

## Characterization of the *dUTPase- Integrase* Region of the Pol Gene of Small Ruminant Lentiviruses from Greece

Y.M. Eltahir

Department of Preventive Medicine and Veterinary Public Health, Faculty of Veterinary Science, University of Nyala, Nyala, Sudan

**Abstract:** Despite the wide spread of Small Ruminant's Lentiviruses (SRLV), Maedi Visna Virus (MVV) and caprine arthritis-encephalitis (CAEV) in Greece, sequence information on the pol gene of Greek SRLV (GrSRLV) is not reported before. The present study was aimed to establish a sequence database in a conserved region of the viral genome which can then serve to perform phylogenetic analysis. Three hundred and seventy six nucleotides (*dUTPase- integrase*) region of the pol gene of GrSRLV from different mixed flocks were obtained by semi-nested PCR using degenerate primers. GrSRLV showed 79 and 81% homology in their nucleotides and deduced amino acids sequences respectively. Molecular epidemiology based on the pol sequences indicated that GrSRLV contains both type A and B viruses and the occurrence of five genetically different clusters of SRLV circulating in Greece. The results suggest that GrA-SRLV have a different origin from that of British, Icelandic and South African SRLV reference strains. It also gives additional evidence of interspecies transmission of these viruses between sheep and goats.

**Key words:** Caprine arthritis encephalitis, maedi visna, phylogeny, pol, small ruminat lentiviruses

### INTRODUCTION

Small Ruminants Lentiviruses (SRLV) Maedi Visna Virus (MVV) and Caprine Arthritis-Encephalitis (CAEV) cause life long persisting infections. These include interstitial pneumonia, arthritis, mastitis, emaciation and rarely meningoencephalomyelitis in sheep and goats. The transmission of SRLVs among goats or sheep occurs through the ingestion of virus-infected colostrum or milk by the newborn. Interspecies transmission has been demonstrated through the detection of particular SRLV subtypes in both goats and sheep (Leroux *et al.*, 1997; Seroude *et al.*, 2002; Shah *et al.*, 2004a, b; Pisoni *et al.*, 2005; Ramírez *et al.*, 2010).

Phylogenetic classification of SRLV showed that: MVV prototypes isolated from sheep are referred to as group A. Which then further divided into several subtypes isolated also from sheep (A1, A2,A10), goats (A5, A7,A8) or both species (A3, A4, A6,A9) (Shah *et al.*, 2004, b; Grego *et al.*, 2007; Laamanen *et al.*, 2007). CAEV prototypes 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, b). Strains, isolated from a Norwegian goat, Swiss goat and Italian goat are referred to as groups C, D and E (Pisoni *et al.*, 2005; Shah *et al.*, 2004b; Grego *et al.*, 2007; Reina *et al.*, 2010).

In Greece, seropositive sheep using the Agar Gel immunodiffusion Test were first identified in 1980s' during a serological screening, which was performed at

different geographic regions of the country. Later the detection of SRLV proviral DNA by PCR is reported by Karanikolaou *et al.* (2005). The phylogenetic analysis of Greek SLRV (GrSRLV) strains which was based on the gag gene of five strains isolated from four sheep and one goat originating from the north of the country suggested that Greek strains cluster into a unique group; separate from, but related to other ovine prototype sequences (Angelopoulou *et al.*, 2005).

Despite the high prevalence (42%) of the SRLV infection, no compulsory eradication program currently exists in Greece (Karanikolaou *et al.*, 2005) and the amplification of the pol gene of GrSRLV has never been reported before. The main objective of this research is to establish a sequence database in a conserved region of the viral genome which can then serve to perform phylogenetic analysis and be representative of the SRLV strains now circulating among sheep and goats in Greece. This would in turn add new sequence information of GrSRLV which could be used to perform further molecular and epidemiological studies.

### MATERIALS AND METHODS

**Animals:** Seventy four animals (55 sheep and 19 goats) originating from five different mixed flocks from Nea Horouda (Nh) , Kavala (Ka), Attiky (At), Grevena (Gv) and Kalamata (Kl), (n = 15, 15, 15, 15, 14) villages representing northern, central and south Greece with history of SRLV infection were used in this study. The

study was conducted in 2006 in the laboratory of microbiology and infectious diseases, School of Veterinary Medicine, Aristotle University of Thessaloniki, Greece. Serology was carried out with the Agar gel immunodiffusion test (AGID) using Maeditec kit (Maeditec, Veterinary Laboratory Agency, Weybridge, UK). Formal published procedures were followed throughout AGID (Cutlip *et al.*, 1977). Twenty one seropositive animals (6 Goats and 15 sheep) from Nea Horouda (n = 9), Kavala (n = 1), Attiky (n = 1), Grevena (n = 1) and Kalamata (n = 1) were then used for the amplification of pol gene by PCR.

