

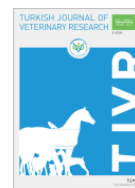


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First genetic characterization and phylogenetic analysis of Bovine Rhinitis A Virus in Türkiye

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ABSTRACT

Objectives: Bovine Rhinitis A Virus (BRAV) is a significant respiratory pathogen which plays a role in the development of bovine respiratory disease complex (BRDC) in cattle worldwide, however, genomic data regarding its prevalence and genetic diversity in Türkiye remain scarce. This study describes the first molecular characterization and phylogenetic analysis of BRAV in Türkiye.

Materials and Methods: Nasal swab samples which were positive for BRAV in real-time RT-PCR screening were tested via conventional RT-PCR targeting a partial region of the viral 3D polymerase (3Dpol) gene of BRAV. Seven samples were sequenced using the Sanger method.

Results: The obtained BRAV sequences were deposited to GenBank (accession numbers PZ150730-PZ150736). The sequence data were analyzed phylogenetically and the Turkish BRAV isolates showed high nucleotide identity (97.91-100%) with each other, forming a distinct cluster when compared to global BRAV isolates. The nucleotide identity between Turkish BRAV isolates and global isolates ranged between 86.82% and 92.23%, while the nucleotide identity with the reference BRAV strain Sd-1 ranged from 86.93% to 88.21%.

Conclusions: This study provides the first genetic characterization of BRAV strains circulating in the cattle population in Türkiye, filling a significant gap in the global and national molecular epidemiology of BRAV. These data will serve as a baseline for future large-scale surveillance in our country.

Keywords: Bovine Rhinitis A Virus, 3D polymerase gene, Türkiye, Phylogeny, Molecular characterization, bovine respiratory disease complex

INTRODUCTION

Bovine respiratory disease complex (BRDC) is one of the most significant challenges in the global cattle industry, leading to substantial economic losses due to decreased productivity, treatment costs, and high mortality rates and especially affecting calves. BRD imposes substantial economic burdens through mortality, decreased weight gain, and lower carcass yield, and also has effects including trade limitations and reduced long-term productivity (Taylor et al., 2010; Peel, 2020; O'Donoghue et al., 2025). The etiological agents of BRDC consist of many bacterial and viral pathogens. The leading

viral agents are bovine herpesvirus-1 (BoHV-1), bovine respiratory syncytial virus (BRSV), parainfluenza-3 virus (PI-3V), bovine viral diarrhea virus (BVDV), influenza viruses, and bovine coronavirus (BCoV) (Zhang et al., 2019; Gaudino et al., 2022; Donbraye et al., 2026). While these viral agents have been extensively studied globally, the role of emerging or neglected viruses in the BRDC pathogenesis is gaining increasing attention currently as metagenomic surveillance expands (Mitra et al., 2016; Ng et al., 2016; Werid et al., 2026). The significance of neglected viruses such as bovine rhinitis A virus (BRAV) within the BRDC is

becoming progressively more recognized currently (Hause et al., 2015; Zhang et al., 2019; Zhang et al., 2021; Ambrose et al., 2023).

BRAV is a member of the *Aphthovirus* genus within the *Picornaviridae* family, and currently named as *Aphthovirus bogeli* (ICTV, 2024). The genome of BRAV consists of a single-stranded, positive-sense RNA of approximately 7.5 to 8.5 kb. Similar to other picornaviruses, the polyprotein is processed into structural (P1) and non-structural (P2 and P3) regions. The 3D polymerase (3Dpol), located at the 3' end of the genome, functions as the RNA-dependent RNA polymerase (RdRp) responsible for viral replication. Due to its highly conserved motifs and essential functional role, the 3Dpol gene is a primary target for molecular detection and phylogenetic studies, providing critical insights into the evolutionary history and taxonomic classification of bovine picornaviruses (Hause et al., 2015; Rai et al., 2016; Brito et al., 2023; Koizumi et al., 2025).

