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BRIEF REPORT

# Molecular characterization of a rabies virus isolate from a rabid dog in Hanzhong District, Shaanxi Province, China

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**Abstract** A canine rabies virus, Shaanxi-HZ-6, was isolated in Shaanxi Province, China, in 2009. Its genome has been completely sequenced and found to be closely related to the China I rabies virus strains widely circulating in China. The genomic length was 11,923 base pairs, and the overall organization of the genome was similar to that of other rabies virus isolates. Compared with isolates CQ92 and J, 84 amino acid substitutions (7 in the N gene, 15 in P, 6 in M, 25 in G, 31 in L) were observed in strain Shaanxi-HZ-6. Amino acid substitutions of $R_{264}H$ and $V_{332}I$ were noted in the G protein antigenic site I and site III, respectively. Residue 333 of the G protein, which is considered to be associated with pathogenicity, was Arg in Shaanxi-HZ-6. These and other substitutions may help provide an explanation why the China I lineage strain maintains its prevalence in China.

**Keywords** Rabies virus · Street isolate · Molecular characterization

**Abbreviations**

| | |
|---|---|
| RT-PCR | Reverse transcription polymerase chain reaction |
| IGRS | Intergenic signals |

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## Introduction

Rabies is one of the oldest zoonotic diseases, causing a fatal infection with lethal encephalomyelitis. Dogs are the main reservoir and vector of rabies in the developing countries, especially in Asia and Africa [1, 2]. In 2009, there was an outbreak of rabies in both humans and dogs in Hanzhong District, Shaanxi Province, China. About 7,300 humans were bitten by dogs, and 26 died of rabies due to failure to perform post-exposure prophylaxis [3]. There had been no previous human rabies in Shaanxi Province since 1992. Hanzhong District is situated in a basin surrounded by mountains. Its south borders Sichuan Province, the only province neighboring Hanzhong with enzootic rabies. To analyze the cause of this outbreak of human rabies as well as the origin of the virus, dog brain samples were collected from this district, and one rabies virus (Shaanxi-HZ-6) was isolated [3]. Phylogenetic analysis showed that its N and G gene sequences were closely related to those of the rabies virus strain SC-GY from Sichuan Province [3].

In recent years, several epidemiological studies on rabies virus within China have been performed. However, there has been no extensive investigative focus on why China I rabies virus strains are widely circulating in China. Therefore, to investigate this question further, we sequenced the complete genome of representative isolate Shaanxi-HZ-6 and compared it with Chinese street isolates and vaccine strains.

## Materials and methods

Total RNA of Shaanxi-HZ-6-infected mouse brains was extracted with TRIzol (Invitrogen, Carlsbad, CA). Reverse transcription (RT)-PCR was performed with nine pairs of

**Table 1** Primers used

| Primer name | Sequence (5′ to 3′) | Base position[#] | Length (nt) |
|---|---|---|---|
| F1-F | CTATAGTCACGCTTAACAACAAAAC | 0-17 | 1367 |
| F1-R | CTTGATGATTGGAACTGACTGAAAC | 1343-1367 | |
| F2-F | GTCCTGAGGCTGTCTATACTCGAAT | 1272-1296 | 2182 |
| F2-R | GATTGTTCGGACAGCTGAGATGATG | 3430-3454 | |
| F3-F | AATTCCCCATTTACACGATACCAGAC | 3374-3399 | 1549 |
| F3-R | TTAGGAGATGAGGTCTTCGGG | 4918-4938 | |
| F4-F | AAGAACAACTAGCAACACT | 5365-5383 | 1474 |
| F4-R | TCTCGTTCTGGTGAAAGAGTGTGAC | 6852-6876 | |
| F5-F | ATACATGGCATAAACTCCCAATCAC | 6773-6797 | 1480 |
| F5-R | ATCCGGGTTCCCTGCTTCCTGACAC | 8229-8253 | |
| F6-F | TTTGGCTGAGCTCCCATGAATCTTG | 8192-8216 | 1118 |
| F6-R | GTACTAGTTCAGATGTCCATGTTTGCGC | 9283-9310 | |
| F7-F | TGAACTAGTACAAAGGGACACCAGG | 9300-9324 | 1472 |
| F7-R | CGTTAACCTTTCTTTGGACTGACTG | 10750-10772 | |
| F8-F | AGACCTGAGGAACTTGACAACATGG | 10714-10738 | 1210 |
| F8-R | GACCCACGCTTAACAAATAAAC | 11908-11924 | |
| W-F | GCGGAAGTGAGACCAAGC | 4867-4884 | 590 |
| W-R | CTCAATCGGATCAACTGG | 5439-5456 | |

