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Letter

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

Xiaohui Zan [a], Shirong Wang [a], Tianqi Zhang [a], Ying Li [a], Chunge Zhang [b,d], Cun Fu [a], Jialei Wang [a], Youzhi Wu [a], Yanhua Ma [a,e], Zhifei Li [a], Yan Wang [a], Hao Wang [a], Yuhai Bi [b,d,*], Wei Wang [a,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

Dear Editor,

Akabane disease is an arthropod-borne viral disease caused by the Akabane virus (AKAV) that can affect cattle, sheep, and goats. Inapparent infections in adults can lead months later to abortions, stillbirths, congenital defects, and death in newborns (Kurogi et al., 1976). Wild ruminants can also be affected, but abnormalities of the offspring in these species have not been reported. One report suggests that this virus can also cause clinical signs in pigs (Yanase et al., 2018).

The virus is an arbovirus in the genus *Orthobunyavirus* of the family *Bunyaviridae*. Bunyaviruses are spherical or oval particles, 90–100 nm in diameter. The AKAV is an enveloped virus with three segments of the single-stranded negative-sense RNA genome, including small (*S*), medium (*M*), and large (*L*), which differ in size by approximately 0.86, 4.3, and 7 kb, respectively. The *S* and *L* RNA segments are relatively conserved among field-isolated AKAV, but high sequence variability is observed in their *M* RNA segments (Tang et al., 2019). The *S* segment encodes the nucleocapsid protein (N) and a non-structural protein (NSs) from overlapping open reading frames, whereas the *M* and *L* segments encode the membrane glycoproteins and RNA-dependent RNA polymerase (RdRp), respectively. The virus was projected through the cell membrane with glycoproteins G1 and G2 (Ariza et al., 2013).

AKAV is mainly transmitted by the bite of arthropod vectors. The true vector has not yet been identified, but mosquitoes and biting midges are the most likely suspects (Dagalp et al., 2021). Akabane disease firstly broke out in Akabane Village of Japan in 1949. The virus was first isolated in Japan from mosquitoes in 1959 (Oya et al., 1961). It has existed in East Asia (Japan, South Korea, China), West Asia (Saudi Arabia, Yemen, Oman, Bahrain, Syria, Jordan, Turkey, and Israel, etc.), Southeast Asia (Vietnam, Indonesia, Thailand, and the Philippines, etc.), Oceania (Australia) and Africa (Kenya, Zimbabwe, South Africa, and Zambia, etc.) (Kurogi et al., 1976; Al-Busaidy et al., 1987; Liao et al., 1996; Uchida et al., 2000; Bryant et al., 2005; Oem et al., 2012; Purnomo et al., 2017; Sevik, 2017). The neutralizing antibody has been found in sera collected from cattle and sheep of 24 provinces in China from August 2006 to September 2015, indicating a wide distribution of the AKAV among cattle and sheep in China (Wang et al., 2017). However, only a few AKAV strains were isolated from the field in China (Liao et al., 1996).

In September 2020, cattle from a cattle farm in Inner Mongolia Autonomous Region, China, showed flexor spasm. The skin tissue with blood was collected from the cattle, and were then homogenized and centrifuged for supernatant collection. The supernatant filtrate was serially diluted and cultured in Vero cell monolayers. On the first blind passage, CPE characterized by cell rounding, detachment, and cell death was observed on 4th-day post-infection (dpi) in one of the inoculated flasks suggesting adaptation of the virus to Vero cells. Then, the indirect immunofluorescence assay (IFA) was carried out using the monoclonal antibody against the AKAV N protein. As shown in Supplementary Fig. S1, the green fluorescence signals of the AKAV N protein were observed in Vero cells. And the fragment of 850 nt of the *S* gene was amplified from the sample. The results showed that a strain of AKAV was isolated and named LK07.

To clarify the genetic relationship of this new AKAV LK07 strain to other strains reported, the whole genome of the LK07 strain was sequenced. The sequences of the *S*, *M*, and *L* segments were submitted to GenBank (accession OP432312, OP432313, and OP901841). Then, the phylogenetic analysis of the *S*, *M*, and *L* genes of LK07 was constructed by the software MEGA7 and the reliability of the phylogenetic trees was assessed by bootstrap analysis with 1000 replicates. Based on *S* genes (Fig. 1A), LK07 is genetically closest to the ISR-170/18 strain (GenBank

* Corresponding authors.
E-mail addresses: beeyh@im.ac.cn (Y. Bi), wangwei@imu.edu.cn (W. Wang).

