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ZIKA virus isolated from mosquitoes: a field and laboratory investigation in China, 2016

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A field investigation of arboviruses was conducted in Dejiang, Guizhou Province in the summer of 2016. A total of 8,795 mosquitoes, belonging to four species of three genera, and 1,300 midges were collected. The mosquito samples were identified on site according to their morphology, and the pooled samples were ground and centrifuged in the laboratory. The supernatant was incubated with mosquito tissue culture cells (C6/36) and mammalian cells (BHK-21) for virus isolation. The results indicated that 40% (3,540/8,795) were *Anopheles sinensis*, 30% (2,700/8,795) were *Culex pipiens quinquefasciatus*, and 29% (2,530/8,795) were *Armigeres subbalbeatus*. Furthermore, a total of eight virus isolates were obtained, and genome sequencing revealed two Zika viruses (ZIKVs) isolated from *Culex pipiens quinquefasciatus* and *Armigeres subbalbeatus*, respectively; three Japanese encephalitis viruses (JEVs) isolated from *Culex pipiens quinquefasciatus*; two Banna viruses (BAVs) isolated from *Culex pipiens quinquefasciatus* and *Anopheles sinensis*, respectively; and one densovirus (DNV) isolated from *Culex pipiens quinquefasciatus*. The ZIKVs isolated from the *Culex pipiens quinquefasciatus* and *Armigeres subbalbeatus* mosquitoes represent the first ZIKV isolates in mainland China. This discovery presents new challenges for the prevention and control of ZIKV in China, and prompts international cooperation on this global issue.

arbovirus surveillance, mosquito-borne arbovirus, Japanese encephalitis virus, Zika virus

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INTRODUCTION

Hematophagous insects such as mosquitoes, ticks, midges (Culicoides) and sandflies can harbor arboviruses that cause viral zoonoses, which can be transmitted by insect bites and blood sucking (Weaver and Reisen, 2010). In 1992, there were 535 species of arboviruses registered in WHO, 128 of which were likely pathogenic to humans and animals (Karabatsos, 1985). Among these registered arboviruses, more than 300 species are mosquito-borne viruses (Karabatsos, 1985), including dengue virus (DENV) (http://www.who.int/csr/resources/publications/dengue/whocdsdenic20001. pdf?ua=1), Japanese encephalitis virus (JEV) (Hills and Phillips, 2009), West Nile virus (WNV) (Mackenzie et al., 2004), Zika virus (ZIKV) (Franca et al., 2016), and other viruses that can lead to serious infectious diseases worldwide. Therefore, mosquito-borne viruses and their related diseases present important problems directly related to public health (Liang et al., 2015; Gould et al., 2017; Li et al., 2016b).

Guizhou Province (east longitude 103°36′–109°35′, latitude 24°37′–29°13′), located in southwest China, has complex natural physiographic conditions, including mountains, rivers, and river basins; it has a sub-tropical climate and abundant rainfall suitable for the breeding of a variety of hematophagous insects. Since 1950, Guizhou Province has had a high-prevalence of Japanese encephalitis (JE) cases (Gao et al., 2010; Ye et al., 2010). In addition, many JEVs (Wang et al., 2007) and Getah viruses (Li et al., 2012) have been isolated from mosquito samples collected in Guizhou Province. IgM antibodies for JEV and human herpesvirus have been identified in patients with viral encephalitis in Guizhou Province (Gao et al., 2010), and IgM antibodies for Barmah Forest and Ross River viruses have been detected in local febrile patients (Ye et al., 2012). These data suggest that there are a variety of arboviruses and insect-borne viral diseases in Guizhou Province.

In view of the complex physiographic conditions of Guizhou Province and the wide variety of arboviruses and infected cases in the region, we conducted an investigation in Dejiang, Guizhou Province in the summer of 2016 to understand the distribution of mosquitoes and insect-borne viruses. In this study, we systematically introduce the collection of samples and virus isolation in Dejiang, including the isolation of ZIKV from *Armigeres subbalbeatus* samples for the first time as well as other viruses, such as JEV and BAV.

