Microbes and Infection xxx (xxxx) xxx

Contents lists available at ScienceDirect

Microbes and Infection

journal homepage: www.elsevier.com/locate/micinf

Original article

Genomic and phylogenetic profiling of RNA of tick-borne arboviruses in Hainan Island, China

Gaoyu Wang [b, 1], Xiuying Tian [b, 1], Ruoyan Peng [b, 1], Yi Huang [b, 1], Youyou Li [b], Zihan Li [a, b], Xiaoyuan Hu [b], Zufen Luo [c], Yun Zhang [b], Xiuji Cui [b], Lina Niu [b], Gang Lu [b], Fan Yang [a], Lei Gao [a], Jasper Fuk-Woo Chan [d], Qi Jin [a], Feifei Yin [b, 2], Chuanning Tang [b, ***, 2], Yi Ren [e, **, 2], Jiang Du [a, b, *, 2]

[a] NHC Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, 100005, China
[b] Hainan Medical University-The University of Hong Kong Joint Laboratory of Tropical Infectious Diseases, Key Laboratory of Tropical Translational Medicine of Ministry of Education, Hainan Medical University, Haikou, 571199, China
[c] Department of Infectious Disease, the Second Affiliated Hospital of Hainan Medical University, Haikou, 570216, China
[d] State Key Laboratory of Emerging Infectious Diseases, Department of Microbiology, School of Clinical Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong Special Administrative Region, China
[e] Haikou Maternal and Child Health Hospital, Haikou, 570102, China

ARTICLE INFO

Article history:
Received 1 June 2023
Accepted 11 September 2023
Available online xxx

Keywords:
Arbovirus
Hainan island
Phylogenetic analysis
Tick
Virome

ABSTRACT

Ticks act as vectors and hosts of numerous arboviruses. Examples of medically important arboviruses include the tick-borne encephalitis virus, Crimean Congo hemorrhagic fever, and severe fever with thrombocytopenia syndrome. Recently, some novel arboviruses have been identified in blood specimens of patients with unexplained fever and a history of tick bites in Inner Mongolia. Consequently, tick-borne viruses are a major focus of infectious disease research. However, the spectrum of tick-borne viruses in subtropical areas of China has yet to be sufficiently characterized. In this study, we collected 855 ticks from canine and bovine hosts in four locations in Hainan Province. The ticks were combined into 18 pools according to genus and location. Viral RNA-sequence libraries were subjected to transcriptome sequencing analysis. Molecular clues from metagenomic analyses were used to classify sequence reads into virus species, genera, or families. The diverse viral reads closely associated with mammals were assigned to 12 viral families and important tick-borne viruses, such as Jingmen, Beiji nairovirus, and Colorado tick fever. Our virome and phylogenetic analyses of the arbovirus strains provide basic data for preventing and controlling human infectious diseases caused by tick-borne viruses in the subtropical areas of China.

In the nineteenth century, ticks were already known as blood-sucking parasites that bite humans and domesticated animals. However, the innovative work of Smith and Kilbourne on tick-transmitted Texas fever in cattle in 1893, Rickett's discovery of Rocky Mountain spotted fever caused by *Dermacentor andersoni* in 1907, and Stockman's identification in 1918 of the tick-borne Louping ill virus, which causes severe encephalitis in sheep and other livestock, provided solid evidence that ticks can serve as vectors for transmitting multiple pathogens among both humans and animals [1–3].

Arboviruses are among the most common tick-borne pathogens [4]. For example, the tick-borne encephalitis virus (TBEV), which causes human meningitis, encephalitis, and meningoencephalitis in the northern European subcontinent, is transmitted by *Ixodes persulcus*. It has been estimated that there are at least 10,000 clinical cases of TBEV annually in Europe and that the morbidity

* Corresponding author. NHC Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, 100005, China.
** Corresponding author.
*** Corresponding author.
E-mail addresses: chuanningtang@hotmail.com (C. Tang), renwing1981@163.com (Y. Ren), dujiangemail@163.com (J. Du).
[1] These authors contributed equally to this work.
[2] These authors contributed equally to this work and are co-senior authors.

https://doi.org/10.1016/j.micinf.2023.105218

Please cite this article as: G. Wang, X. Tian, R. Peng *et al.*, Genomic and phylogenetic profiling of RNA of tick-borne arboviruses in Hainan Island, China, Microbes and Infection, https://doi.org/10.1016/j.micinf.2023.105218

and mortality rates vary according to virus subtype [5]. Crimean Congo hemorrhagic fever (CCHF) often leads to sporadic cases or outbreaks of severe illness among humans in western China, the Middle East, southeastern Europe, and most parts of Africa [6]. CCHF is transmitted via vertical and horizontal transmission cycles in domesticated and wild animals. *Hyalomma asiaticum*, a vector of CCHF, is the main source of infections in humans, which causes clinical symptoms such as hemorrhagic fever, multiple organ failure, and shock [6]. Severe fever with thrombocytopenia syndrome (SFTS) was first identified in rural China in 2010 and has been reported in multiple provinces, with an average fatality rate of 7.3%. SFTS cases have also been reported in Japan and South Korea in 2012. SFTS is a zoonotic disease caused by the SFTS virus (SFTSV), characterized by a close homology to the Heartland virus found in the United States [7].

In addition, ticks have also been established to be vectors of the Powassan virus, Colorado tick fever virus (CTFV), Jingmen tick virus (JMTV), Guangxi tick virus (GXTV), Bourbon virus, and Louping ill virus [8–11]. Compared with other vector viruses, the unique evolutionary status of tick-borne viruses presents a considerable challenge, given their horizontal or vertical transmission. To obtain and maintain the ability to replicate in different vertebrate hosts, tick-borne viruses must overcome the various barriers encountered in different host environments [12]. Consequently, tick-borne viruses have developed a strong transmission capacity, thereby representing a considerable threat to human life and health and presenting substantial challenges regarding preventing and controlling infectious diseases. Accordingly, studies on tick-borne viruses are a major focus of infectious disease research.

This study collected 855 *Rhipicephalus sanguineus* and *Rhipicephalus microplus* ticks from Tunchang County, Danzhou City, Lingao County, and Qiongzhong City in Hainan Province, which is the only tropical island province in China. The ticks were combined into 18 pools according to genus and location. Viral RNA-sequence libraries were subjected to transcriptome sequencing analysis using an Illumina HiSeq2500 sequencer. Molecular characteristics identified based on metagenomic analyses were used to classify sequence reads into virus families or genera using MEGAN. Representative reads for important arboviruses and arthropod viruses were selected for genome sequencing and used for read-based polymerase chain reaction (PCR) to identify partial genomes and screen for positive rates. Our results provide a profile of the virome composition of ticks and a baseline for tick-borne viruses in Hainan Island, China. Furthermore, phylogenetic analysis of arboviruses characterized their representative biodiversity, evolution, and potential transmission to humans and domesticated animals. The findings of this study also provide basic data that will contribute to the prevention and control of human infectious diseases caused by arboviruses on Hainan Island.

1. Materials and methods

1.1. Collection of ticks

Between May 21 and June 3, 2020, we collected 855 ticks from canine and bovine hosts in four different locations (Tunchang County, Danzhou City, Lingao County, and Qiongzhong City) in Hainan Province, China (Table 1). Before experiments, these specimens were maintained in cases at room temperature. Groups of ticks [15–20] were subsequently transferred to 2 ml tubes according to collection location and species. Initially, identification was performed by a professional technician based on morphology and subsequently confirmed based on 12S rDNA and 16S rDNA gene sequence analyses. The ticks were prepared for analysis by rinsing three times with sterile phosphate-buffered saline (PBS, pH 7.4) [3], followed by homogenization with 1 ml of PBS per group. The homogenates were centrifuged for 10 min at 5000×*g* and 4 °C to remove debris, and the supernatants were stored at −80 °C until further analysis. The flowchart outlining the bioinformatics analyses conducted on tick-borne arboviruses in this study is shown in Fig. 1. These sampling procedures were approved by the Ethics Committee of Hainan Medical University (approval number: HMUEC20180059).

