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Human Metapneumovirus Infection in Japanese Children

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Human metapneumovirus (hMPV) has been recently discovered as an etiological agent of acute respiratory infections. Our purpose was to asses the virological and clinical features of children with respiratory infections caused by hMPV. We examined 658 nasopharyngeal swab samples obtained from 637 children with respiratory infections for hMPV by using reverse transcription-PCR (RT-PCR). A total of 268 samples from 637 children were inoculated onto tertiary monkey kidney cells. A total of 36 serum samples (26 in the acute phase and 10 in the convalescent phase) from the 26 hMPV-positive children were tested for immunoglobulin G (IgG) and IgM antibodies to hMPV by using an indirect immunofluorescence assay. We detected hMPV in 57 (8.9%) of the 637 samples by using RT-PCR and isolated 7 (2.6%) hMPV strains of the 268 samples in cell cultures. A total of 12 (46.2%) of 26 hMPV-positive children were suspected to have primary infection with hMPV as determined by an indirect immunofluorescence assay. The infected children were diagnosed as having wheezy bronchitis (36.8%), upper respiratory tract infection (26.3%), bronchitis (22.8%), and pneumonia (14.0%). We showed that two hMPV groups were circulating in different regions during the same period and that reinfection with hMPV frequently occurs in childhood. The RT-PCR test is the most sensitive test for detection of hMPV, and a serological test may be useful to differentiate between primary infection and reinfection with hMPV.

Human metapneumovirus (hMPV) was recently isolated in The Netherlands and found to be a new paramyxovirus belonging to the genus *Metapneumovirus* of the subfamily *Pneumovirinae* of the family *Paramyxoviridae* by virological data, sequence homology and gene constellation (18). hMPV is genetically related to human respiratory syncytial virus (hRSV) (19). Detection of hMPV by using reverse transcription-PCR (RT-PCR) in several countries indicates that the virus is widespread and causes respiratory infections (1, 4, 11, 12, 15, 17). hMPV generally causes upper respiratory tract infection and flu-like illness (1, 17) but is also associated with lower respiratory tract infections, such as wheezy bronchitis, bronchitis, bronchiolitis and pneumonia, in very young children, elderly persons, and immunocompromised patients (2, 5, 8, 14). Some patients with severe acute respiratory syndrome have been found to be positive for hMPV, although it is not clear whether infection with hMPV aggravates illness in patients with severe acute respiratory syndrome or whether its presence is mere coincidence (16). We examined the virological and clinical features in Japanese children with respiratory infections associated with hMPV infection.

MATERIALS AND METHODS

Patients and sample collection. We collected 658 nasopharyngeal swab samples from 637 children with respiratory infections in three different regions of Japan. The male/female ratio was 1.5 to 1. Twenty-one samples from 16 RT-PCR-positive patients were subsequently obtained. The samples collected in three different regions of Japan included one group of 246 stored nasopharyngeal swab samples randomly obtained during the period from June 2000 to October 2002 from 246 outpatients (aged 1 month to 13 years) with respiratory infections at Suzuki Pediatric Clinic in Yamaguchi and one group of samples obtained during the period from October 2002 to May 2003 from 306 hospitalized patients and 47 outpatients (aged 1 month to 12 years) with respiratory infections who were treated at seven hospitals in Sapporo. All of the samples in Sapporo were collected from patients in whom the possibility that the infection was caused by influenza virus A or B or by hRSV had been ruled out by the results of rapid antigen detection tests. The third group included 38 samples obtained from 38 outpatients with respiratory infections under the age of 6 years in Hiroshima between March and May 2003, the same period as that during which an outbreak of hMPV infections in children occurred in Sapporo. After we confirmed that all of the samples in Hiroshima were negative for influenza viruses A and B and for hRSV by rapid antigen detection tests and negative for other viruses by culture on Madin-Darby canine kidney, rhesus monkey kidney (LLC-MK2), buffalo green monkey kidney, human epidermoid laryngeal carcinoma (HEp-2), and rhabdomyosarcoma (RD-18S) cells, the samples were examined for hMPV by using RT-PCR. Yamaguchi and Hiroshima are located in the southwestern region of Honshu, the main island of Japan, and Sapporo is located in Hokkaido, the northernmost island of Japan. The clinical data of patients from whom the samples were collected in Sapporo and Hiroshima were available (Table 1).

