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Phylogenetic Analysis and Genetic Characterization of Bovine Respiratory Syncytial Virus G Glycoprotein of Cattle in Burdur Province

Ali Küçük [1a]*, Özge Sevinç Korkmaz Akar [1b], Yakup Yıldırım [1c], Kamil Atlı [1d]

[1] *Department of Virology, Faculty of Veterinary Medicine, Burdur Mehmet Akif Ersoy University, Burdur-Türkiye*

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ORCIDS:
[a] 0000-0001-9929-1378
[b] 0000-0001-8854-0420
[c] 0000-0003-4299-4712
[d] 0000-0002-2266-4372

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Abstract:

This study aimed to molecularly detect Bovine Respiratory Syncytial Virus (BRSV) in cattle with clinical signs of respiratory system infection and to conduct a phylogenetic analysis of the detected strains. To detect the pathogen, nasal swab samples collected from 96 cattle were subjected to Reverse Transcriptase – nested PCR using specific primer sets. As a result of the molecular techniques, viral nucleic acid was detected in 2.08% (2/96) of the samples. Additionally, sequence and phylogenetic analyses of the detected local variant were performed and compared with various BRSV strains identified in different regions of Turkey and around the world. The genetic data obtained revealed that the isolates identified in this study belong to Subgroup III, alongside BRSV strains reported from various regions of Turkey, France, and the USA. Immunologically significant point mutations were detected in the immunodominant G glycoprotein region of the variant Asn(N)$^{179}\longrightarrow$Asp(D) / Ala(A) $^{205}\longrightarrow$Thr (T)) This result indicates that BRSV strains circulating among cattle can escape the host immune system without losing their pathogenicity and is considered to be one of the important factors that can cause respiratory system infection outbreaks in cattle.

Keywords: Cattle, Bovine Respiratory Syncytial Virus, Reverse Transcriptase – nested PCR, Genetic Characterization

Burdur İlindeki Sığırlarda Bovine Respiratory Syncytial Virus G Glikoproteininin Filogenetik Analizi ve Genetik Karakterizasyonu

Özet:

Bu araştırmada solunum sistemi enfeksiyonu klinik bulguları gösteren sığırlarda Bovine Respiratory Syncytial Virus (BRSV)' un moleküler tespiti ve tespit edilen lokal suşların filogenetik analizlerinin gerçekleştirilmesi hedeflenmiştir. Etken tespiti için 96 sığırdan toplanan nazal sürüntü örneklerine spesifik primer setleri kullanılarak Reverz Transkriptaz-nested PCR (RT-nPCR) yöntemi uygulanmıştır. Uygulanan moleküler teknikler sonucunda % 2.08 (2/96) oranında viral nükleik asit tespiti gerçekleştirildi. Bununla birlikte tespit edilen lokal suşların sekans ve filogenetik analizleri gerçekleştirilerek Türkiye ve Dünya'nın farklı bölgelerinden tespit edilmiş farklı BRSV suşları ile karşılaştırmaları yapıldı. Elde edilen genetik verilerden, bu çalışmada tespit edilen izolatların, Türkiye, Fransa ve ABD' nin farklı bölgelerinden bildirilen BRSV suşları ile birlikte, Subgrup III içerisinde yer aldığı belirlenmiştir. Ayrıca izolatların immünodominant G glikoprotein bölgesinde immünolojik açıdan önemli nokta mutasyonları tespit edilmiştir (Asn(N)$^{179}\longrightarrow$Asp(D) / Ala(A) $^{205}\longrightarrow$Thr (T)). Bu sonuç, sığırlar arasında dolaşan BRSV suşlarının patojenitelerini kaybetmeden konak bağışıklık sisteminden kaçabildiklerini ve sığırlarda solunum sistemi enfeksiyon salgınlarına neden olabilecek önemli faktörlerden biri olarak değerlendirilmiştir.

