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RESEARCH ARTICLE

Molecular Investigation of Viral Agents in Cattle with Respiratory System Problems#

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Abstract

This study was carried out to investigate viral etiology using molecular methods in cattle with respiratory system problems. The animal material of the study consisted of 200 cattle of different breeds, age and sex, which were not vaccinated against bovine parainfluenza virus 3 (BPIV-3), bovine viral diarrhoea virus (BVDV), bovine respiratory syncytial virus (BRSV) and Bovine adenovirus-3 (BAV-3), brought to the clinics of Kafkas University Faculty of Veterinary Medicine, Department of Internal Medicine, with respiratory system complaints and found to have abnormal respiratory system findings after clinical examination. Blood and swab (if animals had nasal discharge) samples were collected. PCR was performed, positive samples were sent for sequence analysis and the data were subjected to phylogenetic analysis. BVD was detected in 3 of the blood samples collected. From the swab samples; BVD was detected in 8 animals, BPIV-3 in 3 animals, and BRSV in 4 animals. BAV-3 was not detected in any of the collected samples. As a result of the phylogenetic analysis, it was determined that BVD viruses were classified as BVDV-1a, BPIV-3 viruses as genotype C and BRSV viruses as subgroup III (subgroup A). It is thought that these data will be help in guiding in the struggle against viral agents that frequently cause respiratory system diseases.

Keywords: Cattle, Respiratory system, BPIV-3, BVDV, BRSV, BAV-3

Introduction

The etiology of respiratory system diseases in cattle has a complex structure. This disease can be caused by bacteria and viruses and is called Bovine respiratory disease complex [1-3]. The diagnosis of this disease complex is usually made by multiplex ELISA kits [4,5]. Viral agents frequently involved in this disease complex generally include bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus 3 (BPIV-3), bovine viral diarrhea virus (BVDV) and bovine adenovirus (BAV) [4,6].

Bovine adenovirus (BAV) is a non-enveloped DNA virus and belongs to the *Adenoviridae* family. BAV type-3 is found within *Mastedenovirus* genus [7,8]. The agent, which has ten serotypes (BAV 1-10), plays a role in respiratory and enteric infections causing clinical findings such as conjunctivitis, pneumonia, polyarthritis, and diarrhea in calves [9].

BRSV is also known as Bovine orthopneumovirus. Virus is classified in genus *Pneumovirus* which belongs to the *Pneumoviridae* family. Its genetic material is a single-stranded enveloped RNA. The most severe clinical signs of Bovine respiratory syncytial virus (BRSV) are observed in calves aged 2 weeks to 6 months [10-12]. BRSV can be asymptomatic or cause respiratory infections in varying severities. Although cattle under six months of age are more susceptible to the disease, it can be seen frequently in cattle aged 3-12 months [13].

Bovine Parainfluenza type 3 (BPIV-3) is located in the *Respirovirus* genus in the *Paramyxoviridae* family [14-16]. The causative agent is an enveloped RNA virus. It causes clinical outcomes ranging from asymptomatic infections to serious respiratory problems. Cough, fever and nasal discharge are the main clinical findings [17].

Bovine Viral Diarrhea (BVD) virus which is classified

in the genus *Pestivirus* and *Flaviviridae* family [18,19], has an RNA genome [20]. It can cause abortion, diarrhea, respiratory system symptoms or severe pneumonia by involving in mix infections with other agents [18].

The aim of this study was to determine the presence of viral agents in cattle with respiratory system problems and make molecular characterization of these strains.

MATERIAL AND METHODS

Ethical Approval

This study is approved by Kafkas University Local Ethics Committee of Animal Experiments with permission number (KAÜ-HADYEK/2020-031).

Sampled Animals

The study included 200 cattle of different age, sex and breed with clinical respiratory system symptoms that were brought to the clinics of the Department of Internal Medicine, Faculty of Veterinary Medicine, Kafkas University. These animals were not vaccinated against the viruses that were investigated in the study (BPIV-3, BVDV, RSV and BAV-3). From these cattle, 200 blood and 63 nasal swab samples were collected. Swab samples were collected from animals with nasal discharge.

Blood Samples

Blood was collected from the *vena jugularis* into tubes with EDTA (BD Vacutainer®, BD, UK). Blood samples in EDTA tubes were centrifuged at 2000 rpm for 10 minutes. The leukocyte layer was removed with a Pasteur pipette and transferred to stock tubes containing 2 mL Phosphate Buffer Saline (PBS). The samples were stored in a -20°C deep freezer until testing.

Nasal Swab Samples

The swabs were taken by pressing and rubbing to the nasal cavity. Samples were delivered to the laboratory in cold chain. The sticks of the swabs were discarded, tips were put in stock tubes with 2 mL PBS and vortexed vigorously. Afterwards, they were centrifuged in a refrigerated centrifuge at 3000 rpm for 10 min at +4°C. The supernatant was transferred to clean stock tubes and stored in -20°C deep freezer for PCR applications.