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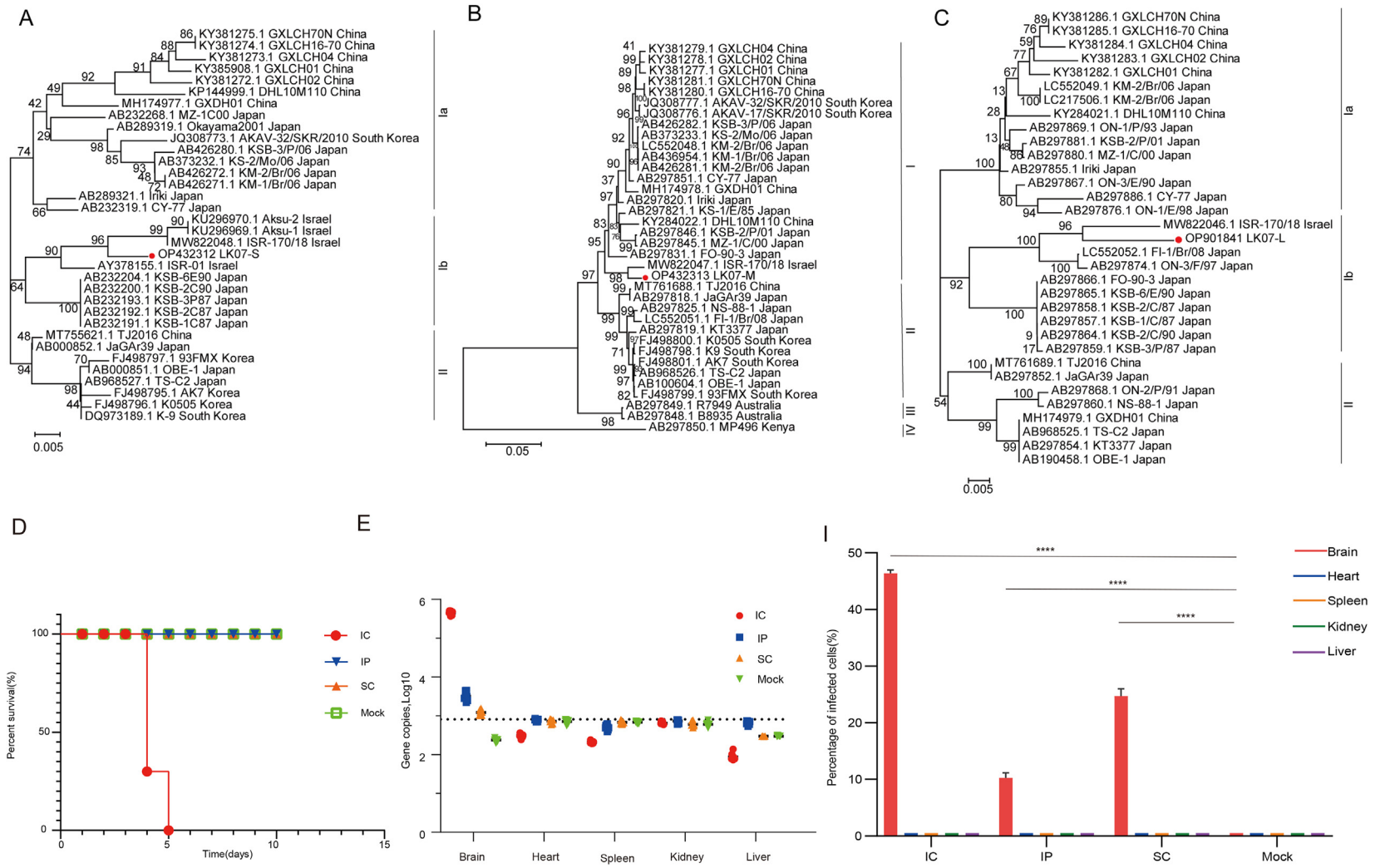

