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Geographical distribution and phylogenetic analysis of Jingmen tick virus in China

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SUMMARY

Jingmen tick virus (JMTV) is a novel tick-borne segmented RNA virus that is closely related to un-segmental RNA virus in evolution. It has been confirmed that JMTV could be a causative agent of human disease. In this study, a total of 3658 ticks were sampled from 7 provinces of China and then divided into 545 pools according to the location and species. QRT-PCR and nested PCR were performed to confirm the presence of JMTV. The results showed JMTV was identified in 5 out of 7 provinces with an average infection rate of 1.4% (51/3658). Phylogenetic analysis indicated that all JMTV strains identified in this study were closely related to each other and formed a well-supported sub-lineage. Our results provide molecular evidence of JMTV in different species of ticks from endemic and non-endemic regions and demonstrate that JMTV, as a natural foci pathogen, may be widely distributed all over China.

INTRODUCTION

Jingmen tick virus (JMTV) is a recently identified segmented positive-sense ssRNA virus, which has a close evolutionary link with un-segmented Flaviviridae viruses (Qin et al., 2014). The viral genome consists of four linear RNA segments, denoted segments S1, S2, S3, and S4, respectively. S1 and S3 encode two nonstructural proteins (NSP1 and NSP2) and are genetically related to the NS5 and NS3 sequences of the genus *Flavivirus*, while S2 and S4 encode three structural proteins (VP1, C and VP3) and share no known homology. JMTV is the first segmented RNA virus that originated from an un-segmented virus (Vandegrift and Kapoor, 2019), which expands our knowledge about the variety of genome recombination and evolution of positive-strand RNA virus.

Recently, a growing number of novel tick-borne viruses (TBVs) have been identified around the world, such as severe fever with thrombocytopenia syndrome virus (SFTSV) and Dabieshan tick virus (DTV) in China (Shao et al., 2020; Yu et al., 2011), South Bay Virus and Bourbon virus (BRBV) in the USA (Savage et al., 2017; Tokarz et al., 2014), Timbillica virus and Hunter island group virus (HIGV) in Australia (Gauci et al., 2015; Harvey et al., 2019), Muko virus (MUV) and Kabuto Mountain virus in Japan (Ejiri et al., 2015, 2018), Dugbe virus in Ghana (Kobayashi et al., 2017), and so on. Many of these viruses can bring a series of symptoms to humans and animals, ranging from mild to severe and even death. As a novel TBV, JMTV is classified as a new member of Flavivirus genus, Flaviviridae, for the reason that two segments encoding nonstructural proteins are highly homologous with the sequences of flavivirus members. JMTV is the first segmented flavivirus that has been identified and confirmed, revealing an unexpected connection between segmented and un-segmented RNA viruses. Subsequently, many viruses with a similar genomic structure to JMTV, which are referred to as JMTV-like viruses or Jingmenvirus group, have been identified in arthropods both inside and outside of China. For instance, Alongshan virus from ticks and humans in China, Russia, Finland, France, Serbia, and Germany (Kholodilov et al., 2020; Kuivanen et al., 2019; Paraskevopoulou et al., 2021; Stanojević et al., 2020; Wang et al., 2019), Wuhan flea virus and Yanggou tick virus from ticks in China and Russia (Kholodilov et al., 2021; Qin et al., 2014; Shi et al., 2016), Guaico Culex virus from mosquitoes in Central America and South America (Ladner et al., 2016), Charvil Virus from Drosophila melanogaster in the United Kingdom (Webster et al., 2015). At present, segmented flaviviruses have attracted more attention, and Jingmenvirus group has become a hot point in recent research.

