Distribution of Mosquitoes and Mosquito-Borne Arboviruses in Yunnan Province near the China–Myanmar–Laos Border

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Abstract. Economic development and increased tourism in the southern region of Yunnan Province in China, adjacent to several countries in Southeast Asia, has increased the likelihood of import and export of vectors and vector-borne diseases. We report the results of surveillance of mosquitoes and mosquito-borne arboviruses along the border of China–Myanmar–Laos in 2005 and 2006, and information associating several arboviruses with infections and possibly disease in local human populations. Seventeen mosquito species representing four genera were obtained, and 14 strains of mosquito-borne viruses representing six viruses in five genera were isolated from *Culex tritaeniorhynchus*. In addition, IgM against Japanese encephalitis virus, Sindbis virus, Yunnan orbivirus and novel Banna virus was detected in acute-phase serum samples obtained from hospitalized patients with fever and encephalitis near the areas where the viruses were isolated. This investigation suggests that Japanese encephalitis virus, Sindbis virus, and lesser-known arboviruses circulate and may be infecting humans in the China–Myanmar–Laos border region.

INTRODUCTION

Yunnan Province, located in southwestern China, has a 4,061 km border with Myanmar, Laos, and Vietnam. This area is characterized by low elevation and a tropical to southern subtropical climate with high temperatures, abundant rain, and high humidity. A great diversity of hosts and mosquito vectors are found in Yunnan Province, which support transmission of numerous arboviruses.^{1–7} Surveillance conducted in the area showed that Japanese encephalitis (JE) and dengue (DEN) fever cases have been frequently reported in recent years.^{5,8–10} In addition, fevers and encephalitis of unknown etiology are commonly reported in this area during the JE and DEN epidemic seasons.¹¹

Previous investigations in this area also indicated that antibodies against multiple arboviruses, including Japanese encephalitis virus (JEV), dengue virus, chikungunya virus, Sindbis virus (SINV), and Batai virus (BATV), were detected in serum samples of local residents and animals.^{11,12} However, no investigations of arboviruses and related infections in humans have been conducted specifically in the border of southern Yunnan Province.

Therefore, to describe the vectors and arboviruses in this area and provide a scientific basis for further investigations of fever and encephalitis, we collected mosquito samples in the border area in 2005 and 2006 and identified the mosquito species and the viruses in those specimens. In addition, we collected serum samples from hospitalized patients with fever and encephalitis in the Xishuangbanna region of the border between China, Myanmar, and Laos.

MATERIALS AND METHODS

Mosquito collection. Mosquito samples were collected during July 2005 and July 2006 in residential regions of three

villages of Manxi (Daluo Town) (21°42'16.01"N, 100°02'50.09"E, altitude = 676 meters); Manguo (Menghun Town) (21°48'05.53"N, 100°23'28.11"E, altitude = 1,203 meters); and Manen (Mengzhe Town) (21°56'38.35"N, 100°21'50.75"E, altitude = 1,195 meters) in Menghai County, Xishuangbanna, in southern Yunnan Province (Figure 1).

Mosquitoes were collected from dwellings and from chicken and bovine shelters at night by using light traps (12 V, 300 mA; Wuhan Lucky Star Environmental Protection Tech Co., Ltd., Hubei, China). Traps were used from 9:00 PM to 5:00 AM the next morning, which spanned the period from sunset to sunrise. Captured mosquitoes were killed by freezing at –20°C for at least 40 minutes. Specimens were placed on a chill plate, and species were identified by using morphologic characteristics.¹³ Mosquitoes were combined into pools of ≤ 100 specimens and stored in liquid nitrogen until virus testing was conducted.

Virus isolation. Pools of mosquitoes were homogenized in a sterile glass grinder and centrifuged as reported.^{14,15} To isolate the virus, 150 µL of supernatant from each pool was inoculated onto monolayers of BHK-21 cells (2 × 10⁵ cells/mL) and C6/36 cells (1 × 10⁶ cells/mL) in a 1-mL tube. After seven days, the 150-µL supernatant from the previous passage was inoculated onto the new passage in BHK-21 cells or C6/36 cells as above. The cells were maintained at 37°C or 28°C, respectively, and examined daily for cytopathic effect (CPE) from day 1 through day 7 post-inoculation. A specimen was regarded as a positive isolate if it caused CPE in three successive cell passages. Infected cell supernatants were harvested and stored at –80°C until further identification.