1.2. RNA-seq library construction and next-generation sequencing

Based on collection location and tick species, tick specimens were grouped into 19 batches (Table 1), and we randomly selected a supernatant as one pool from the same batch. We used a 300-μl aliquot of supernatant for each pool to extract total RNA using TRIzol LS reagent (Invitrogen, Carlsbad, CA). Nineteen corresponding RNA-seq libraries were constructed using the Illumina protocol [13]. These viral nucleic acid libraries were analyzed using an Illumina HiSeq3000 sequencer (Illumina, USA), and the sequencing data thus obtained have been deposited in the National Center for Biotechnology Information sequence reads archive under the accession number PRJNA793118. Raw sequence reads were filtered using previously described criteria to obtain valid sequences [14].

Table 1
Summary of tick sample collections in Hainan Province.

Number	District	Location	Time	Tick Species	Quantity	Host
69	Danzhou	Xiqing farm E	2020.05.26	*Rhipicephalus sanguineus*	40	canine
70	Tunchang	Nankun town G	2020.05.25	*Rhipicephalus sanguineus*	39	bovine
71	Tunchang	Nankun town M	2020.05.25	*Rhipicephalus sanguineus*	48	bovine
73	Tunchang	Nankun town A	2020.05.25	*Rhipicephalus sanguineus*	81	bovine
74	Tunchang	Nankun town C	2020.05.25	*Rhipicephalus sanguineus*	54	bovine
75	Tunchang	Nankun town D	2020.05.25	*Rhipicephalus sanguineus*	43	bovine
76	Tunchang	Nankun town E	2020.05.25	*Rhipicephalus sanguineus*	50	bovine
77	Tunchang	Nankun town F	2020.05.25	*Rhipicephalus microplus*	38	bovine
78	Tunchang	Nankun town I	2020.05.25	*Rhipicephalus microplus*	55	bovine
79	Tunchang	Nankun town J	2020.05.25	*Rhipicephalus microplus*	42	bovine
80	Danzhou	XiQing farm A	2020.05.26	*Rhipicephalus sanguineus*	50	canine
81	Danzhou	XiQing farm B	2020.05.26	*Rhipicephalus sanguineus*	44	canine
82	Danzhou	XiQing farm C	2020.05.26	*Rhipicephalus sanguineus*	52	canine
83	Danzhou	XiQing farm D	2020.05.26	*Rhipicephalus sanguineus*	39	canine
84	Linggao	Jinmo village A	2020.05.27	*Rhipicephalus sanguineus*	48	bovine
85	Linggao	Jinmo village B	2020.05.27	*Rhipicephalus sanguineus*	46	bovine
86	Linggao	Jinmo village C	2020.05.27	*Rhipicephalus sanguineus*	41	bovine
87	Qiongzhong	Luodan village A	2020.06.03	*Rhipicephalus microplus*	45	bovine

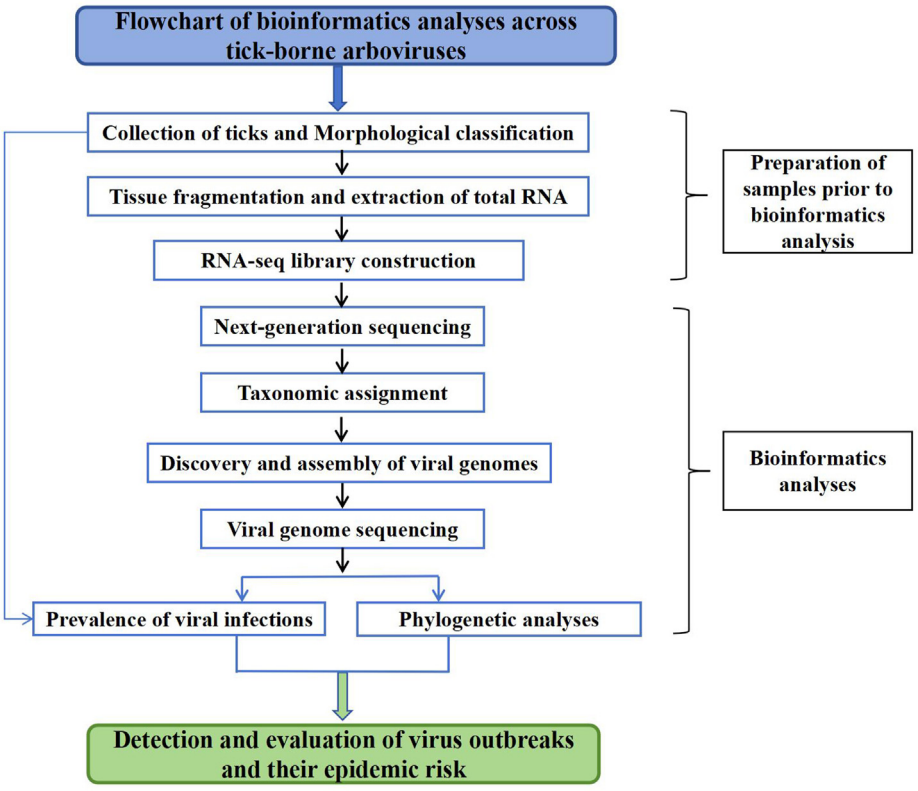

Fig. 1. Flowchart of bioinformatics analyses across tick-borne arboviruses.

1.3. Taxonomic assignment

Sequence similarity-based taxonomic assignments were conducted as previously described [14]. Briefly, each read was evaluated for viral origin by conducting alignments against the National Center for Biotechnology Information (NCBI) non-redundant nucleotide (NT) and protein (NR) databases, using BLASTn and BLASTx (E: expected value < 10^{-5}, −F: filter query sequence, default = T). The taxonomies of aligned read with the best BLAST scores (E-value <10^{-5}) from all lanes were parsed and exported using the MEGAN 6- MetaGenome Analyzer [15].

1.4. Metagenomic sequencing

The abundance of mapped viral sequences was estimated and characterized by normalized reads to avoid any bias of unequal sequencing depth or gene length between different libraries. Heatmaps representing virus abundance in each pool were generated using TBtools (https://github.com/CJ-Chen/TBtools/releases). The reads of each viral family in each pooled sample were normalized by reads per kilobase million of the total virus reads, as described in our previous study [14]. The normalized abundance of each viral pool is shown as the $\log_{10}$ scale of read values in the heatmap. The identities and coverage rates of the viral read sequences compared with those of the reference viruses were also calculated using a heatmap [16].

1.5. Viral genome sequencing

Molecular characteristics determined based on metagenomic analyses were used to classify sequence reads into virus families or genera using MEGA 6. Representative viral reads were selected for open reading frame (ORF) sequencing and used for read-based PCR to identify the partial genomes. Viral RNA was isolated from 300-μl aliquots of the supernatants of each pool using TRIzol LS reagent (Invitrogen, Carlsbad, CA). The remaining genomic sequences were analyzed based on genome walking and 5'- and 3'-rapid amplification of cDNA ends (Invitrogen/USA, Takara/Japan). All primer sequences were based on the newly obtained reads and newly amplified sequences and are shown in Supplementary Table S1 (Not shown).

1.6. Prevalence of viral infections among ticks

To screen for the Jingmen tick virus in each supernatant, we designed specific semi-nested primers targeting the non-structural gene for PCR, using the complete genomic sequences of the viruses obtained based on genome sequencing (Table 2). PCR was performed using a 2 × Taq PCR Mastermix (Tiangen, China). Two microliters of the first-round PCR product were used as the template for the second round of PCR. The thermal cycling conditions for both PCRs were as follows: an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 45 s, and a final elongation step at 72 °C for 10 min. PCR products were analyzed using 1% agarose gel electrophoresis and ultraviolet imaging.

Table 2
Primers used for assessing the prevalence of Jingmen tick virus infections in ticks.

Pathogen	Primer name	Sequence	Size (bp)
Jingmen tick virus	S1−F1	TCGGCGATAAATAGGAGAGGTGCCAT	486
	S1-R1	TCTGCGTAGAGTCGGTAGAGGTGGTG	
	S1−F2	GGACTGGAGACAAGACGTCAACACG	370
	S1-R2	CGCCATTTCTTCATCCTCCGCTAG	

1.7. Genome annotation

The nucleotide sequences of the genomes and the amino acid sequences of the ORFs were deduced by comparing the sequences with those of astroviruses. Conserved protein families and domains were predicted using Pfam, Blastp (https://blast.ncbi.nlm.nih.gov), and InterProScan 5 (http://www.ebi.ac.uk/services/proteins). Routine sequence alignments were performed using Clustal Omega (http://www.ebi.ac.uk/Tools/).

