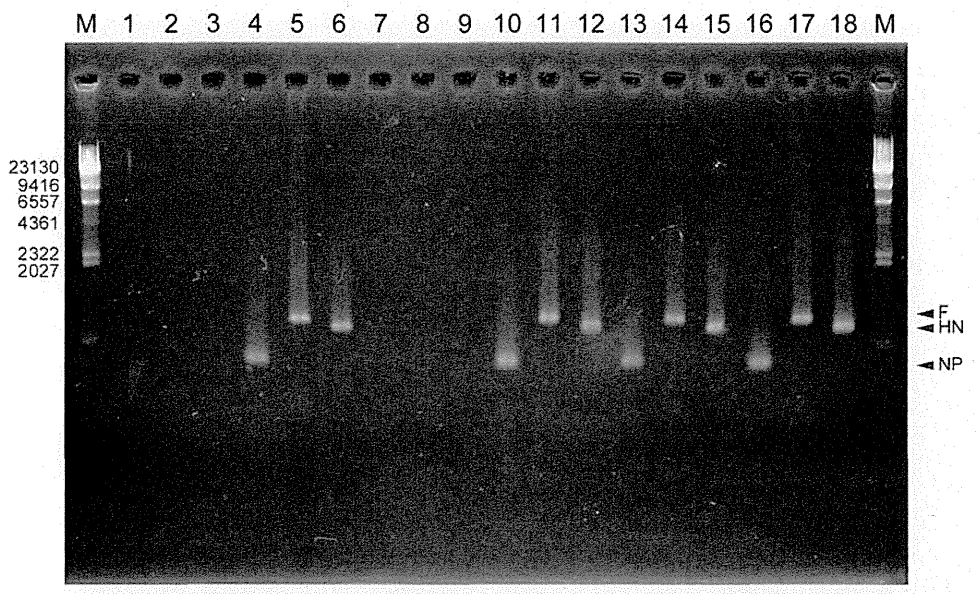

Fig. 1. RBV inhibits viral mRNA synthesis. cDNA was synthesized using oligo dT primer and PCR was carried out using NP, F and HN specific primers. Lane M, marker (base pair); lanes 1–3, non-infected cells; lanes 4–6, hPIV-2-infected cells; lanes 7–9, hPIV-2-infected cells cultured with RBV; lanes 10–12, hPIV-2-infected cells cultured with acyclovir; lanes 13–15, hPIV-2-infected cells cultured with lamivudine; and lanes 16–18, PIV-2-infected cells cultured with AZT. Lanes 1, 4, 7, 10, 13 and 16, NP; lanes 2, 5, 8, 11, 14 and 17, F; lanes 3, 6, 9, 12, 15 and 18, HN.

Figure 2a–c shows NP, F and HN protein expression in hPIV-2-infected cells, respectively. NP, F and HN proteins were observed in almost all hPIV-2-infected cells: NP protein was observed in many strong fluorescent dots mainly in the cytoplasm, whereas F and HN proteins were observed in small dots in the cytoplasm and on the cell surface. RBV almost completely inhibited expression of NP (Fig. 2d), F (Fig. 2e), and HN (Fig. 2f) proteins. However, expression of NP, F and HN proteins was not inhibited by acyclovir (Fig. 2g, h, i, respectively), lamivudine (Fig. 2j, k, l, respectively) or AZT (Fig. 2m, n, o, respectively). As shown in Figure 1, mRNA was not detected in RBV-treated infected cells, indicating that inhibition of protein synthesis by RBV was mediated by inhibition of transcription.

Entry and cell-to-cell spreading of hPIV-2

The above results show that RBV inhibits viral genome RNA synthesis and almost completely inhibits protein synthesis. In the subsequent experiment, the effects of the selected four antiviral drugs on entry and cell-to-cell spreading of hPIV-2 in the cells was determined using rhPIV-2ΔMGFP (Fig. 3). Figure 3a shows a negative control: there are no fluorescent giant cells. Figure 3b shows a positive control: there are multinucleated giant cells and the cells have strong fluorescence. Figure 3c–f shows the infected cells cultured with RBV, acyclovir, lamivudine and AZT, respectively. Figure 3c shows several fluorescent cells with a single nucleus or a few nuclei, indicating that RBV did not completely inhibit virus entry but did inhibit multinucleated giant cell formation. Acyclovir, lamivudine and AZT did not inhibit either entry or cell-to-cell spreading of rhPIV-2ΔMGFP.

The effect of the drugs on cytoskeleton

The selected four drugs were added to the cells, and the cytoskeletons observed by fluorescence microscopy at 20 hr of cultivation. Figure 4a, b shows actin microfilaments and microtubules, respectively, in $LLCMK_2$ cells. As shown in Figure 4, the four drugs did not disrupt either actin microfilaments or microtubules, indicating that the inhibitory effect of RBV is not attributable to disruption of the cytoskeleton. At 20 hr, the virus had no effect on the cytoskeleton (data not shown).

DISCUSSION

In the present study, RBV was the only one of the eight antiviral drugs tested that had an inhibitory effect on

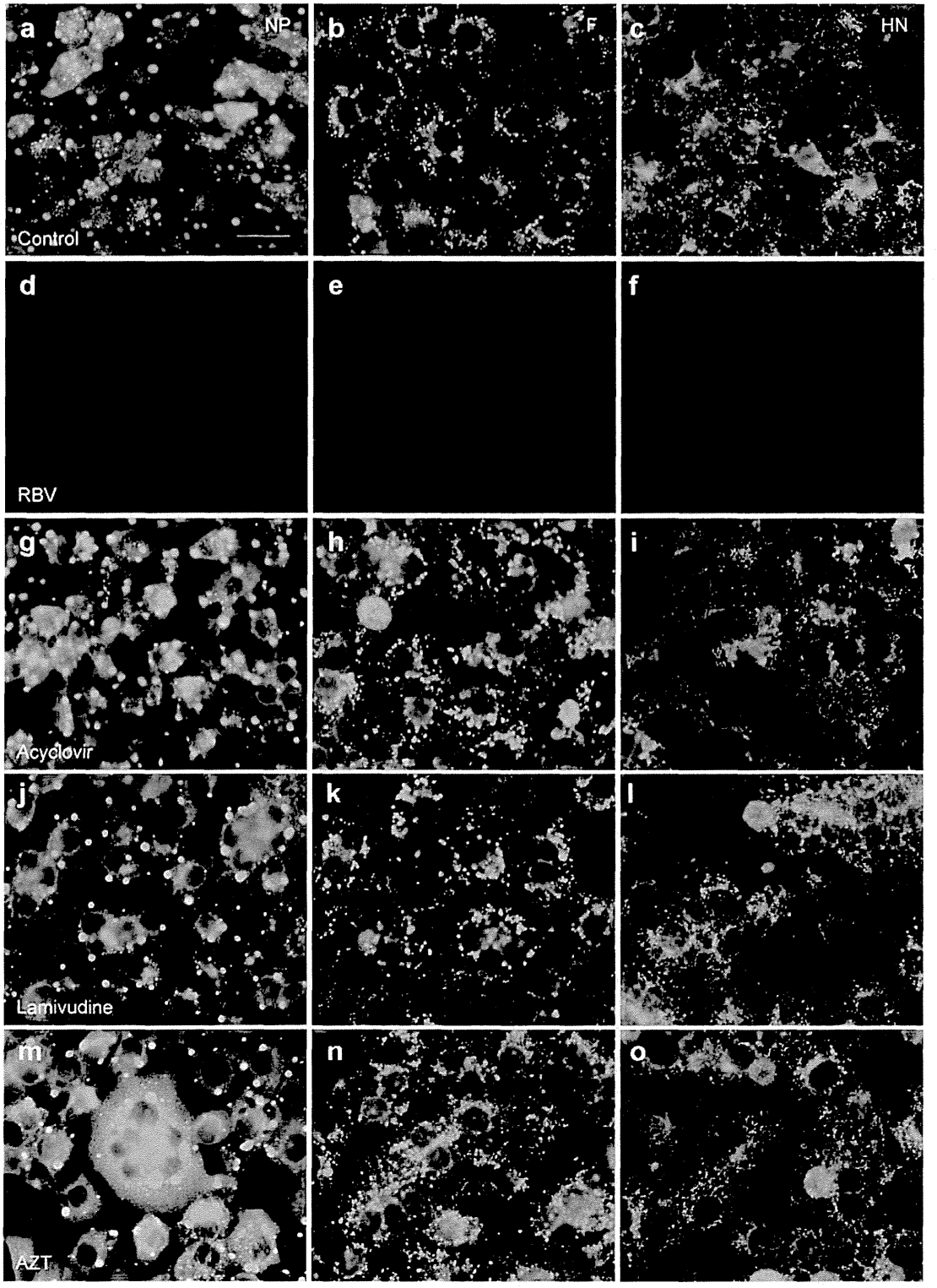

Fig. 2. RBV is the only one of the four antiviral drugs tested that inhibits expression of NP, F and HN proteins of hPIV-2. Expression of (a) NP, (b) F and (c) HN proteins of hPIV-2-infected cells. RBV inhibits expression of (d) NP, (e) F and (f) HN proteins. Acyclovir does not inhibit expression of (g) NP, (h) F and (i) HN proteins, lamivudine does not inhibit expression of (j) NP, (k) F and (l) HN protein, and AZT does not inhibit expression of (m) NP, (n) F and (o) HN protein (bar: 50 μm).

