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Research Article

Molecular Detection of Picornaviruses in Diarrheic Small Ruminants at a Glance: Enterovirus, Hunnivirus, and Kobuvirus in Türkiye

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Abstract: Enteric diseases are considered an important factor affecting the livestock industry; however, there are limited data on viruses that cause enteric diseases in small ruminants in our country. In this study, we focused on enterovirus (EV), hunnivirus (HuV), and kobuvirus (KoV), which are members of the family of *Picornaviridae*. In order to provide further information on the prevalence and the molecular epidemiology of these viruses, a total of 73 fecal samples or gut contents from diarrheic sheep and goats were screened for EV, HuV, and KoV. For this purpose, RT-PCRs were performed by using the specific primers for each virus. The prevalence rate determined in the sampled population was 2.7% (2/73) for each virus. In detail, EVs (2/60, 3.3%) and HuVs (2/60, 3.3%) were each detected in only sheep samples while KoVs were identified in a goat sample (1/13, 7.6%) and a sheep sample (1/60, 1.6%). There was no evidence of coinfection with these viruses in the tested animals. According to the results of the molecular analyzes, our EVs were clustered in caprine/ovine-specific EV-G and HuV strains retrieved in this study were grouped along with the other caprine/ovine origin sequences in Hunnivirus A2 genotype. Moreover, it was observed that the detected KoVs clustered in distinct species: Aichivirus B and Aichivirus C. In conclusion, this study, which reported the detection of EVs and HuVs from sheep as well as KoVs from sheep and goats in our country, provides valuable data on the epidemiology and molecular characteristics of these viruses.

Keywords: *Enterovirus, Goat, Hunnivirus, Kobuvirus, Sheep*

Bir Bakışta İshalli Küçük Ruminantlarda Picornavirusların Moleküler Tespiti: Türkiye'de Enterovirus, Hunnivirus ve Kobuvirus

Öz: Enterik hastalıklar, hayvancılık endüstrisini etkileyen önemli bir faktör olarak kabul edilmektedir, ancak ülkemizde küçük ruminantlarda enterik hastalıklara neden olan viruslara ilişkin veriler sınırlıdır. Bu çalışmada *Picornaviridae* ailesinin üyeleri olan enterovirus (EV), hunnivirus (HuV) ve kobuvirus (KoV) üzerinde durulmuştur. Bu virusların prevalansı ve moleküler epidemiyolojisi hakkında daha fazla bilgi elde etmek amacıyla, ishalli koyun ve keçilerden toplam 73 dışkı örneği veya bağırsak içeriği EV, HuV ve KoV için test edilmiştir. Bu amaçla, her bir virus için spesifik primerler kullanılarak RT-PCR'lar yapılmıştır. Örneklenen popülasyonda tespit edilen prevalans oranı her bir virus için %2.7 (2/73) olarak hesaplanmıştır. Detaylı olarak değerlendirildiğinde, EV (2/60, %3.3) ve HuV (2/60, %3.3) yalnızca koyun örneklerinde saptanırken, KoV ise bir keçi (1/13, %7.6) ve bir koyun örneğinde (1/60, %1.6) tespit edilmiştir. Bununla birlikte, test edilen hayvanlarda bu viruslarla herhangi bir koenfeksiyon saptanmamıştır. Moleküler analizlerin sonuçlarına göre, EV suşlarının keçi/koyuna özgü EV-G'de kümelendiği ve bu çalışmada elde edilen HuV suşlarının ise Hunnivirus A2 genotipinde diğer keçi/koyun kökenli dizinlerle birlikte gruplandığı ortaya konulmuştur. Ayrıca tespit edilen KoV suşlarının Aichivirus B ve Aichivirus C olmak üzere farklı türlerde kümelendiği gözlemlenmiştir. Sonuç olarak, ülkemizde koyun ve keçilerde KoV'ların yanı sıra koyunlarda EV ve HuV'un tespitini bildiren bu çalışma, bu virusların epidemiyolojisi ve moleküler özelliklerine ilişkin değerli veriler sunmaktadır.