1.8. Phylogenetic and data analyses

We used the MUSCLE package and default parameters in MEGAX to align nucleotide sequences and deduce amino acid sequences. A phylogenetic tree was constructed using the maximum-likelihood method. Modeling based on the substitution model rtREV with the Freqs. (+F) model was performed using the model selection function of MEGA6.0 with 1000 bootstrap replicates. Pairwise amino acid alignment between the novel viruses and other reference sequences was performed using the NCBI Basic Local Alignment Search Tool. TBtools software (https://github.com/CJ-Chen/TBtools/releases) was applied to generate a heatmap representing the abundance of viruses in each pool and the sequence identity and percentage coverage of the corresponding reference virus. The ggplot2 package in R studio was used to quantify selected viruses from ticks [17].

2. Results

2.1. Collection of ticks from canine and bovine hosts

We collected 855 tick samples from canine and bovine host animals in Danzhou City (Xiqing Farm), Tunchang County (Nankun Town), Linggao County (Jingmo Village), and Qiongzhong County (Luodan Village) (Fig. 2A and Table 1). The tick species were initially identified based on morphology with subsequent confirmation based on 12S rDNA and 16S rDNA gene sequencing, which indicated that the collected specimens comprised two species, namely, *R. sanguineus* (78.95%) *and R. microplus* (21.05%) (Fig. 2B and C). Supernatants obtained from 12 to 20 homogenized ticks based on the sampling location and species were grouped into 18 pools. 198.4 GB of nucleotide data (1,145,387,778 valid reads, 150 bp in length) was obtained. Reads with no significant similarity to any amino acid sequence in the viral RefSeq database were discarded. The remaining 479,746 reads were searched again using DIAMOND against the NCBI non-redundant database. The final 181,324 reads best matched the available viral proteins (approximately 0.016% of the total sequence reads). The number of virus-associated reads in each lane, representing a pool, varied between 19 and 57,923.

2.2. Metagenomic analysis and virome overview

The virus-associated reads were assigned to 17 families classified as double-stranded (ds) DNA viruses, dsRNA viruses, retro-transcribing viruses, single-stranded (ss) DNA viruses, and ssRNA viruses. Following a further screening of fungal viruses, protozoal viruses, and phage, as described previously, viral reads (178,648 sequence reads, approximately 98.5% of the total viral hits) were assigned to 12 families and unclassified viruses in each pooled sample, namely, *Flaviviridae, Chuviridae, Rhabdoviridae, Retroviridae, Phenuiviridae, Nairoviridae, Orthomyxoviridae, Totiviridae, Papillomaviridae, Reoviridae, Luteoviridae,* and *Poxviridae*. A comparison of the total reads, and the proportion of viral reads from the two tick species and the four sampling locations is presented in Fig. 3. The viral composition of each pooled sample is shown in Fig. 4A.

The normalized abundance of viral species in tick pools was analyzed by hierarchical clustering based on a Euclidean distance matrix calculated from the normalized read count. The generated heatmap indicated that viromes hosted by canine animals and bovine-derived ticks might be associated with their geographical distribution (Fig. 4B). Based on the similarities among viral species and normalized abundance, a high abundance of Jingmen tick virus, rhabdo-like virus, Mivirus Wuhanense, Wuhan alpharicinrhavirus, Lihan uukuvirus, Manly virus, Changping mivirus, belonging to families *Flaviviridae, Rhabdoviridae, Chuviridae, Phenuiviridae,* and unclassified viruses, respectively, were found clustered in the sampled areas (Danzhou 80–83, Ledong 84–86, and Qiongzhong 87) and tick species (*R. sanguineus* and *R. microplus*). The pooled samples of ticks (*R. microplus* 78-79 and *R. sanguineus* 70-73) collected in Tunchang had greater viral diversity than samples from other regions of Hainan Province. Viruses belonging to the families *Chuviridae* and *Luteoviridae* and unclassified viruses, such as Bole tick virus and Hubei toti-like virus [24], *Ixodes scapularis*-associated virus [2], Lesnoe mivirus, and Norway luteo-like virus [2], although not as widely distributed, were identified in *R. sanguineus* ticks collected from a few locations (Tunchang 71-70, 73, 75-76 and Danzhou 69) with a high viral abundance. In addition, Betapapillomavirus 1, Cowpox virus, CTFV, and Beiji nairovirus from the families *Papillomaviridae, Poxviridae, Reoviridae,* and *Nairoviridae*, respectively, were present in tick species and individuals in certain areas in Hainan Province with a low normalized abundance.

2.3. Analysis of important viruses

2.3.1. Jingmen tick virus (JMTV)

Jingmenviruses (JMVs) are a group of segmented, positive-sense, single-stranded RNA viruses. Based on homology with NS3 and NS5 of the *Flaviviridae*, JMV is currently classified in the Jingmenvirus group of this family [18]. JMV was the first reported positive-strand (+) RNA virus and has contributed to filling the gap between segmented and unsegmented RNA viruses. The main members of the Jingmenvirus group include JMTV, Guaico Culex virus (GCXV), Mogiana tick virus (MGTV), and apple latent spherical virus (ALSV) [8,9,11,19]. The main hosts of JMV are arthropods, including ticks, mosquitoes, cricks, fleas, and aphids. JMV can infect cattle, monkeys, and other mammals. Notably, a human case of Jingmenvirus infection has recently been reported. JMTV, a JMV representative characterized by significant genetic diversity and intercontinental geographical clustering, was first identified in *R. microplus* in the Jingmen area of Hubei Province, China, in 2010 and has subsequently been reported worldwide.

To identify partial genomes, representative reads for JMTV in *R. sanguineus* ticks collected from Danzhou and Lingao were selected for genome sequencing and used for read-based PCR. We obtained the complete genome sequence of novel JMTV, termed JMTV-HMU, which included segments 1, 2, 3, and 4. Based on BLAST analysis, the nucleic acid sequence of segment 1 was established to have less than 93% homology with the NS5-like protein gene of known members of JMV. Similarly, the nucleic acid sequence of segment 2 had less than 92% homology with the putative glycoprotein gene, whereas the nucleic acid sequence of segment 3 had less than 93% homology with the NS3-like protein gene, and that of segment 4 had less than 92% homology with the putative capsid protein gene of known members of JMV.

Phylogenetic analysis revealed that this novel JMTV-HMU is closely related to other JMTVs and MGTV isolated from ticks or rodents in Southeast Asia (Fig. 5). JMTV, MGTV, and GXTV formed an independent branch separated from the ALSV in the deep root of the phylogenetic tree. In epidemiological analysis, the 855 ticks were combined into 57 pools based on genus and location and were

