BMC Veterinary Research

RESEARCH

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Viromics-based precision diagnosis of reproductive abnormalities in cows reveals a reassortant Akabane disease virus

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Abstract

Background At the end of 2021, an epidemic of reproductive abnormalities in cows occurred in Jilin Province, China, posing an urgent need for a rapid diagnosis.

Results To identify the cause of the disease, a total of 172 samples were collected from 21 dead calves and 45 aborting or pregnant cows in 10 farms across the province. Routine PCR or RT-PCR detection did not find any common abortion-related agents. We then employed the viromics-based precision diagnosis method to analyze these samples, and the read-based annotation showed signals of an Akabane disease virus (AKAV) in some libraries. To further identify the virus, nested RT-PCR detection revealed that 52.3% (11/21) of dead calves and 26.6% (12/45) of cows were positive for the virus. Phylogenetic analysis of the partial fragments showed that the S segment of the virus was 100% identical to the Chinese strain TJ2016, but its M and L segments shared 94.3% and 96.5% identities with an Israeli strain.

Conclusions The viromic and molecular results suggested that these animals were infected with a reassortant AKAV. Coupled with the clinical signs, the virus should be responsible for the epizootic, highlighting that molecular and serological surveys of the virus in cows during early pregnancy, as well as ecological investigation in its arthropod vectors, are necessary.

Keywords Virome, Akabane disease virus, Arbovirus, Cattle, Reassortment

Introduction

Constituting a substantial part of animal husbandry, cattle industry provides an important source for the daily food supply [1]. Located in the geographical center of northeast China, Jilin Province has diversified structures of the cattle industry with co-existence of free-range mode and intensified industrialized production, which, in turn, contributes essential drivers to the economic growth of the province. However, because of the frequent introduction of breeder cattle and trade of live cattle, infectious diseases pose a stubborn obstacle to the regeneration of the cattle industry of Jilin, even across the nation [2]. In addition to sporadic outbreaks of diseases

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caused by foot-and-mouth disease virus (FMDV), bovine ephemeral fever virus (BEFV) [3], bovine viral diarrhea virus (BVDV), and infectious bovine rhinotracheitis virus (IBRV) [2, 4], exotic diseases have also caused devastating consequences to cattle farmers, e.g., the incursion of lumpy skin disease virus (LSDV) in 2019 and its subsequent spread over 14 provinces [5]. Particularly, cattle are also an important source of zoonoses, such as brucellosis [6] and tuberculosis [7]. Therefore, to address these concerns, measures like virus surveillance, vaccine development and application, and the practice of healthy feeding concepts are necessary.

Belonging to the genus *Orthobunyavirus* within the family *Peribunyaviridae*, the only member of the species *Orthobunyavirus akabaneense*, a.k.a., Akabane virus (AKAV), is an important pathogen of herbivore livestock that can invade the blood-placenta barrier to disturb the fetal development, causing hydranencephaly with a virtual absence of the cerebral hemispheres, even accompanied by arthrogryposis [8]. The genome of AKAV is ~12 kb in length and is divided into small (S), medium (M), and large (L) segments. As an arbovirus, AKAV is mainly transmitted by biting midges of the genus *Culicoides* and mosquitos of the family *Culicidae* [9]. Hence, the incidence of the disease depends on the distribution of its vectors and is closely related to the seasonality. Cows are most susceptible to AKAV infection during the first three to six months of gestation, with the outcomes being most severe when infected between the third and fourth months [10]. The virus was first identified in Japan in 1959 and has subsequently been reported in several other countries [11]. A large-scale serological survey of the virus in cattle, yak, sheep and goats from 24 provinces in China between 2006 and 2015 revealed that AKAV infection was prevalent in 23 provinces, with the seroprevalence in cattle in Jilin and the neighboring Liaoning Province being 20.1% (15/72) and 18.3% (22/120), respectively [12]. However, no Akabane disease has been reported in China.

From September 2021 to January 2022, an outbreak of reproductive abnormalities in cows occurred across Jilin Province, which has also spread to the neighboring Inner Mongolia, Liaoning, and Heilongjiang provincial regions, raising severe concerns among cattle farmers and local governments. In this study, we report how the disease was diagnosed using the viromics-based precision diagnosis of animal infectious diseases. The results not only help to take timely measures to control and prevent the disease but also exemplify that the precision surveillance strategy can be used to diagnose those unusual infectious diseases.

