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RESEARCH ARTICLE

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

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Solunum sistemi enfeksiyonu olan buzağılarda mastadenovirusların tespiti ve moleküler karakterizasyonu

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Öz

Amaç: Bovine adenoviruslar (BAdV), birçok viral etkenle birlikte sığırlarda solunum sistemi hastalığına neden olan patojenlerden biri olarak kabul edilmektedir. Ülkemizde BAdV prevalansı, yapılan serolojik çalışmalarla tespit edilmiş olmasına rağmen, solunum sistemi örneklerinde BAdV'nin saptanmasına ilişkin veriler sınırlıdır ve moleküler karakterizasyonları ile ilgili herhangi bir çalışma bulunmamaktadır. Bu çalışmada *Mastadenovirus* genusunda bulunan BAdV'ların tespiti ve moleküler karakterizasyonu amaçlanmıştır.

Gereç ve Yöntem: Bu çalışmada, solunum sistemi hastalığı semptomları olan farklı yaştaki 65 sığırdan alınan toplam 64 burun sürüntüsü ve bir akciğer örneği kullanıldı. Viral DNA'nın ekstraksiyonunu takiben, örnekler hekzon gen bölgesini hedefleyen primerler kullanılarak PCR ile test edildi ve beklenen büyüklükteki amplikonların dizin bilgisi elde edildi.

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

Öneri: Bu çalışmada BAdV'lerin ülkemizde solunum sistemi enfeksiyonuna katkıda bulunan ajanlar olduğu ortaya koyulmuştur. Ayrıca bu, Türkiye'de solunum sistemi enfeksiyonu olan buzağılarda BAdV-2 ve BAdV-3'ün moleküler karakterizasyonunu bildiren ilk çalışmadır.

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

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.

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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 Hacioğlu 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 included 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 (8.1-87.87%) 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 Oguzoglu 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-spesific 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 $MgCl_2$ (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

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

City	Year	Number of farms sampled	Number of Samples	Age
Ankara	2017	1	3	2-4 months
	2018	4	20	10 days-10 months
	2019	1	7	10-15 days
	2020	1	2	≥ 5 years
Denizli	2018	1	5	7-30 days
İzmir	2017	1	4	4 years
	2018	1	3	≥ 5 years
	2019	1	2	15 days
	2020	1	4*	0-3 months
Tokat	2018	2	5	15 days-8 months
	2019	1	10	1-3 months

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

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 BAdV was clustered in the BAdV-2 serotype while the other was grouped in the BAdV-3 serotype. They were named BAdV-2/9640/TUR/2019 (Accession No: OL513130) and BAdV-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 BAdVs sequences available from GenBank. Data revealed that these two Turkish BAdVs shared 64.82% nt and 68.39% aa identity to each other. When the sequence of BAdV-2/9640/TUR/2019 compared to those of other viruses in BAdV-2 serotype, the nt and aa identity were found 95.39-100% and 97.34-100%, respectively. Moreover, the nt and aa identities of BAdV-3/Mrt/TUR/2020 hexon gene with other viruses in

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

BAdV	Name	Accession No	City	Year	Age
BAdV*	-	-	Ankara	2018	3 months
BAdV-2	BAdV-2/9640/TUR/2019	OL513130	Tokat	2019	3 months
BAdV-3	BAdV-3/Mrt/TUR/2020	OL513131	İzmir	2020	3 months

* The serotype of the detected BAdV is unknown as it cannot be sequenced.

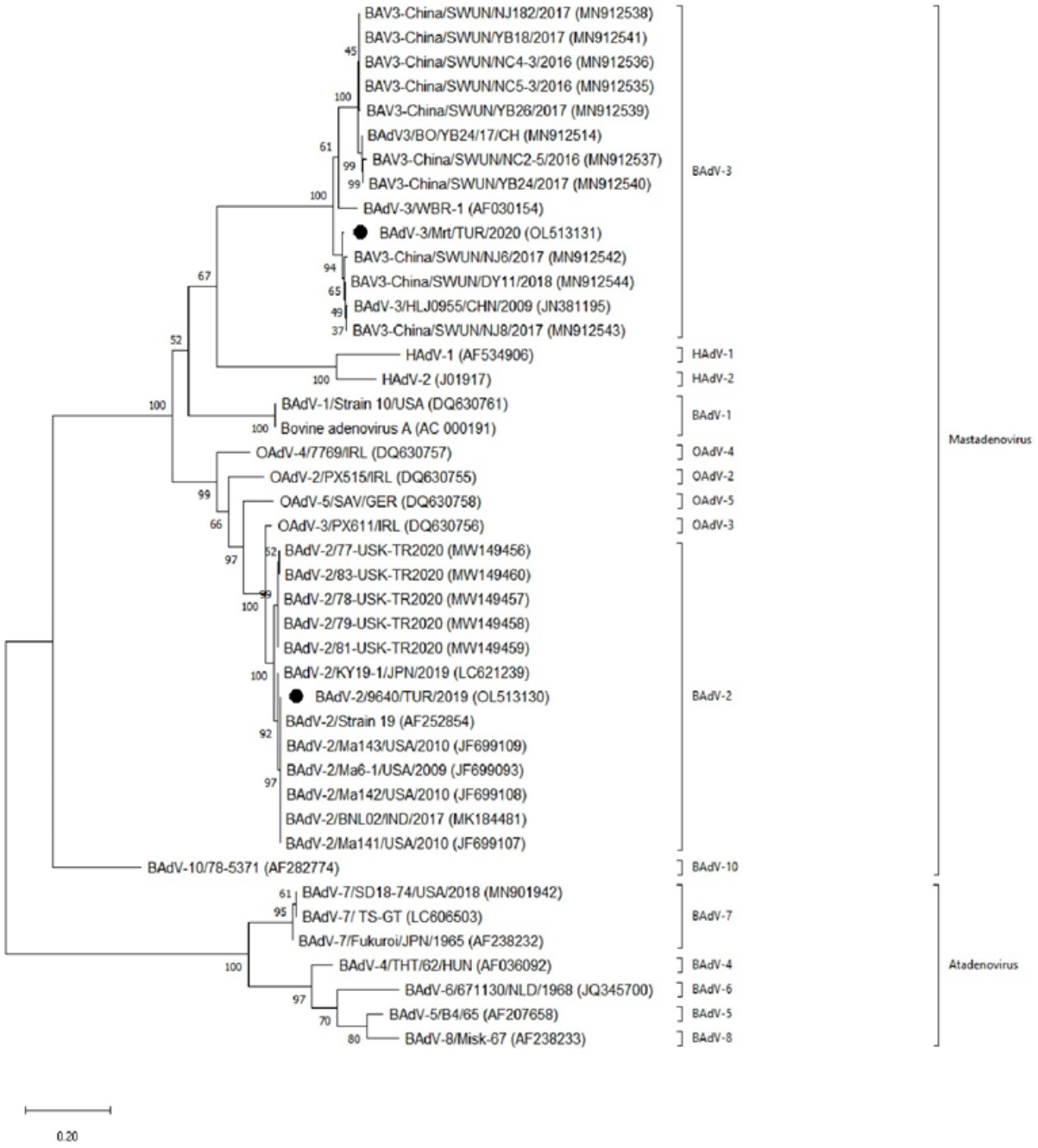


Figure 1. Neighbour-Joining phylogenetic tree based on the partial nucleotide sequences (580 bp) of the hexon gene. The strains investigated in this study are indicated by black dots.

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, Özgünlük 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, Özgünlük 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 that 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, Özgünlük 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/NJ8/2017 (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.

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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.

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Ethical Approval

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

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