First Isolation, Molecular Characterization, and Serological Survey of Bovine Adenovirus Type 2 in Japan

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Research Article

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Abstract

Bovine adenovirus type 2 (BAdV-2), a member of the Mastadenovirus genus of the Adenoviridae family, is involved in mild respiratory disease and is rarely isolated from cattle in the world. Here, we isolated BAdV-2 for the first time in Japan, from the feces of cattle with severe respiratory symptoms. The isolate, designated strain KY19-1, was identified morphologically and genetically. Electron microscopic observation of infected cells showed intranuclear, non-enveloped virus particles, approximately 70 nm in diameter and with typical adenoviral morphology. Whole-genome sequencing revealed that the viral genome is 33,175 bp long, with 105 bp inverted terminal repeats and encoding 32 predicted genes. KY19-1 has a similar genomic organization to the prototype BAdV-2 strain No. 19 with overall 99.1% nucleotide identity. Notable differences were found in the E3 region of the genome, which may affect various biological properties, including pathogenicity. The seroepidemiology of BAdV-2 was also investigated, using a virus-neutralization test, and 92.8% of the Japanese cattle (n=1,325) were seropositive, with the positivity rate increased by age. Further experimental infections with KY19-1 may elucidate the pathogenic properties of BAdV-2 and the importance of this virus as a causal agent of bovine respiratory disease.

Introduction

Bovine adenoviruses (BAdVs) are members of the Mastadenovirus genus of the Adenoviridae family and are widespread in cattle throughout the world. Affected animals are mostly asymptomatic but sometimes show various clinical signs, including pyrexia, rhinitis, pneumonia, diarrhea, conjunctivitis, and polyarthritis [1, 2]. Serologically, BAdVs are divided into ten types (BAdV-1 to 10) [3]. The most well studied serotype is BAdV-3, whereas the biology and seroepidemiology of BAdV-2 have been reported less frequently. BAdV-2 was first isolated in 1959 in Hungary from the feces of clinically healthy cattle [4]. And, later, similar viruses were isolated from lambs with pneumoenteritis [5]. The isolates from cattle and sheep can be differentiated, based on restriction endonuclease digestion patterns and haemagglutination characteristics, and serotype BAdV-2 is divided into two groups, subtypes A and B [5, 6]. Viruses of each subtype are capable of infecting both cattle and sheep, but the pathogenicity varies, depending on the subtype. The prototype strain No. 19 of subtype A usually is apathogenic but sometimes causes mild respiratory and enteric symptoms in experimentally infected calves and sheep, whereas strain ORT/111 of subtype B is more pathogenic and causes severe pneumoenteritis in both species [7, 8].

A serological survey in 1998 revealed that BAdV-2 is the most prevalent adenovirus serotype in Hungarian cattle and sheep [9]. Since then, the isolation and serological survey of BAdV-2 in cattle and sheep has not been reported. Only partial genomic sequences of the hexon gene were obtained from environmental samples in Spain, the US, and India, and currently are available in the GenBank database (accession nos. AY288817, JF699093, JF699107-9, and MK184481) [10, 11].

Up to now BAdV-3, 4 and 7 have been isolated from cattle in Japan with respiratory and enteric diseases and the prevalence of these viruses was also determined by serological surveys [12–15]. There has been
no reported isolation of or pneumonia cases potentially caused by BAdV-2 infection. Here, we describe for the first time the isolation and molecular characterization of BAdV-2 from a calf with severe pneumonia, and we clarify the seroprevalence of the serotype in calves and cows in Japan.

**Materials And Methods**

**Case description**

During February and March in 2019, an epidemic of pneumoenteritis in cattle occurred on a dairy farm in Kyoto, Japan. In this farm, a 20-month-old Holstein-Friesian calf (#1) exhibited typical symptoms, including loss of appetite, slight fever (40.1°C), dyspnea with cough, and conjunctival hyperemia. This and other affected animals were treated with antibiotics but recovered very slowly, therefore, viral agents were suspected to be involved in the disease. Clinical samples, including sera, nasal swabs, and stools were collected from the animals, and referred to the Chutan Livestock Hygiene Service Center for viral and bacterial examination. The clinical condition of calf #1 had improved two weeks after the onset.

