Genetic and pathogenic characterisation of a virulent Akabane virus isolated from goats in Yunnan, China

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Abstract

Introduction: Akabane virus (AKAV) has been detected in a variety of host species in China, but there are only limited records of its occurrence in goats. However, more attention needs to be paid to understanding the diversity of viruses in this species. The aim of the study was to explore the genotype characteristics and variation trend of AKAV and their relationship with virulence in Yunnan, China. **Material and Methods:** Blood samples were collected from goats during routine surveillance of goat diseases in Yunnan province in 2019. The AKAV CX-01 strain was isolated using BHK-21 cells. To understand pathogenicity, the virus was intraperitoneally (IP) and intracerebrally (IC) inoculated into suckling mice and tissue samples were subsequently analysed histopathologically and immunohistochemically. **Results:** Akabane virus CX-01 strain induced encephalitis and impairment of the central nervous system with fatal consequences. Phylogenetic analysis based on the ORF sequences of the small segments indicated that the AKAV isolate used was most closely related to the GD18134/2018 Chinese midge and bovine NM BS/1strains, while phylogenetic analysis based on the medium segments showed a close relationship between CX-01 and the Chinese GLXCH01 strain. **Conclusion:** The CX-01 isolate was related to AKAV genogroup Ia and probably originated from a recombination of different strains.

Keywords: goat, Akabane virus, CX-01 strain, pathogenicity, phylogenetic analysis.

Introduction

Akabane disease is a viral disease primarily affecting ruminants and manifesting most clearly in pregnant animals, where it is characterised by abortions, stillbirths, and premature births frequently of offspring with congenital defects (12, 14). The causative agent of the Akabane disease comes from a group of viruses termed arboviruses (from "arthropod-borne virus") and is called Akabane virus (AKAV) or *Akabane orthobunyavirus*, which is a species belonging taxonomically to the *Orthobunya virus* genus of the *Peribunyaviridae* family (28). There are three segments of single-stranded negative-sense RNA in the AKAV genome: small (S), medium (M) and large (L) (28). Recently, AKAVs have been classified into four genetically distinct groups (genogroups I–IV), and genogroup I has been further subdivided into two subgroups (Ia and Ib) (19). The virus is primarily transmitted by biting midges belonging to the *Culicoides*

genus and by mosquitoes (29). A wide range of wild and domesticated animals are susceptible to AKAV infection and symptomatic infections have been observed in bamboo rats, cattle, swine, sheep and goats (7, 13, 18, 26, 30, 32, 33). In most cases, transient fever has been shown in animals without any other apparent clinical symptoms. Serum antibodies against AKAV have been detected in buffalo, camels, deer, horses and donkeys, suggesting that more animal species can be infected by this virus (2, 8). Infections have also been reported in several regions across Australia, Southeast Asia, East Asia, Africa, and the Middle East (19, 25, 34). Disease induced by AKAV has only been reported in bamboo rats, although several AKAV strains have been isolated from cattle and goats in regular disease surveillance in China (31, 32). Although no outbreak of this disease has been reported in China, it is still listed as one of the seven epidemic diseases to which there applies mandatory quarantine of imported cattle, sheep and goats.

A total of 2,731 serum samples were tested from cattle, sheep and goats from 24 provinces of China during the national survey from 2006 to 2015. The samples were analysed by serum neutralisation. The overall seroprevalence of AKAV was 21.3% in cattle (471/2215), 12.0% (17/142) in sheep or goats, and 0% in yak (0/374) (35). In the prevailing circumstances of no vaccine use against the disease caused by AKAV in China, the results indicated that AKAV infection was a widespread problem. Another survey carried out on 420 serum samples collected from the Yunnan province indicated that the prevalence in cattle was much higher than that in sheep and goats, where seroprevalence was 30% and 20% in cattle and goats, respectively. Seroprevalence in tropical and subtropical Yunnan province indicated that this infection was much more common than the nationwide average. In this study we aimed to characterise the AKAV infecting goats in the Yunnan province of China, and to investigate the genotype characteristics and variation pattern of AKAV and their relationship with virulence.

Material and Methods

Laboratory animals, positive serum and cells. Seven-day-old BALB/c mice from the Laboratory Animal Research Center of Kunming Medical University were used for animal trials in this study. All animal experimentation was approved by and conducted according to the requirements of the Yunnan Laboratory Animal Administration Authority. Positive serum samples were collected from convalescent sheep as part of surveillance and assayed to detect antibodies against the Akabane virus. Baby hamster kidney cells (BHK-21) were obtained from Kunming Institute of Zoology, Chinese Academy of Sciences and cultured in Eagle's minimum essential medium (MEM, Sigma-Aldrich, St. Louis, MO, USA).

Virus isolation and immunofluorescence assay. The BHK-21cells used for virus isolation were cultured in MEM supplemented with antibiotics (100 IU/mL penicillin and 10 µg/mL streptomycin) and 5% heat-inactivated foetal bovine serum (Everygreen, Hangzhou, China). Blood samples were inoculated with 1 mL of supplemented cell culture for 1 h at 37℃, then washed three times with MEM, and the flasks were incubated at 37℃ in 5% CO_2 for 3 days. The viruses were harvested and inoculated into fresh BHK-21 cells for the second passage, when cytopathic effects (CPEs) were observed. Samples without developing CPEs were considered AKAV-negative. Viral titration was determined in 96-well microplates by using 10-fold serial dilutions, and CPEs were observed under a microscope on day 2 post inoculation. Viral titres were calculated according to the Reed and Muench method and expressed as the 50% tissue culture infectious dose ($TCID_{50}$/0.1mL).

