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Molecular Characterization of Viral G Gene in Emerging and Re-emerging Areas of Rabies in China, 2007 to 2011*

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Abstract: In recent years (2007 to 2011), although the overall number of rabies cases in China has decreased, there is evidence of emerging or re-emerging cases in regions without previous rabies cases or with low incidence of rabies. To investigate the origin and the factors affecting the spread of rabies in China, specimens were collected from 2007 to 2011 from provinces with emerging and re-emerging cases and tested for the presence of the rabies virus. Positive specimens were combined with sequences from GenBank to perform comparisons of homology and functional sites, and to carry out phylogenetic analyses. Out of these regions, five provinces had 9 positive specimens from canine and cattle, and 34 canine or human specimens were obtained from previously high-incidence provinces. Complete sequences of G gene were obtained for these samples. Homology of the sequences of these 43 specimens was 87%-100% at the nucleotide level and 93.7% -100% at the amino acid level. These G gene sequences were combined with reference sequence from GenBank and used to construct a phylogenetic tree. The results showed that 43 specimens were all assigned to China clade I and clade II, with all specimens from emerging and re-emerging areas placed within clade I. Specimens isolated from Shanxi and Inner Mongolia in 2011 were distinct from previously-isolated local strains and had closer homology to strains from Hebei, Beijing and Tianjin whereas new isolates from Shanghai were tightly clustered with strains isolated in the 1990s. Finally, Shaanxi isolates were clustered with strains from adjacent Sichuan. Our results suggest that the rabies cases in emerging and re-emerging areas in China in the last 5 years are a consequence of the epidemic spreading from of neighboring provinces and regions experiencing a serious epidemic of rabies.

Key words: Rabies virus; G gene; Genetic variation; Molecular characteristics

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Rabies is a zoonotic infectious disease caused by rabies virus and its mortality rate is nearly 100% when infected [6]. Rabies is widely distributed across the globe. More than 55,000 people die of rabies each

year [12]. About 95% of human deaths occur in Asia and Africa. India is the most severely affected, and China is the next.Since 1996 human rabies cases in China have risen continuously until it has become one of the countries experiencing the largest number of cases. Since 2007, although the number of human cases in China has declined overall [8], some provinces and regions have continued to experience a rise and recently some regions have even seen a re-emergence in number of cases. Thus, rabies prevention and control in China continues to be a matter of great importance [8,24].

Rabies virus is a negative single-strand RNA virus, belonging to genus Lyssavirus, family Rhabdoviridae[12]. The overall length of viral genome is about 12 kb, and genes from 3' to 5' terminal include N(Nucleoprotein), P(Phosphoprotein), M(Matrix protein), G(Glycoprotein) and L(RNA-dependent RNA polymerase) [18]. Of these, the G gene-encoding the glycoprotein is located on the viral envelope surface, and it can effectively stimulate virus neutralizing antibodies [6]. In addition, G is the binding site of virus and host cell receptors, determining viral tissue tropism and is related to viral virulence and pathogenicity[21]. Thus, the G protein is a good choice for examining the relationship between different viral isolates.

As part of a national surveillance program of the rabies epidemic from 2007 to 2011, this study collected rabies specimens in emerging and re-emerging areas and carried out G gene sequencing of positive samples. In order to investigate the epidemic spread of rabies in China we carried out analyses of the molecular characteristics of these sequences combined with sequences from high-incidence provinces which have seen a reduction in their epidemic situations in the last five years. Our findings provide data that should contribute towards determining rabies prevention and control strategies in the future.

MATERIALS AND METHODS

Epidemic information

Epidemic information was obtained from the "China information system for disease control and surveillance" program of the China CDC. Excel2007 was used for statistical analysis, and MapInfo7.0 software was used to draw the geographical distribution of national rabies cases.

Specimen source

On the basis of the national and provincial rabies epidemiology analysis from 2007 to 2011, the ranges of emerging and re- emerging areas of rabies in recent five years were determined. Rabies specimens were collected in these regions, screened for the presence of the rabies virus and combined with specimens previously collected from high-incidence provinces.

