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Detection of Human Coronavirus NL63 and OC43 in Children with Acute Respiratory Infections in Niigata, Japan, between 2010 and 2011

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Human coronavirus (HCoV) is a member of the respiratory viruses that includes HCoV-229E, HCoV-OC43, and the severe acute respiratory syndrome (SARS) CoV (1). Recently, new types of HCoVs, such as NL63 and HKU-1, have also been described (1). Historically, HCoV research has been hampered by poor growth and lack of cytopathic effect in cell culture (1). The development of polymerase chain reaction (PCR) technology had allowed the field of coronavirology to develop widely and rapidly (1). However, according to the Infectious Agents Surveillance Report, only 54 and 59 HCoV-positive cases, including 18 cases from Niigata, were reported in Japan in 2010 and 2011, respectively (2). Although we have succeeded in isolating HCoV-229E viruses from children with nasopharyngitis using CaCo-2 cells in March 2008 and April 2010, we failed to isolate any further HCoVs thereafter (3). We therefore used reverse-transcription PCR (RT-PCR) methods in a screening analysis for HCoV in order to clarify the epidemiology of this virus in Niigata, Japan.

Between August 2010 and July 2011, 507 throat and nasal swab specimens were collected from patients with upper or lower acute respiratory infections at pediatric clinics working in collaboration with the Niigata Prefectural Health authorities as part of the national surveillance of viral diseases in Japan. Specimens were transported to the Virology Section of the Niigata Prefectural Institute of Public Health and Environmental Sciences for virus isolation. We were able to isolate respiratory viruses, including influenza virus, parainfluenza virus, RS virus, human metapneumovirus, rhinovirus, adenovirus, and enterovirus, from 376 specimens using 6 cell lines (MDCK, LLC-MK2, CaCo-2, HEp-2, Vero9013, and RD-18S). Therfore, we investigated the presence of HCoV in the 131 specimens from which no other respiratory virus was isolated.

Viral nucleic acid was extracted from the specimens using the QIAamp Viral RNA Mini Kit (Qiagen, Germantown, Md., USA), suspended in AVE buffer, and applied to RT reactions using a PrimeScript™ RT Reagent Kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Then, we screened for the amplification of 4 HCoVs (HCoV-229E, HCoV-OC43, NL63, and HKU-1) by performing multiplex PCR using outer sense and antisense primers and by heminested PCR, using inner sense and outer antisense primers, as a previously reported method (4) with some modifications. Direct sequencing was used to determine the nucleic acid sequence of the 443-bp and 328-bp PCR products for identifying HCoV-OC43 and NL63, respectively. When the screening identified a HCoV-positive sample, we also amplified a portion of the spike glycoprotein region by performing PCR using our original primers to construct a phylogenetic tree. We prepared the following primer pairs: 1st PCR primers (OC-SP1F: 5′-ATGGTGGATAATGTTACTAGGCT-3′ and OC-SP1R: 5′-TAGTACCTGCAGGACAAGTG C-3′) and 2nd PCR primers (OC-SP2F: 5′-ATAATGT TACTAGGCTGCATGA-3′ and OC-SP2R: 5′-CAGG ACAAGTGCCTATACCA-3′) for HCoV-OC43 based on the reference strain (AY391777) and 1st PCR primers (NL-SP1F: 5′-TGAGTTTGATTAAGAGTGGTAGG-3′ and NL-SP1R: 5′-CAAACTGCAAGTGCTCACA C-3′) and 2nd PCR primers (NL-SP2F: 5′-GATTAA GAGTGGTAGGTTGTTG-3′ and NL-SP2R: 5′-GCT

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Table 1. Clinical characteristics of patients and detected human coronaviruses

Patient	Sex	Age[1)]	Sampling date	Clinical condition and diagnosis	NL63 strain	GenBank accession no.	
						Spike glycoprotein	Nucloeocapsid protein
1	M	7 Y	1 Dec. 2010	Fever, URI[2)]	HCoV-NL63/Niigata.JPN/10–1575	AB695183	AB695176
2	M	1 Y	13 Dec. 2010	Fever, bronchitis	HCoV-NL63/Niigata.JPN/10–1606	AB695184	AB695177
3	F	4 M	22 Dec. 2010	Fever, URI	HCoV-NL63/Niigata.JPN/10–1697	AB695185	AB695178
4	F	2 M	22 Dec. 2010	URI	HCoV-NL63/Niigata.JPN/10–1698	AB695186	AB695179
5	M	7 Y	27 Dec. 2010	Fever, URI	HCoV-NL63/Niigata.JPN/10–1708	AB695187	AB695180
6	M	8 M	4 Jan. 2011	Fever, otitis media	HCoV-NL63/Niigata.JPN/11–22	AB695188	AB695181
7	F	1 Y	19 Jan. 2011	Fever, URI	HCoV-NL63/Niigata.JPN/11–119	AB695189	AB695182

