Kocatepe Veterinary Journal

https://doi.org/10.30607/kvj.1819411

Research Article

Molecular Detection of Influenza D Virus in Cattle with Clinical Signs of Respiratory Infection in Burdur Province, Türkiye

Burdur İlinde Solunum Sistemi Enfeksiyonu Klinik Bulguları Gösteren Sığırlarda Influenza D Virusunun Moleküler Tespiti

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Received: 07.11.2025
Accepted: 12.05.2026
Published: 15.06.2026

Citation:
Küçük A. Yıldırım Y. Usta A. Korkmaz Akar ÖS. Molecular Detection of Influenza D Virus in Cattle with Clinical Signs of Respiratory Infection in Burdur Province, Türkiye. Kocatepe Veterinary Journal (2026) 19(2):200-209

Abstract

Influenza D virus (IDV) is an important viral pathogen commonly associated with the etiology of Bovine Respiratory Disease Complex (BRDC). This study aimed to investigate the role and prevalence of IDV in the etiology of respiratory system infections in cattle bred by farmers in the region. Therefore, molecular detection of the agent was carried out using the conventional RT-PCR method with oligonucleotide primers specific to the Hemagglutinin-Esterase-Fusion (HEF) gene region in nasal swab samples obtained from 200 cattle of different breeds, ages, and sexes with clinical symptoms of respiratory system infection in the research area. As a result, a prevalence rate of 1.5% (3/200; %95 CI: 0.3-4.3) was reported. Furthermore, it was determined that affected herds contained imported animals and that newly introduced animals had not undergone an appropriate quarantine procedure. The research results are consistent with prevalence and experimental clinical studies conducted worldwide. Considering the limited molecular studies investigating IDV circulation in cattle in Türkiye, this study provides additional epidemiological data from the province of Burdur.

Key Words: Cattle, Influenza D Virus, Prevalence, RT-PCR

Öz

Influenza D virus (IDV) çoğunlukla Bovine Respiratory Disease Complex (BRDC) etiyolosinde yer alan önemli bir viral patojendir. Bu araştırmada halk elinde yetiştirilen sığırların solunum sistemi enfeksiyonu etiyolojisinde IDV'nin rolü ve prevalansının belirlenmesi amaçlanmıştır. Bu nedenle araştırma bölgesindeki solunum sistemi enfeksiyonu klinik bulguları gösteren farklı ırk, yaş, cinsiyete sahip ve etkene karşı aşılanmamış 200 sığırdan toplanan nazal swap örneklerinde etkenin Hemagglutinin-Esterase-Fusion (HEF) gen bölgesine özgü oligonükleotid primer çifti kullanılarak konvensiyonel RT-PCR yöntemi ile moleküler tespiti gerçekleştirilmiştir. Sonuç olarak 1,5% (3/200; %95 CI: 0,3-4,3) prevalans oranı tespit edilmiştir. Bununla birlikte etken tespiti yapılan işletmelere ithal hayvan alımı yapıldığı ve sürüye yeni katılan hayvanların uygun bir karantina dönemi geçirmediği tespit edilmiştir. Araştırma sonuçları dünya genelinde gerçekleştirilen prevalans ve deneysel klinik çalışmalarla benzerlik göstermektedir. Türkiye'de sığırlarda IDV dolaşımını araştıran moleküler çalışmaların sınırlı sayıda olması göz önüne alındığında, bu çalışma Burdur ilinden ek epidemiyolojik veriler sunmaktadır.

Anahtar Kelimeler: Influenza D Virus, Prevalans, RT-PCR, Sığır

Introduction

Bovine Respiratory Disease Complex (BRDC) is a multifactorial disease that causes severe pneumonia and bronchopneumonia in large herds, threatening animal health and involving by many viral, bacterial, and parasitic factors (Küçük and Yıldırım 2022). The primary pathogens in the etiology of BRDC include Bovine Parainfluenza Virus 3 (BPIV3), Bovine Respiratory Syncytial Virus (BRSV), Bovine Herpes Virus-1 (BHV-1), Bovine Viral Diarrhea Virus (BVDV), *Pasteurella multocida*, and *Mannheimia haemolytica* (Kirchhoff et al. 2014; Küçük and Yıldırım 2022). Recent studies have attempted to clarify the role of IDV in the etiology of BRDC (Yu et al., 2021).

