Short Communication

Longitudinal Study on Respiratory Viral Co-Infections in the Presence or Absence of Clinical Manifestation in Infants Aged 0–2 Years

Takako Kawabuchi-Kurata^{1*}, Takako Misaki², Yutaka Suehiro³,
Atsushi Jun Komano¹, Tetsuo Kase¹, and Kazuo Takahashi¹

¹*Division of Virology, Department of Infectious Diseases, Osaka Prefectural Institute of Public Health, Osaka 537-0025;*

²*Kawasaki City Institute of Public Health, Kanagawa 210-0821; and*

³*Department of Pediatrics, Saiseikai Nakatsu Hospital, Osaka 530-0012, Japan*

(Received August 28, 2013. Accepted December 25, 2013)

SUMMARY: We conducted a long-term follow-up study between December 2005 and February 2007 on 4 immunocompetent infants, who repeatedly presented with respiratory symptoms, using PCR-based techniques targeting 14 viruses related to acute respiratory tract infection. Of 38 specimens, 30 were collected from symptomatic infants and 8 were collected when respiratory symptoms were absent. Overall, one or more respiratory viruses were detected in 94.7% (36/38) of the specimens. Of the 36 PCR-positive specimens, 77.8% (28/36) were positive for more than one virus. Most of these co-infections were double infections (55.6% or 20/36). Of note, co-infections with 4 and 3 viruses were observed in 3 (8.3% or 3/36) and 5 (13.9% or 5/36) specimens, respectively. Of the 8 specimens collected from the 4 infants when apparent respiratory symptoms were absent, 7 (87.5%) were positive for respiratory viruses. Respiratory viral co-infections were also frequent and found in 5 of the specimens (62.5%). However, apparent correlation between disease severity and co-infection was undetectable due to the limit of the number of cases studied. Taken together, this longitudinal study revealed that respiratory viral co-infections were not infrequent in infants aged 0–2 years, regardless of the presence of respiratory symptoms (62.5–77.8%).

Advances in nucleic acid-based technology have resulted in rapid and sensitive diagnoses of respiratory infections and the identification of potential human pathogens from clinical specimens (1). Several viruses are known to cause respiratory infections in humans, including the influenza virus (IFV), respiratory syncytial virus (RSV), parainfluenza virus (PIV) 1–4, human rhinovirus (HRV), enterovirus (EV), and human adenovirus (HAdV) (2). In addition, recent studies have shown that the human metapneumovirus (hMPV), human bocavirus (HBoV), and human coronavirus (HCoV) also cause respiratory infections (2,3). PCR-based analyses have shown that respiratory viral co-infections are quite frequent; for example, approximately 15% of patients with respiratory symptoms were found to be positive for respiratory viral co-infections in one study (4). However, the clinical impacts of such co-infections remain poorly understood, partly because most of the previous studies were cross-sectional studies (4–6). The significance of respiratory viral co-infections, especially in infants without immunological disorders, could be investigated by assessing various populations over a period of time. In the present study, we addressed the same by monitoring 4 infants (age, 0–2

years) without underlying diseases longitudinally between 2005 and 2007. The presence of 14 respiratory viruses was examined in these infants, regardless of whether they presented with respiratory symptoms. Unexpectedly, respiratory viral co-infections, including quadruple infections, were detected more frequently than expected on the basis of previously reported rates (5,7,8). The clinical significance of these viral co-infections was assessed by examining the correlation between co-infection profiles and clinical symptoms.

Four healthy infants were enrolled in this study, including a newborn female (Pt 1), a 9-month-old female (Pt 2), a 9-month-old male (Pt 3), and a 21-month-old female (Pt 4). All the four infants showed no clinical signs indicative of primary immunodeficiency, as diagnosed by a physician, and attended nursery in the Saiseikai Nakatsu Hospital in Osaka between December 2005 and February 2007. The infants repeatedly presented with respiratory symptoms, including fever (over 37.5°C), cough, wheezing, chest retraction, nasal discharge, and decreases in arterial oxygen saturation during the study period. Appearance of any of these symptoms defined the onset of respiratory disease in this study. In total, 38 nasal aspirates were collected for the diagnosis of respiratory infections using an immunochromatographic test kit, which was employed for the rapid diagnosis of respiratory viral infections during onset and after recovery from the disease for the purpose of infection control in the hospital. The remaining portions of the specimens were used as detailed (Table 1). Pt 2, Pt 3, and Pt 4 were vaccinated against IFV in October 2006. RNA and DNA were extracted from the

