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RESEARCH LETTER

A longitudinal study on respiratory viral infection for healthy volunteers

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1 | BACKGROUND

Human-to-human transmission by means of droplets is an important route of infection for respiratory viruses.[1,2] Therefore, outbreaks are thought to spread via asymptomatic and/or mildly symptomatic infected persons. We reported previously that several viruses were detected in children regardless of their health condition.[3] Respiratory tract viral infection is one of the most common and important diseases of children. When we consider their range of movement, adults might spread viruses to the community more easily than children. Although asymptomatic rates may vary depending on the host's age and the virus, little is known about what respiratory viruses are present in adults with subclinical or mild symptoms.

In this study, we investigated how often, how long, and what respiratory viruses were present in asymptomatic adults. Gargle samples obtained by rinsing the throat with distilled water were collected from participants once a week and subjected to two-step real-time PCR to detect respiratory viruses. Sixteen singleplex real-time PCRs were employed for the detection of the following respiratory viral pathogens: parainfluenza viruses (PIVs) 1, 2, 3, and 4, respiratory syncytial virus (RSV), human metapneumovirus (hMPV), enterovirus (EV)/rhinovirus (RV), human bocavirus (hBoV), human parechovirus (hPeV), adenovirus (AdV), human coronaviruses (hCoV) OC43, NL63, 229E, and HKU-1, and influenza virus (FluV) type C, and one-step real-time reverse transcription (RT)-PCR was used for detection FluV A and B.[3–5]

2 | METHODS

2.1 | Subjects

Before beginning the study, we explained the study plan and methods to the healthy volunteers of one office and obtained written informed consent from them. From week 45 of 2017 through week 12 of 2018, throat gargle samples were obtained from 11 healthy participants once a week. The first collection of samples in 2018 took place on January 4 after the New Year holidays. If the gargling specimen could not be collected on the scheduled day, a viral detection test was conducted if it could be collected the next day. When we could not obtain a specimen the next day either, the subject was not tested (NT) in the week concerned. To preserve personal profile, each participant was assigned a code ID number and the analysis was conducted using only this number for identification, although the age and sex were linked to the code number as indicated in Table 1. In addition, we prepared a questionnaire that the participants used each time to answer questions about their respiratory and systemic symptoms in the week prior to sampling. We also obtained some information about the existence of respiratory and/or systemic symptoms in their family members from the questionnaire.

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TABLE 1 Prevalence of respiratory viruses in gargle specimens of participants

ID	Age (years)	Sex	Total no. of samples	Condition (n)	Prevalence% (positive samples)	Sampling dates (M/D), viral species, and Ct value (viral load Log10 copies/uL)		
01	41	F	19	Asymptomatic (5)	20.0 (1)	3/26 Ad Ct: 42.25 (0.92)		
				Symptomatic (14)	14.3 (2)	1/4 HKU-1 Ct:37.99 (6.16)	1/9 HKU-1 Ct:40.05 (4.87)	
02	29	F	21	Asymptomatic (0)	0.0 (0)			
				Symptomatic (21)	14.3 (3)	11/8 RVA24 Ct:34.86 (7.18)	11/27 RVA24 Ct:32.84 (8.55)	12/4 RVA24 Ct: 34.96 (7.11)
03	43	F	21	Asymptomatic (9)	0.0 (0)			
				Symptomatic (12)	8.3 (1)	1/4 PIV2 Ct:36.99 (6.92)		
04	40	F	21	Asymptomatic (12)	8.3 (1)	2/19 229E Ct:24.47 (14.70)		
				Symptomatic (9)	22.2 (2)	12/25 HKU-1 Ct:35.95 (7.43)	1/22 FluA Ct:40.92 (2.50)	
05	47	F	20	asymptomatic (14)	0.0 (0)			
				symptomatic (6)	7.4 (2)	2/13 EV/RV Ct:38.03 (5.03)	3/5 FluB Ct:35.88 (6.19)	
06	54	F	20	Asymptomatic (15)	6.7 (1)	3/12 PeV Ct:39.96 (6.12) and Ad Ct:39.96 (2.48)		
				Symptomatic (5)	20.0 (1)	2/19 FluB Ct:26.70 (13.04)		
07	41	M	21	Asymptomatic (16)	0.0 (0)			
				Symptomatic (5)	40.0 (2)	12/4 RVA103 Ct:26.08 (13.13)	12/11 RVA103 CT:42.94 (1.70)	
08	30	M	21	Asymptomatic (16)	37.5 (6)	12/11 RVC8 Ct:29.90 (10.54)	12/18 RVC8 Ct:33.98 (7.77)	12/25 RVC8 Ct:33.37 (8.19)
						1/9 RVC8 Ct:33.18 (8.32)	1/22 EV/RV Ct:36.99 (5.73)	3/26 RVC11 Ct:27.57 (12.12)
				Symptomatic (5)	60.0 (3)	11/27 RSV CT:37.95 (5.69)	12/4 RVC8 Ct:32.12 (9.04)	2/5 RVC8 Ct:32.98 (8.45)
09	49	M	21	Asymptomatic (14)	0.0 (0)			
				Symptomatic (7)	42.9 (3)	3/5 EV/RV Ct:38.99 (4.34)	3/19 RVC44 Ct:37.13 (5.64) and Ad Ct:37.90 (3.88)	
						3/26 RVC44 Ct:25.75 (13.36)		
10	47	F	21	Asymptomatic (6)	33.3 (2)	2/5 Ad Ct: 40.97 (1.79)	2/13 229E Ct:37.90 (5.61)	
				Symptomatic (15)	13.3 (2)	12/4 RVC15 Ct:30.98 (9.81)	1/4 EV/RV Ct:37.97 (5.07)	
11	41	F	20	Asymptomatic (12)	0.0 (0)			
				Symptomatic (8)	25.0 (2)	12/11 EV/RV Ct:37.65 (5.28)	12/18 RVC9 Ct:31.28 (9.61)	

