



## RESEARCH ARTICLE

### Molecular Characterization of Important Viruses Contributing to Bovine Respiratory Disease Complex in Türkiye

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#### ABSTRACT

Bovine respiratory disease complex (BRDC) is one of the most common cattle diseases, causing widespread effect in worldwide. That pathogens causing BRDC occur with coinfections may even lead irreversible losses. That is why, in this study, it is aimed to investigate crucial viral pathogens associated with BRDC [Bovine herpesvirus-1 (BoHV-1); bovine respiratory syncytial virus (BRSV), bovine viral diarrhoea virus (BVDV), Hobi-like pestivirus, bovine parainfluenza-3 virus (BPIV-3)] in the same sample simultaneously. Aligned with the aim, 129 nasal swabs were collected from cattle with clinical symptoms of respiratory system disease in specific provinces of eastern and southeastern Türkiye. Firstly, viral RNA and DNA were isolated from the samples. Subsequently, PCR and RT-PCR analyses were conducted using specific primers for the viral pathogens. The results illustrated 10.85 BRSV, 1.55 BVDV-1 and 3.8% BPIV-3 positivity rates. BoHV-1, Hobi-like pestivirus, and mixed infection were not detected in any of the samples. Samples that tested positive were sequenced to represent each province for molecular characterisation and phylogenetic analysis. They were determined that the BRSV strains (TR/Bngol-04/BRSV, TR/Dyrbkr-17/BRSV, and TR/Elz-23/BRSV) obtained from this study are a part of subgroup III, BVDV strains (BVDV-1/TR-Malatya-1 and BVDV-1/TR/Elazig-29) belonged to subgroup BVDV 1I, and the BPIV-3 strains (BPIV3/M17/TUR and BPIV3/M19/TUR) were within genotype C. The findings of these studies can be valuable to minimise the losses attributed to BRDC by facilitating the development of diagnostic, molecular, and epidemiological approaches. These insights can also serve as a foundation for establishing targets for vaccine development, ultimately contributing to the reduction of BRDC.

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#### INTRODUCTION

Bovine Respiratory Disease Complex (BRDC), responsible for bronchopneumonia, has a multifactorial etiology consisting of infectious pathogens, host-related factors, environmental stressors, and their intricate interactions. Viral pathogens that cause BRDC include bovine herpesvirus-1 (BoHV-1), bovine coronavirus (BCV), bovine respiratory syncytial virus (BRSV), bovine viral diarrhoea virus (BVDV), parainfluenza-3 virus (BPIV-3), bovine adenoviruses (BAV), bovine enterovirus-1, 2 and 3 (Earley *et al.*, 2017).

The Pestivirus genus, located within the Flaviviridae family, comprises 11 species. Six of these, namely bovine viral diarrhoea virus 1 (BVDV-1 or Pestivirus A), bovine

viral diarrhoea virus 2 (BVDV-2 or Pestivirus B), border disease virus (Pestivirus D), pronghorn virus (Pestivirus E), HoBi-like pestivirus (Pestivirus H) and Aydin-like pestivirus (Pestivirus I), are identified in members of the Bovidae family (Smith *et al.*, 2017). The pestivirus genome is a single-stranded, positive-sense RNA molecule containing an open reading frame (ORF) with untranslated regions (UTRs) at the 5' and 3' ends. The 5'UTR region of pestiviruses is highly conserved and is usually used for classification of strains. N-terminal autoprotease (Npro) and envelope glycoprotein E2 are utilised in phylogenetic analyses (Hoppe *et al.*, 2019).