As its initial identification in the Jingmen region of Hubei province in 2010 and description in 2014, JMTV has been confirmed in several other regions in China, such as Zhejiang, Guizhou, Yunnan, Guangxi, Henan, Xinjiang, and Heilongjiang province (Guo et al., 2020; Jia et al., 2019; Meng et al., 2019; Shi et al., 2021; Xu et al., 2020; Yu et al., 2020). Subsequently, JMTV was reported around the world, including Kenya, the UK, the USA in 2015, Uganda in 2016, Kosovo and Brazil in 2018, Trinidad and Tobago, Turkey, Laos, and French ¹School of Public Health, Weifang Medical University, Weifang 261053, China

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Table 1. Tic	k species and	I number collected fro	m seven provinces	of China				
Collection site	Origin	No. of individuals (adults/nymphs/ larvae)	Haemaphysalis longicornis (adults/ nymphs/ larvae)	Rhipicephalus microplus (adults/ nymphs/ larvae)	lxodes sinensis (adults/ nymphs/ larvae)	Hyalomma asiaticum (adults/ nymphs/ larvae)	lxodes ovatus (adults/ nymphs/ larvae)	Haemaphysalis campanulata (adults/ nymphs/ larvae)
Henan	Dog	212/0/0	61/0/0	151/0/0	/	/	1	/
	Grassland	412/31/0	342/22/0	70/9/0	/	/	/	/
Guizhou	Cattle	161/0/0	78/0/0	1	83/0/0	/	/	/
	Grassland	337/13/0	166/8/0	51/0/0	120/5/0	/	/	1
Yunnan	Cattle	123/0/0	28/0/0	40/0/0	55/0/0	/	/	/
	Grassland	306/16/24	98/6/8	121/10/16	87/0/0	/	/	/
Xizang	Sheep	52/0/0	1	52/0/0	/	/	/	/
	Grassland	135/20/5	/	50/20/5	/	/	85/0/0	/
Xinjiang	Dog	50/0/0	1	1	/	50/0/0	/	/
Anhui	Dog	63/0/0	43/0/0	/	20/0/0	/	/	/
Shandong	Sheep	141/0/0	99/0/0	42/0/0	/	/	/	/
	Grassland	1175/150/232	973/102/212	145/48/20	/	/	/	57/0/0
Total		3167/230/261	1888/138/220	722/87/41	365/5/0	50/0/0	85/0/0	57/0/0

Antilles in 2019 (Dincer et al., 2019; Temmam et al., 2019; Zhang et al., 2020). Unlike most classical members of *Flavivirus*, which only utilize a certain arthropod as a transmitting vector, JMTV has been detected in many species of ticks, and other insects, such as *Armigeres* sp. and *D. melanogaster* (Webster et al., 2015), indicating that this virus may use a variety of arthropods as a transmission vector. In addition, it has been reported that JMTV could infect a broad range of mammals, including cattle, rodents, bats, and humans (Guo et al., 2020; Jia et al., 2019). Moreover, JMTV has been proved to be stable during passage in the tick embryo-derived cell line BME/CTVM23 (Jia et al., 2019). Most importantly, JMTV has been demonstrated to be associated with human febrile diseases in China and Kosovo, and is becoming a potential threat to public health and safety (Emmerich et al., 2018; Jia et al., 2019). Hence, it is of great significance to strengthen the epidemiology, biodiversity, ecology, and pathogenicity research of JMTV for a better understanding of this novel virus and to deal more effectively with a related disease.

Herein, we intend to gain more knowledge about the geographical distribution and diversity of JMTV by performing a molecular epidemiology survey of JMTV in ticks sampled from seven provinces of China (Guizhou, Yunnan, Henan, Shandong, Anhui, Xinjiang, and Xizang) to investigate the prevalence and the phylogenetic relationships of different strains.

