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Molecular characterization of rabies virus isolates in China during 2004

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Abstract

Human rabies cases have been on the rise during the past few years in China and a total of 2651 cases were reported in 2004. To better understand the current rabies epidemics in China, we isolated rabies viruses from dogs and humans from five provinces and characterized these isolates genetically by sequencing the entire nucleoprotein (N) gene. Comparison of the N genes among these isolates revealed 86.6–99.9% homology and these viruses can be grouped into three lineages. Phylogenetic analysis indicates that all the Chinese isolates have a close relationship with viruses circulating in Asian canine population. When compared with rabies viruses isolated previously, the three lineages were similar to three of the four groups. Thus, our data suggest that rabies viruses currently circulating in China were similar, if not identical, to those reported in the previous epidemics.

Keywords: Rabies; Molecular epidemiology

1. Introduction

Rabies is one of the ancient zoonotic diseases with an almost invariably fatal encephalomyelitis (Dietzschold et al., 1996). Rabies virus (RV) is transmitted usually through a bite of rabid animals. Although human rabies is rare in Europe and North America due to massive vaccination of domestic animals and the availability of post-exposure prophylaxis for humans, rabies remains a neglected disease and a serious public health threat in many developing countries (Cleaveland et al., 2002; Rutebarika et al., 2000; WHO, 1998, 2002). It has been reported that rabies causes about 55,000 human deaths annually throughout the world, and occurs primarily in Asia and Africa because animal control, vaccination programs and effective human post-exposure prophylaxis are either not widely available, or not effectively applied (Knobel et al., 2005; WHO, in press).

Rabies has been known in China for more than 2000 years (Wang and Huang, 2001). There have been three major rabies epidemics in China since the 1950s (Zhang et al., 2005). One of the major epidemics occurred during the 1980s with 5000–7000 cases per year (Zhang et al., 2005, 2003). Due to dog population control and the availability of rabies post-exposure prophylaxis in human, rabies cases were reduced dramatically during the decade of the 1990s with only 159 cases reported in 1996 (Kureishi et al., 1992; Lin, 1990; Lin et al., 1986, 1988; Lin and Lina, 2000; Tang et al., 2001; Yu, 2002; Zhang et al., 2005). Unfortunately, this decline reversed its course during the turn of the new century. The number of human rabies cases increased consecutively during the past 5 years with 2651 cases reported in 2004 (Zhang et al., 2005). The peak of the current epidemic is yet to be reached.

RV is the prototype of the *Lyssavirus* genus within the rhabdoviridae family (de Mattos et al., 2001; Warrell and Warrell, 2004). It contains a single-stranded and negative-sense genomic RNA encoding five structural proteins, i.e., nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and

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RNA-dependent RNA polymerase (L) (Tordo and Kouknetzoff, 1993; Wunner et al., 1988). RV N gene has been used extensively for epidemiological and evolutionary studies (Bourhy et al., 1993, 1999; Cisterna et al., 2005; David et al., 2000; Hyun et al., 2005; Smith et al., 1992; Wunner et al., 1988; Xu et al., 2002). To investigate factors contributing to the current rabies epidemic in China, we collected specimens from dogs and humans during 2004 and characterized the Chinese isolates by sequencing the entire N gene and comparing the N sequence from the current epidemic with that of RV isolates from previous epidemics. It was found that these viruses can be grouped into three lineages, all of which have been reported previously in China.

2. Materials and methods

2.1. Epidemiological and clinical rabies history

The epidemiological data for human rabies cases were obtained from the surveillance database of reportable diseases of China Center for Disease Control. The rabies diagnosis in humans reported to the national surveillance data bank was based on clinical criteria including history of animal bite(s), intense anxiety, nervousness, paresthesia in the area of the bite, hydrophobia, and finally death. The history of animal bite was confirmed by subsequent case epidemiological surveys from various county CDC offices.

