The Role of Respiratory Viruses in Lower Respiratory Tract Illnesses in Early Life

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De rol van respiratoire virussen in lagere luchtwegaandoeningen vroeg in het leven

(met een samenvatting in het Nederlands)

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Herinner je gisteren
Droom van morgen
Maar leef vandaag!

Contents

Chapter 1

Introduction

Introduction

Respiratory illness is the most important cause of morbidity and mortality in infancy and early childhood[1,2]. Viral infections play a key role in respiratory illnesses in young childhood. In up to 85% of children viruses can be detected during respiratory tract illnesses[3-5]. Viruses cause a variety of respiratory diseases in children ranging from common cold[6] to life threatening pneumonia and bronchiolitis[7-9]. Viral infections also play an important role in both childhood and adult asthma[5]. On the one hand, the hygiene hypothesis suggests that children who have frequent infections during early life have a reduced risk for allergies and asthma[10]. On the other hand viruses may play an important role in eliciting symptoms in those with constitutional asthma[11]. Although the importance of respiratory virus infections during respiratory illness is beyond any doubt, many questions on the prevalence of specific viruses and on the association between viruses and symptoms still remain unanswered. By gaining more insight in the interaction between virus infections and development of chronic respiratory illness, we hope to improve management and prevention of these frequent illnesses in the future.

Prevalence of respiratory viruses

The most commonly known viruses responsible for respiratory illnesses include influenza viruses (IFVs), respiratory syncytial virus (RSV), parainfluenza viruses (PIVs) 1-4, enteroviruses, human rhinoviruses (HRVs) adenoviruses (AdVs) and the human coronaviruses (HCoVs) 229E and OC43[3,4]. HRV and RSV are reported as the most frequent causing agents of respiratory illness[9]. Data on the occurrence of pathogens in children with respiratory illness are mainly based on epidemiological studies in selected populations of hospitalized children and in children at high risk for atopic diseases. Most of these studies have used rather insensitive conventional methods for virus detection. Since some pathogens are difficult to detect using classic detection techniques like culture or serological testing, the introduction of PCR can importantly change the view on the epidemiology of pathogens responsible for respiratory illnesses[12].

One example of an under exposed pathogen found during respiratory illness is HRV. Until last decade little attention was paid to HRV since it was thought to be limited to the upper respiratory tract, causing only mild symptoms. During the last decade the perspective on HRV infections has importantly changed. The detection rate of HRV in patients with respiratory infections has increased to up to 50% and HRVs also seem to be able to cause lower respiratory tract infections[8]. Moreover, some studies show that HRV infections might play a role in recurrent wheezing later on in life[7,13]. Further, besides this increasing awareness on the relevance of HRVs from epidemiological studies there is growing evidence for the importance of different HRV subtypes. For example, a newly identified HRV species, HRV-C,

was found as an important cause of febrile wheeze and asthmatic exacerbations in children requiring hospitalization[14]. Moreover, HRV is a member of the *Picorna Viridae* family and more than 100 genetically and serologically different HRV subtypes have been described[15]. It can be questioned whether the high prevalence rates of HRVs found in epidemiological studies is due to long-term individual carriership with the same subtype or whether it is a result of highly frequent subsequent infections with different HRVs subtypes. Longitudinal data on the diversity of HRVs in individuals are lacking.

Last decade several new viruses with possible respiratory impact were identified using molecular detection techniques. Recently, human metapneumovirus (hMPV)[16], novel strains of coronaviruses (SARS-CoV, HCoV-NL63 and HKU1)[17], human bocavirus[18] and the novel polyomaviruses[19,20] (WUPyV and KIPyV) have been described. HMPV and HCoV-NL63 are recognized as being etiological agents of lower respiratory tract illness[21,22]. For both bocavirus and the new polyomaviruses the role of these pathogens in respiratory illness is not yet established. In a Swiss birth cohort study bocavirus was found in 4.5% infants at the onset of respiratory symptoms, but many of these children had co-infections with other pathogens[23]. WUPyV and KIPyV have been reported in respiratory samples of uncontrolled studies of small groups of hospitalized patients, but data in large unselected groups of subjects are lacking[24-26].

The association between respiratory viruses and symptoms

The introduction of sensitive molecular detection techniques has raised new questions. Whether pathogens are actually the cause of the respiratory symptoms or are simply colonizing the respiratory tract during symptomatic episodes is unclear. Most studies have focused

Table 1. Review of studies on viral detection in asymptomatic children.

	N	Virus %	Age	Population	Viruses
Jartti et al 2004(27)	79	16%	0-16 y	Hospitalization for surgery	HRV/EV
Noko-Koivisto et al 2002(28)	107	5%	0-17 y	Hospitalization for surgery	HRV/ EV/ HCoV
Van Benten et al 2003(4)	221	16-33%	0-2 y	Birth-cohort	HRV/EV/HCoV/ RSV/ PIV/ IFV/ CMV/ AdV
Johnston et al 1993(29)	65	12%	9-13 y	Follow-up study for asthma like- symptoms	HRV/ EV
Winther et al 2006(30)	410	9%	1-9 y	Healthy	HRV/ EV
Rakes et al 1998(9)	17	41%	0-2 y	Emergency room for non-respiratory illness	HRV/ EV/ RSV/ HCoV
	42	36%	2-16y		HRV/ EV/ RSV/ HCoV
Van Gageldonk et al 2005(31)	541	68%	<5 y	General practitioner	HRV/ EV/ RSV/ hMPV/ HCoV/ PIV/ IFV/ AdV/ MP/ CP
		40%	5-14y		
		15%	>14		

Abbreviations: HRV, human rhinovirus; EV, enterovirus; HCoV, human coronavirus; RSV, respiratory syncytial virus; PIV, parainfluenzavirus; IFV, influenzavirus; CMV, cytomegalovirus; AdV, adenovirus; hMPV, human metapneumovirus; MP, Mycoplasma pneumoniae; CP, Chlamydophila pneumoniae.

on the prevalence of pathogens in respiratory illness. Scarce data are available on the prevalence of pathogens in asymptomatic children. Table 1 reviews the studies reporting data on viruses in asymptomatic children. In these studies the prevalence of virus detection ranges between 5 and 68%[4,9,27,29-32]. This wide range in prevalence is probably due to differences in study design, study population, definition of symptoms and viral detection methods. Most studies used a control group of children admitted in the hospital for elective surgery. It can be speculated that these infants are in a setting where more pathogens circulate compared to the 'normal' household setting. Further most studies only investigated a small subset of pathogens responsible for respiratory illness. In order to study the association between pathogens and respiratory symptoms a careful surveillance on viruses in unselected infants is needed during both symptomatic and asymptomatic periods.

Factors influencing the association between viruses and respiratory symptoms

All infants encounter viral infections during the first years of life, however, some infants have more symptoms than others. The mechanisms which underlie the inter-individual variability in the sensitivity to viral infections are unclear. It has been speculated that host-, pathogen- and environmental factors might influence the emergence of respiratory symptoms.

Host factors

Host factors of importance might be age, gender, prematurity, small lung size and genetic predisposition to allergies. In young children the respiratory and immune systems are immature and it has been suggested that infants might be more susceptible to respiratory pathogens. A recently published Finnish study showed that most young children with HRV were symptomatic, while nearly half of the older children and adults were asymptomatic[33]. Presumably, children develop immunity to many respiratory viruses over time[34]. In addition, some studies found a different immune response in younger children during a RSV infection compared to older children[35].

Most epidemiological studies have shown that boys have more respiratory symptoms during the first years of life compared to girls[36,37], however this relationship is reversed in adulthood. One explanation for this relationship has been that boys initially have smaller airway diameters compared to lung volume (dysanapsis) [38]. Another possible explanation was found by Wright et al. who showed that boys with wheeze were more likely to visit the physician compared to girls, resulting in a higher diagnostic rate in boys compared to girls[37].

Prematurity is also suggested to be a risk factor for increased respiratory illnesses in childhood[39]. Broughton et al. have shown that premature born infants, with or without broncho-

pulmonary dysplasia (BPD), who had a RSV lower respiratory tract infection had more days of cough and wheeze at follow up at 1 year of age[40]. Notably, premature delivery has been associated with diminished lung function[41]. There is some debate about whether prematurity alone is a determinant of respiratory illness or an intermediate for lower levels of lung function.

Several studies have shown that lower levels of lung function shortly after birth and prior to any respiratory illness are associated with the occurrence of wheezing illnesses during the first years of life[42-44]. These studies have mainly focussed on infants with RSV infections and recurrent wheezing later on in life. To study possible relationships between congenital lung function, virus infections and later respiratory diseases, longitudinal birth cohort studies in unselected children are necessary and sensitive detection tools for viral pathogens have to be used. It has also been suggested that reduced lung function early in life predisposes infants to wheezing during viral respiratory infections, but the association between neonatal lung function, subsequent confirmed viral infections and respiratory symptoms has never been investigated.

A number of studies have evaluated parental effects on the development of wheezing illnesses and asthma in their offspring. Recently, a Canadian trial showed that paternal history of asthma and airway responsiveness is an important determinant of disease severity among children with asthma[45]. There is strong evidence that a maternal history of asthma or atopy has more influence than paternal history over subsequent allergic phenotypes in offspring[46]. Besides, maternal history of asthma and atopy also appear to modify the effects of environmental factors that promote the development of asthma[47]. Paternal history of asthma is less well studied.

Pathogen factors

Some pathogens are considered to be more pathogenic than others. RSV is most often mentioned to cause severe respiratory tract illness in young children, and can result in prolonged post viral wheezing[48,49]. Other viruses frequently mentioned to be associated with wheezing illnesses include IFV, PIVs and hMPV[50,51]. Recent years HRVs have raised increasing interest as they seem to be responsible for a wide range of respiratory illnesses. There are several studies which suggest that symptomatic HRV infections might play an important role in recurrent wheezing later on in life or even in development of asthma. Other studies suggest that disease severity of viral pathogens is associated with viral load. An Italian study showed that a high viral load of HRVs were associated with increased disease severity, while a low viral load was associated with mild disease or was found in immunocompromised patients[52].

Further, the importance of multiple infections has been increasingly recognized. The exact mechanism is unknown and reports are not conclusive on this issue. Some argue that multiple viruses cause more severe disease[53-55], whereas others report no difference between

single and multiple infections in disease severity[56,57]. One explanation could be that multiple viruses give rise to a different reaction of the immune system with another cytokine pattern. The cytokines could then cause more severe damage to the respiratory system and therefore more often lead to symptomatic illness.

Environment factors

Environmental factors which are suggested to be associated with disease include smoking during pregnancy, day care visits and siblings. Since the hygiene hypothesis[10] many studies have focussed on the association between daycare attendance, siblings[58,59] and pet exposure[60] and respiratory illness. There is no agreement on the association of these factors, which is probably due to the heterogeneity of the various populations studied. A recent Dutch birth cohort study[58] showed that early daycare attendance was associated with more respiratory symptoms until the age of 4 years, but unless they had siblings, they did not develop less asthma symptoms or allergies at the age of 8 years.

Infants of mothers who smoke have reduced respiratory function and are more likely to develop wheezing. A recent study showed that maternal smoking during pregnancy was represented by changes in airway obstruction parameters, which appeared especially in the youngest group of children[61].

Overall, the association between the various host-, pathogen- and environmental factors in the occurrence of respiratory illness are not clearly defined yet (Figure 1).

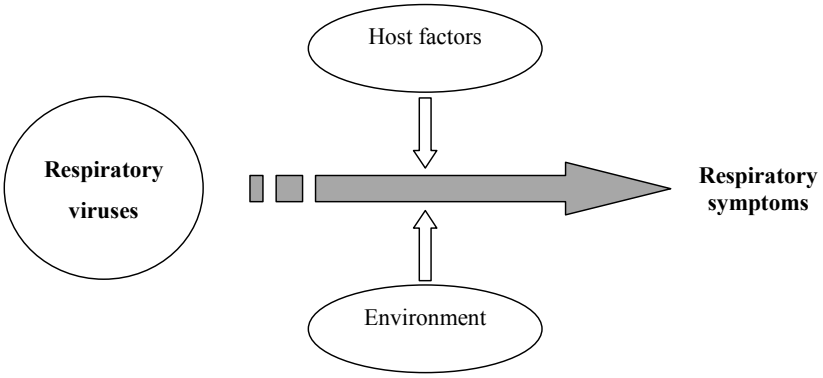

Figure 1. The role of respiratory viruses in lower respiratory tract illnesses in early life

Outline of this thesis

In summary, the precise role of respiratory viruses during early life is unclear. In this thesis we focus on three important epidemiological questions:

1. **What is the prevalence of respiratory viruses, including newly discovered viruses in early life?**
2. **What is the association between respiratory viruses and respiratory symptoms?**
3. **Which factors influence the occurrence of virus-associated respiratory symptoms?**

Chapter 2 describes the prevalence and pathogenicity of respiratory pathogens during the first year of life. The methodology of respiratory viral sampling used in this thesis is outlined in **Chapter 3**. We questioned whether the high prevalence rates of HRVs found in epidemiological studies is due to long-term individual carriership with the same HRV subtype, or whether it is a result of highly frequent subsequent infections with different HRVs subtypes. Therefore we described the longitudinal diversity of HRV in **Chapter 4**. The prevalence and pathogenicity of the newly discovered WU and KI polyomaviruses is described in **Chapter 5.** The second part of this thesis focuses on the association between respiratory pathogens and symptoms. **Chapter 6** describes the differences in the prevalence of respiratory pathogens between children with and without symptoms.
The last part focuses on additional factors associated with development of respiratory symptoms. Reduced lung function early in life is related to wheezing illnesses during the first years of life. However, nothing is known about the effect of neonatal lung function on confirmed respiratory tract infections. We investigated the effect of neonatal lung function in HRV associated wheeze in **Chapter 7**. In **Chapter 8** the association between respiratory symptoms and the presence of respiratory pathogens is studied in infants during the first year of life. The main findings are discussed in the last chapter (**Chapter 9**), followed by a summary in Dutch in **Chapter 10**.

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Chapter 2

Respiratory pathogens in respiratory tract illnesses during the first year of life

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Abstract

Background

Respiratory virus infections are the most important trigger of respiratory illnesses in childhood. Data on the occurrence and the clinical impact of respiratory pathogens in the general population of infants are scarce. Therefore, we described the occurrence and clinical impact of respiratory pathogens in infants with respiratory tract infections during the first year of life.

Methods

In a prospective birth cohort study, infants were followed from birth through the first year of life with daily questionnaires about respiratory symptoms. Nose and throat swabs were collected during episodes with respiratory symptoms. Polymerase chain reaction was used to detect an extensive panel of respiratory pathogens.

Results

The parents reported a median of 5 respiratory episodes per infant per year. A total of 668 respiratory samples were collected in 305 infants. One or more respiratory pathogens were detected in 85% of the samples. The most common respiratory pathogens were human rhinovirus (HRV) (73% of the samples), respiratory syncytial virus (RSV) (11%), and coronavirus (8%). HRV infections were associated with a prolonged period of symptoms compared with RSV ($P = 0.03$). Infections with RSV were associated with more physician visits than HRV infections ($P = 0.06$).

Conclusion

We found a high prevalence of respiratory pathogens among infants with parent-reported respiratory illnesses in the first year of life, with HRV being the most prevalent. Although RSV infections seemed to be responsible for the most severe symptoms compared with HRV, the overall burden of disease was highest for HRV infections.

Keywords
respiratory tract infections, children preschool, rhinovirus, respiratory syncytial virus

Introduction

Respiratory virus infections are the most important trigger of respiratory illnesses in young childhood. Human rhinovirus (HRV) and respiratory syncytial virus (RSV) are reported as the most frequent causing agents of respiratory illness in selected populations of hospitalized children and in children at high risk for atopic diseases.[1–4] Data on the occurrence and clinical impact of respiratory pathogens during respiratory tract infections in unselected infants are scarce. Most of these studies have used rather insensitive conventional methods for virus detection. The development and use of polymerase chain reaction (PCR) methods have markedly increased the identification of viral pathogens.[5,6]

Because of the scarcity of data on the prevalence of respiratory pathogens in young children, prospective data on the clinical impact of respiratory pathogens during respiratory tract infections are almost lacking. For a long time, it has been suggested that some respiratory pathogens cause more severe respiratory illness than others. RSV is most often mentioned to cause severe respiratory tract illness in young children, resulting in prolonged postviral wheezing.[7,8] HRV, on the contrary, was thought to be limited to the upper respiratory tract, causing only mild symptoms.[9]

During the last decade the perspective on HRVs has changed, as HRVs seem to be able to infect the lower respiratory tract as well.[10] Moreover, some symptomatic HRV infections could play an important role in recurrent wheezing later on in life.[11]

In view of this, we questioned whether there is a difference in the clinical impact between the various respiratory pathogens. In this prospective population based birth cohort study, we described the occurrence and clinical impact of respiratory pathogens in infants whose parents reported respiratory infection episodes during the first year of life.

Methods

Study Population

The study was done as part of the ongoing wheezing illnesses study leidsche rijn, a prospective ongoing population-based birth cohort study on determinants of wheezing illnesses. Study design and rationale of wheezing illnesses study leidsche rijn were described elsewhere.[12]

Briefly, healthy infants were enrolled in this study at the age of 2 to 3 weeks, before any respiratory symptoms had occurred and followed until they reached the age of 1 year. Exclusion criteria were gestational age <36 weeks, major congenital abnormalities and neonatal respiratory disease. Inclusion of infants that participated in this part of the study was done from October 2003 to September 2006. In total, 450 parents were asked to participate in this part of the study. Hundred thirty-seven parents did not return a sample and 8 infants

were lost to follow-up. The study was approved by the local medical ethics committee (UMC Utrecht) and the parents gave written informed consent.

Data Collection

During the first year of life all parents filled in a daily questionnaire with regard to respiratory symptoms in their baby. The respiratory symptoms considered were: cough, wheeze (a whistling noise coming from the chest and not the nose), with or without fever (temperature above 38°C). To relate respiratory virus positivity to specific respiratory symptoms parents were asked to take nose and throat swabs at the second day of a reported episode with respiratory symptoms. Parents were instructed by research physicians on how to recognize the various respiratory symptoms. A symptomatic episode was defined by the presence of cough and/or wheeze, with or without fever, for a period of 2 days or longer. The end of a symptomatic episode was defined as having a period of at least 2 days without any respiratory symptoms. Primary care visits and physicians-diagnoses during the first year of life were recorded according to the International Classification system of Primary Care (ICPC).[13] We defined visits to the physician as the occurrence of a "respiratory ICPC," ie, dyspnea (R02), wheezing (R03), cough (R05), acute upper tract infection (R74), acute bronchi(oli)tis (R78), pneumonia (R81), asthma like symptoms (R96), or other less prevalent respiratory ICPC's (breath problems [R04], sneeze [R07], other symptoms of the nose [R08], symptoms of the throat [R21], abnormal sputum [R25], concern about respiratory illness [R27], acute laryngitis [R77], influenza [R88], other infection of the airways [R83], and other respiratory diseases [R99]).

Virus Detection

Respiratory pathogens were detected from the respiratory samples by PCR. After receiving precise instruction at the beginning of the study, parents collected the samples by rubbing one of the nostrils and posterior oropharynx using separate cotton-tipped swabs. After sampling, the 2 swabs were collected into a single vial containing gelatine, lactoalbumine, yeast viral transport (GLY) medium with pimaricin 0.1 mg/mL as viral transport medium and sent to our laboratory via mail. Samples were stored at -20°C until analysis. Sampling of respiratory pathogens by the parents using nose and throat swabs has been shown to be feasible and reliable. Both the sampling frequency and the viral recovery rate in parental samples are at least as efficient compared with sampling by a dedicated research nurse.[14]

The respiratory pathogens HRV and enterovirus, human metapneumovirus (hMPV), human coronaviruses OC43 and 229E, and *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* were analyzed as described.[15] The PCR for adenovirus was performed on conventional PCR (PE 9700) and analyzed by gel electrophoresis.[16] The realtime PCR for coronavirus NL63,

influenzavirus A and B, RSV A and B was performed using the Lightcycler 2.0 format with Lightcycler Taqman Mastermix (Roche).[16] Sensitivity and specificity are state of the art as monitored by Quality Control Molecular Diagnostics (www.qcmd.org) (QCMD) panels.

Statistical Analysis

The independent *T* test was used to compare the number of reported episodes between children with or without siblings. Kruskal-Wallis test was used to compare the number of reported episodes between children born in different seasons. Logistic regression was used to investigate the clinical impact of different respiratory pathogens. An infection with a single respiratory pathogen was compared with a single infection with all other pathogens. The clinical impact was assessed using 5 different parameters; duration of the reported respiratory episode, percentage of cough and percentage of wheeze during each episode, the presence or absence of fever, and physician visits related to the respiratory episode. Information of physician visits was available for 95% of all single infections. Results are presented as odds ratios (OR) with their 95% confidence interval (CI) and *P* value. A $P < 0.05$ was considered significant. All analyses were performed using SPSS, 2001, version 15.0 (SPSS Institute, Inc, Chicago, IL).

Results

Three hundred five infants participated in this study (152 girls and 153 boys) (Figure 1). Of these infants we had a mean documented observed period of 11.5 months (96% follow-up; range: 1–12). These infants reported a median of 5 episodes of respiratory illness per infant/y (range: 1–35; Table 1). Figure 2 shows the number of episodes reported by the

Table 1. Characteristics of the study group.

	N=305
Gender (female)	152 (49.8%)
Siblings (yes)	154 (50.5%)
Birth season	
Winter	83 (27.2%)
Spring	86 (28.2%)
Summer	61 (20.0%)
Fall	75 (24.6%)
No. episodes per child/ yr	5.0 (1-35)
No. samples per child	2.0 (1-9)
Episode number of first sample	1.0 (1-18)
Physician visits for respiratory symptoms per child/ yr*	1.0 (0-25)

* Data on physician visits were available for 289 children.

Data are expressed as median with range between brackets. Absolute values are presented with percentages between brackets.

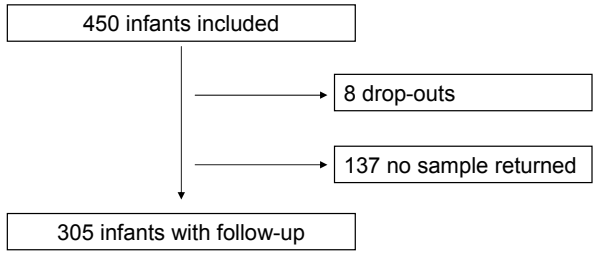

Figure 1. Flowchart of infants included in the study.

