



Letter

Diversity analysis of tick-associated viruses in northeast China

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Dear Editor,

The ongoing threat of tick-borne diseases, especially the increasing rate of new infections caused by tick-borne viruses has drawn wide attention in recent years (Madison-Antenucci et al., 2020; Yang et al., 2023). Therefore, there is a need to strengthen the surveillance of a wide range of viruses carried by ticks to prevent outbreaks of tick-borne diseases.

In this work, a total of 533 ticks from six species including *H. longicornis*, *D. silvarum*, *D. nuttalli*, *D. niveus*, *D. sinicus*, and *D. marginatus* were collected from farming cattle and sheep, vegetation in grassland and forest areas in eight regions of Liaoning Province and Inner Mongolia Autonomous Region (Fig. 1A, Supplementary Fig. S1) during May to June in 2021 and 2022, the peak season for tick activity (Yu et al., 2010). Differences between tick species may result in different regional distributions of tick-borne viruses (Fig. 1B and C). *H. longicornis*, the dominant tick species in Liaoning Province, from which we identified a total of 10 viral strains [Bole tick virus 4 (BTV-4), Xinjiang tick associated virus 1 (XJTAV-1), Shanxi tick virus 2 (SXTV-2), Thogoto virus (THOV), Hubei sobemo-like virus 15 (HBSLV-15), Dabieshan tick virus (DBTV), Hepelivirales sp. (HPLVS), Alongshan virus (ALSV), Huangpi tick virus 1 (HPTV-1) and Densovirinae sp. (DSVS)] based on NGS technology (Table 1, Supplementary Tables S1 and S2). In particular, TcTV-2 was detected in *D. niveus* ticks for the first time from Arxan City. We attempted to isolate the 11 viral strains identified in this study but failed.

Seven of the eleven viruses were found in Huludao City (HPTV-1, DBTV, SXTV-2, HBSLV-15, HPLVS, THOV and DSVS), while only one strain (XJTAV-1) was found in Xiqi County (Fig. 1A, Table 1). Novel species of ALSV and TcTV-2 were found in Liaoning Province and Inner Mongolia Autonomous Region, with ALSV (Yu et al., 2010) and TcTV-2 (Wang et al., 2019) strains previously reported in both provinces. BTV-4 was identified in Arxan City.

The main collection areas of this study are rural areas of Huludao City, Liaoning Province, where farmers are engaged in livestock raising or grazing, and other areas of Liaoning Province and Inner Mongolia

Autonomous Region, where grasslands, forests and vegetation grow abundantly. The majority of the ticks collected were in the adult stage (Supplementary Table S2). Different sampling times, tick populations, climate change and geographical differences may lead to varied spectrum of tick-borne viruses between regions. And due to increases of rural grazing behavior, the natural habitat of tick changed, which may affect the distribution and transmission pattern of tick-borne virus.

ALSV belongs to the Jingmenvirus group within the *Flaviviridae* family with segmented RNA genomes. It has been identified in *I. persulcatus* in China, *Ixodes persulcatus* in Russia, *Ixodes ricinus* in southeastern Finland, and *Ixodes ricinus* in France (Kholodilov et al., 2020; Kuivanen et al., 2019; Temmam et al., 2019). In this research, the virus was identified for the first time from *H. longicornis* ticks in Shenyang City, implying that ALSV strain is broadly distributed in various tick species. The obtained 2048-bp ALSV segment 2 (C_AA001280.1) was annotated to VP1 glycoprotein corresponding to sequence 41–1411 aa. We were unable to identify the signal peptide in VP1a using TMHMM 2.0, although two potential N-glycosylation sites were identified. C_AA001280.1 ALSV strain shared 85.92%, 75.24%, and 74.98% nucleotide identity to previous ALSV strains found in Liaoning Province (MZ676705.1), Heilongjiang Province (MT536950.1), and Russia (MW525295.1), respectively. The phylogeny indicates that C_AA001280.1 ALSV strain is located in the same branch as MZ676705.1, and ALSV strains from other regions form a monophyletic group (Fig. 1D, Table 1, Supplementary Table S3). Based on the relevant classification principles, we speculate that ALSV strain identified in Liaoning Province is a novel species in the Jingmenvirus group and that the virus has the potential to become widespread in northeastern China.

