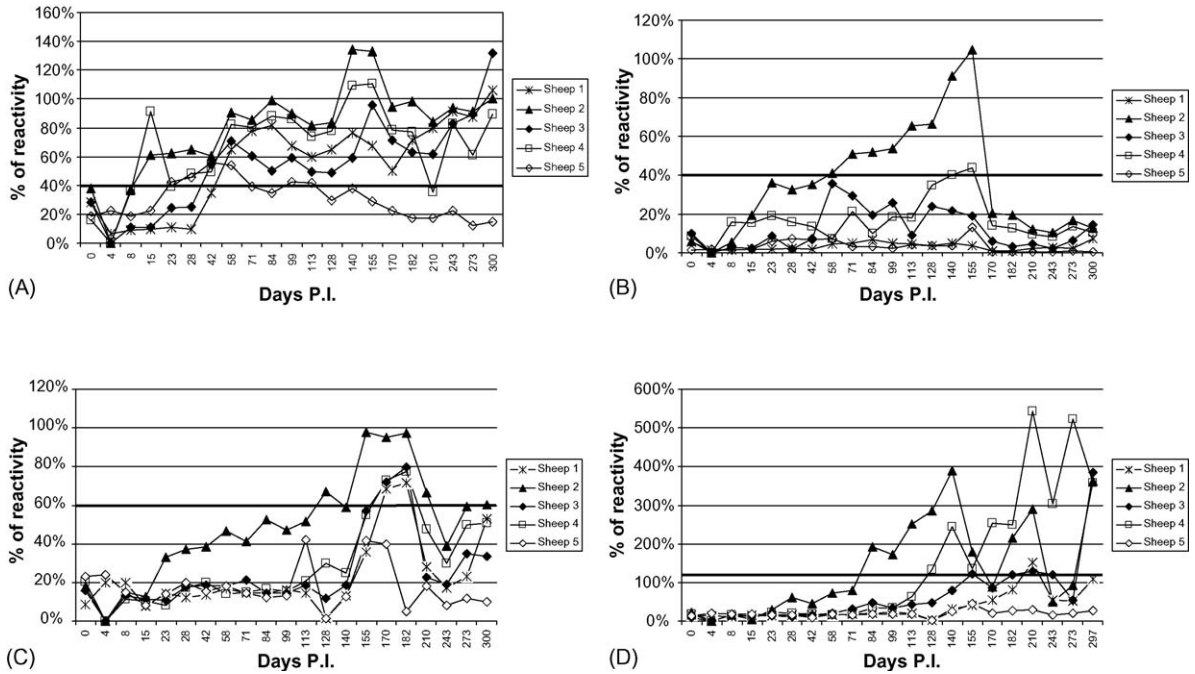


Fig. 2. Time pattern and percentage of antibody reactivity in five sheep experimentally infected with SRLV strain It-561 (phylogenetic group A3). (A) Recombinant P16/P25 homologous antigen (It-561); (B) recombinant P16/P25 heterologous antigen (It-Pi1); (C) WVcomELISA; (D) RcomELISA. Bold lines are cut off values for each test.

Since a clear type specific response was detected in sheep infected with A3 genotype well in advance as regards cross reacting response, a subunit ELISA was carried out to define epitope reactivity in group A. Table 1 shows the number of positive reactions at different time points for each subunit. Subunit B of capsid antigen (p25b) was the peptide most frequently recognised followed by p25c, p16b and p25a subunit.

Table 1

Sera from group A, collected at different time points ( $n = 50$ ) were analysed against seven subunits of matrix protein and capsid antigen derived from A1 prototype K1514 strain

Ag subunit	Days p.i.								
	15	42	58	84	99	113	128	140	155
p16a	0	1	0	0	0	0	0	1	2
p16b	1	1	1	1	1	1	1	1	1
p16c	1	0	0	1	1	1	1	1	1
p25a	1	1	1	1	1	1	1	1	1
p25b	3	3	3	3	4	2	2	2	3
p25c	2	2	2	1	1	1	1	1	1
p25d	0	1	0	1	0	0	0	0	0

Number indicate positive reactions against each subunit ELISA.

However, the higher response was detected against p25b and p16b subunits, based on absorbance value. There were also a huge difference among animals in terms of quality (recognised epitopes) and quantity (antibody concentration) of antibody response. One sheep was negative against all subunits, another was slightly positive against p16c and p25c subunit, two sheep were reactive only against p25b subunit, although with different titres, and the fifth animal was reactive against a wide range of peptides, but highly reactive against p16b. Epitope reactivity of two sheep of group A are shown in Fig. 4a and b as an example.