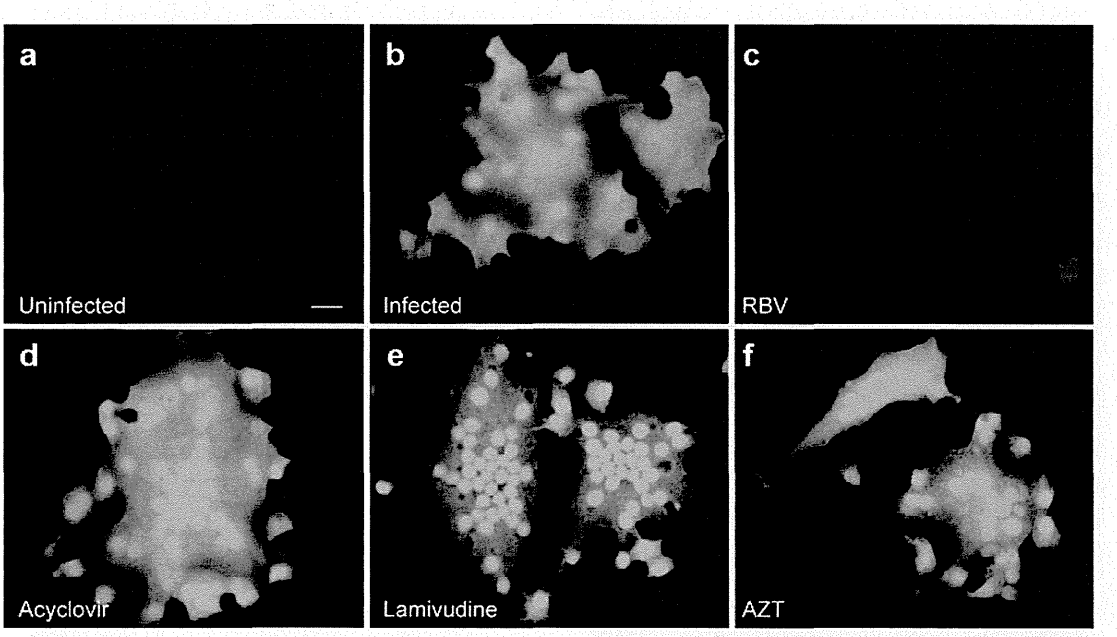

Fig. 3. Effects of the four selected antiviral drugs on hPIV-2 entry and cell-to-cell spreading. (a) Uninfected cells. (b) Cells infected with rhPIV-2ΔMGFP. (c) rhPIV-2ΔMGFP-infected cells cultured with RBV, (d) acyclovir, (e) lamivudine and (f) AZT. Fig. 3c shows several fluorescent cells with a single or a few nuclei, but no multinucleated giant cells, indicating that RBV does not inhibit viral entry but does inhibit cell-to-cell spreading of the virus (bar: 50 μm).

hPIV-2 replication in $LLCMK_2$ cells. Almost no virus was found in the culture medium of RBV-treated cells infected with hPIV-2. Viral genome RNA was not detected in the RBV-treated infected cells. In addition, viral proteins were not observed in the RBV-treated infected cells. Viral mRNA was not detected in RBV-treated cells, indicating that the inhibition of viral protein synthesis was mediated by inhibition of transcription by RBV. Using rhPIV-2ΔMGFP, it was shown that RBV did not completely inhibit entry of hPIV-2, but did suppress multinucleated giant cell formation. The inhibitory effect of RBV was not mediated by disruption of the cytoskeletons: the antiviral drugs used in the present study did not affect either actin microfilaments or microtubules. Taken together, the present findings suggest that the inhibitory effect of RBV on hPIV-2 growth is mediated by inhibition of virus replication in the cells, mainly by inhibition of transcription, resulting in inhibition of viral protein synthesis, of cell-to-cell spread of virus and virus budding from cells.

RBV is a synthesized nucleoside analog that has broad antiviral activities against many DNA and RNA viruses. RBV in combination with IFN is reportedly an effective treatment for hepatitis C virus (10, 11), respiratory syncytial virus (12), Lassa fever virus (19), yellow fever virus (20), Andes virus (21), flaviviruses and paramyxoviruses (13). Shah *et al.* examined the activity of RBV against vesicular stomatitis virus and Sendai virus in various cell types and found that RBV had an inhibitory action only on cells that could take up RBV (22). Upon uptake, RBV is phosphorylated into mono-, di- and tri-phosphate by cellular kinases. The mechanism of RBV action on the viruses has not yet been fully elucidated. However several mechanisms have been proposed (13, 19, 22). RBV inhibits the host enzyme inosine monophosphate dehydrogenase, which is essential for *de novo* synthesis of GTP. Phosphorylated RBV directly inhibits viral RNA polymerase. RNA chain elongation is terminated by incorporation of RBV tri-phosphate into viral RNA. RBV tri-phosphate incorporation as a substitute for guanosine or adenosine leads to lethal mutagenesis.

In summary, the present investigation showed that RBV inhibits hPIV-2 replication in $LLCMK_2$ cells by inhibition of viral genome RNA synthesis and transcription inhibition, resulting in inhibition of viral protein synthesis, giant cell formation and viral release from the infected cells into culture medium. Our group's next aim is to elucidate some of the mechanisms of hPIV-2 growth inhibition by RBV.

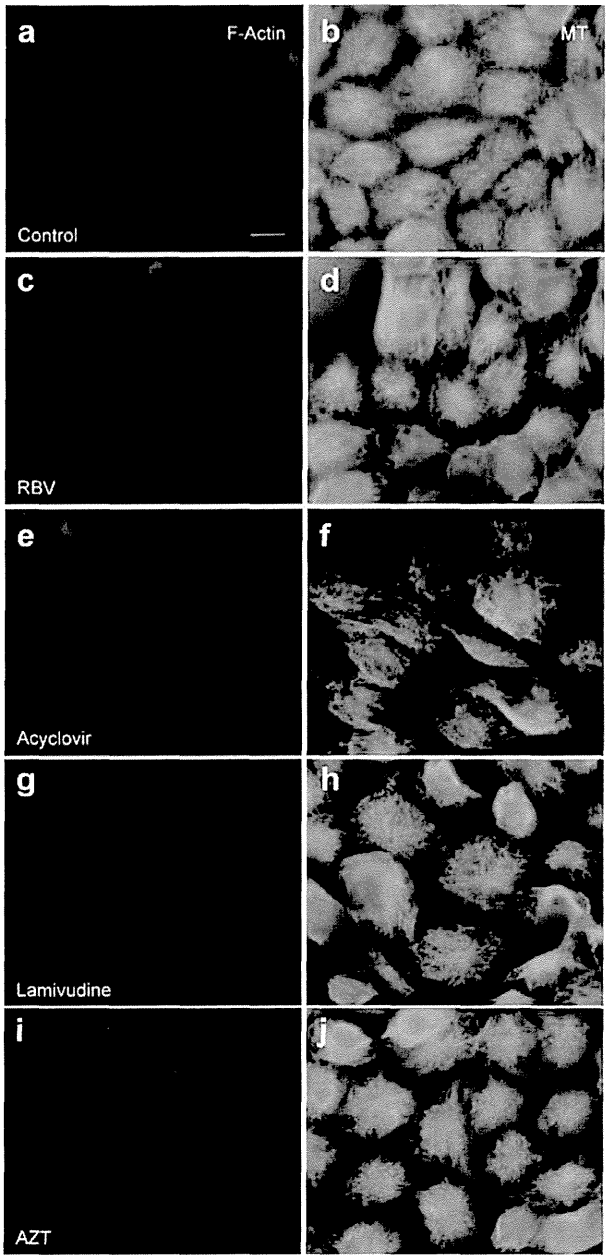

Fig. 4. The four selected antiviral drugs had no effect on actin microfilaments and microtubules. The cells were cultured with the drugs for 20 hr and actin microfilaments and microtubules stained with rhodamine phalloidin and anti-tubulin α mAb, respectively. (a) Actin microfilaments in control cells and in (c) cells cultured with RBV, (e) acyclovir, (g) lamivudine and (i) AZT. (b) Microtubules in control cells and in (d) cells cultured with RBV, (f) acyclovir, (h) lamivudine and (j) AZT (bar: 50 μm).

