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Detection, Genetic Characterization, and Evolutionary Analysis of Jingmen Tick Virus in Ticks from Border Regions of Yunnan Province, China

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Abstract:Background: Jingmen tick virus (JMTV), an emerging tick-borne arbovirus of global public health concern, is a segmented positive-sense RNA virus assigned to the genus Flavivirus of the family Flaviviridae. Although JMTV has been detected in ticks and mammals across Asia, Africa, Europe, and the Americas, epidemiological data from the border regions of southwestern China remain limited. This study investigated the prevalence and genetic characteristics of JMTV in ticks collected from border areas of Yunnan Province, China.**Methods**: A total of 1,035 ticks infesting cattle were collected from five border regions of Yunnan (including Ruili and Malipo) between May 2024 and August 2025. Tick species were identified by PCR amplification and sequencing of the mitochondrial *COI* and 16S rDNA genes. JMTV was detected by RT-qPCR, and genetic and phylogenetic analyses were performed on near-full-genome sequences of positive samples; attempts were made to isolate the virus in Vero, BHK-21, and C6/36 cells and in Kunming mice.**Results**: Three tick species were identified: *Rhipicephalus microplus*, *Haemaphysalis longicornis*, and *Haemaphysalis bispinosa*. The overall JMTV-positive rate was 70.82% (733/1,035), with JMTV detected exclusively in *R. microplus*; the regional positive rates were 93.90% in Ruili and 71.33% in Malipo, a statistically significant difference ($P < 0.001$). Phylogenetic analysis placed all four genome segments (NS1–NS4) of the detected strains within the Asian reference clade, where they formed an independent lineage; the four segments did not cluster entirely on the same branch, indicating host-associated crossover together with a degree of geographic clustering. Attempts to isolate live virus in the three cell lines and in mice were unsuccessful.**Conclusions**: JMTV is highly prevalent in *R. microplus* along the China–Myanmar and China–Vietnam borders of Yunnan, posing a potential public health risk and providing epidemiological evidence to support local JMTV prevention and control. Because sampling was uneven across regions, more balanced future surveys are needed to refine regional epidemiological estimates.

Keywords: RT-qPCR; Tick; Jingmen tick virus; Phylogenetic analysis; Yunnan Province

Jingmen tick virus (JMTV) is an emerging arthropod-borne virus of global public health concern, attracting considerable international attention owing to its unique genomic structure, broad host range, and potential human pathogenicity. JMTV is a segmented positive-sense RNA virus, first isolated and identified from *Rhipicephalus microplus* tick samples in the Jingmen area of Hubei Province, China, and subsequently detected in ticks in the Mogiana region of Brazil (Qin et al. 2014; Maruyama et al. 2014). The viral genome comprises four segments of positive-sense single-stranded RNA. According to the current classification of the International Committee on Taxonomy of Viruses (ICTV), this virus is classified as a member of the genus *Flavivirus* within the family *Flaviviridae*, and belongs to a virus group within this genus that has not yet been assigned to a specific species (Simmonds et al. 2017). Genomic sequence analysis indicates that JMTV is closely related to members of the genus *Flavivirus* in the family *Flaviviridae*, such as Dengue virus (DENV) and Japanese encephalitis virus (JEV). In contrast, flaviviruses possess a single-segment genome of approximately 10-11 kb, that encodes seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Malet et al. 2007), whereas JMTV typically has four genome segments of similar size, with only segments 1 and 3 encoding the two non-structural proteins NSP1 and NSP2, which share homology with the flavivirus NS5 and NS2B-NS3, respectively (Qin et al. 2014; Gao et al. 2020). To date, no sequences homologous to the structural proteins encoded by JMTV segments 2 and 4 have been identified in other known viruses, which appears to be a genetic feature unique to the Jingmen virus group (Mwasi et al. 2025).

JMTV has a wide geographic distribution, with confirmed cases reported in Asia (e.g., China, Japan, and Cambodia) (Kobayashi et al. 2021; Lloyd-Smith et al. 2009), Europe (e.g., Turkey, Corsica in France, Finland, and Kosovo) (Cicculli et al. 2024b; Kuivanen et al. 2019; E et al. 2019), Africa (e.g., Kenya and Uganda) (Ergunay et al. 2022; Ladner et al. 2016), and the Americas (e.g., Brazil and the United States) (Maruyama et al. 2014; Webster et al. 2015).

JMTV has a broad host range, encompassing arthropods, mammals, and various other animals (Zhen et al. 2023; W. Li et al. 2025). Ticks serve as the primary biological vectors and long-term reservoir hosts of JMTV. To date, the virus has been detected in multiple tick genera, including *Rhipicephalus* (Linyu et al. 2023; Lin et al. 2023), *Haemaphysalis* (Dincer et al. 2023; Jian et al. 2024), *Hyalomma* (E et al. 2019; Cicculli et al. 2024a), and *Amblyomma* (Zhang et al. 2022; Matsumura et al. 2024). Furthermore, in studies conducted in Japan, all detected viral strains originated from *Amblyomma testudinarium*, suggesting that this tick species may constitute a key vector for JMTV transmission within Japan (Matsumura et al. 2024). Furthermore, this virus has been successively detected in a variety of mammals, including humans, domestic animals (such as cattle and sheep), and wild animals (such as wild boars, bats, and multiple rodent species) (Ogola et al. 2022; Liu et al. 2024; Xia et al. 2024). Studies have indicated that bats may serve as the natural hosts or original reservoir hosts of JMTV, playing an important ecological role in the long-term persistence and evolution of the virus (Guo et al. 2020). Wild boars, whose activity ranges span both natural habitats and areas of human activity, may act as key "bridge hosts," facilitating cross-species transmission of the virus from wild animals to domestic animals and human populations (Liu et al. 2024). In addition, other studies have shown that reptiles may also participate in the transmission cycle of JMTV. For example, in a study conducted in Kenya, high JMTV positivity rates were detected in both leopard tortoises and their parasitic ticks, indicating that reptiles and their ectoparasites may play a role in the maintenance and spread of the virus in

local areas (Ogola et al. 2022).