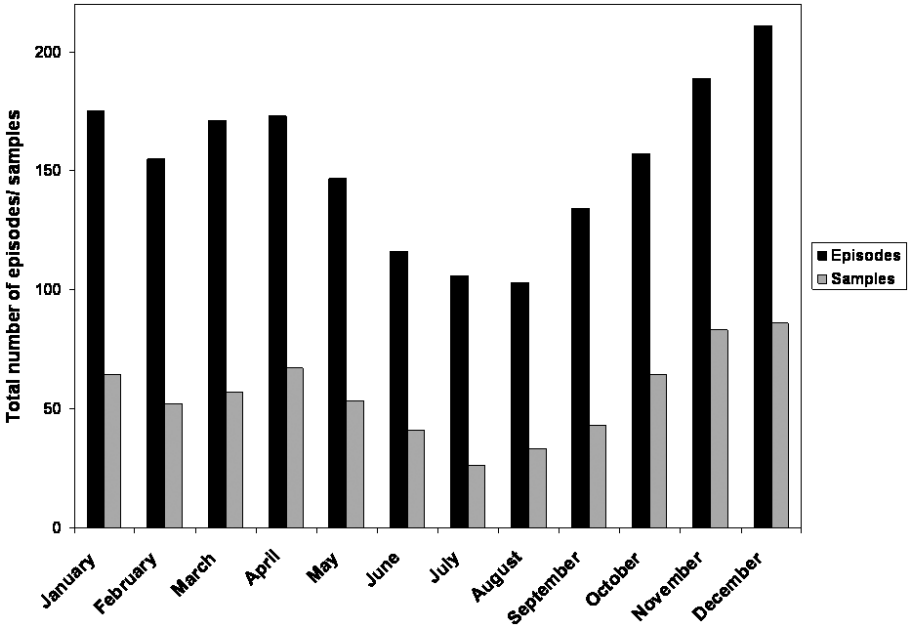

Figure 2. Number of respiratory episodes reported by parents and number of respiratory samples collected throughout the year.

parents throughout the year and the number of samples collected each month. There was no significant difference in number of reported episodes in children with or without siblings (Independent *T* test, $P = 0.23$) or children born in the winter compared with other seasons (Kruskal-Wallis test, $P = 0.88$).

In total, 668 samples were collected. The first sample was generally taken during the first episode at a median age of 4.5 months (interquartile range: 3.0 –7.0). Table 2 shows the PCR results of all collected samples. In the majority of the samples 1 or more viruses were detected (85%). HRV was by far the most common pathogen, it was found in 72% of the samples followed by RSV in 11% and coronaviruses in 8% of the samples. Coronavirus OC43 was the most common type of the coronaviruses followed by NL63 and 229E (80%, 18%, and 2%, respectively). For RSV, the types A and B were equally often found (53% and 47%, respectively). The majority of the influenzavirus infections were caused by influenzavirus type A

Table 2. Respiratory viruses identified in episodes with respiratory symptoms.

	All specimens n=668	Numbers detected as single virus n=468
Any virus-positive	566 (84.7)	-
Human rhinovirus	485 (72.6)	399 (85.3)
Enterovirus	6 (0.9)	4 (0.9)
Coronaviruses	51 (7.6)	18 (3.8)
Respiratory syncytial virus	70 (10.5)*	31 (6.6)
Influenzavirus	18 (2.7)	8 (1.7)
Human metapneumovirus	11 (1.6)	6 (1.3)
Adenovirus	5 (0.7)	0
Mycoplasma pneumoniae	16 (2.4)	1 (0.2)
Chlamydophila pneumoniae	11 (1.6)	1 (0.2)
Multiple viruses	98 (14.7)	-

* RSV A and B were once detected together and were counted as one.

Numbers of specific viruses detected with PCR techniques, with percentages of all samples. The first column is the number of each virus found. The second column is the number of viruses we found as single virus.

(83% and 17%, for influenza virus type A and B, respectively). Multiple respiratory pathogens were found in 15% of the samples. *M. pneumoniae* and *C. pneumoniae* were predominantly found as a coinfection and not as a single pathogen. Adenovirus was exclusively found as a coinfection.

The seasonal distribution of the pathogens is shown in Figure 3. HRV infections occurred throughout the year. RSV infections occurred mainly during the winter months (from November to January). The influenza virus showed a peak incidence in the beginning of the year (late winter) until April.

The clinical impact of the various respiratory pathogens was studied in episodes with single pathogen detections (468 samples). The contribution of days with cough or wheeze within each episode was calculated to correct for the varying duration of the episodes. Further, the presence or absence of fever was reported during each respiratory episode. Details of the respiratory episodes, including duration, type of symptoms, and health care usage, are provided (Table 3). The median duration of symptoms during a pathogen- positive episode was 9.5 days. Cough was almost always present during the entire episode for all respiratory pathogens. Single hMPV infections were observed in only 6 cases. Episodes with hMPV infections were associated with the longest duration and the highest proportion of wheeze. Wheeze was also frequently reported in infections with RSV. Influenza virus was associated with a shorter duration of the episode, yet fever was present during all episodes. The duration of an episode with multiple viral infections was shorter compared with episodes with a single virus infection.

The number of physician visits for respiratory symptoms were recorded during the first year of life. Follow-up data on physician visits were vailable of 289 (95%) infants; the other (5%) infants had a physician outside the research district. In total, 777 visits were made for respiratory symptoms (range: 0-25 visits), with a median of 1 visit per infant/y. A hundred one visits

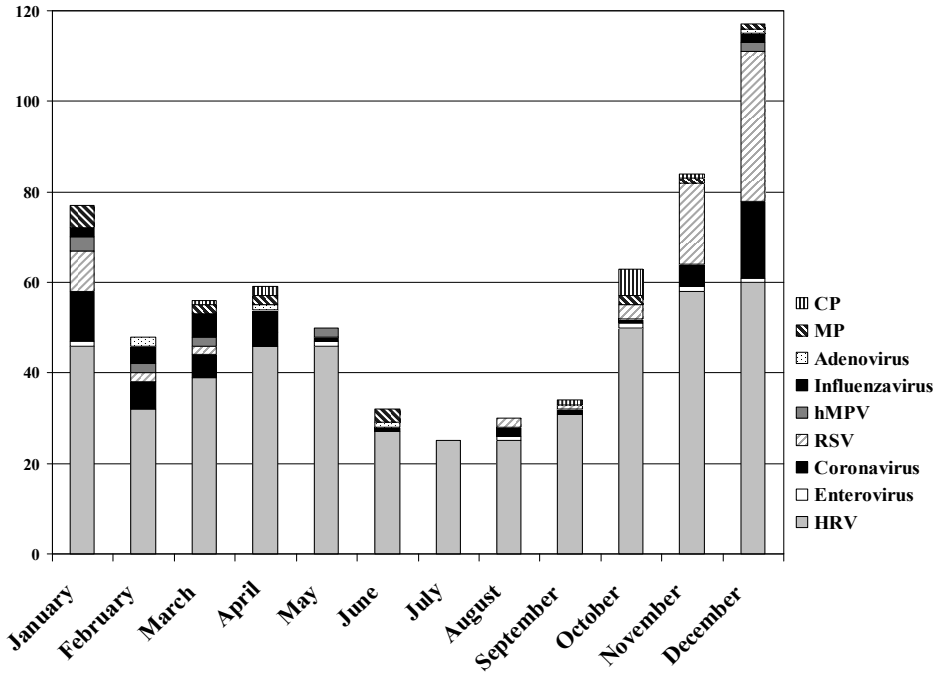

Figure 3. Distribution of respiratory pathogens detected throughout the year.

to the physician with respiratory complaints could be linked to a sampled episode (13% of the physician visits). In 88% (89 of 101) of the samples, which was linked to physician visits, 1 or more viruses were detected. In Table 3, we compared the number of physician visits for the different pathogens. Physician visits occurred most often for infections with RSV, hMPV,

Table 3. Baseline table single virus infections and clinical symptoms

	Duration of episode	% of days with cough	% of days with wheeze	Fever present	Physician visit †
All single pathogens (n=468)	9.5 (5.0-18.0)	100.0 (97.1-100.0)	13.3 (0-72.4)	162 (34.6%)	73 (16.4%)
Human rhinovirus (n=399)	10.0 (5.0-20.0)	100.0 (96.0-100.0)	12.9 (0-75.0)	129 (32.3%)	60 (15.9%)
Enterovirus (n=4)	9.0 (5.3-22.5)	100.0 (88.5-100.0)	3.8 (0-51.9)	2 (50.0%)	1 (33.3%)
Coronavirus (n=18)	10.0 (5.8-14.3)	100.0 (97.5-100.0)	0 (0-75.0)	6 (33.3%)	0
Respiratory syncytial virus (n=31)	8.0 (5.0-11.0)	100.0 (100-100.0)	30.0 (0-100)	15 (48.4%)	9 (30.0%)
Influenzavirus (n=8)	4.5 (3.3-13.3)	100.0(100-100.0)	0 (0-71.4)	8 (100%)	1 (12.5%)
Human metapneumovirus (n=6)	11.5 (8.8-15.0)	100.0 (98.2-100.0)	44.8 (25.0-70.0)	1 (16.7%)	2 (33.3%)
Multiple pathogens (n=98)	8.5 (5.0- 14.0)	100.0 (97.1-100.0)	5.0 (0-77.8)	38 (38.8)	16 (16.3)

† Numbers are corrected for the availability of the data from physician visits. (All pathogens n=444; HRV n=378; enterovirus n=3; coronavirus n=17; RSV n=30; Influenzavirus n=8; hMPV n=6; multiple pathogens n=91)

Results are presented as a median with interquartile range (IQR) or with numbers and percentages between brackets. Mycoplasma pneumoniae and Chlamydophila pneumoniae were both responsible for one single infection.

Table 4. Comparison clinical symptoms between single infections with RSV and single infections with HRV (n=430)

	RSV versus HRV	
	OR (95% CI)	P value
Duration of episode	0.95 (0.91- 1.00)	**0.03**
Proportion of episode with cough	1.01 (0.99- 1.02)	0.42
Proportion of episode with wheeze	1.00 (0.99- 1.01)	0.63
Fever (y)	1.96 (0.94- 4.09)	0.07
Physician visit (y)†	2.21 (0.97- 5.07)	**0.06**

Logistic regression; RSV compared to HRV (HRV was taken as the reference group). Only samples with a single detection with HRV or RSV were selected.

Abbreviations: OR= Odds Ratio, CI= Confidence Interval.

† Numbers are corrected for the availability of the data from physician visits (n=408).

and enterovirus. The number of physician visits in episodes with multiple virus infections was comparable to episodes with a single virus.

Finally, we compared the clinical impact of infections with HRV to infections with RSV (Table 4). The duration of infections with RSV was shorter compared with infections with HRV, this difference was statistically significant (OR: 0.95, 95% CI: 0.91–1.00, *P* =0.03). There was no statistically significant difference in the proportion of cough and wheeze during episodes with a RSV and HRV infection. Fever was reported more frequently in episodes with RSV than in episodes with HRV, this difference was borderline significant (OR: 1.96; CI: 0.94–4.09, *P* = 0.07). More physician visits were linked to episodes with RSV infection than episodes with HRV infection (OR: 2.18; CI: 0.96–4.99, *P* = 0.06).

Discussion

This study shows that respiratory viruses and atypical bacteria are frequently found in infants with lower respiratory tract symptoms during the first year of life. HRV was the most frequent detected respiratory pathogen followed by RSV and coronavirus. We observed that infections with RSV were more frequently associated with wheeze, fever, and physician visits compared with infections with HRV. Nevertheless, the high prevalence and duration of HRV infections results in a higher burden of disease compared with RSV infections.

Some methodologic aspects need to be considered. In this large prospective study, viral samples were only taken during respiratory episodes. Recent literature shows that respiratory viruses are also frequently found in children without respiratory symptoms.[15,17] Further, molecular detection techniques have increased viral pathogen identification, but it is uncertain whether is represents true infections. A longitudinal study with regular viral sampling regardless of respiratory symptoms could give a more detailed picture of the occurrence of respiratory pathogens in the first year of life. However, besides description of the prevalence of different viruses, the focus of the present study was to compare the relative pathogenicity

of different viruses. Although we have no data on the absolute pathogenicity of viruses, the study is useful to compare different viruses.

In the present study, parents sampled their infants during respiratory episodes. In general, the first sample was taken during the first reported episode at a median age of 4.5 months. In a recently published Swiss birth cohort,[18] the first acute respiratory tract illness was reported at a median age of 6 months and respiratory samples were collected by research nurses. Even though parent-sampling led to earlier sampling in this study, a proportion of parents did not sample at all. These results show that the method of sampling by either parents or research nurse is never complete and respiratory episodes are missed. Systematic sampling could be essential to further unravel the importance of respiratory pathogens during episodes with symptoms and in episodes without symptoms.

The most common respiratory pathogens we found were HRV, coronavirus, and RSV. HRV was by far the most prevalent virus in this study, as it was identified in nearly three-quarter of the samples. Besides, the contribution of HRV as single virus infection was even higher (85%). Overall, this study confirms the high occurrence of HRVs in respiratory tract infections during the first year of life.[3,4] Interestingly, *C. pneumoniae, M. pneumoniae*, and adenovirus were predominantly found as a coinfection and not as a single pathogen. This result might indicate that the role of these pathogens in young infants with respiratory symptoms is limited.[19,20]

The clinical impact of the respiratory pathogens was derived from the duration, type of symptoms, and health care utilization. Influenza virus was associated with the shortest duration of illness. All influenza-positive episodes were presented with fever. In the literature, fever is a well known symptom of influenzavirus but usually the duration is also thought to be extended.[21] Infections with hMPV resulted in the longest duration of symptoms and the highest proportion of wheeze. Further, a high proportion of the hMPV infections, although the numbers were small, led to a physician visit. These findings were comparable to other studies for the impact of hMPV in children.[21,22] Cough was not a good discriminatory symptom as it was reported during the complete episode regardless of type of pathogen. The results show that the pattern of response to a pathogen was too diverse to draw conclusions for the clinical impact of single pathogens.

Until recently, HRVs have been thought to cause only mild symptoms in contrast to RSV, which is seen as a virus causing more severe illness.[23] In some studies RSV was detected in up to 80% of the bronchiolitis cases,[24] which could be suggestive for a high pathogenicity of this virus. We compared the clinical impact of HRV infections to infections with RSV and observed some differences. HRV infections were associated with a longer period of illness. Infections with RSV, on the other hand, were more often associated with wheeze, fever, and physician visits. This might indicate that infections with RSV cause more severe illness compared with infections with HRV. However, the occurrence of single-HRV infections is almost 13 times greater than to RSV infections. In addition, the absolute number of physician visits made

during episodes with a HRV infection is nearly 7 times greater than to RSV infections. So, even though RSV infections might lead to more severe respiratory illness, due to the presenting symptoms, the occurrence, and the duration of HRV infections lead to a higher burden of disease. These results are in line with the growing evidence of the importance of HRV infections in respiratory illnesses. Although HRVs also seem to be associated with recurrent wheezing later on in life,[11] more follow-up studies are needed to further investigate the long-term effects of rhinovirus infections early in life.

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Chapter 3

Viral specimen collection by parents increases response rate in population-based virus studies

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Viral specimen collection by parents increases response rate in population-based virus studies

Recently, Lemanske et al[1] described an impressive study in the Journal with regard to the role of viral respiratory infections in the development of wheezing and allergic diseases in childhood. In a prospective cohort study in 285 high-risk newborns, nasopharyngeal mucus samples for viral diagnostics were collected at 5 scheduled clinic visits in the first year of life. In addition, parents were asked to notify the study coordinator during times of acute respiratory illnesses to schedule an extra visit with nasopharyngeal sampling. A total of 1668 nasal specimens were obtained, 1102 during scheduled clinic visits and 566 during sick visits. Hence, the study by Lemanske et al[1] reports 566/285 = 1.99 respiratory viral episodes per infant per year, which is considerably lower than expected rates from other studies[2]. We argue that the study design might have influenced the response rate of parents during periods of respiratory symptoms. Nowadays, parents might be too busy to call the study coordinator, leading to underreporting of viral episodes. Parents might also simply forget to call the study coordinator, which possibly leads to bias to periods with more severe symptoms.

We tested this hypothesis in the Wheezing Illnesses Study Leidsche Rijn (WHISTLER) study currently being conducted in The Netherlands[3]. In this study, infants are followed from birth through the first year of life with daily questionnaires about respiratory symptoms. We asked parents of 50 children to participate in a 6-month pilot study into the diagnosis of viruses during periods of upper for lower respiratory tract infections. A random half of the parents were asked to call the study coordinator to schedule a sick visit at home (group A), and the other half of the parents were asked to take a nasopharyngeal mucus sample from their child themselves and to send it to the study center by mail (group B). All samples were analyzed using PCR techniques for respiratory syncytial virus, rhinovirus, enterovirus, corona OC43/229E, human metapneumovirus influenza type A and B, adenovirus, Chlamydia pneumoniae, and Mycoplasma pneumoniae. During the observation period, the 2 groups had an equal number of symptomatic periods according to their diaries (respectively, 49 vs 47 periods in group A and B). In group A, 12 sick visits with sampling were obtained (24% of periods), and in group B, 24 samples were obtained (43% of periods; = 0 .07). No differences between the groups were observed for severity of symptoms or for viral recovery rate (respectively, 67% vs 80%; P = 0.44).

We conclude that study design can improve the response rate in population-based virus studies. As much as 76% of symptomatic periods can be missed when parents have to call a study coordinator for virus sampling. This will importantly influence the numbers on prevalence of virus infections in population-based studies. Our data show that these numbers can be significantly reduced to 57% if parents are asked to sample their children themselves, without any loss of viral recovery rate.

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Chapter 4

Frequent infections with human rhinovirus in young children; a longitudinal cohort study

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Submitted

Abstract

Background

There is growing evidence that human rhinoviruses (HRVs) are an important cause of respiratory tract infections. We questioned whether the high prevalence rates of HRVs found in epidemiological studies is due to long-term individual carriership or a result of frequent infections with different HRV subtypes.

Methods

In a 6-month winter period 18 healthy controls, aged 0-7 years, were at least sampled biweekly for HRV-PCR, irrespective of respiratory symptoms. All HRV positive samples were genotyped to determine HRV diversity.

Results

In total 271 samples were collected. HRV was found in 95/271 (35%) samples. Genotyping revealed 32 different HRV subtypes. A median of 3.0 different HRV subtypes were found per child. Re-infections and persistence with identical HRV sequences were observed. The number of HRVs were higher in the youngest age group ($p= 0.03$) and they had more different HRV subtypes ($p= 0.02$) compared to oldest age group.

Conclusion

We found a high HRV exposition with a considerable diverse population of HRV subtypes in young children. These results have major implications for future research into the pathogenic role of HRV in respiratory diseases. Characterisation of subtypes will be necessary to discriminate between prolonged carriership and re-infections in patients with respiratory diseases.

Introduction

During the last decade human rhinoviruses (HRVs) have raised increasing interest as they seem to be responsible for a wide range of respiratory illnesses. HRVs are frequently found in asymptomatic children and adults [1,2], but are also detected in patients with symptoms ranging from mild common colds [3] to serious lower respiratory tract disease [4,5]. Since the development of molecular assays for the detection of HRVs, the detection rate of HRV in patients with respiratory infections has increased to up to 50% [6].

Besides an increasing awareness from epidemiological studies regarding the high prevalence of HRV there is growing evidence for the importance of different HRV subtypes. HRV is a member of the *Picornaviridae* family and more than 100 genetically and serologically different HRV subtypes have been described [7]. HRV subtypes can be classified according to several parameters, including receptor specificity, antiviral susceptibility and nucleotide sequence homologies [8]. Taxonomically HRV subtypes can be distinguished accurately by sequencing the 5'NCR [9] and divided in clades, like HRV A, HRV A2 and, HRV B. It has been suggested that some HRV subtypes might be associated with more severe or different respiratory disease patterns than others [10-12].

Despite this increasing knowledge from epidemiologic and basic studies, longitudinal data on the diversity of HRVs in individuals are lacking. We questioned whether the high prevalence rates of HRVs found in epidemiological studies is due to long-term individual carriership with the same subtype [13] or whether it is a result of highly frequent subsequent infections with different HRVs subtypes. Therefore, we performed a prospective longitudinal cohort studying young children to closely monitor the occurrence of HRV subtypes over time.

Patients and methods

Study population

We conducted a prospective longitudinal cohort study during a 6-month period (from November 2004 through April 2005) in 19 healthy children aged 0-7 years. One of them (male, almost 3 years old) failed to complete the study after the ninth week and was excluded from the analysis. None of the children had a history of asthma or recurrent respiratory complaints. At the beginning of the study, parents were instructed to take samples for virus detection by rubbing one of the nostrils and posterior oropharynx of their child using separate cotton-tipped swabs. The two swabs were collected into a single vial containing GLY medium containing 0.1 mg/ml pimaricine as viral transport medium and sent to our laboratory via regular mail. Samples were stored at –20°C until analysis. Samples were taken every two weeks regardless of any respiratory symptoms and additional samples were taken when respiratory

symptoms were present for more than two days. Sampling of respiratory pathogens by the parents using nose and throat swabs has been shown to be feasible and reliable. Both the sampling frequency and the viral recovery rate in parental samples are higher compared to sampling by a dedicated research nurse [14;15].
The study was approved by the local Medical Ethics Committee (University Medical Center, Utrecht) and all parents gave written informed consent.

PCR, sequencing and phylogenetic analysis

Viral RNA was isolated from 200µl of the original sample using the High pure RNA isolation kit (Roche, Germany). cDNA synthesis, nested PCR and Southern blotting were carried out to detect HRV [16]. In case of a positive PCR for HRV, the amplicon was extracted from gel and purified with Qiaquick gel extraction kit (Qiagen® Germany). These amplicons from the inner primer set (approximately 310 nucleotides of the 5'NCR region) were sequenced using capillary DNA sequencer (ABI model 3700). When sequencing failed initially, nucleic acid isolation, PCR and sequencing were repeated once on the original sample. Sequence data were blasted against Genbank and analyzed with BioNumerics 4.6 (Applied Matths, Gent, Belgium) with a maximum parsimony algorithm performing 100 bootstraps. Subtypes were defined as different when sequence homologies were < 90%.

Statistical analysis

Statistical analysis was performed using SPSS Inc., 2001, Chicago USA, version 12.0. Comparisons of the distributions of categorical variables between groups were examined using a two-tailed Chi-square and the medians of continuous variables using the nonparametric Mann Whitney U test. A significance level of $p \leq 0.05$ was used throughout.

Results

Eighteen children were longitudinally followed during a 6-month study period. The median age of the children was 3.6 years. Further characteristics of the group are shown in Table 1.
In total 271 samples, regardless of symptoms, were collected and tested for the presence of HRV. We observed a high prevalence of HRV in our population, HRV was found in 95/271 (35%) samples. All children had at least one HRV positive sample during the study period with a maximum of ten (median number of HRV positive samples 3.5).
To investigate whether this high frequency of HRV positive samples in our study is due to frequent infections with different HRV subtypes or of persistence of the same subtype, we performed sequencing on HRV positive samples. Sequencing was successful in 72/95 (76%)

Table 1. Characteristics of the study group

	N=18
Gender (male/ female)	3/ 15
Age (yr)	3.6 (2.7- 5.0)
Number of samples/ child	15.5 (13.8-17.0)
Number of different HRV subtypes/ child	3.0 (2.0- 4.3)

Data are expressed as median with interquartile range (IQR).

of the HRV positive samples. In total 32 different HRV subtypes were found. A median of 3.0 different HRV subtypes were found per child (interquartile range IQR 2.0- 4.3). A maximum of 6 different HRV subtypes was found in 2 children.

To study the distribution and diversity of HRV subtypes a dendrogram of the HRV subtypes was constructed (Figure 1). The majority of the HRV sequences we found can be grouped into the HRV A strain (64/72; 89%). A smaller proportion of the HRV subtypes belonged to the HRV A2 strain (3/72; 4%) and the HRV B strain (5/72; 7%).