BRAV was first identified as bovine rhinovirus 1, with the prototype BRAV Sd-1 strain isolated in Germany in 1962 (Bögel and Böhm, 1962). Despite its global distribution, the molecular epidemiology of BRAV remains under-reported in many countries and epidemiological data on BRAV are limited globally. In a study conducted in China, BRAV positivity was detected at a rate of 22.09% in nasal swab samples (Zhou et al., 2023). In the United States, metagenomic analysis of calves with bovine respiratory disease (BRD) revealed a BRAV prevalence of 24% (Ng et al., 2015). In Canada, a metagenomic study involving both BRD-affected and healthy calves reported a BRAV detection rate of 4.8% (Zhang et al., 2021). Another metagenomic study from Canada found BRAV in 12% of BRD-affected calves and 3% of healthy animals, with the difference between the groups being statistically significant (Zhang et al., 2019). Additionally, a metagenomic analysis conducted in feedlot cattle from the United States and Mexico, including both BRD-affected and clinically healthy animals housed in the same facilities, reported a BRAV prevalence of 52.7% (Mitra et al., 2016). In our previous study, we demonstrated the presence of BRAV in cattle for the first time in Türkiye, with an overall prevalence of 24.5%, including 40% in BRD-affected animals and 9% in asymptomatic animals (Aksoy et al., 2025). Recent metagenomic studies in respiratory diseases in cattle have led to increasing scientific interest in BRAV currently.

Genomic investigations of BRAV including whole-genome sequencing and phylogenetic analyses, have been conducted in several countries such as USA, Mexico, China, Canada (Mitra et al., 2016; Zhang et al., 2019; Zhou et al., 2023). Although, our group have reported the presence of BRAV in both symptomatic and asymptomatic cattle in Türkiye for the first time (Aksoy et al., 2025), the genetic characterization of the circulating BRAV strains remained unexplored in our country. To address this knowledge gap, the present study aims to provide molecular analysis of the 3D polymerase (3Dpol) gene from representative isolates, facilitating an understanding of the genetic diversity of BRAV in Türkiye.

MATERIALS AND METHODS

Samples

A total of 100 nasal swab samples were collected from cattle showing respiratory symptoms in our previous study (Aksoy et al., 2025), and real-time RT-PCR assays for BRAV was performed with the primers and probe that were developed by Ambrose et al. (2023). In this study, for genetic characterization of BRAV, a total of seven samples, previously identified as BRAV-positive via real-time RT-PCR in our initial survey (Aksoy et al., 2025), were selected for partial genomic sequencing (Table 1).

Conventional RT-PCR

Total RNA was extracted from the nasal swab samples using a commercial viral RNA isolation kit (High Pure Viral RNA Kit, Roche) following the manufacturer's instructions. cDNA synthesis was performed with using random hexamer primer (Thermo Scientific, USA), dNTPs (Thermo Scientific, USA), RNase inhibitor (Thermo Scientific, USA), RT buffer and reverse transcriptase (New England Biolabs, USA), and incubated at 25°C for 5 min, 42°C for 1 hour and 65°C for 20 min. Following cDNA synthesis, to detect BRAV via conventional RT-PCR, a partial region of the 3Dpol gene was amplified using previously described primers (Table 2). PCR conditions were optimized as follows: initial denaturation at 94°C 3 min; 36 cycles of 94°C 30 s, 60°C 30 s, 72°C 30 s; final extension at 72°C 10 min. The expected 252 bp PCR products were visualized by 2% agarose gel electrophoresis, and positive samples showing the expected band size were sent to Sentebiolab Genetics (Ankara, Türkiye) for Sanger sequencing.

Table 1. Animal- and symptom-related information of the samples that analyzed in this study. Fever status, respiratory symptoms, and nasal discharge findings were recorded at the time of sampling.

Sample No	Age	Fever	Respiratory Symptoms	Nasal Discharge
1	6-8 months	None	Coughing and respiratory depression	Present
2	6-8 months	None	Coughing and respiratory depression	None
3	6-8 months	None	Coughing and respiratory depression	None
4	9 months	None	Coughing	None
5	2-3 months	Present	Coughing	Present
6	2-3 months	None	Coughing	None
7	2-3 months	None	Coughing	None

Table 2. Primer sequences used in conventional RT-PCR in this study. The forward primer is also used in Sanger sequencing.

