

Molecular-Biological Analysis of the First Imported Rabies Case in Taiwan

Abstract

Taiwan is free from rabies. No cases have been reported in the last 43 years. In late April 2002, a 45-year-old woman surnamed Lo of Hunan Province, Mainland China, was accidentally bitten at home by a domesticated dog. She came to Taiwan in May to visit relatives. On June 29, she was admitted to hospital for clinical symptoms such as difficulty swallowing, fear of wind (aerophobia), and numbness of the arms. The case was diagnosed as rabies. On July 1, saliva and CSF specimens, and on July 3 suctioned tracheal specimens of the woman, were sent to the Division of Research and Laboratory Testing of the Center for Disease Control for confirmation. A preliminary analysis by the RT-PCR method found the specimens positive. The findings further underwent sequencing analysis, and according to DNA sequencing they were confirmed rabies viruses. For reconfirmation, 433bp of the RT-PCR section was used for TA cloning by the pGMT-EASY method to further confirm that they were rabies viruses. The saliva, CSF and TS extracts of the woman were inoculated into suckling mice. All the mice died on the sixth day.

Introduction

Rabies was introduced for the first time from Shanghai to Taiwan in 1947. In 1948, the first case of rabies occurred in Taipei. More cases were reported subsequently, peaking at 238 and 102 cases in 1951 and 1952 respectively. Inoculation of domestic dogs against rabies, elimination of wild dogs, and quarantine of dogs imported from epidemic areas began in 1957. No human rabies cases since 1959 and no animal rabies cases since 1961 had been reported. Taiwan is one of a few countries in the world to be free of rabies.

Rabies virus is of the genus *Lyssavirus*. All members of the genus are antigenically related, but use of molecular antibodies and nucleotide sequencing of the virus demonstrates differences according to the affected animal species or the geographic location from which they originate⁽¹⁾.

Urban rabies infection is spread primarily by dogs and cats; wildlife rabies most commonly involves spread by wild *Canidae* and bats, and occasionally dogs, cats and livestock. Domestic livestock such as cows, sheep and pigs become infected by a bite or scratch of infected animals, and under herding conditions, they can transmit the infection to other animals or to man by biting. Virus-laden saliva of a rabid animal is introduced by a bite or scratch (or, very rarely, into a fresh break in the skin or through intact mucous membranes). Rabies is an acute viral encephalitis. Onset begins with a sense of apprehension, fever, headache, malaise, loss of appetite, and vomiting, sensory changes often around the site of the wound. The disease progresses to paralysis, or spasm of swallowing muscles leading to fear of water (hydrophobia); delirium and convulsions follow; agitation and aerophobia are also frequent symptoms. Without medical intervention, death is often due to

respiratory paralysis. The infection is worldwide, with a World Health Organization estimate of 35,000-50,000 deaths a year, almost all occurring in developing countries. In Taiwan, no human rabies since 1959, and no animal rabies since 1961 have been reported. The areas currently free of rabies in the animal population include Australia, New Zealand, New Guinea, Japan, Hawaii, Taiwan, Oceania, the UK, Ireland, Iceland, mainland Norway, Sweden, Finland, Portugal, Greece and some of the West Indies and Atlantic islands⁽¹⁰⁾.

Materials and Method

Symptoms of patient and sources of specimens

A 45-year-old woman surnamed Lo of Hunan Province, Mainland China, was bitten by a dog that was a house pet in late April 2002. She came to Taiwan on 22 May to visit relatives. She was admitted to hospital for difficulty swallowing (dysphagia), fear of wind (aerophobia), numbness of arms, and apprehension. Specimens of saliva, CSF and TS were collected from the woman for testing. The woman died on 10 July.