*Corresponding author: Mailing address: Division of Virology, Department of infectious Diseases, Osaka Prefectural Institute of Public Health, 3-69 Nakamichi 1-chome Higashinari-ku, Osaka 537-0025, Japan. Tel: +81-6-6972-1321, Fax: +81-6-6972-2393, E-mail: kurata@iph.pref.osaka.jp

Respiratory Viral Co-Infections in Infants

Table 1. Summary of respiratory viruses detected in this study

Patient no.	Age at specimen collection	Sample ID	Days after onset	Virus detected									Severity of clinical signs ¹⁾	Hospitalization
				HAdV	HBoV	IFV	PIV	RSV	HMPV	HRV	EV	HCoV		
Pt 1	0 m	P1-1	2	+ ²⁾	—	—	+ ⁵⁾	—	—	+	—	—	++	+
	4 m	P1-2	2	+ ³⁾	+	—	—	—	—	+	—	—	++	
	5 m	P1-3	34	+ ³⁾	—	—	—	—	—	—	—	—	—	
	6 m	P1-4	2	+ ³⁾	—	—	—	+ ⁷⁾	—	—	—	—	++	+
		P1-5	3	+ ³⁾	—	—	—	+ ⁷⁾	—	+	—	—	++++	
		P1-6	8	+ ³⁾	—	—	—	+ ⁷⁾	—	—	—	—	+++	
Pt 2	9 m	P2-1	5	—	—	—	—	+ ⁸⁾	—	—	—	—	+++++	+
	11 m	P2-2	13	+ ³⁾	—	—	—	—	+	—	—	—	+++	
	1 y	P2-3	16	+ ³⁾	—	—	—	—	—	—	—	+	+	
		P2-4	13	+ ³⁾	+	—	—	—	—	—	—	—	—	
	1 y3 m	P2-5	8	+ ³⁾	+	—	—	—	—	+	—	—	++	
		P2-6	8	—	+	—	+ ⁵⁾	—	—	—	—	—	+	
	1 y8 m	P2-7	4	—	—	—	+ ⁶⁾	—	—	—	—	—	++	
		P2-8	11	—	+	—	—	—	—	—	—	—	—	
	1 y9 m	P2-9	12	—	—	—	—	—	—	—	—	—	—	
		P2-10	2	+ ⁴⁾	—	—	—	—	—	+	—	—	++++	+
	1 y10 m	P2-11	3	+ ⁴⁾	—	—	—	—	—	+	—	—	+++	
		P2-12	2	+ ⁴⁾	+	—	—	+ ⁷⁾	—	—	—	—	+++	
Pt 3	9 m	P3-1	4	+ ⁴⁾	—	—	—	—	+	—	—	—	++++	
		P3-2	18	+ ⁴⁾	+	—	—	—	—	—	—	—	—	
	10 m	P3-3	12	+ ⁴⁾	+	—	—	—	—	—	—	—	+	
		P3-4	2	+ ⁴⁾	+	—	—	—	—	—	—	—	++++	
	1 y	P3-5	4	+ ⁴⁾	+	—	—	—	—	—	—	—	+++	
	1 y1 m	P3-6	26	+ ⁴⁾	—	—	+ ⁵⁾	—	—	—	—	—	+	
		P3-7	40	+ ⁴⁾	+	—	+ ⁵⁾	—	—	+	—	—	+	
	1 y2 m	P3-8	2	+ ³⁾	—	—	—	—	—	+	—	—	—	
	1 y6 m	P3-9	4	+ ⁴⁾	—	—	—	—	—	—	—	—	+++	
		P3-10	10	+ ⁴⁾	+	—	+ ⁶⁾	—	—	+	—	—	—	
	1 y7 m	P3-11	2	+ ³⁾	—	—	—	+ ⁷⁾	—	—	—	—	+++	
Pt 4	1 y9 m	P4-1	7	—	+	—	+ ⁶⁾	—	—	—	—	—	+++	
	1 y10 m	P4-2	3	—	+	—	+ ⁶⁾	—	—	+	+ ⁹⁾	—	—	
		P4-3	8	—	+	—	—	—	—	+	—	—	+	
		P4-4	25	—	—	—	—	—	—	—	—	—	+	
		P4-5	3	—	+	—	—	—	—	+	—	—	++++	+
	1 y11 m	P4-6	4	—	—	—	—	—	—	+	—	—	+++	
		P4-7	6	—	+	—	—	—	—	—	—	—	++	
	2 y	P4-8	3	—	—	—	—	+ ⁷⁾	—	—	—	—	+++	
	2 y1 m	P4-9	4	+ ⁴⁾	—	—	—	—	—	+	—	—	++	