Primer	Sequence (5'→3')	Product length	Reference
6550–6570 F	ATGATTGAGGCCGTGTTCTCG	252 bp	Koizumi et al., 2025
6782–6801 R	GTCGTCCCCATAAGCAACCA		

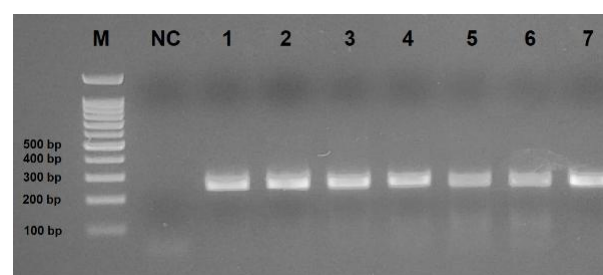
Phylogenetic analysis

The alignment and phylogenetic analysis of the seven Turkish BRAV isolates were analyzed using MEGA 12 software. Nucleotide sequences were aligned using the MUSCLE algorithm with codon-based alignment to maintain the correct reading frame of the 3Dpol gene. The best-fit evolutionary model for the dataset was determined to be the Kimura 2-parameter (K2) model with a discrete Invariant Sites (+I) distribution, based on the Bayesian Information Criterion (BIC) in MEGA 12 software. Following alignment, sequences were manually trimmed to remove poorly aligned regions and to ensure equal sequence length across all samples. The final sequence dataset with no gaps or missing data, which was used for all downstream analyses and GenBank submission. The phylogenetic tree was constructed using the Maximum Likelihood method based on the Kimura 2-parameter model with 1000 bootstrap replicates. Pairwise nucleotide distances were calculated using the Kimura 2-parameter model, and nucleotide similarities between sequences were calculated as percentages in Microsoft Excel.

RESULTS

A total of seven Bovine rhinitis A virus (BRAV) sequences obtained from cattle in Türkiye were successfully amplified and analyzed based on a 252

bp region of the 3D pol gene via conventional RT-PCR (Figure 1). The 195-nucleotide sequences obtained from all Turkish BRAV strains were submitted to GenBank under accession numbers PZ150730–PZ150736. Sequence alignment revealed no insertions or deletions, and all sequences were of equal length, allowing the inclusion of all positions in subsequent analyses.

**Figure 1.** Conventional RT-PCR results of seven BRAV-positive samples on 2% agarose gel. M: Marker (Bioron, Germany), NC: negative control (dH₂O), 1-7: BRAV isolates from this study.

Pairwise nucleotide identity analysis demonstrated that the Turkish BRAV isolates shared a high degree of identity among themselves, ranging from 97.91% to 100%, indicating a close genetic relationship (Table 3). In contrast, the nucleotide identity between the Turkish BRAV isolates and international strains ranged from 86.82% to 92.23% (Table 4).

Table 3. Pairwise nucleotide sequence identity (%) matrix of Turkish BRAV strains and selected global strains, based on a 195-nucleotide fragment of the 3Dpol gene. Pairwise nucleotide distances among BRAV sequences were calculated using the Kimura 2-parameter model in MEGA and nucleotide identity percentages were calculated in Excel. The diagonal values represent internal identity.

BRAV Isolates	TR_1	TR_2	TR_3	TR_4	TR_5	TR_6	TR_7	Sd-1	WBYDN	H-1
TR_1	100									
TR_2	100	100								
TR_3	98.44	98.44	100							
TR_4	98.44	98.44	98.96	100						
TR_5	97.91	97.91	99.48	98.44	100					
TR_6	97.91	97.91	99.48	98.48	98.96	100				
TR_7	97.91	97.91	99.48	99.48	98.96	100	100			
Sd-1*	87.57	87.57	88.21	86.93	87.57	87.57	87.57	100		
WBYDN**	91.77	91.77	91.17	91.77	90.56	91.17	91.17	87.62	100	
H-1***	89.90	89.90	91.73	90.52	91.13	91.13	91.13	84.82	91.77	100

*Reference BRAV strain NC_038303.1, **PX941976.1 (China), ***JN936206.1 (Japan)

Table 4. Nucleotide identity ranges between Turkish BRAV isolates and other strains from different countries.