The hamster cells were plated and infected with the CX-01 strain in 6-well plates and fixed in cold acetone (−20℃) for 20 min to observe the virus proliferation. After three successive washes with PBS (pH 7.2), the cells were treated with an AKAV-positive sheep serum for 30 min at 37℃ and then stained with a 100-fold dilution of fluorescein isothiocyanate–conjugated rabbit-anti-sheep antibody (BBI, Shanghai, China). After washing the plates with PBS, the BHK-21 cells were air-dried and examined at 200 × magnification under a fluorescence microscope. The hamster cells in which the virus was not propagated were used as the negative control. Samples exhibiting specific fluorescence in the cytoplasm were identified as positive.

Electron microscopy. Cells infected with the CX-01 isolate were harvested 36 h post inoculation and were frozen and thawed three times. After centrifugation at 4,000 × g for 30 min, cell debris was removed, and viral supernatant was treated with 10% polyethylene glycol (MW 8000; Sigma-Aldrich) and 0.5 M NaCl and precipitated overnight at 4℃. After precipitation, the pellet was collected and dialysed with PBS. The particles were examined under a model 7100 transmission electron microscope (Hitachi, Tokyo, Japan).

PCR amplification and sequencing. Viral nucleic acids were extracted from virus-containing cell culture supernatant using a MiniBest Viral RNA/DNA Extraction Kit (TaKaRa, Dalian, China) and stored at −80℃ until analysis. The PCR primers were designed based on the sequences of the DHL10M110 virus strain (Table 1). Reverse transcriptase PCR procedures were performed in a one-tube system for sequencing. A 702 bp fragment covering the ORF sequence of the S RNA segment was amplified, as were full-length M and L RNA segments, and the amplified DNA fragments were purified using an Agarose Gel DNA Extraction Kit and cloned into pMD-19T cloning vector (TaKaRa, Dalian, China) according to the manufacturer's instructions. All nucleotide positions were confirmed by three independent sequencing reactions in both directions. The nucleotide sequences determined in this study were deposited in GenBank (accession numbers MW194115–MW194117).

Genome alignment, phylogenetic analysis and recombinant analysis. The sequences of CX-01 ORFs and the deduced proteins were analysed using DNAstar 7.0 software (DNASTAR Inc., Madison, WI, USA). For the construction of the neighbour-joining phylogenetic tree, 31 S gene segments and 28 M gene segments of AKAV's reference genomic sequences were downloaded from GenBank. The phylogenetic trees of the coding regions in the S and M RNA segments were constructed using the neighbour-joining method in the Molecular Evolutionary Genetics Analysis program version 6.06 with bootstrap values based on 1,000 replicates, the Kimura 2-parameter and a nucleotide substitution model (31). Whole genomic sequences of the CX-01 field strains and four other representative strains were aligned using MEGA 6.06 and analysed using SimPlot (21) with a 200 bp window that slides along the genome (20 bp step size).

Table 1. The primers used for the amplification of the small (S), medium (M) and large (L) segments of the Akabane virus genome

Gene	Primer	Sequence (5′–3′)	Position	Product size (bp)
S	AKVS1	CTCCACTATTAACTACGCAT	9–26	804
	AKVS2	GGTGTGCACCACATAGACAT	793–812	
M	AKVM1	AGTAGTGAACTACCACAACAAAATG	1–25	4,308
	AKVM2	AGTAGTGTTCTACCACAACAAATAATTAT	4,280–4,308	
L	AKVL1	AGTAGTGTACCCCTAAATACAACATACA	1–28	4,151
	AKVL2	GTCAGCTTGCTTAAATCCC	4,133–4,151	
	AKVL3	GTGATTGTGCATTCCTTGG	3,764–3,782	3,106
	AKVL4	AGTAGTGTGCCCCTAAATGCAATAATAT	6,842–6,869	

The oligonucleotide position is based on the sequences of the DHL10M110 Akabane virus strain

Experimental infection and immunohistochemistry. In order to evaluate the pathogenicity of the AKAV isolate CX-01, one-week-old suckling BALB/c mice were divided into three groups and inoculated with the CX-01 strain or saline. There were 10 suckling mice in each experimental group and each was either inoculated intraperitoneally (IP) with 0.1 mL virus ($10^{-4.85}$ TCID$_{50}$/0.1 mL) or inoculated intracerebrally (IC) with 10 μL virus and observed for 6-7 days. One-week-old suckling BALB/c mice were inoculated with 0.85% saline instead of virus solution as the control group. When the experimental mice inoculated with the virus died after 7 days, all the mice were sacrificed. All experimental procedures were conducted as described in previous reports (23). Brain tissue samples were collected immediately after the death of the mouse, fixed with 10% buffered formalin and processed for paraffin-wax embedding. The tissue sections from each paraffin block were stained with haematoxylin and eosin for histopathological examination. The section was incubated with AKAV-positive sheep serum (1:500 dilution) against the AKAV CX-01 strain, containing a biotinylated secondary antibody for anti-sheep IgG and streptavidin-conjugated peroxidase.

