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RESEARCH ARTICLE

Investigation of Some Neuroinfectious Viral Agents in Turkish Cattle: First Detection and Molecular Characterization of Bovine Herpesvirus Type 5 (BoHV-5)

Kezban Can-Sahna[1], Hasan Abayli[1], Mehmet Ilgin[2] and Mehmet Aksoy[2]

[1]Faculty of Veterinary Medicine, Department of Virology, Fırat University, Elazığ, Turkiye
[2]Elazığ Veterinary Control Institute, Elazığ, Turkiye
*Corresponding author: kcansahna@firat.edu.tr

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ABSTRACT

Neuroinfectious diseases in cattle, which result in significant economic losses and pose a serious threat to animal health, often go undiagnosed due to the challenge of identifying their underlying cause. In this study, a total of 180 brain samples from cattle with neurological symptoms were collected from various provinces in Türkiye during 2021-2022. Viral nucleic acids were extracted, followed by PCR and RT-PCR assays targeting specific gene regions of BoHV-1, BoHV-5, OvHV-2, and BoAstV.BoHV-1 was detected in 7.22% (13/180) of the samples, BoHV-5 in 5.00% (9/180), and OvHV-2 in 0.55% (1/180). BoAstV, however, was not detected in any of the samples. Analysis of the gB gene region sequences revealed a high nucleotide identity (100%) between BoHV-1 strains from America, Israel, Sweden, and Italy, and BoHV-5 strains (S9 and S24) from Brazil. The OvHV-2 strain obtained from this study clustered within the OvHV-2.1 cluster along with some Asian strains (Iraq, Pakistan, Mongolia, India, Turkey). Studies focused on neuroinfectious diseases in cattle, especially those related to BoAstV and BoHV-5, are relatively scarce. This study represents one of the first investigations into the majority of neuroinfectious viruses in Turkish cattle, with the identification of BoHV-5 being reported for the first time here. It is anticipated that the data obtained will significantly contribute to our understanding of the pathogenesis and molecular epidemiology of these studied viruses.

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INTRODUCTION

Neuroinfectious diseases, arising from viral, bacterial, fungal, and parasitic sources in farm animals, can lead to significant productivity losses and fatalities. It is imperative to prevent these losses and take preventive measures (Truchet *et al.*, 2017). Bovine Herpesvirus 1 (BoHV-1), Bovine Herpesvirus 5 (BoHV-5), Bovine Astrovirus (BoAstV), and Ovine Herpesvirus 2 (OvHV-2) are among the viruses that cause neuroinfectious diseases (*Favier et al*., 2014; Hierweger *et al*., 2021; Hirashima *et al*., 2018). Upper respiratory tract infections caused by BoHV-1, known as infectious bovine rhinotracheitis, genital tract infections referred to as infectious pustular balanoposthitis/ vulvovaginitis, immunosuppression, and conjunctivitis are commonly observed among cattle worldwide (Del Medico Zajac *et al*., 2010). A prominent manifestation in infections caused by BoHV-5 in cattle is meningoencephalitis, which can culminate in fatal outcomes (Favier *et al*., 2014).

BoHV-1 and BoHV-5 persist latently in the trigeminal ganglia of the natural host for an extended duration, periodically reactivating depending on environmental, physical, and hormonal factors (Oliveira *et al*., 2015; Ostler and Jones, 2023). According to the classification by the International Committee on Taxonomy of Viruses, BoHV-1, which shares high genetic similarity (>80% amino acid identity) with BoHV-5, has been classified within the Varicellovirus genus of the Alphaherpesvirinae subfamily within the Herpesviridae family. Both viruses have a long double-stranded DNA genome (135-140 kb) consisting of unique long (UL), unique short (US), internal repeat (IR), and terminal repeat (TR) sequences, as well as over 70 open reading frames (ORFs). Restriction endonuclease motifs differentiate BoHV-1 into BoHV1.1, BoHV 1.2a, and BoHV 1.2b subtypes (Khan *et al.*, 2024), and BoHV-5 into 5a, 5b, and 5c subtypes (Del Medico Zajac *et al*., 2010).

Malignant catarrhal fever (MCF) causes significant economic losses in several major ruminant species.

