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Abstract

Small ruminant lentivirus (SRLV) belonging to the highly divergent genotype E has recently been identified in the Italian goat breed Roccaverano. In this report we have developed a specific serological test based on recombinant matrix/capsid antigen fusion protein. Performance has been evaluated and compared with a similar test based on genotype B antigen. Herds under study were selected according to the infectious status characterized by blood PCR and sequencing. Results clearly showed that B and E based recombinant ELISA only detected homologous infection and an apparent cross-reactivity was recorded in a herd in which co-infection was present. Three commercially available ELISAs showed different abilities in detecting genotype E infection, being the whole virus- based immunoassay the best choice. Genotype E-recombinant antigen was not detected in ELISA by three commercially available Mabs known to be cross-reactive among CAEV and MVV capsid antigens, further supporting the high divergence of the E genotype from others. Finally, a SRLV-free herd according to commercial ELISA testing, was analysed in the same area where genotype E was identified and few animals belonging to Roccaverano breed were found slightly reactive with the E antigens. Our results suggest that the prevalence of genotype E in other small ruminant populations may be conveniently estimated using a comparative assay based on a combination of genotype specific recombinant antigens and may highlight a wider space in which SRLVs evolve.

1. Introduction

Small ruminant lentiviruses (SRLVs) are a heterogeneous group of viruses affecting sheep and goat and are responsible for chronic debilitating diseases known as Maedi Visna (MV) and caprine arthritis-encefalitis (CAE) (Pepin et al., 1998). Viral isolates characterized so far, show different genetic, antigenic and biological properties and are no longer considered species-specific (Pisoni et al., 2005; Shah et al., 2004b). From an antigenic point of view, most SRLVs can be classified as MVV-like or CAEV-like, corre- sponding to genotype A and B respectively (Shah et al., 2004a). Previous studies have suggested that early serolo- gical diagnosis can be achieved using homologous antigen (Lacerenza et al., 2006). Nevertheless, most of the currently available diagnostic tests are produced using a single strain- based antigen preparation which is believed to detect cross- reacting antibodies against epitopes located in structural proteins (Gogolewski et al., 1985). Recently, a novel genotype E has been identified in the local breed Roccaver- ano in north-west Italy. First sequences were obtained by chance in a caprine herd, using a set of degenerated primers designed to amplify a gag fragment from the majority of known genotypes, encompassing major linear capsid antigen epitopes. Following a preliminary sequence screen- ing, it seemed quite clear that this viral cluster

might have escaped diagnosis in the field using conventional antigen preparations, likely due to the low similarity found in the major immunodominant regions (Grego et al., 2007). A viral strain was subsequently isolated from an apparently healthy goat highly reactive by ELISA against genotype E- derived major capsid antigen epitopes (Reina et al., 2009). Genetic features of this genotype have been described in three epidemiologically unrelated herds. The complete genome (8.4 Kb) presented two major deletions corre- sponding to the dUTPase subunit of the pol gene and to the vpr accessory gene. Based on previous studies in which such subunits were independently deleted from a pathogenic infectious clone, these deletions could explain why the viral cluster is not related to any known clinical signs, represent- ing a natural, well host-adapted, low pathogenic lentivirus (Harmache et al., 1996; Turelli et al., 1996; Zhang et al., 2003). Moreover, preliminary epidemiological data suggest that infection can persist in the population through familiar lineage, with a low tendency to spread horizontally. To date, no information is available on the prevalence of genotype E in larger goat populations due to the lack of a specific antibody detection system. To address this issue, in this report we have developed genotype E specific recombinant antigens which were tested with a panel of monoclonal antibodies (Mabs) known to be reactive against CAEV and MVV capsid antigens. These genotype E derived antigens were also used to develop an indirect ELISA in order to test a panel of goat sera belonging to herds in which the SRLV infectious status was determined by PCR product sequence analysis. Serological and sequence data were in agreement highlighting the importance of using genotype specific tests when determining SRLV seroprevalence, assessing SRLV- free status and searchng for epidemiological information.