BTV-4 is a *Flaviviridae*-like virus with a genome analogous to that of flaviviruses, although it is not currently classified by ICTV in any genus of the *Flaviviridae* family. BTV-4 was widely distributed among ticks and was previously found in *H. asiaticum* ticks in China (Shi et al., 2016),

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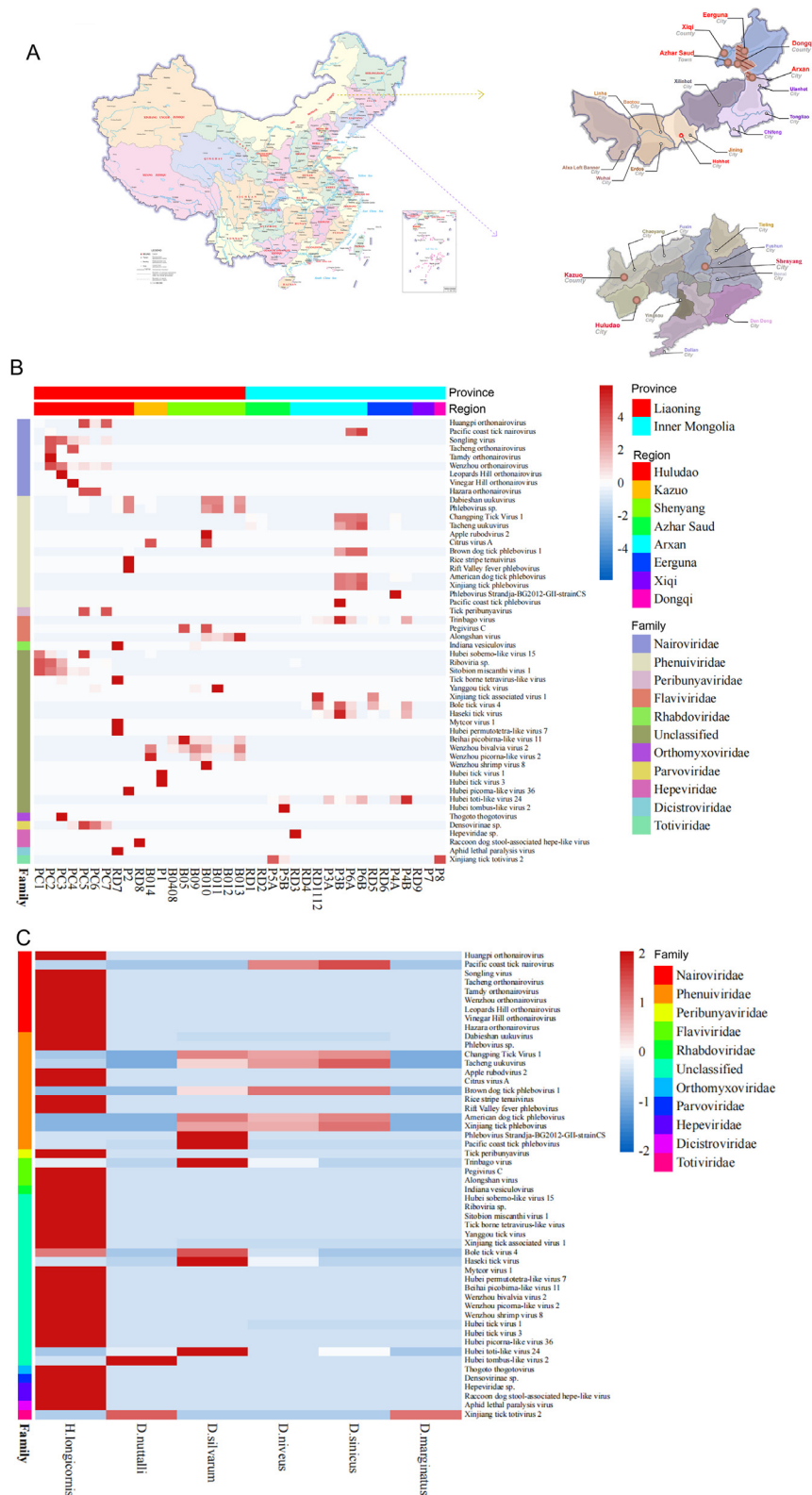


