NOTE

Virology

Isolation, genetic analysis of the first Akabane virus from goat in China

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ABSTRACT. Akabane virus (AKAV) is an important insect-borne virus belonging to the genus Orthobunyavirus, the Peribunyaviridae family. An AKAV defined as GXDH 01 here, was isolated for the first time from blood from a sentinel goat in China in 2016, and its full-length open reading frames (ORFs) were sequenced in this study. Sequence analysis suggested that the isolate GXDH 01 probably had undergone a reassortment incident and acquired L segments from other strain originating from an attenuated vaccine, such as OBE-1. This study aims to provide more understanding as to the origin and epidemiology of AKAV in China.

KEY WORDS: Akabane virus, complete open reading frame, goat, phylogenetic analysis

Akabane disease is an important arthropod-borne disease of cattle and sheep caused by Akabane virus (AKAV). AKAV belongs to the species Akabane orthobunyavirus, the genus Orthobunyavirus, the Peribunyaviridae family [15]. AKAV is an enveloped virus with three segments of single-stranded negative-sense RNA genome, small (S), medium (M), and large (L), which differ in sizes with approximately 0.86, 4.3, and 7 kb, respectively [20].

Diseases caused by AKAV in cattle, goats and sheep are associated with abortions, premature delivery, stillbirths, and fetal malformation, mummified fetus, neonatal joint flexion, without brain and encephalitis [7]. The disease was prevalent in cattle and sheep in Australia in the 1930s and was subsequently reported in Japan. From 1972 to 1975, more than 42,000 calves were lost in the herds west of Kanto, Japan, associated with economic losses of more than five billion Japanese Yen [4, 13, 17]. Apart from Japan and Australia, Israel, Saudi Arabia, Kuwait, Yemen, Bahrain, Turkey, Indonesia, South Korea and the United Arab Emirates had reported this disease successively [1, 3, 6, 8, 19, 23]. It has caused serious economic losses to animal husbandry and posed a great threat to the development of cattle and sheep breeding [11]. It is classified as a classII epidemic disease that must be in quarantine in the importing of cattle and sheep in China. Because of its wide distribution and damaging effects, AKAV is one of the key quarantine objects in international animal trade.

A previous serological survey of AKAVs in cattle, goats and sheep in Shaanxi, Inner Mongolia, Hunan, Hebei, Shandong, Anhui, Zhejiang, Fujian, and Shanghai displayed that the total neutralizing antibody positive rate of cattle reached 39.18%, and the neutralizing antibody positive rate of goats was 12.66% [14]. A large-scale serological survey of Akabane virus infection in cattle, yak, sheep and goats were performed in China. Results showed that the total neutralizing antibody positive rate of cattle reached 21.3%, and the neutralizing antibody positive rate of goats was 7.7% [24]. Tang et al. first reported natural infection of bamboo rats by AKAVs in Guangxi province, southern China [22]. The results indicated a widespread AKAV infection among cattle and sheep in China. Asymptomatic infection of AKAV has been demonstrated in wild animals such as impala, blue wildebeest, warthog and elephant [2]. Akabane disease rarely occurs in other animals. However, in the brain samples collected previously from domestic bamboo rats after death we confirmed that the bamboo rats were infected with AKAV [22]. So far, there has never been a report of direct isolation of AKAV from cattle, goat and sheep in China. The present study describes the first goat-derived AKAV isolate reported in China.

In this study a survey on sera from 14 cities in Guangxi of China was conducted (Table 1). Serum samples were collected from cattle (983) and goats (1,428) in 2014 and 2015. All serum samples were collected randomly from animals over 6 months of age on large-scale farms or from free-range cattle or goats that had no record of AKAV vaccination. The serum antibody to Akabane disease is an important arthropod-borne disease of cattle and sheep caused by Akabane virus (AKAV). AKAV belongs to the species Akabane orthobunyavirus, the genus Orthobunyavirus, the Peribunyaviridae family [15]. AKAV is an enveloped virus with three segments of single-stranded negative-sense RNA genome, small (S), medium (M), and large (L), which differ in sizes with approximately 0.86, 4.3, and 7 kb, respectively [20].

