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A Fatal Case of Encephalopathy Possibly Associated with Human Metapneumovirus Infection

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Encephalitis and encephalopathy are neurological disease entities that are associated with significant morbidity and mortality, and their etiology often remains unknown. Glaser et al. (1) analyzed 1,570 cases of neurological disease and demonstrated that a confirmed or probable etiologic agent was identified in 16% of cases, of which 69% were viral. Etiology was identified in an additional 13% of cases, yet these agents have not yet been definitely demonstrated to be encephalitogenic. Four cases of human metapneumovirus (HMPV) were included in the latter cases.

HMPV was first reported in 2001 from the Netherlands (2); since then, the virus has been isolated all over the world (3). The virus is a member of the *Metapneumovirus* genus, which belongs to the family *Paramyxoviridae*. The negative-stranded RNA genome of HMPV is comprised of N, P, M, F, M2, SH, G, and L genes. Based on genomic sequence determination and phylogenetic analyses, two major genotypes of HMPV have been recognized, each genotype with two subgroups, designated A1 and A2, and B1 and B2, respectively (4,5). HMPV causes lower and upper respiratory tract diseases and induces severe symptoms in infants, children, elderly, and immunocompromised persons (3). While respiratory symptoms are the most common manifestations of HMPV infection, it is possible that HMPV affects the human central nervous system. To date, two cases with encephalitis have been reported (6,7) in addition to the four cases mentioned above (1). In this report, we described a fatal case of encephalopathy that could be attributed to HMPV infection.

The case is a 6-month-old girl, who first visited a local pediatric clinic with the complaints of fever, cough, and feeding difficulty. Medications for fever and cough brought her no significant improvement. The next day she was referred to Toyohashi Municipal Hospital and was immediately hospitalized because her blood test showed elevated creatinine kinase and a low platelet count. Five hours after admission, generalized convulsion was observed, although her electroencephalogram and cerebrospinal fluid findings were normal. Several hours later the patient began Cheyne-Stokes respiration and was diagnosed as having acute encephalopathy. A computed tomography (CT) scan of the brain showed low-density areas (LDA) in the white matter. On the next day the baby fell into a coma. She died 9 days after admission. All bacterial culture tests turned out negative and no sign of metabolic abnormality was found.

The specimens collected at the outpatient ward, i.e., throat swab, cerebrospinal fluid, serum, and urine, were transferred to Aichi Prefectural Institute of Public Health for the surveillance of infectious agents. Each specimen was subjected to routine examination of virus isolation by inoculating onto Vero, HeLa, and RD-18s cells. During 4 weeks of culture, no specimen induced cytopathic effects (CPEs) on cultured cells. In addition, RT-PCR analysis for enterovirus was done and turned out to be all negative. A part of each specimen was also tested for the presence of the HMPV gene by RT-PCR as described below. Total RNA was extracted from each specimen with a High Pure Vial RNA Kit (Roche Applied Science, Penzberg, Germany). Semi-nested RT-PCR was carried out to amplify the F gene of HMPV using primers described by Takao et al. (8). The first PCR was done by the One-step RT-PCR Kit (Invitrogen, Carlsbad, Calif., USA) with primers MPVF1f and MPVF1r at 50°C for 30 min for reverse transcription, and 94°C for 2 min for denaturation, followed by 40 cycles of 94°C for 15 s, 50°C for 30 s, 68°C for 2 min. Second PCR was done using primers MPVFf2 and MPVF1r with LA Taq polymerase (Takara, Otsu, Japan) at 94°C for 3 min, followed by 40 cycles of 94°C for 15 s, 50°C for 30 s, and 70°C for 1 min. A specific band for the F gene of HMPV was detected in throat swab and urine specimens while cerebrospinal fluid and serum were negative. Portions of the specimens (throat swab and urine) judged positive for HMPV F gene were inoculated onto LLC-MK2 cells in a 24-well plate. The infection was performed with 50 μl of specimen into 0.5 ml of DMEM/0.2% BSA containing 5 μg of trypsin (Sigma-Aldrich, St. Louis, Mo., USA). After a 1-week culture, CPEs were observed in the cells inoculated with the throat swab. Viral RNA was extracted from these LLC-MK2 cells showing CPE, and the HMPV F gene was detected by RT-PCR. The urine specimen was cytotoxic to the cells and therefore virus-specific CPE failed to be detected. Direct sequencing of the amplified fragment was performed with a Model-4200 automated DNA sequencer (Li-cor, Lincoln, Nebr., USA). An identical 340-bp sequence of the F gene was amplified from both the respiratory specimen and the infected LLC-MK2 cells. As for the urine specimen, the result of direct sequencing suggested it to be the mixture of different viruses. Therefore, we cloned PCR products with the pGEM-T vector system (Promega, Madison, Wis., USA) and further determined the base sequences of 10 clones. Each of

