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Molecular monitoring of causative viruses in child acute respiratory infection in endemo-epidemic situations in Shanghai

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ABSTRACT

Background: Numerous viruses are responsible for respiratory infections; however, both their distribution and genetic diversity, in a limited area and a population subgroup, have been studied only rarely during a sustained period of time.
Methods: A 2-year surveillance program of children presenting with acute respiratory infections (ARIs) was carried out to characterize the viral etiology and to assess whether using gene amplification and sequencing could be a reliable approach to monitor virus introduction and spread in a population subgroup.
Results: Using multiplex RT-PCR, 15 different respiratory viruses were detected within the 486 nasopharyngeal positive samples collected among 817 children aged <9-year old who presented with ARI during October 2006 to September 2008. A single virus was detected in 373 patients (45.7%), and two to four viruses in 113 patients (13.8%). The most frequent causative viruses were respiratory syncytial virus (RSV) (24.7%), human bocavirus (24.5%), and human rhinovirus (HRV) (15%). RSV was more prevalent in winter and among young infants. Cases of seasonal influenza A and B viruses were reported mainly in January and August. An increase in adenovirus infection was observed during the spring of the second year of the study. Sequence analyses showed multiple introductions of different virus subtypes and identified a high prevalence of the newly defined HRV-C species. A higher viral incidence was observed during the winter of 2008, which was unusually cold.
Conclusions: This study supports the usefulness of multiplex RT-PCR for virus detection and co-infection, and for implementation of a molecular monitoring system for endemic and epidemic viral respiratory infections.

1. Background

Acute respiratory infections (ARIs) are the leading cause of pediatric morbidity worldwide.[1,2] Many viruses are associated with ARIs: influenza viruses A, B and C (IAV, IBV and ICV); respiratory syncytial virus (RSV); human metapneumovirus (HMPV); human coronaviruses (HCoV) NL63, 229E, OC43, and HKU1; parainfluenza viruses (PIV) 1–4; human rhinovirus (HRV); human enterovirus (HEV); and adenovirus (ADV).[3] A new pathogen, human bocavirus (HBoV) has been shown to be associated with respiratory illnesses, mainly when it is present at a high viral load.[4–7]

Since the epidemic of severe acute respiratory syndrome (SARS) in 2003, and the recent attention on possible influenza pandemics, sustained surveillance project was required to detect endemic, epidemic and newly emerging respiratory pathogens.

The diagnosis of respiratory viruses mainly relies on molecular techniques. Multiplex RT-PCR (mRT-PCR) techniques allow identification of a majority of respiratory viruses[8–10] as well as co-infections.[11,12]

2. Objectives

In the present 2-year study, we used a five-tube mRT-PCR assay we implemented in the Pasteur Institute network in the Asian region (http://www.pasteur-international.org/ip/easysite/pasteur-international/activites-scientifiques/projets/tous-les-projets/sisea), which covered 17 common respiratory viruses,[10] to identify viruses in nasopharyngeal specimens in 817 children with

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Table 1
Criteria of patient enrollment.

Criteria	
Inclusion	Exclusion
Children younger than 9-year old, first onset within 48 h,	Subjects already under antiviral treatment for any prophylactic or curative purpose
AND	
Fever ($T \geq 38\,^{\circ}C$) plus cough and/or sore throat	
AND/OR	
Dyspnoea or tachypnea, cyanosis, cough, pleuritic chest pain, hypoxemia	
AND	
Signature of the patient consent agreement	

ARI. Sequencing primers specific to other fragments of viral genes were employed to amplify the positive samples for sequencing and phylogenetic analysis. The sequences showed the genetic variation of viruses circulating in the region, and identified the virus evolution and introduction of new variants. This study allowed cartography of viral etiology of a large panel of viruses that were co-circulating in ARI in children in a district of Shanghai, with the aim of implementing a monitoring system for endemic or epidemic viral respiratory infections.

3. Study design

3.1. Patient population

Between October 1, 2006 and September 30, 2008, all subjects aged <9 years, who presented with an ARI syndrome and attended the outpatient ward of the pediatric department of Shanghai Nanxiang Hospital, China, were enrolled prospectively. The protocol was approved by the Ethical Committee of Shanghai Nanxiang Hospital and by the Biomedical Committee of Institut Pasteur in Paris. Written informed consent of a parent or a legal guardian was required. ARI was defined as the presence of fever (at least 38 °C) plus cough and/or sore throat (Table 1). Study enrollment was organized twice weekly (every Monday and Thursday); all patients who consulted on Monday and Thursday and presented with the above case definition were included in the study.