[#] All sequence positions are given relative to the sequence of isolate BD06 (GenBank accession number EU549783)

primer sets designed based on rabies virus strain BD06 (GenBank no. EU549783) sequences available from GenBank (Table 1) [4]. The PCR products were purified and cloned into the pMD18-T vector (TaKaRa, Dalian, China). Selected positively identified clones were sequenced at least twice in both directions (Nanjing Genscript Biological Technology Co., Ltd., China).

Multiple alignments of complete genome sequences from GenBank (Fig. 1) were performed using CLUSTAL W [5]. Similarity scores and percentage identities were determined using DNASTAR. Neighbor-joining (NJ) analysis was performed using MEGA 4.0 [6]. Bootstrap support was estimated for 1000 replicates.

Substitutions detected in the deduced amino acid sequences of Shaanxi-HZ-6 were compared with the genomic sequences of CQ92 (GenBank no. GU345746) and J (GenBank no. GU345747) (Table 2).

## Results and discussion

The entire genomic length of Shaanxi-HZ-6 virus was 11,923 nucleotides, with a genomic organization similar to previously sequenced rabies virus genomes: N gene, 1,353 nt; P gene, 894 nt; M gene, 609 nt; G gene, 1,575 nt; L gene, 6,387 nt. Intergenic signals (IGRS) were as follows: a 3' leader region of 58 nt (nt 1–58), N–P (nt 1,484–1,485), P–M (nt 2,476–2,480), M–G (nt 3,284–3,288), G–L (nt 5356–5,378), with a 5' trailer region of 70 nt (nt 11,854–11,923).

The rabies virus N protein is generally highly conserved among lyssaviruses. The N ORF of Shaanxi-HZ-6 encoded 450 aa, and substitutions of $T_{332}$ A and $V_{379}$ L were noted in antigenic sites III and IV, respectively (Table 2).

Of the five structural proteins of RABV, the P protein has been found to be the least conserved, and this might explain the lower bootstrap value in the P protein phylogeny. Conserved domain I (position 1–50), conserved domain II (position 201–245), variable domain I (position 61-80) and variable domain II (position 134–180) of Shaanxi-HZ-6 and other strains were analyzed by multiple alignment [7, 8]. Three substitutions, $A_{280}$P, $I_{286}$V and $N_{292}$S, were found in RNA-protein binding sites (261-293) (Fig. 2, Table 2).

The M ORF of the isolate encoded a protein of 202 aa. The PPEY L-domain motif of the M protein, which is located at the N-terminus of the protein at amino acids 35–38 [12] and is involved in virion release and RV pathogenicity, was conserved.

The G protein is recognized to play an important role in viral pathogenicity and elicits neutralizing antibodies. The main antigenic sites (I–IV and "a") of G are responsible for virus attachment to cells and host-cell receptor recognition [13, 14]. Compared with strains CQ92 and J, 25 aa

