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Genetic analysis of strains of Japanese Encephalitis Virus isolated from swine in central China

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Abstract In this study, four strains of Japanese Encephalitis Virus (JEV) were isolated from the cerebrospinal fluid of aborted fetuses or stillborn piglets collected randomly from a number of piggeries in central China. The E genes were cloned by RT–PCR and sequenced. Phylogenetic analysis was performed with 48 JEV isolates previously reported in China and other countries, and showed that all four isolates can be classified into the subcluster of genotype III. The results strongly suggest that the genotype III of JEV is the major variant currently circulating in the swinery of central China.

Keywords Japanese Encephalitis Virus · Swine · Cerebrospinal fluid · Genotype III

Japanese Encephalitis Virus (JEV) is a mosquito-borne flavivirus that causes severe encephalitis in humans [1, 2] and is the most important and widespread cause of endemic viral encephalitis in the South-East Asian, western Pacific Ocean, Indian subcontinent, and China. Since the first report in 1949, JE epidemics have occurred in China for over 50 years and the virus can be detected in many parts of the country. JEV is a flavivirus primarily existing in an enzootic cycle of transmission between water-birds and/or pigs, and rice-field breeding mosquitoes, *Culex Tritaeniorhynchus.* Domestic and migrating birds as well as pigs are effective amplifying hosts with occasional infection of humans.

Using highly variable short sequence in the capsid/pre-membrane (C/prM) gene region [3–5], JEV strains can be grouped into four geographically distinct genotypes by phylogenetic analysis based on nucleotide differences of more than 12%. Genotype I is found primarily in Laos, northern Thailand, Cambodia, and Malaysia; Genotype II contains isolates from southern Thailand, Malaysia, and Indonesia; Genotype III appears to be the most widely distributed and includes isolates from Japan, Korea, China, Malaysia, Philippines, Indonesia, India, and Sri Lanka; Genotype IV appears to be restricted to Indonesia. However, it is possible that analysis based on short sequences leads to somewhat unclear and unreliable results [6]. Recently, the E gene of JEV was shown to be a good candidate for phylogenetic analysis and produced division into five genotypes, with one genotype represented by a single isolate from Singapore [7–9].

Recently, a number of JEV strains isolated from the mosquito and human patients have been reported in China [10]. However, the prevalence of JEV in the pig population is still unclear. In the present study, JEV strains isolated from swine in central China were characterized and using a 1,450-nucleotide sequence from the E gene region, compared with a group of previously reported isolates, particularly those from China.

Jian-Ming Fan and Jun Luo contributed equally to this work.

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Table 1 Japanese encephalitis virus isolates analyzed in this study

Strain	Year	Geographical location	Source	Genotype	E gene GenBank accession no.
TS00	2000	Australia	Mosquito	I	EF434785
JKT5441	1981	Indonesia	Mosquito	II	U70406
FU	1995	Australia	Human serum	II	AF217620
M859	1967	Cambodia	Mosquito	II	U70410
P20778	1958	India	Human brain	III	AF080251
GP78	1978	India	Human brain	III	AF075723
JKT1724	1979	Indonesia, Java	*Culex tritaeniorhynchus*	III	U70404
Ishikawa	1994	Japan, Ishikawa	Swine mononuclear cells	I	AB051292
K87P39	1987	Korea	Mosquito	III	AY585242
K94P05	1994	Korea	Mosquito	I	AF045551
KV1899	1999	Korea	Pig serum	I	AY316157
B2524	1985	Nepal	Human cerebrospinal fluid	III	U70392
PhAn1242	1984	Philippine, Santo Cristo	Pig serum	III	U70417
691004	1969	Sri Lanka	Human blood	III	Z34097
H-49778	1987	Sri Lanka	Human brain	III	U70395
HK8256	1972	Taiwan	*Culex annulus*	III	U70396
Th2372	1979	Thailand	Human brain	I	U70401
B-1065	1983	Thailand, Chumporn	Pig blood	II	U70388
VN-118	1979	Vietnam	*Culex fatigans*	III	U70420
Ha-3	1960s	China, Heilongjiang	Human brain	III	AY243842
SA14	1960	China, Shanxi	Mosquito pool	III	AY243850
SA14-14-2	2000	China	Vaccine	III	AF315119
SH-3	1987	China, Shanghai	Human brain	III	AY243826
SH-53	2001	China, Shanghai	*Culex tritaeniorhynchus*	I	AY555757
SH-96	2001	China, Shanghai	*Culex tritaeniorhynchus*	I	AY555760
SH-101	2001	China, Shanghai	*Culex tritaeniorhynchus*	I	AY555761
TLA	1971	China, Liaoning	Human brain	III	AY243832
G35	1954	China, Fujian	Mosquito pool	III	AY243831
LYZ	1957	China, Fujian	Human brain	III	AY243834
02-29	2002	China, Fujian	Human cerebrospinal fluid	III	AY555762
JKT9092	1981	Indonesia,	Mosquito	IV	U70409
JKT7003	1981	Indonesia	Mosquito	IV	U70408
JKT6468	1981	Indonesia	Mosquito	IV	U70407
SH04-5	2004	China, Shanghai	*Culex tritaeniorhynchus*	III	DQ404106
GZ04-4	2004	China, Guizhou	*Armigeres*	III	DQ404110
FJ03-31	2003	China, Fujian	Human blood	III	DQ404117
YNDL04-29	2004	China, Yunnan	*Culex theileri*	III	DQ404139
HLJ02-134	2002	China, Heilongjiang	*Genus culicoids*	III	DQ404081
HLJ02-170	2002	China, Heilongjiang	*Aedes vexans*	III	DQ404084
GZ04-71	2004	China, Guizhou	*Armigeres*	III	DQ404114
FJ03-97	2003	China, Fujian	Human blood	III	DQ404127
YNDL04-19	2004	China, Yunnan	*Culex theileri*	III	DQ404147
HN04-11	2004	China, Henan	*Culex*	I	DQ404087
HN04-40	2004	China, Henan	*Culex*	I	DQ404089
SC04-25	2004	China, Sichuan	*Culex*	I	DQ404094
SH03-103	2003	China, Shanghai	*Culex tritaeniorhynchus*	I	DQ404096