**DNA extraction:** According to manufacturer's instructions, DNA was extracted from peripheral blood mononuclear cells (PBMCs) from seropositive animals using the commercially available Flexi Gene DNA Kit (QIAGEN GmbH, Germany). The DNA was then quantitated and stored at -20°C until PCR was performed.

**Semi-nested PCR:** PCR reactions and conditions were carried out as described previously (Eltahir *et al.*, 2006) in a T-CY thermocycler (Creacon, The Netherlands). Briefly proviral sequences of 404 base pairs (bp) from the pol gene were amplified by the semi-nested PCR using primers LenUp1 5'-ARGGIGGIATMATAGAYTCIGG-3' and LenDo2 5'-ARTGIGTRTARTCIACYTGC CA-3' for the first round, and LenNestUp3 5'-GGGATMATAGAYTCGGGRTA TCARGG-3' and LenNestDo4 5'-TGGGTRTARTCGACYTGCCARTG-3' for the second round. DNA of the WLC-1 Weybridge MVV strain was used as positive control and DNA extracts from a seronegative animal or a non-infected tissue culture were used as negative controls. The semi-nested PCR products were analyzed by electrophoresis in 1.5% agarose gels in TAE buffer, stained with ethidium bromide and visualized under UV light. For all animals the isolation of the DNA and PCR amplifications were

carried out twice, to confirm the reproducibility of the results.

**DNA sequencing:** Specific semi-nested PCR products obtained from seropositive animals were purified using the agarose gel with the QIAquick Gel Extraction Kit (Qiagen GmbH, Germany). Both strands of the DNA were directly sequenced using the dideoxynucleotide termination cycle method with an ABI3730XL sequencer, using a Big Dye Terminator v 3.1 (Macrogen Inc. Seoul, Korea) and the internal primers "LenNestUp3" and "LenNestDo4".

**Analysis of the sequence data:** Alignments of nucleotide sequences were initially performed with CLUSTAL X (Thompson *et al.*, 1997) and later edited manually. The corresponding amino acid sequences were obtained according to the Expasy (ExpertProtein Analysis System) server. Further, Phylogenetic and molecular evolutionary analyses were carried out using MEGA version 3.1 (Kumar *et al.*, 2004). Phylogenetic analysis of nucleic acid sequences was done with the neighbor-joining method, Kimura 2-parameter model. The aa sequences were also analyzed with the neighbor-joining method, with the Poisson correction. Bootstrap values (1000 replications) were indicated on each tree. Confirmation of tree topology was carried out with Maximum Likelihood using PHYLIP (Phylogeny Interface Version 3.5c). To reduce redundancy, relevant one-two sequences representing types (A-D) and subtypes (A1-A7 and B1-B2) SRLV were used (Shah *et al.*, 2004b). Subtypes (A8-A10) were excluded from analysis since they do not contain the amplified part of the pol gene.

## RESULTS

**PCR:** Specific PCR product (404 bp) was detected in 21 seropositive animals out of the 74 animals under the study (Fig. 1).

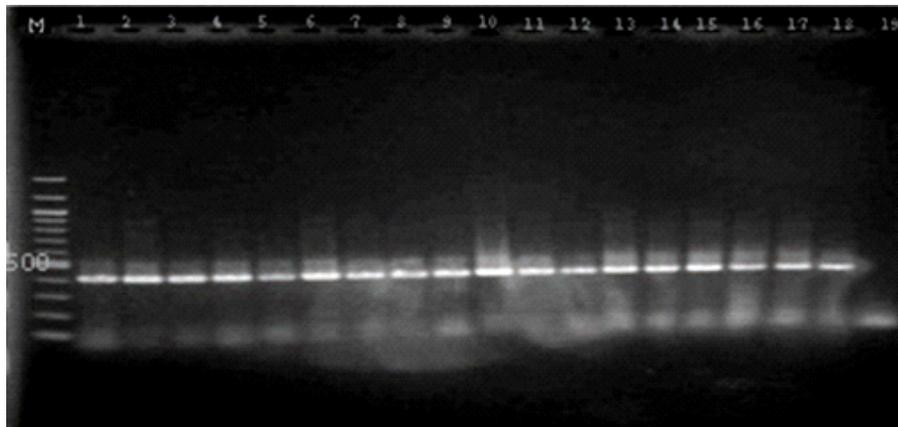


Fig. 1: Specific PCR product (404-bp); (M) 100 bp DNA ladder marker (Biolab). Lane 1 positive control, lanes 2-18 samples detected positive, lane 19 negative control