Specimen detection and sequencing

For brain tissue specimens direct immunofluorescence assay (DFA) was used for detection [9]. For saliva and cerebrospinal fluid specimens from rabies cases, after RNA was extracted with a QIAamp Viral RNA Mini Kit (QIAGEN company), they were reverse transcribed into cDNA with a Ready-To-Go You-Prime First-Strand Beads Kit (GE Healthcare company), and nested-PCR was used for detection .

For positive brain tissue specimens as determined by DFA assay, after Trizol reagent (Invitrogen) was used to extract total RNA, cDNA was obtained by reverse transcription. RNA and cDNA of positive saliva or cerebrospinal fluid specimens were jointly

used to carry out amplification and determination of G gene sequences. The following two pairs of primers were used for G gene amplification G-20F (5′-CTCAA AAGACTCAAGGAAAGATG-3′, 3298-3320), G966R (5′-ATGACTAAGACGTCTGAAACTCAC-3′,4260-4283) and G822F-(5-′TCAGCTAGTAAATCTACATG ACTTTC-3′, 4139-4164), GR-85-(5′-GAGGACTGT TAAATCCAGAGATG-3′, -4955-4977). Primer sites corresponded to the complete genome sequence of rabies virus PV strain (M13215). The cycling parameters were one cycle at 94 ºC for 3 min for an initial denaturation, then 35 cycles at 94 ºC for 30 s, 30 s for 52 ºC, 70 s for 72 ºC, and a final extension at 72 ºC for 10 min[18]. PCR amplification products were sent to the Beijing Biomed Company for bidirectional sequencing. Sequencing primers were identical to the amplification primers.

Sequence analysis

ATGC soft (Genetyx Co., Tokyo, Japan) was used for joining and calibration of sequencing segments; Clustal X v2.1 software was used to complete the sequence alignment [19]; BioEdit v7.0.4 software [3] was used to uniformly shear nucleotide sequences into the coding region and translation into amino acids; the MegAlign software in the DNAstar (5.01 version, Laser gene, USA) package was used for homology analysis for both the nucleotide and amino acids; The Neighbor-Joining (NJ) method in the MEGA4 software package[17] was used to construct a phylogenetic tree and 1000 bootstrap(BP) replicates were used to estimate the reliability of the tree. A phylogenetic tree was constructed with the whole gene sequence data, BP values > 70% were shown in the tree and taken to represent significant support for a clade [4].

RESULTS

Analysis of current rabies epidemic in China and specimen detection

Investigation of the geographical distribution of rabies cases in recent years (Fig. 1) shows that most cases occurred in southeast regions of China (Fig. 2). After 2007, the epidemic situation in the southeast and the overall number of cases at the national peaked and subsequently reduced year by year [24]. However, this reduction was accompanied by the emergence of cases

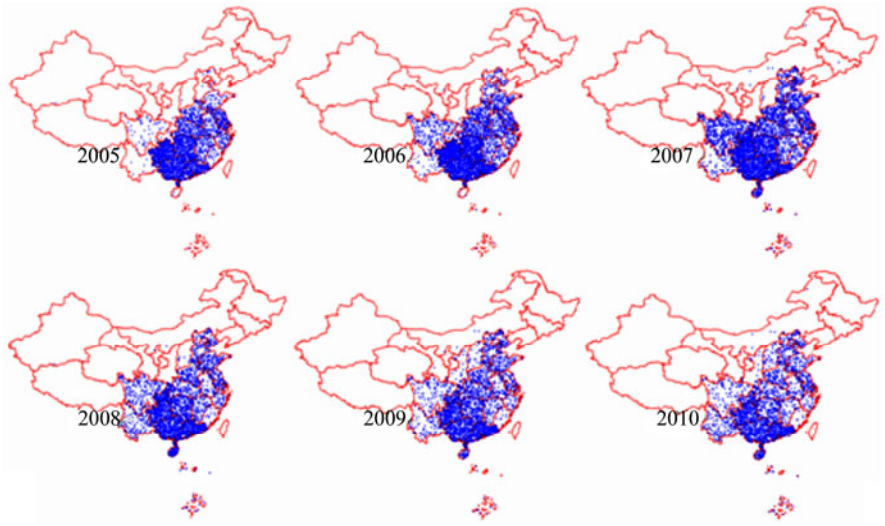

Fig. 1. Geographical distribution of human rabies cases in China from 2005 to 2010. Geographical expansion is shown by changes of dots.