A total of 36 serum samples obtained from the 26 hMPV-positive children, including 26 samples obtained in the acute phase of infection and 10 samples obtained during the convalescent phase, were used for detection of antibodies to hMPV. Totals of 19 and 7 serum samples of 26 samples in the acute phase were collected within 8 days after onset of illnesses in Sapporo and Hiroshima, respectively. All 10 serum samples in the convalescent phase were collected in Sapporo. Serum samples obtained randomly from 100 Japanese children aged from 1 month to 10 years were also examined for the presence of immunoglobulin M (IgM) antibody to hMPV as controls. All samples were collected after obtaining informed consent from the children's parents.

RT-PCR test and sequencing. The 658 samples obtained from 637 children were examined for the presence of RNA sequence of hMPV by using RT-PCR based on the fusion glycoprotein (F) gene. Twenty-one hMPV-positive samples were subsequently reexamined. Total RNA was extracted from each sample by using the RNAzol B (Tel-Test, Inc.) method according to the manufacturer's protocol. Approximately 0.1 μg of each RNA sample was incubated in a solution containing 100 ng of random hexadeoxynucleotides and 200 U of Moloney murine leukemia virus reverse transcriptase (First-Strand cDNA synthesis kit; Amersham Pharmacia Biotech) in a final volume of 15 μl at 37°C for 1 h to synthesize cDNA. The cDNA (0.2 μl) was subjected to PCR analysis to deter-

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TABLE 1. hMPV detection from 391 children with acute respiratory infections in Sapporo and Hiroshima

Parameter	No. (%) of patients hMPV-RNA positive/no. (%) of patients examined		
	Sapporo (n = 353)	Hiroshima (n = 38)	Total
Age			
<6 mo	0/38	0/0	0/38[a]
6 mo to 1 yr	7/75 (9.3)	0/1	7/76 (9.2)
1 to 3 yr	17/158 (10.8)	13/24 (54.2)	30/182 (16.5)
3 to 5 yr	6/57 (10.5)	8/10 (20.9)	14/67 (20.9)
5 to 10 yr	1/22 (4.5)	3/3 (100)	4/25 (16.0)
>10 yr	0/3	0/0	0/3
Male or female			
Male	18/211 (8.5)	10/20 (50.0)	28/231 (12.1)
Female	13/142 (9.2)	14/18 (77.8)	27/160 (16.9)
Inpatients or outpatients			
Inpatient	25/306 (8.2)	0/0	25/306 (8.2)
Outpatient	6/47 (12.8)	24/38	30/85 (31.6)[b]
Clinical diagnosis			
Wheezy bronchitis	16/166 (9.6)	4/10 (40.0)	20/176 (11.4)
Upper respiratory tract infection	3/108 (2.8)	12/20 (60.0)	15/128 (11.7)
Pneumonia	7/36 (19.4)	1/1 (100)	8/37 (21.6)
Bronchitis	5/25 (20.0)	7/7 (100)	12/32 (37.5)[c]
Laryngotracheitis	0/15	0/0	0/15
Bronchiolitis	0/3	0/0	0/3

[a] Significantly different from the results of patients aged 1 to 3 years, 3 to 5 years, and 5 to 10 years by Fisher exact test at $P = 0.0070$, $P = 0.0018$, and $P = 0.021$, respectively.
[b] Significantly different from the results of patients by Fisher exact test at $P = 6.0e{-}09$.
[c] Significantly different from the results of patients with wheezy bronchitis and upper respiratory infection by Fisher exact test at $P = 0.00065$ and $P = 0.0013$, respectively.

mine the expressions of the hMPV F gene. On the basis of published data, a set of primers was designed for amplification of the hMPV F gene to detect two groups of hMPV (00-1 [GenBank accession number NC_004148] and 99-1 [GenBank accession number AF371344]). The forward primer sequence was 5′-GC TTCAGTCAATTCAACAG-3′ (00-1 [NC_004148; nucleotides 3626 to 3644]), and the reverse primer sequence was 5′-CCTGCAGATGTTGGCATGT-3′ (00-1 [NC_004148; nucleotides 3767 to 3749]). PCR products were subjected to electrophoresis through a 1.5% agarose gel. After the primer set had been used for screening, PCR was carried out to detect the two groups (00-1 and 99-1). A forward primer with a sequence of 5′-CTGTTCCATTGGCAGCAATA-3′ (JPS03-139, corresponding to 00-1 [NC_004148; nucleotides 4218 to 4237]) and a reverse primer with a sequence of 5′-TCAAAGCTGCTTGACACTGG-3′ (JPS03-139, 00-1 [NC_004148; nucleotides 4391 to 4372]) were used to detect group of 00-1 strain, and a forward primer with a sequence of 5′-AATCGGGT TGGAATCATCAA-3′ (99-1F [AF371344; nucleotides 18 to 37] corresponding to 00-1 [NC_004148; nucleotides 4234 to 4253]) and a reverse primer with a sequence of 5′-GCTGTTCACCTTCAACTTTGC-3′ (99-1F [AF371344; nucleotides 138 to 118] corresponding to 00-1 [NC_004148; nucleotides 4354 to 4334]) were used to detect group of 99-1 strain.

