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Short Communication

Molecular Epidemiology of Human Metapneumovirus from 2009 to 2011 in Okinawa, Japan

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SUMMARY: To clarify the molecular epidemiology of human metapneumovirus (HMPV) in Okinawa Prefecture, located in a subtropical region of Japan, we performed genetic analysis of the *F* gene in HMPV from patients with acute respiratory infection from January 2009 to December 2011. HMPV was detected in 18 of 485 throat swabs (3.7%). Phylogenetic analysis showed that 17 strains belonged to subgroup A2 and 1 strain belonged to subgroup B1. We did not observe seasonal prevalence of HMPV during the investigation period. A high level of sequence identity was observed in the strains belonging to subgroup A2 (>95%), and no amino acid substitution was found compared with other strains detected in Japan and other countries. The pairwise distance values among the present strains belonging to subgroup A2 were short. Our results suggest that the predominant HMPV strains belonging to A2 are highly homologous and seasonal epidemics were not seen in Okinawa during the investigation period.

Human metapneumovirus (HMPV) is a member of the family *Paramyxoviridae*, subfamily *Paramyxovirinae*, and genus *Metapneumovirus*, and is an important causative agent of acute respiratory infection (ARI) in humans (1). Recent studies suggest that HMPV affects both children and adults, including the elderly (2,3). In addition, recurrent HMPV infection occurs throughout life (4,5).

Okinawa Prefecture is located in a subtropical region of Japan, and the prevalence of various respiratory viruses might be unique to the area. For example, the prevalent season of respiratory syncytial virus (RSV) infection in Okinawa differs from that of mainland Japan (6). In addition, the epidemic pattern of influenza virus (Flu) is distinct (7–9). However, the epidemiology of HMPV in subtropical regions such as Okinawa remains unknown. Here, we analyzed the *F* gene detected in patients with ARI to address the molecular epidemiology of HMPV in Okinawa.

From January 2009 to December 2011, 485 throat swab samples were collected from 417 patients with ARI at Awase Daiichi Clinic (located in the central region of Okinawa island), Aozora Pediatric Clinic (located in a southern region of Okinawa island), and Gushi Kodomo Clinic (also in a southern region of Okinawa island). This was conducted as part of a collaboration with the local health authority of Okinawa Prefecture for the surveillance of viral diseases in Japan. Three hundred sixty-nine of the 485 samples were single samples from 369 patients. The remaining 116 samples were collected as follows: 36 patients were samples twice (72 samples), 7 patients were sampled 3 times (21 samples), 3 patients were sampled 4 times (12 samples), 1 patient was sampled 5 times (5 samples), and 1 patient was sampled 6 times (6 samples). Samples were not collected from the same patient in the same month and year. Informed consent was obtained from all patients or the parents/guardians of underage patients for the donations of throat swab samples used in the study.

Patients were aged from 0 to 73 years (5.9 ± 12.2 years; mean ± standard deviation [SD]) and were residents of Okinawa Prefecture, Japan. Of the 485 samples, 195 were collected from patients diagnosed with upper respiratory infection (URI) and 290 with lower respiratory infection (LRI), including bronchitis and pneumonia. One hundred seventy-two of the 290 LRI patients were diagnosed with wheezy LRI, and 118 were diagnosed with non-wheezy LRI.

Viral RNA was extracted from 140 μL of samples using the QIAamp Viral RNA Mini Kit (Qiagen, Tokyo, Japan) and suspended in DNase/RNase-free water. After RNA extraction, cDNA was synthesized using a PrimeScript RT reagent kit (Takara, Shiga, Japan), and

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PCR was performed using the primers MPVF1f and MPVF1r, as described previously (10). Amplification products were separated by electrophoresis on a 2.0% (w/v) agarose gel stained with ethidium bromide. Amplicons were purified using a QIAquick PCR Purification Kit (Qiagen), and nucleotide sequences were determined by direct sequencing. Sequence data were registered under accession numbers AB683044–AB683047 and AB683238–AB683251 at DDBJ/EMBL/GenBank. Phylogenetic analysis of the partial nucleotide (nt) sequences (321 nt) of the *F* region of HMPV was achieved using Molecular Evolutionary Genetics Analysis (MEGA) software, version 5 (11). Evolutionary distances were estimated using Kimura's two-parameter method, and phylogenetic trees were constructed using the neighbor-joining method (12). The reliability of the tree was estimated using 1000 bootstrap replications. In addition, the pairwise distances for the present strains were calculated to assess the frequency distribution, as previously described (13).

