

Diversity and circulation of Jingmen tick virus in ticks and mammals

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Abstract

Since its initial identification in ticks in 2010, Jingmen tick virus (JMTV) has been described in cattle, rodents, and primates. To better understand the diversity, evolution, and transmission of JMTV, we sampled 215 ticks, 104 cattle bloods, 216 bats, and 119 rodents in Wenzhou city, Zhejiang Province, China as well as 240 bats from Guizhou and Henan Provinces. JMTV was identified in 107 ticks (from two species), 54 bats (eleven species), 8 rodents (three species), and 10 cattle, with prevalence levels of 49.8, 11.8, 6.7, and 9.6 per cent, respectively, suggesting that bats may be a natural reservoir of JMTV. Phylogenetic analyses revealed that all the newly identified JMTVs were closely related to each other and to previously described viruses. Additionally, all tick and mammalian JMTV sampled in Wenzhou shared a consistent genomic structure, suggesting that the virus can cocirculate between ticks and mammals without observable variation in genome organization. All JMTVs sampled globally could be divided into two phylogenetic groups, Mantel tests suggested that geographic isolation, rather than host species, may be the main driver of JMTV diversity. However, the exact geographical origin of JMTV was difficult to determine, suggesting that this virus has a complex evolutionary history.

Key words: Jingmen tick virus; ticks; mammals; diversity; evolution; transmission.

1. Introduction

In recent decades, emerging and reemerging infectious diseases, including severe acute respiratory syndrome (SARS), avian influenza, and Zika virus disease have appeared regularly, often with substantial impacts on public health (Guan et al. 2003; Gao et al. 2013; Fauci and Morens 2016). The emergence of these infectious diseases results from the spillover or cross-species transmission of zoonotic agents from their natural reservoir hosts to humans (Wolfe et al. 2007; Lloyd-Smith et al. 2009). This process is highlighted by the ongoing pandemic of pneumonia (COVID-2019), caused by a novel coronavirus (SARS-CoV-2), that is posing a major threat to global public health (WHO, 2020; Wu et al. 2020; Zhou et al. 2020). Although greater efforts are needed to determine its natural reservoir hosts, it is believed that SARS-CoV-2 likely arose in bats and then either passed directly, or via mammalian intermediate hosts, to humans (Lam et al. 2020; Wu et al. 2020; Zhang and Holmes 2020).

The evolutionary history of RNA viruses is characterized by a complex combination of long-term virus–host co-divergence and frequent host switching (Kitchen et al. 2011; Li et al. 2015; Shi et al. 2016a). For example, both patterns are apparent in viruses of the order *Bunyavirales*. Although these viruses have a broad spectrum of hosts, including different phyla and even different kingdoms (Animalia and Plantae), there are clear host boundaries at either the virus genus or family levels (Guo et al. 2013; Li et al. 2015; Shi et al. 2016a, 2018). Notably, at the species level, while some viruses (e.g., Seoul virus) appear to infect multiple closely related rodent taxa, no viruses have been found to circulate in both invertebrates and vertebrates or both arthropods and plants. In contrast, viruses of the family *Flaviviridae* exhibit more complex virus–host relationships (Gaunt et al. 2001; Kitchen et al. 2011; Blitvich and Firth 2015). For example, some viruses (such as dengue virus and yellow fever virus) are transmitted among mammals by arthropod vectors, whereas others (like hepatitis C virus) have no known vector cycle. As the emergence of novel infectious diseases in humans are often due to the spillover or cross-species transmission of zoonotic agents from their natural reservoir hosts, a better understanding of the association between viruses and their hosts may help prevent future emergence events, especially for those viruses that may have pathogenic potential in humans.

Jingmen tick virus (JMTV) was first identified in *Rhipicephalus microplus* ticks sampled from Jingmen city in Hubei Province and Wenzhou city of Zhejiang Province, China, in 2010 (Qin et al. 2014). The viral genome comprises four separate segments—denoted S1–S4—of single-stranded positive sense RNA. These segments encode five proteins: the flavivirus NS5-like protein (NSP1), the nonstructural protein resembling the NS2b–NS3 complex of flaviviruses (NSP2), the envelope protein (VP1), the capsid protein (VP2), and the membrane protein (VP3). Although the S1 and S3 segments are closely related to the nonstructural protein genes (NS3 and NS5) of flaviviruses, the remaining two segments (S2 and S4) are unique to this virus and have no known homologs (Qin et al. 2014).

Since its initial identification, JMTV has been identified in several regions of China (Jia et al. 2019; Yu et al. 2020) as well as in Brazil, Kosovo, Uganda, Turkey, French Antilles, and Trinidad and Tobago (Ladner et al. 2016; Emmerich et al. 2018; Souza et al. 2018; Dincer et al. 2019; Pascoal et al. 2019; Sameroff et al. 2019; Temmam et al. 2019). In addition to ticks and mosquitoes, JMTV has been identified in cattle, rodents, and primates (Qin et al. 2014; Ladner et al. 2016; Emmerich et al. 2018; Souza et al.

2018; Jia et al. 2019; Yu et al. 2020), suggesting that it may infect a broad range of animals. Recently, it was suggested that JMTV was associated with febrile disease in China and Kosovo (Emmerich et al. 2018; Jia et al. 2019), suggesting a possible role in public health. It is therefore of clear importance to better understand the diversity and transmission of JMTV in nature.