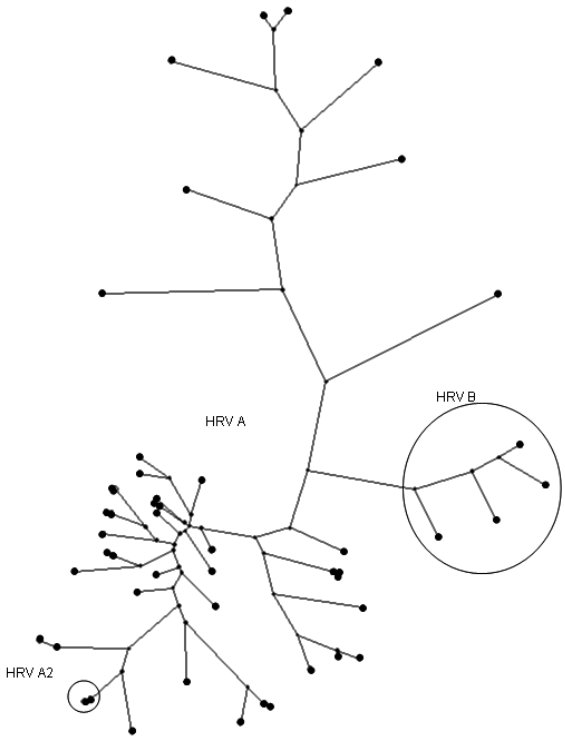

Figure 1: Phylogenetic analysis of HRV positive samples. Circles refer to HRV clades described in literature (HRV A2, HRV B); all other sequences belong to clade HRV A. Sequence data were analyzed with maximum parsimony algorithm performing 100 bootstraps.

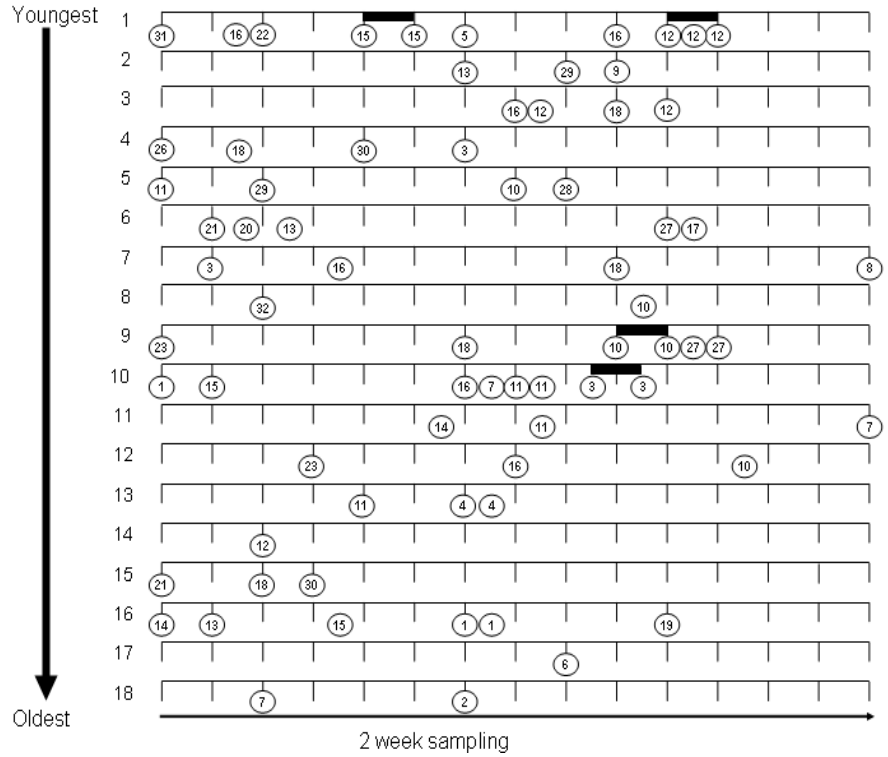

Figure 2: Timelines of detected HRV subtypes for all children during the study period. Each line represents a child in order of increasing age; the time between two vertical lines accounts for approximately 2 weeks. The numbers are the 32 different HRV subtypes found. The black bars refer to a period of persistence (≥ 2 weeks) with the same HRV subtype.

To visualize the dynamics of HRV infections in children during the observation period, we constructed individual timelines of the HRV positive samples in Figure 2. During the observation period we observed re-infections with the same HRV subtype in two children (children 1 and 3). These children showed re-infection with an identical HRV subtype with periods of other HRV subtypes in between. In most instances HRV positivity concerned a new infection with a new HRV subtype. Persistence of identical HRV subtypes for 14 days or more was observed in 3 children (children1, 9 and 10).

Finally, we analysed the influence of age on the occurrence of HRV and the diversity of HRV subtypes. In the youngest age group (children <5 years, children 1-10; Figure 2) 40% of the samples taken were HRV positive, compared to 28% of the samples in the oldest age group (children ≥5 years, children 11-18) (Chi-square test, p=0.03). The number of different HRV subtypes found was higher in the youngest age group with a median of 4.0 (IQR 3.0- 5.3) compared to the oldest group with a median of 2.5 (IQR 1.3- 3.0) (Mann Whitney U test p= 0.02). All children with a re-infection and/ or a prolonged infection with an identical HRV subtype belonged to the youngest age group (children <5 years). We could not find any relationship between specific HRV subtypes and age.

Discussion

This longitudinal study shows that HRV is highly prevalent in young children due to a high infection rate with a huge diversity of HRV subtypes. We also observed re-infections and persistence of identical HRV subtypes during this 6-month observation period. Younger age is associated with a higher infection rate with HRV.

This is the first study using longitudinal analysis and phylogenetic subtypes to describe the dynamics of HRV infections over time. The high sampling frequency and sequencing of HRV subtypes gives a detailed picture of HRV acquisition of young children and makes it possible to distinguish prolonged infection with the same subtype from repeated infections with different HRV subtypes. In this study we sampled from November through April, the "respiratory season" but still this could limit the generalizability of our results. Besides not all HRV-PCR positive samples were successfully sequenced, possibly due to low RNA concentrations in samples that were nevertheless positively identified by Southern blotting. Successful sequencing in 76% seems acceptable to draw conclusions and is even higher compared to results of other studies [17].

In this study, HRV was found in 35% of all samples. This high detection rate is in line with a similar designed longitudinal study [18] where picornavirus was detected in 26% of the samples from periods of both illness and wellness in children. In addition, in cross-sectional studies HRVs are also frequently found in up to 50% of the children during respiratory tract illnesses [19-21]. The systematic surveillance for HRVs in this study revealed that the occurrence of HRV is even higher than thought before with an average of almost 4 HRVs in a six-month period.

Our data show that the prevalence of HRV is high due to a high infection rate with a huge diversity of HRV subtypes. A recent study [14] showed that HRVs are frequently transmitted from children to other family members and multiple HRV types circulated simultaneously within these families. In this study children had a median of 3 different HRV subtypes with a maximum of 6 during a 6-month period. The majority of the HRV sequences we found belonged to HRV A species; this is in line with other studies [22;23].

We investigated the longitudinal course of infections with HRV subtypes. In our study re-infection with the same HRV subtype was seen in 2 children with a period of other HRV subtypes in between. Apparently, re-infections with the same HRV subtype occur during a winter season. It would be interesting to observe whether different HRV subtypes can be found in successive seasons or if HRV subtypes disappear and/ or reappear in following years [24]. Persistence of the same HRV subtype was seen in 3 different children. The maximum period for a prolonged infection with an identical HRV subtype was 14 days. Persistence of HRV was also described in a few others studies. Our data shed new light on these studies because sequence data have been lacking in all studies until now. The Finnish study [13] longitudinally followed children for the persistence of HRV after hospitalisation for wheezing illness. Follow up was done after 2, 5 and 8 weeks; HRVs could be detected until 2-5 weeks after onset of

symptoms. In another study [18] samples were taken weekly to identify picornavirus infections in children. Here, detection of the picornavirus was episodic lasting for a period of 1-3 weeks. In the third study [25], HRV-RNA was detected in 40% of the asthmatic children till 6 weeks after an acute exacerbation. Considering the high number of different HRV subtypes we found in our study, only genetic analysis can prove persistence of HRV subtypes instead of the discovery of different subtypes.

Finally, we studied the influence of age on the dynamics of HRV infections. The frequency of symptomatic viral respiratory tract infections is higher in young children compared to adults [2;14;26]. Therefore, we hypothesised that young children might be more often infected with HRV than older children. In this study we found that children <5 years of age acquire significantly more HRV infections compared to children from ≥5 years of age (40% versus 28%, respectively). Moreover, they have significantly more different HRV subtypes, although we could not find a relationship between age and specific HRV subtypes. Perhaps the higher infection rate in younger children is due to differences in immune reaction between younger and older children. Several studies have shown that there are differences in cytokine profiles, T cell proliferation and Natural Killer Cell (NKC) activity between young children and adults [27-29]. This is supported by the fact that both re-infection and prolonged infection of HRV subtypes were only seen in the youngest children of the study population. Further studies are needed to unravel the association between age and the diversity of HRVs.

In conclusion, we found that there is a high HRV exposition with a considerable diverse population of HRV subtypes in young children. Future studies into the pathogenic role of HRV should differentiate for HRV subtypes to discriminate between persistence and re-infections of viruses.

Acknowledgements

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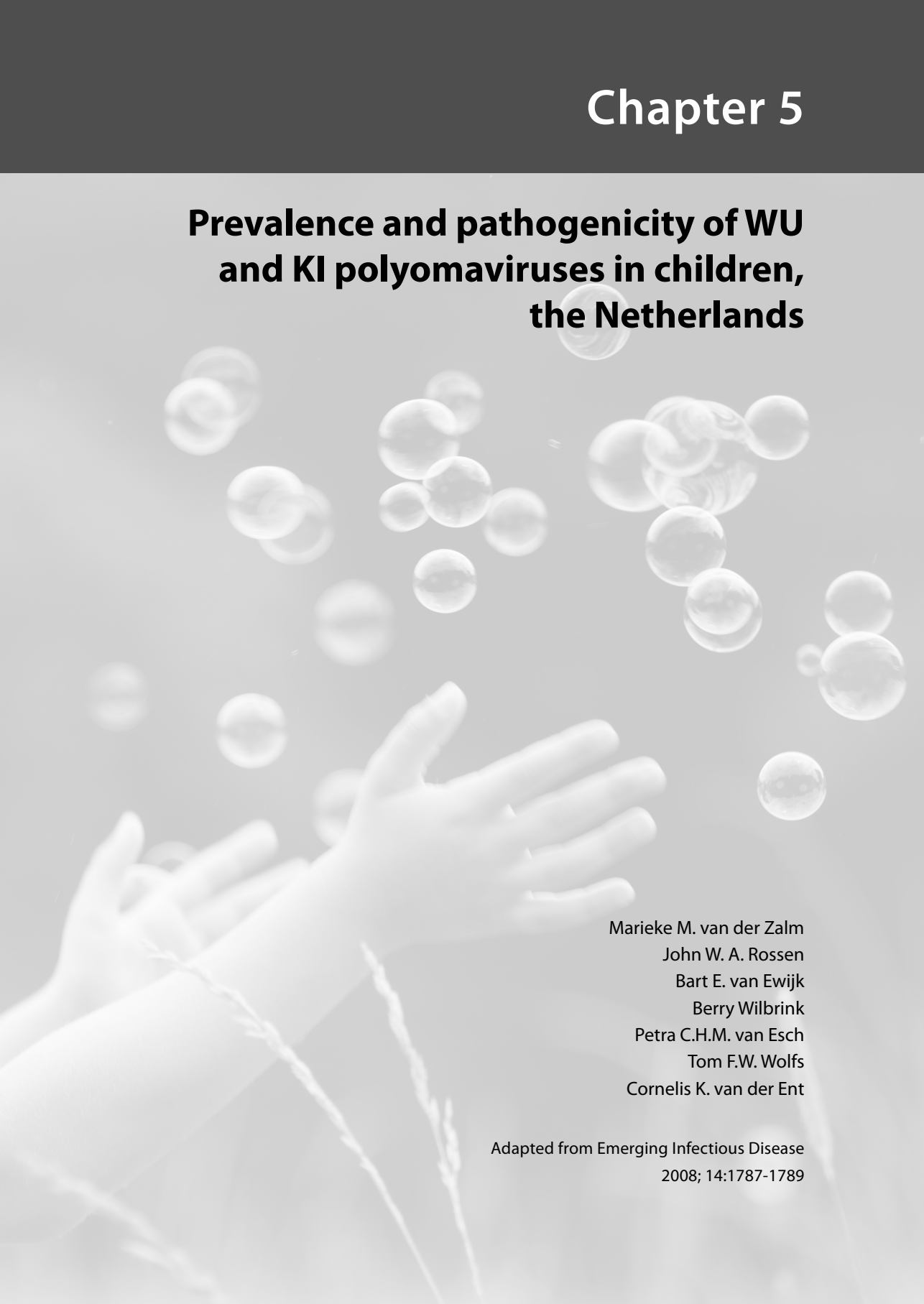
Chapter 5

Prevalence and pathogenicity of WU and KI polyomaviruses in children, the Netherlands



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Abstract

Recently, two new polyomaviruses called WU virus (WUPyV) and KI virus (KIPyV) were identified in patients with respiratory tract symptoms. We prospectively studied the prevalence of WUPyV and KIPyV in children with and without respiratory symptoms.

During a 6-month winter period we bi-weekly sampled the nose and throat in 18 healthy children aged 0-7 years, irrespective of respiratory symptoms. Real-time polymerase chain reaction was used to detect WUPyV and KIPyV and to rule out other causative respiratory pathogens.

WUPyV and KIPyV were found in 9% and 3% of all samples, respectively. In 5 and respectively 2 cases the viruses were detected in symptomatic children in whom no other causative respiratory pathogen could be detected. Repeated positivity for both viruses was observed in this winter period.

We conclude that WUPyV and KIPyV frequently occur and recur in young children, and suggest that WUPyV and KIPyV might be associated with respiratory disease.

Introduction

Respiratory viruses are responsible for the majority of the respiratory illnesses in young childhood. Since the introduction of molecular detection techniques, like polymerase chain reaction (PCR), the percentage of pathogens found in children with respiratory tract symptoms in published studies has increased till up to 85% [1;2]. Maybe polyomaviruses play a role in the remaining group of symptomatic children without known causative agents [3].
Recently, high throughput sequencing techniques revealed two new polyomaviruses called WU virus (WUPyV) [4] and KI virus (KIPyV) [5]. A few studies described the presence of WUPyV and KIPyV in respiratory samples in uncontrolled small groups of hospitalized patients [4-10]. However, the clinical relevance of these viruses in humans is unclear, because data on these viruses in otherwise healthy subjects outside a hospital setting are lacking [11-13]. Until now it is unknown whether these newly identified viruses also occur in healthy children and whether they have to be observed as causative agents for clinical respiratory disease.
In this study, we questioned how often WUPyV and KIPyV occur in young children and whether these viruses are associated with clinical respiratory symptoms. Therefore we performed a systematic surveillance for WUPyV and KIPyV and closely monitored respiratory symptoms in a prospective longitudinal cohort study in young children.

Material and methods

Study design and subjects

We conducted a prospective longitudinal cohort study during a 6-month winter season (from November through April). A total of 19 healthy children aged 0-7 years were enrolled; one of them failed to complete the study. There were two sibling pairs, all other children were unrelated. None of the children had a history of asthma or recurrent respiratory complaints.
The study was approved by the local medical ethics committee (University Medical Center, Utrecht) and all parents gave written informed consent.

Study protocol

Parents were contacted twice a week via telephone or e-mail by the study coordinator to ask for the presence of any symptoms of respiratory tract illness. Respiratory symptoms were defined as symptoms of coryza (rhinorrhea or nasal congestion), sore throat, earache with or without ear discharge, cough, sputum production or dyspnoea, all with or without a temperature above 38°C. Every two weeks samples for virus detection were collected regardless of any respiratory symptoms. A sample was defined as 'asymptomatic' if there were no respi-

ratory symptoms during a complete period of one week prior to, one week after sampling. A sample was defined as 'symptomatic' if there were any respiratory symptoms during the period of one week prior to, one week after sampling.

Virus detection

After receiving precise instruction at the beginning of the study, parents collected the samples for virus detection by rubbing one of the nostrils and posterior oropharynx of their child with separate cotton-tipped swabs. After sampling the two swabs were collected into a single vial with GLY medium containing 0.1 mg/ml pimaricine as viral transport medium and sent to our laboratory via regular mail. Samples were stored at -20°C until analysis. Feasibility of virus sampling by the parents was described earlier [14].

We performed real-time PCRs (rtPCRs) for WUPyV and KIPyV at the laboratory of Medical Microbiology and Immunology in Tilburg. We extracted viral nucleid acids from 200µL of the original sample using the MagNa Pure LC total nucleid acid isolation kit (Roche Diagnostics, Basel, Switzerland) [15]. Subsequently, rtPCR was performed on a ABI 7500 rtPCR Instrument (Applied biosystems, Foster City, CA) [15;16]. Positive controls for the WUPyV and KIPyV PCR were a kind gift of Dr. Bialasiewicz and Dr. Sloots (University of Queensland, Australia).

To rule out other respiratory pathogens as a possible cause for respiratory symptoms, an additional broad panel of PCR's was used, containing rhinovirus, enterovirus, respiratory syncytial virus (RSV) A and B, coronaviruses OC43, 229E and NL63, influenza viruses A and B, human metapneumovirus, adenovirus, *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae*.

Statistical analysis was performed using SPSS Inc., 2001, Chicago USA, version 12.0. A two-tailed Fischer's exact test was used to compare differences between groups. Differences were considered statistical significant if the p-value was <0.05.

Results

The median age of the children was 3.6 years. Further characteristics of the group are presented in Table 1.

In total 230 samples were collected and tested for the presence of WUPyV and KIPyV (Figure

Table 1. Characteristics of the study group.

	N=18
Age (yrs)	3.6 (2.7-5.0)
Gender (male/female)	3/15
Number of samples from symptomatic episodes/ child	10.0 (7.0-15.0)
Number of samples from asymptomatic episodes/ child	5.0 (3.0-10.5)

Characteristics of the study group. Data are expressed as a median, with interquartile range (IQR) between brackets.

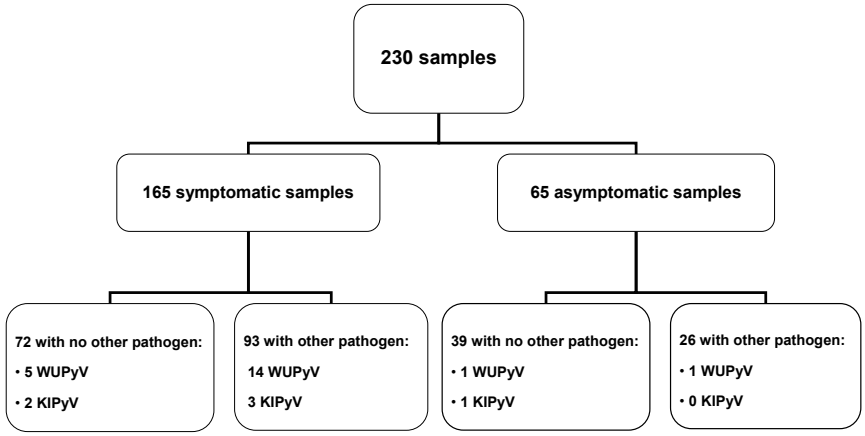

Figure 1. Flow chart of the respiratory samples taken in the study. Samples were collected during November 2004- April 2005, throughout the Netherlands. Samples were taken during symptomatic and asymptomatic episodes. Results show WU polyomavirus (WUPyV) and KI polyomavirus (KIPyV) detections in samples simultaneously negative for other respiratory pathogens and in samples in wich ≥ 1 other respiratory pathogen(s) were detected.

1). In 119 samples (52%) non-polyomavirus respiratory pathogens were detected ((rhinovirus; 31.7%), enterovirus (3.0%), RSV A and B (2.2%), coronaviruses OC43, 229E and NL63 (16.5%), influenza viruses A and B (0.9%), human metapneumovirus (0.9%), adenovirus (0.4%), *Mycoplasma pneumoniae* (2.6%) and *Chlamydophila pneumoniae* (5.2%)).

WUPyV was found in 21/230 (9%) samples (Figure 1) in 8/18 (44%) children. In 5 episodes WUPyV might be responsible for respiratory symptoms, because in these symptomatic samples no other causative respiratory pathogen could be detected. WUPyV was detected twice in asymptomatic samples. KIPyV was found in 6/230 samples (3%) in 3/18 (17%) children. In two symptomatic samples KIPyV might be the causative pathogen, because no other pathogens were detected. Only one KIPyV positive sample was asymptomatic. WUPyV and

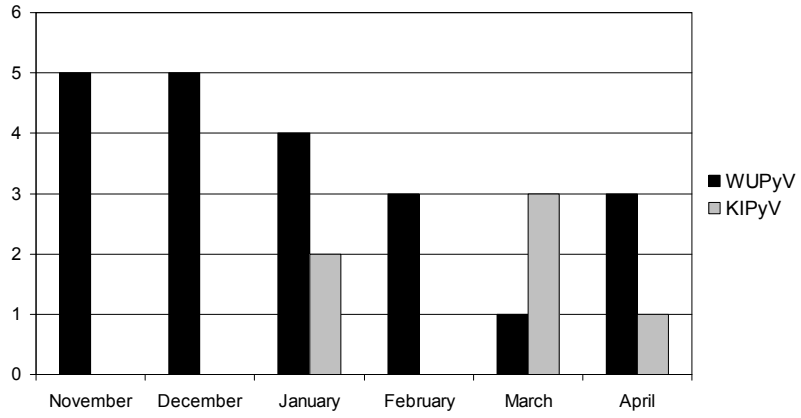

Figure 2. Seasonal distribution of WUPyV and KIPyV positive samples detected in this study. Number of samples found positive for WUPyV and KIPyV during the months of the study period (November 2004- April 2005).

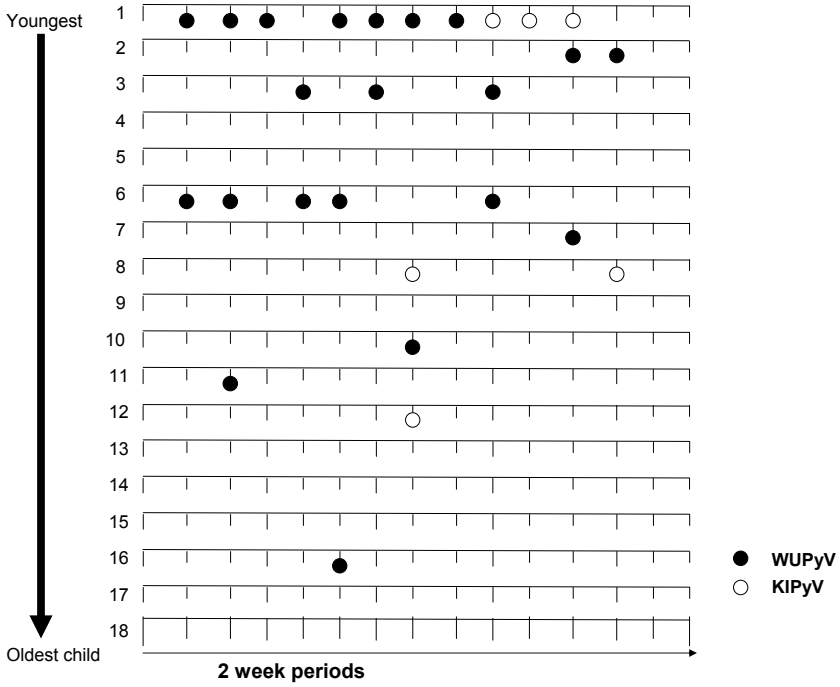

Figure 3. Timelines of WU polyomavirus (WUPyV) and KI polyomavirus (KIPyV) in 2-week samples, taken regardless of symptoms. Samples were collected during November 2004- April 2005, throughout the Netherlands. Each line represents a child in order of increasing age (patients 1-18, aged <1-7 years); the time between two vertical lines accounts for approximately 2 weeks. The solid symbols are WUPyV infections; the open symbols are KIPyV infections.