3.2. Data and specimen collection

Upon enrollment, systematic recordings were made of the patients' demographic characteristics and medical history using a standardized questionnaire. The questions included detailed signs and symptoms, laboratory and radiology examinations; the presence of a chronic underlying disease; and family smoking history. After a complete physical examination, the children were classified into three different disease groups on the basis of signs and/or symptoms indicating the inflammation site: upper respiratory tract infection, bronchitis and pneumonia. Patients and their parents were interviewed by the same doctor to obtain demographic data and information about their clinical presentation.

For each ARI case, nasopharyngeal swabs (NPSs) were obtained by the same nurse. Specimens were collected with sterile cotton-tipped swabs that were introduced into the nostril and the pharyngeal areas, and then placed in 2 ml viral transport medium. The NPSs were then transported at 4 °C to the Virology Department of the Institut Pasteur of Shanghai, where they were divided into aliquots, and stored at −80 °C.

RNA extraction—Total RNA from NPS aliquots was extracted using a QIAamp viral RNA Minikit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol. Purified RNA was frozen at −80 °C in aliquots.

Multiplex RT-PCR—A mRT-PCR previously published was improved and employed in this study for virus detection. It was initially described by Bellau-Pujol et al. (2005) for 12 virus identification including IAV, IBV, ICV, RSV, HMPV, PIV1–4, HCoV-OC43 and 229E, and HRV in three tubes with hemi-nested PCR[8] then later improved by Vabret et al. for 14 viruses plus with HCoV-HKU1 and HCoV-NL63 in four tubes.[13] Besides, the primers used for HRV detection could also detect HEV, and the two viruses were then differentiated based on the sizes of the amplified products. The DNA band with the size of 550–574 bp corresponded to rhinovirus while the band with the size of 600–700 bp corresponded to enterovirus. Hence, the mRT-PCR could detect 15 viruses. The mRT-PCR multiplex 1 and the hemi-nested multiplex 1 detected 1 and 0.1 TCID50 of RSV A, respectively, and 0.01 and 0.001 TCID50 of influenza virus A/H3N2, respectively.[8]

We have improved the method to detect 17 viruses in a five-tube mRT-PCR assay by introducing specific primers to ADV and HBoV.[14,15] Moreover, we eliminated previous hemi-nested PCR step to avoid cross-contamination but introduced sequencing of amplification products. The assay was compared with commercialized Resplex II assay (Qiagen) in previous study and its sensitivity reached 0.01 TCID50 of RSV B, 0.3 TCID50 of influenza virus A/H1N1, and 0.001 TCID50 of IBV, respectively.[12]

Tube 1 targeted IAV, IBV, RSV, HMPV; tube 2, PIV1–4; tube 3, HRV and ICV; and tube 4, HCoV-229E, HCoV-OC43, HCoV-NL63 and HCoV-HKU1.[8,10,12] Tube 5 targeted ADV and HBoV using previously published primers: HBoV (188F, 5′-GASCTCTGTAAGTACTATTAC-3′; 542R, 5′-CTCTGTGTTGACTGAATACAG-3′); ADV (ADHEX1F, 5′-CAACACCTAYGASTACATGAA-3′; ADHEX2R, 5′-ACATCCTTBCKGAAGTTCCA-3′).[14,15] RNA was amplified using a one-step RT-PCR kit (Qiagen) as previously described.[12] In brief, 2.5 μl of extracted RNA was mixed with a 5× buffer and 0.2 mM dNTP, 0.2 μM of each primer, and l μl of enzyme mix, and DEPC-treated ultrapure water was added to a final volume of 25 μl. Amplification programs included reverse transcription at 50 °C for 30 min, inactivation at 95 °C for 15 min, followed by 40 cycles at 94 °C for 30 s, 50 °C (tubes 1, 2 and 5) or 55 °C (tubes 3 and 4) for 30 s, 72 °C for 45 s and final extension at 72 °C for 10 min. The amplified DNA products were detected by 0.5 g/l ethidium bromide/2% agarose gel electrophoresis.

Cloning and sequencing—Sequencing primers were designed based on the conserved region of each virus or on previous publications[16–21] (Table 2). Extracted RNA was amplified by specific monoplex RT-PCR following the same protocol as above, but with a hybridization temperature of 53 °C. The DNA products were purified from agarose gels using QIAquick Gel Extraction Kit (Qiagen), and were ligated into pMD20-T vector (Takara Biotechnology, Dalian, China). Recombinant plasmids were sequenced by Biosune Sequence Company and Invitrogen Biotechnology Company in Shanghai, China.