2. Material and methods

2.1. Virus and plasmids

Roccaverano strain was originally isolated using mam- mary gland explants from an adult goat and a complete proviral sequence was obtained (Genbank accession number EU293537). The gag gene was amplified by concatenating overlapping PCR fragments and cloned in pCRTopoXL (Invitrogen). The gene fragment coding for Matrix (P16) and major Capsid Antigen (P25) was subsequently amplified and was subcloned between the BamH1/EcoR1 sites of pGEX6His following site-directed PCR-mediated mutagenesis suppressing an internal EcoR1 restriction site. This plasmid, derived from pGEX6P prokaryote expression vector (GE Healthcare), was mod- ified by inserting an in frame 6Xhis-tag between EcoR1 and Sal1 restriction sites thus allowing a double step affinity purification.

2.2. Expression and purification of P16–25 recombinant antigens and P25-B3 subunit epitopes

Transformed E. coli BL21 bacteria were induced at early log phase for 2 h with 0.5 mM IPTG under agitation. Bacterial cells were recovered by centrifugation and lysed by physicochemical methods. Recombinant GST/P16–25/ 6H fusion protein was recovered in the soluble fraction and the first affinity step was carried out in batch using glutathione Sepharose 4B (GE Healthcare). GST cleavage was achieved in pooled eluted fractions using PreScission Protease (2 U/mg) (GE Healthcare). Solution containing GST and P16–25/6H was dialyzed for 24 h to remove reducing and chelating agents and loaded into a Hi-Trap chelating HP column (GE Healthcare), positively charged with nickel ions. Following immobilised metal chelate affinity chromatography, purity and yield of recombinant antigen was estimated by SDS–PAGE and DC protein assay (BioRad).

Using the same protocol, the same gag antigens derived from strain It-Pi1 (genotype B) were employed to generate the antigenic CAEV-like counterpart.

Subunit immunodominant capsid antigen epitopes of MVV and CAEV had previously been characterised. In this study a third version using genotype E (P25-B3) derived sequence was

produced, generating a GST fusion protein as previously described (Grego et al., 2002; Rosati et al., 1999).

2.3. Blood samples, polymerase chain reaction and sequencing

Twelve caprine herds were selected in this and in a previous study (Grego et al., 2007). Heparinized blood samples were obtained from a number of adult animals, representative of each herd: and DNA was extracted from white blood cells using DNA blood kit (Qiagen). A gag nested PCR previously developed (Grego et al., 2007) and known to detect the highest number of SRLV genotypes/ subtypes was applied to each sample and all positive results with suitable bands were sequenced. After, this preliminary screening, five herds of Roccaverano breed were selected: a first herd, BL (n=52) in which only genotype E was detected; a second herd, NG (n = 40) in which genotype B and E were detected; and a third group of goats, TM (n=20), BM (n=18) and CF (n=6) in which only genotype B was present. In this study, all caprine herds were retested when possible at completion and additional sequences were obtained.

To date, the presence of genotype E had been limited to few herds of the Roccaverano breed. Therefore, an additional three-breed long term SRLV negative herd (n = 400) was also included for serological testing. The herd consisted of 109 Roccaverano, 107 Saanen and 184 French Alpine goats.

2.4. Serum samples and ELISAs

For P16–25 recombinant ELISA, microplates (Immuno- maxi TPP) were coated with 100 ng of P16–25 derived from B and E genotypes or water as negative antigen (Fig. 1). Plates were allowed to dry overnight at 37 8C and then blocked with 2.5% bovine casein for 1 h at 37 8C. After four washes, serum samples were diluted 1/20 in phosphate- buffered saline containing 1.25% casein and incubated for 1 h at 37 8C. Subsequently to the washing step, anti-sheep/goat IgG peroxidase labelled Mab diluted in the same buffer was added and the plates incubated as above. After a final washing step, the reaction was developed with 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 and visualized by box plots. Cut off was previously defined for genotype B as having a reactivity of >40% relative to the positive control serum reactivity included in each plate (Lacerenza et al., 2006). The same absorbance value was applied for genotype E, due to the lack of a truly negative flock regarding this genotype.

For subunit ELISA (P25-B3 epitope), microplates were coated with 220 ng of GST-B3 derived from genotype B (sequence KLNEEAERWRRNNPPPP), genotype E (sequence KLNKEAETWMRQNPQPP) and an equimolar amount of GST as negative control. Net absorbances were obtained by subtracting the GST antigen absorbance from that of each recombinant subunit.

