PLOS ONE

RESEARCH ARTICLE

Aetiology of Acute Respiratory Tract Infections in Hospitalised Children in Cyprus

Jan Richter[1]*, Christakis Panayiotou[1], Christina Tryfonos[1], Dana Koptides[1], Maria Koliou[2], Nikolas Kalogirou[2], Eleni Georgiou[2], Christina Christodoulou[1]

1 Cyprus Institute of Neurology and Genetics, Department of Molecular Virology, Nicosia, Cyprus,
2 Archbishop Makarios III Hospital, Department of Pediatrics, Nicosia, Cyprus

* richter@cing.ac.cy

Abstract

In order to improve clinical management and prevention of viral infections in hospitalised children improved etiological insight is needed. The aim of the present study was to assess the spectrum of respiratory viral pathogens in children admitted to hospital with acute respiratory tract infections in Cyprus. For this purpose nasopharyngeal swab samples from 424 children less than 12 years of age with acute respiratory tract infections were collected over three epidemic seasons and were analysed for the presence of the most common 15 respiratory viruses. A viral pathogen was identified in 86% of the samples, with multiple infections being observed in almost 20% of the samples. The most frequently detected viruses were RSV (30.4%) and Rhinovirus (27.4%). RSV exhibited a clear seasonality with marked peaks in January/February, while rhinovirus infections did not exhibit a pronounced seasonality being detected almost throughout the year. While RSV and PIV3 incidence decreased significantly with age, the opposite was observed for influenza A and B as well as adenovirus infections. The data presented expand our understanding of the epidemiology of viral respiratory tract infections in Cypriot children and will be helpful to the clinicians and researchers interested in the treatment and control of viral respiratory tract infections.

Introduction

Viral Respiratory tract infections (RTI) represent a major public health problem because of their world-wide occurrence, ease of transmission and considerable morbidity and mortality effecting people of all ages. Children are on average infected two to three times more frequently than adults, with acute RTIs being the most common infection in childhood [1,2]. Illnesses caused by respiratory viruses include, among others, common colds, pharyngitis, croup, bronchiolitis, viral pneumonia and otitis media. Rapid diagnosis is important not only for timely therapeutic intervention but also for the identification of a beginning influenza epidemic and the avoidance of unnecessary antibiotic treatment [3,4].

RTIs are a major cause of morbidity and mortality worldwide. Acute RTI is most common in children under five years of age, and represents 30–50% of the paediatric medical admissions, as well as 20–40% of hospitalizations in children. Respiratory infections cluster during winter and early spring months. The leading viral agents include respiratory syncytial virus (RSV), influenza A and B (INF-A, INF-B) viruses, parainfluenza viruses (PIVs), and human

adenoviruses (HAdVs). In addition, there is a continuously increasing list of new respiratory viruses that contribute significantly to the burden of acute respiratory infections, such as the recently identified human metapneumovirus (HMPV) and human Bocavirus (HBoV) [5].

Acute RTIs are classified as upper (UTRIs) and lower RTI (LRTIs), according to the involved anatomic localization. URTIs cause non-severe but widespread epidemics that are responsible for continuous circulation of pathogens in the community. LRTIs have been classified as frank pneumonia and bronchiolitis with clinical, radiological and etiological features that usually overlap [6,7]. Viruses are again the foremost agents of LRTIs often misdiagnosed as bacterial in origin and hence treated with antibiotics unnecessarily [8].

The main aim of this study was to determine the aetiology of acute respiratory tract infections in Cypriot children and assess the epidemiology of the identified viral pathogens over three epidemic seasons.

Materials and Methods

Patients and clinical specimens

The study was approved by the Cyprus National Bioethics Committee. Accordingly, written informed consent was obtained from parents prior to sample taking. Between November 2010 and October 2013, 485 nasopharyngeal swab samples were collected from children up to 12 years of age, who had been hospitalized with acute respiratory tract infection at the Archbishop Makarios III hospital, Nicosia. Clinical and demographic information including symptoms, duration of hospitalisation, diagnosis and treatment were recorded. Nasal swab samples were collected using the BD Universal Viral Transport Collection Kit. Viral RNA/DNA was extracted from 400 μl sample using the iPrep PureLink Virus Kit on an iPrep purification instrument (Invitrogen).

Virus detection

A set of four multiplex Real-Time RT-PCR assays was established and validated for the detection of the 15 most common respiratory viruses as follows: assay 1: influenzaviruses A and B, RSV, assay 2: parainfluenzaviruses 1–4, assay 3: HAdV, enteroviruses, HMPV and HBoV and assay 4: rhinoviruses and the human coronaviruses OC43, NL63 and 229E (Table 1).

Published primer and probe sets were used as a basis for designing the assays, however, all primer/probe sequences were checked against newly build sequence alignments of all viruses tested and were modified, if necessary, to account for possible sequence variations. For this purpose, all available complete genome sequences were obtained for each virus from GenBank, imported into the BioEdit Sequence Alignment Editor v7.1.7 and aligned using ClustalX. In case of mismatches between published primers/probe and target sequences, modifications were applied, as indicated in Table 1. The alignments for the viruses, which necessitated changes to the primers/probe are available in Fasta-Format as supplement S1–S4 Files.

