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Abstract
Recent worldwide serological and genetic studies of small ruminant lentiviruses (SRLV) have led to the description of new genotypes and the development of new diagnostic tests. This study investigated the detection and molecular characterization of visna/maedi virus (VMV) infection in serum and blood samples from pure and mixed sheep breeds acquired from different regions in Turkey using ELISA and PCR techniques. The prevalence of VMV was 67.8 % by ELISA and/or LTR-PCR with both assays showing a medium level of agreement (kappa: 0.26; ± 0.038 CI). Positivity of VMV in sheep increased according to the age of the animal, although PCR positivity was higher than ELISA in young individuals. Phylogenetic analysis of 33 LTR sequences identified two distinct clades that were closely related to American and Greek LTR sequences. Phylogenetic analysis of 10 partial gag gene sequences identified A2, A3, A5, A9, A11 subtypes of genotype A SRLVs. In vitro culture of all isolates in fetal sheep lung cells (FSLC) showed a slow/low phenotype causing less or no lytic infection compared with infection with the WLC-1 American strain characterized by a rapid/ highly lytic phenotype. Phylogenetic analysis revealed that Turkish VMV sequences preceded the establishment of American or Greek strains that were associated with the migration of sheep from the Middle East to Western Europe several centuries ago. This is the first study that describes Turkish VMV sequences with the molecular characterization of LTR and gag genes, and it strongly suggests that SRLV-genotype A originated in Turkey.

Introduction

Small ruminant lentiviruses (SRLV) include visna/maedi virus (VMV) and caprine arthritis encephalitis virus (CAEV) and belong to the genus Lentivirus of the family Retroviridae. SRLV infection is persistent and chronic, characterized by slow progressive degenerative inflammation in multiple organs, including lungs, brain, udder, and joints in sheep and goats. SRLV infection results in performance losses, decreased milk production and sudden deaths due to encephalitis [1]. Transmission of SLRV occurs during colostral uptake (lactogenic transmission) or by direct contact between infected animals through respiratory secretions (horizontal transmission). Diagnosis of the infection is usually performed by serological tests or detection of the viral genome. Although agar gel immunodiffusion (AGID) has been used for diagnosis for many years and is the World Organization for Animal Health (OIE) reference method for serological diagnosis, various ELISA

methods have been developed using different antigen preparations. Available commercial ELISA kits include whole virus preparations (AG-Chekit CAEV/MVV kit; IDEXX, Switzerland), single recombinant proteins (Institut Pourquier, Montpellier, France) or a combination of synthetic peptides (Elitest) [2]. However, intermittent or weak antibody responses may cause false-negative results in serological assays. Molecular techniques may also be used for SRLV diagnosis and show an added value for detecting early infections, especially those caused by divergent strains [3– 5]. Molecular epidemiological studies have resulted in the classification of SRLV into five genotypes (A-E) using genetic sequences from the gag, pol, and long terminal repeat (LTR) regions [5–10]. Turkey contains approximately 29 million small ruminants [11] but does not yet have control measures for SRLV. Anatolia is a part of the ‘‘fertile crescent’’ where the transition of humans from hunting to manipulating the behaviors of certain animals is known to have led to the process of domestication more than 10,000 years ago [12, 13]. The seroprevalence of VMV in Turkey has been reported in many studies [14–18]. Most of them were local reports, and no molecular characterization of circulating field viruses has been reported.
In this study, 911 serum and blood-DNA samples from different pure and mixed sheep breeds in Turkey were analyzed using a commercial ELISA and LTR-PCR, respectively. Turkish sheep showed a high seroprevalence rate, and PCR detected mostly early infections. Nucleotide sequences of the LTR and gag gene regions demonstrated the presence of two different clades related to VMV-like viruses and strongly suggested that SRLV-genotype A originated in Turkey.

Material and methods

Samples

A total of 911 sheep sera and 757 EDTA-treated blood samples from 23 flocks (identified as I through XXIII) were collected for serological studies and virological diagnosis of SRLV, respectively, in Turkey from 2006 to 2009 (Table 1). The animals ranged from 1 to 7 years of age. Individual sera were used for antibody determination by ELISA, and leukocytes were obtained from the buffy coats for proviral DNA detection by PCR. Animals included eight different breeds, namely Karayaka, White Karaman, Turkish Merino (cross of Kivircik 90 % + German Mutton Merino 10 %), Anatolian Merino (cross of German Mutton Merino 80 % + White Karaman 20 %), Acipayam (cross of Awassi 50 % + Ost Fries 25 % + Daglic 25 %), Anatolian Merino 9 Awassi, Anatolian Merino 9 Chios, and White Karaman 9 Awassi. Clinical signs characterized by respiratory disease (coughing, sneezing, nasal flow, and pneumonia), weakness, and mastitis that sporadically led to deaths were observed in animals from 13 flocks (Table 1). Flocks II, III and VI had been reported previously as seropositive for VMV infection [19] where no control measures or eradication programs have been applied and were included this study. Furthermore, flock II was sampled twice, with a four-month interval. Finally, only serum samples were available from flocks I, VII and XXI, and thus, these flocks were evaluated only by ELISA.

Antibody detection (ELISA)

A commercial ELISA kit (Institute Porquier, Montpellier, France) that employs recombinant proteins from the capsid antigen and transmembrane (TM) region of the env gene as coating antigens was used. ELISA was performed, and the results were interpreted according to the manufacturer’s instructions.