RESULTS

Sample collection

A total of 8,795 mosquitoes belonging to four species of three genera were collected from houses, pigsties, cowsheds, sheepfolds, and hen houses in Dejiang, Guizhou Province; 40.25% (3,540/8,795) were *Anopheles sinensis*, 30% (2,700/8,795) were *Culex pipiens quinquefasciatus*, 29% (2,530/8,795) were *Armigeres subbalbeatus*, and 25 were *Culex fuscanus*. In addition, 1,300 midges were collected.

Virus isolation

The samples were divided into 121 pools before processing. The C6/36 and BHK-21 cells were incubated with the sample supernatants and observed daily for CPE. Finally, eight virus isolates were obtained. Among them, five virus isolates (GZDJ1666-2, GZDJ1685 (Song et al., 2017), GZDJ1608, GZDJ1609, and GZDJ1648) caused CPEs in both C6/36 and BHK-21 cells, manifesting as cell aggregation, cell rounding, and shedding. Three other virus isolates (GZDJ1601-1, GZDJ1610, and GZDJ1621-1) caused CPEs only in C6/36 cells, manifesting as cell aggregation and shedding. Details of the virus isolates are listed in Table 1. All viruses were isolated from mosquito samples, while no isolates causing CPEs were obtained from midges.

Identification of virus isolates

For preliminary identification of the eight virus isolates, gene amplification and sequencing were conducted using a variety of viral gene universal and specific primers. The results showed that five virus isolates (GZDJ1666-2, GZDJ1685, GZDJ1608, GZDJ1609, and GZDJ1648) were positive for PCR amplification using flavivirus-specific primers. Sequence analysis and alignment results of the positive PCR products indicated that the sequences of GZDJ1666-2 and GZDJ1685 (Song et al., 2017) exhibited high homology with ZIKV, while those of GZDJ1608, GZDJ1609, and GZDJ1648 were highly homologous to JEV. GZDJ1610 and GZDJ1621-1 were positive for BAV virus-specific primers, and sequencing analysis results confirmed them as BAV viruses. GZDJ1601-1 was positive for DNV-specific primers and sequencing analysis confirmed it as DNV. The identification results are shown in Table 1.

Molecular biological characteristics of virus isolates

ZIKV

GZDJ1685 was identified as ZIKV using universal primers for flavivirus. We further verified the result using ZIKV-specific primers (Table S2 in Supporting Information); two sequences of the coding region (GZDJ1666-2: MF964216 and GZDJ1685: MF099651) were obtained. The length of both sequences was 10,296 base pairs (bp), with a single open reading frame (ORF) encoding 3,423 amino acids. The homology analysis of nucleotide and amino acid of the two ZIKVs were 100%, respectively.

These two ZIKVs exhibited nucleotide homology greater than 88% with African-lineage ZIKVs, and the amino acid

Table 1 Background information of the virus strains isolated from mosquitoes in Guizhou, China[a)]

Strains	Host (number)	Breeding ground	The CPE time (day)		Initial positive by PCR*	Identification of virus
			C6/36	BHK-21		
GZDJ1666-2	Armigeres subalbatus (50)	Pigsty	2	6	Flavivirus	Zika virus
GZDJ1685	Culex pipiens quinquefasciatus (50)	Pigsty	2	2	Flavivirus	Zika virus
GZDJ1608	Culex pipiens quinquefasciatus (50)	Pigsty, Cowshed	3	3	Flavivirus	Japanese encephalitis virus
GZDJ1609	Culex pipiens quinquefasciatus (50)	Pigsty, Cowshed	3	3	Flavivirus	Japanese encephalitis virus
GZDJ1648	Culex pipiens quinquefasciatus (50)	Pigsty	3	3	Flavivirus	Japanese encephalitis virus
GZDJ1610	Culex pipiens quinquefasciatus (50)	Pigsty, Cowshed	3	−	BAV-12 segment	Banna virus
GZDJ1621-1	Anopheles sinensis (50)	Pigsty, Cowshed	3	−	BAV-12 segment	Banna virus
GZDJ1601-1	Armigeres subalbatus (50)	Pigsty	4	−	DNV-nonstructural 1	Densovirus

a) *, Virus initially identified using 14 pairs of gene amplification primers (Table S1 in Supporting Information). −, No signs of CPE were observed after five days.

homology varied from 96.4% (Africa I) to 96.5% (Africa II). The nucleotide and amino acid homologies among these two ZIKVs and Asian-lineage ZIKVs ranged from 95% (P6-740, Malaysia) to 99% (SZ-WIV01, American-Samoa), and from 98.6% (P6-740, Malaysia) to 99.8% (SZ-WIV01, American-Samoa), respectively (Table S3 in Supporting Information).