The influenza D virus is a member of the *Deltainfluenzavirus* genus within the *Orthomyxoviridae* family and causes disease in farm animals such as pigs and cattle, which are bred worldwide (Liu et al. 2020). IDV, which has a filamentous morphology and a diameter of 80–120 nm, is enveloped by a lipid envelope composed of a large glycoprotein structure. The pathogen contains a 10-14 kb length, 7-segmented, single-stranded, negative-sense RNA genome. (Taubenberger and Morens 2008; Sederdahl and Williams 2020). Although cattle are the primary host of IDV, it is known that this virus can be transmitted from cattle to pigs (Hause et al. 2013). Furthermore, serological studies have reported the detection of antibodies against IDV in humans, pigs, small ruminants, horses, and buffalo species. This finding has been interpreted as indicating that IDV has a broad host spectrum and may pose a zoonotic threat (Yu et al. 2021). The influenza D virus, like other types of influenza viruses, is usually transmitted through aerosols and droplets. Research has revealed that this virus, first reported in the United States, has spread to almost every region of the world where pigs and cattle are farmed. (Ducatez et al. 2015; Salem et al. 2017). It was assumed that cattle were not susceptible to influenza viruses until IDV was identified. However, with the isolation of this agent from cattle with clinical symptoms of respiratory infection, it was considered a new viral agent for BRDC (Ruiz et al. 2022). Macroscopic findings of the infection include patchy atelectasis, collapse of the parenchymal tissue, and reddish discoloration, particularly in the cranial lobes of the lungs. (Salem et al. 2017). The agent can be detected in samples collected from the upper and lower respiratory tracts of infected animals. Nasal/nasopharyngeal swab materials are commonly used for molecular, virological, and histopathological diagnosis. (Rosignoli et al. 2017). Although various techniques are utilized to diagnose IDV, the most commonly used techniques are conventional and real-time RT-PCR. RT-PCR is performed to detect the viral genome and has significantly higher specificity and sensitivity than other virological and serological methods (Ducatez et al. 2015).

This study aimed to detect IDV molecularly and determine its prevalence in cattle of different breeds, ages, and sexes with clinical signs of respiratory infection, bred by local producers in Burdur province.

Materials and Methods

Animals and Sampling Used in the Study

The study was performed using nasal swab samples collected from 200 cattle of different breeds, ages and sexes with respiratory infection symptoms (dyspnoea, mucopurulent nasal secretion, conjunctival secretion). It was determined that 24.5% (49/200) of the sampled animals were between 0-6 months old, 36.5% (73/200) were between 1-3 years old, and 39% (78/200) were over 3 years old. On the other hand the gender and breed composition of the animals was 45.5% (91/200) female, 54.5% (109/200) male, 54%

(108/200) Simmental, and 46% (92/200) Holstein. Sampling was conducted from cattle farms with 50 to 100 head of cattle located in the city center and districts of Burdur province. The majority of the sampled herds represented combined production systems. Due to local trade practices, cattle movement between farms was common, and strict quarantine procedures were generally not applied to newly introduced animals. Biosecurity measures varied between farms, and most herds were managed in semi-open barns conditions. Study samples collected from cattle were placed in transport tubes containing penicillin (500 IU/mL) + streptomycin (20 mg/mL) and Phosphate Buffer Saline (PBS, 1.5 mL) and brought to the diagnostic laboratory in accordance with the cold chain protocol and the RNA extraction stage was initiated promptly.

A photograph showing bilateral mucopurulent nasal discharge in one of the animals diagnosed with IDV is presented in Figure 1.