#### 4. Discussion

In this study we provided evidence that lentiviruses of different genotype may drive the antibody response of experimentally infected sheep against type specific epitopes, at least in the early stage of infection. Previous studies have described the presence of variable epitopes in *env* and *gag* encoded proteins,

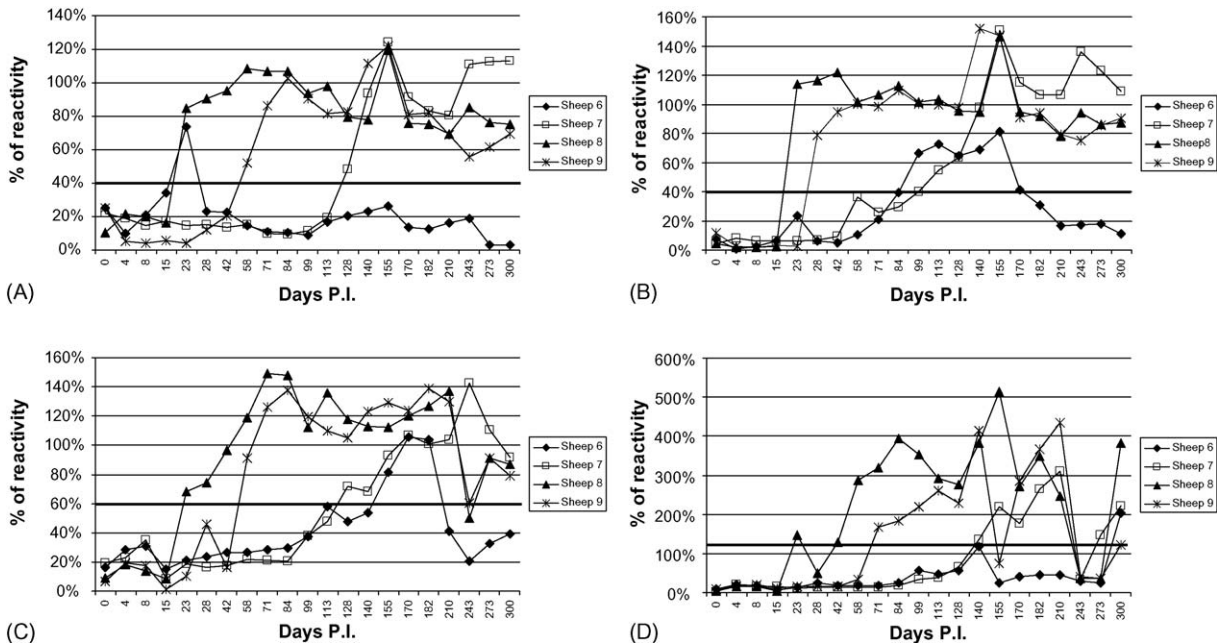


Fig. 3. Time pattern and percentage of antibody reactivity in four sheep experimentally infected with SRLV strain It-Pi1 (phylogenetic group B2). (A) Recombinant P16/P25 heterologous antigen (It-561); (B) recombinant P16/P25 homologous antigen (It-Pi1); (C) WvcomELISA; (D) RcomELISA. Bold lines are cut off values for each test.

but so far, a strain specific antibody response was associated with the early stage of infection only for the SU antigen (Bertoni et al., 2000). Among gag encoded proteins, capsid antigen and matrix protein are frequently recognised in the early stage of infection but the current concept of the universality of single strain immunoassays are based on a wide range of cross reacting linear epitopes in these structural proteins (Gogolewski et al., 1985). We had previously investigated the variability of immunodominant epitopes of matrix protein and capsid antigen among SRLVs roughly classified as MVV-like and CAEV-like strains (Grego et al., 2005). At least for group A and B, the most widespread genotypes, recombinant subunits of matrix protein and capsid antigen had been identified, able to classify genotypes circulating in the field, based on differential antibody reactivity. For the first time, this study reports that early antibody response against gag encoded structural proteins are directed to type specific epitopes. This was particularly evident with sheep infected with group A genotype, where homologous antigen classified infected animals months before heterologous antigen

or commercially available ELISAs which are supposed to have been developed using group B strains. This is consistent with the finding that the same commercial ELISAs were more able to classify sheep infected with It-Pi1 strain which is a B2 genotype. However, even the heterologous antigen It-561 performed better in group B than did antigen It-Pi1 in group A, leading to the supposition that group B strains might drive antibody response against more conserved (cross reacting) epitopes. This observation may explain why in a previous study, 46 out of 50 sera from CAEV infected goats (genotype B1) were reactive against p25b recombinant subunit derived from strain K1514 (genotype A1) (Rosati et al., 1999). It is debatable whether different viral titre in the inoculum might have influenced seroconversion. However, reactivity against homologous antigen was detected slightly earlier in group A which received a 10-fold lower dose of virus than group B. Moreover, the antibody concentration, in terms of percentage of reactivity, was quite similar in both groups. We therefore, believe that viral titre in the range of  $10^3$ – $10^4$  TCID<sub>50</sub> could not have affected results in this experiment.