Anahtar sözcükler: *Enterovirus, Keçi, Hunnivirus, Kobuvirus, Koyun*

Introduction

Picornaviruses, which belong to the family *Picornaviridae*, are icosahedral, non-enveloped viruses with a single-stranded positive-sense RNA genome [1]. Despite the fact that the great majority of picornavirus infections are asymptomatic, several picornaviruses cause disorders of the central nervous system, respiratory and gastrointestinal

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tracts, as well as some other organs, such as the heart and liver in humans and animals [2].

The picornavirus genomic RNA (6.7-10.1 kb), commonly contains a single large open reading frame (ORF) flanked by 5'- and 3'-UTRs. A large polyprotein precursor produced by the single ORF is post-translationally cleaved into three distinct P regions (P1-P3), which encode the structural proteins and the non-structural proteins. In detail, P1 encodes the viral capsid proteins, while proteins involved in protease processing and genome replication are encoded by P2 and P3. Also, in many viruses, such as kobuviruses, P1 is preceded by a leader protein (L) [1].

The genus *Enterovirus* consists of 12 species of enterovirus (A-L), and three species of rhinovirus (A-C) [3]. Out of them the three enterovirus (EV) species, EV-E, EV-F, and EV-G are most closely associated with the diseases affecting the livestock industry [4-6]. EV-E and EV-F, previously known as bovine enterovirus A and B, are the causative agents of infections in cattle that display clinical indications ranging from respiratory diseases to enteritis, reproductive disease, and infertility [7-9]. In addition, they have also been detected in asymptomatic animals as well as in the environmental samples [10-12]. EV-G, which was previously referred to as porcine enterovirus B (PEV-B), comprises viruses isolated from pigs, wild boars, and small ruminants such as sheep, goats, and Sichuan takins [4,13]. Previous studies revealed that natural infection with EV-G can cause severe diarrhea with high morbidity and mortality rates as well as neurological disorders, fertility disorders, diarrhea, and dermal lesion [4,10,14,15].

Hunnivirus is a novel picornavirus genus that was established by the International Committee on Taxonomy of Viruses (ICTV) in 2013 [16]. This genus is comprised of a single species, which is known as Hunnivirus A and it has been classified into at least nine genotypes: hunnivirus A1 (formerly bovine hungarovirus 1) [2], hunnivirus A2 (formerly ovine hungarovirus 1) [2], hunnivirus A3 (isolated from sheep cell cultures) [17], hunnivirus A4 (Norway rat hunnivirus) [18], and hunnivirus A5-A9 [3,19,20]. After the first discovery of hunnivirus (HuV) in sheep cell cultures in 1965 [17], it was detected in cattle, sheep, goats, water buffalo, rats, and cats [2,19-21]. However, *Hunnivirus* genus is poorly understood, and it is unknown what clinical signs they can cause in which animal species and its potential risks to human health.

Kobuvirus (KoV) was first reported in a fecal sample from a human with gastroenteritis in 1989 [22]. Subsequently, an increasing number of novel KoVs have been frequently detected in humans and a wide variety of domestic and wild animals with or without clinical signs [23-28]. The genus *Kobuvirus* is grouped into six species, Aichivirus A to F, and 20 genetic types. In addition to these, there are three unclassified KoVs [3]. There have been several reports of KoVs in sheep and goats, and so far, KoVs detected in sheep have been grouped into the Aichivirus B and Aichivirus D species [27,29,30] whereas goat KoVs have been classified into the Aichivirus B and Aichivirus C species [24,31,32]. However, the data of their pathogenicity and epidemiological distribution is still limited.

Considering the circulation of mentioned viruses worldwide among many animal species and the detection of them in some other animal species except small ruminants in Türkiye, fecal samples or gut contents from diarrheic sheep and goats were screened for EV, HuV, and KoV in order to fill the gap in this regard and to provide further information on the molecular epidemiology of these viruses.

Material and Methods

Ethical Statement

The study was approved by the Ankara University Animal Experiments Local Ethics Committee (Decision No: 2022-10-93).

