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Identification of Rabies Virus from Chinese Ferret-Badger and Analysis of its P and M Genes

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ABSTRACT

Infection of rabies virus (RV) was inspected in 23 captured wild Chinese Ferret-badgers by direct fluorescence immunoassay. One of the Ferret-badger was identified to be RV antigen positive. Existence of the RV was confirmed by the RT-PCR. The P and M genes of the RV from the badger (CZJF01) was amplified and the genetic variability on functional positions were studied on molecular level. The homology comparison and evolution analysis of the P and M genes among the rabies virus isolated from Chinese Ferret-Badger, human, dog, vaccine strains were performed. As the results, the CZJF01 strain shared with the RV from other sources in nucleotide sequences a homology of 80.6％～98.7％ and 84.2％~97.2％ for the P and M gene, respectively, and a homology of 86.2％～96.6％ and 89.2％～96.6％ in the relevant amino acid sequences, respectively. Phylogenic analysis of the P and M gene nucleotide sequence revealed that the CZJF01 strain was closely related with the genotype 1 RV strains. The CZJF01 has the closest evolution relation with a vaccine strain, HEP-Flury. The functional area of P and M gene is highly conserved among all the genotype 1 RV strains. There were no variations on functional positions of P and M proteins that affect its biological functions. The vaccine developed from HEP-Flury may have better protective effect in Zhejiang, China.

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Key Words: Chinese Ferret-badger, Rabies virus; P gene; M gene; Phylogenic tree

Abbreviations: RV, Rabies Virus type 2; DFA, Direct Flouorescence ImmunoAssay; RT-PCR, Reverse Transcription Polymerase Chain Reaction;

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Introduction

Rabies disease once under control in China has a rapid and significant rebound since 1995. Rabies disease is now becoming a threat to the public health in certain areas of China. Rabies virus (RV), the pathogen for the rabies disease, is a single strand negative-sense RNA virus. Rabies virus has tendency to infect the nerve tissues and cause disease. While rabies virus can be hosted in various warm-blooded animals including human beings, they are more likely to infect and to be carried in the wild carnivorous animals. For the stocking animals, animals belong to the families of *felidae* and *canidae* are most susceptible to be infected and to carry the virus. In addition to the biting by the dogs, biting and assaulting from other potential rabies virus carriers such as Chinese Ferret-Badger is another important source of the pathogen. So far there are several reports described cases of human rabies disease, which were caused from the biting by the Chinese Ferret-Badger. During Aug.1994 to July, 1995, a hospital in Huzhou, Zhejiang has treated 6 human rabies diseases patients, who were bitten by the Chinese Ferret-Badger. During 2002-2004, 8 cases of confirmed rabies disease were reported and 7 of them were caused potentially by the biting of the Chinese Ferret-Badger in Chun-an, another county of Zhejiang Province. There are also cases that the povetry and cattles (pigs, dogs and cats) were bitten by the Chinese Ferret-Badger resulting in death possibly due to the infection of the rabies virus. Such cases has been reported and summarized in the recent report[1,2].

The rabies virus RNA has five coding regions which encode the N, P, G, M and L genes[3]. The virus replication is mainly attributed to the viral polymerase complex, composed from the L and P proteins[4] . The P protein is expressed in large quantity by the infected host cells. Comparing to the extremely high conservation for the N and L genes, P gene is relatively variable, and therefore is often used to discriminating among different rabies virus[5,6]. On the other hand, M protein is critical for the viral assembly and budding in the virus replication[7]. Both genes have been used for the comparison of the phylogenic relation and cluster analysis of different rabies virus from different sources and different regions.

To identify the rabies virus from the Chinese Ferret-Badger and to understand its molecular epidemiology characters, we have collected tissue samples from 23 Chinese Ferret-Badger, and screened for rabies virus. By using the Direct Flouorescence ImmunoAssay (DFA) and RT-PCR, we have identified a rabies virus (CZJF01) from one of the samples. We further analyzed the P and M genes of the virus and conducted comparison of the

genes among this strain and the other known rabies virus strains. To our knowledge, there are no reports of isolation and analysis of rabies virus from the Chinese Ferret-Badger, and this is a first report of detection of rabies virus from Chinese Ferret-Badger.

