**Virologica Sinica**

**Supplementary Data**

**Isolation and characterization of Akabane virus Ib substrain and its pathogenesis in sulking mice**

Xiaohui Zana, Shirong Wanga, Tianqi Zhanga, YingLia, Chunge Zhangb,d, Cun Fua, Jialei Wanga, Youzhi Wua, Yanhua Maa,e, Zhifei Lia, Yan Wanga, Hao Wanga, Yuhai Bib,d,\*, Wei Wanga,c,\*

*a State Key Laboratory of Reproductive Regulation & breeding of grassland livestock, College of Life Sciences, Inner Mongolia University, Hohhot, 010070 , China*

*b CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Center for Influenza Research and Early-warning (CASCIRE), CAS-TWAS Center of Excellence for Emerging Infectious Diseases (CEEID), Chinese Academy of Sciences, Beijing, 100101, China*

*c Inner Mongolia Mengwei Biotech Co. Ltd, Hohhot, 012000, China*

*d University of Chinese Academy of Sciences, Beijing, 100049, China*

*e College of Basic Medicine, Inner Mongolia Medical University, Hohhot, 010080, China*

\* Corresponding authors.

\**E-mail address*: [wangwei@imu.edu.cn](mailto:wangwei@imu.edu.cn) (W.Wang), [beeyh@im.ac.cn](mailto:beeyh@im.ac.cn) (Y.Bi)

**Sample collection and virus isolation**

The geographic distribution of sampling locations in this study was Inner Mongolia Autonomous Region, China. The samples collected from cattle were homogenized in 1.0 mL Dulbecco's Modified Eagle Medium (DMEM), containing 3.5% serum with penicillin (100 U/mL), streptomycin (100 mg/mL), using one stainless steel bead in a TissueLyser (Qiagen, Hilden, Germany) set to 26 Hz for four min. Then samples were filtered and clarified with a 0.45-µm nylon syringe filter. The supernatant filtrate was serially diluted and cultured in Vero cell monolayers in DMEM medium containing 3.5% serum and incubated at 37 °C. When cells developed CPE, they were stored frozen at –80 °C.

**Indirect immunofluorescence assay**

Vero cells were grown in six-well plates to 90%, and were then infected with the LK07 strain for 48 h. The supernatants were removed and cells were washed with PBS three times. The cells were fixed with paraformaldehyde for 10 min at 4 °C and permeabilized with 0.1% Triton X-100 followed by blocking with 5% BSA in PBS for 30 min. Then the cells were incubated with AKAV anti-N monoclonal antibody which was made in our lab for 1 h at 37 °C. The samples were then washed again and incubated for 1 h at 37 °C with fluorescent-labeled secondary antibodies (Invitrogen). Then cells were washed three times with PBS and were observed under fluorescence microscopy ((Nikon, Tokyo, Japan).

**PCR amplification**

Total RNA was extracted from 600 μL of clinical samples using a viral RNA mini kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. The viral dsRNA was subjected to reverse transcription using a PrimeScript II First-Strand cDNA Synthesis Kit (Takara, Dalian, China). The complete *S* genes of AKAV were amplified using the primers (Supplementary Table S2). Amplification was done under the following conditions: initial denaturation cycle at 95 °C for 5 min, 35 cycles (denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1.5 min), followed by a final extension cycle at 72 °C for 7 min. The PCR assay was performed in 25 μL volume, including 12.5 μL Dream Taq green PCR master mix (2×), 1 μL of each primer (10 pmol/μL), 8.5 μL deionized water, and 2 μL of DNA template.

**Nucleotide sequencing and phylogenetic analysis**

In order to further confirm the identity of LK07 strain, duplicate samples were submitted for full genome sequencing and only high-quality sequences were submitted to the Genbank database. The alignment and neighbor-joining (NJ) phylogenetic trees were constructed using MEGA 7.0 software. The reliability of phylogenetic relationships was evaluated using bootstrap analysis with 1,000 replicates. DNASTAR lastergene was used to analyze homologous comparison analysis in this study.