Figure 1. Image of animal number 84, nasal bilateral mucopurulent secretions (original)

Total RNA Extraction and RT-PCR

Total RNA was isolated from nasal swab specimens using the acid-guanidinium-phenol method described by Rio et al. 2010. Upon arrival at the laboratory, samples in antibiotic-containing PBS were vortexed thoroughly to ensure homogeneity, followed by centrifugation at 3000 rpm for 20 minutes at 4°C. After centrifugation, 250 μL of the clarified supernatant was carefully transferred into 2 mL RNase/DNase-free microcentrifuge tubes. Subsequently, 750 μL of Hibrizol reagent (MG-TAQMX-03, Hibrigen, Türkiye) was added to each tube, and the mixtures were stored at -80°C until RNA extraction. The extraction procedure was conducted according to the manufacturer's instructions. Following extraction, the lyophilized RNA pellets were reconstituted in 55–60 μL of DEPC-treated water and preserved at -80°C until use in complementary DNA (cDNA) synthesis. Prior to PCR, viral cDNA was synthesized from extracted viral RNA using the ABT one-step cDNA synthesis kit (A.B.T., Türkiye, Cat No: C07-01-20), following the protocol provided by the manufacturer. The Hemagglutinin-Esterase-Fusion (HEF) gene region of IDV

was amplified using the conventional RT-PCR method. Amplification was carried out with gene-specific primers described by Ducatez et al. 2015. The RT-PCR amplification was performed under the following cycling conditions: initial denaturation at 95 °C for 10 min**,** followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 **s**, and extension at 72 °C for 5 **s**, with a final extension step at 72 °C for 10 min**.** Information about oligonucleotides is provided in Table 1.

Each RT-PCR assay included a positive control containing IDV RNA and a negative control (nuclease-free water) to monitor for potential contamination. The prevalence of IDV in the collected samples was determined using the Clopper-Pearson precision method and an estimated confidence interval.

To detect the PCR products, gel electrophoresis was performed. The resulting amplicons were separated on a 1.5% agarose gel at 90 V for 1 hour and visualized under UV illumination using a transilluminator.

Table 1. Information on primers used in amplification

Primer	Oligonucleotid Primers (5′→3′)	Product Size (bp)	Genomic Region	Reference
FluD-HE-667F	GTTTGTGGGACTGAGCAATC	683 bp	HEF	(Ducatez et al. 2015)
FluD-HE-1350R	CCCTGCTTGCGGTATTATC			

Results

Using the conventional RT-PCR technique performed with oligonucleotide primers utilised in the amplification of the conserved position in the HEF gene region of IDV, viral nucleic acid was detected in 1.5% (3/200; %95 CI: 0.3-4.3) of the collected samples (3/200) (Fig. 2).

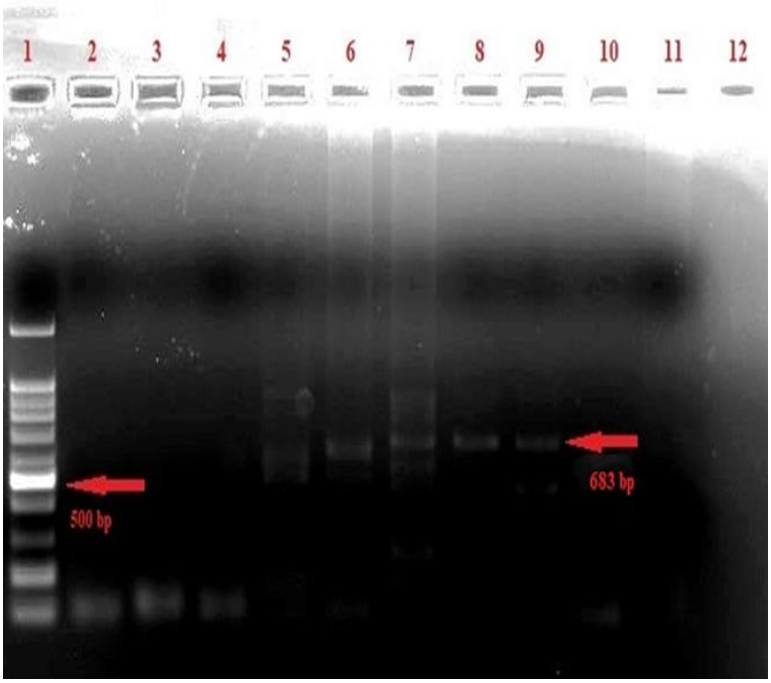

Figure 2. IDV gel electrophoresis image 1: DNA ladder (100 bp); 2-5,11,12: Negative Samples, 7,8,9: Positive Samples, 10: Negative Control, 6: Positive Control (683 bp)

It has been observed that both beef and dairy cattle are raised in herds where Influenza D Virus has been detected. Additionally, it has been noted that animals are included in and excluded from these herds at specific time intervals, but quarantine procedures are not applied to newly included animals. Furthermore, it has been observed that repeated and costly vaccination programs are implemented against viral and bacterial respiratory pathogens frequently isolated in cattle populations. However, it has been observed that the animals in which the virus was detected were housed in semi-open barns characterized by poor air quality and natural yet insufficient ventilation conditions. The clinical and demographic characteristics of IDV-positive animals are summarized in Table 2.