Anahtar kelimeler: Sığır, Bovine Respiratory Syncytial Virus, Reverz Transkriptaz - nested PCR, Genetik Karakterizasyon

Sorumlu Yazar / Corresponding Author: alikucuk@mehmetakif.edu.tr, alikucuk13@gmail.com

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Introduction

Bovine Respiratory Disease Complex (BRDC) is an infection that causes large-scale economic losses to cattle breeders due to reasons such as calf deaths due to severe pneumonia, decrease in milk yield and carcass quality, abortion, reproductive problems and veterinary health expenses (Sermiento-Silva et al., 2012). BRSV one of the major factor that cause this disease complex, is widely seen all over the world due to cattle mobility between countries (Ellis, 2013; Nath et al., 2017). The virus that causes infection, especially in cattle aged 2-6 months, during winter and autumn months, is primarily transmitted by aerosol. On the other hand, it is known that it is indirectly transmitted through contaminated feeders and lickers (Ohlson et al., 2010). Since it is difficult to diagnose the agent using classical clinical diagnostic methods, many laboratory diagnostic techniques such as virus isolation, Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), Enzym Immuno Assay, Direct Immunofluorescence have been developed (Ellis, 2013; Maclachlan et al., 2017).

According to the taxonomy prepared by International Committee on Taxonomy of Virus, the name of the agent, which was named Bovine Respiratory Syncytial Virus (BRSV) in the Pneumoviridae family Orthopneumavirus genus until 2015, was changed to Orthopneumovirus bovis after 2016 (Rima et al., 2017; Postler et al., 2021). The virus has an envelope and pleomorphic morphology and negative polarity single-stranded (ss) non-segment RNA genome of approximately 15.2 kb in length. Ten Open Reading Frames (ORFs) on the viral genome encode 11 proteins (3'-NS1-NS2-N-P-M1-SH-G-F-M2.1-M2.2-L-5'). Among these proteins, Small Hydrophobic Protein (SH), Glycoprotein (G) and Fusion Protein (F) are transmembrane glycoproteins its located on the viral envelope. Other proteins are non-structural NS1 and NS2, nucleoprotein (N), phosphoprotein (P), Matrix protein (M), RNA-dependent polymerase protein (L), M 2.1 and M.2.2 proteins (Valacher and Taylor 2007; Ellis, 2013). While Glycoprotein (G), which has an important immunodominant region, is involved in the adsorption of the virus to the host cell surface (Larsen, 2000; Valarcher and Taylor, 2007). Genetic characterization and detected to heterogeneity of BRSV isolates were performed by phylogenetic analysis of G, N and F genomic regions (Langedijk et al., 1997; Valarcher et al., 2000). Studies on the sequence of the G glycoprotein region have revealed that BRSV is genetically divided into ten (I-X) subgroups, and studies using monoclonal antibodies have revealed that the agent has four serotypes (A, B, AB, untyped) (Klem et al., 2014; Kumagai et al., 2021).

This study aimed to molecular diagnosis of BRSV and determined its etiological role in cattle respiratory system infections. The G glycoprotein region, which is an important immunodominant region responsible for the adsorption of the virus to the susceptible cell, was analyzed in this study to evaluate the cellular infection of the virus. In addition, it was aimed to amplify the G glycoprotein region of the detected local isolates and sequence analysis, thus determining the subtypes of BRSV strains circulating among cattle in the region, their phylogenetic characterization and their differences with other strains.

Material and Method

Animals Used in the Study and Sampling

In the study, nasal swap samples were obtained from 96 nonvaccinated cattle of different breeds, ages and genders with clinical signs of respiratory system infection (dyspnea, mucopurulent nasal discharge, conjunctival discharge) and fever > 40°C. Samples were collected by random sampling method, constituting 10% of the number of infected animals from four different cattle herds. Three of the ranches where the samples were collected are combined animal breeding and one is dairy cattle breeding.

The general characteristics of the animals that constitute the

Table 1. General characteristics of the sampled animals

Data of Sampled Animals	n (%)
Number of Animals Sampled	96 (%100)
Age	
< 9 months	30 (%31.25)
10-24 months	43 (%44.79)
> 24 months	23 (%23.95)
Gender	
Female (♀)	43 (%45.8)
Male (♂)	53 (%55.2)
Breed	
Simmental	66 (%68.75)
Holstein	30 (%31.25)

Nasal swap samples obtained from animals were placed in transport tubes containing penicillin (500 IU/mL) + streptomycin (20 mg/mL) and Dulbecco's Modified Eagle Medium (DMEM, 1.5 mL) and brought to the diagnostic laboratory in accordance with the cold chain protocol.