The PCR mixture consisted of 100 μmol of each deoxyribonucleotide, 1.0 U of Ampli*Taq* Gold, 50 mmol of potassium chloride/liter, 10 mmol of Tris-HCl (pH 8.3)/liter, 1.5 mmol of magnesium chloride/liter, 0.01% (wt/vol) gelatin, 10 pmol of each primer, and cDNA in a volume of 25 μl. The PCR conditions were as follows: 94°C for 9 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. A total of 80 bp (00-1 [NC_004148; nucleotides 4254 to 4333] and 99-1 [AF371344; nucleotides 38 to 117]) of the PCR products were sequenced directly by using a BigDye terminator cycle sequencing ready reaction kit (Perkin-Elmer Applied Biosystems, Tokyo, Japan) with an ABI Prism 310 genetic analyzer (Perkin-Elmer Applied Biosystems). Both sense and antisense strands of the PCR products were sequenced directly.

Phylogenetic tree analysis. Nucleotide sequences of amplified F gene products were aligned by using CLUSTAL W software. Phylogenetic trees were constructed by neighbor joining by the use of the DNADist and Neighbor software package of the Phylip 3.6 (alfa3) program using 100 bootstraps with random sequence addition. Bootstrap values were computed for consensus trees created with the Consensus package by applying a 50% majority consensus rule.

Virus isolation. A total of 268 nasopharyngeal swab samples from 268 children in Sapporo were collected in Eagle minimum essential medium and placed on ice, and the samples were inoculated onto tertiary monkey (*Macaca mulatta*) kidney cells within 3 h after collection. The cells were cultured in Eagle minimum essential medium with a weekly medium change for 3 to 4 weeks. Trypsin (Sigma-Aldrich) was added to the medium at a concentration of 1 μg/ml. Isolation of hMPV was confirmed by immunofluorescence assays with guinea pig anti-hMPV-specific serum against two different serotype isolates provided by Albert Osterhaus (Erasmus University, Rotterdam, The Netherlands) and by PCR tests.

Detection of antibody to hMPV. Indirect immunofluorescence assays with two isolates of hMPV-infected cells were carried out as serological tests for hMPV in the present study, as described previously (3, 18). Two isolates of hMPV, JPS02-76 and JPS03-178, were used as representatives of two distinct groups. Serum samples that reacted to hMPV antigens at a dilution of more than 1:10 were considered positive for IgG and IgM antibodies to hMPV.

Statistical analysis. For statistical analyses, we used the Fisher exact test or the Student t test to determine the significant differences between two groups. Differences at P values of <0.05 were considered significant.

RESULTS

RT-PCR test. RNA sequences of hMPV were detected in samples from 57 (8.9%) of the 637 children by direct gel analysis. The male/female ratio in the 57 patients was 1 to 1. Furthermore, two primer sets selectively amplified F genes of two groups of hMPV. The 57 hMPV-infected children were between 6 months and 6 years of age (7 children aged less than 1 year, 30 children aged 1 to 3 years, 15 children aged 3 to 5 years, and 5 children aged more than 5 years) with a mean age of 2 years and 6 months. Of the 57 RT-PCR-positive samples, 47 (82.5%) were collected during the period from March to April in 2003. hMPV RNA was detected in only 2 (0.8%) of the

