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Research Article

First Molecular Characterization of Caprine Arthritis Encephalitis Virus in Blood and Milk Samples from Goats in Turkey Based on Gag Gene Sequence Analysis

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ABSTRACT

This study aimed to determine the presence of CAEV infection in blood and milk samples of goats collected from various regions of Turkey, using ELISA and PCR techniques and molecular characterization of local viruses. Data was collected from 435 blood samples and 285 milk samples from 8 goat production companies in 6 provinces (Ankara, Eskişehir, Kütahya, Antalya, Burdur, Kırklareli) in Central Anatolia, Aegean, Mediterranean and Marmara regions. All blood and serum samples were examined for antibodies against ELISA and CAE viruses. Nested PCR tests were done on 70 blood samples and 16 milk samples, using specific primaries in the gag region to detect CAE virus nucleic acid.

Of the 435 blood samples, 37 (8.5%) tested positive by ELISA and 14 (4.9%) of the 285 milk samples. PCR results were positive for 14 of the selected 70 leucocyte samples and 8 of the selected 16 milk samples. Two blood samples and 3 milk samples that tested positive with PCR in the field were cloned in plasmide, and the gag gene region sequence of the virus was analyzed. The results were consistent, and similar phylogenetically to CAEV and SRLV viruses in terms of the known partial gag gene levels in Turkey.

This study suggests that it is necessary to determine CAEV infection using ELISA techniques at regular intervals in order to identify the molecular characteristics of circulating viruses rather than detecting infection with PCR tests. The study also indicates that milk samples are important for detecting CAEV infection, and can be used in for diagnosing infection.

Key words: CAEV, ELISA, PCR, gag, detection, Turkey

INTRODUCTION

Caprine Arthritis Encephalitis Virus (CAEV) of goats and Maedi Visna Virus (MVV) of sheep, from the lentivirus genus of the Retroviridae family, are known as "Lentiviruses of small ruminants" (Small Ruminant Lentivirus-SRLV). They reduce milk production, birth weight and weight gain by generating multisystemic inflammatory disease, which causes significant economic losses due to high mortality rates (Saman *et al*., 1999; Peterhans *et al*., 2004; Lamara *et al*., 2013). Cross-species infection has been reported in mixed herds of sheep and goats (Shah *et al*., 2004). Subclinical persistent CAEV infection in goats causes indurative mastitis resulting in encephalitis, non-suppurative polyarthritis and "hard udder" syndrome (Adams *et al*., 1983; Reina *et al*., 2009).

The most important forms of transmission are oral intake of infected goat milk or colostrum, and viral contamination of milking equipment (Rowe *et al*., 1992; Pisoni *et al*., 2004). In addition, transplacental, aerosol, direct contact, and seminal plasma transmission are also possible (Rowe and East, 1997; Blacklaws *et al*., 2004; Lara *et al*., 2005; Ali Al Ahmad *et al*., 2008; Peterson *et al*., 2008). Agar Gel Immunodiffusion (AGID), ELISA and Immunoperoxidase tests can be used to detect the presence of specific antibodies against CAEV (Herrmann *et al.,* 2003; De Andres *et al.*, 2005; Yavru *et al*.; 2006) while polymerase Chain Reaction (PCR), Southern Blotting and In Situ Hybridisation techniques can be used for direct detection of the virus (Rowe and East, 1997; Eltahir *et al*., 2006; Li *et al.,* 2013).

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The aims of this study are to determine the prevalence of CAEV infection in Turkey (as previous studies have revealed its presence), determine the suitability of blood and milk samples for diagnosing CAEV infection, assess the diagnostic value of ELISA and PCR, and provide a genetic characterization of local strains.

MATERIALS AND METHODS

To conduct serological and virological diagnosis of CAEV infection, 435 goat serum samples were collected in EDTA tubes and 285 goat milk samples were collected during the lambing periods between 2007 and 2010. Samples were collected from 8 farms (named A to H) from four regions (Central Anatolia, Mediterranean, Aegean and Marmara). All sampled animals were older than 6 months. Goats from A and B farms were Saanen breed, while the others were Angora. CAEV antibody results from the blood and milk samples were analyzed using ELISA, while 70 blood samples and 16 milk samples, chosen to represent animals with or without antibodies, were evaluated for CAEV nucleic acid. For biological diagnosis, blood samples in EDTA tubes were centrifuged for 10 minutes at 2000 rpm. Blood serum samples collected for serological diagnosis were stored at -20ºC until testing. The oily layer formed above milk serum was centrifuged for 10 minutes at 2000 rpm before removal for serological testing.