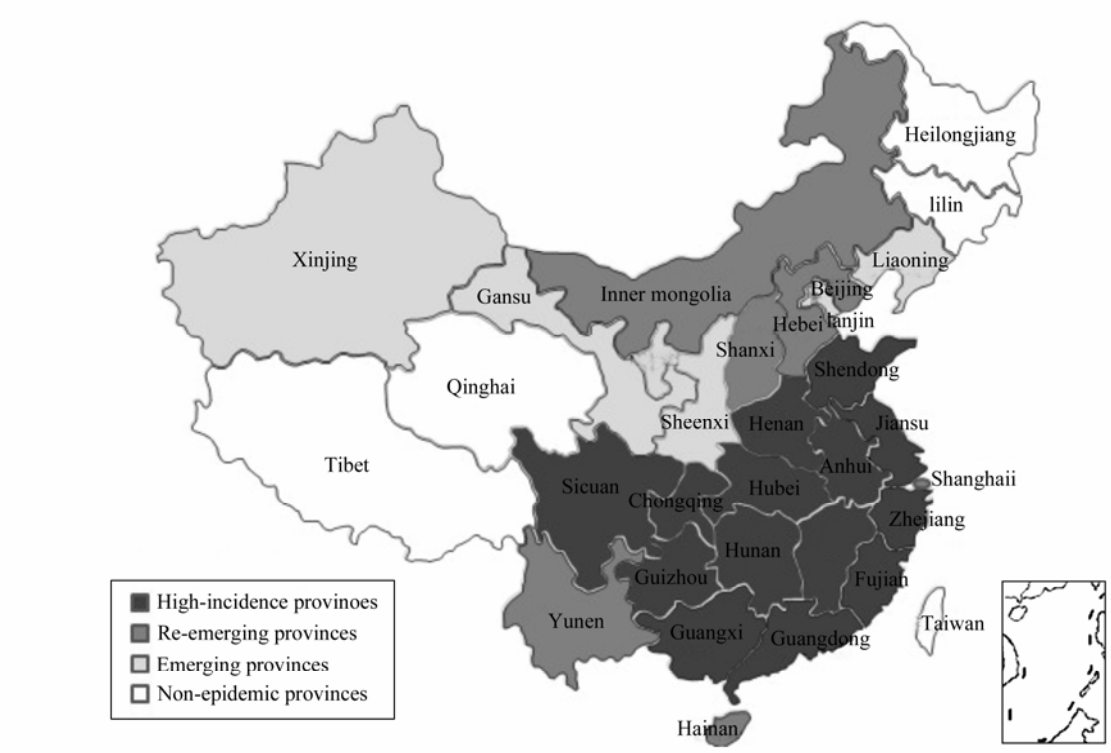

Fig. 2. The rabies epidemic status of each province in China, 2007-2011. Epidemic regions of different kind are marked by responding color.

in regions that were previously free of rabies and re-emergence in regions that previously had a low incidence of rabies cases. Specifically, new rabies cases were recorded in Shaanxi(XiAn) province, Gansu province, Liaoning province, Ningxia Autonomous Region, Xinjiang Autonomous Region (Table 1) and in six other low-incidence provinces and municipalities (Beijing, Shanxi, Inner Mongolia, Shanghai, Yunnan and Hainan) the number of cases were significantly reduced (Fig. 3).

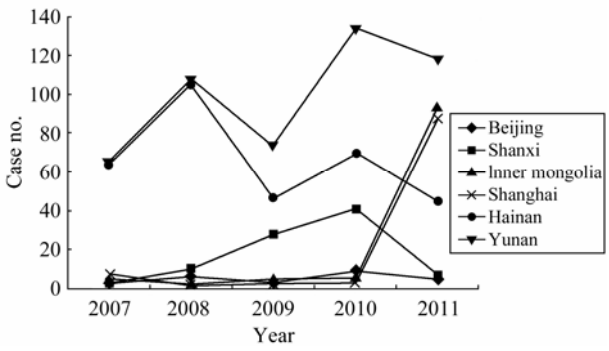

Fig. 3. Reported rabies cases in six low-incidence provinces or municipalities from 2007 to 2011.