Fig. 1. A Tick collection area. Different colors show the regional distribution of the two provinces, with red highlighted marks representing the areas where ticks were captured in this study. **B** Virus abundance maps for 37 libraries. **C** The distribution of viruses in six tick species. **D** Phylogenetic tree based on ALSV VP1 (457 aa) and other representative viral glycoproteins of the *Flaviviridae* family. **E** Phylogenetic tree based on PB1 protein sequence of THOV (111 aa) and other representative viruses of the *Orthomyxoviridae*. **F** Phylogenetic tree based on RdRp of TcTV-2 (2195 aa), DBTV (2148 aa) and other representative viruses of the *Phenuiviridae*. The viruses obtained here are marked with red circles, and the branches of the genus are displayed in different colors. Phylogenetic tree with 1000 bootstrap replicates was generated using the MEGA 11.0 NJ method, and the numbers above the branches indicate the NJ bootstrap values (only $\geq 70\%$ are shown).

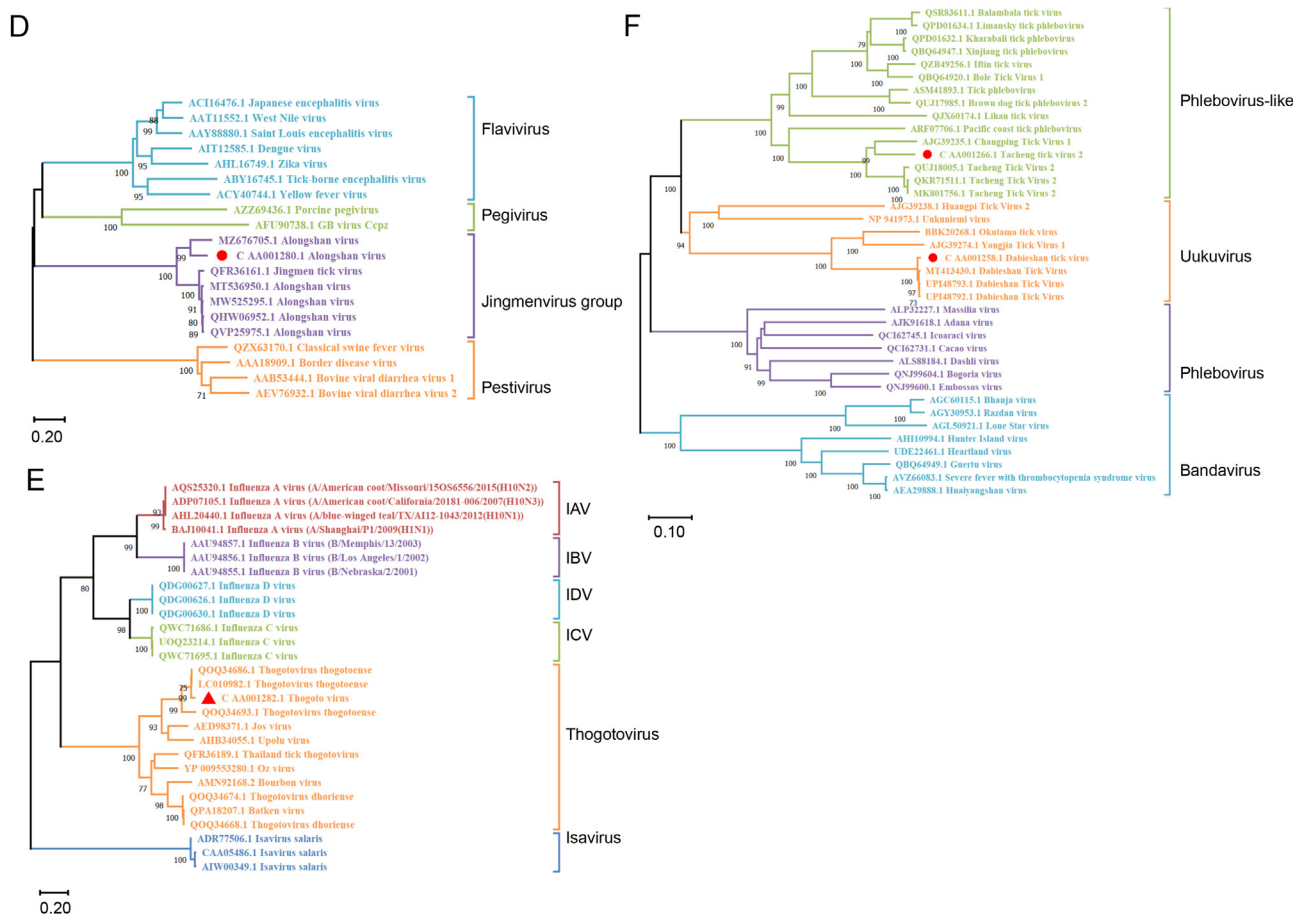


Fig. 1. (continued)

R. sanguineus ticks in Trinidad and Tobago (Sameroff et al., 2019), and *H. truncatum* ticks in Kenya (Sameroff et al., 2019). We found the BTV-4 reads concentrated in *H. longicornis*, *D. silvarum*, and *D. niveus* ticks in Arxan and Erguna cities. The BTV-4 (C_AA001274.1) genome sequence obtained in this study is 16,335 bp in length and contains an ORF (5211 aa) encoding a polyprotein. It shared 97.34% nucleotide sequence identity and 98.07% amino acid sequence identity with ON408075.1 Bole tick virus 4 strain