Short Communication

Sequence and Phylogenetic Analysis of the Nucleoprotein (N) Gene in Measles Viruses Prevalent in Gunma, Japan, in 2007

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(Received June 15, 2007. Accepted August 31, 2007)

SUMMARY: In 2007, relatively large outbreaks of measles occurred in the Kanto region of Japan, including Gunma Prefecture. We performed sequence and phylogenetic analysis of the nucleoprotein gene (N gene) of measles viruses from 3 measles patients in this area in May 2007. The N gene sequences of the present strains were identical to each other, and phylogenetic analysis showed these viruses were classified into genotype D5. The results suggest that highly homologous measles viruses may be associated with outbreaks of measles in Gunma, Japan.

The measles virus (MV), genus Morbillivirus, family Paramyxoviridae, causes acute and highly contagious measles infections in humans (1,2). Measles presents as an unpleasant mild to moderately severe illness, and the most serious complications include blindness, encephalitis (a dangerous infection of the brain causing inflammation), severe diarrhea (possibly leading to dehydration), ear infections, and severe respiratory infections such as pneumonia, the most common cause of death associated with measles. Encephalitis is estimated to occur in 1/1,000 cases, while otitis media (middle ear infection) reportedly occurs in 5 - 15% of cases and pneumonia in 5 -10%. The case fatality rate in developing countries is generally in the range of 1 to 5% but may be as high as 25% in populations with high levels of malnutrition and poor access to health care (http://www.who.int/mediacentre/factsheets/fs286/en/print.html).

The measles vaccine was introduced in the 1978 as a regular vaccination in Japan. According to the World Health Organization’s field guidelines for measles elimination (http://www.wpro.who.int/publications/pub_929061126x.htm), over 95% population immunity is needed to interrupt transmission and hence eliminate measles. However, the actual vaccination rate in Japan is thought to be about 70 to 90%, and thus, relatively large outbreaks of measles still occur every 5 to 7 years (3,4).

Between March and June 2007, outbreaks of measles occurred in the Kanto region of Japan, which includes Tokyo, Saitama, Chiba, Kanagawa, Ibaragi, Tochigi, and Gunma Prefectures. The MV prevalent in 2007 in Gunma, Japan, was characterized using the nucleoprotein (N) gene. The findings are presented herein.

In Gunma Prefecture, which has a population of approximately 2 million, 131 cases were reported during the period from January to May 2007, compared to only 6 cases in 2006, representing a dramatic increase in the incidence of measles in this area (Fig. 1A). Most patients were teenagers aged 15 to 19 years (22%) and young adults in their twenties (27%) (Fig. 1B). In May 2007, throat swab samples were collected from 3 measles patients showing typical clinical symptoms such as a high fever, cough, conjunctivitis, Koplik’s spots inside the mouth, and a rash on the face, trunk, upper neck, back, and, eventually, hands and feet. All patients gave written informed consent prior to participation in this study. One patient (M01) was a senior high school student (17 years old), and the remaining 2 were junior high school students (13 and 15 years, respectively). The 2 junior high school students (M02 and M03) were residents of Tatebayashi City, and the senior high school student lived in Maebashi City; the distance between the two cities is about 50 km. None of the 3 patients had a history of contact with each other. One patient (M03) was immunized in May 1993 (about 13 years before contracting measles), but the remaining 2 were unvaccinated or had an unknown vaccination history, respectively.

Throat swabs were centrifuged at 3,000 × g at 4°C for 30 min, and the supernatants were used for RT-PCR and sequence analysis performed as previously described (12). Briefly, MV RNA was extracted from 140 μl of the swab supernatants using a QIAamp Viral RNA Mini Kit (Qiagen, Germantown, Md., USA). The extracted RNA was then suspended in 60 μl of DNase/RNase-free water. To amplify the N gene, we used a one-step RT-PCR kit (Qiagen) with reaction mixture (total volume, 50.0 μl) consisting of 10.0 μl of template RNA, 2 μl of pMvGTf1 and pMvGTr1 primers (20 pmol each), 10.0 μl of 3 OneStep RT-PCR Buffer, 2 μl of dNTP Mix (containing 10 mM of each dNTP), 2 μl of OneStep RT-PCR enzyme mix, 0.5 μl of RNase inhibitor (containing 10 units/μl), and 23.5 μl of RNase-free water. The samples were incubated for 30 min at 50°C then 15 min at 95°C followed by 40 cycles at 94°C for 1 min, 53°C for 1 min, and 72°C for 1 min, ending with elongation for an additional 10 min at 72°C (574 bp). Nested PCR (533 bp) was then used...
Fig. 1. (A) Epidemic curve showing breakdown by age for patients with measles in Gunma Prefecture, 2007. (B) Breakdown of patients with measles by age and number of cases (n = 131).