Alcelaphine herpesvirus 1 (AlHV-1) identified from antelope-associated MCF, which is economically important in Africa, and Ovine herpesvirus 2 (OvHV-2) identified from sheep-associated MCF, which is widespread in other countries, are undoubtedly the most common (Headley *et al*., 2020; Hussain *et al*., 2017). These viruses belong to the Macavirus genus within the Gammaherpesvirinae subfamily of the Herpesviridae family. MCF cases in Türkiye are attributed to OvHV-2. When OvHV-2, which typically shows asymptomatic behavior in sheep, is transmitted to cattle, it causes a disease characterized by high fever, mucopurulent nasal discharge, ocular manifestations, generalized lymphadenopathy, erosions in the upper respiratory tract and gastrointestinal system, sometimes accompanied by diarrhea and neurological signs (Sood *et al*., 2017). The tegument gene open reading frame (ORF) 33 and ORF 75 of OvHV-2 encode crucial proteins and are frequently targeted in viral DNA detection and phylogenetic studies (Headley *et al*., 2020).

Bovine astrovirus (BoAstV) is a virus belonging to the Mamastrovirus genus of the Astroviridae family, characterized by an unenveloped, positive-sense, single-stranded RNA genome (Wildi and Seuberlich 2021). To date, the International Committee on Taxonomy of Viruses (ICTV) has included 19 genotypes within the Mamatrovirus genus, with an additional 14 new genotypes proposed (Wildi and Seuberlich 2021). While astroviruses are generally known to cause diarrhea, since 2010, new strains have been isolated from the brain tissues or cerebrospinal fluid of humans and animals presenting neurological symptoms and encephalitis (Giannitti *et al*., 2019; Lee *et al*., 2021).

Molecular studies play a crucial role in determining the etiologies and pathogenesis of neuroinfectious diseases and identifying risk factors. Research on disease etiologies has predominantly been conducted in South American countries such as Brazil, Argentina, and Uruguay, with fewer studies in countries like Australia, Switzerland, and India (Kessel *et al*., 2011; Maidana *et al*., 2011; Clarke *et al*., 2019; Giannitti *et al*., 2019; Kumar *et al*., 2020; Pinheiro *et al*., 2024).

In Türkiye, no study has been conducted to investigate viral agents in neuroinfectious diseases in cattle. This study is the first to investigate the presence of BoHV-1, BoHV-5, OvHV-2, and Bovine Astrovirus in the brains of cattle presenting neurological symptoms.

MATERIALS AND METHODS

History of samples: In this study, 180 brain samples from cadavers who died showing neurological symptoms and were sent to the Elazığ Veterinary Control Institute for diagnosis were used. The brain samples used were selected from the years 2021-2022 and encompassed 12 provinces of Türkiye (Elazığ, Malatya, Muş, Bingöl, Tunceli, Diyarbakır, Mardin, Bitlis, Siirt, Van, Batman, Şırnak).

The sections of the dorsolateral and posterior cortex, cerebellum, and medulla oblongata were dissected into longitudinal sections of 1 to 2 cm thickness and placed in petri dishes. These samples were stored at -20°C until analyzed. The brain samples were suspended in PBS (Phosphate Buffer Saline) at a ratio of 10-20% and vortexed thoroughly, then centrifuged at 3000 rpm for 5 minutes at +4°C, and stored at -80°C.

Nucleic acids extraction, PCR and RT-PCR: Supernatants prepared from brain samples were used to extract the DNA and RNA of viral agents according to the instructions of the IndiSpin Pathogen Kit (QIAGEN GmbH, Hilden, Germany Cat. No: SP54106). The extracted material was stored at -20°C until analysis.

Following extraction, the viral RNA obtained was used to generate cDNA for Bovine Astrovirus using the Revert Aid™ First Strand cDNA Synthesis Kit (Lot: #K1622). The kit's usage instructions were strictly followed. The PCR primers used for the diagnosis of BoHV-1, BoHV-5, OvHV-2, and BoAstV are provided in Table-1. PCR conditions followed those in the corresponding reference without modification.

Sequencing and phylogeny: The PCR products subjected to sequencing by a commercial service provider (Macrogen Europe). The resulting nucleotide sequences were aligned, edited, verified using BLASTN, and subsequently deposited into the GenBank database (PP336320-33;PP341419). Phylogenetic trees were generated using Molecular Evolutionary Genetics Analysis software version X (MEGA X) and the Maximum Likelihood method with 1000 bootstrap replicates.