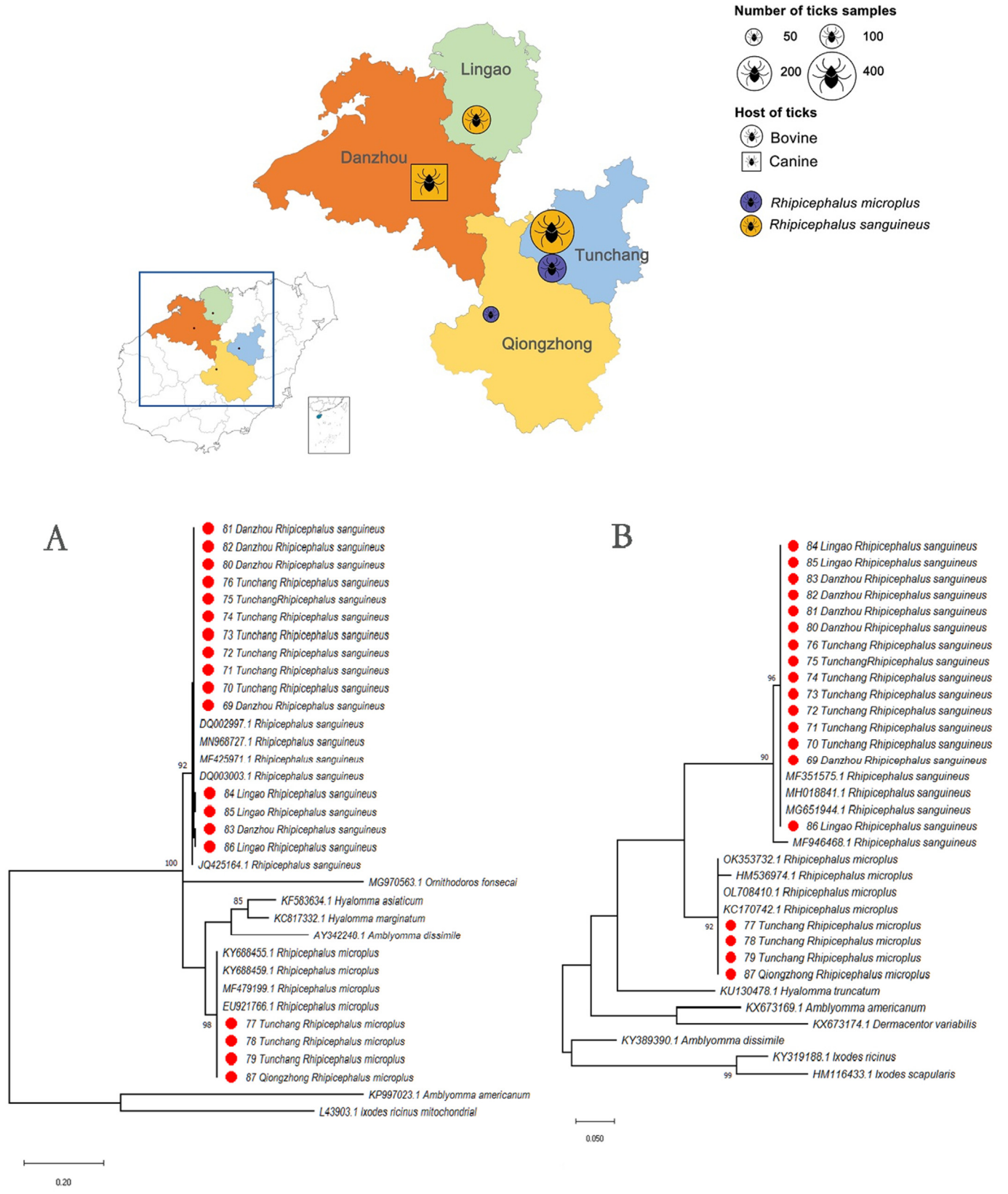

Fig. 2. Collection of ticks from Hainan Island. (**A**) Tick collection in Hainan Island and the metagenomics of ticks according to species and location. A map of Tunchang County, Danzhou City, Lingao County, and Qiongzhong City in Hainan Island shows the samples' locations. Tick species and the number of ticks and animals are also shown on the map, denoted by different shapes, colors, and sizes. (B and C) Molecular identification of tick samples. Phylogenetic analysis was performed using the maximum-likelihood method based on (**B**) 12S rDNA and (**C**) 16S rDNA gene sequences. The ticks collected in this study are indicated by red circles (●).

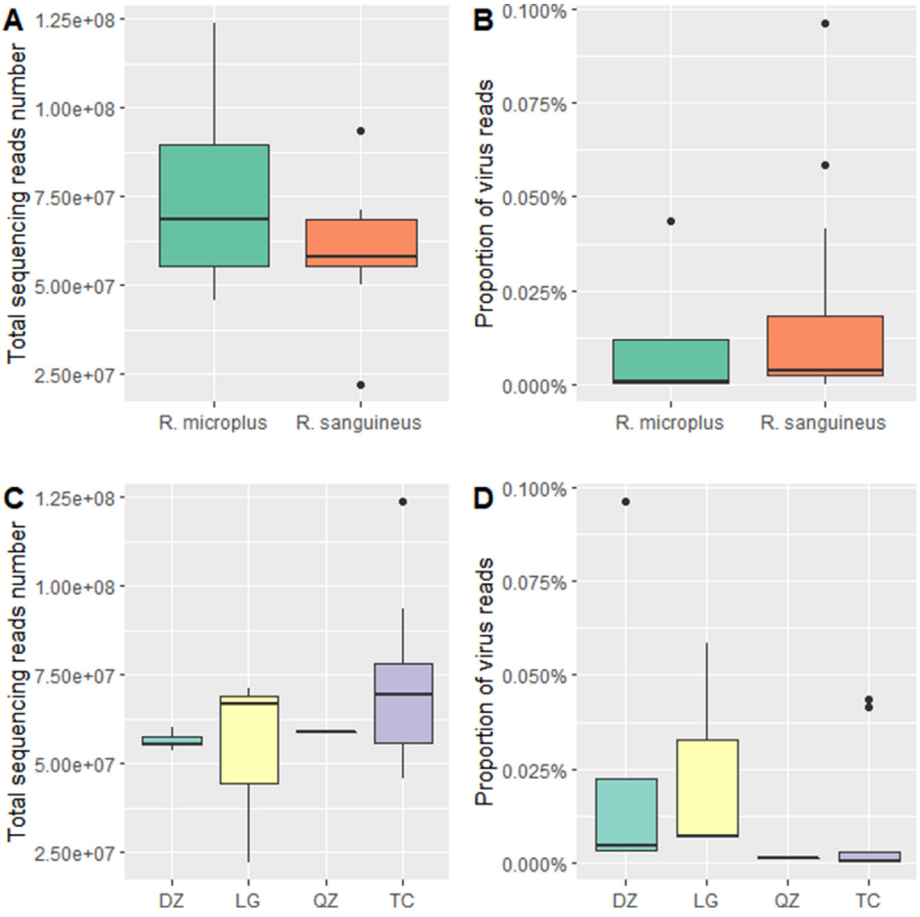

Fig. 3. (**A and B**) Comparison of the number of total reads and proportions of viral reads from the two tick species. (**C and D**) Comparison of the numbers of total reads and proportions of viral reads from the four sampling locations. Danzhou City (DZ), Lingao County (LG), Qiongzhong City (QZ), and Tunchang County (TC).

screened for JMTV-HMU infection. Thirty-three pooled samples were found to be strongly positive for JMTV-HMU in all three genera, with a positive rate of 58%.

2.3.2. Brown dog tick Phlebovirus 1 (BDTPV1)

The genome of *Phlebovirus* is a negative-stranded, linear segmented RNA genome, with large (L: 6.4 kb), medium (M: 3.2 kb), and small (S: 1.7 kb) segments. The brown dog tick *Phlebovirus* 1 (BDTPV1) is currently classified as an unclassified *Phlebovirus* and *Phenuiviridae* of the Bunyavirales. The main hosts of *Phlebovirus* are ruminants, camels, and humans, with the main vectors being mosquitoes and ticks. BDTPV1 was first found in ticks (*R. sanguineus*) collected from animals throughout Trinidad and Tobago in 2017 and 2018 [20]. The L and S segments of these viruses are similar to those of known phleboviruses, although they lack an M segment, a component of the *Phlebovirus* genome that encodes the viral glycoprotein.

Representative reads for BDTPV1 carried by *R. microplus* collected from Tunchang were selected for genome sequencing and used for read-based PCR to identify partial genomes. We obtained a BDTPV1 partial L segment genome sequence (BDTPV1-HMU) of 3888 bp, which, based on a Blast analysis, was found to have 96% nucleic acid sequence homology with the polymerase protein gene of the BDTPV1 isolate TTP-Pool-12 L segment. BDTPV1-HMU had the highest similarity [69.11% nucleotide (nt)] to the L segment of the *Phlebovirus* identified in *R. sanguineus* ticks collected in Turkey [21] and to Bole tick virus 1 (65.86% nt), which was recently identified in *H. asiaticum* ticks in China [13]. We also identified two short contigs of the S segment (293 and 245 bp, respectively) of BDTPV1-HMU, which showed the closest similarity (100% and 98% nt, respectively) to the S segment of BDTPV1.

In the phylogenetic analysis, the representative species of 20 genera in *Phenuiviridae* and the representative unclassified *Phlebovirus* were considered reference sequences. BDTPV1-HMU was found to be closely related to BDTPV1 and formed separate clusters with *Phlebovirus* and Bole tick virus 1 isolated from ticks in Trinidad and Tobago among the unclassified *Phlebovirus* group from the other genera of *Phenuiviridae* (Fig. 6).