Results

Overview of the reproductive abnormalities in cows

The reproductive abnormalities in cows were featured by abortion, stillbirth, or delivery of deformed fetuses (Fig. 1A). Some cows with abortions were accompanied by fevers. Deformed fetuses were difficult to deliver. Although successfully born, calves showed neurological symptoms such as astasia-abasia and died within a few days. Necropsy of dead calves revealed significant cerebral edema with shallowed or disappeared gyri and some calves were even born with the absence of brain tissues and/or with forelimb joint deformities (Fig. 1B). A variety of clinical samples were collected for molecular diagnosis. In total, 104 samples of the heart, liver, spleen, lung, kidney, lymph node, intestine, and blood from 21 dead calves and 2 placenta tissues, 21 vaginal swabs, and 45 blood samples from aborted or pregnant cows were collected from 10 scaled farms (Fig. 1A). We performed PCR or RT-PCR detection of viral and bacterial pathogens that are known to cause abortion in cows, including BVDV, bluetongue virus (BTV), epizootic hemorrhagic diseases virus (EHDV), *Brucella* spp., and the results were all negative. Hence, it prompted us to investigate the causative agents using the viromics-based precision diagnosis strategy [13], which enables us to identify new, mutated, and co-infected pathogens within a single test.

Viromics-based precision diagnosis

By mixing the same tissues of dead calves into 22 pools and serum samples, vaginal swabs, and placenta from cows into 3 pools, we prepared 25 libraries for a combination of multiple displacement amplification (MDA) and meta-transcriptomics (MTT) viromic analyses [13]. The two methods generated 237.7 gigabases (Gb) and 264.1 Gb of clean data for the follow-up bioinformatic analysis. These data were sequentially subjected to the removal of the host genome, classification of the bacteria and fungi genomes, *de novo* assembly, and virus annotation. At the contig-level annotation, a total of 854 eukaryotic virus-like sequences with 308-7,320 bp in length were obtained. They were assigned into 11 families, including *Adenoviridae*, *Circoviridae*, *Herpesviridae*, *Genomoviridae*, *Polyomaviridae*, *Papillomaviridae*, *Smacoviridae*, *Astroviridae*, *Caliciviridae*, *Tobaniviridae*, and *Picornaviridae* (Fig. 1C). Compared to these RNA viruses, circoviruses and genomoviruses were prevalent across these pools. These RNA viruses were much more conserved, showing 78–100% nucleotide (nt) identities with reference sequences. On the contrary, some DNA viruses were very divergent, with 27–55% amino acid (aa) identities to known viruses (Fig. 1C). Although some viruses have been reported to cause diarrhea and respiratory diseases in livestock, such as astrovirus (AstV), norovirus (NoV), aphthovirus, and kobuvirus, but none

