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A Comparison of Complete Genome Sequences of a Rabies Virus Chinese Isolate SH06 with the Vaccine Strains*

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Abstract: In this study, we determined the complete nucleotide and deduced amino acid sequence of a primary isolate of rabies virus (SH06) obtained from the brain of a rabid dog. The overall length of the genome was 11 924 nucleotides. Comparison of the genomic sequence showed the homology of SH06 at nucleotide level with full-length genomes of reference vaccine strains ranged from 82.2% with the PV strain to 86.9% with the CTN strain. A full-length genome-based phylogenetic analysis was performed with sequences available from GenBank. Phylogenetic analysis of the complete genome sequences indicated that the SH06 exhibited the highest homology with rabies street virus BD06 and CTN vaccine strain originated from China.

Key words: Rabies virus; Complete genome sequence; Phylogenetic analysis

Rabies virus belongs to the genus Lyssavirus of Rhabdoviridae and infects a variety of mammals including humans and causes fatal viral encephalitis (3). The rabies virus genome consists of single-stranded, unsegmented, negative-sense RNA of about 12 kb, which encodes five viral proteins (3'-N-P-M-G-L-5') and is contained in a bullet-shaped, bilayered envelope(15). The RNA polymerase (L) and phosphoprotein (P) complex with the nucleoprotein (N) form the nucleocapsid (NC), and the matrix protein (M) and the glycoprotein (G) form the inner and outer layers of the envelope, respectively (16).

Currently, through comparing the N, G gene and non-coding region between the G and L genes, known as the G-L intergenic or pseudogene (Ψ) region, the Lyssavirus genus have been resolved into seven genotypes, which can be divided into two major phylogroups with high bootstrap support. Phylogroup I comprises Rabies virus (RV; genotype 1), Duvenhage virus (DUVV; genotype 4), European bat lyssavirus 1 (EBLV-1; genotype 5), European bat lyssavirus 2 (EBLV-2; genotype 6), and Australian bat lyssavirus (ABLV; genotype 7). Phylogroup II comprises Lagos bat virus (LBV; genotype 2) and Mokola virus (MOKV; genotype 3) (2). Four recently described lyssaviruses (Aravan, Khujand, Irkut and West Caucasian Bat Virus) recovered from bats in Eurasia still await formal classification but may constitute several new genotypes (6).

According to World Health Organization (WHO) estimates, 55 000 deaths due to rabies are reported worldwide every year; the majority of them being in

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the developing countries of Asia and Africa (12). In China, human rabies cases have been on the rise during the past few years and since 2003 the number of cases each year have been above 2000 with a total of 2 381 cases reported in 2008. Molecular techniques combining amplification by polymerase chain reaction (PCR) and direct sequencing offer a very powerful method for evaluating the epidemiology of viral diseases and therefore could yield a clearer understanding of the origin and transmission patterns of the rabies. Ultimately, data obtained from molecular epidemiological studies can lead to a better understanding and more effective strategies to control the spread of rabies (1).

In this study, we report a full-length genomic sequence of a dog rabies virus (SH06) isolated in China. This street rabies virus genome sequence has been compared with the sequences of all other complete lyssavirus genomes as well as the complete gene sequences of many other vaccine strains available from GenBank (Table 1).

MATERIALS AND METHODS

Virus isolate

The SH06 sample was isolated from a rabid dog in Shanghai in 2006. This SH06 sample was confirmed as rabies positive by detection of rabies virus antigen using both the direct immunofluorescent antibody (DFA) test (1) and a rapid rabies enzyme immunodiagnosis (RREID) method employing anti-nucleocapsid and anti-gly-coprotein monoclonal antibodies. Anti-rabies monoclonal antibodies were obtained from Center