RESULTS

Tick collection

From July to August 2018 and 2019, 802 ticks of the species *Haemaphysalis longicornis* (309, 38.5%), *Rhipicephalus microplus* (285, 35.5%), *Ixodes sinensis* (158, 19.7%), and *Hyalomma asiaticum* (50, 6.3%) were collected from domestic animals (dog, cattle and sheep, Table 1). Meanwhile, 2856 ticks were collected by dragging a cloth through vegetation, including *H. longicornis* (1937, 67.8%, 1579 adults, 138 nymphs, and 220 larvae), *R. microplus* (565, 19.8%, 437 adults, 87 nymphs, and 41 larvae), *I. sinensis* (212, 7.4%, 207 adults, 5 nymphs, and 0 larvae), *Ixodes ovatus* (85, 3%, 85 adults, 0 nymphs, and 0 larvae) and *Haemaphysalis campanulata* (57, 2%, 57 adults, 0 nymphs, and 0 larvae). Geographically, 655 ticks in Henan province, 511 ticks in Guizhou province, 469 ticks in Yunnan province, 212 ticks in Xizang province, 50 ticks in Xinjiang province, 63 ticks in Anhui province, and 1698 ticks Shandong province were sampled (Table 1, Figure 1). *H. longicornis* was the dominant species (61.4%) and was distributed across most sampling sites. *H. asiaticum* (1.4%), *I. ovatus* (2.3%), and *H. campanulata* (2%) were only found in Xinjiang, Xizang, and Shandong province, respectively.



Table 2. Positive rates for JMTV in ticks collected from seven provinces of China							
Collection site	No. of individuals	No. of pools	qRT-PCR positive	Nested-PCR positive	MIR		
Henan	655	105	9	3	1.4%		
Guizhou	511	82	12	5	2.4%		
Yunnan	469	75	8	3	1.7%		
Xizang	212	35	0	0	0		
Xinjiang	50	16	0	0	0		
Anhui	63	22	2	1	3.2%		
Shandong	1698	210	12	3	0.7%		
Total	3658	545	51	15	1.4%		

In total, 3658 ticks from six species were collected in this study. The ticks were identified and grouped into 545 pools by site, species, host, and life stage. The nucleotide sequence of an 887-bp fragment of the COI region was amplified and determined for all pools.

Detection of Jingmen tick virus RNA in ticks

RT-PCR-based screening of all samples showed that JMTV was distributed in 5 of the 7 provinces included in this study. Of the six tick species examined, only *H. longicornis, R. microplus,* and *I. sinensis* adults tested positive for JMTV. However, JMTV was not detected in *H. asiaticum, I. ovatus,* and *H. campanulata* ticks. JMTV were detected with a minimum infection rate (MIR) of 1.4% in this study (Table 2). Geographically, the MIR of JMTV in ticks originating from the five provinces ranged from 0.7% (12/1698) to 3.2% (2/63). No significant difference was found in the prevalence of JMTV among ticks collected from the five positive provinces except Guizhou province.

JMTV was initially isolated using invertebrate cell lines C6/36 and mammalian cell lines DH82; however, virus replication in these two cell lines is limited, and the viral genome can only be detected in the first two passages (Dincer et al., 2019; Qin et al., 2014). Similarly, these cells were not proved to be consistently suitable for isolation in our study. There are reports already that the embryo-derived tick cell line BME/CTVM23 and Vero E6 cells were successful in supporting JMTV replication. Subsequently, Vero E6 cells were used in this study. Unfortunately, no JMTV strain was isolated from any positive tick samples. The failure may have resulted from a few copies of live virus in the sample.

Detection of Jingmen tick virus RNA in human specimens

109 blood specimens of febrile patients from Laizhou and Weifang city were screened for JMTV infection. Among them, 70 samples have been diagnosed with severe fever with thrombocytopenia syndrome (SFTS) in the laboratory and the other 39 samples were collected from unknown fever cases with a tick bite history. Nevertheless, JMTV was negative through qRT-PCR in all human blood samples. In view of the unknown fever, we are trying to further analyze the etiology of patients by second-generation sequencing technology.