Antibody detection

Presence of CAEV antibodies in blood and milk was determined using commercial ELISA kit (Institute Porquier, Montpellier, France), according to kit procedure. Microplates were read in an ELISA reader with a 450 nm wavelength filter. The resultant absorbance values were evaluated according to kit protocol.

DNA extraction and nested PCR technique

The guanidine thiocyanate-phenol extraction method of Chomczynski and Sacchi (1987) was used to obtain proviral DNA. To fix CAEV nucleic acid, primers specific to the gag gene region of the virus were used [10]. The primers GEX5 (5'-GAAGTGTTGCTGCGAGA GGTCTTG-3') and GEX3 (5'-TGCCTGATCCATGTTA GCTTGTGC-3') were used for the first amplification, while primers GIN5' (50-GATAGAGACATGGCGAGG CAAGT-3') and GIN3 (5'-GAGGCCATGCTGCATTG CTACTGT-3') were used for the second amplification. A modification of the method reported by Al Ahmad *et al.* (2008) was used to perform the nested PCR reaction. For internal control, nested PCR was performed under the same conditions using primers specific to the 40. exon region of the β-actin gene (Bouzar *et al*. 2007). Primer sequences were as follows: ES3' (50-TCATGTTTGAGA CTCAACACCCCAG-3') and ES32 (5'-CG GGGAAGG CTGGAAGAGTGCC- 3') for the first, and ES31 (5'-CCCCAGCCATGTACGTTGCTATCC-3') and ES33 (5'-GCCTCAGGGCACGGAACCGCTCA-3') for the second round of amplification, respectively. A field isolate, which was isolated in GSM cell at the Joseph Fourier University-Jean Roget Institute, was used as a control to optimize the PCR tests (Figure 1). PCR was performed in a 50-µL reaction consisting of 10 X reaction

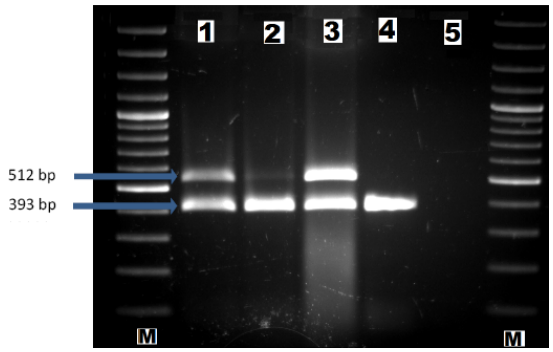


Fig. 1: Ethidium bromide stained agarose gel electrophoresis of Nested PCR products; [M: Marker (100 bp DNA ladder) 1,2: spesific product for CAEV, 3: postive control, 4: negative sample (where no virus was detected), 5: negative control (Distile water)].

buffer, 25 Mm MgCl2, 10 mM dNTP, 0,2 pmol of each primer, and 5U/ µL Taq DNA polymerase (Sigma, Lithuania). The PCR cycling profile included a denaturation stage at 94ºC for 5 min, followed by 35 cycles of 94mC for 1 min, 46ºC for 5 min, 60ºC for 2.5 min, and a final extension step at 60ºC for 15 min. PCR products were analyzed after electrophoresis in 1% agarose gel containing ethidium bromide, and visualized on a UV transilluminator (Kodak, Gel Logic 100, USA).

Cloning of PCR product

Purification of the products determined to be positive for CAE virus nucleic acid by nested PCR was performed using commercial purification kit (High Pure PCR Cleanup Micro Kit, Roche, Germany) according to the procedure recommended by the company. Then purified products were then cloned using a suitable vector before sequence analysis (pGEMt Easy Vector System, Promega, USA).

Nucleotide sequences and phylogenetic analysis

The PCR products were purified using a commercial kit (Gel and PCR purification kit, GenMark, Taiwan) before samples confirmed as a loading agarose gel were subjected directly to sequencing in a CEQ 8000 Genetic Analyzer (Beckman Coulter, USA) using the Dye Termination Cycle Sequencing Kit (DTCS, Beckmann Coulter, USA). Resultant sequences were compared using the CLC Bio DNA Workbench (Denmark) analysis program, and by sequence analysis of gag gene regions of different strains of reference CAE virus obtained from the Gene Bank. The results were presented on a phylogenetic map using the same program.