Several viruses with similar genome structures to JMTVs have been identified in arthropods both inside and outside of China (Ladner et al. 2016; Shi et al. 2016b). However, these viruses were phylogenetically distinct from JMTVs, exhibiting >20 per cent amino acid (aa) difference and falling as a well-supported clade in flavivirus phylogenies (Ladner et al. 2016; Shi et al. 2016b). These included Alongshan virus, identified in ticks and humans from China, Russia, and Finland (Kuivanen et al. 2019; Wang et al. 2019; Kholodilov et al. 2020), Yanggou tick virus identified in ticks from China (MH688538.1), and JMTV/*Pteropus lylei*/Cambodia found in the urine of bats from Cambodia (Temmam et al. 2019). Due to the large genetic differences between these viruses and JMTV, they are referred to 'JMTV-like viruses' in the current study and the 'Jingmenvirus group' elsewhere (Shi et al. 2016b).

Herein, we tested bats, cattle, rodents, and ticks collected in three Chinese Provinces (Zhejiang, Guizhou, and Henan) for the presence of JMTV. Using the viral genome sequences recovered, we explored the diversity, evolution, and transmission of JMTV in these animals.

2. Materials and methods

2.1 Specimen collection

This study was approved by the ethics committee of the National Institute for Communicable Disease Control and Prevention of the Chinese Center for Disease Control and Prevention (CDC). Under the protocols authorized by the National Institute for Communicable Disease Control and Prevention, all animals were treated strictly according to the Guidelines for Laboratory Animal Use and Care of the Chinese CDC and the Rules for the Implementation of Laboratory Animal Medicine (1998) from the Ministry of Health, China.

During 2012–6, numerous samples, including ticks, bats, rodents, and cattle blood, were collected in Guizhou, Henan, and Zhejiang Provinces, China (Fig. 1). Ticks, some of which had evidence of blood meals, were directly collected from dogs, goats, and cattle in Wenzhou city, Zhejiang Province in 2012. Cattle blood samples were obtained from local farms or slaughterhouses before butchery in Wenzhou city in 2014. Bats were captured with mist nets or harp traps in caves of nature roosts in Wenzhou city in 2016 or in Anlong country (Guizhou Province) and Neixiang country (Henan Province) in 2012. Rodents were trapped using baited cages in Wenzhou city in 2016. All animals were kept alive after capture. Species were initially identified by morphological examination, which was further confirmed by sequencing the mitochondrial cytochrome oxidase subunit I gene of ticks and the mt-cyt b gene of bats, rodents, and cattle (Folmer et al. 1994; Guo et al. 2013). To minimize suffering, bat and rodent liver and lung tissue samples were collected after anesthetizing. All samples were stored at -80°C until DNA and RNA were extracted as described below.



Figure 1. A map of the world showing the location of the trap sites (red solid circle) in which ticks, bats, rodents, and cattle blood were sampled in China in this study, as well as the location of known JMTVs and their associated hosts (red hollow circle).

2.2 Sample processing, DNA and RNA extraction, polymerase chain reaction, and sequencing

Individual ticks were homogenized with a mortar and pestle on ice in 0.5 ml phosphate-buffered saline (PBS) solution after washed three times with PBS. Total DNA and RNA was then extracted from suspension using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) and TRIZOL LS Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total DNA and RNA were extracted from liver and lung samples from bat and rodent using DNA or RNA isolation kits (Omega bio-tek, USA). The Nucleo Spin RNA Blood (MN, Düren, Germany) and QIAamp DNA Mini Kits (Qiagen GmbH, Hilden, Germany) were used to extract RNA and DNA from cattle blood, respectively.

The JMTV and JMTV-like viruses were detected by nested polymerase chain reaction (PCR) targeting the conserved regions of the known JMTV and other members. To obtain complete virus genome sequences, primers were designed based on the known JMTV sequence as described previously (Qin et al. 2014; Shi et al. 2016b; Yu et al. 2020). The termini of the four segments of JMTVs were obtained by using a RACE kit (TaKaRa, Dalian, China).

PCR amplicons were separated by agarose gel and purified using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Amplicons <700 bp in length were subjected to direct Sanger sequencing, whereas those >700 bp were cloned into pMD18-T vector (Takara, Dalian, China) and then transformed into JM109-143 competent cells. Finally, at least three positive clones were chosen for genome sequencing.

2.3 Sequence analysis

The SOSUI (http://harrier.nagahama-i-bio.ac.jp/sosui/sosui_submit.html) (Hirokawa et al. 1998) and SignalP v5.0 (<http://www.cbs.dtu.dk/services/SignalP/>) (Almagro Armenteros et al. 2019) packages were used to determine the location of transmembrane domains and signal peptide, respectively, as described previously (Qin et al. 2014; Shi et al. 2016b).

2.4 Phylogenetic analyses

All the viral sequences were aligned using the L-INS-i algorithm implemented in MAFFT version 7 (Katoh and Standley 2013). Phylogenetic trees of these data were then estimated using the maximum likelihood (ML) method implemented in PhyML version 3.0 (Guindon et al. 2010) with bootstrap support values calculated from 1,000 replicate trees. The best-fit model of nucleotide (nt) substitution was identified using MEGA version 7.0 (Kumar et al. 2016). Sequences similarity measures at nt and aa levels were calculated by MegAlign program implemented in the Lasergene software package version 5.0 (DNASTar).