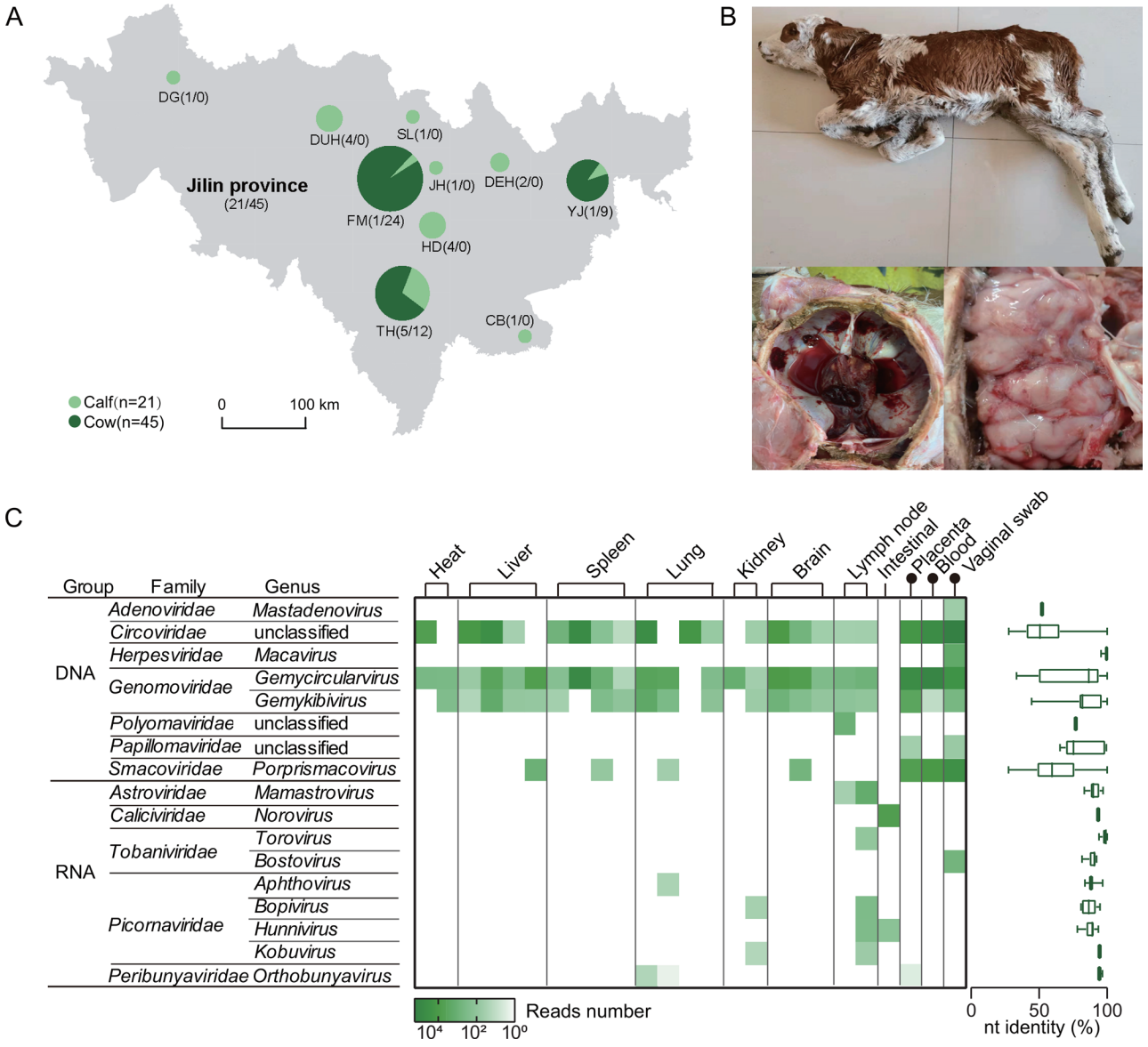

Fig. 1 Sampling sites, pathological changes, and virome overview of this study. (**A**) Sampling sites in Jilin province (FM = Fengman, DUH = Dunhua, DEH = Dehui, JH = Jiaohe, HD = Huadian, YJ = Yanji, CB = Changbaixian, TH = Tonghua, DG = Dagangzi, SL = Shulan). The pie charts are scaled to indicate the sample size of calves and cows (#/#: the sample number of calves/that of cows) (**B**) The dead calves with arthrogryposis and cerebral edema or absence of the cerebral hemispheres with replacement by fluid-filled sacs. (**C**) Overview of the virome. The heatmap shows the number of viral reads in each pool. These boxplots show nt identities of viral contigs with reference sequences. Due to the damage of the DNA sequencing data of the intestinal sample, the numbers of DNA viral reads are not shown. Libraries of cows are marked using filled black circles

were associated with the reproductive abnormalities. Therefore, we re-annotated the virome at the read level. The results were almost consistent with the contig-level annotation, but unexpectedly, we found 23 reads related to an Akabane disease virus (AKAV) in two lung pools and one placenta pool (Fig. 1C), which were further assembled into 149- and 256-nt-long L sequences and a 245-nt-long M sequence.

Molecular detection and phylogenetic analysis of AKAV

To confirm the presence of AKAV and investigate its prevalence, we first conducted molecular detection of AKAV in these samples using a nested RT-PCR method targeting a 448-nt-long conserved S segment. Overall, 11 out of the 21 dead calves were positive for the virus (52.3%), with varied positive rates (10–47%) in their internal organs (Fig. 2A). Notably, most (n = 8) of these calves were positive in their multiple organs, suggesting systematic AKAV infection of them (Fig. 2B). On the contrary,

