Title: Viral Pathogens Associated with Acute Respiratory Infections in Central Vietnamese Children

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Keywords: viral; respiratory pathogens; multiplex PCR; pediatric; Vietnam

Abbreviated title: Viral Pediatric Acute Respiratory Infections in Vietnam

Running title: Pediatric Viral Respiratory Agents, Vietnam

ABSTRACT

Hospitalized Vietnamese children with acute respiratory infection (ARI) were investigated for 13 viral pathogens using multiplex-polymerase chain reaction. We enrolled 958 children of whom 659(69%) had documented viral infection: rhinovirus (28%), respiratory syncytial virus (23%), influenza virus (15%), adenovirus (5%), human metapneumo virus (4.5%), parainfluenza virus (5%) and bocavirus (2%). These Vietnamese children had a range of respiratory viruses which underscores the need for enhanced ARI surveillance in tropical developing countries.

Acute respiratory tract infections (ARI) are a major cause of morbidity and mortality in children worldwide (1). Viruses are common cause of lower respiratory tract disease in infants and young children and represent a major public health problem in children (2). Because there are limited resources and facilities for virus isolation and detection in the developing countries, the role of viral respiratory pathogens in the tropical region has not been well-studied. To investigate the magnitude of viral respiratory infections among hospitalized infants and children in a tropical country, we applied a newly developed multiplex-polymerase chain reaction (PCR) technique into an active ARI surveillance program conducted in south central Vietnam.

METHODS

The study was conducted from February 1, 2007 to March 31, 2008 at Khanh Hoa General Hospital (KHGH) which is a tertiary care facility located in Nha Trang city, Khanh Hoa Province. According to the field site census survey in July 2006, the study catchment area of 16 communes in Nha Trang city, had 198,729 residents including 13,952 children less than 5 years of age. An ARI case was defined as any child presenting with cough or/and difficulty in breathing. Before study enrollment, informed consent was obtained from parents of children who presented with ARI and lived in the

study catchment area. Patient clinical information, chest radiographs (CXR), laboratory data and nasopharyngeal (NP) samples were collected from all enrolled patients. Radiograph interpretations were standardized using MiASoft, Ltd. (Faringdon, UK) radiology training modules. Acute Respiratory Infection patients with normal CXR were categorized as upper respiratory tract infection (URTI) and patients with abnormal CXR were categorized as lower respiratory tract infection (LRTI). Bronchiolitis was defined as ARI patient ≤ 2 years old presenting with wheezing and abnormal CXR without evidence of pulmonary consolidation.

Nasopharyngeal (NP) samples were collected at the time of admission and viral nucleic acid was extracted using QIA viral RNA minikit (QIAGEN Inc., Valencia, CA, USA). Four multiplex-PCR assays (1: influenza A, influenza B, RSV, hMPV; 2: PIV-1, -2, -3, and -4; 3: rhinovirus, coronavirus 229E, coronavirus OC43; 4: adenovirus and bocavirus) were performed to detect 13 respiratory viruses in each NP sample. A second confirmatory-PCR was performed for samples positive on the initial PCR test (Supplementary table 1., online only). Samples positive for both PCR assays were defined as positive. Reverse transcription-PCR (RT-PCR) assays were performed using one-step RT-PCR kit from QIAGEN. For the multiplex PCR and hemi-nested PCR assays, Taq DNA polymerase (Promega, San Luis Obispo, CA) was used. Twenty-eight

primers used were established in this study and 11 were from the published studies (3-5). Positive templates were used in each assay for quality control.

RESULTS

During the 14-months study period, a total of 1,014 pediatric patients from the catchment area were admitted to KHGH, of which 958 (95%) were enrolled in the study. Males comprised 58% of patients and 94% of the patients were less than 5 years old (median age: 1.4 years). The results showed that one or more respiratory viruses were found in 69% of patients: 11% had dual and 1.4% had triple infection. Eighty six percent of the viral ARI patients were less than 3 years old (detail information of age breakdown is shown in supplementary table 2, online only).

