



RESEARCH ARTICLE

Metagenomic Profiling of Viruses Associated with *Rhipicephalus microplus* Ticks in Yunnan Province, China

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Abstract

Ticks are well known as vectors of many viruses which usually do great harm to human and animal health. Yunnan Province, widely covered by flourishing vegetation and mainly relying on farming husbandry, is abundant with *Rhipicephalus microplus* ticks. Therefore, it is of great significance to characterize the viral profile present in *R. microplus* parasitizing on cattle in Yunnan Province. In this study, a total of 7387 *R. microplus* ticks were collected from cattle and buffalo in the northwest and southeast areas of Yunnan Province from 2015 to 2017. We investigated the virome of *R. microplus* using next-generation sequencing (NGS) and the prevalence of important identified viruses among tick groups by RT-PCR. It revealed the presence of diverse virus concerning chu-, rhabdo-, phlebo-, flavi- and parvo- viruses in Yunnan. These viruses consist of single-stranded, circular and segmented sense RNAs, showing a greatly diversity in genomic organization. Furthermore, continuous epidemiological survey among ticks reveals broad prevalence of three viruses (Yunnan mivirus 1, Wuhan tick virus 1 and YN tick-associated phlebovirus 1) and two possible prevalent viruses including a flavivirus-like segmented virus (Jingmen tick virus) and a bovine hokovirus 2 in Yunnan. Serological investigation among cattle indicates that these identified viruses may be infectious to cattle and can elicit corresponding antibody. Our findings on *R. microplus*-associated viral community will contribute to the prevention of viral disease and tracking the viral evolution. Further analysis is needed to better elucidate the pathogenicity and natural circulation of these viruses.

Keywords Viral profile · *R. microplus* ticks · China · Diversity

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Introduction

Ticks are one of the most important vectors being notorious for spreading etiological agent from wild hosts to domestic animals and humans. Since the first identification of Louping ill virus in ticks, which can cause severe encephalitis in sheep and other livestock (Stockman 1918), a large group of tick-borne viruses (TBVs) have been recognized, presenting great genetic diversity and various properties. Recently, the whole-genome sequencing of six ixodid tick species from China has been completed revealing rich variety of tick-associated pathogens (Jia *et al.* 2020). So far, TBVs are involved in at least 12 genera of nine families and two orders, and also include members that haven't been assigned (Shi *et al.* 2018).

TBVs that can replicate and maintain in ticks and infest vertebrate hosts pose severe threat to animal and human health (Horak *et al.* 2002). In China, it has long been plagued by TBVs including tick-borne encephalitis virus

(TBEV) and Crimean-Congo hemorrhagic fever virus (CCHFV) in the northern provinces where fatal cases in several epidemics and sporadic outbreak were noted with a very high mortality rate decades ago (Moming *et al.* 2018; Zhang *et al.* 2012). In recent years, a novel TBV called severe fever with thrombocytopenia syndrome virus (SFTSV) has been identified in China, which is the causative agent of severe fever with thrombocytopenia syndrome (SFTS) disease to humans with a high initial mortality due to the lack of specific drugs and vaccines (Zhang *et al.* 2017). From 2010 to 2016, over 10,000 SFTS cases have been reported in 23 provinces of China (Zhan *et al.* 2017). In 2018, China suffered an unprecedented livestock disaster implicating over 5000 pigs caused by African swine fever virus (ASFV) which transmitted among pigs, wild boar via soft ticks (Wang *et al.* 2018). So it is of great significance to investigate the tick-borne viromes in natural habitat for better control and prevention of large epidemic caused by TBVs.

The next-generation sequencing (NGS) has become a powerful technique used for investigating viromes of many species. Numerous novel viruses and virus-related sequences have been found from various arthropods using NGS, which largely boosted the current viral community (Tokarz *et al.* 2014; Li *et al.* 2015; Brinkmann *et al.* 2018). Novel viruses have also been identified or isolated from different tick species in recent years, indicating that the current knowledge of TBVs may be a tip of the iceberg.

Yunnan Province locates in the southwest of China, bordering on Myanmar, Laos and Vietnam. It is a quite suitable habitat for ticks in most areas of Yunnan Province, because of the abundant rainfall, lush vegetation, diverse wildlife and livestock (Xia *et al.* 2015). A variety of tick species have been identified in Yunnan Province, where *Haemaphysalis* and *Rhipicephalus* spp. ticks are commonly found (Chen *et al.* 2008). The *Rhipicephalus (Boophilus) microplus* tick is widely distributed in the world, and is an important vector for many viral pathogens, including Thogoto virus (*Orthomyxoviridae*, genus *Thogoto*), Wad Medani virus (*Reoviridae*, genus *Orbivirus*), CCHFV (*Nairoviridae*, genus *Orthonairovirus*), and Kadam virus (*Flaviviridae*, genus *Flavivirus*), which can cause diseases in livestock or humans (Labuda and Nuttall 2004). A number of researches have reported the viral diversity of *R. microplus* from different locations, such as Brazil and Colombia. And both common virus as well as region specific virus were identified (Gómez *et al.* 2020; Souza *et al.* 2018; Zhao *et al.* 2020). Previous study on *R. microplus* collected from different host shows that comparing to natural host of tick, the geographic location has more influence on virus profile (Zhao *et al.* 2020). *R. microplus* is also the dominant tick species in Yunnan Province, however, its virome profiling has not been fully

characterized. In this study, *R. microplus* ticks were collected from domestic cattle surface from 2015 to 2017 in Yunnan Province. We investigated the virome of *R. microplus* using NGS and the prevalence of important identified viruses among tick groups by RT-PCR. Serological survey was performed to reveal the potential transmission and infection of these identified viruses to human and cattle. The data will contribute to the understanding of the TBVs community and also reveal the potential risks from TBVs being vectored by *R. microplus* ticks in Yunnan Province, China.

Materials and Methods

Sample Collection and Preparation

From 2015 to 2017, 7387 live adult ticks were plucked from infested cattle and buffalo in Nujiang Lisu Autonomous Prefecture (NJ), Dali Bai Autonomous Prefecture (DL) and Luxi County (LX) in Yunnan Province (Supplementary Figure S1 and Table 1), maintained alive in cases at room temperature before study. The ticks were identified morphologically as *R. microplus* by a trained expert and further confirmed by sequencing internal transcribed spacer 2 gene (*ITS2*). These ticks were washed three times using sterile phosphate-buffered saline (PBS, pH7.4), grouped (n = 11–20) depending on their body size and homogenized with 1 mL PBS for each group as described (Zhang *et al.* 2018). Homogenates were centrifuged at 5000 ×g for 10 min at 4 °C to remove debris, and supernatants were subpackaged and stored at – 80 °C until further processing.