DNA/RNA Extraction

The method described by Sambrook et al.[21] was used to extract both DNA and RNA. Extracts of the samples were stored at -20°C until molecular analyses.

Molecular Procedures

After extraction of viral nucleic acids from the samples, complementary DNA synthesis and PCR for BPIV-3, BRSV, BVDV viruses was achieved with 2x One-step RT-PCR master mix (Hibrigen) enzyme kit according to the manufacturer's instructions. For BAV-3, Taq polymerase kit (Hibrigen, Türkiye) was used for PCR after extraction. Primer pairs and optimizations for PCR were applied according to references [9,17,22,23].

Sequencing and Phylogenetical Analyses

Positive samples obtained after PCR/RT-PCR procedures were selected and sent for sequence analysis to a commercial company (BM Lab, Ankara, Türkiye). Raw data from sequence analysis were processed and phylogenetic trees were constructed for each virus. Sequence alignment was performed with Bioedit (Version 7.0.5.3) [24]. Sequence similarities were compared by using the GenBank database and BLAST software (NCBI) [25]. Phylogenetic analysis of gene sequences was performed using MEGA7 software [26]. Neighbor-joining method was chosen for comparison and sequence divergence was calculated by Kimura two-parameter model; confidence level was assessed by bootstrapping using 1000 replicates.

RESULTS

A total of 200 animals were included in the study. The ages of the animals were ranged from 1 day to 7 years old. The age distribution of the animals included in the study is shown in *Table 1*. Of these animals, 84 were female and 116 were male. The breed distribution was 178 Simmental, 3 Holstein hybrids, 5 local breed, 1 Zavot, 12 Brown Swiss and 1 hybrid breed. It was noteworthy that the majority of the animals included in the study were of Simmental breed. All animals had clinical respiratory symptoms (nasal discharge, cough, dyspnea etc.)

It was observed that respiratory system infections were more common in animals up to 1 year of age than in older animals.

A total of 200 blood and 63 swab samples were collected from 200 animals. While three of the blood samples were positive for BVDV, no positivity for other viral agents was detected in the blood. In comparison, BVDV was detected in eight samples, BPIV-3 in three samples and BRSV was detected in four samples from the collected swabs. While three blood samples were positive for BVDV, swab samples of the same animals were also positive. Consequently, eight animals were found to be positive for BVDV.

Table 1. *Age distribution of the animals included in the study*

Age	Number	Ratio (%)
1 day-1 month	54	27%
>1month-≤3 months	49	24.5%
>3months-≤1 year	55	27.5%
>1 year-≤3 years	19	9.5%
>3 years	23	11.5%

Given the unavailability of a positive control, BAV-3 was analyzed without the benefit of such a control. The results demonstrated that BAV-3 was not detected in any of the collected samples.

Of the animals positive for viral agents, 13 were Simmental (7 BVDV, 3 BPIV-3, 3 BRSV), 1 was Brown Swiss (BRSV) and 1 was local breed (BVDV).

It was noted that 6 of the BVD positive animals were 4-7 months old, 1 was 18 months old and 1 was 2 years old; BPIV-3 positive animals were 15 days, 3 months and 4 months old; and 4 BRSV positive animals were 2-7 months old.

It was observed that 13 of the 15 animals positive for viral agents were male and 2 were female.

No mixed infection with respect to viral agents was found in any of the positive animals. Respiratory distress, nasal discharge, coughing, and anorexia were observed in all positive animals.

Table 2. *Vital signs of the virus positive animals*

Viral Agent	Mean Rectal Temperature (°C)	Mean Pulse Rate (Frequency/min)	Mean Respiratory Rate (Frequency/min)
BVDV (swab) (n=8)	39.45	98	42.5
BPIV-3 (n=3)	39.8	82.67	50.67
BRSV (n=4)	39.35	111.5	44