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A classical operation procedure for AKA V isolation was performed on these serum samples as described by Gard et al. [5]. Briefly, embryonated chicken eggs were inoculated with AKA V antibody-positive serum collected from sentinel cattle. Brains, lungs, spleens and livers were harvested from dead embryos at day 3–5 post inoculation and were homogenized, after which the supernatants of organization homogenates were passaged 3–5 times on BHK-21 cells. The culture virus was tested with ID Screen® Akabane Competition ELISA Kit (ID-VET, Montpellier, France) following the manufacturer’s instructions. A total of 2411 serum samples obtained from cattle and goats in Guangxi were tested for the presence of antibodies to AKA V. There was evidence of AKA V antibodies in cattle and goats from the 14 cities. Overall AKA V seroprevalence in cattle was 63.89% (628/983 tested). And AKA V antibodies were 36.48% positive in goat serum samples from all 14 cities (521/1428 tested). AKA V seroprevalence was higher in cattle than in goats in the χ² test (P<0.01) (Table 1). The positive rate of our detection of AKA V antibodies is higher than previously reported. It is possible that because of the previously reported focus on neutralizing antibodies of AKA V, we detected the multiple antibodies of the virus. In addition, these differences may also have a certain relationship with the region. Previous reports were mainly distributed in the northern China, and our tests were in southern China. The climatic reasons determine the difference in the type and quantity of Culicoides biting midges.

The AKA V positive serum samples collected from sentinel cattle and goat in Guangxi Province in 2015 and 2016 were for AKA V isolation and identification. A classical operation procedure for AKA V isolation was performed on these serum samples as described by Gard et al. [5]. Briefly, embryonated chicken eggs were inoculated with AKA V antibody-positive serum collected from sentinel cattle. Brains, lungs, spleens and livers were harvested from dead embryos at day 3–5 post inoculation and were homogenized, after which the supernatants of organization homogenates were passaged 3–5 times on BHK-21 cells. The culture virus was monitored daily for cytopathic effect (CPE). The cultured supernatants were harvested at 2–3 days post-inoculation and stored at −70°C as a viral stock. An isolate of AKA V was obtained from these serum samples by testing cell culture supernatants using a reverse transcription polymerase chain reaction (RT-PCR) method [22]. The AKA V isolate defined as GXDH 01, then 6-well tissue culture plates were used for plaque purification of the isolate in which each well was seeded with 1 × 10⁶ BHK-21 cells in 10% growth medium and incubated at 37°C. GXDH 01 was serially diluted 10⁻¹ from to 10⁻⁶ in Minimum Essential Medium (MEM), in six wells, monolayer of five wells were infected with 0.5 ml of different virus dilution starting from 10⁻² to 10⁻⁶ and 6th well was kept as cell control. This plate was kept in incubator with 5% CO₂ and 6th day, plaques which are more distinct and isolated from others were collected with the help of micropipette in 200 µl of 2× MEM, 50% Fetal Bovine Serum (FBS); 2% plaque agarose mixture in 1:1 ratio. Finally, overlayed plates were allowed to solidify and then transferred to incubator. For further analysis, twelve primer pairs were designed based on the published genome sequence of the KU375444, AB190458 and AB297832 strains and were used to amplify the whole AKA V genome (Table 2). For amplification of the complete genome segments of the isolate GXDH 01, total RNA was extracted from infected BHK-21 cells with RNA Kits (TransGen Biotech, Beijing, China) according to the manufacturer’s instructions. The cDNA was synthesized using 1.0 µg total RNA, 1 µl (20 pmol/µl) forward primer, and 100 U Moloney murine leukemia virus (MMLV) reverse transcriptase in a 25-µl reaction volume. PCR products were purified using a DNA purification kit (TianGEN, Beijing, China) according to the manufacturer’s instructions and were submitted to Sangon Biotech Co., Ltd. (Shanghai, China) for sequencing, then the genome sequences were assembled using SeqMan software (DNASTAR Inc., Madison, WI, U.S.A.). The sequences of 3 genome segments of AKA V isolate GXDH 01 were submitted to GenBank under accession No. MH174977–MH174979. The S, M and L segments of AKA V isolate was 0.744, 4.190, and 6.816 kb in length excluding the primer binding sites respectively. Goat AKA V shared 95.6–98.3, 88.2–96.6 and 92.2–99.1% homology with the sequences of the same segments of the AKA V isolate GXDH 01 from Guangxi.