Extraction of the viral nucleic acid

Total RNA extraction from nasal swab samples was performed by the acid-guanidine-phenol method (Rio et al., 2010). The samples brought to the laboratory were thoroughly vortexed and then centrifuged at 3000 rpm +4°C for 20 minutes. After centrifugation, 250 µL of supernatant was taken into RNase/ DNase free microcentrifuge tubes (2mL) and 750 mL of Hibrizol (Hibrigen/Türkiye) was added and stored at -80°C until the extraction stage. Total RNA extraction was carried out as specified by the manufacturer, and after extraction, the lyophilized RNA was rehydrated with 55-60 mL of DEPC water and stored at -80°C until the complementer DNA (cDNA) stage.

Reverse transcription-nPCR

Before the PCR, viral cDNA synthesis was performed from viral RNA using the iScript cDNA synthesis kit (Biorad, USA, Cat No: 1708891). cDNA synthesis was performed as specified by the manufacturer. G glycoprotein region of BRSV were amplified using the RT-nPCR technique. Amplifications were performed with gene-specific oligonucleotide primers and PCR conditions reported by Vilcek et al. (1994). Gel electrophoresis method was used to observe the amplicons. PCR amplicons of the samples were run on a 1.5% agarose gel at 90V for 1 hour and visualized under UV light with the utilized of a transilluminator. Information about oligonucleotide primers, gene regions and amplicon sizes is given in Table 2.

Table 2. G glycoprotein oligonucleotide primers used in RT-nPCR (Vilcek et al., 1994)

BRSV G	B5A	CCACCCTAGCAATGATAACCTTGAC	603 1st
	B6A	AAGAGAGGATGC(T/C)TTGCTGTGG	
	B7A	CATCAATCCAAAGCACCACACTGTC	371 2nd
	B8	GCTAGTTCTGTGGTGGATTGTTGTC	

Sequencing

With the oursourcing bi-directional (Forward and Reverse primers) sequence analysis performed of the amplicons showing the clearest band image after gel electrophoresis with the Sanger sequencing method (Letgen, Türkiye). Sequence data were assembled and edited using Aliview 1.27 sequence analysis software (Larsson, 2014). The edited sequence data were compared to the nucleotide sequence database using the Basic Local Alignment Search Tool, a module of the National Center of Biotechnology Information. Phylogenetic analyzes of G gene sequence were carried out with the MegaX software program based on the maximum likelihood method using the Tamura 3 model (Kumar et al., 2018). The confidence level of the Maximum Likelihood tree was determined as 1000 bootstrap. Evolutionary closeness between isolates were determined with Sequence Demarcation Tool v. 1.2 (SDT) software. Sequences were recorded in the GenBank database and accession numbers were obtained.

Results

As a result of the RT-nPCR technique performed using primers specific to G glycoprotein region, the presence of nucleic acid belonging to BRSV was detected in two of the ninety-six nasal swap samples. The animals from which the samples were obtained, in which the BRSV genome was detected, were younger than 9 months old and with clinical signs of acute respiratory tract infection. In addition, it was determined that the animals in which the causative agent was detected were in an enterprise where combined animal breeding was carried out.

The sequences reported in this article were submitted to the GenBank, with accession numbers as follows OR797906 and OR797907 for G gene.

Partial sequencing G glycoprotein region of the our BRSV isolates in our research was performed and phylogenetic analyzes were performed. In this context, BRSV subgroups of our local BRSV isolates were identified and their genetic similarities with strains isolated from Türkiye and other countries and nucleotide/amino acid differences on their partial genomes were revealed. Our phylogenetic analysis on the gene region coding the G protein revealed that our Burdur/ BRSV NS/13 and Burdur/BRSV NS/14 strains were included in BRSV Subgroup III, together with BRSV strains isolated from England, Belgium, France and Türkiye. In this study, the nucleotide similarity rate between our two local strains was determined to be 98.8%. In contrast, the nucleotide similarities of our strains to those with accession numbers MW881234-MW881233, identified in Afyonkarahisar, and MH133326-MH133327, identified in Eskişehir, were found to be approximately 88-89%. Meanwhile, the similarity rates to the strains with accession numbers MW711876-MW711875, isolated in Hatay, and MW892047, detected in Izmir, were determined to be around 92-93%. On the other hand, the similarity was found to be 85.2-85.8% with the strains identified in France with accession numbers AF188579-

AF188581, and 89.9% with the BRSV strain identified in Belgium with accession number U24716. Additionally, a similarity of 86.4% was revealed with the strain identified in England with accession number M58307. On the other hand, the similarity between our field strains and a vaccine isolate, L27802, was revealed as 86.7%.