Phylogenetic analysis—Multiple sequences were aligned using Clustal X (v1.83). The multiple-sequence alignment was subjected to phylogenetic analyses using programs in the PHYLIP package (v3.6). Bootstrap analysis was performed using SEQBOOT, in which replicate number was 1000. DNADIST and NEIGHBOR were used to obtain a distance matrix; in DNADIST, the transition/transversion ratio was 4. Consensus trees were computed by CONSENSE, and then rerooted with RETREE. The final tree was visualized and edited with MEGA version 4.

3.3. Statistical analysis

The patient data were analyzed using Statistical Package for the Social Sciences (SPSS) for Window version 17.0 (SPSS Inc. Chicago,

Table 2
Sequencing primers.

Virus	Primer name	Sequence 5′ → 3′	Region	Position	Reference strain	Reference
229E	229E/SPIKE-F	CTACAAATGGGMTGAACACTAGTYACTC	Spike	53–80	AY386395	This study
	229E/SPIKE-R	TACGTGGTTGAACAGCAATTATAG		1679–1702		This study
NL63	NL63/SPIKE-F	GAGTTTGATTAAGAGTGGTAGGTTG	Spike	20391–20415	AY567487	[16]
	NL63/SPIKE-R	ACACGGCCATTATGTGTGGTGAC		21049–21073		[16]
HKU-1	KHKU1-SPIKE-F	CYTCACCTCTTAATTGGGAACG	Spike	23920–23939	DQ415914	This study
	KHKU1-SPIKE-R	AAAACRCAACCAAGATAACTATC		25067–25045		This study
PIV1	PIV1-HNF	CAACCTRYAAGGMAACARCATCYG	HN	1–24	M91648	This study
	PIV1-HNR	TCTATTTGTCATATAAATRTCTATTCATGC		1835–1864		This study
PIV3	PIV3-HNF	AAATCCKAGGATCTCTCATAYTTTY	HN	7522–7545	EU424062	This study
	PIV3-HNR	GCCYTTGTCAACAACAATRATRG		7946–7966		This study
PIV4	PIV4-HN-S	GGAACRCRCTTCTCAGCTC	HN	65–81	M34033-4A	This study
	PIV4-HN-ANTI	GAGAAGTAAGTTATTGTGTATGAGTC		1417–1439		This study
ADV	ADHEX1F	CAACACCTAYGASTACATGAA	Hexon	19002–19022	FJ169625	[15]
	ADHEX2R	ACATCCTTBCKGAAGTTCCA		19255–19274		[15]
HMPV	F698	ACATGCCAACATCTGCAGGACAAATAAAAC	Fusion	698–727	EU857610	[17]
	F1285	ACATGCTGTTCACCTTCAACTTTGC		1282–1307		[17]
IBV	B/HA98	ATAACATCGTCAAACTCACC	HA	64–83	EU852039	[18]
	B/HA836	GCACCATGTAATCAACAACA		780–799		[18]
IBV	B/NA1	GCTACCTTCAACTATACAAACG	NA	3–24	EU852040	[19]
	B/NA2	AACGAGGGTATGTCCACTCC		233–253		[19]
IAV	43f	GTCTGGTTTTCGCTCAAAAACTTCC	HA	35–59	EU716524	This study
	1129r	GAATTTTGATGCCTGAAACCGTACC		1097–1121		This study
IAV	32f	GATTGGCTCTGTTTCTCTCACC	NA	27–48	CY031565	This study
	984r	CTGGGTGTGTCTCCAACAAGTCCTG		956–980		This study
HRV	P1-1 F	CAAGCACTTCTGTYWCCCC	5′UTR	163–181	L24917	[20]
	P3-1 R	ACGGACACCCAAAGTAG		536–552		[20]
	VP4/2 F	GGGACCAACTACTTTGGGTGTCCGTGT	VP2/4	528–554		[21]
	VP4/2 R	GCATCIGGYARYTTCCACCACCANCC		1061–1086		[21]
RSV	RSV-GLYCO-AF	ATCATATTCATAGCCTCRGCAAAC	Glycoprotein	4833–4856	NC_001803	This study
	RSV-GLYCO-AR	ACACTTCAAAGTGAAAATCATTAT		5160–5132		This study
	RSV-GLYCO-BF	TATTCATCATCTCTGCGAATCAC		4868–4890	NC_001781	This study
	RSV-GLYCO-BR	AATCATCTTTGGGTTTTTTTGGTGG		5176–5150		This study

IL, USA). A p value of <0.05 was considered statistically significant. Categorical variables were assessed for statistical significance by Pearson χ^2 test. Fisher's exact test was used if the expected values were <5.

