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Full Length Research Paper

Isolation and molecular characterization of Japanese encephalitis virus GZ strain from piglets, China

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JEV GZ strain was isolated from piglets. The fragments of *E* and *NS1* gene of GZ strain were successfully amplified by reverse transcription-polymerase chain reaction (RT-PCR), then cloned into pMD19-T vector and sequenced. The homology of *E* and *NS1* gene of GZ strain and amino acid sequence were analyzed using the software's DNAStar Clustal_1.81 and Mega. Compared with other JEV strains, the homology of *E* gene ranges from 88 to 99.1% and 97.6 to 99.8% on the level of nucleotide and amino acid sequences, respectively. The homology of *NS1* gene ranges from 90.7 to 98.1% and 97.6 to 99.8% on the level of nucleotide and amino acid sequences, respectively. Phylogenetic analysis of *E* gene of GZ strain shows that the isolate strain belongs to genotype III and it is most closely related to the VN50/Viet Nam/1989/Human brain strain. In conclusion, results of this study show that JEV GZ strain was a wild virulent strain. Genotype III of JEV still circulates in Guizhou Province and it is thus important for active surveillance on genotype III of JEV from the swine population. Future study should be aimed at investigating the efficacy of current widely used JEV vaccines against the GZ strain and control the JE of pigs in order to prevent human JE.

Key words: Japanese encephalitis virus, *E* gene, *NS1* gene, cloning, sequence analysis.

INTRODUCTION

Japanese encephalitis (JE) is mostly prevalent in eastern and southern Asia, such as China, India and Japan (Williams et al., 2000; Ghosh et al., 2009), with an estimated 30000 to 50000 reported cases and 10000 deaths occuring worldwide annually. Japanese encephalitis virus (JEV) exists in a zoonotic cycle between mosquitoes and pigs and/or water birds (Monath and Heinz, 1996). JEV can caused an acute infectious in central nervous system which seriously threats to human and animal health; the main symptoms of this disease are high fever, violent or depressed in sentiment (Yin and Liu, 1997). In China, JE is one of the most important viral encephalitis and has been reported in most provinces of mainland China except for Tibet, Xinjiang Uygur Autonomous Region and Qinghai Provinces (Vaughn and Hoke, 1992; Li et al., 2011).

Japanese encephalitis virus is a *flavivirus*, belonging to the family *Flaviviridae*, JEV genome is a single-stranded positive sense RNA molecule, encoding three kinds of structural proteins including capsid protein (C), membrane (M, a mature form of itsprecursor protein prM), protein (E), and seven nonstructural proteins (NS1, NS2A, NS2B, NS3,NS4A, NS4B and NS5). There is no overlap in every genome, and the *E* gene is closely related to the virus absorption, penetration and pathopoiesia and the organism's immune response, in addition. The nucleotide sequence of *E* gene was targeted for JEV phylogenetic analyses and JEV strains can be divided into five genotypes (Solomon et al., 2003; Nga et al., 2004; Li MH et al., 2011); The *NS1* gene has soluble complement binding activity and good immunogenicity (Lindenbach et al., 2007).

Swine is an important reservoir and overwintering host

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for JEV and thus plays a critical role in the human encephalitis epidemics. JEV is one of the major pathogens which can cause reproductive failure in sows. In the present study, we reported the isolation of a strain of JEV from piglets and the virus which is designated GZ strain. To investigate its molecular characters, the *E* and *NS1* gene of JEV GZ strain were sequenced, analyzed and compared with a large group of previously published JEV strains. Phylogenetic analysis indicated that GZ strain is a wild virulent strain.

MATERIALS AND METHODS

Collection of samples and virus isolation

Ten (10) swine brain samples were collected from piglets which were suspected to be infected with JEV in Anshun, Guizhou Province 2010. The virus was isolated on BHK-21 cells. Briefly, the swine brain samples were grind into homogenate, the mixture was filtrated to remove bacteria by 0.22 μm germtight filter, then the filtrate was inoculated onto the monolayer of BHK-21 cells, and the cytopathic effects (CPE) was observed daily under a microscope. Culture supernatants were harvested and re-inoculated onto fresh BHK-21 cells until the typical CPE of JEV appeared.

Virus purification

The culture supernatants were propagated by injection into the brains of suckling mice. The brain tissue was collected and ground while the first signs of paralysis emerged. The sample was centrifuged (12000 r/min, 30 min) and the supernatant was stored at -80°C as a virus stock. Mice were bought from Guiyang Medical University.