Identification of virus. Immunofluorescent assay. Cell cultures that exhibited 75% CPE were treated with trypsin and collected by centrifugation at 1,000 rpm for 10 minutes. They were washed three times with 0.01 M phosphate-buffered saline (PBS). After re-suspension in PBS, aliquots of the cell suspension were placed on multi-well slides, dried at room temperature, and fixed in acetone for 15 minutes at 4°C. The fixed slides were probed with a panel of mouse antisera against alphaviruses and flaviviruses and incubated at 37°C for 30 minutes as described.^{16,17} The antisera were prepared in our laboratory and included broadly reactive

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FIGURE 1. Mosquito collection sites in Menghai County of Yunnan Province, China. The star indicates location of the mosquito collection sites and shading indicates location of the serum collection sites. Distance between Manguo Village and Manen Village is 20.2 km, distance between Manguo Village and Manxi Village is 52.2 km, and distance between Manen Village and Manxi Village is 69.0 km.

immune ascites against alphaviruses, specific antisera to SINV, Getah virus, Mayaro virus, chikungunya virus, and Simliki Forest virus, and antisera against the flaviviruses JEV, dengue virus, and West Nile virus. Antisera were diluted 1:50 in PBS before use. After subsequent rinses in PBS and sterile water, the slides were probed with the secondary rabbit anti-mouse monoclonal fluorescein isothiocyanate conjugate (Sigma, St. Louis, MO) at 37°C for 30 minutes before visualization with a fluorescent microscopy. Positive and negative controls of immune ascites against alphaviruses were SINV (YN87448) and JEV (P3), respectively. Positive and negative controls of immune ascites against flavivirus antisera were JEV (P3) and SINV (YN87448), respectively.

Polyacrylamide gel electrophoresis, RNA extraction, cDNA synthesis, reverse transcription-polymerase chain reaction, and sequence analysis. RNA extraction and polyacrylamide gel electrophoresis were performed as described.¹⁸ Briefly, double-stranded RNA was extracted from approximately 400 µL of cell suspension with phenol/chloroform. Each RNA sample was mixed with sample buffer and subjected to electrophoresis at room temperature on a standard discontinuous 7%, 10%, or 15% acrylamide (acrylamide/bisacrylamide 29:1; Bio-Rad Laboratories, Hercules, CA) slab gel (18 × 16 × 0.075 cm) (Hoefer Pharmacia Biotech Inc., San Francisco, CA) with a 3.5% acrylamide stacking gel in Tris-glycine buffer (25 mM Tris, 192 mM glycine, pH 8.3) (Bio-Rad Laboratories). After electrophoresis, virus double-stranded RNAs were visualized by staining with silver nitrate.

Viral RNA was extracted by using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA), and cDNA was synthesized by using Ready-To-Go You-Prime First Strand Beads (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's procedure. Samples were tested by polymerase chain reaction (PCR) with flavivirus-specific primers,¹⁹

alphavirus-specific primers²⁰ and primers to detect the SINV nonstructural protein 2 (NS2) gene, the JEV envelope (E) gene, the 12th segment of Banna virus (BAV),²¹ the VP7 gene of Yunnan orbivirus (YUOV),²² and the densovirus (DNV) partial NS1 gene¹⁵ (Table 1).

The reverse transcription-PCR conditions were 1 hour at 37°C, 5 min at 94°C, and 35 cycles of amplification (30 seconds at 94°C, 30 seconds at 55°C, and 2 minutes at 72°C), and final extension at 72°C for 10 minutes. Amplified products were separated by electrophoresis on 1% agarose gels in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), visualized by staining with ethidium bromide, and viewing under ultraviolet light. The PCR products were purified by using the TaKaRa DNA fragment Purification Kit Version 2.0 (TaKaRa, Dalian, China), and sequenced by the Beijing Genome Institute, Beijing, China.

Initial sequence assembly and analysis were conducted by using the ATGC software package Version 4.0 (GENETYX Corp, Tokyo, Japan). Homology and alignment analysis was carried out by using the Clustal X (Version 1.83) and MegAlign (DNASTAR, Madison, USA). MEGA 3.1²³ was used for phylogenetic analysis and tree construction was based on the neighbor-joining assay. The bootstrap value (the number of replications) was 1,000.

Serologic investigation. Sample collection. Serum specimens from patients with acute fever or encephalitis were obtained from hospitals and clinics in the Xishuangbanna region near locations where mosquito samples were obtained. Specimens were sent to the Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention for testing.