Statistical Analysis: The data were analyzed using the "SPSS for Windows 22.0" software package. Each parameter was expressed as a percentage. Chi-square test was applied for the comparison of parameters. Since the distribution was not in accordance with the chi-square distribution, some groups were analyzed together, taking this into consideration. A p-value of <0.05 was considered statistically significant.

RESULTS

PCR and RT-PCR: Out of the 180 brain samples, positivity for viruses other than BoAstV was detected in 18 samples (10%). The geographical distribution of samples is shown in Fig. 1. BHV-1 was detected in 13 samples (7.22%), BHV-5 in 9 samples (5.00%), and OvHV-2 in 1 sample (0.55%) (Table 2). Five samples were positive for both BHV-1 and BHV-5 (2.77%). There was no statistically significant difference found in terms of age, breed, and gender among the positive samples ($p > 0.05$) (Table 2).

Sequencing and phylogeny: Among the 13 BHV-1 strains obtained from the study, partial gB genes exhibited nucleotide identities ranging from 95.28% to 100% within their own group, while the identities with strains from the same and different geographical locations ranged from 54.23% to 100%. Some of the strains uploaded to GenBank (TR-11-BHV-1gB, TR-12-BHV-1gB, TR-13-BHV-1gB) shared 100% nucleotide identity with vaccine strains (Nasalgen_IP_MLV, TSV-2_Nasal_MLV, BoviShield_ Gold_FP5_MLV_vaccine, BoviShield_IBR_MLV, Vista_ IBR_MLV, Pyramid_IBR_MLV, Express_1_IBR, Titanium_IBR, Arsenal_IBR_MLV, PA1, MN1, ATCC: VR-188_strain_Los_Angeles, Cooper), American strains

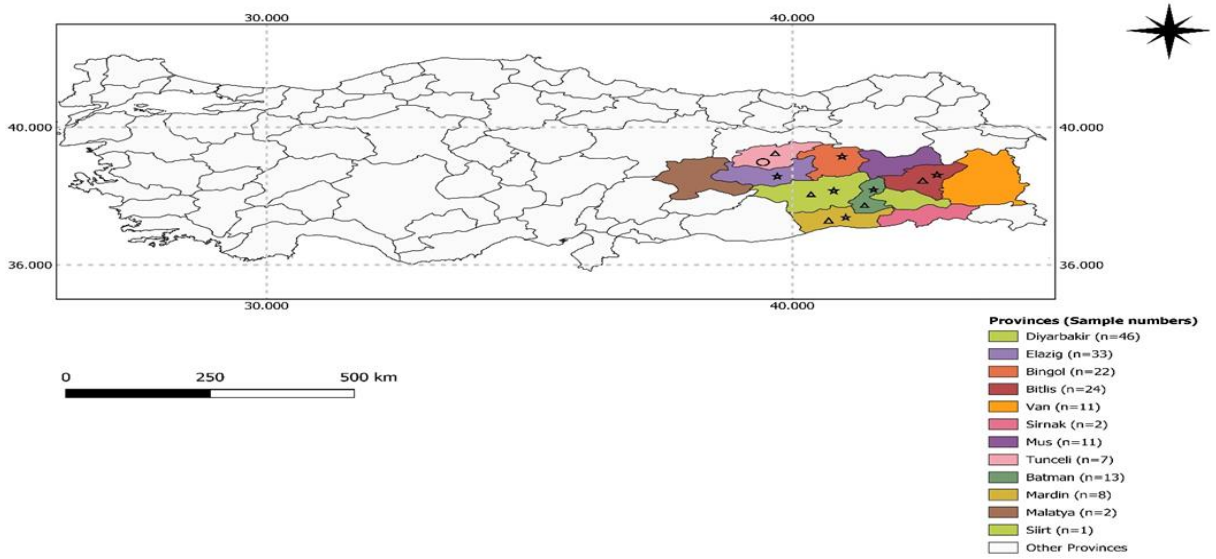

Fig. 1: Geographical distribution of clinical samples and BoHV-1, BoHV-5 and OvHV-2 positivity. On the map, BoHV-1, BoHV-5, and OvHV-2 are represented by a star, triangle, and circle, respectively.

