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Detection and Genetic Characterization of Rabies Virus from Human Patients*

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Abstract: Saliva and blood were collected from two patients who had not received post exposure prophylaxis in the cities of Wenzhou and Xinning respectively. Both patients were confirmed as positive for rabies by detection of rabies virus specific nucleoprotein antibodies in the sera by Western Blot. However, rabies virus specific RNA was only identified in the saliva collected from the patient in Wenzhou. Furthermore, the isolate Zhejiang Wz0 (H) was obtained by inoculating one-day-old suckling mice. Both nucleoprotein (N) and glycoprotein (G) genes from the isolate were amplified by RT-PCR and sequenced. Phylogenetic analysis indicated that the isolate belonged to classic rabies virus, and shared a higher homology with the street viruses from dogs in the main endemic areas in China and the street virus from dogs in Indonesia than with other known strains. Further comparison of the deduced amino acid sequences between the isolate and the vaccine strains used in China showed that the virus had a higher level of homology with the vaccine strain CTN than with the other vaccine strains (3aG, PV, PM and ERA). In particular, amino acid residues substitutions located in antigenic site Ⅲ in the G protein, which could react with the neutralizing antibodies, were observed. These results suggested that the virus belonged to the classic rabies virus, and both N and G genes diverged from the current vaccine strains used in China at either the nucleotide or the amino acid level.

Key words: Rabies Virus; Nucleoprotein Gene; Glycoprotein Gene; Phylogenetic Analysis

Rabies is one of the most important and widespread zoonotic diseases. The disease is caused by the rabies virus in the genus *lyssavirus*, family *Rhabdoviridae*. Rabies virus possesses a single-stranded, negative-sense RNA genome consisting of five genes in the order 3' N-P-M-G-L 5', which encode nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA-dependent RNA polymerase (L) respectively (19, 22). The virus is transmitted usually through a bite of rabid animals. Exposure to the virus among

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animals and human will result in an almost invariably fatal encephalomyelitis (8). Although an effective vaccine is available, worldwide human deaths caused by canine rabies were estimated at 55,000 people each year with 56% of these deaths estimated to occur in Asia and 44% in Africa (21).

Rabies remains a serious public health problem in China as in many developing countries. During the past ten years, human rabies cases have been increasing gradually, from 159 in 1996 to 2,548 in 2005. Furthermore, the third rabies epidemic apparently has not reached its peak (26), and the number in 2006 increased by almost 30% when compared with that in 2005. Moreover, human rabies suddenly occurred in some areas in 2004, where no human rabies cases had been reported over the ten years, and the incidence rate reached up to 10.73 per 100,000 habitants (25, 28). In addition, the percentage of the cases with an incubation period of ≤20 days apparently increased in the endemic areas (27, 28). Although rabies has been prevalent in China, knowledge of molecular epidemiology and the genetic characteristics of the virus is scant (18, 26), and there are particularly few virus isolations from patients. In this study, we confirmed two patients as positive for rabies, and characterized the rabies virus derived from one patient.

MATERIALS AND METHODS

Patients, sample collection and virus isolation

The first patient was a young boy who lived in Wenzhou, Zhejiang province. He was bitten by a dog, and did not receive post exposure prophylaxis in 2004. The second one was a peasant who lived in Xinning, Hunan province. He was also bitten by a stray dog in 2004, and did not receive post exposure prophylaxis. According to the clinical signs, both patients were suspected as being positive for rabies in their local hospital.

Saliva and blood sample were collected from two patients by the local physicians in The Affiliated Hospital of Wenzhou Medical College and Xinning Center of Disease Control and Prevention respectively. Saliva samples were supplemented with 0.75% bovine serum albumin (BSA), penicillin (500U/mL) and streptomycin (2mg/mL), frozen immediately at -70℃, and transported to the laboratory for further analysis. One-day-old suckling mice were inoculated with the saliva by the intracerebral route and were observed for 30 days.