GZDJ1666-2 and GZDJ1685 shared the same five amino acid differences (Pettersson et al., 2016) compared with the sequence of ZIKV strains that caused severe epidemics in South America in 2015. The analysis of the amino acid differences in the two ZIKVs is shown in Table S3 in Supporting Information.

Phylogenetic analysis based on the complete genome of ZIKV revealed that the GZDJ1666-2 and GZDJ1685 virus strains belonged to the Asian lineage, genotype-2 ZIKV, along with the strains isolated since 2007 from Yap Island (ECMN2007, Micronesia), French Polynesia (1_0199_PF), Brazil (SSABR1) in South America, Puerto Rico in the Caribbean (PRVABC59), and Haiti (Haiti1225/2014). In addition, the two Chinese strains had the closest relationship with ZIKVs isolated in Samoa and French Polynesia in the Pacific region. The phylogenetic tree of ZIKVs is shown in Figure 1.

Other arboviruses

GZDJ1608, GZDJ1609, and GZDJ1648 were identified as JEVs using universal primers for flavivirus. Therefore, we obtained and analyzed three nucleotide sequences of the envelope (E) gene region using JEV-specific primers. The nucleotide lengths of the three viral E genes were 1,500 bp, encoding 500 amino acids. The nucleotide and amino acid homologies of the E genes from the three strains were between 97.0%–98.4% and 97.2%–99.4%, respectively. GZDJ1608 had a 97.8% nucleotide homology and 99.6% amino acid homology with genotype-1 JEV (GZ56 strain) isolated in Guizhou; on the other hand, it had a 87.9% nucleotide homology and 97.4% amino acid homology with genotype-3 JEV (P3 strain).

Phylogenetic analyses based on the JEV E gene nucleotide sequences registered in GenBank from various countries and genotypes over several years indicated that these three JEV isolates, GZDJ1608, GZDJ1609, and GZDJ1648, were located in the same evolutionary branch: genotype-1 JEV.

Phylogenetic analyses of the 12th segment of BAV, Liaoning virus (LNV), and Kadipiro virus (KDV) belonging to Seadornavirus showed that GZDJ1610 and GZDJ1621-1 isolated in Dejiang County, Guizhou Province in 2016 were BAVs that belonged to genotype-A2 BAVs.

Phylogenetic analysis of the nonstructural (NS) 1 and NS2 genes of DNV, brevidensoviruses (BreviDNV), pefudensovirus, and iteravirus indicated that GZDJ1601 was a member of BreviDNV.

DISCUSSION

ZIKV is a mosquito-borne flavivirus. In 2015–2016, South American countries such as Brazil suffered severe epidemics of ZIKV infection, which infected almost 2 million people in more than 60 countries (Franca et al., 2016). During the epidemics, many patients infected with ZIKV manifested congenital ZIKV syndrome (CZVS) (Franca et al., 2016; Mlakar et al., 2016), Guillain-Barre syndrome (GBS) (Cao-Lormeau et al., 2016), or non-vector-borne transmission stillbirth (Ogden et al., 2016). ZIKV cases transmitted through sex were also reported (Enserink, 2015; Rahman and Huhtaniemi, 2017). As ZIKV infection has caused an enormous public health burden due to various virus transmission modalities combined with the lack of vaccine and treatment,