Samples

A total of 73 fecal samples or gut contents from small ruminants (60 sheep and 13 goats) with diarrhea from herds in several provinces of Türkiye were used in this study *(Fig 1)*. Samples were collected by the field veterinarians and sent to our laboratory for routine diagnosis.

Viral RNA Extraction and RT-PCRs

The viral RNA was extracted from sample suspensions (1:10, w/v) using QIAamp Cador Pathogen Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and stored at -80°C. Reverse transcription was performed using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. For the detection of KoV and EV, the generic KoV primer pair UNIV-kobu-F/R [33] and EV primer pair targeting the conservative 5' UTRs (Non-HumanEntero-5' UTR-R/F) [13] were used, respectively. In order to identify HuV, a generic primer pair (Hungaro-3D-F/R) [2], which were designed based upon the nucleotide sequences of the 3D region of hunnivirus A1 (formerly bovine hungarovirus 1), was used. The RT-PCRs were performed using DreamTaq DNA polymerase (Thermo Fisher Scientific, USA) with the following thermal conditions: denaturation at 95°C for 3 min, 40 cycles of denaturation at 95°C for 60 sec, annealing at (48°C for KoV, 51°C for HuV, and 57°C for EV) for 30 sec, extension at 72°C for 60 sec, and final extension at 72°C for 10 min. The products were run in 1% agarose gel stained with SafeView Classic (ABM, Canada), and visualized under UV light.

Sequencing and Phylogenetic Analysis

Nucleotide sequencing of the amplicons with expected sizes (~220 bp for EV, 465 bp for HuV and 217 bp for KoV) were performed using the same primers used in the RT-PCRs. The MUSCLE algorithm as implemented in Aliview Software was used to create multiple sequence alignments [34,35]. Cognate sequences of each viruses representing different genotype/serotype were retrieved from GenBank through the BLAST engine. The identities of nucleotides (nt) and amino acids (aa) were determined using the SIAS online program *(http://imed.med.ucm.es/Tools/sias.html)*. Phylogenetic analyses were conducted using MEGA X software by applying the maximum likelihood (ML) method [36]. Measuring the best fit model with the "Find Best DNA/Protein Model" feature of the MEGA X software was performed before applying the ML method and Bayesian information criteria (BIC) was used to determine the nucleotide substitution model. Accordingly, the ML phylogenetic trees were constructed using the T93 (Tamura Nei) + G (for EV and KoV) and T92 (Tamura-3) + G (for HuV) nucleotide substitution models. The nucleotide sequences of viruses detected in this study were deposited into the GenBank database under the following accession numbers: ON316762-ON316767.

Results

In this study, a total of 73 samples from diarrheic small ruminants, containing 60 sheep and 13 goats, were screened for EV, HuV and KoV. A total of six samples (8.2%) were confirmed to be positive for any one of the viruses studied. Overall, in our survey, the prevalence rate determined in the population was 2.7% (2/73) for each virus. Specifically, EVs (2/60, 3.3%) and HuVs (2/60, 3.3%) were each detected in only sheep samples while KoVs were identified in a goat sample (1/13, 7.6%) and a sheep sample (1/60, 1.6%). No coinfection with these viruses was detected in tested animals. A map of the sample collection sites and the locations of samples positive for the viruses are shown in *Fig. 1*.

In this study two samples from sheep produced the expected size amplicons of the 5'UTR of EVs while there was no positive sample from goats. These sheep were from farms located in different provinces of Türkiye, Cankiri (A1G) and Eskisehir (KRM33). Sequence comparison for the partial 5'-UTR between the A1G and KRM33 strains revealed 92.61% nt identity to each other. Both strains also shared the highest nt identity (A1G 92.21% and KRM33 90.47%) to the corresponding region of ovine enterovirus 2019-00927 isolate [14]. The phylogenetic tree based on the partial 5'-UTR sequences showed that our EVs were clustered in caprine/ovine-specific EV-G *(Fig. 2)*.