The phylogenetic tree created based on the G protein-coding gene region is shown in figure 1. In additionally, the similarity rates of the G Glycoprotein region of BRSV are given in figure 2.

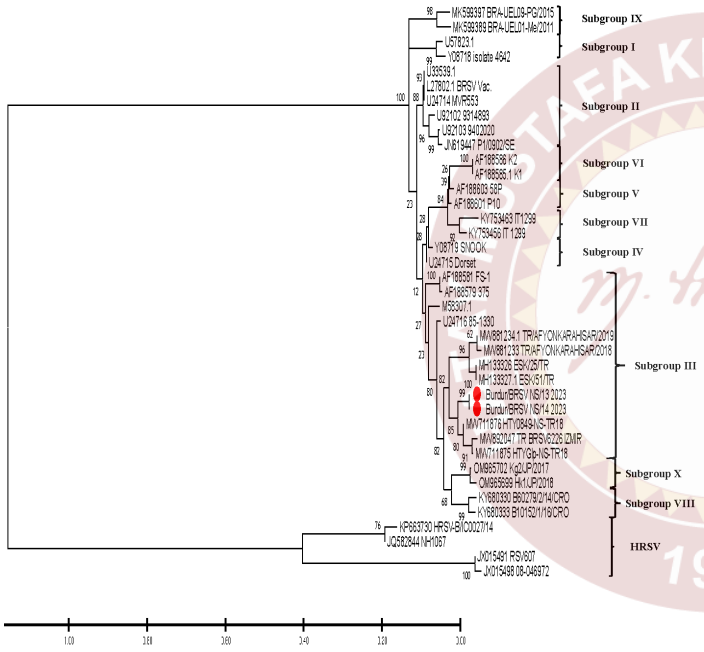

Figure 1. Phylogenetic tree of G gene; red circles indicate strains local strain in this study

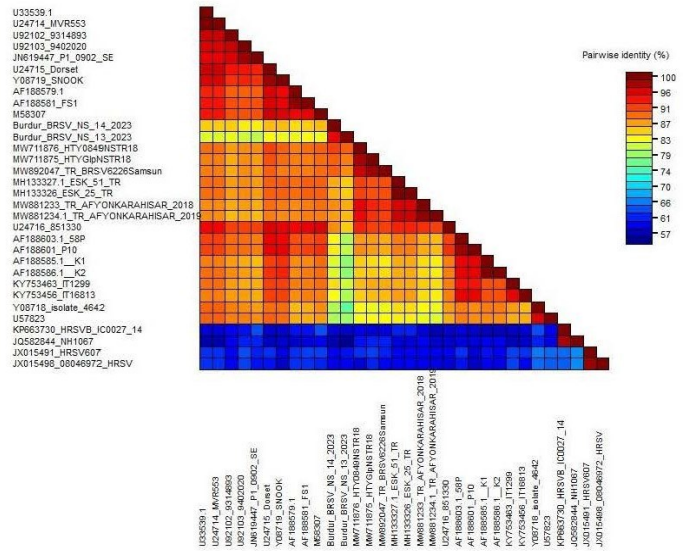

Figure 2. The sequence demarcation tool (SDT) results of G gene

G protein amino acid sequences of Burdur/BRSV NS/13 and Burdur/BRSV NS/14 isolates were compared with BRSV strains isolated from different countries and Türkiye, and it was determined that both isolates preserved four cystine residues in the immunodominant region.