DNA extraction and PCR

Proviral DNA was extracted from buffy coat samples using a phenol-chloroform extraction method, as described previously [20] with slight modification. Briefly, 300 µL denaturing solution (4 M guanidinium thiocyanate, 0.75 M sodium citrate, 0.6 % N-lauryl sarcosine, 0.2 M 2-mercaptoethanol) was added to 300 µL of buffy coat and mixed vigorously by vortex. The buffy coat suspension was mixed with 325 µL phenol (pH 5.0) and 325 µL chloroform–isoamyl alcohol solution (24:1, v:v). After centrifugation at 12,000 9 g for 10 min, the upper phase was precipitated with 3 M sodium acetate and isopropanol at -80 °C for 1 h. After washing with 70 % ethanol, the pellet was dried and then resuspended in deionized water.
LTR and gag gene regions were amplified by PCR using specific primer pairs reported by Extramania et al. [21] and Grego et al. [5], producing fragments of approximately 300 and 800 nt, respectively. LTR-PCR was performed in a 30-lL reaction consisting of 10 9 reaction buffer, 2 mM MgCl2, 0.2 mM dNTP, 0.2 pmol each primer and 1.25 U Taq DNA polymerase (Fermentas, Lithuania). The PCR cycling profile included a denaturation stage at 96 °C for 6 min, followed by 35 cycles of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 10 min. Gag-PCR was performed using the same mix but employing a nested protocol, as described previously [5]. PCR products were analyzed after electrophoresis in 1 % agarose gels containing ethidium bromide and visualized on a UV transilluminator (Kodak, Gel Logic 100, USA).

Sequencing and phylogenetic analysis

PCR fragments were purified using a High Pure PCR Cleanup Micro Kit (Roche, Germany), and sequenced. Sequencing of partial LTR and gag gene regions was carried out in a Beckman Coulter CEQ 8000 genetic analyzer (Beckman Coulter, USA) using a dye terminator cycle sequencing kit (GenomeLab DTCS-Quick Start Kit, Beckman Coulter, USA) according to the manufacturer's protocol. The LTR and gag sequences were evaluated and identified using the Basic Local Alignment Search Tool (BLAST) web service of the National Center for Biotechnology Information (NCBI). Sequence analysis of 300 bp of the LTR and 800 bp of gag was performed using the MEGA version 5.1 [22]. Phylogenetic trees were constructed by the neighbor-joining method with bootstrapping of 1000 replicates. The final phylogenetic trees of LTR and gag gene sequences were performed using 11 and 29 reference sequences available from the GenBank database, respectively. Multiple sequence alignments of Turkish SRLVs were performed using ClustalW [23] in the BioEdit program (version 7.0.5.3) using reference sequences (AY101611-85/34, DQ084441-GR-LTR6 and DQ64767710-blood for the LTR gene region and M316646-SAOMVV, AY101611-85/34, AY454686-SNCR5586, AY45 4210-SNCR5693, AY454175-SNCR5560, EF676006-It0 30g03, EF676017-It060s01, M33677-Cork, AY454218SCNR5720, JF502418-TR-DM, AF322109-1GA and EU29 3537-Roccaverano for the gag region) obtained from GenBank. Aligned LTR sequences were evaluated for conservation of AP1, AML (vis), AP4, and TATA box regions within U3, and the compositions of the R regions and conservation of the inverted repeat (IR), and primerbinding site (PBS) regions within U5. Aligned gag sequences were investigated for immunodominant epitope motifs.

Virus isolation and immunochemistry

Fetal sheep lung cell (FSLC) cultures were prepared from 102to 120-day-gestation-age sheep fetuses obtained from a local abattoir as described previously [24] to isolate field viruses selected from PCR-positive flocks. Cells were prepared using Dulbecco's modified Eagle medium (DMEM;

Biochrom, Germany) containing 25,000 U/mL penicillin, 20 mg/mL streptomycin and 10 % fetal calf serum (FCS; Biochrom, Germany) and checked for pestivirus and VMV infections by RT-PCR [25] and PCR [21], respectively. The VMV American strain WLC-1, obtained from Weybridge Laboratory Agency (Veterinary Research Institute, UK) was used as a positive control for PCR.
To detect virus-infected cells after a 10to 15-day incubation period, an immunoperoxidase assay (IPA) was performed. Briefly, cells, after five passages, were fixed in 10 % formalin for 1-2 h. After washing with PBS-Tween 20 (0.01 %), cells were treated for 1 h with a monoclonal antibody raised against VMV p25 protein (kindly supplied by Dr. B. Blacklaws, Department of Veterinary Medicine, University of Cambridge, UK). After washing, rabbit IgG secondary antibody horseradish peroxidase (HRP)conjugated anti-sheep was incubated for 1 h at 37 °C. After washing the cells with PBS, a substrate solution was added (combination of 3-amino-9-ethyl carbazole ? dimethyl formamide ? H2O2 in sodium acetate buffer), and the cells were then incubated for 30 min at room temperature.

Statistical analysis

To evaluate the agreement between ELISA and PCR values, the kappa value [26] was determined, and sensitivity/ specificity and chi-square tests were performed using SPSS (version 15.0; IBM, USA).

Nucleotide sequence accession numbers

The nucleotide sequences from this study have been submitted to GenBank and assigned the accession numbers GQ862782 to GQ862813 and JQ898288 for the LTR gene regions and JQ898278 to JQ898287 for the partial gag gene regions.

Results

ELISA and PCR

Using ELISA and/or PCR, 618 of 911 (67.8 %) samples were found to show evidence of infection (Table 1). Furthermore, 465 positive animals were determined to be seropositive by ELISA, but only 414 (54.7 %) were detected by PCR. The seroprevalence within flocks (n = 22) varied between 8 and 100 %, and for PCR, the proportion of positive samples ranged from 22.2-100 %, reaching a medium level of agreement between the two assays (kappa: 0.26; ± 0.038 CI). The seroprevalence within flocks II, III, and VI in this study was found to be 75, 33.3, and 84 %, respectively, by ELISA and 45, 40.2, and 49.2 % by PCR, and the cumulative prevalence of infection reached 80, 62.1, 87.3 %, respectively (Fig. 1).
The prevalence of SRLV infection was evaluated according to the age of animals and, as expected, it increased with age (Table 2). The prevalence was also analyzed between breeds, and the Karayaka breed appeared to have a lower rate of infection compared with the others (Table 1). Indeed, flock VII was composed of Karayakabreed sheep and was evaluated only by ELISA, but it showed no evidence of infection. However, five VMVinfected flocks containing Karayaka-breed sheep (n = 91) showed a prevalence of 70.3 %. The prevalence in other breeds ranged between 66.7 and 98 %.
Forty positive animals from flock II were sampled twice, with a four-month interval (Fig. 2). The prevalence of infection detected by ELISA and PCR was found to be 85 % (34) and 17 % (6), respectively, in the first sampling and 87.5 % (35) and 55 % (22) in the second sampling,. Four animals were found to be positive in the first sampling and then negative in the second sampling.
Comparison between ELISA and PCR