Both HuVs reported in this study were identified in sheep samples from Ankara (KD6) and Kirsehir (KRM2); however, none was detected in goat samples. The molecular analysis of the partial 3D gene region showed that our HuV strains shared 91.18% nt and 94.83% aa identity to each other. Interestingly, our strains displayed higher genetic identities with previously identified several HuV strains from small ruminants than each other. Specifically, the KRM2 strain was most closely related to Hungarian sheep and Chinese goat and sheep strains (94.62-96.55% nt and 97.41-98.7% aa identity). The KD6 strain had the highest identity to Chinese goat strain (91.39% nt and 96.77% aa identity). Also, the phylogenetic tree showed that HuV sequences retrieved in this study were grouped along with the other caprine/ovine origin sequences in Hunnivirus A2 genotype *(Fig. 3)*.

Partial nucleotide fragments of the 3D gene region of KoVs were detected in two samples, one from sheep (KRM21) and one from a goat (OBI), and both were obtained from different provinces: Bartin and Hatay, respectively. By sequence analysis of the partial 3D gene,

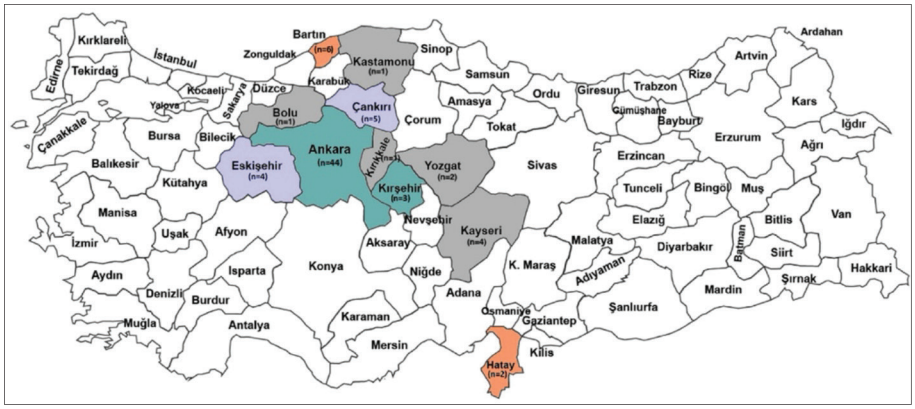


Fig 1. The map showing the distribution of the samples according to sampled provinces. Purple, green, and orange colors indicate the positive provinces EV, HuV, and KoV were detected, respectively. The grey color indicates the provinces of negative samples