Table 1. Lyssavirus isolates compared in this study

Strain	Host species	Location	Year of isolation	GenBank accession no.	Reference
8743THA	Human	Thailand	1983	EU293121	(1)
8764THA	Human	Thailand	1983	EU293111	(1)
9147FRA	Fox	France	1991	EU293115	(1)
9001FRA	Dog bitten by a bat	French Guyana	1990	EU293113	(1)
9704ARG	Bat	Argentina	1997	EU293116	(1)
SHBRV-18	Bat	USA	1983	AY705373	(2)
NNV-RAB-H	Human	India	2006	EF437215	(3)
RABV	Human	India	2004	AY956319	(1)
SADB19	Vaccine	USA	1935	M31046	(4)
PV	Vaccine	France	1882	NC_001542	(5)
DRV	Deer	China: Jilin		DQ875051	
ON-99-2	raccoon	Canada	1999	EU311738	(6)
ERA	Vaccine	USA	1935	EF206707	(7)
BD06		China		EU549783	
CTN	Vaccine	China: Shandong	1956	EF564174	(8)
RV-97	Vaccine	Russian		EF542830	(9)
Rb/E3-15-5	Vaccine			EU182347	
RC-HL	Vaccine	Japan		AB009663	(10)
Nishigahara	Vaccine	Japan		AB044824	(10)
HEP-Flury	Vaccine	USA	1939	AB085828	(11)
MOKV	Cat	Zimbabwe	1981	NC_006429	(13)
aG N	Vaccine/Dog	China:Beijing	1931	AF155039	
aG P	Vaccine/Dog	China:Beijing	1931	DQ646875	
aG M	Vaccine/Dog	China:Beijing	1931	DQ490077	
aG G	Vaccine/Dog	China:Beijing	1931	L04522	
aG L	Vaccine/Dog	China:Beijing	1931	EF564173	

for Rabies Diagnosis, Wuhan Institute of Biological Products, China (13). A small amount of the tissue was homogenized using PBS and a 10% homogenate was prepared. For isolation of the virus, 100 μL of the homogenate was inoculated into 3-day-old Bab/c mice intracerebrally. After the development of symptoms in the mice the brain was harvested and the presence of rabies viral antigen was confirmed by ELISA and the tissue was stored in the -70℃.

RNA extraction

Viral RNA was extracted from 0.1 g samples using 1mL TRIzol® (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. In brief, 250μL Chloroform was added into homogenized tissues, mixed gently, and centrifuged at 13 000 r/min for 15 min. The top aqueous layer of about 450μL was collected and RNA was precipitated by using 500μL of isopropyl alcohol and then centrifuged at 13 000r/min for 10 min and the supernatant was discarded. RNA was washed with 1.2 mL of 75% alcohol, centrifuged at 10 000 r/min for 5 min, and the pellet was air-dried. The pelleted RNA was dissolved in 20μL of diethyl pyrocarbonate (DEPC)-treated water, then vortexed softly and frozen at -70℃.

Primer design

Primers employed for the amplification are listed in Table 2. The primers were designed based on the full-length genomes of the PV strain (GenBank accession number NC_001542) and RC-HL (GenBank accession number AB009663) by using freely available primer designing software (''GeneFisher'' Interactive PCR primer design software, http:// bibiserv.techfak.uni-bielefeld.de/genefisher2/). At the same time, we referenced many known primers (8, 11). The 11 bases in the two UTRs, which have no variation, were used as templates to design the forward and reverse primers to amplify both the 3' and 5' ends, respectively (4, 11).

Synthesis of cDNA

For reverse transcription, 1μL total RNA, 1μL Random Hexamer Primer (0.2μg/μL) and 4.5μL DEPC-treated water were heated at 100℃ for 1 min, and then quickly chilled on ice for at least 2 min. The cDNA was prepared by using 0.5μL Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) (200U/μL) (TaKaRa), 2μL reaction buffer and 0.5μL dNTP mix (10 mM each) at 30℃ for 30 min, then incubated at 42℃for 90 min, before heat-inactivation of the MMLV-RT Reverse Transcriptase at 95 ℃ for 5 min in DNA Thermal Cycler (Perkin Elmer Cetus).