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DISCLOSURE

The authors declare they have no conflict of interests.

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Short Communication

Phylogenetic Analysis and Seroprevalence of Influenza C Virus in Mie Prefecture, Japan in 2012

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SUMMARY: Reverse transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR were used to detect 14 (6.6%) influenza C virus (InfC) among 213 clinical samples collected from children with respiratory symptoms in Mie Prefecture, Japan, between January 2012 and December 2012. Virus isolation using Madin-Darby canine kidney cells and/or embryonated chicken eggs was also successful for 3 of the 14 PCR-positive samples. Eleven patients (78.6%) were aged <3 years. Phylogenetic analysis of the hemagglutinin-esterase gene showed that the InfC detected in Mie Prefecture belonged to the C/Sao Paulo/82-related lineage. To determine the seroprevalence of InfC, a total of 575 serum samples from patients aged 1 month to 69 years in Mie Prefecture were screened by hemagglutination inhibition test using the C/Mie/199/2012 (C/Sao Paulo/82-related lineage) strain as the antigen. The samples with an antibody titer of $\geq 1:16$ were designated as antibody-positive. The results showed that 53.7% of the 296 serum samples collected in 2011 and 85.3% of the 279 samples collected in 2012 were positive for antibodies against InfC, suggesting that an outbreak of InfC infection occurred in Mie Prefecture in 2012. Therefore, continuous and proactive monitoring is important to determine the number of InfC-infections and to better understand the epidemiology.

Influenza C virus (InfC) is a causative agent of acute respiratory tract infections in children. It was first isolated from a patient with a slight head cold by Dr. Taylor in the United States in 1947 (1). Reportedly, most children acquire antibodies against InfC by approximately 6–7 years of age and children and adults are repeatedly infected with InfC (2–5). In Japan, InfC is isolated mainly from January to June and, where prevalent, particularly between early spring and early summer (6–8). Furthermore, a biennial increase and decrease in the number of InfC infections detected has been observed, suggesting the possibility of periodic outbreaks (9).

According to the National Epidemiological Surveillance of Infectious Diseases (NESID) system, InfC infection was detected in only 226 cases between the 2002–2003 and 2012–2013 seasons in Japan, whereas there were 79,169 and 15,386 type A and B influenza cases, respectively. Therefore, the number of InfC infections reported to the NESID was extremely low compared with the number of type A or B influenza infections (10). However, it is believed that this is due to the fact that few laboratories perform proactive surveys of InfC infection. Thus, at present, the precise distribution of InfC among cases of respiratory tract infections is not well understood. In this study, we report 14 cases of InfC detected in Mie Prefecture, Japan, in 2012 and provide seroprevalence results of InfC among residents of Mie Prefecture.

Clinical samples (nose discharge and pharynx wipes) were collected from pediatric patients who presented at either of two medical institutions with respiratory symptoms in Mie Prefecture between January 2012 and December 2012. Among the 237 clinical samples that were transported to the Mie Prefecture Health and Environment Research Institute, 24 were positive for influenza A and B viruses using rapid antigen tests. Therefore, 213 samples were analyzed for InfC, exempting the 24 influenza A- and B-positive samples. All 213 patients lived in Kameyama City or Tsu City, which are located in the north-central region of Mie Prefecture. Of the 213 samples, 80 were collected from patients diagnosed with upper respiratory tract infections and 133 from patients with lower respiratory tract infections. Each sample was subjected to reverse transcription polymerase chain reaction (RT-PCR) for hemagglutinin-esterase (HE) gene amplification and real-time RT-PCR with a TaqMan probe targeting the NP gene using the methods described by Kimura et al. (11) and

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Matsuzaki et al. (12), respectively. Of the 213 tested samples, 14 (6.6%) were InfC-positive by both RT-PCR and real-time RT-PCR. Of the 14 InfC-positive samples, 12 were obtained from patients residing in Kameyama City and 2 from patients residing in Tsu City. PCR was used to detect the presence of other viruses, as described previously (13-20). In addition to InfC, a total of 155 viruses were detected, which included the following: human rhinovirus (n = 43), human metapneumovirus (n = 36), human parainfluenza virus (n = 30), RS virus (n = 25), human coronavirus (n = 11), human bocavirus (n = 8), and influenza virus (AH3) (n = 2). The remaining 44 samples were negative for all tested viruses.

Next, we isolated viruses from the InfC-positive samples using Madin-Darby canine kidney (MDCK) cells. In brief, the cells were inoculated and then incubated for 7-10 days, and the conditioned media collected from the cultures were then examined for hemagglutination activity (HA) using 0.75% guinea pig erythrocytes and 0.5% chicken erythrocytes. Only samples that were HA-positive in chicken erythrocytes and HA-negative in guinea pig erythrocytes were considered as positive for InfC. Two samples were found to be HA-positive in chicken erythrocytes after propagating twice or thrice in MDCK cells (C/Mie/185/2012 and C/Mie/199/2012). A hemagglutination inhibition (HI) assay was performed using antiserum against C/Ann Arbor/1/50, which was obtained from Yamagata University Faculty of Medicine, to identify the InfC strain. Moreover, 2 InfC strains were isolated from clinical samples using embryonated chichen eggs (C/Mie/199/2012 and C/Mie/231/2012).

The clinical characteristics of the 14 InfC-positive children are presented in Table 1. InfC infections were detected in children aged 4 months to 6 years. Eleven patients (78.6%) were aged <3 years old. The ratio of males to females was 1:1. Body temperatures on initial examination ranged from 36.5°C to 39.8°C. These patients were diagnosed with bronchitis (n = 2), bronchiolitis (n = 7), laryngitis (n = 2), pharyngitis (n = 2), and tonsillitis (n = 1). InfC was detected in 6.8% (9 cases in 133 samples) of patients diagnosed with lower respiratory tract infections, which was similar in frequency among patients diagnosed with upper respiratory tract infections (6.2%; 5 cases in 80 samples). A previous report indicated that most patients with InfC infections were aged <6 years and many patients aged <2 years (approximately 30%) required hospitalization, mainly for pneumonia, bronchitis, or bronchiolitis (6). Therefore, it is essential to consider the potential for increased severity in patients with InfC infection, as InfC was detected in patients with pneumonia from Mie Prefecture in 2011 (21).

The HE gene was sequenced in the 14 InfC-positive samples to determine the lineage of the circulating viruses. Total RNA was extracted from each clinical sample for sequencing analysis; however, RNA extraction from C/Mie/185/2012 and from C/Mie/199/2012 and C/Mie/231/2012 were performed with each virus isolated from MDCK cells and chicken eggs, respectively. Amplification and sequencing of the region from nucleotide 19 to 1083 of the HE gene were performed using the methods described by Kimura et al. (11). The nucleotide sequences were submitted to the DDBJ/GenBank databases and assigned the accession numbers AB751364-AB751365, AB751594-AB751603, and AB811846-AB811847. A phylogenetic tree of the HE gene was constructed using these 14 sequences together with previously published sequences (Fig. 1). Matsuzaki et al. classified the InfC HE gene into five lineages, which are represented by C/Yamagata/26/81 (D28971), C/Kanagawa/1/76 (D63470), C/Aichi/1/81 (D28970), C/Mississippi/80 (M11640), and C/Sao Paulo/378/82

Table 1. Characteristics of patients with laboratory-confirmed influenza C virus infection