Reverse transcription-polymerase chain reaction (RT-PCR) detection

Total viral RNA was extracted from the supernatant of infected cells or the collected supernatant from the brain tissue of the suckling mice inoculated with the virus using TRIZOL reagent (Invitrogen, USA), according to the manufacturer's protocol. Briefly, 200μl of sample was mixed with 1.0 ml of TRIZOL reagent and 0.2 ml of chloroform. After incubation on ice (10 min), the aqua phase was separated by centrifugation (12,000×*g*, 15 min) at room temperature (RT) and 500 μl of isopropyl alcohol was mixed with 80% of the aqua phase in a fresh tube. After incubation (10 min) at RT, the RNA was precipitated (12,000 ×*g*, 10 min) at RT. After washing once with 80% ethanol, the pellets were briefly dried at RT and then dissolved with RNase free water and stored at -80°C until use. One pair of specific primers was designed based on *E* gene of JEV SA14 strain and SA14-14-2 strain published on GenBank using software Oligo6.0 and Primer5.0 for the detection of Japanese encephalitis virus. The forward primer5'-TTTAATTGTCTGGGAATGGGCAATC-3' and the reverse primer 5'-AGCATGCACATTGGTCGCTAAGAAC-3' targeted a 1500 bp fragment of *E* gene. Standard precautions were taken to avoid PCR contamination and no false-positive was observed in negative controls. The extracted total viral RNA was used as a template for cDNA synthesis using avian myeloblastosis virus (AMV) reverse transcriptase (Toyobo, Japan). The cDNA was subsequently used for PCR amplification with LA PCR Kit Ver.2.1 (TaKaRa, Dalian), according to the manufacturer's protocol. The PCR products were gel-purified using the QIAquick gel extraction kit (Qiagen), then the purified products were sent for sequencing(Invitrogen, Shanghai), the sequencing was performed in both directions, both strands of the PCR products were sequenced at least twice to avoid artefacts. The sequencing results of the PCR products were compared with known sequences of *E* gene of Japanese encephalitis virus in the GenBank database. The isolated virus was a strain of Japanese encephalitis virus; a field isolate strain which was named GZ strain.

Cloning and sequence analysis of *E* and *NS1* genes of JEV GZ strain

Two pairs of specific primers were designed using softwares Oligo6.0 and Primer5.0, based on *E* and *NS1* gene of JEV SA14 and SA14-14-2 strain that were published on GenBank. The oligonucleotides sequences are as follows: JEV-E BamHI F (upstream primer) CG*GGATCC*TTTAATTGTCTGGGAATGGGCAATC (contain a BamHI site in 5' terminal), JEV-E EcoRI R (downstream primer），CG*GAATTC*AGCATGCACATTGGTCGCTAAGAAC (contain a EcoRI site in 5' terminal), JEV-NS1 BamHI P1 (upstream primer), CG*GGATCC*GACACTGGATGTGCCATTGAC (containing a BamHI site in 5' terminal), JEV-NS1 EcoRI P2 (downstream primer), CG*GAATTC*AGCAGCGACTAGCACCACATACCTC (containing a EcoRI site in 5' terminal). The primers were synthesized by Takara Bio Inc. The amplified fragments were 1500 and 1300 bp, respectively.

The above extracted total viral RNA was used as a template for cDNA synthesis. The RT reaction system was: 10 μL of PCR reaction mixture consisting 5 μL total viral RNA, 2 μL 5×superscript Ⅲ buffer, 0.5 μL 0.1 M dithiothreitol (DTT), 0.5 μL 10 M dNTPs, 0.25 μL RNase-inhibitor, 0.5 μL superscript Ⅲ reverse transcriptase, 1.25 μL 10 M downstream primer and reaction time at 42°C for 1 h, to get the cDNA template. The reaction system of PCR was: 10×PCR buffe,r 5 μL; 10 M dNTPs 10 μL; upstream primer, 2.5 μL; downstream primer, 2.5 μL; Taq polymerase, 1 μL; cDNA, 2 μL; adding ddH_2O to 50 μL in total. *E* and *NS1* gene amplification reaction conditions were: denaturation for 5 min at 94°C, then 35 cycles was carried out (denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 90 s), the final chain extension step was at 72°C for 10 min. 50 μL PCR products were mixed with 10 μL 6xloading buffer together after the reaction, electrophoresis on 1% agarose gel at 100 V for 30 to 50 min, of which the results observed, was used to recover the gene fragment with Gel Extraction Kit (Qiagen), according to the procedures of specification. The recovered target gene products were cloned into pMD19-T vector, respectively, to construct pMD19-T-E recombinant plasmids and pMD19-T-NS1 recombinant plasmids. The reaction conditions were: pMD19-T vector 1 μL, target gene fragments 4 μL and solution 15 μL, then transformed into competent cell Top10 after connecting at 16°C overnight. The Top10 in 2×YT agar plate was supplemented with IPTG and X-gal and 50 μg/mL ampicillin was used to coat it. The agar plat was then cultivated for 12 to 16 h at 37°C. A white single colony was picked and inoculated into the 2×YT liquid medium (containing 50 μg/mL ampicillin), then the culture was shaken under 120 rpm for 12 to 16 h at 37°C. The extract Plasmid DNA was digested using BamH I and EcoR I. If the double digestion results were correct, then both kind of recombinant plasmids bacteria liquid (10 samples) were sent for sequencing (TaKaRa, Dalian). The sequencing was performed in both directions at least twice to avoid artefacts.