Major viruses detected were rhinovirus (28%), RSV (23%) and influenza A (15%). This was followed by adenovirus (5%), hMPV (5%), PIV3 (4%) and bocavirus (2%). Other viruses (PIV1, PIV2 and influenza B) were detected in a small proportion (1.5%) of ARI patients. Across age, sex, and case categories, there were no significant differences between proportion of virus positive and negative patients.

The pattern of virus detection did not differ between URTI and LRTI patients. A total of 268 radiologically-confirmed pneumonia (RCP) patients and 195 bronchiolitis

patients were identified. PIV3 detection was significantly associated with hospitalized LRTI (p=0.016) and bronchiolitis (p=<0.001). Similar to previous reports, we found that RSV infection was significantly associated with bronchiolitis (p=0.002) (6). We also found that a significantly higher proportion of patients (n=119) with multiple virus infections had radiological-confirmed pneumonia (p=0.05 by Fisher's exact test) (supplementary table 2, online only). None of the cases with multiple virus infection were malnourished.

Influenza A infection predominated during the 2007 cool season, from February through June 2007 (Figure 1) while RSV patients were detected largely during warmer months, from July to November with the peak in August 2007. In contrast to influenza A and RSV, rhinovirus was detected year round with no distinct seasonality. In early 2008, ARI patients were infected with rhinovirus but not influenza A virus.

To calculate the incidence rates, we used only one year data from March 1st 2007 to February 28th 2008 during which 812 children less than 5 years old were admitted to KHGH (Table 1). The hospitalized ARI incidence for children less than 5 years living in Nha Trang was 58 of 1000 children-year. The highest hospitalized ARI incidence was observed in children less than 1 year age group (130/ 1000 children-year) while the highest age group for RCP and LRTI were among 1-2 year age group (39 and

85/ 1000 children-year respectively). The highest incidence of rhinovirus- and influenza-associated ARI hospitalization were found among 1-2 year age group (41/1000 children-year and 18/1000 children-year) while highest RSV-associated ARI incidence (41/1000 children-year) occurred in infant less than 12 months.

DISCUSSION

In this study, we demonstrated pediatric ARI was associated with a wide variety of viruses in a tropical developing country setting such as south central region of Vietnam. Ten different respiratory viruses were associated with approximately two-thirds of the hospitalized patients with ARI. Recently discovered viruses, including hMPV (7) and bocavirus (8) were associated with a substantial number of pediatric ARI hospitalizations. It is plausible that prevalence of viral pathogens associated with ARI may vary in different countries with different geographic and climatic characteristics. The spectrum of major respiratory pathogens identified in our study was similar to previous studies in Germany and South Korea (9, 10). However, there were some differences in terms of proportion and seasonality of detected viruses. Our study showed a higher proportion of influenza-associated ARI compared with either Germany or South Korea.

We detected no viruses in one-third of children hospitalized with ARI. It is possible that some children may have had infection with other viruses not included in our assays (e.g., enteroviruses, other types of coronaviruses like NL63 and HKU1). In addition, some patients may have cleared their viral infection before the collection of clinical samples or the viruses circulating in our study population may carry polymorphisms in the primer binding sequences of the our target genes.

Although our study showed a high incidence of respiratory viral infections among hospitalized ARI patients, we believe that bacterial pathogens account for a substantial proportion of ARI among hospitalized children. Since this study was only 14 months, we cannot conclude on the seasonality of the viruses that we detected. However it was interesting that a different pattern of influenza was observed in the last 2 months compared to the same period of previous year. For the above reasons, we believe that further studies are warranted to investigate the relation between viral infection and bacterial ARI, and the interaction among different respiratory viruses, and to elucidate seasonal patterns and annual variations of viral infections in tropical developing countries like Vietnam.

In conclusion, a high incidence of respiratory virus-associated ARI was found among hospitalized children in central Vietnam. In this study, rhinoviruses, RSV and

influenza A viruses were the commonest respiratory viruses detected among children. The identification of recently identified respiratory pathogens such as human metapneumovirus and bocavirus in central Vietnam underscore the need to enhance existing surveillance systems to understand the full burden of respiratory pathogens.