As a newly emerged tick-borne virus, JMTV poses a public health threat that cannot be ignored, given its global distribution, broad host range, potential for transovarial transmission, and confirmed pathogenicity in humans (Zhen et al. 2023; Xia et al. 2024; Zheng et al. 2022). To date, human cases of JMTV infection have been reported in both China and Kosovo (Cicculli et al. 2024b; W. Li et al. 2025). Infected individuals may present with viremia and varying degrees of clinical symptoms (Zhen et al. 2023). Symptoms following human infection include fever, headache, lymphadenopathy, and fatigue, among others (Linyu et al. 2023). A retrospective study described the clinical characteristics of patients in detail: all patients developed pruritic or painful eschars at the tick bite site, with some accompanied by lymphadenopathy; local histopathology revealed a pronounced inflammatory response predominantly characterized by neutrophilic infiltration (Na et al. 2019). Currently, JMTV infection can be diagnosed from patient samples such as skin biopsy tissue and blood using methods including high-throughput sequencing, RT-PCR, and serological testing (Na et al. 2019). Studies have also been conducted to develop universal RT-qPCR detection methods to facilitate vector surveillance and early clinical diagnosis (Cicculli et al. 2024b).

Despite the expanding global documentation of JMTV, systematic surveillance of the virus in ticks along the border regions of Yunnan Province—a gateway for transboundary animal and vector movement—remains limited. In this study, we investigated the prevalence, genetic diversity, and evolutionary relationships of JMTV in ticks collected from five border areas of Yunnan between May 2024 and August 2025, and attempted virus isolation, to provide epidemiological evidence for the prevention and control of JMTV in the region.

1 Samples and Materials

1.1 Sample collection

From May 2024 to August 2025, a total of 1,035 parasitic ticks were collected from cattle in border areas of China-Myanmar, China-Laos, and China-Vietnam, specifically in Ruili, Wenshan, Honghe, Xishuangbanna Prefecture, and Tengchong, Yunnan Province, China (Fig. 1). After species identification, the tick samples were stored at -80 °C for subsequent pathogen detection.

1.2 Materials

The Vero, BHK-21, and C6/36 cells lines used in this study were preserved by Kunming Customs Technical Center. Kunming mice (KM mice) were purchased from Kunming Medical University.



Fig 1 Sample geographical distribution and quantity

Remark：From May 2024 to August 2025, the research group collected a total of 1,035 bovine parasitic ticks from five border regions: Tengchong (China-Myanmar border), Ruili (China-Myanmar border), Xishuangbanna (China-Myanmar and China-Laos borders), Honghe (China-Vietnam border), and Wenshan (China-Vietnam border).

2 Methods

2.1 Molecular identification and phylogenetic analysis of tick species

The collected ticks were classified and numbered by region, yielding a pool of 1,035 samples. Each tick was rinsed with autoclaved ultrapure water, then cut into two portions: one portion was stored in a liquid nitrogen tank for subsequent testing, and the other was minced and processed with a conventional genomic DNA purification kit to extract tick tissue DNA.

Primers targeting the tick mitochondrial cytochrome C oxidase subunit I (*COI*) gene were designed according to the standard SN/T 5337-2021, *Technical Specification for Identification of Tick Species as Vectors of Animal Epidemics*, and the 16S rDNA gene sequence was synthesized by Sangon Biotech based on the primers described in reference (Min et al. 2023). Primer information is shown in Table 1. PCR amplification was performed using the PrimeScript™ One Step RT-PCR Kit Ver.2 (Takara Biomedical Technology Co., Ltd.) according to the manufacturer's instructions.

The PCR-amplified *COI* gene products were subjected to gel electrophoresis and recovered using a gel extraction kit, followed by paired-end sequencing by Sequencing Biotechnology. The sequenced gene sequences were assembled using DNASTAR software; the assembled *COI* sequences were then aligned against the sequences stored in the BOLD SYSTEM barcode database. The sequence with the highest alignment score and a sequence similarity of ≥ 97% was identified as the tick sequence, thereby enabling tick species identification. *COI* and 16S rDNA

reference sequences were downloaded from the GenBank database maintained by NCBI. Phylogenetic trees were constructed using MEGA 7.0 software with the maximum likelihood (ML) method, and the bootstrap replication parameter was set to 1,000.

Table 1 Primer information for molecular identification of ticks

Target Gene	Primer Names	Primer Sequence（5′-3′）	Expected Fragment Size
COI	LCO1490	GGT CAA CAA ATC ATA AAG ATA TTG G	710 bp
	HCO2198	TAA ACT TCA GGG TGA CCA AAA AAT CA	
16S rDNA	16S-F	CTG CTC AAT GAT TTT TTA AAT TGC TGT GG	460 bp
	16S-R	CCG GTC TG AAC TCA GAT CAA GT	

2.2 Detection of JMTV by RT-qPCR

The primers and probes for JMTV (Cicculli et al. 2024b) and the EF1A reference gene (Salata et al. 2020) were synthesized according to the respective references. The RT-qPCR reaction mixture was prepared as described in Table 2. The reaction procedure was as follows: reverse transcription at 55 ℃ for 15 min, pre-denaturation at 94 ℃ for 1 min, followed by 45 cycles of denaturation at 94 °C for 10 s and annealing at 60 °C for 31 s. This method was used to detect JMTV in the collected ticks.

Table 2 RT-qPCR reaction system

Component of Reaction	Amount of Reagent（μL）
2× One Step U+ Mix	10
One Step U+ Enzyme Mix	1
50× ROX Reference Dye 1	0.4
JMTV Gene Specific Primer Forward(10 μM)	0.4
JMTV Gene Specific Primer Reverse(10 μM)	0.4
JMTV Taq Man Probe(10 μM)	0.2
EF1A Gene Specific Primer Forward(10 μM)	0.4
EF1A Gene Specific Primer Reverse(10 μM)	0.4
EF1A TapMan Probe(10 μM)	0.4
Template	5.0
RNase-free ddH2O	1.4

2.3 Detection of JMTV by RT-PCR

JMTV whole-genome amplification primers were synthesized according to reference (Linyu 2023), and the positive samples screened by RT-qPCR were verified. The RNA extracted from 8 positive samples from different regions was used as the template for PCR amplification using PrimeScript ™ One Step RT-PCR Kit Ver.2, following the manufacturer's recommendation. (Annealing temperature and extension time were adjusted according to the size of primers and amplified fragments).