Polymerase chain reaction (PCR) and cloning

PCR amplification of the defined stretches of the genome was carried out by using different sets of primer pairs. The polymerase chain reaction (PCR) was performed on a DNA Thermal Cycler (Perkin Elmer Cetus) in a 50μL reaction mixture that consisted of 1.5μL cDNA, 5μL 10×Ex Taq buffer, 20pmol of forward and reverse primers, 100μmol/l of each dNTP and 1 unit of Ex Taq (TaKaRa). The initial denaturation was carried out at 94℃for 4 min followed by 39 cycles using initial denaturation at 94℃ for 1 min, annealing at 55℃ for 50 s, and extension at 72℃ for 90 s. The final extension step was performed at 72℃ for 10 min. To confirm the 3' and 5' sequence of the genomic RNA by rapid amplification of cDNA ends (RACE) method we also used 3'-Full RACE Core Set Ver.2.0 and 5-Full RACE Kit from TaKaRa following manufacturer's instructions. PCR products were confirmed visually by GoldView (SBS Genetech) staining of 1% agarose gels after electrophoresis. The

Table 2. Primers used for amplification and sequencing of the SH06 strain

Fragment	Primer name	Sequence(5'-3')	Sense	Base Location [a]	Length	Position
3'	RV1F	GTACCTAGACGCTTAACAAC	+	Beginning	499	1-499
	RV1R	AAGACCGACTAAGGACGCAT	−	470-499		
a	N127	ATGTAACACCTCTACAATGG	+	55-74	1531	55-1586
	N8m	CAGTCTCYTCNGCCATCTC	−	1586-1567		
b	RHN-6	GACTCTTAAAGAGTTGAACAA	+	1415-1435	1087	1415-2502
	RHN-7	GTTCATTTTATCAGTGGTGTT	−	2502-2482		
c	RHNS-1	TGAATCGtTATACATCTTGC	+	2386-2405	1019	2386-3405
	RGP-13	AGTTTGTCTGGTATCGTGTA	−	3405-3386		
d	M220	TGGTGTATAACATGRAYTC	+	3000-3019	1096	3000-4097
	G780	ACCCATGTYCCRTCATAAG	−	4096-4077		
e	MSL	TGGATTTGTGGATKAAAGAGGC	+	3995-4016	1541	3995-5536
	L1	GAGTTNAGRTTGTARTCAGAG	−	5536-5516		
f	RHL-4	AGATTTCCGTCATGATGATCG	+	5398-5418	805	5398-6103
	RHL-5	GAGTAAACAAAGTCCTTTGTC	−	6103-6083		
g	RHL-6	AAATATGGGGACTACTAATTG	+	6061-6081	700	6061-6761
	RHL-7	TACTATATGTTTGGGTGGCC	−	6761-6742		
h	RHL-8	TGACTCCTTATGTCAAAACTC	+	6715-6735	705	6715-7420
	RHL-9	CCGATGAGGTCTGATCTGTC	−	7420-7401		
i	RHL-10	AGAAGTCTTGGATCTATTATTC	+	7378-7399	723	7378-8101
	RHL-11	GAAGGGTCTAGATAGATTATCC	−	8101-8080		
j	RHL-12	AACTTTCTCCTAGCGATGTC	+	8058-8077	924	8058-8982
	RHL-13	TTTTCACCACATGAACATTGG	−	8982-8962		
k	RHL-14	CATCCACCTCCATGTCAACC	+	8914-8933	1607	8914-1052
	RHL-15	CAACCACAAGGCATAAGGATG	−	10521-10501		
l	M3F	ATGTTCCAGCCMTTGATGCTT	+	9262-9283	1537	9262-10799
	M3R	ATGTCAGTRACTTCTGCATCAC	−	1077-10799		
5'	12F	GAAGTYACTGACATTGCATC	+	10821–10841	1113	10821-11924
	END Oligo	CGACGTTGTAAAACGACGGCCAGTACG CTTAACAA	−	End		

[a] The positions of primers are based on the RC-HL strain of rabies virus.