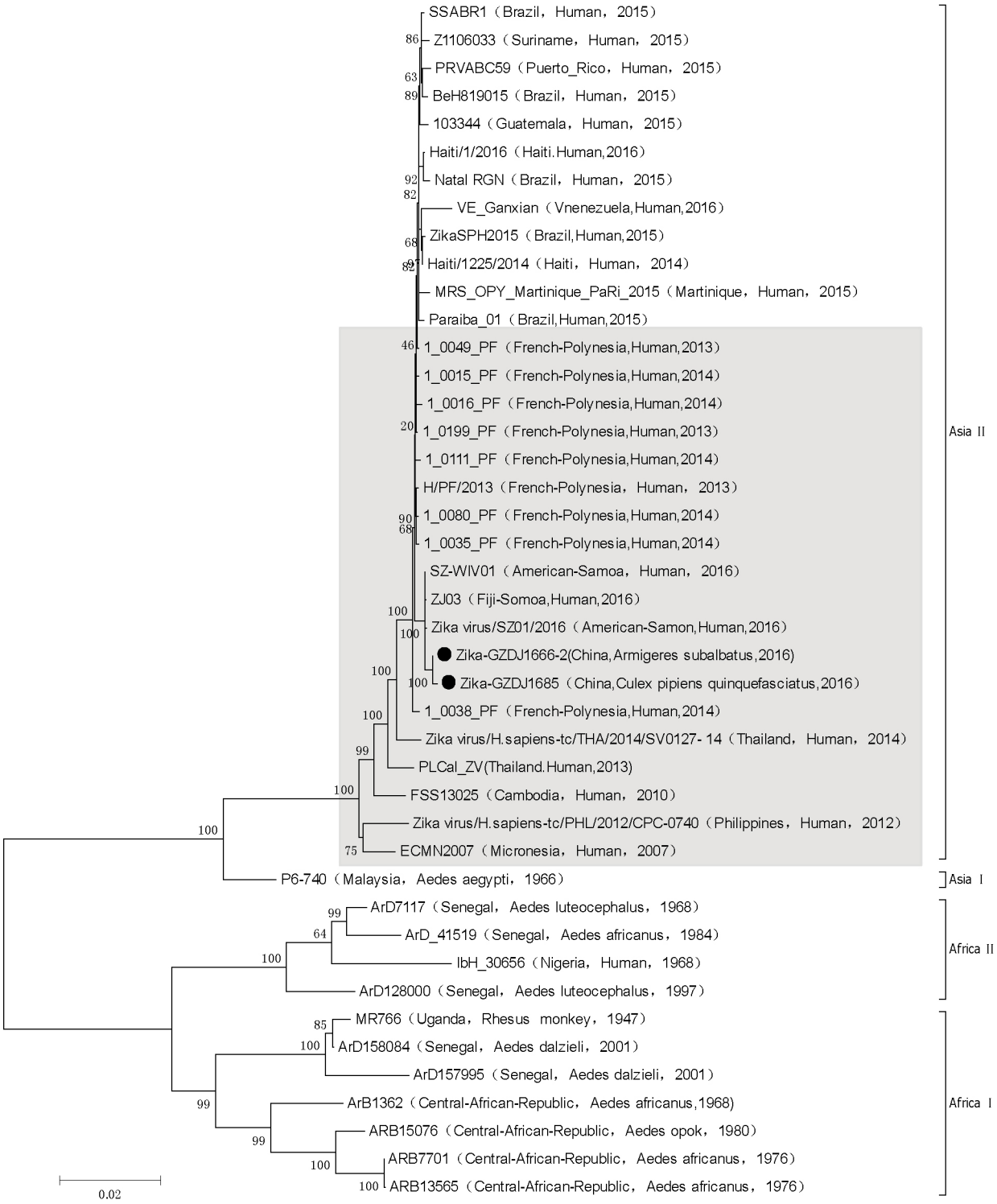

Figure 1 Phylogenetic analysis of ZIKV isolates based on the complete CDS. This analysis employed a maximum likelihood method using the MEGA7.0 software . Bootstrap probabilities for each node were calculated using 1,000 replicates. Scale bars indicate the number of nucleotide substitutions per site. GZDJ1666-2,GZDJ1685 strains were marked with the black spots.

knowledge of ZIKV vectors and the biological characteristics of the virus are critical for the prevention and control of ZIKV infection.