Results

Virus isolation and titration. In this study, blood samples were collected from goats suspected of suffering from Akabane disease which were manifesting subclinical symptoms with transient fever and occasional neurological signs. Five blood samples were found positive in the subsequent RT-PCR detection based on the S segment gene-specific primers. A CX-01 isolate was successfully cultured after 48 h from the blood samples inoculated into BHK-21 cells. Specific CPEs characterised by large cells containing multiple nuclei and floating cells were observed in the isolates

after the second passage, as shown in Fig. 1B. The control BHK-21 cells without inoculation of the virus are presented in Fig. 1A. Relevant fluorescence was observed in the cytoplasm of BHK-21 cells infected with CX-01 36 h after fixation of the cells and staining with sheep AKAV-positive serum (Fig. 1C). The CX-01 strain titre was $10^{-4.85}$TCID$_{50}$/0.1mL at 36 h post inoculation. The viral particles were 80 to 120 nm in diameter with around shape, indicating a morphological feature typical of *Bunyaviridae* (Fig. 1D).
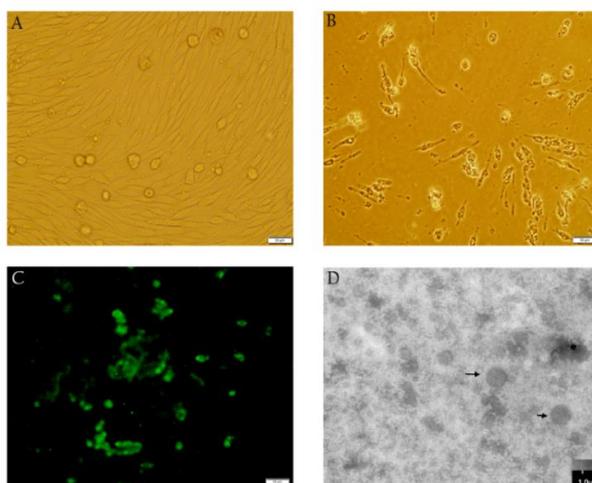
Fig. 1. Histopathological images of baby hamster kidney (BHK)-21 cells infected with Akabane virus strain CX01. A – Control BHK-21 cells; B – Cytopathic effect of CX-01 in BHK-21cells; C – Immunofluorescence results of BHK-21 cells infected with CX-01 after 36 h; D – Negatively stained Akabane virus particles 50–100 nm in diameter. Scale bar - 100 nm

Genome alignment, phylogenetic analysis and recombinant analysis. The nucleotide sequences of the S, M and L RNA segments of the AKAV isolate CX-01 were determined and analysed. The sequenced S RNA segments contained an ORF of 702 nt with no insertions or deletions encoding 233 amino acid residues. The nucleotide and amino acid sequences were compared with Chinese isolates and other reference

strains from GenBank and found to be 94.7%–98.1% and 99.1%–99.6% identical at the nucleotide and amino acid levels, respectively to five Chinese strains. When compared with reference strain of subgroups Ib and other three geno-groups, the CX-01 strain had 83.5%–98.4% and 90.6%–100% identity with the nucleotide and amino acid sequences, respectively. The CX-01 strain shared the highest nucleotide identity with the KM-1/Br/06 strain from outside China (Table 2). The nucleotide sequences determined in this study were deposited in GenBank under accession numbers MW194115–MW194117.

The M RNA segments of CX-01 AKAV isolates contained a 4,203 nt ORF encoding 1,401 amino acid residues without any detectable insertions or deletions. The nucleotide and amino acid sequences of the Chinese isolates exhibited 91.7%–96.8% and 95.6%–97.9% similarity, respectively. The sequences were also compared with strains from outside China and their homology was in the ranges of 70.3%–97.7% and 74.4%–98.4% at the nucleotide and amino acid levels, respectively. The CX-01 strain showed the highest similarity with the AKAV-32/SKR/2010 strain (Table 2).

The M segment was encoded in two virion surface glycoproteins, Gc and Gn, and a nonstructural protein, NSm. Among all of the Chinese strains listed in Table 2, Gc shared 91.3%-97.2% at the nucleotide and 78.0%-93.2% at the amino acid levels, Gn shared 92.0%-96.8% at the nucleotide and 78.9-91.4% at the amino acid levels. The NSm ORF sequences were highly

variable when compared with the local isolate DHL10M110, whereas field isolates of CX-01 shared 90.5%–96.7% and 75.7%–91.7% similarity at the nucleotide and amino acid levels, respectively.

The ORF sequence of the L RNA segment of CX-01 was 6,756 nt and encoded 2,252 amino acid residues. The nucleotide and amino acid sequences of the L RNA segment were compared with reference strains from GenBank. The sequence identities showed 91.8%–97.5% nucleotide similarity and 95.8%–99.4% amino acid similarity (Table 2).