2.3.3. Beiji nairovirus (BJNV)

Members of the genus *Orthonairovirus* in the family *Nairoviridae* produce enveloped virions with genomes consisting of three negative-sense, single-stranded RNA segments: S (1.7–2.1 kb), M (4.4–6.3 kb), and L (11.2–14.4 kb). These viruses are maintained in arthropods or transmitted among mammals (including bats) and birds by ticks and have endemic potential in Asia, Africa, and Southern and Eastern Europe [22]. Beiji nairovirus (BJNV) is currently classified as an unclassified nairovirus, *Orthonairovirus, and Nairoviridae* of the Bunyavirales. The genome sequence of Beiji nairovirus YKS59 was isolated from a patient with febrile illness and a history of tick bites in Yakeshi, Inner Mongolia [23]. The genome sequence of BJNV is highly homologous to that of the Gakugsa tick virus and Norway nairovirus isolated from *Ixodes persulcatus* and *Ixodes ricinus* in Russia and Norway, respectively.

In this study, 829 representative reads for *Orthonairovirus* of the *Nairoviridae* family identified in *R. sanguineus* ticks collected from Tunchang were extracted for assembly. All reads were assembled

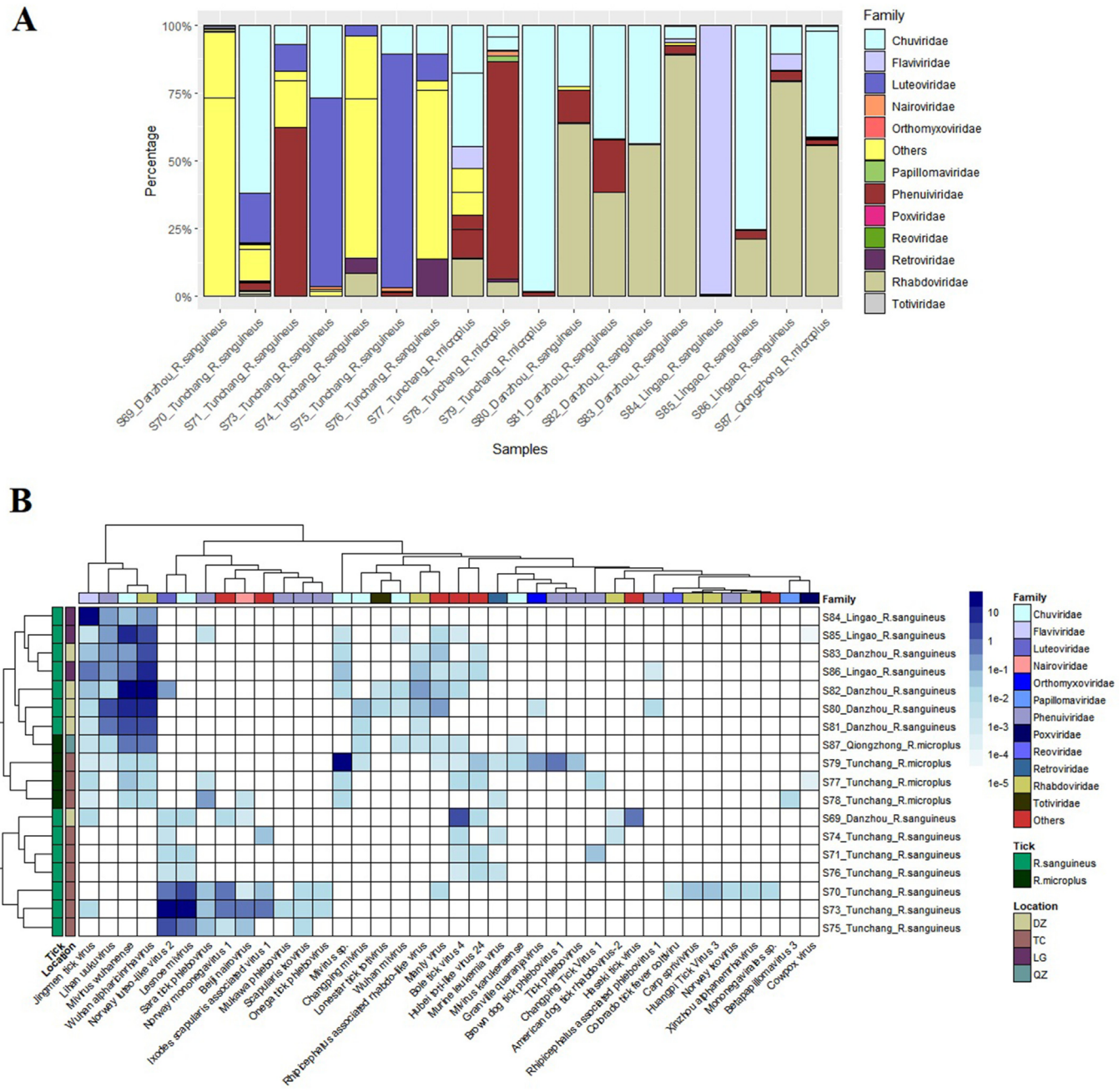

Fig. 4. Metagenomic analysis. (**A**) The proportions of the number of reads assigned to each viral family in each of the 18 pools are shown in the bar graph. Danzhou City (DZ), Tunchang County (TC), Lingao County (LG), and Qiongzhong City (QZ). (**B**) Normalized abundance of viral species in tick pools. Comparative abundances of different viral species reads were normalized based on RPKM (Reads Per Kilobase Million) counts, and th $\log_{10}$ scale of RPKM was calculated for hierarchical clustering using a Euclidean distance matrix and displayed as a heatmap. The names of viral species identified by taxonomic annotation using BLASTx are sorted by viral family in the heatmap. The sampling locations and species of ticks are marked above and below the heatmap.

into six contigs, the longest and shortest of which were 701 and 314 bp, respectively. Among these, four contigs were matched to the RNA-dependent RNA polymerase of BJNV with 98–100% nucleotide identity and two contigs were aligned to the RNA-dependent RNA polymerase of the Gakugsa tick virus with 99–100% identity. These newly discovered BJNV contigs also belong to unclassified nairoviruses.

2.3.4. *Unclassified* Quaranjavirus

Members of the genus *Quaranjavirus* of the *family* Orthomyxoviridae produce segmented ssRNA (−) linear-genome virions encapsidated by a nucleoprotein. The genome contains six segments of differing sizes (ranging from 0.9 to 2.3 kb), which code for six proteins. The total genome size is approximately 10 kb. *Quaranjavirus* contains two species, *Quaranfil quaranjavirus* (QRFV), Johnston Atoll quaranjavirus (JAV), and unclassified *Quaranjavirus*. The main hosts of *Quaranjavirus* are humans, and the main vectors are ticks. *Q. quaranjavirus* was initially isolated from ticks (*Argas arboreus*) collected near Cairo, Egypt, in 1953 [24] and has subsequently been isolated from ticks and birds in multiple geographical areas. In addition, the unclassified *Quaranjavirus,* Zambezi tick virus 1, was isolated from *R.* in Mozambique in 2014.