Fig. 1. Phylogenetic tree of the AKAVs based on partial *S* (**A**), *M* (**B**) and *L* (**C**) segment. The alignment and neighbor-joining (NJ) phylogenetic trees were constructed using MEGA 7.0 software. The reliability of phylogenetic relationships was evaluated using bootstrap analysis with 1000 replicates. The AKAV LK07 strain was marked as a red dot. 7-day-old Kunming white mice were inoculated with AKAV LK07 (10 μL) by intracranial, subcutaneous, and intraperitoneal routes. Mice were injected with physiological saline by intracranial, subcutaneous, and intraperitoneal routes as a negative control (n = 10). **D** Survival rate (log-rank test, $^{****}P < 0.0001$). **E** Brain, kidney, spleen, liver, and heart of sucking mouse were obtained when they died or 10 days after infection. Viral load was measured by qPCR. The dashed line is the detection limit of the assay. **F** The morphology of brain, heart, kidney, spleen, and liver of suckling mouse in each group. **G** Sections of brain, heart, kidney, spleen, and liver of each group were stained with HE and magnified 200 fold (scale bar = 100 μm). Histopathological results show that the local structure of brain tissue of IC group is loose, the arrangement of neurons is irregular, and more irregular holes can be seen (yellow arrow). A small number of neurons can be seen in the cortex with punctate necrosis, nuclear fragmentation (black arrow), and a small amount of neuronal degeneration. The cytoplasm is loose and lightly stained (blue arrow), accompanied by a small amount of glial cell proliferation (red arrow). In brain of IP group, there were more hyperchromatic nuclei and eosinophilic cytoplasm (orange arrow) in the cortex and more small vacuoles (green arrow) in the nerve fibers. In SC group, a large number of nuclei of neurons in the cortex were hyperchromatic, the cytoplasm was eosinophilic (purple arrow), and there were many small vacuoles in the nerve fibers (gray arrow). **H** Immunohistochemical staining of AKAV antigen in brain, heart, spleen, kidney, and liver. The magnification of the sectional image is 400× (bar = 50 μm), and the magnification of the zoomed-in image in the top right corner is 800×. Red arrow represents positive signal of AKAV antigen. **I** Quantitative analysis of AKAV-positive cells (three experiments, $^{****}P < 0.0001$, one-way ANOVA).