2.4 Phylogenetic analysis of JMTV

The PCR products of JMTV were purified by gel extraction kit and subjected to sequencing. The sequencing results were compared by BLAST against the NCBI database, and reference sequences of JMTV whole genome from different regions were downloaded from GenBank database. MegAlign Pro software was used to analyze the nucleotide homology of each sequence

to determine the degree of sequence homology and variation among different strains. On this basis, MEGA 7.0 software was used to construct the phylogenetic tree by Maximum Likelihood (ML) method, and the Bootstrap test repetition number was set to 1000 times (Bootstrap=1000) to evaluate the confidence and reliability of the phylogenetic tree branches. The phylogenetic relationship and genetic evolution of different strains were analyzed.

2.5 Isolation of JMTV

In this study, Vero, BHK-21 and C6/36 cells were selected as in vitro culture systems to isolate and proliferate JMTV. According to the growth characteristics of different cell lines, appropriate culture conditions and media systems were used. Before virus inoculation, 5 JMTV nucleic acid positive original samples were filtered through 0.22 μm sterile filter membrane to remove bacteria, fungi and particulate impurities, thereby preventing microbial contamination from interfere with cell culture. The treated samples were then inoculated into six-well plates containing monolayers of the three cell lines in the logarithmic growth phase and in good condition, at a volume of 500 μL per well. The virus particles were allowed to adsorb for 1 h under the appropriate culture conditions for each cell type, ensuring full adsorption and invasion of the host cells. After incubation, the unadsorbed sample solution was discarded, and 1 mL of cell growth medium was added to each well. The plates were then returned to the corresponding culture conditions for further culture and observation. Cell status was monitored under a microscope. After three consecutive blind passages, the presence of JMTV in the cell supernatant was detected by RT-qPCR.

Meanwhile, the filtered JMTV nucleic acid-positive samples were injected intramuscularly into mice, and control mice received an equal volume of normal saline. Rectal temperature was measured and recorded daily after inoculation, and blood was collected from the tail vein for RT-qPCR on day 5 post-inoculation. After 7 days, the mice were dissected to examine for tick swelling, and the heart, liver, spleen, lung, and kidney were collected for RT-qPCR to determine whether the mice were infected with JMTV.

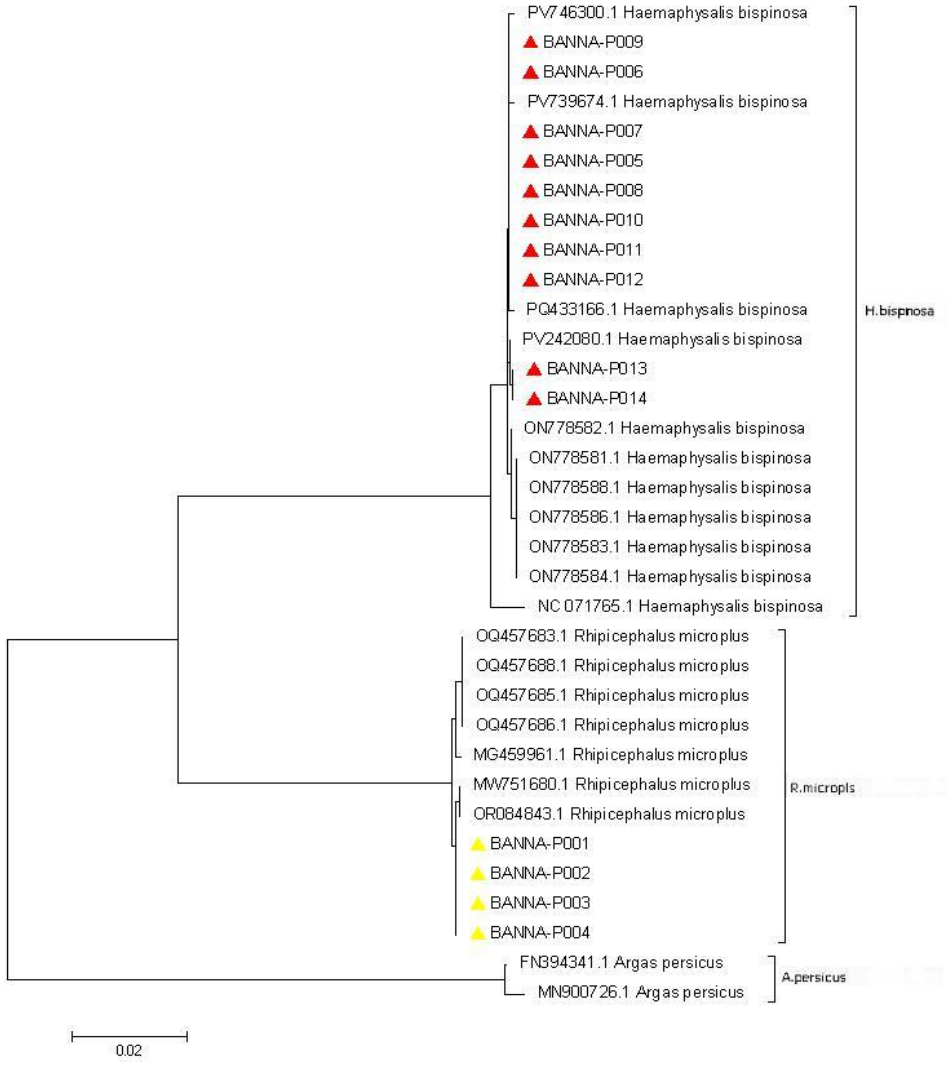
3 Results

3.1 Molecular Identification and Phylogenetic Analysis of Tick Species

Specific fragments of *COI* and 16S rDNA were amplified by PCR, yielding target bands of approximately 710 bp and 460 bp, respectively.