**Table 1.** Distributions of the seroprevalence of Akabane virus in Guangxi province

| Location     | Cattle (%) | Goat (%) | Total (%) |
|--------------|------------|----------|-----------|
|              | 2014       | 2015     | 2014      | 2015     |          |
| Liuzhou      | 40.42 (19/47) | 14.00 (7/50) | 29.74 (69/232) | 30.00 (6/20) | 28.94 (101/349) |
| Guilin       | 65.21 (30/46) | 56.67 (17/30) | 12.85 (9/70) | 13.33 (4/30) | 34.09 (60/176) |
| Yulin        | 58.33 (35/60) | 87.5 (35/40) | 18.18 (4/22) | 47.56 (39/82) |
| Hezhou       | 87.5 (35/40) | 60.00 (62/100) | 42.10 (8/19) | 66.04 (105/159) |
| Beihai       | 100 (30/30) | 68.33 (41/60) | 84.89 (88/180) | 58.99 (164/278) |
| Qinzhou      | 80.00 (48/60) | 41.58 (42/101) | 55.90 (90/161) |
| Laibin       | 50.00 (15/30) | 25.00 (25/100) | 23.33 (7/30) | 29.38 (47/160) |
| Fangchenggang | 55.00 (33/60) | 14.00 (7/50) | 10.00 (2/20) | 24.56 (28/114) |
| Chongzuo     | 43.18 (19/44) | 60.00 (30/50) | 66.67 (20/30) | 62.50 (50/80) |
| Wuzhou       | 67.24 (39/58) | 80.25 (126/157) | 44.19 (76/172) | 50.00 (115/230) |
| Baise        | 84.73 (111/131) | 24.46 (23/94) | 40.00 (4/10) | 67.35 (264/392) |
| Nanning      | 26.67 (16/60) | 35.00 (7/20) | 32.50 (13/40) | 33.63 (37/110) |
| Guigang      | 84.73 (111/131) | 80.25 (126/157) | 44.19 (76/172) | 50.00 (115/230) |
| Hechi        | 35.00 (7/20) | 34.00 (17/50) | 32.50 (13/40) | 33.63 (37/110) |
| Total        | 74.80 (190/254) | 60.08 (438/729) | 35.54 (365/1027) | 38.90 (156/401) | 47.66 (1,149/2,411) |

The seroprevalence of the virus was collected from an 8 month old sentinel goat kept in Danzhang tun, Chengnei Village, Dahua County, Hechi City, Guangxi in April 2016. For further analysis, twelve primer pairs were designed based on the published genome sequence of the NU375444, AB190458 and AB297832 strains and were used to amplify the whole AKA V genome (Table 2). For amplification of the complete genome segments of the isolate GXDH 01, total RNA was extracted from infected BHK-21 cells with RNA Kits (TransGen Biotech, Beijing, China) according to the manufacturer’s instructions. The cDNA was synthesized using 1.0 µg total RNA, 1 µl (20 pmol/µl) forward primer, and 100 U Moloney murine leukemia virus (MMLV) reverse transcriptase in a 25-µl reaction volume. PCR products were purified using a DNA purification kit (TianGen, Beijing, China) according to the manufacturer’s instructions and were submitted to Sangon Biotech Co., Ltd. (Shanghai, China) for sequencing, then the genome sequences were assembled using SeqMan software (DNASTAR Inc., Madison, WI, U.S.A.). The sequences of 3 genome segments of AKA V isolate GXDH 01 were submitted to GenBank under accession No. MH174977–MH174979. The S, M and L segments of AKA V isolate was 0.744, 4.190, and 6.816 kb in length excluding the primer binding sites respectively. Goat AKA V shared 95.6–98.3, 88.2–96.6 and 92.2–99.1% homology with the sequences of the same segments of the AKA V isolate GXDH 01 from Guangxi.
of nucleotide identity with the known AKAV isolates in S, M and L genes, respectively. The putative proteins of goat AKAV was 233 amino acids (aa) for the nucleoprotein (N), 1401 aa for the polyprotein and 2251 aa for the RNA-dependent RNA polymerase protein respectively. Goat AKAV shared 97.9–99.1, 92.3–98.5 and 97.8–99.5% of amino acid identity with the known AKAV isolates in S, M and L genes, respectively.