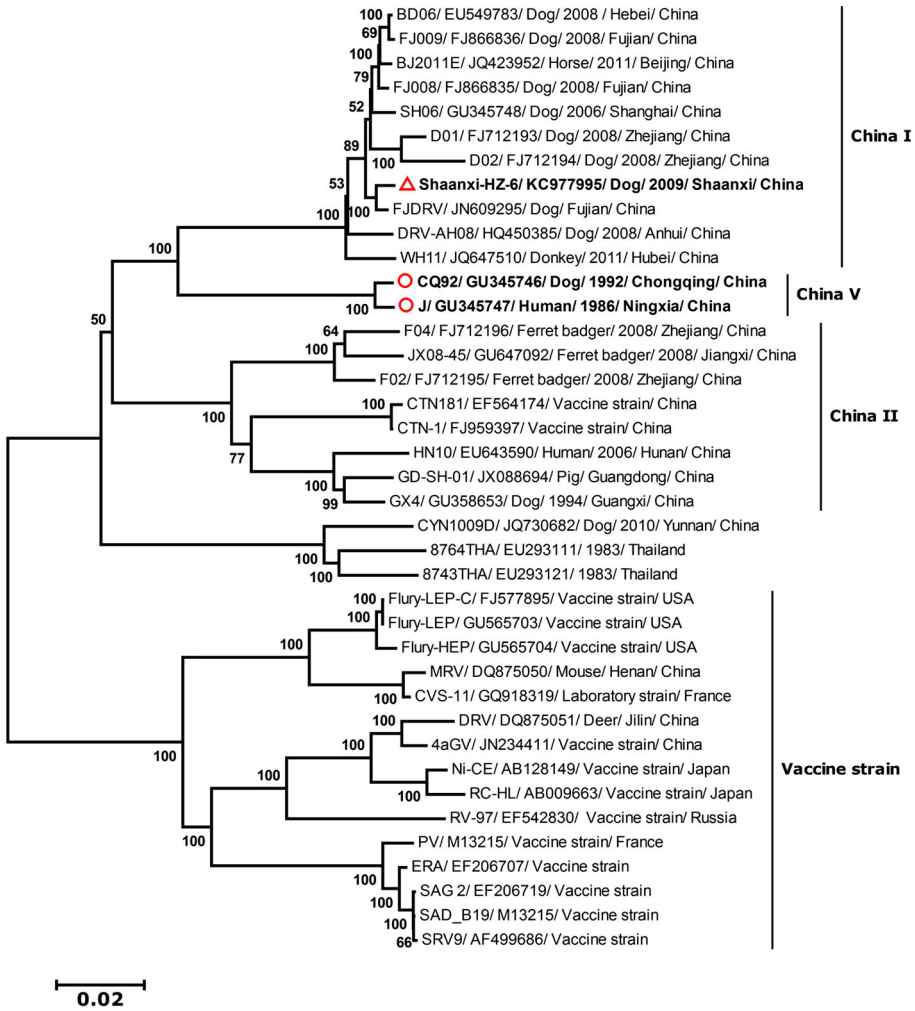


**Fig. 1** Neighbor-joining phylogenetic tree constructed using the nucleotide sequences of the complete genomes of the isolates Shaanxi-HZ-6 (red triangles), CQ92 and J (red circles) in Mega v 4.0 software. The numbers above the branches are bootstrap values (%) for 1000 replicates. Each strain name is followed by the GenBank accession number, host, year of detection, and country of origin

substitutions were observed in the G protein, including $R_{264}H$ in antigenic site VI, $V_{332}I$ in antigenic site III, and eight substitutions in cytoplasmic domain (Table 2).

The L protein contains six conserved domains (I-VI) [15–17], some of which have been characterized as functional motifs [18]. Six substitutions were observed in these domains, and 25 outside there (Table 2).

Compared with Chinese street virus isolates and vaccine strains, Shaanxi-HZ-6 showed 83-99 % nucleotide sequence identity. Phylogenetic analysis indicated that Shaanxi-HZ-6 is most closely related to Chinese epidemic isolates (FJDRV, BD06, SH06, D01) from Fujian, Hebei, Shanghai, and Zhejiang in China I [19]. It is notable that China I is the younger lineage, originating around 1992, and members of this lineage have properties that closely match the observed spread of recent epidemic strains [20]. China I viruses spread from southeastern to western and northern China, constantly encountering new hosts; e.g., WH11 from a donkey in Hubei [21] and BJ2011E from a horse in Beijing.

