Detection and molecular characterization of mastadenoviruses in calves with respiratory system infection

İlke Karayel Hacıoğlu1*, Selda Duran Yelken2, Feray Alkan3

1Ankara University, Veterinary Faculty, Department of Virology, Ankara, Turkey
2Siirt University, Veterinary Faculty, Department of Virology, Siirt, Turkey

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*ilkeekarayel@gmail.com

Solunum sistemi enfeksiyonu olan buzaţlarda mastadenovirüslerin tespiti ve moleküler karakterizasyonu

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Abstract

Aim: Bovine adenovirus (BAdV) is one of the viral agents that causes respiratory illness in cattle, along with numerous other viral agents. Although the prevalence of BAdV has been determined by serological studies conducted in our country, there are limited data on the detection of BAdVs in respiratory system samples, and there is no study on their molecular characterization. The aim of this study was to detect and characterize BAdVs of the Mastadenovirus genus.

Materials and Methods: In this study, a total of 64 nasal swabs and one lung sample from 65 cattle of different ages with respiratory system disease symptoms were used. After the extraction of viral DNA, they were tested by PCR using primers targeting the hexon gene region and the expected size of amplicons were sequenced.

Results: According to the PCR results, three samples were found positive and the positivity rate was detected as 4.6%. Out of positive samples, two were able to be sequenced and one clustered in the BAdV-2 serotype while the other was grouped in the BAdV-3 serotype.

Conclusion: In this study, it was revealed that BAdVs are contributing agents in respiratory system infection in our country. Also, this is the first study reporting the molecular characterization of BAdV-2 and BAdV-3 in calves with respiratory system infection in Turkey.

Keywords: Bovine adenovirus, PCR, respiratory system, serotype.

Öz

Amaç: Bovine adenoviruslar (BAdV), birçok viral etkenle birlikte sığrılarda solunum sistemi hastalığı neden olan patojenlerdi biyoloji olarak kabul edilmiş tedyic Ülkenizde BAdV prevalansı, yapılan serolojik çalışmalarla tespit edilmiş olmasına rağmen, solunum sistemi örneklerinde BAdV’nin sıptanmasına ilişkin veriler sunulmadı ve moleküller karakterizasyonları ile ilgili herhangi bir çalışma bulunmamaktadır. Bu çalışmada Mastadenovirus genosunda bulunan BAdV’ların tespiti ve moleküller karakterizasyonu amaçlanmıştır.

Gereç ve Yöntem: Bu çalışmada, solunum sistemi hastalığı semptomları olan farklı yaşta 65 sığırdan alınan toplam 64 burun sürümü ve bir akıkçğer örnekleri kullanıldı. Viral DNA’nın elektroforize edildiğinde, örnekler hekzon gen bölgesini hedefleyen primerler kullanılarak PCR ile test edildi ve beklenen büyükülük amplikonlarının dizin bilgisi elde edildi.

Bulgular: PCR sonucunda üç örnek pozitif bulunmuş ve pozitiflik oranı %4,6 olarak tespit edildi. Dizin bilgileri elde edilen iki örnekten birinin BAdV-2 serotipinde, diğerinin ise BAdV-3 serotipinde yer aldığı saptanmıştır.

Öneri: Bu çalışmada BAdV’erin ülkemizde solunum sistemi enfeksiyonuna katkıda bulunan ajanlar olduğu ortaya koyulmuştur. Ayrıca bu, Türkiye’de solunum sistemi enfeksiyonu olan buzaţlarda BAdV-2 ve BAdV-3’ün moleküller karakterizasyonu hakkında ilk çalışmadır.

Anahtar kelimeler: Bovine adenovirus, PCR, solunum sistemi, serotip
**Introduction**

Bovine respiratory system disease (BRD) is one of the most important problems of livestock breeding worldwide. BRD is defined as a multifactorial disease as it develops due to the interaction between many factors including the host, environment, herd management as well as viral and bacterial infectious agents (Amat 2019). Viruses such as Bovine Respiratory Syncytial virus (BRSV), Bovine Parainfluenza 3 virus (BPIV3), Bovine Herpesvirus-1 (BHV-1), Bovine Coronavirus (BCoV), and Bovine Adenovirus (BAdV) are considered to be the main viral agents involved in this disease (Alkan 1998, Ng et al 2015, Timurkan et al 2015, 2019, Karayel Haciöglu et al 2019, Sevinc Temizkan and Alkan 2021, Toker and Yeşilbağ 2021).

