## Letter to the Editor

# Epidemic of Rabies and Effect of Its Vaccine against a Dog That Consecutively Attacked Ten People in One Day*

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**On December 21, 2010, a stray dog consecutively attacked 10 people in Lengshui Village, Ningyuan County, Yongzhou City, Hunan Province, China. The dog was killed by the local CDC staff and vicinity villager, its brain tissue sample was taken within 24 h. The epidemic focus was disinfected and the injured people received post exposure prophylaxis (PEP). Pathogens were detected in the tissue sample by the provincial CDC. The immunity and safety of rabies vaccine were assayed after PEP, the injured people were regularly followed up in the following 2 y and 6 mon.**

As an epidemic viral disease in animals, rabies poses a potential threat to 3.3 billion people around the world. The rabies virus (RABV) circulating in dogs are responsible for over 99% of human cases[1-2]. China is one of the severely affected countries in the world[3]. Hunan Province, located in South-Central China, covers an area of 210 800 square kilometers with 70 million people including 53.5 million agricultural populations. It is the province with the most severe rabies epidemic in China, where 12 856 rabies cases were reported from 1979 to 2012[4], and the number of deaths of rabies decreased to 229 in 2008, 200 in 2009, 152 in 2010, 162 in 2011, and 118 in 2012 due to the effective control measures taken by the local government.

Rabies is also known as hydrophobia in Hunan Province where the local people are accustomed to feeding or eating dog meat, 70% of the households feed several dogs in their family with an approximately 3% vaccination rate, and the villagers are frequently assaulted by dogs[5], which often causes public panic and scare. This stray dog without owners suddenly attacked 10 people in succession from 7:00 am of December 21 to 3:30 am of December 22, 2010. The average age of the 10 people was 35 y (9-66 y), including 5 students and 5 peasants. Of them, 3 had multiple transdermal bites or scratches with 14 category III exposure sites. The accident was defined as a severe public health event by the local government.

The brain, hippocampus, brainstem and corebellar tissue samples were taken from the dog on December 22, 2010, and tested using the rabies DFA reagent (Millipore, USA). Total RNA was extracted from the brain tissue sample by Trizol (Invitrogen, USA) and specific primers were designed for oligonucleotides according to the nucleoprotein (N) and glycoprotein (G) sequences of PV strain (No.M13215) and other domestic prevalent strains in GenBank by Primer Premier 5.0. Both N and G genes were amplified by RT-PCR consisting of 45 min at 42 °C for denaturation, 3 min at 95 °C for heating, followed by 30 cycles, each consisting of 25 s at 95 °C for denaturation, 30 s at 45 °C for anneal and 1 min at 72 °C for extension, and 5 min at 72 °C for a final extension.

The amplified products of N and G genes were purified with the min elute PCR purification kit (Qiagene, Germany), cloned into the pGM-T vector and transformed to *E.coli* TOP10 competent cells. The recombinant plasmids pGM-T/N and pGM-T/G were further identified by white-blue plaque selection and sequenced with the BigDye terminator (3.1/1.1 version) sequencing kit according to its manufacturer's instructions. Data from raw sequencing were edited and named by Chromas software (version 2.24), the final sequences of N and G genes were aligned to the PV, 3aG, HEP-Flury, and CTN-1 strain using Clustal W (version 1.83 package) and DNAStar software (Lasergene®v8.0), the

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alignments were used to build a neighbor-joining phylogenetic tree according to the Kimura 2-parameter evolutionary model and 500 bootstrap repetitions for statistical support with Mega5.0.4.

The serum was divided into two groups according to the age and sex. The immunity effect was analyzed and the neutralizing antibody titers were tested in the 10 persons by RFFIT.

The people potentially exposed to RABV were identified by epidemiological investigation. The wounds in 10 people with category III exposures were immunized at the local health clinic. According to the Essen-5 dose regimen, 0.5 mL vaccine (speeda, lot 201004047, Liaoning Cheng Da Co., Ltd., Shengyang, China) was injected into their upper arm (deltoid region) on days 0, 3, 7, 14, and 28 after attack. Various human rabies immunoglobulin doses were injected on 0 day after attack according to the injured people weights, and all procedures were conducted according to The National Guideline for PEP issued by Ministry of Health, PRC.