Multiple alignments and phylogenetic analysis

The JEV strains used in multiple sequence alignments and phylogenetic analyses in this study are listed in Tables 1 and 2. Multiple sequence alignments and phylogenetic analysis were

Table 1. Details of E gene of JEV strains used for analysis in this study.

Strain	*E* gene GenBank accession No.	Origin	Year of isolation	Genotype	Source
SC04-27	DQ404095	China,Sichuan	2004	1	*Culex*
SC04-25	DQ404094	China,Sichuan	2004	1	*Culex*
SC04-17	DQ404093	China,Sichuan	2004	1	*Culex tritaeniorhynchus*
SC04-16	DQ404092	China,Sichuan	2004	1	*Armigeres*
SC04-15	DQ404091	China,Sichuan	2004	1	*Culex tritaeniorhynchus*
SC04-12	DQ404090	China,Sichuan	2004	1	*Culex*
GZ04-2	DQ404109	China, Guizhou	2004	3	*Armigeres*
GZ04-71	DQ404114	China, Guizhou	2004	3	*Armigeres*
GZ04-43	DQ404113	China, Guizhou	2004	3	*Culex*
GZ04-29	DQ404111	China, Guizhou	2004	3	*Culex*
GZ04-36	DQ404112	China, Guizhou	2004	3	*Culex*
GZ04-4	DQ404110	China, Guizhou	2004	3	*Armigeres*
GZ04-89	DQ404115	China, Guizhou	2004	3	*Armigeres*
VN50/Viet Nam/1989/ Human brain	AY376463	Vietnam	1989	3	Human brain
SA14	AY243842	China	2003	3	mosquito pool
P3	AY243844	China	2003	3	Patient brain sample
SA14-14-2	AF315119	China	2000	3	vaccine
WHe	EF107523	China	2006	3	brain tissue
KV1899	AY316157	Korea	1999	1	Pig serum
YNDL04-39	DQ404142	China, Yunnan	2004	3	*Culex tritaeniorhynchus*
YNDL04-45	DQ404145	China, Yunnan	2004	3	Mosquito pool
YNJH04-25-3	DQ404148	China, Yunnan	2004	3	*Culex*
YN	DQ404127	China, Yunnan	1954	3	Human brain
YN83-Meng83-54	DQ404130	China, Yunnan	1983	1	*Forcipomyia taiwana Shiraki*
YN-Xiang JE	DQ404135	China, Yunnan	1986	1	Human blood
YUNNAN0902	JQ086763	China, Yunnan	2009	3	*Sus scrofa*
YUNNAN0901	JN864064	China, Yunnan	2009	3	mosquito
Beijing-1	L48961	China	1950s	3	Human brain
A2	AY243843	China, Beijing	1949	3	patient brain sample
JaGAr01	AF069076	Japan, Gunma	1959	3	*Culex tritaeniorhynchus*
Muar	HM596272	Malaysia	1952	5	brain
SX09S-01	HQ893545	China	2009	1	Swine brain
GZ56	HM366552	China	2008	1	cerebrospinal fluid
XZ0934	JF915894	China	2009	5	mosquito
MVE-1-51*	NC_000943	Australia	1951	–	Human brain

Murray Valley encephalitis (MVE) virus used as an outgroup for the phylogenetic analysis.

performed by the Clustal W method using the DNAStar software. The bootstrap probabilities of each node were calculated using 1000 replicates. Phylogenetic tree was drew using the softwares DNAStar Clustal_1.81 and Mega.

RESULTS

Viral isolation

In order to isolate the pathogen, brain samples were ground and the supernatant was inoculated onto the fresh BHK-21 cells. BHK-21 cells appeared round, shrinked and dislodged from the cell surface under a microscope on days three to four after inoculation, and typical cytopathic effect (CPE) of JEV was observed, meanwhile the normal BHK-21 cell was set as control (Figures 1a and b). The suckling mice which emerged showed shivers, tonic, limb spasms, and excitability of nervous system enhanced symptoms after the culture supernatants was injected, and the symptoms gradually

Table 2. Details of NS1 gene of JEV strains used for analysis in this study.