Phylogenetic analyses of the S and M RNA segments. To establish the genetic relationships of CX-01 with other AKAV strains, we constructed phylogenetic trees based on ORF sequences of the S and M RNA segments of 32 and 29 reference AKAV strains previously reported (4). Based on the sequences of the S genes, the field isolate CX-01 strain belonged to genogroup Ia, clustering with all Chinese strains (Fig. 2A). This isolate also belonged to genogroup Ia when aligned by the sequences of the M genomic segments. However, all of the lineage strains have been reported in China since 2004 and showed evidence of evolutionary divergence in the local isolate CX-01 and, to some extent, in DHL10M110. Although the cattle isolate NM/BS/1 fell within genogroup Ia, it had the lowest identity with the CX-01 strain (Fig. 2B). These results may indicate that recombination events had occurred in the genomes of the local isolates, especially in the M segment.

Table 2. Comparison of the three segments S, M and L (ORF) and the coding region of the M segment among AKAVs

Genotype	Strain	Geographic origin and host	Pairwise % identity (nt/aa)					
			S	M	M			L
					Gn	Gc	NSm	
Genogroup Ia	DHL10M110	China *Anopheles vagus*	94.7/99.5	91.7/95.6	92.7/80.7	91.3/78.4	90.5/75.7	97.5/99.4
	NM/BS/1	China *Bovine*	98.1/99.1	91.8/96.2	92.6/80.7	91.5/78.0	91.9/79.6	-
	GXLCH01	China *Rhizomys pruinosus*	97.6/99.6	96.6/97.9	96.8/91.4	96.4/91.1	96.7/91.7	94.5/98.4
	GXLCH70N	China *Rhizomys pruinosus*	97.7/99.6	96.8/97.9	96.4/90.7	97.2/93.2	95.4/89.0	94.6/98.7
	HN10174	China *Culex quinquefasciatus*	-	91.7/96.3	92.0/78.9	91.5/78.0	91.9/79.6	95.1/98.8
	KM-1/Br/06	Japan *Bovine*	98.4/100	97.3/97.9	97.4/93.6	97.2/93.5	97.1/92.3	96.2/98.9
	AKAV-32/SKR /2010	Korea *Bovine*	94.9/98.7	97.7/98.4	98.1/95.4	97.7/94.3	97.2/92.8	-
	IRIKI	Japan *Bovine*	95.0/98.7	94.7/97.0	94.9/81.6	94.5/86.0	93.4/82.9	-
Genogroup Ib	FO-90-3	Japan *Culicoides spp.*	95.3/98.3	91.7/95.6	91.0/71.0	91.8/79.6	92.3/81.2	-
Genogroup II	OBE-1	Japan *Bovine*	95.3/98.7	87.5/93.8	89.3/66.8	86.4/66.7	89.4/71.8	91.8/97.6
Genogroup III	B8935	Australia *Bovine*	92.7/97.4	84.4/91.9	85.7/57.6	84.1/63.2	83.9/58.6	96.4/95.8
Genogroup IV	MP496	Kenya *Anopheles funestus*	83.5/90.6	70.3/74.4	75.2/-	69.9/38.3	65.9/30.4	-
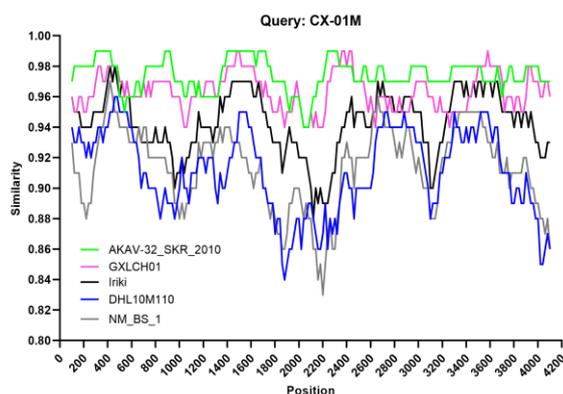
Fig. 3. Recombination analysis of the CX-01 strain using a 200-bpsliding window and a 20-bp step. The y-axis indicates the percentage similarity between the query sequence and the reference sequences. Comparison of genome scale similarity of CX-01 (query) with DHL10M110 (green), GXLCH01(pink), Iriki (black), AKAV-32/SKR/2010 (blue) and NM/BS/1 (grey)
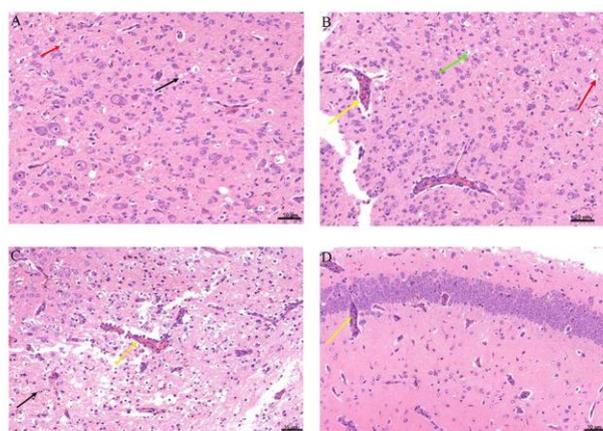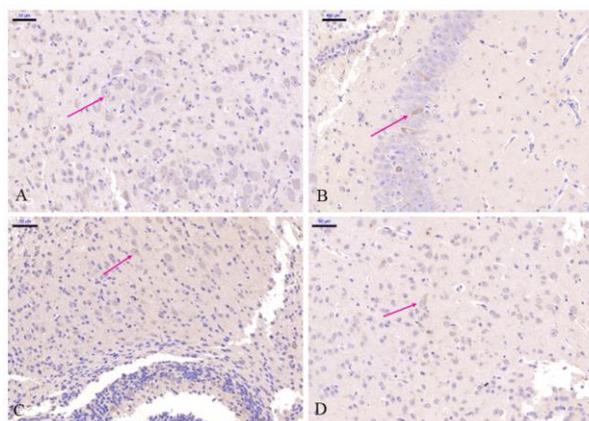

