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Molecular Epidemiology and Sequencing of the G-L Intergenic Region of RabiesViruses Isolated in China*

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Abstract: A group of 25 rabies viruses (RABVs), recovered from 24 dogs and one human case, were collected from various areas in China between 2004 and 2006. Genetic and phylogenetic analyses of the G-L intergenic region were carried out in 25 street RABV isolates and CTN vaccine strains of 7 generations. The study was based on the comparison of a 519 bp nucleotide sequence, encompassing the G-L intergenic region. The nucleotide sequence homologies of Chinese street strains were from 95.5% to 100%. The phylogenetic analysis showed that all Chinese isolates clearly supported the placement of all Chinese viruses in Lyssavirus genotype 1 and they were distributed according to their geographical origins. All of the Chinese strains were closely related but they could still be divided into two groups: group of street strains and group of CTN strains. This study presents details about the molecular epidemiology of rabies viruses based on the sequences of the G-L Intergenic region.

Key words: Rabies virus; Molecular Epidemiology; G-L intergenic region; China

Rabies is an acute, progressive, incurable viral encephalitis, caused by a single stranded RNA virus belonging to the genus Lyssavirus of the family Rhabdoviridae. Human mortality from endemic canine rabies is estimated to be 55 000 deaths per year with 56 % of the deaths estimated to occur in Asia and 44% in Africa (14). While many distinct RABV variants are harbored by a variety of mammalian host species, the main transmission route for human rabies in the developing world is a rabid dog bite: between 94% and 98% of human rabies deaths are due to canine rabies (2).

In China, there were 110 983 human rabies cases and three major epidemic peaks between 1950 and 2005. These human cases occurred mainly in poor, rural areas and were transmitted by dogs. The first epidemic occurred in the mid 1950s when cases rose to a peak of about 2 000 annually. After a decline in

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the 1960s, cases again started to increase in the early 1970s reaching a peak in 1982 but remaining at levels of 5-6 000 cases per year till the end of the decade(16) . Since the vast majority of cases have been due to canine rabies, an extensive dog vaccination and culling program was initiated in 1987 and between the years 1990 and 1996 canine rabies decreased. It is important to note that 98% of the people who died of rabies in China had not received any anti-rabies vaccine treatment and 70% of the people who died of rabies between 1996 and 2002 lived in Guangxi, Jiangxi, Hunan, Guangdong and Jiangsu provinces. There were 2 651 and 2 571 cases in 2004 and 2005, respectively, which suggest the new epidemic peak is coming. All warm-blooded mammals are susceptible to RABV infection, but dogs represent the major host of rabies virus in China. The main transmission route for rabies is canine bites. 95% rabies deaths are due to canine bites and rabid cats are responsible for 4% human cases in China. Increased dog populations and limited vacci-nation against rabies together with improved transportation may have resulted in this recent increase in rabies cases.

The lyssavirus particle has a bullet-shaped form, 100-300 nm in length and 75 nm in diameter (7). The RABV genome consists of a 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 and bilayered envelope. The RNA polymerase(L) and phos-phoprotein(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.

Evolutionary studies of lyssaviruses have tended to focus on the N protein, a well conserved structural protein, and the G protein which forms the envelope and capsid of the virus. The G protein contains domains responsible for host cell receptor recognition and membrane fusion and is the major target for the host neutralizing-antibody response (1), and the G-L intergenic region or pseudogene (Ψ) which is a noncoding region, highly variable, not subject to immunological selective pressure, and has therefore been used in studies of molecular epidemiology of the rabies virus (11).

Sequence comparison of several regions of the genome, including the N, P, and G genes, has provided consistent evidence for division of the Lyssavirus genus into two major phylogroups and seven established genotypes as follows: Phylogroup I comprises Rabies virus(RABV; genotype 1), Duvenhage virus(DUVV; genotype 4), European bat lyssavirus 1 (EBLV-1; genot-ype 5), European bat lyssavirus 2 (EBLV-2; genotype 6), Australian bat lyssavirus(ABLV; genotype 7); Phylogroup II contains Lagos bat virus(LBV; genotype 2), Mokola virus, (MOKV; genotype 3)(1, 8). Four recent lyssavirus isolates from bats of Eurasia, designated Aravan virus(ARAV), Khujand virus(KHUV), Irkut virus(IRKV) and West Caucasian bat virus(WCBV), have been described (6). While these viruses await formal classification, their genetic diversity between each other and currently classified lyssaviruses would suggest that they represent several additional genotypes.

