Praca oryginalna *Original paper*

Molecular analysis of tick-borne Bole Tick Virus 1 in southern Xinjiang, China

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Received 27.07.2025 *Accepted 16.10.2025*

Zhang X., Zhang L., Li W., Feng Q., Zhao W., He W., Nuermaimaiti N., Dai Y., Sun L., Zhou K., Zhao X., Wu J.
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Summary

Ticks serve as vectors for the transmission of pathogens through bites, causing diseases in wildlife, domestic animals, and humans. Although Xinjiang, China, exhibits an exceptional diversity of tick-borne pathogens, limited data exists regarding the association between Bole Tick Virus 1 (BLTV1) and the local tick populations. A total of 319 ticks were collected across 15 sites in southern Xinjiang between 2023 and 2024. The ticks were identified according to their morphological characteristics and confirmed as three genera encompassing five species according to mitochondrial 16S rDNA sequencing. Molecular detection of BLTV1 in ticks was performed using nested RT-PCR targeting the L gene segment of the virus. BLTV1 was detected in 48 out of 319 ticks collected, accounting for 15.05% (48/319) of the sample. This study provides basic evidence of the existence of BLTV1 in ticks in southern Xinjiang. To our knowledge, BLTV1 was detected for the first time in *Dermacentor pavlovskyi*, a rare tick species in Xinjiang. Moreover, this is the first time that BLTV1 has been detected in *Rhipicephalus turanicus*, also in southern Xinjiang. Therefore, the potential threat of ticks to livestock and humans should not be ignored.

Keywords: tick, Bole Tick Virus 1, RT-PCR, southern Xinjiang

Worldwide, about 900 species of ticks have been recorded (11), among which more than 700 species of hard ticks and 193 species of soft ticks have been identified (1, 21). There are 111 species of hard ticks in China, nearly half of which (45) have been detected in Xinjiang (7, 26). Common tick species in Xinjiang include *Hyalomma asiaticum*, *Rhipicephalus turanicus*, and *Dermacentor marginatus* (13). Rare species include *Dermacentor pavlovsky* and *Dermacentor kaiseri*. Ticks play a key role in the transmission of diseases between humans and livestock, leading to a range of tick-borne diseases. With the continuous development and utilization of the natural environment by humans, and the increasing frequency of outdoor activities, the opportunity for people to come into contact with ticks has increased significantly. Therefore, it is very important to strengthen the surveillance of tick-borne pathogens.

Bole Tick Virus 1 (BLTV1) is an important pathogen of *Phlebovirus* transmitted by *Ixodes* in Xinjiang, China. This virus, belonging to the Phenuiviridae family, is a segmented, single-stranded negative-sense RNA virus. The viral genome is composed of large (L), medium (M), and small (S) RNA segments (17). BLTV1 is transmitted mainly by ticks, and current studies have shown that BLTV1 is associated with human-related diseases. In 2016, high-throughput sequencing technology was employed in China to identify 1,445 novel RNA viruses from 220 invertebrate species, including BLTV1 (18). In 2017, positive nucleic acids were also detected in *Hyalomma asiaticum* from Turkey through next-generation sequencing, and evolutionary analysis formed a sister branch with BLTV1 from Xinjiang, China (3). In 2022, BLTV1 was detected in the blood of a patient in northern Xinjiang, China, who had symptoms including fever, headache,

sore throat, full-body rash, and muscle soreness (25). Furthermore, studies have revealed an overall BLTV1 positivity rate of 17.7% in ticks from the northern Xinjiang border areas, with a concurrent detection of the virus in the internal organs of wild gerbils in the region. The current research shows that BLTV1 has the capacity for infecting animals and human (22) and for sustained ecological circulation through „tick-tick/offspring" and „tick-natural host/human" transmission cycles, establishing it as a potential arthropod-borne zoonotic pathogen (25). In addition, the virus is expanding in prevalence, host diversity and geographic distribution, and there is evidence of its potential for zoonotic transmission, which increases health risks for humans and animals.