When the results of ELISA and PCR assays were compared for 757 samples, detection of VMV infection (77.9 %) varied, with 57.7 % positive by ELISA, 54.7 % positive by PCR, and 34.5 % positive by both assays (Table 2). Prevalence based on a single test was 23.2 % by ELISA alone but was 20.2 % by PCR alone. As shown previously, prevalence increased with age, but when compared by ELISA and PCR, the prevalence in young animals was higher by PCR (age \ 1=38.8 %; age B2=47.7 %) than by ELISA (age \1=15.1 %; age B2=42.7 %). The results of the two tests showed a lower agreement value when analyzing animals less than 2 years of age (kappa1age: 0.18; kappa2age: 0.20) or more than 5 years (kappaC5age: 0.30).

Sequence analysis and phylogenetic tree

The sequences of 33 VMV LTRs and 10 partial gag gene regions were determined from 15 and 6 different flocks, respectively, and compared. LTR and gag-region sequences showed 26.3-98.4 % and 70.2-88.9 % identity, respectively.

LTR regions were predicted to contain three AP-1, two AML (vis), and one AP-4 putative motifs without any duplication or deletion in their U3 regions compared with reference sequences (Fig. 3). Putative promoter and transcription-factor-binding domains within the U3 region, AP-1, AP-4, AML and AML (vis) were conserved with respect to similar reference strains, excluding the K18, K23, K24, t2-4, and t2-10 sequences (Fig. 4). The composition of the R region in the LTR sequences differed from that of other field viruses from Greece (DQ084441/GR-LTR6, DQ647677/10-blood), Portugal (AF479638/P1OLV), North Africa (SA-OVMV) and North America (AY101611-85/34) for which insertions or deletions in certain regions had been reported. The K18, K23, K24, t2-4, and t2-10 sequences contained a 12to14-nucleotide insertion, and the B29, B37 and B40 sequences had a 7-nucleotide insertion within the R region. The PBS region in the U5 region of the LTR was highly conserved among the field isolates and was similar to that of American strain 85/34 (Fig. 3).

A phylogenetic tree of the Turkish LTR sequences comparing it to other reference sequences of genotypes A, B, C, and E available in the GenBank database formed a separate branch in which only the American 85/34 and Greek sequences were included, suggesting a common origin. The p25 capsid antigen epitope and major homology region (MHR) of the10 gag sequences obtained were conserved and corresponded to genotype A (Fig. 5). Sequences from the gag region from Turkish isolates grouped with other reference VMV-like viruses within the A2, A3, A5, A9, A11 subtypes in the phylogenetic tree (Fig. 6).

Agreement of the LTR gene region with in vitro culture of field strains in IPA

Blood samples from VMV-positive animals were screened for virus isolation in FSLCs. Co-culture and IPA revealed that seven positive isolates showed evident syncytia formation and the presence of spindled cells (Fig. 7). The LTR region of three isolates (P73, P79 from flock III, and G49 from flock XIX) were sequenced and were found to possess high homology within the R region, with no insertions or deletions. P73 and G49 were cultivated without evident cytopathic effect in FSLC; however, P79 developed a cytopathic effect in FSLC after five passages. When all isolates were compared with the control virus, a
slow/low phenotype with less or no visible cell lysis in vitro was detected.

Discussion

SRLV causes a persistent immunopathogenic infection characterized by progressive degenerative inflammation in multiple organs of sheep and goats [1] that is only controlled by early diagnosis

and culling [27]. SRLV infection results in reductions in birth weight, weight gain, and milk yield, leading to significant economic losses in small-ruminant production [1]. Serological diagnosis can be achieved by different types of ELISA [2]; however, the use of new serological and/or molecular assays has resulted
in the description of new genotypes/subtypes [5, 7, 9, 10, 28].

The presence of VMV infection in Turkey, which is considered a bridge between Asia and Europe, has been shown by several serological or virological studies since the 1970 s [14–18]. However, molecular characterization of field isolates of SRLV has not yet been reported. In this study, we investigated the occurrence of VMV infection in sheep in Turkey by serology and PCR. Molecular characterization of field viruses according to LTR and gag gene sequences provided evidence that is compatible with genesis of the VMV epidemic in the Middle East, followed by spreading throughout the world due to human migrations.

The serological diagnosis of infection has some disadvantages, such as the presence of infected animals with a low antibody titer or the absence of an antibody response during early infection stages, and the lack of cross-reacting antibodies from different genotypes of SRLVs, leading to false-negative results [4]. These infected animals that are not detected by serology may then act as carriers of VMV infection, resulting in outbreaks of disease [29]. Another difficulty with overreliance on serological diagnosis is that the antigens used to design the tests can show differences in sensitivity and specificity. For the detection of SRLV infections, current tests are better at detecting genotype B
infections, although genotype-A-derived tests are able to detect cross-reacting antibodies, but the sensitivity is quite low when used in genotype-Bor E-infected populations [30]. Furthermore, it may be possible to genotype isolates by serology using genotype A or B versions of the same epitope [5, 30–32]. Nonetheless, recent studies suggest that using a combination of two or more techniques to achieve a ‘‘gold standard’’ is preferable than trusting a single assay [27, 33]. ELISA has been commonly used in recent years [3], and when combined with molecular assays, it provides excellent diagnostic capabilities, especially for detecting early infections [33].