Fig. 4. Histopathological characteristics in the central nervous tissues of mice with AKAV CX-01 strain. A – brain of a 7-day-old mouse inoculated intraperitoneally (IP) with the CX-01 virus. Neuronal degeneration, necrosis (red arrow) and cavities are visible in the brain stem tissue (black arrow); B–brain of a 7-day-old mouse inoculated intracerebrally (IC) with the CX-01 virus. Enlarged vascular endothelial cells and perivascular infiltration of mononuclear cells (yellow arrow), gaps in the neuron cells (green arrow) and neuronal degeneration and necrosis are visible (red arrow); C – brain of a 7-day-old mouse inoculated IP with the CX-01 virus. The cortex, thalamus and hypothalamus on one side of the tissue have extensive necrosis, and fragmented nuclei are visible (black arrows); D – brain of a 7-day-old mouse inoculated IC with the CX-01 virus. Hippocampal pyramidal cells are regularly arranged, with clear demarcation, and round nuclei with white blood cells in the vascular cavity (yellow arrow). Haematoxylin and eosin staining. Scale bar– 50 μm

Recombination analysis. Possible recombination events were detected using SimPlot. This analysis revealed that the M genomic segment showed remarkably high degrees of variability. Five recombination breakpoints within the M segment of CX-01 were identified from the similarity plot, which were located at nt 1,001, nt 1,881, nt 2,197, nt 3,103 and nt 4,035. Genome scale similarity comparisons of CX-01 (query) with another three strains besides AKAV-32/SKR/2010 (to which CX-01 is most similar) located recombination in the region of nt 1,881–2,301 and a break-point at nt 3,103 (Fig. 3). The breakpoints in the CX-01 strain showed that recombination had mostly taken place with

the DHL10M110, Iriki and NM/BS/1strains. Among the cited strains, recombination breakpoints were found to be located in the NSm non-structural protein. This is consistent with the genome analysis based on the M gene segment (Table 2). These results indicated that the strains shared the same pattern of recombination and acquired different virulence through recombination events from the M segment, especially controlled by the NSm region.

Experimental infection, histopathology and immunohistochemistry analysis. One-week-old mice were inoculated with the CX-01 strain *via* either the IP or IC route and were observed three times a day. All mice inoculated IP and six out of the ten inoculated IC died after 6 days. The mice remaining alive showed weak pathological signs and died after a further 4 days. Brain tissue samples were collected from mice immediately after death and fixed with formalin for histopathological analysis. Microscopy revealed tissue damage, mainly as neuronal degeneration, necrosis, enlarged vascular endothelial cells and perivascular infiltration of mononuclear cells, most evident in IP inoculated mice (Fig. 4A). Furthermore, circular cavities were visible in the brain stem tissue, a small number of nerve cells were present around the gap and a proportion of the neurons were necrotic. Nuclei were fragmented or dissolved and a limited number of white cells were also contained in the IC-inoculated lumen (Fig. 4B). The thalamus and hypothalamus were necrotic, with a loose structure, eosinophilic reticulum, pyknosis and nuclear fragmentation. Nuclei were large and round with less chromatin and obvious nucleoli in IP-inoculated mice tissue (Fig. 4C). A significant number of white blood cells were seen in the IC-inoculated vascular cavity (Fig. 4D).

Fig. 5. Immunohistochemical characteristics in the central nervous tissues of mice with AKAV CX01 strain. A – brain stem of a mouse inoculated intraperitoneally (IP). Cells detected as antigen positive were observed as dark brown (red arrow); B – brain of a mouse inoculated intracerebrally (IC). Viral antigen is present mainly in neurons of the hippocampus, of which some cells were detected as antigen positive (red arrow); C – brain of a mouse inoculated IP. The hippocampal pyramidal cells were virus antigen–positive (red arrow); D – brain of a mouse inoculated IC. The hippocampal pyramidal cells were virus antigen positive (red arrow). Haematoxylin and eosin staining. Scale bar– 50 μm

Immunohistochemical examination was performed to detect AKAV antigens in neurons and brain tissue. Positive antigens were found in the neurons and the vascular endothelial cells of the cerebrum, cerebellum and IP-inoculated brain stem (Fig. 5A). Severe neuronal necrosis, mild neuronal degeneration, necrosis and perivascular infiltration of mononuclear cells with virus antigens were present in the brains of mice inoculated IC with the CX-01 strain (Fig. 5B). The brain of a 7-day-old mouse inoculated IP as well as that of one inoculated IC with the CX-01 virus contained hippocampal pyramidal cells which were virus antigen–positive (Figs. 5C and 5D).