Three commercially available ELISAs, based on whole virus (brand A), double recombinants (brand B) or recombinant and synthetic (brand C) antigens, were used to detect and quantify the infection status in BL herd. Assays were carried out according to the manufacturer's protocols.

Three commercially available monoclonal antibodies (Mabs), namely 5A1, 10A1 and 8B1, known to be reactive against CAEV and MVV capsid antigen (McGuire et al., 1987) were obtained from VMRD, Inc. (Pullman WA, USA) and tested in P16–25 recombinant ELISA. Mabs were used at dilution of 1 mg/well, using mouse-specific secondary antibody. In the latter experiment, a previously produced recombinant capsid antigen from the MVV strain K1514 reactivity between genotype A and B.

2.5. Western blot

The genotype B and E recombinant P16–25 proteins were also tested by Western blot using the same three Mabs (5A1, 10A1 and 8B1), as well as a polyclonal serum from mice immunized with recombinant P16–25 of genotype E (positive control) and serum from the

same goat from which the Roccaverano strain (genotype E prototype) had originally been isolated.

2.6. Sequence analysis and phylogenetic trees

In order to create phylogenetic trees, a model of molecular evolution was estimated using a hierarchical likelihood ratio test approach and the Akaike information criterion (Akaike, 1973) implemented in the software ModelTest ver. 3.7 (Posada and Crandall, 1998, 2001). Bayesian methods implemented in the computer program MrBayes ver. 3.1.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) were used to create phylogenetic trees and to assess statistical support for clades. A Markov chain Monte Carlo search for 1,000,000 generations using two runs with four chains (tempera- ture = 0.05) was performed and results were represented as a 50% majority rule consensus tree. Tree statistics and phylogenetic manipulations were calculated from the computer program PAUP* ver. 4.0b10 (Swofford, 2000). Genetic diversity was expressed as nucleotide diversity (Nei, 1987), or the mean proportion of nucleotide differences among sequences.

2.7. Statistical analysis

Maximum expected prevalence of both genotypes in the herds was estimated using WinEpiscope 2.0 (http:// www.clive.ed.ac.uk/winepiscope/). Agreement beyond chance between genotype E specific ELISA and commercial assays was analysed using Fleiss'k coefficient (Fleiss, 1981). Differences in O.D. values between tests were evaluated using Wilcoxon test.

2.8. Nucleotide sequence accession numbers

Nucleotide sequences reported in this paper were deposited in the GenBank database with accession numbers FJ547242–46. A subset of additional sequences (EF675997–6026; EU726488–525) had been generated in previous studies (Grego et al., 2007; Reina et al., 2009).

3. Results

Recombinant antigen derived from genotype E was successfully expressed and purified. SDS–PAGE of the purified product revealed a single protein band of a molecular weight corresponding to the expected size of matrix and capsid antigen fusion protein (41 kDa) (Fig. 1).

Out of 124 blood DNA samples, 56 were PCR positive and the 39 suitable for sequence analysis, were used to construct a phylogenetic tree using CAEV-CO (M33677), Roccaverano (EU293537) and K1514 (M10608) as refer- ence strains. As shown in Fig. 2, all sequences, representa- tive of herd BL belonged to genotype E (8 sequences out of 12 positive PCR reactions). This sample size allowed us to estimate a maximum expected prevalence of B genotype lower than 25% (CI 95%) within this herd.

Conversely, sequences obtained from herds TM, BM and CF were grouped into the B1 subtype (15 sequences out of 22 PCR reactions), allowing an estimation of a maximum expected prevalence of E genotype lower than 13.5% (CI 95%). Finally, both infections were detected in herd NG with a high degree of co-infections (B and E genotypes) within the same animal, as a previously described genotype specific PCR had revealed (Grego et al., 2007).