Bole Tick Virus (BLTV), belonging to the order Bunyavirales and the family Phenuiviridae, is one of the viruses transmitted by hard ticks (16). Reclassified in 2020, the genus *Phlebovirus* contains 60 species of viruses, including 37 in the Americas, 11 in Africa, 8 in Asia, and 4 in Europe (23). Currently, the genus includes 9 definitive and 33 tentative species, at least 11 of which are associated with human disease (4). These viruses can cause fever, abdominal pain, diarrhoea, rash, thrombocytopenia and leukopenia, meningitis, hepatitis, haemorrhagic fever, and retinal vasculitis in both animals and humans (9). Globally, the hosts of the viruses of the *Phlebovirus* genus include humans, cats, dogs, mice, cows, sheep, and various wild animals. In 2015, Tacheng Tick Virus and BLTV types 1-3 were detected by metagenomic sequencing technology in ticks collected in Xinjiang, China, for the first time (2). In 2023, a total of 1,337 ticks were collected from four countries in Eastern Europe and the Black Sea region, and multiple variants, such as Bole Tick Virus 2 (BLTV2), Bole Tick Virus 3 (BLTV3), and Bole Tick Virus 4 (BLTV4), were detected in these samples (5). These studies suggest that BLTV may spread through multiple hosts, adding complexity to the spread of the virus in different animal populations. These findings also demonstrate that BLTV may exist globally and pose a threat to public health.

Xinjiang, located on the northwestern border of China, is the largest provincial-level administrative region in China, accounting for one-sixth of China's total land area (19). This vast land is rich in natural resources and diverse ecosystems (12). Southern Xinjiang is one of China's five major pastoral areas, characterized primarily by cattle and sheep farming. The distribution of ticks in southern Xinjiang is closely related to the density of the local livestock breeding. Given the large number of livestock in the area, ticks thrive by feeding on the blood of these livestock, increasing the risk of disease transmission. In recent years, cases of tick-borne diseases have frequently been reported all over the world (24). These not only seriously endanger human and animal health, but also affect the development of animal husbandry. In addition, the important geographical location of southern Xinjiang, bordering Kazakhstan, Kyrgyzstan, Afghanistan, Pakistan, and India, means that the region plays an important role in the international joint prevention and control of infectious diseases (10).

In this study, nucleic acids of BLTV1 were detected in ticks collected from 15 sites in southern Xinjiang. This study provides data on the prevalence of BLTV1 in southern Xinjiang and contributes to our understanding of the diversity of tick-borne pathogens. Although the pathogenicity of BLTV1 is still unknown, this study is of great significance for the surveillance and early warning of arboviruses in southern Xinjiang. The molecular detection of BLTV1 in ticks to identify the prevalence, genetic evolution, and pathogenic characteristics of these viruses is also of great social significance for public health, wildlife resource protection, harmonious development of animal husbandry, and the diagnosis and treatment of severe tick bite patients.

Material and methods

Sampling area. From March 2023 to mid-September 2024, a total of 319 free and parasitic ticks were collected from the environment and body surfaces of sheep, camels, and red deer in 15 regions of southern Xinjiang (Tab. 1, Fig. 1). Before collecting the samples, the researchers ensured that the environment around the sampling site had