Previous studies investigating the transmission rate of ZIKV by mosquitoes under laboratory conditions have indicated that *Aedes aegypti* and *Aedes albopictus* can be vectors of ZIKV (Lourenço-de-Oliveira and Failloux, 2017; Liu et al., 2017a; Liu et al., 2017b). Besides, ZIKV-positive virus particles were detected in the salivary glands of *Culex pipiens quinquefasciatus* infected orally with ZIKV in the

laboratory (Guedes et al., 2017; Guo et al., 2016). In addition, after these mosquitoes fed on laboratory rats, ZIKV gene amplification was positive in the animal brain tissue (Guo et al., 2016). These results indicate that *Culex pipiens quinquefasciatus* has the ability not only to replicate ZIKV but also to spread ZIKV to animals.

ZIKV has been isolated in a variety of mosquito species collected in nature except Aedes, although it is an *Aedes*-transmitted pathogen. For example, 31 strains of ZIKV were isolated from mosquitoes collected in Seychelles; 28 strains were isolated from 10 species of *Aedes albopictus* and three ZIKVs were isolated from *Ma. uniformis*, *Cx. perfuscus*, and *An. Coustani* (Diallo et al., 2014). In addition, during the ZIKV epidemic in Brazil from February to May 2016, a total of 1,496 *Culex pipiens quinquefasciatus* and 408 *Aedes aegypti* were collected in the Metropolitan Region of Recife. Quantitative reverse transcription PCR performed in 270 pools of *Cx. quinquefasciatus* and 117 pools of *Ae. aegypti* revealed that three pools of *Cx. quinquefasciatus* and two pools of *Ae. aegypti* were PCR-positive for ZIKV. Moreover, two ZIKVs isolated from *Cx. quinquefasciatus* were shown to be stable in Vero cells (Guedes et al., 2017). The present study represents the first isolation of ZIKV from *Armigeres subalbatus* (Table 1 and Figure 1). The above results indicate that ZIKV can be detected and isolated in many species of mosquitoes, not just in *Aedes aegypti*. Whether these mosquitoes are involved in the prevalence of ZIKV in local regions requires further experimental studies involving the identification of ZIKV-infected mosquitoes and the monitoring of animal serum epidemiological results.

Phylogenetic analysis suggested that ZIKVs can be divided into three different evolutionary clusters: ZIKVs circulating in Africa, ZIKVs endemic in the South Pacific region between 2007 and 2013, and ZIKVs emerged and spread widely in the Americas since 2015 (Pettersson et al., 2016; Qin, 2016). The ZIKVs isolated in China in 2016 showed the closest evolutionary relationship with the strains isolated in the South Pacific region between 2007 and 2013 (grey area in Figure 1), indicating that the ZIKVs isolated from *Culex pipiens quinquefasciatus* and *Armigeres subbalbeatus* in this study may have spread to China before 2016.

The ZIKV strains isolated since 2015 that have induced many severe syndromes, including microcephaly and GBS in South America, have five unique amino acid mutations compared with the ZIKV isolates obtained before the epidemic (Pettersson et al., 2016). These differences may provide an important basis for the large-scale infection and transmission of ZIKV. The amino acid change (S139N) in the virus M protein may be directly related to the microcephaly and GBS cases (Pettersson et al., 2016). The ZIKV isolated in our study had the same five unique amino acid mutations with that in 2015, suggesting that the ZIKV isolated from *Culex pipiens quinquefasciatus* (Song et al., 2017) and *Armigeres subbalbeatus* in China may have the molecular basis for causing CZVS. Apart from ZIKV, we also isolated JEV, BAV, and DNV from the samples. Guizhou Province has been a high prevalence area of JEV infections annually since 1950 (Gao et al., 2010; Ye et al., 2010). Many genotype-1 JEV strains were isolated in *Culex pipiens quinquefasciatus* and *Armigeres subbalbeatus* collected in Guizhou Province in 2004 (Wang et al., 2007); moreover, it was isolated from cerebrospinal fluid samples collected from patients with viral encephalitis in Guizhou Province (Wang et al., 2010). In our study, we isolated three genotype-1 JEVs (Table 1) in *Culex pipiens quinquefasciatus*, reflecting the activity of JEV in the local region. BAV is considered as an emerging pathogen that can cause human infection (Attoui et al., 2005; Liu et al., 2010). BAV has been isolated from mosquitoes of three genera and 10 species, suggesting that the spread of BAV may involve a variety of mosquito vectors (Liu et al., 2010; Liu et al., 2016b). In our study, we isolated BAV in *Culex pipiens quinquefasciatus* and *Anopheles sinensis*, representing the first isolates identified in Guizhou Province. DNV belongs to the family Parvoviridae, genus *Densovirus*, which has been isolated in many mosquitoes, such as *Cx. pipiens pallens*, *Cx. pipiens quinquefasciatus*, *Cx. tritaeniorhynchus*, and *An. sinensis*. The virus was isolated in *Culex*. sp. in Guizhou Province in 2005 (Zhai et al., 2008.). This study represents the first isolation of DNV in *Armigeres subalbatus*. Up until now, there have been no reports on the relationship between DNV and zoonosis disease.