NE-ShL2. Phylogenetic analysis showed that BTV-4 strain was closely related to Haseki tick virus and Dermacentor reticulatus pestivirus-like virus 1 (Supplementary Fig. S2, Table 1, Supplementary Table S3). It forms an evolutionary branch similar to the genus *Pestivirus* of the *Flaviviridae*.

DBTV is classified as a new member of the genus *Uukuvirus* in the family *Phenuiviridae* (Abudurexiti et al., 2019) and was first discovered in 2015 in *H. longicornis* ticks in Hubei Province (Li et al., 2015). DBTV strains were subsequently found in Jiangsu, Zhejiang, Shandong, Yunnan, and Guizhou provinces in China (Shao et al., 2020, 2021; Wang et al., 2021; Zhu et al., 2020). Recent studies have shown that DBTV strain is closely related to the Yongjia tick virus, Okutama tick virus, and Uukuniemi virus, though its pathogenicity in animals and humans is unclear (Shao et al., 2021; Zhu et al., 2020). And DBTV species is evolutionarily conserved. In this study, the whole genome of the DBTV (L segment: C_AA001258.1, S segment: C_AA001259.1) strain was identified from *H. longicornis* in Huludao City and is closely related to the Yongjia tick virus (Fig. 1F, Table 1, Supplementary Table S3). In addition, DBTV reads were available from four areas in the Inner Mongolia Autonomous Region, but could not be assembled. This may be due to the contamination of certain segments of the metagenome with extraneous samples. In Liaoning Province, the sequences of DBTV shared over 95% nucleotide identity with each other. Virus evolution is therefore relatively smooth.