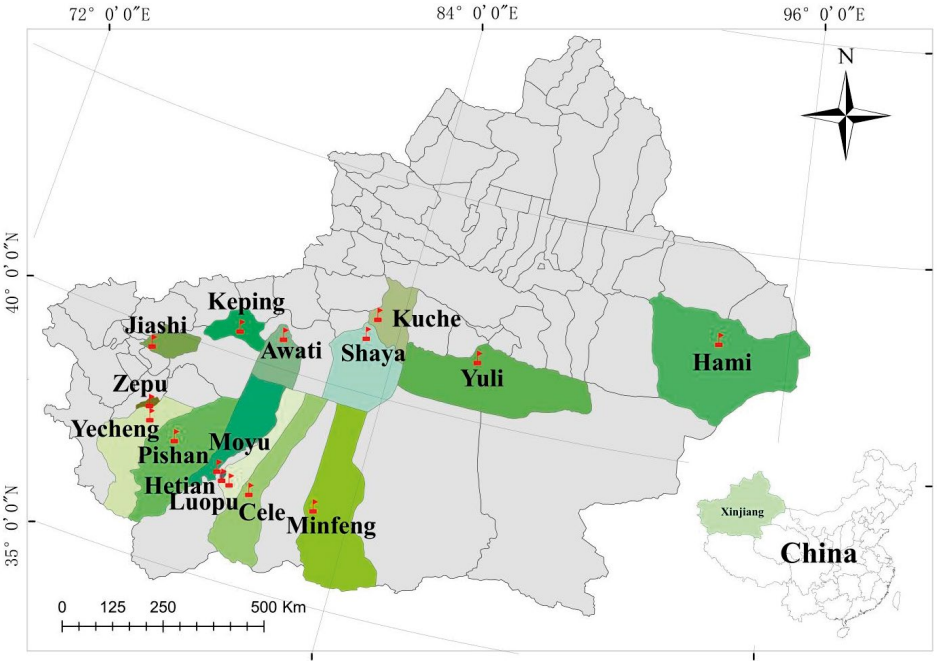

Fig. 1. Map of sample collection sites. A. The fifteen sampling sites are marked in different colors on the map of Xinjiang. B. Red flags are used to indicate where the samples were collected

Tab. 1. BLTV1 infection rates for ticks in southern Xinjiang from 2023 to 2024

Cities	Sampling sites	N (2023 + 2024)	Sex (♂/♀)	Tick species	Quantity	Positive for BLTV1 N (%)
Hetian	Hetian	113 + 0	55/58	*Hyalomma asiaticum*	79	24 (30.38)
				Rhipicephalus turanicus	32	0
				Dermacentor marginatus	2	0
	Pishan	28 + 0	12/16	*Hyalomma asiaticum*	26	4 (15.38)
				Rhipicephalus turanicus	2	0
	Minfeng	33 + 0	14/19	*Hyalomma asiaticum*	15	2 (13.33)
				Rhipicephalus turanicus	18	0
	Moyu	4 + 0	3/1	*Rhipicephalus turanicus*	4	0
	Cele	6 + 0	5/1	*Hyalomma asiaticum*	6	2 (33.33)
	Luopu	25 + 0	14/11	*Hyalomma asiaticum*	25	1 (4.00)
Akesu	Kuche	14 + 0	5/9	*Rhipicephalus turanicus*	14	2 (14.29)
	Awati	8 + 0	6/2	*Rhipicephalus turanicus*	8	1 (12.5)
	Shaya	2 + 14	7/9	*Hyalomma asiaticum*	14	6 (42.86)
				Rhipicephalus turanicus	2	0
	Keping	28 + 0	14/14	*Dermacentor pavlovskyi*	27	1 (3.70)
Kashi	Zepu	20 + 0	15/5	*Rhipicephalus turanicus*	20	1 (5.00)
	Yecheng	6 + 0	2/4	*Rhipicephalus turanicus*	6	0
	Jiashi	4 + 0	2/2	*Rhipicephalus turanicus*	4	1 (25.00)
Bazhou	Yuli	10 + 0	2/8	*Hyalomma asiaticum*	10	3 (30.00)
Hami	Hami	4 + 0	3/1	*Dermacentor nuttalli*	5	0
Total		319	159/160		319	48 (15.05)

not been exposed to acaricides, and consent was obtained from the farmers and ranchers. Ticks were collected from the environment at each sampling site by the marker method. Parasitic ticks were collected directly from the body surfaces of animals in the following order: ears, chest, neck, mouth, nose, armpit, abdomen, forehead, limbs, perianal area, tail, etc. After tick collection, detailed data on each sample were recorded, including the area, time, place, and quantity of the sample. After preliminary morphological identification and careful selection, the ticks were divided into bottles according to preliminary species morphology. They were then labeled and stored at –80°C.

Tick morphological identification and total DNA extraction. The morphological features, including capitulum, mouthpart, legs, scutum, conscutum, punctuation, and anal groove, were identified under a Leica stereo microscope M165C (Solms, Germany). The ticks were washed with 90%, 70%, 50%, 30%, and 10% ethanol and sterile distilled water under a constant temperature culture oscillator for 30 min to eliminate contamination with tick faeces and other contaminants. In a sterile environment, each tick sample was cut into halves in a microtube with disposable sterile scissors. The two halves were placed in separate microtubes: one half was used for DNA extraction and the other for RNA extraction. Total DNA was extracted using a TIANamp Genomic DNA Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. Each half-tick sample was minced with a disposable sterile scalpel in a microtube and digested with proteinase K (20 μg/mL). Then, the lysate was transferred to the columns of the TIANamp kit for DNA absorption. Finally, DNA was eluted in 200 μL of the buffer provided with the kit and stored at –20°C to avoid degradation. DNA was quantified using a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), with a total DNA concentration threshold > 50 ng/μL to ensure species identification.

