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Molecular detection of novel Jingmen tick virus in hard ticks from diverse hosts in Guangxi, southwestern China

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Abstract

Background Ticks are the second most important vectors of arboviruses after mosquitoes, and they also serve as reservoir hosts for some zoonotic diseases. It is essential to understand the prevalence of tick-borne viruses in ticks from different sampling sites and vectors, as this information can facilitate the surveillance and prevention of arboviral infectious diseases.

Methods We systematically collected ticks from a variety of animals, including wildlife and domestic livestock, across 18 distinct regions in Guangxi Zhuang Autonomous Region(Guangxi). We then identified the ticks using traditional morphological classification and molecular biology methods to investigate the diversity of ticks in the regionWe also systematically examined the diversity of viruses carried by ticks using comprehensive virological methods based on viral metagenomics. We performed phylogenetic and recombination analyses for the assembled viral sequences.

Results We collected 1286 *Ixodidae* from 18 sampling sites in 17 districts of Guangxi. We identified 4 genera and 6 species of *Ixodidae*. We annotated 2 unclassified viruses and 13 known viral families. We assembled 208 nucleotide sequences and obtained six near full-length sequences of Jingmen tick virus (JMTV). Among these sequences, GXTV-PC4.2 and GXTV-43 were new mutant strains of JMTV. We detected genetic recombination of JMTV in segments 2, 3, and 4 of JMTV.

Conclusions Our study uncovers a diverse tick fauna in Guangxi, including 4 genera and 6 species, and a broad virome with 13 viral families and 2 novel viruses. The JMTV, in particular, shows significant genetic diversity and potential for cross-species transmission, marked by new strains and recombination events. These findings underscore the need for vigilant tick-borne disease surveillance in Guangxi.

Keywords Ixodidae, Diversity, Viral Metagenomics, Jingmen tick virus, Genetic evolutionary analysis

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Background

Ticks are a group of arthropods that exclusively parasitize various terrestrial vertebrates. They feed on blood and serve as vectors for a range of arthropod-borne infectious diseases, as well as reservoir hosts for some zoonotic viruses [1]. Ticks can be classified into three families based on their morphological characteristics: Ixodidae, Argasidae, and Nuttalliellidae [2]. Ticks, as the second most important vectors of arboviruses after mosquitoes, are abundant and widely distributed. They harbor numerous zoonotic pathogens and surpass all other arthropods in their ability to transmit diseases. Ticks can transmit a diverse array of pathogens, including bacteria, fungi, viruses, and protozoa [3, 4]. Tick-borne viruses (TBVs) can be transmitted between ticks and their vertebrate hosts. These viruses can infect and replicate in both arthropod and vertebrate cells, thus playing a crucial role in tick-borne transmission [5]. Currently, TBVs, totaling over 160 and with approximately 25% pathogenic to humans and domestic animals, can be classified into two orders, nine families, at least 12 genera, and include several unclassified viruses [6–8].

JMTV is a new tick-borne RNA virus that was first detected in a pool of *Rhipicephalus microplus* ticks collected from the Jingmen region of Hubei Province, China in 2010 [9]. JMTV belongs to the Family *Flaviviridae*, which is characterized by its enveloped spherical structure. The JMTV genome consists of four independent linear single-stranded positive-sense RNA segments, named Segments 1 (S1), Segment 2 (S2), Segment 3 (S3), and Segment 4 (S4). S1 and S3 encode nonstructural proteins 1 (NSP1) and 2 (NSP2), respectively, which are homologous to the nonstructural protein genes (NS3 and NS5) of classical flaviviruses within the Family *Flaviviridae*. In contrast, S2 and S4 encode structural proteins (VP1, VP2, and VP3), which have no sequence similarity to any known viruses. This finding challenges the previous notion of flaviviruses as non-segmented viruses [9].

Mogiana tick virus (MGTV), identified in Brazilian *R. microplus* ticks, was initially documented in 2014. This virus falls under the Family *Flaviviridae* and exhibits a notable resemblance to the JMTV [10]. The partial protein sequences of MGTV display a 92.4%-96.8% amino acid identity and an 88%-90.3% nucleotide similarity with JMTV, indicating a potential shared identity between the two viruses [11]. Subsequently, JMTV has been identified in diverse arthropods, including ticks and mosquitoes, as well as in vertebrates such as rodents, humans, monkeys, cattle, and tortoises, spanning various countries such as China, Uganda, Brazil, Kosovo, France, Turkey, and Kenya [12, 13]. Of greater significance, JMTV has been confirmed to infect humans and induce a range of mild to severe diseases, as evidenced by high-throughput sequencing analysis of skin biopsies and blood samples from infected individuals [14]. To gain insights into the tick virome in Guangxi, researchers collected ticks from wild animals, as well as ticks from cattle and dogs in the region. These ticks were then subjected to high-throughput sequencing to determine their viral composition. The analysis involved the application of phylogenetic trees and recombination analysis to explain JMTV diversity and genetic characteristics within the region.

Materials and methods

Ticks collection and morphological identification

Tick samples were systematically collected from 17 counties (districts) in Guangxi spanning the period from January 8, 2019, to March 6, 2022, utilizing a simple random sampling method. The direct method was employed to gather parasitic ticks from the body surfaces of wild animals or naturally grazing domestic animals. Subsequently, the collected ticks were carefully placed in sterile 5 ml centrifuge tubes, meticulously labeled, and pertinent details such as sampling time, location, and host information were diligently recorded. To uphold the sample quality, a rigorous preservation approach was adopted: initially, the samples were preserved on dry ice during transportation and later stored in a laboratory refrigerator at -80 °C. Due to the incomplete nature of tick sequences currently available on NCBI, the identification of tick species primarily relies on morphological classification. This involves observing external characteristics such as mouthparts, scutum, eyes, leg segments, idiosoma and sexual dimorphism to determine the species. We referred to the identification keys by Guglielmone AA et al., 2014 [15], and also considered the descriptions provided by Hoogstraal et al., 1979 [16] and Horak et al., 2000 [17] for different tick species. Morphological identification was conducted under a stereomicroscope with guidance from an experienced tick expert. Following morphological identification, the ticks were grouped into pools, each containing 3 to 7 individuals, based on tick species (as inferred from morphology), sex, sampling time, sampling location, and host information. For pools necessitating further molecular biological identification, one complete tick was extracted from each pool for DNA analysis, ensuring the accuracy of the morphological classification.