Primer concentrations and reaction conditions for the four assays were subsequently optimised for multiplexing. In order to assess the sensitivity and specificity of the assays, the laboratory enrolled for two consecutive years in Quality Control for Molecular Diagnostics (QCMD) external quality assessment schemes for all viruses, except Bocavirus, which was unavailable. In summary, the established assays were able to correctly identify all viruses tested, proving their suitability for diagnostic application.

Statistical Analysis

A possible correlation of virus prevalence and age of infection was assessed using univariate analyses. The Fisher's exact test was used where cell counts below 5 were encountered;

Table 1. Primers and probes used in four Multiplex-assays for the detection of 15 different viral pathogens.

	Virus	Probe label	Gene targeted	Reference	Comments
Assay 1	Inf A	FAM	Matrix protein 2	CDC H1N1[9]	
	Inf B	Yakima Yellow	Nuclear export protein	Selvajaru et al. [10]	
	RSV	TAMRA	Matrix protein	Fry et al. [11]	Probe modified*
Assay 2	PIV 1	FAM	HN	Watzinger et al. [12]	
	PIV 2	JOE	HN	Watzinger et al. [12]	
	PIV 3	TAMRA	HN	Watzinger et al. [12]	
	PIV 4	Cy5	Phosphoprotein	Templeton et al. [13]	Probe modified*
Assay 3	HCoV OC43	FAM	Nucleocapsid protein	Tiveljung et al. [14]	
	HCoV NL63	Yakima Yellow	Nucleocapsid protein	Tiveljung et al. [14]	
	HCoV 229E	TAMRA	Nucleocapsid protein	Tiveljung et al. [14]	
	Rhinovirus	Cy5	5'UTR	Lu et al. [15]	Additional F primer*
Assay 4	Enterovirus	FAM	5'UTR	Tryfonos et al. [16]	
	HBoV	Yakima Yellow	Nonstructural protein	Tiveljung et al. [14]	
	HAdV	TAMRA	Hexon protein	Heim et al. [17]	Probe modified*
	HMPV	Cy5	Nucleoprotein	Tiveljung [14]	

*The sequences of the modified primers/probes are shown in supplements S1–S4 Files.

doi:10.1371/journal.pone.0147041.t001

otherwise, the chi-squared test was performed. The same statistical tests were used to compare the frequency of subjects with single or multiple infections between age groups. In addition, Pearson correlation was used to examine co-infections of different viruses. All statistical analyses were performed using StataSE 12 (StatCorp. 2007. College Station, TX, USA).

Results

The present study was a prospective investigation of children hospitalized with acute respiratory tract infections between November 2010 and October 2013 in Cyprus. The median age of the children was 15 months (range: 0–140 months) with 243 being male and 181 female (male/female ratio 1.34). The age distribution is shown in Fig 1.

Viral aetiologies

Out of the 424 samples analysed, 364 (85.8%) were positive for one or more viruses. Results are summarized in Table 2.The most commonly detected viruses were RSV, which was found in 129 (30.4%) patients and rhinoviruses in 116 (27.4%) accounting together for almost 60% of all detections. With moderate frequency have been detected HAdV in 31(7.3%) patients, influenza A in 28 (6.6%), HBoV in 24 (5.7%), enteroviruses and PIV 3 in 23 (5.4%) of patients respectively, and Influenza B in 21 (5.0%). A low frequency was exhibited by HMPV with 16 (3.8%) positive samples, human coronavirus OC43 with 13 (3.1%), PIV 1 with 12 (2.8%), PIV 4 with 9 (2.1%), PIV 2 with 7 (1.7%) and HCoV NL63 with 6 (1.4%). Coronavirus 229E could be detected only in a single sample.