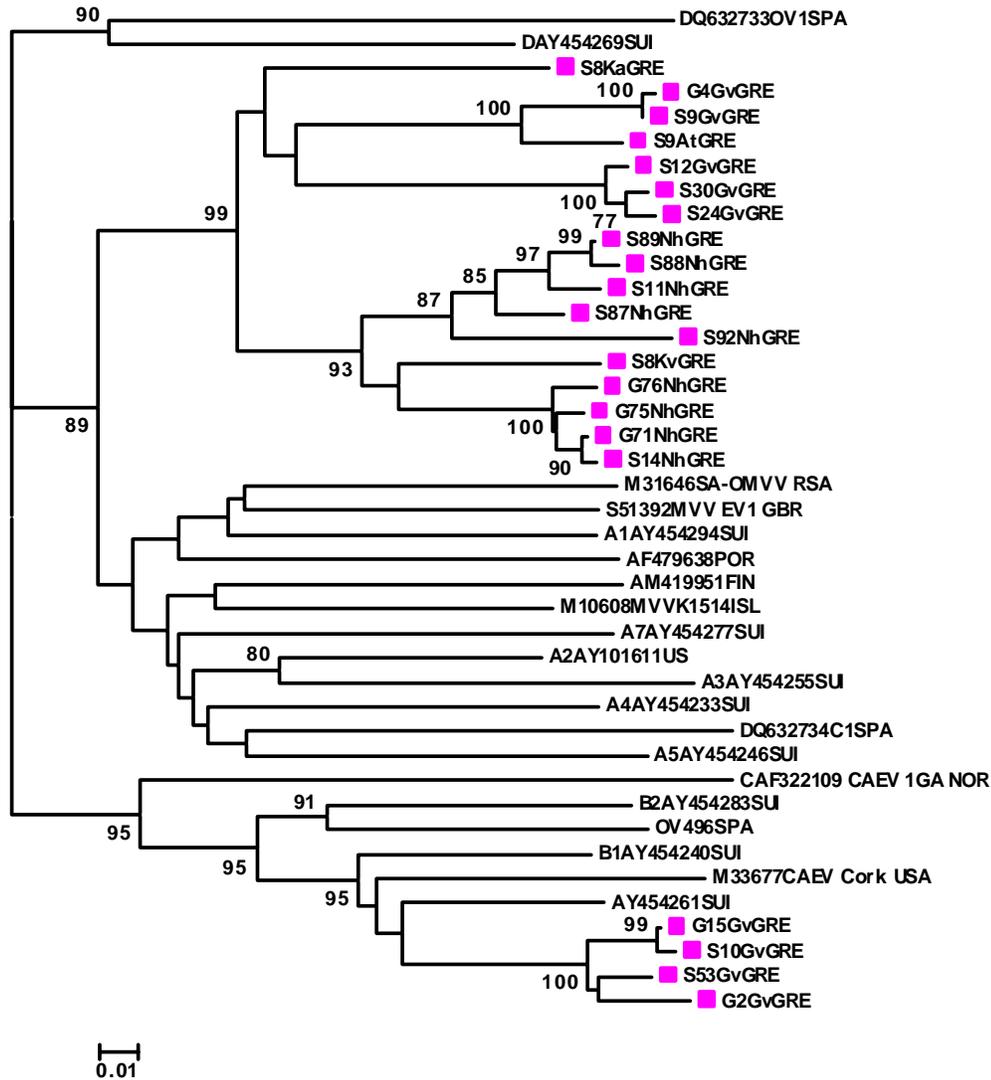


Fig. 2: Neighbour-joining tree of a 376 nt fragment of the pol (dUTPase-integrase) region from isolates of the present studied population and GenBank selected sequences representing SRLV subtypes (A1-A7), (B1 and B2), types C and D labeled according to (Shah *et al.*, 2004b). In addition to SRLV prototypic strains and isolates from Finland, Norway, Portugal and Spain. The tree was constructed with the distance matrix calculated with the Kimura's two-parameter model. Bootstrap testing of phylogeny was performed with 1000 replications and values equal or greater than 70 are indicated on the branches (as percentage). Sequences from the present study are named as follows G/S-Pp GRE where, G: goat; S: sheep; Pp: area of origin; GRE: Greece. Closed symbols correspond to the new Greek isolates. M311646 corresponds to SA-OMVV, M10608 IL to K1514, S51392 to EV1 and M33677 to CAEV-Cork. Country abbreviations IN: Finland; GBR: Great Britain; ISL: Iceland; NOR: Norway; POR: Portugal; RSA: South Africa; SUI: Switzerland; USA: United States of America.

**Nucleotide sequence accession numbers:** All Greek SRLV pol gene nucleotide sequences were deposited in the GenBank and are available under accession numbers AM230934 to AM230945 and AM233492 to AM233508.

**Phylogenetic analysis:** Phylogenetic analysis using the neighbor-joining algorithm of GrSRLV along with the

selected sequences obtained from the data base, which correspond to our PCR product 376bp after the removal of primers sequences, showed that, GrSRLV isolates could be divided into two main groups. Group1 MVV related or type A Greek SRLV (GrA-SRLV) and group2 as CAEV-Cork like or type B Greek SRLV (GrB-SRLV). GrA-SRLV which was established from 17 isolates