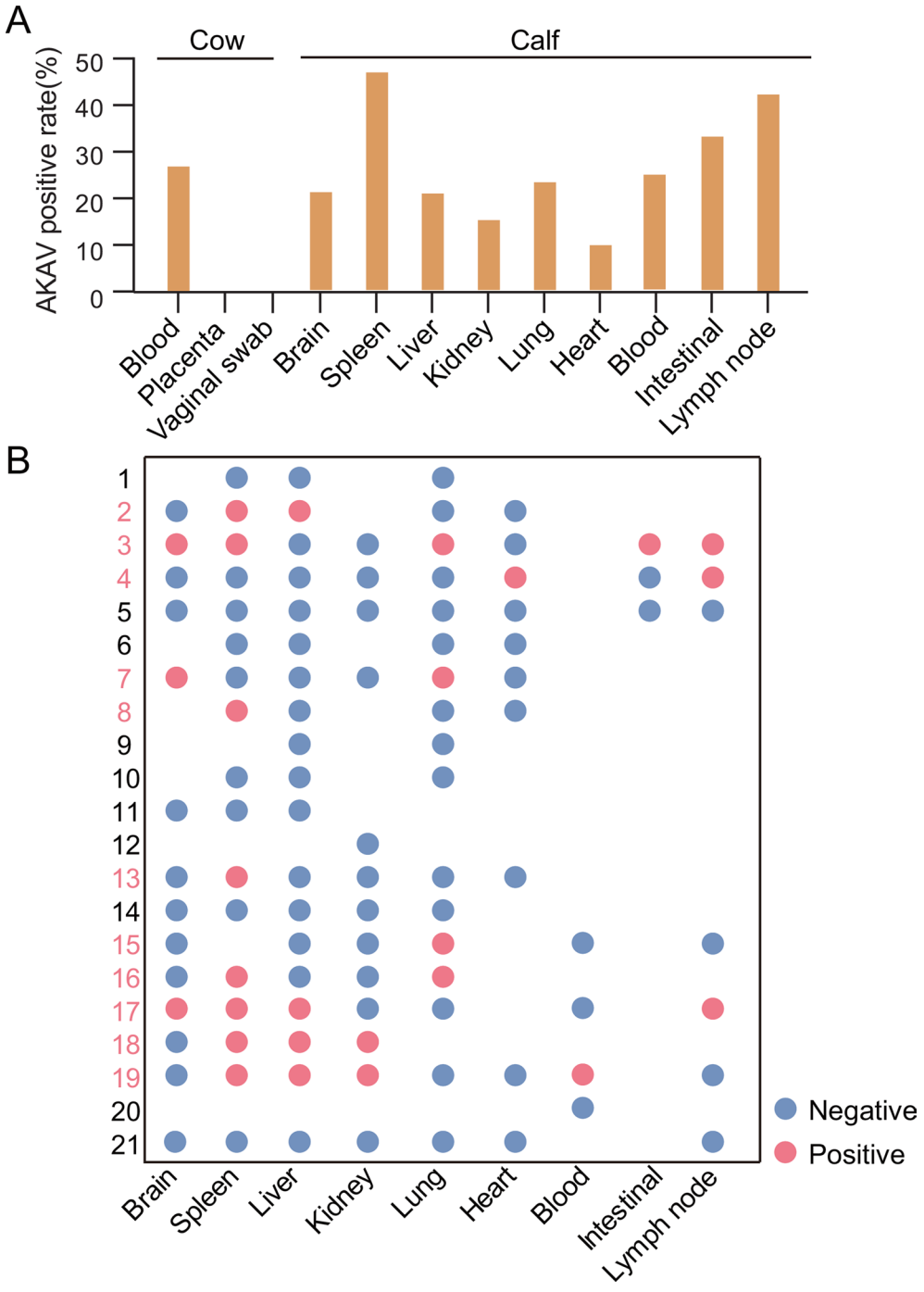

Fig. 2 RT-PCR results of AKAV in these samples. (**A**) The overall positive rates across different tissue types of cow and calves. (**B**) The positive details at the individual calf level

the virus was only detected in the blood of cows, with a positive rate of 26.6% (12/45) (Fig. 2A). These amplicons were ≥99.6% nt identical between each other, suggesting that these strains were the same virus, and hence, were named AKAV/JL01/*Bos taurus*/CHN/2021. To recover more AKAV genomic sequences, we tried nested RT-PCR detection using other primer pairs targeting the remaining genomic regions, as well as using individual MTT sequencing of positive samples, but unfortunately, these approaches did not result in any positives or additional AKAV-like reads. We tried to isolate the virus using Madine-Darby bovine kidney (MDBK) and baby hamster kidney (BHK) cells with RT-PCR-positive spleen and lung samples, but five blind passages achieved no cytopathogenic effects nor RT-PCR positives. Intracranially inoculated suckling mice did not show any abnormal

signs during the 7-day observation, and their internal organs were all negative for AKAV. These results indicate that the AKAV was not isolated.

The RT-PCR detection and viromic sequencing recovered four fragments (Fig. 3). Three fragments, including the amplicons, were longer than 200 nt and were used for preliminary phylogenetic analyses. These 448-nt-long amplicons were clustered closely with the S segment of the strain TJ2016/*Bos taurus*/CHN/2016 from cattle of Tianjin, China, with 100% nt identity, and related to