**Viral isolation and bacterial culture**

Approximately 1 g of fecal samples was homogenized in 9 mL of maintenance medium (MM), minimal essential medium (MEM) containing 10 mg/ml gentamicin (Takata Pharmaceutical, Saitama, Japan), and centrifuged at 3,000 rpm for 20 min. The supernatant was filtrated through a 0.45 mm cellulose acetate filter membrane (Advantec, Tokyo, Japan) and stored at -80°C until use. Madin-Darby bovine kidney (MDBK) cells were used for virus isolation. Cells and all chemicals used in this study were checked beforehand by RT-PCR [16] and all were pestivirus-free. Fecal homogenates were inoculated into MDBK cells. The inoculated cells were incubated at 37°C, 5% CO₂ for 1 h, then MM was added and the cells were maintained in the same culture conditions. The cells were checked daily using an inverted microscope (Nikon, Tokyo, Japan) and monitored for the appearance of cytopathic effects (CPE). When 80% of the cells showed CPE, the cell culture was harvested, vortexed vigorously, and centrifuged at 3,000 rpm for 10 min to remove cellular debris. The supernatants were stored at -80°C.

The fecal homogenates were also inoculated onto blood agar (supplemented with 5% horse blood) and deoxycholate-hydrogen sulfide-lactose agar and incubated aerobically at 37°C for 1-2 days.

**Transmission electron microscopic analysis**

For transmission electron microscopy, infected MDBK cells were detached from the petri dish using a rubber policeman. The cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4), pelleted by centrifugation, post fixed with 1% osmium tetroxide in 0.1 M PB, dehydrated in graded ethanol, and embedded in LUVEAK-812 resin (Nacarai Tesque Inc., Kyoto, Japan). Ultrathin sections were stained with uranyl acetate and lead solutions and observed using a JEM-1400Flash transmission electron microscope (Jeol Ltd., Tokyo, Japan).
PCR detection

Total DNA and RNA were extracted simultaneously from the fecal homogenates and cell culture supernatants using a MagDEA Dx SV magtraction reagents (Precision System Science Co. Ltd., Matsudo, Japan) according to the manufacturer's instructions. A nested PCR assay was conducted to detect the BAdV hexon gene, as described previously [10]. The amplicons were gel purified using a QIAquick Gel Extraction kit (Qiagen) and direct sequencing was performed with forward and reverse primers using an ABI PRISM Big Dye Terminator cycle sequencing kit (Applied Biosystems) and an ABI 3130 genetic analyzer (Applied Biosystems). The nucleotide sequences of both strands were obtained for verification and the finalized sequences were used for a BLAST search. Other respiratory viruses, including bovine viral diarrhea virus (BVDV), bovine infectious rhinotracheitis virus (IBRV), bovine respiratory syncytial virus (BRSV), bovine parainfluenza 3 virus (BPI3V), and bovine coronavirus (BCoV), were also investigated by PCR or RT-PCR, as described previously [16-19].

Complete genome sequencing and phylogenetic analysis

The infected cell culture supernatants were collected six days after virus inoculation and centrifuged to remove cellular debris. The stocks were further semi-purified by discontinuous sucrose density gradient ultra-centrifugation and dissolved in phosphate-buffered saline (pH 7.4). DNA was extracted using a QIAamp MinElute Virus Spin kit (Qiagen) and submitted to Macrogen Japan Co. Ltd (Tokyo, Japan) for whole-genome sequencing. Briefly, sequence libraries were constructed using a Nextera XT DNA library preparation kit (Illumina, Tokyo, Japan). DNA sequencing was performed with a deep sequencing protocol using Novaseq 6000 (Illumina). De novo assembly of the resultant reads and determination of 5’ and 3’ terminal sequences were performed as described previously [20]. The sequences were aligned using a Muscle program available in MEGA X (https://www.megasoftware.net/) with reference adenovirus strains retrieved from GenBank. Phylogenetic trees were constructed using MEGA X with maximum-likelihood methods and 1,000 bootstrap replicates.