Table 1: Primer sets using in PCR and RT-PCR

Target gene	Primer	Primer Sequence (5'→3')	Product (bp)	Reference
Bovine Herpesvirus-1	gB-F	AAGCGCAAAAACGTGTG		Santurde *et al.* 1996
gB	gB-R	TGCAGGTACAGCTTGGC	323	
Bovine Herpesvirus-5	gB-F	GTGGTGGCCTTTGAC CGCGAC	273	Vogel *et al.*
gB	gB-R	GCTCCGGCGAGTAGCTGGTGTG		2003
Ovine Herpesvirus-2	556-F	AGTCTGGGTATATGAATCCAGATGGCTCTC	422/238	Baxter *et al.* 1993
Tegument	755-R	AAGATAAGCACCAGTTATGCATCTGATAAA		
	555-R	TTCTGGGGTAGTGGCGAGCGAAGGCTTC		
Astrovirus	Nsp1ab –F	GAYTGGACHMGNTWYGAYGG	432	Turan and Isıdan.
Nsp1ab	Nsp1ab –R	KYTTRACCCACATNCCAA		2018

Table 2: Prevalence and risk factor analysis in bovine samples BoHV-1, BoHV-5 and/or OvHV-2

	Groups	BoHV-1(%)	BoHV-5	OvHV-2
Age (Month)	12-24	4/78(5,13)	2/78(2,56)	1/78(1,28)
	25-36	4/28(14,28)	3/28(10,71)	-
	37-48	2/23(8,7)	-	-
	49-60	-	3/17(17,64)	-
	Above 60	3/34(8,82)	1/34(2,94)	-
Race	Simmental	8/83(9,63)	6/83(7,22)	1/83(1,20)
	Anatolian Black	2/54(3,70)	1/54(1,85)	-
	Brown Swiss Mix Breed	2/34(5,88)	2/34(5,88)	-
	Holstein	1/9(11,11)	-	-
Gender	Male	2/20(10,0)	1/20(5,0)	-
	Female	11/160(6,87)	8/160(5,0)	1/160(0,62)

(MN1, NVSL_challenge_97-11, C36_876-459, C33), Israeli strain (BoHV-1-1643-12), Swiss strain (Ac number: AJ004801), and Italian strain (16453/07_TN). Although the current strains showed higher identity with BHV-1.1, the phylogenetic tree analysis did not allow for a distinction between subtypes BHV-1.1 and BHV-1.2 (Fig. 2).

The partial gB genes of the two BoHV-5 strains (named TR-0006-BHV-5 and TR-0013-BHV-5) obtained from this study exhibited 99.32% nucleotide identity. When compared, these two Turkish strains showed 95-100% identity to 16 BoHV-5 strains from different geographical locations. Among these, the Brazilian strains S9 and S24 were 100% identical to the Turkish BoHV-5 strains (Fig. 3). The phylogenetic tree divided the Argentine strains (166-84, A663, 674/10) and others into two distinct lineages. Unique substitutions were identified in the gB protein of the Argentine strains, specifically at positions 245 and 270 (from glutamic acid to aspartic acid) and at position 323 (from aspartic acid to glutamic acid). In contrast, the TR-0013-BHV-5 strain obtained from this study exhibited a unique substitution at position 333 (from serine to threonine) not observed in the other strains.

The partial tegument gene of the virus strain obtained in this study (TR-232-OvHV2) exhibited nucleotide identities ranging from 95.47% to 98.5% with 28 selected Ovine Herpesvirus-2 strains/isolates from different countries. Among them, those from Türkiye (OvHV2/EA-2/TUR, KarsSh1_SW), Pakistan (Pak-MCF6-OvHV-2), India (IndAp_Nel02), Mongolia (OvHV2_Mongolia_1), Egypt (Egy-BSU), and the United Kingdom (BJ1035) shared high nucleotide identity (98.5%). The strains from Norway (KX012003) and Iraq (MF004402) showed slightly lower genetic similarity (96% and 97.8%, respectively) compared to others. Following phylogenetic analysis, the OvHV-2 strains/isolates were grouped into two clusters (OvHV-2.1 and OvHV-2.2). The TR-232-