Both TcTV-2 and DBTV species belong to the genus *Uukuvirus*. TcTV-2 strain was isolated from the blood of patients with a record of tick bites in Xinjiang in 2019, demonstrating the risk of infection in humans (Dong et al., 2021). In this study, TcTV-2 reads were mainly found in Azhar Saud town, Arxan city, and Erguna city in the Inner Mongolia Autonomous Region. The whole genome of the TcTV-2 strain (L segment: C_AA001266.1, S segment: C_AA001267.1) in this research shared 75.51% and 78.29% nucleotide identity to the TcTV-2 strain (L segment: MK801756.1, S segment: NC_055426.1) isolated from patient's blood, respectively. According to the principles of classification of the *Phenuiviridae* family, TcTV-2 identified in this research may represent a new viral species of the genus *Uukuvirus* (Fig. 1F, Table 1, Supplementary Table S3). In particular, TcTV-2 was identified in *D. niveus* ticks from Arxan city for the first time. The highest number of TcTV-2 reads was obtained from Arxan city, with 99%–100% nucleotide identity. The Inner Mongolia Autonomous Region and Xinjiang are adjacent to each other and the two provinces are mainly based on animal husbandry, which may facilitate tick migration and transmission of tick-borne virus.

The *Nairoviridae* family was classified as three main genera of *Orthonairovirus*, *Shaspivirus*, and *Striavivirus* (Garrison et al., 2020). In our research, three complete sequences of HPTV-1 in the genus *Orthonairovirus* were obtained from *H. longicornis* ticks in the city of Huludao, Liaoning Province including L (11,866 bp), M (4041 bp) and S (1807 bp) segments. (C_AA001260.1, C_AA001261.1, C_AA001262.1), sharing 95.40%, 90.15%, and 94.25% nucleotide identity with Huangpi Tick Virus 1 strain (MW721865.1, MW721867.1, NC_031137.1) found in *H. longicornis* ticks (Xu et al., 2021) in Hubei Province, China, respectively. The phylogeny suggests that HPTV-1 forms a relatively independent single cluster lineage. It also formed an evolutionary branch similar to Tamdy Orthonairovirus with Tacheng Tick Virus 1, Wenzhou Tick Virus,

Table 1
Information on the 11 viral sequences obtained in this research.

Classification	Virus (abbreviation)	Sampling site	Tick species	Accession number	Genome (bp)	Closest relative (% nt identity)	Percentage of positive libraries corresponding to viruses
Flaviviridae							
Pestivirus like	Bole tick virus 4 (BTV-4)	Arxan City	<i>H.longicornis</i>	C_AA001274.1	16,335	ON408075.1 Bole tick virus 4 (97.34%)	9/37, 24.32%
Unclassified Flaviviridae	Alongshan virus (ALSV)	Shenyang City	<i>H.longicornis</i>	C_AA001280.1	2048	MZ676705.1 Alongshan virus (85.92%) (new viral species)	4/37, 10.81%
Phenuiviridae							
Uukuvirus	Dabieshan tick virus (DBTV)	Huludao City	<i>H.longicornis</i>	C_AA001258.1	6521	MT413430.1 Dabieshan Tick Virus (96.01%)	18/37, 48.65%
	Tacheng tick virus 2 (TcTV-2)	Huludao City	<i>H.longicornis</i>	C_AA001259.1	1753	MT413431.1 Dabieshan Tick Virus (94.37%)	18/37, 48.65%
		Arxan City	<i>D.niveus</i>	C_AA001266.1	6641	OP312998.1 Changping Tick Virus 1 (81.26%) (new viral species)	7/37, 18.92%
		Arxan City	<i>D.niveus</i>	C_AA001267.1	2122	NC_055426.1 Tacheng Tick Virus 2 (78.29%) (new viral species)	7/37, 18.92%
Nairoviridae							
Orthonairovirus	Huangpi tick virus 1 (HPTV-1)	Huludao City	<i>H.longicornis</i>	C_AA001260.1	11,866	MW721865.1 Huangpi Tick Virus 1 (95.40%)	4/37, 10.81%
		Huludao City	<i>H.longicornis</i>	C_AA001261.1	4041	MW721867.1 Huangpi Tick Virus 1 (90.15%)	4/37, 10.81%
		Huludao City	<i>H.longicornis</i>	C_AA001262.1	1807	NC_031137.1 Huangpi Tick Virus 1 (94.25%)	4/37, 10.81%
	Shanxi tick virus 2 (SXTV-2)	Huludao City	<i>H.longicornis</i>	C_AA001263.1	12,083	MZ244235.1 Shanxi tick virus 2 (95.32%)	5/37, 13.51%
		Huludao City	<i>H.longicornis</i>	C_AA001264.1	4228	MZ244236.1 Shanxi tick virus 2 (95.67%)	5/37, 13.51%
		Huludao City	<i>H.longicornis</i>	C_AA001265.1	1554	MZ244237.1 Shanxi tick virus 2 (95.40%)	5/37, 13.51%
Orthomyxoviridae							
Thogotovirus	Thogoto virus (THOV)	Huludao City	<i>H.longicornis</i>	C_AA001282.1	334	LC010982.1 Thogoto virus (85.84%)	1/37, 2.70%
Densovirinae							
Unclassified Densovirinae	Densovirinae sp. (DSVS)	Huludao City	<i>H.longicornis</i>	C_AA001281.1	1310	MW353163.1 Densovirinae sp. (95.49%)	4/37, 10.81%
Hepelivirales							
Unclassified Hepelivirales	Hepelivirales sp. (HPLVS)	Kazuo County	<i>H.longicornis</i>	C_AA001275.1	5401	MW722060.1 Hepelivirales sp. (96.70%)	8/37, 21.62%
Unclassified							
Unclassified virus	Xinjiang tick associated virus 1 (XJTAV-1)	Arxan City	<i>H.longicornis</i>	C_AA001284.1	2588	ON408204.1 Xinjiang tick associated virus 1 (98.69%)	6/37, 16.22%
Unclassified RNA viruses	Hubei sobemo-like virus 15 (HBSLV-15)	Huludao City	<i>H.longicornis</i>	C_AA001276.1	3035	KX882895.1 Hubei sobemo-like virus 15 (98.02%)	5/37, 13.51%
ShiM-2016							