Nucleic acid extraction and molecular biological characterization of ticks

Each tick was individually placed in a sterile, enzyme-free 2 ml centrifuge tube and immersed in 75% ethanol for 5 min. Subsequently, it underwent three washes with sterile deionized water and was dried on sterile filter paper to eliminate surface impurities. Genomic DNA

from the ticks was then extracted following the instructions provided with the E.Z.N.A.® Insect DNA Kit (Omega, USA). We employed molecular biology techniques to amplify 16S rRNA gene sequences using PCR for molecular identification, a method that is critical for distinguishing species with ambiguous morphological characteristics, identifying suspicious new species, and resolving easily confused species. The 16S rRNA gene sequences of mitochondrial genes from various tick species were sourced from GenBank, and generalized primers were designed within conserved sequences in the coding region of these genes. The primers, synthesized by Sangyo Bioengineering (Shanghai) Co. Ltd., had the following sequences: 16S rRNA-F: 5' to 3' CTGCTCAAT GATTTTTTAAATTGCTGTGG, 16S rRNA-R: 5' to 3' CCGGTCTGAACTCAGATCAAGT. The PCR products were sequenced via Sanger sequencing at Bioengineering (Shanghai) Co., and the sequences were assembled and edited using Contig Express (Version 9.1). Concurrently, reference sequences of various tick species were downloaded from the NCBI database (Table S1). To facilitate molecular biological identification, a phylogenetic tree was constructed using MEGA 7.0 software, employing the Maximum-Likelihood (ML) method with the Tamura-Nei model, which is appropriate for nucleotide sequences. A bootstrap value of 1000 was used to ensure statistical robustness.. In the phylogenetic tree construction, *Dermanyssus gallinae* served as an outgroup (GenBank accession no. L3432) [18].

Extraction and reverse transcription of viral nucleic acids

The tick samples were systematically organized into multiple pools based on the sampling point, sampling time, tick species, sex, developmental stage, and reference host. Approximately 30%-40% of the samples from each pool were randomly chosen for tick RNA extraction, while the remaining samples were diligently preserved at -80°C for future utilization. Before RNA extraction, aseptic processing and grinding of tick surfaces were conducted to ensure the integrity of the extracted RNA. Following the grinding of ticks, the resulting solution underwent centrifugation to obtain the supernatant. The supernatant was then filtered through a 0.22 μm needle-type filter to eliminate any debris or impurities. For RNA extraction, the QIAamp Viral RNA Mini Kit (QIAGEN, Germany) was employed, adhering to the provided instructions. This kit ensures efficient purification of viral RNA from the tick samples. To convert the extracted RNA into complementary DNA (cDNA), reverse transcription was conducted using the PrimeScriptTM IV 1st strand cDNA Synthesis Mix (TaKaRa, Japan) kit, following the operating instructions. The reverse transcription reaction was carried out at 42℃ for 20 min, followed by a denaturation step at 70℃ for 15 min, and then maintained at 4℃. Upon completion of the reaction, the resulting cDNA was stored at -80℃ in a refrigerator for subsequent analysis.

Construction of RNA library

The construction of the RNA library (Table S1) was executed utilizing the VAHTSTM Total RNA-seq (H/M/R) Library Prep Kit for Illumina® (Novaseq, Nanjing, China), following the provided operating instructions. To optimize the detection of DNA viruses, DNase I digestion of DNA was intentionally omitted from the process. Subsequently, the constructed RNA libraries were dispatched to Arnold UDA Genentech (Beijing) Co., Ltd. for high-throughput sequencing, employing the Illumina Novaseq6000 platform. The chosen sequencing strategy was PE150, resulting in a data volume of 20G/sample.

Processing and analyzing of high-throughput sequencing data

Following the initial data preprocessing steps, a supplementary trimming and filtering process was conducted on raw reads through the utilization of the Cutadapt [19] program. Reads originating from potential PhiX Control v3 adapter-ligated libraries were eliminated using BWA-mem and SAMtools [20, 21]. The resultant clean fastq reads were subsequently aligned to the virus database of NCBI/refseq employing the bwa-mem algorithm. For paired-end mapping reads, if only one of the reads met the filtering criteria, both reads were excluded. The paired-end sequences were aligned to a reference sequence, and each read was assigned an alignment score. A tab-separated file was generated for the virus database, encompassing details about the reference sequence hits and statistics of reads at the strain level. The strains were named based on the header names from the fasta file used for database generation. This file also provided read count statistics and annotations, including taxid and taxonomy clade. To establish positive taxonomy, the following criteria were applied: (1) A minimum count of reads exceeding 10; (2) Mismatch ratio (Mismatches/Nucleotides) less than 0.01; (3) Size-normalized abundance exceeding 0.01; (4) Fraction of unique read count greater than 0.5%. For virus sequence assembly, fastp (version 0.23.4) was employed to remove joints and low-quality bases, while bowtie2 (version 2.5.1) was used to compare the reference genome. The mapping reads were sorted by site using SAMtools (version 1.18), and the sorted bam files underwent consensus sequence assembly through Kindel (version 0.4.5).As the length and sequencing depth of genes in different samples can influence the number of reads generated, FPKM (Fragments Per Kilobase of exon model per Million mapped fragments) was employed to standardize the raw sequencing data.

RT-PCR for detection of JMTV

To affirm the presence of JMTV in six sample pools (Tick-PC4.2, Tick-1.2, Tick-43, Tick-77H, Tick-1, Tick-2.1), RT-PCR analysis was executed, yielding a sequence coverage exceeding 85%. The primer design was predicated on the reference provided by Qin et al. (2014), and the specific primer sequences can be found in Table S2. The PCR amplification reaction comprised two steps. In Step 1, a pre-denaturation was conducted at 94℃ for 1 min. Step 2 involved denaturation at 98℃ for 10 s, annealing at 53℃ for 15 s, and extension at 68℃ for 1 min. A total of 35 cycles were performed, followed by a final maintenance temperature of 4℃. The PCR amplified products were subsequently sent to Shanghai Sangong Bioengineering Co. for Sanger sequencing. The resulting sequencing data underwent comparison and analysis using the Blast nucleotide database of NCBI to validate the presence of JMTV in the respective sample pools.

Gap region filling of JMTV sequence

Following RT-PCR validation, we identified positive samples with known sequences flanking the gap regions within the JMTV genome. To fill these gaps, we designed primers targeting the sequences immediately upstream and downstream of each gap. The targeted gap regions were amplified, re-sequenced, and the resulting sequences were assembled using MEGAHIT (v1.2.9) to complete the JMTV genome sequence. Specific primers were designed using Primer Premier 5.0 and synthesized by Guangzhou Kengke Biotech Co. The primer sequences can be found in Table S2. The subsequent PCR amplification was carried out using the 2×Phanta® Master Mix from Nanjing Novelty Zan Company. The reaction program involved three steps. In Step 1, incubation was conducted at 95℃ for 3 min. Step 2 comprised cycling through denaturation at 95℃ for 15 s, annealing at X℃ for 15 s (where X represents the specific annealing temperature for each primer as listed in Table S3), and extension at 72℃ for 30 s. This cycle was repeated for 35 cycles. Step 3 included a final extension step at 72℃ for 5 min, followed by maintenance at 4℃.

