

# Serological and Epidemiological Investigation of Bluetongue, Maedi-Visna and Caprine Arthritis-Encephalitis Viruses in Small Ruminant in Kirikkale District in Turkey <sup>[1]</sup>

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## Summary

Viral infections cause important problems and significant economic losses in sheep and goats that can be protected by an investigation of infections. This study aimed to determine the sheep and goat viral infections including bluetongue virus (BTV), maedi-visna virus (MVV) and caprine arthritis-encephalitis virus (CAEV) in Kirikkale city located in Central Anatolia region of Turkey. Blood samples collected from 279 sheep and 146 goats were tested by ELISA, RT-nested PCR and nested PCR. It was detected that antibody response to BTV (49.8%), CAEV (7.5%) and MVV (19.4%) were seropositivity values in all serum samples. BTV (2.87%) and MVV (9.25%) antigens were detected in seropositive whole blood samples via RT-nested PCR and nested PCR but not CAEV. It was found that double positive animals (14.6%) for MVV and BTV in sheep but not goat. As a conclusion sheep and goat were infected with naturally BTV, CAEV and MVV in Kirikkale region. This study showed that this is the first report for BTV, MVV and CAEV infections from Kirikkale region. Therefore infections are needed to be further investigations to determine detailed survey studies.

**Keywords:** Sheep, Goat, Bluetongue virus, Caprine arthritis-encephalitis virus, Maedi-visna virus

## Kırıkkale Yöresinde Bulunan Küçük Ruminantlarda Mavidil, Maedi-Visna ve Caprine Arthritis-Encephalitis Enfeksiyonlarının Serolojik ve Epidemiyolojik Araştırılması

### Özet

Viral enfeksiyonlar, koyun ve keçilerde yapılan araştırmalar ile önlenemeyen önemli sorunlara ve ekonomik kayıplara neden olur. Bu çalışmada mavidil virüsü (BTV), maedi-visna virüsü (MVV), caprine arthritis-encephalitis virüsü (CAEV)'lerini içeren viral enfeksiyonların, İç Anadolu bölgesinde yer alan Kırıkkale ilinde bulunan koyun ve keçilerde belirlenmesi amaçlanmıştır. Kan örnekleri 279 koyun ve 146 keçiden toplandı ve ELISA, RT-nested PZR ve nested PZR ile test edildi. Serum örneklerinde antikor cevabı BTV (%49,8), CAEV (%7,5) ve MVV (%19,4) seropozitiflik oranı ile tespit edildi. Seropozitif tam kan örneklerinde RT-nested PZR ve nested PZR ile, BTV ve MVV antijenleri sırasıyla (%2,87) (%9,25) oranlarında belirlendi fakat CAEV belirlenemedi. Koyunlarda hem MV hem de BT'ye karşı seropozitif hayvanların oranı %14,6 oranında saptandı. Sonuç olarak Kırıkkale yöresinde koyun ve keçilerde BTV, CAEV ve MVV enfeksiyonlarının doğal varlığının ortaya konulduğu bu çalışma, bu yörede sözü geçen enfeksiyonlarla ilgili yapılan ilk çalışmadır. Bu nedenle enfeksiyonların belirlenmesi için daha detaylı survey araştırmalarına ihtiyaç vardır.

**Anahtar sözcükler:** Koyun, Keçi, Mavidil virüsü, Caprine arthritis-encephalitis virüsü, Maedi-visna virüsü

## INTRODUCTION

Bluetongue (BT) is an arthropod-transmitted disease and bluetongue virus (BTV), the etiological agent of bluetongue disease of wild and domestic ruminants which

results in significant economic losses. Etiological agent is a non-enveloped double-stranded RNA virus belonging to genus orbivirus of the family Reoviridae <sup>1</sup>. Twenty-four