By analyses of the nucleotide sequence data of the non-coding G-L intergenic region, this study provides a new description of the molecular epidemiology of rabies in China and a regional comparison of virus relationships.

1. Materials and methods

1.1 Virus isolates

Between 2004 and 2006, brain samples from 24 dogs and one human were collected from 5 provinces

in China: Anhui, Hubei, Jiangsu, Hunan, and Jiangxi provinces. The CTN vaccine strains of 7 generations from 7 to 35 passages were also included in the analysis. The species origin, year of isolation and geographical distribution of the rabies virus samples used in this study are described in and Table 1.

1.2 Diagnostic tests

All the brain samples were tested for rabies virus by direct immunofluorescence antibody(IFA) test and enzyme-linked immunosorbent assay (ELISA), which are the standard diagnostic tests currently used in China. The anti-rabies nucleoprotein monoclonal antibodies and FITC- labelled monoclonal antibody were manufactured in Wuhan Institute of Biological Products, China.

1.3 Viral RNA extraction

Total RNA was extracted by TRIzol(Invitrogen, Carlsbad, CA, USA), from brain tissue of naturally infected animals for PCR assay according to the manufacturer's instructions.

1.4 cDNA synthesis

Reverse transcription(RT) of viral RNA was Per-

Table 1. List of rabies virus Chinese strains employed for evolutionary analysis

Virus name	City/ province	Host	Year	GenBank accession number.
FY1	Fuyang / Anhui	Dog	2004	DQ836077
FY2	Fuyang / Anhui	Dog	2004	DQ836078
FY3	Yingshang /Anhui	Dog	2004	DQ836079
FY4	Funan / Anhui	Dog	2004	DQ836080
FY5	Fuyang / Anhui	Dog	2004	DQ836081
FY6	Fuyang/Anhui	Dog	2004	DQ836082
FY7	Lixing /Anhui	Dog	2004	DQ836083
FY8	Funan/Anhui	Dog	2004	DQ836084
FY9	Funan / Anhui	Dog	2004	DQ836085
FY10	Yingshang/ Anhui	Dog	2004	DQ836086
FY12	Fuyang /Anhui	Dog	2005	DQ836087
FY13	Fuyang/ Anhui	Dog	2005	DQ836088
FY14	Fuyang /Anhui	Dog	2005	DQ836089
FY15	TaiHe/Anhui	Dog	2005	DQ836090
FY16	Fuyang /Anhui	Dog	2005	DQ836091
WH5	Wuhan/ Hubei	Dog	2005	DQ836092
HN06	Wuhan /Hubei	Dog	2005	DQ836093
WG430	Wugang /Hunan	Dog	2005	DQ836094
WG432	Wugang /Hunan	Dog	2005	DQ836095
JSS62	Suqian /Jiangsu	Dog	2005	DQ836096
JSL26	Ganyu/ Jiangsu	Dog	2005	DQ836097
JSL27	Ganyu/Jiangsu	Dog	2005	DQ836098
JSL29	Ganyu/Jiangsu	Dog	2005	DQ836099
QC	Qichun/ Hubei	Human	2006	DQ836010
NC	Nanchang /Jiangxi	Dog	2004	DQ836011
CTN-7	Shandong	Mouse	2006	DQ836012
CTN-27	Shandong	Mouse	2006	DQ836013
CTN-28	Shandong	Mouse	2006	DQ836014
CTN-29	Shandong	Mouse	2006	DQ836015
CTN-30	Shandong	Mouse	2006	DQ836016
CTN-33	Shandong	Mouse	2006	DQ836017

formed in a 100μL reaction volume using 1 μL extracted RNA, 10units AMV Reverse Transcriptase (Promega), 10 units RNasin ribounclease inhibitor, 2 μL RT reaction buffer (250 mmol/L Tris-HCl [pH 8.3, 25°C],250 mmol/L KCl, 50 mmol/L $MgCl_2$, 2.5mmol/L spermidine and 50mmol/L ditheothreitol), 250mmol/L for each of the four deoxynucleotides, and 30 pmol sense primerGH3.3(5’-GAYTACACCATC-TGGATGCC-3’) corresponding to nucleotides 3894-3913 of the PV genome. RT reactions were incubated at 42°C for 90 min before heat-inactivating AMV Reverse Transcriptase at 100°C for 3min. The cDNA product was stored at -20°C or PCR amplification was done at once.