Multiple Infections

Co-infections with two or more viruses were observed in 84 out of the 364 positive samples (see Table 2). Dual infections accounted for 17% of all positive samples and three viruses were detected in 2.7% of samples). A single patient sample displayed a quadruple infection being simultaneously positive for RSV, rhinovirus, HBoV and influenza B. Table 3 summarizes the
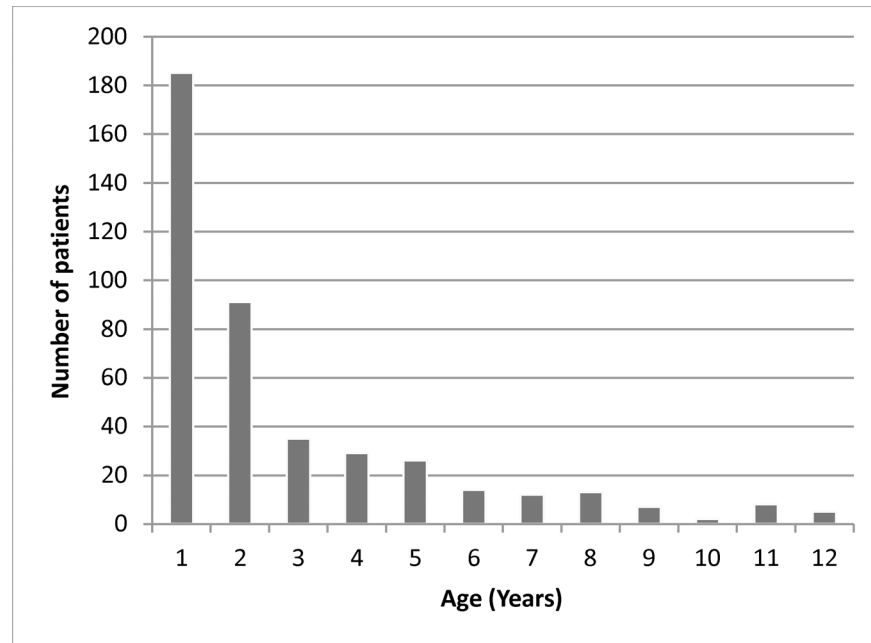
Fig 1. Age distribution of paediatric patients hospitalised with acute respiratory infections.

doi:10.1371/journal.pone.0147041.g001

frequency of each virus in single vs. multiple infections as well as the number of co-occurrences of viruses for each possible virus combination. In absolute terms the most common combination observed was RSV/rhinovirus. As a percentage, however, the virus appearing most often in co-

Table 2. Distribution of virus infection by patient age.

Virus	0–3 months, n = 74		4–12 months, n = 108		13–36 months, n = 127		3–12 years, n = 115		Total, n = 424		P-value[α]
RSV	43	58.1%	41	38.0%	30	23.6%	15	13.0%	129	30.4%	**<0.0001**
HRV	15	20.3%	33	30.6%	42	33.1%	26	22.6%	116	27.4%	0.121
HAdV	0	0.0%	11	10.2%	14	11.0%	6	5.2%	31	7.3%	**0.005**
Inf A	2	2.7%	3	2.8%	9	7.1%	14	12.2%	28	6.6%	**0.021**
HBoV	1	1.4%	12	11.1%	9	7.1%	2	1.7%	24	5.7%	**0.006**
HEV	2	2.7%	7	6.5%	10	7.9%	4	3.5%	23	5.4%	**0.033**
PIV 3	6	8.1%	7	6.5%	7	5.5%	3	2.6%	23	5.4%	0.339
Inf B	0	0.0%	2	1.9%	4	3.1%	15	13.0%	21	5.0%	**<0.0001**
MPV	3	4.1%	3	2.8%	8	6.3%	2	1.7%	16	3.8%	0.298
HCoV OC43	1	1.4%	4	3.7%	2	1.6%	6	5.2%	13	3.1%	0.365
PIV 1	1	1.4%	3	2.8%	5	3.9%	3	2.6%	12	2.8%	0.816
PIV 4	3	4.1%	1	0.9%	1	0.8%	4	3.5%	9	2.1%	0.249
PIV 2	0	0.0%	2	1.9%	3	2.4%	2	1.7%	7	1.7%	0.695
HCoV NL63	2	2.7%	0	0.0%	3	2.4%	1	0.9%	6	1.4%	0.671
HCoV 229E	0	0.0%	0	0.0%	0	0.0%	1	0.9%	1	0.2%	0.700
Negative	7	9.5%	9	8.3%	19	15.0%	25	21.7%	60	14.2%	**0.020**
Multiple infection	11	14.9%	27	25.0%	32	25.2%	13	11.3%	83	19.6%	**0.014**

[a] The correlation of virus prevalence and age was assessed using univariate analyses. P-values were calculated using the chi-squared test or Fisher's exact test, where cell counts below 5 were observed. P-values less than 0.05 are indicated in bold.

doi:10.1371/journal.pone.0147041.t002

Table 3. Rates of detection of 15 viral pathogens in children hospitalized with acute RTI (Nov. 2010—Oct. 2013) and occurrence of multiple infections.