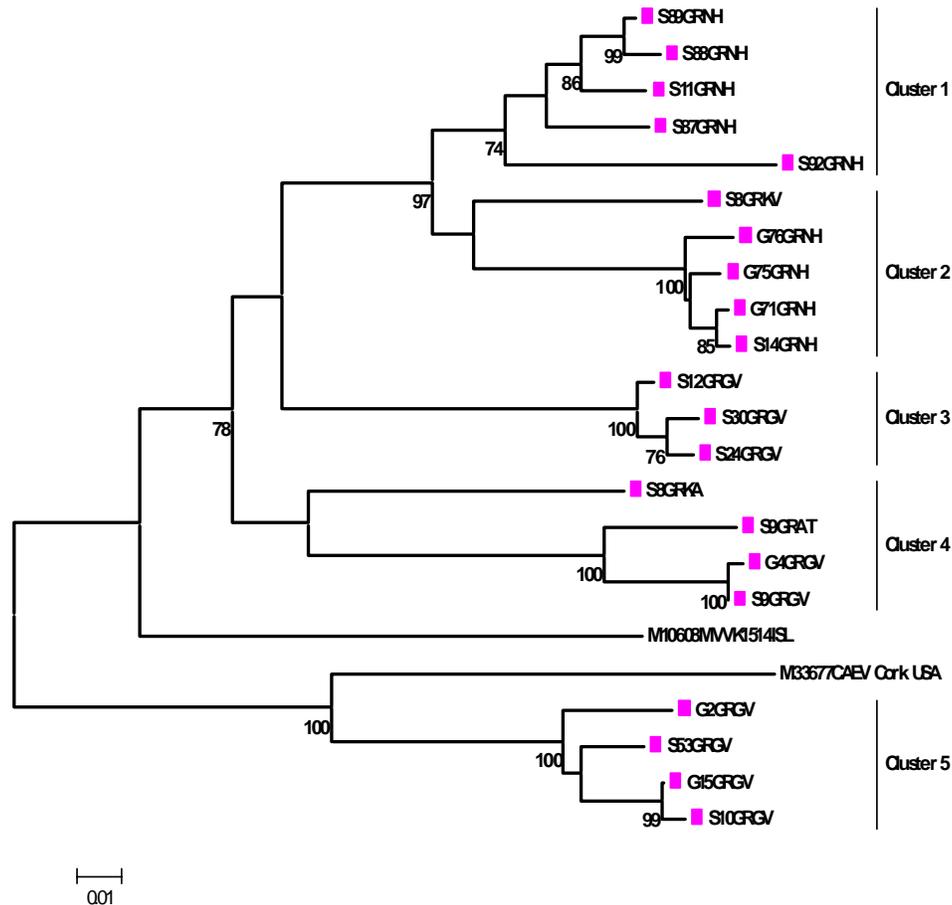


Fig. 3: Neighbour-joining tree of a 376 nt fragment of the pol (dUTPase-integrase) region from isolates of the present studied population and GenBank sequences representing the CAEV-CO and K1514 prototypic strains. Greek isolates were chosen as representative of the five flocks of origin. The tree was constructed with the distance matrix calculated with the Kimura's two-parameter model. Bootstrap testing of phylogeny was performed with 1000 replications and values equal or greater than 70 are indicated on the branches (as percentage). Sequences from the present study are named as follows G/S-Pp GRE where, G: goat; S: sheep; Pp: area of origin; GRE: Greece. Closed symbols correspond to the new Greek isolates .M10608 IL corresponds to K1514 and M33677 to CAEV-Cork

(13 sheep and 4 goats) isolated from north (Nh, Kv and Gv), central (At) and south (Ka) of Greece contained highly divergent isolates related to the type A-SRLV, which contained MVV prototypic strains as they were separated by a bootstrap value of 89 from SRLV types B, C and D.

Four isolates 2 goats and 2 sheep (GrB-SRLV) originating from one mixed flock (Gv), clustered with type B- SRLV, which contains CAEV-Cork, had low divergence and were separated by a bootstrap value of 95 from other types (A, C and D) of SRLV (Fig. 2). Similar phylogenetic tree topology was obtained using maximum likelihood algorithm. However, lower boot strap values of 72 and 85 were observed to separate GrA-SRLV and GrB-SRLV respectively (data not shown).

The epidemiological analysis of GrSRLV isolated in this study was performed by the reconstruction of a phylogenetic tree which included all Gr SRLV along with the K1514 and CAEV-Cork reference strains. Gr SRLV formed five different clusters characterized by low divergence with mean similarity degrees that ranged from (94-100%). Four clusters that contain isolates from Nh and Gv flocks were located within GrA-SRLV. Whereas, one cluster which established by isolates from Gv flock which was located within GrB-SRLV(Fig. 3).