A total of 30 cerebrospinal fluid (CSF) samples collected from aborted fetuses and stillborn piglets from swine in Henan province, central China, were subjected to virus isolation as described previously [11]. Susceptible PK-15 cells were exposed in infection experiments and any subsequent virus infection were detected by conventional immunohistochemistry assays after 72 h post-infection (p.i.). The total cellular RNA was extracted from the JEV-infected PK-15 cell cultures using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription polymerase chain reaction (RT–PCR) was used to amplify the specific E gene regions. Briefly, purified RNA was used as the template for cDNA synthesis using reverse transcriptase M-MLV (TaKaRa). The complete E gene was amplified using a specific primer pair JEV-EP1 (5′-GAGGACACTATCACGTACG-3′) and JEV-EP2 (5′-CGTTTCTGGCAAATATTTATACC-3′). The PCR products were sequenced using an ABI PRISM 3100 DNA sequencer. Finally, the results of RT–PCR and DNA sequencing showed that four field isolates, nominated as CSF.ZMD-3, CSF.ZMD-4, CSF.BY-1, and CSF.ZY-2, were characterized as JEV strains (GenBank ACC. No. GQ336809, GQ845081, GQ845082, and GQ845083, respectively).

Using the E gene sequences of the four isolates and 48 reference strains listed in Table 1, a phylogenetic tree was constructed. The phylogenetic status was assessed using MEGA-3 software according to the methods described previously [12, 13]. The neighbor-joining tree (Fig. 1a) and maximum parsimony tree (Fig. 1b) were generated using

