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Detection of respiratory viruses in gargle specimens of healthy children

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ABSTRACT

Background: Respiratory tract viral infection is one of the most common and important diseases in children. Polymerase chain reaction (PCR) tests are often used to detect viruses in samples, it is difficult to interpret the clinical significance of PCR positivity, which may reflect a past, imminent or active asymptomatic infection due to their high sensitivity. Although single respiratory viruses have been detected in samples from children with symptoms, other respiratory viruses can also be detected simultaneously. However, the clinical importance of these findings for the symptoms is not known.
Objectives: To investigate the prevalence of respiratory viruses among children without any symptoms such as acute respiratory illness and/or fever.
Study design: From week twenty-five 2013 to week twenty-six 2014, gargle samples were collected from children once a week and these samples were subjected to real-time PCR to detect respiratory viruses. On each sampling day, we asked the parents about their children's health condition.
Results: Among the 286 samples collected, 200 were from asymptomatic children. In the asymptomatic condition, human parechovirus, adenovirus, enterovirus, rhinovirus, coronavirus 229E and HKU1 were observed in 45 episodes. In samples from symptomatic children, parainfluenza viruses, respiratory syncytial virus and coronavirus OC43 were detected in addition to those mentioned above.
Conclusions: Various viruses of different species were detected in the specimens from the children regardless of their health status. It might be speculated that host factors such as the function of the immune system influence the clinical outcome of the infection. However, this needs to be studied further.

1. Background

Respiratory tract viral infection is one of the most common and important disease conditions in children. Recently, PCR based assays have made it possible for novel viruses to be discovered, leading to appraisal of the clinical impacts of these viruses and several other well-known respiratory viruses [1–4]. Some of these viruses are detected alone in specimens from patients with respiratory symptoms (sometimes in those of inpatients) but their pathogenicity is not clear because they are detected simultaneously with other viruses in many cases [5–7]. As a result, the clinical importance of these findings for the symptoms is not known.

2. Objectives

In this study, we investigated how often and what respiratory viruses were detected in specimens from asymptomatic children. Gargle specimens (obtained by rinsing the throat with distilled water) were collected from children once a week and the samples were subjected to two-step real-time PCR to detect respiratory viruses. Singleplex real-time PCR procedures were employed for detection of the following 15 respiratory viral pathogens: parainfluenza viruses (PIV) 1–4, respiratory syncytial virus (RSV), human metapneumovirus (hMPV), enterovirus (EV)/rhinovirus (RV), human bocavirus (hBoV), human parechovirus (hPeV), adenovirus (AdV), and human coronaviruses (hCoV) OC43, NL63, 229E, and HKU-1 (Table 1), and one-step real-time reverse transcription (RT)–PCR was used for detection of influenza viruses (FluV) A and B (Table 1).

Abbreviations: PCR, polymerase chain reaction; PIV, parainfluenza virus; RSV, respiratory syncytial virus; hMPV, human metapneumovirus; EV, enterovirus; RV, rhinovirus; RVA, rhinovirus genogroup A; RVB, rhinovirus genogroup B; RVC, rhinovirus genogroup C; hBoV, human bocavirus; hPeV, human parechovirus; AdV, adenovirus; hCoV, human coronavirus; FluV, influenza virus; RT, reverse transcription.

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Table 1
Primers and probes used in this study.