In the phylogenetic tree constructed based on the *COI* gene (Fig. 2), all four geographic strains of *Rhipicephalus microplus* clustered within the same clade, showing close genetic proximity to the Sichuan strain (OR084843.1) and the Yunnan strain (MW751680.1), while being distantly related to foreign strains. For *Haemaphysalis bispinosa*, the 10 geographic strains fell within the same major branch, which consisted exclusively of foreign geographic strains. In the major clade of *Haemaphysalis bispinosa*, BANNA-P009 clustered within the same subclade as the Indian geographical strain (PV746300.1), BANNA-P007 clustered within the same clade as the Indian geographical strain (PV739674.1), and BANNA-P013 and BANNA-P014 clustered within the same clade as the Singapore geographical strain (PV242080.1). In the phylogenetic tree based

on the 16S rDNA gene (see Fig. 3), the 16S rDNA gene sequence of *Rhipicephalus microplus* clustered with those from Yunnan Province (JX051072, JX051062), Guizhou Province (PQ198484), and India (OQ683871, HM536970); *Haemaphysalis longicornis* clustered with those from Sichuan Province (KJ710087, KX083342); and *Haemaphysalis bispinosa* clustered with those from Pakistan (PV686643), Bangladesh (OP349640), and India (MK621326, KC853419, MK617560). Overall, the 16S rDNA phylogenetic tree showed no significant geographic differentiation ,in contrast to the *COI* phylogenetic tree.



▲ ：Rhipicephalus microplus ▲ ：Haemaphysalis bispinosa

Fig 2 Phylogenetic tree of ticks based on the COI gene

Remark：Phylogenetic tree inferred from cytochrome c oxidase subunit I (*COI*) gene sequences. For *Rhipicephalus microplus*, four geographical isolates clustered into a single clade, which was closely related to the Sichuan isolate (OR084843.1) and Yunnan isolate (MW751680.1), but genetically distant from foreign isolates. For *Haemaphysalis bispinosa*, all 10 geographical isolates were grouped into a large clade consisting exclusively of foreign isolates. Within this clade, BANNA-P009 formed a subclade with the Indian isolate (PV746300.1), BANNA-P007 clustered with the Indian isolate (PV739674.1), and BANNA-P013 and BANNA-P014 grouped with the Singaporean isolate (PV242080.1).

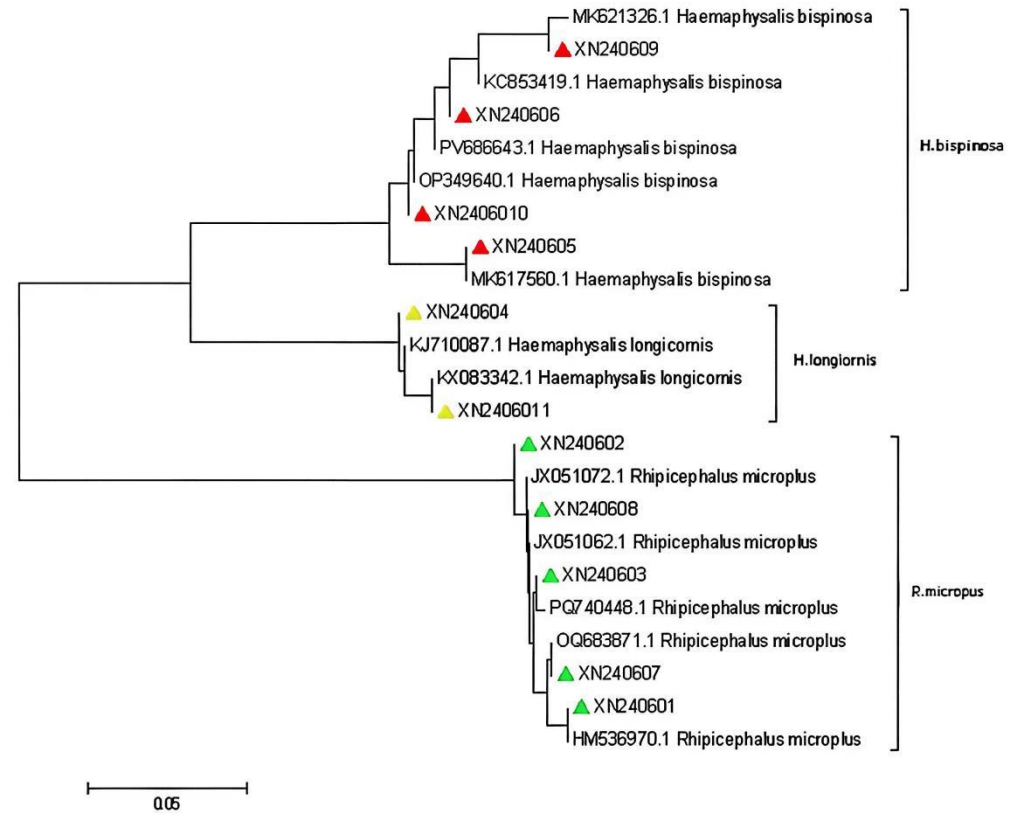


Fig 3 Phylogenetic tree of ticks based on 16S rDNA genes

Remark：Phylogenetic tree inferred from 16S rDNA gene sequences. For *Rhipicephalus microplus*, its 16S rDNA sequences formed a single clade with isolates from Yunnan (JX051072, JX051062) and Guizhou (PQ198484) of China, as well as India (OQ683871, HM536970). For *Haemaphysalis longicornis*, sequences clustered into a clade with Sichuan isolates of China (KJ710087, KX083342). For *Haemaphysalis bispinosa*, sequences grouped with isolates from Pakistan (PV686643), Bangladesh (OP349640) and India (MK621326, KC853419, MK617560).

The bovine parasitic ticks collected in this study were identified as *Rhipicephalus microplus*, *Haemaphysalis longicornis*, and *Haemaphysalis bispinosa*. Among them, *Rhipicephalus microplus* was the most abundant species and was detected in all collection areas, whereas *Haemaphysalis longicornis* was identified in the Dehong and Wenshan regions, while *Haemaphysalis bispinosa* was detected only in the Xishuangbanna area.