Discussion

AKAV infections have been widely reported in Australian and Asiatic countries from tropical Indonesia to temperate countries such as Japan and Korea. The prototype strain of AKAV, JaGAr39, was first isolated in Japan from mosquitoes in 1959 (24). Since then, this disease has been detected in other countries as well. The first Chinese AKAV isolation took place in 2004 from a mosquito in the southwestern Yunnan province (10); however, other hosts both from Yunnan province and other parts of China harboured all AKAVs associated with genogroup Ia (5, 32, 33). Although the first described isolation in China was from a mosquito, the main transmission modes in China will only be ascertained after further investigation. However, recent entomological studies have revealed that *Culicoides* species serve as the principal vector of AKAVs (20, 36), mosquitoes nevertheless being important transmission agents of AKAV to susceptible hosts (3, 16). Yunnan province is located in the southwestern region of China and borders Vietnam, Laos and Myanmar. The climate of Yunnan is the monsoon type of the subtropical plateau. Vectors are observed to be present year-round in some tropical regions, whereas the number of adult *Culicoides* decreases or drops virtually to zero during the dry season in most temperate climate regions. However, *Culicoides* and mosquito species are widely distributed from low-latitude valleys to high mountain plains (9).

A survey in Yunnan in the 1980s revealed that seroprevalence of AKAVs infections was high in cattle, sheep and goats. In this study, we collected suspicious blood samples from surveyed farm goats with mild symptoms for virus isolation and characterisation. Previous studies of the Chinese isolates were mainly focused on the analysis of the S and M gene segment variation. We cloned the whole genome in the full complement of three segment genes to analyse the evolution and mutation of CX-01 isolated from goats. Analysis of the S and M segments indicated that AKAV isolates from East Asia clustered together in three clades corresponding to the previously described genogroups Ia, Ib and II. Genogroup Ia included AKAV isolates from Japan, China and South Korea from 1984 to 2016, while

genogroup Ib comprised only six viruses isolated in Japan (4, 17, 37). However, all the Chinese isolates fell within genogroup Ia (11, 32, 33). In this study on the CX-01 virus strain isolated from goat samples, that strain had 94.7%–98.1% nucleotide similarity and 99.1%–99.6% amino acid similarity of the S segment. Only one amino acid substitution was detected at site 310, where Valine (Val) was substituted by Isoleucine (Ile). These results showed that the S segment was the most conserved region. Analysis of the NSm gene in this segment of the AKAV genome also showed that the S segment was highly conserved. Our findings were consistent with previous studies that found a low degree of variability in the S segment (1, 6). The M segment was encoded into two virion surfaces, Gn and Gc, as well as NSm (22). Glycoprotein NSm is known for virus attachment to insect cells and Gc for neutralisation and virus attachment to mammalian cells (27). Analysis of these genes showed that their nucleotide and amino acid sequence identities were low. The strong mutation in the NSm region may indicate the source of the difference in virulence and growth character, suggesting that NSm plays a role in virus replication. Further analysis also demonstrated that NSm affects AKAV replication *in vitro* as well as *in vivo* and that it may be a virulence factor (15). However, further reverse genetics investigation is still needed to assess for CX-01 whether the virulence is in correlation with NSm variation.

Classical clinical symptoms of AKAV infection mainly include reproductive failures, such as abortion, stillbirth, premature birth and congenital deformities known as arthrogryposis-hydranencephaly syndrome (19). Although high seroprevalence has been detected in China, the disease usually takes an asymptomatic or mild course with only a transient fever and mild neurological disorder in the rainy season when the proliferation of *Culicoides* species and mosquitoes is at its peak. An outbreak of this disease causing abortion and death has never been reported in Yunnan province. In demonstration of the pathogenicity of the CX-01 strain, suckling mice inoculated with the CX-01 strain *via* the IP and IC routes at the age of one week developed encephalitis resulting in death and indicating that the strain was highly virulent. There are considerable variations in antigenic and pathogenic properties among field isolates of AKAV (19). To study the tissue distribution of the viral antigen, tissue samples were immediately collected after the death of the mice and fixed for pathology and histopathological analysis. Immunohistopathological examination results showed the virus mainly damages brain tissue, especially in the brain stem and the hippocampal pyramidal region. However, no obvious pathological changes in muscle, liver or spleen tissue were observed. The results also showed that AKAV can break through the blood–brain barrier effectively, which is a prevalent characteristic of *Bunyaviridae*; however the neuropathogenic mechanism is poorly understood.

In conclusion, this study describes the molecular characterisation of a novel goat AKAV isolate, CX-01,

and demonstrates its affiliation to genogroup Ia. The isolate CX-01 can cause encephalomyelitis and finally lead to the death of the mouse inoculated either IP or IC. Sequence analysis also indicates that new mutations are present in this field isolate, especially in the M segment, when compared to local Chinese strains.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Animal Rights Statement: All procedures and animal care provided in the study were in accordance with the guidelines approved by the Yunnan Laboratory Animal Administration Authority.

References

1. Akashi H., Kaku Y., Kong X.-G., Pang H.: Sequence determination and phylogenetic analysis of the Akabane bunyavirus S RNA genome segment. J Gen Virol 1997, 78, 2847–2851, doi: 10.1099/0022-1317-78-11-2847.