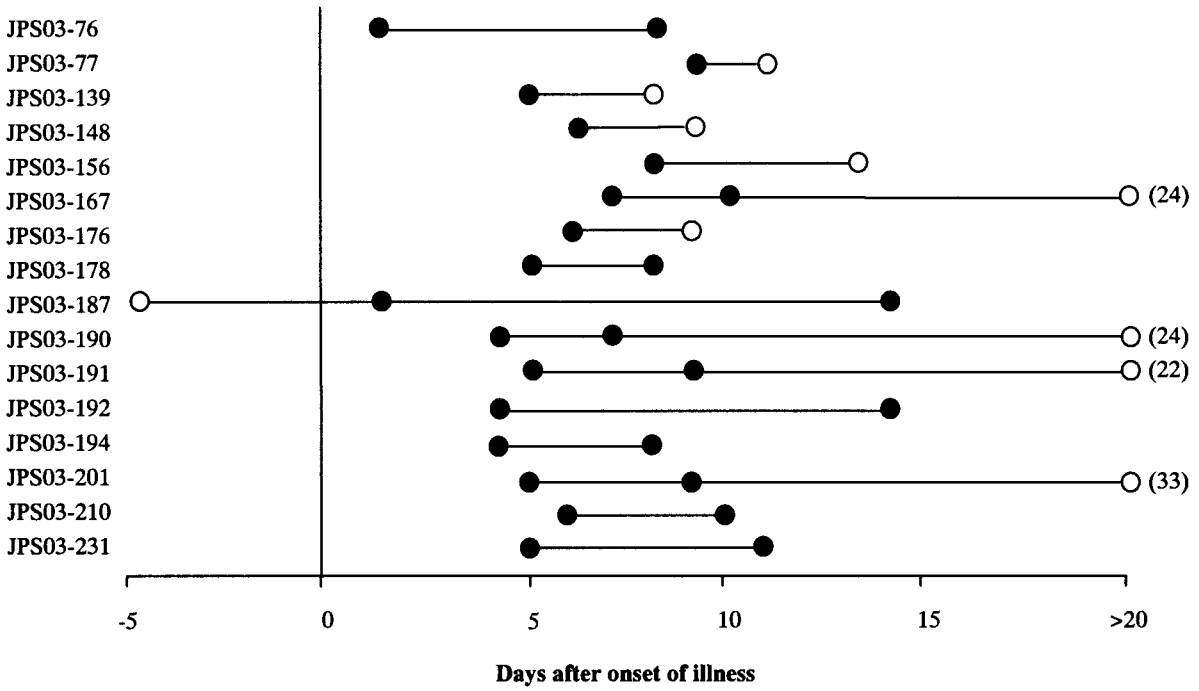

FIG. 1. Analysis for hMPV by RT-PCR. Symbols: ●, positive for hMPV; ○, negative for hMPV. JPS, isolate from Sapporo. The numbers in parentheses are the numbers of days from onset to sampling.

246 samples collected randomly during a 3-year period from the 246 outpatients with respiratory infections in Yamaguchi. Of the 353 samples obtained from children in Sapporo after the possibility of infection having been caused by influenza virus or hRSV infection had been ruled out, 31 (8.8%) were positive for hMPV. In Sapporo, the positive rates were 8.2% (25 of 306) in hospitalized patients and 12.8% (6 of 47) in outpatients. The prevalence of hMPV infection in hospitalized patients was not significantly different from that in outpatients in Sapporo. The number of cases of detected hMPV in Sapporo increased dramatically during the period from March to May in 2003. Of the 38 samples obtained from patients in Hiroshima in whom the possibility of infection having been caused by other respiratory viruses had been ruled out, 24 (63.2%) were positive for hMPV. hMPV was detected in many of the samples in the Hiroshima group during the same outbreak period as that in Sapporo.

For Sapporo and Hiroshima, where all of the clinical data of the 391 patients were available, the rates of RT-PCR-positive samples in various patients' groups are summarized in Table 1. The distributions of age and sex of the patients were similar between in Sapporo and Hiroshima. Of 114 patients aged less than 1 year, 7 (6.1%) were infected with hMPV. This rate of RT-PCR-positive samples was significantly lower than that in patients aged more than 1 year ($P = 0.0036$ [Fisher exact test]). Because in Hiroshima, where the rate of RT-PCR-positive samples was very high, all samples were collected only from outpatients, in outpatients was significantly higher than that in inpatients. The rate of RT-PCR-positive samples in the patients' group of bronchitis was significantly higher than other patients' groups (wheezy bronchitis and upper respiratory tract infections) in Sapporo and Hiroshima.

As shown in Fig. 1, virus shedding continued up to 1 to 2 weeks in most patients. Although one case was examined before the onset of disease, hMPV was not detected 5 days before the onset of disease.