Patient	Gender	Age[1)]	Date of onset	Date of sample collection	Clinical diagnosis	Body temperature (°C)	Laboratory examination			Strain name (GenBank accession no.)
							Virus isolation		RT-PCR	
							MDCK[2)]	Egg[3)]		
1	M	1Y	19 Jan. 2012	24 Jan. 2012	Bronchiolitis	38.8	Negative	Negative	Positive	C/Mie/67/2012 (AB751600)
2	M	3Y	12 Mar. 2012	13 Mar. 2012	Bronchiolitis	39.3	Negative	Negative	Positive	C/Mie/180/2012 (AB751603)
3	F	6Y	8 Mar. 2012	14 Mar. 2012	Bronchiolitis	39.5	Negative	Negative	Positive	C/Mie/182/2012 (AB751602)
4	F	1Y	14 Mar. 2012	15 Mar. 2012	Bronchiolitis	38.5	Positive	Negative	Positive	C/Mie/185/2012 (AB751598)
5	F	5Y	14 Mar. 2012	15 Mar. 2012	Laryngitis	36.9	Negative	Negative	Positive	C/Mie/186/2012 (AB751364)
6	M	1Y	14 Mar. 2012	15 Mar. 2012	Laryngitis	39.8	Negative	Negative	Positive	C/Mie/188/2012 (AB811846)
7	F	2Y	17 Mar. 2012	19 Mar. 2012	Tonsillitis	39.6	Negative	Negative	Positive	C/Mie/193/2012 (AB751597)
8	M	4M	19 Mar. 2012	21 Mar. 2012	Bronchiolitis	37.9	Negative	Negative	Positive	C/Mie/198/2012 (AB751601)
9	F	2Y	19 Mar. 2012	21 Mar. 2012	Bronchitis	36.5	Positive	Positive	Positive	C/Mie/199/2012 (AB751596)
10	M	2Y	23 Mar. 2012	23 Mar. 2012	Pharyngitis	39.2	Negative	Negative	Positive	C/Mie/204/2012 (AB751599)
11	F	1Y	2 Apr. 2012	3 Apr. 2012	Pharyngitis	38.6	Negative	Positive	Positive	C/Mie/231/2012 (AB811847)
12	M	8M	17 Apr. 2012	19 Apr. 2012	Bronchitis	38	Negative	Negative	Positive	C/Mie/265/2012 (AB751595)
13	M	1Y	18 Apr. 2012	23 Apr. 2012	Bronchiolitis	39	Negative	Negative	Positive	C/Mie/275/2012 (AB751594)
14	F	1Y	21 Apr. 2012	23 Apr. 2012	Bronchiolitis	39.7	Negative	Negative	Positive	C/Mie/277/2012 (AB751365)

[1)]: Y, year old; M, month old.
[2)]: MDCK, Madin-Darby canine kidney.
[3)]: Egg, embryonated chicken eggs.
For sequencing, RNA was extracted from the clinical sample; however, RNA extraction from C/Mie/185/2012 and from C/Mie/199/2012 and C/Mie/231/2012 were performed with each virus isolated using MDCK cells and chicken eggs, respectively.

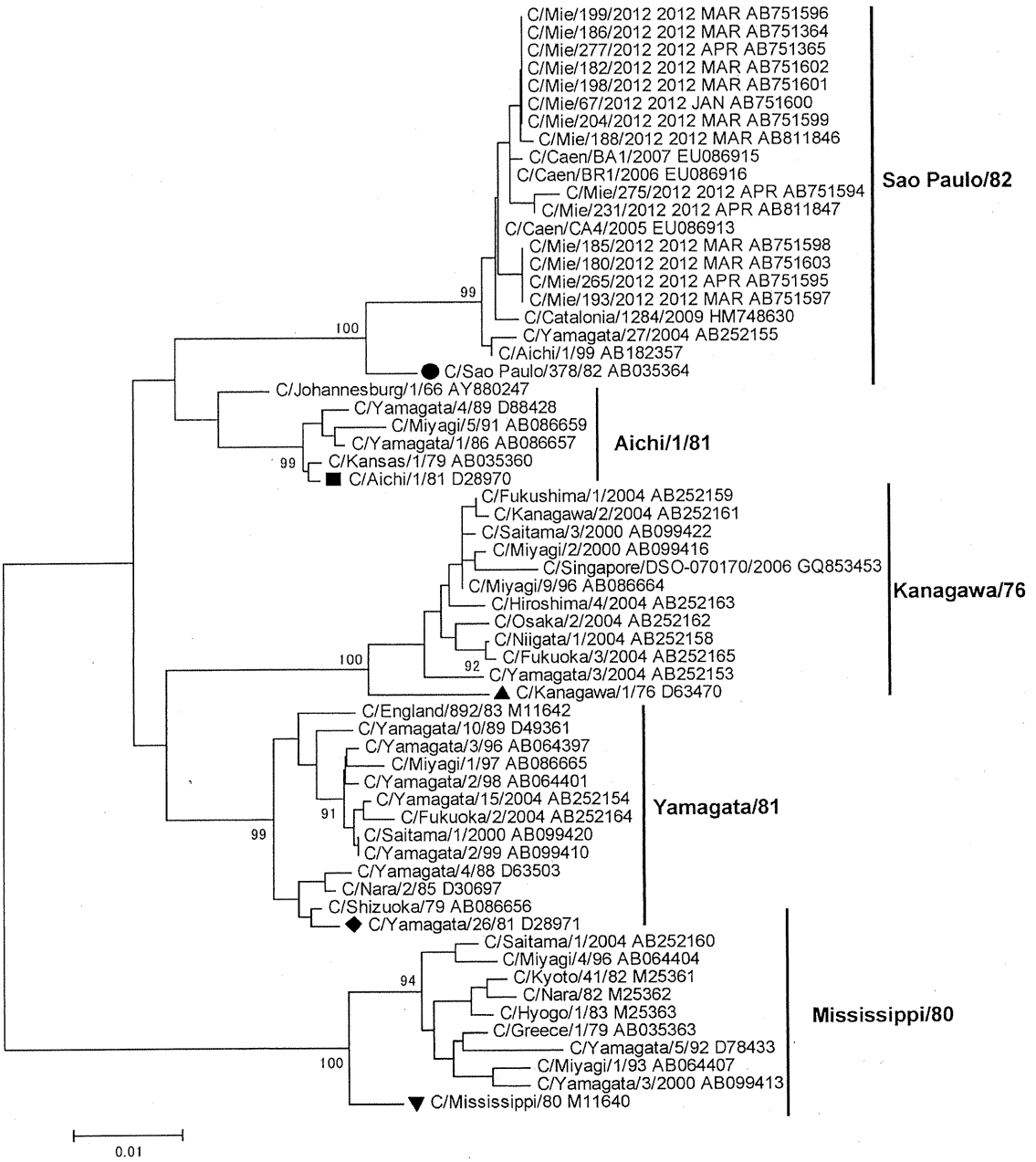

Fig. 1. Phylogenetic analysis of the HE genes of influenza C viruses detected in Mie Prefecture, Japan, in 2012. The region from nucleotides 64 to 965 was used for analysis. The tree was constructed using neighbor-joining method. Figures at the nodes indicate confidence levels of bootstrap analysis using 1,000 replicates as percentage value. Reference strains: Sao Paulo/378/82 (AB035364), Aichi/1/81 (D28970), Kanagawa/1/76 (D63470), Yamagata/26/81 (D28971), Mississippi/80 (M11640).

(AB035364) strains, and reported that multiple lineages of InfC were co-circulating in a community (22,23). Our results showed that the 14 strains detected in Mie Prefecture in 2012 belonged to the Sao Paulo/82-related lineage, which is represented by the C/Sao Paulo/378/82 strain. Although InfC belonging to the Sao Paulo/82-related lineage was detected in Yamagata in 2004, InfC belonging to the Kanagawa/76-related lineage was predominantly detected throughout Japan around 2004 (24). InfC belonging to the Sao Paulo/82-related lineage was also detected in France from 2005 to 2007 (25), and in Spain in 2009 (26).

Serological analysis of InfC was performed using serum samples collected from 575 persons aged 1 month to 69 years who visited hospitals located in Mie Prefecture for a medical examination (249 persons) or medical checkup (326 persons) between April and September 2012 (279 samples) and from April to September 2011 (296 samples). All serum samples were tested after obtaining informed consent from the patients or their guardians. This seroprevalence study was conducted while protecting the personal information of each participant. Antibody levels were determined by measuring serum HI titers, which were expressed as the reciprocal of the highest serum dilution that inhibited hemagglutination. The C/Mie/199/2012 strain propagated in chicken eggs was used as the antigen for the HI test and HI titration was performed as described previously (2).