Table 3 Distribution of tick species collected from border areas of Yunnan Province

Sampling site	Neighboring country	*Rhipicephalus microplus* (individuals)	*Haemaphysalis longicornis* (individuals)	*Haemaphysalis bispinosa* (individuals)
Nongdao Town, Ruili, Dehong	Myanmar	284	29	-

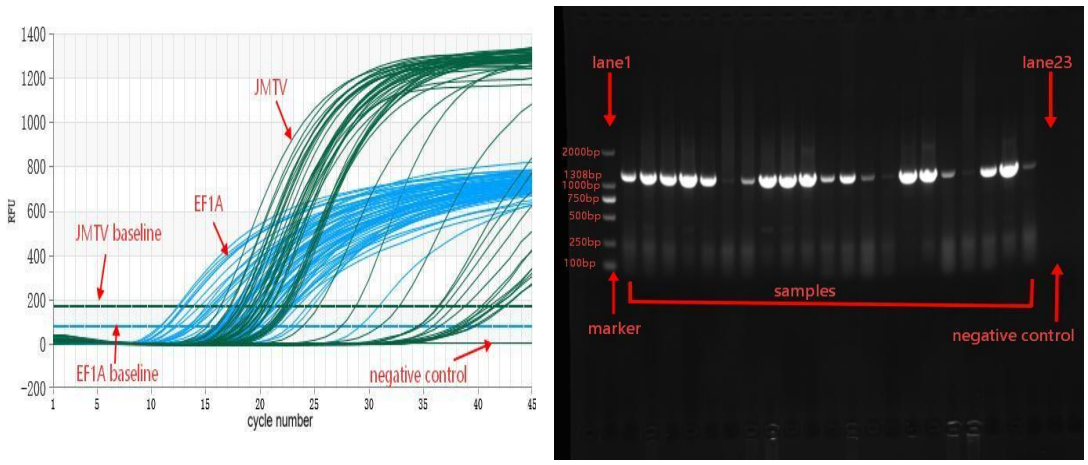
Sampling site	Neighboring country	*Rhipicephalus microplus* (individuals)	*Haemaphysalis longicornis* (individuals)	*Haemaphysalis bispinosa* (individuals)
Jiexiang Town, Ruili, Dehong	Myanmar	283	27	-
Malipo County, Wenshan	Vietnam	98	4	-
Maguan County, Wenshan	Vietnam	19	-	-
Lüchun County, Honghe Prefecture	Vietnam	35	10	-
Tengchong, Baoshan	Myanmar	44	8	-
Menglong Town, Jinghong City, Xishuangbanna, Yunnan Province	Myanmar, Laos	8	-	4
Menghai County, Xishuangbanna	Myanmar	23	-	11
Mengman Town, Xishuangbanna	Laos	6	-	10
Mengban Town, Xishuangbanna	Laos	27	-	25
Total		827	78	25

3.2 RT-qPCR and PCR Identification of JMTV

A total of 1035 cattle parasitic ticks collected from border areas of Yunnan Province were screened for JMTV. Of the 1,035 ticks collected in this study, 733 tested positive for JMTV by real-time fluorescence quantitative PCR, yielding an overall positive rate of 70.82%. Among these, 631 positive ticks were identified from samples collected in Ruili City, Dehong, with a positive rate of 93.90% (631/672), and 102 positive ticks were identified from cattle parasitic tick samples collected in Malipo, Wenshan, with a positive rate of 71.33% (102/143). The differences in positive rates among regions were confirmed to be statistically significant by chi-square test ($P < 0.001$). Combined with the tick species identification results, positive samples were detected in *Rhipicephalus microplus*. No positive samples were detected in the remaining regions.

Primers targeting the JMTV-NS1 gene were used to validate 733 positive samples identified by RT-qPCR. Of these, 706 samples tested positive for JMTV, with a single band of the expected size (1308 bp) amplified, and no bands observed in the negative controls. The amplification results confirmed the accuracy and feasibility of the established method. Furthermore, near-full-genome amplification was performed on 8 selected JMTV strains using JMTV whole-genome sequences.

Agarose gel electrophoresis showed that all 8 samples produced a single band of the expected molecular size, whereas no amplification bands were observed in the negative controls.



A B

Fig 4 Partial amplification results of JMTV by RT-qPCR and RT-PCR

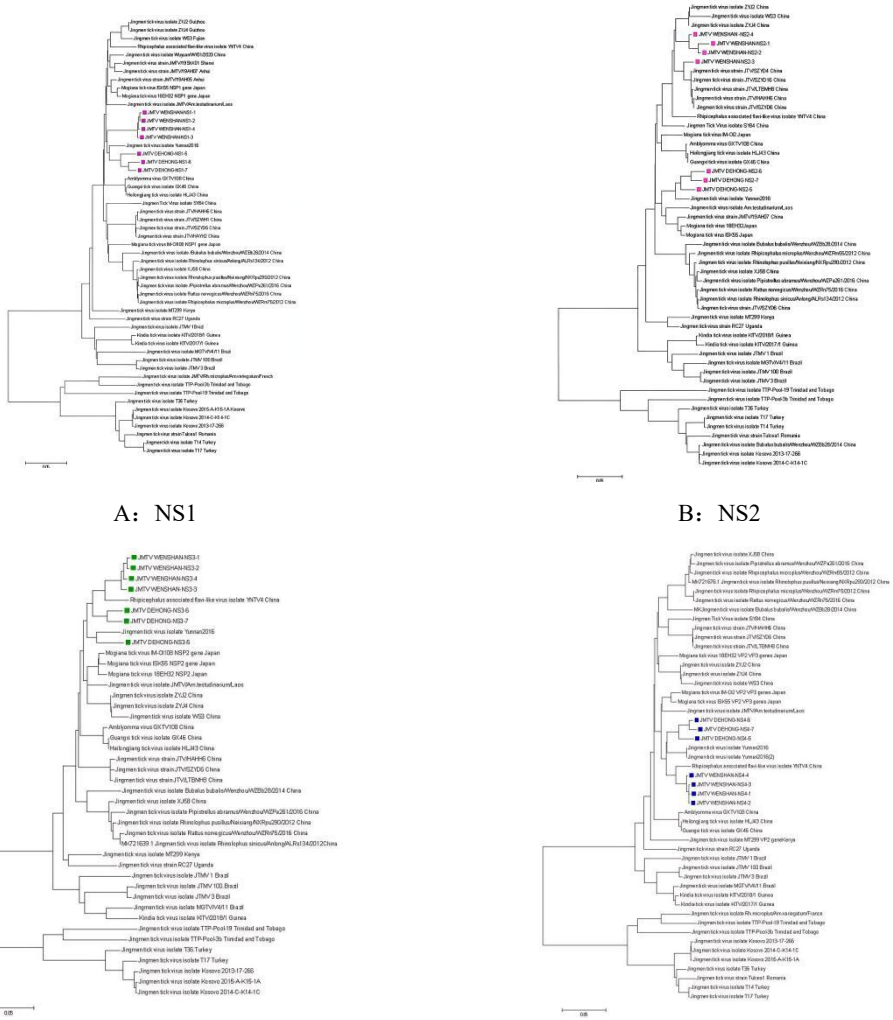
Remark：A total of 733 JMTV-positive samples were identified by RT-qPCR. These positive samples were further validated by RT-PCR using the JMTV-NS1 gene primer set. Among them, 706 samples were confirmed as JMTV positive, with a single amplified band at the expected molecular size of 1308 bp, and no band was detected in the negative control.