The findings of this study support the idea that none of the available strategies, when used alone, are able to detect the entire infected population. Rather, a combined strategy may be more suitable for the diagnosis of SRLV. PCR techniques were designed with specific primers that are able to detect current SRLVs genotypes A, B, and E [5], even though the proviral load may not be high enough to ensure amplification in all infected animals. Thus, seroconversion and viral load may be variable parameters in blood, depending on the infection stage [34]. The results of the ELISA and PCR assays in this study show discrepancies that may be associated with factors such as the period of early infection, animal age, genetic variability, virus load, antibody level, and sampling time. Conflicting results about the positivity of VMV infection can be obtained using serological and virological methods. In young animals, the PCR assay provided an important alternative to the antibody-based methods and helped to prevent false negative results. The current study sampled three flocks that were previously reported to be seropositive [19], and no eradication program for SRLV infections had been implemented since. We show that the prevalence of VMV infection in these flocks increased with time (Fig. 1), and PCR complemented the ELISA results for the overall determination of prevalence.

The relationship between breed and VMV infection has been emphasized previously [35–39]. The Finnish, Border Leicester, and Awassi breeds have been found to have a high infection rate, whereas Roumbouillet and Iceland 9 Border Leicester sheep have shown low infection rates. In

Turkey, the Awassi, Chios, Daglic, Kivircik, and Merino breeds have been shown to have high seroprevalence [14, 19]. All eight breeds in the present study were positive, with prevalence ranging from 30.3 to 98 %, although the positivity rate in pure-breed animals (69.3 %) was lower than for mixed breeds (80 %).

The U3 region of the SRLV LTR contains promoter/ enhancer sequences, AP-1, AP-4 and AML(vis), which are transcription-factor-binding sites that play a regulatory role in virus transcription [40, 41]. The presence of deletions in U3 has been shown to be associated with slow virus replication in vitro [40–43]. As in many other field isolates, LTR regions sequenced in this study possessed the normal binding sites without repetition in the U3 region. Sheep numbers K18, K23, K24 (flock VI) showed no clinical symptoms, whereas t2-4, t2-10 samples (flocks XIV and XV, respectively) had evidence of chronic respiratory disease, and both clustered together in the LTR phylogenetic tree, suggesting a low association of pathogenic potential and LTR sequences. Variations in the R region of the LTR have been reported, and although a deletion was supposed to be present in asymptomatic sheep [44], recently it has also been found in diseased animals [28]. Thus, VMV strains growing slowly in cell cultures or showing low cytopathic effect may lead to mild disease, and lytic strains can cause severe disease in sheep [28, 42].

Animal trade is known to be a factor involved in increased transmission [1]. Phylogenetic analysis in this study revealed that Turkish VMV sequences might have
existed before the American or Greek strains. This would correspond to the migration of sheep from the Middle East to Western Europe several centuries ago. In contrast, increasing numbers of animals have been imported into Turkey in recent years. The phylogenetic evaluation of LTR sequences, in particular, showed more variability within subbranches. Animal interactions may be affecting the variability of the field viruses. Phylogenetic estimation using LTR sequences identified Turkish sequences as the common ancestor of European or American ones, indicating that SRLV of genotype A may have arisen from exportation during different historical ages (i.e., during the colonization of the Mediterranean, and the Ottoman Empire), together with human migration from the Fertile Crescent to Western Europe.

In vitro cultivation of Turkish isolates in FSLC showed a slow/low phenotype when compared with the WLC-1 American strain, which is characterized by a rapid/high phenotype. Therefore, one can tentatively hypothesize that the LTR affects in vitro virus replication, but recent findings clearly demonstrate that the LTR is not a limiting factor in in vitro virus production [45]. Alternatively, other factors such as receptor usage or the presence of host-cell restriction factors should be further explored.

The gag region of the viral genome is commonly used in the phylogenetic classification of SRLV [5–10]. VMV-like viruses originally isolated from sheep are referred to as genotype A and can be further subdivided into 13 subgroups (A1-A13). CAEV-like viruses originally isolated from goats are referred to as genotype B and can be further subdivided into three subgroups (B1-B3). Genotypes C, D, and E have been reported to be geographically limited to Norway, Switzerland and Spain [6, 46], and Italy [5], respectively. In Turkey, genotype B (subtype B3) has been described previously from a single sheep, showing a close relationship to Italian gag sequences from within the Mediterranean Basin [8]. In the present study, ten partial gag sequences were identified as genotype A, including A2, A3, A5, A9, and A11 subtypes. This likely reflects differences in circulating viruses in the sampling area, since the PCR assay is capable of amplifying sequences from both genotypes.

This study is the first to describe the occurrence of SRLV in Turkey from both the biological and molecular perspective to investigate the field virus genotype A with a slow/low pattern of in vitro growth. In addition, phylogenetic analysis provided evidence of the origin of SRLV, identifying Turkish, European, and American strains as the originators of current SRLV infections worldwide. Further studies are required that include the collection of additional sequences from other genetic regions of the virus and from all described genotypes.

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Table 1 The data of sampled sheep in this study. Flocks are identified with romanic numbers, seroprevalence and PCR incidence are indicated between brackets