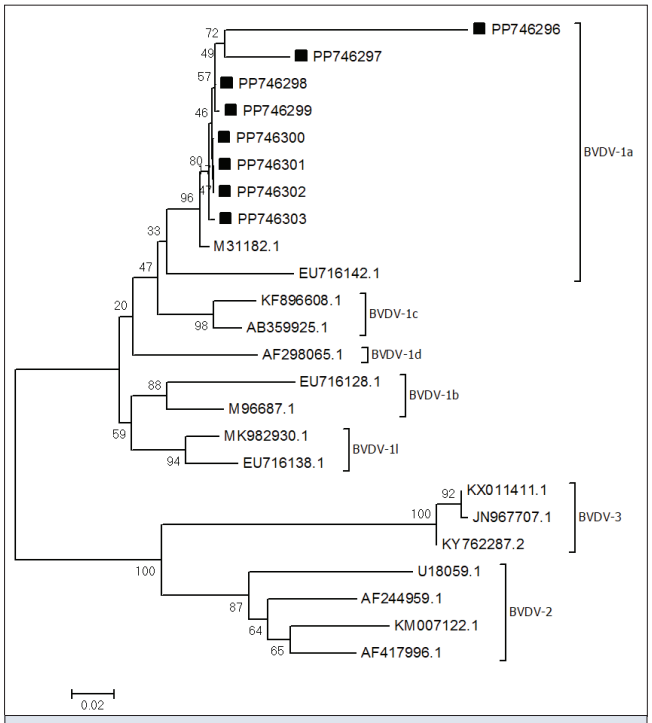

Fig 1. Phyogenetic tree constructed for BVDV using study's sequence data. Study isolates are shown with a black squares

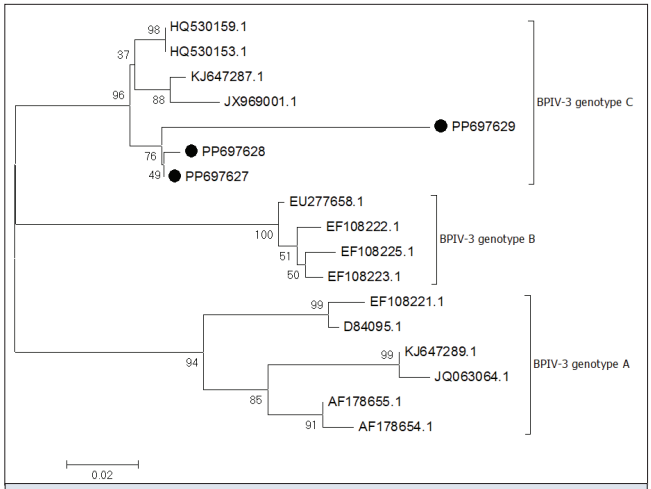

Fig 2. Phyogenetic tree constructed for BPIV-3 using study's sequence data. Study isolates are shown with a black dots

The mean rectal temperature, pulse rate and respiratory frequency of all animals included in the study (n=200) were 39.02°C, 101.38/min and 43.07/min, respectively. The mean rectal temperature, pulse rate and respiratory frequency of the animals (n=185), which were included in the study but had no virus infection, were 38.98°C, 101.61/min and 42.95/min, respectively. The vital signs of the positive animals are shown in *Table 2*.

It was found significant that 6 of the animal included in the study belonged to the same owner and 3 swab samples belonging to these animals were tested positive for BVDV.

Positive amplicons were subjected to sequence analysis and obtained sequences were submitted to GenBank for archiving. The accession numbers of three isolates of BPIV-3 were PP697627, PP697628, PP697629; four isolates of BRSV were PP719911, PP718912, PP718913, PP718914; eight isolates of BVDV were PP746296, PP746297, PP746298, PP746299, PP746300, PP746301, PP746302, PP746303. Given that the sequence data obtained from the blood and swab samples of the same animals were identical, no accession number was assigned to the sequence derived from the blood samples.

As a result of the phylogenetic analyses, the BVDV isolates found in the study were classified as BVDV-1a *(Fig. 1)*, BPIV-3 isolates were classified as genotype C *(Fig. 2)* and BRSV isolates were classified as subgroup III (subgroup A) *(Fig. 3)*. It was determined that the viruses found were similar to the strains previously encountered in Türkiye.

DISCUSSION

Respiratory diseases can cause severe economic losses in sheep [27] and cattle [28]. Among these, viral agents have an important place in cattle and are widely observed in Türkiye [28]. In addition to the disease symptoms they

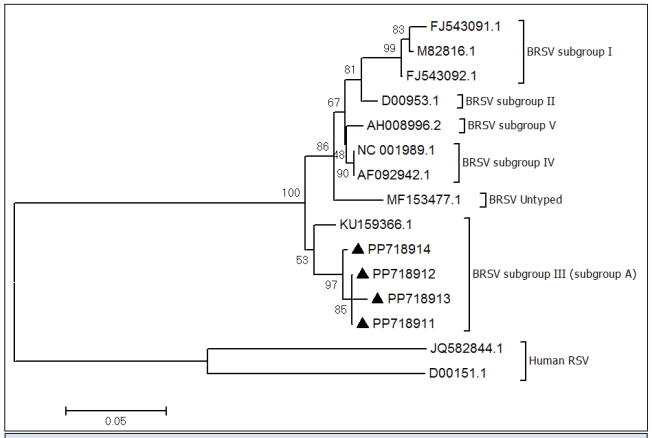

Fig 3. Phyogenetic tree constructed for BRSV using study's sequence data. Study isolates are shown with a black triangles

cause, viral agents that cause respiratory system infections can also suppress the immune system by altering immune mechanisms [29-31].