Bovine herpesvirus type 1 is a member of the Varicellovirus genus belonging to the Herpesviridae family, Alphaherpesvirinae subfamily. The genome of

BoHV-1 comprises a double-stranded DNA with 73 Open Reading Frame (ORF) regions. The BoHV-1 genome encodes 10 glycoproteins: gK (UL53), gC (UL44), gB (UL27), gH (UL22), gM (UL10) and gL (UL1) are located in the long region of the genome, U<sub>L</sub>; gG (US4), gD (UL6), gI (US7) and gE (US8) are located in the short region (U<sub>S</sub>) of the genome (Muylkens *et al.*, 2007; Sobhy *et al.*, 2014). Infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV), and infectious pustular balanoposthitis (IPB) are the three main clinical syndromes primarily associated with BoHV-1 (Nandi *et al.*, 2009). Erosion of mucosal surfaces and compromised immune response due to virus infection can cause a secondary infection, leading to BRDC, a vital disease complex in cattle (Fu *et al.*, 2019). Like many RNA viruses, BRSV has high genetic diversity and rapid evolution, forming distinct variations within a single host. The viral G protein (glycoprotein) has been identified as the main binding protein. Genetic and antigenic variety in fusion, glycoprotein, and nucleoprotein genes led to the inclusion of BRSV strains in eight subgroups designated I–VIII (Bertolotti *et al.*, 2017; Leme *et al.*, 2020).

Bovine parainfluenza virus-3 (Respirovirus, Paramyxoviridae) was initially isolated from the nasal discharge of cattle experiencing respiratory disease. BPIV-3 is a crucial virus linked to BRDC globally and a 150–200nm diameter pleomorphic virion containing a (-) ssRNA genome and an envelope. The genome includes six large ORF regions consisting of six gene regions coding for nine different proteins [nucleoprotein (N), phosphoprotein (P), matrix protein (M), homotrimeric fusion glycoprotein (F), homotetrameric hemagglutinin-neuraminidase (HN), large protein (L), nonstructural V, C and D proteins] (Macias-Rioseco *et al.*, 2018; Alatorre-Garcia *et al.*, 2021).

Epidemiological research on BRDC pathogens in cattle in Türkiye with in the last five years indicates that 59.78% (İnce *et al.*, 2022), 71.2% (Kadiroğlu *et al.*, 2020) for BRSV; 40.2% (Kadiroğlu *et al.*, 2020), 56.2% (Muftuoğlu *et al.*, 2021) for BPIV-3; 11.11% (Aktaş and Çelik, 2021), 50.23% (İnce and Ayaz, 2023) for BVDV and 14.28% (Aktaş and Çelik, 2021), 39.66% (İnce and Şevik, 2022) for BoHV-1 seropositivity rates were reported.

Understanding the genotypic characteristics of viral pathogens contributing to the development of BRDC in cattle exhibiting respiratory system infections is crucial. This knowledge, along with the continual identification of new variants and strains resulting from mutations, is essential in the challenge against the disease. Moreover, it is central in developing vaccines and control strategies for effective protection and control measures. For these purposes, the current study were investigated BVDV-1, BVDV-2, BVDV-3, BRSV, BPIV-3, and BoHV-1, vital

pathogens of viral-induced severe respiratory system infections in cattle in Türkiye using molecular techniques, and were presented their molecular characterization and phylogeny.

## MATERIALS AND METHODS

**Ethical approval:** This study was performed under the rules of the Local Animal Ethics Committee of Fırat University (Approval Number: 877).

**Clinical sampling:** The study selected cattle exhibiting pneumonia, preferably older than two months and representing various breeds (Montafon: 3; Simmental: 56; Holstein: 11; mixed breed: 59) and genders (male: 68 female: 61). In the general examination, nearly all the animals displayed signs of breathing problems, coughing, weakness, high fever, and nasal discharge. For this study, sampling was conducted in five provinces-Malatya (n: 40), Elazığ (n: 40), Diyarbakır (n: 31), Bingöl (n: 13), and Sivas (n: 5)- in the east and southeast of Türkiye from 2020 to 2023. Nasal swabs were taken from the animals after their general examination; following this process, nasal swabs were immersed in 2mL sterile PBS and stored at -20°C. The samples were carefully transported to Fırat University Faculty of Veterinary Medicine, Department of Virology, maintaining cold conditions throughout the process.