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serotypes of BTV have been recognized world wide. The disease can cause up to 100% morbidity and with 0-50% case fatality rates in sheep <sup>2</sup>. The vast majority of BT infections are clinically unapparent. The virus is known to be distributed around the world in countries located in the tropics and subtropics, where *Culicoides* species are present. Only around 50 of the 1.500 known *Culicoides* species have been shown to be capable of developing a fully disseminated transmissible BTV infection <sup>3</sup>. BTV was first described in the Cape Colony of southern Africa after merino sheep were introduced into the region in the late 18<sup>th</sup> century, and was subsequently recognized in other parts of Africa, Europe, the Middle East and Indian sub-continent, the Americas, and Asia <sup>4</sup>. Bluetongue disease has been almost found all region of Turkey since the first outbreak seen in Turkey was in Syria border 1944-1947 <sup>5-7</sup>. Survey studies are the important to epidemiology of BTV because it was hypothesized that Turkey could a possible the BT gateway through European countries <sup>3,8</sup>. The distribution of *Culicoides* biting midges is known to be influenced by weather and climate conditions such as temperature, humidity, and rainfall <sup>9</sup>. To date there is no evidence that any outbreak of BTV or any study BTV infection and scientific data about kind of *Culicoides spp* in the Kirikkale city where located near the Kizilirmak river that is the longest river in Turkey. Diagnosis of BTV disease is used many techniques such as virus isolation detection of viral RNA with PCR or RNA hybridization, antigen detection IFAT, ELISA, antibody detection ELISA <sup>10,11</sup>. Although there is a vaccine for preventing BTV infections, an animal vaccinated and protected from infection with one serotype of BTV is not protected from subsequent infections with other viral serotypes <sup>12</sup>.

Small ruminant lentiviruses (SRLV) cause two different diseases that are called maedi-visna virus (MVV) in sheep and in goat's caprine arthritis-encephalitis virus (CAEV) are considered genetically as a single pathogen causing a multisystemic inflammatory disease <sup>13,14</sup>. MVV and CAEV are a single-stranded RNA virus of the genus lentivirus in the family Retroviridae <sup>15</sup>. SRLVs include cause persistent infections in target organs for instance the mammary gland, the carpal joints, the central nervous system and the lungs <sup>16</sup>. While MVV recognized in the Iceland in 1933, CAEV first recognized in the United States in 1974 <sup>17</sup>. Infection is distributed almost worldwide and is responsible for economic losses related to the drop of milk production, lameness and interstitial pneumonia <sup>18,19</sup>. In Turkey, the presence of a MVV infection was first revealed among sheep based on pathological observation conducted in 1975. Following first observation numerous studies have demonstrated that infection is widespread in Turkey <sup>20-22</sup>. In Turkey, even though CAEV was firstly reported in 1994 there have been documented a few case and sero-epidemiological studies about CAEV <sup>23-27</sup>. To date, there is no evidence that any outbreak of MVV or any CAEV infection and scientific data in the Kirikkale, Central Anatolia Turkey.

## MATERIAL and METHODS

### Study Area and Sample Collection

The present study was conducted on two hundred and seventy nine sheep and a hundred forty six goats during the period from April to December 2010 at the Kirikkale, Turkey. Samples were collected from all sheep and goat within herds under investigation at the same time of clinical examination. However animals used in this study did not show any apparent clinical signs of BTV, MVV and/or CAEV. Sera and whole blood samples were collected from sheep and goat in Kirikkale province. While whole blood samples were collected in EDTA coated tubes (Vacutest, K3 EDTA; Arzergrande, Italy), blood samples were collected into tubes (Vacutest, Arzergrande, Italy). Blood samples allowed to clot at room temperature and centrifuged at 1.200 rpm for 10 min on the same day and sera were collected and kept at -20°C until ELISA work.

### Enzyme Linked Immunosorbent Assay (ELISA)

A commercial ELISA kit (Pourquier ELISA Maedi-Visna/CAEV serum Screening, France) was used for the detection of MVV antibody response according to the manufacturer's instructions. A commercial ELISA kit [Pourquier Bluetongue Competitive ELISA version: P00450/06 (Institute Pourquier, France)] was used for the detection of BTV antibody response according to the manufacturer's instructions. Samples were analyzed and calculated with an automated ELISA reader at 450 nm (SIRIO S® Elisa Reader, Indonesia).