¹⁾: A plus sign represents one of the following clinical signs; fever, cough, wheezing, chest retraction, nasal discharge, or decreased arterial oxygen saturation density.

²⁾: HAdV serotype 5.

³⁾: HAdVserotype 2.

⁴⁾: HAdV serotype 1.

⁵⁾: PIV serotype 3.

⁶⁾: PIV serotype 2.

⁷⁾: RSV genotype B.

⁸⁾: RSV genotype A.

⁹⁾: Coxsackievirus A9.

HAdV, human adenovirus; HBoV, human bocavirus; IFV, influenza virus; PIV, parainfluenza virus; RSV, respiratory syncytial virus; HMPV, human metapneumovirus; HRV, human rhinovirus; EV, enterovirus; HCoV, human coronavirus.

specimens using QIAamp viral RNA and QIAamp DNA mini kits[®], respectively (Qiagen, Hilden, Germany). Twelve respiratory RNA viruses were tested using three sets of multiplex RT-PCR, each targeting four viruses: set 1, PIV 1–4; set 2, IFV A, IFV B, RSV, and HMPV; and set 3, HRV/EV, IFV C, HCoV-OC43, and HCoV-229E. The two respiratory DNA viruses, HAdV and HBoV, were examined by conventional PCR. The primers used in this study are listed in Table 2. The detection limits of the in-house PCR were comparable to those obtained by the methods described previously (9, 10). HAdV and HBoV were 10 copies/ μ l, respectively. The amplicons were sequenced using BigDye Terminator Cycle Sequencing Ready Reaction kits (Applied Biosystems, Foster City, CA, USA).

In total, 38 specimens were collected. Of these, eight were collected in the absence of respiratory symptoms. Overall, one or more respiratory viruses were detected in 94.7% (36/38) of the specimens. The most frequently detected virus was HAdV (69.4% or 25/36), followed by HBoV (47.2% or 17/36), HRV (38.9% or 14/36), PIV (22.2% or 8/36), RSV (19.4% or 7/36), HMPV (5.6% or 2/36), EV (2.8% or 1/36), and HCoV-OC43 (2.8% or 1/36). RSV and PIV 2 were detected during the winter season, whereas HBoV, HAdV, and HRV were detected throughout the year. The genomic sequences of all HBoV isolates were identical to a previously reported sequence (accession no. FJ548924). On the other hand, the genomic sequence identities of the HRV isolates ranged from 67.3% to 81.2%, suggesting that each HRV infection was caused by an independent virus. On an average, 2.0 ± 0.9 viruses were detected per sample.

Of the 36 PCR-positive specimens, 77.8% (28/36) were positive for more than one virus. Most of these co-infections were double infections (55.6% or 20/36). Of note, co-infections with four and three viruses were observed in three (8.3% or 3/36) and five (13.9% or 5/36) specimens, respectively. The most frequent double infection occurred with HAdV and HBoV (17.9% or 5/28), followed by HAdV and HRV (14.3% or 4/28).

Seven events requiring hospitalization were reported in the four infants, and five specimens were obtained during hospitalization, when symptoms were present (Table 1). Each sample was collected 2–5 days after the onset of disease. Respiratory viruses were detected in all the events, three of which presented with co-infections of HAdV and HRV. An average of 2.0 ± 1.0 viruses was detected per sample.