**Nucleic acid extraction:** Nucleic acids (DNA and RNA) of the viral pathogens investigated in the study (BoHV-1, BPIV-3, BRSV, BVDV-1, BVDV-2, BVDV-3) were extracted from the QIAamp MinElute Virus Kit (Qiagen, USA). During extraction, the guidelines for the use of the kit were followed entirely. The received viral nucleic acid was measured with a Nanodrop2000 spectrophotometer (Thermo Fisher, USA) and stored in the freezer until utilised, used as templates for RT-PCR and PCR procedures.

**PCR and RT-PCR:** The primer sets and their source references used in the current study are listed in Table 1. The PCR and RT-PCR procedures in the references were followed identically. RT-PCR was applied to amplify specific gene regions of pestiviruses, BRSV and BPIV-3. Firstly, cDNA was synthesized from the extracted RNA samples using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems®, Foster City, CA, USA). PCR amplification targeting specific gene regions of these viruses was then performed. For the amplification of the BoHV-1 specific gene region, the protocol as indicated in the reference was pursued. Amplified PCR products were visualized under UV on a 1% low-melting agarose gel containing ethidium bromide.

**Table 1:** PCR primer sets and references used in the study

Virus	Target Gene	Primer	Primer sequence (5'-3')	Reference
Pestiviruses (BVDV-1, BVDV-2, Hobi-like)	5'UTR	324	ATGCCCWTAGTAGGACTAGCA	Vilcek <i>et al.</i> , 1994 (a)
		326	TCAACTCCATGTGCCATGTAC	
BRSV	Gene F	B1	AATCAACATGCAGTGCAGTTAG	Vilcek <i>et al.</i> , 1994 (b)
		B2A	TTTGGTCATTCTGTTATAGGCCAT	
BPIV-3	Gene M	M1	AGTGATCTAGATGATGATCCA	Maidana <i>et al.</i> , 2012
		M2	GTTATTGATCCAATTGCTGT	
BoHV-1	Glycoprotein B	gB1	AAGCGCAAAAACGTGTG	Santurde <i>et al.</i> , 1996
		gB2	TGCAGGTACAGCTTGGC	

**Sequencing and phylogenetic analysis:** Some of the PCR-positive amplicons, especially from different provinces, were confirmed by sequencing. The PCR products of the expected size were sequenced dually at the MacroGen laboratory (Netherlands). For this process, the Sanger sequencing method was preferred and ABI 3730XL Sanger sequencing device (Applied Biosystems, CA, USA) and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) were utilised. After the nucleotide sequences were confirmed with the Basic Local Alignment Search Tool-nucleotide (BLASTn) (nih.gov), the data were transferred to the Molecular Evolutionary Genetics Analysis version X program (MEGA X) (Kumar *et al.*, 2018). The nucleotide sequences obtained from the present study were aligned and compared to other strains/isolates from similar or different geographic locations selected from GenBank. This comparison was conducted using the CLUSTAL-W algorithm (Thompson *et al.*, 1994). The aligned sequences of BRSV and BPIV-3 were performed to phylogenetic analysis using the Maximum Likelihood method and the Tamura Nei model (Tamura and Nei, 1993). This analysis involved 1000 replicates and was conducted using the MEGA X program. Phylogenetic analysis of the aligned pestivirus sequence data was performed in 1000 replicates using the MEGA-X program, the Neighbor-joining method and the Tamura Nei model by MEGA-X program. The confirmed sequence data were submitted to the GenBank database (OR026643-9).

## RESULTS

According to the molecular test results, the viruses investigated in the study were detected in 21 of 129 clinical samples (16.2%). Among the positive samples, 5 (3.87%) were detected as BPIV-3, 14 (10.85%) as BRSV and 2 (1.55%) as BVDV-1. Hobi-like pestivirus, BoHV-1 and mixed infection were not detected in any of the samples.