Materials and Methods

1. Chinese Ferret-Badger

The 23 Chinese Ferret-Badgers used in this study were captured during April 2008 ~ June 2015 by trapper from wild. One of them was caught when the animal invaded into a resident house. This animal (Figure 1) was apparently sick comparing to the healthy normal animal. The animal died about half day after capture. The brain tissues were collected from the animals and frozen at -70 ^{0}C for further studies.

2. Direct flouorescence immunoAssay

Brain tissue samples of various part of the brain tissues of the Chinese Ferret-badger (brain, mesencephalon, cerebellum and hippocampus) were smeared on the slide glasses. After drying, the slides were fixed at 4^{0}C in acetone for 10 min. Monoclonal antibodies were then applied to the slides and incubated for 30 min at 37^{0}C. The slides were then washed with phosphate buffer and distilled water for 2 times, respectively. After drying, the slides were covered with glycerin and analyzed under fluorescent microscopy. DFA reagent kit was purchased from Chemicon (USA).

3. RT-PCR to identify rabies virus

Total RNA was extracted with TRIzol reagent (Invitrogen, USA) from the whole brain tissue which was confirmed to be rabies virus positive by the DFA analysis. cDNA was synthesized by using the random primers pd(N)6 (TaKaRa, Japan) with a Ready-To-Go RT-PCR kit from Amersham (Germany). N Gene of the rabies virus was amplified by using the GoTaq Green Master Mix (Promega, USA) following the instruction of the kits. The amplified fragments were analyzed in a 1% agarose gel (Sigma, USA).

4. Amplification and analysis of M and P genes:

M and P genes of the rabies virus from the Chinese Ferret-Badger was amplified from the cDNA with two pairs of the primer sets using the GoTaq Green Master Mix (Promega, USA) following the instruction of the kits. The amplified products were separated by agarose electrophoresis and DNA was recovered by the PQIAquick Gel Extraction Kit (Qiagen, Germany). The sequences of the genes were then determined and compared with P and M genes of the rabies virus isolated from human (type 1) and canine and other vaccine strains. The homology and phylogenic tree of P and M genes of the rabies virus from the Chinese Ferret-Badger in comparison with the genes from other sources were analyzed by using Clustalx 1.83, DNAStar and MEGA 3.1.

A: B:

Figure 1. Photos of the captured Chinese Ferret-badger.

A: The Chinese Ferret-Badger that was infected with the rabies virus.

B: A representative healthy Ferret-Badger.

A： B:

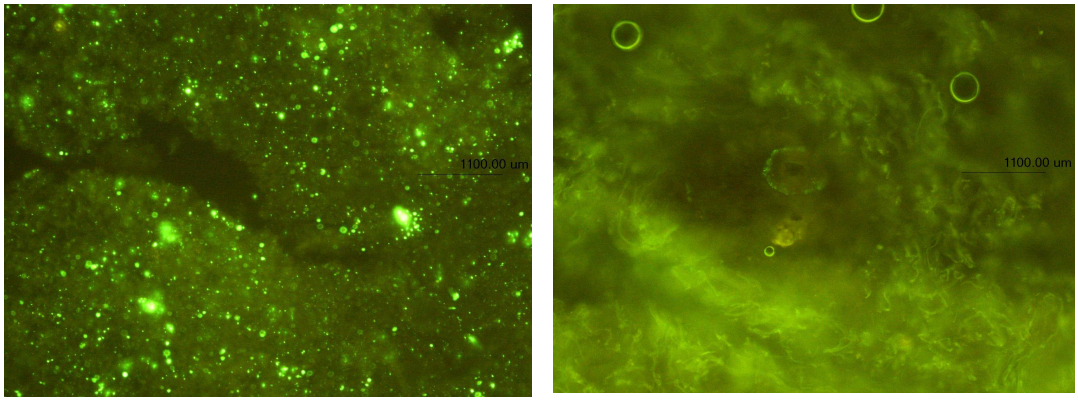

Figure 2. Direct detection of rabies virus from the brain tissue of the Chinese Ferret-Badger infected by the Rabies Virus using the DFA.

A: The brain tissue of the Chinese Ferret-Badger infected with the rabies virus.