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the 10 clones belonged to one of the four different types, which differed from each other in one or two nucleotides throughout the sequenced region. All four differed in two nucleotides from the strain obtained from the throat specimen. Phylogenetic analysis was performed on the nucleotide sequences using GENETYX software (Ver 7; GENETYX Corp., Tokyo, Japan). The result indicated that all belonged to genetic lineage A2 (Fig. 1).

HPMV is reportedly associated with neurological manifestations. Schildgen et al. detected HMPV RNA in brain and lung tissues of a 14-month-old boy, who died of encephalitis in Germany (6). Kaida et al. in Japan also detected HMPV RNA in respiratory samples from a 1-year-old girl with encephalitis (7). The HMPV strains detected in these two cases were classified as A1 and A2, respectively. These findings could suggest a possible link between the A strain and the neurological diseases. However, more epidemiological studies are needed to clarify the association.

Nucleotide substitutions were observed among the F gene fragments detected from the urine and the throat swab. The results suggest that HMPV may have mutated and generate quasispecies in vivo, although the high viral diversity was observed only in the urine-derived clones.

In conclusion, this case adds another example of possible involvement of HMPV in fatal neurological disease. Further surveillance studies are necessary to define the full spectrum of clinical manifestations caused or induced by HMPV infection.
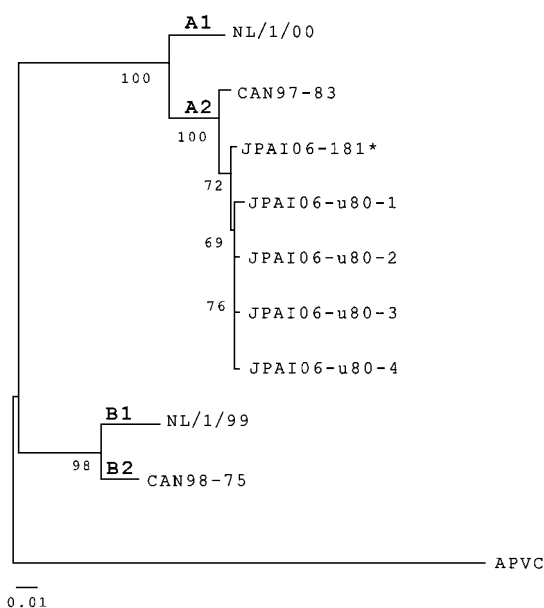
Fig. 1. Phylogenetic tree of the F genes of HMPV detected in the throat swab and urine from a patient of fatal encephalopathy. The virus strain isolated from the throat swab was marked by *, JPAI06-181 (GenBank accession no. AB302895). Clones from urine were JPAI06-u80-1 to 4 (AB302935 to AB302938). Reference strains are A1, NL/1/00 (AF371337); A2, CAN97-83 (AY297749); B1, NL/1/99 (AY525843); B2, CAN98-75 (AY297748). The out-group in the tree is APVC (AY579780). The numbers at branching points are bootstrap values based on 100 replicates.

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