The identity rate between the BPIV3/M17/TUR and BPIV3/M19/TUR strains from this study was 98.72%. The nucleotide identity of the two new Türkiye strains of BPIV 3 and the 60 strains/isolates selected in this study was between 81.65-99.39% (Fig. 1). The BPIV3/M17/TUR strain exhibited a high nucleotide identity (>99.3%) with Turkey\_S1 and BUR-BPIV-3. Likewise, the BPIV3/M19/TUR strain displayed high nucleotide identity (>99%) with Turkey\_S1, ICSA-4/France/2013, ICSA-6/France/2013, and ICSA-11/France/2013. The phylogenetic tree revealed the genotypes a, b, and c; the strains/isolates of Türkiye were grouped within "genotype-C".

The strains TR/Bngol-04/BRSV, TR/Dyrbkr-17/BRSV and TR/Elz-23/BRSV obtained from the present study showed a high identity of 99.34-100%. Three new BRSV strains of Türkiye were compared with 22 other strains/isolates selected in the study, and the identity rate was revealed to be 72.91-99.56%. Three BRSV strains were highly identical (>99.3%) with isolates 07TR2019, 43TR2018, and 34TR2018 from Türkiye. As a result of the phylogenetic tree, five distinct subgroups were revealed, and the new and other BRSV strains of Türkiye

obtained from this study were categorized within subgroup III (Fig. 2).

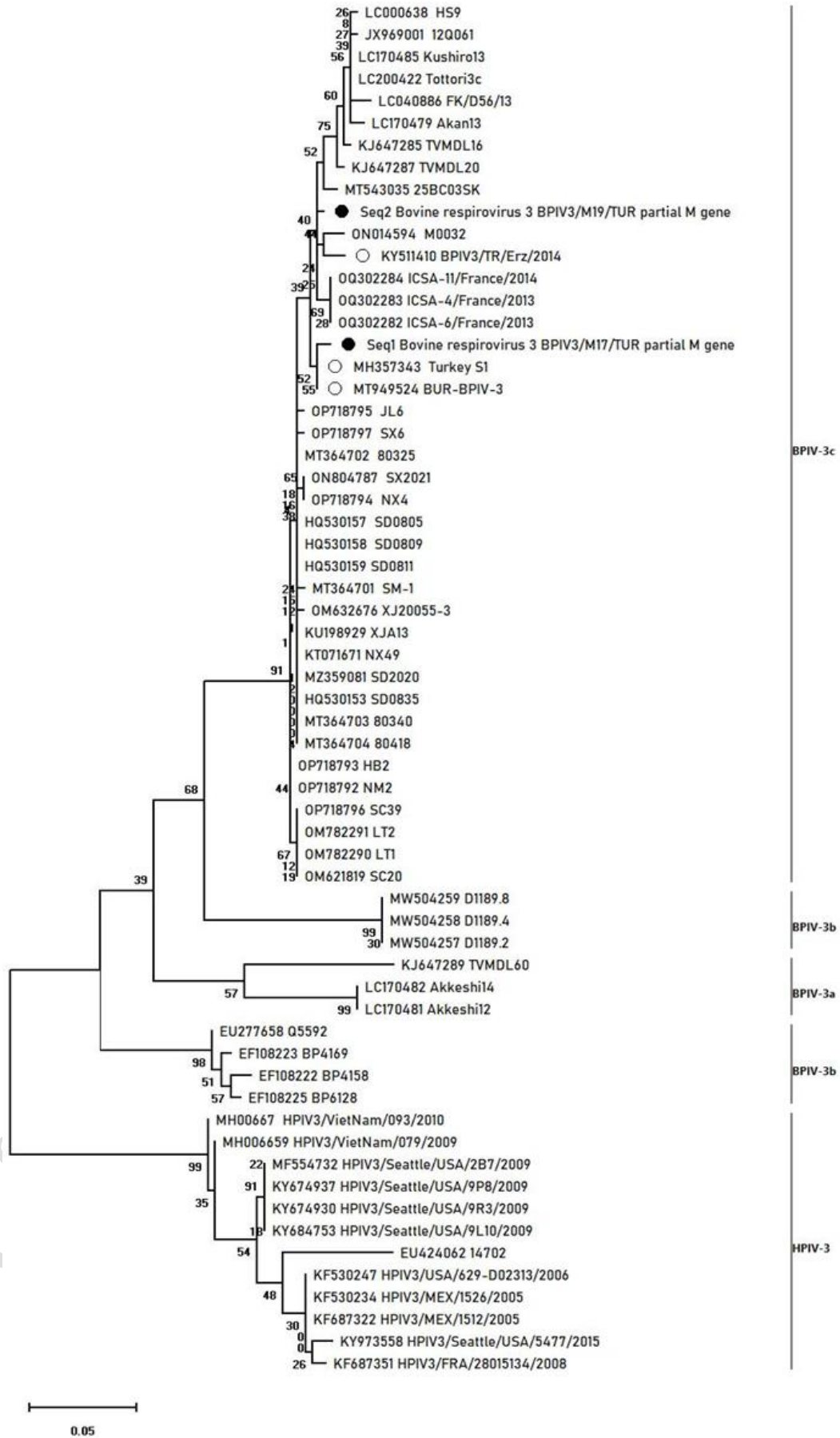
The nucleotide identity ratio between the BVDV-1/TR/Malatya-1 and BVDV-1/TR/Elazig-29 strains received in this study was 98.95%, and they were found genetically close to BVDV-1 strains in the nBLAST screening. It exhibited an identity of 70-98.2% with the selected BVDV-1 strains in the study while displaying an identity of 64.82-75.52% with BVDV-2 and BVDV-3 strains. The phylogenetic analysis result confirmed this result, both strains were identified as BVDV-11 variants (Fig. 3).