### PCR Technique

To detect MVV or CAEV antigen, firstly DNA was extracted from blood using a spin column system (DNAsy Blood and Tissue Kit, Qiagen, Germany) according to the manufacturer's instructions. To detect CAEV antigen, the first round PCR was run in reaction mixture contained 5 µl cDNA, 25 mM Tris-HCl pH 8.9, 50 mM KCl, 3 mM MgCl<sub>2</sub>, 2 mM of dNTP mix, 10 pmol of each external primer 5'-CAAGCAGCAGGAGGAGAAGCTG-3' and 5'-TCCTACCC CCATAATTTGATCCAC-3' and 3 U Taq DNA polymerase (MBI Fermentas) the expected PCR product size 296 bp. Ten microliters nested PCR reactions were performed in a reaction mixture with the same reagent composition using the internal primers 5'-GTTCCAGCA ACTGCAAACA GTAGCAATG-3' and 5'-ACCTTTCTGCTTCTTCATTTAATTT CCC-3' the expected PCR product size 196 bp <sup>15</sup>. To detect MVV antigen, the first round PCR was run in reaction mixture contained 5 µl cDNA, 25 mM Tris-HCl pH 8.9, 50 mM KCl, 2 mM of dNTP mix, 10 pmol of each external primer 5'-CAACARGGGIATMATAGAYTCIGG-3' and 5'-AR TGIGTRTARTCIACYTGCCA-3' and 3 U Taq DNA polymerase (MBI Fermentas) the expected PCR product size 412 bp. Ten microliters nested PCR reactions were performed in a reaction mixture with the same reagent

composition using the internal primers 5'-GGG ATMATA GAYTCGGGRTATCARGG-3' and 5'-TGGGTRTARTCGACYTGC CARTG-3' the expected PCR product size 404 bp<sup>28</sup>. MVV positive control was provided kindly by Dr. Dilek Muz that is worked in the Department of Virology Faculty of Veterinary Medicine University of Mustafa Kemal, Turkey. Reaction conditions for nested PCR were as follows; 95°C for 2 min, followed by 32 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min and finally a cycle of 72°C for 5 min. To detect BTV antigen, firstly RNA was extracted from blood using a spin column system (QIAamp Viral RNA mini kit, Qiagen, Germany) according to the manufacturer's instructions. Random primer (1.25 mM random primer; Promega, Madison, WI, USA) was used in the first step of cDNA synthesis. For this purpose, 10 µl viral RNA was combined with 1 µl (0.5 µg) random primer and preheated at 70°C for 10 min to denature secondary structures. The mixture was cooled rapidly, and 2 µl (100 mM) dNTPs, 5 µl 5X RT buffer, 1 µl M-MLV RT (200 IU/µl Promega, USA), 1 µl RNasin (MBI Fermentas) and 5 µl H<sub>2</sub>O were added reaching to the total volume of 25 µl. The RT mix was incubated at 37°C for 60 min and stopped by heating at 95°C for 10 min. The yield of cDNA was checked by PCR signal generated from the internal standard housekeeping gene, *Ovies aries* glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (GenBank accession number: DQ386891). The GAPDH forward primer was 5'-AGCTCGTCATCAATGGAAAGGC-3', and the GAPDH reverse primer was 5'-ATGGCGTGGACAGTGGTCATAA-3'. The cDNA stock was stored at -20°C until study. The first round PCR was run in reaction mixture contained 5 µl cDNA, 25 mM Tris-HCl pH 8.9, 50 mM KCl, 2 mM of dNTP mix, 10 pmol of each external primer 5'-GTTC TCTAGTTGGCAACCACC-3' and 5'-AAGCCAGACTGTTCCC GAT-3' and 3 U Taq DNA polymerase (MBI Fermentas) the expected PCR product size 274 bp. 10 µl nested PCR reactions were performed in a reaction mixture with the same reagent composition using the internal primers 5'-GCAGCATTTTGGAGAGCGA-3' and 5'-CCC GATCATACATTGCTTCT-3' the expected PCR product size 101 bp<sup>29</sup>. Reaction conditions nested PCR reactions were as follows; 95°C for 2 min, followed by 32 cycles of 95°C for 40 s, 55°C for 1 min, 72°C for 40 s and finally a cycle 50°C 3 min and of 72°C for 5 min. BTV vaccine strain was using as positive control provided kindly by Prof. Dr. Aykut Ozkul that is worked in the Department of Virology Faculty of Veterinary Medicine University of Ankara, Turkey. All nested-PCR reactions were carried out in BO-PCR-5 thermal cyler (Hamburg, Germany). The InGenius LHR (Syngene, Cambridge, UK) was used to see PCR amplicons by using ethidium bromide staining after 1.5% agarose gel electrophoresis.