KIPyV were never found simultaneously. Figure 2 shows the distribution of WUPyV and KIPyV positive samples over the months during the study. WUPyV was found in all study months (November through April), with a maximum in November and December. KIPyV was found in January, March and April.

To visualize possible re-infections and persistence of WUPyV and KIPyV during the observation period, we constructed individual timelines of the subsequent two-weekly samples in Figure 3. Re-infections with WUPyV were suspected in different children during this study (1, 3 and 6). These children showed WUPyV positive samples with WUPyV negative intervals. Persistence of WUPyV (detection of the virus in 2 or more successive samples, equal to <2 weeks) was seen in 3 children (1, 2 and 6). The youngest child of the group (1), had 2 periods of a prolonged infection with WUPyV: one period of 3 successive positive samples and one with 4 positive samples. Re-infections with KIPyV were never observed in our study. The youngest child (1) had 3 successive KIPyV positive samples; in the other children KIPyV positivity was limited to 1 period. Figure 3 also demonstrates that most infections with WUPyV and KIPyV are seen in the youngest children; 95% of the WUPyV infections and 83% of the KIPyV infections were seen in children <4 years. The median age of the children with a WUPyV infection was 2.6 years (interquartile range IQR 0.7-3.9), for a KIPyV infection the median age was 3.0 years (IQR 0.4- 4.0).

Discussion

This unique prospective longitudinal cohort study shows a high occurrence of WUPyV and KIPyV in children. Both WUPyV and KIPyV were repeatedly observed as the only detectable pathogen in children with respiratory symptoms, which suggest that both viruses might have pathogenic capacities. Re-infections with WUPyV and persistent infection with WUPyV or KIPyV are seen during this relative short study period. In addition, younger age is associated with a higher occurrence of WUPyV and KIPyV infections.

The uniqueness of our study is the longitudinal surveillance for WUPyV and KIPyV in healthy children at home, combined with close monitoring of respiratory symptoms.

This way we got a detailed picture of the frequency and course of infections with WUPyV and KIPyV during a winter season in young children. Although we studied a relatively small number of children, the number of samples taken is large which enables to get insight into the occurrence and dynamics of these polyomaviruses. A second drawback of this study is that our observation is limited to the winter season. Future studies might reveal additional data on this topic.

The overall percentage of WUPyV in our study (9%) is somewhat higher than percentages seen in most other studies (0.4- 7%) [4-6;8-13]. Because our study has a longitudinal design, prevalences cannot be compared to prevalences of other studies with a cross sectional design. Though a substantial proportion (44%) of the children in this study was at least once infected with WUPyV. KIPyV was found in 3% of all samples in 17% of the children. Other studies report frequencies of KIPyV between 0.6% and 6.8% [5-7;10;13]. In our study WUPyV was found as the third most prevalent pathogen; after rhinoviruses (32%) and coronaviruses (17%). KIPyV was found as the fifth most prevalent pathogen, comparable with enteroviruses. All of these viruses were found in both symptomatic as well as in asymptomatic episodes, so probably other factors, presumably host factors play a role in the pathogenicity of these viruses. The number of WUPyV and KIPyV detections confirms the results of other studies that these viruses are prevalent in children worldwide.

In literature, limited data are available on the seasonal distribution of WUPyV and KIPyV. Some studies examined respiratory samples of a period for at least one year [6;8;12]. They showed that WUPyV and KIPyV infections were present throughout the year with a peak of WUPyV from late winter to early summer. In our study WUPyV was found throughout the winter season. For KIPyV the numbers were relatively small to draw definite conclusions about seasonality, but we did not observe KIPyV during the last months of the year.

There is some dispute about the pathogenic role of WUPyV and KIPyV in respiratory disease. Some studies suggest that there might be an association between WUPyV and KIPyV and respiratory symptoms [4-9], whereas others question the association between these viruses and disease [11-13]. Norja and colleagues [13] found no evidence for an association between infections with WUPyV and KIPyV and respiratory disease. This conclusion was based on the fact that

they found more WUPyV and KIPyV infections in the control group compared to the subjects with respiratory symptoms. Besides, the infections with WUPyV and or KIPyV were found in either immunosuppressed subjects or in immunocompetent subjects with a co-infection. There is some discrepancy between their results and the results of our study. Firstly, in our study we found several cases in which WUPyV and KIPyV were the only causative agents that could be found. Further, the majority of these cases were found in samples of symptomatic episodes. These findings are suggestive for a pathogenic role of both viruses. The difference in results between our study and the study of Norja and colleagues might be explained by differences in control samples and definition of asymptomatic episodes. In our study a period of 2 weeks around the moment of sampling was used to define an episode as symptomatic, while in the study of Norja a sample was defined as 'asymptomatic' if no symptoms existed at the single day of sampling.

Since latent infections with subsequent asymptomatic reactivation are described as a feature of the polyomaviruses BK and JC [17;18], we were interested in the longitudinal course of WUPyV and KIPyV infections. Re-infections and/ or persistence of WUPyV infections were seen in this study. In one child WUPyV was detected in 4 successive samples; this means that the virus was found for a period of 6 weeks. There were no reinfections with KIPyV, but persistence was seen in one child. Because most other studies have a cross-sectional design re-infections and persistence of WUPyV and KIPyV are usually missed. One study [8] reported that WUPyV was detected in sequential samples of 2 immunocompromised patients for a period of 6-8 weeks; one child was 16-months and the other was 4 years of age. In our study we show that persistence is also seen in immunocompetent individuals. However larger numbers are needed to investigate whether this observed persistence is a common feature of WUPyV and/ or KIPyV.

WUPyV and KIPyV infections were more often found in the youngest children of the study group (< 4 years). This is comparable to other studies were WUPyV and KIPyV infections were consistently more often found in the youngest children [4;6;7;13]. One explanation for this age-tendency could be that the underdeveloped respiratory and immunological system in young children can result in a different immune response and an increased susceptibility for viral infections [19]. Moreover, it is possible that older children have already developed immunity against WUPyV and KIPyV. In conclusion, our study shows that WUPyV and KIPyV frequently occur and recur in young children, and suggests that WUPyV and KIPyV might be associated with respiratory disease.

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Chapter 6

Respiratory pathogens in children with and without respiratory symptoms

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Abstract

Objectives

To investigate the occurrence of respiratory pathogens in samples from children with and without respiratory symptoms and to identify whether age and/ or coinfections modify the impact of respiratory pathogens on symptoms.

Study design

In a prospective longitudinal study, 18 children were sampled biweekly for respiratory pathogens, irrespective of respiratory symptoms. Polymerase chain reaction was performed for 13 respiratory pathogens. Episodes were defined "asymptomatic" if no symptoms of any respiratory tract illness were present between 1 week before and 1 week after sampling.

Results

A total of 230 samples were collected. In 56% of the symptomatic episodes, a pathogen was detected, compared with 40% of the asymptomatic episodes (P =.03). Rhinovirus and coronaviruses were most prevalent in both symptomatic and asymptomatic episodes. In the youngest children, 9% of the pathogen-positive episodes were asymptomatic, compared with 36% in the oldest children (P = .01). Multiple pathogens were found in 17% of the symptomatic episodes and in 3% of the asymptomatic episodes (P = .02).

Conclusions

Respiratory pathogens are frequently detected in samples from children with no respiratory symptoms. Symptomatic cases occurred more often in younger children and with detections of more than 1 respiratory pathogen.

Introduction

Respiratory tract infections occur frequently in early infancy and account for a major percentage of morbidity and mortality in childhood. Virus infections seem to be responsible for most of this burden. Since the introduction of molecular detection techniques, such as polymerase chain reaction (PCR), the percentage of pathogens found during respiratory tract illnesses in published studies has increased dramatically, up to 85%.[1-4]

Although many studies have investigated the prevalence of respiratory pathogens during respiratory illnesses, little is known about the prevalence of pathogens in non-symptomatic children. Whether pathogens are actually the cause of the respiratory symptoms or are simply colonizing the respiratory tract during symptomatic episodes remains unclear. It can be speculated that not every infection with a pathogen leads to respiratory symptoms and that pathogenicity might depend on host or environmental factors. In young children, the respiratory and immune systems are immature and may be more susceptible to respiratory pathogens.[5] We hypothesized that infections with respiratory pathogens are likely to have the most serious effect on young children with developing respiratory and immunologic systems. Furthermore, we hypothesized that infection with multiple respiratory pathogens will more often lead to respiratory symptoms compared with infection with a single pathogen.[6-9]

The aims of our study were to determine the prevalence of respiratory pathogens in the presence or absence of respiratory tract symptoms in prospectively sampled young children, and to identify whether age and coinfections modify the impact of a pathogen on illness. Sensitive PCR techniques were used to detect 13 common respiratory pathogens, 11 viruses, and 2 atypical bacteria.

Methods

Study Design and Subjects

A prospective longitudinal study was conducted during a 6-month winter season (November 2004 through April 2005). A total of 19 healthy children age 0 to 7 years were enrolled, 1 of which failed to complete the study. None of the children had a history of asthma or recurrent respiratory complaints. Parents were contacted twice a week by telephone or e-mail by 1 of the 2 study coordinators to determine the presence of any symptoms of respiratory tract illness. Respiratory tract symptoms were defined as symptoms of coryza (rhinorrhea or nasal congestion), sore throat, earache with or without ear discharge, cough, sputum production, or dyspnea, all with or without temperature above 38°C.

Samples were collected every 2 weeks regardless of the presence or absence of respiratory symptoms. The biweekly sampling frequency during the study resulted in about 13 subse-

quent observation episodes per child. An episode was defined as "asymptomatic" if there were no respiratory symptoms during a complete period of 1 week before to 1 week after sampling. An episode was defined as "symptomatic" if there were any respiratory symptoms during the period of 1 week before to 1 week after sampling.
The study design was approved by the local Medical Ethics Committee, and all parents gave written informed consent.

Detection of Respiratory Pathogens

Respiratory pathogens were detected by PCR. After receiving precise instruction at the beginning of the study, parents collected the samples by rubbing 1 of the child's nostrils and the posterior oropharynx using separate cottontipped swabs. After sampling, the 2 swabs were collected into a single vial containing GLY medium with 0.1 mg/mL of pimaricine as a viral transport medium and sent to our laboratory by regular mail. Samples were stored at -20°C until analysis. Sampling of respiratory pathogens by parents using nose and throat swabs has proven feasible and reliable. Both the sampling frequency and the viral recovery rate in parental samples are higher compared with sampling by a dedicated research nurse.[10,11]
PCR was performed at the National Institute of Public Health and the Environment, Bilthoven, The Netherlands. The respiratory pathogens human rhinovirus and enterovirus, human metapneumovirus, human coronaviruses OC43 and 229E, *Chlamydophila pneumoniae*, and *Mycoplasma pneumoniae* were analyzed by conventional PCR, essentially as described previously.[3]
The PCR for adenovirus consisted of 40 cycles of 1 minute at 94°C, 1.5 minute at 45°C, 1 minute at 72°C with a final extension of 10 minutes at 72°C (PE 9700) with the following primers: forward, 5′-GCCGCAGTGGTCTTACATGCACAT- 3′; reverse, 5′-ARCACICCICGRATGTCAAAG- 3′ and 5′-CAGCACGCCGCGGATGTCAAAGT- 3′ targeting the hexon gene. Amplicons were analyzed by gel electrophoresis.
Real-time PCR for human coronavirus NL63, influenza viruses A and B, and respiratory syncytial virus (RSV) A and B was performed using the Lightcycler 2.0 format with Lightcycler Taqman Mastermix (Roche, Germany). A separate reverse-transcription step with avian myeloblastosis virus reverse transcriptase was used for 60 min at 42°C for NL63 and for 60 minutes at 50°C for influenza and RSV.
The reaction for NL63 consisted of 1 cycle of 10 minutes at 95°C and 45 cycles of 5 seconds at 50°C and 10 seconds at 72°C, with primers 5′-AACCTAATAAGCCTCTTTCTC- 3′ and 5′-TTTGGCATCACCATTCTG- 3′ and probe 5′-6FAM-AGTGCTTTGGTCCTCGTG-Tamra-3′ targeting the nucleocapsid gene, as provided by L. van der Hoek.[12]
The reaction for influenza consisted of 1 cycle of 10 minutes at 95°C, and 45 cycles of 10 seconds at 95°C, 20 seconds at 50°C, and 10 seconds at 72°C, with primers 5′-AAGACCAATCCTGTCACCTCTGA- 3′ and 5′-CAAAGCGTCTACGCTGCAGTCC-3′ with probe 5′-6Fam-

TTTGTGTTCACGCTCACCGTGCC- BHQ1-3′ for influenza A, targeting the *M pneumoniae* gene, and 5′-TGAAGGACATTCAAAGC- 3′ and 5′-ACCAGTCTAATTGTCTC-3′ with probe 5′-YY-AGCACCGATTACACCAG-BHQ1-3′ for influenza B, targeting the NS gene. The reaction for RSV consisted of I cycle of 10 minutes at 95°C and 45 cycles of 15 seconds at 95°C and 47 seconds at 60°C with primers 5′-TGAACAACCCAAAAGCATCA- 3′ and 5′-CCTAGGCCAGCA GCATTG-3′ with probe 5′-6Fam-AATTTCCTCACTTCTCCAGTGTAGTATTAGG- BHQ1-3′ for RSV A and 5′-TGT-CAATATTATCTCCTGTACTACGTTGAA- 3′ and 5′-GATGGCTCTTAGCAAAGTCAAGTTAA- 3′ with probe 5′-YY-TGATACATTAAATAAGGATCAGCTGCTGTCATCCA- BHQ1-3′ for RSV B, both targeting the nucleocapsid gene.

Each sample was spiked with the equine arteritis virus as an internal control to detect inhibition of RNA isolation and PCR.[13]

Statistical Analysis

All statistical analyses were done using SPSS version 12.0 (SPSS Inc, Chicago, Illinois). A 2-tailed χ^2 test was used to compare differences between groups. Differences were considered statistically significant at a P value ≤ .05.

Results

During a period of 460 child-weeks, respiratory symptoms in were recorded in 18 children. There were 2 sibling of the study group are presented in Table 1.

A total of 230 biweekly samples from both symptomatic and asymptomatic episodes were tested for the presence of respiratory pathogens; 119 samples (52%) were positive for at least 1 respiratory pathogen. Table 2 gives the results of the PCR testing of samples obtained during symptomatic and asymptomatic episodes. Significantly greater numbers of pathogens were found in symptomatic episodes compared with the asymptomatic episodes. A pathogen was detected in 56% of the symptomatic episodes, compared with 40% of the

Table 1. Characteristics of the study group.

	N=18
Gender (male/female)	3/15
Age (yr)	3.67 (0-7)
0- 2 years	8
3-4 years	6
5-7 years	4
Number of siblings/ child	1.50 (0-4)
Number of symptomatic episodes/ child	9.50 (4-15)
Number of asymptomatic episodes/ child	3.00 (0-9)

Data are expressed as a median, with a range between brackets.

Table 2. Single or multiple respiratory pathogens detected in asymptomatic and symptomatic episodes in young children.

	Asymptomatic samples N=65	Symptomatic samples N=165	P value
Any pathogen	26 (40)	93 (56)	0.03
Human rhinovirus	14 (22)	38 (23)	0.88
Enterovirus	2 (3)	7 (4)	NA*
Coronaviruses (total)	5 (8)	15 (9)	0.38
HCoV OC43	1 (2)	6 (4)	
HCoV 229E	4 (6)	6 (4)	
HCoV NL63	0	3 (2)	
Respiratory syncytial virus B	0	1 (1)	NA
Influenza virus A	0	1 (1)	NA
Human metapneumovirus	0	0	NA
Adenovirus	0	1 (1)	NA
Chlamydophila pneumoniae	3 (5)	2 (1)	NA
Mycoplasma pneumoniae	0	0	NA
Multiple pathogens	2 (3)	28 (17)	0.02

*NA = not applicable.

Data are presented as numbers with percentages of the samples between brackets. P-value is calculated with a two-tailed chi-square.

asymptomatic episodes (P = .03). Rhinovirus was the most prevalent virus, found in 23% of the symptomatic episodes and in 22% of the asymptomatic episodes; this difference was not significant (P = .88). The second most prevalent pathogens were the coronaviruses, which also were detected in comparable numbers in symptomatic and asymptomatic episodes (P = .38).

Table 3. Combination of respiratory pathogens found in samples with multiple pathogens detections.

	Asymptomatic episodes N=2	Symptomatic episodes N=28
HRV + CP	1	3
HRV + HCoV OC43	1	3
HRV + MP		4
HRV + HCoV 229E		5
HRV +IVB		1
HRV + RSVB		1
HRV + CP + MP		1
HRV + CP + RSVB		1
HRV + MP + HCoV OC43		1
HCoV 229E + HCoV OC43		1
HCoV 229E + HCoV NL63		1
HCoV 229E + CP		1
HCoV 229E + hMPV + EV		1
HCoV 229E + HCoV OC43 + hMPV		1
HCoV 229E + HCoV OC43 + EV		1
HCoV OC43 + RSVA		1
HCoV OC43 + RSVB		1

Abbreviations: HRV human rhinovirus, CP Chlamydophila pneumoniae, HCoV human coronavirus, MP Mycoplasma pneumoniae, IVB influenzavirus B, RSVA/ B respiratory syncytial virus A or B, hMPV human metapneumovirus, EV enterovirus.

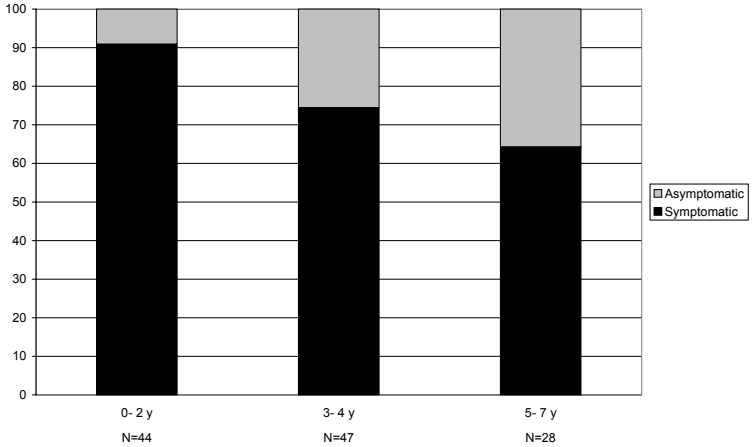

Figure 1. A selection of pathogen- positive episodes. The proportion of symptomatic and asymptomatic episodes was studied in 3 different age categories.

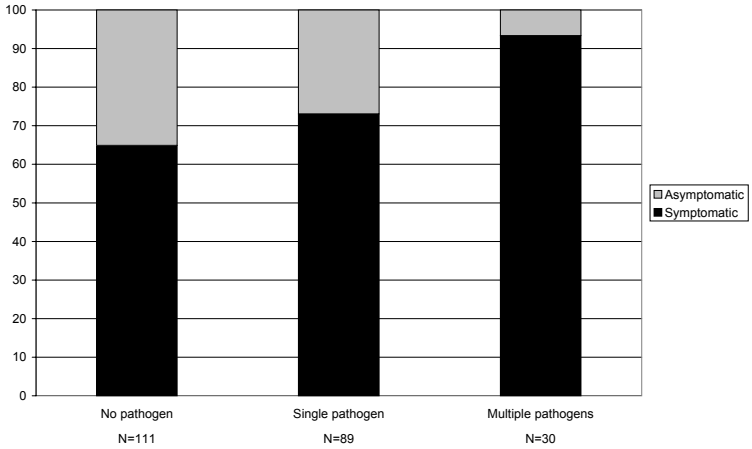

Figure 2. Percentages of the symptomatic and asymptomatic samples related to the number of pathogens detected.

Human metapneumovirus and *M pneumoniae* were never detected as a monoinfection, but only as a coinfection with another pathogen (Table 3).

We analyzed the influence of age on the occurrence of symptoms during an episode. For this reason, we selected the episodes in which at least 1 pathogen was detected and divided them into 3 age categories. Figure 1 shows the distribution of symptomatic and asymptomatic episodes in the different age categories. With increasing age, pathogen-positive children are increasingly asymptomatic. Only 9% of the pathogen-positive episodes were asymptomatic in children age 0 to 2 years, compared with 26% in those age 3 to 4 years and 36% in those age 5 to 7 years ($P = .04$ and .01, respectively; χ^2 test). Multiple pathogens were seen in 17% of the symptomatic episodes, compared with 3% of the asymptomatic episodes ($P = .01$). Figure 2 shows the distribution of symptomatic and asymptomatic episodes when

no pathogen, a single pathogen, or multiple pathogens were detected. If no pathogen was found, then 35% of the episodes were asymptomatic, compared with 7% of the episodes with multiple pathogens ($P < .01$).

Discussion

Our data indicate that respiratory pathogens are frequently found in samples from children with no respiratory symptoms (~40%). Rhinovirus and coronaviruses were found in most of the symptomatic cases and asymptomatic cases. Symptomatic cases were more often associated with detection of more than 1 respiratory pathogen.

Two limitations of this study are the small sample size and the fact that we sampled only during the winter season. These aspects limit the generalizability of our results. Nevertheless, the longitudinal sampling of healthy children at home and the use of a broad panel of respiratory pathogens for detection gives a detailed picture of the prevalence of respiratory pathogens during a winter season in young children.

The most prevalent virus in our study was rhinovirus, found in both symptomatic and asymptomatic episodes. The fact that rhinovirus is often found in asymptomatic children is not surprising, because it is generally a relatively mild pathogen that can colonize the nasal mucosa without causing symptoms.[4] On the other hand, recent studies attribute a more important role to rhinovirus in both upper and lower respiratory tract infections.[2,14] Presumably host and environmental determinants play roles in the pathogenicity of this virus.

Coronaviruses were the second most prevalent single virus in both asymptomatic and symptomatic children. This is in keeping with studies in which coronaviruses accounted for approximately 1/3 of common colds in children.[15] Coronaviruses often were found in multiple infections,[15-17] which suggest a relatively mild pathogenicity of coronaviruses. The recovery of both RSV and influenza virus was remarkably low in our study, possibly due to the small number and the varying ages (0 to 7 years) of the children enrolled in this study.

Of the asymptomatic children, 40% carried 1 or more pathogens. In the literature, the prevalence of respiratory pathogens in samples from asymptomatic children ranges from 5% to 68%.[3,18-21] This wide range may be explained by differences in study populations, definitions of symptoms, and sampling and virus detection methods. Most of these studies were performed in older children, usually hospitalized for elective surgery. Furthermore, most of the studies are of a cross-sectional design comparing single symptomatic and asymptomatic episodes in different subjects. Such a design disregards the natural variation of virus colonization in an individual during a certain period. Winther et al[20] also sampled longitudinally and found a 9% prevalence of picornavirus in asymptomatic children. This discrepancy with our findings may be explained by the fact that those authors used a period of 4 weeks around the onset of respiratory illness to define symptomatic episodes.

In our study, the youngest children were more often symptomatic when a pathogen was detected compared with older children. This finding is in agreement with the results of a recently published Finnish study on the transmission of rhinovirus within families.[11] In that study, most rhinovirus infections in young children were symptomatic, and nearly half of the infections in older children and adults were asymptomatic. Presumably, older children have developed immunity against most respiratory pathogens.