Virus	Target	Product size(bp)	Specific primers and probes	Detection limit (copy/uL)	Reference
PIV1	HN	135	Antisense 5' GTCCTTCCTGCTGGTGTGTTAAT 3'	6.55×10^2	
			Sense 5' CCAACCTACAAGGCAACAACATC 3'		[27]
			Probe 5' (FAM)CAAACGATGGCTGAAAA(TAMRA) 3'		
PIV3	HN	161	Antisense 5' TTGTTATAGTGTGTAATGCAGCTCGT 3'	5.30×10^2	
			Sense 5' GGGAGCATTGTGTCATCTGTCA 3'		[27]
			Probe 5' (FAM)CCCAGTCATAACTTACTC(TAMRA) 3'		
PIV2	NP	65	Antisense 5' TCYTCAGCTAATGCTTCRAARGC 3'	1.0×10^2	
			Sense 5' ATTCCAGATGCTCGATCAACTATG 3'		[28]
			Probe 5' (FAM)AGCACYTCTCCTCTGG(TAMRA) 3'		
PIV4	NP	123	Antisense 5' ATGTGGCCTGTAAGGAAAGCA 3'	1.0×10^1	
			Sense 5' CAAAYGATCCACAGCAAAGATTC 3'		[29]
			Probe 5' (FAM)GTATCATCATCTGCCAAATCGGCAATTAAACA(TAMRA) 3'		
RSV	F	89	Antisense 5' CGATTTTTATTGGATGCTGTACATTT 3'	2.22×10^2	
			Sense 5' AACAGATGTAAGCAGCTCCGTTATC 3'		[30]
			Probe 5' (FAM)TGCCATAGCATGACACAATGGCTCCT(TAMRA) 3'		
hPMV	M	152	Antisense 5' CATCAGCCYYATCWGTGTTTCTTAAAA 3'	2.47×10^2	
			Sense 5' GGCTCCATGCAAATATGAAGTG 3'		[31]
			Probe 5' (FAM)CTAACGAGTGTGCGCAAG(TAMRA) 3'		
EV/RV	5'NTR[b]	203	Antisense 5' GAAACACGGACACCCAAAGTAGT 3'	Echo 9.76×10	
			Sense 5' AGCCTGCGTGGCKGCC 3'	RVC 2.98×10^2	[32]
			Probe 5' (FAM) CTCCGGCCCCTGAATGYGGCTAA(TAMRA) 3'		
hBoV	NP-1	75	Antisense 5' TGGACTCCCTTTTCTTTTGTAGGA 3'	5.05×10^2	
			Sense 5' GCACAGCCACGTGACGAA 3'		[33]
			Probe 5' (FAM)TGAGCTCAGGGAATATGAAAGACAAGCATCG(TAMRA) 3'		
hCoV229E	NC	80	Antisense 5' TCTTTTCCACCGTGGCTTTT 3'	1.0×10^2	
			Sense 5' CTGCCAAGAGTCTTGCTCGTT 3'		[28]
			Probe 5' (FAM)AGAACAAAAGCATGAAATG(TAMRA) 3'		
hCoVNL63	NC	61	Antisense 5' CGAGGACCAAAGCACTGAATAA 3'	1.17×10^2	
			Sense 5' AACCTCGTTGGAAGCGTGTT 3'		[28]
			Probe 5' (FAM)ATTTTCCTCTCTGGTAG(TAMRA) 3'		
hCoVOC43	NC	67	Antisense 5' GCTGAGGTTTAGTGGCATCCTT 3'	2.19×10^2	
			Sense 5' GACATGGCTGATCAAATTGCTAGT 3'		[28]
			Probe 5' (FAM)TCTGGCAAAACTTGG(TAMRA) 3'		
hCoV HKU	ORF 1a/b	61	Antisense 5' CATTCATTCGCAAGGCGATA 3'	1.11×10^2	
			Sense 5' CCCGCAAACATGAATTTTGTT 3'		[28]
			Probe 5' (FAM)AATCTATCACCATGTGAA (TAMRA) 3'		
hPeV	5'NTR	194	Antisense 5' GGCCCCWGRTCAGATCCAYAGT 3′	1.0×10^2	
			Sense 5' GTAACASWWGCCTCTGGGSCCAAAAG 3′		[34]
			Probe 5'(FAM)CCTRYGGGTACCTYCWGGGCATCCTTC(TAMRA) 3′		
AdV(ACDF)	Hexon	85	Antisense 5' AAACTTGTTATTCAGGCTGAAGTACGT3'	1.0×10^2	
			Sense 5' CCAGGACGCCTCGGAGTA 3'		[35]
			Probe 5' (FAM)AGTTTGCCCGCGCCACCG(TAMRA) 3'		
AdV(BE)	Hexon	81	Antisense 5' CTTGTTCCCCAGACTGAAGTAGGT 3'	1.0×10^2	
			Sense 5' GGACAGGACGCTTCGGAGTA 3'		[35]
			Probe 5' (FAM)CAGTTCGCCCGYGCMACAG(TAMRA) 3'		
FluV typeA	MP	149	Antisense 5' TGACAGRATYGGTCTTGTCTTTAGCCAYTCCA	7.5[a]	
			Sense 5' CCMAGGTCGAAACGTAYGTTCTCTCTATC		[36]
			Probe 5' (FAM)ATYTCGGCTTTGAGGGGGCCTG(MGB) 3'		
FluV AH1pdm09	HA	187	Antisense 5' TGTTTCCACAATGTARGACCAT	6.8[a]	
			Sense 5' AGAAAAGAATGTAACAGTAACACACTCTGT		[36]
			Probe 5' (FAM)CAGCCAGCAATRTTRCATTTACC(MGB) 3'		
FluV AH3	HA	178	Antisense 5'GTCATTGGGRATGCTTCCATTTGG	7.1[a]	
			Sense 5' CTATTGGACAATAGTAAAACCGGGRGA		[36]
			Probe 5' (FAM)AAGTAACCCCKAGGAGCAATTAG(MGB) 3'		
FluV B	NS	105	Antisense 5'GTKTAGGCGGTCTTGACCAG	8.2[a]	
			Sense 5' GGAGCAACCAATGCCAC		[37]
			Probe 5' (FAM)ATAAACTTTGAAGCAGGAAT(MGB) 3'		