Comparative analysis of the Jingmen tick virus genomes

Direct NGS of the JMTV-positive tick pools (ZYJ-2, ZYJ-4) from Guizhou province provided 7.98 G and 9.55 G bases data with total read numbers of 26.59 and 31.84 M, respectively. The complete nucleotide sequences of the four genomic segments of the JMTV genome in these two samples (ZYJ-2, ZYJ-4) were obtained, those were, S1: 3115bp, S2: 2845bp, S3: 2824bp, S4: 2796bp. The result of individual segment alignment demonstrated maximum sequence diversity of 0.7% on nucleotide level and 100% identity on the amino acid level. The sequences have been uploaded to GenBank with the accession numbers OM_459841-OM_459848.

The result of functional analysis of the JMTV-ZJY-2 genomes was similar to that of reported isolates. Segment 1 encoded the nonstructural protein 1 (NSP1), which was an NS5-like protein with conserved domains for the SAM binding site and nucleic acid substrate binding site of a typical flavivirus. Segment 3 encoded the second nonstructural protein (NSP2) that resembled the NS2b-NS3 complex of flaviviruses









Figure 1. Map of the sampling regions

Location of seven provinces in China and the location of sampling sites, within the province where tick samples were collected, in 2018-2019. JMTV was found positive in Henan, Guizhou, Yunnan, Anhui, and Shandong provinces (red circle), and negative in Xizang and Xinjiang provinces (white circle) in this study.

with conserved domains for the ATP binding site. The non-flavivirus proteins of this virus were viral protein 1 (VP1), C, and VP3. VP1 was encoded by Segment 2 and C, VP3 was encoded by overlapping ORFs of Segment 4. The protein sequences of JMTV representative strains in 9 different regions were then collected, and further compared and analyzed for the differences in important protein binding sites in these conserved regions. The results showed that these sites were very conservative, and the related amino acids had almost no change in the sequences from all over the world (Figure 2), demonstrating that these core loci were very slow in evolution, regardless of their locations.

Phylogenetic analysis

After being aligned with reported global JMTV genomes, the JMTV sequences obtained in this study had 92-95.2% nucleotide identity with JMTV strains detected in other regions of China and Lao PDR, 90.7-93.2% with JMTV R27 from Uganda, 78-90.9% with JMTV from Brazil and Trinidad and Tobago, while relatively low nucleotide identity with Turkey and Kosovo strains (77-80.6%). In the phylogenetic trees based on four segments, all reported JMTVs were divided into two phylogenetic groups (Figure 3). The first group consisted of the strains from China, Lao PDR, Uganda, and Brazil, while the second group contained those identified in Trinidad and Tobago, French Antilles, Kosovo, Romania, and Turkey. All Chinese strains clustered with JMTV from Lao PDR and formed a separate clade, sharing a common ancestor with viruses identified in Uganda and Brazil. However, the genomes of ZYJ-2 and ZYJ-4 strains were distinct from previously characterized viruses in Turkey and Kosovo.

In this study, the partial S1 segment encoding NSP1 of JMTV (1071 bp) was successfully amplified and sequenced from 15 of 51 qRT-PCR-positive samples. Pairwise distances analysis showed they had a



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Figure 2. The functional analysis of the JMTV-ZJY-2 genome

Alignment of the important protein binding sites of conserved domains identified on viral genomic segments 1 and 3 of JMTV. Regions and residues corresponding to the conserved motifs of the virus are indicated with different frames.

93.4-100% nucleotide identity and shared more than 94% amino acid identity with each other. Phylogenetic tree was estimated using a neighbor-joining (NJ) approach based on 15 amino acid sequences (Figure 4). Similarly, all related sequences could be roughly divided into two major phylogenetic groups. Notably, except for HHY-20, the other 14 sequences from seven provinces clustered together and showed a closer evolutionary relationship with JMTV_SY84 identified in *R. microplus* from Hubei province. Meanwhile, the HHY-20 sequence from Yunnan province, which was the only one amplified from *R. microplus*, formed another sub-lineage with other JMTVs identified in Lao PDR, Uganda, and Brazil.