We detected other respiratory viruses in the present samples using (RT)-PCR methods for RSV (14), human parainfluenza viruses type 1–3 (HPIV1–3) (15), human rhinoviruses (HRV) (16,17), and human bocaviruses (HBoV) (18). In addition, to isolate adenoviruses (AdV), enterovirus (EV), and Flu (subtype A to C), we applied cell culture methods using 3 cell lines (HEp-2, RD18S, and MDCK cells), as previously described (19). The cells were checked daily for cytopathic effect (CPE), and culture supernatant fluids were harvested when CPE was clearly observed. The culture supernatants were examined for AdV and EV by (RT)-PCR methods, as previously described (16,17,20).

Statistical analysis was performed by a χ^2 test using Statcel (OMS, Tokyo, Japan). A P value of <0.05 was regarded as statistically significant.

Eighteen HMPV strains were detected in the 485 samples (3.7%); the 18 HMPV positive samples were collected from 18 different patients. In addition, other respiratory viruses such as RSV, HPIV1–3, HRV, and HBoV were detected or AdV and EV strains were isolated (Table 1). All 18 HMPV strains were detected in the 389 samples collected from patients aged 0–6 years (4.6%). Four of 18 strains were detected in 117 samples collected from patients under 1 year old (3.4%), and 4 were detected in 165 samples collected from patients aged 1 year (2.4%), and 5 were detected in 58 samples collected from patients aged 2 years (8.6%), and 3 were detected in 21 samples collected from patients aged 3 years (14.3%), and 1 was detected in 12 samples collected from patients aged 5 years (8.3%), and 1 was detected in 5 samples collected from patients aged 6 years (20.0%). HMPV strains were not detected in the 96 patients aged >6 years. Five HMPV strains were detected in 195 URI patients (2.6%), and 13 HMPV strains were detected in 290 LRI patients (4.5%). There was no significant difference between the detection rates in URI and LRI (χ^2 test; $P = 0.27$). Of the 13 HMPV strains detected in LRI patients, 9 strains were detected in the 172 wheezy LRI patients (5.2%) and 4 strains were detected in the 118 non-wheezy LRI patients (3.4%). There was no significant difference between the detection rates (χ^2 test; $P = 0.45$). HMPV alone was detected in 16 of the 18 patients, while HMPV plus HRV was detected in 1 patient and HMPV plus EV in the remaining patient. In each month, 0–3 HMPV strains were detected (0–15%) (Fig. 1). There was no significant difference between the detection rates (χ^2 test; $P > 0.05$), and no seasonal prevalence was found during the investigation periods.

On the phylogenetic tree, 17 strains were classified into subgroup A2 and a single strain detected in May 2011 was classified into subgroup B1 (Fig. 2). Strains belonging to subgroup A2 were subdivided into 3 clusters: A2a, A2b, and A2c. Cluster A2a included 2 strains detected in March 2009 and 1 strain detected in July 2009. Cluster A2b included 1 strain detected in July 2009 and another in April 2011. Cluster A2c included 9 strains detected in 2010, 1 strain detected in March

Table 1. Detection of the respiratory viruses in the present study

	No. of detection (%)
HMPV	18 (3.7)
RSV	55 (11.3)
HPIV1–3	28 (5.8)
HRV	25 (5.2)
HBoV	18 (3.7)
AdV	39 (8.0)
EV	24 (4.9)
Flu	0 (0)

HMPV, human metapneumovirus; RSV, respiratory syncytial virus; HPIV1–3, human parainfluenza virus type 1–3; HRV, human rhinovirus; HBoV, human bocavirus; AdV, adenovirus; EV, enterovirus; Flu, influenza virus.