The success of vaccination against diseases is affected by environmental factors such as proper vaccination procedures and herd management [32]. In addition, the protection of vaccines may change due to the emerging of new strains with ongoing mutations in viruses [33,34]. Phylogenetic reports obtained from regional studies are important for the evaluation of vaccine efficacy. For this reason, it is an important strategy in the fight against diseases to continuously identify and reveal field strains by competent laboratories and to revise vaccine preparations according to this data if different strains are found [32]. The phylogenetic analysis results obtained from this study contributed to the literature in this regard.

One of the problems that occur in multiple infections, since it can be caused by many microbial agents (such as bacteria and viruses) [4], the role of some agents in the clinical picture may be overlooked. Even if one of the agents in infection has a minor role, prognosis may be worsened if that agent is not diagnosed and treated accordingly. Mixed viral infection was not found in this study, this situation has been reported in seroprevalence studies [4,35-38]. Although seroprevalence values vary in studies [36,37], it is seen that viruses infecting the respiratory system frequently coexist. As vaccines against respiratory viruses are usually in combined form, they offer protection against more than one disease. In this regard this situation seems to be advantageous for current picture of the field.

Serologic presence of BVDV has been known for a long time, and its contribution to respiratory system problems has been reported by previous studies, according to these data, the virus is widespread in Türkiye [30,39,40]. Molecularly, BVDV is classified as BVDV-1 (Pestivirus A), BVDV-2 (Pestivirus B) and BVDV-3 (Pestivirus H- also known as HoBi-like), and there are subtypes of these genotypes too [41]. In Türkiye, BVDV-1 is found predominantly, BVDV-2 and BVDV-3 types have also been reported [41-46]. The isolates found in our study were found to be the common BVDV-1 genotype and all isolates were clustered in BVDV-1a subtype. According to literature review this is the first time that molecular characterization has been performed in the study location and this report has made an important contribution to the literature in this respect.

There are serological and molecular reports of infection with BRSV and BPIV-3. The seroprevalence of these two viruses and their effect on respiratory problems have been demonstrated [3-5,11,14,33,39,40,47]. Molecularly, BPIV-3 has three genotypes named as genotype A, genotype B and genotype C, while BRSV is divided into eight subgroups. These are I (former subgroup B), III (former subgroup A), II, IV, V and VI (former subgroup AB), subgroup VII, VIII and a ninth subgroup has been proposed [11,14]. Molecular data from previous studies indicate that field isolates are molecularly similar and that different types have not yet been encountered in circulation. In the reported previous studies, genotypes were identified as genotype C for BPIV-3, while BRSV isolates were reported as subgroup III [3,5,11,14, 33]. The results of our study were similar to those of previous studies and BPIV-3 isolates were characterized as genotype C while BRSV isolates were found as subgroup III. Our study made a contribution by characterizing these two viruses for the first time in the study area.

When the vaccines available in our country and possible antibody responses are examined, it is thought that there will be no problem in protecting against BVDV and BRSV strains that are currently circulating in the field in terms of BVD and BRS viruses [32]. However, with BPIV-3, it has been reported that there may be problems in providing adequate protection against genotype C strains since the vaccines in use are genotype A-based [33,47]. The results of the study are similar to this finding and suggest that genotype C-based vaccines should be imported/produced for more effective immunity. Further studies investigating the immune response are needed in this regard. It would be most effective to repeat the studies for all three viruses at regular intervals to determine the new strain situation in the field and to update the control strategies according to the results obtained.

In conclusion, the viral agents BPIV-3 (n=3), BVDV (n=8) and BRSV (n=4) were molecularly detected in cattle with respiratory problems in the Kars region and were found to play a role in the etiology of respiratory diseases. The fact that the majority of cattle with respiratory problems were less than 1 year old indicates that these animals need more attention in terms of protection and control measures. Phylogenetic analysis indicated that BRSV subgroup III, BPIV-3 genotype C and BVDV-1a are the pathogens circulating in the region. It is considered that

these data will help to guide the fight against viral agents that frequently cause respiratory diseases.

Declarations

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author (EEE) on reasonable request.

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Ethical Statement: The study has been approved by the ethics committee of Kafkas University (Approval No. KAÜ-HADYEK/2020-031).

Conflict of Interest: The authors declared that there is no conflict of interest.

Declaration of Generative Artificial Intelligence: The article and/or tables and figures were not written/created by AI and AI-assisted technologies.

Author Contributions: EEE, NC: Idea and study design. EEE, MS, AHK, EA: Sample collection; NC, VY: Laboratory analyses; EEE, NC: Manuscript preparation. EEE, NC, AHK, VY, EA, MS have read and approved the final manuscript.

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