## DISCUSSION

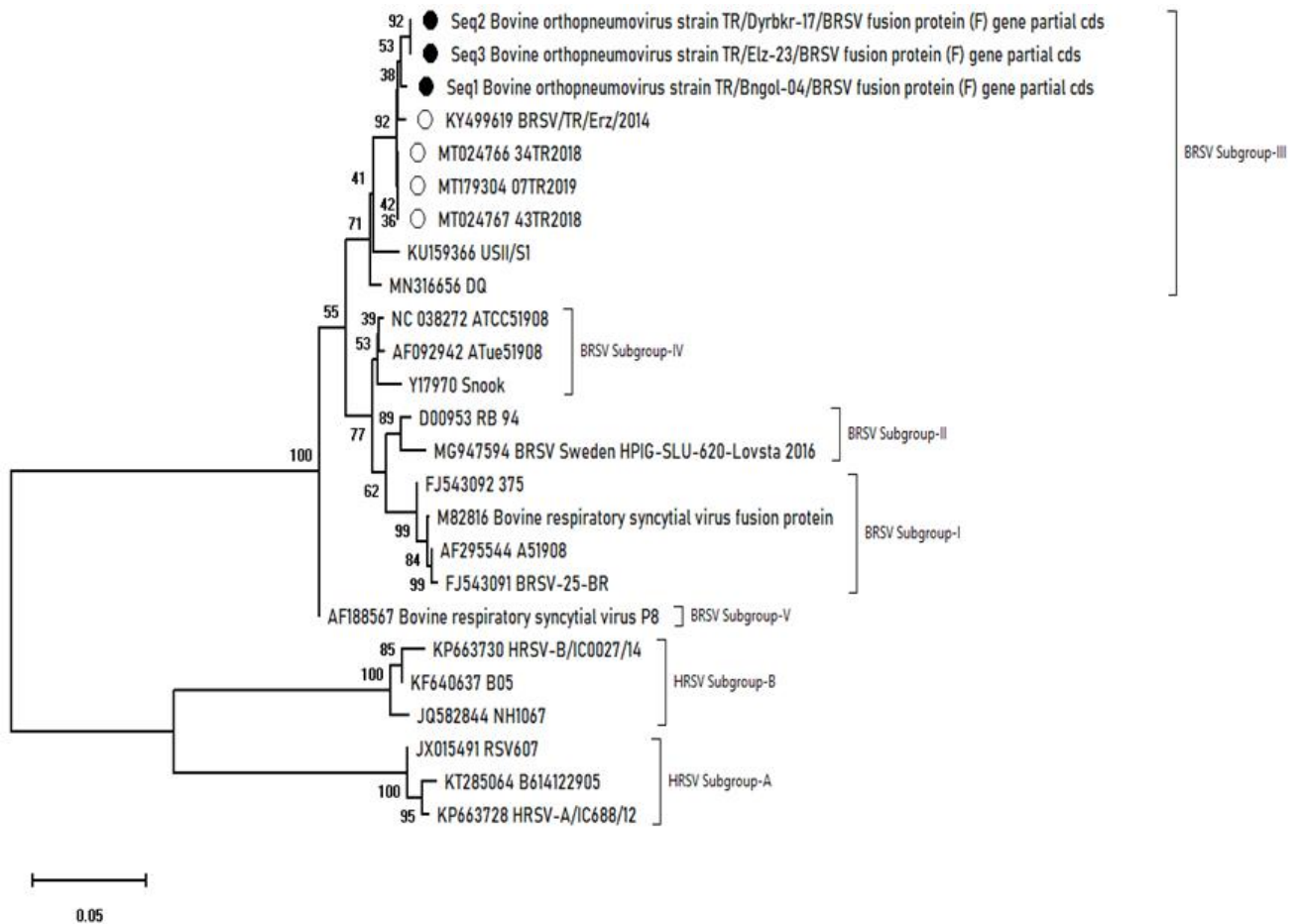
The pathogens causing BRDC often synergize with various viral and bacterial pathogens during an outbreak, increasing the severity of the disease (Calderon Bernal *et al.*, 2023). Based on this, the current study, we aimed to simultaneously investigate the key viral pathogens contributing to BRDC in animals exhibiting respiratory system infection symptoms using molecular techniques. There are extremely limited simultaneous studies of BRDC pathogens in Türkiye available (Timurkan *et al.*, 2019; Toker and Yeşilbağ, 2021; İnce *et al.*, 2022).

