Detection of Japanese Encephalitis Virus Antibodies in Bats in Southern China

Jie Cui, Dorian Counor, Di Shen, Guya Sun, Hongxuan He, Vincent Deubel, and Shuyi Zhang

School of Life Science, East China Normal University, Shanghai, China; Emerging Viruses, Institute Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai, China; College of Life Science, Hebei Normal University, Shijiazhuang, China; National Research Center for Wildlife Born Disease, Institute of Zoology, Chinese Academy of Sciences, Beijing, China

Abstract. In this study, 336 bats of eight species were collected during 2004–2007 in six provinces of Southern China. Antibodies against Japanese encephalitis virus (JEV) were detected by ELISA in 43 of 336 (12.8%) serum samples. Among those ELISA-positive samples, 11 serum samples had neutralizing antibodies against JEV. No JEV was detected in brain and liver samples using specific real-time reverse transcriptase-polymerase chain reaction. This is the first report of JEV neutralizing antibodies in bats in China, which reinforces that bats can be involved in the life cycle of this virus.

INTRODUCTION

Japanese encephalitis virus (JEV) is a zoonotic arbovirus belonging to the genus Flavivirus with mosquito-borne or tick-borne characteristics. The whole genome of JEV is ~11 kb and encodes three structural proteins (capsid protein; precursor membrane protein; envelope protein) and seven non-structural (NS) proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5). The JEV serologic group includes eight virus species and two subtype viruses: JEV; West Nile virus (WNV); Kunjin virus (a subtype of WNV); Murray Valley encephalitis virus (MVEV); Alfuy virus (a subtype of MVEV); St. Louis encephalitis virus (SLEV); Usutu virus (USUV); Koutango virus; Yaounde virus; and Cacipacore virus.

Each year, 30,000–50,000 cases of Japanese encephalitis occur in Asia, and most are related to Culex mosquitoes. JEV exists in a zoonotic cycle among Culex mosquitoes and pigs, and some birds are disseminating and maintaining hosts, with human and horses being dead-end hosts. JEV antibodies were detected in a few bat species including Hipposideros armiger, H. pomona, H. speoris, H. bicolor, H. cineraceus, Rhinolophus comutus, R. rouxi, R. ferrumequinum, Vesperilio superans, Myotis macrourus, and Miniopterus schreibersii. JEV was isolated from M. schreibersii and R. comutus in Japan. WNV, which belongs to the JEV serologic group, was once isolated from Rousettus leschenaulti. Antibodies against WNV were detected in the following bat species: Eptesicus fuscus, Myotis lucifugus, and M. septentrionalis. SLEV was isolated from Tadarida brasiliensis. JEV in Southern China is high, and ~8,000 cases break out in Guizhou, Sichuan, Chongqing, Yunnan, Shanxi, and Guangxi provinces annually (Dr. Guodong Liang, personal communication). In this study, we collected bat samples in Southern China with the aim to determine whether these mammals play certain roles of JEV circulation in China.

MATERIALS AND METHODS

Collection of bat specimens. From December 2004 through August 2007, 336 bats were collected from nine locations in six provinces in Southern China (Figure 1). For surveillance, sites were chosen only where there were large sums of bat colonies (> 1,000 individuals) for bat conservation. Insectivorous bats were captured in caves, huts, or trenches using mist net or butterfly nets during the day; fruit bats were acquired in caves, old buildings, or under Chinese fan-palms (Livistona chinensis) using mist-nets near sunset or before dawn.

The following procedures were approved by Animal Care Ethics Committee, East China Normal University. Bats were anesthetized with 1% pentobarbital sodium (40 mg/kg weight, IP) and killed by intracardiac exsanguination. All blood samples were kept in the freezer until centrifugation. Sera were decanted into plastic vials and frozen in liquid nitrogen for long-time transportation and stored at -80°C back from the field. Bats were identified to species using the mitochondrial cytochrome b (cytb) gene extracted from wing membrane tissue biopsies (data not shown). Morphologic statistics such as sex and weight were recorded individually. Brain and liver organs were collected surgically and treated as sera.