Box plots of serum reactivity against B and E recombinant antigens are shown in Fig. 3. In herd BL and the TM–BM–CF herd group, only one homologous antigen was clearly detected among infected animals based on infectious status determined by phylogenetic analysis, while an apparent cross-reactivity between the two antigens was present only in herd NG. To further study the serological reactivity against genotype specific gag antigens, sera were also tested by subunit ELISA, using P25-B3 epitope from genotypes B and E. Indeed, several samples reacted in a type specific manner (Fig. 4). Serum samples belonging to herd BL showed a distribution mainly oriented towards the E derived epitope and on the contrary, B1

infected herds (TM–BM–CF) were clearly reactive only with the B derived epitope (Fig. 4a and c). Herd NG showed a wider distribution of absorbances reaching high values against both peptides (Fig. 4b).
Genotype E derived p16–25 antigen reacted also with few sera from the long term negative herd tested and these sera were from Roccaverano goats, while no reactivity was recorded in Saanen and French Alpine goats cohabiting in the same herd (not shown).
Commercial ELISAs showed a different capacity in detecting infection in herd BL, being the whole virus ELISA in perfect agreement with genotype E derived ELISA (k = 1), followed by the double recombinant ELISA (k = 0.83) and the recombinant/synthetic ELISA (k = 0.47). Distribution of absorbance values in infected animals of the same herd revealed that the best signal-to-noise ratio belongs to genotype E antigen and is significantly higher than those obtained from the three commercial ELISAs ($p < 0.05$) (Fig. 5).
None of the three Mabs showed reactivity against recombinant antigen derived from genotype E in ELISA while antibody cross-reaction was confirmed when using CAEV and MVV capsid antigens (Fig. 6a). Western blot analysis (Fig. 6b) showed a strong reaction when using p16–25 from genotype B and a weak signal against p16–25 of genotype E, likely due to intrinsic higher sensitivity of WB compared to ELISA under the test conditions applied.

4. Discussion

In this study we demonstrate that the highly divergent SRLV genotype E can be detected in a type specific manner by a comparative assay using gag derived antigens from different genotypes. The true prevalence of viral E genotype might have been underestimated so far for two reasons: (i) commercial ELISAs may detect infection without yielding specific serotype information and (ii) some animals may escape diagnosis based on immuno- domiant epitopes highly divergent from E genotype antigens. The development of a first serological tool specific for genotype E has become therefore essential to monitor natural infection by this genotype in the field. We chose gag derived structural proteins since it is well documented that anti-gag derived antibody response remains detectable for a long time, if not life long (de Andres et al., 2005), and cross-reactivity between MVV and CAEV P16–25 is acceptable except in the early stage of infection when homologous antigen is more quickly recognised (Lacerenza et al., 2006). Lack of sensitivity of CAEV P16–25 in detecting genotype E infection and vice versa was not surprising. All the diagnostically relevant epitopes characterized so far in the gag encoded proteins (summarized in Table 1), show divergences between genotype E and CAEV or MVV. Commercial ELISA based on whole virus preparations demonstrate that genotype E can be detected, likely due to the presence of a larger panel of cross-reacting epitopes which may compensate the high variability between genotype E and ELISA antigen. The other commercial ELISAs used are based on a more restricted antigen display and we cannot exclude that, for example, the TM epitope of genotype E might elicit cross-reacting antibodies recognised by heterologous antigen preparations.
We previously described P25 immunodominant epitope and matrix protein as specifically detecting A or B genotype infections (Rosati et al., 1999; Grego et al., 2005). Here, we show that genotype E derived P25 immunodominant epitope also recognises only homologous infection. Lack of reactivity of three Mabs, known to be reactive against CAEV and MVV, with genotype E P25 protein was surprising and demonstrates that there are at least three different type specific epitopes in P25. This is relevant when aiming for antigenic detection of a wide spectrum of SRLVs. The hypothesis that genotype E infection could have escaped through traditional diagnostic pressure (test and slaughter policy), was evaluated in a SRLV free herd in which few old aged animals of the Roccaverano breed were slightly reactive against genotype E recombinant antigen. We cannot rule out a false positive reaction since an attempt to amplify viral sequences from the same animals gave inconclusive results. It should