DISCUSSION

It was reported that JMTV was present in bats from Henan and Guizhou; however, it was further confirmed that this virus was also prevalent in ticks of these two provinces through our study (Guo et al., 2020). Meanwhile, JMTV was first identified in unrecognized endemic regions including Shandong and Anhui province, demonstrating that JMTV may be widely distributed in eastern and central China and this virus has formed a stable ecological cycle in the local area. In addition, JMTV was detected in three common ticks in China in our study including *H. longicornis, R. microplus,* and *I. sinensis,* which further verified the previous reports (Qin et al., 2014). Since discovered in 2010, epidemiological investigations about JMTV have been implemented not only in multiple Chinese regions but also in Asia and Europe. In comparison with the previous survey about JMTV in ticks from Heilongjiang and Zhejiang province of China, and French Antilles (Guo et al., 2020; Meng et al., 2019; Temmam et al., 2019), the positive rate of JMTV in ticks from these areas was more than 30%; however, the MIR was relatively low (1.4%) in this study. This may be owing to the wide sampling range and large number of samples in our study. Additionally, our results were consistent with those of a large-scale tick survey conducted in Turkey, in which the JMTV prevalence in ticks was less than 4% in two areas (Dincer et al., 2019). It seemed probable that the positive rate of JMTV obtained from large scale and detailed epidemiological investigation was generally low, which may be closer to the actual prevalence of virus in nature.

Two complete JMTV genomes from direct NGS on qRT-PCR positive tick pools were sequenced and analyzed. Genome topology and functional organization of the viruses from Guizhou province were







Figure 3. Phylogenetic analysis of the complete genomic sequences of JMTV strains ZYJ-2 and ZYJ-4

(A): segment 1, (B): segment 2, (C): segment 3, (D): segment 4. The sequences identified from ticks in the current study were marked black with a rhombus. Phylogenetic trees were constructed by the NJ method using MEGA 5.1, with 1,000 replicates with bootstrap values > 70% considered significant. GenBank accession numbers are listed for each strain/isolate/amplicon. Descriptions of the sequences published in GenBank were reproduced without changes.

identical to those from other regions, with the highest sequence identities to JMTV strains from Lao PDR and China, and the lowest to Turkey and Kosovo strains. This was also consistent with the results of phylogenetic analysis, that all four fragments of ZYJ-2 and ZJY-4 in this study clustered with the isolates found in Lao PDR and China, and exhibited remote relationships with Turkey and Kosovo strains. Comparison of the complete genomes also revealed spatial segregation of viral sub-clades, indicating local adaptive processes. In the meantime, significant similarities in amino acid sequence were noted among these viruses from different regions of China, which demonstrates that JMTV strains within a specific geographical range may originate from a common ancestor or undergo gene recombination in the same way, and were relatively conserved in evolution. Other reports have suggested that geographic isolation, rather than host species, may be the main driver of JMTV diversity (Guo et al., 2020; Meng et al., 2019). These findings indicated that JMTV distributed globally were well-adapted to local tick populations and had a relatively low mutation rate in evolution, especially in the core functional sites.

JMTV has been recently documented to cause human diseases and reveal viremia in some individuals (Jia et al., 2019). In addition, JMTV was found to be present in CCHFV-infected patients, revealing the possibility of JMTV co-infection with other arboviruses (Emmerich et al., 2018). Hence, we tested blood specimens from acute febrile patients, including SFTS confirmed patients and unknown fever cases with tick bites history, to identify







H0.02





Figure 4. Phylogenetic analysis of the partial sequences of JMTV obtained in this study with representative viruses of the Jingmen virus group in the family Flaviviridae

The phylogenetic tree was constructed based on a 415aa fragment of the nonstructural protein (NSP1). Trees were generated using an NJ method, with 1,000 replicates with bootstrap values > 70% considered significant. Black triangles indicate JMTV sequences identified in the current study.