RESULTS

The ELISA analysis detected the presence of CAE virus specific antibodies in 37 (8.5%) of 435 blood serum samples and in 14 (4.9%) of 285 milk serum samples. Comparing matched blood and milk samples from 285 animals, seropositivity was detected in the milk of 4 (1.4%) and in the blood of 20 (7.0%), while 10 (3.5%) animals had specific CAEV antibodies in both samples. Seropositivity was detected in 15% (27/180) of Saanen strain animals and 3.9% (10/255) of Angora strain animals (Table 1).

Table 1: Number of tested blood and milk samples ELISA and Nested PCR, ELISA and Nested PCR positivity rates in blood and milk samples

Flock No	Number of blood samples tested by ELISA	ELISA (+) (%) in blood samples	Number of blood samples tested by PCR	PCR (+) (%) in blood samples	Number of milk samples tested by ELISA	ELISA (+) (%) in milk samples	Number of milk samples tested by PCR	PCR (+) (%) in milk samples
A	105	22 (20,9)	19	8	91	9 (9,8)	7	7
B	75	5 (6,6)	12	-	68	1 (1,4)	2	-
C	152	6 (3,9)	21	4	52	4 (7,6)	2	-
D	20	1 (5)	5	1	19	-	-	-
E	30	1 (3)	2	-	29	-	2	1
F	23	-	3	-	4	-	1	-
G	22	1(4,5)	5	-	22	-	2	-
H	8	1 (12)	3	1	-	-	-	-
Total	435	37(8,5)	70	14(20,0)	285	14(4,9)	16	8(50,0)

Table 2: Comparation of ELISA ve Nested PCR results

Tested of samples	Tested number of samples	ELISA(+) PCR (+)	ELISA (-) PCR(+)	ELISA(+) PCR(-)	ELISA(-) PCR (-)
Blood	70	3 (% 4,2)	11 (% 15,7)	20 (%28,5)	36 (%51,4)
Milk	16	1 (% 6,2)	7 (% 43,7)	4 (% 25,0)	4 (% 25,0)

Table 3: CAEV-gag gene partial sequence name and accession number

Flock Code	Name of Sequence	Accession Number
A	L-114 (Leukocyte)	HM534737
A	LS-105 (Milk)	HM534738
A	LS-107 (Milk)	HM534739
A	L-107 (Leukocyte)	HM534740
E	BUS-27 (Milk)	HM534741

As shown in Table 1, nucleic acid analysis for CAEV by nested PCR demonstrated positivity for CAE virus nucleic acid in 14 (19.9%) of 70 blood samples (comprised of 3 (4.2%) / 23 seropositive + 11 (15.7%) / 47 seronegative) and in 8 (49.9%) of 16 milk samples (comprised of 1 (6.2%) / 5 seropositive + 7 (43.7%) 11 seronegative).

Obtained nucleotide sequences of local CAEV gag amplicons, which were purified by placing them on plasmid vector, were sent to the World Gene Bank to obtain sequence registration numbers. Three milk samples and 2 blood samples from two different sample herds (Table 3) were compared with other CAE viruses present in the Gene Bank to determine the degree of similarity. Table 4 shows the origins of the reference viruses obtained from the Gene Bank and their Gene Bank registration numbers. Multiple comparisons of resultant gag sequences detected nucleotide changes between the sequences of some gene regions and the presence of deletions in some sequence regions. Specifically, there were similarities between deletions beginning from 144, 378, 452 and 483. base levels, which include about 10 bases, and CAEV strains from Italy, Poland, Norway and Iceland, SRLV strains from Spain, and the CAEV-CO (USA) reference strain (Figure 2).

A phylogenetic map (Figure 3) was created by comparing resultant gag gene sequences with each other and with reference CAEV, MVV and SRLV viruses obtained from the Gene Bank. This revealed that sequences of samples from farm A had formed a branch between themselves, while sequences of samples from farm E were making another branch. The map demonstrated that the viruses were phylogenetically close to other CAEV and SRLV viruses that have been reported as present in Turkey, at the level of partial gag gene.