According to molecular test findings, crucial viral pathogens associated with BRDC investigated were found in 21 of 129 clinical samples (16.2%). The positive samples consisted of 3.87, 10.85, and 1.55% for BPIV-3, BRSV, and BVDV-1, respectively. The absence of Hobi-like pestivirus, BoHV-1, or mixed infections were observed across all samples analyzed. As the samples of the study context were not tested for the presence of other BRDC-related viral, bacterial, and mycotic pathogens mentioned above, it is also possible that the negative samples were infected with one or more of these pathogens.

Bovine parainfluenza virus-3, an important pathogen associated with the bovine respiratory disease complex, was initially reported in the United States in 1959; subsequently, antibodies have been reported in most countries worldwide (Wang *et al.*, 2022). As a result of phylogenetic analysis conducted with the genes of BPIV-3, three distinct genotypes, BPIV-3a, BPIV-3b, and BPIV-3c, were identified. Based on the current data, Genotype A has been detected in the United States, China, Argentina, and Japan, Genotype B in Australia and Argentina, and Genotype C in China, South Korea, the United States, and Japan (Ren *et al.*, 2022). In different studies conducted in Türkiye (Albayrak *et al.*, 2019; Timurkan *et al.*, 2019; Toker and Yeşilbağ, 2021), the BPIV-3c genotype was identified. Although sampling was conducted in different regions in our study, it was observed that the clustering strains/isolates of Türkiye within genotype C. The identity rate between BPIV3/M17/TUR and BPIV3/M19/TUR strains obtained from the study was determined as 98.72%. The nucleotide identity between the two new BPIV-3 strains of Türkiye and the 60 selected strains/isolates from the GenBank ranged from 81.65% to 99.39% was detected. The BPIV3/M17/TUR strain demonstrated a high nucleotide identity (>99.3%) with both the Turkey\_S1 and BUR-BPIV-3 strains. The BPIV3/M19/TUR strain exhibited a



**Fig. 1:** Based on the M gene, the phylogenetic tree includes strains/isolates from GenBank (60) and obtained from this study (Seq1: OR026648; Seq2: OR026649). The phylogenetic analysis involved 1000 replicates using the Maximum Likelihood method and the Tamura Nei model through the MEGA X program. Filled circles represent BPIV-3 strains/isolates of Türkiye obtained from this study, and empty circles represent other BPIV-3 strains/isolates of Türkiye.