Flock no.	Region	Breed	Sampling date	Age	Number of sheep sampled	ELISA (+) (%)	PCR (+) (%)	ELISA and/or PCR (+) (%)
I[a]	Amasya	Karayaka	December-2006	1-4	24	24 (100)	-	24 (100)
II[b]	Ankara	Acipayam	June-2007 and October- 2007	2-5	100	75 (75)	45 (45)	80 (80)
III	Ankara	Anatolian Merino	November-2007	2-6	87	29 (33.3)	35 (40.2)	54 (62.1)
IV	Antalya	Anatolian Merino × Chios	February-2008	2-4	25	2 (8)	13 (52)	15 (60)
V	Antalya	Anatolian Merino + Chios	April-2009	3-7	5	5 (100)	5 (100)	5 (100)
VI	Bursa	Turkish Merino	November-2007	2-6	63	53 (84)	31 (49.2)	55 (87.3)
VII[a]	Giresun	Karayaka	December 2006	1-4	120	0 (0)	-	0 (0)
VIII[b]	Hatay	White Karaman + Awassi	April-2008	2-7	49	32 (65.3)	40 (81.2)	48 (98)
IX	Konya	Anatolian Merino + Awassi	December -2007	2-4	93	80 (86)	56 (60.2)	85 (91.4)
X[b]	Konya	Anatolian Merino	January-2008	2-4	20	17 (85)	13 (65)	18 (90)
XI[b]	Kütahya	White Karaman	April-2008	2-4	10	9 (90)	8 (80)	10 (100)
XII[b]	Kütahya	White Karaman	April-2008	2-4	5	2 (40)	3 (60)	4 (80)
XIII[b]	Kütahya	White Karaman	April-2008	2-4	9	6 (66.7)	2 (22.2)	7 (77.8)
XIV[b]	Tokat	Karayaka	September-2007	2-4	12	9 (75)	4 (33.3)	10 (83.3)
XV[b]	Tokat	Karayaka	September-2007	2-4	11	10 (90.1)	6 (54.6)	11 (100)
XVI[b]	Tokat	Karayaka	September-2007	2-4	27	6 (22.2)	9 (33.3)	12 (44.4)
XVII[b]	Tokat	Karayaka	November-2007	2-4	17	4 (23.6)	6 (35.2)	7 (41.2)
XVIII	Şanlıurfa	Anatolian Merino	February-2008	2-6	135	78 (57.8)	90 (66.7)	113 (83.7)
XIX	Şanlıurfa	Anatolian Merino	March-2008	0-1	9	1 (11.1)	2 (22.2)	3 (33.3)
XX[b]	Aydın	Anatolian Merino	April-2009	1-4	47	7 (14.9)	32 (68)	35 (74.5)
XXI[a,b]	Sivas	Whitekaraman	January-2008	1-4	10	4 (40)	-	4 (40)
XXII[b]	Hatay	Whitekaraman	June -2009	2-4	15	6 (40)	5 (33.3)	8 (53.3)
XXIII	Ankara	Anatolian Merino	May-2009	2-4	18	6 (33.3)	9 (50)	10 (55.6)
TOTAL					911 (757[c])	465 (51)	414 (54.7)	618 (67.8)

[a] Only serum samples were collected, [b] chronic respiratory tract disease was noticed, [c] number of samples analysed by PCR

Table 2 ELISA, PCR or combined positivity rates comparison regarding animal's age

Age	Number of animals sampled (%)	ELISA and/or PCR (+) (%)	ELISA (+) PCR (-) (%)	ELISA (-) PCR (+) (%)	ELISA (+) PCR (+) (%)	ELISA (-) PCR (-) (%)
1	139 (18.4)	62 (44.6)	8 (5.8)	41 (29.4)	13 (9.4)	77 (55.4)
2	297 (39.2)	223 (75)	69 (23.2)	58 (19.5)	96 (32.3)	74 (24.9)
3	231 (30.5)	218 (94.3)	68 (29.4)	41 (17.7)	109 (47.2)	13 (5.6)
4	74 (9.8)	71 (95.9)	26 (35.1)	10 (13.5)	35 (47.3)	3 (4)
≥ 5	16 (2.1)	16 (100)	5 (31.3)	3 (18.7)	8 (50)	0 (0)
Total	757	590 (77.9)	176 (23.2)	153 (20.2)	261 (34.5)	167 (22)

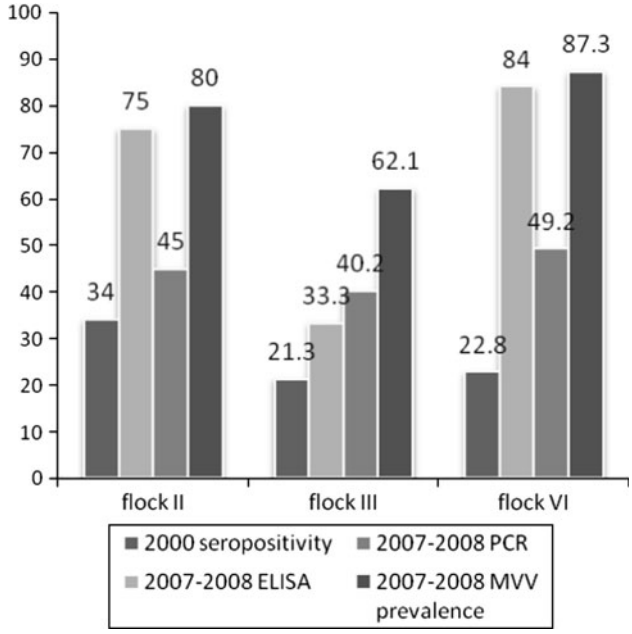


Fig. 1 Evolution of VMV seroprevalence in flocks II, III and VI over a period of seven years, as determined by ELISA. Both PCR and ELISA results from the last sampling are indicated

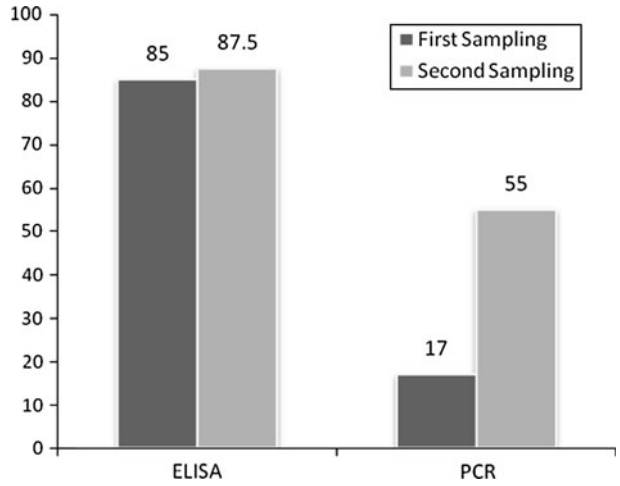


Fig. 2 Difference in MVV detection rates for positive animals of flock-II depending on the time of sampling