2.5 Correlations of viral genetic, geographic, and host genetic distance matrices

To determine the relationship between viral genetic, geographic, and host genetic distance, we compiled a data set containing the JMTVs recovered in this study. We then used the Patristic v1.0 program to produce genetic distance matrices from pairwise comparisons, either in the form of uncorrected percentage differences or calculated from the phylogenetic trees (patristic distance) (Fourment and Gibbs 2006). Geographic distances (Euclidean distance) were calculated using the following formula: $\text{distance} = (\text{acos}((\sin(\text{latitude}1) \times \sin(\text{latitude}2)) + (\cos(\text{latitude}1) \times \cos(\text{latitude}2) \times \cos(\text{longitude}2 - \text{longitude}1)))) \times 6,371$, with the spatial coordinates of the samples derived from the geographic location information. Following this, we used Mantel correlation analyses to measure the association between these matrices as described previously (Mantel 1967; Tao et al. 2017). Both simple and partial Mantel's tests were performed, with the correlation evaluated using 10,000 permutations. All statistical analyses were performed using the Ecodist package implemented in R 3.0.2 (Goslee et al. 2007), and statistical results were considered significant when $P < 0.05$.

2.6 Analyses of selection pressures

The numbers of synonymous nt substitutions per synonymous site (d_s) and the numbers of nonsynonymous substitutions per

nonsynonymous site (d_N) for each coding region of tick and mammalian JMTV were estimated using the Kimura 2-parameter method implemented in MEGA version 7.0 (Kumar et al. 2016). Additionally, site-specific selection pressures were assessed using four methods in the HyPhy software package as implemented in the Datamonkey online tool (<http://www.datamonkey.org>): Single Likelihood Ancestor Counting (SLAC), Fixed Effects Likelihood (FEL), Fast Unconstrained Bayesian Approximation (FUBAR), and Mixed Effects Model of Evolution (MEME). Codons identified by at least three of these methods at a significance level of $P < 0.05$ or a posterior probability > 0.95 were considered to subject to positive selection (Castel et al. 2014).

3. Results

3.1 Collection of ticks, cattle blood, bats, and rodents and the identification of JMTV

During 2012–2016, 215 ticks, 104 blood samples from cattle, 216 bats, and 119 rodents were collected in the city of Wenzhou, Zhejiang Province, China. Additionally, 240 bats were sampled from Anlong country, Guizhou Province and Neixiang country, Henan Province (Fig. 1). The details of numbers, species, and geographic locations of these samples are described in Table 1. All these samples were then screened for Jingmenviruses.

Viral RNA was identified in 107 ticks (49.8%), 10 cattle (9.6%), 54 bats (11.8%), and 8 rodents (6.7%) using nested PCR targeting the conserved region of the Segment 1 (Yu et al. 2020). Genetic analysis of the recovered viral sequences revealed that they were closely related to each other (93.7–100% nt similarity) and to known JMTVs (76.0–99.8% nt similarity). Other Jingmenviruses were not identified in these samples. Combined with previous studies (Qin et al. 2014; Jia et al. 2019; Yu et al. 2020) and phylogenetic analysis (see below), these data indicate that JMTV is present in a broad range of hosts including ticks and mammals with a wide geographic distribution in China.

3.2 Diversity and genomic features of JMTVs in different hosts

To better understand the distribution of JMTVs in a broad range of hosts, six complete and twenty-six near complete genome sequences, reflecting different host species and sampling sites, were successfully recovered from viral RNA positive samples. Their genome size is described in Supplementary Table S1. The viruses newly identified in ticks, cattle, bats, and rodents shared >93 per cent nt identity and >95 per cent aa identity in all four segments. The nt similarity between viruses in ticks, cattle, bats, and rodents from Wenzhou was 93.7–100 per cent. Notably, the identity of some viruses (JMTV/*Apodemus agrarius*/Wenzhou/WZAa112/2016 and JMTV/*Rhipicephalus microplus*/

Table 1. Prevalence of JMTV in ticks, cattle, bats and rodents by species and locations in China.

Species	Guizhou Anlong	Henan Neixiang	Zhejiang Wenzhou	Total (%)
Ticks				
<i>Haemaphysalis hystricis</i>	–	–	47/102	47/102 (46.1)
<i>Rhipicephalus microplus</i>	–	–	60/113	60/113 (53.1)
Subtotal (%)	–	–	107/215 (49.8)	107/215 (49.8)
Cattle				
<i>Bubalus bubalis</i>	–	–	10/104	10/104 (9.6)
Bats				
<i>Aselliscus stoliczkanus</i>	0/1	–	–	0/1 (0)
<i>Eptesicus andersoni</i>	–	–	2/10	2/10 (20.0)
<i>Hipposideros armiger</i>	0/1	–	–	0/1 (0)
<i>Minopterus fuliginosus</i>	–	–	1/4	1/4 (25.0)
<i>M. schreibersii</i>	–	0/2	0/15	0/17 (0)
<i>Murina leucogaster</i>	–	0/1	–	0/1 (0)
<i>Myotis davidii</i>	11/63	0/1	2/10	13/74 (17.6)
<i>M. laniger</i>	1/9	–	2/42	3/51 (5.9)
<i>M. siligorensis</i>	2/23	–	–	2/23 (8.7)
<i>Nyctalus noctula</i>	–	–	3/6	3/6 (50.0)
<i>Pipistrellus abramus</i>	–	–	9/123	9/123 (7.3)
<i>Rhinolophus affinis</i>	–	0/1	–	0/1 (0)
<i>R. ferrumequinum</i>	–	4/10	–	4/10 (40.0)
<i>R. luctus</i>	–	0/1	–	0/1 (0)
<i>R. macrotis</i>	0/7	0/3	–	0/10 (0)
<i>R. monoceros</i>	–	0/3	0/6	0/9 (0)
<i>R. pearsonii</i>	–	1/13	–	1/13 (7.7)
<i>R. pusillus</i>	0/1	12/85	–	12/86 (14.0)
<i>R. sinicus</i>	4/15	–	–	4/15 (26.7)
Subtotal (%)	18/120 (15.0)	17/120 (14.2)	19/216 (8.8)	54/456 (11.8)
Rodents				
<i>Apodemus agrarius</i>	–	–	1/49	1/49 (2.0)
<i>Rattus tanezumi</i>	–	–	2/30	2/30 (6.7)
<i>R. norvegicus</i>	–	–	5/40	5/40 (12.5)
Subtotal (%)	–	–	8/119 (6.7)	8/119 (6.7)

Note: ‘–’ indicates that no animals were captured.