Comparison of amino acids produced by translation of gag gene partial sequences of field viruses showed that the LS-107 virus differed from other viruses detected in the same herd. Whereas amino acid changes in this virus generally occur via one hydrophilic amino acid changing into another, in the present study a hydrophilic amino acid (T-Threonine) located in 48th place on the amino acid sequence changed into a hydrophobic amino acid (A-Alanine) (Figure 4).

DISCUSSION

CAEV infection causes a disease condition in goats characterized by immunosupression and latent infection after a long incubation period. CAEV virus was first isolated from a joint of an arthritic goat and the brain of a kid goat with encephalitis (Crawford *et al*., 1980; Narayan *et al*., 1980). After the viral genome was cloned in 1990 the complete genomic sequence determined and the genes coding viral proteins were defined (Saltarelli *et al*., 1990).

Previous studies evaluating the presence and prevalence of CAE virus infection in Turkey reported infection rates of 1.03-6% (Burgu *et al.*, 1994; Çimtay *et al*., 2004; Yavru *et al.*, 2006; Aslantaş *et al*., 2005). In this study, however, a seropositivity rate of 8.5% (37/435) was detected in 8 local farms located in Ankara, Eskişehir, Kütahya, Antalya, Burdur and Kırklareli.

The replication features of retroviruses and infection pathogenesis should both be considered while evaluating CAEV seropositivity rates. It is possible that in Lentivirus infections the immune system cannot identify the mutant genotypes formed during transformation of RNA to DNA by reverse transcriptase, which prevents the host from producing neutralizing antibodies (De Andres *et al*., 2005; Brinkhof *et al.,* 2010). The preparation protocols of ELISA kits commonly used in serological diagnosis of CAEV infection are also valuable for serological studies (De Andres *et al*., 2005). Brinkhof *et al.* (2006) reported that ELISA kits prepared using recombinant membrane proteins and synthetic transmembrane proteins have high sensitivity and specificity. In this study, test data reliability was ensured by using ELISA kits covered by viral capsid protein (p28) specific antibodies.