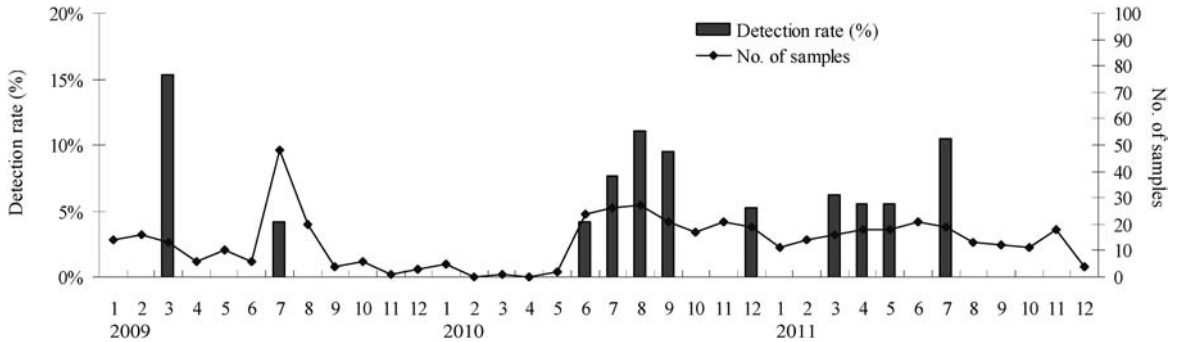

Fig. 1. Monthly distribution of HMPV strains detected in Okinawa Prefecture, Japan, from January 2009 to December 2011.