Wenzhou/WZRM55/2012) sampled from mice and ticks in Wenzhou was 100 per cent. Moreover, all viruses newly identified in this study shared high sequence similarity with known Chinese JMTVs: 92.1–99.7 per cent at the nt level and 95.1–99.9 per cent at the aa level in all four segments (Supplementary Tables S2 and S3). In addition, the similarity between JMTVs identified inside and outside of China was 76.3–95.0 per cent at the nt level and 81.2–99.1 per cent at the aa level in all four segments. Notably, however, all JMTVs discovered were genetically distinct from Alongshan viruses (Wang et al. 2019), sharing only 57.3–72.1 per cent nt and 61.7–79.5 per cent aa sequence identity.

All tick and mammalian JMTVs shared similar genomic organizations, with four segments encoding five proteins (Table 2 and Fig. 2). Indeed, all viruses identified in bats, cattle, rodents, and ticks collected in Wenzhou shared the same genomic structure (Table 2). However, viruses sampled from different geographic regions exhibited differences in the length of VP1, NSP2, and VP3 genes, with great variation between viruses sampled in China and other countries including Uganda, French Antilles, Turkey, and Kosovo (Fig. 2). Variation in the length of VP1, NSP2, and VP3 is due to deletions in some regions (Supplementary Fig. S1). The signal peptide in both Segments 1 and 2 was only found in the primate and tick associated JMTVs (Fig. 2). In addition, two open reading frames (ORFs) encoded by Segment 4 have different organizations in JMTVs. Notably, two ORFs are overlapping in JMTVs sampled from China, Turkey, French Antilles, Kosovo, and Uganda, but not so in JMTVs sampled from Brazil, Guinea and Trinidad, and Tobago (Figs 2 and 3 and Supplementary Fig. S2). All these data suggest that the overlapping/nonoverlapping ORFs encoded by Segment 4 might have been acquired multiple times during JMTV evolution.

3.3 Evolutionary relationships of JMTV from different hosts

Phylogenetic trees were estimated using an ML approach based on six complete genome sequences and nine nearly complete genomes recovered in this study, combined with reference sequences retrieved from the GenBank (Fig. 3). Although all known JMTVs, including those identified in this study, exhibit high genetic diversity, they can be divided into two major phylogenetic groups (Fig. 3). The first group comprised JMTVs sampled from Asia, Africa, and Brazil, exhibiting >87.6–99.7 per cent nt similarity, whereas the second group contains viruses sampled from the French Antilles, Trinidad and Tobago, Kosovo, and Turkey, with 75.9–96.7 per cent nt similarity.

As showed in Fig. 3, all viruses of the first phylogroup could be further divided into two subgroups. All Chinese viruses clustered together and formed a subgroup regardless of whether they were sampled from ticks, cattle, bats, and rodents. As Lao People's Democratic Republic (Lao PDR) and China share a border, it is not surprising that the viruses identified in *Amblyomma testudinarium* ticks sampled from Lao PDR fell into this subgroup and were phylogenetically close to with those sampled from the neighboring region—Yunnan Province, China. Interestingly, JMTV_RC27, sampled from a red colobus monkey in east Africa (Uganda) (Ladner et al. 2016) fell in the basal position, followed by viruses from southwestern China (Yunnan and Guangxi Provinces) and Lao PDR. In addition, this subgroup contained the viruses identified in ticks sampled from Turkey (Dincer et al. 2019) in the tree based on the Segment 3 sequences (Supplementary Fig. S2). The second subgroup comprised of the viruses sampled from Brazil. Notably, MK673133–MK673136

identified in ticks sampled from West Africa (Guinea) also fell into this subgroup. Probably as only a few of Guinean JMTV sequences was available, its position was variable in the trees based the four segments (Fig. 3).

The second group could also be divided into two distinct subgroups, corresponding to viruses sampled from Kosovo and Turkey and parts of the West Indies (French Antilles and Trinidad and Tobago), respectively. The two viruses sampled from Trinidad and Tobago (Sameroff et al. 2019) exhibited interesting phylogenetic relationships in the trees based on Segments 2, 3, and 4 (Supplementary Fig. S2). Specifically, TTP-Pool-19 showed a closer evolutionary relationship to the virus from French Antilles rather than to that sampled in the same locality (TTP-Pool-3b), whereas in the tree based on the Segment 1 TTP-Pool-3b was more closely related to the virus from French Antilles. Remarkably, TTP-Pool-3b showed a closer evolutionary relationship with viruses sampled from Kosovo, suggestive of a possible reassortment event. In sum, all these data suggest a complex evolutionary history of these viruses.

3.4 Association between JMTV diversity and hosts and geographic distributions

Both host and geography play an important role in shaping the viral diversity (Guo et al. 2013; Shi et al. 2016a, 2018). Indeed, the identification of JMTVs in Asia, Africa, Europe, and Central and South America (Fig. 1) indicates that they have a worldwide geographic distribution. We identified JMTVs in two genera of ticks, five genera of bats (from one family), two genera of rodents, and domestic cattle in Wenzhou city. Similarly, JMTV was found in seven bat species from two families sampled in both Guizhou and Henan Provinces, indicating that the virus can infect a broad spectrum of hosts.