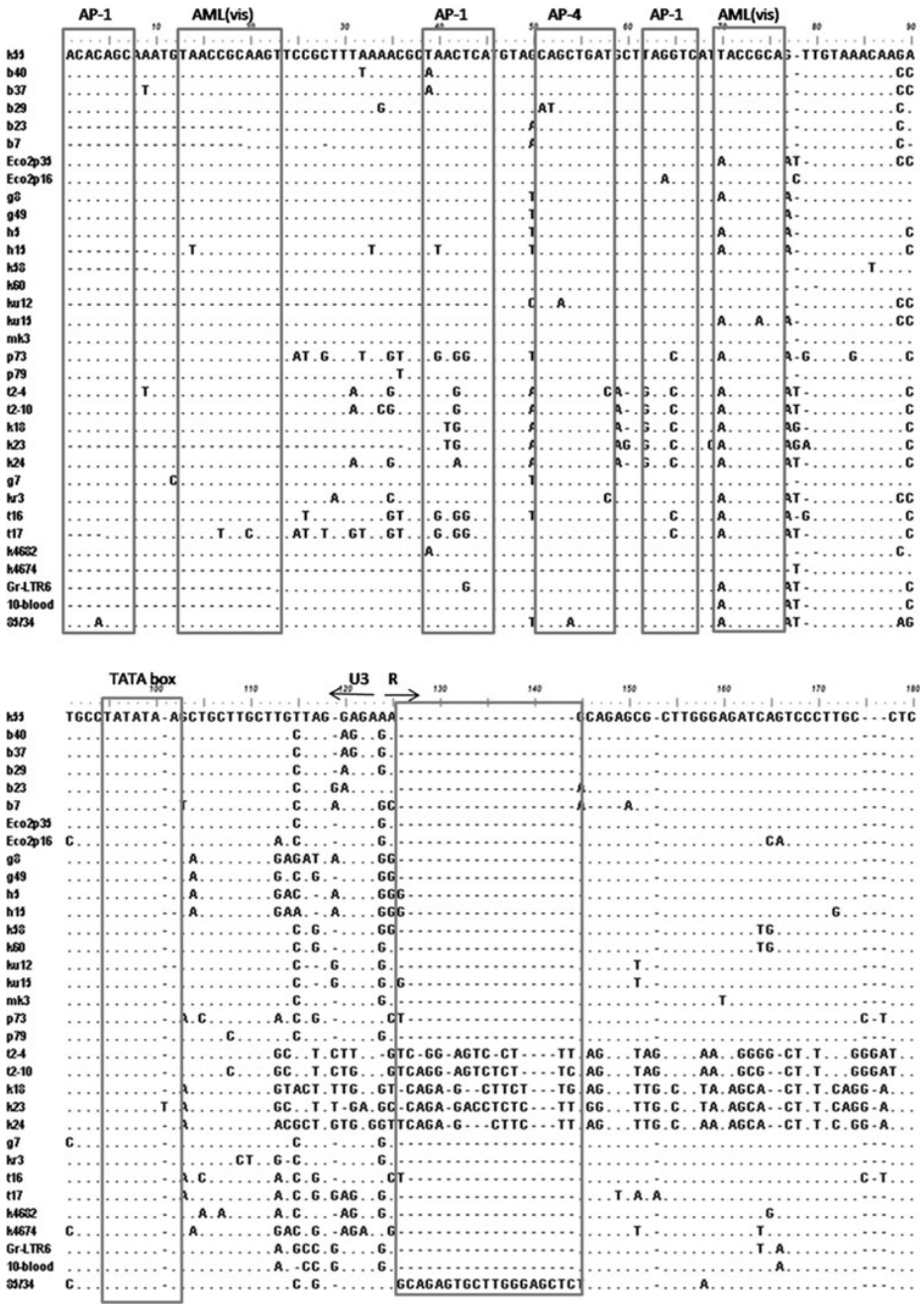
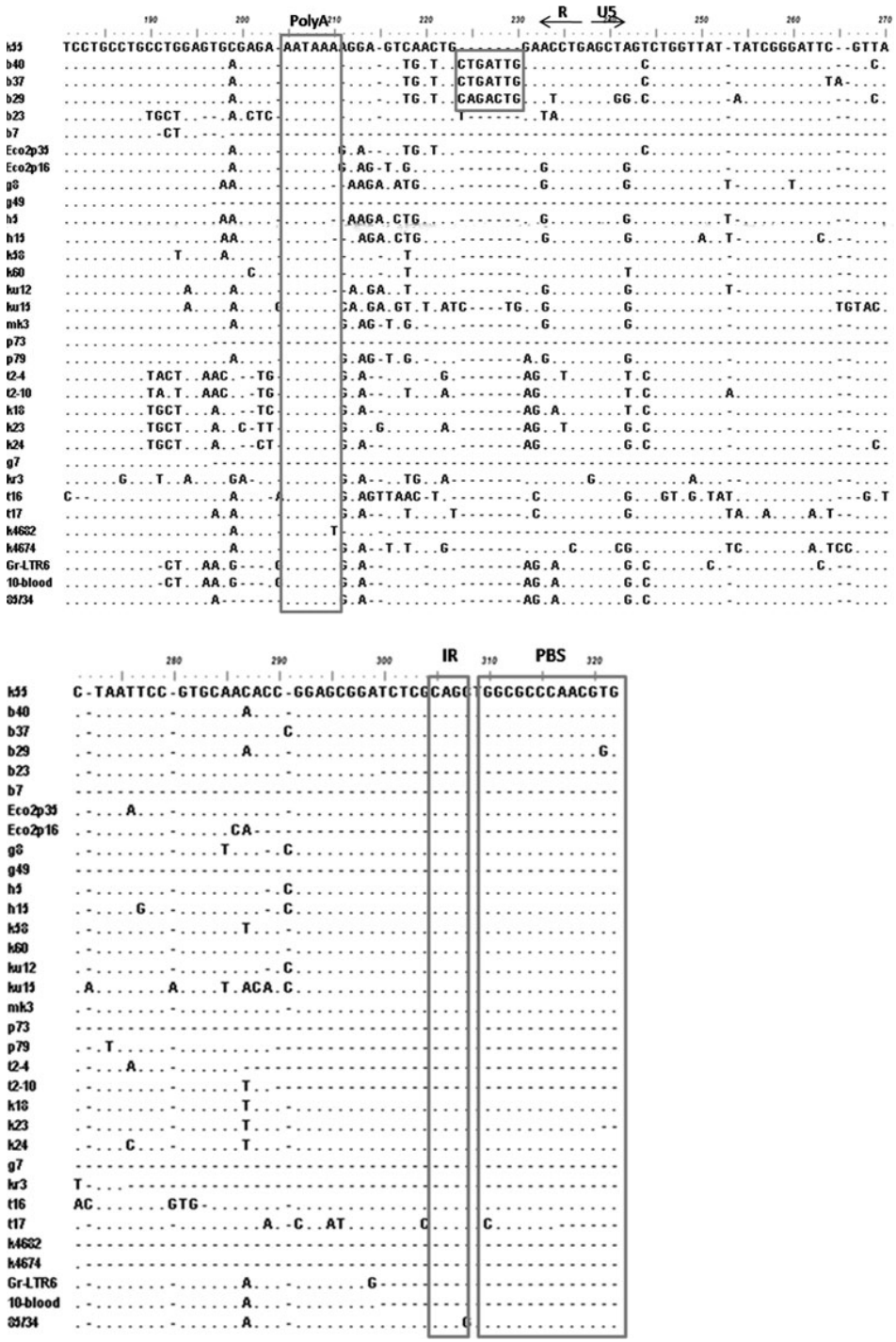