|	Total	Single	Double	Triple	Quad	Mixed infection	RSV	HRV	HAdV	Inf A	HBoV	HEV	PIV3	Inf B	MPV	HCoV OC43	PIV1	PIV4	PIV2	HCoV NL63	HCoV 229E
RSV	129	94	28	6	1	27.1%		17	2	2	9	2	2	2	1	3	1	2	0	0	0
HRV	116	62	46	7	1	46.6%	17		6	2	7	12	4	3	2	3	4	1	2	0	0
HAdV	31	19	9	3	0	38.7%	2	6		1	1	0	0	0	1	2	0	0	1	0	1
Inf A	28	21	7	0	0	25.0%	2	2	1		0	0	0	0	0	1	0	0	0	1	0
HBoV	24	7	12	4	1	70.8%	9	7	1	0		1	0	1	0	1	0	1	1	1	0
HEV	23	10	9	4	0	56.5%	2	12	0	0	1		1	0	0	0	1	0	0	0	0
PIV3	23	17	5	1	0	26.1%	2	4	0	0	0	1		0	0	0	0	0	0	0	0
Inf B	21	17	3	0	1	19.0%	2	3	0	0	1	0	0		0	0	0	0	0	0	0
MPV	16	10	6	0	0	37.5%	1	2	1	0	0	0	0	0		0	0	0	0	2	0
HCoV OC43	13	5	6	2	0	61.5%	3	3	2	1	1	0	0	0	0		0	0	0	0	0
PIV1	12	7	4	1	0	41.7%	1	4	0	0	0	1	0	0	0	0		0	0	0	0
PIV4	9	6	2	1	0	33.3%	2	1	0	0	1	0	0	0	0	0	0		0	0	0
PIV2	7	4	2	1	0	42.9%	0	2	1	0	1	0	0	0	0	0	0	0		0	0
HCoV NL63	6	2	4	0	0	66.7%	0	0	0	1	1	0	0	0	2	0	0	0	0		0
HCoV 229E	1	0	1	0	0	100.0%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	
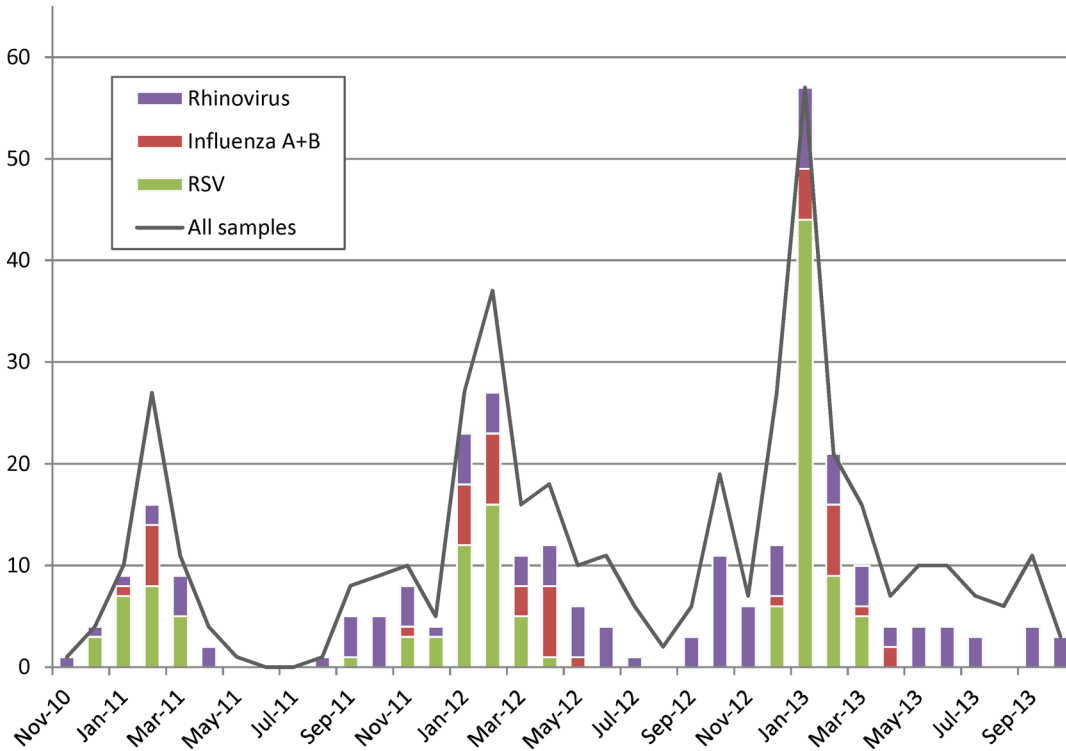
Fig 2. Seasonality of RSV, rhinovirus and influenza virus infections.

doi:10.1371/journal.pone.0147041.g002

infections was HBoV, which was found in more than 70% of cases together with another virus, followed by coronaviruses HCoV OC43 and HCoV NL63 with 61% and 67%, respectively. On the other hand, the viruses most rarely seen in co-infections were influenza viruses A and B as well as RSV. Pearson correlation coefficients were calculated to examine the likelihood of co-infections of different viruses. The results of the analysis are summarized in Table 1 in S1 Table. Significant correlation (P-value < 0.05) was seen mostly for co-infections with RSV, however correlations were very weak (r<0.3) and negative. This finding can probably be explained by the fact that RSV infections occurred predominantly in the very young, where co-infections were less frequently observed. On the other hand, a significant positive correlation was observed for enterovirus and rhinovirus co-infection hinting maybe at similarities in circulation patterns and/or transmission modes.