We found a greater prevalence of multiple pathogens in samples from symptomatic children compared with samples from asymptomatic children. There is some debate regarding the association between infections with multiple pathogens and disease severity. Some have argued that multiple pathogens cause more severe disease,[6-9] whereas others have reported no difference between single and multiple infections in terms of disease severity.[8,22,23] Our findings support the former assertion and indicate an association between multiple infections and illness severity. During our 6-month study period, the children had a median of 9.5 (range, 4 to 15) symptomatic episodes, supporting the fact that apparently normal children can appear "chronically or repeatedly" infected during the respiratory season.

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Chapter 7

The influence of neonatal lung function on rhinovirus associated wheeze

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Abstract

Rationale

Several studies have shown that the occurrence of wheezing illnesses during the first year of life is associated with lower levels of lung function shortly after birth and prior to any respiratory illness. It has been suggested that reduced lung function early in life predisposes infants to wheezing during viral respiratory infections, but the association between neonatal lung function and subsequent confirmed viral infections has never been investigated.

Objectives

To study the influence between neonatal lung function and the occurrence of human rhinovirus (HRV) associated wheeze.

Methods

In a prospective birth cohort study, infants were followed from birth through the first year of life with daily questionnaires about respiratory symptoms. Neonatal lung function was performed within the first 2 months of life. Nose and throat swabs were collected during episodes with respiratory symptoms. Polymerase chain reaction was used to detect single HRV-infections.

Measurements and main results

In 176 of the 202 infants (87%) with a single-HRV infection, valid lung function measurements were obtained. The risk of wheeze was 1.49 times higher for each standard deviation increase of airway resistance. The adjusted risk (corrected for possible important confounders) for wheeze was 1.77 (95% CI 1.16-2.69, p=0.01) times higher for each standard deviation increase of airway resistance.

Conclusions

This study showed that total lung resistance is clearly associated with HRV-associated wheeze. Moreover, HRV-associated wheeze might be the first sign to recognize infants with reduced neonatal lung function.

Introduction

Respiratory virus infections are the most common trigger of wheezing illnesses in young childhood. Human rhinovirus (HRV) and respiratory syncytial virus (RSV) are reported as the most frequent causing agents. Since the development of molecular assays for the detection of HRVs, the detection rate of HRV in patients with respiratory infections has increased to up to 50% [1]. Last decade HRVs have raised increasing interest as they seem to be responsible for a wide range of respiratory illnesses. HRVs are frequently found in asymptomatic children and adults [2;3], but are also detected in patients with symptoms ranging from mild common colds [4] to serious lower respiratory tract disease(5). More recently there are several studies that suggest that symptomatic HRV infections might play an important role in recurrent wheezing later on in life [6] or asthma [7-9].

However, the mechanisms which underlie the inter-individual variability of HRV associated respiratory morbidity are unclear. HRV is also found in up to 65% of the asthma exacerbations in children and adults [10], suggesting that underlying disease might be an important determinant of HRV associated symptoms. Several studies have shown that lower levels of lung function shortly after birth and prior to any respiratory illness are associated with the occurrence of wheezing illnesses during the first years of life [11-13]. Previous studies have mainly focussed on infants with RSV infections and recurrent wheezing later on in life. From these studies it is recognized that infants with a proven RSV infection have a diminished lung function after this infection compared to aged matched peers [14]. To study possible relationships between congenital lung function, virus infections and respiratory diseases, longitudinal birth cohort studies in unselected children are necessary and sensitive detection tools for viral pathogens have to be used. Until now, the association between neonatal lung function and subsequent confirmed HRV infections has never been studied prospectively.

In this study we hypothesised that diminished neonatal lung function might be related to a higher occurrence of HRV-associated wheeze. We conducted a community-based birth cohort study to investigate the role of neonatal lung function on the occurrence of PCR proven HRV-associated wheeze during the first year of life. Some of the results on the prevalence of different viruses in this cohort have been previously reported [15].

Methods

Study design and subjects

All infants were participants of the WHeezing Illnesses STudy LEidsche Rijn (WHISTLER), a prospective population-based birth cohort study on determinants of wheezing illnesses (including early life lung function) [16].

Briefly, healthy infants were enrolled in this study at the age of two to three weeks, before any respiratory symptoms have occurred and followed until they reached 1 year of age. Exclusion criteria were gestational age < 36 weeks, major congenital abnormalities and neonatal respiratory disease. At enrolment a questionnaire filled in by the mother was used to gather information on gestational age, birth length and weight and exposure to tobacco smoke. Maternal smoking during pregnancy was considered present if the mother smoked at least one cigarette per day. Data on maternal asthma (questionnaire) was obtained from the linked database of the Utrecht Health Project (Dutch acronym LRGP: Leidsche Rijn Gezondheids Project), a large health monitoring study in Leidsche Rijn, which aims to generate valuable data from all inhabitants on determinants of health and disease [17].
The study was approved by the local medical ethics committee (University Medical Center, Utrecht) and all parents gave written informed consent.

Lung Function test

Lung function was measured before the age of two months during natural sleep and without the use of any sedation. Lung function was assessed from measurement of passive respiratory mechanics (resistance (R_{rs}), compliance (C_{rs}) and time constant (τ_{rs}) of the total respiratory system) using the single breath occlusion technique (SOT). Further details about the lung function measurement were previously reported [18].

Outcome variable: wheeze during a HRV infection

Follow up for respiratory symptoms like wheeze during the first year of life was achieved by a prospectively scored questionnaire filled in by the parents on a daily basis. Parents were instructed by research physicians on how to recognize symptoms of cough and wheeze at the start of the study. In order to relate wheezing symptoms to virus positivity parents were asked to collect respiratory virus samples on the second day of each episode with wheeze and/or cough. For the present study we selected all infants with the first single HRV-PCR positive episode without the detection of any other pathogen. The primary outcome measures were 1) did the patient wheeze (yes or no) during the episode and 2) how long (number of days) did the patient wheeze during the HRV positive episode?

Viral sampling and analysis

Parents were asked to take nose and throat swabs at the second day of a reported episode with respiratory symptoms. Viral samples were collected by the parents with a cotton-tipped swab from both the nose and posterior oropharynx. Both swabs were collected into a single vial containing GLY medium containing 0.1 mg/ml pimaricine as viral transport medium and

sent to our laboratory via regular mail. Samples were stored at –20°C until analysis. Sampling of respiratory pathogens by the parents using nose and throat swabs has been shown to be feasible and reliable. Both the sampling frequency and the viral recovery rate in parental samples are higher compared to sampling by a dedicated research nurse [19-21].
Viral RNA was isolated from 200µl of the original sample using the High pure RNA isolation kit (Roche, Germany). cDNA synthesis, nested polymerase chain reaction (PCR) and Southern blotting were carried out to detect HRV [3]. To rule out other respiratory pathogens as a possible cause for respiratory symptoms, an additional broad panel of PCR's was used, containing enterovirus, respiratory syncytial virus (RSV) A and B, coronaviruses OC43, 229E and NL63, influenza viruses A and B, human metapneumovirus, adenovirus, *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* [3;22].

Statistical analysis

Logistic regression analysis was used to investigate the effect of neonatal lung function on HRV-associated wheeze. Outcome was defined as wheeze yes or no during a HRV positive episode. We decided to maximize the duration of the reported respiratory episodes to a maximum of 30 days based on studies in literature [23] and our own anticipation of 30 days being the maximum period for a virus to cause symptoms. Lung function was assessed using the parameters C_{RS} and R_{RS}. First, we used univariate analysis to investigate the effect of C_{RS} and R_{RS} separately. Then, multivariate analysis was used to adjust the effect for possible confounders influencing both wheezing complaints (outcome) as well as lung function (determinant). Results are presented as odds ratio (OR) with a 95% confidence interval (CI) and P values. Poisson regression analysis was used to investigate the role of lung function on the duration of HRV-associated wheeze. Outcome was defined as number of days with wheeze during a HRV positive episode. Results are presented as incidence rate ratios (IRR) with a 95% confidence interval (CI) and P values. Statistical analysis was performed using SPSS Inc., 2001, Chicago USA, version 15.0.

Results

Three hundred and five infants were prospectively followed for respiratory symptoms during the first year of life. Of these infants 254 infants had a HRV positive infection. Fifty-two of them were infections with multiple pathogens, leaving 202 infants with a single-HRV infection. In 176 of the 202 infants (87%) with a single-HRV infection, valid lung function measurements were obtained (Figure 1). Failure of a valid lung function was mainly due to failure to fall asleep naturally during the scheduled time for inclusion (1.5 hours). In total 92 (45.5%) infants wheezed during HRV infection. Other characteristics of the infants are presented in Table 1,

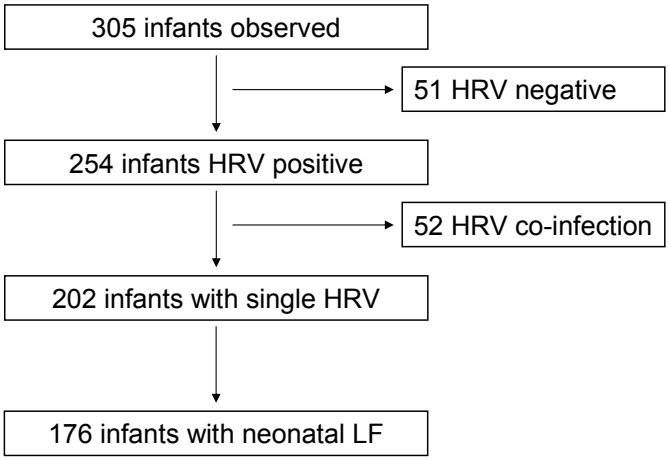

Figure 1. Flow chart of the infants included in the study. Finally, 176 infants were included with a single HRV infection and a succesful lung function measurement.

Table 1. General characteristics of the study population.

	Total study group (n=1105)	Single HRV infections (n=202)		
	All infants	All infants	Wheeze during HRV (n=92)	No wheeze during HRV (n=110)
Wheezing episodes in first year (yes)	597/869* (68.7%)	161 (79.7%)	NA	NA
Gender (male)	548 (49.6%)	100 (49.5%)	49 (53.3%)	51 (46.4%)
Age at lung function test (wks)	7.3 (3.9)	7.2 (2.0)	7.3 (2.0)	7.2 (2.1)
Age at infection (weeks)	NA	38.4 (19.3)	37.0 (18.9)	41.4 (19.6)
Birth length (cm)	50.9 (2.3)	51.2 (2.2)	51.1(2.3)	51.4 (2.0)
C_{RS} (mL/kPa)	43.6 (10.9)	44.3 (9.4)	44.0 (9.0)	44.6 (9.8)
R_{RS} (kPa/L/s)	7.3 (2.3)	7.2 (2.4)	7.7 (2.3)	6.8 (2.4)
Maternal smoking in pregnancy (y)	244 (22.1%)	23 (11.4%)	16 (17.4%)	7 (6.4%)
Maternal asthma (y)	71/861 (8.2%)	15/174 (8.6%)	10/73(10.9%)	5/101 (4.5%)

Data are expressed as a mean (SD) and number (%).

Table 2. Respiratory viruses identified in episodes with respiratory symptoms.

	Total samples n=668	Single pathogens n=468
Any virus-positive	566 (84.7)	-
Human rhinovirus	485 (72.6)	399 (85.3)
Enterovirus	6 (0.9)	4 (0.9)
Coronaviruses	51 (7.6)	18 (3.8)
Respiratory syncytial virus	70 (10.5)	31 (6.6)
Influenzavirus	18 (2.7)	8 (1.7)
Human metapneumovirus	11 (1.6)	6 (1.3)
Adenovirus	5 (0.7)	0
Mycoplasma pneumoniae	16 (2.4)	1 (0.2)
Chlamydophila pneumoniae	11 (1.6)	1 (0.2)
Multiple viruses	98 (14.7)	-

Data are expressed as a number with a percentage between brackets.

Table 3. Relative numbers of samples of infants with wheezing symptoms during infections with different single respiratory viruses

Pathogen	Sampled periods with wheeze/ samples with single virus
Human rhinovirus	220/ 399
Enterovirus	2/4
Coronaviruses	8/18
Respiratory syncytial virus	17/31
Influenzavirus	3/8
hMPV	5/6
Adenovirus	-
Mycoplasma pneumoniae	1/1
Chlamydophila pneumoniae	0/1

Data are expressed as total number of samples during wheezing episodes divided by total number of samples with single pathogen detection.

including the characteristics of the whole study population. There was not much difference between the total study group and the group of infants with a single HRV infection. Table 2 shows the virus PCR results of the samples in the whole study population (n=668 samples). Table 3 shows the relative numbers of patients with wheezing symptoms during infections with different respiratory virus

In the univariate regression analysis the risk of HRV-associated wheeze was increased in infants with higher values of R_{rs}. The risk of wheeze was 1.49 times higher for each standard deviation increase in airway resistance (Table 4). The risk of wheezing during HRV was 32% in those with low airway resistance (R_{rs}< -1 SDS) compared to 67% in those with high airway resistance (R_{rs}> +1 SDS) (Figure 2). In the univariate analysis HRV-associated wheeze was also increased in boys (OR 1.32, 95% Confidence Interval 0.76-2.30, p-value=0.33), in infants

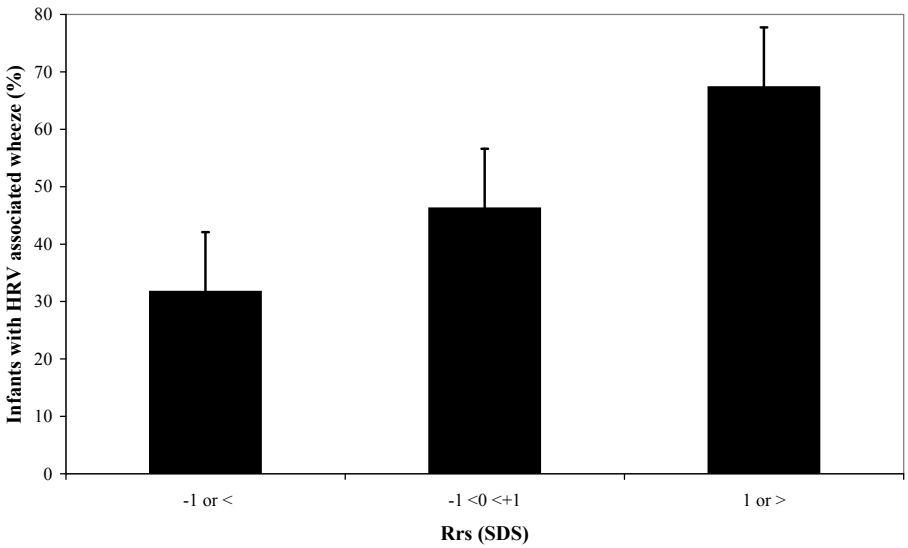

Figure 2. Mean percentage (with standard error) of wheezing infants during a Human Rhinovirus infection according to airway resistance (R_{rs}). Groups of infants are divided according to airway resistance measured shortly after birth (Infants with airway resistance less than −1 SD, between −1 and +1 SD and infants with an airway resistance of more than +1 SD).

Table 4. Association between neonatal lung function and wheezing (yes/no) in children afflicted with proven Human Rhinovirus infections.

	Univariate analysis				Multivariate analysis *		
	OR	95% CI	p value		OR	95% CI	p value
C_{RS} (SDS)	0.94	0.70- 1.27	0.69	C_{RS} (SDS)	0.91	0.70- 1.19	0.49
R_{RS} (SDS)	1.49	1.08- 2.04	0.02	R_{RS} (SDS)	1.77	1.16- 2.69	0.01

* In multivariate analysis C_{rs} and R_{rs} are adjusted for gender, maternal smoking during pregnancy, maternal history of asthma and birth length and weight. Odds Ratios (OR) indicate the risk associated with one standard deviation (SDS) increase in lung function characteristic.

of mothers who smoked during pregnancy (OR 3.10, CI 1.22-7.90, p=0.02) and in infants of mothers who reported to have asthma (OR 3.05, CI 1.00-9.34, p=0.05).

In multivariate analysis we adjusted the risk of HRV-associated wheeze for gender, maternal smoking during pregnancy, maternal history of asthma and birth length and weight. After adjusting, the observed association between R_{rs} and wheeze did not substantially change. The adjusted risk for wheeze was 1.77 (CI 1.16-2.69, p=0.01) times higher for each standard deviation increase of R_{rs} (Table 4). Airway compliance was not associated with wheeze.

Further we investigated whether the duration of wheezing episodes was related to lung function. Each standard deviation increase of R_{rs} was associated with 24% more days of wheeze during a HRV infection ($p<0.01$).

Discussion

This study shows that increased airway resistance measured before the age of 2 months is associated with an increased risk on the occurrence and duration of HRV-associated wheeze during the first year of life. The association we found was not influenced by gender, maternal smoking during pregnancy or maternal history of asthma. To our knowledge this is the first study to investigate the effect of neonatal lung function on wheeze during confirmed HRV infections.

When interpreting the results of our study some aspects need to be considered. The fact that parents had to recognize a respiratory episode and to decide to take a respiratory sample could have led to collection of the more severe respiratory tract infections. On the other hand, the clinical relevance of respiratory episodes parents that did not sample is probably marginal. Another consideration should be made about our lung function technique. A major strength of this study is that we carefully determined the effect of neonatal lung function on wheezing illness in a population-based group of infants with a confirmed HRV infection. Several studies have shown that reduced lung function early in life is associated with wheezing illnesses early in life [12;13;24-26]. However, none of these studies investigated the association between neonatal lung function and subsequent confirmed viral respiratory tract infections. Airway resistance was associated with HRV-associated wheeze, however for airway compliance this was not the case. Since the endpoint of our study is wheeze, which is a sign of lower

airway obstruction, we suggest that this is best measured by airway resistance. Compliance tells us more about the other parts of the respiratory system like chest-wall characteristics and is therefore less associated with wheezing.

Reduced lung function and wheezing in infancy have been associated with gender, maternal smoking during pregnancy and maternal history of asthma. Several studies have shown that lung function is significantly lower in boys compared to girls during infancy [27;28], others state that boys have larger lung values but smaller airways for given lung size [29]. In addition, studies report that boys have an increased incidence of respiratory illnesses in comparison to female infants [30]. Maternal smoking during pregnancy is known to influence lung growth and therefore cause impaired lung function early in life and increases wheezing in infancy [31-33]. Finally, infants with a maternal history of asthma had significantly impaired lung function and are more likely to develop subsequent wheezing [25;31]. In this study we corrected our analysis of the relationship between lung function and HRV-associated wheeze for these factors. The association between early life lung function and the occurrence of wheeze was independent of gender, maternal smoking during pregnancy and maternal history of asthma. Maybe other genetic or environmental factors influence lung growth and development during pregnancy and early life.

HRVs are increasingly recognized as an important respiratory pathogen. Previous studies have shown that HRV infections in infancy are linked with recurrent wheezing and asthma [6-9]. However, all most all infants are infected with a HRV during the first year of life [15]. A few mechanisms are suggested for the development of recurrent wheezing in infancy, one of these suggest a role for genetic predisposition through lung function. Our study shows that HRV is associated with wheeze in infants with congenital small airways. This indicates that reduced lung function is an important determinant in HRV-associated wheeze in infancy. In addition, low lung function at birth is shown to be a major risk factor for low lung function in later life [34;35]. The Tucson birth cohort study followed the lung function of infants shortly after birth to the age of 22 years. Infants with a low lung function early in life remained in the group with lower lung function in adulthood [34]. In the Melbourne asthma study children were followed from the age of 7 till adult life with lung function measurements and questionnaires [36]. In this study, it was shown that children with frequent or persistent wheeze in childhood continue to have abnormal lung function into mid-adult life. These studies indicate that individuals with reduced lung function at birth will be more likely to remain in the lower end of the lung function distribution until adulthood. It might be speculated that these lower levels of lung function in early childhood lead to increased respiratory morbidity during adulthood. In a recent Norwegian birth cohort reduced lung function shortly after birth was associated with an increased risk of asthma at 10 years of age [7]. In our study we show that both the prevalence and severity of virus associated symptoms are importantly influenced by congenital lung function. Whether such an increased risk persists during later childhood and adulthood remain to be studied.

In conclusion, this study showed that total lung resistance is clearly associated with HRV-associated wheeze. Premorbid lung function is an important determinant for respiratory disease in later life. Moreover, HRV-associated wheeze might be the first sign to recognize infants with congenital smaller airways.

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Chapter 8

The association between respiratory symptoms and the presence of respiratory pathogens in infants during the first year of life

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Abstract

Background:

Respiratory virus infections are the most important trigger of respiratory illnesses in childhood. Data on the occurrence and the clinical impact of respiratory pathogens in the general population of infants are scarce. Therefore, we described the occurrence and clinical impact of respiratory pathogens in infants with respiratory tract infections during the first year of life. In addition, we wanted to identify which host, pathogen and environment factors modify the impact of pathogens on respiratory illness. Finally, we studied the phenomenon of post-viral symptoms after RSV and non-RSV infections.

Methods:

In a prospective birth cohort study, infants were followed from birth through the first year of life with daily questionnaires about respiratory symptoms. Nose and throat swabs were collected during episodes with and without respiratory symptoms. Polymerase chain reaction was used to detect an extensive panel of respiratory pathogens.

Results:

One hundred and forty-four infants completed the study and had a mean documented observed period of 10.3 months. In total 1425 samples were collected, 245 symptomatic and 1180 asymptomatic samples. In 85% of the symptomatic cases and 51.1% of the asymptomatic cases a pathogen was detected (p=<0.01). HRV was the most prevalent pathogen. RSV and PIV were significantly more often found in symptomatic episodes compared to asymptomatic episodes. In multivariate airway resistance and the presence of single or multiple pathogens were significantly associated with respiratory symptoms. During the 3-months period after proven RSV-infection children had a mean of 10.9 days of cough and 4.7 days of wheeze. This was not significantly different from the symptoms after random non-RSV infections (cough 9.2 days, p = 0.83; wheeze 3.1 days, p= 0.46)

Conclusions:

We found that respiratory viruses play a huge role during the first year of life especially during symptomatic periods. Important factors that influence the presence of respiratory symptoms include airway resistance and the number of pathogens detected. RSV and PIV infections were frequently associated with respiratory symptoms. Finally, we could not confirm the exclusive phenomenon of post-RSV infection wheeze.

Introduction

Respiratory tract infections occur frequently during early infancy and account for a major part of morbidity and mortality in childhood. Virus infections seem to be responsible for the majority of this burden. In up to 85% of children respiratory pathogens can be detected during respiratory tract illnesses[1-4].

Although many studies have investigated the prevalence of pathogens during respiratory illnesses in early life, little is known about the prevalence of pathogens in asymptomatic children. Furthermore, little is known which factors determine whether respiratory symptoms arise during a virus infection or not. It has been speculated that host, pathogen and environmental factors can be associated with respiratory symptoms.

A few host factors have been suggested as important in the pathogenicity of viruses such as age, prematurity, small lung size and genetic predisposition [5,6]. For example, premature delivery has been associated with diminished lung function[7]. However, there is some debate about whether prematurity alone is a determinant of respiratory illness or an intermediate for lower levels of lung function.