PCR products with expected size were purified using a Agarose Gel DNA Fragment Recovery Kit (TaKaRa) following the manufacturer's instructions. The purified product was then ligated directly into the pMD18-T cloning vector system (TaKaRa) at 16℃ for 1 h. The ligated product was transformed into competent *E. coli* DH5α cells by heat shock method, following the manufacturer's instructions. The transformed colonies were screened by both ampicillin as resistant marker and blue white color selection using X-gal, IPTG containing LB medium.

Sequencing and phylogenetic analysis

Sequencing of rabies virus genes cloned in the plasmid was carried out with an Applied Biosystems 3770 DNA automated sequencer (Applied Biosystems Inc, Foster City, CA, USA). The complete genome sequences of 22 isolates were used for the multiple alignments and to understand the phylogenetic relationships among isolates (Table 1). The N, P, M, G, L gene sequences and complete genome sequences was modeled for phylogenetic tree reconstructions. Phylogenetic analysis of complete genome sequences, including phylogenetic tree construction and calculation of nucleotide and amino acid identities were performed using MEGA version 4.0 (14). Phylogenetic trees were constructed using the neighbour joining (NJ) method

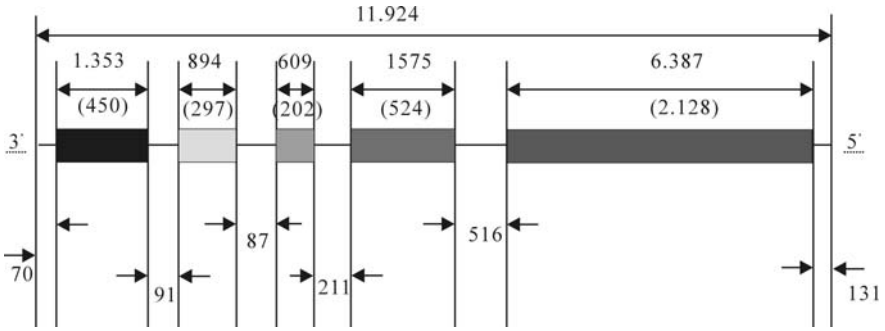

Fig.1. The genome structure of the SH06 strain. Boxes represent open reading frames (ORFs) for five structural proteins, and lines between ORFs show noncoding regions. Numbers in parentheses indicates the number of amino acids in the translated ORF.

with Kimura evolutionary distance correction statistics (9). The statistical significance of the phy- logenies constructed was estimated by bootstrap analysis with 1000 (NJ) replicates. Bootstrap values above 70% were considered significant (7). Complete nucleotide sequences of the SH06, aG, CTN, PV, HEP-Flury and ERA (reference vaccine strains) were carefully analyzed, which are the current strains used in the production of human anti-rabies vaccines and veterinary vaccine production in China.

RESULTS

Genomic structure of the SH06 isolate

The complete genome sequences of the SH06 isolate was composed of 11 924 bases (bp). The five structural genes are similar to other rabies viruses, without any insertion and deletion. The open reading frame (ORF) sizes of N, P, M, G and L were 1 353bp (71-1483), 894bp (1515-2408), 609bp (2496-3104), 1 575bp (3316-4890) and 6 387bp (5407-11793) respectively. The full genome encodes five structural proteins of N (450 aa), P (297 aa), M (202 aa), G (524 aa) and L (2 128 aa) (Fig.1). All five genes are initiated with AACA and terminated with poly (A) 7 (Table 3). The non-coding sequence sizes of 3' UTR, N-P, P-M, M-G, G-L and 5' UTR were 70bp (1-70), 91bp (1424-1514), 87bp (2409-2495), 211bp (3105-3315), 516bp (4891-5406) and 131bp (11794-11924), respectively.