Adenoviruses are non-enveloped viruses containing linear and double-stranded DNA. Their icosahedral capsid possesses 240 hexon proteins, the main capsid component (919-968 amino acids), and 12 fiber attachment proteins linked to 12 penton base proteins involved in recognition and interaction with cellular receptors (ICTV 2020). There are six genera (Mastadenovirus, Aviadenovirus, Atadenovirus, Siadenovirus, Ichtadenovirus and Testadenovirus) in the Adenoviridae family (ICTV 2020). Mastadenovirus and Atadenovirus are the two genera that ten identified BAdVs have been assigned in so far (Paim et al 2021). BAdVs in deduced in Mastadenovirus (subgroup I) are currently BAdV-1 (type Bovine mastadenovirus A), BAdV-2 (type Ovine mastadenovirus A), BAdV-3 (type Bovine mastadenovirus B), BAdV-9 (type Human mastadenovirus C), and BAdV-10 (type Bovine mastadenovirus C), while those within Atadenovirus (Subgroup II) are classified as BAdV-4, -5, -8, and -Rus strain (type Bovine atadenovirus D). BAdV-6 and BAdV-7 serotypes, are members of the genus Atadenovirus, however they have not been approved as species (ICTV 2020). Within the host species, adenoviruses are divided into serotypes according to their immunological characteristics determined by quantitative cross-neutralization tests (Lehmkuhl and Hobbs, 2008). In addition to serological tests, determination of phylogenetic relationships by comparison of sequence data plays an important role in classifying viruses within a genus (Reddy et al 1998).

BAdVs are usually associated with mild respiratory and gastrointestinal infections in cattle, they can also cause more serious respiratory system infections, conjunctivitis, keratitis, and pyrexia in certain cases (Mattson et al 1988, Yagubi et al 1998, Lehmkuhl et al 1999). Generally, young animals are more susceptible and the symptoms are more severe. Clinical symptoms may include nasal and ocular discharge, cough, anorexia, fever, pneumonia, diarrhea and polyarthritis (Mattson et al 1988, Zhu et al 2011, Ng et al 2015, Shen et al 2020). Studies have shown that BAdV is widely distributed worldwide (Zhu et al 2011, Kubota et al 2021, Paim et al 2021). In Turkey, BAdV-1,-2,3 infections were first reported by Burgu and Toker (1985). Subsequently, various seropositivity rate (81.4%-87.87%) was detected in many serological studies (Öztürk et al 1992, Erol et al 2007, Yazici et al 2007, Duman et al 2009, Özgünlük and Gür, 2012, Kale et al 2013, Alpay et al 2014, Avci et al 2014, Koç and Oguzoğlu 2018) whereas there is only one study (Alkan 1998) that reports the detection of subgroup I adenoviruses (BAdV-1, -2, -3,-9, and -10), from nasal discharge of cattle with respiratory disease. However, to date, no reports have focused on the molecular characterization of BAdVs in our country. Therefore, the aim of this study was to investigate and molecularly characterize BAdVs in the Mastadenovirus genus in the samples from cattle with respiratory system disease symptoms.

**Material and Methods**

**Samples**

Out of 65 samples tested in this study, 64 were nasal swabs from cattle, ages 7 days to ≥5 years, with clinical symptoms of respiratory system infection, and one was a lung sample from calves with age 0-3 months. Respiratory disease was defined as the presence at least one of the following signs: nasal discharge, abnormal breathing, respiratory distress, increased respiratory rate and cough. These samples were collected from cattle housed in 15 farms located in four provinces (Ankara, İzmir, Denizli, Tokat) between 2017-2020 (Table 1) by the field veterinarians and were sent to our laboratory in accordance with the transport conditions.

The study protocol was approved by the Ankara University Animal Experiments Local Ethics Committee (Decision No: 2021-22-201).

**Extraction of viral DNA and PCR**

Viral DNA was extracted by using the method by Sambrook et al. (1989) and subjected to semi-nested PCR using primer sets targeting the hexon gene, coding the hexon protein contain type-specific epitopes, as described elsewhere (Sibley et al 2011) with some modifications. PCRs were performed using a Taq DNA Polymerase (Thermo Fisher Scientific, USA) and the reaction mixture consisted of 18.35 μl nuclease free water, 2.4 μl of MgCl₂ (25 mM), 3 μl of 10×Taq buffer, 1.25 μl of each primer, 1 μl of dNTP (10 mM each), 0.25 μl of Taq polymerase (500 U/μl), and 3 μl of DNA. For both rounds of semi-nested amplification, the following PCR protocols were used: initial denaturation for 5 min at 94°C, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, finishing with a final extension at 72°C for 7 min. The amplification products were analyzed by 1% agarose gel.
electrophoresis and visualized under ultraviolet light.