To better understand the effect of host and geography on the diversity of JMTVs, we performed a series of Mantel tests. Notably, both simple and partial Mantel analyses across the four virus segments provided positive and significant values in the comparisons of the virus genetic and geographic distance matrices ($P < 0.05$) (Table 3). In contrast, there was no significant correlation between JMTV diversity and host genetic distance matrices. Accordingly, the genetic diversity of JMTV is largely related to host geographic location rather than host genetic distance.

3.5 Selection pressures on the JMTV genome

Both coding and noncoding regions are highly conserved between the tick and mammalian JMTV genomes, especially in China, suggesting that these viruses may have experienced long-term purifying selection. To better evaluate the selection pressures acting on the JMTVs, we estimated d_N , d_S , and d_N/d_S ratios across the whole viral genome and codon-specific selection pressures in the Chinese tick and mammalian JMTVs (Tables 4 and 5). Notably, all d_N/d_S ratio estimates were lower than 1, revealing that purifying selection is the dominant evolutionary pressure acting on the viral genome. However, codon-specific analyses provided evidence of positive selection acting on mammalian JMTVs (Table 5). Interestingly, one aa—position 892 in NSP1 was identified positively selected codons by three methods (FEL, FUBAR, and MEME). Similarly, two positively selected codons in VP1 (positions 11 and 38) were identified by four methods. The functional importance of these mutations should now be assessed experimentally. In contrast, there was no evidence for positive selection on the tick JMTVs.

Table 2. Key genomic features of JMTV from different hosts.

Characteristic	Tick ^a				Cattle ^a		Bat ^a		Rodent ^a		Primate ^a	
	JTMV_1/ Brazil	KITV/ Guinea	JMTV_T36/ Turkey	JMTV/ Trinidad and Tobago	JMTV/ French Antilles	WZBb40/ Wenzhou ^b	WZPa261/ Wenzhou ^b	NXRpu290/ Neixiang ^b	ALMd414/ Anlong ^c	WZRn75/ Wenzhou ^c	2013/ Kosovo	JMTV_RC27/ Uganda ^c
Segment 1												
Length (nt) ^d	3,019	2,968	2,947	3,012	2,995	3,073	3,073	3,073	3,073	3,073	2,962	2,950
5'UTR (nt)	93	104	86	100	33	104	104	104	104	104	99	57
NSP1 (nt/aa)	2,745/914	2,745/914	2,745/914	2,745/914	2,745/914	2,745/914	2,745/914	2,745/914	2,745/914	2,745/914	2,745/914	2,745/914
3'UTR (nt)	181	119	116	167	217	224	224	224	224	224	118	148
Segment 2												
Length (nt) ^d	2,820	2,805	2,556	2,814	2,309	2,785	2,785	2,785	2,785	2,785	2,657	2,264
5'UTR (nt)	163	156	170	201	53	173	173	173	173	173	142	98
VP1 (nt/aa)	2,264/753	2,264/753	2,235/744	2,235/744	2,235/744	2,265/754	2,265/754	2,265/754	2,265/754	2,265/754	2,235/744	1,815/604
3'UTR (nt)	395	387	151	378	21	347	347	347	347	347	280	351
Segment 3												
Length (nt) ^d	2,741	2,667	2,555	2,667	2,537	2,805	2,805	2,805	2,805	2,805	2,647	1,996
5'UTR (nt)	102	97	97	77	99	127	127	127	127	127	56	24
NSP2 (nt/aa)	2,427/808	2,427/808	2,427/808	2,334/777	2,427/808	2,427/808	2,427/808	2,427/808	2,427/808	2,427/808	2,427/808	1,974/657
3'UTR (nt)	212	143	31	256	11	251	251	251	251	251	164	NA
Segment 4												
Length (nt) ^d	2,738	2,725	2,603	2,701	2,654	2,751	2,751	2,751	2,751	2,751	2,611	2,698
5'UTR (nt)	128	130	111	190	38	144	144	144	144	144	125	92
VP2 (nt/aa)	765/254	765/254	765/254	765/254	765/254	765/254	765/254	765/254	765/254	765/254	765/254	765/254
VP3 (nt/aa)	1,506/501	1,506/501	1,617/538	1,506/501	1,617/538	1,617/538	1,617/538	1,617/538	1,617/538	1,617/538	1,617/538	1,617/538
Overlapping ORFs	No	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
3'UTR (nt)	259	244	141	160	265	256	256	256	256	256	135	255

^aHost of virus.

^bThe JMTVs newly identified in this study are shown in bold.

^cPartial sequences.

^dLength of segment does not include the polyadenylated tail at the 3' terminus; UTR, untranslated region.

NA, not available; KITV/Guinea, KITV_2017_1/Guinea; JMTV/Trinidad and Tobago, TTP-Pool-3b/Trinidad and Tobago; 2013/Kosovo, 2013-17-266/Kosovo.