Virus neutralization tests

Calves and cows from the 36 dairy farms were selected randomly for sampling. A total of 1,325 serum samples and the isolated virus were used for virus neutralization (VN) tests. Serial two-fold dilutions of sera, heat-inactivated at 56°C for 30 min, were incubated in 96-well tissue culture plates with an equal volume of a viral suspension containing 200 median tissue culture infective doses (TCID50)/0.05 ml of the isolate at 37°C for 1 h. An MDBK cell suspension containing 1.2 x 10^5 cells/ml was added with 0.1 ml of each serum-virus mixture, and the mixtures were incubated at 37°C for 7 days. VN antibody titers are expressed as the reciprocal of the highest dilution of serum that inhibited CPE completely.

Results

Viral and bacterial isolation
Fecal samples collected from a group of dairy cattle with acute respiratory and enteric disease were inoculated onto cultures of MDBK cells. The third passage of one sample from the calf #1 displayed obvious CPE after two days of MDBK cell culture (Fig. 1A). The cells were rounded and shrunken, and eventually detached from the plastic surface. Infective titers of the isolates were a constant $10^{4.5}$ to $10^{5.0}$ TCID$_{50}$/ml after passage three. Viral isolation from other samples, such as nasal swabs, was unsuccessful. No CPE was observed in the negative control cells (Fig. 1B). On the other hand, respiratory and enteric disease related bacteria could not be isolated from the fecal samples using common culture methods.

**Morphological and genetic identification of the isolate**

Ultrastructurally, the nuclei of infected MDBK cells contained numerous nonenveloped viral particles (Fig. 2). The particles show concentric morphology and are approximately 70 (from 65 to 80) nm in diameter, consistent with the characteristics of adenovirus virions.

PCR revealed that both the cell culture supernatant and its inoculum were positive for BAdV, and negative for BRSV, BCoV, BPIV3, IBRV, and BVDV (data not shown). A BLAST search revealed that the sequence of the amplified fragment was related to the BAdV-2 strain No. 19 (GenBank accession number AF252854) with 99.5% nucleotide identity. The isolate was designated BAdV-2 strain KY19-1 and its partial hexon gene sequence was deposited in the GenBank database with the accession no. LC528154.

**Whole genome sequencing**

The complete genome of the KY19-1 is 33,175 bp in length with a G+C content of 43.7%, 105 bp inverted terminal repeats (ITRs) at both end of the genome, and 32 predicted coding genes. The locations and the predicted functions of all the ORFs in the KY19-1 genome are summarized in Table 1. The full-length genome sequence identity between the KY19-1 and the No. 19 is 99.1%. The overall genome organization of KY19-1 exhibits generally a colinear gene arrangement similar to that of No. 19, with minor differences (Fig. 3A). Most significant differences were found in the coding sequences in the E3 region. A 95 bp insertion, 3 single nucleotide indels, and 11 mutations were found within the 1.9 kb E3 region (data not shown). Consequently, the KY19-1 genome encodes one large E3 ORF, 1,902 bp in size, whereas, the No. 19 genome encodes five E3 small open reading frames (ORFs), with a minimum of 117 bp (38 aa) and a maximum of 537 bp (178 aa) in size, in the homologous region (Fig. 3B). The ORF coverage is 89.2% in total and amino acid identities between the two viral strains are 86.4% on average in the E3 region.

**BAdV-2 subtyping using phylogenetic analysis**

A phylogenetic tree constructed with the complete hexon genes of different adenoviruses showed that KY19-1 clustered with BAdV-2 No. 19 with a 100% bootstrap value (Fig. 4A). The other BAdVs in the phylogenetic tree clustered within four different genera of the *Adenoviridae* family, namely *Mastadenovirus*, *Atadenovirus*, *Siadenovirus*, and *Aviadenovirus*, and their branching patterns were in agreement with the presently accepted classification of the family *Adenoviridae*. However, it was
impossible to determine whether KY19-1 is subtype A or B from this phylogeny, because hexon gene sequences of subtype B are not currently available. To determine the subgenotype of KY19-1, a phylogenetic analysis was carried out using the complete fiber genes of different adenoviruses. The analysis showed that KY19-1 clustered with No. 19, with a 100% bootstrap value, and so was classified into subtype A of BAdV-2 (Fig. 4B).