Comparative analysis of coding genes sequences

The comparison of the complete genomic sequence of SH06 with aG, CTN, PV, HEP-Flury and ERA were used for molecular characterization and to understand their relative similarity. The homology of the SH06 with full-length genomes of reference vaccine strains at nucleotide level ranged from 82.2% (with PV) to 86.9% (with CTN). Among the five genes, N gene was the most conserved according to the overall amino acid diversity of the five structural genes, the homology of nucleoprotein gene of SH06 with reference vaccine strains ranged from 84.5% to

Table 3. Transcriptional start and stop signals of the SH06 isolate

Gene	mRNA				Coding sequence (CDS)			
	Start	Position	Stop	Position	Start	Position	Stop	Position
N	AACACCCCT	59-67	ATG(A)7	1474-1483	ATG	71	TAA	1423
P	AACACCCCT	1486-1494	ATG(A)7	2466-2475	ATG	1515	TGA	2408
M	AACACCACT	2481-2489	GTG(A)7	3274-3283	ATG	2496	TAG	3104
G	AACATCCCT	3289-3297	GAG(A)7	5346-5355	ATG	3316	TGA	4890
L	AACACTTCT	5380-5388	TAG(A)7	11845-11854	ATG	5407	TAA	11793

88.9% at nucleotide level (nt) and from 94.5 to 98.2% at amino acid level (aa), followed by L gene (nt/aa: 81.9%-87.0%/94.2-97.8%), M gene (85.2%-89.1%/ 92.8%-97.0%), G gene (78.6%-85.5%/86.8%-92.3%) and P gene (78.6%-86.0% / 87.1%-93.0%) (Table 4); the extent of genetic diversity, reflected in percentage identity of five structural proteins, is in the order N>L>M>G>P. Amino acid similarity is dramatically higher than the nucleotide similarity, which means that many of the nucleotide mutations are synonymous.

Phylogenetic analysis

Phylogenetic analysis was conducted with the NJ method using Maximum Composite Likelihood and bootstrapped with 1000 replicates using software Mega 4.0. To understand the genetic relationships and evolution of rabies virus strains, the entire genome sequence of the SH06 was aligned with 21 complete genome sequences of Lyssavirus Genotype 1 and five structural genes sequences of aG available in the GenBank, Mokola virus was used as the outgroup (Fig.2). At the same time, the coding regions of N, P, M, G and L gene sequences were aligned using Clustal W, and subjected to tree reconstructions with the NJ method. The topology of the phylogenetic trees generated by the five structural genes was of the same shape, and agreed with phylogenetic tree of the complete genome (not shown).

Among vaccine strains, six different phylogroups (Fig. 2) could be found, and the grouping was supported by high bootstrap values (BT=100). The Chinese vaccine strains (CTN and aG) forms two different groups. The SH06 is most closely related to Chinese vaccine strain CTN, which is in concordance with previous research (10). At the same time, SH06 is also in the same group as isolates from Thailand.

DISCUSSION

Though the rabies N and G gene has been used extensively for epidemiological and evolutionary studies, similar analyses were seldom carried out at the whole-genome-scale, especially in China. To gain a better understanding of the molecular characteristics of circulating rabies virus strains in China, we undertook the complete genome sequence analysis of a rabies virus isolate obtained from a rabid dog, and undertook a phylogenetic analysis of 21 complete genome sequences of Lyssavirus Genotype 1 and five structural genes sequences of aG.

The SH06 we studied was closely related to Chinese strains BD06 and vaccine strain CTN, as well as to Thailand strains (8743THA and 8764THA), this was supported by a high bootstrap value (BT=100). However, SH06 was in a different group with other vaccine strains and street strains (Fig. 2). In previous

Table 4. Homology comparisons and overall diversity of nucleotide and amino acid sequences of N, P, M, G, L and complete genome of SH06 with five rabies virus vaccine strains.

Strain	N (%)	P (%)	M (%)	G (%)	L (%)	Genome
aG	84.5 (94.5)	79.7 (87.5)	86.9 (93.7)	78.6 (86.8)	81.9 (94.2)	
CTN	88.9 (97.8)	86.0 (93.0)	89.1 (97.0)	85.5 (92.3)	87.0 (97.8)	86.9
PV	85.7 (96.8)	79.1 (87.5)	85.2 (92.8)	79.3 (90.0)	82.6 (95.6)	82.2
HEP-Flury	86.8 (98.2)	78.6 (87.1)	86.0 (94.4)	81.2 (90.0)	82.9 (96.5)	82.6
ERA	86.5 (97.3)	79.3 (88.2)	85.4 (93.3)	80.2 (89.5)	82.6 (95.9)	82.3
overall diversity	10.9 (3.0)	16.0 (10.7)	12.6 (6.7)	14.6 (9.5)	13.4 (4.2)	

Note: The number outside the bracket is nucleotide similarity and the number inside the bracket is amino acid similarity.