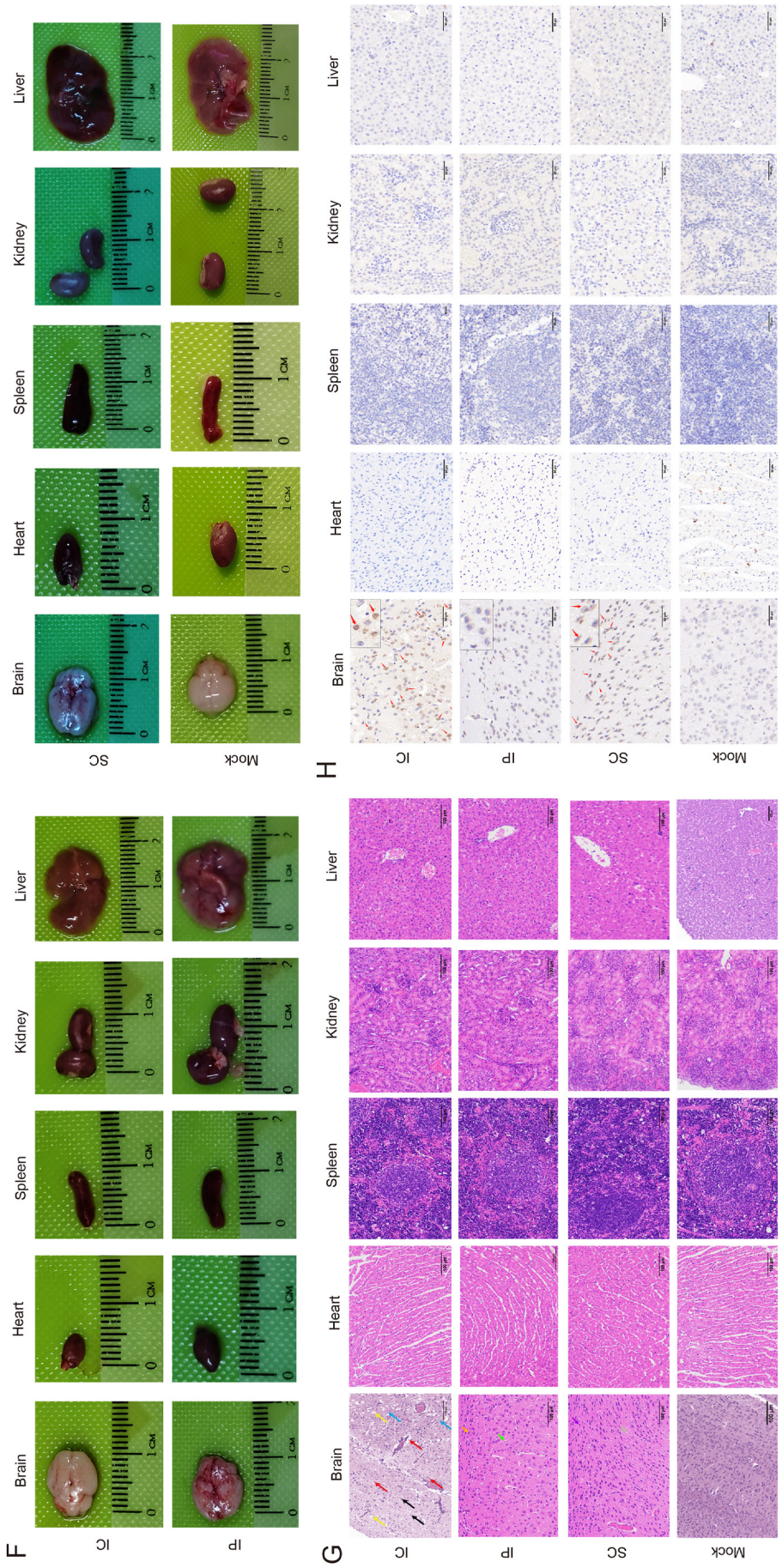

Fig. 1. (*continued*).

accession MW822048.1), clustering with the strains from Israel and Turkey. For *S* gene, LK07 strain shared 97.4%–98.5% nt identity with the AKAV strain isolated from Israel and Turkey (Supplementary Table S1). Based on *M* genes (Fig. 1B), LK07 is genetically closest to the ISR-170/18 strain (GenBank accession MW822047.1), and they are located on a separate branch. The GXLCH01 (GenBank accession KY381277.1), GXLCH02 (GenBank accession KY381278.1), GXLCH04 (GenBank accession KY381279.1), GXLCH16-70 (GenBank accession KY381280.1) and GXLCH70N (GenBank accession KY381281.1) strains belong to groups Ia. Similar to the phylogenetic tree of the *S* gene, in the phylogenetic tree of the *M* gene, the B8935 strain (GenBank accession AB297848.1) and R7479 strain (GenBank accession AB297849.1) belong to group III, while the MP496 strain (GenBank accession AB297850.1) belongs to group IV. For *M* gene of AKAV, LK07 shared 95.3% nt identity with ISR-170/18 strain from Israel (Supplementary Table S1). Based on *L* genes (Fig. 1C), LK07 strain also belongs to the same branch as the ISR-170/18 strain, and the nt identity is 95.7% between them. The present results indicate that the field strains most likely originated from the Middle East (Supplementary Fig. S2). And the field strains are genetically closely related to the ISR-170/18 strain. Notably, the AKAV strains isolated in China were previously reported to be assigned to genogroup I subgroup Ia (Gao et al., 2022) and genogroup II (Tang et al., 2019). The LK07 strain isolated in this study belongs to the genogroup I subgroup Ib. To our knowledge, this is the first report on a potential middle-east-lineage transmission route of AKAV in China.

To explore the pathogenicity LK07 in mice, a total of 40 Kunming white suckling mice (7-day-old) of either gender, free of antibodies against the AKAV, were randomly divided into four groups, including the intracerebral injection group (IC), subcutaneous injection group (SC), intraperitoneal injection group (IP), and normal control group (mock). In this study, 10 μL of LK07 stain ($10^{6.19}$ $TCID_{50}$/mL) was injected into each suckling mouse for each injection group. At 4 dpi, ten suckling mice of IC group had clinical symptoms such as stopping sucking, showing spasms, twitching, and dying. All suckling mice of IC group died after 5 days (Fig. 1D). The suckling mice in IP, SC and mock groups have no clinical symptoms. All mice were euthanized at 10 dpi. Results of previous reports have shown that the suckling mice inoculated with the OBE-1 strain have no clinical symptoms, and the survival rate is 100%. Fatal neurological signs due to severe virus infection were observed sooner in mice inoculated with the Iriki strain compared with the OBE-1 strain, indicating that the Iriki strain was more neurotoxic to suckling mice than the OBE-1 strain (Murata et al., 2015).