### Experimentally infection

7-day-old Kunming white suckling mice were purchased from Spelfa (Beijing) Biotechnology Co., Ltd. A total of 40 suckling mice of either gender without antibodies against AKAV were randomly divided into four groups, including intracerebral injection group (n = 10), intraperitoneal injection group (n = 10), subcutaneous injection group (n = 10) and control group (n = 10). The experimental mice were injected with 10μL of LK07 strain containing 106.19 TCID50/mL, while mice in control group were inoculated with the 10 μL physiological saline via intracerebral, intraperitoneal, and subcutaneous routes. The clinical signs were observed daily.

**Quantitative real-time polymerase chain reaction (RT-qPCR)**

The brain, heart, spleen, kidney and liver of all suckling mice were collected. The tissue samples were centrifuged at 5,000 rpm for 10 min at 4 °C and supernatant was collected for total RNA extraction. Total RNA was extracted from 600 μL of the samples using a QIAamp RNA Mini Kit following the manufacturer’s instructions. cDNA was synthesized using the PrimeScript™ II 1st Strand cDNA Synthesis Kit. The following primer pairs were used: 5′-CCC CTG GTG CTG AGA TGT TT-3′; 5′-CTT CCT CAT GTT GAC ATC CAT-3′. The obtained Cq value was calculated and copied according to the standard curve equations (y=-3.1286x+40.044). Three independent tests were performed on each organization.

**Histopathological examination**

The gross lesions in brain, heart, spleen, kidney, and liver of suckling mice were observed and examined at necropsy. Brain, heart, spleen, kidney, and liver were collected and fixed in 4% formaldehyde, treated routinely, and then embedded in paraffin. Tissue sections (6 μm) were prepared using a microtome (HM-340E, Thermo Fisher Scientific, MA), placed on glass slides, and stained with hematoxylin and eosin (H&E) according to standard procedure.

**Immunohistochemical evaluation**

The paraffin sections were dewaxed to water, treated with3% H2O2 and washed twice with 0.01 mol/L PBS. The slides were put into the citric acid buffer, heated by steam, and kept at 95–100 °C for 30 min, and were then removed for natural cooling. After PBS washing, the slides were blocked with 5% BSA for 30 min, and incubated with N protein monoclonal antibody which was made in our lab (1:3200) at 4 °C overnight. HRP-labeled secondary antibody (Sigma) was added after PBS washing for 30 min at room temperature. DAB was stained after PBS washing. Hematoxylin staining was performed. And sections were mounted in neutral gum. The images were observed and collected by an optical microscope and image acquisition system (Pannoramic MIDI).

**Statistical analysis**

GraphPad Prism 8™ (GraphPad Software, USA) was used for data analysis. One-way ANOVA is used to analyze differences between two groups. Survival curves were constructed using the Kaplan-Meier method and differences between curves were assessed by log-rank test.

