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Short communication

Isolation and characterization of a Far-Eastern strain of tick-borne encephalitis virus in China

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ABSTRACT

Tick-borne encephalitis virus (TBEV) is a leading cause of human neurological infection in many parts of Europe and Asia. Although several TBEV isolates have been reported, current understanding of the biological characteristics of a Chinese strain is limited. In this study, a Far-Eastern strain of TBEV designated WH2012 was isolated in northern China. Its genome has been sequenced and found to be closely related to other Chinese TBEV isolates. Human cell lines of neural origin exposed to WH2012 showed cytopathic effects and WH2012 replicated most efficiently in human neuroblastoma cells SK-N-SH. In addition, WH2012 possessed a pathogenic potential in the mouse model, characterized by inducing a complete paralysis in the hindlimbs with a fatal outcome. We herein describe the first data regarding biological properties of TBEV from China. This study may help future research on pathogenic mechanisms of the neurological disease induced by TBEV infection in China.

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Tick-borne encephalitis virus (TBEV) is an important causative agent of central nervous system (CNS) infections in humans and a member of the genus *Flavivirus* within the family *Flaviviridae*. The TBEV genome is an approximately 11 kb long positive single-stranded RNA molecule, consisting of a single open reading frame encoding three structural proteins (C, prM/M and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). Based on phylogenetic analysis, TBEV has been subdivided into three subtypes, namely, European (TBEV-Eu), Siberian (TBEV-Sib) and Far-Eastern (TBEV-FE). The dominant vector of TBEV-Eu is *Ixodes ricinus*, and *Ixodes persulcatus* for the other two subtypes (Lindquist and Vapalahti, 2008; Mansfield et al., 2009).

TBE cases exhibit a variety of clinical symptoms in humans, including high fever, headache, vomiting and acute or chronic progressive encephalitis, with or without a fatal outcome (Gritsun et al., 2003b). TBEV is endemic over a wide area of Europe and Asia and causes more than 10,000 cases of neurological manifestations annually (Holzmann et al., 2009). In China, the first human case of TBE was reported in 1943 and TBEV was isolated from patients and ticks in 1952. So far all TBEV strains isolated in China belong to the FE subtype, which usually is associated with severe encephalitis signs with fatality rates of 20–40% (Gao et al., 2010; Lu et al., 2008). Although several TBEV isolates have been reported (Si et al., 2011; Zhang et al., 2012), information regarding biological characterization of Chinese strains is currently lacking. Here, we report the isolation of a FE strain of TBEV from ticks collected in northern China and investigate its pathogenesis potential in both cellular and mouse models.

Ticks (*I. persulcatus*) were collected in the wood-steppe region of northern China. Each 50 individual ticks were pooled into the same group and processed as previously described (Golovljova et al., 2004). Then the supernatant samples of tick pool suspenetons were used for further RNA extraction using an EZNA viral RNA kit (Omega Bio-Tek, Inc., GA, USA), according to the manufacturer's protocol. Viral RNA was detected by a TBEV-specific nested RT-PCR as previously described (Schrader and Süss, 1999). Virus isolation was carried out in suckling mice with tick pool samples which were found positive by TBEV-specific PCR mentioned above. Briefly, litters of specific-pathogen-free (SPF) two-day-old BALB/c mice were inoculated intracerebrally with 20 μl samples using Hamilton syringe (Hamilton Co., Switzerland) and observed for 14 days. When a majority of the suckling mice in one litter

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Table 1
Chinese TBEV isolates used in the study and comparison of identify for full-length sequences with strain WH2012.

Isolate	Place of origin	Year of isolation	WH2012	
			% Identity of nt sequence	% Identity of aa sequence
WH2012	Northern China	2012	100	100
Xinjiang-01	Northwestern China	2012	99.4	99.4
MDJ01	Northeastern China	2001	99.3	99.3
MDJ-02	Northeastern China	2010	98.2	99.2
MDJ-03	Northeastern China	2010	98.2	99.1
Senzhang	Northeastern China	1953	98.3	99.1

showed clear generalized clinical signs, they were sacrificed and the brains were aseptically removed. Then brains were pooled, mechanically homogenized and centrifuged. The samples were further passaged intracerebrally in suckling mice until the clinical signs became stable and evident. The brain supernatant was diluted with 10% solution in RPMI 1640 medium supplemented with 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin, followed by filtration through 0.22-μm-pore-size sterile membranes. For virus proliferation and purification, diluted supernatant was added to BHK-21 monolayers. When a strong cytopathic effect (CPE) appeared, supernatants of infected cultures were collected and viruses were purified by ultracentrifugation through a 30% sucrose cushion at 30,000 rpm for 2 h by using a Ty70 rotor (Beckman Coulter, CA, USA). The pelleted viruses were resuspended in PBS and stored in aliquots at −80 °C. Virus titers were determined as the 50% tissue culture infectious dose ($TCID_{50}$) in Vero cells using the Reed–Muench formula. Handling of the infectious material was performed in Biosafety level 3 facilities.