1.5 PCR amplification

In a 100μL eppendorf tube, add the following components in the indicated order: 37μL dd H_2O, 1μL sequence-specific sense primer MSL(5’-TGGATTTGTGGATKAAAGAGGC-3’), and 1μL anti-sense primer L1(5’-GAGTTNAGRTT GTART-CAGAG-3’) (30.3 pmol/μL), corresp- onding to bases 3995-4016 and 5516-5536 of the PV genome, respectively, 2 μL template cDNA, 5μL 10×PCR buffer(100mmol/L Tris-HCl[pH9.0], 500 mmol/L KCl, 0.5% Tween-20), 3μL 20mM $MgCl_2$, Mix gently and collect drops by brief centrifugation, then incubate the mixture at 100℃ for 3min. For collecting drops, chill on ice and spin down by brief centrifugation, then place the tube on ice and add 0.5μL Taq DNA polymerase(Generay Biotech Shanghai, 5U/μL). Collect drops by brief centrifugation, then PCR amplify. Thermal cycling conditions were: 1 cycle at 94°C for 1min; 40 cycles at 94°C for 1min, at 58°C for 50s and at 72°C for 90s, followed by 1 final incubation at 72℃ for 10 min; holding at 4℃. The PCR product was analyzed on a 1% agarose gel containing ethidium bromide.

1.6 Sequencing and phylogenetic analysis

PCR products were purified using the quick-spin PCR Purification Kit and sequenced using the Taq Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an Applied Biosystems 3770 DNA automated sequencer(Applied Biosystems Inc. Foster City, CA, USA). A multiple alignment of the nucleotide sequence of G-L intergenic region (519 bp) was generated with ClustalW1.8 (12). Distance calcula-tions were done using the Kimura 2-parameter model (4). At the same time, for construction of the phylogenetic trees, the Neighbour Joining(NJ) and Maximum Likelihood (ML) method were conducted using MEGA version 3.1 (3, 5). The statistical significance of the phylogenies constructed was estimated by bootstrap analysis with 1000 replicates, and bootstrap values above 70% were considered significant. The new nucleotide sequences obtai- ned in this study have been submitted to GenBank and their accession numbers are listed in Table 1.

2. Results

Between 2004 and 2006, from the total of 381 dogs, 80 cats, 100 bats and a human brain samples examined, the rabies infection were confirmed in 24 dogs and one human brain samples (Table 1) by FAT, ELISA and RT-PCR.

The G-L intergenic region of 25 street rabies virus isolates from China and CTN vaccine strains of 7 gene-rations were amplified by the RT-PCR and sequenced. A 519 nucleotides sequence of the non-coding G-L intergenic region was analyzed with the related sequences of other rabies viruses obtained from GenBank (Table 2). The sequence homologies of the 25 Chinese strains against other non-Chinese rabies virus strains were at least 67.5%, and the pairwise distances between strains were calculated using the Kimura 2-parameter model in MEGA 3.1 (data not shown). Analysis of the sequences within the G-L intergenic region showed that street rabies viruses isolated from China had the closest relationship to the Thailand strain (THA1-HM) and the Malaysia strain (MAL1-HM). The homologies were at least 82% and 82.3%, respectively. However, there were higher nucleotide homologies of the sequences of the G-L

intergenic region among all rabies virus isolates from China, ranging from 95.5% to 100%. In addition, alignment of the G-L intergenic region of Chinese street strains showed that nucleotide homologies were ranging from 85.5% to 86.4%, 68.5% to 69.8%, 68.1% to 69.8%, 70.7% to 73.2% and 69.9% to 72.8% as compared with 4tive results of nucleotide homologies are shown in Table 3.

For a better understanding of phylogeny of Chinese strains, pairwise comparisons of the G-L intergenic region were performed. One consensus tree (not shown) was constructed with the NJ method in rabies viruses isolated from China, showing the Chinese strains segregated into two groups (Ⅰ and Ⅱ) with

Table 2 Sequences of rabies virus strains obtained from GenBank in this G-L intergenic region analysis

Year	Country	Host	Strain name	Reference	Accession no.
1882	France	Cow	PV	Tordo *et al*. (1986)	M13215
1935	America	Dog	SAD	Conzelmann *et al*. (1990)	M31046
1939	America	Human	HEP-Flury	Morimoto *et al*. (1989)	M32751
1983	Thailand	Human	THA1-HM	Badrane *et al*. (2001)	AF325488
1985	Malaysia	Human	MAL1- HM	Badrane *et al*. (2001)	AF3254871
1989	Brazil	dog	BRdg12	Sato *et al*. (2004)	AB110659
1998	South Korea	Cattle	SKRBV9801YC	Hyun *et al*. (2005)	DQ076108
2002	South Korea	Dog	SKRDG0203CW	Hyun *et al*. (2005)	DQ076108
	India	Buffalo	IIL R2	Nagarajan *et al*.(2005)	DQ255915
	India	Buffalo	IIL R4	Nagarajan *et al*.(2005)	DQ255917
	Canada	Canis	ArcticA1-1090DG	Nadin-Davis *et al.* (1994)	U03766
	Germany		PM1503	Stallkamp *et al*.(2005)	DQ099525