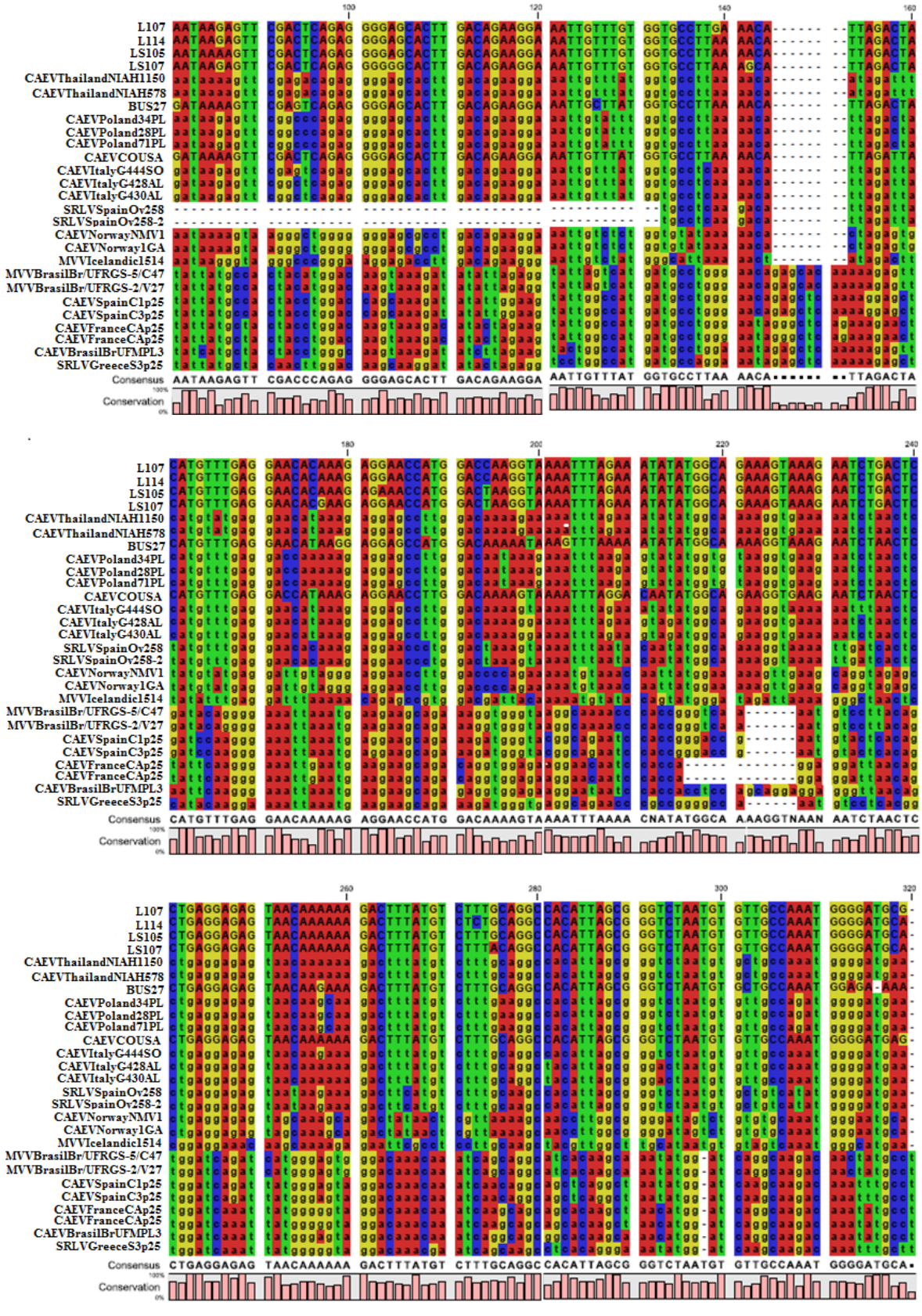
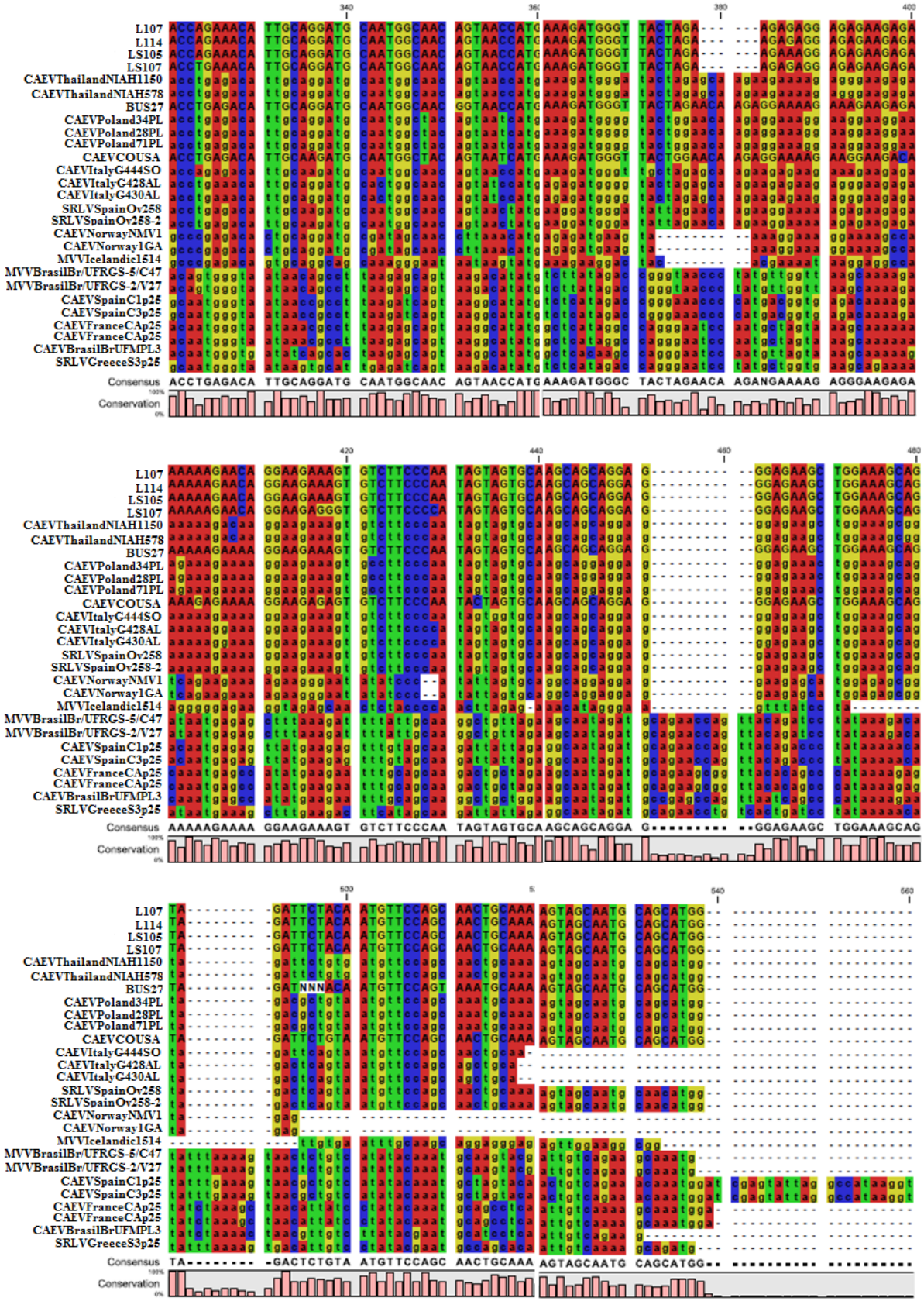