Phylogenetic tree analysis. Phylogenetic trees were constructed on the basis of the F gene sequences of 57 hMPV Japanese isolates (GenBank accession numbers AY312177 to AY312233). As shown in Fig. 2, all of the 57 isolates were classified into two distinct genetic clusters or groups that were tentatively named group 1 (99-1 group) and group 2 (00-1 group). Nucleotide comparison of the 57 hMPV F gene sequences of 80 bases revealed the existence of two major phylogenetic groups with 70.0 to 77.5% similarity among groups versus 91.3 to 100% (group 1) and 93.8 to 100% (group 2) similarity within groups. Additional variability was also observed within the individual groups. Of the 57 isolates, 13 belonged to group 1 and 44 belonged to group 2. Totals of 6 of the 24 isolates in Hiroshima and 7 of the 31 isolates in Sapporo belonged to group 1. There was no significant regional difference between percentages of isolates in the two groups.

Virus isolation. Seven hMPV strains (JPS02-76, JPS03-176, JPS03-178, JPS03-180, JPS03-187, JPS03-194, and JPS03-201) were isolated from 22 RT-PCR-positive patients.

Serological analysis. As shown in Table 2, although 12 of the 26 serum samples obtained from patients during the acute phase of infection were negative for the IgG antibody to hMPV, the IgM antibody was positive in 11 of these 12 serum samples. Of these 12 serum samples, 3 samples that were tested were seroconverted in the convalescent phase. Although the IgG antibody was already present in serum samples obtained from 14 children in the acute phase of infection, the titers in five patients that were tested were more than fourfold