Serologic testing. IgM against JEV was detected by using the JEV-IgM enzyme-linked immunosorbent assay (ELISA) Kit (Beixi, Shanghai, China) as described in the manufacturer's protocol. Serum samples were not tested for IgG against JEV because JE was an important disease in the China-Myanmar-Laos border region in previous surveillance, and serum specimens were obtained from patients with acute fever or encephalitis within one week. After identification of the viruses isolated from mosquitoes in the area, ELISA detection of IgM or IgG was performed on the specimens that were negative for IgM against JEV as described.²⁴

Briefly, isolate MX10 (SINV) was inoculated into BHK-21 cells and isolates MZ93 (YUOV), MZ65 (novel Yunnan orbivirus [NYUOV]) and MX6 novel BAV virus (NBAV) were propagated in C6/36 cells. Culture supernatants were obtained when they showed 75% CPE and centrifuged for 15 minutes at 5,000 rpm at 4°C. The supernatant was filtered through a 0.22-µm filter, and the filtrate was concentrated by using a 100k Millipore tube (Millipore Corporation Billerica, MA). The virus was inactivated at 56°C for 30 minutes and stored at -70°C.

Human serum samples were tested for virus-specific IgM and IgG against SINV, NBAV, YUOV, or NYUOV by using the MX10, MZ93, MZ65 and MX6 antigens produced in cell culture.²⁴ Box titration was performed to determine the optimal antigen dilution (1:400 in bicarbonate buffer, pH 9.6). After incubation with 100 µL of antigen dilution overnight at 4°C, 96-well plates were washed five times with PBS, 0.05% Tween 20 (PBS-T) and blocked with 200 µL of 5% skim milk in PBS for 2 hours at 37°C. Diluted sera (1:200 in PBS-T and 5% skim milk) were incubated for 60 minutes at 37°C on

TABLE 1
Primers used to identify and sequence viruses in this study*

Primers	Primers sequence (5'→3')	Size, basepairs (reference)
Alphavirus		
M2w	YAGAGCDTTTTCGCAYSTRGCHW	430 ⁽¹⁷⁾
cMw3	ACATRAANKGNGTNGTRTCRAANCCDAYCC	
SINV NS2 gene primers		
SINV-NS2F (1761–1780)	CACCGACCTCTGTACTGAAG	1,438
SINV-NS2R (3198–3179)	GGGAACAACCTCACTCCACTG	
Flavivirus		
Fu1	TACCACATGATGGGAAAGAGAGAGAA	310 ⁽¹⁸⁾
cFD3	GTGTCCCAGCCGCGGTGTCTATCAGC	
JEV E gene primers		
JEV-EF (955–974)	TGYTGGTCGCTCCGGCTTA	1,526
JEV-ER (2481–2461)	GATGTCAATGGCACATCCAGT	
BAV S12 gene primers		
12-854-S	AAATTGATAGYGYTTGCGTAAGAG	845 ⁽¹⁹⁾
12-B2-R	GTTCTAAATTGGATACGGCGTGC	
YUOV VP7 gene primers		
YUOV57F	AGCATTCGGTACGCAGTATCTCG	470 ⁽²⁰⁾
YUOV57R	GCCGAGCCGATCATGTACAGTGT	
DNV NS1 gene primers		
DNVNS1F (298–317)	GGTGATTCTGGTTCTGACTCTTG	1,035 ⁽¹³⁾
DNVNS1R (1332–1354)	GARRGVCARAGYGAGWDATG	

* SINV = Sindbis virus; NS = nonstructural; JEV = Japanese encephalitis virus; E = envelope; BAV = Bannan virus; YUOV = Yunnan orbivirus; DNV = densovirus

antigen-coated wells, washed five times with PBS-T, and incubated for 60 minutes at 37°C with 100 µL of horseradish peroxidase-labeled rabbit-anti-human IgM (diluted 1:50,000) or IgG (diluted 1:4,000) (Sigma), and washed. The substrate reaction with 100 µL of A and B solutions (Sigma) at room temperature for 10 minutes was stopped by addition of 4N H₂SO₄. Optical density was measured at 450 nm. A serum sample was considered seropositive if the positive/negative value was > 2.1.²⁵

Serum samples that had had high antibody neutralization titers were used as positive control serum samples for the ELISA. Mixtures of 20 serum specimens obtained from healthy persons of difference ages were use as controls. All specimens were negative for IgM and IgG against viruses isolated from the China–Myanmar–Laos border region by ELISA and neutralization tests.

RESULTS

Mosquito collection. A total of 7,952 mosquitoes were obtained in July 2005, and 6,771 mosquitoes were obtained in July 2006 (Table 2). Mosquitoes obtained in the three sites represented 17 species belonging to four genera (*Culex*, *Linnaeus*; *Anopheles*, *Meigen*; *Armigeres*, *Theobald*; and *Lutzia*, *Theobald*). In Manxi, 11 species were collected, among which *Cx. tritaeniorhynchus* Giles was the dominant species (63.2% in 2005 and 61.1% in 2006), followed by *Ar. subalbatus* (Coquillett) (12.1% in 2005 and 12.6% in 2006) and *Cx. bitaeniorhynchus* Giles (9.9% in 2005 and 11.4% in 2006). The other eight species accounted for less than 9% of the total. In Manen, 11 species were collected. *Culex tritaeniorhynchus* was the dominant species (90.4% in 2005 and 91.7% in 2006) and *Anopheles sinensis* Wiedemann was the next highest (4.7% in 2005 and 4.3% in 2006). The other nine species accounted for less than 3% of the total. Only six mosquito species were obtained in Manguo and the dominant species was *An. sinensis* (66.5% in 2005 and 68.1% in 2006). *Culex tritaeniorhynchus* comprised 26.4% in 2005 and 25.2% in 2006, and the remaining species accounted for less than 7% of the total obtained in Manguo.