Detection of specific nucleoprotein antibody with Western Blot

The recombinant nucleoprotein of rabies virus was separated by SDS-PAGE. A stacking gel containing 5% polyacrylamide and a resolution gel containing 12% polyacrylamide were run in a vertical slab gel apparatus (Bio-Rad). Western blot was performed by using a semi-dry transfer cell (Bio-Rad). The blotted nitrocellulose sheet was quenched by incubation in blocking solution containing 5% non-fat dry milk. After washing twice in TBST (10 min per washing), the sera from the patients were added, and incubated at 37℃ for 2 h. After washing in TBST again, the strips were incubated at 37℃ for 1 h with affinity-purified peroxidase-conjugated goat polyclonal antibodies specific for human IgG (1:2,000) (Sigma Chemical Co. St. Louis, MO, US), and developed with 4-chloro-1-napthol (4CN) (Sigma Chemical Co. St. Louis, MO, US). The strips were dried and photographed.

RT-PCR, DNA cloning and sequencing

Total RNA was extracted from saliva or virus-infected mouse brain with Trizol reagent according to the manufacturer's instructions (In-vitrogen, Beijing). RNA was also extracted from the normal saliva or the uninfected mouse brains for use as negative controls. RNA was used to amplify N and G gene sequences by RT-PCR. cDNA from the N gene was obtained with primer RHN1 (12). The initial amplification of N gene was performed with primers RHN1 and RHN3 (12), the nested PCR with primers AraN-O1 (1) and 304 (10). The conditions of PCR reaction were as follows: incubated at 94℃ for 5 min, 94℃ for 1 min, 37℃ for 1 min, 72℃ for 3 min (with a 2s auto extension) for 30 cycles and a final extension at 72℃ for 10 min.

In order to amplify the G gene sequences, primer P3 (15) was used for cDNA synthesis of the G gene. Nested PCR for amplification of the G gene was performed with two pairs of primers P3 and P4 (15) as outer primers, and PIMC (17) and XCYW1 (5' TC (T/C) TGAATCTACCCTGTTGC 3') as inner primers, which was designed by the authors. The condition of PCR reactions was identical to those used for amplifying N gene sequences.

The final PCR products were purified by using the Agarose Gel DNA Purification Kit (TaKaRa Biotechnology Co., Ltd, Dalian) according to the manufacturer's instructions. Purified DNA fragments were cloned into pMD18-T vector provided by TaKaRa. The ligated products were transformed into JM109 competent cells (Promega, Beijing). DNA sequencing was performed with the ABI-PRISM Dye Termination Sequencing kit and an ABI 373-A genetic analyzer.

Phylogenetic analysis

Sequences were edited and aligned with the DNAStar software package (version5.01). The nucleotide and amino acid identities were calculated with the same software. The PHYLIP program package (3.65) was used to construct the phylogenetic trees by using the maximum likehood (ML) method with 1,000 replicates. The rabies virus sequences used in the comparisons were obtained from GenBank. The N and G gene sequences used in this study are listed in Table 1.

RESULTS

Detection and isolation of rabies virus

The two patients died at 17 days (in Wenzhou) and 20 days (in Xinning) after exhibiting the clinical signs respectively. Rabies virus specific RNA was identified in the saliva collected from the patient in Wenzhou, but not in the saliva from the patient in Xining. However, the sera from both patients reacted with the recombinant nucleoprotein and appeared a specific band with a molecular weight of 50 kDa that is consistent with the predicted molecular weight of the nucleoprotein (Fig.1).

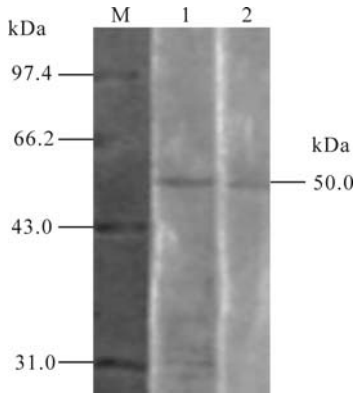

Fig. 1. Detection of anti-nucleoprotein antibody in the sera from the two patients by using Western Blot. M, Marker; 1, The sera from the patient in Wenzhou; 2, The sera from the patient in Xinning.