2.2 | Molecular analysis

Nucleic acids were extracted from 200-μL aliquots of specimens as 50 μL of elution volume using the Magtration System with a MagDEA viral DNA/RNA 200 kit (Precision System Science Co., Ltd. Chiba, Japan). RT reactions were performed using a ReverTra Ace qPCR RT kit (Toyobo Co., Ltd. Osaka, Japan) by following the manufacturer's instructions. Then, the cDNA was amplified to a total volume 25 μL using Realtime PCR Master Mix (Toyobo). The sensitivity of each of the real-time PCR methods was described previously.[3] The copy number of the viral genome in the sample was estimated from the calibration curve obtained by measuring the positive plasmid by each PCR method. For the detection of FluV types A and B, we used one-step real-time RT-PCR because of its increased sensitivity.[5,6] The RNA samples that tested positive for EV/RV were further classified into individual enterovirus and rhinovirus genotypes by direct sequencing. The amplification of the VP4/VP2 region of each enterovirus or rhinovirus for typing was performed using semi-nested RT-PCR as previously described.[7] Sequence analysis was performed using DNADynamo (Blue Tractor Software, UK). For RV genotyping, phylogenetic trees were constructed in MEGA7 (Tamura et al[8], Ver 7.0.16) by the neighbor-joining method with the VP4/VP2 region (420 nt) sequences of prototype isolates of each rhinovirus type commonly used in studies of human rhinovirus epidemiology and new types of them proposed previously.[9–12]

2.3 | Ethical approval

This study was approved by the Osaka Institute of Public Health ethical committee (No. 1701-02).

3 | RESULTS

We defined the term "asymptomatic" of which the criteria were the absence of respiratory symptoms (cough, sniffles, or sore throat) and systemic symptoms (fever or rash) up to 1 week before through 2 days after sampling similar to the experiment that we reported previously.[3] Cases in which the RNA was EV/RVpositive in real-time PCR but for which the viral VP4/VP2 region could not be amplified by semi-nested RT-PCR because of low viral loads were designated "EV/

