Characterization of Akabane virus (KV0505) from cattle in Korea

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Abstract: Akabane disease is caused by an arthropod-borne viral pathogen and leads to congenital abnormalities of the central nervous system in infected ruminants. One isolate, KV0505, showed cytopathic effect in Vero cells. The KV0505 isolate was obtained from plasma, which was collected from a cattle raised on Jeju Island in May 2005. Jeju Island is located near the southern part of the Korean peninsula. The isolate was confirmed as Akabane virus (AKAV) by immunofluorescence assay using AKAV specific monoclonal antibodies and reverse transcription polymerase chain reaction (RT-PCR). Suckling mice inoculated with the isolate showed signs of paralysis and died within 10 days post-inoculation. Comparisons of the KV0505 N gene sequence with 39 other known AKAV strains revealed nucleotide homologies ranging from 83.6% (MP496 strain) to 99.7% (M171 strain). When compared with the K-9 strain, which was isolated from a cow in Korea in 1994, the nucleotide sequence homology with the N gene was 99.7%. Thus, genes of the KV0505 isolate were closely related to those of the M171 strain, which were clustered into the Ic group of AKAV.

Keywords: Akabane virus, cattle, isolation, nucleotide sequence

Introduction

Akabane virus (AKAV) is a mosquito-borne pathogen that affects cattle, sheep, and goats. Akabane viral infection mainly occurs in ruminants during the summer rainy season when transmission vectors are active [5]. Several AKAVs have been isolated from mosquitoes and the infected materials of cattle in Japan, Taiwan, Australia, Israel, and Korea [1, 4, 6, 13, 14, 18]. In general, AKAV infection in susceptible ruminants causes short-duration subclinical viremia, which does not demonstrate significant clinical signs. Infection within the first 3 months of pregnancy results in a relatively low incidence of the disease; however, infection during mid- to late gestation results in stillbirths, abortions, and congenital arthrogryposis-hydranencephaly syndrome [11, 15]. Recently, Akabane disease was reported in a 5-month-old calf and adult cow [12, 14, 20]. AKAV is transmitted via the bites of midges of the family Culicoides; depending on the geographical region, this may include the species C. brevitarsis, C. ovisomata and C. nebeaulosus [12]. The diagnosis of Akabane viral infection is based on clinical, pathological, and epidemiological observations and may be confirmed by the detection of antibodies in the thoracic fluid of aborted fetuses [4]. Several methods, including serum neutralization (SN) tests, hemagglutination inhibition tests, and enzyme linked immunosorbent assay, have been used to detect specific antibodies against AKAV [10, 19]. In addition, RT-PCR and real-time RT-PCR methods have been used for the confirmatory diagnosis of Akabane disease by identifying the viral genome [5, 18].

AKAV belongs to the Simbu serogroup, genus Orthobunyavirus, family Bunyaviridae, and consists of three segmented RNA genomes designated L (large), M (medium), and S (small). The L segment RNA encodes the polymerase gene, which plays a role in transcriptase activity [3], whereas the M segment RNA encodes two envelope glycoproteins, Gn and Gc, which are responsible for viral neutralization. The S segment RNA contains nucleotides 858 bases in length and encodes two proteins: nucleocapsid (N) and nonstructural
(NS) proteins [7, 8]. AKA viruses, including field isolates of AKAV, display considerable variation in amino acid and nucleotide sequences. Based on the phylogenetic analyses of deduced amino acid and nucleotide sequences, AKA viruses have been grouped into four or more clusters [1, 18, 21]. It is important to analyze the genetic variation of AKAV isolates to improve the preventive measures and diagnostic methods available to combat the disease.

Our aim was to report the molecular characteristics of a strain of AKAV that was isolated from the plasma of a native Korean cow that resulted in an abortion in Jeju, March 2005.

Materials and Methods

Virus and cells
The Akabane, Anio, and Chuzan viruses used for SN test were K-9, KSA9910, and K-47 strain, respectively. The three viruses were propagated in Vero cells cultured in α-minimum essential medium (MEM; Gibco BRL, USA) with antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin), 5% fetal bovine serum (FBS) free of BVDV and antibodies against BVDV (Gibco BRL, USA). Uninfected cell cultures were used as negative controls.