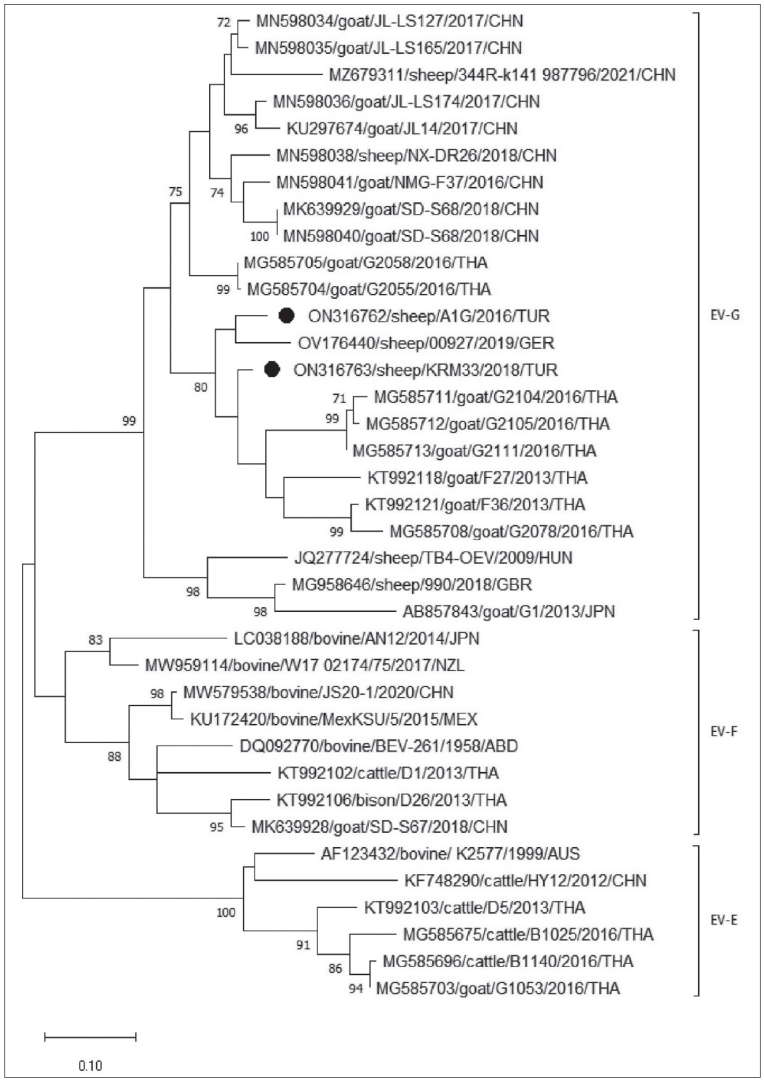
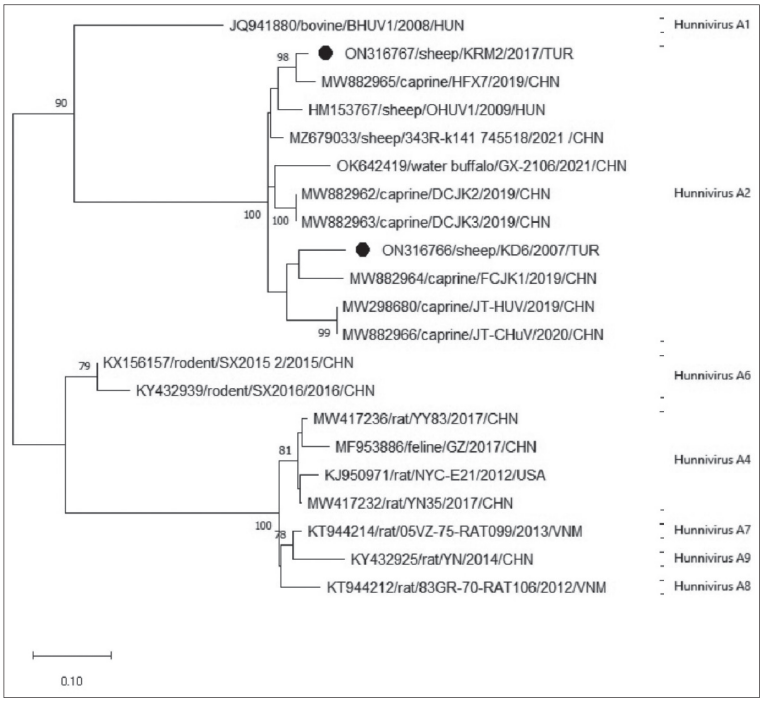


Fig 2. Phylogenetic tree based on the nucleotide of ~270 bp 5'UTR of EV. Our strains are indicated by black dots. The phylogenetic trees were constructed using the Maximum-Likelihood method with bootstrap of 1000 replicates. Numbers to the left of node indicate bootstrap values. Bootstrap values < 70% are not shown. Scale bar indicates nucleotide substitutions per site

Fig 3. Phylogenetic tree based on the nucleotide of 465 bp 3D gene region of HuV. Our strains are indicated by black dots. The phylogenetic trees were constructed using the Maximum-Likelihood method with bootstrap of 1000 replicates. Numbers to the left of node indicate bootstrap values. Bootstrap values < 70% are not shown. Scale bar indicates nucleotide substitutions per site