Table 1. Sequences of rabies virus strains used in this study

Strain	Location	Host	Accession No of N gene	Accession No of G gene
Zhejiang Wz0(H)	Zhejiang/China	Human	EF556197	EF556198
CTN	Shandong/China	Vaccine strain	AF367863	AY009100
3aG	Beijing/China	Vaccine strain	AF155039	L04522
PM	France	Vaccine strain	DQ099525	DQ099525
PV	France	Vaccine strain	M13215	M13215
ERA	United States	Vaccine strain	AF406695	AF406693
CVS	France	Laboratory strain	AF406696	AF406694
Guizhou Al0(H)	Guizhou/China	Human	DQ666288	-
Guizhou Qx2	Guizhou/China	Dog	DQ666295	-
Guizhou Al01	Guizhou/China	Dog	DQ666289	-
Guangxi Yl66	Guizhou/China	Dog	DQ666287	-
Jiangsu WX1	Jiangsu/China	Dog	DQ666321	-
Jiangsu Yc63	Jiangsu/China	Dog	DQ666322	-
Jiangsu Wx0(H)	Jiangsu/China	Human	DQ666320	-
Hunan Wg430	Hunan/China	Dog	DQ666315	-
Henan Sq10	Henan/China	Dog	DQ666300	-
CNX8511	Ningxia/China	Human	-	AY009099
CGX89-1	Guangxi/China	Dog	-	L04523
CHI1-BK	Henan/China	Buck	-	AF325471
JSS62	Jiangsu/China	Dog	-	DQ849065
CQ92	Chongqing/China	Dog	-	DQ849072
FEIDONG	Anhui/China	Dog	-	DQ849073
NC	Jiangxi/China	Dog	-	DQ849064
WG430	Hunan/China	Dog	-	DQ849060
QC	Hubei/China	Human	-	DQ849063
YUE1	Guangxi/China	Dog	-	DQ849070
MAL1-HM	Malashiya	Human	-	AF325487
HM88	Thailand	Human	AY219002	AY257982
SN01-23	Indonesia	Dog	AB154236	AB115921
Ballina	Australia	Pteropid Bat	AF006497	AF006497
Mokola	Eidolon helvum	Nigeria	NC006429	NC006429

To further characterize the virus from the patient in Wenzhou, the saliva was used to infect suckling mice in order to isolate the rabies virus. Inoculated mice developed clinical signs of rabies within 12 days after infection. The isolate was designated as Zhejiang Wz0 (H).

Genetic analysis of N gene

The complete N gene of the Zhejiang Wz0 (H) virus was amplified and sequenced (GenBank accession No EF556197)**.** The entire N gene had a total of length of 1425 nucleotides, including an open reading frame (ORF) encoding a nucleoprotein of 450 amino acids. Comparison of the Zhejiang Wz0(H) isolate with other known classic rabies viruses in Table 1, revealed that the isolate shared 86.2%-97.8% nucleotide identities and 94.0%-98.4% amino acid identities with classic rabies viruses respectively. Sequences analysis indicated that Zhejiang Wz0 (H) isolate was closely related with the street viruses (Guangxi Yl66, Guizhou Qx2, Hunan Wg430, Henan Sq10, and Jiangsu Wx1) from dogs in China (29), with 97.3%-97.8% identities at the nucleotide level and 97.8%-98.4% at the amino acid level respectively. However, lower nucleotide sequence identities were observed with other Chinese street viruses, such as: Guizhou AL01 and Jiangsu Yc63 (isolated from dogs), and Jiangsu Wx0(H) and Guizhou AL0(H) (isolated from human) (29), with 86.5%-88.8% nucleotide

identities and 95.6%-96.5% amino acid identities respectively. Comparison of the Zhejiang Wz0 (H) isolate with non-Chinese rabies viruses, indicated that the virus shared the closest relationship with the Indonesia street virus (92.0%-97.0%). Comparison of the Zhejiang Wz0 (H) isolate with current strains (CTN, 3aG, PV, PM and ERA) which are used for the production of human and veterinary vaccine in China, revealed that the strain had a relatively high level of homology with CTN, with 89.3% at the nucleotide level and 97.1% at the amino acid level respectively. However, the Zhejiang Wz0 (H) isolate shared only a low level of similarity to Ballina (77.3%/91.1%) and Mokola (72.1%/ 80.3%).