Molecular identification of tick species. Three ticks from each specimen collection site, making a total of 45 representative samples, were selected for molecular identification. These ticks were adults that had not consumed blood. They were subjected to PCR amplification with primers targeting a 460-base-pair (bp) fragment of the tick mitochondrial 16S rDNA gene. PCR amplification was performed using 2 × Taq PCR Master Mix (Tiangen) according to the manufacturer's instructions. The reaction mixture was initially denatured at 94°C for 5 min, then cycled 37 times through denaturation at 92°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 30 s, and finally, extension was performed at 72°C for 8 min. The amplicons were visualized by electrophoresis on 1% agarose gel containing GelStain (TransGen Biotech, Beijing, China). Bands of DNA of the correct size were purified and sequenced by GENEWIZ (Suzhou, China), and the 16S rDNA sequencing results were submitted to the GenBank database.

RNA extraction and reverse transcription (RT). In a sterile environment, the remaining half of the tick was cut into pieces with sterile disposable scissors. Following the procedure for extracting tissue RNA with Trizol reagent, RNA was extracted from treated tick samples using Trizol (Life Technologies Cat No. 15596-018), chloroform (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China), isopropanol, 75% ethanol, and RNase-free water (commercial) or autoclaved DEPC-treated water reagents. The concentration and purity of the extracted RNA were determined with a spectrophotometer by measuring absorbance at 260 nm (A260) and 280 nm (A280). RNA samples with A260/A280 ratios between 1.8 and 2.1 were considered qualified (or acceptable). To obtain cDNA templates, tick RNA was reverse-transcribed into cDNA using EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix kits (TransGen Biotech Co., Ltd, Beijing, China, AE311-3). Before nested RT-PCR, cDNA was stored at –80°C.

Detection of BLTV1. To identify whether the 319 ticks carried BLTV1, the L gene fragment was amplified by nested

Tab. 2. Virus name, target gene, nucleotide sequence, and product size of nested RT-PCR assays

Virus name	Target gene	Primer	Nucleotide sequences (5'-3')	Product size (bp)
BLTV1	L	BL1-F	ACACTTTCATTCATGATTTCAC	250 bp
		BL1-R1	CTGAACGTGACCGGCAGGCTGGC	
		BL1-F	ACACTTTCATTCATGATTTCAC	492 bp
		BL1-R	GCTTTCAGTTACCTCCCAGTTG	

Tab. 3. Amplification conditions (temperature and time) corresponding to each set of primers

Primer	Initial denaturation	Denaturation	Annealing	Extension	Cycles	Final extension
BL1-F BL1-R1	95°C/5 min	95°C/30 s	54°C/30 s	72°C/15 s	35	72°C/5 min
BL1-F BL1-R	95°C/5 min	95°C/30 s	50°C/30 s	72°C/20 s	35	72°C/8 min

RT-PCR. According to the information provided in reference (25), the optimal reaction conditions for the amplification of each pair of primers of BLTV1 were finally determined through continuous optimization of experiments. The primer sequences and reaction conditions used for amplification of the 492 bp and 250 bp fragments are shown in Table 2 and Table 3. The reaction system included 2 × Taq PCR Master Mix (Tiangen) 13 μL, 1 μL of forward and reverse primers each (10 μM final concentration), and 2 μL of tick cDNA as the template for the first round. For the second round, 0.5 μL of the first round PCR product was used as the template. Then, the volume of the reaction mixture was adjusted to 25 μL with nuclease-free sterile distilled water. To avoid false-positive results, PCR was performed at least twice; PCR amplification included a negative control (nuclease-free water) and a positive control (laboratory-maintained plasmid carrying the complete target gene). For each experiment, 5 μL of the PCR product was applied to 1% agarose gel that contained GelStain (TransGen Biotech Co., Ltd, Beijing, China) for gel electrophoresis. Experimental data were evaluated using a gel documentation system (FluorChem, ProteinSimple, CA, USA). The positive PCR products were sent to Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China) for sequencing.