Table 2. Demographic and clinical data of animals identified with IDV

Herds No.	Positive SamplesNo.	Sex	Breed	Age	Symptoms	Onset of Infection	Time to Entry the Herd
7	**82**	♂	Simmental	12 Months	Dyspnea, Fever	< 1 week	< 1 week
7	**84**	♂	Simmental	12 Months	Dyspnea, Fever, Mucopurulent nasal secretion	> 1 week	< 1 week
11	**134**	♂	Simmental	12 Months	Mucopurulent nasal secretion	< 1 week	> 1 week

Discussion

Respiratory system infections in cattle are predominantly multifactorial in nature, with the aetiology involving viral pathogens such as BHV-1, BRSV, BPIV3, BVDV, BAV (Bovine Adenovirus), and BCoV (Bovine Coronavirus); *Mannheimia haemolytica*, *Pasteurella multocida*, *Mycobacterium bovis* (Irsik et al. 2006; Fulton 2009). Environmental and meteorological conditions under which herds are maintained, transportation, inadequate care and nutrition, and other stress factors, together with individual factors such as age and the host's immune status and level, may lead to the emergence of infections in epidemic form and pose a threat to herd health. Recent studies have revealed that IDV is increasingly involved in the aetiology of respiratory system infections in cattle. Surveillance studies conducted in many countries where cattle breeding activities are carried out professionally have detected IDV in the majority of Bovine Respiratory Disease (BRD) outbreaks. This indicates that the virus has become a factor that cannot be ignored in terms of herd health and management (Ruiz et al. 2022; Kwasnik et al. 2023). In this regard, IDV should be considered a new and important factor affecting cattle health on a global scale.

The respiratory mucosa represents the primary replication site of viral respiratory pathogens, and early sampling is critical for successful detection. Therefore, most samples in this study were collected within the first 10 days of clinical symptom onset, when viral shedding is expected to be highest (MacLachlan et al. 2017; Pardon et al. 2020; Küçük and Yildirim 2022).

In this study, IDV RNA was detected in 1.5% of the cattle population in the sample (3/200; 95% CI: 0.3–4.3) using the conventional RT-PCR method; the confidence interval was estimated using the Clopper–Pearson exact method. This result indicates a relatively low detection rate, which may reflect regional epidemiological characteristics or differences in the sampling methodology. Molecular investigations on IDV in Türkiye are still scarce. Although all positive samples were examined for partial sequence alignment, suitable data for phylogenetic analysis could not be acquired. This limitation may be related to various technical factors, such as low viral RNA concentration in clinical samples, possible RNA degradation during sample preservation or transport, or insufficient amplicon quality for sequencing reactions. Therefore, phylogenetic characterization of the IDV strains identified in this study could not be achieved. This represents a limitation of molecular analysis, as the lineage of circulating IDV strains could not be identified. Due to the limited number of positive samples detected in this study, statistical analyses investigating possible associations between IDV infection and demographic variables such as age, breed and sex were not considered statistically meaningful.

The first national report, by Yilmaz et al. (2020), identified viral RNA in 2.2 % (3/76) of cattle nasal swabs collected from various regions. Yesilbag et al. (2022) investigated the molecular presence and genetic characteristics of Influenza D virus in cattle with respiratory disease in Türkiye. In their study, IDV RNA was detected in two nasal swab samples and phylogenetic analysis revealed the circulation of two genetic lineages, D/Yama2019 and the tentative lineage D/Bursa2013. Together, these findings suggest that IDV has been circulating in Turkish cattle populations for several years, although available data remain limited. The present data provide additional epidemiological evidence for the presence of IDV among cattle raised under small-scale husbandry systems in Burdur Province and contribute to a better understanding of the virus's local circulation within Turkish herds.