Fig. 2. Phylogenetic tree based on the nucleotide protein (N) gene sequences of various strains of the measles virus. The distance was calculated using Kimura's two-parameter method, and the tree was plotted using the neighbor-joining method. Numbers at each branch indicate the bootstrap values of the clusters supported by that branch. Numbers in parenthesis are GenBank accession numbers. The genotype of reference strains and the present strains are represented in bold type.
to detect the N gene. The PCR reaction mixture contained 5 µl of template DNA, 2 µl of pMvGTf2 and pMvGTr2 primers (20 pmol each), 25 µl of PCR Master Mix (Promega, Madison, Wis., USA), and 18 µl of DNase- and RNase-free distilled water (total volume, 50 µl). The PCR protocol included incubation for 3 min at 94°C followed by 30 cycles at 94°C for 2 min, 55°C for 3 min, and 72°C for 2.5 min, ending with elongation for an additional 5 min at 72°C. The sizes of the amplified DNA fragments were confirmed by electrophoresis using 3% agarose gel. The DNA fragments were purified using a QIAquick PCR Purification kit (Qiagen), and the nucleotide sequence was determined with an automated DNA sequencer ABI PRISM™ 310 Genetic Analyzer and the nucleotide sequence was determined with an automated DNA sequencer ABI PRISM™ 310 Genetic Analyzer.

The nucleotide sequences of the partial N gene of the MV (positions 1302 to 1686: 385 bp) were analyzed phylogenetically using the CLUSTAL W program on the DNA database of Japan (DDBJ) homepage (http://clustalw.ddbj.nig.ac.jp/top-e.html) and TreeExplorer (Version 2.12) (http://evolgen.biol.metro-u.ac.jp/TE/). Evolutionary distances were estimated using Kimura’s two-parameter method, and phylogenetic trees were constructed using the neighbor-joining (NJ) method (13). Reliability of the trees was estimated using 1,000 bootstrap replications.

The nucleotide sequences of the N gene in the obtained MV samples were identical to each other. In addition, these sequences were identical to those of another strain detected in Okinawa in 2006; this case was epidemiologically confirmed during an outbreak in Tokyo. The results suggest that the MVs detected in Gunma are highly homologous to each other as well as strains detected in other areas.

Based on the N gene sequences, the genotypes were classified as A to H then were further classified into subgenotypes. A detailed phylogenetic tree based on the N gene sequences is shown in Fig. 2. It has been suggested that there is an epidemiological link between certain genotypes/subgenotypes and geographical area (5-11,14,15). The present strains were classified into the same cluster as D5, and they were highly homologous compared to other MV strains detected in areas of Taiwan (Mvs/Taichung.TWN/45.03 [D5]), Australia (Mvi/Queensland.AU/37.03 [D5]), and England (Mvs/Luton.GBR/06.05 [D5]) (Fig. 2). Recently, genotype D5 has mainly been detected in Asian countries including Taiwan, Cambodia, Thailand, and Japan (3,6,10), and recurrent outbreaks have been particularly apparent in Japan in recent years (3). For example, a large measles outbreak that occurred in Gunma in 1998 was due to the D5 virus (16). The results suggested that Japanese outbreaks of measles during the past 2 years were at least partially due to a highly homologous virus(es) belonging to the D5 genotype.

In Japan, during the past 10 years, domestic measles outbreaks occurred in 1998, 2001, 2006, and 2007 (3,4,14-16). In June 2007, outbreaks were still being confirmed in various areas in Japan (http://idsc.nih.go.jp/disease/measles_e/idwr200719.html). Additional information regarding the epidemiological status of measles continues to be needed, since there is a high likelihood of the disease spreading to other areas.

REFERENCES