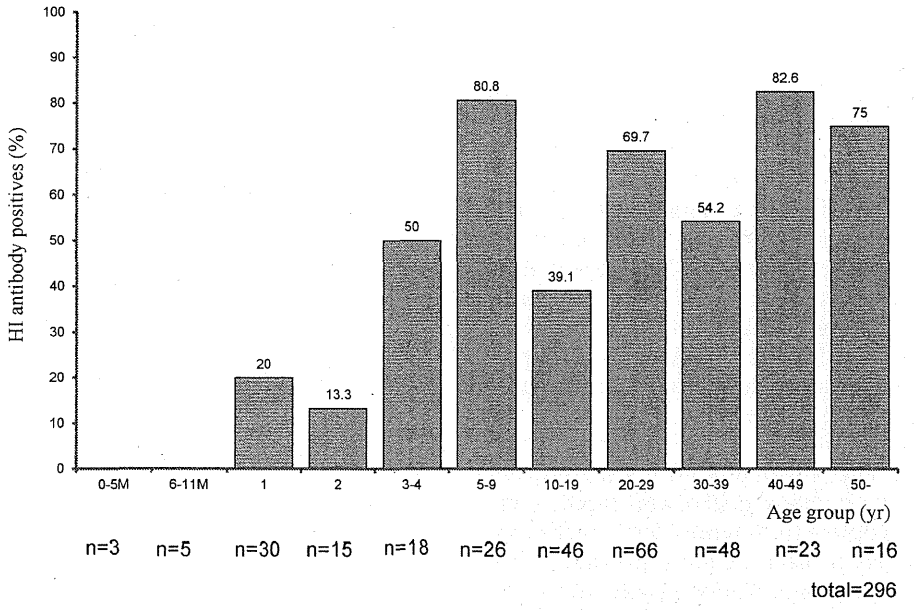

Fig. 2. Age group distribution of InfC hemagglutination inhibition (HI) antibody positives (≥1:16) in 2011.

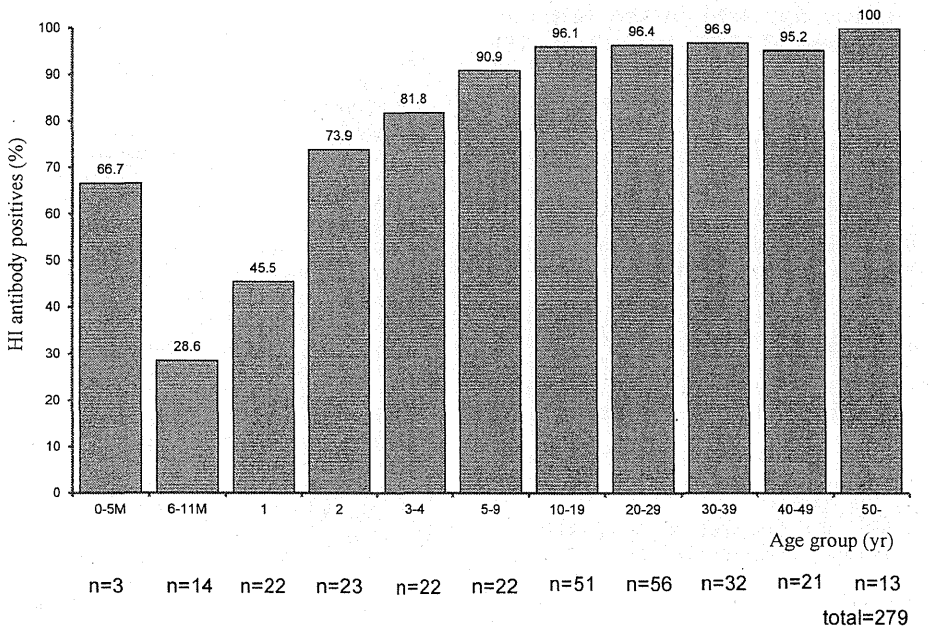

Fig. 3. Age group distribution of InfC hemagglutination inhibition (HI) antibody positives (≥1:16) in 2012.

The samples with an antibody titer of ≥1:16 were designated as antibody-positive. The results showed that 53.7% of the 296 serum samples collected in 2011 and 85.3% of the 279 samples collected in 2012 were InfC antibody-positive (Figs. 2 and 3). This finding appears to reflect an outbreak of InfC within Mie Prefecture in 2012, although too few serum samples were obtained from infants aged 6–11 months in 2011 to claim that InfC was not in circulation in 2011. Because the serum samples in Fig. 3 were collected from the period immediately after the circulation of InfC in Mie Prefecture in 2012, we believe that this sample size was sufficient to confirm an outbreak of InfC. Although further annual seroprevalence data are needed to define the periodicity of InfC transmission, this is the first seroepidemiological study of InfC performed over two continuous seasons. As shown in Fig. 3, the antibody retention ratio in 2012 was lowest (28.6%) in children aged 6–11 months and increased with age until it reached 96.1% in the 10–19 years age group, suggesting that all the children had been exposed to InfC by the age of 10 years. Although a relatively small number of serum samples were obtained from infants aged 0–5 months, there was a considerable difference in the seroprevalence in this age group from 2011 to 2012. Because the prevalence of antibodies in infants aged 0–5 months is influenced by maternal antibodies, the difference in the prevalence of antibodies in this age group (66.7% in 2012 vs. 0% in 2011) may have been due to the difference in seroprevalence among the mothers in the age group 20–29 and 30–39 years. The high prevalence of antibodies among adults in 2012 indicates that reinfection must have been

common, an observation that supports findings previously reported in Japan (2,27-29).

A local InfC outbreak occurred in the north-central region of Mie Prefecture in 2012, a year in which only 60 cases of InfC infection were reported throughout Japan (30). Patients with InfC infection were reported in Yamagata, Niigata, Hiroshima, and Mie Prefectures and Shizuoka City. In Mie Prefecture, 13 of 14 cases of InfC infection were detected between March and April 2012. There have been no reports of InfC cases after May 2012 in our prefecture, although 19 cases were detected between May and July 2012 in Yamagata Prefecture. Thus, the mechanism of nationwide epidemics of InfC infection is not yet sufficiently understood. The identification of InfC infection based on clinical symptoms is difficult (6). The real-time RT-PCR method (12) used in this study was highly sensitive and rapid; therefore, it is a useful method to diagnose InfC infections. InfC infection is likely to become more prevalent, thus continuous and proactive monitoring studies are highly important to monitor the number of patients with InfC infection and to understand its epidemiology.

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Conflict of interest None to declare.

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三重県における急性呼吸器症状を呈した小児から検出されたコロナウイルス（HCoV-OC43）

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短　報

三重県における急性呼吸器症状を呈した小児から検出されたコロナウイルス（HCoV-OC43）

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Key words: Human coronavirus, acute respiratory infection (ARI), child

序　文

コロナウイルス（Human coronavirus：HCoV）は4つの血清型（α，β，γ，δ）に分類され，現在，ヒトにおいて主に流行しているHCoVはα・コロナウイルスの229EおよびNL63，β・コロナウイルスのOC43およびHKU1の4種である[1]．本ウイルスは普通感冒の主要なウイルスとされ，上気道炎症状および鼻症状を主とする呼吸器症状[2][3]を呈するとされているが，血清型別の流行状況等，依然として不明な点が多い感染症である．そこで我々は，流行疫学を把握するために，2013年に流行[1]を捉えたHCoV-OC43に関して遺伝子系統樹解析を実施したので報告する．

対象と方法

2013年1～6月に三重県感染症発生動向調査において県内の定点医療機関を受診した小児呼吸器感染症患者128名の患者検体（鼻汁，咽頭拭い液，気管吸引液）を呼吸器系ウイルス検査の対象とした．HCoVは，従来法の細胞によるウイルス分離が困難であり[5]，今回，RT-PCR法によるHCoV遺伝子検査[5][7]を実施した．患者検体から抽出したRNAは使用時まで－80℃に保存した．Spike glycoprotein領域の系統樹解析は一部のHCoV-OC43についてKonらの方法[5]により実施した．なお，対象とした小児の保護者から患者情報および患者検体の使用に関する承諾書への署名を得た．検査依頼医療機関記入の調査票（患者情報）の使用にあたっては，個人情報保護等について倫理的配慮をした．