4. Results

A viral etiology could be determined in 486 of the 817 (59.5%) patients. Multiple viral infections were detected in 113 (13.8%) patients (94 with two pathogens, 18 with three pathogens, and one with four pathogens) (Table 3). Among 486 virus-positive cases, 346 (71.2%) were diagnosed by clinicians as bronchitis, 135 (27.8%) as pneumonia, and 5 (1.0%) were diagnosed with upper respiratory tract infection.

Overall, 618 viral pathogens were detected: RSV was the most frequent pathogen (n = 120, 19.4%). The second to the fifth most frequent pathogens were HBoV (n = 119, 19.3%); IV (IAV and IBV) (n = 108, 17.5%); PIV1, 3 or 4 (n = 77, 12.5%), and HRV (n = 73, 11.8%). HMPV (n = 44, 7.1%), ADV (n = 43, 7.0%), HCoV-OC43, 229E, NL63 or HKU1 (n = 24, 3.9%). HEV (n = 11, 1.8%) were also detected occasionally.

Among the 113 patients diagnosed with a multiple viral infection, the most frequent pathogens were HBoV, RSV, HRV and ADV (data not shown).

4.1. Seasonality of viruses

The monthly distribution of viruses is presented in Figs. 1 and 2. Viruses were detected significantly more often during fall or winter than during other seasons (71% and 49%, respectively, $p < 0.01$). RSV and HBoV identifications were most frequent in the fall and winter. IV showed a biannual distribution, one peak in winter and another in the summer. One important increase in ADV infection from March to June in 2008 was recorded. Twenty-two ADV strains among 118 samples were detected compared to five strains among 52 samples in the same period of 2007. A small number of HCoV-NL63 was detected during the summer of 2007. HRV, PIV and HMPV were detected continuously throughout the year.

4.2. Impact of age distribution

Patients included in this study were aged from 1 month to 9 years. The median age was 3 years. RSV was more frequent in younger children ($p = 10^{-7}$) with 12 out of 23 virus-positive cases from 6 months to 2 years age group. No IAV or IBV infection was detected in children younger than 1-year old (Table 4). Only 8 patients from 1 to 6 months age group were enrolled in the study.

4.3. Prevalent types/subtypes of viruses

To identify the prevalent subtype of different viruses and the similarity of the virus strains, virus-positive samples were sequenced for target genes, when the amount of genetic material amplified was sufficient (Table 5), and phylogenetic trees were constructed (data not shown; dendrograms are available on request to

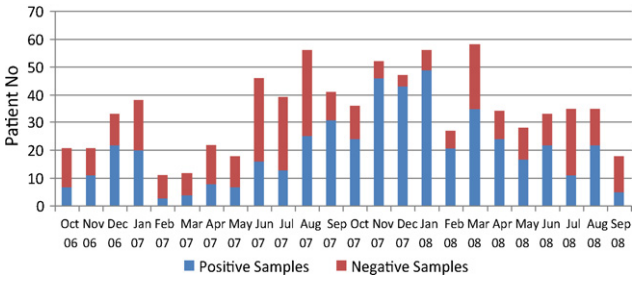

Fig. 1. Monthly distribution of ARI cases with mRT-PCR results, in 817 outpatient children, Shanghai Nanxiang Hospital, October 2006 to September 2008.

Table 3
Viral etiology of ARI in 817 outpatient children, Shanghai Nanxiang Hospital, October 2006 to September 2008.

Virus	Virus detection[a]		Total virus strains
	Single infection	Co-infection	
Human RSV	71	49	120
IV	80	28	108
IAV	44	9	53
IBV	36	19	55
HMPV	33	11	44
PIV	52	25	77
PIV1	27	18	45
PIV3	23	6	29
PIV4	2	1	3
HRV	43	30	73
HEV	5	6	11
HCoV	17	7	24
HCoV-OC43	1	0	1
HCoV-229E	1	4	5
HCoV-NL63	14	3	17
HCoV-HKU1	1	0	1
ADV	18	25	43
HBoV	54	65	119

[a] Case number.

the authors). Identity among isolates of each virus type or subtype was calculated by pairwise algorithm and their nearest reference strains are shown in Table 4.