Sequencing and phylogenetic analysis. The results of bidirectional sequencing were used to identify nucleotide sequences, and the sequences were compared with reference sequences from the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/). The amino acid sequence of BLTV1 was deduced from the corresponding genome sequence using the EditSeq software (DNASTAR, Inc.), and amino acid sequence comparison was performed using MEGA 11.0 (https://megasoftware.net/). A phylogenetic tree of 1000 bootstrap replicates was built in MEGA 11.0 using the maximum likelihood algorithm. The nucleotide sequences obtained in this study were deposited in the GenBank nucleotide database; the accession numbers are PQ309526-PQ309531, PQ330214-PQ330235, PQ530058, and PV208195.

Results and discussion

Tick collection and identification. After two years of intermittent collection, 319 ticks were captured from 15 counties or cities in southern Xinjiang. Based on morphological identification and molecular analysis, the tick species identified in this study included *Hyalomma asiaticum*, *Rhipicephalus turanicus*, *Dermacentor pavlovskyi*, *Dermacentor nuttalli*, and *Dermacentor marginatus*. The corresponding genetic sequences have been deposited in the GenBank database under accession numbers PV207527–PV207538 and PV217825–PV217827. A maximum likelihood phylogenetic tree was constructed using the MEGA11.0 software (version 11.0.13) to evaluate the biogeographical divergence among the tick populations (Fig. 2).

Analysis of BIVT-1. From March 2023 to mid-September 2024, BLTV1 nucleic acid was detected in 48 ticks from 15 sampling sites in southern Xinjiang, accounting for 15.05% (48/319) of all ticks collected. Among the 319 ticks collected (175 *Hyalomma asi-*

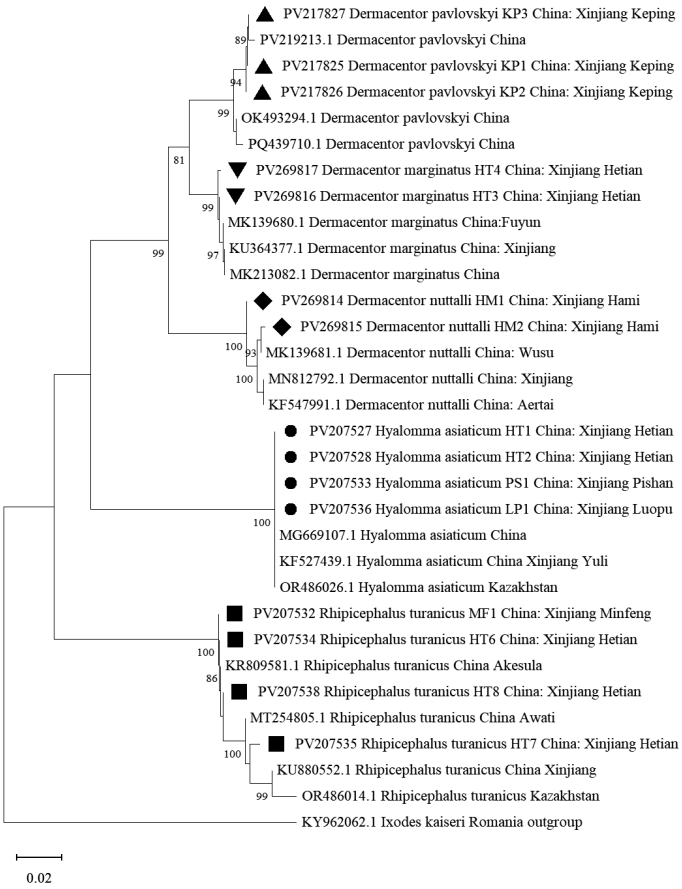

Fig. 2. Phylogenetic analysis of tick species based on the tick mitochondrial 16s rDNA gene from southern Xinjiang, China
Explanations: The tree was constructed by maximum likelihood phylogenetic analysis with 1000 bootstrap replicates using the MEGA 11 software. Sequences of the *Dermacentor pavlovskyi*, *Dermacentor marginatus*, *Dermacentor nuttalli*, *Hyalomma asiaticum*, and *Rhipicephalus turanicus* identified in this study are shown as (▲), (▼), (◆), (●) and (■), respectively