B. Brain tissue of the normal healthy Chinese Ferret-Badger.

Results

1. Identification of Rabies Virus

For the 23 captured Chinese Ferret--badger, we used the DFA method to directly screen and identify the rabies virus antigen from the brain tissues of the animals. As shown in Figure 2, from the animal which was captured in a resident house, there is a clear sign of the virus in the brain tissue as shown as dotted points with apple-green fluorescence. While from the rest tissues, we were unable to confirm other rabies virus positive tissues. To further confirm the existence of the rabies virus in the antigen positive animal, we used RT-PCR to amplify the N gene. We confirmed that a 260 bp of the characteristic fragment was identified from the sample of the antigen-positive animal (data not shown). We named the virus in this animal as CZJF01.

2. Sequencing of P and M Genes of CZJF01

To compare and analysis of the P and M genes from CZJF01 with the known rabies virus, we amplified the two target genes. After amplification, we identified two expected fragments of about 1000 and 600 bp from the CZJF01 strain. The sequences of the two genes were then determined after extraction of the amplified products. The sequences have been registered in the GenBank and the access numbers are FJ0322324 and FJ032330, respectively. We compared the sequences of the P and M gene and the corresponding protein sequences with those from HEP-Flury strain, which has most high homology with CZJF01 (see below). We found that most of the critical locations for the P protein to inter act with Larger Protein (LP) and Nucleus protein (NP) are conserved except that the codon in resiude 19 of G is changed to C. Meanwhile, the residue 58 of M protein in CZJF01 is E instead of G. We did not find any changes which will cause changes in its critical biological functions.

3. Homology Analysis of the P and M Genes

We analyzed the homology of the two genes of CZJF01 with the corresponding genes from the rabies virus isolated from human beings, canine and the vaccine strains. As shown in table 1, there is about 80.6~98.7% of homology in the P gene nucleotides and even higher homology of 86.2~96.6% in the putative amino acid sequences among the CZJF01 and the other rabies virus. The highest homology was observed for CZJF01 vs. the animal vaccine strain HEP-Flury (98.7% and 96.6% for bases and amino acid sequence, respectively) and PM1503 (94.3% and 93.3%, respectively). Higher homology was also observed for CZJF01 vs. the human vaccine strains of aG (88.1% and 87.6%, respectively) and CTN(88.1% and 87.6%, respectively). The homology of the M gene was around 84.2-97.2% for nucleotides and 89.2~96.6% for amino acid sequences (table 2). CZJF01 has the

Table 1. Comparison of the Nucleotide and Amino Acid Sequence of P Gene of The Rabies Virus from Various Sources

Strain	F01	HEP Flury	PM1503	ERA	PV	aG	CGX 0511D	CHN 0610H	CTN	CJS 0635D	CGZ 0622D	CYN 0601H
F01	***	98.7	94.3	89.6	89.4	88.1	80.6	81.1	82.2	82.2	81.8	82.9
HEP Flury	96.6	***	94.7	90.3	90	88.9	81	81.4	82.6	82.6	82.1	83.6
PM1503	93.3	93.6	***	89.3	88.9	87.7	81.1	81.9	82.6	83.3	83.4	83.6
ERA	89.3	89.9	91.3	***	99	89.8	81.7	82.6	83	83.1	82.9	84.2
PV	89.3	89.9	91.3	97.7	***	89.7	81.3	82.2	82.7	82.9	82.4	84.1
aG	87.6	88.6	88.9	89.9	90.6	***	81.7	82.3	83.6	83.1	82.6	84.3
CGX0511D	87.6	87.9	89.3	89.9	89.3	88.9	***	97.3	92.2	87.9	86.2	86.6
CHN0610H	87.6	87.9	89.3	89.9	89.3	88.3	98.7	***	92.4	88.1	86.5	87
CTN	86.2	86.2	87.6	88.6	87.9	87.2	96.6	96	***	87.1	86.6	87.2
CJS0635D	86.9	87.6	87.9	88.6	87.9	87.6	93.3	93.6	92.6	***	97.1	85.8
CGZ0622D	86.6	87.2	87.9	88.3	87.6	87.2	93	93.3	92.3	98.7	***	85.1
CYN0601H	86.6	87.2	87.9	88.3	89.3	88.3	93	91.9	91.3	91.6	91.3	***

Note: The right upside part are the nucleotide sequence homology, and the left downside part are the amino acid sequence homology.