Phylogenetic and genetic recombination analysis of JMTV: elaboration on sequence collection and search strategy

We expanded our analysis to encompass a comprehensive view of JMTV evolution and genetic recombination, we included additional JMTV sequences retrieved from the NCBI Nucleotide database, ensuring a broader representation of the virus's diversity (Table S4). To construct a robust phylogenetic framework, we aligned these sequences alongside those obtained in our study using the Muscle method in MEGA software (version 7.0) [22], which is adept for Multiple Sequence Alignment (MSA). This approach facilitated a more inclusive analysis, strengthening the reliability of our phylogenetic inferences. To further substantiate the representativeness of our phylogenetic tree, we also reviewed the literature for existing phylogenetic studies related to "Jingmen tick virus or JMTV," incorporating relevant sequences and insights. Moreover, our search in the NCBI database for tick-borne viruses closely related to JMTV, based on sequence homology and nomenclature cited in the literature, enriched our analysis with additional comparable sequences. The construction of the phylogenetic tree was executed using the Maximum-Likelihood method(Tamura-Nei model) in MEGA software, with a bootstrap value of 1000 to ensure statistical robustness. This methodological choice was pivotal for the accurate depiction of evolutionary relationships among JMTV strains. In parallel, to scrutinize potential genetic recombination events within JMTV strains, we employed the RDP4 software [23], which integrates a suite of nine assays including RDP, Geneconv, Bootscan, Maxchi, Chimaera, 3Seq, Siscan, Phylpro, and LARD. We set a stringent *P*-value threshold of 0.01 and configured the Bootscan method's Bootstrap parameter to 1000. For the RDP subroutine, we selected "Internal and external references" to ensure a comprehensive evaluation of recombination events. These stringent parameters ensured the validity and reliability of detected recombination events. To visually represent the genetic similarities and recombination patterns, we utilized SimPlot software (version 7.0) [24, 25] to generate nucleotide similarity and Bootscan plots, providing a graphical interpretation of the genetic data.

Results

The species composition of ticks collected from 18 sites in Guangxi

Morphological identification and statistical analysis were conducted on 1,286 ticks collected from 18 sites in Guangxi between January 8, 2019, and March 6, 2022. The analysis unveiled the presence of four genera: *Amblyomma, Haemaphysalis, Rhipicephalus, Dermacentor*. Within the collected hard tick samples, the *R. microplus* constituted the highest proportion (45.33%), followed by the *Amblyomma javanense* (27.60%). The remaining four tick species were ranked as follows: the *Haemaphysalis longicornis* (12.52%), the *Haemaphysalis hystricis* (10.11%), the *Rhipicephalus sanguineus* (3.03%), and the *Dermacentor tamokensis* (1.40%) (Fig. 1A). The predominant tick species observed in Guangxi were the *R. microplus* and the *A. javanense*. For more intricate insights into the distribution and prevalence of these tick species across different collection sites in Guangxi (Fig. 1B). The

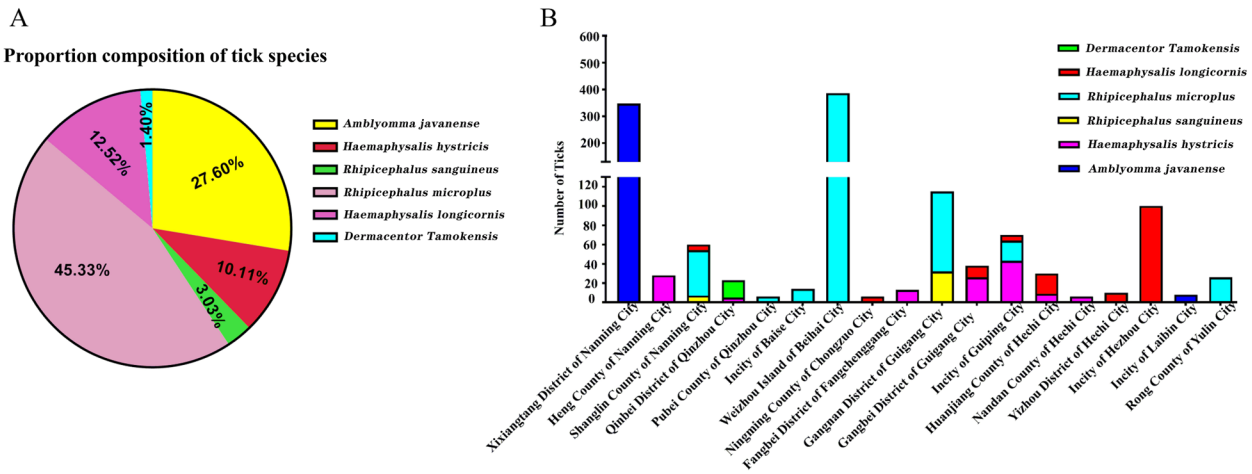

Fig. 1 The composition of ticks from 2019 to 2022 in this study. **A** The proportion of diverse ticks of all the sampling in this study. **B** The distribution of tick species collected in various regions

details of the sequencing library for all the collected ticks are presented in Table S1.

Phylogenetic analysis of ticks

After PCR and Sanger sequencing, the 16S rRNA sequences of these 14 tick samples were acquired and subjected to nucleotide/NCBI blasting. The sequences for *A.javanense*, *H. hystricis*, *H. longicornis*, and *R. microplus* exhibited 100% similarity with existing sequences. Notably, the 16S rRNA sequence of *Ixodes tamoxiflorus* is currently unavailable in public databases. Our obtained sequences demonstrated a similarity ranging from 89.78% to 93.72% when compared to other sequences within the same genus. These sequence comparison results align with the outcomes of morphological classification. To further elucidate the evolutionary relationships, a phylogenetic tree was constructed for 40 tick species, comprising 26 tick species retrieved from the Nucleotide database and 14 sequences determined in this study (Table S2). The phylogenetic tree revealed distinct monophyletic grouping for each tick genus. *Rhipicephalus* formed a separate branch, initially clustering with *Hyalomma* and subsequently with *Dermacentor* and *Haemaphysalis* in larger branches. According to our constructed phylogenetic tree, Argasidae exhibited the closest relationship to *Ixodes* and the furthest relationship to *Rhipicephalus*. Furthermore, all 16S rRNA gene sequences obtained in this experiment were observed to cluster with known sequences of the same species in the database, aligning with the morphological identification. Individuals of the same tick species clustered together on the tree, showcasing clear distinctions among tick species from different genera. Notably, there was an absence of clustering between individuals from different tick species (Fig. 2).