There have been more than 117,500 recorded human rabies cases in China since 1950, with three major epidemics (1956–1957, 1980–1990 and 1997 to the present), and in the third epidemic, which that peaked in 2007, 3,301 cases have been recorded. Although the numbers have decreased in recent years, there are ~2,000 cases reported every year [22], and rabies remains a public health concern in China. The number of domestic dogs in China was estimated to be 80 to 130 million [23], while the annual production of rabies vaccines in China plus the amount of the annually imported rabies vaccines are estimated to be at most 20 million doses [24]. Therefore, it is hard for the rabies vaccination coverage in dogs to reach 70 % of the total dog population. Also, poor management of the dog population results in a large number of roaming dogs in rural areas in China, which makes the spread of rabies in the dog population easier, and currently, 85 %–95 % of human rabies cases are attributed to dog bites [25]. The current rabies epidemic is also likely to be the result of inadequate rabies prevention education and lack of

**Table 2** Substitutions in genome sequence of rabies virus strain Shaanxi-HZ-6, compared with genome sequences of strains CQ92 and J

| Protein | Amino acid substitution | Site/domain/region of protein |
|---|---|---|
| N | $T_{42}S$ | |
| | $D_{110}E$ | |
| | $A_{135}S$ | |
| | $I_{179}V$ | |
| | $T_{332}A$ | Antigenic site III |
| | $V_{379}L$ | Antigenic site IV |
| | $E_{403}G$ | |
| P | $S_{63}P$ | Variable domain I |
| | $D_{65}G$ | Variable domain I |
| | $T_{71}A$ | N protein binding site in variable domain I |
| | $L_{91}F$ | |
| | $E_{98}D$ | |
| | $P_{134}S$ | Variable domain II |
| | $N_{135}T$ | Variable domain II |
| | $A_{149}V$ | Variable domain II |
| | $P_{158}L$ | Variable domain II |
| | $F_{168}S$ | Variable domain II |
| | $A_{174}V$ | Variable domain II |
| | $I_{257}L$ | |
| | $A_{280}P$ | RNP binding region |
| | $I_{286}V$ | RNP binding region/STAT binding region |
| | $N_{292}S$ | RNP binding region/STAT binding region |
| M | $Q_{17}H$ | |
| | $S_{46}G$ | |
| | $A_{100}D$ | |
| | $D_{145}N$ | |
| | $V_{168}I$ | |
| | $S_{191}P$ | |
| | $E_{192}G$ | |
| G | $A_{-15}P$ | Signal peptide |
| | $L_{-4}S$ | Signal peptide |
| | $T_{90}M$ | |
| | $S_{156}G$ | |
| | $Y_{168}C$ | |
| | $V_{193}T$ | |
| | $S_{204}G$ | |
| | $K_{220}R$ | |
| | $T_{249}I$ | |
| | $P_{253}S$ | |
| | $R_{264}H$ | Antigenic site VI |
| | $S_{289}T$ | |
| | $V_{332}I$ | Antigenic site III |
| | $Q_{382}H$ | |
| | $I_{405}V$ | |
| | $V_{427}I$ | |
| | $M_{442}I$ | Transmembrane domain |
| | $D_{465}N$ | Cytoplasmic domain |
| | $T_{467}A$ | Cytoplasmic domain |
| | $G_{468}E$ | Cytoplasmic domain |
| | $T_{470}I$ | Cytoplasmic domain |
| | $H_{471}Q$ | Cytoplasmic domain |
| | $Q_{486}H$ | Cytoplasmic domain |
| | $A_{492}S$ | Cytoplasmic domain |
| | $G_{501}S$ | Cytoplasmic domain |
| L | $S_5P$ | |
| | $T_{65}A$ | |
| | $V_{146}I$ | |
| | $R_{432}K$ | |
| | $T_{437}N$ | |
| | $A_{533}P$ | |
| | $N_{561}S$ | Conserved domain II, RNA-binding region |
| | $I_{661}V$ | Conserved domain III |
| | $F_{1000}S$ | Conserved domain IV |
| | $M_{1137}T$ | Conserved domain V |
| | $R_{1216}K$ | Conserved domain V |
| | $P_{1333}S$ | |
| | $R_{1363}H$ | |
| | $G_{1412}S$ | |
| | $V_{1470}I$ | |
| | $S_{1562}N$ | |
| | $M_{1570}V$ | |
| | $H_{1601}R$ | |
| | $T_{1618}I$ | |
| | $V_{1654}F$ | |
| | $A_{1661}T$ | |
| | $K_{1664}R$ | |
| | $R_{1665}K$ | |
| | $K_{1723}R$ | Conserved domain VI |
| | $K_{1786}R$ | |
| | $E_{1822}D$ | |
| | $V_{2012}M$ | |
| | $M_{2016}T$ | |
| | $G_{2098}R$ | |
| | $I_{2100}V$ | |
| | $I_{2117}L$ | |

