Comparison of genomic and amino acid sequences of eight Japanese encephalitis virus isolates from bats

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Abstract We compared nucleotide and deduced amino acid sequences of eight Japanese encephalitis virus (JEV) isolates derived from bats in China. We also compared the bat JEV isolates with other JEV isolates available from GenBank to determine their genetic similarity. We found a high genetic homogeneity among the bat JEVs isolated in different geographical areas from various bat species at different time periods. All eight bat JEV isolates belonged to genotype III. The mean evolutionary rate of bat JEV isolates was lower than those of isolates of other origin, but this difference was not statistically significant. Based on these results, we presume that the bat JEV isolates might be evolutionarily conserved. The eight bat JEV isolates were phylogenetically similar to mosquito BN19 and human Liyujie isolates of JEV. These results indicate that bats might be involved in natural cycle of JEV.

Introduction

Japanese encephalitis (JE) is a severe zoonotic disease with a high fatality rate, ranging from 25 % to 50 % in humans. Nearly 50 % of survivors suffer from persistent neurological sequelae [22]. JE mainly occurs in China, India and Southeast Asia [12, 17, 35]. An estimated 67,900 cases occur globally each year (overall incidence: 1.8/100,000 people), of which only about 10 % are reported to the World Health Organization [4].

JE is caused by Japanese encephalitis virus (JEV), which belongs to the genus Flavivirus of the family Flaviviridae. JEV is an enveloped virus with a single-stranded, positive-sense RNA genome approximately 11 kb in length. The virus contains a single open reading frame (ORF) flanked by 5’ and 3’ nontranslated regions (NTRs). The 5’ one-third of the ORF encodes three structural proteins, named capsid protein (C), precursor membrane protein (PrM), and envelope protein (E), whereas seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) are encoded by the remaining 3’ region [38]. Five genotypes (GI -V) of JEV have been identified based on the nucleotide sequence of the envelope (E) gene [25, 42]. The predominant genotypes of JEV isolates exhibit geographical and temporal differences [6, 21, 29, 52]. GI includes isolates isolated in India, Southeast Asia, Australia, Korea and Japan from 1967 to the present, while GII includes isolates isolated from Korea, southern Thailand, Malaysia, Indonesia, Papua New Guinea and northern Australia between 1951 and 1999 [34]. In China, the dominant JEV isolates belong to GI and GIII. GIII JEV has been circulating in China since 1949, while GI began to replace GIII to become the dominant genotype in the 1980s [28, 47].

JEV can infect humans and a variety of vertebrate animals including pigs, horses, birds, sheep, dogs and...
monkeys [13, 28, 43]. However, only pigs and water birds are considered reservoirs of the virus [2, 22, 33, 43]. Bats are recognised as important reservoirs of a large number of zoonotic viruses [1, 3, 30, 45]. Some species of bats can maintain viruses for long periods of time [16, 37]. Previous studies have shown that JEV and/or serum antibody against JEV may exist in bats in Japan and China [10, 23, 28, 36, 48]. However, the role of bats in the JEV life cycle is unknown.

Limited information is currently available about bat-derived JEV isolates. The full-length nucleotide sequences of four JEV isolates (B58, GB30, HB49 and HN97) derived from bats in Yunnan Province, China, were determined [28, 48]. The B58 and GB30 isolates were isolated from a *Rousettus leschenaultia* bat in 1989 and a *Murina aurata* bat in 1997, respectively. The HB49 and HN97 isolates were isolated from a *R. leschenaultia* bat in 1990. These four bat JEV isolates belonged to GIII [28, 48].

In recent years, we collected four JEV isolates from bats captured in Guangdong, Hainan and Hunan provinces. The genetic relationship between the genomes of these bat-derived JEV isolates and the previously collected bat-derived isolates remains unknown. Here, we compared the genetic characteristics of eight bat JEV isolates and compared them to those of other original JEV isolates available from GenBank.

Materials and methods

Bats were sampled at four natural habitats in three regions in Guangdong, Hainan and Hunan provinces of southern China between July 2007 and August 2009. Bats were captured using mist nets at natural habitats of bats (e.g., caves or palm trees). The sampling method was followed as described previously [31]. Bat brain samples were taken in the laboratory, immediately placed into tubes containing 300 µl of RNAlater (QIAGEN, Hilden, Germany), and stored at −80 °C until used.