Table 2. Comparison of the Nucleotide and Amino Acid Sequence of M Gene of The Rabies Virus from Various Sources

Strain	PM1503	HEP Flury	ERA	PV	aG	F01	CHN 0610H	CTN	CGX 0511D	CJS 0635D	CGZ 0622D	CYN 0601H
PM1503	***	95.4	91.8	91.5	91.8	90.6	84.9	85.9	86	86.7	87.2	87.2
HEPFlury	93.1	***	92.8	92.4	92.8	92	86	86.2	87.2	87.8	87.5	87.5
ERA	92.1	93.1	***	97.7	92.4	90.6	84.1	84.4	84.7	86.7	85.9	85.9
PV	91.6	92.6	94.1	***	92.1	90.3	83.6	83.9	84.2	86.7	85.4	85.4
aG	92.6	94.1	91.6	91.1	***	97.2	84.2	83.9	84.7	85.6	86.5	86.9
CZJF01	90.6	93.1	89.7	89.2	96.6	***	84.6	84.2	85.7	87.4	86.5	86.5
CHN0610H	94.6	96.1	94.6	94.1	94.6	93.6	***	95.4	96.2	90	90.8	91.1
CTN	92.6	94.1	92.6	92.1	93.1	92.1	97.5	***	94.1	90.3	90.6	91
CGX0511D	94.1	96.6	94.1	93.6	94.1	94.1	99	97	***	92.4	90.8	91.1
CJS0635D	93.1	95.6	93.1	92.6	93.1	94.1	97	95.6	97.5	***	89.8	89.5
CGZ0622D	93.6	94.1	92.6	92.1	92.6	91.6	97.5	95.6	97	96.1	***	99.7
CYN0601H	94.1	94.6	93.1	92.6	93.1	92.1	98	96.1	97.5	95.6	99	***

Note: The right upside part are the nucleotide sequence homology, and the left downside part are the amino acid sequence homology.

most high homology with the human vaccine strain aG (97.2% and 96.6%), and higher homology with the animal vaccine strain HEP-Flury (92.0% and 93.1%). Again, it shows the lowest homology with that of the strain isolated from human beings.

To understand the phylogenic relation of the CZJF01 with other known rabies viruses, we aligned the P and M genes for human and animal vaccines and those isolated from other sources. As shown in Figure 3, CZJF01 belong to the same branch with the gene type 1 animal vaccine strains (HEP-Flury, PM1503, ERA and Nishigahara), human vaccine strains (aG and CTN), Pasture strain (PV) and isolated human rabies virus from China. It has only remote connection with the other strains. This suggests that the CZJF01 strain belong to gene type 1 and located in the same small cluster with the animal vaccine strains.