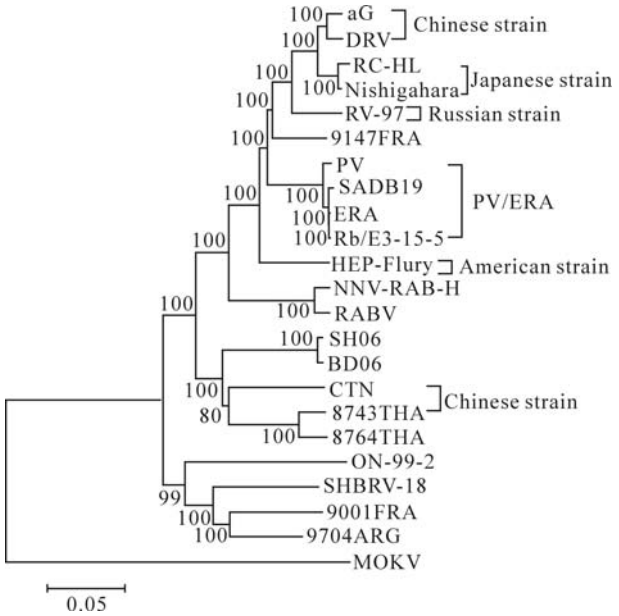

Fig.2. Phylogenetic relationships of 21 complete genome sequences of Lyssavirus Genotype 1 and five structural genes sequences of aG. The Phylogeny was inferred using an NJ method.

research, the predicted G protein of the CTN vaccine strain and Chinese street rabies virus strains shared a high identity (10). Based on the complete genome sequences analysis alone, the SH06 isolate had 86.9% homology with the CTN vaccine strain but <82.6% with other vaccine strains at the nucleotide level. Generally, the nucleoprotein and glycoprotein are considered to be the most important for immunogenicity. 97.8% homology of the nucleoprotein and 92.3% homology of the glycoprotein were observed between the SH06 and the CTN vaccine strain respectively. The SH06 shares 86.8%-90% amino acid identity of glycoprotein with other vaccine strains. By analyzing other genes, we observed similar results: SH06 is most closely related to the CTN vaccine strain than any other vaccine strain. Thus, it seems the CTN strain should be most suitable for use in China as a vaccine strain; however, direct vaccine efficiency trials should be undertaken to confirm this hypothesis.

Both the 3'and 5' UTR have conserved signals that play a role to modulate replication and transcription (5) . The 3' UTR of the SH06 comprises 70 nt and includes the leader regions potentially transcribed into the leader RNA. Our data also reveals a strict complementary sequence of the 11 terminal nucleotides as well as nucleotide positions 13, 14, 15 and 16 from both ends of the genome (Fig. 3). The rabies virus genome consists of a single-stranded RNA virus, but we also found that at least 11 nucleotides of the 3'and 5' UTR sequence of the SH06 isolate are the reverse complement of the sequence; other researches have obtained similar results (3). These observations indicate the genome of the rabies virus is possibly a single-stranded RNA molecule with partial double-strands in the UTR; however, further experiments are necessary to confirm this hypothesis.

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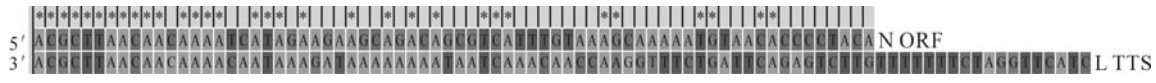

Fig.3. Comparison of the 5' and the reverse complementary 3' genomic termini of the antigenomic (+) sense RNA of strain SH06. Identical nucleotides are indicated by a *. TTS: transcription termination signal.

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