Virus isolation and identification. A total of 14 strains were isolated from the 152 pools of mosquitoes obtained in three sites during July 2005 and July 2006. Isolate MX10 showed a CPE in BHK21 cells; 11 isolates (MX6, MZ11, MZ13, MZ20, MZ25, MZ30, MZ31, MZ43, MZ65, MH76, and MZ93) showed a CPE only in C6/36 cells, and the remaining 2 isolates (MZ24 and MZ66) showed a CPE in BHK-21 cells and C6/36 cells. All 14 strains of viruses were isolated from *Cx. tritaeniorhynchus* (Table 3).

Japanese encephalitis virus. Virus isolates MZ24 and MZ66 reacted with antibody against JEV by immunofluorescent assay (IFA), suggesting that these isolates were JEV or a related flavivirus. Polymerase chain reaction and sequence analysis showed that that these 2 isolates contained gene sequences indicative of JEV. The E gene sequence of MZ24 and MZ66 was amplified and produced a 1,500-nucleotide segment that belonged to a JE virus genotype similar to JEV YN03-A151 (percentage nucleotide identities for MZ24 and MZ66 were 99.1%) and YN04-25-3 (percentage nucleotide identities for MZ24 and MZ66 were 97.1%) strains isolated in China²⁶ and showed the closest relationship with the JEV-P3 (percentage nucleotide identities for MZ24 and MZ66 were 98.9%) strain isolated in 1949, which also belonged to genotype III (Figure 2).

Sindbis virus. The IFA results demonstrated that MX10 had a strong reaction with antibodies against alphavirus and SINV and a weak reaction with antibodies against chikungunya, Mayaro, and Getah viruses. Identification of MX10 as an alphavirus was confirmed by PCR and sequence analysis, which showed that the sequence was highly homologous to the prototype of Oriental/Australian genotype SINV MRE16 (90.8%). Phylogenetic analysis of the MX10 NS2 gene sequence (1,000 nucleotides) (Figure 3) showed that MX10 can be placed within a cluster formed with other SINV sequences. Two relatively independent branches, including the Paleoarctic/Ethiopian genotype represented by SAAR86 virus and Oriental/Australian genotype represented by MRE-16 virus isolated in Malaysia, have been described.²⁷ MX10 was in the Oriental/Australian branch, and the other isolates

TABLE 2
Mosquito abundance in border areas in Yunnan, China–Myanmar–Laos, July 2005 and July 2006*

Genus	Species	Manxi				Manguo				Manen				Total			
		2005		2006		2005		2006		2005		2006		2005		2006	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
<i>Culex</i>	<i>Cx. tritaeniorhynchus</i>	1,364	63.2	474	61.1	912	26.4	463	25.2	3,434	90.4	2,490	91.7	5,710	60.7	3,427	64.38
	<i>Cx. whitmorei</i>	23	1.07	6	0.7	0	0	0	0	5	0.1	2	<0.1	28	0.2	8	0.1
	<i>Cx. pseudovishnui</i>	0	0	0	0	0	0	0	0	32	0.8	18	0.6	32	0.3	18	0.3
	<i>Cx. fuscocephala</i>	23	1.0	7	0.9	5	0.1	3	0.1	81	2.1	50	1.8	109	1.1	60	1.1
	<i>Cx. gelidus</i>	0	0	0	0	0	0	0	0	11	0.2	6	0.2	11	0.1	6	0.1
	<i>Cx. nigropunctatus</i>	0	0	0	0	3	<0.1	1	<0.1	0	0	0	0	3	<0.1	1	<0.1
	<i>Cx. pallidothorax</i>	5	0.2	2	0.2	0	0	0	0	0	0	0	0	5	<0.1	2	<0.1
	<i>Cx. bitaeniorhynchus</i>	215	9.9	89	11.4	0	0	0	0	0	0	0	0	215	2.2	89	1.6
<i>Lutzia</i>	<i>Lu. fuscus</i>	1	<0.1	1	0.1	0	0	0	0	0	0	0	0	1	<0.1	1	<0.1
<i>Anopheles</i>	<i>An. sinensis</i>	164	7.6	63	8.1	2,294	66.5	1,250	68.1	181	4.7	117	4.3	2,639	28.0	2,050	38.5
	<i>An. vagus</i>	0	0	0	0	0	0	0	0	8	0.2	4	0.1	8	<0.1	4	<0.1
	<i>An. peditaeniat</i>	53	2.4	21	2.7	225	6.5	114	6.2	31	0.8	19	0.7	309	3.2	154	2.9
	<i>An. maculatus</i>	0	0	0	0	0	0	0	0	2	<0.1	1	<0.1	2	<0.1	1	<0.1
	<i>An. tessellatus</i>	0	0	0	0	8	0.2	3	0.1	7	0.1	4	0.1	15	0.1	7	0.1
	<i>An. annularis</i>	36	1.6	11	1.4	0	0	0	0	0	0	0	0	36	0.3	11	0.2
	<i>An. barbirostris</i>	11	0.5	3	0.3	0	0	0	0	0	0	0	0	11	0.1	3	<0.1
	<i>Ar. subalbatus</i>	261	12.1	98	12.6	0	0	0	0	5	0.1	3	0.1	266	2.8	101	1.9
Total		2,156		775		3,447		1,834		3,797		2,714		9,400		5,323	