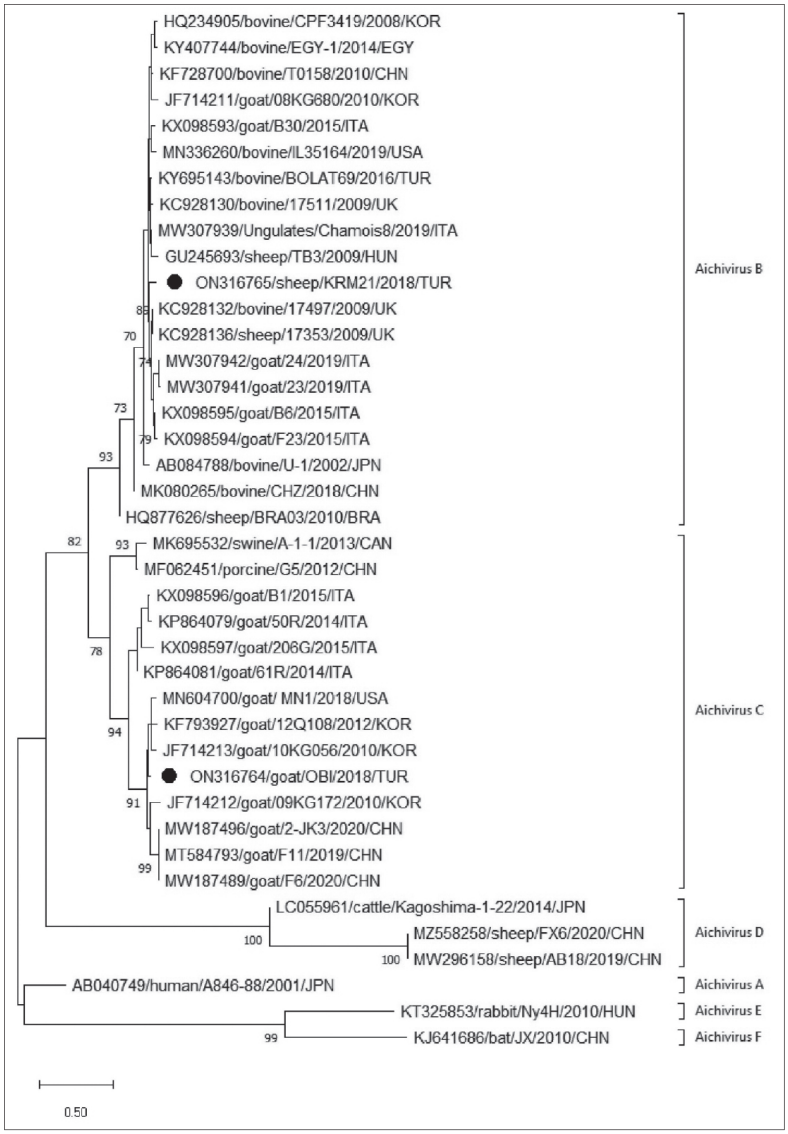


Fig 4. Phylogenetic tree based on the nucleotide of 217 bp 3D gene region of KoV. Our strains are indicated by black dots. The phylogenetic trees were constructed using the Maximum-Likelihood method with bootstrap of 1000 replicates. Numbers to the left of node indicate bootstrap values. Bootstrap values < 70% are not shown. Scale bar indicates nucleotide substitutions per site

our strains shared 76.05% nt and 84.28% aa identity with each other. The phylogenetic tree revealed that the detected both KoVs clustered in distinct species: the KRM21 strain within the species Aichivirus B and the OBI strain in the species Aichivirus C *(Fig. 4)*. The strain KRM21 displayed a close relatedness, 91.82-94.83% nt and 94.28-95.71% aa identity, to KoV sequences clustered in the species Aichivirus B. The strain OBI demonstrated a significant relation to the goat strain detected in USA (94.47% nt and 98.59% aa) and black goat strain from South Korea (94.47% nt and 100% aa identity).

Discussion

According to the data of the Turkish Statistical Institute, as of the end of June 2021, the total number of sheep and goats was determined as approximately 57.4 million (45.2 million sheep and 12.2 million goats), and approximately 10% of the total red meat and milk production were provided from small ruminants [37].