3.3 Genetic Evolutionary Analysis of JMTV

As shown in Fig. 5, the four gene segments of JMTV (NS1–NS4) all cluster into two major branches in the phylogenetic tree. From a geographic distribution perspective, the phylogenetic analysis results indicate that the first branch comprised reference sequences from Asia (China, Japan, and Laos), Africa (Kenya, Uganda, and Guinea), and South America (Brazil), whereas the second branch consisted primarily of tick- and human-derived JMTV from Europe (France, Turkey, Kosovo, etc.). From the perspective of parasitic hosts, phylogenetic tree analysis results show that JMTV, represented by the first branch of the phylogenetic tree, has a broad host range, including ticks from multiple genera within the family Ixodidae, various species of rodents and mice, as well as mammals such as monkeys, bats, and wild boars; whereas the JMTV in the second branch has hosts primarily comprising ticks and humans.

In this study, the four segments of JMTV NS1, NS2, NS3, and NS4 all clustered within the Asian reference strains of the first evolutionary branch, forming a distinct clustered independent branch. Regarding the NS1 segment, the seven isolates clustered closely with the JMTV/Yunnan2016 reference strain from ticks of the genus *Amblyomma testudinarium* in Yunnan Province, China, showing a close phylogenetic relationship. Among them, the seven isolates in this study clustered geographically, with the isolates from Ruili being closely related to the JMTV/Yunnan2016 reference strain and grouped within the same sub-branch, while the isolates from Malipo formed a separate sub-branch on their own. Regarding the NS3 segment, the four isolates from Malipo and two isolates from Ruili were closely related to the Chinese *Rhipicephalus microplus*-associated flavivirus reference strains JMTV/YNTV4, while the two Ruili isolates did not form a separate clade. One isolate from Ruili clustered in the same clade as the JMTV/Yunnan2016 reference strain. Regarding the NS4 segment, the four Malipo isolates, as

with the NS3 segment, clustered in the same clade as the JMTV/YNTV4 reference strain. The three Ruili isolates were closely related to the JMTV/Yunnan2016 reference strain. Overall, the four segments of the 7 JMTV isolates did not cluster entirely on the same branch, and there was cross-species host distribution; however, to some extent, the four segments exhibited geographic clustering.



A：NS1　　B：NS2

C：NS3　　D：NS4

Fig 5 Phylogenetic tree of the whole-genome sequences of JMTV

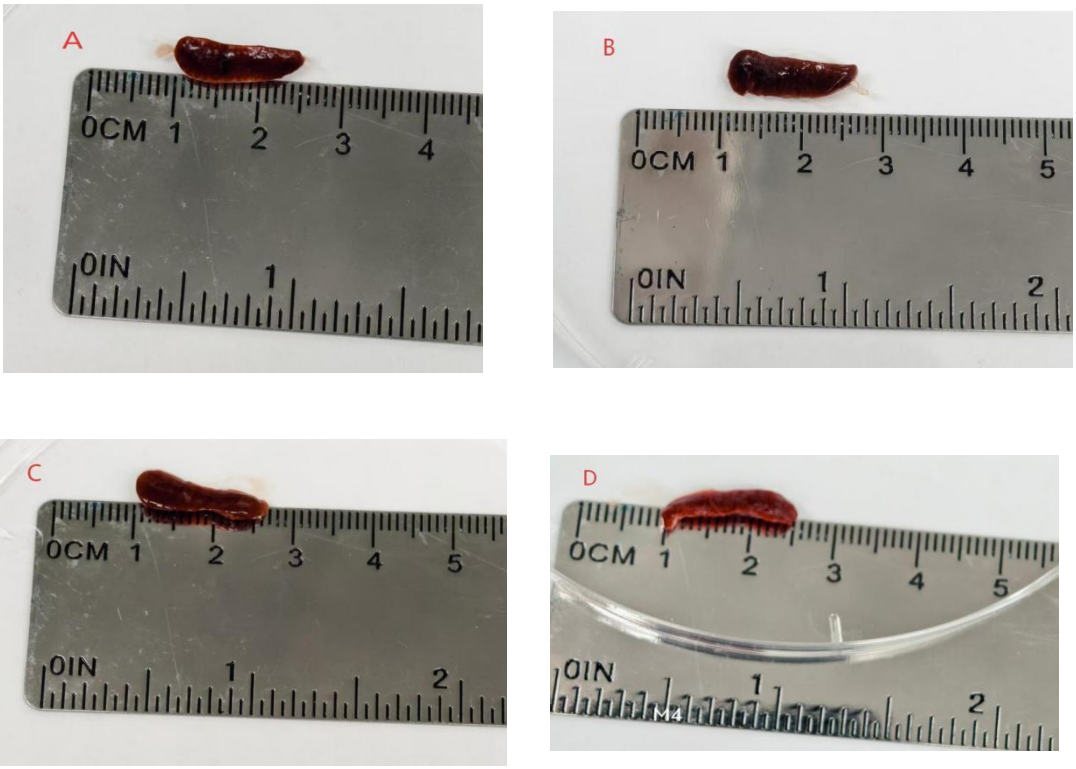
Remark：The color markings indicate the genes detected in this study's genome