Seasonality

Regarding seasonality, different patterns of circulations could be observed for RSV, rhinoviruses and influenzaviruses (A and B combined) (Fig 2), with RSV and influenza exhibiting a clear seasonality with marked peaks in January/February, while rhinovirus infections did not exhibit a pronounced seasonality being detected almost throughout the year. However, as more than 100 different rhinovirus strains have been identified to be circulating worldwide in parallel and successively, a potential seasonality of individual rhinovirus serotypes may be masked by overlapping patterns [18,19].

Age group characteristics of viral infection

The data was further analysed with regard to the age distribution of virus infection (see Table 2). In infants up to 3 months old, RSV was by far the most common pathogen (58.1%),
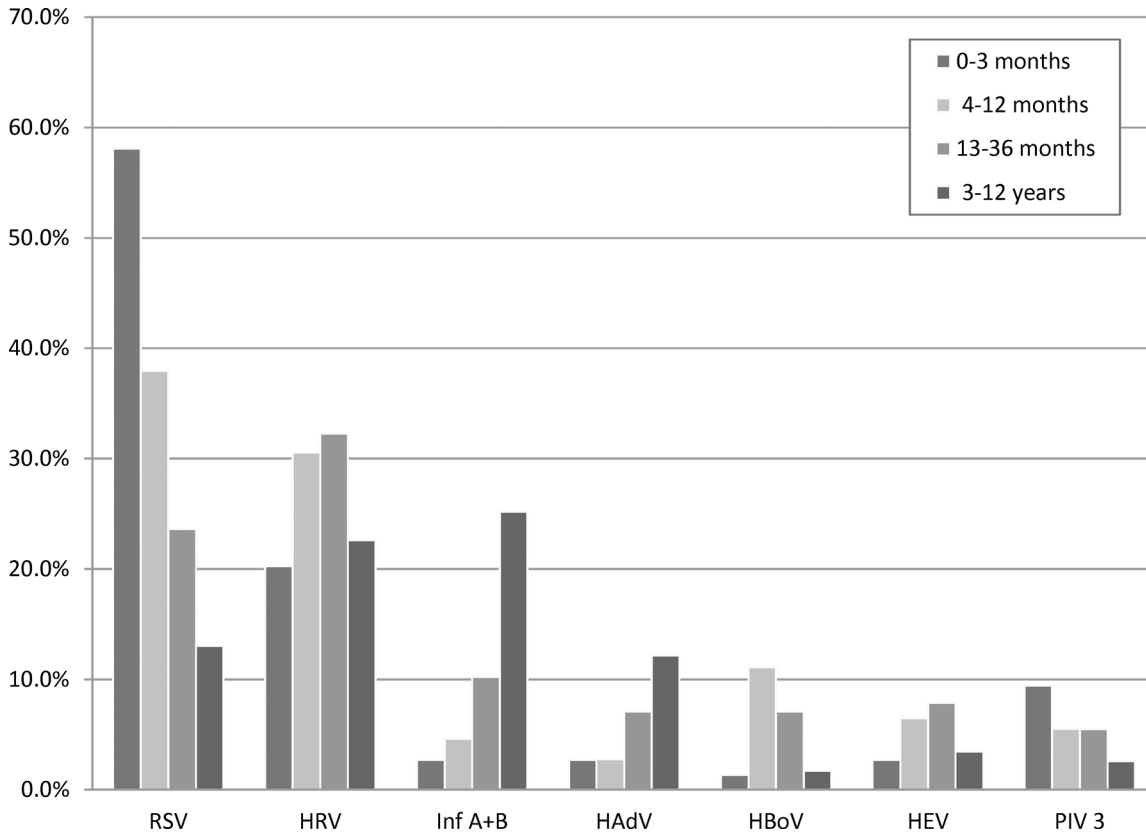
Fig 3. Distribution of virus infection by patient age for the 7 most frequently detected viruses.

followed by rhinovirus (20.3%) and PIV3 with 8.1% each. The incidence of RSV, however, decreases significantly with increasing age (p-value < 0.0001) dropping to 13% in children older than 3 years old, while the reverse relationship is observed for Influenza A and B and HAdV. Rhinoviruses, HBoV and enteroviruses are most frequently observed in children from 4 months to 3 years of age. The age dependency of the virus incidence is visualized in Fig 3 for the seven most frequently observed viruses. The positivity rate also showed a trend according to the age group dropping from 90.5% in the under 3-month old to 78.3% in the 4–12 years old (p-value = 0.020). This may point to an increasing role of pathogens not included in the assays, such as bacterial infections in older children.

Regarding multiple infections, children less than 3 month of age and those older than 4 years had a significantly smaller risk to present with multiple infections as compared to the other two age groups (p-value = 0.014).

A reason for this could be that very young children have limited contact to others reducing thereby the chance for a co-infection, whereas children older than 3 years already established immunity to an increasing number of viruses encountered previously.

Discussion

This study for the first time examined the aetiology of acute respiratory tract infections in hospitalised children in Cyprus. Four multiplex Real-Time RT-PCR assays were developed in order to detect the most common respiratory viral pathogens in a fast and cost-effective way. The high rate of positive samples (85.8%) is evidence of the high sensitivity of the Multiplex-

assays used and that the range of viruses included in the analysis is comprehensive. Many previous studies have shown detection rates ranging from below 50% to 75% [20–24].