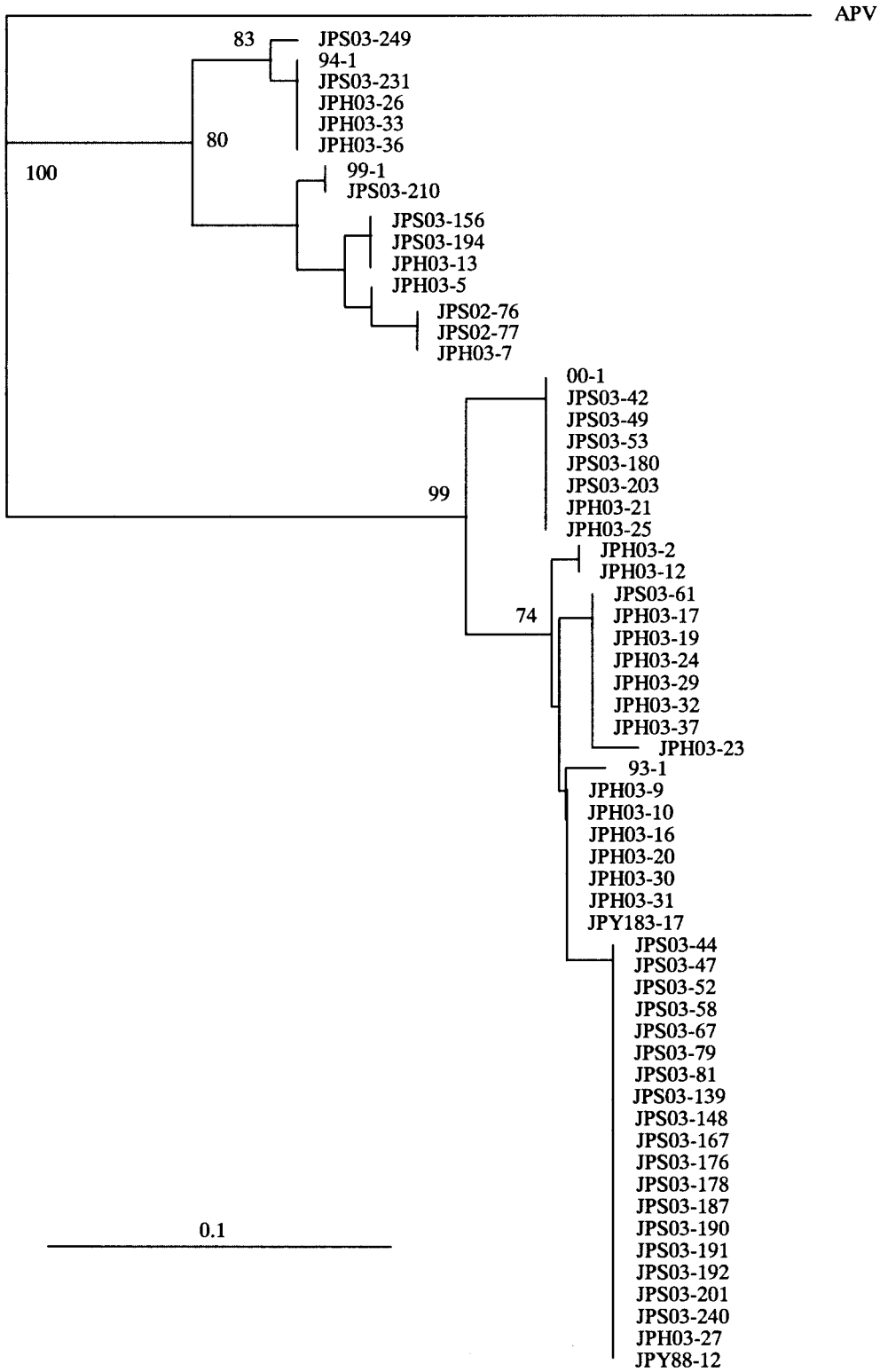

FIG. 2. Phylogenetic analysis of hMPV fusion nucleotide sequences. A tree was constructed by the neighbor-joining method. The length of the horizontal line represents number of substitutions/site. Bootstrap values of >70% are shown. Avian pneumovirus subgroup C (GenBank accession no. AF187152 [APV]) was the outgroup and used to root this tree. 94-1, 99-1, 00-1, and 93-1, isolates from The Netherlands (18); JPS, isolate from Sapporo; JPH, isolate from Hiroshima; JPY, isolate from Yamaguchi. The GenBank accession numbers are AY312177 to AY312233.

TABLE 2. IgG and IgM antibody titers to hMPV

Age	Sex	Titer of IgG antibody		Titer of IgM antibody		Virus no.[b]
		Acute	Convalescent	Acute	Convalescent	
6 m	F	<10	NT[a]	80	NT	JPS03-44
11 m	M	<10	NT	20	NT	JPS03-49
1 yr 3 m	M	<10	1280	40	40	JPS03-167
1 yr 5 m	M	<10	NT	20	NT	JPS03-47
1 yr 7 m	F	<10	NT	10	NT	JPH03-29[c]
1 yr 8 m	M	<10	NT	20	NT	JPS03-139
2 yr 3 m	F	<10	NT	20	NT	JPS03-52
2 yr 5 m	M	<10	640	40	40	JPS03-42
2 yr 5 m	F	<10	80	20	20	JPS03-178
3 yr 1 m	M	<10	NT	20	NT	JPH03-33
4 yr	F	<10	NT	<10	NT	JPH03-32
5 yr 9 m	F	<10	NT	20	NT	JPH03-37
9 m	F	20	160	<10	40	JPS03-231
1 yr 2 m	M	20	640	20	<10	JPS03-176
6 yr 11 m	F	40	NT	10	NT	JPH03-26
8 m	M	80	640	40	20	JPS03-187
4 yr 8 m	M	80	NT	10	NT	JPS03-58
7 m	F	160	640	160	40	JPS03-148
10 m	F	320	NT	40	NT	JPS03-201
4 yr	F	320	NT	10	NT	JPH03-31
4 yr 5 m	M	320	NT	10	NT	JPH03-27
1 yr 11 m	M	640	NT	40	NT	JPS03-192
3 yr 4 m	M	640	NT	<10	NT	JPS03-53
3 yr 3 m	M	640	5120	<10	<10	JPS03-190
3 yr 10 m	M	2560	2560	160	10	JPS03-194
5 yr 6 m	M	20480	40960	40	20	JPS03-191

[a] NT, not tested.
[b] JPS, isolate from Sapporo; JPH, isolate from Hiroshima.

higher in the convalescent phase. Eleven children of the fourteen children also had IgM antibody in the acute phase. Overall, 22 (84.6%) of the 26 patients were IgM positive in the acute phase. Of the 100 control serum samples, 11 (11.0%) samples were positive for the IgM antibody to hMPV. There was no regional statistical difference of antibody titers because the number of serum samples was too small to clarify regional difference.