Strain	*NS1* gene GenBank accession No.	Origin	Year of isolation	Genotype	Source
Muar	HM596272	Malaysia	1952	5	Brain
HB49	JF706284	China	1990	3	Blood
SA14	JEU14163	China	1954	3	Mosquito
SA14-14-2	AF315119	China	2000	3	Vaccine
WHe	EF107523	China	2006	3	Brain tissue
KV1899	AY316157	Korea	1999	1	Pig serum
YUNNAN0902	JQ086763	China, Yunnan	2009	3	*Sus scrofa*
YUNNAN0901	JN864064	China, Yunnan	2009	3	Mosquito
CH2195SA	AF095793	Taiwan	1998	3	Small plaque variant
CH2195LA	AF095156	Taiwan	1998	3	Large plaque variant
TC	AF098736	Taiwan	1998	3	*Aedes albopictus*
Beijing-1	L48961	China	1950s	3	Human brain
JaGAr01	AF069076	Japan, Gunma	1959	3	*Culex tritaeniorhynchus*
HN2	JN711459	China	2008	3	*Scotophilus kuhlii*
SD0810	JF706286	China	2009	1	*Culex tritaeniorhynchus*
YN83-Meng83-54	DQ404130	China, Yunnan	1983	1	*Forcipomyia taiwana* Shiraki
GZ56	HM366552	China	2008	1	cerebrospinal fluid
SX09S-01	HQ893545	China	2009	1	Swine brain
XZ0934	JF915894	China	2009	5	mosquito
MVE-1-51*	NC_000943	Australia	1951	–	Human brain

Murray Valley encephalitis (MVE) virus used as an outgroup for the phylogenetic analysis.

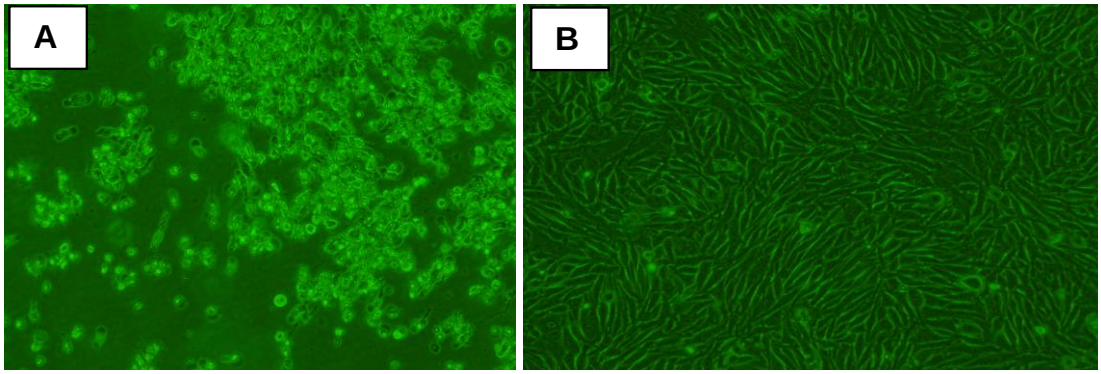

Figure 1. A. Cytopathic effect of BHK-21 cell. **B.** The normal BHK-21 cell.

aggravated with the passage of time, resulting in appearance of typical symptoms of Japanese encephalitis (Figure 2). RT-PCR with the E primers resulted in the amplification of a 1500 bp band (Figure 3).

Cloning of *E* gene and *NS1* gene of JEV GZ strain

The RT-PCR results of *E* and *NS1* gene of JEV GZ strain are shown in Figures 4a and 4b. The amplified target gene fragments were about 1500 and 1245 bp from JEV GZ strain, in accordance with the expected results, indicating that *E* and *NS1* gene of JEV were successfully amplified.

After the recovery, connection and transformation of the target gene, the constructed recombinant plasmid was identified by PCR confirmation, and *EcoR* I/*BamH* I double enzyme digestion and DNA sequencing. Both the *E* and *NS1* gene were obtained from the corresponding target gene fragments, indicating the inserted target gene fragment and the vector, respectively. The electrophoresis results were shown in Figures 5a and b.

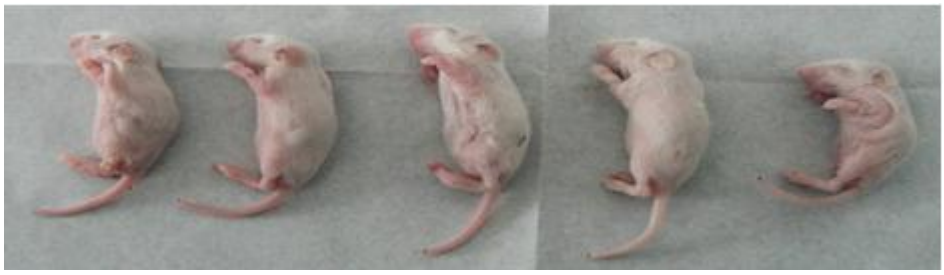

Figure 2. Suckling mice inoculated culture supernatants.