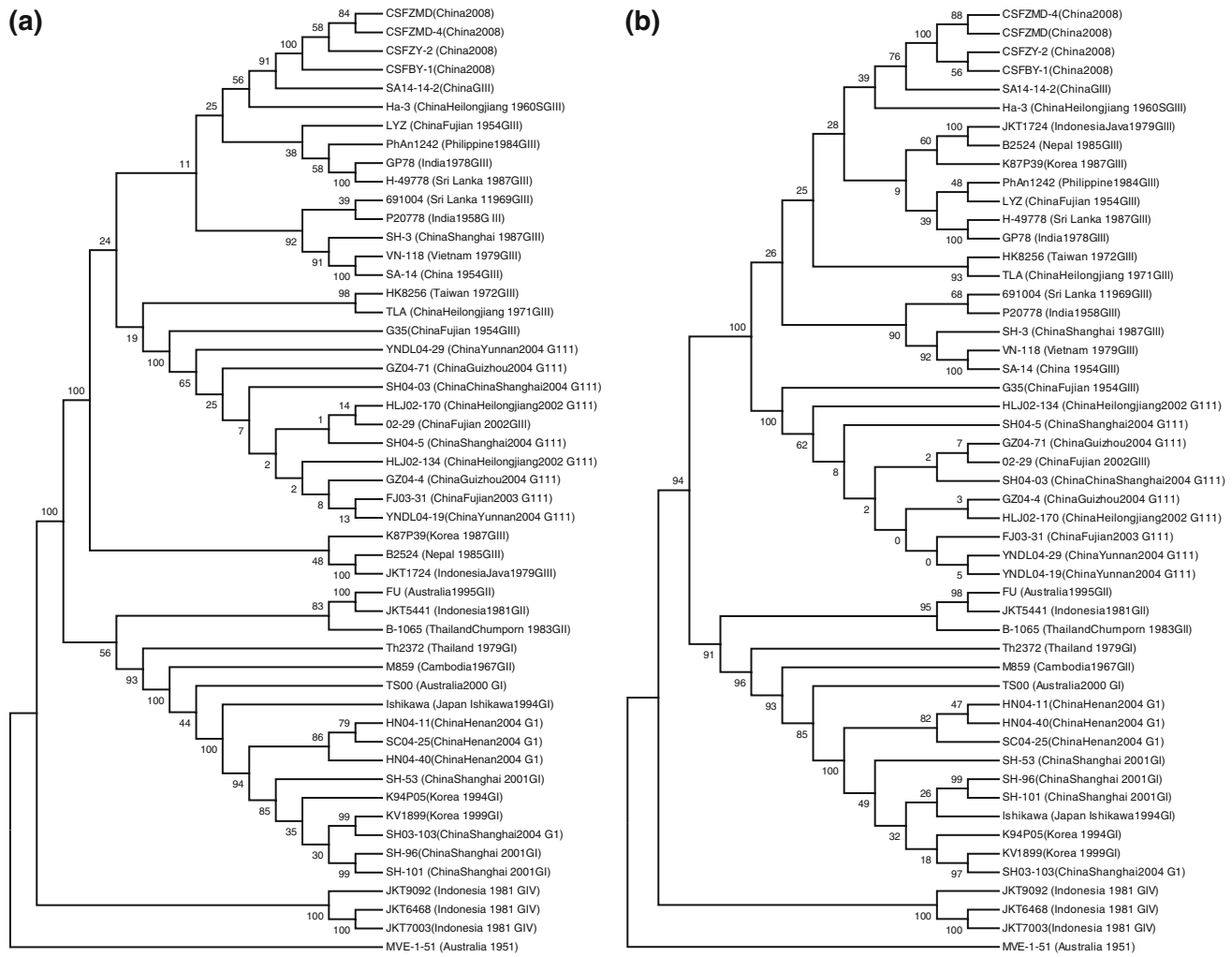

Fig. 1 Phylogenetic analysis of JEV strains predicted from the E gene sequences. Four isolates of JEV collected in central China are CSF.ZMD-3 (GQ336809), CSF.ZMD-4 (GQ845083), CSF.BY-1 (GQ845081), and CSF.ZY-2 (GQ845082). The neighbor-joining tree (**a**) and maximum parsimony tree (**b**) was generated using CLUSTAL_X. Phylogenetic groups are given on the left of each tree, indicated according to Uchil and Satchidanandam [17]. The tree was rooted using Murray Valley encephalitis virus (MVEV) sequence information. Bootstrap confidence limits for 1,000 replicates are indicated above each branch. Horizontal branch lengths are proportional to genetic distance and vertical branch lengths have no significance

CLUSTAL_X. The reliability of different phylogenetic groupings was evaluated using the bootstrap test (1,000 bootstrap replications) available in MEGA. The genome sequence of Murray Valley encephalitis virus (NC_000943) was used as the outgroup in all analyses. A bootstrap value of >70% was defined as the criterion for phylogenetic grouping. All of the phylogenetic trees were drawn using TreeView software [12]. On the basis of the criteria of Chen and others, phylogenetic analysis based on the E region demonstrated that the four newly isolated JEV strains from central China belong to genotype III (Fig. 1a, b). Further analysis showed that these new isolates belong to a discrete subcluster. The isolates were close to SA-14 and closer to SA-14-14-2, a live vaccine virus derived from the parental SA-14. It is hard to believe that SA-14 virus genome is so naturally stable that it has survived all this time, so we have speculated that the virus re-appeared from natural environment. We, therefore, point out one possibility is that the strains originated from immunization of humans or pigs by the SA-14-14-2 virus. We examined the four isolates biological characters and found that all of them caused cytopathogenic effect (CPE) in PK15 cells, and were fetal to suckling mice. The four isolates obtained from aborted fetuses and stillborn piglets from swine in Henan province, central China. These swines were vaccinated using the live attenuated vaccine strain SA14-14-2 to provide immunity against these related viruses and reduce their circulation in vaccinated regions. However, if the incidence of the apparent infection with JEV genotype III increases among the vaccinated swines population, development of a new bivalent vaccine that contains both the genotype I and III strains might be necessary.

Our results indicate that genotype III is the major subtype of JEV, circulating in central China, Fujian, and Heilongjiang. Genotype III was the major genotype of JEV in Shanghai until 1987, but the isolates recently reported in Shanghai in 2001 belongs to genotype I, while those isolated in Fujian Province in 2002 belong to genotype III [14, 15]. Genotype I strains distribute throughout the Southeast Asian countries, whereas, genotype III strains are distributed throughout a broader region encompassing Northern Asia.

The phylogenetic tree showed that the JEV strains recently isolated from China are grouped into the same subclusters of genotypes I or III. Genotype I JEV was first isolated in Cambodia in 1967 and has only been isolated in China since 1979 while genotype III strains were isolated before the 1970s, suggesting that genotype I strains were introduced into China around 1979 [10]. The genotype distribution of JEV strains seems to be changing in various regions but the genotype III appears to be the major subtype currently circulating in central China. During the last several years from 2003 to 2008, the average official numbers of human patients and death caused by JEV infection per year are around 6,703 and 306, respectively. It is apparent that JEV is a serious threat to human health, although declining in recent years [16]. Thus, efforts for further investigating the distribution and seasonality of JEV in China need to be continued.

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