* Values are no. mosquito adults collected and percentage of the total collection for each species within the location and year.

of SINV isolated in China (YN87448 virus from the border area of China/Laos in 1987^{28,29} and XJ-160 virus from Xinjiang Uighur Autonomous Region of Western Province in China³⁰) belong to the Palearctic/Ethiopian genotype.

Orbivirus. Eleven isolates that caused a CPE in C6/36 cells but did not react with alphavirus and flavivirus antisera by IFA were identified by polyacrylamide gel electrophoresis. Results for seven strains (MZ11, MZ20, MZ30, MZ25, MZ43, MZ65, and MZ93) showed that all the virus isolates were 10-segment double-stranded RNA viruses (Figure 4). The migration pattern of bands for MZ65 distinguished it from the other six strains. Six strains (MZ11, MZ20, MZ30, MZ25, MZ43, and MZ93) were amplified by using primers derived from segment 7 of YUOV, but MZ65 failed to amplify. The phylogenetic tree based on the six new isolates and other orbiviruses demonstrated a close genetic relationship between the six new isolates and the YUOV 77-2 strain,¹ nucleotide sequence identity of > 93%, and a distant relationship with the other seven orbiviruses analyzed, indicating that the six new

isolates from the China–Myanmar–Laos border area were strains of YUOV (Figure 5).

Strain MZ65 is also a 10-segment double-stranded RNA virus, but was distinguished from the other six orbivirus strains by the band migration pattern, and the observation that MZ65 virus was not amplified with the primers designed for the segment 7 of YUOV.²¹ Further sequence analysis showed that segment 10 had a low level of nucleotide sequence identity with YUOV (54.4%) and formed relatively independent branches in a phylogenetic tree based on the MZ65 and other orbiviruses, suggesting that MZ65 virus is considerably different from YUOV. This virus is referred to as NYUOV.

TABLE 3

Virus isolates from *Culex tritaeniorhynchus* mosquitoes collected in the Manxi, Manen, and Manguo areas of Yunnan Province, China*

Isolate	Location	Habitat	Cell line	Year of isolation	Virus
MX6	Manxi	Cattle pen	C6/36	2005	BAV
MX10	Manxi	Cattle pen	BHK-21, C6/36	2005	SINV
MZ11	Manen	Cattle pen	C6/36	2005	YUOV
MZ13	Manen	Cattle pen	C6/36	2005	DNV
MZ20	Manen	Cattle pen	C6/36	2005	YUOV
MZ24	Manen	Cattle pen	BHK-21, C6/36	2005	JEV
MZ25	Manen	Cattle pen	C6/36	2005	YUOV
MZ30	Manen	Cattle pen	C6/36	2005	YUOV
MZ31	Manen	Cattle pen	C6/36	2005	DNV
MZ43	Manen	Cattle pen	C6/36	2005	YUOV
MZ65	Manen	Cattle pen	C6/36	2006	NYUOV
MZ66	Manen	Human house	BHK-21, C6/36	2006	JEV
MH76	Manguo	Cattle pen	C6/36	2006	DNV
MZ93	Manen	Cattle pen	C6/36	2006	YUOV

* BAV = Banna virus; SINV = Sindbis virus; YUOV = Yunnan orbivirus; DNV = Dengue virus; JEV = Japanese encephalitis virus; NYUOV = Novel Yunnan orbivirus.