Comparison of the deduced amino acid sequences of Zhejiang Wz0 (H) with those of other street viruses and vaccine strains are listed in Table 1. 8 amino acid residues (S_{42}, K_{183}, Y_{236}, P_{251}, S_{275}, G_{347}, S_{360} and L_{379}) were substituted. In particular, the substitution (L_{379}) was located in antigenic site Ⅰ. Furthermore, 12 amino acid residues in the Zhejiang Wz0(H) amino acid sequences were different from those in the CTN sequence (K_9, S_{42}, T_{90}, E_{110}, N_{157}, K_{183}, Y_{237}, P_{250}, R_{254}, S_{275}, G_{247} and S_{360}), and more divergent than those of the other vaccine strains (3aG, PV, PM and ERA). It was noticeable that the substitution at position 360 was located in both antigenic site Ⅰ and antigenic site Ⅳ.

Genetic analysis of G gene

The entire G gene of the Zhejiang Wz0 (H) virus was amplified and sequenced as well (GenBank accession No EF556198)**.** The entire G gene had a total of 2067 nucleotides, including an ORF encoding a glycoprotein of 524 amino acids with the signal peptide of 19 amino acids. Comparison of the Zhejiang Wz0 (H) isolate with other known classic rabies viruses (Table 1), revealed that the identities for the G gene ranged from 83.0% to 99.4% nucleotide identities and from 88.1% to 99.4% amino acid identities respectively. Sequence comparison indicated that Zhejiang Wz0 (H) isolate have a higher homology with JSS62, NC, YUE1, FEIDONG, and WG430 (derived from dogs in Jiangsu, Jiangxi, Guangxi, Anhui and Hunan, respectively) and QC (derived from human in Hubei), shared 98.1%-99.4% nucleotide identities and 98.4%-99.4% amino acid identities with these strains. However, the isolate shared a relatively low level of homology with a Chinese isolate CHI1-BK (85.2% nucleotide identity and 92.9% amino acid identity). Compared Zhejiang Wz0 (H) with non-Chinese rabies viruses. Conversely, the virus had maximum homology with SN01-23 isolated from a dog in Indonesia (92.4%-96.0%). Similar to the genetic results obtained from the N gene, the isolate shared a higher level of homology with the vaccine strain CTN (87.5%-92.5%) than the other vaccine strains (3aG, PV, PM and ERA). The identities of nucleotide and amino acid sequences with other genotypes were less than 71.7% and 77.9%, respectively.

Genetic analysis of the deduced amino acid sequences showed that the following substitutions occurred in the Zhejiang Wz0 (H) isolate when compared with other classic rabies viruses (Table 1): M_{90}, A_{167}, C_{168}, G_{204}, I_{249}, S_{253}, T_{289}, S_{311}, I_{332}, H_{382}, I_{427}, T_{454}, P_{474}, H_{486} and K_{501}. In particular, the substitution (I_{332}) was located in antigenic site Ⅲ, which is critical for rabies virus pathogenicity and is responsible for induction and binding of rabies virus-neutralizing antibodies (2, 3, 13). Further analysis

showed that only two potential glycosylation sites (Asn-X-Ser/Thr) were found at position 37 and 319 amino acid residues, while a site was lost at position 247. Although the isolate had the highest level of homology with CTN, there were a total of 37 amino acid residues differences between the isolate and CTN, and these amino acid residues are K_1, F_2, P_3, I_4, K_{10}, M_{90}, A_{96}, L_{132}, G_{156}, C_{168}, G_{204}, D_{247}, I_{249}, S_{253}, K_{278}, T_{289}, K_{294}, S_{295}, H_{303}, S_{311}, I_{332}, R_{333}, N_{336}, G_{349}, H_{382}, A_{412}, I_{427}, Y_{440}, I_{443}, S_{444}, A_{447}, T_{454}, K_{463}, P_{474}, H_{486}, N_{487} and S_{501}, and more differences from other vaccine strains (3aG, PV, PM and ERA). Furthermore, the substitutions at position 332, 333 and 336 amino acid residues were located in the antigenic site Ⅲ, which could react with the neutralizing antibodies (3, 24).

Phylogenetic analysis

A phylogenetic analysis based on the entire N nucleotide sequences was shown in Fig.2. It indicates that the Zhejiang Wz0 (H) isolate belong to classic rabies virus and is closely related to the Chinese strains (Guizhou Qx2, Guangxi Yl66, Hunan Wg430, Jiangsu Wx1 and Henan Sq10) and the Indonesia strain (SN01-23). It is notable that the vaccine strain CTN is also located in this clade. However, the isolate was distantly related to other vaccine strains, such as: 3aG, PV, PM, and ERA, which formed a separate clade. The phylogenetic tree constructed with the entire G sequences displayed a similar topology to that constructed with the N gene nucleotide sequences (Fig. 3).