Burana virus, and Tamdy virus (Supplementary Figs. S3A–3C, Table 1, Supplementary Table S3).

Among the *Nairoviridae* family, apart from Crimean Congo haemorrhagic fever virus (CCHFV) and Nairobi sheep disease virus (NSDV), which can cause serious diseases in humans and animals, the newly discovered Songling virus in the *Orthonairovirus* genus has also been associated with febrile disorders in humans in China (Ma et al., 2021). In this study, three complete genome sequences of SXTV-2 were obtained from *H. longicornis* ticks in Huludao city, Liaoning Province, including L (12,083 bp), M (4228 bp) and S (1554 bp) segments, sharing 95.32%, 95.67% and 94.40% nucleotide identity with Shanxi tick virus 2 (MZ244235.1, MZ244236.1, MZ244237.1) identified in *H. longicornis* ticks in China, respectively. Interestingly, SXTV-2 strain L segment has 76.50% nucleotide identity to Songling virus (ON408076.1), but with only 13% sequence coverage. Phylogenetic trees were constructed based on the RdRp (3969 aa), glycoprotein (G) protein (1248 aa), and N protein (490 aa) of SXTV-2, respectively (Supplementary Figs. S3A–3C, Table 1, Supplementary Table S3). The analysis showed that SXTV-2 was similarly related to the Burana virus and distantly related to the Wenzhou tick virus and Songling virus. In addition, according to the BLASTx results, the sequence of SXTV-2 RdRp shares 73.96%, 70.32%, and 65.91% amino acid identity with NC_043439.1 Burana virus, NC_031291.1 Wenzhou Tick Virus and ON408076.1 Songling virus, respectively, confirming the relatedness in the phylogenetic tree. Furthermore, the sequences of SXTV-2 identified in this study shared around 96% nucleotide identity with each other, suggesting a relatively smooth differences between sequences.