Virus diversity based on large scale viral metagenomics

We obtained a total of 1,993 gigabytes of clean data through high-throughput sequencing. The data exhibited an average Q30 rate of 96.31%. The overall number of reads amounted to 5,428,751,184, with 32,575,680 reads annotated to viruses. To minimize the false-positive rate, we performed a screening of viruses based on a coverage of 0.05% or higher and a read count of 20 or higher. The categorized viruses were further analyzed based on their natural host types, encompassing vertebrate viruses (0.03%), arboviruses (52.64%), plant viruses (15.80%), protozoan viruses (31.44%), and phages (0.08%). This categorization is visually represented in Fig. 3A.

Among the ticks in our collection, *R. microplus* and *A. javanense* carried the highest number of virus species, with 17 and 15 species, respectively. *H. longicornis* carried five viruses, *H. hystricis* carried four viruses, and both *R. sanguineus* and *D. tamokensis* carried one virus each. Glypta fumiferanae ichnovirus was the most widely distributed virus and was present in all the ticks we collected. JMTV was found in four tick species, while Mammalian rubulavirus 5 and Mogiana tick virus were found in three tick species each (Fig. 3B). In our study's 74 lncRNA libraries (Table S3), statistically significant differences in virus distribution were observed at both the family level ($F=466.392$, $P<0.001$) and species level ($F=1475.059$, $P<0.001$).

The results of analyzing the relative abundance of each virus family from highest to lowest at the family level are presented in Fig. S1A. Figure S1B illustrates the results at the species level. The levels of *Glypta fumiferanae*

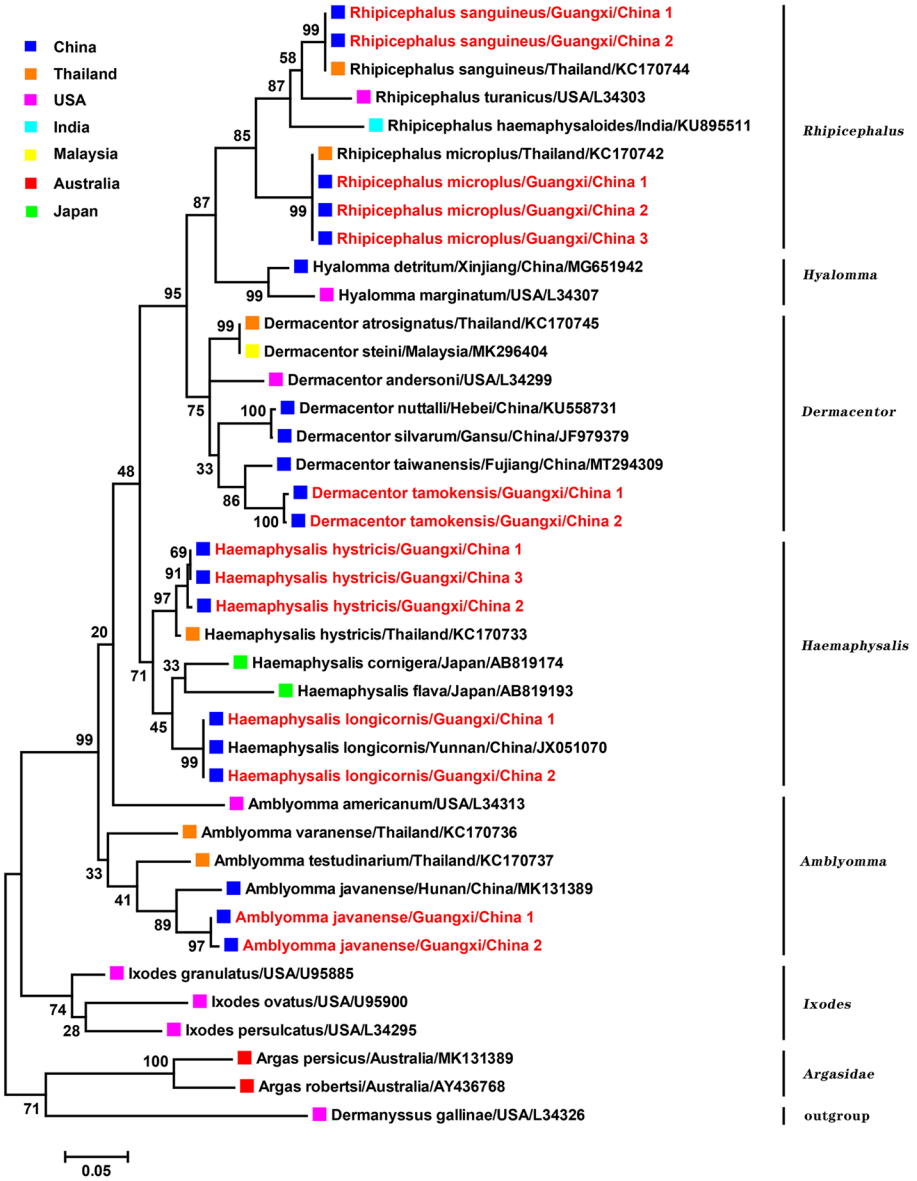

Fig. 2 Maximum likelihood method phylogenetic tree of 16S rRNA gene sequence with Bootstrap = 1000

ichnovirus, JMTV, *Iaco orthobunyavirus*, *Torulaspora delbrueckii Mbarr-1 killer virus*, *Anhembi orthobunyavirus*, *Heterobasidion partitivirus* 1, *Cachoeira porteira orthobunyavirus*, and *Sororoca orthobunyavirus* exhibited higher abundance in all the virus libraries.