Eight specimens were collected from the four infants in the absence of apparent respiratory symptoms, six of the specimens were obtained more than 10 days after the onset of disease, while two (P3-8 and P4-2) were obtained 2 and 4 days after onset, respectively. Of note, seven of the eight specimens (87.5%) were positive for respiratory viruses, including HAdV, HBoV, HRV, and PIV. Co-infections were also frequent; they were present in five of the eight specimens (62.5%). The co-infections of HAdV and HRV or HAdV and PIV were common, with each detected in two of the eight specimens (25.0%). Of note, four viruses, HBoV, PIV 2, HRV, and coxsackievirus A9 were detected in the sample P4-2 from patient 4. An average of 2.0 ± 1.4 viruses was detected per sample. IFV and HCoV229-E were not

detected in this study.

In the present work, the respiratory viral infection profiles of infants were examined over a period of 15 months. The uniqueness of this study arises from the fact that infants under 2 years of age, who lacked any apparent underlying diseases, were subjected to a longitudinal study. Moreover, specimens were recovered from the infants in the absence of respiratory symptoms. Although some reports suggest a link between multiple infections and increased severity of clinical symptoms (5,8), a clear correlation between respiratory viral co-infection and disease severity was not observed in the present study, which is in agreement with a previous report by Paula et al. (11). The unique clinical manifestations associated with a particular respiratory viral infection or co-infection profile could also not be identified. It may be that respiratory viral co-infections only affect disease severity in specific situations where other factors are involved, either by themselves or in combination; these include underlying diseases, genetic backgrounds, and the involvement of particular bacterial infections.

Three of the four patients received IFV vaccine in October 2006, following which IFV was not detected in this study. Although there are only few opportunities for children in the hospital nursery to come in contact with IFV patients, the lack of IFV detection in this study suggests that the vaccination provided protection against IFV infection.

In this study, HRV was detected in 14 of the 36 specimens, mostly as co-infections (13/14); 10 of these 13 specimens were collected from symptomatic patients. Only HRV was detected in P4-6, corresponding to moderate clinical symptoms (Table 1). It has recently been shown that asymptomatic individuals often harbor HRV (12), and Lauinger et al. also reported that the HRV genotype per se was not linked to the severity of clinical symptoms (13). Data from the present study also support the notion that HRV infection is common among the human population. HBoV pathogenesis has been a recent subject of contention; a birth cohort longitudinal study of Danish children detected HBoV frequently in both symptomatic and asymptomatic infants (3,14). In the present study, the pathogenic potential of HBoV in infants with no underlying diseases was unclear. HBoV was intermittently detected for 3–10 months, but the viral genomic sequences that were detected in each infant were identical, suggesting continued viral shedding rather than re-infection with HBoV. This was also observed previously in immunocompromised children and in patients with prolonged respiratory symptoms lasting several months (3,7). It is likely that HBoV establishes a persistent infection in the tonsils (3) without inducing symptoms, such that shedding becomes apparent only when respiratory symptoms are triggered by other viral infections that increase respiratory secretion. It was reported previously that co-infections with HBoV and HAdV are frequent, with HAdV being detected in approximately 30% of HBoV-positive cases (7), suggesting that these viruses may interact in vivo. For example, HAdV infection may re-activate the persistent HBoV infection. It remains unclear whether HBoV is pathogenic in patients with underlying diseases or in patients infected with other respiratory