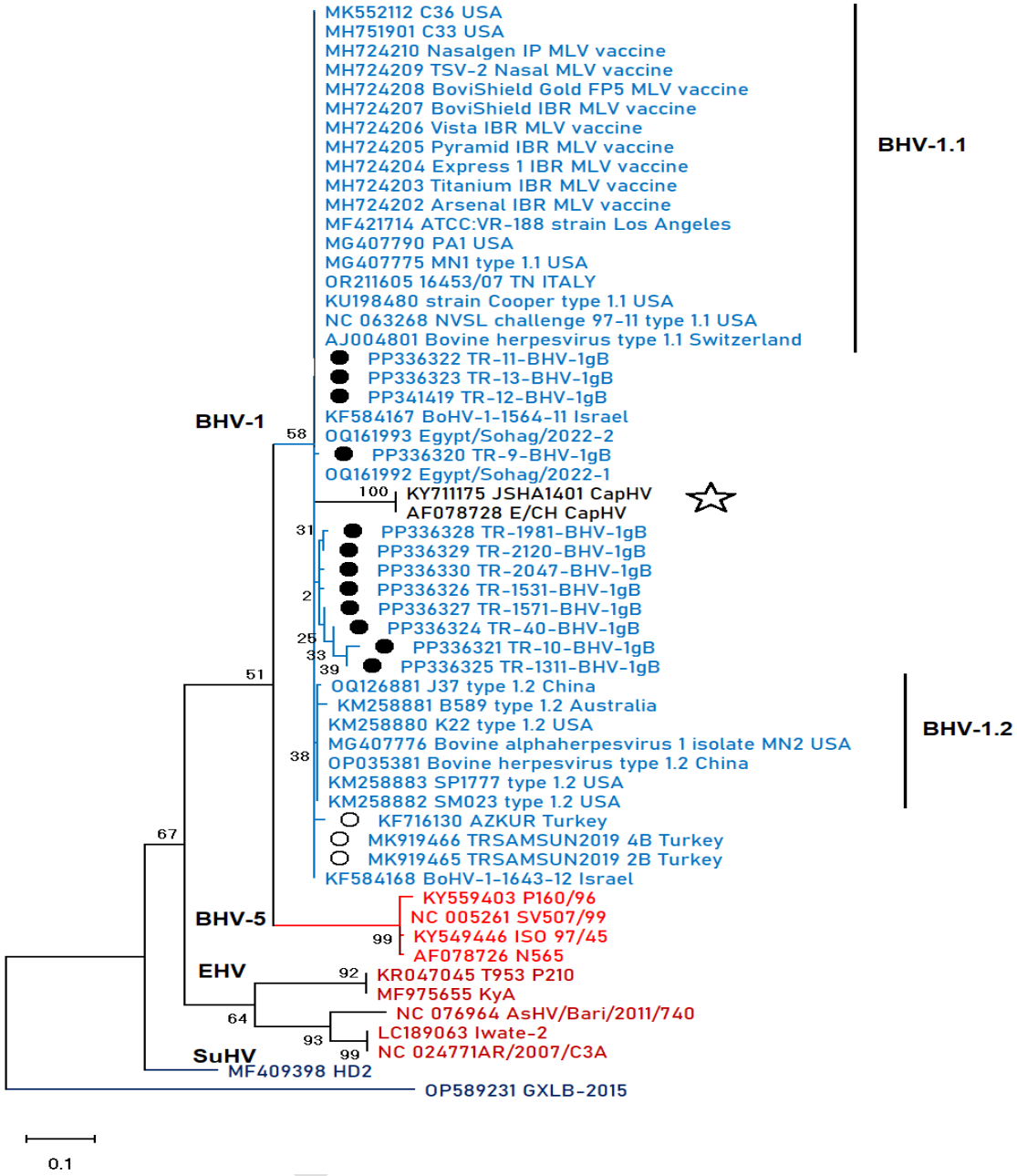

Fig. 2: Phylogenetic tree of partial gB of Bovine alphaherpesvirus-1. Phylogenetic tree was constructed using the using the Maximum Likelihood method and Tamura-Nei model. Bootstrap support values based on 1000 replicates are given at the nodes. Black circles represent the BHV-1 strains in the present study, and white circles represent strains or isolates from Turkey. The white star represents exceptional CapHVs within the BHV-1 phylogroup; CapHV: Caprine herpesvirus; SuHV-1: Suid herpesvirus; EqHV: Equid herpesvirus.