**Supplementary Table S1** The nucleotide homology between LK07 and other Akabane virus gene sequences.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| AKAV strain | Country | Year | Isolation source | Accession No. | | | Homology Analysis | | |
| S | M | L | S | M | L |
| DHL10M110 | China | 2010 | Anopheles vagus (mosquito) | KP144999.1 | KY284022.1 | KY284021.1 | 95.6 | 89.6 | 90.5 |
| GXLCH01 | China | 2016 | Rhizomys pruinosus | KY385908.1 | KY381277.1 | KY381282.1 | 95.6 | 88.5 | 90.0 |
| GXLCH02 | China | 2016 | Rhizomys pruinosus | KY381272.1 | KY381278.1 | KY381283.1 | 95.4 | 89.0 | 89.9 |
| GXLCH04 | China | 2016 | Rhizomys pruinosus | KY381273.1 | KY381279.1 | KY381284.1 | 95.3 | 88.7 | 90.0 |
| GXLCH16-70 | China | 2016 | Rhizomys pruinosus | KY381274.1 | KY381280.1 | KY381285.1 | 95.4 | 88.8 | 90.0 |
| GXLCH70N | China | 2016 | Rhizomys pruinosus | KY381275.1 | KY381281.1 | KY381286.1 | 95.4 | 88.8 | 90.0 |
| GXDH01 | China | 2016 | goat blood | MH174977.1 | MH174978.1 | MH174979.1 | 95.6 | 89.3 | 91.2 |
| TJ2016 | China | 2016 | Bos taurus | MT755621.1 | MT761688.1 | MT761689.1 | 96.4 | 88.8 | 91.7 |
| 93FMX | Korea | 1993 | Bovine fetus | FJ498797.1 | FJ498799.1 | \ | 95.3 | 87.7 | \ |
| K0505 | Korea | 2005 | Bovine plasma | FJ498796.1 | FJ498800.1 | \ | 95.3 | 87.9 | \ |
| AK7 | Korea | 2006 | Bovine fetus | FJ498795.1 | FJ498801 | \ | 95.2 | 88.0 | \ |
| AKAV-32/SKR/2010 | South Korea | 2010 | cattle | JQ308773.1 | JQ308777.1 | \ | 95.2 | 89.0 | \ |
| AKAV-17/SKR/2010 | South Korea | 2011 | cattle | \ | JQ308776.1 | \ | \ | 89.1 | \ |
| K9 | South Korea |  | cloned virus propagated in cells | DQ973189.1 | FJ498798.1 | \ | 95.6 | 88.0 | \ |
| JaGAr39 | Japan | 1959 | Aedes vexans | AB000852 | AB297818.1 | AB297852.1 | 96.6 | 88.8 | 90.4 |
| MP496 | Kenya | 1972 | Anopheles funestus | \ | AB297850.1 | \ | \ | 69.9 | 76.6 |
| OBE-1 | Japan | 1974 | Bovine fetus | AB000851 | AB100604.1 | AB190458.1 | 95.6 | 88.1 | 91.0 |
| KT3377 | Japan | 1977 | Bovine blood | \ | AB297819.1 | AB297854.1 | \ | 88.1 | 89.8 |
| KS-1/E/85 | Japan | 1985 | Bovine erythrocyte | \ | AB297821.1 | \ | \ | 91.1 | \ |
| KSB-3P87 | Japan | 1987 | Bovine plasma | AB232193.1 | \ | AB297859.1 | 96.9 | \ | 90.5 |
| KSB-2C90 | Japan | 1987 | Culicoides oxystoma | AB232200.1 | \ | AB297864.1 | 96.9 | \ | 90.7 |
| KSB-2C87 | Japan | 1987 | Culicoides oxystoma | AB232192.1 | \ | AB297858.1 | 96.9 | \ | 90.7 |
| KSB-1C87 | Japan | 1987 | Culicoides oxystoma | AB232191.1 | \ | AB297857.1 | 96.9 | \ | 90.7 |
| NS-88-1 | Japan | 1988 | Bovine erythrocyte | \ | AB297825.1 | AB297860.1 | \ | 87.6 | 88.6 |
| FO-90-3 | Japan | 1990 | Culicoides spp. | \ | AB297831.1 | AB297866.1 | \ | 91.8 | 90.7 |
| KSB-6E90 | Japan | 1990 | Bovine erythrocyte | AB232204.1 | \ | AB297865.1 | 96.9 | \ | 90.7 |