The phylogenetic trees and recombination analysis of the JMTV

Through RT-PCR validation and gap-filling of sequences, we obtained six near-full-length JMTV sequences from the libraries Tick-PC4.2, Tick-1.2, Tick-43, Tick-77H, Tick-1, and Tick-2.1, designated as GXTV-PC4.2, GXTV-1.2, GXTV-43, GXTV-77H, GXTV-1, and GXTV-2.1, respectively. These sequences were nearly complete, with only partial non-coding regions missing at both ends. The hosts for these isolates were identified as *R. microplus* (GXTV-PC4.2 and GXTV-43) and *H. longicornis* (GXTV-77H, GXTV-1, GXTV-1.2, and GXTV-2.1). The coverage and sequence lengths for each segment (S1-S4) of these isolates are provided in Table S3. Sequence similarity comparisons with known JMTV sequences in the NCBI database revealed that GXTV-PC4.2 and GXTV-43 exhibited less than 96% similarity across all segments, suggesting that these isolates

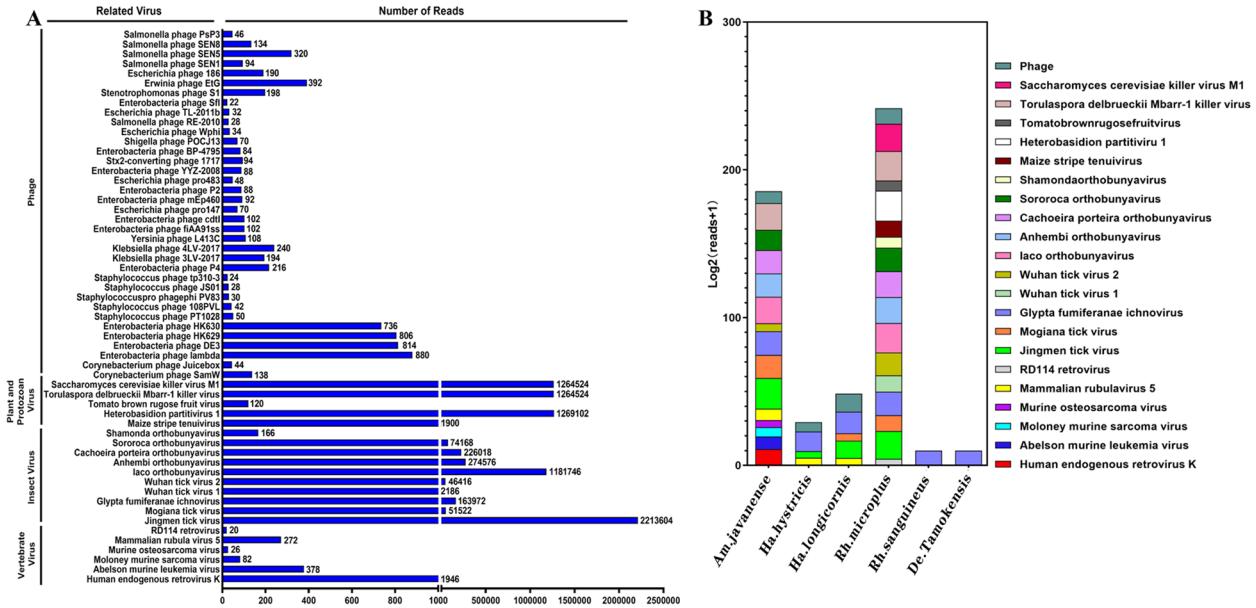

Fig. 3 Virus types and distribution at reads level in this study. **A** Reads-related viruses and number of reads. **B** The viral composition of each tick species

represent novel JMTV variants with genetic divergence exceeding 4%.

JMTV (or JMTV-like) sequences from various countries worldwide and JMTV sequences from different host species were included in the analysis (Table S3). Utilizing MEGA software, we constructed four phylogenetic trees for each of the four JMTV segments using the Maximum Likelihood (ML) method. Each segment encompassed a total of 52 sequences (Table S3). Within cluster I, three sub-branches labeled 1, 2, and 3 were identified. All analyzed phylogenetic trees mostly gave congruent topology. Within cluster I, three sub-branches labeled 1, 2, and 3 were identified. The first sub-branch amalgamated all JMTVs from China, while the second sub-branch contained a single JMTV detected in red colobus monkeys collected from Uganda. The third sub-branch consisted of a singular cluster of JMTVs from Brazil and Guinea. Similarly, cluster II could be further divided into two sub-branches labeled 1 and 2. The first sub-branch clustered JMTVs from France and Trinidad and Tobago, while the second sub-branch grouped JMTVs from Turkey and Kosovo. Cluster III was also divided into two sub-branches labeled 1 and 2. The first sub-branch encompassed JMTVs from Cambodia, while the second sub-branch grouped ALSVs from China and Finland, along with JMTVs from France. Sequences from various related viral strains were used as outgroups to root the phylogenetic trees and ensure accurate topology, although specific outgroup sequences used in the analysis were not explicitly mentioned in the initial explanation (Fig. 4).

We employed two methods, Simplot and Bootscan, to scrutinize potential recombination events in the sequences of GXTV-PC4.2, GXTV-1.2, GXTV-43, GXTV-77H, GXTV-1, and GXTV-2.1 (Fig. 5). However, no evidence of recombination was discerned for these sequences in S1. Nonetheless, recombination was identified in segments 2, 3, and 4 of the viral genome (Fig. 5). Remarkably, segment 2 exhibited a notable event involving strain GXTV-2.1 from Guangxi, acting as a recombinant with strains GXTV-1 from Guangxi and MTV/Yunnan2016 (GenBank accession no. MT080098) from Yunnan serving as parental strains. This recombination event was detected by five tools (RDP, Bootscan, Maxchi, Chimaera, and Phylpro), with identified breakpoints at positions 2316 and 2604 of the sequence. Additionally, utilizing Bootscan, Maxchi, and Chimaera, recombination was observed in segment 4, where the virulent strain GXTV-77H from Guangxi acted as a recombinant with the virulent strains GXTV108 (GenBank accession no. MG703255) from Guangxi and YNTV4 (GenBank accession no. MH814980) from Yunnan serving as parents. The breakpoints for this recombination were located at positions 1936 and 2204 of the sequence. Lastly, segment 3 also exhibited signs of recombination according to the Maxchi and Chimaera tools, involving strains GXTV-77H and GXTV-43 from Guangxi, and the JMTV/Lao PDR (GenBank accession no. MN095529) strain from