The supernatants from brain homogenates were used to inoculate baby hamster kidney (BHK-21) cells and were consecutively passed three times. The virus was isolated as described previously [48]. Four viruses were isolated and designated GD1, HN2, SY87 and YY158 isolates. Full-length genomic sequences were obtained from the GD1 and HN2 isolates, while only the sequence of the E gene was obtained for the SY87 and YY158 isolates. The GD1 isolate was obtained from a *Myotis ricketti* bat collected in Huizhou, Guangdong Province, in 2009, and the HN2 isolate was obtained from a *Miniopterus schreibersii* bat that was collected in Haikou, Hainan Province, in 2008. The SY87 isolate was obtained from a *Rhinolophus affinis* bat and the YY158 isolate was obtained from a *M. schreibersi* bat, both of which were collected in Yueyang, Hunan Province, in 2008.

A total of 105 full-length JEV genomic sequences were downloaded from GenBank, including four bat-derived JEV genomic sequences. Phylogenetic trees were constructed based on these 105 nucleotide sequences and two nucleotide sequences of JEV (GD1, HN2 isolates) determined in this study. Consequently, twenty-seven full-length genomic sequences of JEV isolates were selected from the phylogenetic tree based on region, isolation time, host and phylogenetic position. In addition, fourteen E gene sequences of JEV isolates were selected in the analyses, which included two sequences of the JEV E gene determined in this study. A total of 41 JEV isolates were used for constructing the phylogenetic trees, which contained isolates isolated from mosquitoes (n = 14), humans (n = 12), pigs (n = 3), vaccine (n = 2), midges (n = 2) and bats (n = 8) (Table 1). The JEV isolate that was first isolated from human brain in 1935 (Nakayama strain) was used as prototype strain in sequence comparisons.

Multiple sequence alignments were performed using MEGA 4.0 [40]. The percent identity within the nucleotide sequence alignment was determined using MegAlign (DNASTAR, Madison, WI, USA). Geneious 5.5.6 was used to show differences in the nucleotide and amino alignments.

Phylogenetic trees were constructed based on 41 E nucleotide sequences using the maximum-likelihood method in PHYLIP 3.9.6 [14]. The E gene of West Nile virus was used as an outgroup. In addition, the maximum-parsimony method in PHYLIP 3.9.6, the neighbor-joining method in Mega 4.0 [32], and the Bayesian method in BEAST 1.5.4 [11] were used in the analyses.

The rate of nucleotide substitutions per site was estimated using the Bayesian Markov chain Monte Carlo (MCMC) approach as implemented in the BEAST 1.5.4 package [11]. The analysis was performed by using the HKY substitution model under a coalescent model of constant population size. In each case, the relaxed molecular clock model was used. The resulting convergence was analyzed by using Tracer1.5. A 95 % high-probability density (HPD) was determined to ascertain the uncertainty in the parameter estimates.

Results

The eight bat JEV isolates (GD1, HN2, SY87, YY158, B58, GB30, HB49 and HB97) used were obtained at different times over two decades (1989-2009). Four of these isolates were isolated in Yunnan Province (Fig. 1), which has been a highly epidemic area for JE since the 1990s, with an average incidence of infection greater than
0.5/100,000 people [46, 48]. Two of the isolates were isolated in Hunan Province (Fig. 1), with an average incidence of infection between 0.2/100,000 and 0.5/100,000 people [46, 53]. The other two isolates were from Guangdong and Hainan Province (Fig. 1), respectively, which were once highly endemic areas for JE before the 1990s but are currently low-endemic areas with an incidence of less than 0.2/100,000 people annually [46, 53].