Extraction of RNA

Purification of RNA was made using the QIAmp Viral RNA kit of the QIAGEN company⁽¹²⁾. Specimens of 140 µl each of saliva, CSF and TS were collected from the patient; 560 µl Buffer AVL were added at room temperature for 10 minutes; 560 µl of vortexing was again added. The fluid was put through the QIAmp spin column. The column was washed with Buffer AW twice to dissolve RNA at 80 °C pure water. RNA thus extracted could be used for the reverse transcription polymerase chain reaction (RT-PCR)⁽³⁾.

RT-PCR

I 、Primer: 1-Rabies-F': 5'-CTACAATGGATGVVVGAC-3'

1- Rabies-R': 5'-TTGACGAAGATCTTGATTAT-3'

2- Rabies-F': 5'-TTTGAGACTGCTCCTTTT-3'

3- Rabies-R': 5'-CCCATATAGCATCCTAC-3'

II 、Reverse transcription reaction

10 μ l of the virus RNA was added to 75 mM KCl, 50 mM Tris-HCl, 3 mM MgCl₂, 10 mM DTT, 0.5 mM ATCG mixture, 38 U/ μ l RNasin, and antisense primer: R-RT-probe 50 pmole at 70 °C for 10 minutes; and n 100 units of MMLV-reverse transcriptase, further added at 37 °C for 90 minutes.

III 、PCR

(I) The first round PCR

The cDNA obtained in the reverse transcription reaction was used for PCR. To the cDNA 50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl₂, 0.1% Triton-X 100, 1 mM ATCG dNTP mixture, and 50 pmole each of primer was added: 1-Rabies-F/1-Rabies-R, and 5 units of Taq polymerase (Promega) at 94 °C denature for 3 minutes, and 35 times of reaction at 94 °C for 1 minute, primer matching temperature (T_m differs from each primer) for 1 minute, 72 °C for 2 minutes, and finally at 72 °C for 15 minutes.

(II) The second round PCR

To 5 μ l of the first round PCR product was added 50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl₂, 0.1% Triton-X 100, 1 mM ATCG dNTP mixture, and 50 pmole each of primer:2-Rabies-F/2-Rabies-R; added 5 units of Taq

polymerase (Promega) at 94 °C denature for 3 minutes; and 35 times of reaction at 94 °C for 1 minute, primer matching temperature (T_m differs from primer) for 1 minute, at 72 °C for 2 minutes, and finally at 72 °C for 15 minutes.

Analysis of the phylogenecity tree

The positive control (extract of CVS strain inoculated into suckling mouse brain), CNT-china strain, BRdg12 strain, BRhm6 strain, India, USA2054Colorado, USA-California, and Peru-bat strain and sequencing of saliva, CSF and TS of the patient were used for phylogenecity tree analysis by Molecular Evolutionary Genetics Analysis (MEGA) version 2.1. The analysis used the “Neighbor-joining” method and was calculated 1,000 times for bootstrap.

Results

Nest-RT-PCR

10 µl of the extract virus RNA was made RT-PCR reaction with primer: 1-Rabies-F/1-Rabies-R. After 35 times, 1468 bp of DNA section was obtained. Primer: 2-Rabies-F/2-Rabies-R was then used for the second round PCR. After 35 times, 433 bp of DNA section was obtained. It was found that the saliva, CSF and TS specimens showed 433 bp sections; the positive control was an extract of the CVS strain inoculated into suckling mouse brain. Result of the analysis is shown in Figure 1.

Sequencing analysis

MegAlign soft was used for the RT-PCR analysis of the 433 bp sections of the sequenced specimens. It was found that the rabies virus sequence of the CSF of the patient was 99.7% similar to the nucleic acid of the AVO1 vaccine strain; and the virus sequences of the saliva and TS of the patient were 99.9% similar, and 89% similar in their nucleic acids. By

similarity of amino acid, it was almost 100% similar, indicating that it was a synonymous mutation and that it would not affect the mutation of amino acid. The amino acid of the rabies virus sequence of the CSF of the patient was only 95.2% similar to that of other virus strains (see Table 1).