Country	Number of Sequences	Nucleotide Identity Range (%)
China ¹	3	88.70 - 91.77
USA ²	7	86.82 - 92.23
Mexico ³	3	88.06 - 91.17
Australia ⁴	2	86.88 - 88.79
Germany ⁵	1	86.93 - 88.21
Japan ⁶	1	89.90 - 91.73

¹ PX941976.1, MZ004694.1, MZ004695.1 from China;

² PX680750.1, PX680752.1, PX680761.1, PX680762.1, PX680769.1, PX680771.1, KP264974.1 from the USA;

³ PX680751.1, PX680754.1, KU159363.1 from Mexico;

⁴ OP020162.1, OP020164.1 from Australia; ⁵ NC_038303.1 from Germany (the reference strain); ⁶ JN936206.1 from Japan.

Based on the nucleotide identity matrix, the BRAV sequences from China, Japan, USA and Mexico displayed the most significant genetic proximity to the Turkish isolates (Table 5), revealing a consistent pattern with minor variations among isolates. BRAV_TR_1 and BRAV_TR_2 showed the highest similarity to the Chinese isolate (91.77%), BRAV_TR_3 exhibited the greatest similarity to the Japanese isolate (91.73%). BRAV_TR_4 demonstrated equally high similarity to both the Chinese and one U.S. isolate (91.77%), BRAV_TR_5,

the highest similarity was observed with a U.S. isolate (92.23%), and BRAV_TR_6 and BRAV_TR_7 both showed the greatest similarity to a U.S. isolate (91.63%) (Table 5). In contrast, the greatest genetic divergence was noted against from Australian BRAV strains and German BRAV reference strain Sd-1 (Table 4).

According to nucleotide and amino acid sequence comparison, several nucleotide substitutions were detected between reference Sd-1 strain and Turkish BRAV strains, however these variations did not result in any amino acid changes (data not shown). Although there are many synonymous substitutions in the 3Dpol gene amino acid sequence analysis indicated that the protein still remains highly conserved.

The phylogenetic analysis conducted in this study highlights the evolutionary relationships between the Turkish BRAV isolates and previously identified global strains. Phylogenetic analysis based on a partial sequence of the 3Dpol gene revealed that all BRAV isolates obtained from Türkiye clustered together in a distinct monophyletic group, supported by a high bootstrap value (87%) (Figure 2). This suggests a common evolutionary origin or a localized circulation of BRAV isolates within the region. The BRAV reference sequence Sd-1 (NC_038303.1) and Australian BRAV strains (OP020162 and OP020164) were positioned in more distant branch, indicating the genetic divergence of the Turkish isolates (Figure 2).

Table 5. Nucleotide identity (%) of Turkish BRAV isolates compared to selected global strains showing the highest identity scores. The global reference sequences from China (PX941976.1), USA (PX680769.1, PX680771.1), Mexico (PX680751.1), and Japan (JN936206.1) were selected based on their high genetic similarity and geographical representation.

Turkish Isolates	China (PX941976.1)	USA (PX680769.1)	USA (PX680771.1)	Mexico (PX680751.1)	Japan (JN936206.1)
BRAV_TR_1	91.77	90.56	89.78	89.33	89.90
BRAV_TR_2	91.77	90.56	89.78	89.33	89.90
BRAV_TR_3	91.17	91.17	91.63	91.17	91.73
BRAV_TR_4	91.77	91.77	91.02	90.56	90.52
BRAV_TR_5	90.56	91.77	92.23	91.77	91.13
BRAV_TR_6	91.17	91.17	91.63	91.17	91.13
BRAV_TR_7	91.17	91.17	91.63	91.17	91.13