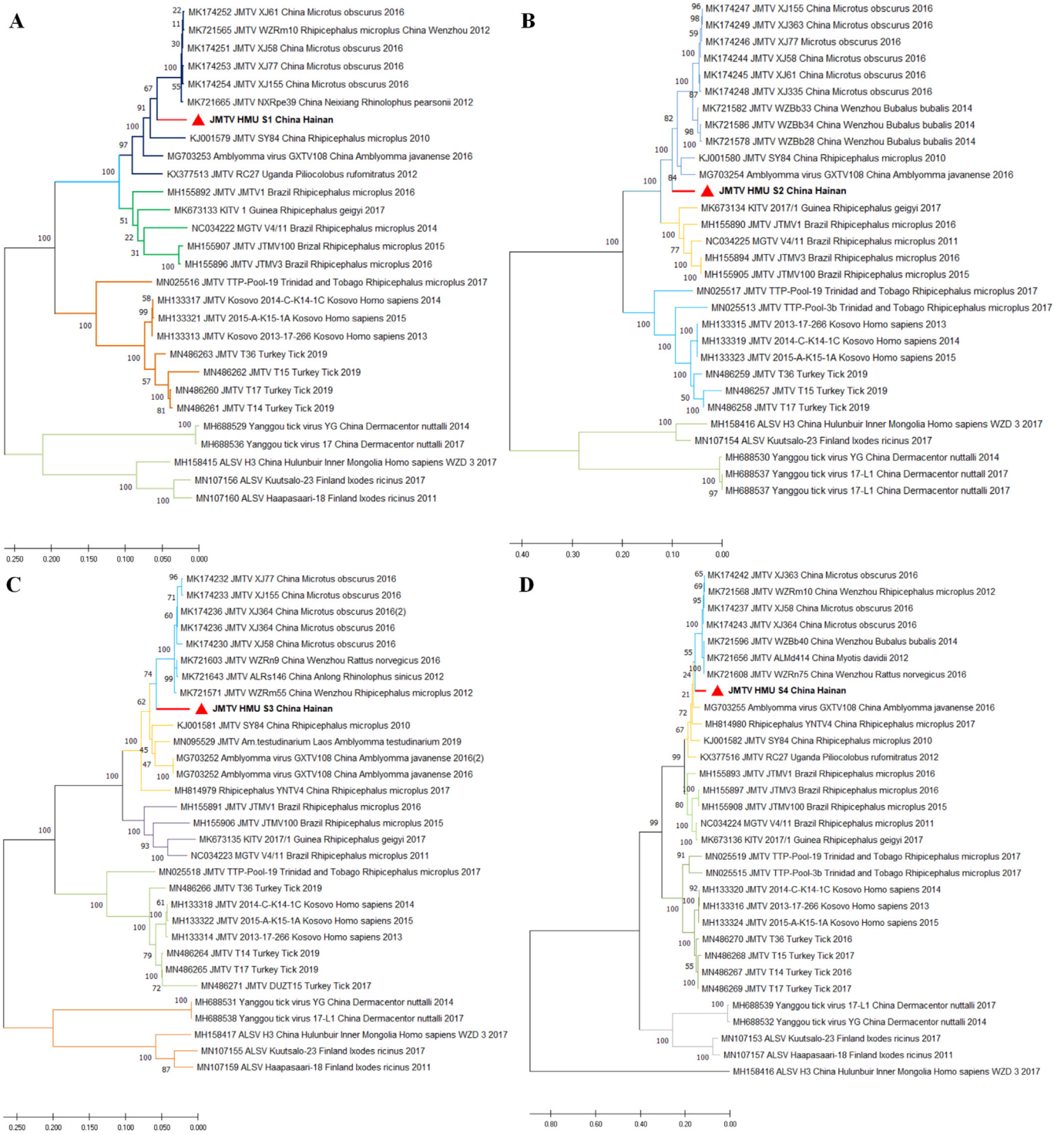

Fig. 5. JMTV phylogenetic tree. A phylogenetic tree based on the complete (**A**) S1, (**B**) S2, (**C**) S3, and (**D**) S4 segment nucleotide sequences of JMTV-HMU. The viruses identified in this study are labeled with a red circle (▲). JMTV, Jingmen tick virus.

In this study, we extracted 219 representative reads for *Quaranjavirus* from *R. microplus* collected in Tunchang. All reads were assembled into five contigs, the longest and shortest of which were 635 and 246 bp, respectively. Among these, three contigs were matched to the polymerase basic protein 1 of Zambezi tick virus 1 with 88.24–91.46% amino acid identity, and two contigs were aligned to the hemagglutinin of the Granville *Quaranjavirus* with 60.71–82.5% amino acid identity. These newly discovered contigs also belonged to unclassified *Quaranjavirus*.

In addition, we identified some mammalian viruses with few annotations, including four reads for *Coltivirus* belonging to *Spinareovirinae* in the family *Reoviridae* carried by *R. sanguineus* ticks collected from Tunchang, which were assembled into two contigs (222 and 337 bp, respectively). These two contigs were matched

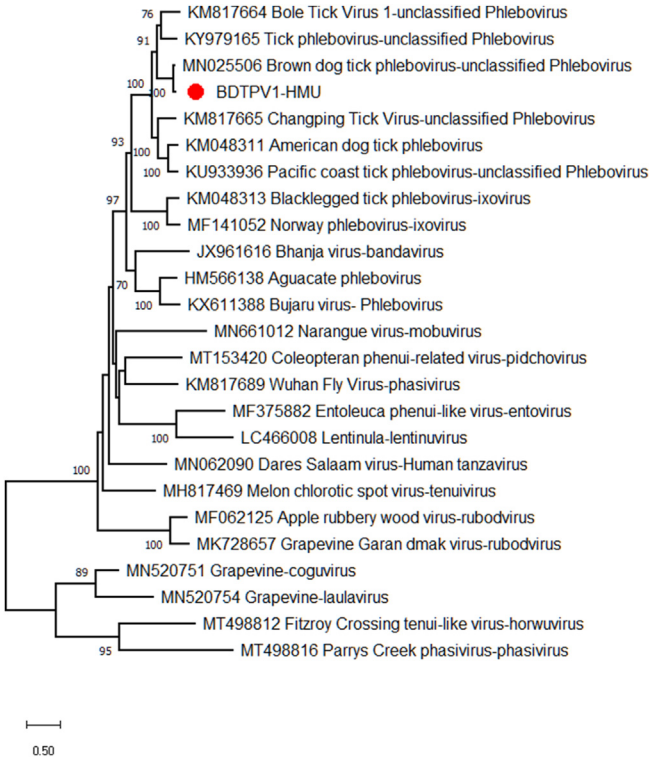

Fig. 6. BDTPV1-HMU phylogenetic tree. A phylogenetic tree based on the almost complete L segment nucleotide sequence of BDTPV1-HMU. The viruses identified in this study are labeled with a red circle (●). BDTPV1, Brown dog tick Phlebovirus 1.

with the VP2 and VP3 proteins of CTFV with 84% and 92% amino acid identities, respectively. A further four reads for Betapapillomavirus belonging to *Firstpapillomavirinae* in the family *Papillomaviridae* were obtained for isolates from *R. microplus* collected from Tunchang and assembled into a single contig (220 bp), which was matched with the L1 protein of human papillomavirus type 49 with 94.44% amino acid identity. In addition, four reads for *Orthopoxvirus* obtained from *R. microplus* collected in Tunchnag and *R. sanguineus* collected in Lingao belonged to *Chordopoxvirinae* in the family *Poxviridae* and assembled into two contigs (183 and 194 bp, respectively), which were matched with the CPXV129 protein of the Cowpox virus with 100% amino acid identity.

3. Discussion

A distinguishing feature of pathogen-transmitting ticks is their extended multi-phase life cycle, which includes four stages (egg, larva, nymph, and adult). Each can infect different host animals and survive for prolonged periods without food, which enables these ticks to be infected at any stage of the life cycle, and once infected, this status persists in each subsequent developmental stage via trans-stadial transmission, thereby providing a stable living environment for the pathogens they carry [25]. Infected ticks can transmit these harbored viruses to mammalian hosts, thereby increasing the hazards of tick-borne pathogens. Accordingly, ticks are efficient carriers of pathogens and excellent hosts [2]. In this study, we aimed to analyze the diversity of the viruses, including arboviruses, vectored by two species of tick collected from mammalian hosts in four geographical locations within Hainan Province. In our analysis, we identified reads that exhibited sequence similarity to 12 viral families and unclassified viruses including *Flaviviridae*, *Chuviridae*, *Rhabdoviridae*, *Retroviridae*, *Phenuiviridae*, *Nairoviridae*, *Orthomyxoviridae*, *Totiviridae*, *Papillomaviridae*, *Reoviridae*, *Luteoviridae*, and *Poxviridae*. Our findings indicate that ticks from these subtropical areas in China harbor many viruses. Consistent with previously reported observations, our findings also tend to indicate that most of the identified viruses are widely distributed among the tick species and are not characterized by any distinct geographical distribution, as many of the identified tick-borne viruses we isolated from subtropical areas of China have also been isolated from northern and western China, as well as other regions worldwide, including America, Turkey, Kenya, Brazil, the Mediterranean Basin, India, and Saudi Arabia [2]. These include the Colorado tick fever virus, Betapapillomavirus, Cowpox virus, Bole tick virus 1, rhabdo-like virus, tick *Phlebovirus*, Lihan uukuvirus, Manly virus, and JMTV. Notably, the BJNV contigs obtained in the present study had 99−100% nucleotide identity with the Russian Gakugsa tick virus, thereby highlighting that these viruses and their hosts are distributed over a wide geographical range.