Enteric diseases are considered an important factor affecting the livestock industry and a large variety of viruses could be responsible for causing diarrhea such as rotavirus, coronavirus, picornavirus, and recently identified an increasing number of novel viruses [8]. However, there is limited information on enteric viruses in small ruminants in our country [38-40]. In this study, we focused on picornaviruses considered as another possible etiological agent for diarrhea cases and reported the prevalence and molecular characterization of EVs, HuVs, and KoVs of small ruminants in different provinces in Türkiye.

In this study, two samples from sheep were found positive for EV, and the detection rate of 3.3% was determined. Although EV previously described in Türkiye in cattle [41,42], and a goat [43], there is no report on the detection of EV in sheep. Nevertheless, there are serological studies revealed the detection of antibodies to EVs by neutralization technique using BEV-1 strain in small ruminants at different

rates, 27.6-71.8% in goats, 32.6-46.5% in sheep [44-46]. In several countries, infection with different genotypes/serotypes (EV-E, EV-F, and EV-G) have been reported in sheep and goats [4-6,10,11,13,14,47]. The detection rates of these studies range from 24-60% in goats and 39.1-44% in sheep, which is quite higher than our detection rate.

The molecular analysis of the partial 5'UTR revealed that our strains were most closely related to ovine enterovirus 2019-00927 isolate which is novel EV isolated from a lamb with progressive neurological symptoms [14]. Also, the phylogenetic tree showed that both EVs detected in this study were clustered in caprine/ovine-specific EV-G and grouped with this ovine strain (2019-00927) as well as EVs detected from fecal samples of goats in Thailand *(Fig. 2)*. The 5'UTR is a reasonably conserved genomic region that differs between EVs, making it valuable for detecting and classifying the *Enterovirus* genus into groups [6,48]. However, the current standard classifies EV species, serotypes, and genotypes based on their capsid and polymerase genes, not on the 5'UTR alone [48]. Therefore, more detailed molecular analyses including the capsid and polymerase genes are required to confirm the serotypes/genotypes of the circulating EV strains in our country. Although the viruses detected in this study were most closely associated with a novel EV isolated from a lamb with progressive neurological symptoms; it is not possible to assess whether these viruses might cause any neurological problem since these EV positive animals were reported to us only having diarrhea. This unexpected finding points out that further studies are needed to understand the host range, pathogenesis, and epidemiology of EVs in animals. Especially, considering the recombinant enteroviruses have been reported previously [5,13] it is particularly suggested that more detailed molecular studies should be conducted in the future.

To date HuVs were detected in cattle, sheep, goats, water buffalo, rats, and recently cats [2,19-21]. However, the detailed data related to HuVs in different host species and different geographic locations are quite limited. To the best of our knowledge, only two sequence data of HuV from sheep were deposited to GenBank so far. In this study, we detected two (2/60, 3.3%) HuVs from sheep samples. The detection rate in our study was quite lower than the rate found in sheep, 25%, in Hungary [2]. In the only study on HuV in our country [41], HuV positivity was reported at a rate of 11.2% in cattle.

The phylogenetic tree demonstrated that the KRM2 and KD6 strains belonged to the genotype of Hunnivirus A2 *(Fig. 3)*. Even though we only analyzed the partial 3D gene region in the current study, the present results will provide a valuable information the epidemiology, molecular characteristics, and evolution of HuVs in sheep in Türkiye, since there is limited data on these viruses. Uncertainty exists regarding the host range of HuVs, as well as their pathogenicity in cattle and small ruminants. These viruses were discovered in young animals (cattle and sheep) that appeared to be healthy at the time of discovery [2]. Later, these viruses were identified from diarrheic calves and a cat [21,41]. Because only diarrheic small ruminants were included in this study, similar to those studies, it is not possible to determine how much of a contribution HuV made to diarrhea cases. Consequently, more investigations are required to determine the geographic distribution, the route of transmission and the link between diarrhea in an animal model, as well as to evaluate its zoonotic potential.