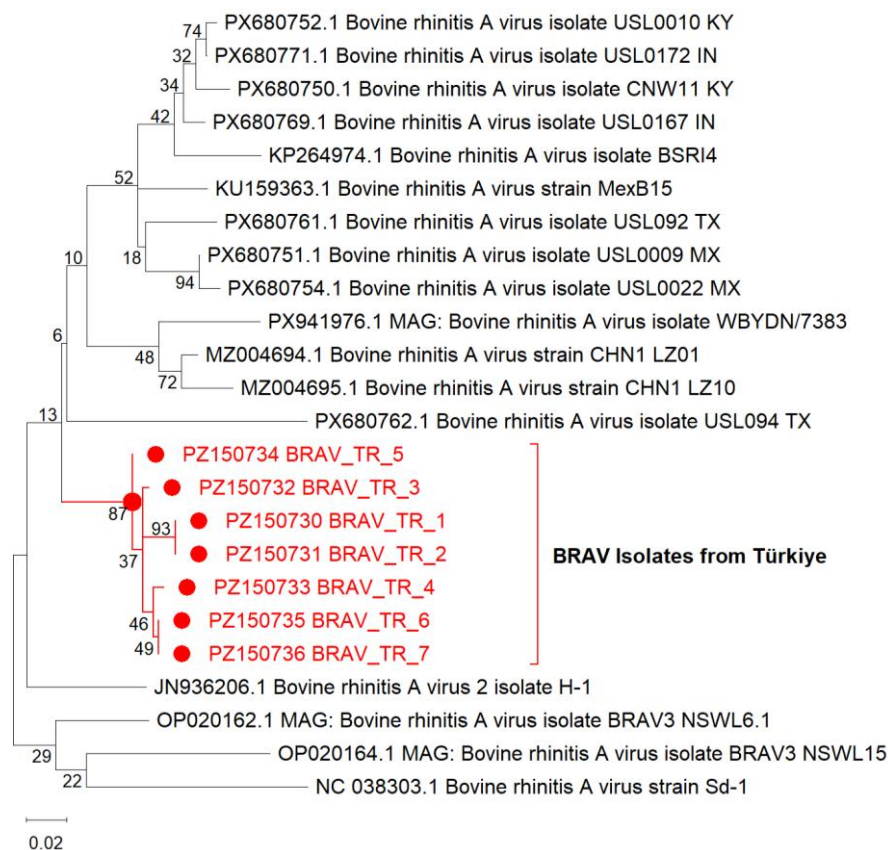


Figure 2. Phylogenetic tree based on partial 3D polymerase gene sequences (195 nt) of bovine rhinitis A virus (BRAV). The tree was constructed using the Maximum Likelihood method with the Kimura 2-parameter model and 1000 bootstrap replicates. Turkish isolates are indicated by filled red circles.

DISCUSSION

Bovine respiratory disease (BRD) still remains a major global challenge for cattle industry due to its association with high mortality rates, particularly in calves, and economic impact. With the increasing

approach of metagenomic analyses, BRAV has been identified among BRD-associated viruses and has recently gained scientific attention. While the first detection and risk factor analysis of BRAV in cattle in Türkiye were previously reported by our group (Aksoy et al., 2025), comprehensive genetic

characterization of the virus has remained unexplored. Although epidemiological, metagenomic, and phylogenetic studies of BRAV have been conducted in various countries—including China, the USA, Mexico, and Australia—the present study provides the first detailed genetic characterization of BRAV isolates in Türkiye, filling a critical gap for molecular epidemiology.

In this study, we report the first molecular and genetic characterization of BRAV in Türkiye. By employing RT-PCR targeting the 3Dpol region followed by Sanger sequencing, we aimed to confirm the presence of the virus and determine its phylogenetic relationship with international isolates. Phylogenetic analysis based on the 3Dpol gene sequences revealed that all seven Turkish BRAV isolates (BRAV_TR_1 to TR_7) clustered together in a distinct monophyletic group with a bootstrap support of 87% (Figure 2). This independent clustering suggests a common evolutionary origin for the circulating strains in the study region, potentially indicating a localized transmission cycle or a single introduction event followed by regional diversification.