be taken into account that no positive reaction was recorded in any of the 292 animals belonging to other breeds of the same herd. On the other hand, animals with a low proviral load could be misdiagnosed using PCR. Interestingly, young animals of the three breeds (Roccaverano, Saanen and French Alpine) were negative, confirming that horizontal transmission is probably hampered due to genetic deletions present in this low pathogenic virus. Thus, artificial feeding of kids, a prevention strategy for SRLV control still used in the herd, is an efficient way to co-eradicate both CAEV and genotype E infections.
To date we could identify only three herds infected with genotype E and no more than about a hundred E-infected animals, all belonging to the Roccaverano goat breed (total heads 1500) which was at risk of extinction in the seventies. We are therefore aware that the geographical distribution of genotype E may have a very limited impact in other countries. However, this study shows that the lack of E specific serological tests does not allow the determination of the true prevalence of genotype E infection. Molecular tools such as PCR are now available for genotype E as well as a congruous number of GenBank deposited sequences. The low agreement between PCR and ELISA in detecting genotype E infected animals was not surprising, since this has been widely demonstrated by other authors regarding A or B genotypes. This is likely due to low viral load or to high heterogeneity at the primer binding site (de Andres et al., 2005). It should also be noted that not all the recently described PCRs developed for generic detection of SRLVs may be used for genotype E identification. Primers described by Shah et al. (2004a) designed to amplify 1.2 kb of pol gene, will result in a product of 0.8 kb, resembling unspecific, due to internal dUTPase deletion. In addition, due to the same deletion, PCR primers described by Eltahir et al. (2006) will fail to amplify as a result of the lack of forward primer binding site.
In conclusion, our results demonstrate that, despite the limited information on the distribution of genotype E in small ruminant population, specific antigen design is required for accurate diagnosis. Subunit ELISA using a panel of A, B and E immunodominant region of capsid antigens can be used as a rapid preliminary screening test that would allow serotype determination. On the other hand, evidence regarding this highly divergent genotype has widened the space in which SRLVs have evolved with potential implications for control strategies.

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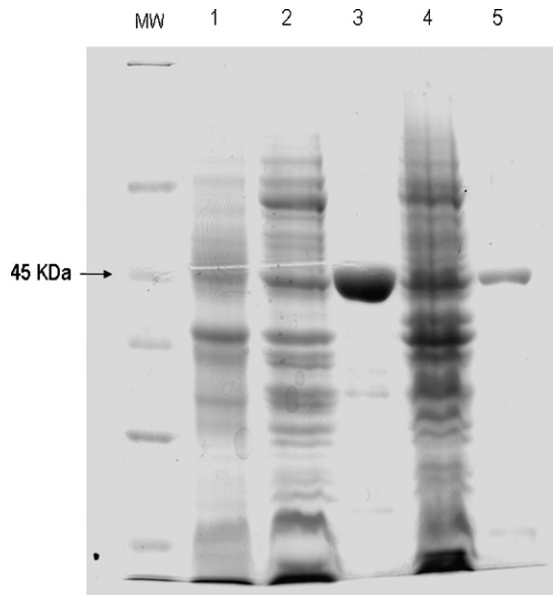

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showing expression and purification of recombinant antigens in E. coli. MW, molecular-weight standard; lane 1 E. coli lysate; lanes 2 and 4 bacterial lysate expressing GST/P16-25 of It-Pi1 (genotype B) and Roccaverano (genotype E) respectively; lanes 3 and 5, affinity purified recombinant P16-25 of the same strains after GST cleavage and IMAC.

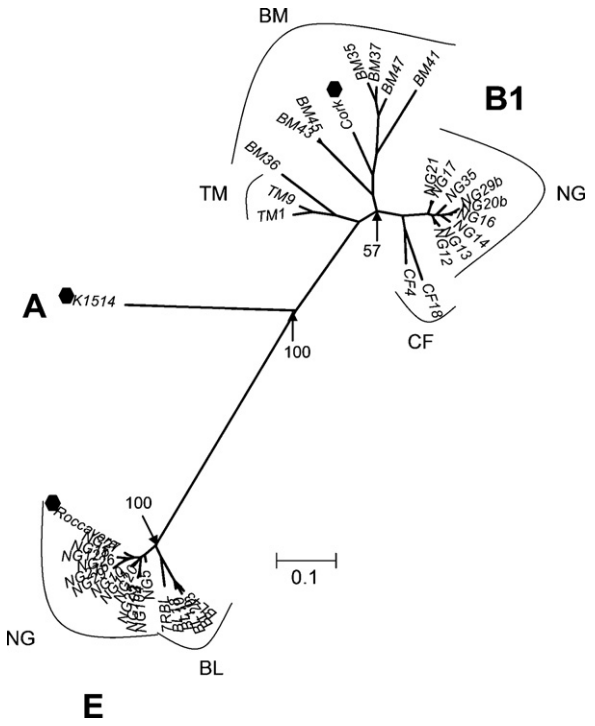

Fig. 2. Phylogenetic tree of amplified gag region from different herds. The reference strains of genotype A (M10608), B (M33677) and E (EU293537) are shown.