Suggested pathogen factors more often associated with disease are number of simultaneous viruses during an infection, although reports are not conclusive on this issue[8-12]. Different viruses might raise different symptoms. For a long time, it has been suggested that some respiratory viruses cause more severe respiratory illness than others. Respiratory syncytial virus (RSV) is most often mentioned to cause severe respiratory tract illness in young children, and can result in prolonged post viral wheezing[13,14]. On the other hand, a recent study found no differences in disease severity between children hospitalized for RSV-positive and RSV-negative respiratory tract infections[15-18]. Data on the differential effects of viruses can be biased, because they all exclusively emerged from clinical studies. Systematic follow up of respiratory symptoms after proven RSV and non-RSV infections was never performed in non-hospitalized infants. Such studies require comprehensive viral surveillance in combination with close prospective monitoring of respiratory symptoms in unselected children. Environmental factors which are suggested to be associated with increased disease include daycare attendance, siblings and smoking during pregnancy. A recent study showed that maternal smoking during pregnancy was represented by changes in airway obstruction parameters, which appeared especially in the group of youngest children[19].

The aim of our study was to assess the association between PCR proven respiratory pathogens and respiratory tract symptoms in infants in an unselected population. In addition, we wanted to identify which host, pathogen and environment factors modify the impact of pathogens on respiratory illness. Finally, we studied the phenomenon of post-viral symptoms after RSV and non-RSV infections.

Methods

Study design and subjects

All infants were participants of the WHeezing Illnesses STudy LEidsche Rijn (WHISTLER), a prospective population-based birth cohort study on determinants of wheezing illnesses (including early life lung function)[20,21].

Briefly, healthy infants were enrolled in this study at the age of two to three weeks, before any respiratory symptoms have occurred and followed until they reached 1 year of age. Exclusion criteria were gestational age < 36 weeks, major congenital abnormalities and neonatal respiratory disease. At enrolment a questionnaire filled in by the mother was used to gather information on gestational age, birth length and weight and exposure to tobacco smoke. Maternal smoking during pregnancy was considered present if the mother smoked at least one cigarette per day. Data on maternal asthma (questionnaire) was obtained from the linked database of the Utrecht Health Project (Dutch acronym LRGP: Leidsche Rijn Gezondheids Project), a large health monitoring study in Leidsche Rijn, which aims to generate valuable data from all inhabitants on determinants of health and disease. Lung function was measured before the age of two months during natural sleep and without the use of any sedation. Lung function was assessed from measurement of passive respiratory mechanics (resistance (R_{rs}) and compliance (C_{rs}) of the total respiratory system) using the single breath occlusion technique (SOT). The study was approved by the local medical ethics committee (University Medical Center, Utrecht) and all parents gave written informed consent.

Viral sampling and analysis

Nose and throat swabs for virus analysis were collected at the start of every month regardless of respiratory symptoms. Samples were taken by the parents with a cotton-tipped swab from both the nose and posterior oropharynx. Both swabs were collected into a single vial containing GLY medium containing 0.1 mg/ml pimaricine as viral transport medium and sent to our laboratory via regular mail. Samples were stored at −20˚C until analysis. Sampling of respiratory pathogens by the parents using nose and throat swabs has been shown to be feasible and reliable[22].

The respiratory pathogens human rhinovirus (HRV) and enterovirus, human metapneumovirus (hMPV), human coronaviruses OC43 and 229E and *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* were analyzed as described[23].

The PCR for adenovirus detection was performed by conventional PCR (PE 9700) and analyzed by gel electrophoresis[23]. The real-time PCR for human coronavirus NL63, influenzavirus A and B, RSV A end B was performed using the Lightcycler 2.0 format with Lightcycler ®Taqman Mastermix (Roche, Germany).All samples were retrospectively tested for human bocavirus

(HboV) and the polyomaviruses WU (WUPyV) and KI (KIPyV), also by using real-time PCR as previously described[24,25]. Amplification was carried out in a 25-µL reaction mixture on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Positive controls for the KIPyV and WUPyV PCR were provided by S. Bialasiewicz and T.P. Sloots, University of Queensland, Queensland, Australia, and the positive control for HBoV was provided by T. Allander, Karolinska Institute, Stockholm, Sweden.

Respiratory symptoms

Respiratory symptoms during the first year of life were achieved by a prospectively scored questionnaire filled in by the parents on a daily basis. The respiratory symptoms considered were: cough, wheeze (a whistling noise coming from the chest and not the nose), with or without fever (temperature above 38˚ Celsius). Parents were instructed by research physicians on how to recognize symptoms of cough and wheeze at the start of the study. Afterwards these questionnaires were correlated with the samples to determine whether samples were collected during symptomatic or asymptomatic episodes. Samples were considered symptomatic whenever respiratory symptoms were present for more than two days in the sampling period.

Statistical analysis

Multilevel analysis was used to investigate the effect of several relevant determinants on the presence of respiratory symptoms. Random effect logistic modelling was used. Outcome was defined as respiratory symptoms for more than two days yes or no during a virus-sampled episode.

First, we used univariate analysis to investigate the influence of the determinants separately. Then, multivariate analysis was used to assess the independence of the associated factors. Determinants with a confidence interval without 1 were considered to be significant. Results are presented as odds ratio (OR) with a 95% confidence interval (CI). Statistical analysis was performed using MlwiN version 2.10.

Finally, for the analysis of post-infection symptoms all single RSV infections were studied for respiratory symptoms reported 3 months after the proven infection. Respiratory symptoms considered were cough and wheeze. As a control group a random group of infants with a single viral infection other than RSV was taken.

Results

One hundred and sixty-six infants participated in the study. Twenty-two infants were lost to follow-up after the first inclusion visit (9 failure of successful lung function and 13 because parents found the study too burdensome). One hundred and forty-four infants completed the study and had a mean documented observed period of 10.3 months (86% follow up; range 1-12 months). These infants reported a mean of 4.7 episodes of respiratory illness per infant/ year (Range 0-18). (Table 1) In total 1425 samples were collected, 245 symptomatic and 1180 asymptomatic samples.

Table 1. Baseline characteristics study population.

	N= 144
Gender (Male)	70 (48.6)
Number of episodes per child/ year	4.7 (0-18)
Age at first sample (months)	1.1 (0.7- 13.0)
Number of samples per child	8.6 (1-19)
Lung function	
Crs (mL/kPa)	47.2 (26.9- 80.1)
Rrs (kPa/L/s)	6.2 (2.2-13.1)
Maternal history of asthma*	116 (92.8)
Siblings (yes)*	81 (57.0)
Pet exposure (yes)	86 (59.7)
Day care visit first 6 months (yes)*	53 (39.8)
Smoking during pregnancy (yes)	5 (3.5)

Data are presented as number with percentages between brackets or as a median with a range between brackets.
* Corrected for missing data.

Table 2a shows the virus PCR results of the samples obtained during symptomatic and asymptomatic episodes. A substantial greater proportion of pathogens was found in symptomatic (85.3%) compared to asymptomatic episodes (51.1%, $p< 0.01$). HRV was the most prevalent pathogen, both in symptomatic episodes (62.4%) and in asymptomatic episodes (36.2%, $p=0.54$). RSV and PIV were significantly more often found in symptomatic episodes compared to asymptomatic episodes (Table 2a). *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* were exclusively found in asymptomatic episodes. Multiple pathogens were significantly more often detected in symptomatic (33.5%) compared to asymptomatic episodes (14.7%, $p<0.01$). The frequency distribution of viruses during symptomatic and asymptomatic periods was not materially different if we exclusively studied single virus infection (Table 2b). The seasonal distribution of the pathogens of both symptomatic as asymptomatic episodes is shown in Figure 1a and 1b. HRV infections occurred throughout the year. RSV infections occurred mainly during the winter months (November till January).

To investigate the influence of infant-, pathogen - and environment related determinants on the occurrence of respiratory symptoms logistic regression was used. In univariate analysis age, airway resistance and the presence of single or multiple pathogens were associated with

Table 2a. Respiratory viruses identified in episodes with and without respiratory symptoms.

	Symptomatic specimens N= 245	Asymptomatic specimens N= 1180	P value
Any virus-positive	209 (85.3)	603 (51.1)	<0.01
Human rhinovirus	153 (62.4)	427 (36.2)	0.54
Enterovirus	3 (1.2)	13 (1.1)	0.77
Coronaviruses	9 (3.7)	25 (2.1)	1.00
Respiratory syncytial virus	26 (10.6)	22 (1.9)	<0.01
Influenzavirus	3 (1.2)	9 (0.8)	1.00
Para-influenzavirus	15 (6.1)	7 (0.6)	<0.01
Human metapneumovirus	6 (2.4)	6 (0.5)	0.09
Adenovirus	18 (7.3)	39 (3.3)	0.35
Bocavirus	36 (14.7)	90 (7.6)	0.44
Wu polyomavirus	30 (12.2)	107 (9.1)	0.28
Ki polyomavirus	19 (7.8)	74 (6.3)	0.26
Mycoplasma pneumoniae	0	5 (0.4)	-
Chlamydophila pneumoniae	0	3 (0.3)	-
Multiple viruses	82 (33.5)	174 (14.7)	<0.01

Table 2b. Single respiratory viruses identified in episodes with and without respiratory symptoms.

	All specimens N= 556	Symptomatic specimens N= 127	Asymptomatic specimens N= 429	P-value
Human rhinovirus	376 (67.6)	85 (66.9)	291 (67.8)	0.91
Enterovirus	12 (2.2)	2 (1.6)	10 (2.3)	1.00
Coronaviruses	19 (3.4)	4 (3.1)	15 (3.5)	1.00
Respiratory syncytial virus	15 (2.7)	11 (8.7)	4 (0.9)	<0.01
Influenzavirus	6 (1.1)	2 (1.6)	4 (0.9)	0.62
Para-influenzavirus	7 (1.3)	5 (3.9)	2 (0.5)	<0.01
Human metapneumovirus	4 (0.7)	2 (1.6)	2 (0.5)	0.23
Adenovirus	14 (2.5)	5 (3.9)	9 (2.1)	0.33
Bocavirus	34 (6.1)	5 (3.9)	29 (6.8)	0.30
Wu polyomavirus	40 (7.4)	4 (3.1)	36 (8.4)	0.05
Ki polyomavirus	26 (4.7)	2 (1.6)	24(5.6)	0.09
Mycoplasma pneumoniae	1 (0.2)	0	1 (0.2)	-
Chlamydophila pneumoniae	2 (0.4)	0	2 (0.5)	-

the occurrence of respiratory symptoms (Table 3). In multivariate analysis the association of all determinants were assed independently of each other. Multivariate analysis revealed that airway resistance and the presence of single or multiple pathogens were significantly and independently associated with respiratory symptoms. One standard deviation increase of airway resistance leaded to a 26% increased risk of having symptoms. In case one or more pathogens were detected the risk of having symptoms was even higher (Odds Ratio (OR) 4.32, Confidence Interval (CI) 95% 2.79-6.71 and OR 6.05, CI 95% 3.62-10.10, respectively).

To study the difference between respiratory symptoms after a proven RSV and non-RSV infection we selected all first single RSV infections (n=41). For the non-RSV infections we selected a random sample of 57 single non-RSV infections. Figure 3 shows the number of days of

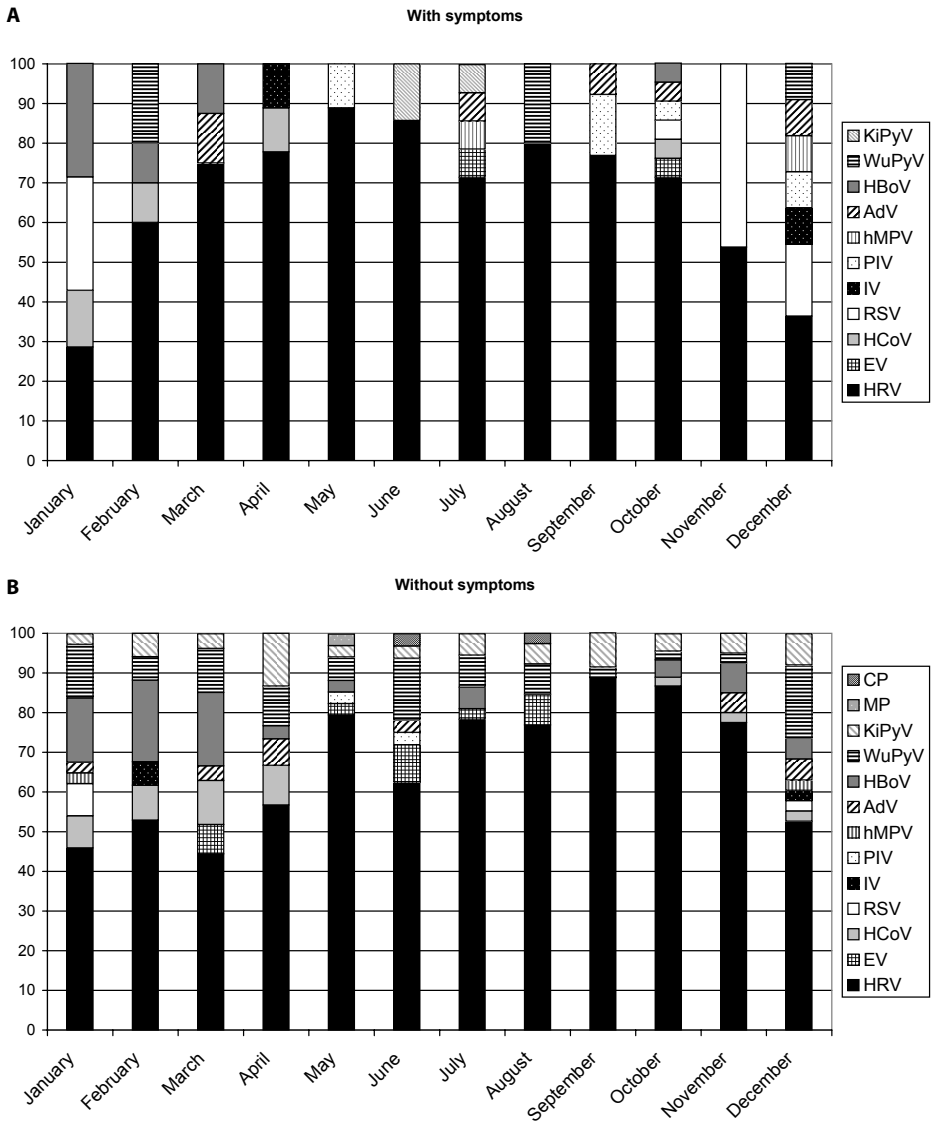

Figure 1a and **b.** The seasonal distribution of respiratory pathogens detected in infants with (a) and without (b) respiratory symptoms.

respiratory complaints the first 3 months after the proven infection. During the 3-months period after proven RSV-infection children had a mean of 10.9 days of cough and 4.7 days of wheeze. This was not significantly different from the symptoms after random non-RSV infections (cough 9.2 days, p = 0.83; wheeze 3.1 days, p= 0.46).

Table 3. The association between the presence of respiratory symptoms and determinants. Univariate and multivariate analysis.

	Univariate		Multivariate	
	OR	**95% CI**	**OR**	**95% CI**
Gender (male)	1.24	0.83-1.86	-	-
Age at sample (months)	1.10	1.05-1.14	-	-
Lung function				
C_{rs} (SDS)	0.99	0.80-1.23	-	-
R_{rs} (SDS)	1.17	0.95-1.43	1.26	1.01-1.57
Maternal history of atopy (y)	1.30	0.57-2.98	-	-
Detection of viruses				
No virus	ref	ref	-	-
Single virus	4.38	2.94-6.54	4.32	2.79-6.71
Multiple viruses	6.63	4.24-10.36	6.05	3.62-10.10
Siblings (y)	1.34	0.89-2.02	-	-
Pets (y)	0.84	0.56-1.26	-	-
Daycare first 6 months (y)	1.39	0.92-2.09	-	-
Smoking during pregnancy (y)	1.48	0.54-4.10	-	-

Multilevel analysis was used to correct for repeated measurements in infants. In multivariate analysis all determinants were placed in the model, only the significant determinants are shown.

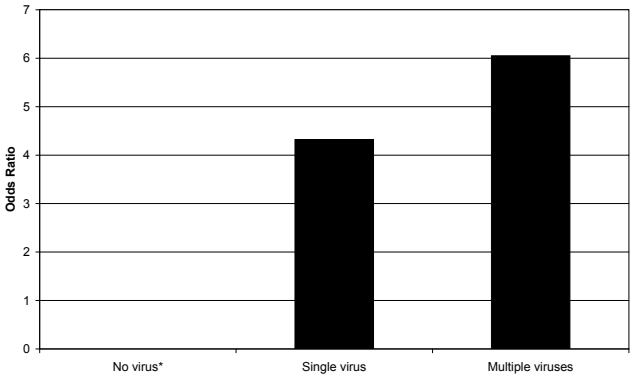

Figure 2. The risk (Odds Ratio) of respiratory symptoms with the detection of one or more respiratory viruses independent of other factors. * No virus was used as reference category.

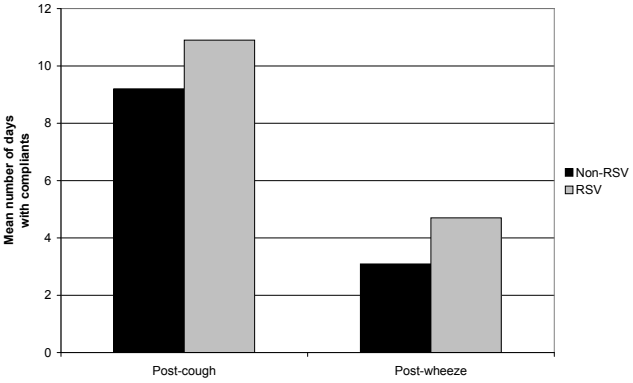

Figure 3. The mean number of days with respiratory complaints in the 3 months after an infection with RSV or non-RSV.

Discussion

In this study we studied the association between respiratory symptoms and the presence of respiratory pathogens during the first year of life in an unselected population. Respiratory viruses frequently occur in both asymptomatic and symptomatic periods, with a substantially higher prevalence in symptomatic periods. Airway resistance and the number of viruses detected are the most important determinants associated with the presence of respiratory symptoms. The phenomenon of post-RSV infection related symptoms could not be found in our study.

This is the first study performing comprehensive respiratory viral surveillance in combination with close prospective monitoring of respiratory symptoms in unselected infants. Besides, sensitive molecular detection techniques were used to detect a broad range of respiratory pathogens including bocavirus and the new polyomaviruses WU and KI. There was a high prevalence of maternal history of asthma in our study population. Parents in the research area were asked to participate in our study, but since the focus of the WHISTLER study was on predicting asthma like symptoms, parents with an atopic constitution might be more eager to participate. Besides this could have lead to a recall bias for a history of atopy in the parents. This influences the generalizability of our study.

In 85% of the samples collected during symptomatic periods a pathogen could be detected. This was significantly higher compared to pathogens found in samples collected during asymptomatic periods. HRV was the most prevalent pathogen in both symptomatic as asymptomatic cases. It was not significantly more often found in symptomatic cases. The role of HRVs is not yet established as HRVs are frequently found in asymptomatic children and adults, but are also detected in patients with symptoms ranging from mild common colds to serious lower respiratory tract disease[26,27]. This study shows that the clinical impact of HRVs is huge as it is seen in the majority of the respiratory tract infections. However we could not find an association with respiratory symptoms. Perhaps, HRV infections are often associated with disease due to high number of infections in the community, though maybe it is only an innocent bystander and revealing those infants at risk for respiratory illness.

We studied a broad panel of respiratory pathogens including the newly discovered Bocavirus and the polyomaviruses WU and KI. These new viruses were more often found in symptomatic infants, however this difference was not significant. Besides, most symptomatic cases were co-infections with other pathogens. As a single pathogen they were more often found in asymptomatic cases. In literature there is some debate about the pathogenicity of these viruses[28,29]. Our study shows that there is no association with these new viruses and disease but the presence of these pathogens may facilitate a secondary infection which eventually might lead to disease. For RSV and PIV there was a significant higher proportion associated with symptomatic cases. This is in line with literature where these viruses are often detected in lower respiratory tract infections[30]. Interestingly, *Chlamydophila pneumoniae (CP)* and

Mycoplasma pneumoniae (MP) were seldom found in this study. This might indicate that the role of these pathogens in young infants with respiratory symptoms is limited.

Respiratory pathogens were found in 51% of the asymptomatic cases. This is a relative high proportion compared to numbers found in other studies in asymptomatic children, where it ranges from 5 till 68%[4,31-34]. One explanation for this difference might be the fact that we studied a broad panel of respiratory pathogens included the newly discovered bocavirus and the polyomaviruses WU and KI. These new viruses comprised 23% of the asymptomatic pathogen detections, so most studies probably have underestimated the numbers of pathogens in asymptomatic children. It is difficult to determine the clinical significance of respiratory pathogens in asymptomatic cases. Although some pathogens do not seem so pathogenic, the presence of these pathogens in the respiratory tract during asymptomatic periods might facilitate other more pathogenic infections[35]. It is therefore important to investigate the role of asymptomatic pathogens in successive infections.

In this study we investigated the association between respiratory symptoms and several infant-, pathogen- and environmental determinants. Infant factors we included were gender, age at sample collection, neonatal lung function and maternal history of asthma. Some studies report that boys have an increased incidence of respiratory illnesses in comparison to female infants[36]. This association was not found in our study. Wright et al have suggested that parents visit the physician more often with boys compared to girls. In our study parents only had to rate symptoms and perhaps therefore this difference was not found. Furthermore, an increased airway resistance was associated with respiratory symptoms. Several studies have shown that the occurrence of respiratory illnesses during the first year of life is associated with lower levels of lung function shortly after birth and prior to any respiratory illness[37-39]. Low lung function at birth is shown to be a major risk factor for low lung function in later life by several studies. These studies indicate that individuals with reduced lung function at birth will be more likely to remain in the lower end of the lung function distribution until adulthood. It might be speculated that these lower levels of lung function in early childhood lead to increased respiratory morbidity during adulthood. In a recent Norwegian birth cohort reduced lung function shortly after birth was associated with an increased risk of asthma at 10 years of age. In our study we show that the occurrence of respiratory symptoms is importantly influenced by congenital lung function. Additionally, previous studies have shown that especially maternal history of asthma is associated with an increased risk of respiratory illness[40]. This study did not show this relation, but that is possibly due to the high prevalence of maternal history of asthma in our cohort.

Since the introduction of more sensitive molecular detections techniques a lot of discussion was focussed on the clinical relevance of the detection of respiratory pathogens. This study showed that the presence of a single virus is highly associated with respiratory symptoms, this association becomes even higher when multiple pathogens were detected. There is some debate about the role of multiple virus infections and disease severity. Some argue

that multiple viruses cause more severe disease[8-10,35], whereas others report no difference between single and multiple infections in disease severity[11,12]. Our findings support the former assertion and point towards an association between multiple infections and respiratory disease. Perhaps multiple viruses give rise to a different reaction of the immune system with another cytokine pattern. The cytokines could then cause more severe damage to the respiratory system and therefore more often lead to symptomatic illness[8].

Since the hygiene hypothesis[41] many studies have focussed on day-care attendance, siblings[42,43] and pet exposure[44]. A recent Dutch birth cohort study[42] showed that early day-care attendance was associated with more respiratory symptoms until the age of 4 years, but unless they have siblings, they do not develop less asthma symptoms or allergies at the age of 8 years. Univariate analysis showed a positive relation for siblings and day-care attendance and respiratory symptoms, however these factors were not independently associated with the presence of symptoms. One explanation could be that day-care attendance and the presence of siblings are an indirect measure for respiratory tract infection (or the presence of a pathogen) and not a risk factor alone. Further, maternal smoking during pregnancy is often associated with an increased risk of wheezing illnesses during the first years of life. Most studies suggest that this is due to a negative effect of smoking on the development op the foetal lungs. Our study could not find an association between both neonatal lung function and maternal smoking during pregnancy. Probably, other genetic and environmental factors play an important role in the vulnerability of an infant for respiratory illness.