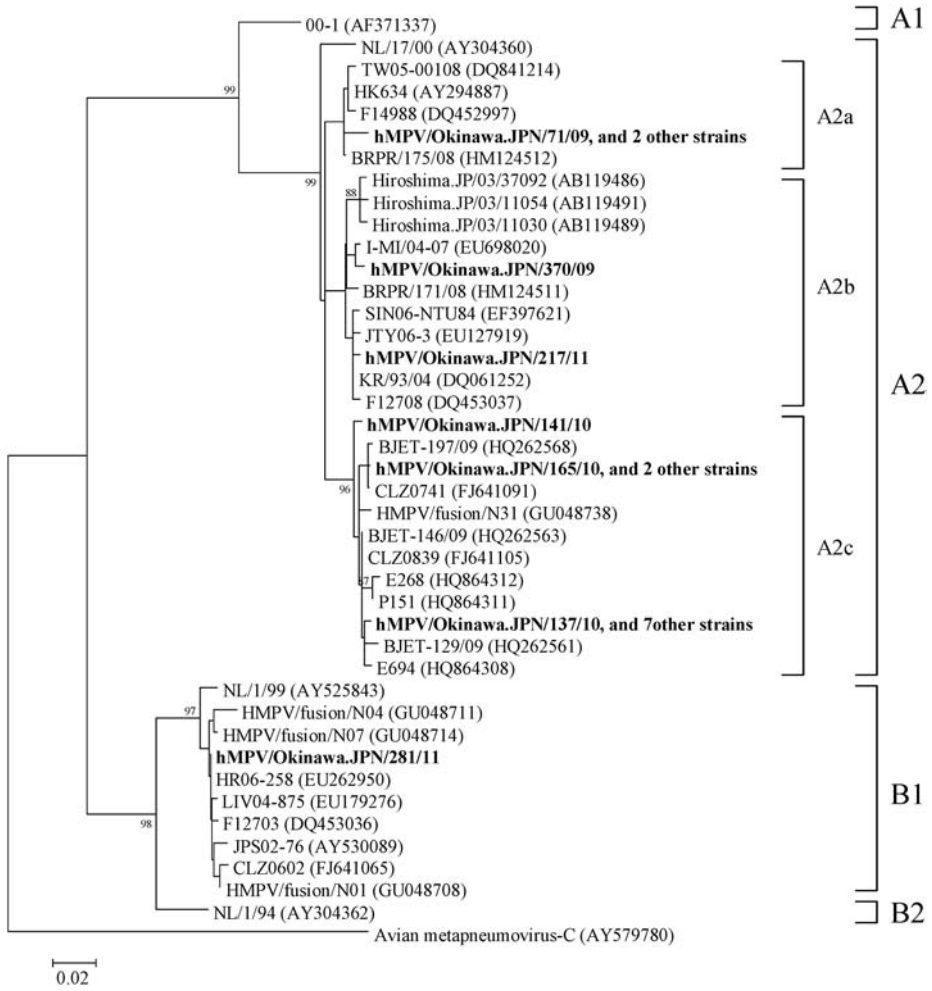

Fig. 2. Phylogenetic tree of HMPV based on the *F* gene sequences. Avian metapneumovirus-C was used as an outgroup. The phylogenetic tree was constructed after neighbor-joining analysis based on the nucleotide sequences of the partial *F* region (321 nt) with 1000 bootstrap replicates. Bootstrap values >70% are indicated at each node. A1, A2, B1, and B2 are the subgroups. A2a, A2b, and A2c are the clusters indicated in this study. Bar indicates nucleotide substitutions per site.

2011, and 2 strains detected in July 2011. Nucleotide identity levels were high (>95%) among the 17 present strains belonging to subgroup A2 and, in comparison with other strains detected in Japan and other countries, no amino acid substitutions were found. The pairwise distances among the present strains belonging to subgroup A2 did not exceed 0.05. These values were not significant compared with those of Japanese and overseas strains.

We detected HMPV in around 4% of samples from patients with ARI in Okinawa from 2009 to 2011 in this study, although a seasonal prevalence was not observed. The phylogenetic tree showed that the HMPV strains detected belonged to subgroups A2 and B1, and A2 was predominant in Okinawa during the investigation period. The present strains belonging to subgroup A2 were subdivided into 3 clusters. Cluster A2a included HMPV strains detected in 2009, and cluster A2c included HMPV strains detected from 2010 to 2011. However, cluster A2b included HMPV strains detected in 2009 and 2011 and the bootstrap value was less than 50% at the nodes of these clusters. Nucleotide identity levels among the present strains belonging to subgroup A2 were high. No amino acid substitution was found, and our results are compatible with earlier reports (21,22). In addition, pairwise distance values were short. The results indicated that the predominant HMPV strains were highly homologous during the investigation period.

Seroepidemiological studies have indicated that in some countries almost all children show evidence of HMPV infection by the age of 5 (23,24). In Japan, Ebihara et al. reported that around 40% of children aged from 4 months to 5 years were seropositive for HMPV (25), and Mizuta et al. reported that the isolation rate of HMPV in patients aged <5 years was higher than that in patients aged ≥5 years (21). In another report from China, HMPV was mainly detected in patients aged <5 years and around 10% of patients were co-infected with other ARI viruses such as HPIV, Flu, AdV, and RSV (26). Moreover, A2 and B2 were the predominant subgroups in Yamagata Prefecture from 2004 to 2009 (21), while A2 was predominant in Yamaguchi Prefecture in 2009 (22). In the present study, 18 HMPV strains were detected in patients aged 0–6 years. In addition, HRV and EV were detected in 2 of these patients (11.1%). Thus, other respiratory viruses might be detected at a steady rate in patients with ARI due to HMPV. These

findings suggested that the molecular epidemiology of HMPV in Okinawa is similar to that of other areas.

Some longitudinal studies suggest that the high season for HMPV in the Northern Hemisphere is from winter to spring (between January and May) and the low season is in the fall (around September and October) (2,21). The high season for HMPV in tropical and subtropical areas varies—winter to spring in Brazil (27), spring and/or summer in Taiwan (28,29), and the rainy season in Vietnam (30). In this study, HMPV did not appear to have a high season in Okinawa. Although the reasons are not yet known, this trend may differ from that of other tropical and subtropical areas. However, our investigation periods may be short. Thus, to clarify the prevalence season of HMPV in Okinawa, more longitudinal studies may be needed. In addition, the present samples were collected from only 3 different areas. Thus, to rigorously investigate the prevalence of HMPV in our prefecture, additional samples from different areas should be tested.

In conclusion, the predominant HMPV strain showed a high degree of genetic homology throughout the investigation period in Okinawa. However, additional studies, including those designed to investigate the clinical features of HMPV infection, are needed to further understand the epidemiology of the virus in Okinawa.

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Conflict of interest None to declare.

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