Seroepidemiological survey of BAdV-2

In a serological survey, 1,230 of 1,325 calves (92.8%) from 36 farms tested were positive for neutralizing antibodies to KY19-1. In a comparison by age group, the antibody prevalence rates were 57.5% for calves below the age of one year, 85.9% for breeding cows up to the age of two years and 96.0% or higher for adult cows over two years (Table 2). Geometric mean antibody titers were relatively low below the age of one year, had increased at two years, and remained at a high level over the age of two years.

Discussion

A virus strain, producing marked CPE in MDBK cells, was isolated from a calf fecal sample collected from a dairy farm in Japan. Typical adenovirus morphology and morphometry were observed in ultra-thin sections of the infected MDBK cells. The isolate was confirmed to be BAdV-2 by genetic analyses, and designated strain KY19-1. Based on the fiber gene sequences, KY19-1 was classified into subtype A of BAdV-2. Previous reports have shown that subtype A viruses are usually isolated from clinically healthy calves but sometimes may be isolated from calves with mild respiratory symptoms [4, 21]. In our study, KY19-1 was isolated from a calf with severe respiratory symptoms. No other viral and bacterial respiratory pathogens were detected in the calf. Although the pathogenicity of the KY19-1 is not proven experimentally, the virus may possibly be involved in bovine respiratory disease.

Sequencing of KY19-1 provides the second complete genome sequence of BAdV-2, following the prototype strain No. 19. Comparative analysis of these genomes revealed that there are crucial differences in the protein coding sequences of the E3 region. Although the ORF coverage and amino acid identities are relatively high, the fragmentation of primarily translated E3 proteins can cause impairment of some of their innate functions. A previous study by Belák et al. also focused on the diversity of this genomic region, they report that the E3 genes in the BAdV-2 genome vary significantly among virus strains [22]. Other reports have revealed that the adenovirus E3 region is not essential for viral growth in cell culture but has important roles in suppression of the cellular immune response, promotion of progeny virus release, and lysis of infected cells [23, 24]. In this way, the adenovirus E3 region can affect pathogenicity in vivo and propagation in vitro. Our isolate KY19-1 produces marked CPE and multiplies efficiently in MDBK cells to generate a high titer virus suspension. Other representative strains, No. 19 and ORT/111, also produce a high titer virus suspension in MDBK cells with marked CPE, at a similar level [25]. Differences in the BAdV-2 E3 region may be more involved in pathogenicity in vivo than propagation in vitro. Further comparative analysis of this genomic region between the KY19-1 and subtype B viruses will contribute to the elucidation of the pathogenic mechanisms of BAdV-2.
A high seroprevalence of BAdV-2 antibodies has been reported in the 3-8-month-old Hungarian calf population, with a serum positivity rate of 55.9% [9]. In the present serological survey, 57.5% of the calves up to one year old were seropositive for BAdV-2 neutralizing antibodies. The positivity rate in Japan is similar to that in Hungary. An increase in antibody titers in the first two years of life implies active infection during this period. The overall positivity rate for all ages was 92.8%, suggesting that BAdV-2 infection is common in cattle in Japan. Our survey data show that the positivity rates and the mean VN antibody titers of BAdV-2 do not decrease with age, the immunity obtained from the initial infection may last for life. In the preliminary test of calf #1 and six cohabiting calves and cows, increases of the serum antibody titers against KY19-1 were observed in all the animals using paired sera, two of these animals showing clinical signs exhibited seroconversion from negative to positive, suggesting that respiratory symptoms are likely to appear in naive cattle at the time of initial infection. Although the significance of BAdV-2 infection in cattle is not yet fully understood, involvement of this virus in respiratory disease is inferred from the epidemiological situation, the clinical course, and the laboratory tests in this case. Therefore, we should pay more attention to BAdV-2 for future diagnosis of bovine respiratory disease.

Declarations

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Author contributions

NK and SH prepared the manuscript. SH designed and coordinated the study. NK, SO, AK and KK collected the samples and performed the experiments. NK, AK and KK designed and performed the data analysis. All authors have read and approved the final manuscript.