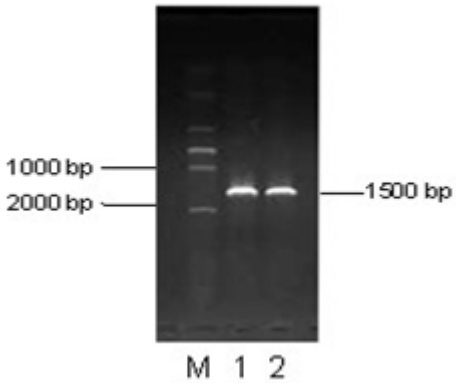

Figure 3. RT-PCR results of E gene of JEV Guizhou strain. M, DL2000 DNA Marker; lane 1, DNA band amplified from culture supernatants; lane 2. DNA band amplified from the brain tissue of suckling mice inoculated culture supernatants.

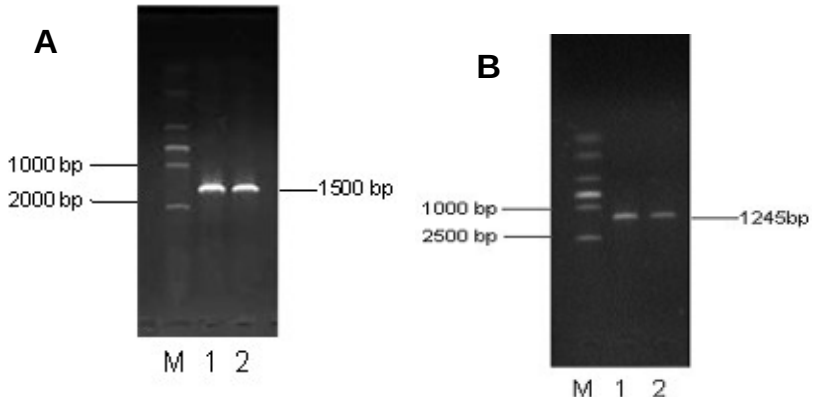

Figure 4. A. RT-PCR results of E gene of JEV GZ strain. **B.** RT-PCR results of NS1 gene of JEV GZ strain. M, DL 2000 DNA marker; lane 1, DNA band amplified from culture supernatants; lane 2, DNA band amplified from the brain tissue of suckling mice inoculated culture supernatants.

The recombinant plasmids were digested, indicating that the objective gene fragments connected were correct.

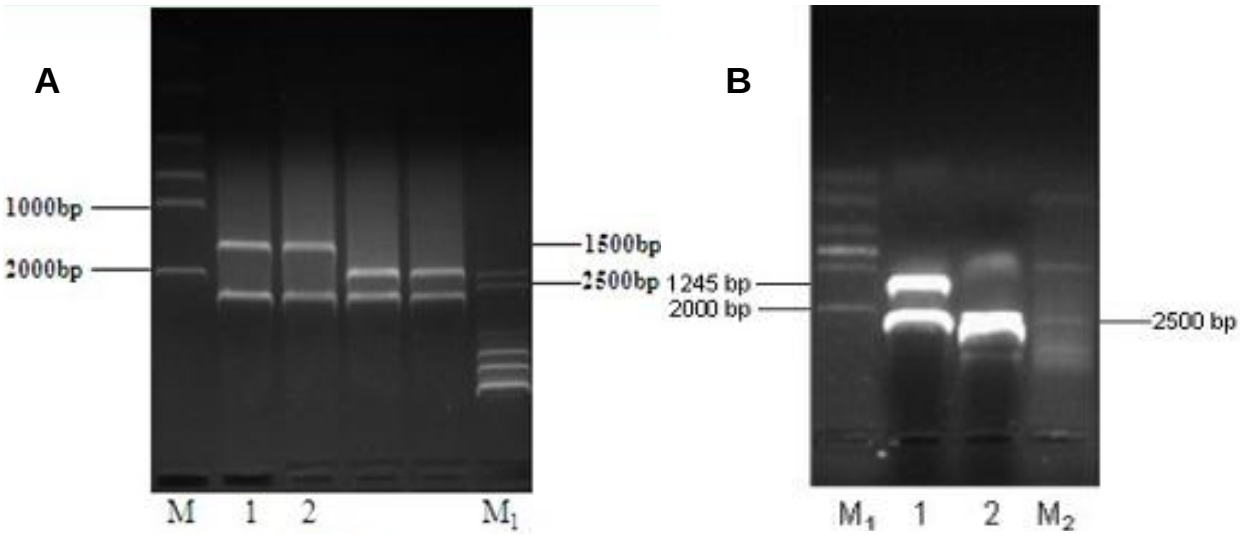

Figure 5 A Identification of recombinant plasmid of E gene of JEV GZ strain. Lane M, DL2000 DNA Marker; lane M1, DL15000+2000 DNA Marker. Lanes 1 and 2, recombinant plasmid of E gene of JEV GZ strain. **b.** Identification of recombinant plasmid of NS1 gene of JEV GZ strain. Lane M_1, DL2000 DNA marker; M2, DL15000+2000 DNA Marker. Lanes 1 and 2, recombinant plasmids of NS1 gene of JEV GZ strain.