In 40 sequenced ADV strains, 37 were species B (92.5%) and three were species C (7.5%). Most of them were similar to serotype ADV-2 (data not shown). In 65 HRV strains, 25 were species A (38.5%), five were species B (7.7%), and 35 were species C (53.8%). HRV showed high variation in nucleotide identity (73–100% in HRV-A, 75–82% in HRV-B, and 68–100% in HRV-C) as previously described (Huang et al.).[26] Among 37 IAV strains, 16 were seasonal H1N1 (95–99% nucleotide identity in the HA gene) and 21 were seasonal H3N2 (92–99% identity) strains. In 36 out of 55 IBV strains, HA and NA gene fragments were sequenced, and comparison showed that the IBV strains were more conserved in the NA gene (96–100% nucleotide identity) than HA gene (87–99% identity). Only 17 out of 119 HBoVs were sequenced but showed high nucleotide identity in the ST2 gene as previously described.[7] The sequences of HCoVs were highly conserved (94–100% nucleotide identity in HCoV-NL63, 99% in HCoV-229E). In 39 HMPV strains, 16 were genotype B1 (41%), 16 were genotype B2 (41%), and seven were genotype A1 (17.9%) with high nucleotide identity (98–99% in A1 and 97–100% in B1 and B2). Only 27 out of 120 RSVs were sequenced and nucleotide sequence analysis of a glycoprotein gene fragment showed all of the strains were classified into RSV A subtype (data not shown). The results indicated a low variability of these viruses that circulated in the region during the 2-year period.

4.4. Clinical features

Among 486 virus-positive cases, 483 (99.4%) patients presented with high fever (>38 °C) and cough, 25 (5.1%) patients with dys-

Table 4
Frequencies of viral pathogens (per age group).

	≤6 months		6 months–1 year		1–2 years		2–4 years		5–9 years		All ages	
	n = 8	%	*n* = 31	%	*n* = 121	%	*n* = 449	%	*n* = 208	%	*n* = 817	%
Single infection	4[a]	50[b]	11	35.5	37	30.6	236	52.6	85	40.9	373	45.7
Co-infection	0		12	38.7	36	29.8	38	8.5	27	13	113	13.8
PCR negative	4	50	8	25.8	48	39.7	175	39	96	46.2	331	40.5
RSV	0		12	52.2	17	23.3	71	25.9	20	17.9	120	24.7
IV (any)	0		3	13	11	15.1	56	20.4	38	33.9	108	22.2
IAV	0		0		5	6.8	31	11.3	17	15.2	53	10.9
IBV	0		3	13	6	8.2	25	9.1	21	18.8	55	11.3
HMPV	1[c]	12.5[d]	2	8.7	6	8.2	28	10.2	7	6.3	44	9.1
PIV (any)	0		5	21.7	10	13.7	49	17.9	13	11.6	77	15.8
Type 1	0		1	4.3	7	9.6	29	10.6	8	7.1	45	9.3
Type 3	0		2	8.7	3	4.1	19	6.9	5	4.5	29	6
Type 4	0		2	8.7	0		1	0.4	0		3	0.6
HRV	2	25	4	17.4	13	17.8	37	13.5	17	15.2	73	13
HEV	0		0		2	2.7	7	2.6	2	1.7	11	2.3
HCoV (any)	0		0		3	4.1	17	6.2	4	3.6	24	4.9
OC43	0		0		0		1	0.4	0		1	0.2
229E	0		0		0		4	1.5	1	0.9	5	1
NL63	0		0		2	2.7	12	4.4	3	2.7	17	3.5
HKU1	0		1	4.3	0		0		0		1	0.2
ADV	0		3	13	5	6.8	19	6.9	16	14.3	43	8.8
HBoV	1	12.5	4	17.4	27	37	61	22.3	26	23.2	119	24.5

[a] Case number.
[b] Case percentage in group.
[c] Detected virus.
[d] Percentage of detected virus in virus infected cases of each group.

Table 5
Genetic variation of circulating viruses.