ACKNOWLEDGEMENTS

We thank Drs. Takato Odagiri, Masahiro Noda from National Institute of Infectious Diseases, Tokyo, Dr.Nobuhisa Ishiguro from Hokkaido University and Dr.Katsumi Mizuta from Yamagata Prefectural laboratory for providing the necessary control viruses. We thank the parents and participants in this study as well as staff from Khanh-Hoa Health Service and medical staff from of Khanh Hoa General Hospital for their support in this study. The primers and multiplex-PCR assays established and used in this study are pending for patent. This work was supported by the program of Founding Research Centers for Emerging and Reemerging Infectious Diseases (MEXT) Japan and Japan Society for Promotion of Science.

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FIGURE LEGEND

Figure 1. Seasonality of leading viral pathogens among hospitalized children in Vietnam,

February 2007- March 2008

SUPPLIMENTAL DIGITAL CONTENT LEGEND

Supplementary Table 1. Primers and PCR assays for multiplex PCR and hemi-nested multiplex PCR

Supplementary Table 2. Characteristics of Hospitalized Pediatric Viral Acute Respiratory Infection patients, February 2007 through March 2008

Table 1. Hospitalized incidence of ARI and major viral agents among children less than five years in Nha Trang (incidence per 1000 children, March 1st 2007 to Feb 28th 2008)

Catchment area Age Population	ARI Incidence(n)	LRI Incidence(n)	RCP Incidence(n)	rhinovirus Incidence(n)	RSV Incidence(n)	InfluA Incidence(n)
0 to 1yr 2250	**129.8** (292)	65.8 (148)	35.1 (79)	30.7 (69)	**40.9** (92)	16.9 (38)
1 to 2yr 2442	127.4 (311)	**84.8** (207)	**38.5** (94)	**41** (100)	31.5 (77)	**18.4** (45)
2 to 3yr 3319	39.5 (131)	24.1 (80)	10.8 (36)	10.5 (35)	8.7 (29)	6.9 (23)
3 to 4yr 2944	19.7 (58)	9.9 (29)	4.8 (14)	4.4 (13)	4.8 (14)	3.4 (10)
4 to 5yr 2997	6.7 (20)	3.3 (10)	1.3 (4)	1 (3)	0.7 (2)	2 (6)
< 5yrs, 13952	58.2 (812)	34 (474)	16.3 (227)	15.8 (220)	15.3 (214)	8.7 (122)

Highest incidence in each group are shown in bold

ARI: acute respiratory infection, LRI: lower respiratory infection, RCP: radiological confirmed pneumonia, RSV: respiratory syncytial virus, InfluA: influenza virus type A