2. Al-Busaidy S., Hamblin C., Taylor W.P.: Neutralising antibodies to Akabane virus in free-living wild animals in Africa. Trop Anim Health Prod 1987, 19, 197–202, doi: 10.1007/BF02242116.

3. Allingham P.G., Standfast H.A.: An investigation of transovarial transmission of Akabane virus in *Culicoides brevitarsis*. Aust Vet J 1990, 67, 273–274, doi: 10.1111/j.1751-0813.1990.tb07791.x.

4. An D.-J., Yoon S.H., Jeong W., Kim H.-J., Park B.-K.: Genetic analysis of Akabane virus isolates from cattle in Korea. Vet Microbiol 2010, 140, 49–55, doi: 10.1016/j.vetmic.2009.07.018.

5. Cao Y.-X., Fu S.-H., Song S., Cai L., Zhang H., Gao L.-D., Cao L., Li M., Gao X.-Y., He Y., Wang H.-Y., Liang G.D.: Isolation and Genome Phylogenetic Analysis of Arthropod-Borne Viruses, Including Akabane Virus, from Mosquitoes Collected in Hunan Province, China. Vector Borne Zoonotic Dis 2019, 19, 62–72, doi: 10.1089/vbz.2018.2267.

6. Chang C.-W., Liao Y.-K., Su V., Farh L., Shiuan D.: Nucleotide sequencing of S-RNA segment and sequence analysis of the nucleocapsid protein gene of the newly isolated Akabane virus PT-17 strain. Int J Biochem Mol Biol 1998, 45, 979–987, doi: 10.1002/iub.7510450515.

7. Charles J.A.: Akabane virus. Vet Clin North Am Food Anim Pract 1994, 10, 525–546, doi: 10.1016/s0749-0720(15)30537-5.

8. Davies F.G., Jessett D.M.: A study of the host range and distribution of antibody to Akabane virus (genus Bunyavirus, family Bunyaviridae) in Kenya. J Hyg (Lond) 1985, 95, 191–196, doi: 10.1017/s0022172400062422.

9. Duan Y.-L., Bellis G., Li L., Li H.-C., Miao H.-S., Kou M.-L.: Potential vectors of bluetongue virus in high altitude areas of Yunnan Province, China. Parasit Vectors 2019, 12, 464, doi: 10.1186/s13071-019-3736-9.

10. Feng Y., He B., Fu S.-H., Yang W.-H., Zhang Y.-Z., Tu C.-C., Liang G.-D., Zhang H.-L.: Isolation and identification of the Akabane virus from mosquitoes in Yunnan Province, China (in Chinese). Bing Du Xue Bao 2015, 31, 51–57.

11. Feng Y., Zhang Y.-Z., Yang W.-H., Zhang H.-L.: Characterization and Analyses of the Full-length Genome of a Strain of the Akabane Virus Isolated from Mosquitoes in Yunnan Province, China (in Chinese). Bing Du Xue Bao 2016, 32, 161–169.

12. Hashiguchi Y., Nanba K., Kumagai T.: Congenital abnormalities in newborn lambs following Akabane virus infection in pregnant ewes. Natl Inst Anim Health Q (Tokyo) 1979, 19, 1–11.

13. Huang C.-C., Huang T.-S., Deng M.-C., Jong M.-H., Lin S.-Y.: Natural infections of pigs with Akabane virus. Vet Microbiol 2003, 94, 1–11, doi: 10.1016/s0378-1135(03)00062-2.

14. Inaba Y., Kurogi H., Omori T.: Letter: Akabane disease: epizootic abortion, premature birth, stillbirth and congenital arthrogryposis-hydranencephaly in cattle, sheep and goats caused by Akabane virus. Aust Vet J 1975, 51, 584–585, doi: 10.1111/j.1751-0813.1975.tb09397.x.

15. Ishihara Y., Shioda C., Bangphoomi N., Sugiura K., Saeki K., Tsuda S., Iwanaga T., Takenaka-Uema A., Kato K., Murakami S., Uchida K., Akashi H., Horimoto T.: Akabane virus nonstructural protein NSm regulates viral growth and pathogenicity in a mouse model. J Vet Med Sci 2016, 78, 1391–1397, doi: 10.1292/jvms.16-0140.

16. Jennings M., Mellor P.S.: *Culicoides*: biological vectors of Akabane virus. Vet Microbiol 1989, 21, 125–131, doi: 10.1016/0378-1135(89)90024-2.

17. Kobayashi T., Yanase T., Yamakawa M., Kato T., Yoshida K., Tsuda T.: Genetic diversity and reassortments among Akabane virus field isolates. Virus Res 2007, 130, 162–171, doi: 10.1016/j.virusres.2007.06.007.

18. Konno S., Moriwaki M., Nakagawa M.: Akabane disease in cattle: congenital abnormalities caused by viral infection. Spontaneous disease. Vet Pathol 1982, 19, 246–266, doi: 10.1177/030098588201900304.

19. Kono R., Hirata M., Kaji M., Goto Y., Ikeda S., Yanase T., Kato T., Tanaka S., Tsutsui T., Imada T.,Yamakawa M.: Bovine epizootic encephalomyelitis caused by Akabane virus in southern Japan. BMC Vet Res 2008, 4, 20, doi: 10.1186/1746-6148-4-20.