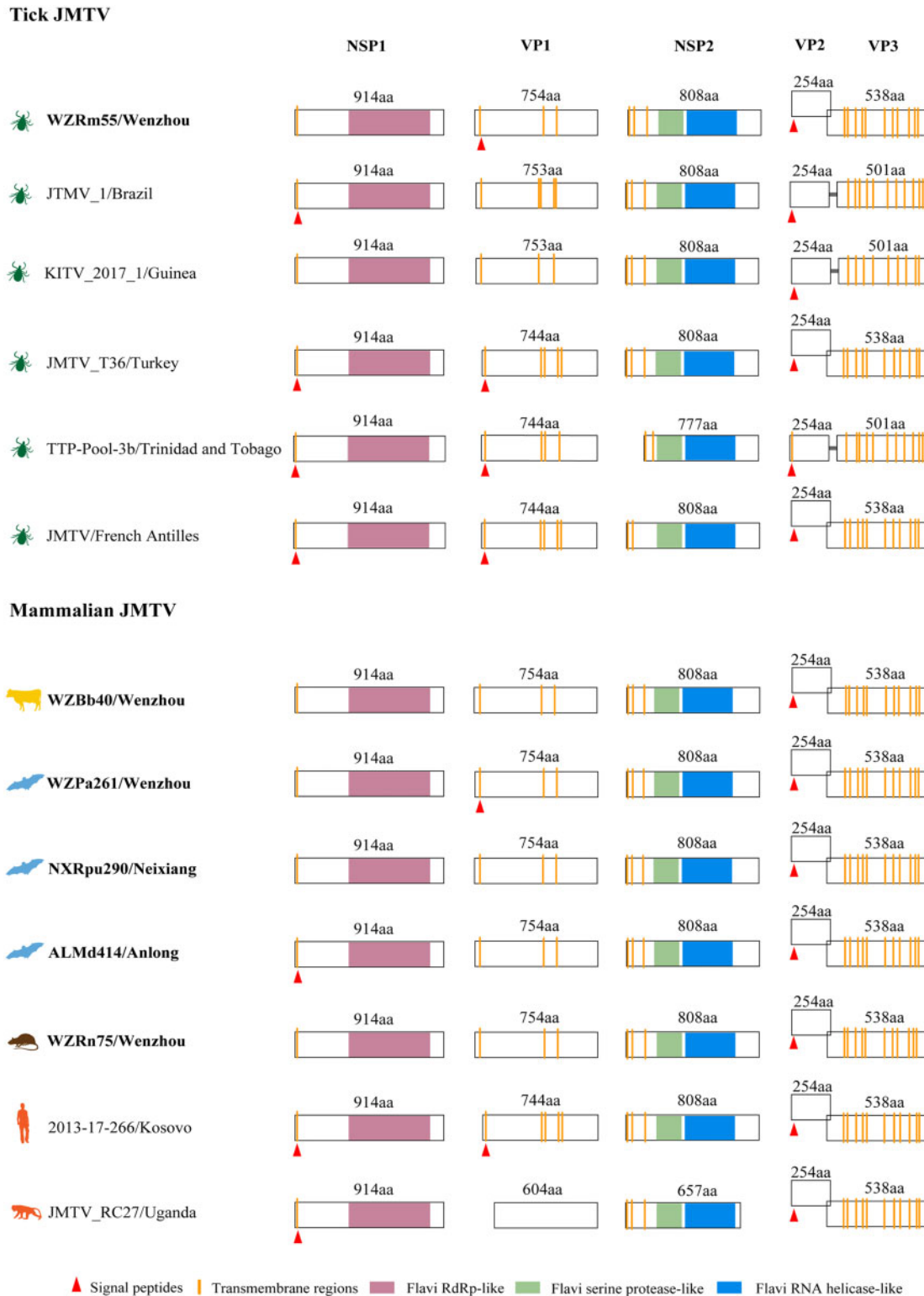


Figure 2. A comparison of protein structures of representative tick and mammalian-associated JMTVs. The viruses recovered in this study are highlighted with bold. A unified length scale is used for all the proteins.

4. Discussion

Since the discovery of JMTV in ticks sampled from Hubei and Zhejiang Provinces, China, in 2010 (Qin et al. 2014), the virus has been found not only in multiple Chinese regions (Jia et al. 2019;

Yu et al. 2020) but also in several countries globally (Ladner et al. 2016; Emmerich et al. 2018; Souza et al. 2018; Dincer et al. 2019; Pascoal et al. 2019; Sameroff et al. 2019; Temmam et al. 2019). In addition to multiple tick species, JMTV has been identified in a red colobus monkey sampled in Uganda (Ladner et al.