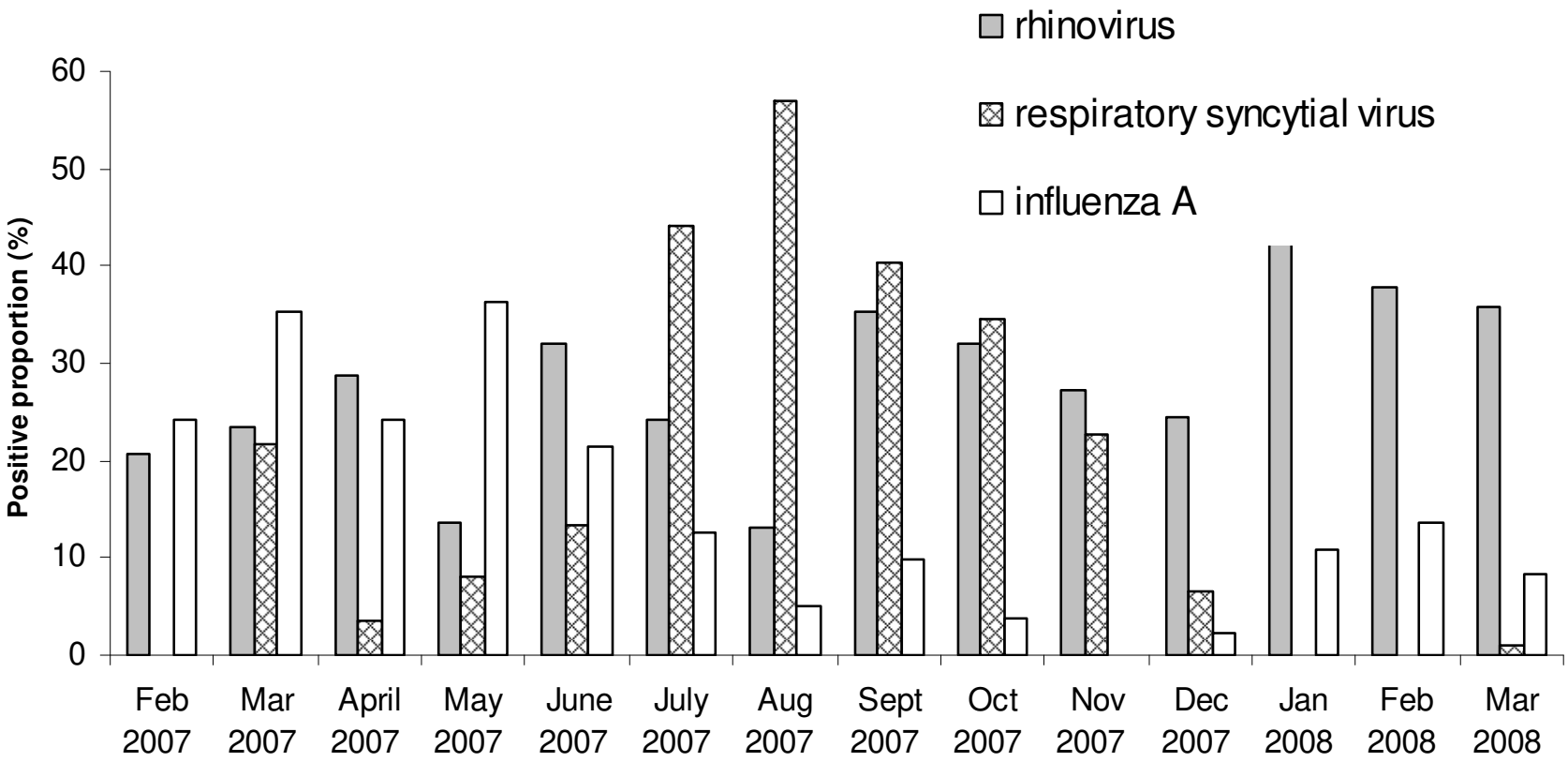

Figure 1. Seasonality of leading viral pathogens among hospitalized children in Vietnam, February 2007- March 2008.

Supplementary Table 1. Primers and PCR assays for multiplex PCR and hemi-nested multiplex PCR