In recent years, many viruses have been detected in ticks, which has enhanced our understanding of the biodiversity and evolution of tick-borne viruses (TBVs). In China, the northern provinces have long been plagued by TBVs, including TBEV and CCHF virus (CCHFV), with fatal cases reported during several epidemics and sporadic outbreaks characterized by high mortality rates [26,27]. From 2010 to 2016, SFTSV was reported to have caused more than 10,000 cases of SFTS in 23 Chinese provinces. Unfortunately, owing to a lack of targeted drugs and vaccines, SFTS is associated with high early mortality in humans [28]. Moreover, in 2018, China suffered a serious livestock disaster in which the African swine fever virus infected more than 5000 pigs and was transmitted between pigs and wild boars via soft ticks [29].

Furthermore, Alongshan virus (ALSV), Songling virus, and BJNV have been identified in blood specimens of patients with unexplained fever in the General Forestry Hospital of Inner Mongolia, China, between 2017 and 2018 [11,23,30]. ALSV and JMTV are the main members of the JMV group, with hosts of the latter being widely distributed in China, Brazil, Laos, Guinea, Kosovo, Uganda, and other regions [10,20,21,31]. JMTV was first detected in a non-human primate in 2016 when Ladner et al. identified a strain of JMTV (RC27) in a serum sample collected from red colobus monkeys in Uganda [19], thereby suggesting that JMTV has the potential to infect humans. A conjecture was subsequently confirmed in 2018 when a JMTV genome sequence was detected in the sera of patients with CCHF. In the present study, we identified JMTV in *R. sanguineus* ticks collected from Danzhou and Lingao and BJNV in the same tick species collected from Tunchang, thereby indicating that JMTV-HMU and BJNV infections of humans and other mammals transmitted by tick bites are distinct likelihood in Hainan Province.

In addition, we detected a variant (BDTPV1-HMU) of the prototype virus BDTPV1 *Phlebovirus* and identified the nearly complete L segments, although only two short contigs of the S segments, of BDTPV1-HMU. In this regard, the findings of a previous study have indicated that BDTPV1, YN-PhelobV1, YN-PhelobV3, and YN-PLV2 are distinct from other bunyaviruses in that they lack a glycoprotein-encoding the M segment [3] This phenomenon has also been identified in *Phlebovirus*-like viruses isolated from ticks in Europe, South America, North America, and Asia [13,32,33]. However, given the diversity of glycoproteins encoded by the M segment, the identification of homologous proteins of this novel virus based on homologous sequence alignment is particularly challenging. Furthermore, we cannot rule out the possibility of bi-segment viruses similar to *Phlebovirus*, as there is evidence of a fully viable two-segment Rift Valley Fever Virus variant that lacks the M segment [34,35].

In 2011, QRFV and JAV were recognized by the International Committee on Taxonomy of Viruses as species belonging to the genus *Quaranjavirus*. Lake Chad virus, Zambezi tick virus, Granville *Quaranjavirus*, and many quaranja-like viruses were tentatively assigned to the unclassified quaranjaviruses. The two newly discovered contigs in this study had a relatively high amino acid identity with the hemagglutinin of the Granville *Quaranjavirus,* and Zambezi tick virus 1. However, we could not determine whether the identified sequences belong to QRFV or unclassified viruses, and consequently, further investigations are warranted. QRFV was originally isolated from two children with self-limiting viral diseases in the Quaranfil area of Egypt. Serological studies in the 1960s revealed that up to 8% of residents were exposed to QRFV [24,36,37]. However, the prevalence and human health implications of QRFV have yet to be sufficiently established.

Nevertheless, identifying QRFV, JAV, and unclassified quaranjaviruses from humans, ticks, and birds in Africa, Central Asia, and other regions confirms that these viruses are distributed over a wide geographical range, and consequently, a large proportion of the human population may be exposed to this group of viruses, including those inhabiting subtropical areas in China. Tick-borne arboviruses are important prevalent infectious agents. Therefore, comprehensive surveillance and characterization of these viruses are crucial to monitor their potential as emerging pathogens concerning their ability to survive, replicate, transmit, and infect tick and human cells. The potentially severe health of infection by these viruses highlights the urgent need for a comprehensive investigation of any potential clinical illness caused by these viruses, which is important as further research is required to determine their potential as emerging pathogens.

4. Conclusions

Our observations on the tick virome in a subtropical area of China provide further insights into the distribution and evolution of tick-borne viruses. Future studies on viruses and their hosts in China may reveal a greater diversity of viral lineages. In addition, identifying tick-borne viruses can contribute to controlling and preventing potential epidemics caused by these viruses.

Data availability

Sequencing data have been deposited in the National Center for Biotechnology Information sequence reads archive under the accession number PRJNA793118. The genome sequences of PicoV-HMU-1 are available at GenBank (https://www.ncbi.nlm.nih.gov/genbank/), with accession number MW883077. The genome sequences of JMTV-HMU, which included segments 1, 2, 3, and 4, are available at GenBank, with accession numbers OM102288, OM102289, OM102290, and OM102291. The genome sequences of BDTPV1-HMU are available at GenBank, with accession number OM102287.

Ethics statement

Animals were treated following the guidelines of the Regulations for the Administration of Laboratory Animals (Decree No. 2 of the State Science and Technology Commission of The People's Republic of China, 1988). The sampling procedure was approved by the Ethics Committee of Hainan Medical University.

Declaration of competing interest

None.

Acknowledgments

This work was supported by the National Key R&D Program of China (Grant No. 2022YFE0210300), the National Natural Science Foundation of China (32060015, 82060378, 81860367, U22A20363), the Key Research and Development Plan of Hainan Province (ZDYF2022SHFZ085), the CAMS Innovation Fund for Medical Sciences (Grant No. 2021-I2M-1-039), the Hainan Provincial Natural Science Foundation of China (820QN267, 822MS076, 820RC628, 821RC1070, 821QN258, 821QN262, 821QN0896, 822RC695), the Research project of Hainan academician innovation platform (YSPTZX202004), the Hainan talent development project (SRC200003), College Cultivation Fund of Hainan Medical University (HYPY201919).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2023.105218.

References

[1] Kazimírová M, Thangamani S, Bartíková P, Hermance M, Holíková V, Štibrániová I, et al. Tick-borne viruses and biological processes at the tick-host–virus interface. Front Cell Infect Microbiol 2017;7:339. https://doi.org/10.3389/fcimb.2017.00339.

[2] Mansfield KL, Jizhou L, Phipps LP, Johnson N. Emerging tick-borne viruses in the twenty-first century. Front Cell Infect Microbiol 2017;7:298. https://doi.org/10.3389/fcimb.2017.00298.

[3] Shi J, Shen S, Wu H, Zhang Y, Deng F. Metagenomic profiling of viruses associated with Rhipicephalus microplus ticks in Yunnan province, China. Virol Sin 2021;36:623–35. https://doi.org/10.1007/s12250-020-00319-x.

[4] Labuda M, Nuttall PA. Tick-borne viruses. Parasitology 2004;129(Suppl): S221–45. https://doi.org/10.1017/s0031182004005220.

[5] Pulkkinen LIA, Butcher SJ, Anastasina M. Tick-borne encephalitis virus: a structural view. Viruses 2018;10:350. https://doi.org/10.3390/v10070350.

[6] Bente DA, Forrester NL, Watts DM, McAuley AJ, Whitehouse CA, Bray M. Crimean-Congo hemorrhagic fever: history, epidemiology, pathogenesis, clinical syndrome and genetic diversity. Antivir Res 2013;100:159–89. https://doi.org/10.1016/j.antiviral.2013.07.006.

[7] Liu Q, He B, Huang SY, Wei F, Zhu XQ. Severe fever with thrombocytopenia syndrome, an emerging tick-borne zoonosis. Lancet Infect Dis 2014;14: 763–72. https://doi.org/10.1016/S1473-3099(14)70718-2.

[8] Maruyama SR, Castro-Jorge LA, Ribeiro JM, Gardinassi LG, Garcia GR, Brandão LG, et al. Characterisation of divergent Flavivirus NS3 and NS5 protein sequences detected in Rhipicephalus microplus ticks from Brazil. Mem Inst Oswaldo Cruz 2014;109:38–50. https://doi.org/10.1590/0074-02760130166.