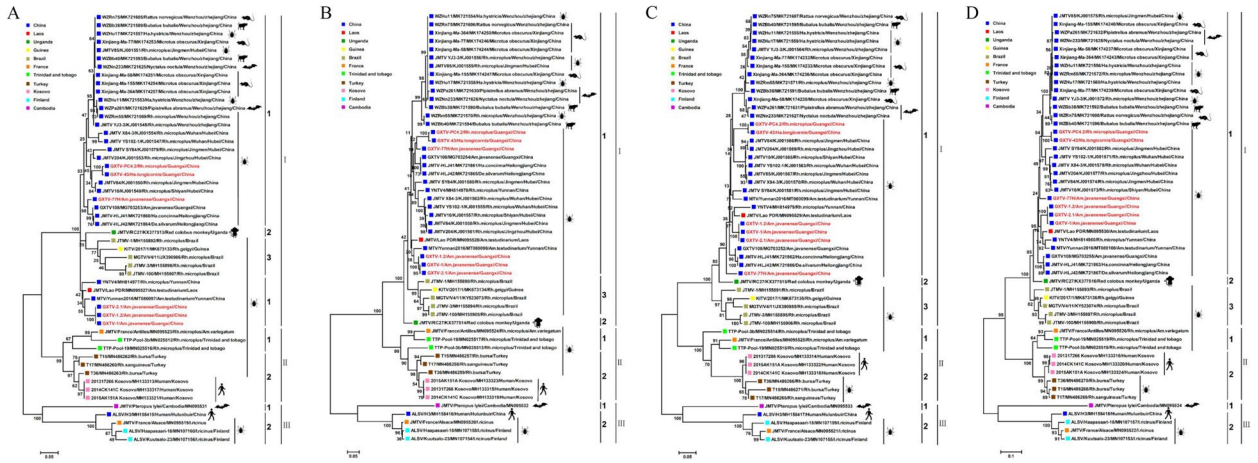

Fig. 4 Phylogenetic analysis of JMTVs based on the nucleotide sequences of segments 1–4. **A** Phylogenetic tree constructed based on the nucleotide sequences of JMTV segment 1. **B** Phylogenetic tree constructed based on the nucleotide sequences of JMTV segment 2. **C** Phylogenetic tree constructed based on the nucleotide sequences of JMTV segment 3. **D** Phylogenetic tree constructed based on the nucleotide sequences of JMTV segment 4. The trees were constructed using the Maximum Likelihood (ML) method with MEGA software. Sequences from this study are highlighted in red. Bootstrap values (1000 replicates) are shown at the nodes. Scale bars indicate the number of nucleotide substitutions per site

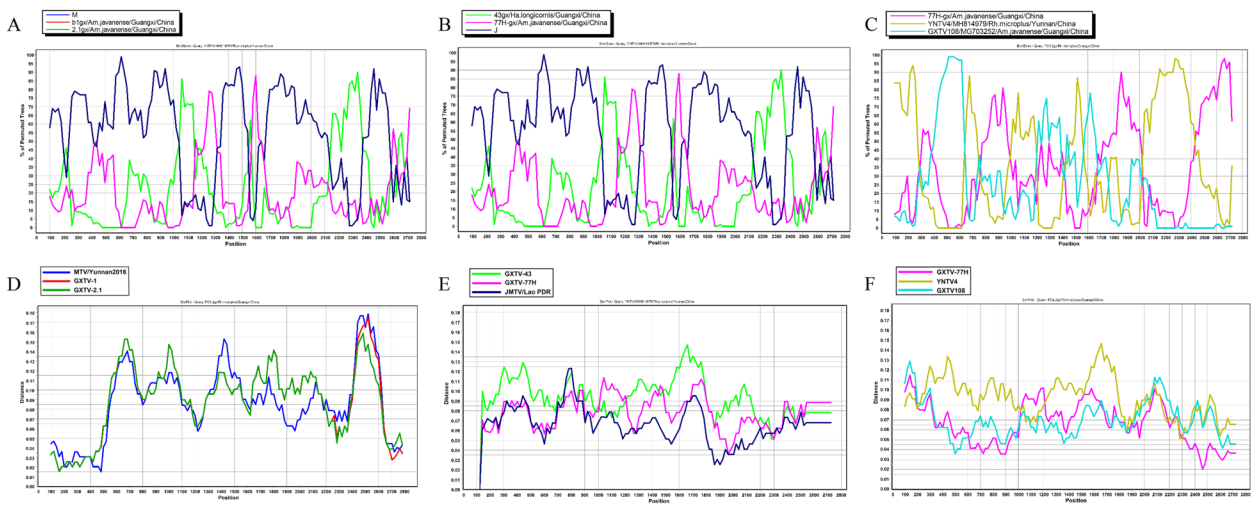

Fig. 5 Similarity and bootscan plots of the alignments of the JMTV genome segments with evidence for recombination. **A**-**C** Using BootScan to analyze the recombination of segment 2–4. **D**-**F** Using SimPlot to analyze the recombination of segment 2–4

Laos. The recombination breakpoints in this case were found at positions 523 and 1102.

Discussion

Guangxi situated in southwestern China, boasts a subtropical monsoon humid climate characterized by abundant heat and rainfall. This favorable environment has fostered a diverse natural ecology abundant in valuable plant and animal resources. However, it also provides conducive conditions for the proliferation of bacteria, fungi, viruses, and parasites [15–17]. With an approximately 1020-km land border, Guangxi experiences active border trade and frequent movement of materials and population, creating circumstances suitable for the transmission of insect-borne infectious diseases.

Given the diverse tick species and the potential variability in the pathogens they harbor [26], it is imperative to delineate the tick types for forthcoming studies on tick macrovirulomics. In this investigation, a straightforward random sampling approach was applied to procure tick samples from 18 collection sites spanning 17 counties (cities) in Guangxi. Morphological and molecular biology

techniques were synergistically employed to ascertain the identity of the collected tick samples. In total, we identified four genera and six species of hard ticks, with the *R. microplus* and *A. javanense* emerging as the predominant tick species. These findings align with prior reports, with the notable inclusion of the *H. hystricis* and the *D. tamokensis*, representing new records of tick species in Guangxi. It is essential to recognize, however, that the detection of only six tick species in our survey does not conclusively imply the exclusivity of these species in Guangxi. Our sampling was confined to a specific area of Guangxi due to the constraints inherent in random sampling. Additionally, factors such as sampling time, vegetation distribution, climate variations, and host adaptation exert considerable influence on tick growth, reproduction, and distribution [27]. Therefore, future research endeavors should prioritize an expanded sampling range and an increased sample size to comprehensively address the complexity of tick diversity in Guangxi.

Traditionally, the identification of tick species has heavily relied on morphological characteristics, particularly during the adult stage. However, challenges arise in identifying ticks during specific morphological stages, such as larvae or when undergoing molting, as well as in instances where their bodies may be damaged due to physical injury or engorgement-induced expansion and deformation [2, 28, 29]. Over the past two decades, molecular markers have gained popularity for tick species identification, owing to advancements in molecular biotechnology. Commonly employed molecular biomarkers for tick identification encompass mitochondrial genes such as 12S rRNA, 16S rRNA, COI, COII, as well as ribosomal genes including 18S rRNA, 28S rRNA, and ribosomal internal transcribed spacer sequences (ITS1, ITS2) [18, 30, 31]. In comparison to traditional morphological classification, molecular biology identification offers a rapid and convenient alternative that is not contingent on intact morphological specimens [32]. However, it is important to note that the DNA sequences of known tick species in the NCBI GenBank database remain incomplete, with less than 20% coverage of tick species sequences. As a result, molecular biological identification still benefits from complementation by morphological classification. The combination of traditional morphological classification and molecular biological identification is recommended to achieve synergistic and comprehensive results. Notably, our study contributes to enhancing and refining tick data in public databases, particularly since the GenBank database currently lacks the 16S rRNA gene sequences of *D. tamokensis*.