Clinical findings of patients infected with hMPV. Symptoms in the 57 hMPV-positive patients included fever (temperature of >38°C) (96.5%), cough (96.5%), rhinorrhea (91.2%), sore throat (33.3%), dyspnea (26.3%), vomiting (8.8%), diarrhea (7.0%), and headache (1.8%). Expiratory wheezing (47.4%) and rales (64.9%) were observed on auscultation. A chest radiograph showed peribronchial and/or alveolar infiltrates in 13 (22.8%) of the 57 patients. Mean (± the standard deviation [SD]) durations of fever and wheezing were 4.8 ± 1.6 and 5.2 ± 3.0 days, respectively. Spiky fever was associated with febrile convulsion in 2 (3.5%) of the 57 patients. Acute otitis media was observed in 9 (15.8%) of the 57 patients. The common clinical diagnoses in patients with 57 hMPV infection were wheezy bronchitis (36.8%), upper respiratory tract infection (26.3%), bronchitis (22.8%), and pneumonia (14.0%). Asthma exacerbation was observed in 5 (8.8%) of the 57 patients. The mean period of hospitalization was 5.7 days (SD 1.7). A total of 7 (53.8%) of the 13 patients infected with group 1 hMPV strains and 17 (38.6%) of the 44 patients infected with group 2 hMPV strains were admitted to the hospital. There is no significant difference between the rates of hospitalization in groups 1 and 2.

Laboratory findings. Despite high fever, 45 (78.9%) of the 57 hMPV-positive patients had no evidence of leucocytosis in peripheral blood tests. The patients had a mean of 7.2×10^9 white blood cells/liter (SD 3.3×10^9). Leukopenia and lymphopenia were present in 5.3 and 17.6% of the patients, respectively. The concentration of C-reactive protein was slightly elevated in 18 (31.6%) of the 57 patients. The mean concentration of C-reactive protein was 1.4 mg/dl (SD 1.8), ranging from 0.0 to 9.4 mg/dl.

DISCUSSION

Viruses most frequently associated with respiratory infections include rhinoviruses, coronaviruses, influenza viruses, parainfluenza viruses, hRSV, and adenoviruses (14). hMPV is also a pathogen causing seasonal outbreaks of respiratory infections in children (5, 13). The proportion of hMPV detected in nasopharyngeal samples from children with unexplained respiratory infections has varied from 1.5 to 10.3% (12, 17, 18). The rate of hMPV in respiratory infections varies depending on several factors, such as age, season, sampling methods, and detection methods. Since the present study is a retrospective study, we cannot rule out the difference of clinical data of patients from whom nasopharyngeal swab samples were obtained in three distinct regions. Therefore, it was difficult to interpret the results that the rates of RT-PCR-positive samples in three groups were different. However, the finding that the RT-PCR-positive rate in samples obtained randomly was the lowest (0.8%) and that the rate in samples obtained from patients in whom the possibility of other respiratory virus in-

fections was ruled out during the same outbreak period as that in Hiroshima was the highest (63.2%) seems reasonable.

We cannot exclude the possibility that the frequency of hMPV infections is underestimated because there is a possibility that the primer set used is not universal for detecting hMPV. However, since we used the well-conserved F gene sequences as three primer sets in the RT-PCR test, false-negative samples should be rare. Furthermore, the low rate of detection of hMPV in a year-round study in Yamaguchi indicates that the rate of coincidental detection of hMPV is low. hMPV had a seasonal peak during the spring in 2003 in Japan. However, one (JPY183-17) of two isolates from a random selection of 246 samples obtained between June 2000 and October 2002 in Yamaguchi was detected in July 2002. Therefore, the possibility of year-round circulation of the virus cannot be excluded. Further year-round study is in progress.

Phylogenetic analysis of 80 base pairs of the F gene of the Japanese isolates showed the presence of two genetic clusters or groups. Our results also showed that both hMPV groups were simultaneously circulating in distinct regions of Japan during the same year. Phylogenetic analysis of the F gene of English, Canadian, and Dutch isolates also showed the same two distinct genetic clusters, suggesting that a relatively homogeneous population of hMPV is circulating throughout the world (15, 17, 19). The genetic diversity of the hMPV F gene may lead to antigenic variability, and the two hMPV genetic clusters may also represent two different antigenic groups. Although we tested for the presence of hMPV antibodies in paired serum samples from patients in the acute phase and convalescent phase by using two respective hMPV strain-infected cells, there was no significant difference between the infected cells in antibody titers. Therefore, it is still not clear whether the differences in the F gene of the hMPV isolates are representative of two distinct hMPV antigenic groups to prevent further hMPV infections in children.

hRSV isolates are separated into subgroups A and B by antigenic and genetic characteristics. This dimorphism is primarily due to variation within the G glycoprotein associated with attachment of the virus (9). Both hRSV subgroups circulate in each season, but the predominant endemic strains vary from season to season (6). Therefore, genetic analysis of hMPV G genes is also necessary to clarify the possibility of reinfection with hMPV. Previous studies have shown that hRSV subgroup A strain infection results in greater disease severity than does subgroup B strain infection (6, 20). However, in the present study, there was no significant difference between clinical findings in the two groups of hMPV.