結　果

HCoVは，HCoV-HKU1およびHCoV-NL63が各々1名から検出された．一方，HCoV-OC43は29名（22.7%）から検出された（RSウイルスとの重複感染2名含む）．これらのHCoV-OC43陽性者の検体採取時期は1月12名，2月8名，3月4名，4月4名，5月1名であった．年齢構成は0歳9名，1歳7名，2歳3名，4歳3名，5歳3名，6歳2名，7歳1名，8歳1名であった．臨床診断名は気管支炎11名（37.9%），咽頭炎10名（34.5%），細気管支炎6名（20.7%），扁桃炎およびRS感染症疑い2名（6.9%）であった．臨床症状は鼻汁が25名（86.2%）で最も多く，咳嗽8名（27.6%），その他（喘鳴，咳，頭痛）が3名（10.3%）でみられ，受診時の平均体温は38.6±1.2℃であった．また，呼吸器症状に加え消化器症状を伴っていた患児は8名（27.6%）であった．対象者29名のうち10名から検出されたHCoVについて遺伝子系統樹解析を実施した結果，2つのGroupに分類され，Group1の検体採取時期は1月3名，2月2名，4月1名の計6名，Group2は1月1名，2月2名，3月1名の計4名であった（Fig. 1）．なお，対象とした128名から検出された他のウイルスは，RSウイルス15名，パラインフルエンザウイルス3型12名，ライノウイルス12名，ヒューマンメタニューモウイルス11名，ボカウイルス5名等であった．

考　察

HCoVは，比較的な軽微な臨床症状を呈する[2][3]ことが多いため，医療機関への受診率の影響も背景にあり，積極的なHCoV動向調査が実施できていない．国立感染症研究所の感染症サーベイランスシステム（NESID）による過去10シーズンのHCoV国内検出報告数[8]は309件（2014年1月末現在）と極めて少数で国内の患者数および流行像は十分に把握されていない．国内ではHCoVの血清型までが明らかにされることは稀で，これまでの調査報告は少数である．本県では

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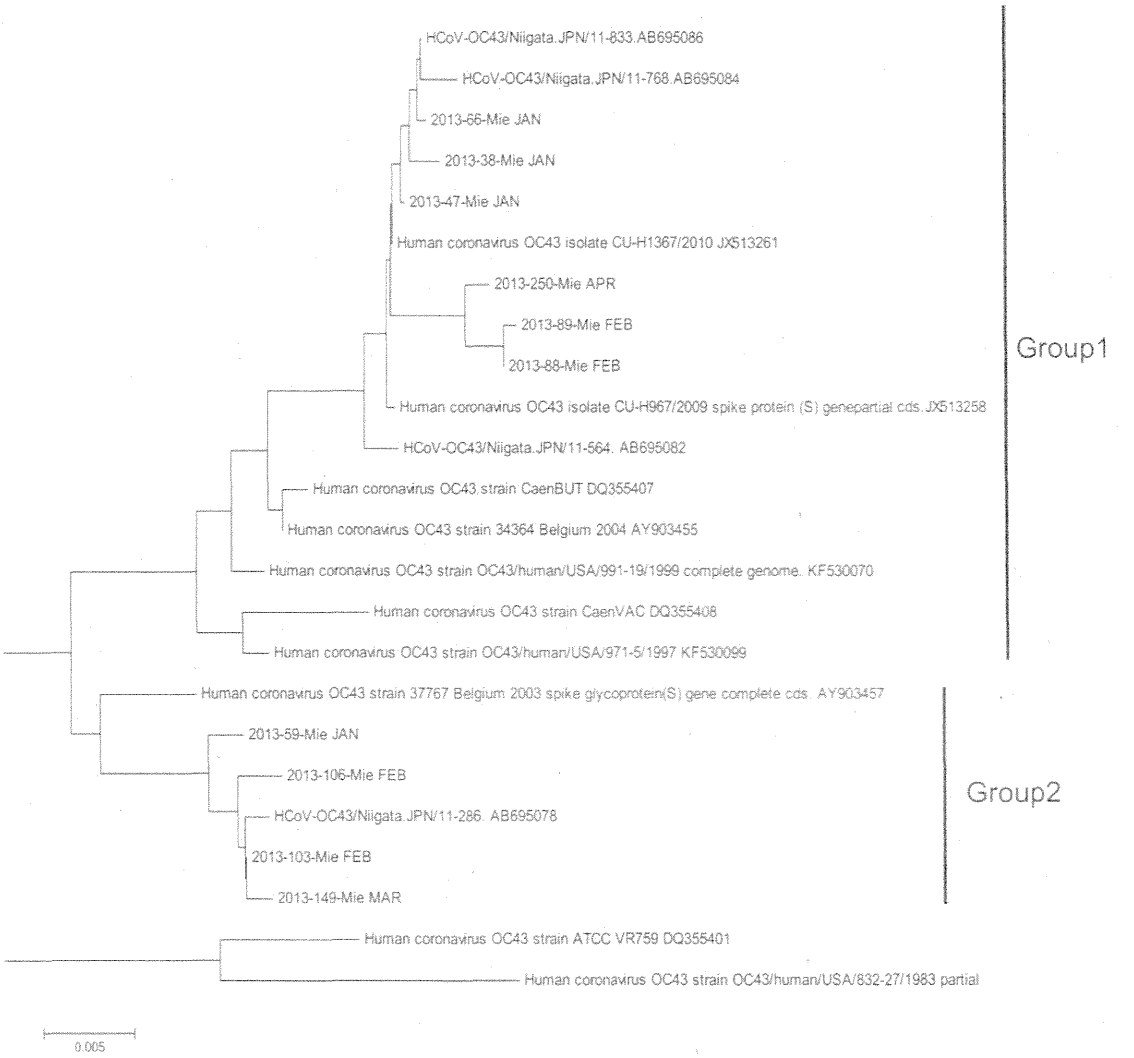
Fig. 1　Phylogenetic tree of HCoV-OC43 Spike glycoprotein genes.



積極的な調査を実施した結果，2013年1～4月にHCoV-OC43が多数検出され，地域流行を捉えたものと推察される．これらのHCoV罹患者の17名(58.6%)は下気道炎症状を呈しており重症化も懸念される．さらに消化器症状を伴った患児が散見されたが，我々は1997～1998年に県内2施設での集団感染事例の急性胃腸炎患者からコロナ様ウイルスを分離[9]している．他の報告事例においても小児患者の糞便から検出[10][11]されていることからも消化器症状についても注視する必要がある．このようにHCoVは様々な臨床症状を呈した患児から検出されており臨床所見による診断は容易ではない．

本事例の遺伝子系統樹解析により，少なくとも2つのGroupが混在流行していたことを示唆する知見を得たが，罹患者の臨床症状，検出時期および年齢には明瞭な差異は認められなかった．HCoV感染症の流行疫学を理解するためには，積極的な全国規模のモニタリングを継続的に実施することが有用であると考えられた．

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利益相反自己申告：申告すべきものなし

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Detection of Human Coronavirus OC43 in Children with Acute Respiratory Infections in Mie, Japan

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呼吸器症状を呈した小児から検出されたヒトボカウイルスの流行疫学および遺伝子系統樹解析（2010～2013年）

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原著

呼吸器症状を呈した小児から検出されたヒトボカウイルスの流行疫学および遺伝子系統樹解析（2010～2013年）

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要旨　三重県感染症発生動向調査事業において，2010～2013年に三重県内の医療機関を受診した小児呼吸器感染症患者763名を対象にHBoVの流行疫学を把握するために調査を実施した．

HBoV陽性者は763名中25名(3.3%)であった．採取年別のHBoV陽性者数は2010年4名，2011年5名，2012年9名，2013年7名であった．HBoV陽性者は春から初夏に多数検出される傾向であった．

はじめに

ヒトボカウイルス（human bocavirus：HBoV）は，パルボウイルス科パルボウイルス亜科ボカウイルス属に分類されており，2005年にスウェーデンの呼吸器感染症患者から初めて発見され[1]，その後，世界的に分布していることが判明した[2~6]急性呼吸器感染症（acute respiratory infections：ARI）の起因ウイルスの一つであるが，依然として不明な点が多くある．現在HBoVは4タイプ（HBoV1，2，3，4）に分類されており，近年国内においてもHBoV検出報告[7~10]がされているが，患者臨床情報などの報告は少なく臨床的意義は明らかではない．そこでわれわれは，三重県内のHBoV流行疫学を把握するために，検出状況（検出時期，臨床診断名など）および遺伝子系統樹解析を実施したので報告する．