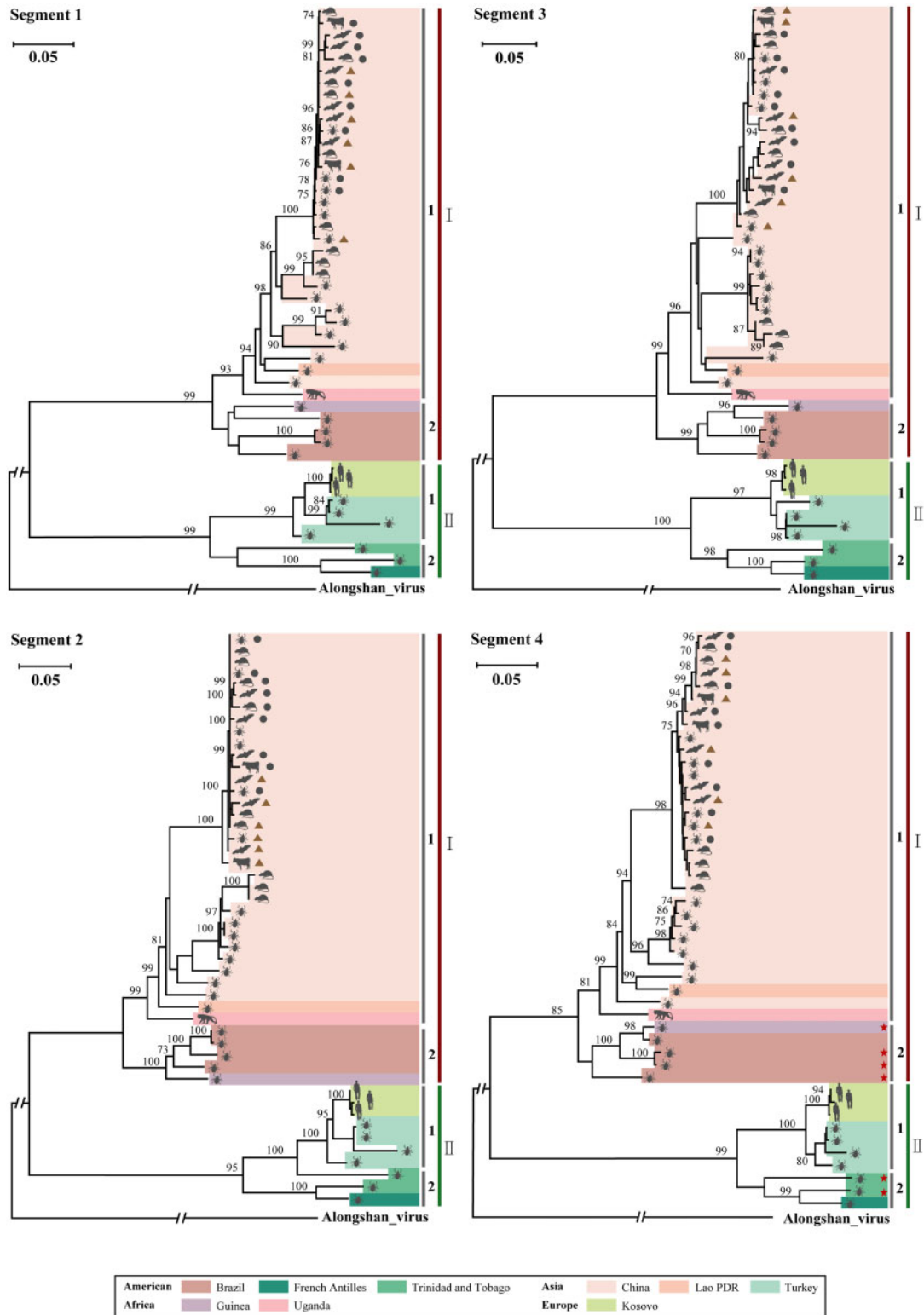


Figure 3. Phylogenetic analysis of the nt sequences of four segments of JMTV. Because of the high sequence similarity of the JMTVs discovered in this study, representative sequences including six complete genomes (brown triangles) and nine nearly complete genomes (gray circles) were used to infer the evolutionary trees. The JMTVs containing three or four segments and available on GenBank were used as reference sequences. Phylogenetic groups, as well as host and geographical origin are indicated. The JMTV whose segment 4 encoded non-overlapping ORFs is marked with red stars in the segment 4 phylogenetic. All trees were rooted using Alongshan virus. Bootstrap values (>70%) are shown at relevant nodes. The scale bar represents the number of nt substitutions per site. The GenBank accession numbers of the viruses used in the trees are found in [Supplementary Fig. 2](#).

Table 3. Results of the Mantel correlation analyses.

Model ^a	r value for JMTV (P value) ^b			
	Segment 1	Segment 2	Segment 3	Segment 4
Host ^c	0.0194 (0.3297)	−0.0192 (0.5784)	0.0152 (0.3543)	0.0292 (0.2790)
Host geography ^d	0.0372 (0.2342)	−0.0135 (0.5376)	0.0318 (0.2780)	0.0487 (0.2113)
Geography ^c	0.6504 (0.0001)	0.7078 (0.0001)	0.6512 (0.0001)	0.5092 (0.0001)
Geography host ^d	0.6508 (0.0001)	0.7078 (0.0001)	0.6515 (0.0001)	0.5103 (0.0001)

^aVertical lines indicate that the first factor excludes the effect of the second.

^bSignificant at P = 0.05.

^cMantel test.

^dPartial Mantel test.

Table 4. Comparison of the mean numbers of nonsynonymous (d_N) and synonymous (d_S) substitutions per site, and their ratios, in the coding region of JMTV from different hosts.

Gene	Tick JMTV			Mammalian JMTV		
	d_N	d_S	d_N/d_S	d_N	d_S	d_N/d_S
NSP1	0.008	0.144	0.056	0.002	0.037	0.054
VP1	0.012	0.126	0.095	0.005	0.028	0.179
NSP2	0.006	0.145	0.041	0.005	0.057	0.088
VP2	0.009	0.156	0.058	0.004	0.045	0.089
VP3	0.010	0.151	0.066	0.003	0.046	0.065

2016) as well as in multiple species of rodents in Xinjiang of China (Yu et al. 2020). Bats (order *Chiroptera*) that comprise more than 1,240 species exhibit remarkable diversity and represent more than 20 per cent of living mammalian species (Nowak 1994). Since the discovery of SARS-related CoV in *Rhinolophus horseshoe* bats in China in 2005 (Lau et al. 2005; Li et al. 2005), they have received increased attention because of their role in the evolution and transmission of a broad range of viruses (Leroy et al. 2005; Drexler et al. 2012; Holmes and Zhang 2015; Nie et al. 2018; Cui et al. 2019). Importantly, SARS-CoV-2 is also believed to have an ultimate bat reservoir (Wu et al. 2020; Zhang and Holmes 2020; Zhou et al. 2020). In this study, we identified JMTV in two species of ticks, six species of bats, three species of rodents, and cattle in Wenzhou (Zhejiang Province), indicating that JMTV is present in both ticks and mammals from the same location. Additionally, JMTV was found in seven species of bats sampled from both Guizhou and Henan Provinces. Because of their global distribution, abundance, ability to fly long distances and often large population densities, bats may play an important role in the transmission of JMTV. In sum, all these data indicate that JMTV is present in a broad range of hosts, comprising both ticks and various species of mammal.