20. Kurogi H., Akiba K., Inaba Y., Matumoto M.: Isolation of Akabane virus from the biting midge *Culicoides oxystoma* in Japan. Vet Microbiol 1987, 15, 243–248, doi: 10.1016/0378-1135(87)90078-2.

21. Lole K.S., Bollinger R.C., Paranjape R.S., Gadkari D., Kulkarni S.S., Novak N.G., Ingersoll R., Sheppard H.W., Ray S.C.: Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J Virol 1999, 73, 1, 152–160.

22. Ludwig G.V., Israel B.A., Christensen B.M., Yuill T.M., Schultz K.T.: Role of La Crosse virus glycoproteins in attachment of virus to host cells. Virology 1991, 181, 564–571, doi:10.1016/0042-6822(91)90889-J.

23. Ogawa Y., Fukutomi T., Sugiura K., Sugiura K., Kato K., Tohya Y., Akashi H.: Comparison of Akabane virus isolated from sentinel cattle in Japan. Vet Microbiol 2007, 124, 16–24, doi: 10.1016/j.vetmic.2007.03.020.

24. Oya A., Okuno T., Ogata T., Kobayashi I., Matsuyama T.: Akabane, a new arbor virus isolated in Japan. Jpn J Med Sci Biol 1961, 14, 101–108, doi: 10.7883/yoken1952.14.101.

25. Qiao J., Meng Q.-L., Zhang Z.-C., Cai K.-J., Zhang J.-S., Ma M.-X., Chen C.-F.: A serological survey of Akabane virus infection in cattle and sheep in northwest China. Trop Anim Health Prod 2012, 44, 1817–1820, doi: 10.1007/s11250-012-0168-3.

26. Şevik M.: Molecular detection and genetic analysis of Akabane virus genogroup Ib in small ruminants in Turkey. Arch Virol 2017, 162, 2769–2774, doi: 10.1007/s00705-017-3398-x.

27. Shi X.-H., Kohl A., Léonard V.H.J., Li P., McLees A., Elliott R.M.: Requirement of the N-Terminal Region of Orthobunyavirus Nonstructural Protein NSm for Virus Assembly and Morphogenesis. J Virol 2006, 80, 8089–8099, doi: 10.1128/JVI.00579-06.

28. Snijder E.J., Brinton M.A., Faaberg K.S., Godeny E.K., Gorbalenya A.E., MacLachlan N.J., Mengeling W.L., Plagemann P.G.W.: Family Arteriviridae. In: *Virus Taxonomy: Eighth Report of the International Committee on the Taxonomy of Viruses*, edited by C.M. Fauquet, M.A. Mayo, J. Maniloff, U. Desselberger, L.A. Ball, Elsevier Academic Press, San Diego, 2005, pp 695–716.

29. St George T.D., Cybinski D.H., Paull N.I.: The isolation of Akabane virus from a normal bull. Aust Vet J 1977, 53, 249, doi: 10.1111/j.1751-0813.1977.tb00203.x.

30. St George T.D., Standfast H.A., Cybinski D.H.: Isolations of Akabane virus from sentinel cattle and *Culicoides brevitarsis*. Aust Vet J 1978, 54, 558–561, doi: 10.1111/j.1751-0813.1978.tb02412.x.

31. Tamura K., Stecher G., Peterson D., Filipski A., Kumar S.: MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 2013, 30, 2725–2729, doi: 10.1093/molbev/mst197.

32. Tang H.-B., Chen F.-L., Rao G., Bai A., Jiang J.-J., Du Y.-C., Ren P.-F., Liu J.-F., Qin S.-M., Yang L., Wu J.-M.: Characterization of Akabane virus from domestic bamboo rat, Southern China. Vet Microbiol 2017, 207, 280–285, doi: 10.1016/j.vetmic.2017. 06.018.

33. Tang H.-B., Ren P.-F., Qin S.-Y., Lin J., Bai A., Qin S.-M., Chen F.-L., Liu J.-F., Wu J.-M.: Isolation, genetic analysis of the first Akabane virus from goat in China. J Vet Med Sci 2019, 81, 1445–1449, doi: 10.1292/jvms.18-0602.

34. Taylor W.P., Mellor P.S.: The distribution of Akabane virus in the Middle East. Epidemiol Infect 1994, 113, 175–185, doi: 10.1017/s0950268800051591.

35. Wang J., Blasdell K.R., Yin H., Walker P.J.: A large-scale serological survey of Akabane virus infection in cattle, yak, sheep and goats in China. Vet Microbiol 2017, 207, 7–12, doi: 10.1016/j.vetmic.2017.05.014.

36. Yanase T., Kato T., Kubo T., Yoshida K., Ohashi S., Yamakawa M., Miura Y., Tsuda T.: Isolation of Bovine Arboviruses from *Culicoides* Biting Midges (Diptera: Ceratopogonidae) in Southern Japan: 1985–2002. J Med Entomol 2005, 42, 63–67, doi: 10.1093/jmedent/42.1.63.

37. Yanase T., Yoshida K., Ohashi S., Kato T., Tsuda T.: Sequence analysis of the medium RNA segment of three Simbu serogroup viruses, Akabane, Aino, and Peaton viruses. Virus Res 2003, 93, 63–69, doi: 10.1016/s0168-1702(03)00066-2.