**Sequencing and phylogenetic analysis**

The expected size PCR products were sequenced in both directions with the same primers used for amplification. Cognate sequences of reference BAdV strains representing different serotypes were retrieved from the GenBank database using the BLAST engine. The nucleotide sequences were aligned by using the Aliview Software (Larsson 2014). Phylogenetic analyses were conducted using MEGA X with the Kimura2+G algorithm and the Neighbour-Joining method and bootstrap testing (1000 replicates) (Kumar et al 2018). Nucleotide (nt) and amino acid (aa) sequence identities between sequences were analyzed using online tools (SIAS, http://imed. med.ucm.es/Tools/sias.html).

**Results**

In this study, out of the 65 samples, three nasal swabs were found positive by PCR thus the positivity rate was detected as 4.6% in samples and 20% (3/15) for sampled farms (Table 1). All of the positive nasal samples were from 3 months old calves (Table 2).

Out of the three amplicons of the expected size (588–714 bp), the two were able to be sequenced and the sequence data were deposited in GenBank. Based on the phylogenetic analysis of the partial hexon gene sequences of these two viruses, one BAadV was clustered in the BAadV-2 serotype while the other was grouped in the BAadV-3 serotype. They were named BAadV-2/9640/TUR/2019 (Accession No: OL513130) and BAadV-3/Mrt/TUR/2020 (Accession No: OL513131), respectively (Table 2 and Figure 1).

The nt and aa identities for the partial hexon gene of the detected viruses was compared with those of other BAadVs sequences available from GenBank. Data revealed that these two Turkish BAadVs shared 64.82% nt and 68.39% aa identity to each other. When the sequence of BAadV-2/9640/TUR/2019 compared to those of other viruses in BAadV-2 serotype, the nt and aa identity were found 95.39-100% and 97.34-100%, respectively. Moreover, the nt and aa identities of BAadV-3/Mrt/TUR/2020 hexon gene with other viruses in

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**Table 1. Detailed information of the samples from cattle used in this study**

<table>
<thead>
<tr>
<th>City</th>
<th>Year</th>
<th>Number of farms sampled</th>
<th>Number of Samples</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ankara</td>
<td>2017</td>
<td>1</td>
<td>3</td>
<td>2-4 months</td>
</tr>
<tr>
<td></td>
<td>2018</td>
<td>4</td>
<td>20</td>
<td>10 days-10 months</td>
</tr>
<tr>
<td></td>
<td>2019</td>
<td>1</td>
<td>7</td>
<td>10-15 days</td>
</tr>
<tr>
<td></td>
<td>2020</td>
<td>1</td>
<td>2</td>
<td>≥ 5 years</td>
</tr>
<tr>
<td>Denizli</td>
<td>2018</td>
<td>1</td>
<td>5</td>
<td>7-30 days</td>
</tr>
<tr>
<td>Izmir</td>
<td>2017</td>
<td>1</td>
<td>4</td>
<td>4 years</td>
</tr>
<tr>
<td></td>
<td>2018</td>
<td>1</td>
<td>3</td>
<td>≥ 5 years</td>
</tr>
<tr>
<td></td>
<td>2019</td>
<td>1</td>
<td>2</td>
<td>15 days</td>
</tr>
<tr>
<td></td>
<td>2020</td>
<td>1</td>
<td>4*</td>
<td>0-3 months</td>
</tr>
<tr>
<td>Tokat</td>
<td>2018</td>
<td>2</td>
<td>5</td>
<td>15 days-8 months</td>
</tr>
<tr>
<td></td>
<td>2019</td>
<td>1</td>
<td>10</td>
<td>1-3 months</td>
</tr>
</tbody>
</table>

*One of the samples was the lung tissue from a calf died following severe respiratory infection.