Patient	Sex	Age[1)]	Sampling date	Clinical condition and diagnosis	OC43 strain	GenBank accession no.	
						Spike glycoprotein	Membrane and nucloeocapsid protein
8	M	1 Y	15 Feb. 2011	Fever, URI, otitis media	HCoV-OC43/Niigata.JPN/11–286	AB695078	AB695068
9	F	2 Y	21 Feb. 2011	Fever, URI	HCoV-OC43/Niigata.JPN/11–335	AB695079	AB695069
10	M	2 Y	23 Feb. 2011	Fever, URI, otitis media	HCoV-OC43/Niigata.JPN/11–343	AB695080	AB695070
11	M	2 M	28 Feb. 2011	URI	HCoV-OC43/Niigata.JPN/11–400	AB695081	AB695071
12	M	3 Y	29 Mar. 2011	Fever, URI, otitis media	HCoV-OC43/Niigata.JPN/11–564	AB695082	AB695072
13	F	3 Y	20 May 2011	Fever, URI, FC[3)]	HCoV-OC43/Niigata.JPN/11–764	AB695083	AB695073
14	M	2 Y	25 May 2011	Fever, bronchitis	HCoV-OC43/Niigata.JPN/11–768	AB695084	AB695074
15	F	8 M	25 May 2011	Fever, bronchitis	HCoV-OC43/Niigata.JPN/11–769	AB695085	AB695075
16	M	9 Y	27 Jun. 2011	Fever, URI	HCoV-OC43/Niigata.JPN/11–833	AB695086	AB695076
17	F	1 Y	27 Jul. 2011	Fever, URI	HCoV-OC43/Niigata.JPN/11–981	AB695087	AB695077

[1)]: Y, year old; M, month old.

[2)]: URI, upper respiratory infection.

[3)]: FC, febrile convulsion.

CACACTGCAACTTTTCA-3′) for NL63 based on the reference strain (AY518894). Sequence data for the spike glycoprotein from the HCoV-OC43 and NL63 strains were added to the DNA Data Bank of Japan under the accession numbers AB695078–AB695087 and AB695183–AB695189, respectively.

We detected NL63 in 7 and HCoV-OC43 in 10 of the 131 specimens (Table 1). Neither HCoV-229E nor HKU-1 was detected. The spike glycoprotein regions of all 17 NL63- or HCoV-OC43-positive specimens were successfully sequenced.

The age distribution of the HCoV-positive patients was between 2 months and 9 years (average, 2.3 years), and most of the patients (14/17; 82%) were less than 4 years old. Clinically, 15 patients presented with fever and 13 had upper respiratory infections.

We found 5 and 2 NL63-positive cases in December 2010 and January 2011, respectively. In 2011, we found 4, 1, 0, 3, 1, and 1 HCoV-OC43-positive patients in February, March, April, May, June, and July, respectively. Although HCoV infections are generally seen from midwinter to early spring, the seasonality varies from year to year (1). Interestingly, the NL63-positive cases were all observed in winter, whereas the HCoV-OC43-positive cases were observed later. According to Gaunt et al. (4) and Kaida et al. (5), the detection rate of HCoVs decreases in the following order: HCoV-OC43 > NL63 > HKU-1 > HCoV-229E. Although we presume that no patient was infected with HKU-1 or HCoV-229E during the study period, further investigation will be required to clarify the seasonality of HCoV infections in Japan.

Phylogenetic analysis indicated that the NL63 strains from patients No. 1–No. 6, detected between December 2010 and January 2011, were closely related to the 25222/2004/SWE strain (DQ231160). Only 1 strain, from patient No. 7, was closely related to the 41687/2007/SWE strain (FJ656160) (Fig. 1A). Four HCoV-OC43 strains, detected in February 2011, were closely related to the 89996 Belgium 2003 strain (AY903454), and 6 strains, detected between March and July 2011, were closely related to the 34364 Belgium 2004 strain (AY903455) (Fig. 1B). Interestingly, these findings suggest that the NL63 and HCoV-OC43 viruses changed over time. Similarly, Vijgen et al. reported that 2 genetically distinct HCoV-OC43 strains circulated in 2003 and 2004 in Belgium (6).

We attempted to isolate 16 of the 17 HCoV-positive specimens using the CaCo-2 cell line, with which we had previously isolated HCoV-229E (3), and the LLC-MK2 cell line, which is commonly used to isolate HCoV (7). Crystal trypsin (final concentration, 0.5 μg/ml) was added to the maintenance media and cells were passaged 5 times. However, we failed to observe any cytopathic effect or to amplify the HCoV genome. HCoVs tend to induce subtle cytopathic effects and many cell types are not susceptible (7). We noticed that HCoV-229E does not grow in the CaCo-2 cell line if trypsin is not present in the maintenance media (3); this finding was a milestone in our attempts to isolate one of the HCoVs. Further study will be required to elucidate the cell lines and the proper maintenance media necessary to isolate different HCoVs.