Ⅰ．材料と方法

2010年1月～2013年12月に三重県感染症発生動向調査事業において，県内の医療機関（8施設）を外来受診した小児呼吸器感染症患者763名から得られた患者臨床検体763件（鼻汁，咽頭拭い液，気管吸引液）をHBoV調査の対象とした．これらの対象者の年齢は0～13歳である．**表1**に年齢区分別の対象者数を示した．採取年別の検査数は2010年88名，2011年225名，2012年213名，2013年237名であった．

患者臨床検体から抽出したDNAは使用時まで−80℃に保存し，HBoV遺伝子検査にはAllanderらのConventional-PCR法[11]およびHBoV-LAMP法[12]を実施した．

Key words：急性呼吸器感染症，ヒトボカウイルス，遺伝子系統樹解析
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表 1 対象者の年齢（2010～2013 年）

年齢（歳）	採取年				計
	2010 年	2011 年	2012 年	2013 年	
0	27	47	37	46	157
1	30	77	88	99	294
2	6	43	33	39	121
3	10	30	22	21	83
4	4	8	16	11	39
5	2	10	5	8	25
6～	9	10	12	13	44
計	88	225	213	237	763

検出された一部の HBoV について，既報[13]に準じて VP1/VP2 領域の遺伝子について塩基配列解析を行った．遺伝子系統解析には，塩基配列解析ソフトウェア Molecular Evolutionary Genetics Analysis (MEGA) version 5 を用いた．遺伝子系統樹作成には，近隣結合法（Neighbor-Joining：NJ 法）により行った．Bootstrap test は 1,000 回行った．HBoV 以外の呼吸器系ウイルスの検出には，既報および国立感染症研究所のウイルス検出マニュアルに準拠し，インフルエンザウイルス[14]，RS ウイルス[15]，ヒトメタニューモウイルス[16]，パラインフルエンザウイルス[17,18]，コロナウイルス[19,20]，アデノウイルス[21]，ライノウイルス[22]を対象とし，各ウイルスの特異的遺伝子を PCR 法により検出を試みた．なお，対象となった小児の保護者から患者情報および患者検体の使用に関する承諾書への署名を得た．検査依頼医療機関記入の調査票（患者情報）の使用にあたっては，倫理的配慮として，個人情報保護などに留意し実施した．

II．結　果

2010～2013 年に採取された 763 名について HBoV の検査を実施した結果，25 名（3.3%）から検出された（表 2，3）．HBoV 陽性者の年齢構成は 0 歳 5 名（20%），1 歳 17 名（68%），2 歳 3 名（12%）であった．年別の HBoV 陽性者数は 2010 年 4 名，2011 年 5 名，2012 年 9 名，2013 年 7 名であった．これらの HBoV 陽性者の検体採取月は主に 4～6 月に多数検出され，各年における検出数（4～6 月）は，2010 年 2 名（50%），2011 年 4 名（80%），2012 年 6 名（67%），2013 年 6 名（86%）と春から初夏に検出される傾向が認められた．なお，HBoV が検出された 25 名中 8 名（32%）は，他のウイルスとの重複検出例であった．その内訳はヒトメタニューモウイルス 6 名，RS ウイルス 1 名，パラインフルエンザウイルス 1 型（1 名）であった．

臨床診断名は気管支炎 12 名（48%），咽頭炎 7 名（28%），細気管支炎 3 名（12%），その他（扁桃炎 1 名，中耳炎 1 名，インフルエンザ様疾患 1 名）が 3 名（12%）であった．

HBoV 陽性者の受診時の平均体温は 38.4±1.1℃であった．37℃台 6 名（24%），38℃台 9 名（36%），39℃台 6 名（24%），40℃台 2 名（8%），不明 2 名であった．37.5℃以上の発熱は 19 名（76%）で，このうち 12 名（48%）は 38.5℃以上であった．

塩基配列解析が可能であった HBoV 陽性者 19 名について VP1/VP2 領域の系統樹解析を実施した結果，すべて HBoV1 に分類された（図 1）．これらの HBoV1 は遺伝子系統樹上では 3 つの Group に分類され，その内訳は Group 1（5 名），Group 2（13 名），Group 3（1 名）であった（図 2）．

III．考　察

2010 年に国立感染症研究所より「ボカウイルス検査マニュアル」[11]が示され，同研究所の感染症サーベイランスシステム（NESID）の集計によると，2010～2013 年までの国内の HBoV 陽性者[8]は 677 名（2014 年 3 月末現在）と少数の報告であり，国内の患者数および流行像は十分に把握されていないと思われる．本県においても，従来から実施してきた呼吸器系ウイルスの感染症発生動向調査[23～26]の検査項目に 2010 年から HBoV 検査を追加したところ，2013 年までの 4 年間に HBoV が 25 名（3.3%）から検出された．NESID の全国集計では，2010～2013 年の HBoV 陽性者 677 名中 383 名（56.6%）は 4～6 月に検出されている．本県においても，HBoV 陽性者 25 名のうち 4～6 月に 18 名（72%）から検出され，主として春から初夏が流行期であることが推察される．重複検出の

表 2　HBoV 陽性者患者情報（2010～2013 年）

	検体 No	年齢	性別	採取日	臨床診断名	受診時体温	検出ウイルス
1	2010-131	0 歳 11 カ月	男	2010.4.21	気管支炎	40℃	HBoV & HMPV
2	2010-150	0 歳 8 カ月	男	2010.5.17	気管支炎	37.4℃	HBoV & HMPV
3	2010-240	1 歳	男	2010.7.14	気管支炎	39.5℃	HBoV & HPIV
4	2010-353	1 歳	女	2010.10.19	咽頭炎	39.5℃	HBoV
5	2011-222	1 歳	男	2011.5.11	気管支炎	38.0℃	HBoV & HMPV
6	2011-228	1 歳	男	2011.5.13	気管支炎	38.4℃	HBoV & HMPV
7	2011-233	0 歳 8 カ月	男	2011.5.18	気管支炎	37.8℃	HBoV & HMPV
8	2011-270	1 歳	女	2011.6.6	気管支炎	39.6℃	HBoV
9	2011-427	1 歳	男	2011.8.2	気管支炎	38.7℃	HBoV
10	2012-137	1 歳	男	2012.2.22	細気管支炎	37.4℃	HBoV
11	2012-213	2 歳	男	2012.3.27	咽頭炎	37.8℃	HBoV
12	2012-245	1 歳	男	2012.4.10	咽頭炎	38.6℃	HBoV
13	2012-256	1 歳	女	2012.4.16	細気管支炎	38.5℃	HBoV
14	2012-289	1 歳	男	2012.5.1	滲出性扁桃炎	38.0℃	HBoV & HMPV
15	2012-300	2 歳	男	2012.5.9	気管支炎	37.2℃	HBoV
16	2012-321	1 歳	男	2012.5.21	インフルエンザ様疾患	不明	HBoV
17	2012-352	0 歳 9 カ月	女	2012.6.12	細気管支炎	不明	HBoV
18	2012-602	1 歳	男	2012.11.15	気管支炎	37.0℃	HBoV
19	2013-240	2 歳	男	2013.4.19	気管支炎	40.5℃	HBoV
20	2013-264	0 歳 11 カ月	男	2013.4.26	咽頭炎	39.0℃	HBoV
21	2013-277	1 歳	男	2013.5.7	気管支炎	39.3℃	HBoV
22	2013-302	1 歳	男	2013.5.13	中耳炎	38.2℃	HBoV
23	2013-377	1 歳	男	2013.6.10	咽頭炎	38.4℃	HBoV
24	2013-421	1 歳	女	2013.6.24	咽頭炎	39.8℃	HBoV & RSV
25	2013-439	1 歳	女	2013.7.3	咽頭炎	38.7℃	HBoV

human bocavirus：HBoV，human metapneumo virus：HMPV，human parainfluenza virus：HPIV，respiratory syncytial virus：RSV

8 名においても 4 月 1 名，5 月 5 名，6 月 1 名，7 月 1 名と同様の傾向を示している．

本研究で解析した HBoV1 の塩基配列相同性は高く，HBoV1 内の genotypes を分類することの臨床的意義などは不明であるが，経年動向を把握するために HBoV1 陽性者の採取年別の Group 分類を実施した．Group 1（5 名）は少数であるが，2010 年 1 名，2011 年 2 名，2012 年 1 名，2013 年 1 名と各年で検出された．Group 2（13 名）は 2010 年 1 名，2011 年 1 名，2012 年 7 名，2013 年 4 名から検出され，2012 年および 2013 年に集中しており，県内での同 Group の流行が推測された．一方，Group 3 は 2011 年（1 名）のみであった．今後の経年的な調査によって HBoV1-genotypes の変遷などが解明されることにより，流行地域の流行疫学的特徴が明らかになることが考えられた．