[a] From reference data.
[b] NTR: non translated region.

3. Study design

3.1. Subjects

Twelve children aged 3–10 years old were enrolled. From week twenty-five 2013 to week twenty-six 2014, throat gargle samples were obtained from the children once a week. Their parents noted the existence of respiratory symptoms (cough, sore throat or nasal mucus) and systemic symptoms (fever or rash) at the time of sampling. Written informed consent was obtained from the parents.

3.2. Molecular analysis

Nucleic acids were extracted from 200 μL specimens using the Magtration System with a MagDEA viral DNA/RNA 200 kit (Precision System Science Co., Ltd., Chiba, Japan) as 50 μL of elution volume. RT reactions were performed using a ReverTra Ace qPCR RT kit (TOYOBO Co., Ltd., Osaka, Japan) following the manufacturer's instructions. The cDNA was then amplified using Realtime PCR Master Mix (TOYOBO) with a total volume of 25 μL. Each sample was amplified containing primers and probes specific for each of the targets as described in Table 1 [27–37]. The sensitivity of each of the

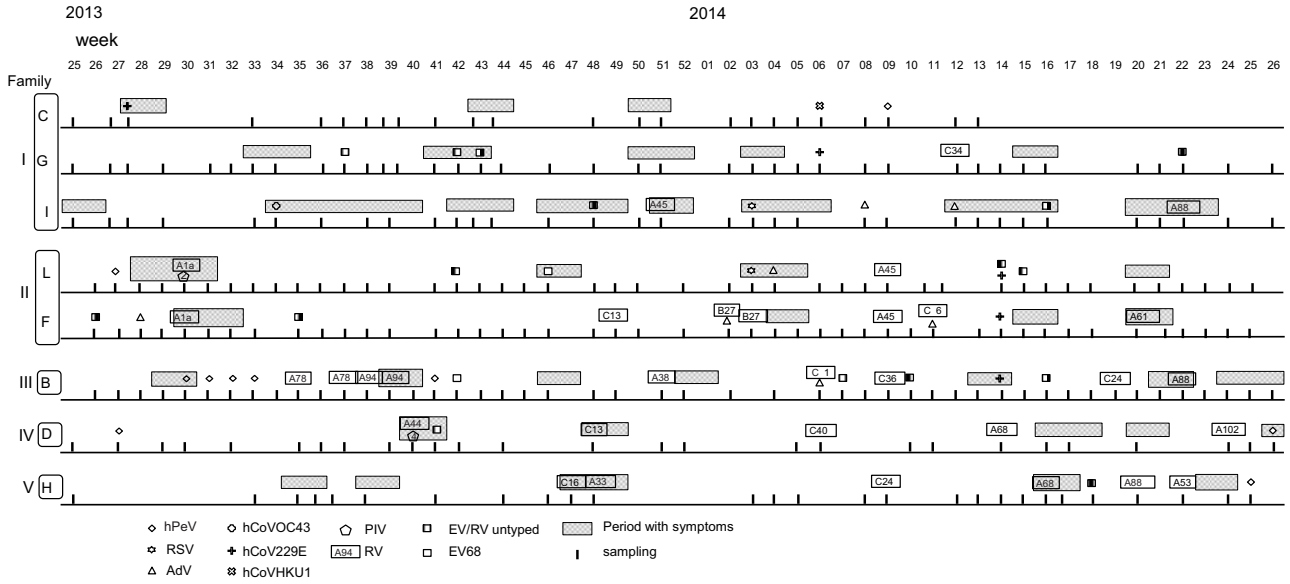

Fig. 1. Relations between viruses detected in gargle specimens of 8 children and respiratory and/or systemic symptoms. Detected viruses are shown using the symbols noted in the explanatory notes. Vertical lines indicate sampling time. The letters and numbers in rectangles indicate RV genotypes. Children C, G and I are three of four siblings. F and L are a girl and her older brother.