The most common viruses detected were RSV and rhinovirus accounting for almost 60% of all cases. Both viruses were reported previously by others as the major aetiology for respiratory viral infections in young children with rhinoviruses being recognized increasingly for their role in lower respiratory tract infections [20,25–30].

Our data support the results of similar studies performed in the Middle East region. A recently published study found that RSV was the most commonly detected virus in nasopharyngeal swabs from children presenting symptoms of RTIs and in addition to that it also showed that RSV infections follow a similar circulation pattern peaking from December to March [31]. Another study has revealed that RSV and PIV3 incidence decreases significantly with age, whereas the opposite is observed for influenza and adenovirus infections, a trend that was also observed in our study [26].

Mixed infections were observed in approximately 20% of all samples, which is in the middle of previously reported rates ranging from 10 to almost 40%. HBoV, HCoV and EV were found most frequently in co-infections. All three subtypes of HCoV were co-detected with several other viruses, while HBoV was co-detected mainly with HRV and RSV. In the case of EV infections, EV were almost predominantly associated with HRV. The rare presence of InfA and InfB viruses in multiple infections witnessed in our study was also observed elsewhere [32,33]. Even though this study did not allow for investigating a possible association between multiple infections and disease severity, a review of the literature shows that such a potential association is still subject to controversy, since there are reports showing no relationship of multiple virus infection with respiratoty illness severity on one hand or a significant association on the other. Studies have shown that viral co-infection was significantly associated with longer duration of illness symptoms, but with a decreased severity in hospitalized children regarding oxygen requirement and intensive care unit admission, whereas the findings of other studies have indicated that severe clinical phenotypes were more prevalent in co-infection patients, especially in RSV co-infections that may increase the severity of RSV associated disease in children [25,34–40].

Viral respiratory infections continue to be a worldwide health concern. As the clinical symptoms of patients with acute respiratory tract infections do usually not allow a discrimination of viral or bacterial aetiology, rapid and reliable diagnostic tools are required for better antibiotic stewardship and the implementation of appropriate infection control measures [4,41]. The data presented expand our understanding of the epidemiology of viral respiratory tract infections in Cypriot children and will be helpful to the clinicians and researchers interested in the treatment and control of viral respiratory tract infections.

Supporting Information

S1 File. Alignment of RSV sequences.
(FAS)

S2 File. Alignment of PIV 4 sequences.
(FAS)

S3 File. Alignment of rhinovirus sequences.
(FAS)

S4 File. Alignment of adenovirus sequences.
(FAS)

S1 Table. Pearson Correlation coefficients for observed viral co-infections.
(PDF)

Author Contributions

Conceived and designed the experiments: JR CC. Performed the experiments: JR CP CT DK. Analyzed the data: JR CC CP DK MK. Contributed reagents/materials/analysis tools: NK EG MK. Wrote the paper: JR CP CC.

References

1. Kesson AM. Respiratory virus infections. Paediatr Respir Rev. 2007; 8:240–8. PMID: 17868922

2. Tregoning JS, Schwarze J. Respiratory viral infections in infants: causes, clinical symptoms, virology, and immunology. Clin Microbiol Rev. 2010; 23:74–98. doi: 10.1128/CMR.00032-09 PMID: 20065326

3. Harper SA, Bradley JS, Englund JA, File TM, Gravenstein S, Hayden FG, et al. Seasonal influenza in adults and children—diagnosis, treatment, chemoprophylaxis, and institutional outbreak management: clinical practice guidelines of the Infectious Diseases Society of America. Clin Infect Dis. 2009; 48:1003–32. doi: 10.1086/598513 PMID: 19281331

4. Low D. Reducing antibiotic use in influenza: challenges and rewards. Clin Microbiol Infect. 2008; 14:298–306. PMID: 18093237

5. Berry M, Gamieldien J, Fielding BC. Identification of new respiratory viruses in the new millennium. Viruses. 2015; 7:996–1019. doi: 10.3390/v7030996 PMID: 25757061

6. Hoare Z, Lim WS. Pneumonia: update on diagnosis and management. BMJ. 2006; 332:1077–9. PMID: 16675815

7. Wenzel RP, Fowler AA III. Clinical practice. Acute bronchitis. N Engl J Med. 2006; 355:2125–30. PMID: 17108344

8. Farley R, Spurling GK, Eriksson L, Del Mar CB. Antibiotics for bronchiolitis in children under two years of age. Cochrane Database Syst Rev. 2014; 10:CD005189. doi: 10.1002/14651858.CD005189.pub4 PMID: 25300167

9. CDC protocol of realtime RTPCR for influenza A(H1N1). (2009) WHO Collaborating Centre for influenza at CDC Atlanta, USA Available: http://www.who.int/csr/resources/publications/swineflu/CDCRealtimeRTPCR_SwineH1Assay-2009_20090430.pdf.