probable JMTV infection in the Shandong province of China. However, no JMTV was detected in sera from 109 individuals. A similar observation was reported from a JMTV screening in Turkey, where the virus was identified in ticks but lacking in 103 specimens from 87 individuals with febrile disease (Dincer et al., 2019). Considering that JMTV infection in humans is not frequent in China with a sero-conversion rate of 1.6% (Jia et al., 2019), it is plausible that we did not detect JMTV from a limited number of patient samples. Therefore, it is necessary to further investigate the ecology and epidemiological of this virus. Meanwhile, the potential pathogenic of JMTV to humans or animals deserves constant attention.

Eventually, phylogenetic analysis showed that these 15 strains of JMTV in this study were closely related to the isolates found in Hubei province (Qin et al., 2014) and shared high homology with each other. The origin of this segmentation has been extensively discussed elsewhere (Ladner et al., 2016; Shi et al., 2016). Our data also suggested that this divergence of JMTV reflected the regionality of virus evolution and high adaptability to the local ecology. Moreover, the possibility of long-distance dissemination carried by flying animals cannot be ruled out. At present, the exact recombination evolution mechanism and phylogeographic origin of JMTV cannot be determined through these existing data, and this segmented flavivirus may have a complex evolutionary history.

In conclusion, our research provided important epidemiological evidence for JMTV in ticks from unrecognized endemic regions and it was estimated that this virus had a wide distribution in China. Despite the lack of detection in human specimens, the viruses originating from ticks were sequenced and the evolutionary relationship between JMTV and related viruses around the world was analyzed in this study. In short, the sero-prevalence survey of JMTV in human and animals are required to elucidate the pathogenicity and transmission cycle of JMTV in nature.

Limitations of the study

No viable virus was successfully isolated from tick samples in this study. However, this result cannot be construed as evidence of the absence of the virus, and it may owing to the extremely low copy number of live virus in these ticks or inappropriate cell lines. In addition, the small number of ticks collected in some regions may lead to sampling deviation and affect the authenticity to a certain extent.

STAR*METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105007.

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AUTHOR CONTRIBUTIONS

G.Y. N.: conceived and designed the study. Y.T. J and M.P conducted experiments and analyzed data. Y.L.Z and Z.W: collected samples and wrote Original Draft. L.L: curated, and interpreted the data. Z.P: wrote a review and supervised the article. All the authors checked and approved the final draft of the article.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR*METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	IDENTIFIER	SOURCE
Critical commercial assays		
RNeasy mini kit	Qiagen	Cat#74104
Transcriptor High Fidelity cDNA Synthesis Kit	Roche	Cat#50293100
Deposited data		
JMTV-ZYJ-1,2,3,4,5, JWJ-1,2,3, ST-2,3,4, LH-1, GY-1,2	This paper	GenBank accession# OM363684 to OM363693 OM363696- OM363700
ZYJ-2,4	This paper	GenBank accession# OM459837- OM459848
Biological samples		
Human serum samples		See Table S1: Human Blood Specimen Table.
Experimental models: Cell lines		
Monkey: Vero cells	ATCC	Cat#CBP60971
Monkey: Vero E6 cells	ATCC	Cat#CBP60972
Mouse: BHK-21 cells	ATCC	Cat#CBP60642
Mosquito: C6/36 cells	ATCC	Cat#CRL-1660
Software and algorithms		
DNASTAR software package	Lasergene	www.dnastar.com/
MEGA7.0	MEGA	https://www.megasoftware.net/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Guoyu Niu (niugy@wfmc.edu.cn).

Materials availability

This study did not generate new unique reagents. All unique/stable reagents used in this study are available from the lead contact with a completed Materials Transfer Agreement.