However, as a result of point mutations in the genomes of both isolates, it was determined that the amino acids Asn^{179} → Asp and Ala^{205} → Thr were synthesized. On the other hand, mutations were detected in the YKST^{98} → CGGL amino acid motifs and it was revealed that another cystine residue was formed in both isolates. The motif mutation detected in Burdur/BRSV NS/13 and Burdur/BRSV NS/14 strains was not found in BRSV field and vaccine strains in different subgroups detected in Türkiye and around the world. On the other hand, point mutations were detected in the form of Ser^{97}-Leu in the strain with accession number KY753456 in Subgroup II and Lys^{96}-Glu in the strain with accession number AF188585 in Subgroup VI.

The differences between the G amino acid sequences of BRSV isolates are shown in figure 3.

Disscussion

BRSV, one of the main causes of BRDC, which is among the multifactorial diseases of cattle, is widespread in the world. It causes producers to happen large-scale economic losses, especially in large herds, directly due to deaths due to pneumonia and bronchopneumonia, and indirectly through abortion, decrease in carcass quality, and prophylactic expenses (Küçük and Yıldırım, 2022).

In this study, molecular diagnosis of BRSV, one of the major viral pathogens causing BRDC, performed the conventional RT-nPCR technique, and phylogenetic characterization of the Glycoprotein region encoding G protein was carried out. Studies on the virology and serological detection of BRSV have shown that the agent is circulating in different species in many countries in Europe, Asia and the South America (Yaegashi et al., 2005; Socha et al., 2009; Kresic et al., 2018; Leme et al., 2020). Likewise, in studies conducted using different diagnostic methods in Türkiye, the prevalence of the agent in cattle was reported to be between 0.9% and 40% (Yaman et al., 2018; Timurkan et al., 2019; Karayel-Hacıoğlu et al., 2019; Yazıcı et al., 2020; İnce et al., 2022). In this study, we determined the prevalence of BRSV in cattle in the research area as 2.08% (2/96). Our prevalence value is parallel to other research results.According to phylogenetic analysis, we determined that our field isolates were included in BRSV Subgroup III, along with BRSV strains isolated from Türkiye, France and the USA. In