## RESULTS

It was collected samples from 425 sheep and goat in the Kirikkale city, Central Anatolia of Turkey. It was found

that rate of antibody response to BTV were in sheep 139 out of 279 (49.8%) seropositivity value. It was found that rate of antibody response to CAEV were 11 out of 146 (7.5%) seropositivity values in the goat. It was determined that rate of antibody response to MVV were 54 out of 279 (19.4%) seropositivity values in the sheep (Table 1). Double seropositive animals 41 out of 279 (14.7%) for MVV and BTV were determined in sheep but not in goat. To determine BTV antigen, 139 whole blood samples (antibody positive for BTV) submitted for BTV diagnosis were tested by RT-nested PCR. Firstly, we checked whether RT-PCR assay was working or not. Therefore *Ovies aries* GAPDH gene was targeted as an internal control. All samples were shown GAPDH signal (Fig. 1A). After confirming RT reaction by using GAPDH primers, first round of nested-PCR was carried out by using external primers which could be amplified at the NS1 of the BTV genome and are considered to amplify all BTV genome. PCR amplicon of NS1 of the BTV genome was not demonstrable in first round nested PCR including positive control for PCR (Fig. 1B). When second round of PCR was carried out internal primers for BTV, 4 out of 139 (2.9%) whole blood samples were detected as a positive (Table 2 and Fig. 1C). Detection of CAEV was carried out eleven samples that are seropositive for CAEV nested PCR. However PCR signal were not detected for CAEV in goat by using CAEV specific primers (Table 2). Detection of MVV was carried out fifty four samples that are MVV positive antibody response nested PCR. PCR amplicon for MVV was not demonstrable

**Table 1.** Prevalence of BTV, MVV and CAEV in sheep and goat from Kirikkale city in Turkey by using ELISA

**Tablo 1.** Kirikkale'de koyun ve keçilerde ELISA ile BTV, MVV ve CAEV prevalansı

Number of Animals Used in This Study	MVV-CAEV	BTV
Number of Goat	146	.*
Number of Sheep	279	279
Number of Seropositives Animals	65	139
Number of Seropositives Goat	11	-
Number of Seropositives Sheep	54	139
Number of Total Animals	425	279

\* Goat did not tested for BTV (Keçiler BTV için test edilmedi)

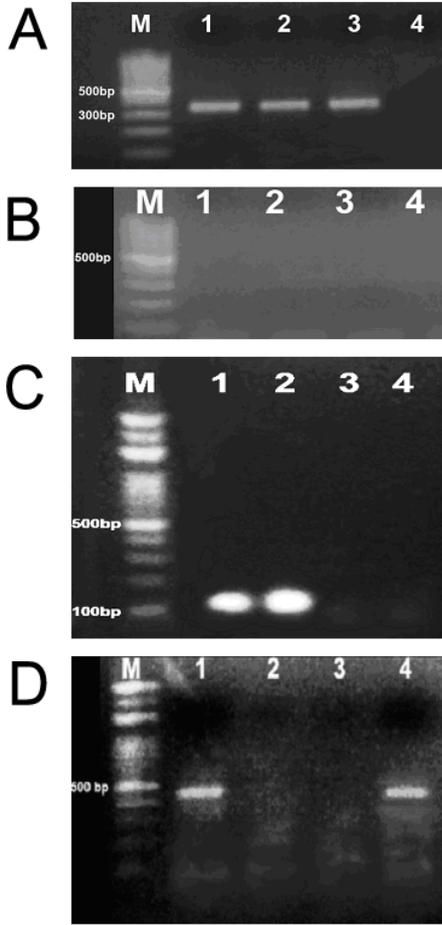
**Table 2.** Number of RT-nested PCR or nested PCR positive samples from whole blood that were coincide with seropositive samples for BTV, CAEV, MVV

**Tablo 2.** BTV, CAEV, MVV seropozitif tam kan örneklerinden, RT-PCR-nested PZR veya nested PZR pozitif örnek sayıları

PCR Results	CAEV	MVV	BTV
Positive Goat	-	-	.*
Positive Sheep	-	5	4
Number of Animals Used in for PCR	11	54	139

\* Goat did not tested for BTV (Keçiler BTV için test edilmedi)

in first round nested PCR including positive control for PCR. When second round of PCR was carried out internal primers for MVV, 5 of 54 (9.3%) (Table 2) samples were shown expected PCR product size (Fig. 1D).