Additionally, 100 serum samples of cattles, 100 serum samples from febrile patients and 100 healthy human serum samples from the same tick sampling location in LX were shipped to the laboratory in a cooler with ice pack or with dry ice, depending on the time required for transportation. Besides, 100 cattle serum samples were collected from a farm of Wuhan, Hubei Province.

Table 1 Summary of total tick sample collections in Yunnan Province from 2015 to 2017

Time	Sampling location	Number of groups	Total ticks
2015	NJ	76	912
	LX	45	540
2016	DL	351	4212
	LX	47	564
2017	LX	79	410
Total		574	7387

Library Preparation and RNA-seq Sequencing

According to our sampling location and time, our tick sample can be classified to four batches. And each 12 ticks from the same batch were divided into one group. For RNA-seq, three groups of ticks were mixed for one RNA-seq library construction, and totally four libraries corresponding to each batches were constructed. Total RNA was purified using TRIzol LS reagent (Invitrogen, Calrsbad, CA) from 300 μ L supernatant of each group. Then equivalent qualities of total RNA from three groups were mixed to generate one RNA pool (3–5 μ g in total). RNA-seq library preparation was performed according to the protocol provided by Illumina as described (Li *et al.* 2015), and was further sequenced by the Hiseq 3000 platform (Illumina) to generate the 150 bp paired-end reads.

Raw sequencing reads with low quality were excluded, trimmed to remove adaptors using Trimmomatic program and then subjected to a basic local alignment search tool (BLASTn) comparing against the tick genome to filter out the abundant host reads so as to simplify the following de novo assembly using Trinity program (Grabherr *et al.* 2011). Subsequently, the contigs were mapped to non-redundant nucleotide database (NT) and non-redundant protein database (NR) downloaded from NCBI using BLASTN and BLASTX respectively by using an E-value cutoff of $1e-5$ to identify potential viral contigs. An overview of contig taxonomy was shown by MEGAN software with the BLASTN or BLASTX comparison results (Huson *et al.* 2007). Bowtie2 were used to obtain the raw reads of contig which mapping to a certain target viral genome and corresponding reads per kilobase million mapped reads (RPKM) were calculated (Langmead and Salzberg 2012).

Viral Full-Length Genome Walking and PCR-Based Epidemiological Survey

Nested RT-PCR was performed to confirm the detection of RNA-seq with primer sets (Supplementary Table S1). That is cDNA from the tick pools which was originally subjected to RNA-seq were used as templates for PCR detection. Only positively detected potential virus would undergo further epidemiological survey.

The contigs which hit viral genome were used as templates for overlapping primers design. And the full-length genome of each virus was determined via genome walking, and 5'- and 3'- rapid amplification of cDNA ends (5', 3' RACE) with commercial kit (Takara) according to the manufacturer's protocol.

All the PCR products were separated on 0.8% agarose gel and purified through agarose gel DNA extraction kit for Sanger sequencing.

Preparation of Polyclonal Antibodies and Immunofluorescence Assay

Firstly, plasmid pET-28a-NP containing partial or complete nucleoprotein of each virus was constructed. Then recombinant plasmid pET-28-NP was electrotransformed into *E.coli* strain BL21 for protein expression induced with IPTG. And expressed NP was purified as described previously (Zou *et al.* 2016), which subsequently used for rabbit immunization to elicit polyclonal antibodies (pAbs) against NP. Western blots were performed to verify the effectiveness of the polyclonal antibody (anti-NP).

The same expression regions of pET-28a-NP were inserted into plasmid pCND3.1+ for eukaryotic expression. Then immunofluorescence assay was performed by using plasmid-transfected HEK293 cells. Cells were fixed with 4% paraformaldehyde for 15 min before being permeabilized with 0.5% Triton X-100 in PBS solution for 10 min. After rinsing with PBS solution, they were blocked with fish gelatin for 1 h, and then washed with PBS solution for another 3 min. The washing steps were repeated three times. Subsequently, bovine sera (diluted 1:20 in PBS solution) or polyclonal antibodies to NP (1:2000) as positive control group were incubated with cells for 2 h and washed as described earlier. Reactions among bovine sera were detected with FITC conjugated protein G (1:2000; Abcam) and the positive controls were detected with Alexa 488-labeled goat anti-rabbit (1:2000; Abcam). Cell transfected with empty pCND3.1+ was severed as negative control.

Phylogenetic and Bioinformatics Analysis

For each potential virus, phylogenetic trees were inferred based on the full-length RNA-dependent RNA polymerase (RdRp) protein which were aligned with their corresponding homologous references sequence. Alignment and phylogenetic analysis were conducted via ClustalW and maximum likelihood (ML) method which both implemented within MAGA version 6.0 (Tamura *et al.* 2013).

Prediction of Protein Functional Domain

We use ORFfinder (<https://www.ncbi.nlm.nih.gov/orffinder>) to predict potential protein, TMHMM v2.0 (<https://www.cbs.dtu.dk/services/TMHMM/>) to predict transmembrane domains, SignalP v4.0 (<https://www.cbs.dtu.dk/services/SignalP/>) to find likely signal sequence, NetOGlyc (<https://www.cbs.dtu.dk/services/NetOGlyc/>) to predict O-GalNAc glycosylation sites and NetNGlyc (<https://www.cbs.dtu.dk/services/NetNGlyc/>) to predict N-glycosylation sites.

Results

Tick Sample Collection and Taxonomic Identification

From 2015 to 2017, a total of 7387 ticks were collected from cattle and buffalo in the northwest (NJ and DL) and southeast (LX) areas of Yunnan Province and were divided into 574 groups (Supplementary Figure S1 and Table 1). Except that samples were collected continuously from LX for 3 years, ticks were collected from NJ in 2015 and from DL in 2016 (Table 1). These ticks were all half or full engorged *R. microplus* identified both by morphology and *ITS2* sequencing (Fig. 1).