GrSRLV examined in this study didn't cluster neither according to the farm of origin nor on the caprine or ovine origins. Indeed isolate S8KvGRE from sheep clustered with sheep and goats isolates from Nh flock. Isolates S9AtGRE and S8KaGRE isolated from sheep

Table 1: Nucleotides (in bold numbers) and deduced amino acids(in plain numbers) comparison between Greek isolates and the SRLV prototypic strains

SRLV	GrA	GrB	K1514	EV1	SA-OVMVV	CAEV-Cork
GrA		29	17	18	19	31
GrB	<b>32</b>		24	27	28	17
K1514	<b>25</b>	<b>32</b>		12	13	26
EV1	<b>28</b>	<b>34</b>	<b>20</b>		10	27
SA-OV MVV	<b>27</b>	<b>33</b>	<b>24</b>	<b>20</b>		31
CAEV-Cork	<b>33</b>	<b>17</b>	<b>28</b>	<b>30</b>	<b>35</b>	

from two different flocks clustered with sheep and goat isolates from Gv flock. Isolates from flock Gv contains both A and B types of GrSRLV( Fig. 3).

The clustering of isolates from sheep and goats together was also clear in both GrA-SRLV and GrB-SRLV groups. This was demonstrated within GrA-SRLV by the clustering of isolate S14NhGRE from sheep with goat's isolates (G71NhGRE, G76NhGR and G71NhGRE) from the same (Nh) flock. Also isolate S9GvGRE from sheep with isolate G4GvGRE isolated from goats from the same (Gv) flock. In contrast goats isolates (G2GvGRE and G15GvGRE) clustered with (S53GvGRE and S10GvGRE) isolated from sheep from the same (GV) flock within GrB-SRLV( Fig. 3).

**Sequence alignment:** GrSRLV showed 79 and 81% homology in their nucleotides and deduced amino acids sequences respectively. Nucleotide sequences comparison with the prototypic strains of SRLV showed that GrA-SRLV were 25, 27 and 28% (nucleotide pairwise distances) divergent from K1514, EV1 and SA-OVMVV reference strains respectively. Whereas, they were 33% divergent from CAEV-Cork reference strain. In contrast GrB-SRLV were 32, 34 and 33% divergent from K1514, EV1 and SA-OVMVV reference strains. Whereas, they were 17% divergent from CAEV-Cork (Table 1).