**Fig. 2:** Based on the F gene, the phylogenetic tree includes strains/isolates from GenBank (22) and obtained from this study (Seq1: OR026643; Seq2: OR026644; Seq3: OR026645). The phylogenetic analysis involved 1000 replicates using the Maximum Likelihood method and the Tamura Nei model through the MEGA X program. Filled circles represent BRSV strains/isolates of Türkiye obtained from this study, and empty circles represent other BRSV strains/isolates of Türkiye.

notable identity (>99%) with Turkey\_S1 from Türkiye and IC5A-4/France/2013, IC5A-6/France/2013, IC5A-11/France/2013 strains from France (Fig. 1). The analyzed sequences were recorded in GenBank (accession numbers: OR026648, OR026649).

Bovine respiratory syncytial virus has a significant viral-induced economic impact of BRDC on the cattle industry globally due to its role in morbidity, mortality, treatment and protection costs, loss of production and reduced carcass value (Bertolotti *et al.*, 2017). In our study, 14 of the 21 positive samples obtained according to molecular test results were BRSV (57.14%). According to findings of the present study, BRSV infection plays a considerable role among the various viral pathogens contributing to BRDC in animals exhibiting clinical symptoms of respiratory system infection in Türkiye. Likewise, previous studies in different countries have reported that BRSV is responsible for approximately 60% of BRD outbreaks observed in dairy cattle and up to 70% in beef cattle herds. Phylogenetic analyzes of BRSV have been reported in Europe, USA, Brazil, Japan (Giammarioli *et al.*, 2020). Previous research examining the genetic characterization of BRSV in Türkiye (Hacıoğlu *et al.*, 2019; Timurkan *et al.*, 2019; Yazıcı *et al.*, 2020; İnce *et al.*, 2022) determined that the genetic subgroup was subgroup III. In our study, analyzing the partial sequence and phylogenetic analysis of the F gene

of BRSV, it was observed that the BRSV subgroups currently present in Türkiye clustered together within subgroup-III, as reported previously in studies. The identity ratio among the strains phylogenetic analyzed, TR/Bngol-04/BRSV, TR/Dyrbkr-17/BRSV, and TR/Elz-23/BRSV, was found between 99.34% and 100% (GenBank accession numbers: OR026643, OR026644, OR026645). Although these three new BRSV strains displayed a high level of identity (>99.3%) with the 07TR2019, 43TR2018, and 34TR2018 isolates in Türkiye, the identity ratio with 22 strains/isolates obtained from other studies worldwide was determined 72.91-99.56% (Fig. 2). Subgroup-III was reported to constitute BRSV strains from America, China, and Brazil in previous studies (Chang *et al.*, 2022). Previous studies reported that Subgroup-III comprised BRSV strains in America, China, and Brazil (Chang *et al.*, 2022).

Pestiviruses and herpesviruses are extremely significant among the pathogens in BRDC. These viruses are highly prevalent worldwide because they are associated with immunosuppression and lead to persistent infection (PI). Additionally, facilitates other pathogens to cause disease (Smith *et al.*, 2017). BVDV which has been an increasing number of subgenotypes is a disease widely distributed worldwide, reported in cattle populations of several countries, including China, Korea, Japan, Thailand, Poland, Brazil, Australia, and the United States



**Fig. 3:** Based on the 5'UTR gene, the phylogenetic tree includes strains/isolates from GenBank (73) and obtained from this study (Seq1: OR026646; Seq2: OR026647). The phylogenetic analysis involved 1000 replicates using the Neighbor-joining method and the Tamura Nei model through the MEGA X program. Filled circles represent pestivirus strains/isolates of Türkiye obtained from this study, and empty circles represent other pestivirus strains/isolates of Türkiye.