### Table 1 Isolates of JEV used in the study

| Isolate name | Place of isolation | Year of isolation | Source | GenBank accession no. | Genotype |
|--------------|--------------------|-------------------|--------|-----------------------|----------|
| GD1†         | China, Guangdong   | 2009              | Bat    | JN711458.1            | III      |
| HN2†         | China, Hainan      | 2008              | Bat    | JN711459.1            | III      |
| SY87†        | China Hunan        | 2007              | Bat    | JX050152.1*           | III      |
| YY158†       | China Hunan        | 2008              | Bat    | JX093498.1*           | III      |
| B58          | China, Yunnan      | 1986              | Bat    | FJ185036.1            | III      |
| GB30         | China, Yunnan      | 1997              | Bat    | FJ185037.1            | III      |
| HB49         | China, Yunnan      | 1990              | Bat    | JF706284.1            | III      |
| HB97         | China, Yunnan      | 1990              | Bat    | JF706285.1            | III      |
| Beijing-1    | China              | 1949              | Human brain | L48961.1        | III      |
| ChiangMai    | Thailand           | 1964              | Human  | U70393.1*             | III      |
| Fj02-76      | China Fujian       | 2002              | Human  | JN381867.1            | III      |
| Fj03-39      | China Fujian       | 2003              | Human  | JN381859.1            | III      |
| Liuyuie      | China, Yunnan      | 1979              | Human  | FJ185039.1*           | III      |
| ML17-live    | Taiwan             | 1981              | Human  | AY508812.1            | III      |
| Nakayama     | Japan              | 1935              | Human brain | EF571853.1        | III      |
| P3           | China              | 1949              | Human brain | U47032.1        | III      |
| P19-Br       | Thailand           | 1982              | Human  | U70416.1*             | I        |
| Vellore      | India              | 1958              | Human brain | AF080251.1       | III      |
| GP78         | India              | 1978              | Human brain | AF075723.1       | III      |
| 057434       | India              | 2005              | Human  | EF623988.1            | III      |
| BN19         | China, Yunnan      | 1982              | Mosquito | FJ185038.1*   | III      |
| DL04-45      | China Yunnan       | 2004              | Mosquito | JN381854.1      | III      |
| JaGa01       | Japan              | 1959              | Mosquito | AF690706.1      | III      |
| JKT5441      | Indonesia          | 1981              | Mosquito | U70406.1*       | II       |
| JKT7003      | Indonesia          | 1981              | Mosquito | U70408.1*       | IV       |
| K94P05       | Korea              | 1994              | Mosquito | AF045551.1     | I        |
| K87P39       | South Korea        | 1987              | Mosquito | AY585242.1     | III      |
| M859         | Cambodia           | 1967              | Mosquito | U70410.1*       | I        |
| SA14         | China              | 1954              | Mosquito | U14163.1        | III      |
| SH04-3       | China              | 2004              | Mosquito | DQ040105.1*     | III      |
| VN118        | Vietnam            | 1979              | Mosquito | U70420.1*       | III      |
| WTP-70-22    | Malaysia           | 1970              | Mosquito | U70421.1*       | II       |
| YN1N04-18    | China              | 2004              | Mosquito | DQ040146.1*     | III      |
| XZ0934       | China Tibet        | 2009              | Mosquito | JF915894.1     | V        |
| SA14-2-8     | China              | NA                | Vaccine | U15763.1        | III      |
| SA14-14-2    | China              | 1954              | Vaccine | AF315119.1      | III      |
| B-2239       | Thailand           | 1984              | Pig    | U70391.1*        | I        |
| JEV/sw/Mie/40/2004 | Japan              | 2004              | Swine  | AB241118.1       | I        |
| WHE          | China              | NA                | Pig    | EF107523.1       | III      |
| YN83-Meng83-54 | China Yunnan      | 1983              | Lasiohelea taiwana (Shiraki) | JF706282.1 | I      |
| HLJ02-134    | China              | 2002              | Heilongjiang | Genus Culicoides | JF706276.1 | III    |

NA, Not available in GenBank
† Isolates sequenced in this study
* Isolates for which E gene sequence information is available
Nucleotide and amino acid sequence analysis

The six complete genomes of bat JEV isolates were analyzed, the length of which ranged from 10,975 to 10,977 nt. All of the isolates had a 95-nt 5' nontranslated region (NTR). The HN2 isolate had a 581-nt 3' NTR, while the other five isolates had a 582-nt 3' NTR. The single ORF encoded a polyprotein of 3,432 amino acid residues, with the ATG start codon at 96-98 nt and the TAG stop codon at 10,392-10,394 nt. The genomes had similar guanine-cytosine content (51.42 % for the GD1 isolate, 51.41 % for the HN2 isolate, 51.44 % for the B58 isolate, 33.33 % for the GB30 isolate, 33.33 % for the HB49 isolate and 33.33 % for the HB97 isolate).