Table 3 Nucleotide homology (%) and Std. Err. of the G-L intergenic regions of some rabies viruses

NO[a].	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1		0.006	0.007	0.007	0.004	0.033	0.034	0.024	0.034	0.031	0.032	0.031	0.030	0.031	0.020
2	98.7		0.007	0.008	0.005	0.035	0.035	0.025	0.034	0.032	0.033	0.031	0.031	0.031	0.021
3	98.4	98.2		0.008	0.006	0.033	0.034	0.025	0.035	0.032	0.032	0.032	0.030	0.032	0.021
4	97.9	97.6	97.4		0.007	0.033	0.033	0.024	0.034	0.031	0.031	0.030	0.030	0.030	0.020
5	99.2	99.0	98.7	98.2		0.033	0.034	0.024	0.034	0.031	0.032	0.031	0.030	0.031	0.020
6	69.8	68.5	69	69.5	69.4		0.009	0.041	0.032	0.029	0.031	0.022	0.024	0.024	0.034
7	69.8	68.4	68.9	69.4	69.3	97.4		0.039	0.032	0.028	0.030	0.024	0.025	0.026	0.034
8	83.7	82.6	82.3	83.0	83.3	64.2	65.6		0.040	0.036	0.035	0.035	0.036	0.033	0.022
9	68.3	68.3	67.9	67.3	67.3	72.0	72.0	65.1		0.021	0.024	0.028	0.025	0.027	0.034
10	72.6	72.2	71.8	72.2	73.0	76.3	76.2	68.5	85.4		0.019	0.024	0.024	0.024	0.030
11	72.2	71.8	71.9	72.7	72.7	74.7	75.4	70.8	82.0	87.9		0.027	0.026	0.029	0.031
12	71.9	71.5	70.3	72.8	71.5	82.5	81.0	69.0	77.3	81.3	79.5		0.023	0.012	0.029
13	72.8	71.6	72.1	71.6	72.4	80.8	79.3	67.9	80.6	81.4	80.0	83.5		0.023	0.030
14	72.7	72.3	71.1	73.2	72.3	79.9	0.216	71.1	76.9	80.9	77.7	93.8	83.1		0.029
15	86.1	85.8	85.5	86.1	86.4	69.9	69.8	84.3	69.5	75.4	73.8	74.2	73.6	75.0	

Upper triangle-Std. Err；Lower triangle-nucleotide homology. Serial Numbers of rabies viruses: 1.WG430.2. WH5. 3. FY1.4. NC. 5. JSS62. 6. SAD. 7. PV. 8. THA1-HM. 9. SKRBV9801YC. 10. IIL_R2. 11. ArcticA1-1090DG. 12. HEP-Flury.13. BRdg12. 14. PM1503. 15. CTN-30.

100% bootstrap support. The CTN vaccine strains formed Group Ⅱ and the Chinese street strains composed of Group Ⅰ, which was further divided into four lineages (Ⅰa - Ⅰd) with at least 80% bootstrap supports and contained rabies viruses originating from different areas of China. The Ⅰa Ⅰd lineage originated from Anhui, Hubei, Jiangsu and Hunan provinces, respectively. As in the Neighbor joining phylogenetic (NJ) tree, the NC specimens lay on a separate branch within group Ⅰ. The nucleotide sequence differences of Chinese street strains are shown in Table 4. The 3 regions(8bp to 49bp, 254bp to 3 08bp, and 505bp to 514bp) are highly diverse. A phylogenetic NJ tree of interior branch test based on alignment of the G-L intergenic region nucleotide sequences and the neighbor- joining method is shown in Fig. 1. A similar ML tree was obtained by the maximum likelihood method (data not shown).