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**Table 2. Detailed information of the positive samples detected in this study**

<table>
<thead>
<tr>
<th>BAadV</th>
<th>Name</th>
<th>Accession No</th>
<th>City</th>
<th>Year</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAadV*</td>
<td>-</td>
<td>-</td>
<td>Ankara</td>
<td>2018</td>
<td>3 months</td>
</tr>
<tr>
<td>BAadV-2</td>
<td>BAadV-2/9640/TUR/2019</td>
<td>OL513130</td>
<td>Tokat</td>
<td>2019</td>
<td>3 months</td>
</tr>
<tr>
<td>BAadV-3</td>
<td>BAadV-3/Mrt/TUR/2020</td>
<td>OL513131</td>
<td>Izmir</td>
<td>2020</td>
<td>3 months</td>
</tr>
</tbody>
</table>

* The serotype of the detected BAadV is unknown as it cannot be sequenced.
the BAdV-3 serotype ranged from 91.65-98.71% and 93.23-100%, respectively.

Discussion

BAdV is considered as one of the important pathogens causing BRD in cattle (Mattson et al 1988, Yagubi et al 1998, Ng et al 2015, Zhang et al 2019, Shen et al 2020). Nevertheless, the extent of BAdV infection in Turkey is still unknown so far. In Turkey, BAdVs have been repeatedly associated with respiratory infections in cattle with different ages, based on serological studies (Öztürk and Yavru, 1992, Erol et al 2007, Yazici et al 2007, Duman et al 2009, Özugül and Gür 2012, Kale et al 2013, Alpay et al 2014, Koç and Oguzoglu 2018). In these studies, various seropositivity rates (8.1-87.87%) for subgroup I viruses (BAdV-1, -2, -3) were reported, in detail, 23.82-87.87% for BAdV-1, 21.9-68% for BAdV-2, 8.1-85.97% for BAdV-3 (Öztürk and Yavru 1992, Erol et al 2007, Yazici et al 2007, Duman et al 2009, Özugül and Gür 2012, Kale et al 2013, Alpay et al 2014). For all that, there is only one
study reporting the presence of BAdV in respiratory system samples from cattle housed in the different herds (Alkan 1998). Since the direct immunofluorescence test using the specific conjugate for subgroup I BAdVs was used as the detection method in this study, no serotype identification was determined for the detected BAdVs. Incidentally, the positivity rate with 4.6% (3/65) of our study is found quite similar to those of Alkan (1998) who also reported that the 4.6% (3/64) subgroup I BAdV positivity in cattle with respiratory system infection. It is known that it is essential to interpret data regarding the population it represents rather than individuals in the epidemiological analyses. It can be considered as remarkable data that BAdV positivity rates for farms were 20% (3/15) and 25% (2/8) in our study and in the previous study (Alkan 1998), respectively. In our opinion, it is not a coincidence that similar results were found in both studies for farms, and this indicates that these viruses are common pathogens in the farms in Turkey, as evidenced by previous studies reporting the high seropositivity rates (Öztürk and Yavru, 1992, Erol et al. 2007, Yazici et al. 2007, Duman et al. 2009, Özgünük and Gür 2012, Kale et al. 2013, Alpay et al. 2014, Koç and Oguzoglu 2018).

For the serotype identification of BAdV, the determination of phylogenetic relationships by comparison of sequence data plays an important role in classifying viruses within a genus (Reddy et al. 1998). In this study, as in other adenovirus studies (Lehmkuhl and Hobbs 2008, Sibley et al. 2011, Shen et al. 2020, Zehra et al. 2020), the hexon gene was selected for molecular analysis because the hypervariable regions of the hexon protein contain type-specific epitopes (Haque et al. 2019). Based on the phylogenetic analysis of the partial hexon gene sequences, one of the positive samples was classified as BAdV-2 and the other as BAdV-3. Both serotypes were determined as contributing agents at the same rate (each 1.5%, 1/65) for respiratory system infections of cattle, although a limited number of samples were used. Unlike BAdV-2, BAdV-3 serotypes have been also reported with different rates in respiratory samples from cattle in several countries. Studies in the United States and China have reported high rates of BAdV-3 detection, with 48% and 78.7%, respectively (Ng et al. 2015, Shen et al. 2020). However, Zhang et al. (2018) reported lower rate (2%) of BAdV-3 in bovine respiratory system samples in China, as in our study (1.5%). We believe that further studies with different population will reveal new epidemiological data.