Fig. 2: Comparision of gag gene sequences of CAEV reference field isolate from genbank



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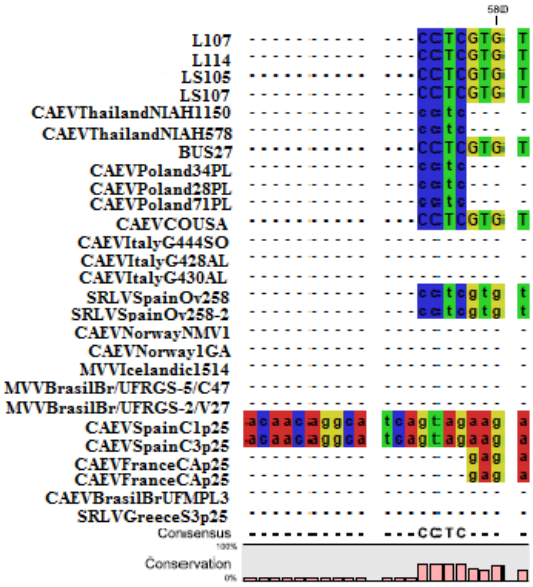

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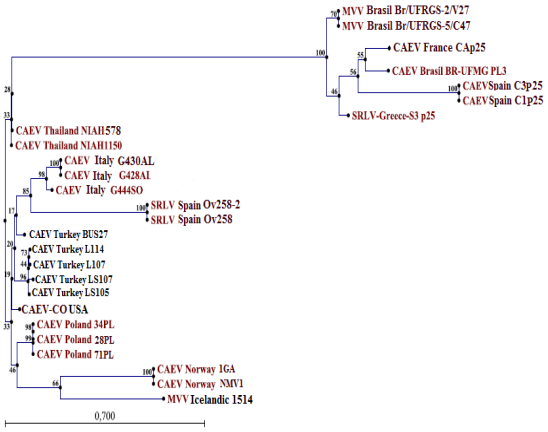

Fig. 3: Phylogenetic tree of sequences from this study and reference gag sequences.

Table 4: gag genes of CAEV used in phylogenetic aanalysis.

Name of sequence	Accession Number	Country of isolation
CAEV-CO	M33677	USA
34PL	DQ456485	Poland
28PL	DQ456484	Poland
71PL	DQ456483	Poland
CAEV-1GA	AF322109	Norway
CAEV-NMV1	L78453	Norway
MVV-1514	M60610	Iceland
SRLV-Ov258	FJ187812	Spain
SRLV-Ov258-2	FJ187811	Spain
C3p25	DQ632735	Spain
C1P25	DQ632734	Spain
G444SO	DQ190051	Italy
G428AL	DQ190038	Italy
G430AL	DQ190037	İtalya
NIAH1150	GQ161215	Thailand
NIAH578	GQ161214	Thailand
Br/UFRGS-2/V27	AJ305039	Brazil
Br/UFRGS-5/C47	AJ305041	Brazil
Br/UFMGPL3	AY101348	Brazil
CAp25	AF015181	France
S3p25	AY530291	Greece