aticum, 110 *Rhipicephalus turanicus*, 27 *Dermacentor pavlovskyi*, 5 *Dermacentor nuttalli*, 2 *Dermacentor marginatus*), the positive rate for BLTV1 carried was the highest for *Hyalomma asiaticum* (24.00%, 42/175), followed by *Rhipicephalus turanicus* (4.55%, 5/110) and *Dermacentor pavlovskyi* (3.70%, 1/27). Among the different regions of southern Xinjiang, the highest positive rate of 30% (3/10) was detected in the Bazhou region, followed by 15.79% (33/209), 15.38% (10/65) and 6.67% (2/30) in the Hetian, Akesu and Kashi regions, respectively; no BLTV1 was detected in tick samples from the Hami region. These data indicate that *Hyalomma asiaticum* may be the main host and carrier of BLTV1. Due to the limited sample collection in Bazhou, the proportion of ticks carrying BLTV1 in this area may be inaccurate. In view of the large number of ticks collected and the wide distribution of ticks carrying BLTV1 in the Hetian area, we speculate that the Hetian area may be the main endemic area of BLTV1 carried by ticks in southern Xinjiang.

Phylogenetic analysis of tick BLTV1. The homology between the uploaded sequence and the known BLTV1 BL-075 (accession numbers: KM817664) sequence in GeneBank was 99.53-100%. The genetic sequences of existing homologous viruses were downloaded from GeneBank. As the viruses in this study belonged to *Phlebovirus* of Phenuiviridae, Phenuiviridae Tacheng tick virus 2 (MN480235.1), Tacheng tick virus (MN480238.1), Changping tick virus 1 (MW561146.1), and Changping tick virus 1 (MW561144.1) were used as outgroups. The MEGA11.0 software was used to construct a molecular genetic evolutionary tree to analyze the BLTV1 carried by ticks from the different regions (Fig. 3).

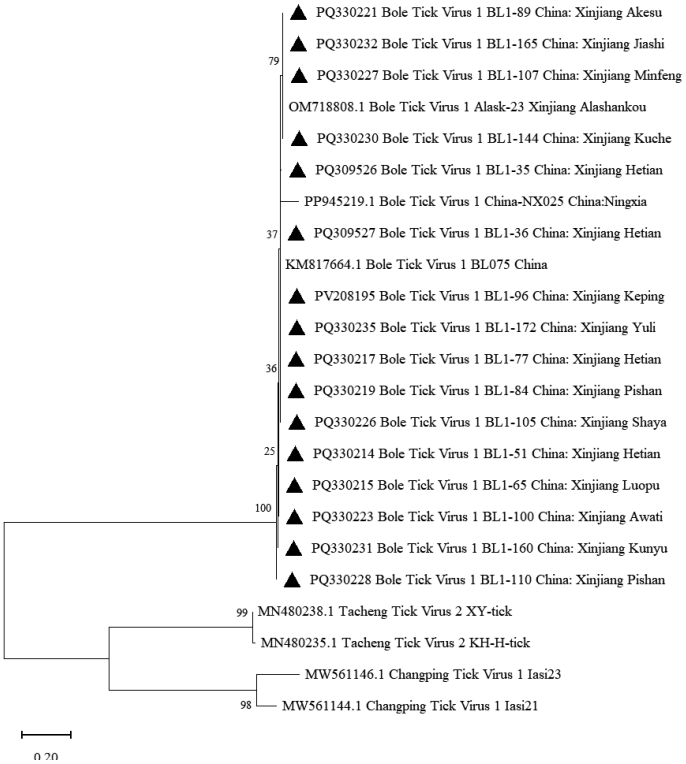

Fig. 3. Phylogenetic analysis of BLTV1 in ticks collected in southern Xinjiang

Explanations: The maximum likelihood (ML) tree with 1000 bootstrap replicates was constructed using concatenated sequence data for the BLTV1 genes in MEGA11.0. Triangles (▲) represent BLTV1 sequences from the ticks investigated in this study