Fig. 3 Alignment of U3-R sequences of the LTR region from field viruses from Turkey. The sequences of 85/34 (AY101611), GR-LTR6 (DQ084441), and 10-blood (DQ647677) were used as references. The boundaries between U3, R and U5 are indicated by arrows. The AP-1, AP-4 and AML(vis) motifs, TATA box, PolyA, IR and PBS are indicated by boxes

Fig. 3 continued



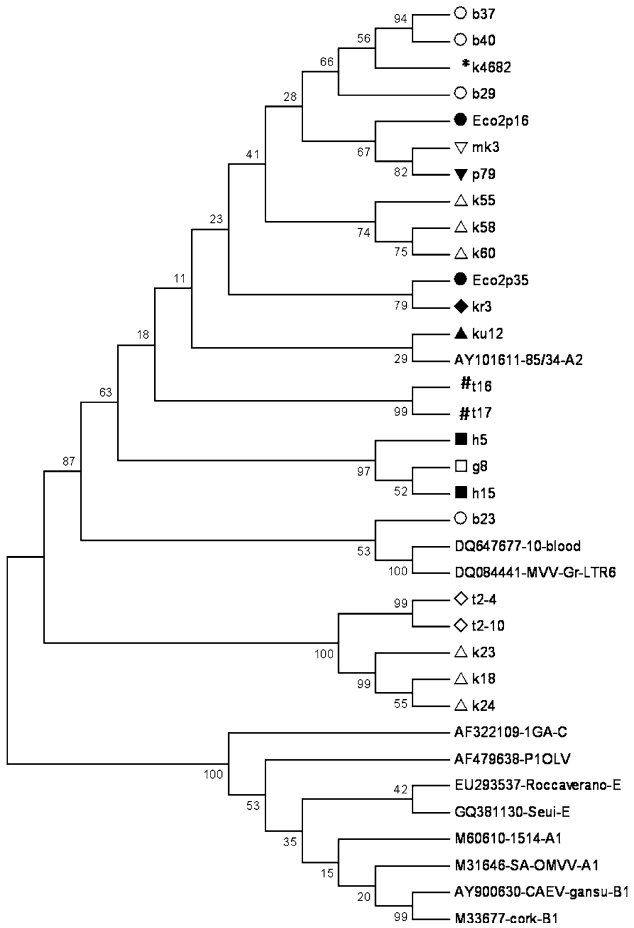


Fig. 4 Phylogenetic tree of sequences from this study and reference LTR sequences. The tree was constructed by the neighbor-joining method with 1000 bootstrap repeats using the Mega (5.1) program. Animals belonging to the same flock are labelled with the same symbol