FIGURE 2. Phylogenetic tree based on the envelope (E) gene of selected Japanese encephalitis virus strains, Yunnan Province, China. Isolates used in this analysis and their GenBank accession numbers are indicated. Murray Valley encephalitis (MVE) E gene was used as an outgroup. Genotypes I–IV are indicated on the right.

FIGURE 3. Phylogenetic tree based on the nonstructural protein 2 gene of Sindbis MX10 virus from Yunnan Province, China. Isolates used in this analysis and their GenBank accession numbers are shown in the tree. SINV = Sindbis virus; CHIKV = chikungunya virus; ONNV = O'nyong-nyong virus; MAYV = Mayaro virus; SFV = Semliki forest virus; MIDV = Middelburg virus; GATV = Getah virus; RRV = Ross River virus; BHV = Barmah forest virus; VEEV = Venezuelan equine encephalitis virus; WEEV = western equine encephalomyelitis virus; EEEV = eastern equine encephalitis virus; P = paleoartic/Ethiopian; O = Oriental/Australian. Genotypes (O and P) are indicated on the right.

Banna virus. Results of polyacrylamide gel electrophoresis demonstrated that the MX6 strain was a 12-segmented double-stranded RNA with migrating bands different from those found in Liaoning virus and Kadipiro virus, but with a pattern more similar to BAV (6-4-2) (Figure 6). Further sequence analysis showed that nucleotide sequence identity of MX6 segment 9 to BAV segment 9, Liaoning virus segment 11 and Kappdio virus segment 10 was 45%, 35.9%, and 37.8%, respectively, and the phylogenetic tree based on the MX6 virus and BAV, Laioning virus, and Kappdio virus further demonstrated that MX6 was placed within a branch formed

FIGURE 4. Electrophoretic migration patterns of genomic double-stranded RNA of orbivirus isolates from Yunnan Province, China, as analyzed by polyacrylamide gel electrophoresis. Lane 1, C6/36 cell control; lane 2, MZ93; lane 3, MZ43; lane 4, MZ65; lane 5, MZ30; lane 6, MZ25; lane 7, MZ20; lane 8, MZ11.

FIGURE 5. Phylogenetic tree based on 430 nucleotides of segment 6 of Yunnan orbivirus isolates from Yunnan Province, China. Strains used in this analysis and their GenBank accession numbers are shown in the tree. YUOV = Yunnan orbivirus; BTV-8 = bluetongue virus 8; BTV-15 = bluetongue virus 15; EHDV = epizootic hemorrhagic disease virus; AHSV = African horse sickness virus; PHSV = Peruvian horse sickness virus; PCV = Palyam chuzan virus; StCRV = St. Croix River virus; CMPV = California mosquito pool virus.

with the BAV sequences (Figure 7), suggesting that MX6 virus may be an NBAV.

Densovirus. Primers specific for densovirus were used to amplify the NS1 gene of MZ13, MZ31, and MH76 isolates to produce 1,000-nucleotide products. Phylogenetic analysis

FIGURE 6. Electrophoretic migration patterns of genomic double-stranded RNA of seadornavirus isolate MX6 from Yunnan Province, China, as analyzed by polyacrylamide gel electrophoresis. Lane BAV, Beijing-75; lane KDV, YN0557; lane LNV, LNV-31.

FIGURE 7. Phylogenetic tree based on 1,085 nucleotides of segment 9 of novel Banna virus isolate MX6 from Yunnan Province, China. Strains used in this analysis and their GenBank accession numbers are shown in the tree. BAV = Banna virus; KDV = Kappdio virus; LNV = Liaoning virus.

using the sequence of the NS1 gene from 14 densoviruses demonstrated that MZ13, MZ31, and MH76 strains are located in the same cluster as *Cx. pipiens pallens* densovirus (CppDNV)-XJ058, CppDNV-YN05217, CppDNV-GZWN1 and CppDNV-JZ-16 (Figure 8), with a nucleotide sequence identity > 99%. These results suggest that MZ13, MZ31, and MH76 are densoviruses and have close genetic relationships with other strains isolated from *Cx. tritaeniorhynchus* in Yunnan and Shanxi provinces.¹⁵

Viral antibodies in human serum samples. The objective of the serum survey in this study was to understand infections with arboviruses, except for JEV. IgM against JEV was first detected in serum samples from patients with acute fever or encephalitis. Detection by ELISA of IgM or IgG against viruses isolated from mosquitoes in the area was then performed on specimens that were negative for IgM against JEV. The results show that of the 184 serum samples obtained from hospitalized patients, 26.6% (49 of 184) were positive for IgM against JEV (Table 4). Of these patients, 19.6% (20 of 102) had fever and 35.3% (29 of 82) had encephalitis. The results suggest that JEV infection persists in the region. Of the remaining 135 serum samples that were negative for antibodies against JEV,