Data and code availability

Sequences of JMTV-ZYJ-1, 2, 3, 4, 5, JWJ-1, 2, 3, ST-2, 3, 4, LH-1, GY-1, 2 are available in GenBank under accession # OM363684-OM363693, OM363696- OM363700. The NGS data used in this study have been deposited in the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under accession OM459837- OM459848 for ZYJ-2, 4. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines

Vero (cat#CBP60971), Vero E6 (cat#CBP60972), BHK-21 (cat# CBP60642) and C6/36 cells (cat#CRL-1660) were purchased from ATCC and maintained in Dulbecco's modified Eagle medium (DMEM, Hyclone, USA) or DMEM/F12 medium. The above cell lines were used directly from the commercial sources and cultured according to manufacturer suggestions.



Patient samples

Human specimens (n = 109) were collected from Laizhou (n = 49) and Weifang city (n = 60) of Shandong province by local center for disease control and prevention (CDC) in 2019. 5 mL of blood were drawn from each person in the early symptomatic phase after obtaining informed written consent from patients. Two groups were set in this study, including individuals with laboratory diagnosis of Severe Fever with Thrombocytopenia Syndrome (SFTS) and individuals with febrile disease of unknown etiology with tick bite history(Yu et al., 2011). Serum was separated by centrifugation and frozen at -80° C until use. Nucleic acid purification and cDNA synthesis were carried out as described for ticks. All specimens were screened for JMTV through qRT-PCR, as described above. All adult subjects provided informed consent and a parent or guardian of any child participant provided informed consent on their behalf, and informed consent given was written. Information on the specimens being analyzed in this study can be found in Table S1:Human Blood Specimen Table.

METHOD DETAILS

Sample collection and nucleic acid extraction

During July to August 2018 and 2019, ticks were collected from 11 sites of 7 provinces in China. The locations and tick species are presented in Figure 1. The engorged ticks were directly picked from domestic animals using forceps and placed in perforated tubes containing a moistened piece of filter paper. After stored in a cool and ventilated place for one week, all the engorged ticks were transferred into liquid nitrogen. Meanwhile, ticks from vegetation were collected by tick drag-flag method using a 100 × 100 cm woolen cloth. All ticks were unfed and stored in liquid nitrogen directly. Ticks collected by both methods were initially identified by a trained expert in morphological field and further verified by sequencing the mitochondrial cytochrome oxidase subunit I gene (COI) of ticks. PCR primers used for COI in this study were LCO1490: GGT CAA CAA ATC ATA AAG ATA TTG G and HCO2198: TAA ACT TCA GGG TGA CCA AAA AAT CA. The cycling conditions were: 94°C for 5 min (initial denaturation) followed by 35 cycles of 94°C for 30 s (denaturation), 55°C for 1 min (annealing), 72°C for 1 min (extension), and a final extension of 72°C for 10 min. A negative control (no DNA template) was included. Successful amplification was determined by 1% agarose gel electrophoresis prior to sequencing. The engorged ticks were pooled individually while all unfed ticks were placed into pools of 5–15 individuals each by collection site, species, host, and stage of development.

After washed three times with PBS, individual or pooled tick specimen was homogenized with a mortar and pestle in 500 μ L of chilled DMEM and centrifuged at 4°C and 10,000 g for 5 min (Eppendorf, Germany). Then, the clarified supernatant of tick homogenates were prepared for RNA extraction(Qiagen, Germany), followed by cDNA synthesis using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Germany) with random hexamer primer, as described by the manufacturers.