Multiple alignments and phylogenetic analysis of JEV sequence

The sequencing results showed that the length of cDNA of *E* gene of GZ strain was 1500 bp and 415 amino acids, and the length of cDNA of *NS1* gene of GZ strain was 1245 bp and 415 amino acids. Phylogenetic analysis of the *E* gene sequence of JEV GZ strain was performed with gene sequences of 35 known JEV strains from different geographical locations (Table 1). As shown in Figure 6, the viruses were classified into three genotypes, and all of these three genotypes have emerged in China. Newly isolated GZ strain was classified as JEV genotype III, and the GZ strain had a highest nucleotide sequence identity with VN50/Viet Nam/ 1989/ Human brain strain. Phylogenetic analysis of the *NS1* gene sequence of JEV GZ strain was performed with gene sequences of 20 known JEV strains from different geographical locations (Table 2). *NS1* gene phylogenetic analysis indicated that JEV GZ strain was genotype III and had more than 99% nucleotide identity with SA strain. This is also confirmed that the isolated GZ strain was a virulent strain from another side.

Analysis of the *E* gene amino acid sequences of GZ strain

JEV E protein is one of the major structural proteins and is closely related to viral virulence, host tropism and antigenicity. Eight amino acid residues of E protein play a critical role in the neurovirulence (McMinn, 1997). To analyze these key amino acids, the E protein of GZ strain were compared with SA14-14-2 (an attenuated vaccine strain), and other virulent strains. Differences among the eight key amino acid residues were found between GZ strain and SA14-14-2 strain, whereas no differences were found between GZ and the compared virulent strains (Table 3). So, we predicted that the GZ strain possessed some typical characteristics of strong virulent JEV.

Analysis of the *NS1* gene amino acid sequences of GZ strain

JEV *NS1* gene encoded a kind of highly conservative secretory glycoprotein and this protein was expressed on the surface of infected cells, and was excreted in the same way of dissolving or connecting it with the cell membrane, but these excreted ways only limited the NS1 protein dimer. In this study, the *NS1* gene of JEV was analyzed on the level of nucleotide and amino acid, compared with the isolated JEV strains in the mainland of China, the homology of *NS1* gene was at least 97.1% on the level of amino acid sequences (Figure 7), proving that the glycoprotein encoded by *NS1* gene is highly conservative and indicating that NS1 protein may be suitable as a kind of vaccine antigen protein.

DISCUSSION

According to Shirish's method of JEV genotyped, there are at least two genotypes of JEV strains in China, including genotype I and III. From 2002 to 2005, JEV isolates from mosquitoes, biting midges, human