Finally, we investigated the mechanism of post-RSV infection related symptoms compared to other pathogen infections. Numerous epidemiological studies have demonstrated an increased risk for recurrent episodes of wheeze following acute RSV respiratory tract infections. In the past this post-infection phenomenon was almost exclusively related to RSV infection[45,46]. In our study we could not find a significant difference between symptoms after a RSV and non-RSV infection. Some studies have investigated the occurrence of bronchial hyperresponsiveness and asthma years after hospitalization for a RSV or non-RSV bronchiolitis[16-18]. These studies too could not find any difference in respiratory symptoms later in life between cases with RSV and cases with a non-RSV infection. We believe that hospitalization with RSV bronchiolitis is simply an early marker of an underlying predisposition for reversible airway disease (i.e., asthma), and that this post-RSV related wheeze is a symptom of bronchial hyperresponsiveness.

In summary, this study showed that respiratory pathogens play a huge role during the first year of life especially during symptomatic periods. Import factors that influence the presence of respiratory symptoms include airway resistance and the number of pathogens detected. RSV and PIV infections were frequently associated with respiratory symptoms. Finally, we could not confirm the exclusive phenomenon of post-RSV infection wheeze.

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Chapter 9

General discussion

The role of respiratory viruses in lower respiratory tract illnesses in early life.

In this thesis we investigated the role of respiratory viruses in lower respiratory tract illness in early life. Epidemiological research on respiratory viruses is mainly focused on hospitalized infants and children. Community based studies are needed to identify determinants of those infants at risk for recurrent respiratory illnesses. The studies of this thesis were performed in a community population. In this thesis we focus on three important epidemiological questions:

1. What is the prevalence of respiratory viruses, including newly discovered viruses in early life?
2. What is the association between respiratory viruses and respiratory symptoms?
3. Which factors influence the occurrence of virus-associated respiratory symptoms?

In this chapter the answers to these questions are summarized and discussed and suggestions are made for further research on this topic.

What is the prevalence of respiratory viruses, including newly discovered viruses in early life?

What was known?

Respiratory viruses are the major cause of respiratory illnesses in infancy and childhood[1]. In older studies data on the prevalence of viruses were mainly based on viral cultures and indirect detection methods. Sensitivity and specificity of these conventional techniques vary between different viruses, laboratory protocols and golden-standards used. The sensitivity estimates of viral culture techniques of several studies ranged from 0.10 to 0.90[2,3]. Specificities ranged from 0.80 to 1.00[2,3]. Routinely, isolation of most respiratory viruses by culture from respiratory secretion will take at least several days. Besides, some respiratory viruses are difficult to grow (e.g. human coronaviruses). Tests for rapid diagnosis of viruses by direct or indirect immunofluorescence assays on exfoliated nasopharyngeal cells have also shown variable sensitivity (40 to 100%) and specificity (86 to 99%)[4]. In general direct immunofluorescence techniques are considered less sensitive compared to culture[4,5].
Polymerase chain reaction (PCR) is a technique to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating millions or more copies of a particular DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a

chain reaction in which the DNA template is exponentially amplified, that can be detected by a fluorescent probe in a real time-PCR (rt-PCR) format.

PCR has proven to be a very sensitive and specific method for the detection of respiratory viruses, mostly RNA viruses so a copy DNA step is needed before the PCR can be performed. Reported sensitivities for PCR range from 95–98% for respiratory syncytial virus (RSV), 94–100% for parainfluenza virus (PIV) and 92–95% for influenza A, which represent a significant improvement over existing methods for these viruses[3]. The application of PCR to the detection of viruses that grow poorly in cell culture represent a significant improvement. Increased sensitivity of PCR for detecting human rhinoviruses (HRVs) has been reported to be 3- to 5-fold greater than cell culture[3].

Before the introduction of new molecular detection techniques prevalences of viruses found in respiratory illnesses ranged from approximately 30 till 52%[3,6]. RSV and PIVs are mentioned as most common respiratory pathogen related to illness using the conventional detection techniques[7]. Dependent on the respiratory illness studied (e.g. bronchiolitis) these viruses were found in varying prevalences ranging from 5-100%[3]. With PCR used as new detection technique HRV and RSV are mentioned most in selected population of children hospitalized for respiratory illness[1,8]. Again these prevalences range depending on the defined respiratory illness of varying study populations.

In epidemiological studies HRV infections are found in up to 50% of the patients with respiratory illnesses[8]. Recently, it has been suggested that some HRV subtypes might be associated with more severe or different respiratory disease patterns than others[9-11], which lead to an increasing awareness of the importance of different HRV subtypes. However, longitudinal data on the diversity of HRVs in individuals are lacking. Jartti et al[12] longitudinally followed children for the persistence of HRV and enterovirus (EV) after the onset of symptomatic respiratory infection in the respiratory tract. They found that HRVs could be detected until 2-5 weeks after onset of symptoms; however the HRVs were not genotyped. It can be questioned whether these repeatedly detected HRVs represents true persistence or whether it can be a result of frequent subsequent infections with different HRVs subtypes.

The percentage of viruses found during respiratory tract illnesses in published studies using PCR has increased till up to 85%[8,13,14]. In a small proportion of the respiratory tract infections no respiratory pathogen is found. These new techniques have lead to the discovery of several new viruses, like human metapneumovirus (hMPV)[15], novel strains of coronaviruses (SARS-CoV, HCoV-NL63 and HKU1)[16] and human bocavirus (HBoV[17,18]). Recently, high throughput sequencing revealed additionally two new polyomaviruses, WU (WUPyV) and KI (KIPyV), most closely related to JC virus (JCV) and BK virus (BKV)[19,20]. Primary infections with the BKV and JCV virus usually occur during early childhood and remain for life clinically unnoticed in immunocompetent individuals[21,22]. Asymptomatic persistent infection in the kidney and peripheral blood is described in 35-85% of the population worldwide[23]. It is unknown however if the newly

discovered polyomaviruses, WUPyV and KIPyV, present the same course of infection. WUPyV and KIPyV were reported in samples of uncontrolled studies of small groups of hospitalized patients worldwide[19,20,24-28]. Despite their clear presence in specimens of patients with respiratory illnesses, the pathogenicity of WUPyV and KIPyV remains speculative. The proposed association of WUPyV and KIPyV with respiratory disease is questionable because the majority of studies have not included specimens from asymptomatic patients. The three studies that included these control groups, viral sequences were detected at similar frequencies in asymptomatic patients[29-31]. More research is warranted to investigate the clinical impact of these viruses.

What is new?

We showed that respiratory viruses and atypical bacteria are frequently found in infants with lower respiratory tract symptoms during the first year of life. The most common respiratory pathogens were HRV, human coronaviruses (HCoV) and RSV. Prevalences found in our community based study are comparable to prevalences in hospitalized infants. The diagnostic yield of pathogens detected during respiratory illnesses has increased from 28% and 50% in respectively culture and DIF[5] to 85% with PCR techniques[32] (see Figure 1).

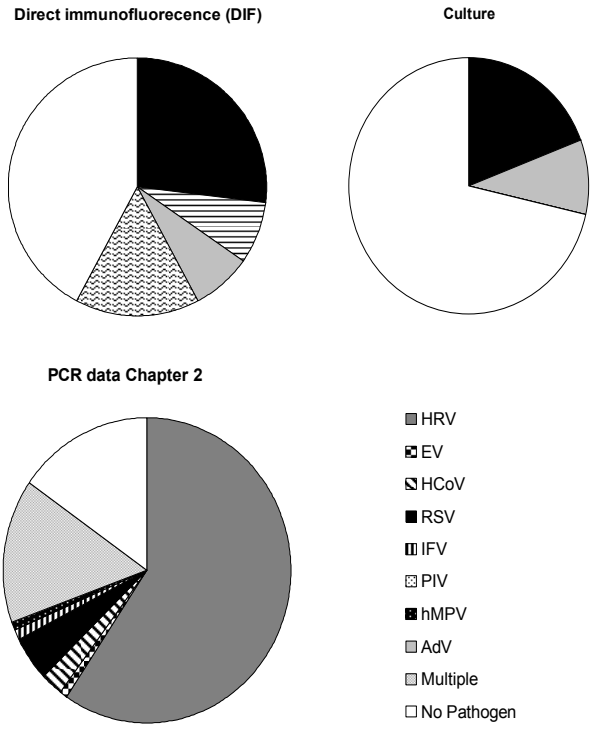

Figure 1. Percentages of Respiratory viruses detected using different detection techniques. Data of DIF and culture were applied from vd Pol et al. 2006, compared to PCR data vd Zalm et al. PIDJ 2009.

Due to diminished detection in culture HRVs were overlooked for a long time. PCR is a very sensitive method and has really increased detection of HRVs[33]. It is now seen as the most common respiratory pathogen. Our study (Chapter 2) showed that it was detected in up to three-quarter of the samples in symptomatic children. Prevalences in hospitalized infants and children, using molecular detection techniques, show comparable high prevalences. These findings have leaded to an increasing awareness of HRV infections during respiratory tract illnesses. Several studies have shown that HRV infections in infancy are linked with recurrent wheezing and asthma[34-37].

In the longitudinal study of HRV infections it was shown that HRV was highly prevalent in young children due to a high infection rate with a huge diversity of HRV subtypes (Chapter 4). The systematic surveillance for HRVs in this study revealed that nearly 4 different HRVs infections occurred in a six-month period. Further, re-infections and persistence of identical HRV subtypes were observed. These data shed new light on the occurrence of HRV infections and persistence. Kling et al[38] described a prolonged persistence of HRV RNA in children with an asthma exacerbation, however HRVs were not genotyped. Another study in lung transplant recipients also found persistence of HRVs in these immunocomprised patients[39]. They proved persistence of a unique strain, which was confirmed by sequence analysis. Our data are suggestive for frequent repeated new infection with HRV. It can be speculated that both prolonged persistence and repeated new infections might cause direct damage in the airways or can modulate immune responses.

RSV was the second most prevalent pathogen related to illness as it was seen in almost 11% of the infants in our study (Chapter 2). Since RSV is easier to detect using the conventional detection techniques the diagnostic yield was not as greatly increased by the use of PCR as for HRV. Earlier studies for the prevalence of RSV infections were mainly performed in hospital settings. In these studies RSV was found in up to 100% of the cases hospitalized with bronchiolitis[3]. Although infections with RSV were less frequent compared to HRV infections, we observed that the clinical impact of RSV was greater compared to HRV. It was more frequently associated with wheeze, fever, and physician visits compared to infections with HRV. These findings confirm earlier studies where it was seen as one of the more severe respiratory pathogens[40,41].

Interestingly, *Chlamydophila pneumoniae (CP), Mycoplasma pneumoniae (MP)*, and adenovirus (AdV) were predominantly found as a co-infection and not as a single pathogen. This result might indicate that the role of these pathogens in young infants with respiratory symptoms is limited[42,43].

Recently found new viruses were also responsible for increasing the diagnostic yield of viruses in respiratory illnesses. Human bocavirus (HBoV) was found as single pathogen in 3.9% of the symptomatic samples. The prevalence of WUPyV and KIPyV was investigated in a prospective longitudinal cohort study of children aged 0-7 years. WUPyV and KIPyV were repeatedly observed as the only detectable pathogen in children with respiratory symptoms.

WUPyV and KIPyV were detected in respectively 5 and 2 cases of the symptomatic children in whom no other causative respiratory pathogen could be detected. This means that the prevalence of viruses detected during symptomatic periods increased from 56% to 61% due to WUPyV and KIPyV detections (Chapter 5).

What needs to be done?

The percentage of viruses found during respiratory illnesses is high, however in a small proportion of the respiratory illnesses still no pathogen is detected. The sensitivity of PCR is probably good enough to detect all known respiratory pathogens clinical relevant for disease, so future research should focus on detection of other unknown viruses. Besides, also bacteria and fungi might play an important additional role Also one should consider the possibility that some respiratory illnesses might not be caused by either respiratory viruses, bacteria or fungi.
Since this thesis showed that persistent and repeated infections with different HRV subtypes are found in children during a winter season more attention must be paid to the longitudinal study of different HRV subtypes. In addition, subtypes of other respiratory viruses also need to be studied in terms of prevalences and seasonal occurrence. Studying the pattern of different virus subtypes occurrence might reveal shifting prevalences of subtypes in subsequent seasons.

What is the association between respiratory viruses and respiratory symptoms?

What was known?

The development of new molecular detection techniques has dramatically changed the ability to detect respiratory viruses. With this improvement questions were raised about the causal relationship between a viral detection and disease. Before the introduction of PCR, Koch's postulates were used to determine whether a pathogen was causally related to disease[44].

Koch's postulate:

i. The microorganism occurs in every case of the disease in question.
ii. The microorganism occurs in no other disease.
iii. After being isolated and repeatedly grown in pure culture, the microorganism can induce disease anew.

PCR is able to detect the genome of a few numbers of microorganisms that may occur in the absence of pathology. In addition, some pathogens are difficult to culture. These technical advances have motivated researchers to propose new guidelines for establishment of causal relations between microbes and diseases. These new guidelines can be summarized as follows[45].

Revised guidelines of Koch's postulate:

i. A nuclei acid detection of a pathogen should be present in most cases of an infectious disease.
ii. Fewer, or no, copy numbers of pathogens should occur in controls without the disease.
iii. With resolution of the disease the copy numbers should decrease.
iv. The causal relationship is more likely whenever the copy number correlates with disease severity.

For the first two (i and ii) statements studies in both symptomatic and asymptomatic children are needed. Since scarce data were available on the prevalence of pathogens in asymptomatic children, causal relation between viruses and disease were to a large extent unanswered. From reported studies of viral prevalences in children without respiratory symptoms a few drawbacks must be addressed. Firstly, most studies lacked a good control group, as many studies used children known with recurrent respiratory episodes or used children hospitalized for elective surgery. In hospitals, it can be suggested that the viral density is higher compared to a home-setting. Secondly, the definition of an asymptomatic episode was not always exactly defined. Some studies only looked at the absence of respiratory symptoms in a period before viral sampling, while others also looked at the period after sampling. Viral infections are thought to have an incubation period in which no symptoms occur. It is therefore very import to clearly define which children are considered asymptomatic. Thirdly, the panel of respiratory pathogens studied was incomplete.
Further, the second (ii) and third (iii) statements need to be studies using rt- PCR. This is a quantative detection technique were viral load of respiratory viruses can be determined.

There is no agreement on the correlation between viral load and clinical symptoms. Some studies have found a positive association between viral load and clinical symptoms[46,47], while others could not confirm this finding[48,49]. Gerna et al. suggest that viral load could also be used to determine the causative pathogen in multiple viral infections. They showed that in case of dual infections with RSV and HRV, RSV was found in higher viral loads compared to HRV. The results are not conclusive and larger numbers are needed to determine whether viral load is associated with disease.

Overall, inconclusive data were available on the association between respiratory viruses and disease. There is an association between viruses and disease, however studies investigating this association should be more stringent and complete.

What is new?

The occurrence of respiratory pathogens in children with and without respiratory symptoms was studied in Chapter 5. All children were followed in home-setting using questionnaires twice a week for respiratory symptoms. Bi-weekly samples were collected regardless of any respiratory symptoms. Asymptomatic cases did not have any respiratory symptoms one week before and one week after sample collection. Furthermore, PCR was used to detect 13 common respiratory pathogens. Our data indicate that respiratory pathogens are frequently found in samples from children with no respiratory symptoms (40%). Though, significantly more viruses were detected in children with respiratory symptoms (56%, p=0.03), which suggest an association between virus detections and disease. When looking at single HRV infections alone, the proportion of symptomatic and asymptomatic cases was comparable. This finding was confirmed in Chapter 8, were again no association between HRV detection and symptoms was observed. HRV can therefore be considered as a relatively mild pathogen. RSV and PIV on the other hand were associated with symptoms. These results are in line with literature where these viruses were commonly associated with severe lower respiratory tract infections[50].

The association between WUPyV and KIPyV and respiratory symptoms are unclear. In Chapter 4, were we longitudinally followed children aged 0-7 years, WUPyV and KIPyV were both more frequently associated with symptoms. In Chapter 8 on the other hand we showed that they were more frequently detected in asymptomatic samples of infants during the first year of life. These inconclusive findings are in line with literature where also conflicting results are found. Norja and colleagues[31] also found more WUPyV and KIPyV infections in the control group compared to the subjects with respiratory symptoms. Besides, the infections with WUV and or KIPyV were found in either immunosuppressed subjects or in immunocompetent subjects with a co-infection. Other studies did find an association between WUPyV and KIPyV and respiratory symptoms[19,20,24,25,27,28].

In conclusion, it can be suggested that respiratory viruses as a whole are associated with respiratory disease, whereby the first point of the revised guidelines of Koch's postulate is fulfilled. Nonetheless, some viruses were equally often found in symptomatic as asymptomatic cases, which suggest that other factors might be important in the pathogenicity of these viruses.

What needs to be done?

The first steps are made towards a better understanding of association between respiratory viruses and disease. However, a few considerations should be made for further research. First of all, the symptoms which comprise respiratory illness are broad. A clear definition should be used in order to compare results of various studies with each other. Secondly, asymptomatic controls should also be more clearly defined. What time period around sampling should be without any symptoms to define it as asymptomatic? Quantative PCR can be used in order to answer this question. Individuals should be sampled daily for a certain period with careful observation of respiratory symptoms. The collection of samples can reveal the moment of virus detection, which in turn can be correlated with disease. How long is the incubation period and is the virus detection the highest whenever symptoms occur? How many days or weeks after disappearance of respiratory symptoms can the virus, or its RNA/ DNA be detected? When both symptomatic and asymptomatic cases are more clearly defined, the association with disease can be further unravelled.
Furthermore, in the 'old' postulates of Koch it was stated that a pathogen should be able to cause disease after being isolated and grown in pure culture. This means that viruses are inoculated in respiratory epithelium, hereafter development of symptoms can be assessed. A limitation of this technique is that viruses must be able to grow in culture.

Which factors influence the occurrence of virus-associated respiratory symptoms?

Despite the fact that all infants encounter viral infections during the first years of life, the clinical impact differs largely among infants. The exact mechanisms which underlie these inter-individual variability in the sensitivity to viral infections are unclear until now. Numerous studies have investigated host-, pathogen- and environmental factors, which might influence the emergence of respiratory symptoms. Nevertheless, the results of these studies are not unambiguous. In this thesis we focused on a subset of these factors, which will be discussed below.

What is known?

There is good evidence that prevalence of respiratory illness vary by gender and these differences change with age[51]. Boys have more respiratory illness during the first years of life, while girls have more respiratory illness in the second decade of life[52]. In addition, several studies have shown that lung function is significantly lower in boys compared to girls during infancy, others state that boys have larger lung values but smaller airways for given lung size[53]. There-

fore it is important to take gender into account whenever investigating the occurrence of respiratory symptoms and lung function. Both can be influenced by gender characteristics.
Results from birth cohort and cross-sectional studies of young children with wheezing have revealed strong associations between lung function in early life and the subsequent development of recurrent respiratory illnesses[54]. Interactions between viral infections, especially those due to HRV and RSV may induce persistent alterations in airway function in susceptible subjects (lung function detracking)[54]. On the contrary, diminished lung function shortly after birth predicts airflow limitation in early adult life (lung function tracking). The Tucson birth cohort study followed the lung function of infants shortly after birth to the age of 22 years[55]. Infants with a low lung function early in life remained in the group with lower lung function in adulthood. In the Melbourne asthma study children were followed from the age of 7 till adult life with lung function measurements and questionnaires. In this study, it was shown that children with frequent or persistent wheeze in childhood continue to have abnormal lung function into mid-adult life[56]. These studies indicate that individuals with reduced lung function at birth will be more likely to remain in the lower end of the lung function distribution until adulthood. The concept of lung function tracking and detracking needs to be explored in combination interacting factors like viral infections, before the occurrence of any respiratory illness.
As mentioned before the effect of parental history of asthma on the development of wheezing illnesses and asthma has been extensively studied. Maternal history of asthma or atopy has more influence than paternal history over subsequent allergic phenotypes in offspring[57]. Besides, maternal history of asthma and atopy appear to modify the effects of environmental factors that promote the development of asthma[58]. Infants with a positive maternal history of asthma might be more vulnerable to viral infections.

Some pathogens are attributed to more severe disease compared to others. RSV is thought to be able to cause severe respiratory illness in young children, and can also result in prolonged post viral wheezing[59,60]. Nevertheless, serological studies show that almost all infants have come across a RSV infection during the first years of life and only a minority experience severe illness. Currently, HRVs have raised increasing interest as they seem to be responsible for a wide range of respiratory illnesses. There are several studies which suggest that symptomatic HRV infections might play an important role in recurrent wheezing later on in life or even in development of asthma[34,35,37,61]. It is however unknown whether this is a virus-induced pathogenic effect (serial hypothesis) or whether these infants were born with congenital smaller airways and therefore develop wheezing episodes (parallel hypothesis).
The importance of multiple infections within disease pathogenicity has been increasingly recognized. The exact mechanism is unknown and reports are not conclusive on this issue. A possible mechanism behind this phenomenon is the immune response patterns elicited by dual respiratory virus infections or successive infections occurring within a short period of

time. Current evidence suggests that early exposure to viral infections is a potent stimulator of postnatal immune maturation[62]. According to the hygiene hypothesis, the infant's immune response is considered immature with diminished capacity to produce cytokines, and exposure to common infectious pathogens might be decisive in determining the type of immune responsiveness by providing a strong stimulus for cytokine production.
Since the hygiene hypothesis many studies have focused on day-care attendance, siblings and pet exposure[63]. Data is not conclusive on whether the high exposure to infections lead to a decrease in the prevalence of asthma. Day-care attendance and having siblings are likely to be proxy's of respiratory infections. In order to unravel the association between these environment factors and respiratory symptoms, these factors need to be assessed independently in an unselected population.

What is new?

Our data showed that increased airway resistance measured before the age of 2 months was associated with an increased risk on the occurrence and duration of HRV-associated wheeze during the first year of life. This association was not influenced by gender, birth length or weight, maternal smoking during pregnancy or maternal history of asthma. Since the lung function was assessed early in life, before the occurrence of any respiratory illness, these results are suggestive for the parallel hypothesis instead of the serial hypothesis. In addition, lower lung function in early life is associated with diminished lung function later in life. This might suggest that wheeze during a HRV infection is the first sign to recognize those infants at risk for recurrent respiratory illnesses later in life. It can be speculated that HRV-associated wheeze is a model for the interaction between lung function and virus infections. Further studies should investigate the association of neonatal lung function and wheezing due to other viruses.
Another host factor often associated with respiratory symptoms was gender. Wright et al[64] have suggested that parents visit the physician more often with boys compared to girls. However, in our study we could not find a significant difference between gender and the presence of respiratory symptoms.
RSV and PIV infections were more frequently associated with respiratory disease compared to other viruses. This is in line with several other studies which have suggested an increased association with these viruses and lower respiratory illness. Moreover, a number of epidemiological studies have demonstrated an increased risk for recurrent episodes of wheeze following acute RSV respiratory tract infections. In the past this post-infection phenomenon was almost exclusively related to RSV infection[59,60]. In our study we could not find a difference between symptoms after a RSV and non-RSV infection. Some studies have investigated the occurrence of bronchial hyperresponsiveness and asthma years after hospitalization for a RSV or non-RSV bronchiolitis[65-67]. These studies also could not find any difference in respira-

tory symptoms later in life between cases with RSV and cases with a non-RSV infection. It can be speculated that hospitalization with RSV bronchiolitis could simply be an early marker of an underlying predisposition for airway disease (i.e., asthma), and that this post-RSV related wheeze is a symptom of bronchial hyperresponsiveness (parallel hypothesis).
The role of multiple infections in the severity of respiratory disease has been argued[68-72]. Our studies showed that the detection of more than one respiratory pathogen was strongly associated with disease. As suggested cytokine profiles of multiple infections might lead to increased disease[68]. At least it can be speculated that the presence of a relatively mild pathogen gives rise to a higher risk of disease.