CONCLUSION

Dejiang, Guizhou, located in the Yunnan-Guizhou Plateau region in southwest China, has an elevation that ranges from 1,000 to 1,500 m. It has a warm and humid climate (subtropical humid monsoon climate) and abundant rainfall. The samples were collected in a mountainous area where residents build houses and terraces on the hillside. Corn and sorghum are the main crops. As the available land area is limited, most local farmers raise pigs, cattle, sheep, chickens, or ducks in their own homes, and the animal waste is conducive to the breeding of *Culex pipiens quinquefasciatus* and *Armigeres subbalbeatus*. As mentioned above, 60% of the collected mosquito samples were *Culex pipiens quinquefasciatus* and *Armigeres subbalbeatus*. In addition, seven of the eight virus strains in this study were isolated from *Culex pipiens quinquefasciatus* and *Armigeres subbalbeatus* (Table 1), suggesting the important role of these two mosquito species in the circulation of local arboviruses. Early in February 2016, the National Health and Family Planning Commission (NHFPC) had released the national strategy for prevention and control of ZIKV infection in China, which includes strengthening the port health and quarantine capacity to make early detection of imported

cases, enhancing the surveillance of transmission vectors of ZIKV nationwide, and promoting the dissemination of knowledge on the prevention of mosquito-borne viral infections among the public (http://www.nhfpc.gov.cn/jkj/s3577/201602/97bc31cc1767485290529a281d11c901.shtml). Due to the effective implementation of these measures mentioned above, no local ZIKV-infection cases were reported by now, despite the occurrence of several imported cases in China (Li et al., 2016a; Su et al., 2016). Further studies are required to determine whether ZIKV circulates between *Aedes aegypti* and non-human primates (Gould et al., 2017), whether ZIKV infection can occur in local non-human primates in Guizhou Province, whether there is an *Aedes aegypti* carrying ZIKV, and whether local ZIKV infections have occurred in Guizhou Province. Meticulous detection and in-depth monitoring are required to identify the public health and disease burden caused by ZIKV infection.

MATERIALS AND METHODS

Sample collection

Blood-sucking insects were collected from houses and feeding pens, such as pigsties, and cowsheds, in Dejiang, Guizhou Province (108.13°E and 28.27°N) in August 2016. Ultraviolet light lamps (Wuhan Lucky Star Environmental Protection Technology Co., Ltd.) were used to collect mosquitoes from 20:00 PM to 7:00 AM. The mosquito samples were classified according to their morphology under ice bath conditions, and numbered and registered according to the collecting environment and species (50 insects per pool). Finally, the samples were stored in liquid nitrogen and transferred to the laboratory (Wang et al., 2011; Zhang et al., 2014; Lu et al., 2011).

Cell culture

C6/36 cells (*Aedes albopictus* cells) and BHK-21 cells (*Mesocricetus auratus* kidney cells) used in this study were kept in our laboratory. C6/36 cells were cultured in 45% RMPI 1640 (Invitrogen, Thermo Fisher Scientific, USA) and 45% Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Invitrogen) and 100 U mL^{-1} penicillin and streptomycin. BHK-21 cells were cultured in 93% DMEM containing 7% FBS and 100 U mL^{-1} penicillin and streptomycin. C6/36 cells were cultured at 28°C, and BHK-21 cells were cultured at 37°C in a 5% CO_2 incubator (Wang et al., 2011; Zhang et al., 2014; Lu et al., 2011).