The diversity of the 27 full-length genomes and the 41 E genes at the nucleotide and amino acid level is shown in Table 2. The isolates generally shared high nucleotide and amino acid sequence identity. The amino acid sequence identities were higher than the corresponding nucleotide sequence identities. The full-length nucleotide sequences of bat JEV isolates shared identities from 99.4 % to 99.9 %, and the E gene sequences shared identities from 99.2 % to 99.9 %. However, all of isolates of full-length nucleotide sequences shared identities from 79.4 % to 99.9 %, and the identities of the E gene sequences ranged from 77.4 % to 99.9 %. When the comparison was restricted to the same isolation time period (1986 to 2009), there was 97.0-99.1 % identity in the nucleotide sequences and 96.7-99.7 % identity in the E gene sequences of JEVs isolated from humans. There was 79.6-97.2 % and 77.9-97.3 % identity in mosquito JEVs in the genomic and E gene sequence, respectively. The gene sequence homology of JEV isolates from bats was higher than those from other hosts (Table 2). When the comparison was restricted to GIII, the genetic homogeneity in the bat JEV isolates was likely higher than those derived from humans and mosquitoes (data not shown).

We compared six bat JEV isolates (GD1, HN2, B58, GB30, HB49 and HB97) with the Nakayama strain on the basis of UTR variation (Table 3). The six bat JEVs shared the same nucleotide changes (C14 → T14 and T49 → C49) in the 5' NTR (Table 3). Two nucleotide changes were
found in the 3’ NTR of the bat isolates: G_{10434}^{10434} → A_{10434}^{10434} and G_{10448}^{10448} → A_{10448}^{10448} (Table 3). Two other nucleotide differences in the 3’ NTR of GD1 and HN2 were also revealed (Table 3). In addition, one nucleotide was absent in the 3’ NTR of the GD1 isolate, and two nucleotides were absent in that of the HN2 isolate (Table 3).

Differences were found in the comparisons of complete sequences between six bat-derived JEV isolates (GD1, HN2, B58, GB30, HB49 and HB97 isolates) and strain Nakayama (Fig. 2). The amino acid substitutions appeared in PrM/M, E, NS1, NS2A, NS2B, NS3, NS4A and NS5 (Table 4). The E protein from six of the bat isolates had 10 unique amino acid mutations. Fewer substitutions were observed in other proteins (Table 4). The six bat isolates had four identical amino acid mutations, which were in the E protein (E-83, K_{377} \rightarrow E_{377}), E-176, T_{470} \rightarrow I_{470}, E-290, R_{584} \rightarrow K_{584}) and the NS1 protein (NS1-8, A_{802} \rightarrow I_{802}) (Table 4).

Phylogenetic analysis

Five genotypes were distinguished based on the E gene nucleotide sequences of the 41 selected JEV isolates (Fig. 3), which is consistent with the classification made by Chen and colleagues [6, 7]. The phylogenetic analysis demonstrated that all bat JEV isolates belonged to GIII. The isolates of GD1, HN2, SY87 and YY158 belonged to the same subgroup (Fig. 3). In addition, these isolates were similar to the GB30, B58, HB49 and HB97 isolates (Fig. 3). Notably, the BN19 isolate, which was isolated from a mosquito in Yunnan Province, China, in 1982 and the Liyujie isolate, which was obtained from a human in Yunnan Province, China, in 1979, were most closely related to the GD1 and HN2 isolates. Similar trees were produced by the neighbor-joining, maximum-parsimony, and Bayesian methods.

Evolutionary analysis of eight E genes for bat JEV isolates showed that the mean evolutionary rate of bat JEV isolates was 1.44 \times 10^{-4} (95 %HPD = 2.33 \times 10^{-7} to 4.41 \times 10^{-4}) nucleotide substitutions per site per year. The mean evolutionary rate previously reported from an analysis of 35 full-length genomes derived from humans, pigs and mosquitoes was 4.35 \times 10^{-4} (95 %HPD = 3.49 \times 10^{-4} to 5.30 \times 10^{-4}) nucleotides substitutions per site per year [24].