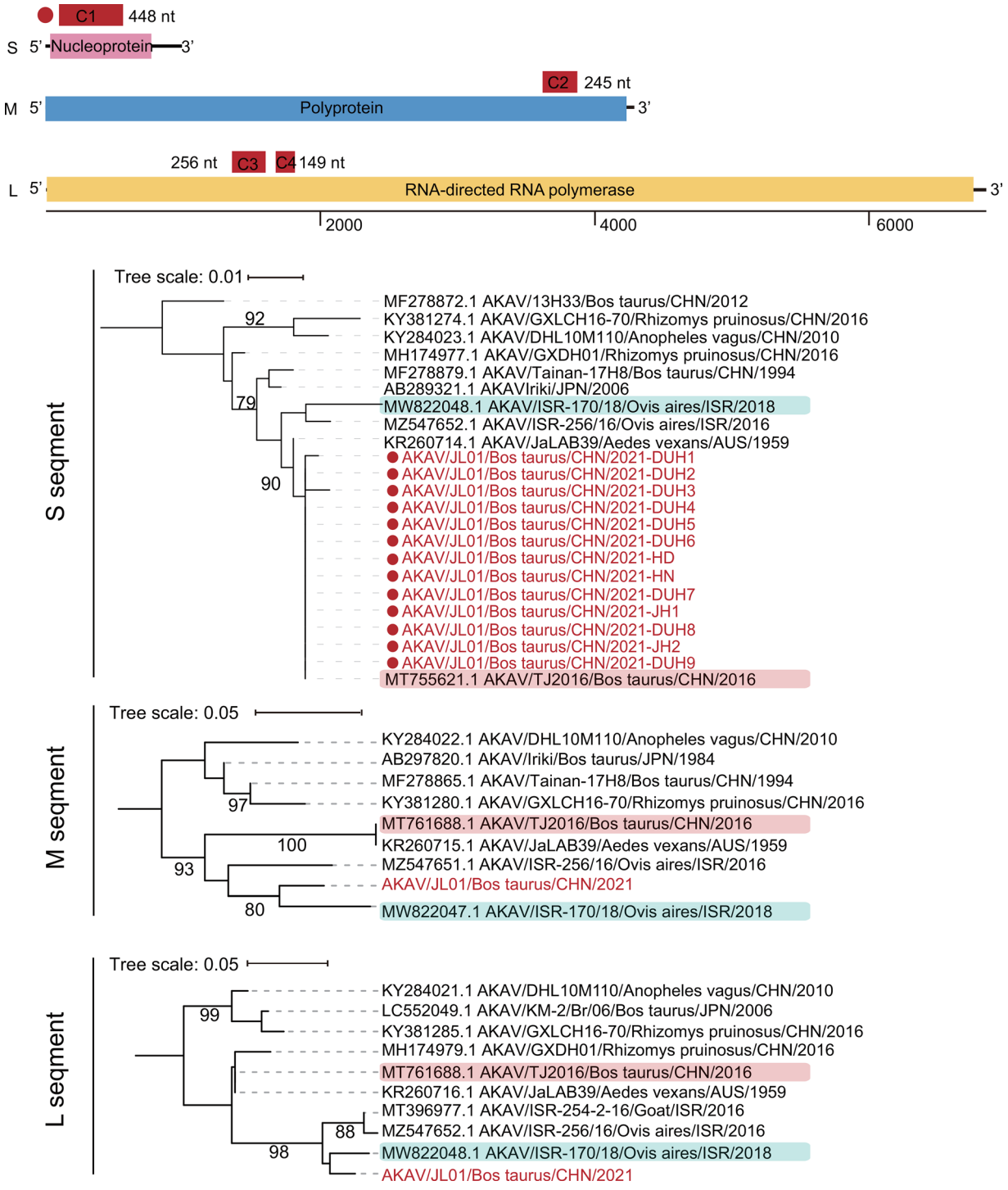

Fig. 3 Phylogenetic analysis of AKAV based on the partial S, M and L fragments. The AKAV sequences obtained in this study are marked in red. All S sequences generated by Sanger sequencing were used for phylogenetic analysis are indicated with filled red circles. The S sequences were clustered with strain TJ2016, while the M and L fragments were clustered with ISR-170-18

an Israeli strain, ISR-170-80/*Ovis aires*/ISR/2018, with 97.0% nt identity (Fig. 3). Surprisingly, the phylogenetic analyses based on the 245 nt-long M fragment and on the 256 nt-long L fragment showed that both were clustered together with ISR-170-80/*Ovis aires*/ISR/2018 with 94.3% and 96.5% nt identity, while only showing 89.4% and 94.1% nt identity with TJ2016/*Bos taurus*/CHN/2016, respectively.

Characterization of astrovirus and norovirus

Viromic sequencing successfully recovered the complete genome sequences of an astrovirus (AstV) and a norovirus (NoV) from lymph nodes and intestinal samples of dead calves, respectively. The complete genome of the recovered AstV (BoAstV/JL01/2021/CHN) was 6,182 nt-long and, encoded three open reading frames (ORFs). ORF1a was 2,406 nt long, encoding an 802 aa-long non-structural protein 1a, which overlapped with the 1,512 nt-long ORF1b that encoded a 504 aa-long polymerase. The 2,235 nt-long ORF2 encoded a 745 aa-long capsid protein precursor. The phylogenetic analysis of the whole genome revealed that the virus was clustered with BoAstV/JPN/Ishikawa9728/2013 with 87% nt identity, which was identified from a calf suffering from diarrhea in Japan (Fig. 4A). The complete genome of the NoV identified here (BoNoV-GIII/JL01/2021/CHN) was 7,320 nt in length, encoding a 1,685-aa-long non-structural protein, a 523-aa-long viral protein (VP) 1, and a 266-aa-long VP2. Phylogenetic characterization based on the full genome showed that BoNoV-GIII/JL01/2021/CHN was closely clustered with the BoNoV-GIII from China, and shared 93% nt identity with BoNoV/HB/BD/2019 and BoNoV-GIII/HB-SJZ-2/2021, both detected from cattle in Hebei Province (Fig. 4B).