Deduced amino acids sequences comparison with the prototypic strains of SRLV showed that GrA-SRLV were less divergent (17%) from K1514, whereas 31% divergent from CAEV-Cork reference strain (Poisson correction). Divergences of (18 and 19%) from, EV1 and SA-OMVV reference strains were observed respectively. GrB-SRLV was less divergent (17%) from CAEV-Cork, whereas 24% divergent from K1514 reference strain. Divergences of (27 and 28%) from, EV1 and SA-OMVV reference strains were observed respectively (Table 1).

## DISCUSSION

This is the first report on the partial characterization of the pol gene of SRLV from Greece. In the present study the SRLV circulation among sheep and goats in Greece was detected by PCR based on the dUTPase-integrase encoding region of the pol gene.

The classification of SRLV proposed by Shah *et al.* (2004b) is open and provides additional room for additional groups and subtypes to be identified in the future. This has been shown by the reporting of new subtypes (A8, A9 and A10) of SRLV (Grego *et al.*, 2007; Laamanen *et al.*, 2007). Thus, we first constructed a phylogenetic tree using neighbor-joining method with the Kimura's two-parameter or Maximum Likelihood models with SRLV pol gene (dUTPase-integrase area) nucleotide sequences which are available from the databases and correspond to the PCR product amplified in this study. The overall tree topology schematically divided SRLV into groups A-D (Shah *et al.*, 2004b). Thereby, indicating that the area selected in this study from the SRLV pol gene is suitable to perform phylogenetic analysis.

Phylogenetic analysis of GrSRLV based on selected sequences in the pol-coding region of naturally occurring SRLV in goats and sheep from mixed flocks indicated that these viruses are highly diverse, neither a separation between goat or sheep lentiviruses, nor a geographical separation could be distinguished. GrSRLV could be divided into two mains groups; group1 MVV related or type A Greek SRLV (GrA-SRLV) and group2 CAEV-Cork like or type B Greek SRLV (GrB-SRLV). GrA-SRLV viral sequences formed four different genetic clusters related to, but were completely distinct from type A-SRLV. This contains the Icelandic K1514, British EV1, and South African maedi visna SA-OMV reference strain. The separation of GrA-SRLV from SRLV type A strains suggests that their origin may be different from that of Icelandic, British and South African viruses. It should be noted that amplifications with primer pairs which were selected according to the prototypic SRLV strains alone or Shah *et al.* (2004b) were not successful. On the other hand, the presence of more than one genetically related viral clusters within the same flock (Neohorouda and Grevena), containing isolates with very low divergence, indicated that different viral strains are simultaneously evolving within the same flock.

GrB-SRLV isolates, characterized by minor genetic distance among them indicating a common source of origin, were closely related to CAEV-cork prototype strain. These isolates came from one mixed flock (Gv) which had documented contact with the importation of

Goats from Switzerland where type B- SRLV is the most dominant one. Evidence for the introduction of SRLV via live animal trade is reported before (Peterhans *et al.*, 2004). At present no control program currently exists in Greece, hence no precise epidemiological information is available with our data concerning this flock, which also harbors GrA-SRLV. Therefore, justification of this infection as a result of live animal trade across herds, which is considered as a major risk in the spread of SRLV, is not clear or conclusive. However, it gives additional evidence of interspecies transmission of these viruses between sheep and goats within the same flock as previously reported by (Leroux *et al.*, 1997; Pisoni *et al.*, 2005; Shah *et al.*, 2004a).

The clustering together of epidemiologically unrelated isolates can be explained by the movement of infected animals from one flock to other. At both nucleotides (25-28%) and deduced amino acids (17-19%) level GrA-SRLV appeared to be closely related to ovine prototypic strains than to the caprine strain CAEV-Co (33 and 31%) respectively. Such findings are also reported before (Angelopoulou *et al.*, 2005). On the other hand GrB-SRLV that closely related to CAEV-Co than to ovine prototypic strains are firstly reported here.

In conclusion this is the first report on partial characterization of the pol gene of SRLV in Greece. GrSRLV contains both type A and B- SRLV. Five genetically different clusters of SRLV are circulating in Greece. The present study confirms also these viruses are not host-specific. Therefore, the interspecies transmission and/or the circulation of the recently reported dUTPase mutant SRLV should be taken into account in control programs.

#### ACKNOWLEDGMENT

Author is highly grateful to Dr. Kagiabakis M. from the State Veterinary Clinic at Grevena, Greece, for providing blood samples and to the Hellenic Scholarship Foundation.