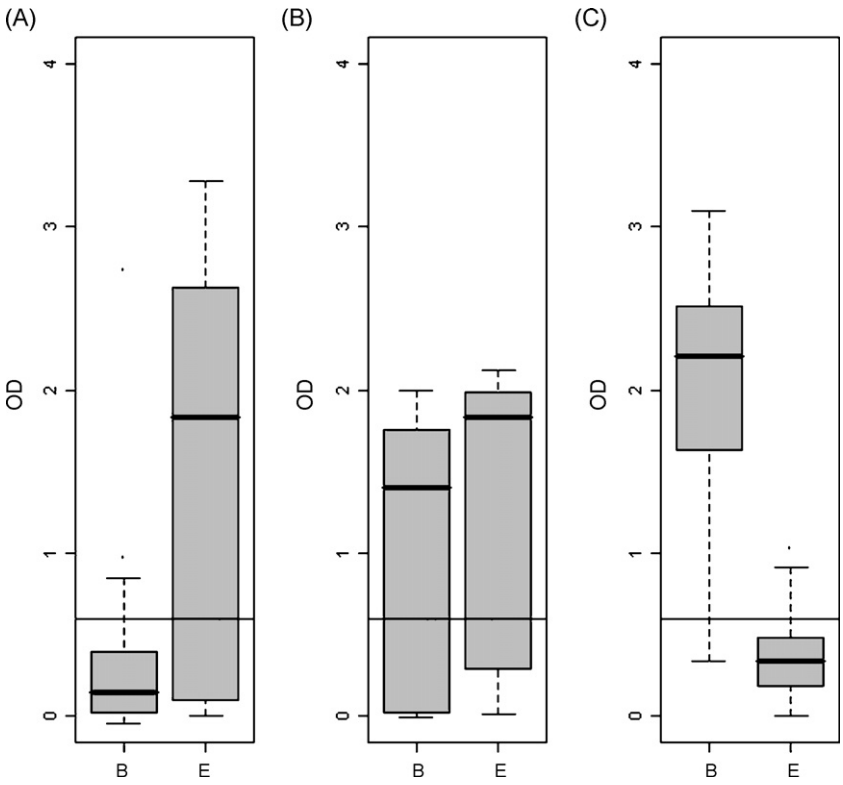

Fig. 3. Box plots of net absorbances in ELISA testing of sera from BL herd (panel A), NG (panel B) and TM–BM–CF group (panel C) against recombinant P16–25 of genotypes B (left box) and E (right box). Bottom and top of each box represent the first and the third quartiles of the distributions. The median is represented by the line inside the box.

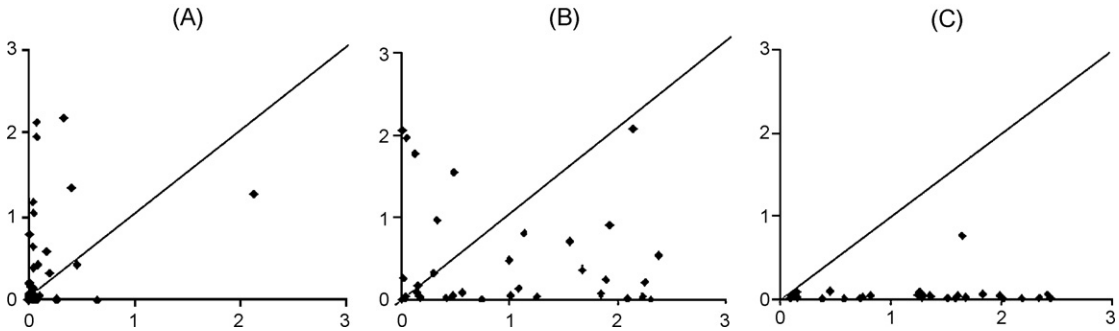

Fig. 4. Net absorbance against P25-B3 subunit ELISA: each serum sample was tested against the B derived epitope (X-axis) and E derived epitope (Y-axis). Samples are from BL herd (panel A), NG (panel B) and TM–BM–CF group (panel C).