10. Selvaraju SB, Selvarangan R. Evaluation of three influenza A and B real-time reverse transcription-PCR assays and a new 2009 H1N1 assay for detection of influenza viruses. J Clin Microbiol. 2010; 48:3870–5. doi: 10.1128/JCM.02464-09 PMID: 20844230

11. Fry AM, Chittaganpitch M, Baggett HC, Peret TC, Dare RK, Sawatwong P, et al. The burden of hospitalized lower respiratory tract infection due to respiratory syncytial virus in rural Thailand. PLoS One. 2010; 5:e15098. doi: 10.1371/journal.pone.0015098 PMID: 21152047

12. Watzinger F, Suda M, Preuner S, Baumgartinger R, Ebner K, Baskova L, et al. Real-time quantitative PCR assays for detection and monitoring of pathogenic human viruses in immunosuppressed pediatric patients. J Clin Microbiol. 2004; 42:5189–98. PMID: 15528714

13. Templeton KE, Scheltinga SA, Beersma MF, Kroes AC, Claas EC. Rapid and sensitive method using multiplex real-time PCR for diagnosis of infections by influenza a and influenza B viruses, respiratory syncytial virus, and parainfluenza viruses 1, 2, 3, and 4. J Clin Microbiol. 2004; 42:1564–9. PMID: 15071005

14. Tiveljung-Lindell A, Rotzen-Ostlund M, Gupta S, Ullstrand R, Grillner L, Zweygberg-Wirgart B, et al. Development and implementation of a molecular diagnostic platform for daily rapid detection of 15 respiratory viruses. J Med Virol. 2009; 81:167–75. doi: 10.1002/jmv.21368 PMID: 19031448

15. Lu X, Holloway B, Dare RK, Kuypers J, Yagi S, Williams JV, et al. Real-time reverse transcription-PCR assay for comprehensive detection of human rhinoviruses. J Clin Microbiol. 2008; 46:533–9. PMID: 18057136

16. Tryfonos C, Richter J, Koptides D, Yiangou M, Christodoulou CG. Molecular typing and epidemiology of enteroviruses in Cyprus, 2003–2007. J Med Microbiol. 2011; 60:1433–40. doi: 10.1099/jmm.0.029892-0 PMID: 21596905

17. Heim A, Ebnet C, Harste G, Pring-Akerblom P. Rapid and quantitative detection of human adenovirus DNA by real-time PCR. J Med Virol. 2003; 70:228–39. PMID: 12696109

18. Richter J, Nikolaou E, Panayiotou C, Tryfonos C, Koliou M, Christodoulou C. Molecular epidemiology of rhinoviruses in Cyprus over three consecutive seasons. Epidemiol Infect. 2014;1–8.

19. Jacobs SE, Lamson DM, St GK, Walsh TJ. Human rhinoviruses. Clin Microbiol Rev. 2013; 26:135–62. doi: 10.1128/CMR.00077-12 PMID: 23297263

20. Pierangeli A, Gentile M, Di MP, Pagnotti P, Scagnolari C, Trombetti S, et al. Detection and typing by molecular techniques of respiratory viruses in children hospitalized for acute respiratory infection in Rome, Italy. J Med Virol. 2007; 79:463–8. PMID: 17311326

21. Hombrouck A, Sabbe M, Van C, V, Wuillaume F, Hue D, Reynders M, et al. Viral aetiology of influenza-like illness in Belgium during the influenza A(H1N1)2009 pandemic. Eur J Clin Microbiol Infect Dis. 2012; 31:999–1007. doi: 10.1007/s10096-011-1398-4 PMID: 21901635

22. Schlaudecker EP, Heck JP, Macintyre ET, Martinez R, Dodd CN, McNeal MM, et al. Etiology and sea-sonality of viral respiratory infections in rural Honduran children. Pediatr Infect Dis J. 2012; 31:1113–8. doi: 10.1097/INF.0b013e31826052eb PMID: 22653487

23. Brittain-Long R, Nord S, Olofsson S, Westin J, Anderson LM, Lindh M. Multiplex real-time PCR for detection of respiratory tract infections. J Clin Virol. 2008; 41:53–6. PMID: 18093871

24. Puzelli S, Valdarchi C, Ciotti M, Dorrucci M, Farchi F, Babakir-Mina M, et al. Viral causes of influenza-like illness: Insight from a study during the winters 2004–2007. J Med Virol. 2009; 81:2066–71. doi: 10.1002/jmv.21610 PMID: 19856468

25. Cho HJ, Shim SY, Son DW, Sun YH, Tchah H, Jeon IS. Respiratory viruses in neonates hospitalized with acute lower respiratory tract infections. Pediatr Int. 2013; 55:49–53. doi: 10.1111/j.1442-200X.2012.03727.x PMID: 22978535