Additionally, 100 serum samples of cattle and febrile patient and healthy human, respectively from Luxing County and 100 serum samples of cattle from Wuhan City in Hubei Province were acquired.

Metagenomic Sequencing Revealed Virome Profile of *R. microplus* Ticks

A total of four tick pools collecting from different regions of Yunnan during 2015 to 2017 generated ~ 28 Gb of data including 2.7×10^9 or so 150-base pair-end reads (Supplementary Table S2). After reads assembly and sequence comparison to the NT or NR database, 143 contigs from the four libraries were identified as viral sequences related to 9 viruses, concerning *Phenuiviridae*

($n = 3$), *Chuviridae* ($n = 2$), *Rhabdoviridae* ($n = 1$), *Parvoviridae* ($n = 1$) and *Flaviviridae*-like ($n = 2$) (Table 2).

RPKM values of the nine viruses suggested that the first five viruses (RPKM > 1.0) had much higher abundance than the last four viruses (RPKM < 1.0) (Table 2), in accordance with the genome coverage of RNA-seq that the former five viruses had several gaps along their genome sequences while dispersed short contigs flanked by large gaps were found along genome of the latter viruses. Genome walking was further performed with the first five viruses in order to acquire the full-length genome. Genome organization of these viruses was shown in Fig. 2, reflecting a great genomic diversity of tick-associated viruses.

Viruses Belong to *Chuviridae*

Viral sequences share 86% and 95% identity at amino acid (aa) level with Wuhan mivirus (WHMV) strain X78-1 and Bole mivirus (BLMV) strain BL199, respectively were detected in our tick pools. WHMV and BLMV are identified in the *R. microplus* and *Hyalomma asiaticum* tick in China, respectively and assigned to the recently-proposed *Mivirus* genus of *Chuviridae* (Li *et al.* 2015). Based on the similarity, we considered virus related to WHMV as a novel chuvirus named Yunnan mivirus strain YN-MV1, and virus related to BLMV as a novel strain of BLTV named YN-MV2. Here we obtained the full-length genome of YN-MV1 (Fig. 2). YN-MV1 (GenBank MH814982) is a

Fig. 1 Molecular identification of tick samples. The phylogenetic analysis was performed via an ML method based on internal transcribed spacer 2 (*ITS2*) gene. The ticks collected in different year in this study were indicated by black dots.

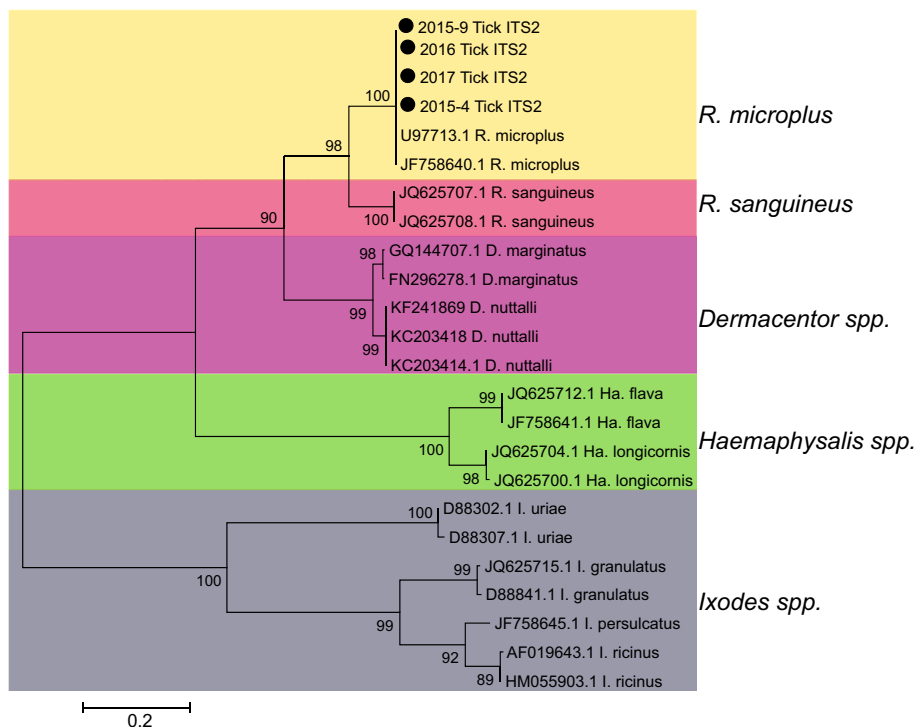


Table 2 Viruses identified in *R. microplus* in our study

No.	Virus name	Closest relative	Identity with relative virus (aa)	Classification of relative virus	Coverage (%)	RPKM
1	Yunnan mivirus 1 (YNMV1) strain YN-MV1	Wuhan mivirus strain X78-1	86%	<i>Mivirus</i>	94.2	4.8
2	Wuhan tick virus 1 (WHTV1) strain YN-rhabdoV1	Wuhan tick virus 1 strain X78-2	97%	Unassigned <i>Rhabdoviridae</i>	83.3	2.5
3	YN tick-associated phlebovirus 1 (YN-PhelobV1) strain YN-PLV1	Lihan tick virus strain LH-1	S segment 89% L segment 88%	<i>Phlebovirus</i>	86	1.57 1.9
4	Jingmen tick virus (JMTV) strain YN-flaviV	Jingmen tick virus isolate SY84	S1 segment 93% S2 segment 99% S3 segment 96% S4 segment 99%	Unassigned (Jingmenvirus)	S1:87.7 S2:84.5 S3:86 S4:87.2	1.33 2.7 1.8 3.0
5	Bovine hokovirus 2 (BHKV) strain YN-BHKV2	Bovine hokovirus 2 strain HK-B38	100%	<i>Tetraparvovirus</i>	97	1.30
6	Bole mivirus (BLMV) strain YN-MV2	Bole tick virus 3 strain BL199	95%	<i>Mivirus</i>	44.1	0.09
7	Bole tick virus 1 (BLTV1) strain YN-PLV2	Bole tick virus 1 strain BL075	S/L segment 98%	<i>Phlebovirus</i>	36.4	0.05
8	YN tick-associated flavi-like virus 1 (YN-FlaviV1) strain YN-FLV 1	Bole tick virus 4	80%	Unassigned (Flavi-like)	30.2	0.03
9	YN tick-associated phlebovirus (YN-PhelobV1) strain YN-PLV3	Blacklegged tick virus	S segment 60% L segment 65%	<i>Phlebovirus</i>	23.2	0.01 0.01

RPKM: Reads Per Kilobase per Million mapped reads

negative-strand circular RNA virus similar to WHMV, with genome length of 11,395 nt which contains three open reading frames (ORFs) encoding RNA-dependent RNA polymerase (RdRp), glycoprotein (G) and nucleoprotein (NP) (Fig. 2). Phylogenetic tree showed that YN-MV1 is a novel member of *Mivirus* (Fig. 3A).