表 3　検体採月別 HBoV 陽性者数（2010～2013 年）

採取月	採取年				計（%）
	2010 年	2011 年	2012 年	2013 年	
1 月					0　(0%)
2 月			1		1　(4%)
3 月			1		1　(4%)
4 月	1		2	2	5　(20%)
5 月	1	3	3	2	9　(36%)
6 月		1	1	2	4　(16%)
7 月	1			1	2　(8%)
8 月		1			1　(4%)
9 月					0　(0%)
10 月	1				1　(4%)
11 月			1		1　(4%)
12 月					0　(0%)
計	4	5	9	7	25

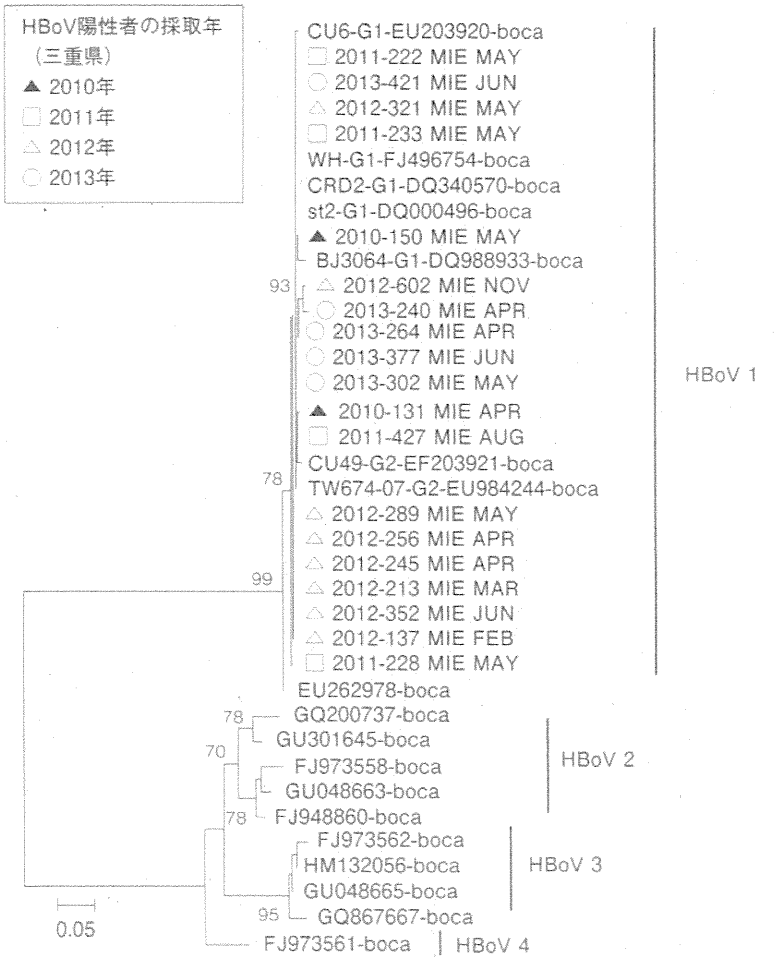

図 1　検出された HBoV の型別（2010～2013 年）

HBoV 遺伝子系統樹内の番号（MIE）は，表 2 の検体 No に一致する．

HBoV は，さまざまな呼吸器症状を呈した患児から検出されており，臨床所見による診断は容易ではないと思われる．呼吸器系症状だけでなく，消化器症状を呈した患者の糞便から検出報告[27,28]があることからも注目する必要がある．また下気道炎症状からの HBoV の検出報告[29]がされており，本調査においても HBoV 陽性者 15 名（60%）が下気道炎症状を呈していたため，注視が必要だと考えられる．

さらに，HBoV は小児の集中治療室での院内感染による ARI 感染症発生時のスクリーニングの対象疾患とすることを考慮すべきと示唆[30]されている．しかしながら，現在のところ医療機関で迅速検査可能な HBoV 簡易診断キットは開発されておらず，苦慮するところである．

また HBoV 血中抗体調査によると，6 歳児までに，ほぼ 100% で HBoV 抗体を獲得しているとの報告[31,32]がある．本県で HBoV が検出された陽性者の年齢分布は 0～2 歳児までの低年齢の小児で多くみられたことは，過去の国内外の報告[1,9,29]と一致しており，小児における HBoV 抗体の獲得が陽性者の年齢分布に反映されていることが示唆された．

本調査では他のウイルスとの重複検出例は，8 名（32%）で確認されている．すべての呼吸器感染症起因病原体の検索には至ってはいないが，

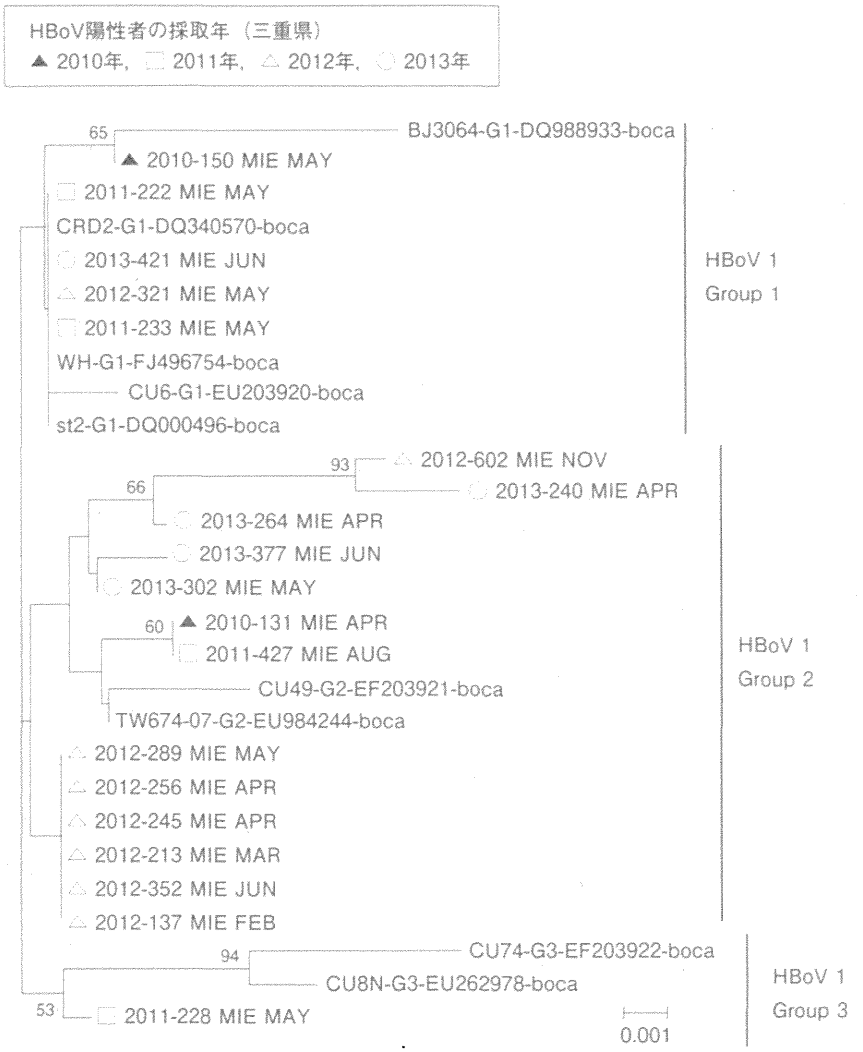

図 2　HBoV1 の遺伝子系統樹解析（2010～2013 年）

HBoV 遺伝子系統樹内の番号（MIE）は，表 2 の検体 No に一致する．

HBoV と他のウイルスの重複検出における臨床的意義を含め不明な点が多いと思われる．HBoV 感染症を理解するためには，全国規模のモニタリングを積極的かつ継続的に実施し，流行疫学および患者臨床情報の蓄積が必要であり，今後の HBoV 調査課題であると考えられた．

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日本小児感染症学会の定める利益相反に関する開示事項はありません．

文　献

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