Comparable prevalence rates have been reported in different geographical regions, including Sweden (2.89%), Pakistan (2.4%), Korea (1.4%), and China (1.8%), while higher values have been observed in some European countries (Chiapponi et al. 2019; Alvarez et al. 2024; Umar et al. 2024; Lim et al. 2023; Gao et al. 2024).

The finding that the prevalence identified in this study is consistent with previously reported global prevalence rates suggests that the observed difference may be due to regional epidemiological conditions or methodological differences. Field surveys have revealed that IDV-positive herds are characterized by inadequate ventilation, frequent animal movement, and deficient quarantine practices; all of which are known risk factors for the transmission of respiratory infections. Additionally, long-term transport and stress-induced immunosuppression may further increase susceptibility to infection (Qi et al. 2024). Clinical findings observed in positive animals, such as dyspnea, mucopurulent nasal discharge, and coughing, were consistent with previous field and experiment studies (Ferguson et al. 2016; Flynn et al. 2018; Lion et al. 2021; Ruiz et al. 2022). Additionally, various studies have reported that IDV frequently co-occurs with other viral and bacterial pathogens related to BRDC, a finding that provides further support for the multifactorial etiology of respiratory diseases in cattle (Ruiz et al. 2022; Vicosa Bauermann et al. 2023). These findings once again highlight the importance of biosecurity measures, particularly the implementation of quarantine for newly introduced animals and the maintenance of appropriate herd management practices (Gaudino et al. 2021) Furthermore, although no definitive evidence has been

reported to indicate that IDV causes severe disease in humans, its zoonotic potential still warrants attention and necessitates further research, particularly regarding individuals with professional contact with cattle (White et al. 2016; Reed 2018; Leibler et al. 2023; Ruiz et al. 2022).

Influenza D virus is increasingly recognized as an important viral contributor to BRDC. Although the prevalence reported in this study was relatively low, the detection of IDV in clinically infected cattle suggests that the virus may play a role in respiratory disease under natural conditions. Therefore, continuous molecular surveillance studies are necessary to better understand the epidemiological role of IDV in BRDC and its circulation dynamics in cattle populations.

Conclusion

In conclusion, this study provides additional molecular evidence regarding Influenza D virus infection in cattle raised under small and medium-scale farm conditions in Burdur province and contributes to the limited epidemiological data on IDV circulation in cattle in Türkiye. The 1.5% (3/200; %95 CI: 0.3-4.3) prevalence identified aligns closely with findings from other countries, suggesting comparable patterns of viral occurrence. The detection of IDV in cattle with clinical symptoms indicates that the virus may contribute to respiratory diseases, particularly in conditions where biosecurity and herd management are inadequate. Its detection in Turkish herds therefore warrants attention in terms of animal health and disease management. Given the economic implications of BRDC—arising from elevated morbidity and mortality rates, increased treatment expenditures, and decreased productivity—the circulation of IDV may contribute to additional financial losses within the livestock sector. Consequently, future investigations should focus on clarifying the epidemiological characteristics of IDV, exploring its interactions with other respiratory pathogens, and supporting the development of effective control and prevention strategies to mitigate its impact.

Declaration of competing 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.

Ethics approval: Ethical approval for this study was obtained from the Animal Experiments Local Ethics Committee of Burdur Mehmet Akif Ersoy University (Approval No:1098)**.** All procedures involving animals were conducted in accordance with national regulations for animal experimentation (MAKÜ-HADYEK 1098/26.04.2023)

Funding: This research was supported by Scientific and Technological Research Council of Türkiye (TÜBİTAK) with 1002/A Rapid support module (Project No: 123O192) as a "Molecular Investigation and Phylogenetic Analysis of Parainfluenza Virus Type 3, Bovine Respiratory Syncytial Virus and Influenza D Virus in Cattle with Respiratory System Infection"

Availability of data and material: All data are presented in manuscript itself. Further details may be obtained through mail alikucuk@mehmetakif.edu.tr

Authors' Contributions: AK and YY contributed to the project idea, design and execution of the study. AK and ÖSKA contributed to the acquisition of data. AK and AU analysed the data. AK, drafted and wrote the manuscript. YY and AK reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

Data availability The data that has been used is confidential.

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