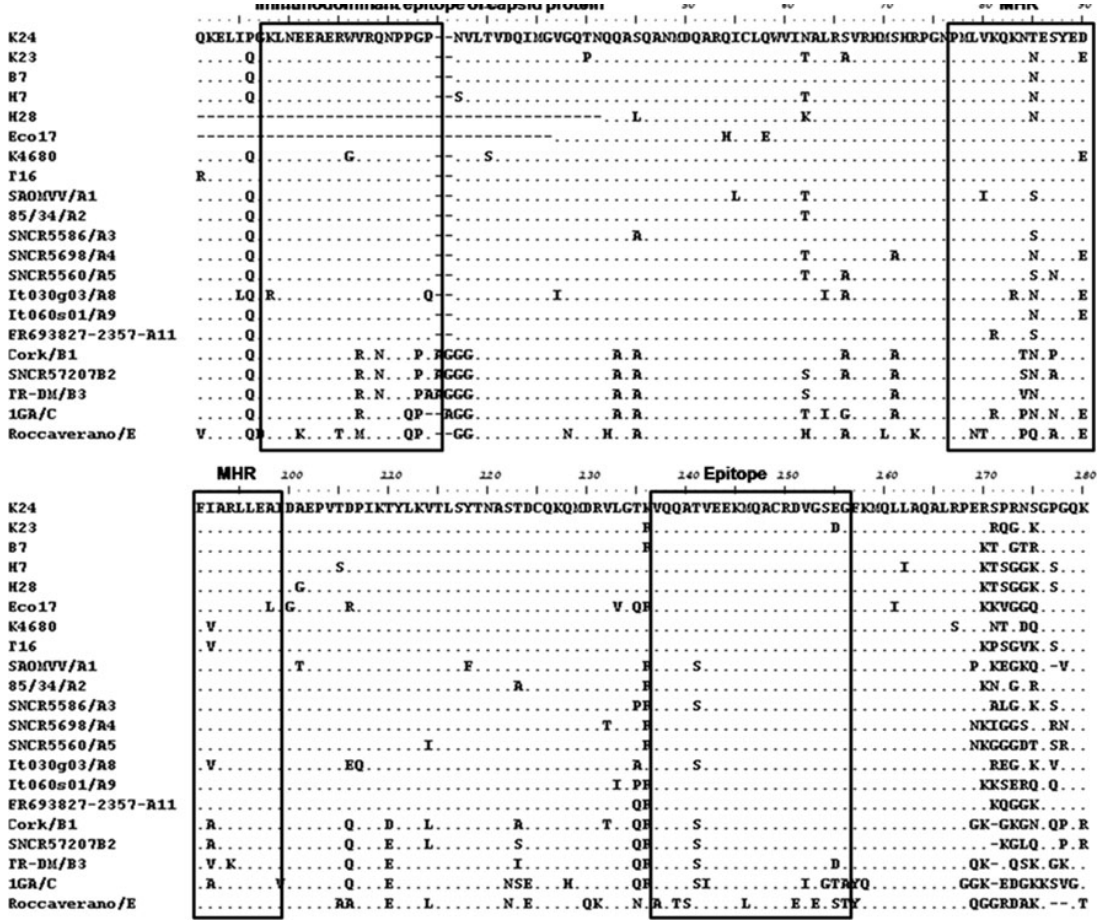


Fig. 5 Aligned amino acid of partial gag sequences obtained in this study and reference SRLV sequences from genotypes A (M316646-SAOMVV; AY101611-85/34; AY454686-SNCR5586; AY454210-SNCR5693; AY454175-SNCR5560; EF676006-It030g03; EF676017-It060s01; FR693827-2357), B (M33677-Cork; AY454218-SCNR5720; JF502418-TR-DM), C (AF322109-1GA), E (EU293537-Roccaverano). İmmunodominant epitopes and major homology region (MHR) are marked by boxes

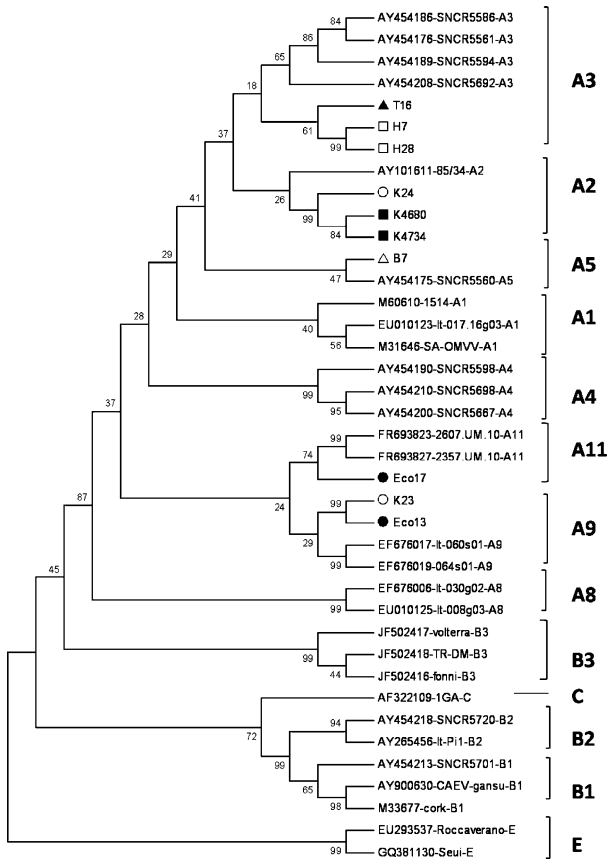


Fig. 6 Phylogenetic tree of partial gag sequences determined in this study. The tree was constructed by the neighbor-joining method with 1000 bootstrap repeats using the Mega (5.1) program. Animals belonging to the same flock are labelled with the same symbol

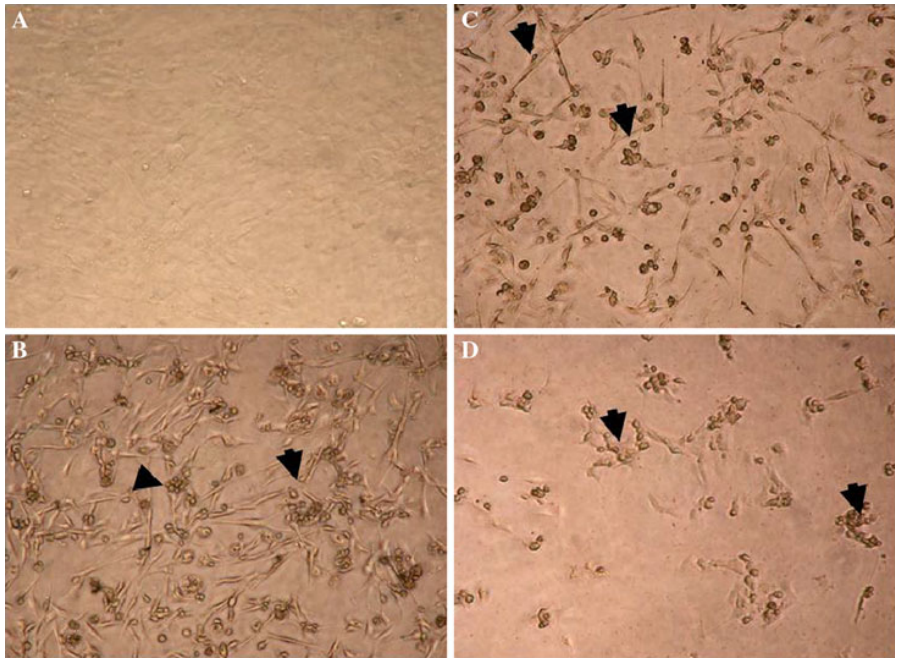


Fig. 7 Immunoperoxidase detection of field isolates in foetal sheep lung cells using a monoclonal antibody against the capsid p25 protein (kindly provided by Dr. B. Blacklaws, University of Cambridge). **A**: negative control, **B**: virus control, **C**, **D**: positive field samples. Giant-cell-like syncytia formation and spindled cells are shown by arrows