Ticks rank as the second most significant vectors of insect-borne infectious diseases, following closely behind mosquitoes. They also function as reservoirs for a diverse range of zoonotic pathogens, capable of vertical transmission to the succeeding generation of ticks or horizontal transmission to vertebrate hosts, thereby causing various zoonotic infectious diseases [5, 33, 34]. In our study, ticks were collected from 18 sampling sites spanning 17 counties (districts) in Guangxi, and a total of 74 libraries were constructed. Our findings unveiled the capacity of ticks to harbor an extensive variety of viruses, encompassing both RNA and DNA viruses. Significantly, RNA viruses exhibited a higher prevalence [8]. We focused our attention on viruses with a coverage of 0.05% or higher and a read count of 20 or more, categorizing them based on their natural host types. Our analysis identified vertebrate viruses (0.03%), arboviruses (52.64%), plant viruses (15.80%), protozoan viruses (31.44%), and phages (0.08%). Among these, arboviruses emerged as the most abundant. The vertebrate viruses were classified into the *Retroviridae* (retroviruses) and *Paramyxoviridae* (paramyxoviruses) families. Intriguingly, only the Tick-1.2 library contained retroviruses, a phenomenon possibly linked to tick behavior [35]. However, the certainty of ticks serving as hosts for these vertebrate viruses remains uncertain, given that this particular sample contained traces of host blood ingested by the ticks.

Among the 74 libraries we constructed, they were classified based on the type of genetic material, revealing a dominance of RNA viruses (97.88%) over DNA viruses (2.12%). These viruses belong to 13 known viral families, inclusive of two viral species from unclassified families. The RNA viruses encompass *Partitiviridae*, *Flaviviridae*, *Retroviridae*, *Virgaviridae*, *Chuviridae*, *Paramyxoviridae*, *Rhabdoviridae*, *Peribunyaviridae*, and *Phenuiviridae*. Conversely, the DNA viruses are associated with *Polydnaviridae*, *Myoviridae*, *Podoviridae*, and *Siphoviridae*. It is noteworthy that *Flaviviridae*, *Elasmobranchiviridae*, *Choumbunyaviridae*, and white *Fibroviridae* viruses are previously known TBVs [8], while *Paramyxoviridae* and *Chuviridae* represent newly discovered tick-borne virus families in recent years [36–38]. Our study disclosed that these viruses were predominantly found in *R. microplus* and *A. javanense*. This distribution pattern can be ascribed to the abundance of arthropod vectors and the geographical prevalence of vertebrate hosts [39]. R. microplus and A. javanense are the dominant tick species in the Guangxi region, exhibiting a wide distribution. Due to their pervasive presence, these *R. microplus* and *A. javanense* are more likely to come into contact with vertebrate hosts, facilitating the transmission of virulence factors.

Guangxi, situated at the southeastern edge of the Yunnan-Guizhou Plateau in the second terrain step of China, lies to the west of two hilly regions. The landscape encompasses six main categories: mountains, hills,

plateaus, plains, rocky mountains, and water surfaces. The topography generally exhibits higher elevation in the northwest and lower elevation in the southeast. Climate, geography, and the presence of animal hosts exhibit variations across different localities, impacting the distribution of arbovirus vectors and resulting in differences in virus abundance based on regional disparities [39, 40]. In the various libraries, significant differences were noted in the relative abundance of viruses at both the family and species levels. Nanning City and Beihai City displayed higher viral abundance, likely attributable to their developed economies, extensive vegetation cover, widespread distribution of vertebrate hosts and ticks, and a higher tick density compared to other areas in Guangxi. Consequently, these regions present a heightened risk of tick bites in natural environments. Furthermore, being the capital of Guangxi, Nanning regularly hosts the ASEAN Expo, fostering frequent trade with ASEAN countries, which increases the potential for cross-border transmission of TBVs. Conversely, Beihai, a coastal city in Guangxi with easy access to the sea, serves as a popular tourist destination. Weizhou Island, the largest island in Guangxi covering an area of 26.63 km^2, features dense jungles with a diverse range of species, particularly birds. Notably, the island is a habitat for over 140 species of migratory birds, contributing to the long-distance spread of TBV [41, 42]. These findings underscore the imperative for enhanced detection and control measures against TBV in Nanning and Beihai in the future.

In our study, we identified JMTV, a recently discovered and significant tick-borne pathogen, distinguished as the first segmented RNA virus ever identified [9]. JMTV showcases distinctive genetic evolutionary characteristics across diverse regions and host sources. The virus can be systematically classified into three major evolutionary clusters based on geographic origin: Evolutionary Cluster I predominantly include JMTVs from China, Laos, Uganda, Guinea, and Brazil; Evolutionary Cluster II is chiefly composed of JMTVs from France, Kosovo, Trinidad and Tobago, and Turkey; while Evolutionary Cluster III predominantly encompasses *Alongshan viruses* (ALSVs) from China and Finland, alongside JMTVs from France and Cambodia. Notably, JMTVs in Cluster III belong to the same family of *Flaviviridae* unclassified JMTVs [43]. The observed clustering patterns at geographic scales within these distinct evolutionary clusters suggest that JMTVs may manifest a diversity of genetic variations influenced by various geographic and environmental factors [37, 44, 45]. Moreover, this observation underscores that similar geographic scales and environmental factors often lead to comparable affinities.

In the evolutionary trajectory of viruses, the coevolution of viruses with their hosts and interspecies transmission across different host species are outcomes of their long-term evolution [37]. We propose that virus-host co-evolution and interspecies transmission could be contributing factors to the genetic diversity observed in JMTV. The phylogenetic tree illustrates that JMTV is associated with various host species, spanning arthropods (ticks), rodents (mice), mammals (bats and cows), non-human primates (monkeys), and humans, encompassing six categories [14, 46]. This broad spectrum of host species and distributions suggests a complex and diverse genetic evolutionary relationship. The JMTV85 strain (GenBank accession numbers: KJ001551, KJ001559, KJ001575), detected in *R. microplus* collected from Jingmen City, Hubei Province, exhibited similarity to strains found in cattle, rats, ticks, and bats collected from Wenzhou City, Zhejiang Province, as well as mice from Xijiang Province. JMTVs identified in rats from Wenzhou City, Zhejiang Province displayed close affinities, and no discernible taxon aggregation pattern was observed among hosts in the evolutionary tree, indicating the ability of JMTVs to horizontally spread across different species. Certain flying vertebrates, such as bats and birds, act as facilitators for cross-border and long-distance transmission of the virus due to their high density, wide distribution, and ability to cover extensive distances in flight [42, 47]. Simultaneously, the illegal smuggling of infected domestic or wild animals for transportation purposes further aids in the virus overcoming geographical barriers and achieving cross-border transmission [48, 49].