According to the national surveillance in Japan (2), HCoV detection has been reported from only 8 prefectures, including Niigata, Osaka City, and Mie (5).

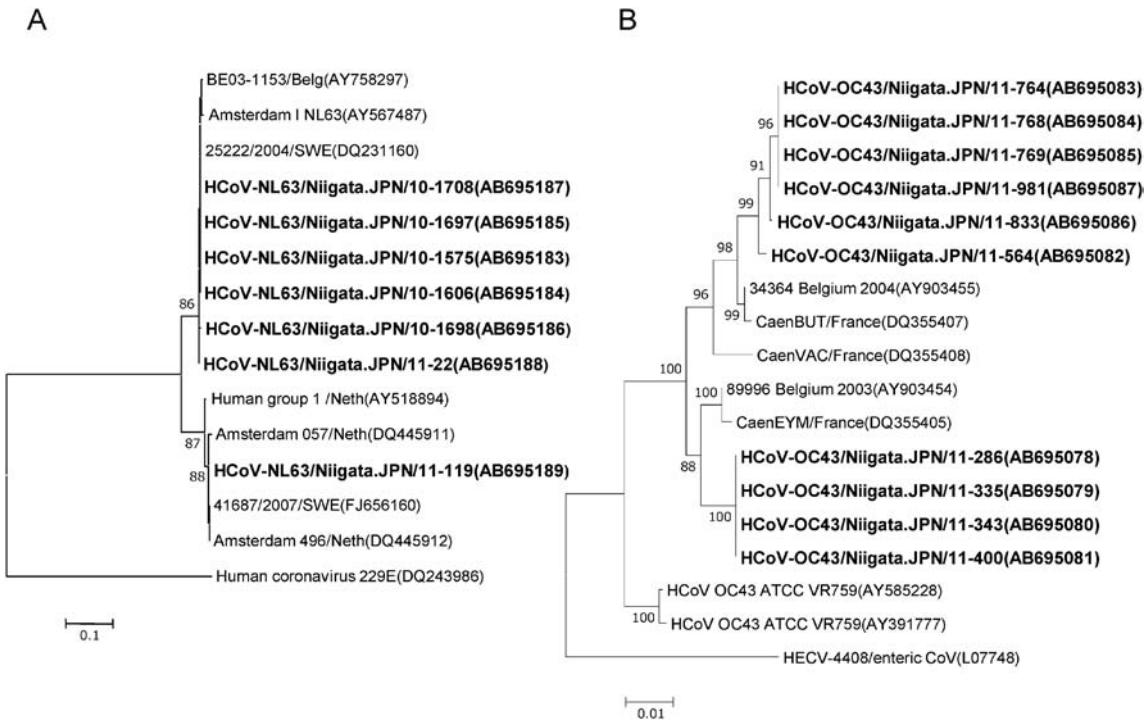

Fig. 1. Phylogenetic trees of the partial spike glycoprotein gene of the human coronavirus NL63 (A) and OC43 (B) strains. The trees of the NL63 (A) and HCoV-OC43 (B) strains were based on 576 bp and 1488 bp nucleotides, respectively. Evolutionary distance was calculated using the Maximum Composite Likelihood method, and the trees were plotted using the neighbor-joining method. Numbers at each branch indicate the bootstrap values of the clusters supported by that branch. The scales of NL63 (A) and HCoV-OC43 (B) indicate 10% and 1% nucleotide differences, respectively. The present strains are represented in bold type.

Therefore, the epidemiology of HCoV in Japan is not well understood and HCoV surveillance may have been ineffective. In this study, we failed to isolate HCoV, but succeeded in detecting the HCoV genome in 13% of specimens (17/131) using RT-PCR. These findings suggest that the difficulties associated with HCoV isolation underscore the importance of HCoV as a causative agent of acute respiratory infections in the national surveillance. Together with parainfluenza virus type 4 and rhinovirus, HCoV is considered one of the most difficult respiratory viruses to identify, despite the fact that these viruses are responsible for a large proportion of viral respiratory tract infections (8). The use of molecular methods may assist in the study of HCoV epidemiology, and our primers designed for sequencing the HCoV-OC43 and NL63 spike glycoprotein gene will be useful for future molecular epidemiological analyses. This does not diminish the need to develop virus isolation technologies and to stock clinical isolates in public health laboratories in order to develop such viral disease control strategies as vaccine development and longitudinal epidemiological studies (8). To conclude, we should continue to clarify the etiology and epidemiology of HCoV infection using a combination of virus isolation techniques and molecular methods such as RT-PCR.

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Conflict of interest None to declare.

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