RABV belongs to the lyssavirus genus of rhabdoviridae family, and has a single stranded-negative sense RNA genome, encoding nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and a viral RNA polymerase (L)[6]. The N gene is widely used for its diagnosis, genetic typing, and evolutionary studies[7]. DFA is recommended by WHO as the gold standard for rabies diagnosis. In our test, the brain tissue sample taken from the dog was stained with anti-rabies conjugate and scanned for fluorescing inclusions at a magnification of approximately 40×. RABV in the brain, hippocampus, brainstem and cerebellar tissue samples produced intracytoplasmic inclusions in various sizes and shapes, showing a glaring and apple green brilliance. The uninfected brain tissue sample was orange red without any specific fluorescence particle (Figure 1). Agrose gel electrophoresis showed that the RT-PCR products of N and G genes were 1 529 bp and 1 642 bp long, respectively, with GoldView staining.

PEP on almost all individuals can induce adequate antibody responses even in a high risk or multiple exposure, and effectively prevent the infection with rabies. The wounds in the 10 sufferers were treated with immunoglobulin and vaccine immunization within 0.5-12 h at the local village medical clinic and followed up by visit and telephone after vaccination. No obvious adverse events occurred except for minor and transient erythema, pruritus at the site of injection, indicating that the vaccine is safe and well tolerated.

Nucleoprotein plays a critical role in RABV replication and transcription. The amino acid homology was 86.4%-92.7% when the N gene sequence of 2010K95 was compared with that of PV, CSV, 3aG, CTN-1, and HEP-Flury strains (Table 1), indicating that most nucleotide variations in N gene coding sequence are synonymous mutations. The primary structure of nucleoprotein has no critical variation, and the current rabies vaccine can effectively protect persons against rabies. Glycoprotein carries the major antigen sites and is related to the virus pathogenicity and neurotropism. It is the unique protein for virus stimulating the host to produce neutralizing antibody[8]. G gene amino acid analysis showed 83%-91.6% homology compared

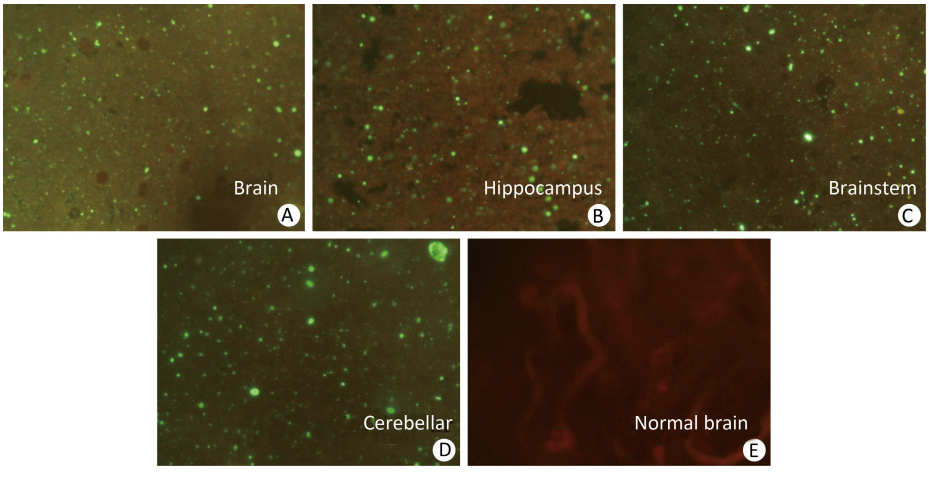


**Figure 1.** Direct fluorescent antibody assay (DFA) showing rabies in the dog brain tissue sample (A), hippocampus tissue sample (B), brainstem tissue sample (C), cerebellar tissue sample (D), and normal brain tissue sample (E).