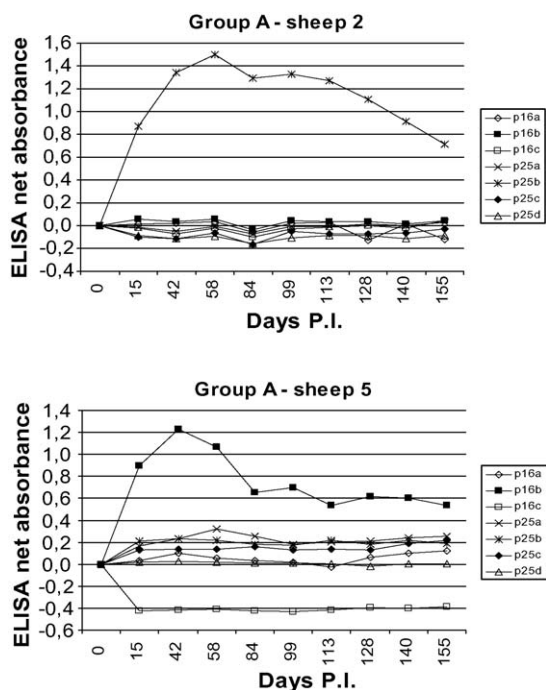


Fig. 4. Antibody response of two animals from group A against each of the seven subunits are presented as an example. Reactivity is expressed as net absorbance, obtained subtracting absorbance against GST as negative antigen and against time point 0 for each animal.

Results shown in Fig. 2 suggested that an unexpected number of sera collected between Day 0 and Day 155 could be used to better define the genotype specific antibody response. Recombinant subunits of capsid antigen, derived from Icelandic strain K1514, had been previously developed and an additional three subunits of matrix protein were developed in this study. We chose the prototype of A1 phylogenetic group, rather than the strain used for experimental infection, to identify (if any) common epitopes shared among group A subtypes. Four out of five animals clearly showed they were reactive against, at least one of such subunits, suggesting that antibody response was directed against genotype A epitopes. Two sheep showed a clear reaction against p16b and p25b subunit, respectively with a peak absorbance value around day 42 and 58, a period where all other tests failed to detect any antibody response. This was not unexpected since the same subunits has been described in previous works as

immunodominant and variable domains of gag encoded proteins (Grego et al., 2002, 2005). Interestingly the animal which was highly reactive against p25b between Day 15 and Day 155 did not react against any other subunit or any other ELISA. This peptide has been shown to share a common N' terminal and variable C' terminal epitopes between MVV-like and CAEV-like strains. When the same samples were tested against the p25b subunit derived from CAEV-like isolate It-128, which shares identical amino acid sequence to It-Pi1, all time points were negative (not shown), suggesting that antibody response was directed versus variable epitope of MVV-like subunit p25b.

One animal was negative against all subunits between 0 and 155 days p.i. This animal showed rapid seroconversion against homologous ELISA but was negative to commercial ELISA until 128–170 days p.i. A subset of time point samples were tested with P25/TM ELISA developed from K1514 strain with positive results, leading to the supposition that reactivity was directed against group A epitope/es not included in the seven subunits rather than against strain specific epitope/es.

In the third end period of the study the commercial ELISAs performed equally well or even better than the homologous antigen. This is consistent with an antibody reactivity directed against other viral proteins, such as transmembrane protein which carries highly conserved linear epitope, well represented in the RcomELISA (Rosati et al., 1995). This may also explain the low sensitivity of both P16-P25 ELISAs obtained in the preliminary evaluation against true positive samples. Thus, the recombinant antigens developed in this study are not supposed to cover a wide period of the infection but rather to highlight the relative insensitive of heterologous antigens in the early stage of infection, when antibody reactivity is mainly directed against gag encoded proteins.

In conclusion, we have demonstrated that sheep experimentally infected with type A and type B lentiviruses seroconverted against homologous recombinant matrix protein and capsid antigen well in advance compared with heterologous antigen. This is mainly due to reactivity against type specific epitopes located in both proteins and fairly conserved among phylogenetically correlated strains. The distribution of type A and type B lentiviruses among sheep and goat



worldwide suggest that a combination of both genotypes may increase the sensitivity of currently available serological tests. Moreover, since lentivirus isolates, such as Norwegian strain, have been described to be equally distant from type A and type B prototypes, further studies are necessary to evaluate the diagnostic capability of current serological tools to detect these infections in the field, in order to avoid the selection of “diagnostic escape” mutants in the future.

### Acknowledgments

This work was supported by Italian Ministry of Health, under project IZS 10/05 RC and Italian MIUR, “fondo ex 60%, 2005”.

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