THOV species belongs to the genus *Thogotovirus* in the family *Orthomyxoviridae* and was mainly found in Africa, southern Europe, and Asia. THOV has been less studied and occasionally causes disease in

humans, with similar symptoms as influenza, which may be accompanied by fever or even meningitis symptoms and, in severe cases, multi-organ failure and death (Peng et al., 2017). *H. longicornis* ticks have been shown to act as a transmission vector for THOV in Japan (Talactac et al., 2018). In this study, a segment 2 of THOV (C_AA001282.1) strain, 334 bp in length, was also detected in *H. longicornis*. It has less than 86% nucleotide identity and about 98% amino acid identity with THOV LC010982.1 and forms a relatively independent branch in phylogenetic tree (Fig. 1E, Table 1, Supplementary Table S3). The seasonal migration of tick-biting birds along the Bohai Strait and the Strait of Japan may accelerate the long-distance transmission of THOV between Japan and Liaoning.

We identified an unclassified virus from the *Densovirinae* subfamily in *H. longicornis* ticks from Huludao City and named it DSVS (C_AA001281.1). The DSVS segment obtained in this study encodes the NS1 protein including DNA and ATP binding regions, the decapping enzyme and the transcriptional activation structural domain (Zhou et al., 2009), which plays an important role in the replication of densovirus. Phylogenetic analysis revealed that DSVS sequence identified in this study shares 95.49% nucleotide sequence identity with DSVS MW353163.1. Moreover, in the phylogenetic tree, C_AA001281.1 and five viruses in the *Iteradenovirus* genus (*Helicoverpa armigera* densovirus, *Dendrolimus punctatus* densovirus, *Papilio polyxenes* densovirus, *Casphalia extranea* densovirus and *Iteravirus* sp.) are located in the same evolutionary branch and have adjacent affinities (Supplementary Fig. S4).

We identified an unclassified virus in the *Hepelivirales* order from *H. longicornis* ticks in Kazuo County, named HPLVS (C_AA001275.1), which shares 95.87% and 96.7% nucleotide identity with HPLVS MW334982.1 and MW722060.1, respectively. HPLVS was only found in

Huludao City and Kazuo County, Liaoning Province, and the virus was mainly found in *H. longicornis* ticks, and the nucleotide sequences of the virus obtained from the two places shared about 95% nucleotide identity to each other. Based on the phylogenetic analysis of viral polyprotein (Supplementary Fig. S5), the sequence identified in this study was most closely related to HPLVS and formed an independent branch. It has a distal relationship with three viruses (Nudaurelia capensis beta virus, Dendrolimus punctatus virus, and Helicoverpa armigera stunt virus) belonging to the *Alphatetraviridae* family (Wang et al., 2012). Therefore, HPLVS may have potential pathogenicity to insects. In conclusion, tick-borne viruses, particularly their ecology, host range, transmission routes, and distribution, have been the subject of little research to date. It is therefore necessary to continue characterizing the virology and epidemiology of the arthropod.

This study fully demonstrates the distribution of tick-borne viruses in Liaoning Province and some areas of Inner Mongolia, China. A total of 11 tick-borne viruses were identified from six species of hard ticks, and the complete genome sequences of 8 viruses were obtained, which greatly enrich the species diversity of tick-borne viruses. The ALSV and TcTV-2 we identified showed low sequence identities (<80%) with previous detected viral strains, which can be considered as new viral species. Of these, TcTV-2 is for the first time detected outside Xinjiang Province and a different tick species namely *D. niveus*. The 11 viruses identified in this study may be potentially pathogenic in the future. Therefore, it is necessary to further strengthen the surveillance of tick-borne viral diseases in Liaoning Province and Inner Mongolia, and conduct in-depth exploration and analysis of the risk, transmission routes, and epidemiological characteristics of tick-borne virus infection, to provide scientific basis for further prevention and control of tick-borne diseases.

Footnotes

The sequences in this study have been deposited in the NGDC (National Genomics Data Center) (<https://ngdc.cnbc.ac.cn/genbase/>) database with Supplementary Table S3. This research was supported by National Key Research and Development Program of China (NO. 2018YFA0903000, 2020YFC2005405, 2020YFA0712100, 2020YFC0840805, 2021YFC0863400). Prof. Yigang Tong is an editorial board member for *Virologica Sinica* and was not involved in the editorial review or the decision to publish this article. All authors declare that there are no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.virs.2023.10.003>.

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