Viruses Belong to *Rhabdoviridae*

Via blastn search, viral sequences demonstrated 97% identity to the Wuhan tick virus 1 (WHTV1) strain X78-2 were found in our tick pools. It is proposed to be a strain of WHTV1 termed YN-rhabdoV1 (GenBank MH814974). WHTV1, originally detected in same ticks in Hubei Province, currently remain unclassified in the family *Rhabdoviridae* and considered as a member of the *dimarhabdovirus* super group (Bourhy *et al.* 2005). The single negative-strand genome of YN-rhabdoV1 with a 10,306 nt in length consists of four ORFs, two of which encodes *RdRp* and *NP* both being homologous with that of rhabdovirus (Fig. 2). The functions of the remaining two ORFs (ORF1 and ORF2) are unknown for no homologous known protein has been found. Phylogenetic analysis with other rhabdovirus shows that YN-rhabdoV1 is closer to Moussa virus, which is an unassigned rhabovirus, than to the other genus and forms a separated branch with a group of novel rhabdovirus-like virus identified mainly in ticks

via NGS (Fig. 3B). This group of novel rhabdovirus-like virus may be considered as a new genus within *Rhabdoviridae*.

Viruses Belong to *Phenuiviridae*

A number of tick-associated phlebovirus sequences were identified in our tick pools, and blastn or blastx search indicate these sequences mostly relate to viruses of *Phlebovirus* genus in *Phenuiviridae*. Viral sequences share 89% aa identity with Lihan tick virus (LHTV) which was originally identified in same tick species in central China, were considered to belong a novel phlebovirus named Yunnan tick-associated phlebovirus 1 strain YN-PhelobV1. Sequences share 98% aa identity with Bole tick virus 1 (BLTV1) strain BL075 are supposed to belong to a novel strain of BLTV1, tentatively named BLTV1 strain YN-PhelobV2. And the remaining short contigs are considered as a novel phlebovirus named Yunnan tick-associated phlebovirus 3, sharing 60% aa identity with a blacklegged tick virus, which was originally identified in blacklegged tick from America. Like other phlebovirus, the genome of YN-PhelobV1 is supposed to consist three negative RNA segments termed *S*, *M* and *L*, respectively (Fig. 2). However, the *M* segment of both YN-PhelobV1 and LHTV haven't been identified yet. Phylogenetic tree shows that YN-PhelobV1 (Genbank No. MH814975 for *S*,

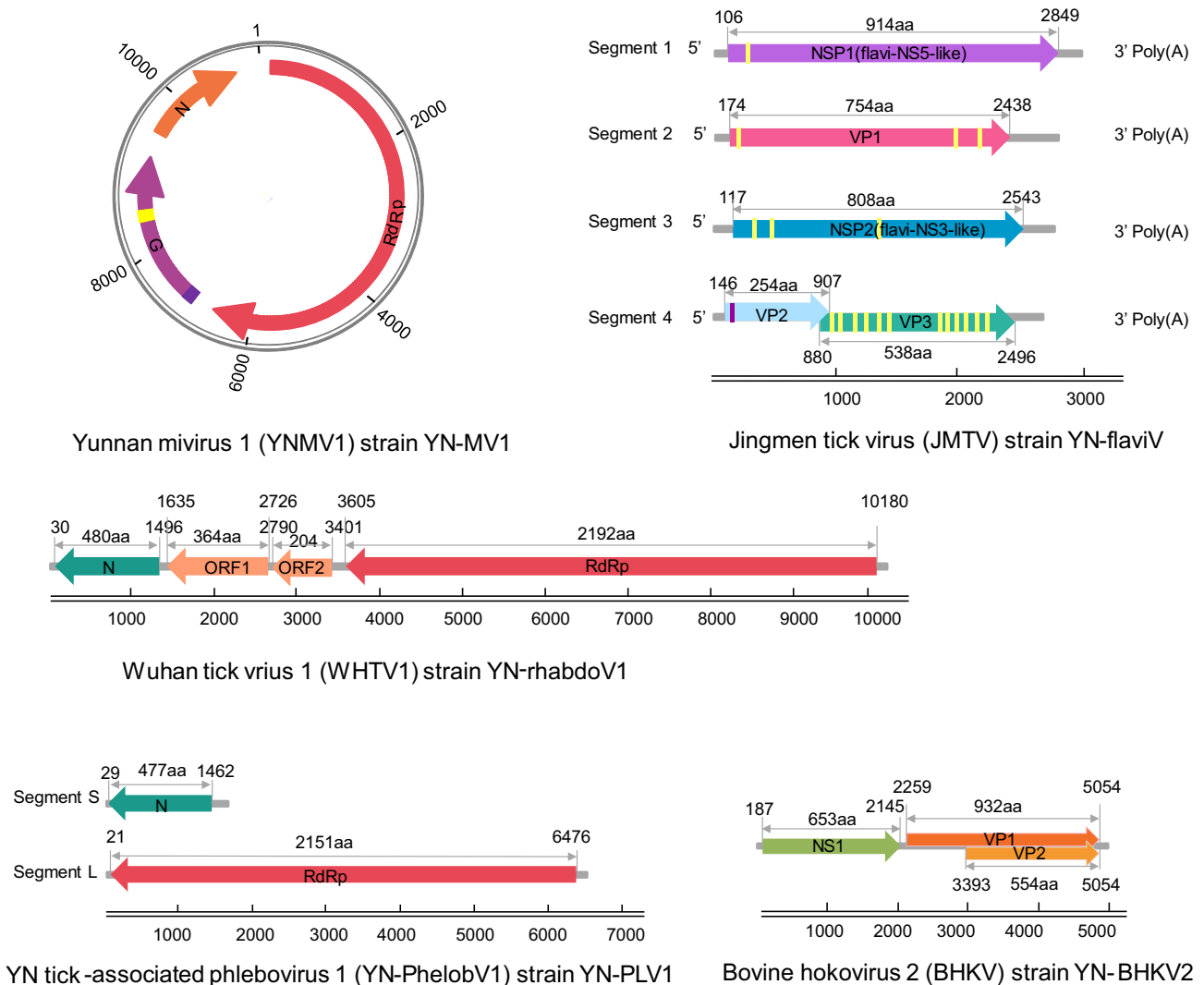


Fig. 2 Schematic genome organization and putative proteomic maps of each virus. Yellow boxes represent the transmembrane regions (TM), and purple boxes represent the signal peptides (SP).