The pairwise identity analysis of the 3Dpol gene sequences demonstrated that the seven Turkish BRAV isolates shared a high degree of nucleotide identity, ranging from 97.91% to 100% (Table 3). Although all Turkish BRAV isolates clustered together in phylogeny; in nucleotide level, they did not cluster exclusively with a single geographic lineage. While BRAV_TR_1, 2, and 4 showed a closer affinity to Asian (Chinese) and North American (USA) strains, BRAV_TR_5, 6, and 7 demonstrated higher identity with the USA lineage. BRAV_TR_3 was uniquely closer to the Japanese reference strain, suggesting a high level of genetic diversity among the circulating BRAV strains in the region (Table 5). The findings of this study demonstrate a notable genetic diversity among the Turkish BRAV isolates, as evidenced by their varying nucleotide identity scores (89.33% to 92.23%, Table 4) against global reference strains. Our analysis indicates that the circulating Turkish strains do not represent a single, uniform introduction; instead, they reflect a complex phylogenetic landscape with multiple geographical affiliations. Furthermore, regarding nucleotide identities, variation was observed among isolates from different states of the USA. Notably, isolates obtained from Indiana displayed higher nucleotide identity compared to those from Texas and Kentucky.

The seven Turkish BRAV isolates displayed a nucleotide identity of 86.93%–88.21% with the prototype Sd-1 strain (NC_038303.1) (Table 3). Notably, the higher similarity observed with the more recent Chinese isolate (91.77%) than with the prototype sequence highlights the ongoing genetic shift and regional diversification of BRAV lineages globally. Comparative identity analysis revealed that Turkish BRAV isolates are genetically more related to currently circulating global BRAV isolates than to the reference Sd-1 strain (Table 3). This indicates a significant genetic divergence from the reference genome and suggests that circulating strains in Türkiye may share a more recent common ancestor with East Asian lineages.

In a comprehensive study conducted in China (Zhou et al., 2023), three near-full-length BRAV genomes (CHN1_LZ01, LZ10, and LZ11) were characterized, showing a nucleotide identity of 79.3–87.5% compared to other available BRAV complete genomes in GenBank. In our study, based on the highly conserved partial 3Dpol gene, we observed a higher identity range (86.38%–92.23%) with global BRAV strains. This discrepancy is likely due to the conserved nature of the 3Dpol region compared to the higher variability found across the entire viral genome (Rai et al., 2016).

Similar to the high intra-group genetic homogeneity (97.3–99.9% nt identity) reported by Zhou et al (2023) for Chinese BRAV isolates, our Turkish isolates exhibited a remarkably stable genetic profile with 97.9%–100% identity. This might be influenced by partial sequence of highly conserved 3Dpol gene was used for phylogeny in this study, while near-full-length sequences were gained for Chinese isolates (Zhou et al., 2023).

The high degree of genetic conservation observed in the 3Dpol region of these Turkish isolates further supports the stability of the virus during local circulation. Sequence analysis revealed that all nucleotide mutations in the Turkish BRAV isolates were phenotypically silent, as no alterations were detected in the corresponding amino acid profiles compared to the reference sequence Sd-1. The lack of amino acid changes underscores the evolutionary stability of the viral polymerase (3Dpol), which is critical for consistent viral replication and pathogenesis, and potential diagnostic targeting (Rai et al., 2016; Koizumi et al., 2025). Such stability in the 3Dpol gene is also significant for the core replication machinery of BRAV remains functionally consistent across different geographical lineages.

CONCLUSION

In the present study, molecular characterization of Turkish BRAV isolates was conducted based on partial 3Dpol gene sequencing, providing essential baseline data on the in Türkiye. Comprehensive complete genome analysis will be crucial for identifying site-specific mutations across the entire viral polyprotein, detecting potential recombination events, and assessing the impact of genetic diversity on viral virulence and diagnostic accuracy. Thus, to fully understand the molecular epidemiology and viral evolution of local BRAV isolates, whole-genome sequencing is imperative in future studies with a national scale.

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Author contribution: EA and AKA designed the study. YŞ collected the samples and clinically examined animals. EA and AKA performed laboratory studies and phylogenetic analysis. EA, YŞ and AKA participated in drafting and revising the manuscript. EA: Emel Aksoy, YŞ: Yasin Şenel, AKA: Ahmet Kürşat Azkur

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Ethical approval: This study consists of the samples from previous study which was approved by Kırıkkale University Animal Experiments Local Ethics Committee (Approval number: 2023/09/32).

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