Viruses are characterized by both virus–host co–divergence and cross–species transmission over evolutionary time scales (Li et al. 2015; Shi et al. 2016a). Despite their ability to jump species boundaries, many viruses show a specific association with their hosts (Guo et al. 2013; Blitvich and Firth 2015). Here, we identified JMTV in ticks, cattle, bats, and rodents in Wenzhou city (Table 1), with the detection rates ranging from 6.7 per cent to 49.8 per cent, again showing that JMTV has the capability to transmit among ticks and mammals. Notably, irrespective of their host species, all viruses circulating in Wenzhou were closely related to each other, with >93 per cent (and sometimes 100%) nt sequence identity. Together, this

suggests that no observable variation in genome structure is needed for the transmission of JMTV between ticks and mammals.

Both host environments and geographic isolation play an important role in shaping the diversity of viruses (Guo et al. 2013; Shi et al. 2016a, 2018). Notably, the data generated here show that JMTVs sampled from ticks and mammals collected in Wenzhou were closely related to each other, with Mantel tests revealing no significant association between JMTV diversity and host genetic distance matrices. Conversely, on a global scale, viruses exhibit strong geographic clustering (Fig. 3), a pattern supported by the Mantel tests which revealed that the geographic distance among host sampling locations has a greater impact on JMTV diversity than host genetic distance (Table 3).

To date, JMTVs have already been identified in Asia, Africa, Europe, and Central and South America (Fig. 1). We were able to classify these viruses into two major groups: the first included viruses identified in Asia, Africa, and Brazil, whereas the second contained viruses identified in islands of Central and South America, South Europe (Kosovo), and Western Asia (Turkey). Although it is notable that both groups of JMTV were found in Turkey, on the currently limited sample of data, it is not possible to determine the exact evolutionary and phylogeographic origin of JMTV, and this virus likely has a complex evolutionary history.

Flaviviruses infect a variety of vertebrates and arthropods (Simmonds et al. 2011). However, some have a limited host range among vertebrates, whereas others can replicate in a wide array of species. Additionally, arthropods are usually infected when they feed on a vertebrate host during viremia, but non–viremic transmission has also been described for tick-borne flaviviruses (Simmonds et al. 2011). Hence, these data indicate that flaviviruses have complex virus–host relationships (Gaunt et al. 2001; Kitchen et al. 2011; Blitvich and Firth 2015). Since its initial discovery in 2010 (Qin et al. 2014), JMTVs have been identified in at least fifteen species of ticks and one species of mosquito (Maruyama et al. 2014; Qin et al. 2014; Souza et al. 2018; Dincer et al. 2019; Jia et al. 2019; Sameroff et al. 2019; Temmam et al. 2019) as well as in cattle, rodents, and primates (Qin et al. 2014; Ladner et al. 2016; Emmerich et al. 2018; Souza et al. 2018; Jia et al. 2019; Yu et al. 2020). In this study, we identified JMTV in a broad range of bat species. Notably, they do not cluster according to animal species in phylogenetic analysis, and the viruses sampled from mammals and ticks share high sequence identities, with identical sequences sometimes detected in ticks and rodents. Hence, these data are compatible with the vector-borne transmission cycle of JMTVs in nature.

Table 5. Selection pressures acting on JMTV from different hosts.

Protein	Model	Tick JMTV			Mammalian JMTV		
		Codons subject to putative positive selection ^a	Codons subject to putative purifying selection ^a	Mean (d_N/d_S)	Codons subject to putative positive selection ^a	Codons subject to putative purifying selection ^a	Mean (d_N/d_S)
NSP1	SLAC	0	7 codons	0.0562	0	14 codons	0.104
	FEL	0	76 codons	0.0529	1 codon: 892	50 codons	0.0993
	FUBAR	1 codon: 892	248 codons	–	3 codons: 306, 755, 892	70 codons	–
	MEME	0	–	0.0529	4 codons: 25, 507, 755, 892	–	0.0993
VP1	SLAC	0	2 codons	0.0941	2 codons: 11, 38	15 codons	0.222
	FEL	0	63 codons	0.0903	2 codons: 11, 38	40 codons	0.215
	FUBAR	0	73 codons	–	4 codons: 11, 14, 38, 472	43 codons	–
	MEME	3 codons: 27, 33, 483	–	0.0903	7 codons: 2, 11, 14, 16, 38, 94, 421	–	0.215
NSP2	SLAC	0	4 codons	0.0395	0	43 codons	0.142
	FEL	0	69 codons	0.0380	0	69 codons	0.136
	FUBAR	1 codon: 216	202 codons	–	6 codons: 27, 161, 461, 781, 804, 805	78 codons	–
	MEME	2 codons: 216, 780	–	0.0380	8 codons: 6, 275, 276, 404, 405, 778, 781, 804	–	0.136
VP2	SLAC	0	0	0.0582	0	2 codons	0.0884
	FEL	0	14 codons	0.0551	0	10 codons	0.0825
	FUBAR	0	19 codons	–	0	14 codons	–
	MEME	0	–	0.0551	0	–	0.0826
VP3	SLAC	0	3 codons	0.0567	0	18 codons	0.0877
	FEL	0	46 codons	0.0544	0	31 codons	0.0831
	FUBAR	0	83 codons	–	2 codons: 42, 389	38 codons	–
	MEME	0	–	0.0544	4 codons: 4, 42, 157, 161	–	0.0831

^aP < 0.05 or PP > 0.95; positions identified as being under positive selection by at least three methods are shown in bold.

Supplementary Data

Supplementary data are available at *Virus Evolution* online.

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Data Availability

All the viral sequences obtained in this study have been deposited in GenBank under accession numbers MK721553–MK721680.

Conflict of interest: None declared.

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