ELISA testing of antibodies against JEV. Bat sera diluted to 1:100 in phosphate-buffered saline (PBS) were screened twice by ELISA in the biosafety level 2 laboratory as described. Domain III of the envelope protein (Ed3) of the JEV SA14 strain was expressed and purified. Briefly, the coding sequence for Ed3 tagged with 6 histidine at its C terminus was cloned into pET-30a vector (Novagen, Darmstadt, Germany) following the manufacturer’s instructions. Purified JEV Ed3 antigen (2 mg/mL) was coated at a dilution of 1:500 in PBS coating buffer. Anti-bat IgG (Bethyl, Montgomery, TX) was used as conjugate. Three negative controls (no bat serum, pre-immunized mouse negative serum in place of bat serum, and no antigen) and one positive control (anti-JEV–positive mouse serum) were included in each test. Parallel test for WNV antibodies was also processed using Ed3 of WNV under the same conditions. The threshold for a positive result was determined as the absorbance values two times higher than the average value of the negative controls.

Microseroneutralization assay. ELISA results were confirmed by using microseroneutralization tests under biosafety level 2 conditions. Except five serum samples (from Miniopterus fuliginosus) for which we did not have enough volume for the test, the remaining 38 serum samples were con-
ducted for the neutralization test. Serum samples were heated for 30 minutes at 56°C and were titrated with three dilutions (1:10; 1:20; and 1:40; 50 μL for each) in Dulbecco modified Eagle medium (DMEM; Gibco, Gland Island, NY) in 96-well microtiter plates, 4 wells per dilution. Positive control (anti-JEV) serum, obtained from a JEV-vaccinated mouse, and blank controls obtained from a pre-immunized mouse and JEV ELISA-negative bat sera were set in each plate. An equal volume of JEV (100 50% tissue culture infective dose [TCID50] in 50 μL) was added to all specimen sera, and the plates were incubated for 1 hour at 37°C. Baby hamster kidney (BHK-21) cells (5 × 10^4 in 100 μL DMEM containing 5% fetal bovine serum [FBS] and penicillin/streptomycin; Gibco) was added to all wells, and the plates were incubated at 37°C for 3 days in a 5% CO2 humid chamber at 37°C. Neutralization titers were defined as the highest dilution in which at least one half of the wells were intact.

**Detection of JEV using real-time RT-PCR.** RNA was extracted from brain and liver organs using TRIZOL Reagent (Invitrogen) according to the manufacturer’s instructions. Total RNA qualities were evaluated by measuring the optical density (OD) value. cDNA was synthesized by a two-step method (Takara, Otsu, Shiga, Japan) and stored at −80°C. The TaqMan assay was performed according to Shirato and others, as well as the JEV primers and probe, using TaqMan universal PCR Master Mix (Applied Biosystems, Foster City, CA) and running on an ABI Prism 7900 (Applied Biosystems) machine. Positive (quantified JEV RNA) and negative controls (RNA omitted) were included in the test. The limit of detection was 3 ng/mL, corresponding to 4.88 copies/mL.

**RESULTS**

**Collection of bat specimens.** Bats were identified to species. All the 336 bats collected belonged to eight species including both Megachiroptera and Microchiroptera (Table 1). A total of 186 (55.4%) bats were female and in a healthy condition.

<table>
<thead>
<tr>
<th>Species</th>
<th>Locations</th>
<th>Seropositive (no. captured)</th>
<th>Real-time positive (no. captured)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ELISA</td>
<td>Neutralization</td>
</tr>
<tr>
<td>Megachiroptera</td>
<td></td>
<td></td>
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</tr>
<tr>
<td><em>Roussetts leschenaulti</em></td>
<td>Guangxi, Guangdong, Hainan</td>
<td>35 (197)</td>
<td>11 (35)</td>
</tr>
<tr>
<td><em>Cynopterus sphinx</em></td>
<td>Guangdong</td>
<td>0 (14)</td>
<td></td>
</tr>
<tr>
<td>Microchiroptera</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Taphozous melanopogon</em></td>
<td>Guangxi</td>
<td>3 (30)</td>
<td>0 (3)</td>
</tr>
<tr>
<td><em>Miniopterus fuliginous</em></td>
<td>Anhui, Guizhou</td>
<td>5 (46)</td>
<td>ND</td>
</tr>
<tr>
<td><em>Pipistrellus abramus</em></td>
<td>Jiangxi</td>
<td>0 (22)</td>
<td></td>
</tr>
<tr>
<td><em>Hippolosderos larvatus</em></td>
<td>Guangxi</td>
<td>0 (2)</td>
<td></td>
</tr>
<tr>
<td><em>Rhinolophas pusillus</em></td>
<td>Guangxi</td>
<td>0 (19)</td>
<td></td>
</tr>
<tr>
<td><em>Rhinolophas macrotis</em></td>
<td>Guangxi</td>
<td>0 (6)</td>
<td></td>
</tr>
</tbody>
</table>

ND = not determined because of poor volume of serum samples.
**ELISA testing of sera.** Of all the samples screened, 43 (12.8%) had positive results (Table 1). Bats with ELISA-positive samples belonged to three species including insectivorous (*Taphozous melanopogon, M. fuliginosus*) and fruit bats (*R. leschenaulti*). All the samples screened with WNV Ed3 were negative, suggesting these bats had not been infected with WNV.