PCR detection of JMTV in ticks

Alignments were performed with Clustal W (BioEdit v7.0.9) using complete genomic sequences of JMTV segment 1 downloaded from the GenBank database. After visual inspection, targeted genomic regions with high conservation were chosen, which located at encoding gene of NS5-like protein. Primer-probe pairs were designed and appraised by Primer Express v3.0 (Applied Biosystem, USA), and BLAST analysis was also performed to confirm the specificities of the primer-probe set. The detection of JMTV was performed by qRT-PCR targeting the NS5-like protein gene located in the genome segment 1, with primer sets JMTV-F and JMTV-R (GTT GGC GGA GGA TGA AGA GAT and AAT CTC CCT CTG GAC CAC CAT, respectively) and probe (Texas Red-CCA CCA CCT CTA CCG GCT TTA CGC AG-BHQ2). All qRT-PCR-positive samples were further verified by nested PCR using primer sets Out-F (ATA GGC TGT CCA ACA CCG TGA T), R (TGG ATC TCA TTG CCG TAC TTC AC) and In-F (CAC ACC ACT GAA CAT GAC CAG AT), R (GTA GAC CCT AGC CTC ATC TCC TCT). The PCR products were visualized by SYBR® Safe (Thermo Fisher Scientific, USA) after 1% agarose gel electrophoresis and subjected to direct Sanger sequencing by Shanghai Sangon Biotechnology Company (Shanghai, China). The partial sequences of segment 1 obtained in this study have been deposited in GenBank and assigned accession numbers (OM363684- OM363693, OM363696- OM363700).

Genome sequencing

Total DNA and RNA of the pool ZYJ-2 and ZYJ-4 were extracted with AllPrep DNA/RNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. Total RNA was used to conduct library preparation





according to a standard Illumina protocol. Briefly, rRNA was removed, and then the remaining RNA was fragmented, reverse-transcribed. After adaptors were added, RNA was purified, and tested by the Agilent 2100 Bioanalyzer. Library pair-end sequencing (150 bp) was performed on the Illumina HiSeq 2500 sequencing platform. The sequencing reads were trimmed and *de novo* assembled by the MegaHit program into contigs (>200 bp). The assembled contigs were then subjected to BLASTx with the nr database (October, 2020). The e-value cutoff was set to 1 when doing the BLASTx searches. Sanger sequencing was performed to confirm the sequences of JMTV genomes obtained from NGS. The Sanger sequencing primers were designed based on deep-sequencing results.

Virus isolation

Processed tick homogenates which were positive for JMTV RNA by qRT-PCR were inoculated onto semiconfluent monolayers of Vero, Vero E6, BHK21 and C6/36 cells. The cells were cultured in DMEM or DMEM/F12 containing 10% heat-inactivated fetal bovine serum (FBS), and maintained at 37°C in 5% CO_2 or at 27°C. Briefly, the specimens were subjected to centrifugation, and the supernatant was passed through a sterile 0.45-µm filter (Merck Millipore, USA). Then, the filtrates (100 µL) were inoculated onto cells in six-well plates. After cell adsorption for one hour, 2 mL of DMEM or DMEM/F12 supplemented with 5% fetal bovine serum, l-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin were added and the cells were incubated under the same conditions for 7–10 days. Cells were monitored daily for cytopathic effects and detected for viral RNA by qRT-PCR on day 6–8 post-infection. After another two blind passages, the supernatants were harvested and preserved at -80° C until further analysis. Virus isolation was performed at a BSL-2 laboratory in School of Public Health, WeiFang Medical University.

QUANTIFICATION AND STATISTICAL ANALYSIS

The prevalence of JMTV in ticks was presented as the minimum infection rate (MIR) and the minimum infection rate per 100 ticks was calculated by no. of positive pools/total no. of ticks assayed. The statistics analyses were calculated and performed by Prism software version 5.00. Fisher's exact test was performed to evaluate the statistical difference in the positive rate. Values of p < 0.05 were considered statistically significant. All viral sequence alignment and pairwise divergences analyses were conducted using MegAlign implemented in DNASTAR software package (Lasergene, USA). Phylogenetic analyses of JMTV-related sequences were constructed with other Flaviviridae nucleotide sequences retrieved from GenBank targeting the NS5-like gene. Phylogenetic trees were generated by MEGA7.0 software through neighbor-joining method with 1,000 replicates with bootstrap values > 70% considered significant.