| CY-77 | Japan | 1993 | Bovine erythrocyte | AB232319.1 | AB297851.1 | AB297886.1 | 95.9 | 90.4 | 88.6 |
| MZ-1/C/00 | Japan | 2000 | Culicoides spp. | AB232268.1 | AB297845.1 | AB297880.1 | 96.3 | 90.0 | 89.6 |
| Okayama2001 | Japan | 2001 | Bovine plasma | AB289319.1 | \ | \ | 95.9 | \ | \ |
| KSB-2/P/01 | Japan | 2001 | Bovine plasma | \ | AB297846.1 | AB297881.1 | \ | 90.1 | 89.5 |
| KS-2/Mo/06 | Japan | 2006 | Bovine medulla oblongata | AB373232.1 | AB373233.1 | \ | 95.9 | 89.8 | \ |
| KSB-3/P/06 | Japan | 2006 | Bovine plasma | AB426280.1 | AB426282.1 | \ | 95.6 | 89.8 | \ |
| KM-1/Br/06 | Japan | 2006 | Bovine brain | AB426271.1 | AB436954.1 | \ | 95.7 | 89.8 | \ |
| KM-2/Br/06 | Japan | 2006 | Bos taurus | \ | LC552048.1 | LC552049.1 | \ | 89.8 | 90.5 |
| KM-2/Br/06 | Japan | 2006 | Bovine brain | AB426272.1 | AB426281.1 | LC217506.1 | 95.7 | 89.8 | 89.6 |
| FI-1/Br/08 | Japan | 2008 | Bos taurus | \ | LC552051.1 | LC552052.1 | \ | 87.3 | 94.3 |
| Iriki | Japan |  |  | AB289321.1 | AB297820.1 | AB297855.1 | 96.2 | 91.2 | 90.4 |
| TS-C2 | Japan |  | bovine fetus | AB968527.1 | AB968526.1 | AB968525.1 | 95.7 | 88.1 | 91.0 |
| B8935 | Australia | 1968 | Culicoides brevitarsis | \ | AB297848.1 | \ | \ | 84.6 | 85.7 |
| R7949 | Australia | 1968 | Culicoides brevitarsis | \ | AB297849.1 | \ | \ | 84.9 | 85.8 |
| ISR-01 | Israel | 2001 | Culicoides imicola | AY378155.1 | \ | \ | 97.4 | \ | \ |
| ISR-170/18 | Israel | 2018 | sheep | MW822048.1 | MW822047.1 | MW822046.1 | 98.5 | 95.3 | 95.7 |
| Aksu-1 | Turkey | 2015 | Sheep Fetal membranes | KU296969.1 | \ | \ | 97.7 | \ | \ |
| Aksu-2 | Turkey | 2015 | Sheep Fetal membranes | KU296970.1 | \ | \ | 97.7 | \ | \ |
| ON-1/P/93 | Japan | 1993 | Bovine plasma | \ | \ | AB297869.1 | \ | \ | 90.0 |
| ON-3/E/90 | Japan | 1990 | Bovine erythrocyte | \ | \ | AB297867.1 | \ | \ | 90.0 |
| ON-1/E/98 | Japan | 1998 | Bovine erythrocyte | \ | \ | AB297876.1 | \ | \ | 89.3 |
| ON-3/F/97 | Japan | 1997 | Bovine fetus | \ | \ | AB297874.1 | \ | \ | 93.6 |
| ON-2/P/91 | Japan | 1991 | Bovine plasma | \ | \ | AB297868.1 | \ | \ | 88.4 |

**Supplementary Table S2** Primers required for the full-length amplification of the *S* gene.

|  |  |
| --- | --- |
| Primer | Sequence |
| S-F | AATAGTGAACTCCACTATTAACTAC |
| S-R | AATTAACTATAAATAATAAAATCCA |

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**Supplementary Fig. S1.** Detection of Akabane virus LK07 strain infected Vero cells by indirect immunofluorescence assay (scale bar =100 μm). **A** CPE on LK07-infected Vero cells. **B** The distribution of AKAV antigen in LK07-infected cells. **C** Normal Vero cell control. **D** IFA negative control.



**Supplementary Fig. S2.** Distribution of AKAV.