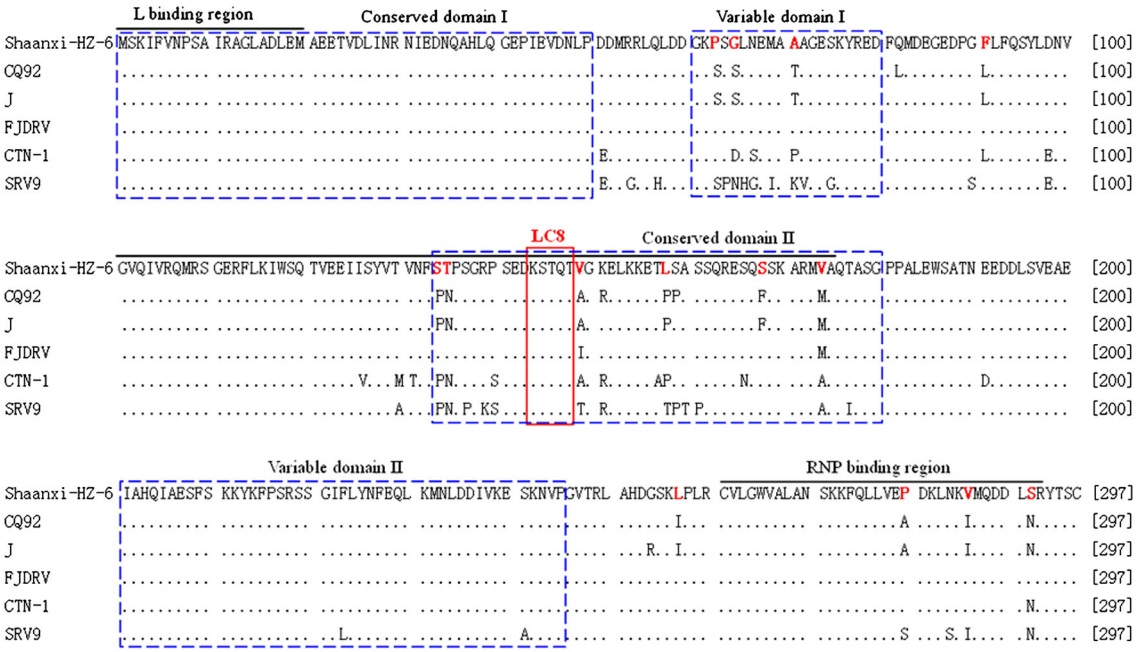


**Fig. 2** Alignment of the P protein amino acid sequences of Shaanxi-HZ-6, Chinese street strains and vaccine strains. Lines above the alignment indicate the L and RNP binding regions

mandatory vaccination of dogs. The consequence has been low vaccination coverage of the dog population, falling far short of the 70 % estimated to be required to impede the spread of the disease sufficiently to prevent major outbreaks [26]. Another factor, however, may be the high efficiency of infection of China I viruses and their high adaptability to cross-species transmission.

Several complete rabies virus genomes from China have been sequenced over the past few years. However, there has been no focus on how the strains of the China I lineage caused the current epizootic in China. For rabies virus, selective pressures such as growth in a new host species favor the emergence of mutants with greater efficiency of infection and transmission [27]. Compared with isolates CQ92 and J, there were 84 aa substitutions in Shaanxi-HZ-6. Rabies viruses of the China I and China V lineages belong to the same phylogenetic cluster, yet China V strains have been found only in Chongqing and Ningxia [19, 21]. The substitutions are likely to include changes that contribute to species adaptation and explain why China I strains maintain their prevalence in China.

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**Conflict of interest** The authors declare that they have no competing interests.

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