### Table 2
Comparisons of nucleotide and amino acid sequence diversities among the JEV isolates isolated from the same source groups

| Isolates      | Full nucleotide sequence | Full amino acid sequence | Isolates      | E gene   | E protein       |
|---------------|--------------------------|--------------------------|---------------|----------|-----------------|
| Bat isolates  | 99.4 %-99.9 %            | 99.4 %-99.9 %            | Bat isolates  | 99.2 %-99.9 % | 99.0 %-99.9 %   |
| (n = 6)       |                          |                          | (n = 8)       |          |                 |
| Mosquito isolates | 79.7 %-98.8 %            | 91.5 %-99.7 %            | Mosquito isolates | 77.4 %-98.9 % | 89.8 %-99.8 %   |
| (n = 6)       |                          |                          | (n = 14)      |          |                 |
| Human isolates| 95.7 %-99.4 %            | 98.6 %-99.9 %            | Human isolates| 87.3 %-99.9 % | 97.0 %-99.8 %   |
| (n = 9)       |                          |                          | (n = 12)      |          |                 |
| Pig isolates  | 89.2 %                   | 98.1 %                   | Pig isolates  | 87.0 %-93.9 % | 96.4 %-98.4 %   |
| (n = 3)       |                          |                          | (n = 3)       |          |                 |
| Midge isolates| 88.4 %                   | 97.7 %                   | Midge isolates| 88.5%     | 97.8 %          |
| (n = 2)       |                          |                          | (n = 2)       |          |                 |
| All isolates  | 79.4 %-99.9 %            | 91.1 %-99.9 %            | All isolates  | 77.4 %-99.9 % | 89.6 %-99.9 %   |
| (n = 25)      |                          |                          | (n = 37)      |          |                 |

### Table 3
Comparison of nucleotide differences in the 3’ nontranslated region (NTR) and 5’ nontranslated region (NTR) between the JEV isolates isolated from bats and the Nakayama strain

| Isolate (origin) | 5’NTR | 3’NTR | 14 | 18 | 49 | 73 | 10048 | 100410 | 100434 | 100448 | 100496 | 100550 | 100554 | 100757 | 100802 | 100953 | 100977 |
|------------------|-------|-------|----|----|----|----|-------|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| GD1 (Bat)        | T     | G     | C  | G  | G  | G  | A     | A      | A     | A     | A     | A     | A     | G     | §      | G      | T      |
| HN2 (Bat)        | T     | G     | C  | G  | G  | G  | A     | A      | A     | A     | A     | A     | A     | A     | §      | §      | G      | C      |
| GB30 (Bat)       | T     | G     | C  | G  | A  | A  | A     | A      | A     | A     | A     | A     | G     | C     | T      | T      | T      |
| B58 (Bat)        | T     | G     | C  | G  | A  | A  | A     | A      | A     | A     | A     | A     | G     | C     | T      | T      | T      |
| HB49 (Bat)       | T     | A     | C  | T  | A  | A  | A     | A      | A     | A     | A     | A     | G     | A     | G      | C      | G      |
| HB97 (Bat)       | T     | A     | C  | T  | A  | A  | A     | A      | A     | A     | A     | A     | G     | A     | G      | C      | T      |
| Nakayama (Human) | C     | A     | T  | G  | A  | G  | G     | G      | A     | A     | G     | G     | C     | G     | G      | T      |

§ Nucleotide sequence is missing
Bats are known to be reservoir hosts for many zoonotic viruses, such as SARS-coronavirus-like viruses of bats [19], Hendra virus [15] and Ebola virus [41]. JEV was isolated from naturally infected Miniopterus schreibersii [3]. There are some features of bats that might help explain the detection of JEV in bats. R. Affinis, M. ricketti and M. schreibersii can migrate hundreds of miles to their hibernation sites. Thus, bats have more opportunities to come into contact with humans or other animals at different geographical locations, which make it possible for interspecies transmission. Secondly, R. leschenaultia and M. schreibersii exhibit an exceptionally long lifespan, ranging up to 14 years. The long lifespan of bats may enhance the persistence of chronic infections [50]. In addition, some bat species also hibernate over the winter [49]. Sulkin et al. [37] found that infectious JEV was recovered from sero-positive bats fifteen weeks after a shift in temperature. The reduced body temperature and metabolic rate may suppress immune responses and reduce the rate of virus replication, and therefore, JEV could persist for extensive periods without evidence of disease [37].