To clarify the virus load of AKAV in different tissues of suckling mice, qPCR was performed. The results showed that the viral load in brain were higher than that in heart, kidney, liver, and spleen among the groups of IC, SC and IP (Fig. 1E). In particular, the viral load in brain was higher in IC group compared to that in SC and IP groups. However, no significant difference was observed in viral load in heart, kidney, liver, and spleen between infection and mock groups. Although the sucking mouse models may not accurately reflect the pathogenesis of the virus in cattle, the establishment of this model provides a good way to understand the neuropathogenesis of AKAV.

Gross dissection find that the brains of the suckling mice inoculated with the LK07 strain showed abnormal shape (Fig. 1F). Histopathological results show that the local structure of brain tissue of IC group is loose, the arrangement of neurons is irregular, and more irregular holes can be seen (yellow arrow). A small number of neurons can be seen in the cortex with punctate necrosis, nuclear fragmentation (black arrow), and a small amount of neuronal degeneration. The cytoplasm is loose and lightly stained (blue arrow), accompanied by a small amount of glial cell proliferation (red arrow). In brain of IP group, there were more hyperchromatic nuclei and eosinophilic cytoplasm (orange arrowheads) in the cortex and more small vacuoles (green arrowheads) in the nerve fibers. In SC group, a large number of nuclei of neurons in the cortex were hyperchromatic, the cytoplasm was eosinophilic (purple arrow), and there were many small vacuoles in the nerve fibers (gray arrow). No abnormalities were found in other tissues (Fig. 1G). No pathological changes were observed in mock group.

To determine the distribution of AKAV antigen in the brain, heart, spleen, kidney, and liver tissues, immunohistochemical analysis was performed (Fig. 1H). The results showed that the positive rate of AKAV antigen in brain tissue was higher in IC, IP and SC groups than that in mock group, with the highest rate of 46.38% ± 0.66% in IC group (Fig. 1I). No positive signals were observed in other tissues of mice from each group. The results of immunohistochemistry and qPCR are consistent.

In previous reports, BALB/cCrSlc mice were infected with the OBE-1 and Iriki strains by intraperitoneal route, and the results showed that the OBE-1 strain caused no mortality and no remarkable clinical signs, whereas the Iriki strain caused high mortality (75%). The virus was found in the hearts of mice infected with the Iriki strain, particularly in the early stages (Takenaka-Uema et al., 2022). Additionally, as previously reported, sibling suckling rats were injected with either KM-1/Br/06 or OBE-1 strains in brain. All rats infected with the KM-1/Br/06 strain died within 72 h post inoculation, whereas rats infected with the OBE-1 virus survived for 7–8 days. Focal immune labeling was observed in the myocardium and femoral muscle of three-day-old rats, but in the OBE-1 infection group, no viral antigen was observed in tissues except for brain (Kimura et al., 2021). In this study, all sulking mice injected intracerebrally with the LK07 strain died within 5 days after inoculation, while mice injected intraperitoneally survived, although viral antigens were detected in the brain. Furthermore, apart from brain tissue, no positive immune labeling was observed in heart, kidney, spleen, and kidney tissues. Previous studies and the epizootic encephalomyelitis in 2006 suggest that the neurovirulence of genogroup Ia strains are significantly higher than that in genogroup II strains (Kono et al., 2008). However, the virulence of strains belonging to Ib is less reported. Therefore, more experimental and clinical data are needed to confirm the precise relationship between pathogenicity and the genome.

In summary, an AKAV strain was isolated from the cattle, and was closely related to the ISR-170/18, ISR-01, Aksu-1, and Aksu-2 strains in genogroup Ib. This is the first detection of the AKAV sub-lineage Ib in China. Infection model of suckling mouse was established. Obvious symptoms were observed in IC group, comparing to other infection routes. Also, brain was more susceptible to AKAV than other tissues. This study enriched the AKAV strain library in China and provided scientific basis for further molecular epidemiology and detection research on AKAV.

Footnotes

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