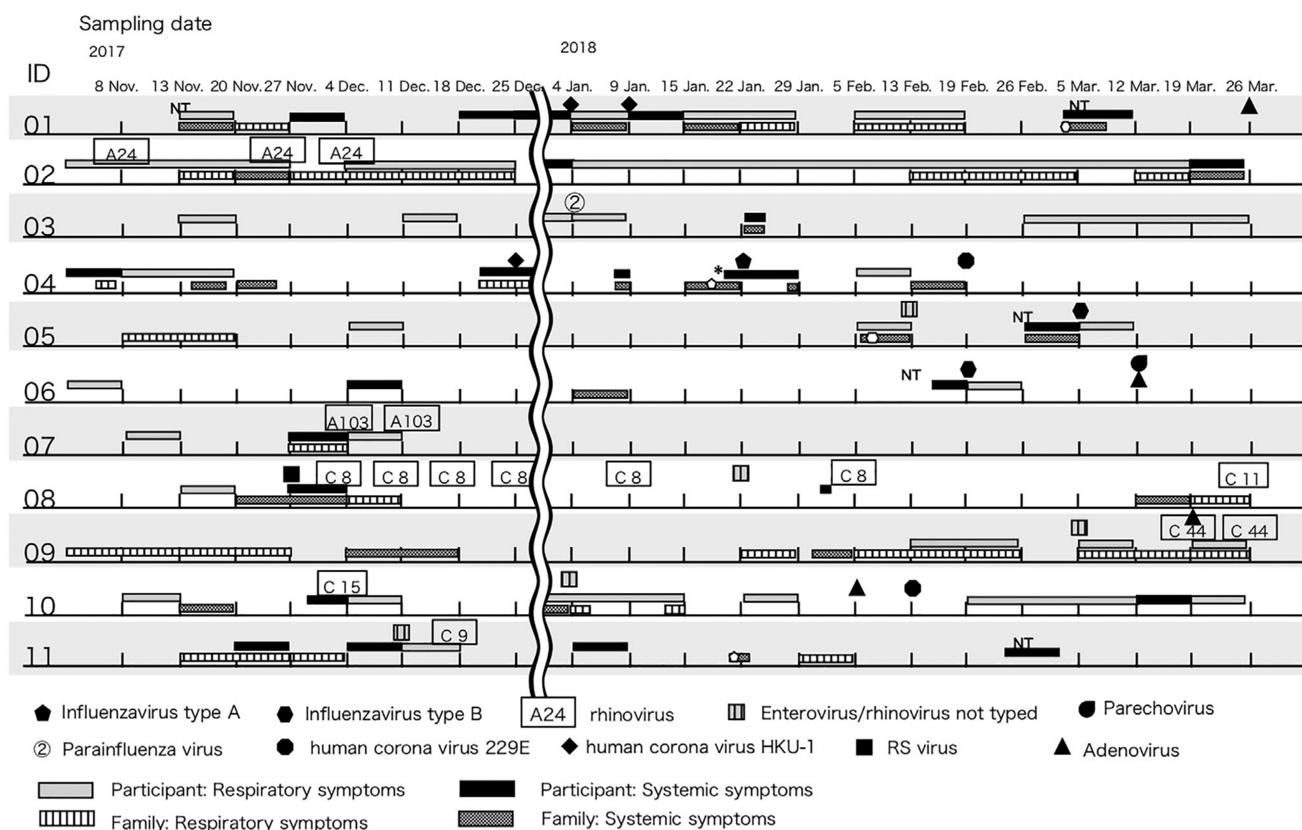

FIGURE 1 Relations between detected viruses from gargle specimens of 11 participants and respiratory and/or systemic symptoms of participants and their family members. The symbols for the detected viruses are shown in the explanatory notes. Vertical lines indicate sampling times. Letters in rectangles indicating RVs show the results for genotyping of the VP4/2 region. The letter in the open circle indicating the PIV shows the type. The open pentagon and the open hexagon indicate that a family member had a diagnosis of "influenza A" or "influenza B," respectively, by a medical institution. Rectangles with gray or black fill, vertical bars, or diagonal grids indicate the period during which any respiratory or systemic symptom was seen in the participants and any respiratory or systemic symptom in their family, respectively

RV untyped." When the same viral type or genotype was detected in one person several times, this was considered a persistent infection.

During the experimental period, 226 specimens were obtained. The submitted specimen and questionnaire were identified by the ID code, sampling month, and sampling day and analyzed. The participants were from 29 years to 54 years old (mean: 42.0 years, median 41.0 years). All participants were married, and eight of them had one to four children (Table 1). During the study period, respiratory viruses were detected in all the participants. Viruses were detected in each participant from one to nine times. We detected 36 viruses in 34 (15.0%) of the 226 samples (two samples showed dual infection). The virus species detected from the participants and the viral load in the sample are also shown in Table 1. For 11 detected specimens (01-3/26, 04-2/19, 06-3/12, 08-12/11, −12/18, −12/25, −1/9, −1/22, −3/26, 10-2/5, and −2/13), the person carrying the virus was asymptomatic for 1 week before and after the sampling day (Figure 1).

Seven RV genotypes were detected in six participants, and four subjects (ID: 02, 07, 08, and 09) were regarded as having persistent RV infection. There were some sampling points when a virus was not detected during the period, but the period from the first to the last detection of the same RV genotype was as long as 9 weeks. Other than the RV, only hCoV HKU-1 was detected in the same participant sequentially. Under asymptomatic conditions, hPeV, RV genotype C11, hCoV 229E, and AdV were detected in 1, 1, 2, and 3 samples, respectively. After RV genotype C8 was detected under the "symptomatic" condition, it was subsequently detected for more than 8 weeks without any symptoms.