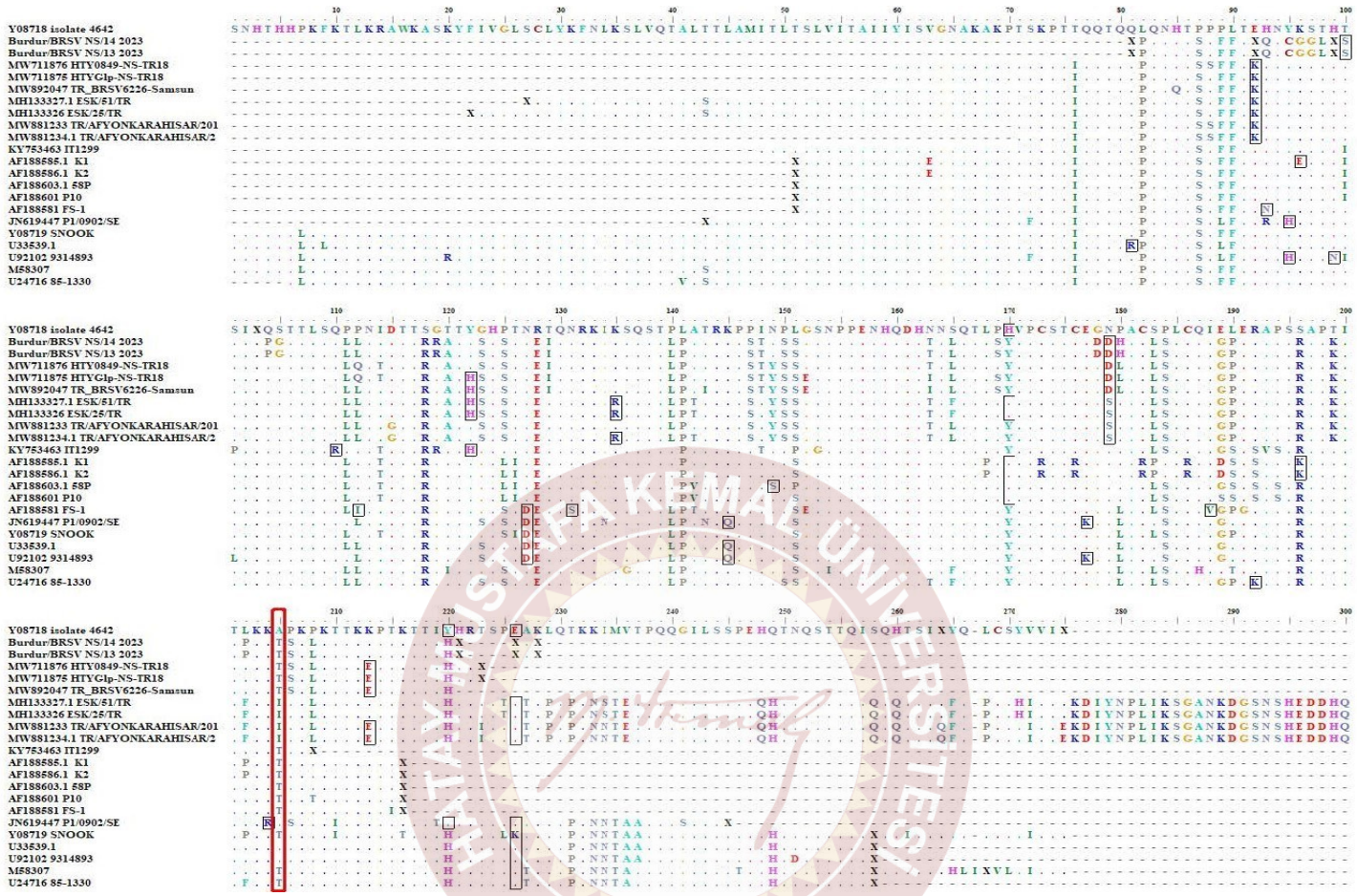

Figure 3. The aminoacids differences between BRSV strains isolated in the study and accessed via GenBank

our research, results similar to the studies conducted on the phylogenetic analysis of BRSV in different regions of Türkiye were obtained and it was revealed that BRSV Subgroup III was in circulation in cattle in Türkiye. Although it was reported that BRSV had a single genotype in the first years when it was detected, with the development of monoclonal antibody techniques and phylogenetic analysis, subtypes with low-grade genetic differences began to be detected (Valarcher et al., 2000) However, it has been suggested that there is a geographical correlation between BRSV subgroups and the regions where they are detected (Yaegashi et al., 2005). In studies which molecular diagnosis and phylogenetic analysis of BRSV were carried out, BRSV subgroup II was detected in Scandinavian countries, while BRSV Subgroups I, II, III, IV, V, VI were found to be widespread in Western and Southern Europe (Valarcher et al., 2000; Bidokhti et al., 2012; Klem et al., 2014; Bertolotti et al., 2018). In addition, ın phylogenetic studies conducted in South America, Subgroup I in Brazil (Spilki et al., 2006); In studies conducted in Asian countries and Türkiye Subgroup III was reported in China and Subgroup X was reported in Japan (Chang et al., 2022; Mitarai et al., 2023). Therefore, the hypothesis that there is a geographical correlation between BRSV subgroups and the regions where they are detected is weakened. On the other hand, it is thought that the reason why BRSV isolates in the same subgroup were detected in molecular and phylogenetic analyzes carried out in countries that are geographically distant from each other may be due to the increase in live animal imports between countries with the development of transportation technologies.

The G glycoprotein in the structure of BRSV, depending on the BRSV isolates, is a type II glycoprotein with 257-263 amino acids and an anchor domain of 38-66 amino acids. In additionally, the genomic region is important for immunization because it contains a cysteine-rich immunodominant region between amino acids 174-188, which is the central binding site of neutralizing antibodies (Valarcher et al., 2000; Valarcher and Taylor, 2007).