Two tick pools collected were found positive by a TBEV-specific nested RT-PCR (data not shown). From one of these two samples, TBEV strain designated as WH2012 was successfully isolated in suckling mice. After further passage and purification, the virus morphology was visualized using electron microscopy, which showed enveloped spherical viral particles approximately 50 nm in diameter (data not shown).

Full-length genome sequencing of the isolated TBEV strain was performed using primer pairs as described previously (Si et al., 2011). Briefly, Viral RNA was extracted from purified viral stock solution, and cDNA synthesis was performed using M-MLV Reverse Transcriptase (Promega, WI, USA). The overlapping DNA fragments were produced by amplifying with TransTaq®-T DNA Polymerase (Transgen Biotech, Beijing, China). The PCR products were then cloned into pGEM®-T Easy vector (Promega) and sequenced by commercial service (Sangon Biotech, Shanghai, China). Sequences were aligned with MegAlign (DNAstar, Madison, WI). The full genome of WH2012 has a length of 10,774 nt with 54% G + C content (GenBank Acc. No. KJ755186). The coding region extends from nucleotide 130 to 10,374 corresponding to a deduced polyprotein of 3414 amino acids. The nucleotide (and deduced amino acid) sequence identities between WH2012 and other fully sequenced TBEV reference strains ranged from 83 (93) to 99 (99)%. In particular, WH2012 showed the lowest identities with Austrian strain Neudoerfl and German strain K23 (data not shown), and highest sequence identities with Chinese strain Xinjiang-01. Analysis of the sequences demonstrated the conservation at the genome and protein levels among WH2012 and other Chinese TBEV isolates, with the sequence variation across the entire polyprotein being <1% (Table 1). This high conservation was observed even for Chinese TBEV isolates collected over a period of 60 years (1953–2012) from distant geographic locations (northwestern to northeastern China). To delineate the genetic variation, we analyzed polyprotein sequences of other Chinese isolates in comparison with WH2012. Single amino acid substitutions observed in WH2012 were not evenly distributed along the polyprotein. Most substitutions were located in the ORF of NS2A, NS3, and NS5. Totally, 21, 25, 27, 31 and 32 amino acid substitutions were found when WH2012 was compared to Xinjiang-01, MDJ01, MDJ-02, MDJ-03 and Senzhang, respectively (Table 2).

Phylogenetic analyses of viral full genome sequences were performed using the neighbor-joining (NJ) method with a bootstrap of 1000 replicates with the MEGA 5 software (Tamura et al., 2011). A phylogenetic tree constructed based on the complete genome sequence (Fig. 1) and polyprotein amino acid sequence (data not shown) indicated that WH2012 strain belonged to the TBEV-FE subtype. Within the FE-subtype, this strain is most closely clustered together with other Chinese TBEV isolates Senzhang, MDJ01, MDJ-02, MDJ-03 and Xinjiang-01.

Using *in vitro* cell culture systems, we investigated the potential tropisms of WH2012 strain in human brain-derived cell lines of three origins. Human neuroblastoma (SK-N-SH; ATCC HTB-11), glioblastoma (T98G; ATCC CRL-1690) and astrocytoma (CCF-STTG1; ATCC CRL-1718) cells were cultured as previously described (Zhang et al., 2013). For virus infection experiments, monolayer cultures of cells were inoculated with virus at a multiplicity of infection (MOI) of 1 in serum-free medium at 37 °C for 1 h. Then free virus was removed by washing with PBS and finally complete medium was added to each well. The virus-mediated CPE was monitored daily using light microscopy. At the indicated time points, culture supernatants were harvested and the titers were determined by $TCID_{50}$ assay in BHK-21 cells. At an MOI of 1, noticeable CPEs, such as cell rounding and detachment, were identified in tested cell lines at 72 h post infection (p.i.). The most significant CPE was observed in virus-infected SK-N-SH cells. By contrast, the CPE in CCF-STTG1 and T98G cells was less advanced compared with that in SK-N-SH (Fig. 2a). To evaluate virus replication efficiency in the three cell lines described above, viral growth curves were measured. WH2012 exhibited efficient viral growth in all cell lines tested, as indicated by a gradual increase of virus

Table 2
Amino acid residue changes in Chinese TBEV isolates compared with strain WH2012.

Isolate	Open reading frame (length in aa)									
	C(117)	prM/M(163)	E(496)	NS1(352)	NS2A(230)	NS2B(131)	NS3(621)	NS4A(149)	NS4B(252)	NS5(903)
Xinjiang-01	0	0	2	1	2	0	6	1	2	7
MDJ01	1	0	2	3	1	1	6	1	2	8
MDJ-02	0	1	2	2	5	0	8	1	3	5
MDJ-03	0	1	4	2	5	0	8	2	3	6
Senzhang	0	1	3	4	5	1	8	1	3	6

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