real-time PCR methods was evaluated by detecting serial dilutions of quantitated plasmids that contained each target DNA clone. For detection of FluV A and B, we used the one-step real-time RT–PCR method because of its increased sensitivity. Enteroviruses and rhinoviruses were genotyped by direct sequencing. Amplification of the VP4/VP2 region of the enterovirus or rhinovirus for typing was performed with semi-nested RT–PCR as previously described [8]. The purified PCR products were subjected to direct sequencing with a BigDye Terminator v1.1 kit as per the manufacturer's instructions (Applied Biosystems, CA, USA). Sequence analysis was performed using the DNADynamo program (Blue Tractor Software, UK). Using MEGA5.2 (Tamura et al., 2011, Ver5.2.2), we employed the neighbor-joining method [14] to construct phylogenetic trees from the VP4/VP2 region (420nt) sequences retrieved from GenBank of prototype isolates of each rhinovirus type commonly used in epidemiologic studies of human rhinoviruses [9–11] and new types proposed previously [9,12,13]. Genotypes were assigned on the basis of their clustering with known prototype reference strains.

4. Results

Four children were excluded because of insufficient sampling frequency. For the asymptomatic condition, the criteria were the absence of respiratory symptoms (cough, sniffle or sore throat) and systemic symptoms (fever or rash) from one week before to two days after sampling. Of the 286 samples, 200 were from children who were asymptomatic (Fig. 1). When RNA was EV/RV positive by real-time PCR but the viral VP4/VP2 region could not be amplified by semi-nested RT–PCR, we defined it as EV/RV untyped. The threshold cycle (Ct.) of real-time PCR is a relative measure of the concentration of the target in the PCR reaction. If the Ct. value of the EV/RV real-time PCR test is high (over 36.0), the nucleic acids cannot be amplified by the semi-nested PCR used for genotyping (data not shown).

Of the 200 samples, 45 (22.5%) were real-time PCR positive. Four of the 45 positive samples contained two viruses. The prevalence of respiratory viruses among asymptomatic children varied from 9.1% (1/11) to 42.9% (15/35) and that in the symptomatic period

Table 2
Prevalence of respiratory viruses in gargle specimens of children.

Family	Child	Age (years)	Sex	Total no. of sample	Condition (n)	Prevalence% (positive sample)
I	C	9	M	24	Asymptomatic (19)	10.5 (2)
					Symptomatic (5)	20.0 (1)
	G	6	F	38	Asymptomatic (27)	14.8 (4)
					Symptomatic (11)	18.2 (2)
	I	3	M	33	Asymptomatic (11)	9.1 (1)
					Symptomatic (22)	31.8 (7)
II	L	6	M	44	Asymptomatic (35)	14.3 (5)
					Symptomatic (9)	44.4 (4)
	F	3	F	45	Asymptomatic (36)	25.0 (9)
					Symptomatic (9)	22.2 (2)
III	B	4	M	48	Asymptomatic (35)	42.9 (15)
					Symptomatic (13)	30.8 (4)
IV	D	3	M	27	Asymptomatic (20)	20.0 (4)
					Symptomatic (7)	57.1 (4)
V	H	5	F	27	Asymptomatic (19)	26.3 (5)
					Symptomatic (8)	37.5 (3)

Table 3
Detection of respiratory viruses in gargle specimens of children.

Virus	Condition	No. of detections(%)
Enterovirus 68	Asymptomatic	1 (0.5)
	Symptomatic	1 (1.2)
Human rhinovirus A	Asymptomatic	10 (5.0)
	Symptomatic	10 (11.6)
Human rhinovirus B	Asymptomatic	2 (1.0)
	Symptomatic	0 (0)
Human rhinovirus C	Asymptomatic	8 (4.0)
	Symptomatic	2 (2.3)
Human parechovirus	Asymptomatic	8 (4.0)
	Symptomatic	2 (2.3)
Human coronavirus HKU-1	Asymptomatic	1 (0.5)
	Symptomatic	0 (0)
Human coronavirus 229 E	Asymptomatic	3 (1.5)
	Symptomatic	2 (2.3)
Human coronavirus OC43	Asymptomatic	0 (0)
	Symptomatic	1 (1.2)
Parainfluenza virus 2	Asymptomatic	0 (0)
	Symptomatic	1 (1.2)
Parainfluenza virus 4	Asymptomatic	0 (0)
	Symptomatic	1 (1.2)
RS virus	Asymptomatic	0 (0)
	Symptomatic	2 (2.3)
Adenovirus	Asymptomatic	5 (2.5)
	Symptomatic	2 (2.3)
EVRV untyped	Asymptomatic	11 (5.5)
	Symptomatic	5 (5.8)

ranged from 18.2% (2/11) to 57.1% (4/7) (Table 2). The most frequently detected virus was RV genogroup A (RVA) ($n = 10$) (Table 3). EV/RV from 11 samples could not be genotyped. Two of the 4 samples with codetection contained RVC and adenovirus, one RVB and adenovirus, and one EV/RV untyped and hCoV 229E.