Langedijk et al. (1997) genetic research on the antigenic structure of the central conserved domain of the G protein of BRSV has been revealed that a change in amino acid sequences 177. and 180. can only cause a local change on the antigenic surface, whereas mutations in positions 183. and

184. can lead to major structural changes. On the other hand, they reported that point mutations in the 180. and 205. amino acid regions played an important role in the preliminary classification of BRSV subgroups. Leme et al. (2020) performed genetic analysis of the F and G genomic regions in BRSV isolates isolated from cattle in Brazil and detected mutations in the cystine residues in the immunodominant region between 170 and 190. On the other hand, many studies have reported that the cysteine-rich immunodominant region located in the central hydrophobic region (CHR; aa 158-189) of the G protein of BRSV isolates is preserved, but point mutations are observed in regions 179 and 205 (Kresic et al., 2018; Karayel-Hacıoğlu et al., 2019; Kumagai et al., 2021; Jia et al., 2021; İnce et al., 2022). In the G protein amino acid sequences of the two BRSV isolates we obtained in this study, it was determined that four cystein residues in the immunodominant region were preserved, similar to the results of previous research. However, Leme et al. (2020) found that different amino acid residues were synthesized instead of the four cystein residues in the BRSV isolates they isolated. Point mutations in the 180. and 205. amino acid regions play an important role in determining BRSV subgroups. However, especially the change in the amino acids Ala$^{205}\longrightarrow$ Thr allows the virus to escape from the host immune system (Langedijk et al., 1997; Valarcher et al., 2000).

Karayel-Hacıoğlu et al. (2019) and İnce et al. (2022) detected the amino acid as Iso^{205} in the BRSV strains they isolated. On the contrary, the change in the Burdur/BRSV NS/13, Burdur/BRSV NS/14 strains isolated in this study and the BRSV strains detected in Hatay was determined to be Thr^{205}. It is thought that these circulating isolates may increase the prevalence of BRSV infection in the region by escaping the immune system developed against the agent in cattle populations.

In the light of this datas, it was evaluated that the BRSV isolates we detected preserved their pathogenicity under in vivo conditions. However, it was concluded that research on this situation should be increased by performing analyzes of BRSV isolates isolated from different regions.

It is known that BRSV causes severe bronchopneumonia in the postnatal period between 2 and 6 months of age. Therefore, the vaccine against the pathogen must have a strong immunostimulatory effect during the first months of life (Valacher and Taylor 2007) Inactivated vaccines (β-propiolactone-inactivated, formalin-inactivated), live vaccines obtained through biotechnological methods (NS deletion mutant, FCS-2 cleavage mutant, SH deletion mutant), and recombinant live vaccines (G protein mutant) have been developed for disease prevention (Scheiber et al., 2000). Studies involving live vaccines produced using G protein deletion have shown that, when experimentally inoculated intranasally in calves, appropriate attenuation of the pathogen was achieved, and neutralizing antibody titers were elevated. However, it has been noted that the serum antibody titers developed due to vaccination were 4-32 times lower than those formed by field strains. On the other hand, although attenuated recombinant BRSV vaccines are considered promising for protection against infection, the divergence of the vaccine virus from the original BRSV field strain due to point mutations and the risk of the virus regaining virulence through serial passage in cell cultures remains a concern (Schmidt et al., 2002).

As a result, in this study, molecular diagnosis made by the RT-nPCR method using primer sets specific to the G Glycoprotein region of BRSV in nasal swap samples obtained from cattle with clinical signs of respiratory system infection. The local BRSV strains we isolated of our research were sequenced and phylogenetic analyzes. As a result of the genetic analysis, differences were detected in the gene region affecting the immunogenicity and pathogenicity of our isolates in Subgroup III. Although there are many studies on virological and serological detection of BRSV, there are a limited number of studies on phylogenetic analysis and revealing the genetic differences of detected local isolates with different BRSV strains. Phylogenetic analysis of BRSV, genetic comparisons with other strains and revealing genome changes in local isolates will shed light on future research on determining the immunological/pathogenic effects of these mutations on isolates. Even, it is concluded that it can provide an infrastructure for genotype-specific vaccine research prepared with local strains that may be needed in the future and pave the way for the development of different diagnostic techniques.

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Ethical Statement: Approval was obtained from the animal testing local ethics committee of Burdur Mehmet Akif Ersoy University. The procedures used in this study adhere to the tenets of the Declaration of Helsinki (MAKU-HADYEK 1045/2023)

Conflict of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions: AK: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. YY: Methodology, Investigation, Data curation. Methodology, Investigation. KA: Writing – original draft, Investigation. ÖSKA: Writing – original draft, Investigation, Methodology.

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