Discussion

Viromic sequencing is a powerful approach to diagnosing emerging infectious diseases, which was successfully used to secure the causative agents of COVID-19 [14], the severe acute hepatitis outbreak in bamboo rats [15], etc. Most viromic analyses were based on the contig level, with its length limited to longer than 1 kb, 1.5 kb, or 3 kb [16]. Indeed, virus loads usually reach a high level during the acute infection phase, which facilitates the assembly of reads into long contigs, even complete genomes. However, when the infection of animals is in latent or convalescent phases, virus loads tend to stay at a trace level; in such cases, viromic annotation at the contig level cannot detect them probably. Hence, to conquer the weakness, read-level viromic annotation is an ideal alternative. With such a strategy, we successfully discovered signals of novel filoviruses in bats, which led us to the identification of Dehong virus, an addition to the family *Filoviridae* [17]. Traditional viromics is widely used to profile DNA or RNA viromes at the contig level, but it cannot capture both within a single test. The viromics-based precision diagnosis is designed for viral disease diagnosis, which incorporates the full-spectrum of viral metagenomic techniques and contig/read-based viromic annotation approaches, hence enabling us to detect DNA and RNA viruses even at the trace level [13]. In this study, routine detection and contig-level viromic profiling did not reveal any possible agents for the reproductive abnormalities in cows, except for such common viruses as AstV and NoV. AstVs is associated with diarrhea or central nervous system (CNS) diseases in cattle [18], and GIII NoVs are the main cause of acute viral gastroenteritis in calf [19]. The detection of AstV and NoVs in the lymph nodes and intestines of dead calves alerts us to the fact that co-infection of multiple viruses in these animals. However, whether these viruses contributed

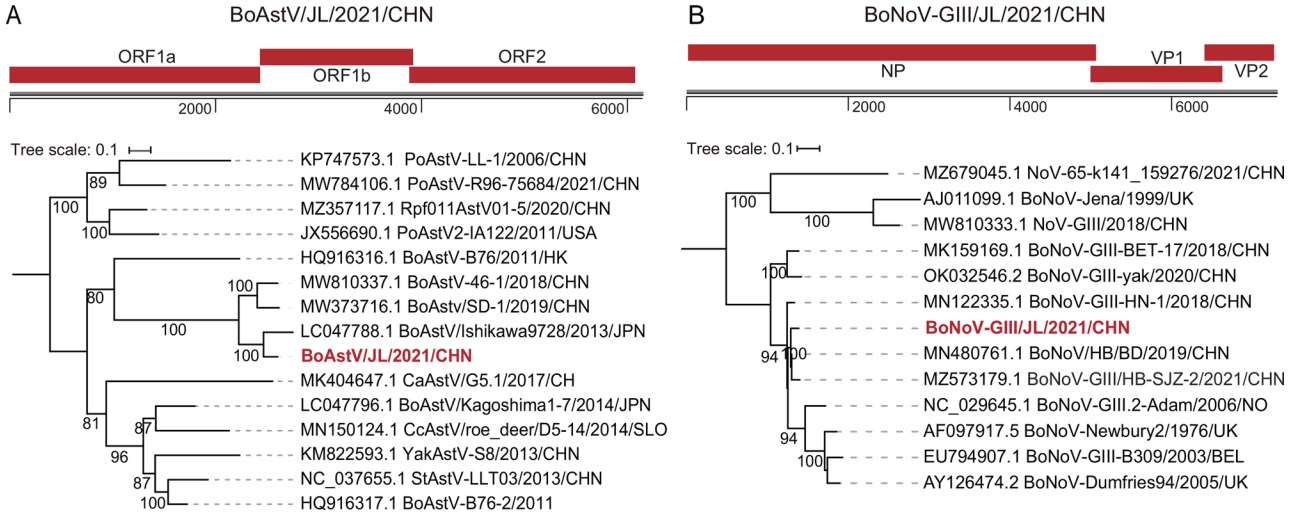

Fig. 4 Genomic and phylogenetic characterization of Astrovirus (**A**); and Norovirus (**B**). Strains obtained in this study are marked in red

to the consequences warrants further investigation. Notably, the read-level annotation detected a few reads related to AKAV, suggesting the virus loads were very low in these samples. It helped us to loop AKAV as the suspicious pathogen. The follow-up molecular detection showed that the virus was very prevalent in these samples, which validated the read-level viromic results and suggested that these animals were infected with the virus. Therefore, this study demonstrates that read-level viromic annotation can be utilized as a complementary approach to contig-level profiling during the diagnosis of emerging infectious diseases.