DISCUSSION

Rabies has been known in China for more than 2, 000 years, and was first described in about 556 BC (24). However, rabies is still prevalent at present in China and presents a major threat to the well being of the general population (29). Despite this, knowledge of the molecular epidemiology and genetic characteristics of the rabies virus is scant (18, 26). Particularly, rabies is still diagnosed only by clinical observation and epidemiological investigation, and no laboratory

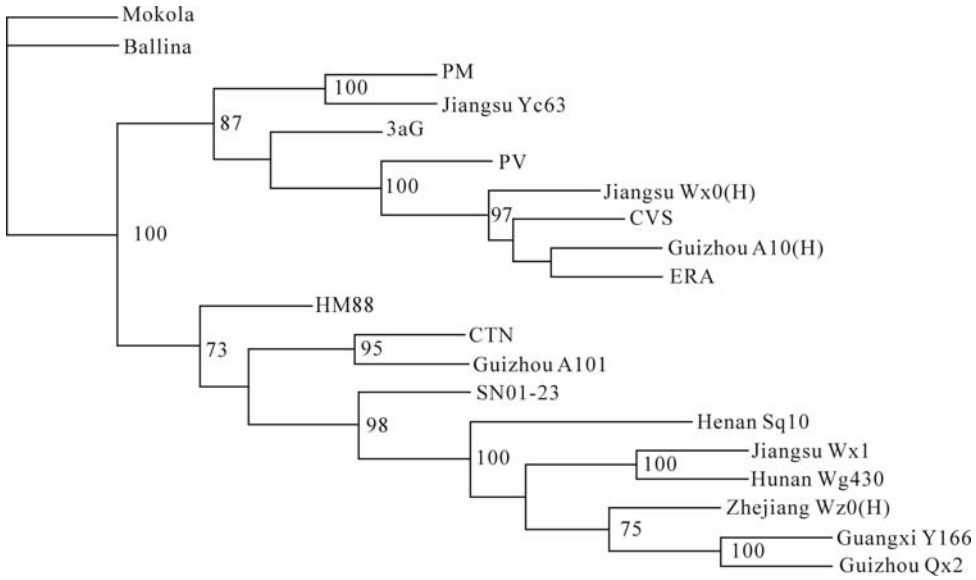

Fig.2. Phylogenetic tree based on the complete N gene nucleotide sequences of Zhejiang Wz0(H) isolate and previously published rabies viruses, produced by ML method by using PHYLIP program package (3.65). Bootstrap values for 1,000 replicates above 70% are shown at the branch nodes. The sequences used in this study were obtained from GenBank and their relative information was described Table 1.