FIGURE 8. Phylogenetic tree based on 903 nucleotides of the non-structural protein 1 gene of densovirus isolate from Yunnan Province, China. Isolates used in this analysis and their GenBank accession numbers are shown in the tree. CppDNV = *Culex pipiens pallens* densovirus; AaeDNV = *Aedes denonucleosis* virus; AalDNV = *Aedes albopictus* densovirus; HeDNV = *Haemagogus equinus* densovirus; DsDNV = *Diatraea saccharalis* densovirus; GmDNV = *Galleria mellonella* densovirus; BmDNV = *Bombyx mori* densovirus; JcDNV = *Junonia coenia* densovirus; PfdNV = *Periplaneta fuliginosa* densovirus.

antibodies against SINV (0.7% IgM, 12.6% IgG), YUOV (0.7% IgM, 5.9% IgG), NYUOV (0% IgM, 3.7% IgG), and NBAV (0.7% IgM, 5.2% IgG) were detected, demonstrating past and potentially some recent infections with these viruses and suggesting the potential that they may have been the disease etiology. Unfortunately, convalescent-phase serum samples were not available and tests to confirm acute infection associated with the symptoms could not be conducted.

DISCUSSION

The topography of the China–Myanmar–Laos border in this area is complex and has numerous mountains, hills, plateaus, and rivers. Characteristic of this area are structures called bazi (small basins) by local residents. These habitats are surrounded by high mountains and are relatively isolated. Although many small basins are only several kilometers apart, their altitude, temperature, and humidity differ and they support distinct biotas.³¹ The three collection sites in this study were located in different basins with different altitudes (600 meters for Manxi, 1,000 meters for Manen, and 1,400 meters for Manguo). The mosquito species distribution varied though the sites, which are only 7–30 km apart. In this investigation, we found 17 mosquito species belonging to four genera. *Anopheles sinensis* was the dominant mosquito species in Manguo. In contrast, *Cx. tritaeniorhynchus* was the dominant mosquito species in Manxi and Manen. In addition, a previous epidemiologic investigation demonstrated that malaria transmitted by *An. sinensis* was prevalent in Manguo.³² Cases of JE were reported in Manxi and Manen.³³ These results are consistent with our observations of the dominant mosquito species in each area.

The local climate characteristics are suitable for survival and reproduction of mosquitoes and conducive to the sustained transmission of arboviruses. Previous studies have demonstrated that the distribution, seasonal peak, and virus infection rate of JEV in *Cx. tritaeniorhynchus* were coincident with the peak of JE in humans.³⁴ In our investigation, all other virus strains isolated were also from *Cx. tritaeniorhynchus*, suggesting that this species is an important vector of SINV, YUOV, and BAV in the border area.

Japanese encephalitis is a mosquito-borne arboviral disease that is prevalent in Asia and Southeast Asia. The annual case rate of JE in this region of China is 5.25/100,000,³⁵ which is approximately twice as high as the average case rate in the rest of Yunnan Province (3.04/100 000).³⁶ In contrast, the JE incidence for China as a whole is 2–3/100 000.³⁷ Our virus isolation results demonstrate that *Cx. tritaeniorhynchus* remains the dominant JEV vector in the region. The prevalence rate of 26.6% for IgM against JEV in serum samples obtained from hospitalized patients suggests that JE remains a public health problem. Although we cannot exclude the possibility that the IgM against JE we detected was from prior vaccination rather than from infection with wild-type JEV, it is unlikely that the IgM was vaccine induced because this area is rural, mass vaccination programs have not been conducted there, and the rate of vaccination is estimated to be < 10% in this area.³⁸

Sindbis virus has a broad distribution and causes fever in humans and symptoms that include arthralgia, rash, and malaise. This virus has caused outbreaks in Finland,^{39,40} Sweden,⁴¹ and South Africa.⁴² It is divided into Paleoarctic/Ethiopian and Oriental/Australian genetic types.⁴³ The SINV strain MX10 isolated from mosquitoes in this study is in the Oriental/

TABLE 4

Percentage of patients with fever and encephalitis from the Xishuangbanna border region, China, with antibodies against selected arboviruses in acute phase serum samples*

Symptom	JEV		SINV (MX10)		YUOV (MZ93)		NYUOV (MZ65)		BAV (MX6)	
	IgM		IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
Fever	19.6% (20/102)		1.2% (1/82)	12.2% (10/82)	1.2% (1/82)	7.3% (6/82)	0 (0/82)	6.1% (5/82)	1.2% (1/82)	7.3% (6/82)
Encephalitis	35.3% (29/82)		0 (0/53)	13.2% (7/53)	0 (0/53)	3.7% (2/53)	0 (0/53)	0 (0/53)	0 (0/53)	1.8% (1/53)
Total†	26.6% (49/184)		0.7% (1/135)	12.6% (17/135)	0.7% (1/135)	5.9% (8/135)	0% (0/135)	3.7 (5/135)	0.7% (1/135)	5.2% (7/135)

* JEV = Japanese encephalitis virus; SINV = Sindbis virus; YUOV = Yunnan orbivirus; NYUOV = Novel Yunnan orbivirus; BAV = Banna virus. Antibodies against JEV were detected by using a commercial assay. Antibodies against other viruses were detected by using virus strain in parentheses as antigen in enzyme-linked immunosorbent assays as described in text.