In the four branches of the evolutionary tree, the GXTV108 strain (GenBank accession numbers: MG703252-MG703255), identified in *A. javanense* collected from Guangxi, and the JMTV-HLJ41 (GenBank accession numbers: MK721860-MK721863) and JMTV-HLJ42 (GenBank accession numbers: MK721864-MK721867) strains detected in *Haemaphysalis Concinna* and *Dermacentor Silvarum* from Heilongjiang Province, China, exhibited closer genetic evolutionary relationships. Remarkably, Heilongjiang is situated over three thousand kilometers away from Guangxi. Considering that ticks, as arthropods, lack proficiency in long-distance migration, it is plausible that JMTVs achieve cross-border transmission through the long-distance migration of flying hosts or by tracking domestic and wild animals that have been artificially transferred. Furthermore, Qin et al. and Dincer et al. also reported the occurrence of genetic recombination in JMTVs [9, 24]. Genetic recombination facilitates the exchange of base sequences between strains, enhancing the virus's flexibility and genetic diversity as it adapts to different hosts, resulting in a broad spectrum of genetic variations [50]. Additionally, two of the six JMTV sequences obtained in

this study were newly identified mutant strains, further emphasizing the characteristic genetic variation diversity of JMTV.

Ticks play a pivotal role as vectors, serving as intermediaries in the transmission of JMTV between arthropods and mammals. Viruses transmitted within the same region and among the same tick species often exhibit similar affinities in the evolutionary tree; however, comparable affinities can also be observed among different tick species. In our evolutionary tree, we noted close relationships between GXTV-PC4.2 from the *R. microplus* of Guangxi and GXTV-43 from the *H. longicornis*. Similarly, GXTV108 (GenBank accession numbers: MG703252-MG703255) from the *A. javanense* of Guangxi exhibited a close relationship with JMTV-HCLJ41 (GenBank accession numbers: MG703252-MG703255) from the swarming *H. concinna* of Heilongjiang, as well as JMTV-HLJ41 (GenBank accession number: MG703252-MG703255) from the *De.silvarum* of Heilongjiang. Additionally, HLJ41 (GenBank accession numbers: MK721860-MK721863) and JMTV-HLJ42 (GenBank accession numbers: MK721864-MK721867) were identified in Heilongjiang. Ticks, being arthropods engaged in various ecological behaviors, exhibit coprophagy [37]. This behavior enables different tick species to parasitize the same vertebrate and feed on its blood, leading to the co-transmission of viruses across tick species. Co-feeding behavior plays a crucial role in facilitating the extensive transmission of TBV between arthropod and vertebrate hosts. Analysis of the phylogenetic tree reveals that nearly every sub-branch encompasses JMTVs found in ticks and mammals, spanning multiple genera and species of ticks. This substantial evidence strongly supports JMTV as an arbovirus primarily transmitted by ticks [9].

With the increasing utilization of high-throughput technologies, more features and properties of JMTV have come to light. In 2016, Ladner et al. identified the JMTV/RC27 strain in red colobus monkeys collected in Uganda, Africa [14]. The virus's gene sequence exhibited a high degree of homology (90.64–92.52%) with the JMTV SY84 strain found on *R. microplus* in Hubei Province, marking the first discovery of JMTV in a non-human primate. This finding further underscores the infectious potential of the virus and its threat to human health. In 2018, Emmerich et al. identified three strains of JMTV in the sera of Crimean-Congo hemorrhagic fever patients in Kosovo through high-throughput sequencing. Although this study was confined to the detection of clinical samples, it provided additional evidence for the potential of JMTV to infect humans and cause health damage [36]. In 2019, Jia et al. demonstrated, through high-throughput sequencing and fluorescence in situ hybridization experiments, that JMTV could replicate locally in the skin of tick-bitten patients and trigger immune responses, particularly neutrophil infiltration. This confirmed that JMTV can directly infect humans and cause localized clinical symptoms [14]. In the same year, Wang et al., a Chinese research team, discovered a JMTV-like virus named ALSV in the serum of patients with unexplained fever in a hospital in Hulunbeier, Inner Mongolia. ALSV belongs to the segmented genus Jingmenvirus and shares a homology rate of 57.4–74.5% with JMTV, making it the world's first segmented flavivirus known to infect humans. This discovery further underscores the ability of JMTV to induce localized clinical symptoms in humans and highlights the potential threat posed by JMTV and its cognate JMTV-like viruses as tick-borne pathogens. Therefore, conducting research on JMTV (or JMTV-like viruses) is strategically important for public health safety to prevent future outbreaks and mitigate the potentially incalculable damages they could cause [43].

Considering the characteristics of JMTV, including its widespread distribution, regional aggregation, diverse host types, cross-border transmission, and genetic variation, our current understanding of JMTV remains limited. Hence, it is imperative to persist in continuous and in-depth research and surveillance on JMTV. These endeavors hold strategic significance in ensuring long-term public health and safety, especially in preventing potential outbreaks of infectious diseases associated with JMTV or JMTV-like viruses in the population.

Conclusions

Guangxi showcases a diverse array of *Ixodidae*, with *R. microplus* and *A. javanense* emerging as the predominant species. Additionally, *H. hystricis* and *D. tamokensis* are newly discovered tick species in this region. The viral composition within these *Ixodidae* is equally diverse, with RNA viruses prevailing as the most common. JMTV exhibits distinctive characteristics, such as widespread distribution, regional clustering, varied host preferences, interspecies transmission, and genetic variability. Enhancing research efforts and surveillance focused on JMTV is of paramount importance for safeguarding public health security and mitigating the risk of infectious disease outbreaks associated with JMTV.

Abbreviations

ALSVs	Aron Mountain viruses
FPKM	Fragments Per Kilobase of exon model per Million mapped fragments
JMTV	Jingmen tick virus
MGTV	Mogiana tick virus
NSP	Nonstructural proteins
TBVs	Tick-borne viruses

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

Supplementary Material 4.

Supplementary Material 5.

Authors' contributions

WJL, YLH, PYC, XHW, and TCQ: participated in the study design and contributed relevant expertise. XHW, TCQ, SSL, YLZ and YJL: gathered and supplied the data. PYC and TCQ extracted, cleaned, and analyzed the data. XHW and TYY generated tables and visualizations. WJL, YLH, TCQ and XHW: drafted and revised the manuscript. All of the authors read and approved the final manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

This project was approved by the Forestry Bureau of Guangxi Zhuang Autonomous Region.Not applicable for consent to participate.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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