Assay	Virus	Direction	Sequence (5' – 3')	Target gene	Amplicon (size, bp)
Multiplex 1					
1st round RT-PCR	InFluA	sense	CCTTCTAACCGAGGTCGAAACG	Matrix protein	241
		antisense	GCATTTTGGACAAAGCGTCTACG		
	InFluB	sense	AGACACAATTGCCTACCTGCTTTC	Matrix protein	352
		antisense	CTGAGCTTTCATGGCCTTCTGC		
	hRSV	sense	CATGACTCTCCTGATTGTGGGATG	Nucleocapsid	271
		antisense	CCTTCAACTCTACTGCCACCTC		
	hMPV	sense	TGAAGTCAATGCGACTGTAGCAC	Matrix protein	371
		antisense	ATGCCTTTGGGATTGTTCATGGTC		
Hemi-nested PCR	InFluA	antisense	ACAGGATTGGTCTTGTCTTTAGCC	Matrix protein	151
	InFluB	antisense	AGTCTAGGTCAAATTCTTTCCCACC	Matrix protein	106
	RSV	sense	AGCAGCAGGGGATAGATCTGGTC	Nucleocapsid	201
	hMPV	antisense	TCACTGCTTATTGCAGCTTCAACAG	Matrix protein	287
Multiplex 2					
1st round RT-PCR	PIV1	sense	ATGATTTCTGGAGATGTCCCGTAGG	HA-NA	300
		antisense	TTCCTGTTGTCGTTGATGTCATAGG		
	PIV2	sense	CAATCAATCCTGCAGTCGGAAGC	HA-NA	386
		antisense	AAAGCGATGCAGACCACCAAG		
	PIV3	sense	GACACAACAAATGTCGGATCTTAGG	HA-NA	230
		antisense	ATACAGCCATCAACAGTCGTTGG		
	PIV4[a]	sense	CTGAACGGTTGCATTCAGGT	Phosphoprotein	451
		antisense	TTGCATCAAGAATGAGTCCT		
Hemi-nested PCR	PIV1	sense	CCACCACAATTTCAGGATGTGTTAG	HA-NA	210
	PIV2	antisense	TAACATAGAGCCTACCTTCTGCACC	HA-NA	329
	PIV3	antisense	GAAGACCAGACGTGCATCTCCA	HA-NA	148
	PIV4[a]	antisense	GTCTGATCCCATAAGCAGC	Phosphoprotein	390
Multiplex 3					
1st round RTPCR	rhinovirus	sense	CCCACAGTAGACCTGGCAGATG	5'-noncoding region	254
		antisense	ACGGACACCCAAAGTAGTTGGT		
	hCV229E[b]	sense	GGTTTTGACAAGCCTCAGGAAAAAGA	Membrane glycoprotein	573
		antisense	GTGACTATCAAACAGCATAGCAGCTGT		
	hCVOC43[b]	sense	GCTAGTCTTGTTCTGGCAAAACTTGGC	Membrane glycoprotein	335
		antisense	TGAATTGCGCTATAACGGCGC		
Hemi-nested PCR	rhinnovirus	antisense	CAGGGTTAAGGTTAGCCGCATTC	5'-noncoding region	175
	hCV 229E[a]	antisense	CCATTGGCCACAACACCTGC	Membrane glycoprotein	230
	hCV OC43[a]	antisense	CTCAGCAAGTAACTAAGCATACTGCC	Membrane glycoprotein	170
Multiplex 4					
1st round PCR	adenovirus	sense	CAAAGCTCCCTAGGAAACGACCT		193
		antisense	GCGGGTATGGGGTAAAGCATGT	Hexon	
	bocavirus[c]	sense	GACCTCTGTAAGTACTATTAC		354
		antisense	CTCTGTGTTGACTGAATACAG	Nonstructural protein-1	
Hemi-nested PCR	adenovirus	antisense	GTTAAAGGACTGGTCGTTGGTGTC	hexon	152
	bocavirus	antisense	CCGATCTACTCTCCCTCGTCTTC	Nonstructural protein-1	303

Notes for supplementary Table 1.

InFluA, influenza A virus; InFluB, influenza B virus; RSV, respiratory syncytial virus; hMPV, human metapneumovirus; PIV, parainfluenzavirus; hCV 229E, human coronavirus 229E; hCV OC43, human coronavirus OC43; RT-PCR, reverse transcriptase polymerase chain reaction; PCR, polymerase chain reaction; HA-NA, Hemagglutinin-Neuraminidase.

In this study a total of four multiplex PCR assays comprising of three multiplex RT-PCR assays targeting 10 RNA viruses and one multiplex PCR assay targeting two DNA viruses were established. Multiplex 1 was to detect influenza A and B, respiratory syncytial virus and human metapneumovirus. Multiplex 2 was to detect parainfluenza 1, 2, 3 and 4. Multiplex 3 was to detect rhinovirus, human corona OC43 and 229E. Multiplex 4 was to detect adenovirus and bocavirus. The sensitivities of the multiplex PCR assays were tested by using known copy number of control templates. The detection limits of the multiplex PCR assays were 10 to 100 copies of template per reaction. The multiplex PCR assays were sensitive enough to detect 10% minor population in mixed template experiments. All the primers were designed in this study, except for PIV4[a], hCoV 229E[b], hCoV OC43[b] and bocavirus[c]:

[a] Bellau-Pujol, S., A. Vabret, L. Legrand, J. Dina, S. Gouarin, J. Petitjean-Lecherbonnier, B. Pozzetto, C. Ginevra, and F. Freymuth. Development of three multiplex RT-PCR assays for the detection of 12 respiratory RNA viruses. J Virol Methods. 2005; 126:53-63.