Experimental testing identified 43 specimens positive for rabies virus. Of these newly collected samples, 9 samples are from emerging and reemerging regions (Shaanxi-XiAn, Shanxi, Ningxia, Inner Mongolia and Shanghai) and the rest are from epidemic regions. For these 43 positive specimens, an overall length of 1575bp for the G gene coding region was obtained. Specific background information of the specimens is shown in Table 2.

Table 1. Rabies cases in five emerging provinces in China from 2007 to 2011

	2007	2008	2009	2010	2011
Shaanxi	0	0	26	21	41
Gansu	0	0	1	0	0
Ningxia	0	0	0	0	2
Xinjiang	0	1	0	0	2
Liaoning	0	0	0	0	2

Table 2. Background information of 43 positive specimens obtained in this study

Strain	Year	Origin	Host	GenBank no.	Strain	Year	Origin	Host	GenBank no.
CHN0506D	2005	Hunan	Dog	JQ699237	CZJ0809D	2008	Zhejiang	Dog	JQ699248
CHN0532D	2005	Hunan	Dog	JQ699236	CZJ0813D	2008	Zhejiang	Dog	JQ699249
CHN0801D	2008	Hunan	Dog	JQ699265	CZJ0817D	2008	Zhejiang	Dog	JQ699250
CHN0802D	2008	Hunan	Dog	JQ699266	CGZ0509D	2005	Guizhou	Dog	JQ699270
CHN1005D	2010	Hunan	Dog	JQ699261	CHB1007H	2010	Hebei	Human	JQ699259
CHN0805D	2008	Hunan	Dog	JQ699232	CNM1101C	2011	Inner mongolia	Cow	JQ699255
CHN0806D	2008	Hunan	Dog	JQ699233	CNM1104D	2011	Inner mongolia	Dog	JQ699256
CHN0807D	2008	Hunan	Dog	JQ699234	CSD0838D	2008	Shandong	Dog	JQ699251
CHN0811D	2008	Hunan	Dog	JQ699235	CSD0840D	2008	Shandong	Dog	JQ699252
CHN1001D	2010	Hunan	Dog	JQ699260	CSD0842D	2008	Shandong	Dog	JQ699253
CHN1002D	2010	Hunan	Dog	JQ699262	CSD0828D	2008	Shandong	Dog	JQ699254
CHN1003D	2010	Hunan	Dog	JQ699263	CSH0406D	2004	Shanghai	Dog	JQ699245
CJX0904D	2009	Jiangxi	Dog	JQ699238	CSH0407D	2004	Shanghai	Dog	JQ699246
CGX0625D	2006	Guangxi	Dog	JQ699271	CNX1101H	2011	Ningxia	Human	JQ699258
CGX0810D	2009	Guangxi	Dog	JQ699272	CHX1101H	2011	Shanxi	Human	JQ699257
CGX0811D	2009	Guangxi	Dog	JQ699267	CSX0902D	2009	Shaanxi	Dog	JQ699239
CGX0812D	2009	Guangxi	Dog	JQ699269	CSX0903D	2009	Shaanxi	Dog	JQ699241
CGX0813D	2009	Guangxi	Dog	JQ699268	CSX0905D	2009	Shaanxi	Dog	JQ699240
CGX0814D	2009	Guangxi	Dog	JQ699264	CSC0801D	2008	Sichuan	Dog	JQ699242
CGX0815D	2009	Guangxi	Dog	JQ699230	CSC0804D	2008	Sichuan	Dog	JQ699244
CGX0816D	2009	Guangxi	Dog	JQ699231	CSC0806D	2008	Sichuan	Dog	JQ699243
CZJ0808D	2008	Zhejiang	Dog	JQ699247					

Analysis of G gene nucleotide and amino acids sequences

The homology of G gene nucleotide sequence of 43 specimens obtained in this study was 87% to 100%, and the amino acid sequence homology deduced from the nucleotide was 93.7%-100%. The nucleotide sequence variation was mainly composed of synonymous mutations and the G gene nucleotide sequences of some specimens from the same province were completely consistent.