OvHV2 strain clustered within the OvHV-2.1 group (Fig. 4). Members of the OvHV-2.2 subgroup exhibited a distinctive amino acid substitution at position 1213 of their tegument proteins, where a lysine (K) was replaced by a threonine (T).

DISCUSSION

The significance of neurotropic pathogens is underscored by the statistic that roughly 25% of notifiable infectious diseases designated by the World Organization for Animal Health (WOAH) fall under this category (Reuter *et al.*, 2018).

The first study supporting the neurotropic potential of Bovine Herpesvirus-1 was conducted in 1962 in Australia. The findings obtained from this study have been further corroborated by similar studies conducted in Europe, Australia, North and South America (Maidana *et al.*, 2011; Rodenbusch *et al.*, 2020). Subsequent studies have revealed antigenic and molecular differences among herpesviruses obtained from respiratory, genital, and neurological diseases following certain experiments such as genomic restriction enzyme analysis (REA), viral polypeptide profiling, specific monoclonal antibody (MAbs) reactivity, and neutralization tests. Based on this analysis, respiratory and genital isolates were classified as BoHV-1.1 and BoHV-1.2 respectively, while neurovirulent isolates were classified as BoHV-1.3. In 1992, the ICTV recognized neurovirulent BoHV 1.3 strains as a separate virus species, subsequently designated as BoHV-5 (Fulton *et al.*, 2016; Khan *et al.*, 2024). Even after this distinction, Bovine Herpesvirus-1 (BHV-1) has been found in the

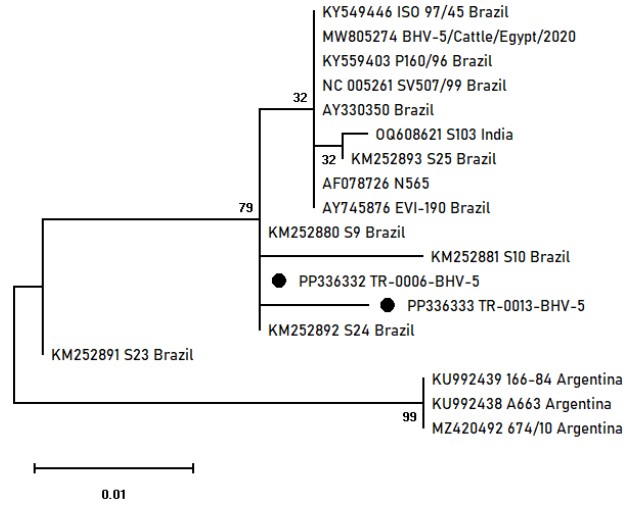

Fig. 3: Phylogenetic tree of partial gB gene of Bovine alphaherpesvirus-5. Phylogenetic tree was constructed using the using the Maximum Likelihood method and Tamura-Nei model. Bootstrap support values based on 1000 replicates are given at the nodes. Black circles represent the BHV-5 strains in the present study.

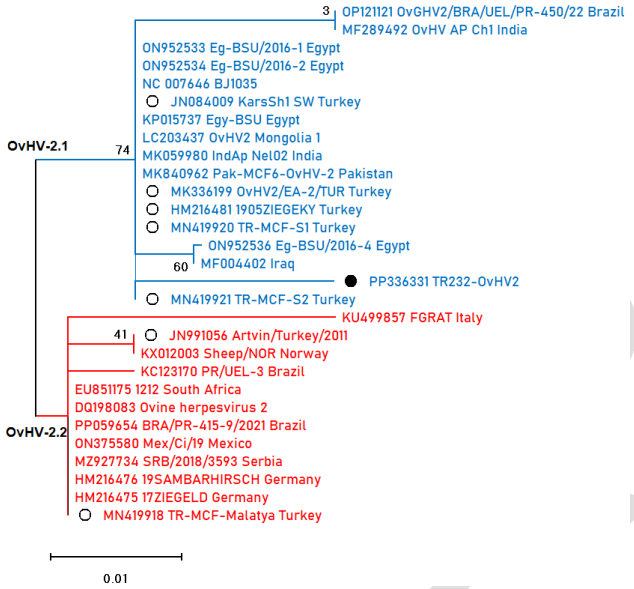

Fig. 4: Phylogenetic tree of partial tegument gene of Ovine herpesvirus-2. Phylogenetic tree was constructed using the using the Maximum Likelihood method and Tamura-Nei model. Bootstrap support values based on 1000 replicates are given at the nodes. Black circles represent the OvHV-2 strains in the present study, and White circles represent strains or isolates from Turkey.