While the prevalence of infection is generally low in meat goat strain and Angora goats, it is quite high in dairy goat strains. Infection causes huge economic losses in farms producing goat milk, with milk yields of CAEV infected female goats being 25% less than those of non-infected animals (Smith and Sherman 1994). In the present study, the seropositivity rate of Saanen goats was 15% (27/180) but only 3.9% (10/255) for other strains. A chi-square test of the seropositivity rates of both strains showed that the difference in rates were statistically significant ($P<0.001$).

In this study, blood and milk serum samples of 285 goats were analyzed by ELISA for the presence of CAEV antibodies and a seropositivity rate of 4.9% (14/285) was detected. Since milk is the most important transmission path for infection, monitoring of individual milk samples or milk tanks is important for detecting infection. Considering reports (Peterhans *et al.*, 1999; Reina *et al.*, 2006) indicating increased viral replication and decreased antibody levels in milk epithelial cells caused by immunosupression during breeding periods, it is important to choose an appropriate time for milk sampling.

In long-term CAEV infections of goats, the mammary gland is the target organ for viral replication, with the largest viral load observed in milk and mammary glands. The virus shelters in mammary tissue epithelial cells and macrophages (Ravazzollo *et al*., 2006). In this study, 8 (50%) of 16 milk samples on which nested PCR was performed showed the presence of CAEV nucleic acid.

Comparing nested PCR and ELISA results in Table 2, shows that ELISA reported the higher CAEV infection positivity rate. It should be remembered that test sensitivity may decrease with age of animal, time since infection transmission to the herd and presence of late seroconversion (Ellis *et al*., 1983; Gil *et al*., 2006). Unlike immunospressive retroviruses, which maintain high titers in blood, detection of the CAE virus is rather difficult. While PCR techniques are more sensitive before seroconversion, serological techniques are more sensitive than PCR tests after seroconversion due to small viral load (Eltahir *et al.*, 2006). Since the CAE virus stays latent as proviral DNA in monocytes, detection of viral nucleic acid is not possible following seroconversion. Rather, differentiation of monocytes to macrophages is necessary for viral replication, which occurs in stressful conditions, during pregnancy or immunosupression, allowing the virus to reach detectable titers in 1 week-10 days (Cheboune *et al.,* 1996b).

Most mutations in Lentiviruses generally occur as antigenic drift or during following phases of viral replication: proviral transcription by RNA polymerase, formation of single stranded viral DNA by viral reverse transcriptase, and synthesis of this genome into double stranded DNA (Kim *et al.,* 1996). CAEV infected animals have hundreds of different genotypes so mutant genotypes may escape from immune system, with only 1-2 genotypes being controlled by immune system. It is possible to detect PCR positivity in seronegative animals (Brinkhof *et al.*, 2010). Instead of comparing the diagnostic values of ELISA and nested PCR techniques, it is therefore recommended to use PCR to detect proviral nucleic acid when no seroconversion occurs and ELISA to follow monitoring programs when infection is present in the herd (Rimstad *et al*., 1993; Gil *et al*., 2006; Reina *et al*., 2009).