In this study, the main purpose is the identification of the local BAdV serotypes. The phylogenetic analysis of the BAdV-2/9640/TUR/2019 showed 100% identity with the reference virus of this serotype, Strain 19, and revealed a formation of two different clusters with other BAdV-2 strains from GenBank; NCBI databases (Figure 1). In contrast to the other BAdV-2 sequences deposited in GenBank from Turkey, BAdV-2/9640/TUR/2019 clustered with the reference strain as well as BAdV-2 strains from field runoff and cattle bedding samples in America (Sibley et al. 2011), from a cattle with pneumoenteritis in Japan (Kubota et al. 2021), and from an environmental water sample in India (Zehra et al. 2020). Although it was observed that the virus caused respiratory system symptoms and diarrhea in experimental infections (Darbyshire et al. 1969, Belák et al. 1977), studies showing that it was also detected in calves with severe or mild respiratory tract infections is limited (Kubota et al. 2021). For this reason, the detection of BAdV-2 in the nasal samples from calves ages 3 months in this study provides an important contribution to the literature.

Although studies (Zhu et al. 2011, Ng et al. 2015, Shen et al. 2020) have shown that BAdV-3 has a very wide geographical distribution since its first detection, there is limited sequence data of the hexon gene region in GenBank. These sequence data consists of BAdV-3 strains detected in China, as well as the one detected in the USA (KP264982), and the strains that are 100% homologous to the BAdV-3 reference virus WBR-1 (AF030154). According to the molecular analysis of the partial hexon gene, BAdV-3/Mrt/TUR/2020 clustered in the same branch with the Chinese strains (Figure 1) and found most closely related to BAV3-China/SWUN/DY11/2018 and BAV3-China/SWUN/NB/2017 (98.71% nt and 100% aa identity). All of these Chinese strains were detected in cattle with respiratory system disease (Zhu et al. 2011, Shen et al. 2020) such as the virus detected in this study. Shen et al. (2020) reported the findings indicating heterogeneity of BAdV-3 in their study. However, due to the fact that the presence of limited sequence data of BAdV-3 as mentioned above and no sequences have been reported in our country before, it was not possible to assess on the origin and evolution of our BAdV-3.

Adenoviruses were frequently detected in the samples in combination with other possible agents (Ng et al. 2015, Zhang et al. 2019). This study was designed to investigate the presence and characterization of BAdV in Mastadenovirus genus in the cattle with respiratory system infection, but not to determine the coinfection with other possible etiological agents. Therefore, there is no conclusion of interest for other possible pathogens that cause the infection and/or the coinfection in sampled animals. It is obvious that these data could have been essential for the improvement of knowledge on respiratory system infections. For this reason, besides BAdVs, other possible etiological agents should be provided in further studies.

Conclusion

Data on the genetic diversity of adenovirus in animals are still limited. In this study, the molecular identification of two different BAdV serotypes in calves with respiratory system infection in our country was revealed. We believe that
this study, which provides valuable data on the molecular characterization of local viruses, will contribute to the development of vaccines and the determination of commercial vaccine preferences to protect against adenovirus infection. Additionally, given that BAdVs are frequently detected as a pathogen of respiratory and digestive system infections, as well as environmental samples, and the evaluation of recombinant BAdV-3 as live vectors for vaccination and gene therapy in animals and humans, more research on these viruses is required.

Conflict of Interest

The authors did not report any conflict of interest or financial support.

Funding

During this study, any pharmaceutical company which has a direct connection with the research subject, a company that provides and / or manufactures medical instruments, equipment and materials or any commercial company may have a negative impact on the decision to be made during the evaluation process of the study or no moral support.

References


Sambrook J, Frisch EF, Maniatis T, 1989. Isolation of...


Authors Contributions

Motivation / Concept: İlke Karayel Hacıoğlu, Selda Duran Yelken, Feray Alkan

Design: İlke Karayel Hacıoğlu, Selda Duran Yelken, Feray Alkan

Control/Supervision: İlke Karayel Hacıoğlu, Feray Alkan

Data Collection and / or Processing: İlke Karayel Hacıoğlu, Selda Duran Yelken

Analysis and / or Interpretation: İlke Karayel Hacıoğlu, Selda Duran Yelken

Literature Review: İlke Karayel Hacıoğlu, Selda Duran Yelken

Writing the Article: İlke Karayel Hacıoğlu, Selda Duran Yelken

Critical Review: İlke Karayel Hacıoğlu, Selda Duran Yelken, Feray Alkan

Ethical Approval

This study was approved by the Ankara University Animal Experiments Local Ethics Committee (Decision No: 2021-22-201).