Respiratory Viral Co-Infections in Infants

Table 2. Primers used in this study

	Target	Primer name	Sequence	Primer usage ¹⁾	Reference
DNA virus					
	Human bocavirus	HBo188F	5′-gag ctc tgt aag tac tat tac-3′	initial	Allander et al. (2005) Proc Natl Acad Sci U S A.
		Hbo542R	5′-ctc tgt gtt gac tga ata cag-3′	initial and nested	
		Hbo NP1-R	5′-cac ttg gtc tga ggt ctt cga a-3′	nested	
	Human adenovirus	hex1deg-F	5′-gcc sca rtg gkc wta cat gca cat c-3′	initial	Allard et al. (2001) J Clin Microbiol.
hex2deg-R		5′-cag cac scc icg rat gtc aaa-3′	initial and nested		
Ad2-F		5′-cca gga cgc ctc gga gta-3′	nested	Wong et al. (2008) J Med Virol.	
Ad4-F		5′-gga cag gac gct tcg gag ta-3′	nested		
RNA virus					
Multiplex set 1	Parainfluenza virus 1	PIP1 +	5′-cct taa att cag ata tgt at-3′	initial	Aguilar et al. (2000) J Clin Microbiol.
		PIP1 −	5′-gat aaa taa tta ttg ata cg-3′	initial	
	Parainfluenza virus 2	PIP2 +	5′-aac aat ctg ctg cag cat tt-3′	initial	
		PIP2 −	5′-atg tca gac aat ggg caa at-3′	initial	
	Parainfluenza virus 3	PIP3 +	5′-ctg taa act cag act tgg ta-3′	initial	
		PIP3 −	5′-ttt aag ccc ttg tca aca ac-3′	initial	
	Parainfluenza virus 4	PI4P +	5′-ctg aac ggt tgc att cag gt-3′	initial	
		PI4P −	5′-ttg cat caa gaa tga gtc ct-3′	initial	
	Parainfluenza virus 1	PIS1 +	5′-ccg gta att tct cat acc tat g-3′	nested	
		PIS1 −	5′-ctt tgg agc gga gtt gtt aag-3′	nested	
	Parainfluenza virus 2	PIS2 +	5′-cca ttt acc taa gtg atg gaa t-3′	nested	
		PIS2 −	5′-gcc ctg ttg tat ttg gaa gag a-3′	nested	
	Parainfluenza virus 3	PIS3 +	5′-act ccc aaa gtt gat gaa aga t-3′	nested	
		PIS3 −	5′-taa atc ttg ttg ttg aga ttg-3′	nested	
	Parainfluenza virus 4	PI4S +	5′-aaa gaa tta ggt gca acc agt c-3′	nested	
		PI4S −	5′-gtg tct gat ccc ata agc agc-3′	nested	
Multiplex set 2	RS virus	hRSV-vrsP1	5′-gga aca agt tgt tga ggt tta tga ata tgc-3′	initial and nested	Bellau-Pujol et al. (2005) J Virol Methods
		hRSV-vrsP2	5′-ttc tgc tgt caa gtc tag tac act gta gt-3′	initial	
		hRSV-vrsi	5′-ggt gta cct ctg tac tct c-3′	nested	
	Influenza virus B	InfluB mib 1	5′-aaa att aca ctg ttg gtt cgg tg-3′	initial and nested	
		InfluB mib 2	5′-agc gtt cct agt ttt act tg-3′	initial	
		InfluB mib 3	5′-cat gaa arc tca cac atc t-3′	nested	
	Human metapneumovirus	hMpv 2	5′-gca gct tca aca gta gct g-3′	initial and nested	
		hMpv 3	5′-agg cca aca cac cac cag-3′	initial	
		hMpv 1-1	5′-agc agt kyt gct yga tca-3′	nested	
	Influenza virus A	InfluA mia4	5′-aay acm gat ctt gag gct ctc atg g-3′	initial	this study
		InfluA mia5	5′-ggc tct cat gga rtg gct aa-3′	nested	
		InfluA mia6	5′-gca ttt tgg aca aag cgt ct-3′	initial and nested	
Multiplex set 3	Human coronavirus OC43	MF3	5′-ggc aaa tct gcc caa gaa ta-3′	initial	Bellau-Pujol et al. (2005) J Virol Methods
		MF1	5′-ggc tta tgt ggc ccc tta ct-3′	initial and nested	
		MF2i	5′-ctc caa aaa ctt cca gtt c-3′	nested	
	Influenza virus C	MIC	5′-tat gag tgc aaa ctg cat ctt gtg g-3′	initial	this study
		CHAD	5′-cct gac agc aac tcc ctc at-3′	initial and nested	
		MICi	5′-gag gat gtg gca act act-3′	nested	
	Human coronavirus 229E	MD1	5′-tgg ccc cat taa aaa tgt gt-3′	initial	Bellau-Pujol et al. (2005) J Virol Methods
		MD3R	5′-cct gaa cac ctg aag cca atc tat g-3′	initial and nested	
		MD2i	5′-ccg tat caa cac tcg tta tgt gg-3′	nested	
	Human rhinovirus	SRH12-1	5′-tcc tcc ggc ccc tga atg ygg-3′	initial	this study
		OL68-1	5′-ggt aay ttc cac cac can cc-3′	initial and nested	
EVP4		5′-cta ctt tgg gtg tcc gtg tt-3′	nested	Ishiko et al. (2002) Inter-virology	