To define the relationship of the newly isolated goat AKAVs within the AKAV family, phylogenetic trees were constructed based on the sequences of the S, M and L genes from the goat AKAV isolate and the known AKAV sequences from the GenBank (Supplementary Table 1). Intact open reading frames (ORFs) sequence alignments were performed by clustal W method using MEGA 5 software [21]. The neighbor-joining (NJ) phylogenetic trees based on the S gene, M gene and L gene were constructed using the MEGA 5.0 software and 1000 bootstrap replicates were used to statistically assess the branching pattern. The phylogenetic trees based on the sequences of the S gene revealed that the goat AKAV isolate from China fell into a single clade (defined as Group Ia) together with the bamboo rat AKAV isolates GXLCH 01, GXLCH 02 and GXLCH 04 from China (97.4–98.8%) (Fig. 1A). The phylogenetic trees based on the sequences of the M gene revealed that the goat AKAV isolate from China fell into the very same clade (defined as Group Ia) as the bovine AKAV isolate AKA V-7/SKR/2010 which caused encephalomyelitis in cattle in South Korea [18] and AKA V isolates GXLCH 01, GXLCH 02 and GXLCH 16–70 were recognized to cause encephalomyelitis in adult bamboo rats from China (95.1–96.6%) (Fig. 1B). The M genes were higher than the 88.3–88.7% homologies observed between the goat AKAV isolate and OBE-1, 93FMX, TS-C2 and K0505 from Japan and Korea. However, based on the sequences of the L genes, the goat AKAV isolate and GXLCH 01, GXLCH 02 and GXLCH 16–70 were classified into two different clades (defined as Group IIa andIIb) (Fig. 1C). The L genes of goat isolate and GXLCH 01, GXLCH 02 and GXLCH 16–70 shared 92.2–92.4% homologies, lower than the 99.0–99.1% homologies observed between the goat AKAV isolate and OBE-1, 93FMX, TS-C2 and K0505. The phylogenetic trees based on the sequences of the L genes suggested that the goat AKAV isolate had close relationship with the OBE-1, 93FMX, TS-C2 and K0505 from Japan and Korea strains recognized to attenuate or were avirulent in mice [12, 16]. It was more distantly related to the GXLCH 01, GXLCH 02 and GXLCH 16–70 isolates (Fig. 1C).

An isolate Tinaroo virus (TINV) from Australia was reported to have genomic reassortment events AKAV S and L RNA segments with an M RNA segment of an unknown orthobunyavirus [10]. The goat AKAV reported here suggests that isolate GXLCH 01 is a reassortant virus which has undergone a reassortment event and has probably obtained L gene segments from other strain originating from an attenuated vaccine, such as OBE-1. To determine the pathogenicity of GXLCH 01, 1day-old suckling mice and adult mice free of AKAV neutralizing antibody were average divided into intracerebral (IC), and negative control groups (n=6 mice/group). The mice were anesthetized by halothane inhalant in a container and then each was inoculated intracerebrally.