Genbank No. MH814976 for *M*) is closest to Uukuniemi virus forming a separated branch with a group of novel putative phleboviruses which are characterized with unknown *M* segment (Fig. 3C).

Flavi-like Viruses and Jingmenvirus

Recently, a group of four segmented single-stranded positive RNA viruses related to flavivirus were identified in various arthropod and the prototype virus Jingmen tick virus (JMTV) strain SY84 was the firstly identified in *R. microplus* ticks collected in Hubei Province, China (Shi *et al.* 2016). Here, we also found the genomic sequences of a new JMTV strain YN-flaviV in the *R. microplus* ticks collected in 2017 in LX of Yunnan Province, which showed 93%–99% nt identity with strain SY84. Like the genome of JMTV, YN-flaviV comprises four segments

(GenBank MH814977–MH814980) (Fig. 2), among which segment 1 and segment 3 respectively code protein related to nonstructural protein NS3 and NS5 of *Flavivirus* (family *Flaviviridae*). Proteins coded by segment 2 and segment 4 are quite unknown because no homologous proteins have been found. Via bioinformatics prediction, two N-glycosylation sites and three transmembrane domains were found on VP1 coded by segment 2, indicating VP1 may be a glycoprotein. Segment 4 has two ORFs coding VP2 and VP3. One signal peptide on the N terminal, one N- and O-glycosylation site was predicted on VP2. And VP3 is largely a membrane protein because 11 transmembrane domains were predicted on it. Phylogenetic tree with members of *Flaviviridae* shows that YN-flaviV clusters with a group of four segmented flavivirus-like viruses named Jingmenvirus group, and were closest to members of *Flavivirus* (Fig. 3D). And this highly divergent lineage

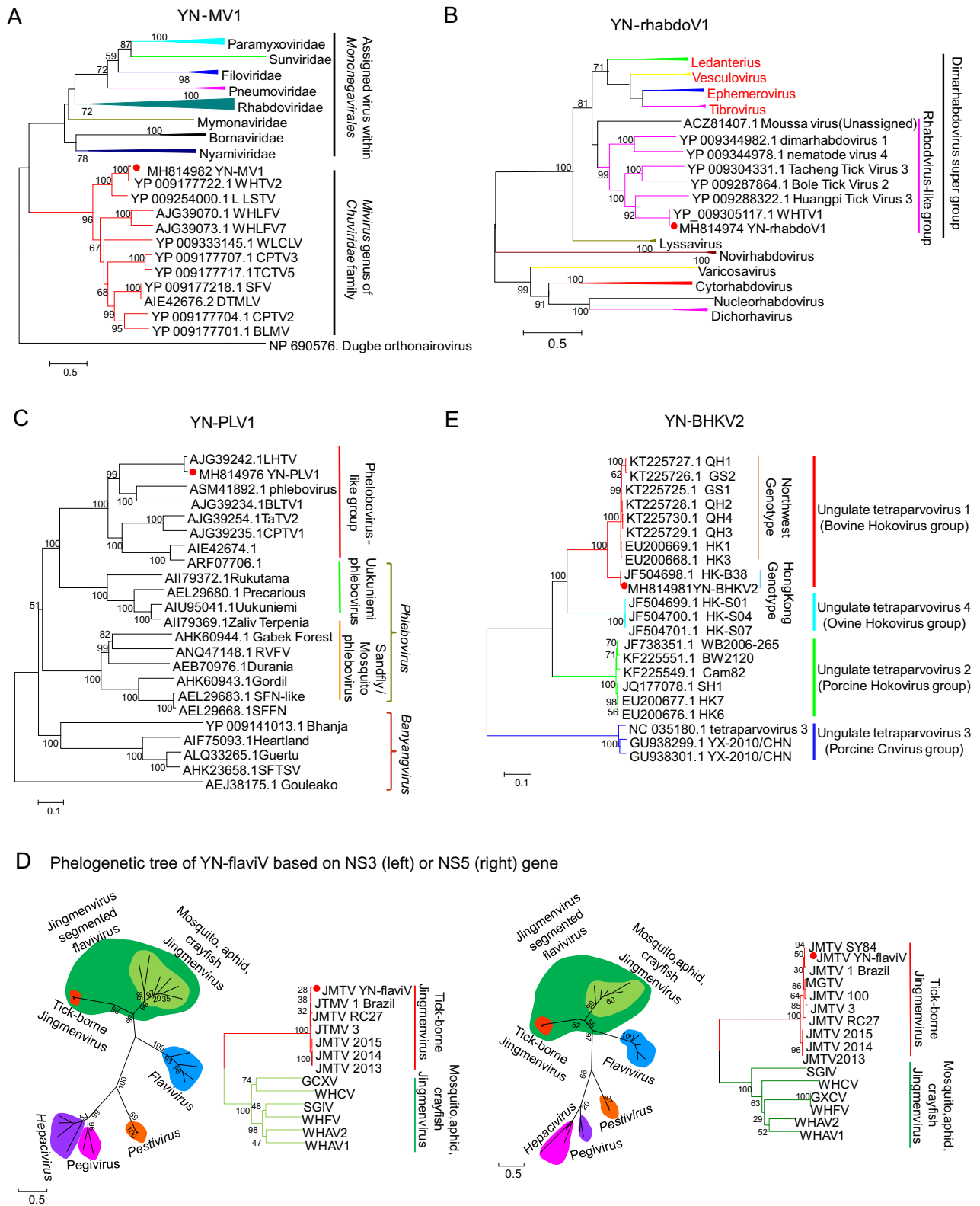


Fig. 3 Phylogenetic relationship of **A** YNMV1 based on RdRp of members within *Mononegavirales*, **B** YN-rhabdoV1 based on RdRp of rhabdovirus, **C** YN-PhleboV1 based on RdRp of bunyavirus, **D** YN-flaviV1 based on NS3 and NS5 of flavivirus, **E** YN-BHKV2 virus

based on RdRp of tetraparavirus (indicated by red dot) with their closest viral family or genus. The trees shown here were inferred using an ML method with 1000 bootstraps.

obviously separates into two sub-clades with one related to ticks and the other one related to insects or arthropods. We also found some short contigs related to NS3 and NS5 protein of flavivirus which share 80% aa identity with Bole tick virus 4 (BLTV4). BLTV4 was originally identified in *Hyalomma asiaticum* in Xinjiang Province of China and remained unclassified (Shi *et al.* 2016). We consider these flavi-like sequences detected in our study belong to a novel virus named Yunnan tick-associated flavi-like virus 1 (YN-FLV1).