2.2. Sample collection

Samples were collected from dogs in five Chinese provinces with high incidence of human rabies. Dogs were trapped and humanely sacrificed. Brains were removed, frozen immediately in liquid nitrogen, and transported to the laboratory for further analysis. Salivary samples were also collected from two human patients, one from Guizhou province and the other from Jiansu province.

2.3. RV antigen detection and virus isolation

RV antigens in brain smears were detected by using direct immunofluorescence assay (IFA) with the fluorescence labeled anti-RV N monoclonal antibodies (Fujirab, Marlvin, PA). For RV isolation, a 20% brain suspension was made by homogenizing brain tissue in PBS supplemented with 0.75% BSA, penicillin (500 U/ml) and streptomycin (2 mg/ml). One-day-old suckling mice were inoculated by the intracerebral route and were observed for 30 days for development of rabies. Brains were removed from mice that succumbed to rabies.

2.4. RT-PCR, DNA cloning and sequencing

RNA was extracted from positive dog brain samples as detected by direct IFA as well as from human saliva samples with TRIzol reagent according to the manufacturer's instructions (Gibco BRL). RNA was used to amplify RV N sequences by RT-PCR as described previously (Bourhy et al., 1999; Kamolvarin et al., 1993). Nested RT-PCR was performed by using RV N-specific primers. Primers RHN1 (nucleotides 28–52) and N8 (nucleotides 1584–1568) (Bourhy et al., 1999; Ito et al., 2001) were used for first amplification and primers N7 (nucleotides 58–73) (Bourhy et al., 1999) and 304 (nucleotides 1539–1516) (David et al., 2000) were used for second amplification. Final products cover the entire N-coding sequence. RT-PCR was performed using the following conditions: 94 °C for 5 min and then 10 cycles of 94 °C for 1 min, 37 °C for 1 min, 72 °C for 3 min and 20 cycles of 94 °C for 1 min, 52 °C for 1 min, 72 °C for 3 min, and subsequently at 72 °C for 10 min. The second amplification was carried out as follows: 94 °C for 5 min and then 20 cycles of 94 °C for 1 min, 52 °C for 1 min, 72 °C for 1.5 min, and subsequently at 72 °C for 10 min.

The final PCR products were separated by gel electrophoresis and purified from gel slices by using the TaKaRa Agarose Gel DNA Purification Kit (TaKaRa Biotechnology Co., Ltd., Dalian) according to the manufacturer's specifications. Purified DNA fragments were cloned into pMD18-T vector provided by TaKaRa. The ligated products were transformed into JM109 competent cells. DNA sequencing was performed with the ABI-PRISM Dye Termination Sequencing kit and an ABI 373-A genetic analyzer. The nucleotide sequence from each isolate was determined from at least two DNA clones.

2.5. Phylogenetic analysis

MEGA3 (3.1) program package was used to construct the phylogenetic trees by using the neighbor-joining (NJ) method with 1000 bootstrap replicates. The nucleotide and amino acid identities were calculated by using DNAStar (Version 5.01). RV N sequences from previous epidemics (Xu et al., 2002) were used for comparison between the current and previous epidemics in China. For comparison between Chinese isolates and RV isolates from all around the world, we used the available full-length N sequences obtained from GenBank (Table 1).

3. Results

3.1. Human rabies cases in China in 2004

During 2004, a total of 2561 human rabies cases were reported to the China Center for Disease Control (Zhang et al., 2005). This represents a 25.7% increase over the number of cases (2037) reported in 2003 (Zhang et al., 2005). The distribution of the human cases from 2004 among different provinces is presented in Fig. 1. Most of the human cases occurred in the South and Southeast. The highest number of cases was reported in Guangxi (601 cases), followed by Hunan (523 cases). There were fewer cases in the North, Northeast, and the West. There was no case reported in Helongjiang, Xinjiang, Xizang, Qinghai, Gansu, Ningxia, Beijing, and Tianjing.

3.2. Virus antigen detection, viral RNA amplification, and virus isolation

To characterize RV during the current rabies epidemics, dog brain samples as well as human salivary samples were collected from five provinces with high incidence. Table 2 summarizes the results of virus antigen detection, viral RNA