Virus	Detected	Sequenced	Gene	Species	Genotype/type	Number	Nucleotide identity % among strains	Reference strain
HMPV	44	39	F		A1	7	98–99	EU698012, EU179277
					B1	16	97–100	EU698017
					B2	16	97–100	EF694069
IAV	53	37	HA		H1	16	95–99	CY031370
					H3	21	92–99	CY040098, CY044788
IBV	55	36	HA				87–99	CY015406, EU982188
			NA				96–100	CY040451, GQ423424
HRV	73	65	VP4	A		25	73–100	EU840871, EU840840
				B		5	75–82	DQ473490, EF173424
				C		35	68–100	EF582385, GQ223227, EF077280, EF582386, EF582387, EF077279, EF186077
ADV	43	40	Hexon	B	Type 2	37	94–100	AY819918, AY819919
				C		3	86–98	AY819936
HCoV-NL63	17	13	Spike				94–100	DQ445912
HCoV-229E	5	5	Spike				99	AF304460
HBoV	119	17	VP2		Type 1	17	99.3	DQ000496
RSV	120	27	Glycoprotein		A	27	94–100	AF512538

pnoea or tachypnea and 46 (9.4%) patients with lymphopenia. Although 346 (71.2%) were clinically diagnosed as bronchitis, 135 (27.8%) were pneumonia and 5 (1.0%) were diagnosed with upper respiratory tract infection by the clinician, we considered that only 25 patients met the severe respiratory infection as showing dyspnoea or tachypnea symptoms. One hundred twenty patients (22.6%) were suggested to be hospitalized after their first consulting but none became inpatients. No correlation was observed between infection with any specific virus (single or co-infection) and clinical severity (Table 6A and B). In addition, the repartition of the different symptoms was statistically insignificant when compared with virus-negative diagnosed patients (Table 6A and B). Interestingly, 30 patients had polynucleosis associated to an inflammatory response may be linked to a bacterial infection. No specific virus was associated to polynucleosis.

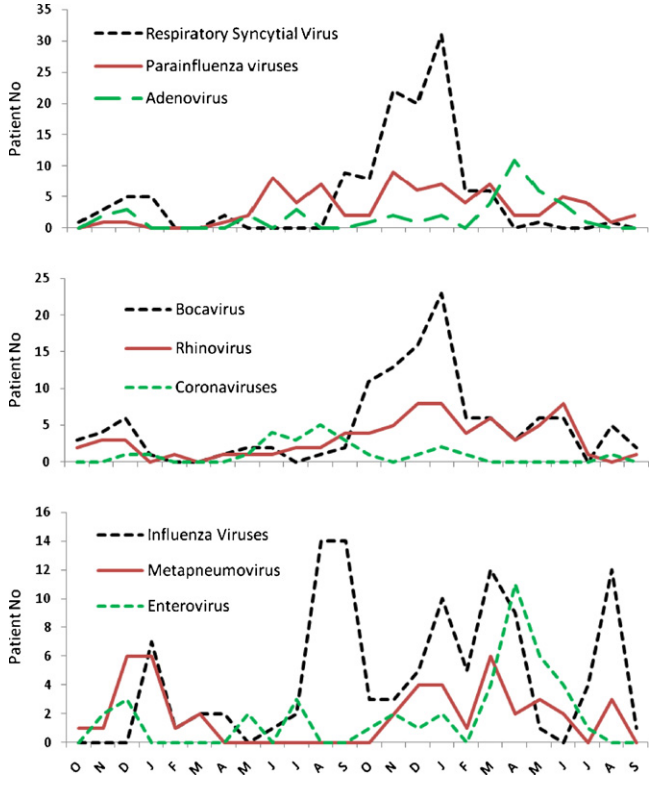

Fig. 2. Monthly distribution of virus strains detected by mRT-PCR, in 817 outpatient children, Shanghai Nanxiang Hospital, October 2006 to September 2008.

5. Discussion

From October 2006 to September 2008, 817 outpatients aged from 1 month to 9 years were included in a surveillance program of viral etiology in ARI. Less than 1% of the children enrolled in this outpatient study were aged of less than 6 months, suggesting that very young children may show more severe symptoms and hospitalized. Fifteen different viruses were detected in 486 samples (59.5%). Thus, respiratory viruses were the major pathogens responsible for ARI in children in Shanghai and multi-infections of different viruses (13.8%) were frequently observed.

Although serotype identification is critical for epidemiological surveillance, the serotyping is time consuming and costly, and limited due to cross-reactivity of the tests. We sequenced the fragments of genes coding for virus antigenic proteins and analyzed by phylogenetic analysis the sequence diversity to monitor the molecular evolution of circulating virus.