† Only serum samples negative for IgM against JEV were tested for antibodies against other agents.

Australian branch, and the other strains of SINV previously isolated in China (YN87448 virus from the border area of China/Laos in 1987^{28,29} and XJ-160 virus from Xinjiang Uighur Autonomous Region of Western Province in China)³⁰ belong to the Paleoarctic/Ethiopian genotype. This finding indicates that two genotypes of SINV are present in China and that both occur in the border area of Yunnan Province. A previous serosurvey in Yunnan showed that prevalence of IgG against SINV was high in serum of patients with fever and in healthy people, and that antibody to SINV could be found in bats and rats in the region.^{36,44} Our results are similar and support this observation, reinforcing the speculation that SINV or a closely related alphavirus may be causing human disease in the area.

Yunnan orbivirus is a 10-segmented double-stranded RNA virus that was first isolated from *Cx. tritaeniorhynchus* obtained in China.²¹ The six strains of YUOV in this study were also isolated from *Cx. tritaeniorhynchus*, suggesting that YUOV persists in this area and that *Cx. tritaeniorhynchus* is likely the main vector. The MPOV isolate, which is similar to YUOV, was isolated from different cattle herds during 1994–2006, including 112 isolates in 2006.⁴⁵ The mosquitoes from which YUOV was isolated in our study were obtained in a bovine shelter, suggesting that cows may be the likely host YUOV in this region. Detection of IgM and IgG against YUOV in serum of one patient suggests that human infections with this virus may occur. Our results also suggest that MZ65 virus is considerably different from YUOV and that detection of antibodies against this strain in local patients suggests an association between MZ65 and human potential infections that should be investigated.

The MX6 strain was a 12-segmented double-stranded RNA with a pattern more similar to BAV (6-4-2). Sequence analysis showed that the nucleotide sequence identity of MX6 with BAV was 45%. The MX6 strain was placed within a branch formed with BAV, suggesting that MX6 virus may be a novel BAV variant and needs further study. Banna virus, considered a potential pathogen by some investigators,⁴⁶ was first isolated in China from patients with encephalitis in Xishuangbanna Autonomous Prefecture, Yunnan Province in 1987.⁴⁷ Subsequently, 10 strains of BAV were isolated from patients with fever and encephalitis and from mosquitoes, pigs, and cows.^{48,49} In addition, Chen and Tao⁵⁰ found evidence of four-fold changes in neutralization antibody titer against BAV in acute-phase and convalescent-phase serum samples from the fever and encephalitis patients, suggesting that BAV was the etiologic agent of their illnesses. Our detection of IgM and IgG against BAV and NBAV support speculation that BAV may be a pathogen in the region. In this study, IgM and IgG against NBAV were detected in serum samples of patients with fever and encephalitis, indicating that NBAV may be causing infections in patients with fever and encephalitis in the border area.

Densoviruses are classified as members of the genera *Densovirus*, *Iteravirus*, *Brevdensovirus*, and *Pefudensovirus* (subfamily *Densovirinae*) within the family *Parvoviridae*.¹⁵ Identification of DNVs in various mosquito cells and wild mosquitoes suggests that the brevidensoviruses have a wide-spread distribution.⁵¹ Densovirus was isolated from several species of mosquitoes, including *Cx. pipiens pallens*, *Cx. pipiens quinquefasciatus*, *Cx. tritaeniorhynchus*, and *An. sinensis* in China.¹⁵ The three strains of DNV in this study were also isolated from *Cx. tritaeniorhynchus*, indicating that these insect viruses may be extensively distributed in China. Because densovirus is not known to cause human infection, testing of the patients for antibodies against densovirus was not conducted in this study.

Our study confirms previous results suggesting that there are well-known arboviruses (e.g., JEV) and several relatively unappreciated arboviruses circulating and possibly causing human infections within a relatively small area of the China–Myanmar–Laos border region. It is essential that monitoring of arboviruses other than JEV should be increased in these areas to gain a better understanding of their contribution to human health in the southern Yunnan Province area.

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