3. Discussion

Since the SARS epidemic of 2003, the Chinese government has set up a systematic surveillance net for zoonotic diseases. Increased surveillance together with increased dog populations may be the principal factors explaining the increasing number of human rabies cases reported in China in the last few years. A

Table 4 The comparison of the G-L intergenic region nucleotide sequences of Chinese street strains

Strain	Position of the G-L intergenic region nucleotide sequences																			
	15	22	36	45	49	115	163	173	256	276	295	302	308	416	417	505	507	511	514
FY1	T	C	G	C	C	G	C	T	A	A	A	T	C	G	T	A	A	T	A
FY2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FY3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FY4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FY5	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FY6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FY7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FY8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FY9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FY10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FY12	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-
FY13	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-
FY14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FY15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FY16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
WH5	-	-	-	-	T	A	-	-	G	-	G	C	T	A	-	G	G	-	G
HN06	-	-	-	-	T	A	-	-	G	-	G	C	T	A	-	G	G	-	G
QC	-	-	-	-	T	A	-	-	G	-	G	C	T	A	-	G	G	-	G
WG430	-	-	A	-	-	A	-	C	G	-	-	-	T	-	G	G	G	-	G
WG432	-	-	A	-	-	A	-	C	G	-	-	-	T	-	G	G	G	-	G
JSS62	C	T	-	T		A	-	-	G	G	-	-	T	-	-	G	G	-	G
JSL26	C	T	-	T		A	-	-	G	G	-	-	T	-	-	G	G	-	G
JS L27	C	T	-	T		A	-	-	G	G	-	-	T	-	-	G	G	-	G
JSL29	C	T	-	T		A		-	G	G	-	-	T	-	-	G	G	-	G
NC	-	-	-	-	-	A	T	-	G	-	-	C	T	-	-	G	G	C	G

Fig.1. Neighbor joining phylogenetic tree based on the G-L intergenic region nucleotide sequences of rabies virus by Kimura two parameters (MEGA version 3.1) .The length of the horizontal branches reflects phylogenetic distance relationship. Percentage bootstrap values above 70% are shown at the branch nodes.

mass vacci-nation of dogs in the epidemic regions, as has been successfully undertaken in certain countries of Latin Ame-rica(13), could virtually eliminate rabies, but the high cost of vaccines currently prevents this undertaking. The data reported here represent an early step in understanding the epidemiology of dog rabies in China. This knowledge will be important to future control efforts.

According to the phylogenetic analysis of the G-L intergenic region in different rabies virus strains, all of the Chinese street isolates and the CTN vaccine strains of 7 generations were closely related but could be divided into two groups. The Chinese street isolates formed Group I, which was further divided into four lineages related to origins of distinct geographic regions. Inte-restingly, we recovered 15 RABV isolates from Fuyang and its surrounding area, and although the homology among these 15 isolates, which comprise lineage 1a, is very high, these isolates could still be further subdivided into several variants. Group II included CTN vaccine strains of different generations, which were originally isolated from Shangdong province in 1956 and became a vaccine strain through serial passages in mouse brain and human diploid cell(KMB-17). Group I and group II were closely related. A previous study on the N genes of Chinese strains has obtained similar phyloge-netic patterns(15).

The G-L intergenic region in different rabies virus strains was highly variable and even the nucleotide numbers of this region were different. In Chinese street strains, this region had 519 nucleotides and only one transcription termination and one polyadenylation(PPT) motif. However, there were two transcription termination and two polyadenylation(PPT) motifs found in this region in the Pasteur strain derived from an 1882 French strain and the SAD strain derived from a 1935 U.S. rabies isolate (10) .

The G-L intergenic region is a non-coding region, which is highly susceptible to random mutations, unrestricted by structure and function requirements or by immunological pressure. Being the most divergent region of the rabies genome, it might be more sensitive in demonstrating recent evolutionary events (9) .

The CTN, PV and PM strains are the human vaccine strains currently used in China. The nucleotide homology between the CTN strains and the Chinese street strains was much higher(at least 12.3%) than that of any other vaccine strains. When the N and G Genes of the same vaccine strains and the same Chinese street strains were sequenced and analyzed, we obtained very similar phylogenetic trees(the data are not showed here and they will be published later). So it is possible that vaccines produced with the CTN vaccine strain might be more efficient than that

produced with any other vaccine strains in China. However, vaccine trials should be undertaken to confirm this hypothesis.

At the same time, we find that SAD, PV, HEP-Flury and PM vaccine strains have very high nucleotide homologies with South Korean, Indian, Canadian and Brazilian isolates. All these vaccine strains originated from America or Europe. For nearly half a century there was virtually no connection between China and South Korea and there is natural barrier between India and Tibet. These might explain the difference in the strains between China and South Korea or India.

In conclusion, the findings from this study confirmed the fact that rabies viruses are genetically variable and the evolutions of different groups are apparently dominated by geographical influences. The nucleotide homology between the CTN vaccine strain and the Chinese street strains is higher than for other vaccine strains. Our study provides new data to support the national rabies control programme.

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