During the outbreak of ADV from March to June in 2008, the majority of strains (37 out of 40) was ADV-B species and of serotype ADV-2. In ADV-B species, only serotype ADV-14 was reported to cause severe infection,[22] which may partly explained that in our study ADV-infected patients showed only mild symptoms.

The IAV strains detected during the period from January 2007 to April 2008 were mainly H3N2 but the strains detected from July 2008 to September 2008 were mainly H1N1. This suggests that subtype H1N1 replaced the H3N2 subtype and predominated during the next year in the region. As vaccination for seasonal influenza (IAV H1N1, H3N2 and IBV) was not included in the children's routine vaccination program in Shanghai, and was usually based on the strains circulating worldwide in the precedent summer, the local population was not protected against the new subtype that emerged in the summer of 2008.

HRV is classified into three species: HRV-A, HRV-B and HRV-C by phylogenetic analysis based on sequences of VP4 gene and/or 5′UTR.[23–25] A predominance of the newly identified species HRV-C (53.8%) and the recombinant strains were observed based on which two new subspecies of HRV-C were proposed as HRV-Ca and HRV-Cc.[26] This suggests the emergence of new variant strains of HRV in future that might cause a new epidemic. Eleven HEV were detected in the study but were not analyzed further.

HMPV is another recently identified respiratory virus[27] and has been found worldwide.[28] It is grouped into four distinct genetic

Table 6
Clinical features in 817 ARI cases.

A	Single infection														
	IAV	IBV	RSV	HMPV	PIV11	PIV3	PIV4	RHV	HEV	OC43	229E	HKU-1	NL63	ADV	HBoV
Total case	44[a]	36	71	33	27	23	2	43	5	1	1	1	14	18	54
Fever (>38 °C)	44	36	71	32	27	23	2	41	5	1	1	1	14	18	54
Cough	44	36	70	32	27	23	2	42	5	1	1	1	14	18	54
Pleuritic pain	0	2	0	0	0	0	0	1	0	0	0	0	0	0	1
Purulent expectoration	5	8	16	8	5	3	2	11	0	1	0	0	3	3	10
Dyspnoea or tachypnea	1 (2.3)	3 (8.3)	2 (2.8)	1 (3.0)	1 (3.7)	1 (4.3)	1 (50)	3 (7.0)	0	0	0	0	0	1 (5.6)	3 (5.6)
Hypoxemia	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Interstitial abnormality at chest X-ray	25 (56.8)[b]	25 (69.4)	47 (66.2)	17 (51.5)	15 (55.6)	14 (60.9)	1 (50)	29 (67.4)	2 (40)	1	1	0	9 (64.3)	11 (61.1)	34 (63.0)
Polynucleosis (>10,000 on absolute count)	3	2	8	1	1	1	1	6	0	0	0	0	1	2	4
Lymphopenia (<1500 on absolute count)	12 (27.3)	4 (11.1)	4 (5.6)	4 (12.1)	0	1 (4.3)	0	2 (9.3)	0	0	1	0	1 (7.1)	1 (5.6)	4 (7.4)
Bronchitis	31 (70.5)	29 (80.6)	46 (64.8)	22 (66.7)	20 (74.1)	19 (82.6)	0	35 (81.4)	5 (100)	1	0	1	10 (71.4)	13 (72.2)	38 (70.4)
Pneumonia	13 (29.5)	7 (19.4)	24 (33.8)	10 (30.3)	6 (22.2)	4 (17.4)	1 (50)	8 (18.6)	0	0	1	0	4 (28.6)	5 (27.8)	16 (29.6)
Upper respiratory tract Infection	0	0	1 (1.4)	1 (3.0)	1 (3.7)	0	1 (50)	0	0	0	0	0	0	0	0
Suggested to be hospitalized	8 (18.2)	10 (27.8)	21 (29.6)	10 (30.3)	5 (18.5)	4 (17.4)	0	8 (18.6)	0	1	0	0	3 (21.4)	5 (27.8)	17 (31.5)

B	All viruses		RT-PCR negative
	Single	Co-infection	
Total case	373	113	331
Fever (>38 °C)	370	113	329
Cough	370	113	330
Pleuritic pain	4	4	4
Purulent expectoration	75	25	50
Dyspnoea or tachypnea	17 (4.6)	8 (7.1)	18 (5.4)
Hypoxemia	1	0	1
Interstitial abnormality at chest X-ray	201 (53.9)	81 (71.7)	174 (52.6)
Polynucleosis (>10,000 on absolute count)	30	11	21
Lymphopenia (<1500 on absolute count)	34 (9.1)	12 (10.6)	21 (6.3)
Bronchitis	270 (72.4)	76 (67.3)	208 (62.8)
Pneumonia	99 (26.5)	36 (31.9)	91 (27.5)
Upper respiratory tract Infection	4 (1.1)	1 (0.9)	32 (9.7)
Suggested to be hospitalized	92 (24.7)	28 (24.8)	88 (26.6)

A: each virus; B: all viruses including single infection and co-infection.