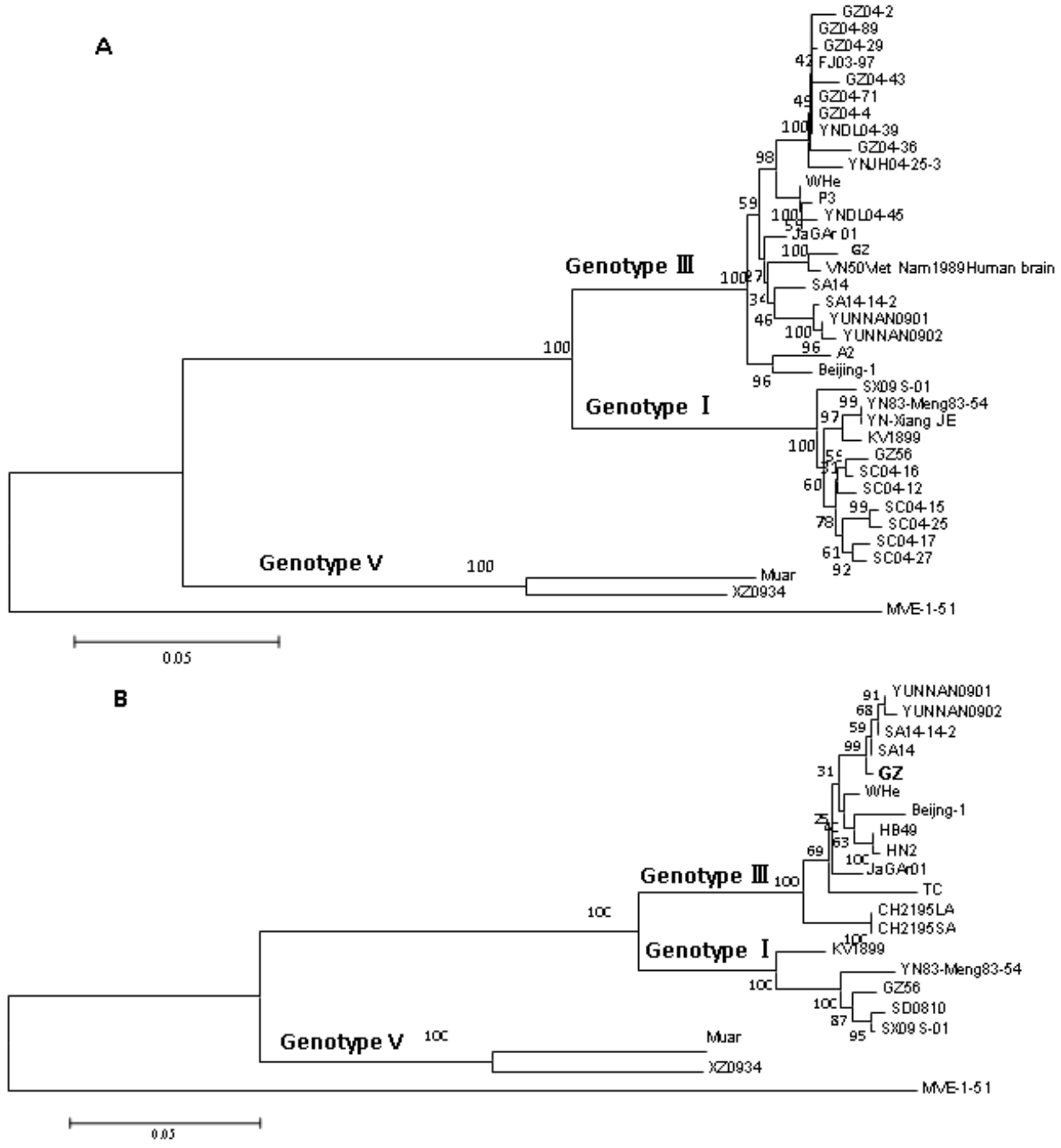

Figure 6. Phylogenetic relationships of JEV GZ strain with other JEV strains based on complete E (A) and NS1 (B) gene sequences. Multiple alignments and phylogenetic analysis were performed by the neighbor-joining (N-J) method using Mega software. The bootstrap probabilities of each node were calculated using 1000 replicates. The phylogenetic trees were generated using Mega software, too. The GZ strain was shown in bold font.

cerebrospinal fluid and human blood samples in China can be divided into two genotypes, genotype I and genotype III (Wang et al., 2007). Genotype I strain was almost isolated from mosquitoes (Wang et al., 2004; An and Wang, 2008) in our country; but in 2011, JEV isolates were divided into Three genotypes, genotype I, genotype III and genotype V (Li et al., 2011). JE is mostly prevalent in eastern and southern Asia, including China, India and Japan. There are many human JE cases reported in recent years (Li et al., 2011; Pan et al., 2011).

Table 3. Comparison of eight critical amino acids closely related to the neurovirulence of JEV in E protein between the isolated GZ strain and other strains.

Strain	E107	E138	E176	E177	E264	E279	E315	E439
SA14-14-2	Phe(F)	Lys(K)	Val(V)	Ala(A)	His(H)	Met(M)	Val(V)	Arg(R)
VN50/VietNam/1989/Human brain	Leu(L)	Glu(E)	Ile(I)	Thr(T)	Gln(Q)	Lys(K)	Ala(A)	Lys(K)
SA14	Leu(L)	Glu(E)	Ile(I)	Thr(T)	Gln(Q)	Lys(K)	Ala(A)	Lys(K)
KV1899	Leu(L)	Glu(E)	Ile(I)	Thr(T)	Gln(Q)	Lys(K)	Ala(A)	Lys(K)
GZ	Leu(L)	Glu(E)	Ile(I)	Thr(T)	Gln(Q)	Lys(K)	Ala(A)	Lys(K)