**Sero logic testing for neutralizing antibodies.** Of the 43 ELISA-positive samples, 38 samples had sufficient volume for neutralization test. Among them, 11 were positive for neutralizing antibodies against JEV. Eight had neutralizing antibody titers at 1:10, two at 1:20, and one at 1:40. All the positive samples came from one species, *R. leschenaulti*, from provinces of Guangxi (seven samples) and Hainan (four samples).

Sera showing neutralization activity were titrated by ELISA using series dilutions. The titration result displayed continuous decreasing of serial dilutions of sera (Figure 2). This result coincided with the neutralization result, which suggested a low titer of antibodies against JEV in bat serum.

**Detection of JEV using real-time RT-PCR.** According to Shirato and others, samples with a Ct value < 40 and ΔRn signal > 0.5 were considered positive using the TaqMan assay. Of all 336 brain and 336 liver samples used, none of them exhibited positive signals.

**DISCUSSION**

Of the five genotypes of JEV, genotype IV was only found in Indonesia and genotype V was isolated in Malaysia; the others (especially genotype III) were distributed widely: genotype I was in Japan, Korea, China, Laos, Cambodia, Vietnam, Thailand, Australia, and Malaysia; genotype II was in Thailand, Malaysia, Indonesia, Australia, and New Guinea; and genotype III was in Japan, Korea, China, India, Sri Lanka, Nepal, Vietnam, Philippines, Indonesia, and Malaysia. Solomon and others estimated that JEV genotypes originated from a common ancestor, which had emerged in the Indonesia-Malaysia region and spread into Asia. The pandemic genotypes I and III have been detected in China since the 1970s and the important reservoir is *T. melanopogon*. All the samples screened with WNV Ed3 were negative, suggesting these bats had not been infected with WNV.

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using human sera for the JEV vaccinated recipient or using JEV- or WNV-infected mouse antisera (unpublished data). In addition, all human sera positive by ELISA were also positive by seroneutralization assay (D. Counor and V. Deubel, unpublished data). Finally, bat sera, which were positive in ELISA but negative in microneutralization, may reflect a previous infection with another JEV genotype. Although genotype III is the major subtype circulating in China (which the SA14 strain belongs to), genotype I has also been recently identified in southern China.16–17

JEV maintenance in bats is questionable because all seropositive bats were found negative for the virus genome in this study. The infectious period of viruses may be short; therefore, the probability of finding a virus-positive bat in such limited number of animals is very low. Similar observation was made when WNV was searched in migratory birds with very low positive results (< 0.3%).32 Because JEV had been isolated in bat tissues such as the brain, brown fat, blood, kidney, or spleen,33–35 the possibility that the virus can sustain in these tissues cannot be excluded in this test. The failure for JEV detection in tissue samples (e.g., brain, liver) using real-time PCR and the low antibody titer may also suggest that these bats had been infected with JEV at an early time. Although it is uncertain that fruit bats can serve as reservoirs or disseminators in viral circulation, the potential threat of JEV-carrying bats to human health should be considered. To avoid human infection, individual prevention awareness should be spread abroad in areas where those bats are inhabited.

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Jie Cui and Dorian Counor contributed equally to this paper.

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Authors’ addresses: Jie Cui, School of Life Science, East China Normal University, 3663 North Zhongshan Road, 200062 Shanghai, China, and College of Life Science, Hebei Normal University, 113 East Yuhua Road, 050016 Shijiazhuang, China. Dorian Counor, Guya Sun, and Vincent Deubel, Emerging Viruses, Institute Pasteur of Shanghai, Chinese Academy of Sciences, 225 South Chongqing Road, 200025 Shanghai, China. Di Shen, College of Life Science, Hebei Normal University, 113 East Yuhua Road, 050016 Shijiazhuang, China. Hongxuan He, National Research Center for Wildlife Born Disease, Institute of Zoology, Chinese Academy of Sciences, Datun Road, 100101 Beijing, China. Shuyi Zhang, School of Life Science, East China Normal University, 3663 North Zhongshan Road, 200062 Shanghai, China.

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