[a] Case number.

[b] Percentage (%).

lineages based on the F gene: A1, A2, B1, and B2.[29] In this study, two strains of HMPV-A1, 11 strains of HMPV-B1 and two strains of HMPV-B2 were detected in the first season, whereas one strain of HMPV-A1, three strains of HMPV-B1 and 12 strains of HMPV-B2 were identified in the second season. No HMPV-A2 lineage was found. Hence, a change of predominant lineage in the seasons was observed, but no association between the severity of infection and genetic drift of HMPV was found, as shown in other previous studies.[30,31] Besides, studies showed more sequence diversity in G and SH genes but not in F gene,[30] which could explain the constant incidence of HMPV infection in the population.

HCoV-HKU1 (in group II with HCoV-OC43) and HCoV-NL63 (in group I with HCoV-229E) are two novel coronaviruses.[32,33] During the present 2-year study, HCoV-NL63 and HCoV-229E were the major HCoV circulating in Shanghai, whereas only one strain of HCoV-OC43 and one of HCoV-HKU1 were detected, indicating a sporadic introduction of group II HCoV to the region. In context that the recent emerged HCoV, like HKU-1 and SARS whose sequence is more homologous to group II virus, could cause severe respiratory infection, the surveillance for emergence of new species of HCoV is necessary.

The co-infection of RSV and HBoV was frequently detected among the samples, whereas these two viruses co-dominated in cold season. HBoV was the second most prevalent virus (24.5%), and the co-infection rate of HBoV with other respiratory viruses was 54.6%, compared to 14% in non-HBoV-infected patients. This was lower than the co-infection rate reported previously, which ranged up to 71%.[34–37] A previous study showed that HBoV increased the severity of bronchiolitis in children less than 1-year-old co-infected with RSV, and that it is not an occasional virus.[38] However, no correlation of HBoV infection with clinical severity was observed in this and its related study.[7]

One study carried out in Wuhan, China analyzed peripheral blood samples by indirect immunofluorescence to detect RSV, IAV, IBV, ADV, PIV1–3, *Chlamydia pneumonia* and *Mycoplasma pneumonia* in children ARI inpatients and used viremia as sign of severe infection.[39] It showed that 36% of cases were co-infected by multiple agents and IAV, IBV and PIV1 were associated with co-infection. In addition, studies showed up to 30% of co-infection in hospitalized children[40] and RSV co-infection was associated with clinical severity.[38,39] However, no such correlation was found in this study. This may be due to differences in the criteria of patient enrollment and in the lower severity of clinical signs observed. Bacteria-virus co-infection was commonly found in inpatients. In our study, only polynucleosis (>10,000 on absolute count) was considered as a sign of bacterial infection and was observed in 30 patients infected with a respiratory virus. Hence, future studies should focus on severe respiratory infection to identify viral determinants of disease severity and should introduce bacteriological test.

Up to 331 specimens were negative in mRT-PCR, despite all of them matched well with the inclusion criteria for ARI. Negative results could have resulted from the low load of viral material in samples, or to infection with bacteria instead of virus. New sensitive tools such as Mass-Tag[41] or high-throughput sequencing[42] have been developed recently to identify new viruses and bacterial pathogens.[43] Implementation of these new molecular techniques for samples that are negative in mRT-PCR might be considered in the future.

This is believed to be the first study in China to characterize 17 common respiratory viruses in pediatric ARI during a 2-year consecutive period in a limited community with an important immigrant population. Using mRT-PCR followed by sequencing and phylogenetic analysis, we could identify a wide variety of agents and differentiate highly pathogenic viruses from less virulent seasonal respiratory viruses. The sequence analysis result could be useful to improve the primer design for RT-PCR and to identify new subtype virus, for example HRV-C. It monitored sustaining virus circulation in the community, which could serve as a baseline of the annual distribution of viruses for surveillance of unusual prevalence of one specific virus.

Conflict of interest

The authors declare no conflict of interest.

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