Phylogenetic trees based on four gene segments (NS1 – NS4) of JMTV consistently displayed two major clades: Clade 1 included reference sequences from Asia (China, Japan, Laos), Africa (Kenya, Uganda, Guinea) and South America (Brazil) infecting a wide range of hosts such as *ixodid* ticks of multiple genera, rodents, primates, bats and wild boars, whereas Clade 2 mainly consisted of tick- and human-origin JMTV strains from Europe (France, Turkey, Kosovo, etc.). All JMTV sequences generated in this study were located in the Asian subclade of Clade 1 and formed a well-supported independent lineage. Specifically, for the NS1 segment, seven isolates clustered closely with the *Amblyomma testudinarium*-derived reference strain JMTV/Yunnan2016 from Yunnan Province, China, among which Ruili isolates shared a subclade with this reference strain while Malipo isolates formed a separate subclade. For NS2, three Malipo isolates exhibited close phylogenetic relatedness to *Haemaphysalis longicornis*-borne strains JMTV/ZYJ4, JMTV/ZYJ and JMTV/WS3 from Guizhou Province, China but did not belong to the same subclade; one additional Malipo isolate clustered with Chinese *H.*

longicornis-derived strains JMTV/SZTD4 and JMTV/HAHH6; and three Ruili isolates grouped with JMTV/Yunnan2016, consistent with the NS1 tree topology. For NS3, four Malipo isolates and two Ruili isolates were closely associated with the *Rhipicephalus microplus*-derived strain JMTV/YNTV4 from China, and one remaining Ruili isolate was assigned to the same subclade as JMTV/Yunnan2016. For NS4, four Malipo isolates clustered with JMTV/YNTV4 as observed in the NS3 tree, and three Ruili isolates showed close genetic relationship with JMTV/Yunnan2016.

3.4 Virus Isolation

JMTV-positive samples were inoculated onto cells and subjected to three blind passages. JMTV genomic RNA was subsequently detected in the culture supernatant. The results showed that JMTV nucleic acid was detected at only low copy numbers in the supernatant of the first two passages of JMTV-inoculated cells, whereas no JMTV nucleic acid was detected in the supernatant of the third passage. Additionally, no obvious cytopathic effect was observed in any cells during this period. In the virus isolation and cultivation trial, mice inoculated with JMTV nucleic acid-positive samples remained in normal condition one week post-inoculation, with no significant changes in body temperature. Seven days after inoculation, mouse blood and heart, liver, spleen, lung, and kidney tissues were collected, and no obvious splenomegaly was observed. RT-qPCR detection revealed that no viral RNA was detected in any of the mouse tissues, indicating that the virus did not replicate in mice.



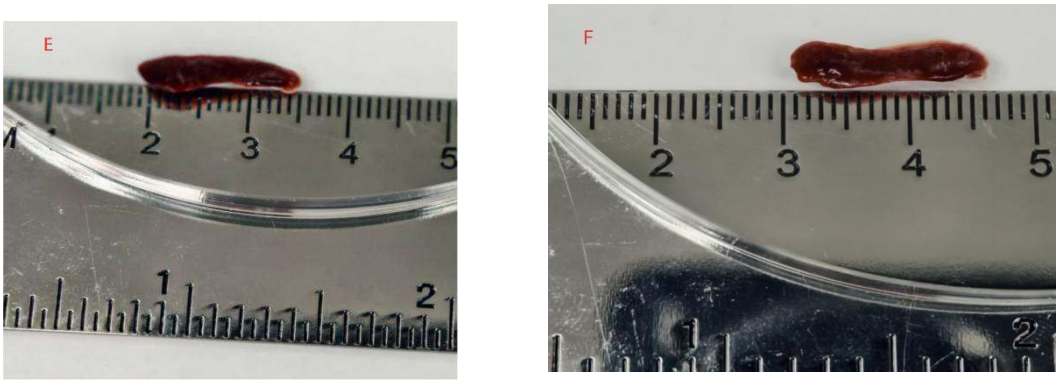


Fig 6 Comparison of spleen between immunized mice and saline control mice

Remark：A~E：The spleen of the poisoned mice　　F：Saline control mice spleen

Seven days post-inoculation with JMTV nucleic acid-positive samples, mouse spleens were harvested for comparative analysis. No significant difference in spleen size was observed between the JMTV-challenged group and the saline-injected control group, and all spleen samples tested negative for JMTV via RT-qPCR.

4 Discussion

JMTV is the first identified member of the Flaviviridae family with a segmented genome, consisting of 4–5 linear RNA segments. This structure stands in stark contrast to the single-stranded, non-segmented RNA genome of classical flaviviruses (such as dengue virus and Zika virus), challenging the traditional understanding of flavivirus genome architecture (Yu et al. 2020). More profoundly, segmented RNA viruses may undergo reassortment or similar genetic exchange mechanisms during evolution, rendering the genetic barriers between different virus families not insurmountable and potentially giving rise to viruses with entirely novel properties (McDonald et al. 2016). Meanwhile, this heightened susceptibility to genetic recombination also poses greater challenges to the prevention and control of segmented viruses such as JMTV (W. Li et al. 2023).

In this study, through phylogenetic analysis, genomic sequence data of 43 JMTV reference strains from different countries and hosts were downloaded, and a systematic analysis was conducted on the global geographic distribution, host range, and genetic evolutionary characteristics of the JMTV obtained in this study.

As a segmented virus, JMTV not only possesses the capacity for gene mutation and recombination but also can undergo genetic reassortment. This unique genetic mechanism allows different segments to evolve independently and exchange segments during mixed infections. In the identification of JMTV variants from Hunan Province, China, it was found that the S1, S3, and S4 segments clustered with strains from Hubei, China, whereas the S2 segment showed higher similarity to Japanese strains (Z. Li et al. 2024). This conclusion bears certain resemblance to the finding in this study that the four segments do not entirely cluster within the same branch. This discovery demonstrates the independent evolutionary trajectories of segments—replication-associated segments remain stable, while certain segments can introduce novel genetic variation through reassortment. However, these changes are largely confined to specific genomic fragments or regions, while core functional genes remain relatively stable.