26. Khadadah M, Essa S, Higazi Z, Behbehani N, Al-Nakib W. Respiratory syncytial virus and human rhino-viruses are the major causes of severe lower respiratory tract infections in Kuwait. J Med Virol. 2010; 82:1462–7. doi: 10.1002/jmv.21823 PMID: 20572084

27. Regamey N, Kaiser L, Roiha HL, Deffernez C, Kuehni CE, Latzin P, et al. Viral etiology of acute respira-tory infections with cough in infancy: a community-based birth cohort study. Pediatr Infect Dis J. 2008; 27:100–5. doi: 10.1097/INF.0b013e31815922c8 PMID: 18174876

28. Kim H, Kim K, Kim DW, Jung HD, Min CH, Kim KH, et al. Identification of Recombinant Human Rhinovi-rus A and C in Circulating Strains from Upper and Lower Respiratory Infections. PLoS One. 2013; 8: e68081. PMID: 23826363

29. Louie JK, Roy-Burman A, Guardia-Labar L, Boston EJ, Kiang D, Padilla T, et al. Rhinovirus associated with severe lower respiratory tract infections in children. Pediatr Infect Dis J. 2009; 28:337–9. doi: 10.1097/INF.0b013e31818ffc1b PMID: 19258921

30. Panayiotou C, Richter J, Koliou M, Kalogirou N, Georgiou E, Christodoulou C. Epidemiology of respira-tory syncytial virus in children in Cyprus during three consecutive winter seasons (2010–2013): age dis-tribution, seasonality and association between prevalent genotypes and disease severity. Epidemiol Infect. 2014; 142:2406–11. doi: 10.1017/S0950268814000028 PMID: 24476750

31. Halasa N, Williams J, Faouri S, Shehabi A, Vermund SH, Wang L, et al. Natural history and epidemiol-ogy of respiratory syncytial virus infection in the Middle East: Hospital surveillance for children under age two in Jordan. Vaccine. 2015.

32. Cui B, Zhang D, Pan H, Zhang F, Farrar J, Law F, et al. Viral aetiology of acute respiratory infections among children and associated meteorological factors in southern China. BMC Infect Dis. 2015; 15:124. doi: 10.1186/s12879-015-0863-6 PMID: 25884513

33. Kim JK, Jeon JS, Kim JW, Rheem I. Epidemiology of respiratory viral infection using multiplex rt-PCR in Cheonan, Korea (2006–2010). J Microbiol Biotechnol. 2013; 23:267–73. PMID: 23412071

34. Richard N, Komurian-Pradel F, Javouhey E, Perret M, Rajoharison A, Bagnaud A, et al. The impact of dual viral infection in infants admitted to a pediatric intensive care unit associated with severe bronchiol-itis. Pediatr Infect Dis J. 2008; 27:213–7. doi: 10.1097/INF.0b013e31815b4935 PMID: 18277932

35. Harada Y, Kinoshita F, Yoshida LM, Minh lN, Suzuki M, Morimoto K, et al. Does respiratory virus coin-fection increases the clinical severity of acute respiratory infection among children infected with respira-tory syncytial virus? Pediatr Infect Dis J. 2013; 32:441–5. doi: 10.1097/INF.0b013e31828ba08c PMID: 23838658

36. Lu Y, Wang S, Zhang L, Xu C, Bian C, Wang Z, et al. Epidemiology of human respiratory viruses in chil-dren with acute respiratory tract infections in Jinan, China. Clin Dev Immunol. 2013; 2013:210490. doi: 10.1155/2013/210490 PMID: 24363757

37. Paranhos-Baccala G, Komurian-Pradel F, Richard N, Vernet G, Lina B, Floret D. Mixed respiratory virus infections. J Clin Virol. 2008; 43:407–10. doi: 10.1016/j.jcv.2008.08.010 PMID: 18829380

38. Bonzel L, Tenenbaum T, Schroten H, Schildgen O, Schweitzer-Krantz S, Adams O. Frequent detection of viral coinfection in children hospitalized with acute respiratory tract infection using a real-time poly-merase chain reaction. Pediatr Infect Dis J. 2008; 27:589–94. doi: 10.1097/INF.0b013e3181694fb9 PMID: 18520973

39. Essa S, Owayed A, Altawalah H, Khadadah M, Behbehani N, Al-Nakib W. Mixed viral infections circulating in hospitalized patients with respiratory tract infections in kuwait. Adv Virol. 2015; 2015:714062. doi: 10.1155/2015/714062 PMID: 25983755

40. Wu X, Wang Q, Wang M, Su X, Xing Z, Zhang W, et al. Incidence of respiratory viral infections detected by PCR and real-time PCR in adult patients with community-acquired pneumonia: a meta-analysis. Respiration. 2015; 89:343–52. doi: 10.1159/000369561 PMID: 25791384

41. Zaas AK, Garner BH, Tsalik EL, Burke T, Woods CW, Ginsburg GS. The current epidemiology and clinical decisions surrounding acute respiratory infections. Trends Mol Med. 2014; 20:579–88. doi: 10.1016/j.molmed.2014.08.001 PMID: 25201713