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Supplementary Table 2. Characteristics of Hospitalized Pediatric Viral Acute Respiratory Infection patients, February 2007 through March 2008

Characteristic	Any virus positive n=659 (68.8)	Virus negative n=299 (31.2)	Viral Pathogens											
			rhino	RSV	Influ A	Adeno	hMPV	PIV 3	boca-virus	PIV 1	PIV 2	Influ B	2 viruses positive	3 viruses positive
Positive proportion (%)			270 (28)	217 (22.7)	146 (15.2)	49 (5.1)	43 (4.5)	36 (3.8)	19 (2)	7 (0.7)	5 (0.5)	2 (0.2)	106 (11.1)	13 (1.4)
Sex, male (%)	59.8	54.5	61.5	55.8	58.9	63.3	60.5	61.1	84.2	57.1	100	50	58.5	84.6
Age, months														
0-6	86	41	37	38	11	6	2	5	1	0	1	0	11	2
6-12	123	73	44	54	29	9	6	3	7	0	0	0	21	4
12-18	137	43	62	42	24	14	14	9	7	1	0	0	30	3
18-24	128	38	52	36	26	10	8	12	3	3	0	1	19	2
24-30	68	34	25	20	19	4	4	6	0	1	1	0	10	1
30-36	32	17	16	9	7	2	2	1	1	0	2	0	6	1
36-42	20	11	7	6	4	2	2	0	0	0	0	1	2	0
42-48	24	9	9	8	8	0	3	0	0	1	1	0	3	0
4-5 yr	11	10	3	2	7	0	0	0	0	1	0	0	2	0
5-15yr	30	23	15	2	11	2	2	0	0	0	0	0	2	0
Median age, yr	1.4	1.4	1.3	1.2	1.6	1.3	1.4	1.5	1.2	2	2.6	2.5	1.3	1.3
Case category														
URTI	261	130	113	93	53	19	15	8	5	3	2	0	37	5
(%)	(39.6)	(43.5)	(41.9)	(42.9)	(36.3)	(38.8)	(34.9)	(22.2)	(26.3)	(42.9)	(40)	(0)	(34.9)	(38.5)
LRTI	392	165	153	123	93	30	27	**28**	14	4	3	2	69	8
(%)	(59.5)	(55.2)	(56.7)	(56.7)	(63.7)	(61.2)	(62.8)	(77.8)	(73.7)	(57.1)	(60)	(100)	(65.1)	(61.5)
Bronchiolitis	150	45	49	**61**	28	8	13	**16**	3	0	2	1	27	2
(%)	(22.8)	(15.1)	(18.2)	(28.1)	(19.2)	(16.3)	(30.2)	(44.4)	(15.8)	(0)	(40.0)	(50)	(25.5)	(15.4)
RCP	183	85	81	52	45	18	13	10	9	3	1	0	37	6
(% of total)	(27.8)	(28.4)	(30)	(24)	(30.8)	(36.7)	(30.2)	(27.8)	(47.4)	(42.9)	(20)	(0)	(35)	(46.2)
(% of RCP)	(68.3)	(31.7)	(30.2)	(19.4)	(16.8)	(6.7)	(4.8)	(3.7)	(3.4)	(1.1)	(0.4)	(0)	(13.8)	(2.2)
Other LRTI	59	35	23	10	20	4	1	2	2	1	0	1	5	0
(%)	(9.0)	(11.7)	(8.5)	(4.6)	(13.7)	(8.2)	(2.3)	(5.6)	(10.5)	(14.3)	(0)	(50.0)	(4.7)	(0)

Bold characters indicate significant associations (p=0.05): PIV3 was significantly associated with LRTI compared with PIV3 negative patients; RSV was significantly associated with bronchiolitis compared with RSV negative patients.

CXR data was not available for 10 patients: 6 patients in virus positive group (1 patients of RSV, 4 patients of Rhino and 1 patient of hMPV) and 4 patients in virus negative group.

URTI, upper respiratory tract infection; LRTI, lower respiratory tract infection; CXR, chest Xray; RCP, radiological confirmed pneumonia; rhino, rhinovirus; RSV, respiratory syncytial virus; InFluA, influenza A; Adeno, adenovirus; hMPV, human metapneumovirus; PIV, parainfluenzavirus; InFluB, influenza B.