Information on the timing of transmission of hMPV from one patient to another, from which the incubation period of the virus could be estimated, was only available for two patients. An 8-month-old boy was admitted to a hospital and stayed for 2 days after admission in a room with an RT-PCR-positive patient (JPS03-190). The boy was negative for hMPV on the first day of contact. However, he developed cough and fever 6 days after the first day of contact and became positive for hMPV (JPS03-187). The 80 bp sequences in the two isolates were completely identical, suggesting that the transmission occurred in the hospital. The incubation period, calculated as the number of days between likely exposure and the onset of symptoms, may have been 4 to 6 days.

Isolation of viruses in cell cultures is considered the "gold standard" for detection. However, hMPV is difficult to detect by cell culture due to its slow growth and mild cytopathic effects without apparent syncytium formation. Only seven hMPV strains were isolated from the 22 RT-PCR-positive samples in our study, indicating that cell culture yields false-negative results and is usually not useful for detection of hMPV.

Generally, serum IgM antibody emerges within a few days and remains detectable for 1 to 2 weeks in primary infection of a virus. IgG antibody appears in the second week, peaks in the fourth week, and declines after 1 to 2 months. In a primary hMPV infection, we do not know when IgG antibody first appears after onset of disease and how long it remains. However, 12 of the 26 serum samples obtained from patients in the acute phase were negative for IgG antibody to hMPV up to 8 days after the onset of disease, indicating that the response of IgG antibody does not occur soon after onset. We set the cutoff point of positivity for IgG antibodies at the titer of 1:10 because all of the children whose acute-phase serum samples were negative ($<$1:10) became seroconverted against IgG antibody ($>$1:10) in the convalescent phase. Although it is difficult to distinguish primary infection and reinfection with hMPV, we assumed that serum samples negative and positive for IgG antibody represent primary infection and reinfection, respectively. According to our criteria, we suspected that 12 cases were primary infection, and 14 cases were reinfection with hMPV. There was no significant difference between the ages of patients with primary infection and the ages of patients with reinfection with hMPV. Of 26 serum samples collected from patients infected with hMPV within 8 days after onset, 4 were negative for IgM antibodies ($<$1:10). This might show that the absence of IgM antibody in acute phase by our method does not exclude the possibility of recent infection with hMPV. Furthermore, since 11 of the 100 control serum samples were positive for IgM antibody to hMPV ($\geq$1:10), the presence of IgM antibody at more than 1:10 does not always seem to indicate the presence of a respiratory infection associated with hMPV. In the present study, paired serum samples were limited. Further analysis was needed to clarify profile of IgG and IgM antibodies.

Clinical manifestations of hRSV infection range from mild upper respiratory tract infection to severe, life-threatening bronchiolitis and pneumonia. The clinical findings associated with hMPV infection are indistinguishable from those caused by other respiratory viruses. Our study showed that wheezy bronchitis was the major diagnosis in the patients with hMPV infection. However, the rate of patients with hMPV infection in the patients' group of wheezy bronchitis was less than the rates in other groups of respiratory infection in Sapporo and Hiroshima. This was possibly due to sampling bias because the numbers of samples collected from acute bronchitis and pneumonia were very small compared to wheezy bronchitis.

We could not detect hMPV-RNA in samples from 38 children under 6 months old in Sapporo and Hiroshima. Only one child required oxygen therapy to support respiration. Although there was no control group of patients with hRSV in the present study, hMPV infection seems to have milder symptoms than does hRSV infection. The role of hMPV in triggering

acute exacerbations of asthma was not clarified in the present study.

We have obtained evidence that two distinct groups of hMPV simultaneously circulate throughout the world and that reinfection with hMPV frequently occurs in children. There was good correlation between results of RT-PCR and serological tests. The RT-PCR test is the best test for detecting hMPV infection, and a serological test may be useful for differentiating between primary infection and reinfection with hMPV. Future studies are needed to clarify the full clinical spectrum of hMPV.

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