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Data availability

The raw read data and the complete genome sequence of BAdV-2 strain KY19-1 have been deposited in the SRA under the accession no. DRA011649 and in GenBank under the accession no. LC621239.

Compliance with ethical standards

Conflicts of Interest
The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Ethics Statements**

Animal housing and sampling in this study conformed to the institutional guidelines approved by the ethics committee of National Institute of Animal Health.

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Tables
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**Figures**

![Figure 1](image)

**Fig. 1**

**Figure 1**

CPE induced by the KY19-1 in MDBK cells 72 h after infection. Infected (A) and noninfected control (B) cells are shown. Bar, 100 μm.
Figure 2

Transmission electron micrograph of MDBK cells infected with KY19-1. Many viral particles with adenovirus morphology may be seen in the nucleus. Bar, 200 nm.
Gene organization of KY19-1 with the E3 ORF highlighted. (A) Predicted protein-coding regions are depicted as black arrows in the appropriate orientation and rectangles denote protein-coding exons. ITR elements located at both ends of the genome are shown as black arrow heads. The E3 ORF is highlighted by a gray arrow. The numbers are nucleotide positions in the KY19-1 genome. (B) E3 ORFs in the KY19-1 and No. 19 genomes are shown by gray and white arrows, respectively. Predicted protein names or functions, coverages and identities of the No. 19 E3 proteins compared with the KY19-1 E3 protein are denoted. The numbers are predicted amino acid positions of the E3 proteins.

**Fig. 3**

**Figure 3**

Gene organization of KY19-1 with the E3 ORF highlighted. (A) Predicted protein-coding regions are depicted as black arrows in the appropriate orientation and rectangles denote protein-coding exons. ITR elements located at both ends of the genome are shown as black arrow heads. The E3 ORF is highlighted by a gray arrow. The numbers are nucleotide positions in the KY19-1 genome. (B) E3 ORFs in the KY19-1 and No. 19 genomes are shown by gray and white arrows, respectively. Predicted protein names or functions, coverages and identities of the No. 19 E3 proteins compared with the KY19-1 E3 protein are denoted. The numbers are predicted amino acid positions of the E3 proteins.
Figure 4

Phylogenetic trees of the complete (A) hexon and (B) fiber genes of KY19-1 with reference adenovirus strains retrieved from the GenBank database. The trees were constructed using the maximum-likelihood method with bootstrap analysis (n=1,000). Boot strap values are shown next to the branches. The GenBank accession numbers of hexon genes of adenovirus reference strains are as follows: PAdV-5 (porcine adenovirus 5; NC_002702), BAdV-1 (AC_000191), BAdV-3 (NC_001876), CAdV-1 (canine adenovirus 1; AC_000003), CAdV-2 (canine adenovirus 2; AC_000020), HAdV-2 (human adenovirus 2; AB330083), HAdV-7 (human adenovirus 7; AB330088), BAdV-4 (NC_002685), BAdV-6 (NC_020074), BAdV-7 (LC597488), FrAdV-1 (frog adenovirus 1; NC_002501), TAdV-3 (turkey adenovirus A; AC_000016), RAdV-1 (raptor adenovirus A; NC_015455), FAdV-1 (fowl adenovirus A; NC_001720), FAdV-2 (fowl adenovirus 2; KT862805). The GenBank accession numbers of fiber genes of adenovirus reference strains are as follows: BAdV-2 strain No.19 (AF308811), BAdV-2 strain ORT/111 (AAN75194), BAdV-1 (AC_000191), HAdV-2 (KC585032), HAdV-7 (KT266800), BAdV-3 (NP_046331), CAdV-1 (KP840544), CAdV-2 (Z37498), PAdV-5 (AF186621), BAdV-4 (NP077404), BAdV-6 (YP_007347014), BAdV-7
(BCO10937), FrAdV-1 (NC_002501), TAdV-3 (AC_000016), RAdV-1 (NC_015455), FAdV-1 (NC_001720), and FAdV-9 (fowl adenovirus 9; AF083975).

**Supplementary Files**

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