Serum neutralization (SN) test
The fifteen serum samples were obtained from a cattle farm showing reproductive disorders. The SN test was carried out in 96-well microplates using Vero cells. A 50 µl aliquot of each dilution of heat-inactivated serum was mixed with an equal volume of 100-200 TCID50 of each virus and incubated at 37°C for 1 h. A total of 100 µl of Vero cells were then added to each well at a concentration of 2 × 10⁴ cells in α-MEM containing 5% FBS. The microplates were incubated for 7 days and examined for CPE, and immunofluorescence antibody assay was conducted using AKAV specific monoclonal antibodies (Data not shown). The virus isolate was cloned three times using the limit dilution method and propagated on Vero cells grown in α-MEM supplemented with 5% FBS.

Mouse inoculation of the virus isolate
To check the pathogenicity of the virus isolate, the suckling mice (ICR strain) were inoculated by intracranial route with 0.03 ml of the isolate and clinical signs of the suckling mice were observed for 15 days.

RNA extraction and RT-PCR
RNA isolation kit (Bioneer, Korea) was used to isolate RNA from 250 µl of cell culture supernatants according to the manufacturer’s instructions. A conventional RT-PCR using specific primers, which amplify a specific region of Akabane viruses, was used for detection and cloning of the viral S RNA segment (Table 1). The RT-PCR was carried out in a reaction mixture containing 10 µl of denaturated RNA, 10 µl of 5 x buffer (12.5 mM MgCl2), 2 µl of enzyme mix (reverse transcriptase and Taq polymerase), 1 µl of each primer (50 pmol) and 26 µl of distilled water (Qiagen, Germany), for a 50 µl final volume. The cycling profile was run as follows: cDNA synthesis at 42°C for 30 min; followed by 35°C cycles with denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, and extension at 72°C for 30 sec; and a final extension at 72°C for 10 min. PCR products were visualized using electrophoresis on 1.5% agarose gel containing ethidium bromide. Purified PCR products using the gel extraction kit (Qiagen, Germany) were ligated with pGEM-T easy vectors (Promega, USA). Plasmid DNA was isolated from transformed Escherichia coli, and recombinant plasmids were identified using EcoR I enzyme digestion.
Sequencing and phylogenetic analysis

Sequencing reactions were performed using recombinant plasmids and the ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer, USA). Phylogenetic analysis was performed on nucleotide sequence data of N genes from two Korean isolates and 38 reference AKAV strains. The sequence data of the reference strains were obtained from GenBank (National Center for Biotechnology Information, NCBI). Phylogenetic tree and sequence pair distances of the nucleotides were obtained using the DNASTAR software program (DNAStar, USA). Homology analysis was performed using DNASIS software (Hitachi software, Japan).

## Results

### Identification of Akabane virus

Abortion associated with fetal deformities was reported in a 6-month-pregnant native Korean cow raised on a farm in Jeju province. RT-PCR using specific primers for the Akabane virus was performed using the homogenate of the aborted fetus to diagnose the cause of the abortion. Amplified gene products corresponding to AKAV were detected using 1.5% agarose gel (Fig. 1). The isolation of the causative agent was attempted in cell culture using the homogenate of fetal organs; however, AKAV could not be isolated. Therefore, 15 plasma samples from the identified farm were obtained and inoculated into Vero cells. One of the 15 plasma samples inoculated into the Vero cells produced obvious cytopathic effect (CPE). The observed CPE, which was characterized by the rounding and detachment of cells, was detected microscopically following an incubation period of 7 days (Fig. 2). The isolate was cloned three times using limiting dilution methods and designated as KV0505, i.e., obtained from the plasma of a Korean native cattle in May 2005. Fifteen serum samples that were collected from animals at the farm from which the AKAV strain was isolated were checked for the presence of AKAV antibodies using SN tests. According to the SN tests, all cattle showed positive antibody titers against Aino virus, ranging from 1:2 to 1:64 (Table 2). In addition, 14 of 15 samples were antibody-positive against Chuzan virus. Eight of the 15 samples were negative against AKAV; however, four of the tested cattle displayed high antibody titers greater than 1:64. The KV0505 isolate was obtained from the plasma sample of a cow designated sample No. 5.