There are currently approximately 105 fully sequenced JEV isolates available from different hosts [28, 48]. Genetic variation has been reported among JEV isolates isolated from widely different time periods and geographical locations [6, 21, 28]. In the present study, we selected JEV isolates with genetic information available from GenBank based on their genotype, time period, geographic region and host from which they were isolated, and we used these reference isolates to compare the genetic variation of the eight bat-derived JEV isolates from China between 1986 and 2009. The isolates showed identities from 79.4 % to 99.9 % at the nucleotide level and identities from 91.1 % to 99.9 % at the amino acid level. Most of the differences were base substitutions and nucleotide changes that did not result in amino acid alterations (Fig. 2), which is consistent with previous findings [6]. The results indicate that most of the nucleotide mutations in the bat JEV isolates are silent. Notably, the bat JEV isolates showed 99.4-99.9 % genetic homogeneity in the full-length nucleotide sequences and 99.2-99.9 % genetic homogeneity in the E gene sequences, which were higher than those from other hosts (Table 2). Also, the results of evolutionary analysis showed that bat JEV isolates probably had slower evolutionary rates than other original JEV isolates. The mean evolutionary rates of bat JEV isolates tended to be lower than those of isolates of other origin, but there was no statistically significant difference. This suggests that JEVs from bats might be more phylogenetically conserved than isolates from humans, swine and mosquitoes (Table 2). Moreover, according to the phylogenetic analysis, the GD1, HN2, SY87 and YY158 isolates were most closely related to the other four bat JEV isolates (B58, GB30, HB49 and HB97), showing a relatively high bootstrap value (Fig. 3). Eight bat JEV isolates were clustered into the same subgroup, although they were isolated from different bat species within separate regions and were originally isolated over the span of more than two decades. The reason for this phenomenon is unclear. It may be attributed to the host preference, with GIII JEVs having adapted to bats. Even though Van den Hurk et al. [44] performed laboratory-based infections on Pteropus alecto (Megachiroptera: Pteropidae) with JEV TS3306 (GII), there was no evidence that bats could harbor other JEV strains in nature except those belonging to GIII. It is unknown
whether the other three genotypes of JEV circulate in bats in nature. In this study, the bats from which JEV isolates were isolated looked healthy, suggesting that the virus is not pathogenic to bats. Since sufficient nucleotide sequence information was not available about human or other host origins in the regions where bat JEVs were isolated, we