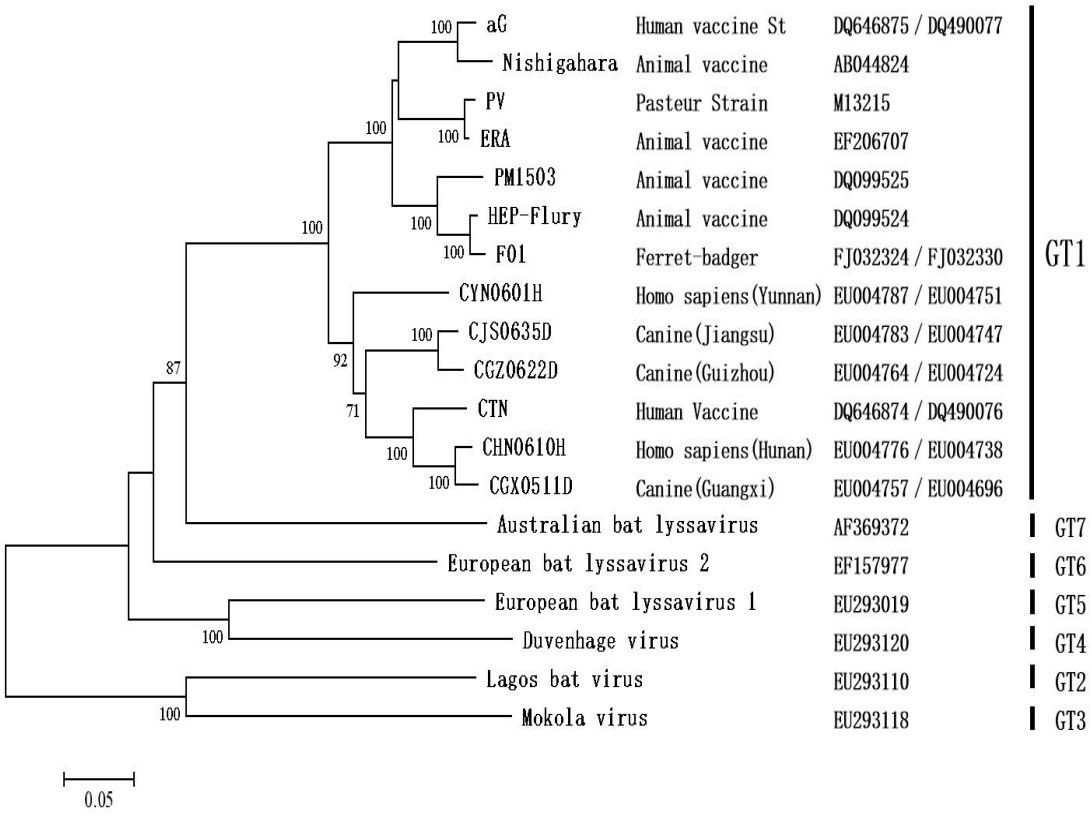

Figure 3. The phylogenic tree of the P and M genes constructed by MEGA 3.1 software.

Discussion

Homology comparison and phylogenic relation analysis using the M and P protein gene sequence indicate that CZJF01 is most closely related to the animal vaccine strain HEP-Flury, and moderately closely related to the strains isolated from human being and canines. The nucleotide homology of CZJF01 with animal vaccine strains, human vaccine strains and the Pastur strain is higher than that of the homology of the protein, indicating that most of the changes are asynonymous mutations. Phylogenic relation analysis indicated that CZJF01 belong to the same branch with the gene type 1 animal vaccine strains (HEP-Flury, PM1503, ERA and Nishigahara), human vaccine strains (aG and CTN), Pasture strain (PV) and isolated human rabies virus from China. It has only remote connection with the other strains. This suggests that the CZJF01 strain belong to gene type 1 and located in the same small cluster with the animal vaccine strains. This is in line with the earlier reports that Asia is the type I rabies virus prevail area and there is no other gene types identified from Asia.

The phosphorylation of the P protein is completed by the Protein Kinase (RVPK) and protein kinase C (PKC) [8]. The phosphrylation sites of RVPK at the residues number 63 and 64 from the N-terminal and sites 162, 210 and 271 that are phosphorylated by PKC in CZJF01 are all conserved in comparison with HEP-Flury.

P protein together with Nucleus Protein (NP) and Large Protein (LP) plays a critical role in regulating the translation and replication of the virus. The initial 19 amino acid residues of P protein are the locations where P protein interacts with the LP protein[9]. We only found one change in the nucleotides at location 10 from G to C. While residues 209-216 are the sequence that P protein interact with Nucleus Protein. The sequence is FSKKKKFP. This sequence is highly conserved and we did not find any change in CZJF01 in this sequence. P protein also interacts with the light chain (LC8) of the cytoplasma dyneins, which affect the activity of the virus in the neuron and to cause pathologic change[10]. The areas where P Protein interacts with LC8 is the residues 143-148, and the sequence is DKSTQT. This area is also highly conserved and we were unable to identify any changes in these locations in CZJF01. M protein is critical in the assembly and budding in the virus replication [7]. The functional structure in M protein contains a sequence called proline structure PPxY. We found that there is a PPEY sequence in residue 35-38 of the M protein in CZJF01. This structure is conserved in all the type I rabies virus M protein structure. The number 58 amino acid residue in M protein contributes to the function of this protein in the regulation of transcription and replication. The strain with the

residue 58 of E (Glutamic) has high activity than those with G (glycine) in virus transcription. For CZJF01, it is R58E.

Rabies disease has a very high mortality and there are no effective cures for the disease so far. Vaccination is the best way to prevent the rabies disease. There are several different vaccine types according to the epidemiology. Meanwhile, due to the frequent usage of vaccine, the immuno--activity of vaccine is decreasing. Therefore, it is critical to choose the correct type for vaccination in the certain regions. Our study indicates that the rabies virus of CZJF01 is most close to HEP-Flury, the animal vaccine strain. We recommend to use the vaccine based on the HEP-Flury for Zhejiang province.

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