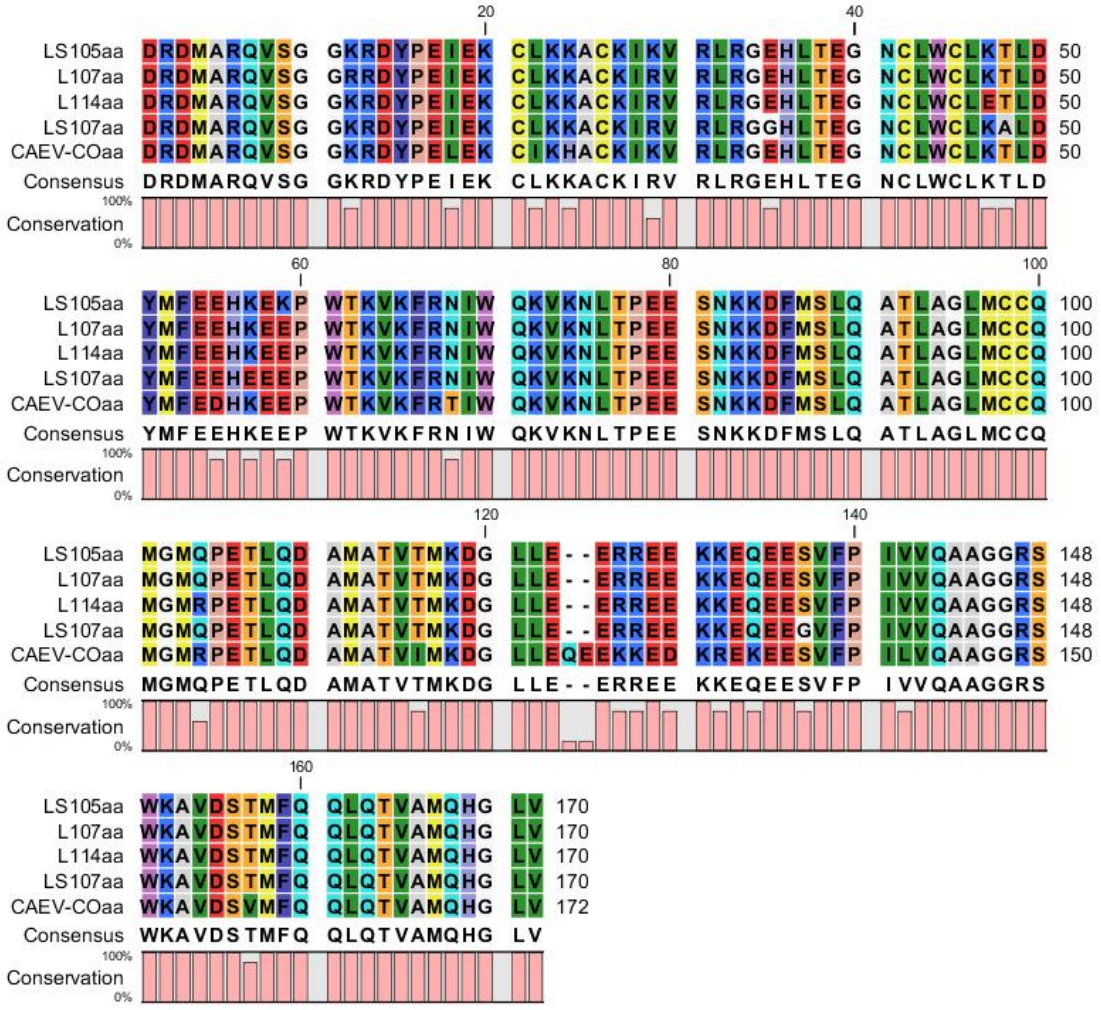

Fig. 4: Aligned amino acid of partial gag sequences obtanied in this study and reference CAEV-CO genotype.

Multiple comparisons of resultant gag gene sequences and reference strains provided from the Gene Bank demonstrated similarities between deletions occurring in some regions in this study and CAEV strains from Italy, Poland, Norway and Iceland, SRLV strains in Spain, and the CAEV-CO (USA) reference strain. Antigenic diversity may occur in major epitopes of SRLV structural proteins. Comparison of French and North American isolates and the CAEV-CO originated strain showed that pol and gag gene regions were protected against mutations and there was genetic diversity in the env gene region (Kuzmak *et al.,* 2007). Comparison of amino acids obtained by translation of gag gene partial sequences of field viruses in this study showed that the LS-107 virus was different from other viruses of the herd, having amino acid alterations. Specifically, a hydrophilic amino acid (T-Threonine) located in 48th place on the amino acid sequence changed to a hydrophobic amino acid (A-Alanine). This may cause a change in the region's conformational structure, indicating that different antigenic viral types may be present in infected animals of the same herd.

Since goat milk is very similar to human breast milk, contains anti-inflammatory fatty acids and has a homogeneous nature, goat milk consumption is recommended for asthma, allergy, gastrointestinal and pulmonary system diseases (Haenlein, 2004). Considering the current increase in the consumption of goat milk, it is clear that infection control program for CAEV infection are needed in Turkey. However, it is important for these practices to have first molecular characterization data supported by serological data on CAEV field strains, which may be helpful in future studies and for developing monitoring program.

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