The oligonucleotide position is based on the sequences of three strains (a) KU375444, b) AB297832, c) AB190458) of Akabane viruses.

| Table 2. The primers amplification the S, M and L segments of Akabane virus isolated from goat, China used in this study |
| --- |
| Gene | Primer | Sequence (5′-3′) | Position | Product size (bp) |
| N | AKVS1 | TGCAATGGCAAATCATATTATTT | 30–51a) | 788 |
|   | AKVS2 | CGAGCAGCTGAACAAAGGTGTG | 796–817 |
| M | AKVM1 | ATGATTATTACAATCCTACACAT | 2–24b) | 816 |
|   | AKVM2 | GCTAAAGAGTGAATTGTT | 799–817 |
|   | AKVM3 | CTTTGTGGGGCATTATCCCTGAC | 673–693 | 1,497 |
|   | AKVM4 | ATTTGGATTGGGACATTCTTGTT | 2,147–2,169 |
|   | AKVM5 | CGTAAATTACCGAACACCTTT | 1,915–1,936 |
|   | AKVM6 | GCCAAGATGTCAATAGAGAC | 3,340–3,361 | 1,447 |
|   | AKVM7 | GCCAAGATGTCAATAGAGAC | 3,340–3,361 | 1,096 |
| L | AKVL1 | TAGTTGACCCCCCTAAATACAAAC | 3–24c) | 1,050 |
|   | AKVL2 | AATGCGTCTAAATATGTC | 1,034–1,052 |
|   | AKVL3 | TTGCAACTATGGACAGAGA | 864–885 | 1,285 |
|   | AKVL4 | TCTTTGGCTTATATGCGTGTA | 2,101–2,121 |
|   | AKVL5 | CGAGCATAGATTGAGGT | 1,886–1,905 | 1,438 |
|   | AKVL6 | AATATGACCCCTCACTACATCAT | 3,302–3,323 |
|   | AKVL7 | AGATGAGATGATTAGCGAC | 2,976–2,996 | 1,286 |
|   | AKVL8 | ACAGAGACGGAGAACGATAC | 4,240–4,261 |
|   | AKVL9 | CAGGCATTCGACTATTTTGCTT | 4,034–4,055 | 1,255 |
|   | AKVL10 | CGCAATGACCGTACGACGCT | 5,269–5,288 |
|   | AKVL11 | CAATTGACCTTCTTGCTGCAATGAT | 5,135–5,156 | 1,219 |
|   | AKVL12 | TCGGATTCCGACTAAGACCTT | 6,334–6,353 |
|   | AKVL13 | AGCCAAAAAGGGTCCAGAGAAG | 5,848–5,869 | 1,013 |
|   | AKVL14 | GCCCCTTAAATGCGAATAATA | 6,840–6,860 |

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with 0.01–0.03 ml of GXDH 01 strain at a titer of $10^{5.2}$ TCID50/ml. After inoculation, the mice were observed daily for clinical signs. The GXDH 01 strain was avirulent or, at most, caused slightly transient weight loss in mice. Jeong et al performed experimental inoculation of AKA V-7 on goats via different routes. During the experimental period, infected goats showed no clinical signs, but some animals showed encephalomyelitis on histopathological examination after IC and IV inoculation and lesions in the brain and spinal cord after IS inoculation [9]. The isolate GXDH 01 of AKA V from southern China maybe have genomic reassortment events AKA V can cause encephalomyelitis in goats S and M RNA segments with an L RNA segment of an avirulent AKA V. The pathogenicity of reassortment AKA V cause attenuate in infection mice or goats. Since GXDG 01 was isolated from asymptomatic sentinel goats of 8 months old, its pathogenicity in sheep and cattle remains unknown. Thus, further study is necessary to evaluate the pathogenicity.

In conclusion, we isolated and sequenced the complete ORFs segments of strain GXDH 01 for the first time from goat in China. The strain was further determined to be a reassortant virus that contained L segments from an isolate of OBE-1 originating from Japan. Further experimental studies should be performed to confirm the biological characteristics of GXDH 01, especially its pathogenicity in sheep and cattle. This study will enhance our understanding of the epidemiology of AKA V in China.

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CONFLICT OF INTEREST. The authors declare no conflict of interests.

ETHICAL APPROVAL. The animal experiments were approved and performed in accordance with the animal ethics guidelines and approved procedures of Guangxi Veterinary Research Institute.

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