**Table 1**. Homologous Partial Nucleotide Sequence in Hunan2010K95 N and G Genes to That in Other Vaccine Strains

| Seq-> | N Genes | | | | | G Genes | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | PV | 3aG | HEP-Flury | CTN-1 | Hunan2010K95 | PV | 3aG | HEP-Flury | CTN-1 | Hunan2010K95 |
| PV | ID | 92.1 | 92.7 | 87.9 | 87.2 | ID | 91.6 | 90.8 | 84.6 | 83.3 |
| 3aG | 92.1 | ID | 91.7 | 87.2 | 86.4 | 91.6 | ID | 89.8 | 84.5 | 83.0 |
| HEP -Flury | 92.7 | 91.7 | ID | 88.5 | 88.3 | 90.8 | 89.8 | ID | 84.8 | 83.8 |
| CTN-1 | 87.9 | 87.2 | 88.5 | ID | 89.8 | 84.6 | 84.5 | 84.8 | ID | 87.6 |
| Hunan2010K95 | 87.2 | 86.4 | 88.3 | 89.8 | ID | 83.3 | 83 | 83.8 | 87.6 | ID |

***Note.*** PV strain: M13215, France, Vaccine strain, Year of 1882. 3aG strain: AF155039 and L04522, China, Vaccine strain, Year of 1931. HEP-Flury strain: AB085828, Japan, Vaccine strain, Year of 2003. CTN-1 strain: FJ959397, China, Vaccine strain, Year of 1956.

with that of PV, CSV, 3aG, CTN-1, and HEP-Flury vaccine strains (Table 1), which is relatively lower than that of N gene, suggesting that variation of 2010K95 G gene is larger than that of N gene.

Forty-five strains isolated from Asia, Africa, Europe and America were clustered into group I and group II. Strians in group I could be further divided into 5 genotypes: G1 (Rabies virus, RABV), G4 (Duvenhage virus, DUVV), G5 (Europe bat lyssavirus, type 1, EBLV-1), G6 (Europe bat lyssavirus, type 2, EBLV-2), and G7 (Australia bat lyssavirus, ABLV), while those in group II contain 9 sequences of rabies genotypes G2 (Lagos bat virus, LBV) and G3 (Mokola virus, MOKV). Hunan2010K95 located in G1 group showed a high nucleotide similarity to Hunan0806 strain (GenBank No. HM756692) isolated from a confirmed rabies human at Yongzhou in 2008 and HuNPN01 strain (GenBank No. DQ496219) isolated from a pig in Xiangxi autonomous prefecture in 2006[9]. It was also found that Hunan2010K95 is closely related with Yunnan strain Zt07, Guizhou strain A148 and Guangxi strain GX074. Yunnan Province, Guizhou Province and Guangxi Zhuang Autonomous Region are adjacent to Hunan Province (Figure 2). Phylogenetic tree based on G segment was similar to that based on N segment, and was equally clustered into groups I and II. Group I contained 29 sequences, including G1 (RABV), G4 (DUVV), G5 (EBLV-1), G6 (EBLV-2), and G7 (ABLV). All isolated China strains and Hunan2010K95 were clustered into G1 group, and group II contained 17 sequences, including G2 (LBV) and G3 (MOKV) (Figure 3). The higher nucleotide similarity of N and G genes to Guangxi strain GXBM, Yunan strain Qj07 and Guizhou strain Qx1 revealed a certain territoriality of the viruses. The China vaccine strains CTN-1 and PG, France vaccine strains PM and PV and Japan vaccine strain HEP-Flury were clustered into G1 group and clearly segregated from the other Lyssavirus genotypes (G2-G7).

According to the requirements of WHO and OIE, the neutralizing antibody should be detected in persons or animals after immunization, and the minimum effective protect titer is 0.5 IU/mL serum. Otherwise, it needs an additional dose of the vaccine. RFFIT is recommended by WHO for detecting serum neutralizing antibody. Theoretically, most individuals can achieve a prompt and high neutralizing antibody response to the vaccine regardless of age or injection of rabies immune globulin[10]. In order to confirm the persistent protective effect of vaccination, the anti-rabies neutralizing antibody titer in serum sample from the 10 subjects was measured 2 y after exposure, showing that the neutralizing antibody titer is 13.91 IU/mL-35.65 IU/mL, significantly higher than the lowest protection titer of 0.5 IU/mL. No significant difference was found in the geometric mean titer (GMT) in the 10 sufferers ($P$>0.05).