**Fig 1.** RT-nested PCR with RNA extracted from clinical samples for BT and nested-PCR with DNA extracted from clinical samples for MVV (whole blood). **A.** RT-PCR control, RT-PCR was carried out *Ovies aries* GAPDH primers. Whole blood samples lane 1 (positive control for BTV), lane 2 (positive clinical sample), lane 3 (negative clinical sample) and lane 4 (dH<sub>2</sub>O), Lanes M: molecular length marker. **B.** First round RT-nested PCR for BTV was carried out BTV external primers. Whole blood samples lane 1 (positive control for BTV), lane 2 (positive clinical sample) and lane 4 (dH<sub>2</sub>O), Lanes M: molecular length marker. **C.** Second round RT-nested PCR for BTV was carried out BTV internal primers. Whole blood samples lane 1 (positive control for BTV), lane 2 (positive clinical sample), lane 3 (negative clinical sample) and lane 4 (dH<sub>2</sub>O), Lane M: molecular length marker. **D.** Second round nested PCR for MVV was carried out MVV internal primers. Sample lane 1 (positive sample), lane 2 (negative clinical sample), lane 3 (dH<sub>2</sub>O) and lane 4 (positive control for MVV), Lane M: molecular length marker

**Şekil 1.** Klinik örneklerden elde edilen RNA'lar ile BTV için RT-nested PZR (tam kan) ve klinik örneklerden elde edilen DNA ile MVV için nested-PZR. **A.** RT-PZR kontrol, RT-PCR *Ovies aries* GAPDH primer ile kuruldu. Tam kan örnekleri hat 1 (BTV için pozitif kontrol), hat 2 (pozitif klinik örnekler), hat 3 (negatif klinik örnekler) ve hat 4 (dH<sub>2</sub>O), hat M: moleküler ağırlık markeri. **B.** BTV için ilk round nested RT-PZR, BTV dış primerleri ile kuruldu. Tam kan örnekleri hat 1 (BTV için pozitif kontrol), hat 2 (pozitif klinik örnekler), hat 3 (negatif klinik örnekler) ve hat 4 (dH<sub>2</sub>O), hat M: moleküler ağırlık markeri. **C.** BTV için ikinci round nested RT-PZR, BTV iç primerleri ile kuruldu. Tam kan örnekleri hat 1 (BTV için pozitif kontrol), hat 2 (pozitif klinik örnekler), hat 3 (negatif klinik örnekler) ve hat 4 (dH<sub>2</sub>O), hat M: moleküler ağırlık markeri. **D.** MVV için ikinci round nested PZR MVV iç primerleri ile kuruldu. Hat 1 (pozitif klinik örnek), hat 2 (negatif klinik örnek), hat 3 (dH<sub>2</sub>O) ve hat 4 (MVV için pozitif kontrol), hat M: moleküler ağırlık markeri

## DISCUSSION

Many disease problems can be solved by an investigation of animal populations rather than the individual. The measurement of the amount of infectious and non-infectious diseases in a population assists in determining their importance and efficacy of control campaign<sup>30</sup>. This study was carried out to determine the BTV, MVV and CAEV infections in Kirikkale city of Turkey.

Currently, most vaccines used are modified live vaccines, but a restricted number of inactivated vaccines are also available and new types of vaccines are being developed. The first epizootic of BT outside Africa was confirmed in sheep on Cyprus in 1943 and a vaccine was successfully used to control outbreaks of BT on Cyprus in 1946 and 1947. At the same time, BT was reported in Israel, Turkey and probably Syria<sup>31</sup>. Prior to 1998, BTV only occurred sporadically in Europe and following 1998 is known as the invasion of the Western and Eastern Mediterranean basin by *Culicoides imicola*, a tropical midge. In 2008, one further step was reached in the invasion of the European continent<sup>32</sup>. It was reported that 14.5% of sampled animals have BT virus specific antibodies in Eastern and South-eastern regions of Turkey<sup>25</sup>. It was announced that BTV seroprevalence was 4.68% in sheep in Konya region of Turkey<sup>33</sup>. Although BTV virus has different 24 serotypes, virus has already been determined on the worldwide with only type 4, 9, 16 recognized within Turkey<sup>2</sup>. Another study showed that BTV seroprevalence was found 91.76% and the specific frequencies were 72.16%, 42.05% and 36.93% for serotypes 4, 9 and 16 in 352 cattle in north-eastern Anatolian cities<sup>34</sup>. It was showed that the seroprevalence of bluetongue was determined as 3% (6/200) in sheep, 11% (22/200) in cattle<sup>35</sup>. The present study was carried out in Kirikkale city that is situated in central the Anatolia region of Turkey in Asia. It lays 39° 52' 53" north of the equator and 33° 26' 46" meridians east of Greenwich. Kirikkale has a coastline with the majority lying along the Kizilirmak River. The northern part of the region is hilly and in many places at altitude ranges between 700 and 1450 meter. Extreme recorded temperatures range from -10°C to 35°C at Kirikkale. Additionally there is no vaccination for BTV in this city in sheep or goat. In present study, 139 out of 279 (49.8%) sheep have shown BTV specific antibodies (Table 1). Unfortunately goat samples did not incorporate in the study in order to limited fund. In this study was found that PCR positive for BTV samples was 4 out of 139 (2.9%) whole blood (Fig 1C and Table 2). It suggested that four sheep is viremic in the sampling time. It was suggested that vaccination of sheep or goat will help to take under control of BTV and *Culicoides spp* will be combated according this results. This is first report BTV infection for Kirikkale. BTV was found by South African, French, American, and Icelandic researchers. Extensive research into the pathology, etiology, and epidemiology of this slowly progressive and ultimately fatal disease was