[9] Jia N, Liu HB, Ni XB, Bell-Sakyi L, Zheng YC, Song JL, et al. Emergence of human infection with Jingmen tick virus in China: a retrospective study. EBioMedicine 2019;43:317–24. https://doi.org/10.1016/j.ebiom.2019.04.004.

[10] Pascoal JO, Siqueira SM, Maia RDC, Juan Szabó MP, Yokosawa J. Detection and molecular characterization of Mogiana tick virus (MGTV) in Rhipicephalus microplus collected from cattle in a savannah area, Uberlandia, Brazil. Ticks Tick Borne Dis 2019;10:162–5. https://doi.org/10.1016/j.ttbdis.2018.10.002.

[11] Wang ZD, Wang B, Wei F, Han SZ, Zhang L, Yang ZT, et al. A new segmented virus associated with human febrile illness in China. N Engl J Med 2019;380: 2116–25. https://doi.org/10.1056/NEJMoa1805068.

[12] Weaver SC, Barrett AD. Transmission cycles, host range, evolution and emergence of arboviral disease. Nat Rev Microbiol 2004;2:789–801. https://doi.org/10.1038/nrmicro1006.

[13] Li CX, Shi M, Tian JH, Lin XD, Kang YJ, Chen LJ, et al. Unprecedented genomic diversity of RNA viruses in arthropods reveals the ancestry of negative-sense RNA viruses. Elife 2015;4. https://doi.org/10.7554/eLife.05378.

[14] Yang J, Yang F, Ren L, Xiong Z, Wu Z, Dong J, et al. Unbiased parallel detection of viral pathogens in clinical samples by use of a metagenomic approach. J Clin Microbiol 2011;49:3463–9. https://doi.org/10.1128/JCM.00273-11.

[15] Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 2013;30:2725–9. https://doi.org/10.1093/molbev/mst197.

[16] Li P, Piao Y, Shon HS, Ryu KH. Comparing the normalization methods for the differential analysis of Illumina high-throughput RNA-Seq data. BMC Bioinf 2015;16:347. https://doi.org/10.1186/s12859-015-0778-7.

[17] Ginestet C. Ggplot2: elegant graphics for data analysis. J Roy Stat Soc A 2011;174:245–6. https://doi.org/10.1111/j.1467-985X.2010.00676_9.x.

[18] Simmonds P, Becher P, Bukh J, Gould EA, Meyers G, Monath T, et al., Ictv report consortium. ICTV virus taxonomy profile: Flaviviridae. J Gen Virol 2017;98: 2–3. https://doi.org/10.1099/jgv.0.000672.

[19] Ladner JT, Wiley MR, Beitzel B, Auguste AJ, Dupuis AP, Lindquist ME, et al. A multicomponent animal virus isolated from mosquitoes. Cell Host Microbe 2016;20:357–67. https://doi.org/10.1016/j.chom.2016.07.011.
[20] Sameroff S, Tokarz R, Charles RA, Jain K, Oleynik A, Che X, et al. Viral diversity of tick species parasitizing cattle and dogs in Trinidad and Tobago. Sci Rep 2019;9:10421. https://doi.org/10.1038/s41598-019-46914-1.
[21] Emanet N, Kar S, Dinçer E, Brinkmann A, Hacıoğlu S, Farzani TA, et al. Novel tick Phlebovirus genotypes lacking evidence for vertebrate infections in Anatolia and Thrace, Turkey. Viruses 2019;11. https://doi.org/10.3390/v11080703.
[22] Walker PJ, Widen SG, Firth C, Blasdell KR, Wood TG, Travassos da Rosa AP, et al. Genomic characterization of Yogue, kasokero, issyk-kul, keterah, gossas, and thiafora viruses: nairoviruses naturally infecting bats, shrews, and ticks. Am J Trop Med Hyg 2015;93:1041–51. https://doi.org/10.4269/ajtmh.15-0344.
[23] Wang YC, Wei Z, Lv X, Han S, Wang Z, Fan C, et al. A new nairo-like virus associated with human febrile illness in China. Emerg Microb Infect 2021;10:1200–8. https://doi.org/10.1080/22221751.2021.1936197.
[24] Presti RM, Zhao G, Beatty WL, Mihindukulasuriya KA, da Rosa AP, Popov VL, et al. Quaranfil, Johnston Atoll, and Lake Chad viruses are novel members of the family Orthomyxoviridae. J Virol 2009;83:11599–606. https://doi.org/10.1128/JVI.00677-09.
[25] Karbowiak G, Biernat B, Werszko J, Rychlik L. The transradial persistence of tick-borne encephalitis virus in Dermacentor reticulatus ticks in natural conditions. Acta Parasitol 2016;61:201–3. https://doi.org/10.1515/ap-2016-0028.
[26] Zhang Y, By Si, Liu BH, Chang GH, Yang YH, Huo QB, et al. Complete genomic characterization of two tick-borne encephalitis viruses isolated from China. Virus Res 2012;167:310–3. https://doi.org/10.1016/j.virusres.2012.05.015.
[27] Moming A, Yue X, Shen S, Chang C, Wang C, Luo T, et al. Prevalence and phylogenetic analysis of crimean-Congo hemorrhagic fever virus in ticks from different ecosystems in Xinjiang, China. Virol Sin 2018;33:67–73. https://doi.org/10.1007/s12250-018-0016-3.
[28] Zhang Y, Shen S, Shi J, Su Z, Li M, Zhang W, et al. Isolation, characterization, and phylogenic analysis of three new severe fever with thrombocytopenia syndrome bunyavirus strains derived from Hubei Province, China. Virol Sin 2017;32:89–96. https://doi.org/10.1007/s12250-017-3953-3.
[29] Wang T, Sun Y, Qiu HJ. African swine fever: an unprecedented disaster and challenge to China. Infect Dis Poverty 2018;7:111. https://doi.org/10.1186/s40249-018-0495-3.
[30] Ma J, Lv XL, Zhang X, Han SZ, Wang ZD, Li L, et al. Identification of a new orthonairovirus associated with human febrile illness in China. Nat Med 2021;27:434–9. https://doi.org/10.1038/s41591-020-01228-y.
[31] Qin XC, Shi M, Tian JH, Lin XD, Gao DY, He JR, et al. A tick-borne segmented RNA virus contains genome segments derived from unsegmented viral ancestors. Proc Natl Acad Sci U S A 2014;111:6744–9. https://doi.org/10.1073/pnas.1324194111.
[32] Pettersson JH, Shi M, Bohlin J, Eldholm V, Brynildsrud OB, Paulsen KM, et al. Characterizing the virome of Ixodes ricinus ticks from northern Europe. Sci Rep 2017;7:10870. https://doi.org/10.1038/s41598-017-11439-y.
[33] Meng F, Ding M, Tan Z, Zhao Z, Xu L, Wu J, et al. Virome analysis of tick-borne viruses in Heilongjiang Province, China. Ticks Tick Borne Dis 2019;10:412–20. https://doi.org/10.1016/j.ttbdis.2018.12.002.
[34] Kortekaas J, Oreshkova N, Cobos-Jiménez V, Vloet RP, Potgieter CA, Moormann RJ. Creation of a nonspreading Rift Valley fever virus. J Virol 2011;85:12622–30. https://doi.org/10.1128/JVI.00841-11.
[35] Wichgers Schreur PJ, Kortekaas J. Single-molecule FISH reveals non-selective packaging of rift Valley fever virus genome segments. PLoS Pathog 2016;12:e1005800. https://doi.org/10.1371/journal.ppat.1005800.
[36] Taylor RM, Hurlbut HS, Work TH, Kingston JR, Hoogstraal H. Arboviruses isolated from argas ticks in Egypt: Quaranfil, chenuda, and nyamanini. Am J Trop Med Hyg 1966;15:76–86. https://doi.org/10.4269/ajtmh.1966.15.76.
[37] Cholleti H, Hayer J, Mulandane FC, Falk K, Fafetine J, Berg M, et al. Viral metagenomics reveals the presence of highly divergent Quaranjavirus in Rhipicephalus ticks from Mozambique. Infect Ecol Epidemiol 2018;8:1478585. https://doi.org/10.1080/20008686.2018.1478585.