brains of cattle infected with IBR (Infectious Bovine Rhinotracheitis) or IPV (Infectious Pustular Vulvovaginitis) occasionally showing neurological symptoms or not. In these cases, the involvement of the lungs or other organs has commonly been observed (Valera *et al*., 2008). In IBR-infected cattle, BHV-1 likely replicates primarily in the respiratory mucosa before potentially invading the brain secondarily, a possibility supported by experimental studies conducted on BHV-1 inoculated into the nasal cavity of cattle (Ostler and Jones 2023; Valera *et al*., 2008). The results of further clinical and histopathological studies on this subject support the hypothesis that Bovine Herpesvirus-1 (BHV-1), akin to BHV-5, could potentially affect the central nervous system (CNS) and lead to neurological disorders. The invasion of BHV-1 into the brain may be influenced by the virus's neurotropism or host factors, or mixed infections may create predispositions. Although infections with BoHV-1 are commonly observed in Türkiye, studies concerning its molecular characterization are limited. To our knowledge, no molecular investigation has been conducted regarding cases of bovine encephalitis. In this study, we investigated the positivity of BoHV-1 in the brain tissues of cattle with neurological disorders using virus-specific PCR and found a rate of 7.22%. Previous studies have reported BHV-1.1 and BHV-1.2 in cattle presenting with different clinical manifestations such as respiratory disease, mastitis, and abortion (Bilge-Dağalp *et al*., 2020; Toker and Yeşilbağ 2021). In this study, although strains obtained from partial gB gene phylogenetic analysis were genetically closer to BHV-1.1, a clear distinction could not be made, and further restriction enzyme analysis (REA) experiments were required. Some of the 13 BHV-1 strains obtained from the study (TR-11-BHV-1gB, TR-12-BHV-1gB, TR-13-BHV-1gB) showed 100% nucleotide identity with field isolates/strains from American, Israeli, Swiss, and Italian origins. Interestingly, these same viruses also shared high nucleotide identity rates (100%) with vaccine strains. Studies have previously reported that BHV-1 obtained from cattle is highly identical to live attenuated BHV-1 vaccines, in investigations conducted on animals carrying infections of the nervous system, respiratory system, or genital system (Fulton *et al*., 2016). Furuoka *et al*. (1995) described an outbreak of meningoencephalitis associated with the use of a live attenuated BoHV-1 vaccine in which the clinical and pathological findings were typical of neurological disease induced by BoHV-5. The virus isolated from the brain of dead animals showed a REA pattern identical to the parental BoHV-1 vaccine strain. In Türkiye, the control of BHV-1 relies on vaccination. Conventional inactivated vaccines or inactivated gE (-) marker vaccines are commonly preferred for vaccination. Therefore, the likelihood of infection originating from a live attenuated vaccine strain appears to be quite low, except for the possibility of entry into the country through animals permitted for import. The occurrence of vaccine-like BHV-1 strains in local cattle breeds across different herds further diminishes this possibility. It is necessary to conduct more detailed studies, including complete genome analyses and detection of genetic recombination, to clarify whether the circulating virus in the field may be a genetically modified form of the vaccine strain or an entirely new virus strain independent of the vaccine strain. In Brazil, BHV-1 was detected in 3.3-14.2% of cattle with encephalitis (Oliveira *et al*., 2015; Rodenbusch *et al*., 2020).