confirmed in several countries<sup>31,36</sup>. BTV infection is need to further investigations to determine detailed sero-epidemiological studies in goat and cattle and the circulated serotypes to found vaccine which should be contain serotype(s).

CAEV and MVV are considered to be genetically distinct but antigenically related pathogens of goats and sheep. SRLV infections seen on farms develop after long incubation and a slow progression of disease to death but in nature they may also have short latency and cause acute leukoencephalitis and/or acute arthritis and pneumonia in young kids or lambs with exceptionally high mortality. SRLV is persistent infection a feature that share with immunodeficiency-causing lentiviruses such as MVV, CAEV<sup>37</sup>. Animals can not be effectively vaccinated against SRLVs and MVV is prevalent in sheep populations worldwide. For this reason seropositive sheep or goat represent a marker of wild type infection. To date, MVV seroprevalence data obtained in Turkey have been found such as 23.9%, from 3.8% to 41.2%, and 1.2%. Recent study showed 15.3% prevalence observed in the Istanbul city<sup>20-22</sup>. Additionally studies with regard to MVV in Turkey that seropositivity was determined 26.7%, 23.9% and 10% respectively<sup>21,38-40</sup>. It was announced that MVV seroprevalence during the study period ranged from 24% to 39% in Latxa sheep and from 77% to 80% in Assaf sheep<sup>41</sup>. In the present study it was found that rate of antibody response to MVV were 54 out of 274 (19.4%) seropositivity values in the sheep (Table 1). PCR finding for MVV showed 5 out of 54 (2.9%) animals were detected as a positive (Fig 1D and Table 2). CAEV infects mostly goats and has a global distribution in the world. Serological data was shown in Australia, USA and, UK prevalence rates of 82%, 73%, 4.3%, respectively<sup>24</sup>. Perspective of in the neighbor of Turkey although there is no data with associated in the Iran, Iraq, Syria, Azerbaijan, Armenia, Bulgaria and Rumania, CAEV was just reported in Greece<sup>42</sup>. To date it was suggested that there is no study in CAEV free countries of neighbor of Turkey. The serologic examination was carried out by AGID technique in 16 out of 808 (1.9%) for CAEV in Turkey<sup>26</sup>. Even though one reported that all of the sampled goats were negative for CAEV antibodies eastern and south-eastern Anatolia, another researchers was reported 7 out of 675 goats (1.03%) sampled were positive for CAEV antibodies with cELISA, but no seropositivity was detected with AGID in the same region<sup>24,25</sup>. Researchers were carried out ELISA for detecting antibody against to CAEV both adult goat (n=75) and their kids (n=70). While adult goat did not show any clinical sign for CAEV and were serological positive for CAEV, their kids showing arthritis and encephalitis were confirmed serological positive for CAEV using ELISA in Nevsehir city that was very close to Kirikkale<sup>23</sup>. In the present study it was found that rate of antibody response to CAEV were 11 out of 146 (7.5%) in the goat (Table 1). However serological finding did not supported by PCR. For diagnosing the

infection, specific antibody detection methods as AGID, ELISA are most frequently used. Molecular biological techniques can be hampered by the low viral load in the blood and the pronounced heterogeneity of the viral genomes<sup>43</sup>. In the present study it was suggested that virus could not be detected for low viral load and maybe real time PCR could be solve this problem. As a conclusion sheep and goat were infected with naturally BTV, CAEV and MVV in the Kirikkale region. Additionally some sheep were co infected with BTV and MVV. But there is no vaccination or prevent to these infection. This study showed that this is first report BTV, MVV and CAEV infection in the Kirikkale and these infections are still available in the Central Anatolia. Therefore infections are needed to be further investigations to determine detailed survey studies.

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