### Table 4 Amino acid sequence differences among bat JEV isolates and the Nakayama strain

| Protein | Amino acid position | Isolates |
|---------|---------------------|----------|
|         |                     | GD1 | HN2 | GB30 | B58 | HB49 | HB97 | Nakayama |
| PreM/M  |                     |     |     |      |     |      |      |          |
| 158 (M-31) | G | G | E | E | E | E | E | E |
| 278 (M-151) | Q | Q | Q | H | Q | Q | Q | Q |
| E       |                     |     |     |      |     |      |      |          |
| 377 (E-83) | E | E | E | E | E | E | K |          |
| 417 (E-123) | S | S | R | R | R | R | S |          |
| 440 (E-146) | T | T | A | A | A | A | T |          |
| 470 (E-176) | I | I | I | I | I | I | T |          |
| 503 (E-209) | R | R | K | K | K | K | - |          |
| 570 (E-276) | N | N | S | S | S | S | N |          |
| 584 (E-290) | K | K | K | K | K | K | R |          |
| 712 (E-418) | A | A | V | V | V | V | A |          |
| 747 (E-453) | F | F | F | F | L | F | F |          |
| 759 (E-465) | G | G | G | G | A | G | G |          |
| NS1     |                     |     |     |      |     |      |      |          |
| 802 (NS1-8) | I | I | I | I | I | I | A |          |
| 971 (NS1-177) | D | G | D | D | D | D | D |          |
| 1004 (NS1-210) | W | W | R | W | W | W | W |          |
| 1014 (NS1-220) | I | I | V | V | V | V | V |          |
| NS2A    |                     |     |     |      |     |      |      |          |
| 1252 (NS2A-43) | V | V | A | V | V | V | V |          |
| 1281 (NS2A-72) | A | A | A | A | T | A | A |          |
| 1332 (NS2A-123) | K | K | R | R | R | R | R |          |
| NS2B    |                     |     |     |      |     |      |      |          |
| 1409 (NS2B-36) | A | A | A | A | A | E | A | A |          |
| NS3     |                     |     |     |      |     |      |      |          |
| 1562 (NS3-58) | I | I | V | V | V | I | I |          |
| 1988 (NS3-484) | G | G | S | S | S | S | S |          |
| 2005 (NS3-501) | L | L | M | M | M | M | M |          |
| 2022 (NS3-518) | F | F | S | S | S | S | S | L |          |
| NS4A    |                     |     |     |      |     |      |      |          |
| 2162 (NS4A-40) | T | T | A | A | A | A | A |          |
| 2269 (NS4A-147) | V | V | V | V | V | V | M |          |
| 2383 (NS4A-261) | F | F | F | F | F | F | V |          |
| NS5     |                     |     |     |      |     |      |      |          |
| 2704 (NS5-177) | R | R | I | R | I | I | R |          |
| 2865 (NS5-338) | V | V | A | A | A | A | A |          |
| 2932 (NS5-405) | V | V | V | V | V | V | V |          |
| 3036 (NS5-509) | V | V | V | V | G | V | V |          |
| 3048 (NS5-521) | L | L | I | L | L | L | L |          |
| 3292 (NS5-765) | - | Q | Q | Q | Q | Q | Q |          |
| 3426 (NS5-899) | T | T | I | I | I | I | I |          |

- Nucleotide sequence is missing
could not determine the relationships between bat JEV isolates and isolates from other host origins in local areas. Further studies are needed to explore the role of bats in the natural cycle of JEV.

However, it is worth noting that the human Liyujie isolate and the mosquito-derived BN19 isolate from Yunnan Province in China were closely related to the six bat isolates (Fig. 3), with high amino acid similarities of 99.0% to 99.6% and 98.8% to 99.8%, respectively. This indicates that a relationship might exist between humans, mosquitoes and bats within the JEV transmission cycle.

Although no amino acid mutations were identified that were previously associated with viral phenotype alteration in the Nakayama isolate, which was isolated from a Japanese patient in 1935 (99.4% to 99.9% at the nucleotide level, 99.4% to 99.5% at the amino acid level, respectively), the comparison of the deduced amino acid sequences of bat JEV isolates and the Nakayama strain demonstrated that there were some variations in amino acid sequences (Fig. 2, Table 4). Amino acid changes in critical determinants of the viral proteins could cause alterations in viral propagation, virulence and neuroinvasion [5, 8, 9, 18, 20, 26, 27, 39, 51]. Even though no amino acid mutations were identified at key positions (E-102 [51], E-138 [39], E-300 [26], E-306 [52], E-308 [26], E-395 [26], C-52 [8, 18], C-109 [8], C-122 [8], NS3-109 [8] and NS3-122 [8]), we cannot exclude the possibility that a combination of these unique amino acids together would contribute to neurovirulence. The biological significance of the differences in amino acid sequences between the bat JEVs and other JEV isolates requires further study.

In conclusion, our study showed that eight bat JEV isolates (GD1, HN2, SY87, YY158, B58, GB30, HB49 and HB97) belonged to GIII of JEV and shared a high degree of genetic identity. We presume that bat JEV isolates might be more evolutionarily conserved than other original JEV isolates. In consideration of the bat JEV isolates being phylogenetically similar to the mosquito isolate (BN19)
and the human isolate (Liyujie) from China and the Nakayama strain, bats might be involved in the JEV cycle in nature. However, we could not conclude whether bats are the hosts for JEV or are occasionally infected by JEV based on current evidence. Further virological and molecular epidemiologic studies of the bat JEVs are still needed.

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