Notably, a study in Henan, China, found that the JMTV nucleic acid sequences carried by sheep and ticks exhibited high consistency and relatively conserved evolution (Xia et al. 2024). Similarly, a study collecting ticks from seven provinces in China for JMTV detection and genetic evolutionary analysis revealed that all identified JMTV strains were closely related and formed a well-supported sublineage (Guo et al. 2020).

These studies indicate that in short-term transmission chains, viral replication fidelity is high and mutations accumulate slowly; whereas during long-term cross-regional transmission, geographical and ecological factors progressively shape local genetic differences. This phenomenon fully demonstrates that the genetic evolution of JMTV genome sequences across different tick species, animal hosts, and geographical regions exhibits a pattern of "overall conservation, local diversity."

Slight discrepancies were observed between the numbers of positive samples detected by RT-qPCR and PCR in this study. This may be attributable to differences in detection sensitivity between the two methods, as PCR may fail to yield positive results for samples with low viral loads.

This study attempted to isolate JMTV and investigate its related characteristics using Vero, BHK-21, and C6/36 cells, but failed to establish a stably passaged live virus strain. Although viral RNA could be detected in the supernatant of the first two passages of cell culture, the viral load was relatively low; by the third passage, viral RNA could no longer detected in the culture supernatant. Previous studies have shown that JMTV can be successfully isolated, stably passaged, and efficiently propagated in tick-derived cell lines (IRE/CTVM19), whereas its replication is markedly restricted in mammalian cells (Kobayashi et al. 2021). In addition, attempts to isolate JMTV from inoculated mice in this study were also unsuccessful. Previous studies have already indicated that the isolation of JMTV is considerably difficult, both in mouse models and in cell culture systems (Qin et al. 2014; Na et al. 2019). It is speculated that tick-derived cell lines may provide replication conditions closer to the intracellular environment of the natural host, thereby facilitating the initial adaptation and sustained replication of tick-borne viruses such as JMTV. In contrast, mammalian cell lines may lack certain essential host factors or exhibit different antiviral responses, resulting in low replication efficiency after viral entry, making it difficult to produce obvious cytopathic effects or yield high-titer viral supernatants, and ultimately leading to the virus being diluted to undetectable levels during passaging.

This study has certain limitations. The number of tick collection sites was limited, and the distribution of tick samples across these sites was uneven. Samples from the Ruili area of Dehong accounted for 64.9% (672/1035) of the total sample size, with a JMTV positivity rate as high as 93.90%. Consequently, the overall positivity rate of 70.82% was substantially driven by data from this region and does not represent the average infection level across the entire Yunnan border area; rather, it primarily reflects the epidemic status in the Ruili region along the China-Myanmar border. Future studies should balance sampling across different regions to obtain more representative regional epidemiological data. Additionally, most ticks collected in this study were engorged ticks, which led to misidentification during morphological classification. Therefore, further improvement in discriminatory capacity is needed.

5 Conclusions

This study provides systematic epidemiological evidence on Jingmen tick virus (JMTV) in ticks along the China–Myanmar and China–Vietnam borders of Yunnan Province, demonstrating that the virus circulates actively in border livestock-associated tick populations. Among 1,035 ticks collected from five border regions, an overall JMTV-positive rate of 70.8% was recorded, with the highest prevalence (93.9%) in the Ruili area bordering Myanmar and a markedly lower rate in Malipo bordering Vietnam; within the examined fauna, *Rhipicephalus microplus* was the only species found to carry the virus. Phylogenetic analysis of the four genome segments placed the circulating strains within the Asian reference clade, where they formed a distinct, locally clustered lineage while showing segment-specific host crossover, consistent with the largely conserved yet locally divergent evolutionary pattern reported for this segmented virus. Taken together, these findings indicate that JMTV is a credible, though currently underrecognized, public health concern along the Yunnan border, and they support the value of routine vector-based surveillance at transboundary points.

The main contribution of this work is therefore epidemiological and practical: it establishes a prevalence and genetic baseline for JMTV in southwestern border areas and underscores *R. microplus* as the principal bridge vector for potential human and livestock exposure. At the same time, the unsuccessful attempts to isolate live virus in the cell lines and mouse model examined reiterate the biological difficulty of JMTV propagation and point to tick-derived cell lines as a more suitable isolation strategy. Because sampling was concentrated in a limited number of sites and relied predominantly on engorged ticks, the regional prevalence estimates should be interpreted with caution. Future surveys with balanced, multi-site sampling and refined morphological identification are needed to delineate the full geographic extent and transmission dynamics of JMTV along the Yunnan border and to inform targeted prevention and control measures.

Declarations

Ethics approval and consent to participate

The animal studies were approved by the Ethics Committee of Kunming Customs Technical Center.The studies were conducted in accordance with the local legislation and institutional requirements.Written informed consent was obtained from the owners for the participation of their animals in this study.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HZ：Conceptualization，Data curation，Formal analysis，Investigation，Software，Validation，Visualization，Writing－original draft,Writing－review & editing.HW:Conceptualization，Data curation，Formal analysis，Investigation，Methodology，Software，Validation，Visualization，Writing－original draft.XJ：Investigation，Project administration，Resources，Validation，Writing－review & editing.MR:Investigation，Project administration，Resources，Validation，Writing－review & editing.SY:Investigation，Project administration，Resources，Validation，Writing－review & editing.DH:Formal analysis,Funding acquisition,Investigation,Project administration,Supervision,Validation,Writing－review & editing.JT:Formal analysis,Investigation,Project administration,Resources,Validation,Writing－review & editing.JA:Conceptualization,Funding acquisitio,Investigation,Methodology,Project administration,Resources,Supervision,Writing－review & editing.ZS:Conceptualization,Funding acquisitio,Investigation,Methodology,Project administration,Resources,Supervision,Writing－review & editing.All authors read and approved the final manuscript.

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