Viruses Belong to *Parvoviridae*

A new strain of Bovine hokovirus 2 (BHKV2) was found in our 2017-YNT RNA-seq library named YN-BHKV2 (GenBank MH814981), the genome of which shares 100% identity with BHKV2 strain BS-S13, which belongs to *Tetraparvovirus* genus of *Parvoviridae*. The genome of YN-BHKV2 consists of two ORFs, with ORF1 encoding the non-structural protein (NS1) and ORF2 encoding the vial capsid protein (VP1) as well as a putative transmembrane protein (VP2) encoded by an overlapping reading frame in ORF2 (Fig. 2). Phylogenetic analysis with other viruses of *Tetraparvovirus* genus shows YN-BHKV2 clustered with bovine hokovirus within ungulate tetraparvovirus group 1. This group of viruses was mainly detected in bovine from Hong Kong and domestic yaks in northwest China. However, different from the genotype epidemic in northwestern of China, another genotype is epidemic in Yunnan Province which was identical to that of Hong Kong (Fig. 3E).

Virus Prevalence Among *R. microplus* Tick and Serum Samples

PCR-based and immunofluorescence-based epidemiological survey was performed to investigate the prevalence of

each virus in natural *R. microplus* tick during 2015–2017 (Table 3) and cattle and human sera (Table 4 and Fig. 4). IFA analysis of the positive control shows that the NP of each virus can be effectively expressed via transfected HEK293 (Fig. 4). YNMV1 virus has 20% or so positive rate among ticks from different landforms in different year. As for WHTV1, significantly high positive rate (42%) was detected in NJ in 2015, and relatively low positive rate (5%) or so was detected in LX in 2016 and 2017. Remarkably, serological assay shows high positive rate as 89% and 94% for YNMV1 and WHTV1, respectively among cattle serum. Comparing the above two viruses, prevalence of YN-PhleboV1 is much lower with 2.6% to 18.4%. Consistently, 8% cattle serum was positively detected with anti-YN-PhleboV1 antibody. And JMTV was only positively detected in one group of ticks from LX collected in 2015. Serological survey shows 18% cattle serum samples were detected positive for JMTV, meanwhile RNA was detected in four of these seropositive samples. Additionally, 63.1% tick samples from LX in 2017 and 40% cattle serum samples are positive for BHKV2. We also performed the same serological survey on 100 bovine serum samples from Hubei Province and healthy/febrile human serum from Yunnan LX, and no positive sample was detected with these five viruses. Collectively, these results suggest a stable and wild existence of YNMV1, WHTV1 and YN-PhleboV1 in *R. microplus* of Yunnan Province via circulation among ticks and cattle or may be other mammals.

Discussion

Our primary objective is to throw light on the virome profile of primary ticks *R. microplus* in Yunnan Province, which is the most important ectoparasite and disease vector of livestock globally. The most notorious pathogens

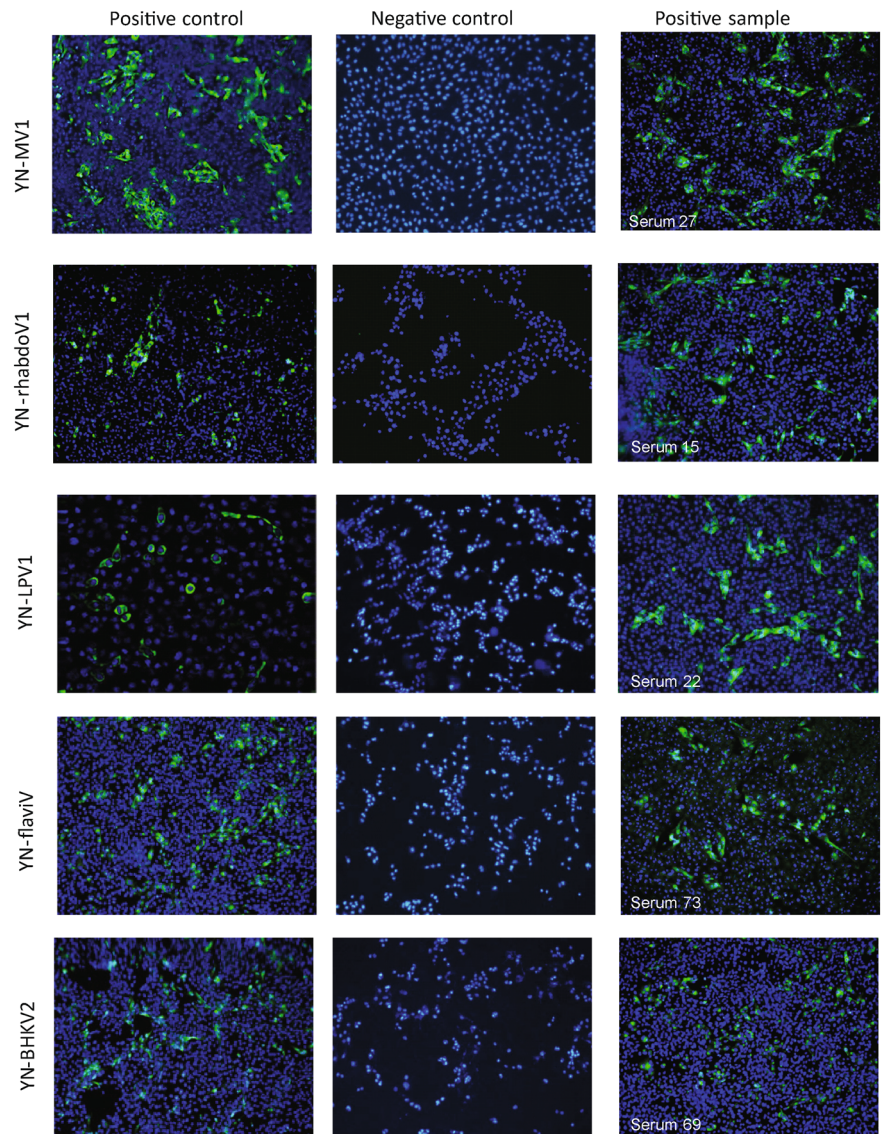
Table 3 PCR-based epidemiological survey of each virus among tick samples