Rabies is not considered as a priority disease in most countries, especially in Asian countries, and is defined as a neglected zoonotic disease by FAO, OIE, and WHO. The only and most cost-effective way for its control and elimination is the widespread dog vaccination programs. However, the mortality of rabies patients is still high at present, which may be related with the local economy, the too low immunization rate in dogs, and the delayed or none regular wound treatment or vaccination after exposure. In addition, it also depends on many respects if we want to protect people and animals against rabies. Any human potential exposure should be investigated to indicate PEP, ensure the reliable diagnostic procedure and improve the cooperation with public departments involved in rabies control.

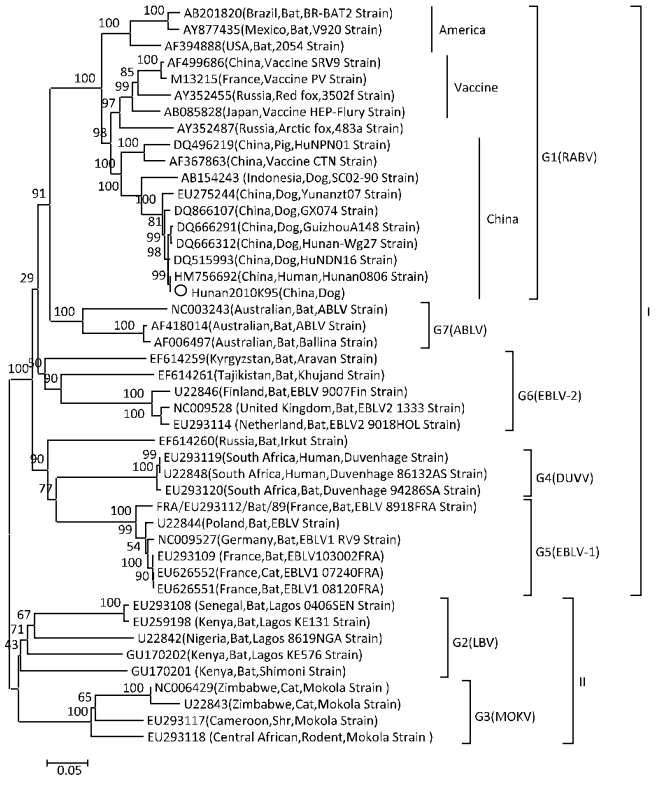


**Figure 2**. Evolutionary taxa of rabies N gene (55 nt-1584 nt).

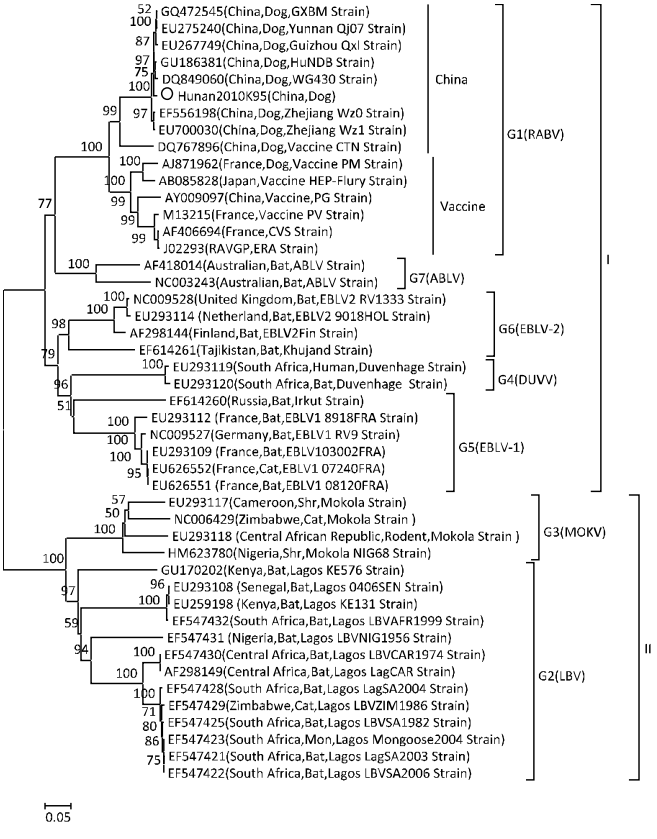


**Figure 3.** Evolutionary taxa of rabies G gene (3894 nt-5536 nt).

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