Although the presence of KoVs in small ruminants has been confirmed in several countries [23,24,29-31,49,50] there has been no information regarding on this topic in Türkiye. Detection rate of KoVs in this study were 1.6% (1/60) and 7.6% (1/13) in sheep and goats, respectively. The level of KoV detected in sheep was similar to the detection rate (2%) in Northern Ireland [50], however it was quite low compared to the rates of 39.1% and 62.5% in Brazil and Hungary, respectively [27,29]. In this study, 7.6% of diarrheic samples were detected as goat KoV positive which was similar to previous reports in diarrheic goats, 6.5% [32] and 9.3% [49], however, there is a report which determined a much higher detection rate, 87.5% [30]. Despite the fact that a small number of goat samples were examined, the findings verified the presence of KoV in Turkish goats. The common finding of KoV among various wild and domestic animals indicates the widespread nature of these viruses and their potential to cause enteric disease [23]. Globally, goat and sheep KoVs have been detected in both diarrheic and asymptomatic animals [23,24,29-31,49,50]. Therefore, the question of whether there is a link between the presence of KoVs in animals and the development of enteric disease has yet to be fully answered and requires further research into this subject.

On the phylogenetic analysis of the partial 3D sequence, the sheep strain, KRM21, clustered within the species Aichivirus B and shared a branch with the other two Italian goat KoVs [24] detected previously *(Fig. 4)*. Also, the phylogenetic analysis revealed that the goat strain OBI was closely related to the other goat KoVs within the species Aichivirus C, which were previously described as being close to porcine kobuviruses rather than to bovine and sheep KoVs [23,31,49]. This result indicates the possibility of the circulation of different KoVs in small ruminants in our country.

In this study, in order to identify HuVs and KoVs the generic primer pairs, Hungaro-3D-F/R [2] were used, respectively. Indeed, using the primer pair, UNIV-kobu-F/R, new viruses classified in the genus *Kobuvirus* have been identified in various animals [21,29,31] however, detection of HuVs in different animal species with these primers

indicated a more generic nature of them [2]. Reuters et al.[2] reported that the generic KoV primers amplified HuV 3D gene region sequences, with 25% sensitivity. Lu et al.[21] further confirmed that the UNIV-kobu-F/R primers determined HuVs with 30% sensitivity. Unlike these reports, in our study, two amplicons detected by these primers were confirmed as KoV by sequencing and none of the HuV positive samples produced the expected fragment size by RT-PCR using UNIV-kobu-F/R. They were only detected by the primers Hungaro-3D-F/R.

It is known that diarrhea outbreaks are often multifactorial, infection with viruses, parasites, or bacteria, as well as dietary factors might contribute to the severity of diarrhea in animals. In Türkiye, the common pasture usage, breeding of animals by families, and management conditions (quality of barns, feeding, and hygiene) may increase the risk of infectious disease. In our country, as in other countries of the world, studies on diarrhea in small ruminants are quite limited. Although other possible enteric agents causing diarrhea and/or coinfection are not the focus of this article, since some of the samples tested in this study had been used in the previous study [40], it is thought that it would be useful to evaluate the results of both studies together. Considering the samples (n=66) used in both studies, coinfection with rota- and picobirnavirus was determined in two animals, KD6 and KRM21, which were positive for HuV and KoV, respectively. Although our study unveiled the circulation of the mentioned picornaviruses in small ruminant animals, it has several limitations. For instance, any clinically healthy animals were not subjected to this study. In addition, this study was carried out on a relatively limited number of samples, especially in terms of goats, and it mostly consisted of young animals.

In conclusion, our study on the detection and molecular analysis of different enteric picornaviruses from diarrheic small ruminants will contribute significantly to the literature and provide valuable data for understanding their epidemiology, molecular characteristics, and evolution.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author (I. Karayel-Hacioglu) upon reasonable request.

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Ethical Statement

The study was approved by the Ankara University Animal Experiments Local Ethics Committee (Decision No: 2022-10-93).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

IKH, SDY, and FA conceived and planned the study design. IKH together with SDY conducted the experiments and performed the molecular biology and bioinformatic analyses (alignments, phylogeny). IKH, SDY, and FA interpreted the obtained data. IKH and SDY drafted and wrote the manuscript; FA reviewed and edited the manuscript. All authors read and approved the final manuscript.

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