	2015		2016		2017
	NJ (76)	LX (45)	DL (353)	LX (41)	LX (79)
Yunnan mivirus 1	28.9% (22/76)	24.4% (11/45)	24.9% (88/353)	19.5% (8/41)	17.8% (14/79)
Wuhan tick virus 1	42% (32/76)	12.9% (4/45)	15.8% (56/353)	4.8% (2/41)	5% (4/79)
YN tick-associated phlebovirus 1	2.6% (3/76)	11.1% (5/45)	18.4% (7/31)	4.8% (2/41)	15% (12/79)
Jingmen tick virus	0	1/45 (2.2%)	0	0	0
Bovine hokovirus 2	0	0	0	0	63.3% (50/79)

Table 4 IFA-based epidemiological survey targeting NP protein of each virus

	Cattle Serum sample		Human serum sample	
	Hubei (n = 100)	Yunnan (n = 100)	Febrile patient (n = 100)	Healthy human (n = 100)
Yunnan mivirus 1	0	89% (89/100)	0	0
Wuhan tick virus 1	0	94% (94/100)	0	0
YN tick-associated phlebovirus 1	0	4% (4/100)	0	0
Jingmen tick virus	0	18% (18/100)	0	0
Bovine hokovirus 2	0	40% (40/100)	0	0

Fig. 4 Representative results of seropositive cattle serum samples detected via IFA. Polyclonal antibody to NP was the primary antibody in positive control, and HEK293 transfected with empty pCDNA3.1+ plasmid in negative control. All the photos were taken at $\times 20$ magnification.



transmitted by *R. microplus* are Babesia bigemina, B. bovis and Anaplasma marginale (Parizi *et al.* 2015), which cause large economic loss in livestock industry.

Comparing to the pathogenic parasites, fewer viruses related to *R. microplus* were reported. Investigating the viral profile of *R. microplus* will improve our

understanding on the viral pathogens of *R. microplus*. Via three consecutive years of metagenomic sequencing, totally nine viruses concerning chu-, rhabdo-, phlebo- and flavi-like viruses were identified indicating the circulation of diverse tick-associated viruses in Yunnan.

The study identified two chuviruses with one novel species (YN-MV1) and one strain of a previously reported Bole mivirus (YN-MV2). And complete genome of YN-MV1 and partial sequence of YN-MV2 were obtained. Chuviruses constitute a large monophyletic group, locating at an intermediated phylogenetic branch between segmented and unsegmented negative-sense RNA viruses. Members of chovirus characterized with diverse genome organization including unsegmented, bi-segmented and circular forms are commonly identified in arthropods like ticks, spiders, true flies and crabs (Li *et al.* 2015; Brinkmann *et al.* 2018). Here, we complementarily provide the evidence that chovirus is ubiquitously prevalent among *R. microplus* ticks in Yunnan, southwest of China.

An increasing number of unassigned rhabdoviruses including the WHTV1 are identified within various arthropods (Li *et al.* 2015; Brinkmann *et al.* 2018). Because only proteins of RdRp and NP similar with that of rhabdovirus could be identified while the remaining corresponding proteins are absent in these group of rhabdovirus, which made it difficult to define their taxon within *Rhabdoviridae*. And it is proposed that these viruses should be considered as a member of the *dimarhabdovirus* supergroup (Bourhy *et al.* 2005; Brinkmann *et al.* 2018) (Fig. 3B). Our viral sequence findings further contribute to the understanding of viral taxon definition. And PCR-based survey for YNMV1 and WHTV1 among ticks provides preliminary evidence for their widespread circulation in *R. microplus*.

Three distinct tick-associated phleboviruses were detected with two novel phleboviruses (YN-PhelobV1, YN-PhelobV3) and one variant (YN-PLV2) of prototype virus Bole tick virus 1. And nearly complete *S* and *L* segment of YN-PLV1 were characterized while only short contigs of YN-PLV2 and YN-PLV3 were found. Phylogenetic analysis consistent with sequence comparison demonstrates YN-PLV1 mostly related to Uukuniemi phlebovirus and clustering with a group of novel phleboviruses which characterized with unknown *M* segment presently (Fig. 3C). The great diversity of glycoprotein coded by *M* segment make it difficult to search homologous protein for this group of novel viruses via homologous sequence alignment. Or, we can't exclude the possibility of the existence of bi-segment viruses which resemble to *Phlebovirus*, because examples of fully viable two-segmented Rift Valley fever virus (RVFV) (*Phenuisridae*, *Phlebovirus*) variant lacking the *M*-segment were documented (Kortekaas *et al.* 2011; Wichgers Schreur and

Kortekaas 2016). We are working on virus isolation and viral particles rescue via reverse genetic system, hoping to throw light on these confused puzzles.

Consecutive positive detection of YNMV1, WHTV and YN-PhleboV1 in ticks strongly indicates a stable and widespread circulation of these viruses among *R. microplus* in Yunnan. And all the ticks in our study are engorged and plucked from infested cattle, along with the significant high seropositive rate of 89% and 94% of YNMV1 and WHTV1 respectively. It's reasonable to speculate that these viruses are circulated via ticks among reservoir host including cattle or even other mammals, which deserve further research. Therefore, all the identified viruses may have the ability to infect cattle and elicit corresponding antibody. However, the lack of observed disease in cattle infected with identified viruses may be a consequence of long-established and stable co-existence of virus and host. But the possibility of changing vector or host adaptation causing by virus innate evolution should be taken into consideration. It would therefore be pertinent to initiate monitoring for the spread of these viruses to other animal species and new geographical locations.