#### REFERENCES

- Angelopoulou, K., K. Karinikolaou, M. Papanastasopoulou, M. Koumpati-Artopiou, I. Vlemmas, O. Papadopoulos and G. Koptopoulos, 2005. First partial characterization of small ruminant lentiviruses from Greece. *Vet. Microbiol.*, 109: 1-9.
- Cutlip, R.C., T.A. Jackson and G.A. Laird, 1977. Immunodiffusion test for ovine progressive pneumonia. *Am. J. Vet. Res.*, 38: 1081-1084.
- Eltahir, Y.M., C.I. Dovas, M. Papanastassopoulou, M. Koumbati, N. Giadinis, S. Verghese-Nikolakaki and G. Koptopoulos, 2006. Development of a semi-nested PCR using degenerate primers for the generic detection of small ruminant lentivirus proviral DNA. *J. Virol. Methods*, 135: 240-246.
- Grego, E., L. Bertolotti, A. Quasso, M. Profiti, D. Lacerenza, D. Muz and S. Rosati, 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.
- Karanikolaou, K., K. Angelopoulou, M. Papanastasopoulou, M. Koumpati-Artopiou, O. Papadopoulos and G. Koptopoulos, 2005. Detection of small ruminant lentiviruses by PCR in field samples of animals from Greece. *Small Rumin. Res.*, 58: 181-187.
- Kumar, S., K. Tamura and M. Nei, 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief. Bioinform.*, 5: 150-163.
- Laamanen, I., M. Jakava-Viljanen and L. Sihvonen, 2007. Genetic characterization of maedi-visna virus (MVV) detected in Finland. *Vet. Microbiol.*, 122: 357-365.
- Leroux, C., C. Lerondelle, J. Chastang and J.F. Mornex, 1997. RT-PCR detection of lentiviruses in milk or mammary secretions of sheep or goats from infected flocks. *Vet. Res.*, 28: 115-121.
- Peterhans, E., T. Greenland, J. Badiola, G. Harkiss, G. Bertoni, B. Amorena, M. Eliazewicz, R.A. Juste, R. Krassnig, J.P. Lafont, P. Lenihan, G. Petursson, G. Pritchard, J. Thorley, C. Vitu, J.F. Mornex and M. Pepin, 2004. Routes of transmission and consequences of Small Ruminant Lentiviruses (SRLVs) infection and eradication schemes. *Vet. Res.*, 35: 257-374.
- Pisoni, G., A. Quasso and P. Moroni, 2005. Phylogenetic analysis of small-ruminant lentivirus subtype B1 in mixed flocks: evidence for natural transmission from goats to sheep. *Virology*, 339: 147-152.
- Ramírez, H., I. Glaria, X.D. Andrés, H.A. Martínez, N.M. Hernández, R. Reina, E. Iráizoz, H. Crespo, E. Berriatua, J. Vázquez, B. Amoren and D.D. Andrés, 2010. Recombinant small ruminant lentivirus subtype B1 in goats and sheep of imported breeds in Mexico. *Vet. J.*, doi:10.1016/j.tvjl.2010.09.005.
- Reina, R., L. Bertolotti, S. Dei Giudici, G. Puggioni, N. Ponti, M. Profiti, C. Patta and S. Rosati, 2010. Small ruminant lentivirus genotype E is widespread in Sarda goat. *Vet. Microbiol.*, 144(1-2): 24-31.
- Seroude, V., G. Audoly, P. Gluschkof and M. Suzan, 2002. Viral and cellular specificities of caprine arthritis encephalitis virus Vif protein. *Virology*, 292(1): 156-161.
- Shah, C., J.B. Huder, J. Boni, M. Schonmann, J. Muhlherr, H. Lutz and J. Schupbach, 2004a. Direct evidence for natural transmission of small-ruminant lentiviruses of subtype A4 from goats to sheep and vice versa. *J. Virol.*, 78: 7518-7522.

Shah, C., J. Boui, J.B. Huder, H.R. Vogt, J. Muhlherr, R. Zanoni, R. Miserez, H. Lutz and J. Schupbach, 2004b. Phylogenetic analysis and reclassification of caprine and ovine lentiviruses based on 104 new isolates evidence for regular transmission. *Virology*, 319: 12-26.

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin and D.G. Higgins, 1997. The CLUSTAL\_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.*, 25(24): 4876-4882.