In recent years, under the influence of ecological environment changes, climate pattern changes, and human intervention, the structure of the tick community has changed significantly. Birds and mammals serve as the primary hosts for most ticks, enabling their spread and, consequently, elevating the risk of tick-borne disease transmission (26). These tick-borne diseases are not only a threat to animal health, but also a major challenge to public health security. In addition to the BLTV1 examined in this study, these ticks can also carry and transmit other viruses associated with fever symptoms. Examples include Crimean-Congo Hemorrhagic Fever virus (CCHFV), SFTSV, and Hinterland virus (HRTV) (14). Given that ticks can serve as vectors for multiple zoonotic pathogens, their transmission potential remains elevated. Consequently, enhanced monitoring and studies of these emerging tick-borne pathogens are critical for effective disease prevention and control strategies.

Given its relatively recent discovery, the bunyavirus BLTV1 remains under active investigation regarding its transmission routes, pathogenic mechanisms, and prevention strategies. Although infected animals may remain asymptomatic carriers, they potentially serve as viral reservoirs, elevating zoonotic transmission risks (20). In this study, the presence of BLTV1 in ticks in southern Xinjiang was confirmed by molecular characterization of the L gene fragment of BLTV1, with studies demonstrating varying infection rates across *Hyalomma asiaticum*, *Rhipicephalus turanicus*, and *Dermacentor pavlovskyi* species. However, China's limited investigations into the BIVT-1 infection mechanisms have resulted in an insufficient understanding of its epidemiology, transmission dynamics, and control measures, which ultimately hinders the development of effective prevention strategies. Consequently, sustained pathogen surveillance is imperative to mitigate zoonotic disease risks.

According to the genetic evolution tree constructed in this study, BLTV1 from various regions of southern Xinjiang and different tick species was closely related, and was clustered with BLTV1BL075 (KM817664), China-NX025 (PP945219), and Alask-23 (OM718808); the homology reached 100%. Consistent with the current study, previous studies have shown that *Hyalomma asiaticum* is the main carrier of BLTV1 and the putative ancestral branch of BLTV1. However, this study is the first to show that BLTV1 is carried by *Dermacentor pavlovskyi* and *Rhipicephalus turanicus* in southern Xinjiang, suggesting that infected ticks may transmit the virus to other ticks that live in overlapping regions (15), either through direct contact with an infected

intermediate host or through trans-species transmission while feeding on a host in close proximity (8). The horizontal transmission of the virus at the tick level has the potential for cross-ground transmission and cross-species transmission phenomena. The detection of the virus at the tick level indicates that the distribution of the virus is the same as the geographical area where the three tick species are distributed. However, the infection rate may vary greatly between different locations and different tick species.

The Tarim River Basin, China's largest, spans over 1 million square kilometers and encompasses much of southern Xinjiang, featuring a diverse terrain with numerous ecological nuances yet to be fully understood. This region offers an ideal environment for argasid ticks to thrive. The majority of residents in rural areas engage in the breeding of cattle and sheep, yet a considerable number of these farmers are either uninformed about technologies for epidemic prevention or lack access to such knowledge. There are very few grassroots service personnel with basic technical skills. Xinjiang's border with multiple nations makes it a vital international gateway for China, facilitating substantial livestock trade which, in turn, increases the risk of tick and tick-borne disease transmission. The frequent movement of livestock within Xinjiang and other provinces further aids in the spread of ticks and associated pathogens (6). In addition, guarding against the influx of tick-borne diseases from abroad is a formidable challenge.

To our knowledge, this is the first time that BLTV1 has been found in *Dermacentor pavlovskyi*. This is also the first time that BLTV1 has been found in *Rhipicephalus turanicus* in southern Xinjiang. This study broadens the spectrum of potential vectors for these pathogens, with the findings highlighting the need to consider the role of ticks in disease transmission. At the same time, this discovery contributes to basic research data on ticks in China. Further research should be conducted in the future to explore the exact transmission routes of these pathogens to reduce the risk of tick-borne diseases.

Financial disclosure statement: This study was supported by grants awarded by the National Natural Science Foundation of China (grants No. 32560863, No. 32160841, and No. 31960705), Key Laboratory of Tarim Animal Husbandry Science and Technology, Xinjiang Production & Construction Group (HS201802).

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