In this epizootic, the clinical signs were highly identical to Akabane disease, particularly the neurological symptoms. Additionally, these samples were negative for those common abortion-associated pathogens but positive for AKAV. This evidence supports that AKAV infection was the cause of the reproductive abnormalities in cows. Unfortunately, we were unable to obtain the complete genome sequence and virus isolation also failed, suggesting no live viruses in these samples. This should be ascribed to that the virus was cleared by maternal immunity in the late pregnancy, with only some viral RNA relics present in the dead calves [10]. Indeed, the presences of viral RNA relics are also common in patients who recovered from the infection of SARS-CoV-2 [20] and Hepatitis C virus [21]. Although we only recovered several fragments, the preliminary phylogenetic analysis based on them revealed that the S fragment of the virus was clustered with TJ2016 but the M and L fragments with ISR-170-18. The phylogenetic discrepancy of the three segments highly suggests that JL01/*Bos taurus*/CHN/2021 was likely a reassortant with its S segment reassorting from TJ2016-like strains and its segments M and L from ISR-170-80-like strains (Fig. 3). Reassortment is an important mechanism for a virus with segmented genomes to evolve and escape from the immunity of host, which increases the complexity of the control and prevention of the disease.

In conclusion, our results provided an important clue to decoding the cause of the epidemic. Nonetheless, it needs additional work to further confirm the virus, such as serological surveys and molecular detection of the virus in cows during early pregnancy. Besides, ecological investigation of the virus in its arthropod vectors around these farms is essential to understanding its circulation dynamics. Arthropod vectors deeply participate in the life cycle of AKAV; the elimination of arthropods is a critical measure to prevent the disease. In addition, vaccination should be implemented to combat the disease. AKAV infection usually brings severe consequences to the primi pregnancy of cows [10]. After the initial infection, cows acquire long-term adaptive immunity [10]. Therefore, cows preparing for primi pregnancy should be emphasized during the vaccination campaign.

Methods

Ethics statement

The procedures for animal experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Changchun Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Authorization permits: AMMS-11-2020-012).

Sample collection and pathogen detection

In those farms where the disease occurred, their in-house veterinarians dissected the dead calves to collect the brain, lung, liver, spleen, heart, blood, kidney, lymph node, and intestinal samples. Blood samples and vaginal swabs were also collected from aborted and pregnant cows if possible. All samples were cryo-transported to Changchun Veterinary Research Institute for molecular detection, where they were stored at -80 ℃. We employed published PCR or RT-PCR methods to detect those pathogens known to cause reproductive diseases, including BVDV [22], BTV [23], EHDV [24], and *Brucella* spp [25]. Total nucleic acids (NAs) of each sample were extracted using RaPure Viral RNA/DNA Kit (Magen, Guangzhou, China). Reverse transcription was effected with a first cDNA synthesis kit (TaKaRa, Shiga, Japan) according to the manufacturer's protocol. PCR was carried out using 2 × Rapid Taq Master Mix (Vazyme, Nanjing, China) with double-distilled water as a negative control.

Sample pretreatment and high-throughput sequencing

Pools were prepared by mixing the same organ samples from every 3–4 calves and were homogenized with sterile PBS (phosphate-buffered saline) at a w/v ratio of 1:5. Placenta and blood samples and vaginal swabs from cows were mixed according to the sample types. Homogenates were centrifuged at 4 °C at 12,000 × g for 5 min, and the supernatants were filtered through 0.45 μm-pore-sized membranes (Merck Millipore, Taufkirchen, Germany), and then digested with DNase I and RNase A (Takara, Shiga, Japan) to eliminate the contamination of foreign NAs. For MTT, RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, California, USA) and rRNA depletion was performed using Ribo-ZeroTM Magnetic Gold Kit (Epicentra Biotechnologies, Madison,, USA), RNA sequencing was performed on an Illumina NovaSeq sequencer using NEBNext Super-Directed RNA Library Prep Reagents cassette (New England Biolabs, Ipswich,, USA). For MDA, DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and amplified using the illustra GenomiPhi V2 DNA Amplification Kit (GE, Fairfield,, USA). Products were purified

Table 1 Primer information for nested RT-PCR of AKAV used in this study

Name	Sequence	Product length
AKAV-SFw	5' AGGGTATGTGGCATTTATCA 3'	466bp
AKAV-SRw	5' AAGTTGACATCCATTCCATC 3'	
AKAV-SFn	5' CACAACCAAGTGTCGATCTTA 3'	285bp
AKAV-SRn	5' CCAGAAACATCTCAGCACC 3'	

using the QIAquick PCR purification Kit (Qiagen, Hilden, Germany), followed by Illumina paired-end sequencing (150 bp) on an Illumina NovaSeq 6000 sequencer.