Remarkably sequences related to parvovirus were only detected in ticks collected in LX in 2017. And the full-length genome of our strain YN-BHKV2 was identical to the prototype Bovine hokovirus 2 (BHKV2) strain HK-B38 which identified in beef from Hong Kong meat market and belonged to Ungulate tetraparvovirus 1 group (Cibulski *et al.* 2016). BHKV2 is a well confirmed infectious agent to cattle, we speculate that the positive tick groups of YN-BHKV2 in 2017 originate from infected domestic cattle, which can also be reflected by the high seropositive rate of 40% among cattle sera. And BHKV2 previously was reported broadly distributed in domestic yaks in northwestern China (Xu *et al.* 2016). Here, we firstly reported BHKV2 circulation in ticks of Yunnan indicating a wider circulation zone. What's more, BHKV2 belongs to the subfamily *Parvovirinae* whose members infect vertebrates while members of subfamily *Densovirinae* infect insects and arthropods (Cotmore *et al.* 2014). However, our findings suggested the probability that members of *Parvovirinae* may infect ticks and even transmitted via it, which deserves further investigation.

Finally, we have identified viral sequences related to flavivirus, which demonstrated 28% or so similarity to NS3 and NS5 protein of flavivirus. Sequences share 80% identity with BLTV4, which originally identified in Xinjiang Province of China in *Hyalomma asiaticum*, and it was proposed as a novel flavivirus-like virus named as YN-FlaviV1. Previously study shows a number of flavi-like viruses are detected via metagenomics in a range of arthropod species, including ticks, true flies, mosquitoes and spiders (Brinkmann *et al.* 2018). Though failed to

obtain the full-length genome of YN-FLV1, our findings supplementarily suggest that tick-associated flavivirus-like viruses are common and ubiquitous in *R. microplus* of Yunnan. The remaining flavi-related sequences show 93%–99% identity with JMTV strain SY84, which is a novel discovered four segmented RNA virus with phylogenetic relationship with flavivirus. Nearly complete genome of JMTV strain YN-flaviV was obtained in our study. Though only one group of ticks from LX in 2015 was detected with YN-flaviV, 18% seropositive results indicate this flavi-related virus may be circulated in Yunnan especially among cattle. And RNA of YN-flaviV was detected in four of the seropositive serum samples which further confirmed the serological result based on IFA. Previous prevalence survey indicated other ticks including *Ixodes* spp., *Armigeres* spp. and *Haemaphysalis* spp. from China and *R. microplus* from Brazil also carry JMTV (Qin *et al.* 2014; Souza *et al.* 2018). And it was subsequently found in the plasma of a red colobus monkey in Uganda (Maruyama *et al.* 2014). In addition, scientists have detected full-length genome of JMTV in three human Crimean-Congo hemorrhagic fever cases though no obvious symptoms were observed (Emmerich *et al.* 2018). And another Jingmenvirus member named Alongshan virus (ALSV) has more direct evidence for infectious to human since it is isolated from serum of a fever patient (Wang *et al.* 2019). And a number of reads with high similarity with Jingmenvirus have been identified in wild *P. leucopus* mice from North America (Vandegrift *et al.* 2020). Combined with our findings, it's reasonable to speculate that JMTV can circulate among domestic cattle and other unidentified vertebrate animals vectored by ticks, especially *R. microplus*. And questions about potential pathogenic effect of JMTV on human and animal health deserve further attention.

Many members of *Flavivirus* are TBVs and pathogenic to human or animals, such as tick-borne encephalitis virus (TBEV), Kyasanur Forest disease virus (KFDV), Louping ill virus (LIV). So, it is important to investigate this virus in respect of both evolution and pathogenicity to public health. We have tried to isolate JMTV via inoculation on new-born mice and kinds of mammal cell lines, including HEK293, BHK-21, Vero, PK15 and DH82 cell lines but failed. Recently, researchers have reported the successful isolation of JMTV via tick cell line BME/CTVM23 or BME26 and Vero cells, however, these cells seem not suitable enough for JMTV consistent maintenance. Very low virus titer and lack of plaque greatly hinder further study on JMTV (Dinçer *et al.* 2019; Jia *et al.* 2019). The investigation on the optimal cell line for JMTV and other Jingmenvirus propagation remains to be resolved. And to obtain in-depth understanding of transmission cycle on this flavi-related virus, further epidemiological survey including other domestic animals and ticks should be considered.

Furthermore, the existence of JMTV in monkey, cattle and febrile patient should be a warning that it may be a potential emerging zoonoses and/or tick-borne virus.

Notably, no positive sample was detected in serum samples from Hubei Province. However, JMTV1 and WHTV1 were all originally identified in *R. microplus* tick in Hubei Province (Li *et al.* 2015). We speculate that it is because the serum samples of Hubei come from the same farm while serum samples of Yunnan from scattering cattle raising households, which are more possible to expose to ticks. Such difference appears to reflect the role of ticks in transmitting viruses. Further surveillance on these viruses in different regions will contribute to the information of their geographical distribution.

Summarily, we provide a comprehensive investigation on virome profiles in *R. microplus* tick in Yunnan. Though no known viruses causing disease to human and animals were detected, our results can confirm the presence of chuo-, rhabdo-, phlebo-, flavi-like and parvovirus in *R. microplus* in Yunnan. And epidemiological survey suggests the stable and widespread circulation of YNMV1, WHTV1 and YN-PhleboV1 among ticks and cattle. Meanwhile, the occasionally positive tick and moderate seropositive cattle serum samples of segmented flavi-related virus and the parvovirus indicating the parasitized vertebrates may be the source in some instances. Our work highlights a remarkable diversity of RNA virus community and extends the current viral diversity. However, further studies are needed to reveal the role of these viruses in the pathogenesis of animals and humans.

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Author contributions SS, YZ and FD conceived and designed the experiments. JS, and HW performed the experiments. JS, SS and FD wrote the paper. YZ and FD finalized the manuscript. All authors have read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

Animal and Human Rights Collections of ticks and serum of cattles were approved by the Medical Ethics Committee of Dali University. Human serum samples were collected from patients and healthy volunteers with informed consent. All the procedures performed in studies involving animals were in accordance with the ethical standards of the Animal Care and Ethics Committees of Wuhan institute of virology, CAS (Approval numbers WIVA01201601).

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