¹⁾: Primers were used for the initial PCR, nested PCR, or both.

viruses. These questions will be addressed in future studies involving frequent samplings of aspirates from infants in the presence or absence of symptoms and comparison with serological diagnosis.

To conclude, a clear correlation between viral co-infection and severity of clinical symptoms was not observed in the present study. However, further research is required to evaluate viral co-infection synthetically. In-

creases in the size of the study group and frequency of sampling, examination of particular bacterial infections, or a combination of these factors need to be incorporated in future studies.

Acknowledgments This study was partly supported by Grant-in-Aid for Young Scientists (B) 19790442. The study protocol was approved by the ethical committee at Osaka Prefectural Institute of Public Health No. 1003-02.

Conflict of interest None to declare.

REFERENCES

1. Olofsson S, Brittain-Long R, Andersson LM, et al. PCR for detection of respiratory viruses: seasonal variations of virus infections. *Expert Rev Anti Infect Ther*. 2011;9:615-26.
2. Mahony JB. Detection of respiratory viruses by molecular methods. *Clin Microbiol Rev*. 2008;21:716-47.
3. Jartti T, Hedman K, Jartti L, et al. Human bocavirus-the first 5 years. *Rev Med Virol*. 2012;22:46-64.
4. Bonzel L, Tenenbaum T, Schrotten H, et al. Frequent detection of viral coinfection in children hospitalized with acute respiratory tract infection using a real-time polymerase chain reaction. *Pediatr Infect Dis J*. 2008;27:589-94.
5. Calvo C, García-García ML, Blanco C, et al. Multiple simultaneous viral infections in infants with acute respiratory tract infections in Spain. *J Clin Virol*. 2008;42:268-72.
6. Franz A, Adams O, Willems R, et al. Correlation of viral load of respiratory pathogens and co-infections with disease severity in children hospitalized for lower respiratory tract infection. *J Clin Virol*. 2010;48:239-45.
7. Martin ET, Fairchok MP, Kuypers J, et al. Frequent and prolonged shedding of bocavirus in young children attending day-care. *J Infect Dis*. 2010;201:1625-32.
8. Richard N, Komurian-Pradel F, Javouhey E, et al. The impact of dual viral infection in infants admitted to a pediatric intensive care unit associated with severe bronchiolitis. *Pediatr Infect Dis J*. 2008;27:213-7.
9. Aguilar JC, Pérez-Breña MP, García ML, et al. Detection and identification of human parainfluenza viruses 1, 2, 3, and 4 in clinical samples of pediatric patients by multiplex reverse transcription-PCR. *J Clin Microbiol*. 2000;38:1191-5.
10. Bellau-Pujol S, Vabret A, Legrand L, et al. Development of three multiplex RT-PCR assays for the detection of 12 respiratory RNA viruses. *J Virol Methods*. 2005;126:53-63.
11. Paula NT, Carneiro BM, Yokosawa J, et al. Human rhinovirus in the lower respiratory tract infections of young children and the possible involvement of a secondary respiratory viral agent. *Mem Inst Oswaldo Cruz*. 2011;106:316-21.
12. Kennedy JL, Tuner RB, Braciale T, et al. Pathogenesis of rhinovirus infections. *Curr Opin Virol*. 2012;2:287-93.
13. Lauinger IL, Bible JM, Halligan EP, et al. Patient characteristics and severity of human rhinovirus infections in children. *J Clin Virol*. 2013;58:216-20.
14. von Linstow M, Høgh M, Høgh B. Clinical and epidemiologic characteristics of human bocavirus in Danish infants: results from a prospective birth cohort study. *Pediatr Infect Dis J*. 2008;27:897-902.