Viromic annotation

Raw data generated by an Illumina sequencer were processed using fastp version 0.19.7, and subjected to host sequence removal by mapping against the whole genome assembly of *Bos turus* (accession number: GCA_002263795.3) using bowtie2 version 2.4.1, followed by a rapid metagenomic classification of bacterial, archaeal, and fungal genomes using kraken2 version 2.0.9. The remaining reads from RNA and DNA viromes were mixed, respectively, and *de novo* assembled using MEGAHIT version 1.2.9. All contigs and unclassified reads were annotated using blastn and diamond blastx searches (e-value $\leq 1\times10^{-10}$) against our refined eukaryotic viral reference database EVRD version 2.0 [26]. To verify the authenticity of these virus-like contigs (VLCs), unclassified reads were mapped back to VLCs, and the vertical and horizontal coverage was determined using samtools version 1.10. For read-level annotation, all clean data were annotated using blastn and diamond blastx searches (e-value $\leq 1\times10^{-10}$) against EVRD version 2.0 [26], with cut off values of 130 bp length and 70% identity.

Molecular detection of AKAV

Nested PCR primers were designed targeting the alignment of segment S of AKAV using Primer Premier 5 (Table 1). Viral RNA was extracted using Simply P Total RNA Extraction kit (BioFlux, Hangzhou, China) and reverse transcribed to cDNA (Takara, Shiga, Japan). PCR amplification was performed using 2 × Rapid Taq Master Mix (Vazyme, Nanjing, China) with the following program, pre-denaturation at 95 ℃ for 3 min; 45 cycles of 95 ℃ for 15 s, 56 ℃ for 15 s, and 72 ℃ for 25 s, and a final extension at 72 ℃ for 5 min. Double-distilled water was used as a negative control. All expected products were directly sequenced on an ABI 3730 Sanger sequencer. We also designed several primer pairs targeting the segments M and L. The RT-PCR methods for them were similar to these described above, with adjusted annealing temperatures.

Virus isolation

MDBK cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Corning, New York, USA) supplemented with 8% fetal bovine serum (FBS) (Cell-Box, Hong Kong, China); BHK cells were cultured in Minimum Eagle Medium (MEM) (Corning, New York, USA) supplemented with 5% fetal bovine serum (FBS) (Cell-Box). AKAV-positive samples were homogenized in PBS. The homogenates were centrifuged at 12,000 × g for 10 min at 4 °C. The supernatants were passed through 0.22 μm-pore-sized membranes (Merck Millipore, Taufkirchen, Germany), and the filtrates were incubated with MDBK and BHK cells at approximately 90% confluence for 1 h. Cells were maintained in DMEM or MEM supplemented with 2% FBS and the cytopathic effects (CPE) were checked daily during incubation with 5% CO_2 at 37 °C. After 5 blind passages, total RNA extraction and RT-PCR were performed to detect the virus replication.

KM mice (SPF) were purchased from Liaoning Changsheng Biotechnology Co., Ltd, and housed separately in the IsoCage biocontainment system (Tecniplast, Varese, Italy). Five to six three-day-old suckling mice were injected intracerebrally with 30 μL supernatants from the positive samples. Two mice were used as a control. They were daily monitored for a week, then were euthanized by inhaling isoflurane inhalation vapor in a fume cupboard. A necropsy was performed to check the gross lesion and to collect their internal organs. All tissue samples were subjected to RNA extraction and RT-PCR detection of AKAV.

Genomic characterization and phylogenetic analyses

Genomic structures were predicted using Geneious Prime 2022.2.2. Seqbuilder 7.1.0 was used to drew the genomic maps. Alignment of viral nucleotide (nt) sequences was performed using MAFFT 7.471. TrimAL version 1.2 was used to clip the ambiguous parts and Modelfinder version 1.2.3 was used to predict the best model. The phylogenetic tree was constructed using IQ-TREE version 1.6.8, and the bootstrap test value was set to 1000.

Acknowledgements

Not applicable.

Author contributions

Y.S. performed the experiments, completed the data analysis, and drafted the manuscript; R.Z., H.W. and Z.S. participated in sample collection; Y.L. provided background materials and the acquisition of data; Y.Y., C.T. and B.H. contributed to the conception, design of this work, supervised the research, analyzed the data, and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding

This research was supported by the National Natural Science Foundation of China (32130104) and the Development Program for Science and Technology of Jilin Province (YDZJ202203CGZH051).

Data availability
The sequences generated in this study were deposited in Genbank under accession numbers OR702990-OR702991 and OR743232-OR743234. The viral metagenomic sequencing raw data were available in the NCBI Sequence Read Archive (SRA) under accession numbers PRJNA1028320 and available at following URL: https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1028320.

Declarations

Ethics approval and consent to participate
The procedures for animal experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Changchun Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Authorization permits: AMMS-11-2020-012). All animals were handled according to the principles and Guidelines for Laboratory Animal Medicine. Informed consent was obtained from all owners.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 21 May 2024 / Accepted: 21 November 2024
Published online: 29 November 2024

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