The G gene of the rabies virus encodes 524 amino acids of which the first 19 amino acids corresponds to a hydrophobic signal peptide. During transcription the signal peptide is removed and the mature glycoprotein composed of 505 amino acids is formed. The Glycoprotein is a crucial protein of the rabies virus in that it stimulates the generation of virus neutralizing antibodies [2] and there are at least three neutralizing antigenic sites (GI, GII and GIII). G II is a typical spatially-configured antigenic site located in an amino acid segment spanning sites 34 to 200. Within this region, two main epitopes are located at sites 34 to 42 and sites 198 to 200 [18]. Other important sites causing antigenic changes include site 147 and site 184; GIII is located in an amino acid segment extending from sites 330 to 357 and is a spatially-dependent antigenic site. Within this segment, sites 333 to 340 are the main binding sites of neutralizing antibodies. Also, amino acids at sites 333, 336 and 339 appear to be the key amino acids [18]. For most street strains, the amino acid at site 333 is Arg (R) which is possibly related to the virulence of virus; in the G protein of all the 43

specimens obtained in this study, R was present at this site. Compared with domestic and international isolates, all amino acids of G protein amino acid sequences of 31 virus specimens (including all the cases from emerging and re-emerging provinces) the amino acids at sites 90, 168 and 332 varied (Table 3). In particular, the hydrophobic amino acid Tyr(Y) at site 168 was replaced by hydrophilic amino acid Cys(C). These sites might be associated with the pathogenicity of rabies virus.

Previous results have shown that the acetylcholine (AchR) receptor acts as the cell receptor of rabies virus in vivo and that the amino acids of the glycoprotein at sites 189 to 214 are related to virus / nerve cell tropism [25]. Rustici *et al.* [14]proved that within the spatial structure of the glycoprotein the amino acids segment from 194-197(Asn-Ser-Arg-Gly) was important, with the spatial configuration of Asn(site 194) and Arg(site 196) residues consistent with the AchR configuration [13]. For the 31 G gene sequences (including all cases from emerging and re-emerging provinces) obtained in this study, only the amino acid at site 204 varied, whence Ser(S) was replaced by Gly(G). This indicates that rabies virus nerve cell tropism did not change significantly.

Table 3. Comparisons of amino acids sequences in the GII and GIII segments of the rabies virus G gene

Strains	G II		G III
	90	168	332
31/44 strains	M	C	I
12/44 strains	T	Y	V
CTN	T	Y	V
PM	T	Y	V
HEP	T	Y	V
3aG	T	Y	V
PV	T	Y	V
ERA	T	Y	V

Note: 9 out of 31 samples are from emerging and re-emerging provinces(Ningxia, Shaanxi-XiAn, Shanxi, Inner Mongolia, Shanghai); the rest are from Hunan，Zhejiang, Shandong, Guangxi and Sichuan provinces.

The glycosylation signal of the G protein is the Asn-X-Thr or Asn-X-Ser motif[10]. At present, additional potential glycosylation sites include amino acids are predicted to be located at sites 37, 70, 247, 319, 465 and 480. In this study, the obtained virus specimens only had two potential glycosylation sites at sites 37 and 319.

Analysis of G gene phylogeny

The phylogenetic analysis of the G gene sequences of 43 positive specimens, as well as representative sequences of Chinese epidemic strains [11] , and one American strain as the outgroup were downloaded from GenBank, aligned and phylogenetic analysis was carried out (Fig. 2). According to the G gene grouping of strains from the current epidemic [11], the China strains can be divided into 6 groupst (clade I-VI)[11] , and the phylogenetic tree showed that the 43 specimens were assigned to clades I and II with all specimens from emerging and re-emerging provinces located in clade IA.

From 2007 to 2011, cases were recorded in five provinces and autonomous regions (Shaanxi, Gansu, Ningxia, Xinjiang and Liaoning) that were previously rabies free (Table 1), and which have been classified as re-emerging areas of rabies. No virus specimens were obtained from Gansu, Liaoning provinces and Xinjiang Autonomous Region. Four canine specimens (there were 3 specimens in this study, and 1 specimen was a previously obtained sample) provided by Shaanxi(XiAn) in 2009 were from Hanzhong, and their homology was very close, belonging to the Clade

IA subgroup. Also, they had close homology with Sichuan specimens (Fig. 4). Onhuman specimen isolated in Ningxia in 2011 had the closest homology with strains from Tianjin and Beijing, whereas two other human specimens from Ningxia that were isolated in 1985 and 1986 were assigned to a different group (clade V).