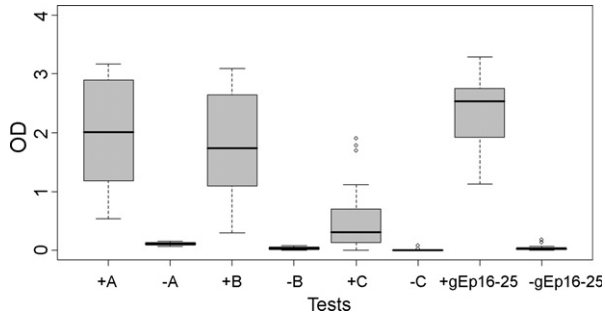

Fig. 5. Distribution of O.D. values in BL herd using different serological tests. Sera which were positive to at least two ELISA tests were considered as true positive. Brand A: whole virus based ELISA (O.D. 450 nm); brand B: double recombinant based ELISA (O.D. 450–595nm); brand C recombinant and synthetic based ELISA (O.D. 450–595 nm); genotype E specific recombinant P16–25 ELISA (O.D. 405 nm). Bottom and top of each box represent the first and the third quartiles of the distributions respectively. The median is represented by the line inside the box.

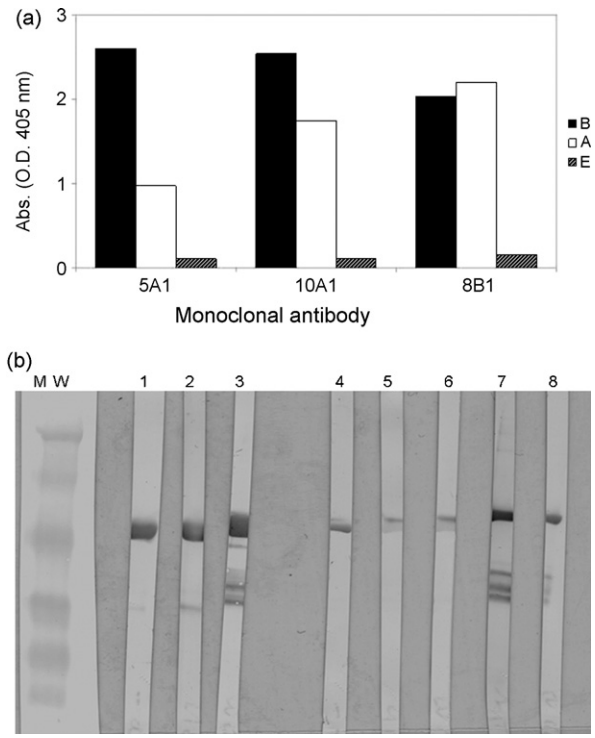

Fig. 6. (a) Monoclonal antibody reactivity in ELISA (O.D. 405 nm) against capsid antigen of CAEV isolate (McGuire et al., 1987). Net absorbances against recombinant P16–25 of genotypes E and B and recombinant P25 of genotype A are shown. (b) Western blot analysis of the same Mabs (5A1, 10A1 and 8B1) against P16–25 of genotypes B (lanes 1, 2 and 3) and E (lanes 4, 5 and 6). Lanes 7 and 8 were developed using a polyclonal mouse antiserum raised against E derived fusion protein, and a serum from an E infected goat respectively. MW, molecular weight marker.

Genotype	GenBank	Matrix (Grego et al., 2005)	Capsid antigen B (Grego et al., 2002)	Capsid antigen D (Rosati et al., 1999)	Nucleoprotein (Lacerenza et al., 2008)	Transmembrane (Bertoni et al., 1994)
B	M33677	KLLTPEESNKKDFMSL	LNEEAERWRRNNPPPPA	VQQASVEEKMQACRDVGSE	GNGRRGIRVVPSAPPME	ELDCWHYHQYCITS
A	M10608	-N———TS-RE-A–	–D———V-Q—G-N	——T—————————	–N—P————————L	——————QH-V–
E	EU293537	RSM——ESR—V–	–K—T-M-Q-Q–GP	A-TST——L——E-E-S	–SQ–P————————Q	-I————G-V–

Table 1
Immunodominant linear epitopes of SRLV identified so far along the structural proteins in genotypes A, B and E. Genbank accession numbers, source references and amino acid sequences from each genotype are indicated. Dashes represent the same amino acid as that of genotype B.