(Ochirkhuu *et al.*, 2016). Thanks to advancements in diagnostic techniques, sequencing and phylogenetic analyses, identified at least 24 subgenotypes of BVDV-1 (BVDV-1a-1x) and four subgenotypes of BVDV-2 (BVDV-2a-2d) (Grange *et al.*, 2023; Giangaspero and Zhang 2023). BVDV-1a and -1b are the most common BVDV-1 subtypes in the world (Mosena *et al.*, 2017). Certain studies (Gallina *et al.*, 2021; Zhou *et al.*, 2022) have demonstrated that BVDV-1b is responsible for gastrointestinal disease in cattle and is correlated to congenital infections or neurological disorders observed in calves. In that context, clinical sampling material (blood, feces, nasal swap, aborted fetus, etc.) is of considerable importance for studies exhibiting the genetic diversity of BVDV. In Türkiye, BVDV-1 includes subtypes 1a, 1b, 1c, 1d, 1f, 1h, 1i, 1l, and 1r, while BVDV-2 includes subtypes 2a and 2b have been identified (Oğuzoğlu *et al.*, 2012; Yılmaz *et al.*, 2012, Timurkan and Aydın, 2019; Toker and Yeşilbağ, 2021). A new BVDV-1 subtype (1v) was also reported in a research conducted in 2019 (Oğuzoğlu *et al.*, 2019). Analysis of partial 5'-UTR sequences from two RT-PCR-positive BVDV samples in our study indicated that they were genetically closer to the BVDV-1 subgroup. This result was supported by the phylogenetic analysis result. According to the results of phylogenetic analysis, both strains were identified as BVDV-1l variant (Fig. 3). This result, combined with previous studies in Türkiye, reveals the predominance of BVDV-1, particularly in cattle displaying symptoms of respiratory system disease, and the dominant subvariant is BVDV-1l. The nucleotide identity was 98.95% between BVDV-1/TR-/Malatya -1 and BVDV-1/TR/Elazığ-29 obtained from this study (accession numbers: OR026646, OR026647). While compared to other BVDV-1 strains selected in the study, they displayed an identity ranging from 70% to 98.2%; they were identical to 64.82% and 75.52% to BVDV-2 and BVDV-3 strains. Since BoHV-1 was not detected in any of the clinical samples, the possibility that BoHV-1 could cause latent infection in animals and that the sample might have been taken at a time when the pathogen was not reactivated.

Due to the severe productivity loss in cattle caused by these investigated viral pathogens in our current study, their consistent mutation potential leading to more virulent strains and the likelihood of current vaccines being ineffective against new strains, regular updates to existing data are recommended. Therefore, in our study, it is crucial to sample from different provinces and farms and individual breeding barns in the same province, sampling especially from animals with clinical symptoms of respiratory disease, and to investigate the significant viral pathogens for BRDC together.

**Conclusions:** BVDV-1, BVDV-2, BVDV-3, BRSV, BPIV-3, and BoHV-1, which are important pathogens of viral-induced severe respiratory system infections in cattle, were investigated by molecular techniques using samples collected from animals with symptoms of respiratory system infection from different provinces, conducted by molecular characterization and phylogenetic analyses.

This study found that the viruses mentioned in the research were circulating at a rate of 16.27% in cattle with severe respiratory system infections. Among the positive samples, 5 tested positive for BPIV-3 (3.87%), 14 for BRSV (10.85%), and 2 for BVDV-1 (1.55%) were detected. BRSV exhibited the highest infection rate among these viruses. As a result, based on the sequence analysis and phylogenetic data obtained from samples representing each province in our study, determined that the identified viruses were highly identical to other viruses circulating in different regions of Türkiye.

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**Authors contribution:** ST is responsible for design of the study. Samples were collected by RO and KS. RO and HA performed the laboratory analyses of the study. All authors interpreted the data. RO, HA, and KS review and report. The manuscript has been revised and approved by all authors.

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