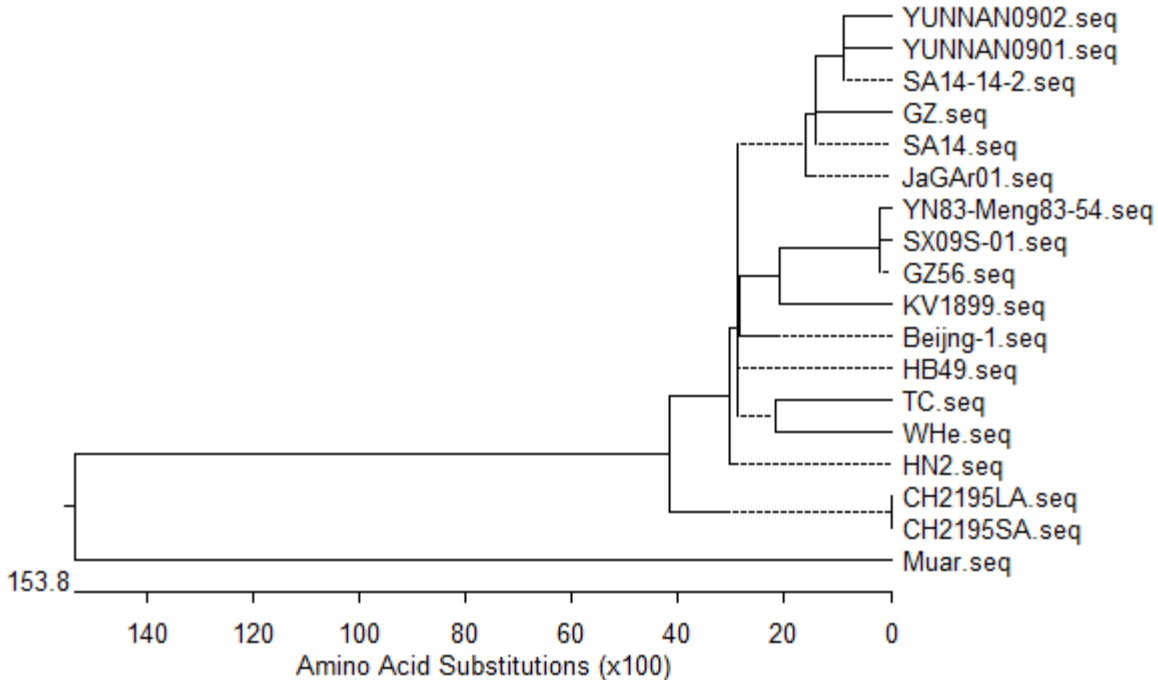

Figure 7. Phylogenetic relationships of JEV GZ strain with other JEV strains based on complete NS1 amino acid sequences. The multiple sequence alignments were obtained by DNAstar software, and the phylogenetic tree was generated using Clustal W method.

However, there is very limited information on JEV strains originated from pigs. Pig is an important reservoir and overwintering host for JEV and thus plays a critical role in the human encephalitis epidemics.In this study, a strain of JEV was isolated from piglets in Anshun, Guizhou Province. The newly isolated JEV, designated GZ strain, belongs to genotype I by E sequences analysis. To our knowledge, it is reported that only HEN0701, SXBJ07 and SX09S-01 strains have been isolated from swine and belonging to the genotype I JEV in China (Zheng et al., 2009; Wang et al., 2009; Qi et al., 2011).

Studies from home and board shows that *E* gene of JEV encoded the viral envelope glycoprotein, its activity area was consisted by Three structurally distinct domains (domains I, domains II and domains III) and 411 amino acid residues. Domains I, E1~E51, E137~E196, E293~E311. Domains II, E52 to E137, E197 to E292, and domains III, E310 to E411 appeared to be associated with viral entry, cell tropism, virulence and protective immunity etc (Kolaskar and Kulkarni-Kale, 1999). Here, the E protein of GZ strain were compared with SA14-14-2 (an attenuated vaccine strain), and other virulent strains. Differences among the eight key amino acid residues were found between GZ strain and SA14-14-2 strain, whereas no differences were found between GZ and the compared virulent strains (Table 3). So, we predicted that the GZ strain has these typical characters of strong virulent JEV.

JEV *NS1* gene encoded a kind of highly conservative secretory glycoprotein which was rich in mannose oligosaccharides groups, which was the first translated non-structural protein. This protein was expressed on the surface of infected cells, and was excreted in the way of dissolving or connecting with the cell membrane, but

these excreted ways only limited in the NS1 protein dimer. Extracellular NS1 protein also called soluble complement antibody fixation (SCF), had the same antigenicity as intracellular NS1 protein (Chen et al., 1996). In this study, the *NS1* gene of JEV was analyzed on the level of nucleotide and amino acid, compared with the isolated JEV strains in the mainland of China, the homology of *NS1* gene at least 97.1% on the level of amino acid sequences, proving that the glycoprotein encoded by *NS1* gene is highly conservative, therefore, NS1 protein may be suitable as a kind of vaccine antigen protein. In summary, results of this study showed that a wild JEV strain, GZ strain was isolated, and it belongs to genotype III, indicating that genotype III JEV still circulates in Guizhou Province. Continuous survey and evaluation of the infectivity and pathogenecity of JEV genotype III are necessary. Future study should be aimed at investigating the efficacy of current widely used JEV vaccines against the GZ strain and control of the JE of pigs in order to prevent human JE.

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