Analysis of the phylogenecity tree

By MEGA 2.1 soft and the “Neighbor-joining” method⁽⁴⁾, it was found that the rabies virus sequences of the saliva and CSF specimens of the patient clustered together on the same branch with the CTN strain of Mainland China. The sequence of the positive control and the rabies virus sequence of the CSF of the patient, though on the same branch, were slightly different, and were clustered with other vaccine strains. The two clusters, however, were quite different. It was speculated that the viruses of the saliva and TS extract of the patient came from different sources than the virus of the CSF. Virus of the CSF could have come from vaccine strains but were not entirely the same^(8,9). Of them, BRdg and BRhm are the Brazilian rabies virus sequence; South Africa is the South African sequence; India, the Indian sequence; USA2054Colorado, USA-California are the US sequence; and Peru-bat, the Peru sequence (see Figure 2).

Discussion

Molecular biological technology improves significantly the sensitivity of laboratory testing and analysis. Clinical laboratory testing often uses serum antibody as the reagent. Testing is not sensitive during the early stage of infection when symptoms have not yet appeared, and treatment is thus delayed. On the second day after specimens of the patient in question were sent to the Division, by RT-PCR method, the 433 bp section of the DNA multiplied by the primers were noted, and it was suspected that the

woman was a rabies positive patient. For confirmation, sequencing analysis was performed on that section. On the third day when the sequenced finding was compared with the gene data bank, the case was confirmed rabies. At the same time, specimens were inoculated by the conventional method into suckling mice for virus culture.

The physician of the Tzuchi Hospital caring for the patient suspected rabies infection from the symptomatology and administered rabies vaccine. An unusual finding of the present analysis was that the virus sequences in the saliva, CSF and TS of the patient were significantly different. Generally, a person would not be infected by two types of rabies viruses at the same time. The similarity of the nucleic acid in vaccines T and vaccine R used on the patient and the CSF of the patient was as high as 98.8%. It was then speculated that the vaccine-like rabies virus sequence isolated from the CSF of the patient was associated with the vaccines. From analysis of the phylogenecity tree, the rabies virus sequences of the saliva and TS extracts were in the same group as the CTN-china strain. It could therefore be speculated that the rabies viruses in the saliva and TS of the woman came from the infection by the dog bite at home ^(2,5,6). Clinically, human rabies appears in five stages: the first stage, the incubation period; the second stage, the prodrome phase; the third stage, the acute neurological phase; the fourth stage, the coma phase; and the fifth stage, possible recovery or death. In the later stages, the saliva of patients will normally contain rabies viruses from bites. It was therefore concluded that the rabies viruses in the saliva and TS of the woman came from the infection of the dog bite at home.

The NEST-RT-PCR method used in the present analysis improved significantly the sensitivity. It can be used with small amounts of specimens, or products of weak RT-PCR signals. One must, however, be

very cautious in using the RT-PCR technology^(3,7). There should be both positive and negative controls. The positive control can tell whether the procedures are correct, and whether enzymes and primers needed are added. The negative control can tell whether the procedures, utensils or primers are contaminated. Contamination is a taboo to PCR.

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Figure 1 Findings of RT-PCR and Positive Controls ; M: marker 100bp ladder; lane 1 is CSF, lane 2 is saliva, lane 3 is TS specimens; P: the positive control, and N: the negative control.

M 1 2 3 P N

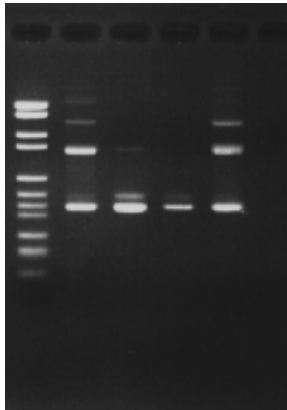


Figure 2 Analysis of the Phylogenecity Tree by MEGA2.1 using the “Neighbor-joining” Method