Human PeV was detected in 8 samples. After hPeV was detected in a sample from a symptomatic child, it was subsequently detected for more than three weeks without any symptoms (Fig. 1, Child B).

In samples from symptomatic children, PIV, RSV and hCoV OC43 were detected in addition to the viruses detected in those from asymptomatic children (27/86; 31.4%). The most commonly detected virus was RVA (10/27; 37.0%). Among the 27 samples, 2 contained PIV and RVA. FluV, hBoV and hMPV were not detected.

5. Discussion

Gargle specimens from 8 children were collected once a week and the samples were subjected to real-time PCR to detect respiratory viruses. RVs and EV/RV untyped were the viruses most frequently detected in samples from asymptomatic children. Current diagnosis of respiratory infections is mainly done using PCR methods. Due to their high sensitivity, it is difficult to determine the exact explanation for positivity in individual participants (e.g., post-viral shedding, asymptomatic infection, or incubation before symptomatic infection). We were able to clarify the active asymptomatic infection by testing gargle specimens of the same children once a week for one year.

RVs are most commonly isolated from persons experiencing mild upper respiratory illness (common cold). Recent studies have reported that those viruses are responsible for severe infections of the lower respiratory tract in children. These viruses play a critical role in exacerbating asthma and chronic lung diseases [15,16]. However, most studies were conducted with symptomatic patients. Few studies have investigated the existence of the viruses in children without any respiratory symptoms [17,18]. One study reported that, after the onset of symptomatic respiratory infection, rhinovirus RNA may take a long time (5–6 weeks) to disappear from nasal mucus [19]. In this study, the children who could gargle might have been relatively older, but RVs were often detected in their throats at a time without symptoms. It seems that RV infection is in most cases asymptomatic or mild. As the sensitivity of the real-time PCR was 100 copies, it can be assumed that the virus might have replicated to some extend. The same RV genotype was detected in two consecutive samples of a child and another RV genotype was detected in the next sample. These findings suggest that RVs do not exist in the upper respiratory tract for a long time even if a child does not show symptoms which were probably the result of interferon response to a virus multiplication.

HPeV was also detected in samples from asymptomatic children. Recent studies have investigated the involvement of hPeVs in respiratory diseases, reporting a low frequency of detection and a lack of clear disease association. In addition to a low hPeV prevalence in respiratory samples, a high rate of coinfection with other respiratory viruses has been observed in hPeV-positive samples [1,20]. With monthly sampling, hPeV was detected in the stools of 48% of healthy Finnish infants by the age of 22 months [21]. In this study, the duration of parechovirus shedding in gargle specimens was calculated to be 3 weeks after the disappearance of the respiratory symptoms.

On the other hand, for PIVs, RSV, and hCoV OC43, which were detected only when clinical symptoms were seen, it is thought that, if these viruses grow in the airway, certain host reactions such as respiratory symptoms or fever will be triggered [22–26].

FluV, hBoV, hMPV and hCoV NL63 were not detected during the study period, probably because the children in this study did not live in a viral epidemic area.

Since various viruses were detected in the children regardless of their health condition, it might be speculated that the clinical outcome of the respiratory viral infection is affected predominantly such as the function of immune system. Most respiratory viruses infect the upper or lower airway and replicate in airway epithelial cells. In patients with normal immunity, these viruses are cleared immediately and it is generally thought that prolonged infection is rare. Therefore these respiratory viruses must repeat human-to-human transmission to continue to be present in the human population. As PCR is a nucleic acid amplification method, it remains unknown whether the respiratory viruses detected in the specimens from asymptomatic children are infective or not. Respiratory viral infection without any symptoms may play an important role in the viral circulation in human populations.

Competing interest

None declared.

Ethical approval

This study was approved by the Osaka Prefectural Institute of Public Health ethical committee (No. 1302-05-01).

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