### Sequence analysis of KV0505 isolate

The gene encoding nucleocapsid protein from the KV0505 isolate was cloned into a pGEM-T easy vector and sequenced. Nucleotide sequence data were deposited in GenBank (NCBI) with the accession number DQ973188. The N gene sequence of all 38 AKAV
viruses obtained from GenBank was compared with those of the Korean isolates to characterize the molecular groups of the KV0505 isolate. Based on a phylogenetic analysis of the Korean isolates and reference strains, the two Korean isolates (KV0505 and K-9) were clustered into group Ic and were closely related to both the M171 and SAB74 isolates with approximately 99.7% nucleotide homology. The KV0505 isolate possessed the highest nucleotide sequence identities with other strains of group Ic at between 97.3 and 99.7%. The nucleotide homologies of the KV0505 isolate with group II, III, and IV strains were 96.8, 93.8, and 83.6%, respectively.

Pathogenicity of KV0505 isolate in mice

The pathogenicity of the KV0505 isolate at a virus titer of $10^{5.0}$ TCID$_{50}$/ml was evaluated in suckling mice via intracranial inoculation. All mice were observed for 15 days. All of the mice inoculated with either the KV0505 or the K-9 isolate showed paralysis at 6 days and died at 10 days post-inoculation.

Discussion

Since AKAV was first isolated in Japan in 1959, several other countries have reported outbreaks and have isolated the virus [1, 18]. Outbreaks of Akabane disease have been reported in Korea [2, 5, 13]. Because the observed reproductive disorders (e.g., abortion and teratogenic malformation in cattle) may also be caused by either Aino or Chuzan virus, additional specific diagnostic methods such as RT-PCR using specific viral primers for each of the three viruses should be performed. The AKAV gene was the only viral gene detected in the organ homogenate of the aborted fetus that we examined. The agent virus was not isolated in
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A virus was isolated in Vero cells from one of the 15 plasma samples taken from cattle from the same farm. These results may indicate that AKAV is highly susceptible to the field environment and was thus rapidly inactivated in the aborted fetus. An additional possibility is that an immune complex that formed in the fetus inhibited the isolation of the virus in cell culture. This case of cattle abortion was diagnosed based on the results of various tests, including virus isolation, FA tests, RT-PCR, and serological examinations.

The genetic relationships among many AKAVs isolated from several countries and sources such as bovine fetuses, bovine blood, mosquitoes, and swine tonsils have been reported in previous studies [1, 9, 18, 21]. The nucleotide sequence of the S RNA genomic segment of the KV0505 isolate was determined and compared with those of other AKAVs. Phylogenetic analyses suggest that there are four groups that reflect different geographical origins. Viruses in group I comprise isolates from Korea, Taiwan, and Japan; viruses in groups II, III, and IV comprised isolates from Israel, Australia, and Kenya, respectively. Because the Korean isolate KV0505 was found to be most closely related to the K-9 strain (isolated in Korea in 1993) and the M171 strain (isolated in Japan in 1974), this genetic feature may reflect more of a geographic change, rather than a chronological change or isolated viral source [5]. Akashi et al. [1] reported that AKAVs may have evolved in multiple lineages and therefore have high antigenic diversity in field isolates. Japanese isolates belong to groups Ia, Ib, and Ic; however, the two Korean isolates were clustered into group Ic. Further systematic and molecular characterizations of AKAVs should be conducted to quantify and describe the exact genetic variation of AKAV in Korea.

AKAV causes histological lesions during the first trimester of pregnancy in cattle [12]. Histological lesions with mild non-suppurative encephalitis were also observed in 4-week-old pigs and in a 5-month-old calf infected with AKAV [9, 20]. AKAV has been isolated using suckling mice that were inoculated with organ
homogenates of aborted fetuses [5] and causes encephalitis in mice and guinea pigs [16, 17]. The two Korean isolates described here had virulence in suckling mice similar to those described in previous reports. Although the pathogenicity of the KV0505 isolate could not be identified in pregnant cattle, lethal doses were obtained in suckling mice via intracranial inoculation. Based on the nucleotide homology and pathogenicity in suckling mice, the KV0505 isolate has biological characteristics similar to those of the K-9 strain, which was isolated in 1993.

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References