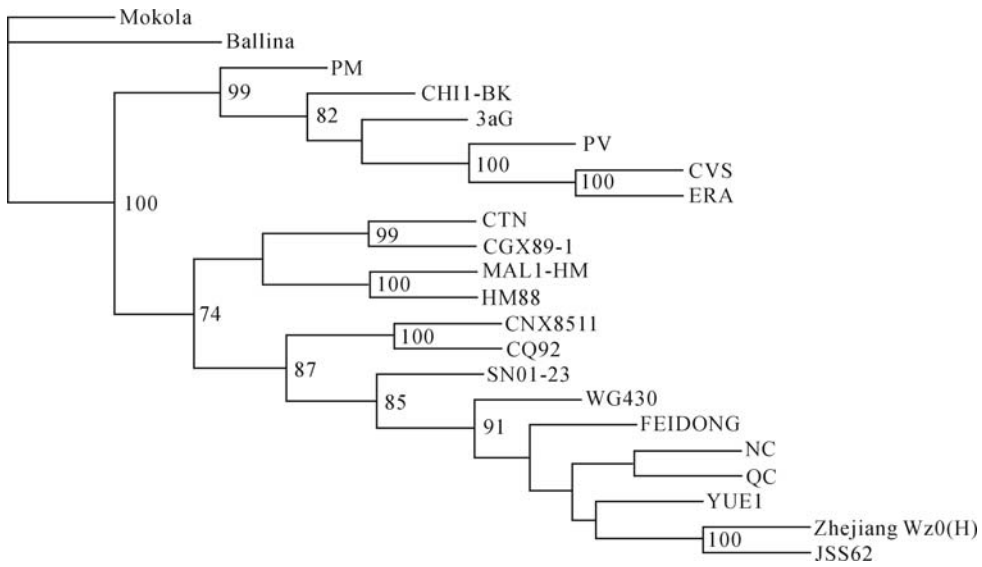

Fig.3. Phylogenetic tree based on the complete G gene nucleotide sequences of Zhejiang Wz0(H) isolate and previously published rabies viruses, produced by ML method by using PHYLIP program package (3.65). Bootstrap values for 1,000 replicates above 70% are shown at the branch nodes. The sequences used in this study were obtained from GenBank and their relative information was described Table 1.

diagnosis has been developed. In the present study, rabies virus specific RNA was only detected in the saliva from the patient in Wenzhou, but not from the patient in Xinning. Although rabies virus specific RNA was not detected in the sera collected from the patient in Xinning, the sera were positive for specific nucleoprotein antibody. Thus both cases were rabies. Other investigators have also reported that the rabies virus could be detected in the saliva from rabies patient (20). In this study, the reasons why virus RNA was not identified in the saliva of Xinning patient might be that the sample was processed improperly and virus RNA was degenerated. The results suggested that the real death number caused by rabies in China might be higher than that reported. Thus the laboratory diagnostic is necessary to be carried out in the endemic areas.

The rabies virus N gene has been used extensively for epidemiological and evolutionary studies (4, 5, 6, 7, 11, 16, 22, 23, 29). Our previous studies revealed that rabies viruses circulating in the dogs in the main endemic areas belonged to classic rabies virus (29). Furthermore, the viruses were divided into the two phylogenetic groups, with at least four sub-genotypes. In the present study, the isolate shared a higher homology with the viruses circulating in the dogs in the main endemic areas (Hunan, Henan, Jiangsu, Guangxi and Guizhou), and was grouped into the same clade with those viruses. Moreover, phylogenetic analysis of G gene sequences also displayed similar results to those for the N gene. Thus, the strain Zhejiang Wz0 (H) also belongs to the classic rabies virus. In addition, previous studies found that the viruses in the clade have a higher level of similarity with the rabies viruses isolated from Indonesia (27, 28, 29). The strain Zhejiang Wz0 (H) isolated from human was also closely related to the Indonesia virus in the present study. Together, these results suggested that the phylogenetic group of rabies viruses circulating in China and South-East Asia countries might have

originated from a common ancient ancestor.

In China, CTN, 3aG, PV and PM are the current strains used in the production of human rabies vaccines, and ERA is the strain used for veterinary vaccine production. It is very important for us to know the level of homology between the street strain and these vaccine strains. Genetic analysis of the nucleotide sequences of the N and G genes indicated that the Zhejiang Wz0 (H) isolate had a higher level of homology with CTN than the PV, PM, PV and ERA vaccine strains. In the phylogenetic trees based on both the N and G gene nucleotide sequences, the isolate formed a group with the rabies street viruses from the main endemic areas and the vaccine strain CTN, while the other vaccine strains (3aG, PV, PM and ERA) were located into another group. When the deduced amino acid sequences of the N and G genes between Zhejiang Wz0(H) and vaccine strains were analyzed, at least 12 amino acids were substituted in the nucleoprotein and 37 amino acids in the glycoprotein. It is well known that the glycoprotein is a major contributor in the neurotropism and the neuroinvasiveness of rabies virus, and plays a key role in the viral pathogenesis (9). Furthermore, the glycoprotein is a main target for virus-neutralizing antibodies (14). Thus, it is worthwhile to further evaluate whether the differences in the amino acid sequences of glycoprotein between the street viruses and vaccine strains influence the protection efficiency of the current vaccines in China.

In conclusion, The Zhejiang Wz0(H) isolate belonged to classic rabies virus and were closely related with the most isolates circulating in the dogs in the main endemic areas in China and in Indonesia. Comparing the strain with the current vaccine strains for vaccine production in China, the Zhejiang Wz0 (H) isolate shared a higher level of homology with CTN than the other vaccine strains. Furthermore, divergence between the street viruses and the vaccine strains at the amino acid level were observed. Thus it is important to investigate the protection efficiency of the vaccine against the street viruses circulating in China.

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