Virus isolation

A total of 50 mosquitoes or 100 midges were added to centrifuge tubes containing 1 mL of medium (90 mL DMEM, 1 mL glutamine (30 g L^{-1}), 8 mL penicillin and streptomycin, and 1 mL $NaHCO_3$ (75 g L^{-1})). Each tube was shaken for 2 min at a frequency of 25 times s^{-1} using a Tissuelyser (Qiagen Co. Ltd., Hilden, Germany) and centrifuged at 4°C at 20,000×*g* for 20 min. The supernatant (100 μL) was used to inoculate C6/36 cells (plated at 70%−80% confluence) and BHK-21 cells in 24-well tissue culture plates. The cells were continuously cultured in incubators for three generations. The cytopathic effect (CPE) of each well was observed under a microscope every 12 h for five consecutive days. The virus suspension was harvested when CPE occurred and stored at –80°C until further identification (Wang et al., 2011; Zhang et al., 2014; Lu et al., 2011).

RNA extraction and complementary DNA (cDNA) preparation

Total RNA was extracted from 140 μL virus-infected cell supernatants using the QIAamp Viral RNA Extract Kit (Qiagen Co. Ltd.) according to the manufacturer's instructions. The nucleic acid was eluted in 50 μL, placed in a water bath at 65°C for 10 min, and centrifuged. RNA (32 μL) was added into the first strand reaction tube provided by the Ready-To-Go kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK), and 1 μL of the random primer pd (N) 6 (50 g μL^{-1}) (TaKaRa, Japan) was added into the tube, for a final volume of 33 μL. After centrifugation, the tube was placed in a water bath at 37°C for 60 min to complete the cDNA preparation and stored at –40°C (Wang et al., 2011; Zhang et al., 2014; Lu et al., 2011).

Virus gene amplification primers

The primers used for gene amplification of the isolates included universal primers for flavivirus, alphavirus, and bunyavirus. In addition, we used primers specific for JEV, Banna virus (BAV), Oya virus, Sindbis virus, Getah virus, Liaoning virus (LNV), Densovirus (DNV), and Tibet orbivirus, and ZIKV whole genome (coding region) primers. The designation, amplification interval, sequence information, and amplified fragment size of the universal and specific primers are listed in Table S1 in Supporting Information. The amplification primers of the entire coding sequence of ZIKV are listed in Table S2 in Supporting Information.

Polymerase chain reaction (PCR) amplification of viral genes

PCR amplification was performed using cDNA as template, GoTaq® Green Master Mix, 2× (Promega, USA), and 10 μmol L^{-1} upstream and downstream primers. Optimum reaction conditions were achieved in consideration of the primers, fragment length, and annealing temperatures to amplify the virus genes.

After the PCR reaction, 5 μL of the PCR products was analyzed by 1% agarose gel electrophoresis to confirm amplification. PCR-positive products were purified using the QIAquick Gel Extraction Kit (Qiagen Co. Ltd.) and inserted

into the pGEM-Teasy vector (Promega) for sequence analysis. The sequences were subjected to a BLAST search in NCBI, and the virus genes were identified according to the GenBank information (Wang et al., 2011; Zhang et al., 2014; Lu et al., 2011).

Sequence analysis

The sequences used for molecular genetic analysis included virus strains isolated in Guizhou and sequences from GenBank that represented strains isolated from various countries in different years. The sequence information and GenBank accession numbers are listed in Table S4 in Supporting Information.

Seqman software (DNAStar, USA) was used for assembly and quality analysis of the nucleotide sequence of the virus genes. BioEdit (version 7.0.5.3; Thomas) was used for multiple sequence alignment. The phylogenetic relationship was inferred by using maximum likelihood method with the bootstrap value set at 1000 by using MEGA7.0 software (Kumar et al., 2016). Differential alignment and homology analyses of the nucleotide and amino acid sequences were analyzed using GeneDOC and MegAlign software (DNAStar) (Wang et al., 2011; Zhang et al., 2014; Lu et al., 2011; Liu et al., 2016a.).

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

Table S1 Primers used for virus identification

Table S2 Primers used to obtain the entire coding sequence of ZIKV (GZDJ1666-2)

Table S3 Homology analysis of nucleotide and amino acid sequences of ZIKV (GZDJ1666-2) and its amino acid substitutions

Table S4 Details of the ZIKV strains used for analysis

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