Two viruses were detected in two samples. AdV and PeV were detected in sample ID: 06-3/12 when the subject was asymptomatic, whereas AdV and RV genotype C44 were detected in sample ID: 09-3/19 when the subject was symptomatic. HMPV, hBoV, PIV 1,3, and 4, hCoV OC43, and FluV type C were not detected during the sampling period.

4 | DISCUSSION

There have been many attempts to detect respiratory viruses in the nasopharyngeal samples of healthy subjects.[13–17] Some studies employed one-point specimen collection, and others continuously collected specimens every week over a long period, but they did not study the shedding period of the participants or examine their familial symptoms. In this study, gargle specimens from 11 healthy workers in one office were collected once a week and samples were subjected to real-time PCR to detect respiratory viruses. We also investigate weekly symptoms of family members and compared them with the virus detection results of participants.

Some respiratory viruses were detected in 11 specimens from persons who were asymptomatic and 23 specimens from those who were symptomatic at the time of sample collection. Because all specimens were obtained after arrival at the office, it was thought that these subjects might have transmitted viruses to other persons at the time of commuting.

We were not able to conduct viral detection for family members with symptoms in many cases because we missed the optimal timing for sampling. Therefore, we could not consider the route of infection in the family and felt the need to obtain specimens from the family in parallel with those from the participants.

In this study, the same viruses detected among the staff of the office in a short period were hCoV 229E in ID 10-2/13 and 04-2/19 and AdV in ID 06-3/12, 09-3/19, and 01-3/26. In the former two subjects, infection between the office staff might have occurred. Because a symptom was found in the family of 04-2/19 before hCoV was detected in the subject, infection between families was also a possibility. Only FluV A with ID 04-1/22 was confirmed to be infected by a family member. Prior to the onset of 04, the child was diagnosed with FluV A using a rapid diagnostic kit, after which the same results were obtained with our real-time PCR method from the child's specimens (data not shown). On the other hand, HMPV, hBoV, PIV 1,3, and 4, hCoV OC43, and FluV type C were not detected during the sampling period. As in previous reports, FluV, HMPV, and RSV were thought to be more frequently associated with symptoms among elderly adults.[18,19]

We detected the same RV genotype infection for more than two consecutive sampling periods. During the detection period, there were times when viral genome could not be detected. Rhinoviruses were detected in gargle samples from ID 02 and 08 over a long term. In ID 08, RV genotype C8 was detected for 9 weeks, although symptoms disappeared after the third week. The RV continued to be detected and seemed to be an "indigenous virus." The detected sequence of RV during this time (approximately 600 bp) was consistent (data not shown). However, the number of viral genome copies at each detection period tended to decrease over time. The next positive sample from the same participant 7 weeks later showed a different RV genotype, C11. Therefore, RVs found in the specimens of asymptomatic participants might not exist latently and could be cleared from the airway finally because the persistent infection appears to be rare.

Many respiratory viruses have seasonal distributions.[20,21] Therefore, it is not clear how a virus is present during a non-epidemic season. The long-term detection of the RV in asymptomatic adults seems to indicate that such persons are carriers of the respiratory virus. Human rhinoviruses must repeat human-to-human transmission to be maintained in the human population.

In this study, we used real-time PCR to detect various viruses at the same time. Because PCR is a nucleic acid amplification method, it remains unknown whether the respiratory viruses detected in the specimens from asymptomatic participants were infectious or not.

5 | CONCLUSIONS

We examined gargle specimens from the same participants every week for 20 consecutive weeks. As a result, it was revealed that even healthy (asymptomatic) adults were able to spread the respiratory virus due to viral infections and sometimes shed the virus for a long time. Respiratory viral infection without any symptoms

may play an important role in the viral circulation in human populations.

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CONFLICT OF INTEREST

The authors report no conflicts of interest relevant to this work.

TRANSPARENCY STATEMENT

Saeko Morikawa affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned have been explained.

AUTHORS' CONTRIBUTIONS

Conceptualization: Saeko Morikawa, Takahiro Yumisashi
Funding Acquisition: Saeko Morikawa
Investigation: Saeko Morikawa, Maki Otsuka
Writing—Original Draft: Saeko MorikawaWriting—Review and Editing: Maki Otsuka, Takahiro Yumisashi, Kazushi Motomura

All authors have read and approved the final version of the manuscript.

Saeko Morikawa had full access to all of the data in this study and takes complete responsibility for the integrity of the data and the accuracy of the data analysis.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article.

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