The tree showed a number of interesting associations (Fig. 4). (i) Only one Beijing sample BeijingHu1 has been released at present and this was assigned to the clade IA subgroup, and it had the closest homology to the Tianjin strain; (ii) The human specimen from Shanxi and human specimens of Hebei in 2011 also formed a branch within the clade IA subgroup; (iii) Canine samples from Shanxi (a low incidence region) collected in 2007 and in 2008 and samples from Hunan (high-incidence region) were all assigned to the clade IC subgroup; (iv) High homology existed between the canine and milk cow specimens from Inner Mongolia in 2011, which was consistent with the infection of the cow by a dog bite; they had high homology with Hebei samples, but greater differences with local wildlife samples collected from raccoon dog in 2007 (clade IA); (v) Samples from Shanghai had a wide range of collection dates spanning 15 years (from 1992 to 2006) but were all placed within clade IA; (vi) The three samples from Yunnan showed significant diversity, and two were closer to a Guangxi strain and an Anhui strain in the clade IC subgroup, yet another was in clade VI and similar to another Guangxi strain.

DISCUSSION

Rabies is an ancient infectious disease, and it has more than two thousand years of epidemic history in China [5,22]. Although rabies has continuously been present in China, the epidemic situation has varied greatly. Most recently, after serious epidemics in the 1970s and 1980s, rabies in China was effectively controlled until the mid 1990s (159 cases) [24] . Subsequently, the number of cases has increased, especially in southern provinces such as Hunan, Guangxi and Guizhou which reported annual cases of five or six hundred. National cases reached a peak of 3302 cases in 2007 and subsequently began to decrease with 1918 cases reported in 2011[15,16].

Although the number of cases decreased nationally from 2007 to 2011, the geographic distribution of the epidemic has expanded. In previously high-incidence provinces, cases were significantly reduced but some provinces, such as western Shaanxi(XiAn), Gansu, Ningxia and Xinjiang and northeastern Liaoning, that had not witnessed rabies in recent years, began to experience their first cases, suggesting that current epidemic is spreading to the west and north. From 1996 to 2008, Shaanxi(XiAn) was a low-incidence region as in 13 years only 22 cases were reported and even in 2007 and 2008 there were no recorded cases; in 2009 however, 26 cases were reported. According to our phylogenetic analysis, the canine brain tissue specimens collected from Hanzhong, Shaanxi in 2009 were highly homologous and were closely related to samples collected from Sichuan (Fig. 2 and 4). The southern border of Shaanxi(XiAn) is adjacent to Sichuan and although mountains and rivers act as a natural barrier, they are well connected by commercial traffic flow which is likely to facilitate the spread of the virus. In Ningxia, there was no reported rabies incidences from 2003 to 2010 but the first cases began to appear in 2011 (2 cases, Table 1).The specimen