While BHV-1 exhibits complexities in its pathogenesis, BHV-5 has been extensively studied and shows a more straightforward course of infection. BoHV-5 infects olfactory receptor neurons and maxillary nerves, using both trigeminal and olfactory pathways to reach the CNS (Del Medico Zajac *et al*., 2010). Following acute infection, the virus can establish latency in the trigeminal ganglia. During periods of immunosuppression, affected animals may develop severe neurological diseases (Cuesta *et al*., 2022). BoHV-5 also establishes latency in bovine tonsils and PBL and possible that infection of these cells might provide an additional way to gain access to the

nervous system (Favier *et al*., 2014). Lesions in the CNS caused by BoHV-5 are characterized by necrotizing meningoencephalitis, areas of astrogliosis, laminar neuronal necrosis, and occasional eosinophilic intranuclear viral inclusions observed in astrocytes and neurons (Rissi and Barros, 2013). While cases of meningoencephalitis caused by BoHV-5 are most prevalent in South America (Argentina, Brazil), reports have also been documented from Australia, North America, and Europe (Hungary, Italy) (Pedraza *et al*., 2010; Cuesta *et al*., 2022; Rodenbusch *et al*., 2020).

Despite the prevalence of BoHV-5 cases in various regions globally, studies specific to Türkiye have been limited, prompting the need for comprehensive investigations. In Türkiye, two different studies targeting the gD and gC gene regions of BoHV-5 did not yield positive results (Aslan *et al*., 2015; Bilge-Dağalp *et al*., 2020). In this study, the positivity rate of BoHV-5 targeting the gB gene region in the brain tissues of cattle displaying neurological symptoms was determined to be 5.0%, and the sequence data of the positive PCR products were submitted to GenBank for the first time.

There have been numerous studies utilizing PCR to detect the presence of OvHV-2 in cattle brains displaying neurological symptoms. Martins *et al.,* (2017) identified OvHV-2 in 4.8% (14/290) of cattle brain samples with neurological symptoms in Brazil. Hierweger *et al*. (2021) determined positivity in 14.4% (18 out of 125) of cattle with neurological symptoms in Switzerland using qPCR. Similarly, Truchet *et al*. (2017) found positivity in 0.38% (7 out of 1816) of cattle brain samples in Switzerland. Although studies investigating the presence of OvHV-2 in Türkiye using serological and virological methods (Uzlu *et al*., 2023; Yazıcı *et al*., 2006) exist, research on the characterization of the virus remains limited (Turan *et al*., 2020). Oğuzoğlu *et al*. (2020) conducted molecular characterization by sequencing the glycoprotein and tegument genes of positive cases detected by PCR. In this study, OvHV-2 was detected in 0.55% (1 out of 180) of brain samples. The partial tegument gene sequence of the obtained strain was sequenced, and phylogenetic analysis with selected strains from GenBank grouped OvHV-2 strains/isolates from Türkiye and worldwide into two clusters. The virus obtained in this study was placed within Cluster 1 (designated as OvHV-2.1), along with Asian strains, while European, South American, and African strains clustered in Cluster 2 (also designated as OvHV-2.1). The tegument protein was conserved among strains, except for a site-specific substitution (K to T) at position 1213, which contributed to the emergence of two clusters (Glišić *et al*., 2023).

On the other hand, astroviruses have been recognized as pathogens causing encephalitis in various animals, including humans, in addition to being detected in fecal samples in recent years. Studies conducted on cattle with neurological symptoms have reported the presence of BoAstV in many countries. In Türkiye, the only study related to BoAstV is by Turhan and Isıdan (2018), who reported the first diagnosis and phylogenetic analysis targeting the partial Nsp1ab gene (ORF1a) region in diarrheic calves. However, in this study, BoAstV positivity was not detected using PCR targeting the same gene region.

Conclusions: Based on the findings presented in this study, it is evident that BoHV-1 and BoHV-5, and OvHV-2 are associated with neurological symptoms in cattle in Turkey. The detection rates of these viruses indicate their presence and potential role in causing neuroinfectious diseases. This study underscores the importance of molecular investigations for understanding the epidemiology, genetic diversity, and pathogenesis of these viruses in cattle populations. Further research and surveillance efforts are essential for developing effective control measures against these neurotropic pathogens in Turkey.

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Author contributions: KCS, MI, and MA were performed the study design. Sampling from cadavers, sample processing and molecular tests were performed MA and MI. Sequencing analysis was performed by HA and MI. The first draft of the manuscript were written by KCS, HA, and MI. All authors read and approved the final manuscript.

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