What needs to be done?

Bacterial co-infections might also play an important role in development of disease. Synergism between viruses and bacteria in inducing infections in respiratory epithelium has been described both in vitro and in vivo studies. Experimental studies have shown that the presence of viruses may contribute to increased bacterial adhesion[73]. In children the nasopharyngeal flora becomes established during the first year of life[74]. The nasopharynx is colonized by a broad variety of microorganisms, including commensal bacteria as well as potential pathogens such as Streptococcus pneumoniae, Haemophilus influenzae and Moraxella catarrhalis. In most cases these organisms are carried without symptoms, however when the condition of the host is changed it may cause disease. Longitudinal follow up of bacterial colonization in unselected infants together with the occurrence of viral infections and symptoms are needed to investigate the exact mechanisms through which bacterial infections and viral infections might interact.
Another important topic for further research into the association between viruses and disease includes innate immunity and especially Toll- like receptors (TLRs). Pathogen-associated molecular patterns initiate the innate immune response against viruses by activating special sensor systems, such as TLRs that recognise viral components. These TLRs have gained interest as orchestras of the initial immune response and as a link to the adaptive immune system[75]. In general, TLR ligation is a strong adjuvant signal to the immune system, increasing the capacity of antigen presenting cells to initiate cellular immune responses. Typically, TLR ligation promotes a Th1 skewed immune response, resulting in a protective antiviral response. There is evidence that TLR systems are immature in infants and therefore result in an increased susceptibility to viral infections. The immaturity of TLRs responses may be a key mechanism underlying the susceptibility of infants to respiratory viral infections.

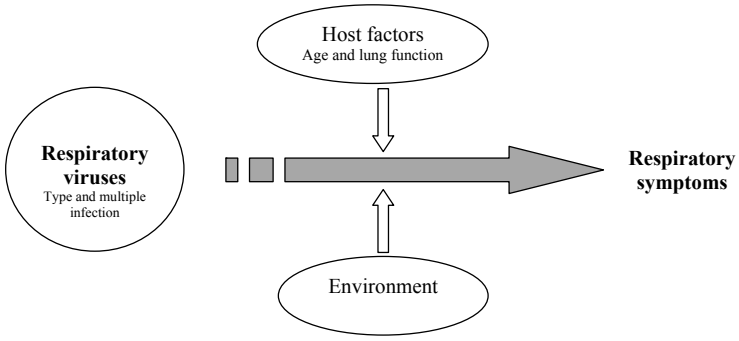

Figure 2. The role of respiratory viruses in lower respiratory tract illnesses in early life.

Conclusions

We conclude that in infants with respiratory symptoms viral pathogens are more frequently present than previously presumed. The development of more sensitive detection techniques and the discovery of new viruses have shed new light on the prevalence of respiratory viruses during respiratory illness. A significant higher proportion of viruses are found in symptomatic compared to asymptomatic infants and children. Nonetheless, viral pathogens are also frequently detected during asymptomatic periods. Factors important in the occurrence of respiratory symptoms include age, lung function and neonatal lung function.

Another important finding of this thesis include that premorbid lung function is associated with HRV-associated wheeze. Since, low lung function at birth is shown to be a major risk factor for low lung function in later life, wheezing during a HRV infection might reveal the infants at risk for recurrent respiratory illnesses later in life.

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Chapter 10

Samenvatting

De rol van de virussen in lagere luchtwegaandoeningen vroeg in het leven

In dit proefschrift is de rol van luchtweg (respiratoire) virussen in lagere luchtwegaandoeningen in het vroege leven onderzocht. Epidemiologisch onderzoek naar respiratoire virussen is voornamelijk gericht op gehospitaliseerde zuigelingen en kinderen. Studies in de "gewone" populatie zijn nodig om factoren te identificeren die een rol spelen in de ontwikkeling van luchtwegklachten.
In dit proefschrift worden drie belangrijke epidemiologische vragen onderzocht:

1. Wat is de prevalentie van respiratoire virussen in het vroege leven, inclusief de recent ontdekte virussen?
2. Wat is relatie tussen respiratoire virussen en luchtwegklachten?
3. Welke factoren beïnvloeden het voorkomen van virusgeassocieerde luchtwegklachten?

Hoofdstuk 1

Dit hoofdstuk geeft een algemene inleiding over de rol van virussen in luchtwegaandoeningen. Luchtwegaandoeningen zijn de belangrijkste oorzaak van ziekte en sterfte van kinderen. Virusinfecties spelen een belangrijke rol tijdens luchtwegklachten. In ongeveer 85% van de kinderen met luchtwegklachten wordt een virus gevonden. Virussen veroorzaken een breed spectrum van luchtwegaandoeningen variërend van verkoudheden tot levensbedreigende longontstekingen. Virussen spelen ook een belangrijke rol in astma op de kinderleeftijd en bij volwassenen. Tijdens astma- exacerbaties wordt in ongeveer 80% van de gevallen een virus gevonden. Aan de ene kant suggereert de hygiënehypothese dat kinderen die frequente luchtweginfecties doormaken in het vroege leven, een verminderd risico hebben op de ontwikkeling van astma en allergieën. Aan de andere kant spelen virussen een belangrijke rol in het uitlokken van klachten bij mensen met astma. Virussen spelen zonder twijfel een belangrijke rol tijdens luchtwegklachten, maar vragen over de prevalentie van verschillende virussen en de associatie tussen virussen en klachten blijven onbeantwoord. Door meer inzicht te krijgen in de interactie tussen virusinfecties en de ontwikkeling van chronische luchtwegaandoeningen, hopen we uiteindelijk de behandeling en preventie van deze veel voorkomende aandoeningen te verbeteren.

Hoofdstuk 2

In dit hoofdstuk is de prevalentie van verschillende virussen en atypische bacteriën bij jonge kinderen met luchtwegklachten onderzocht. Tevens is de klinische impact van de verschillende virussen bestudeerd.

Het onderzoek is gedaan in het WHISTLER geboortecohort. Kinderen zijn gevolgd gedurende het eerste levensjaar met dagboekjes over het voorkomen van luchtwegklachten. Ouders zijn gevraagd om keel en neuswatten af te nemen op het moment dat er meer dan twee dagen klachten waren van hoesten, piepen of zagen. Polymerase ketingreactie (PCR) hebben we gebruikt om de verschillende virussen aan te tonen.
In totaal hebben we 305 zuigelingen gevolgd. Deze kinderen hadden gemiddeld 5 luchtwegepisodes per jaar (periode van meer dan twee dagen klachten van de luchtwegen). Een of meer respiratoir pathogenen werden gevonden in 85% van de monsters. De meest voorkomende pathogenen waren humaan rhinovirus (HRV) (73% van de monsters), Respiratory Syncytial Virus (RSV) (11%) en coronavirus (8%). In vergelijking met RSV waren HRV infecties vaker geassocieerd met langdurige perioden van klachten. Aan de andere kant was RSV vaker geassocieerd met koorts, piepklachten en huisartsbezoeken.
Samengevat hebben we een hoge prevalentie van respiratoire pathogenen gevonden bij zuigelingen met luchtwegklachten gedurende het eerste levensjaar. Het diagnostisch deficit is hiermee verder verkleind van ongeveer 60% vóór de introductie van PCR naar 85% nu. HRV was het meest voorkomende respiratoire pathogeen. Hoewel RSV infecties mogelijk leiden tot ernstigere klachten in vergelijking met HRV infecties, was de impact van de ziekte het hoogst voor HRV.

Hoofdstuk 3

De methode van afname van neus- en keelwatten voor virusonderzoek is beschreven in dit hoofdstuk.
Het hoofdstuk is een reactie op een artikel van Lemanske en collega's die in een prospectieve cohort-studie hebben gekeken naar de rol van virusinfecties in de ontwikkeling van piepklachten en allergische ziektes op de kinderleeftijd. In die studie werden monsters verzameld door een onderzoeksverpleegkundige. Deze manier van monster-verzamelen gaf een frequentie van gemiddeld twee luchtwegepisodes per jaar (periode van meer dan twee dagen klachten van de luchtwegen). We veronderstelden dat deze manier van dataverzameling leidt tot een onderreportage van luchtwegepisodes, wat we getest in ons geboortecohort.
In onze pilot-studie hebben we 50 ouders random verdeeld over twee studiegroepen. Groep A werd gevraagd de onderzoeksverpleegkundige te bellen op het moment dat er luchtwegklachten aanwezig waren zodat er een neus en keelwat kon worden afgenomen. Groep B nam zelf de monsters af.
Beide groepen hadden evenveel luchtwegepisodes genoteerd in de dagboekjes. Groep A had in 24% van de luchtwegepisodes een monster afgenomen tegenover 43% (p=0.07) in groep B. Er was geen statistisch significant verschil in aantal virussen dat werd gevonden tussen beide groepen.

Samenvattend hebben we laten zien dat afname van virusmonsters door ouders, leidt tot hogere monsterfrequentie zonder dat er een verlies van virusdetectie optreedt.

Hoofdstuk 4

Dit hoofdstuk geeft inzicht in het beloop van rhinovirus (HRV) infecties. De laatste jaren is er in toenemende mate aandacht voor HRV infecties. HRVs lijken verantwoordelijk te zijn voor een breed scala aan luchtwegaandoeningen. HRVs worden vaak gevonden bij kinderen zonder luchtwegklachten, maar komen ook voor bij kinderen met klachten variërend van neusverkoudheid tot longontsteking. Sinds de introductie van PCR worden HRVs gevonden in ongeveer 50% van de luchtwegklachten.
Er is in toenemende mate ook aandacht voor de verschillende HRV subtypes die geassocieerd lijken met verschillende ziekteverschijnselen.
We vroegen ons af of de hoge prevalentie van HRVs veroorzaakt wordt door langdurige infectie van eenzelfde HRV subtype of dat het komt door herhaalde infecties met verschillende subtypes.
We hebben 18 gezonde kinderen van de leeftijd van 0 tot 7 jaar gevolgd gedurende een half jaar. Monsters werden minimaal elke twee weken afgenomen. Sequentieanalyses werden verricht om het HRV subtype te bepalen.
HRV werd gevonden in 35% van de monsters. In totaal werden 32 verschillende genotypes gevonden. Zowel herhaalde infecties van het zelfde subtype als aanhoudende infectie van het zelfde subtype werden gezien. Opvallend was dat jonge kinderen vaker met verschillende subtypes in aanraking kwamen dan oudere kinderen.
We concluderen dat HRVs zeer frequent gevonden worden met een zeer diverse populatie van verschillende subtypes. De prevalentie van HRVs is hoog door zowel herhaalde infecties als nieuwe HRV infecties.

Hoofdstuk 5

Recent zijn twee nieuwe polyomavirussen ontdekt bij patiënten met luchtwegklachten. Deze virussen zijn het WU (WUPyV) en KI (KIPyV) polyomavirus. De rol van deze virussen in het ontstaan van luchtwegklachten bij kinderen is nog niet duidelijk.
We hebben 18 gezonde kinderen van de leeftijd van 0 tot 7 jaar gevolgd gedurende een periode van een half jaar. Keel- en neuswatten werden elke twee weken afgenomen ongeacht luchtwegklachten. PCR werd gebruikt om onder andere WUPyV en KIPyV aan te tonen.
WUPyV en KIPyV werden gevonden in respectievelijk 9% en 3% van alle monsters. In respectievelijk 5 en 2 gevallen werden deze virussen gevonden in kinderen met luchtwegklachten waarbij geen enkel ander virus kon worden aangetoond. WUPyV en KIPyV werden vaker gevonden in symptomatische dan in asymptomatische kinderen.

Samengevat hebben we in deze studie aangetoond dat WUPyV en KIPyV frequent voorkomen in jonge kinderen, bovendien lijken deze virussen geassocieerd te zijn met luchtwegklachten.

Hoofdstuk 6

Luchtweginfecties komen vaak voor bij jonge kinderen. De meeste studies hebben de prevalentie van virussen bekeken in groepen kinderen met luchtwegklachten en er is dus weinig bekend over het voorkomen van virussen bij kinderen zonder luchtwegklachten. Om de associatie tussen virussen en klachten te bestuderen is het noodzakelijk om ook te kijken naar de prevalentie van virussen bij kinderen zonder klachten. Verder hebben we gekeken welke factoren een belangrijke rol spelen bij de ontwikkeling van klachten. We veronderstelden dat jonge kinderen vaker klachten hebben van een virusinfectie in vergelijking met oudere kinderen en dat een infectie met meerdere virussen vaker leidt tot klachten.
Deze vragen zijn onderzocht in een groep van 18 kinderen in de leeftijd van 0 tot 7 jaar. Deze kinderen zijn gedurende een half jaar gevolgd. Keel- en neuswatten zijn elke twee weken afgenomen ongeacht luchtwegklachten. PCR is gebruikt om 13 verschillende pathogenen aan te tonen.
We ontdekten dat in 56% van de episodes met luchtwegklachten een virus werd aangetoond, in vergelijking met in 40% van de episodes zonder luchtwegklachten. Dit verschil was statistisch significant. Jonge kinderen hadden vaker klachten en ook de detectie van meerdere pathogenen was vaker geassocieerd met klachten.
Samenvattend hebben we frequent virussen gevonden bij kinderen zonder luchtwegklachten. Toch is de detectie van een virus vaker geassocieerd met luchtwegklachten. Leeftijd en aantal pathogenen spelen een belangrijke rol in de ontwikkeling van klachten.

Hoofdstuk 7

Dit hoofdstuk beschrijft de rol van neonataal gemeten longfunctie op HRV geassocieerd piepen. Verschillende studies hebben laten zien dat het voorkomen van piepklachten in de eerste levensjaren is geassocieerd met lagere longfunctie na de geboorte. Bovendien is gesuggereerd dat verminderde longfunctie vroeg in het leven is geassocieerd met piepklachten tijdens virusinfecties. De associatie tussen neonatale longfunctie en daarop volgende bevestigde virusinfectie is nooit onderzocht.
Deze studie is verricht in het WHISTLER geboortecohort. Zuigelingen zijn gevolgd gedurende het eerste levensjaar met dagboekjes over het voorkomen van luchtwegklachten. Ouders zijn gevraagd om keel en neuswatten af te nemen op het moment dat er meer dan twee dagen klachten waren van hoesten, piepen of zagen. Neonatale longfunctie is gemeten in de eerste 2 maanden.

In totaal werden 176 zuigelingen met een gelukte longfunctiemeting en een HRV infectie gevolgd. Het risico op piepklachten tijdens een HRV infectie was hoger voor zuigelingen met een hogere luchtwegweerstand. Dit risico was onafhankelijk van andere factoren die rol spelen in de ontwikkeling van piepklachten (bijvoorbeeld roken tijdens de zwangerschap en voorgeschiedenis van moeder met astma).
Samengevat was neonataal gemeten luchtwegweerstand geassocieerd met piepen tijdens een HRV infectie. Piepklachten tijdens een HRV infectie is mogelijk het eerste teken bij zuigelingen dat er sprake is van een verminderde longfunctie.

Hoofdstuk 8

Dit hoofdstuk bestudeert de associatie tussen virussen en luchtwegklachten. Het is bekend dat respiratoire virussen de belangrijkste veroorzakers zijn van luchtwegklachten, maar het is onduidelijk welke factoren een rol spelen in het ontwikkelen van luchtwegklachten. We hebben de verschillende gastheer-, pathogeen- en omgevingsfactoren bestudeerd. Tot slot hebben we gekeken naar het "post-viral-wheezing" fenomeen dat vaak geassocieerd is met RSV infecties.
Deze vragen zijn onderzocht in het WHISTLER geboortecohort. Zuigelingen zijn gevolgd gedurende het eerste levensjaar met dagboekjes over het voorkomen van luchtwegklachten. Ouders zijn gevraagd om maandelijks keel- en neuswatten af te nemen op het moment dat er meer dan twee dagen klachten waren van hoesten, piepen of zagen. Polymerase ketingreactie (PCR) is gebruikt om de verschillende virussen aan te tonen.
We ontdekten dat longfunctie (gemeten als luchtwegweerstand) en het aantal virussen dat gevonden werd, geassocieerd waren met luchtwegklachten. We vonden geen verschillen tussen klachten na een RSV of een niet-RSV infectie.

Algemene discussie

In dit hoofdstuk komen we terug op de 3 belangrijke epidemiologische vragen en kijken we naar mogelijkheden voor toekomstig onderzoek.

1. Wat is de prevalentie van respiratoire virussen in het vroege leven, inclusief de recent ontdekte virussen?

In dit proefschrift hebben we laten zien dat respiratoire virussen en atypische bacteriën zeer frequent worden gevonden bij kinderen met lagere luchtwegklachten. De meest voorkomende respiratoire virussen zijn HRV, HCoV en RSV. Het diagnostisch deficit van respiratoire virussen tijdens luchtwegklachten is met PCR verkleind naar ongeveer 15%.

De ontdekking van de nieuwe virussen humaan bocavirus (HBoV), WUPyV en KIPyV heeft ervoor gezorgd, dat er nog vaker virussen worden aangetoond tijdens periodes met luchtwegklachten.
Om het diagnostisch deficit van virussen die worden gevonden tijdens luchtwegklachten nog verder te verkleinen, zal toekomstig onderzoek zich moeten richten de ontdekking van nieuwe virussen. Ook zal het onderzoek zich moeten uitbreiden naar bacteriën en schimmels.

2. Wat is relatie tussen respiratoire virussen en luchtwegklachten?

We hebben laten zien dat de aanwezigheid van virussen is geassocieerd met luchtwegklachten. Voor sommige virussen was er een sterkere associatie met klachten gevonden dan bij andere virussen. Waarschijnlijk spelen andere factoren een belangrijke rol in de pathogeniciteit van deze virussen.
Toekomstig onderzoek zal zich onder andere moeten richten op het duidelijk formuleren van de definitie luchtwegklachten en de definitie van een asymptomatische periode. Om de relatie tussen klachten en virussen verder te ontrafelen, kunnen kwantitatieve detectie technieken, zoals real-time PCR, een belangrijke rol spelen.

3. Welke factoren beïnvloeden het voorkomen van virusgeassocieerde luchtwegklachten?

In dit proefschrift hebben we laten zien dat verschillende gastheer-, pathogeen- en omgevingsfactoren een belangrijke rol spelen in de ontwikkeling van luchtwegklachten.

- Jonge kinderen zijn vaker symptomatisch tijdens infecties (Hoofdstuk 5 en 6)
- Hogere luchtwegweerstand is geassocieerd met (HR)virusgeassocieerde piepklachten en luchtwegklachten in het algemeen (Hoofdstuk 7 en 8)
- RSV en parainfluenzavirussen (PIVs) zijn vaker geassocieerd met luchtwegklachten (Hoofdstuk 6 en 8)
- Detectie van meerdere virussen is vaker geassocieerd met luchtwegklachten dan de detectie van één enkel of geen virus (Hoofdstuk 6 en 8)

Toekomstig onderzoek zou zich moeten richten op de rol van bacteriële co-infecties. Experimentele studies hebben laten zien dat de aanwezigheid van een virus zorgt voor een betere hechting van bacteriën. Ook de rol van het afweersysteem in de associatie tussen virussen en klachten verdiend verder onderzoek.

Conclusie

We concluderen dat respiratoire virussen vaker worden gevonden bij jonge kinderen dan voorheen verondersteld is. De ontwikkeling van nieuwe diagnostische methodes om virussen te detecteren en de ontdekking van nieuwe virussen, hebben geleid tot een verkleining van het diagnostisch deficit. Hoewel de aanwezigheid van virussen duidelijk geassocieerd is met klachten, worden virussen ook vaak gevonden bij kinderen zonder luchtwegklachten. Factoren die een belangrijke rol spelen in de ontwikkeling van klachten zijn: leeftijd, longfunctie, type en aantal virussen.

Dankwoord

Curriculum vitae

List of publications

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Martijn, je vroeg me een alinea aan je te wijden en eigenlijk kan ik wel pagina's vullen over je. In het begin moest ik even aan je wennen, maar al snel bleek je heel erg mee te vallen ;-). Samen hebben we de marathon van Berlijn gelopen. Gaan je toch nog een keer New York meelopen? Alma, wij zaten een beetje op hetzelfde schema en in hetzelfde onderzoeksgebied. We hebben vele koffietjes gedronken en frustraties gedeeld. Ook heb je Martijn en mij met je zwangere buik aangemoedigd in Berlijn. Succes met je verdediging straks, ik heb er alle vertrouwen in!

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Lieve Geek en Saas. Als "Utç" zijn we een aantal keer op vakantie geweest en nu gaan we in december als "Dort" naar Zuid-Afrika. Laat onze vriendschap nog heel lang duren. Ik ga jullie missen straks.

Lieve Annemiek, onze vriendschap begon toen we samen met Jolanda Controversa wilde oprichten. In de afgelopen jaren ben je een dierbare vriendin geworden. Ik vind het super dat je nu mijn paranimf wilt zijn, ondanks het feit dat je dan net moeder bent geworden…ik ben zo benieuwd!

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Marieke

Curriculum vitae

Marieke Margreet van der Zalm werd op 5 april 1979 geboren in Utrecht als jongste van twee kinderen. Na het behalen van haar VWO aan het Paulus lyceum Tilburg, vertrok zij in 1997 naar Maastricht om psychologie te gaan studeren.
In 1999 werd ze ingeloot voor Geneeskunde en startte zij deze studie aan de Universiteit van Utrecht. Na haar keuzecoschappen kindergeneeskunde te hebben gelopen in het Frère ziekenhuis in East London, Zuid-Afrika, en later in De Gelderse Vallei in Ede, was haar keuze voor de kindergeneeskunde definitief.
In 2004 startte Marieke met haar wetenschappelijke stage bij divisie Kinderlongziekten in het Wilhelmina Kinderziekenhuis. Een stage die – mede door het behalen van de MD/PhD beurs – uiteindelijk leidde tot haar promotieonderzoek **"De rol van respiratoire virussen in lagere luchtwegaandoeningen vroeg in het leven"**, onder begeleiding van Prof. Dr. C.K. van der Ent, Prof. Dr. Th.J.M. Verheij, Dr. C.S.P.M. Uiterwaal en Dr. B. Wilbrink.
Marieke begon in 2008 de klinische opleiding tot kinderarts in het Wilhelmina Kinderziekenhuis (opleider Dr. J. Frenkel). Ze begon met het perifere deel van haar opleiding in het St. Antonius ziekenhuis te Nieuwegein (opleider Dr. J.A. Schipper). Naast de opleiding en haar promotieonderzoek heeft ze inmiddels ook de Master Epidemiologie aan de Universiteit Utrecht, specialisatie Klinische Epidemiologie succesvol afgerond.
Eind december 2009 zal Marieke nogmaals naar Zuid-Afrika vertrekken, dit maal om een half jaar van haar opleiding tot kinderarts te doorlopen in Tijgerberg ziekenhuis in Stellenbosch.

Namens Annemiek Evers, paranimf.

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