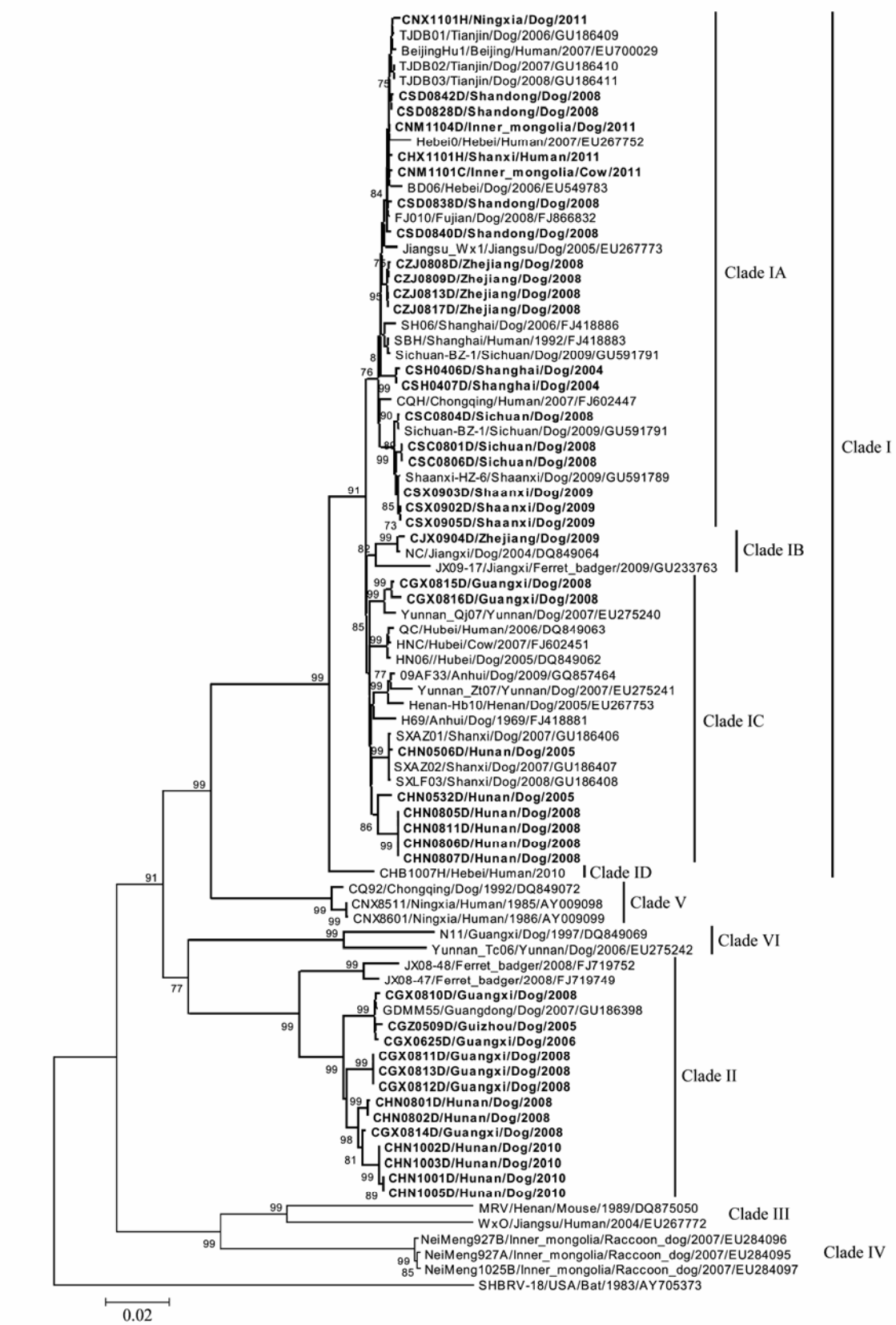

Fig. 4. Phylogenetic tree of rabies street virus G gene sequences isolated in China. Based on a 1575nt (nt1–nt1575) glycoprotein (G) gene of rabies virus rooted with the Pasteur strain of rabies virus (PV). Numbers at each node indicate degree of bootstrap support; only those with support >70% are indicated. New collected sequences are shown in red, sequences from GenBank are shown in black. Provinces, hosts, year of sample collection, and GenBank accession no. are included for each taxa.

obtained from one case in 2011(Fig. 4) was markedly divergent from specimens obtained from patients in Ningxia more than 20 years ago (clade V), and was more homologous with recent epidemic strains obtained from Tianjin and Beijing (clade IA). Both of these results suggest that the virus is spreading from high to low incident regions; however, as virus specimens from the low incidence regions are still relatively few and have yet to be collected in Gansu, Xinjiang and Liaoning provinces, it is not yet possible to carry out a detailed epidemiological analysis.

In summary, we have attempted to investigate the changing dynamics of the rabies epidemic in China in the last five years. While the epidemic appears to have been brought under control in high-incidence provinces, many original low-incidence provinces have experienced an increase in the number of cases. We have obtained a number of isolates from these re-emerging provinces and in this study we have examined their relationship to isolates from high incidence provinces. Our phylogenetic results indicate that these high incidence provinces appear to be 'feeding' adjacent low incidence provinces by spillover. This may well be facilitated by the reduced awareness in provinces which have not experienced rabies cases for several years and therefore are unprepared to counter the risks associated with the disease and fail to take appropriate preventative measures. It is hoped our findings will help towards determining a program for further controlling and working towards eliminating rabies in China.

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