Respiratory viral infections and interaction with bacteria in children with cystic fibrosis

Bart van Ewijk

COLOFON

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Respiratory viral infections and interaction with bacteria in children with cystic fibrosis

Respiratoir virale infecties en interactie met bacteriën
bij kinderen met cystic fibrosis

(met een samenvatting in het Nederlands)

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Chapter 1

Introduction

INTRODUCTION

Cystic fibrosis (CF) is the most common lethal autosomal recessive disorder in the Caucasian population, with an overall birth prevalence of 1 in 4750 live births (1). In the Netherlands currently live about 1300 children and adults with CF (70000 worldwide). It is a chronic disease that affects the lungs and digestive system, caused by mutations of the cystic fibrosis transregulator membrane (CFTR) gene on chromosome 7. Over 1500 CFTR mutations have been described (www.genet.sickkids.on.ca/cftr/). The most frequent mutation is a deletion of phenylalanine at position 508 (dF-508) of the CFTR gene, accounting for 75% of the CFTR mutations in Dutch CF patients (2). This defective CFTR causes the body to produce unusually thick, sticky mucus. In the lungs this results in obstruction of the airways associated with chronic inflammation, bacterial colonisation and recurrent infections of the lungs, which finally leads to irreversible structural abnormalities with deterioration of lung function and early death (3). Average life expectancy is currently around 35 years, although important improvements in treatments result in an increasing survival (4).

Aggressive treatment of pulmonary infections is considered to be one of the major reasons for the recent improvement of life expectancy (5). One of the most important bacterial pathogens recognized in pulmonary morbidity in CF is *Pseudomonas aeruginosa*. Impaired mucociliary transport, decreased anti-pseudomonal defense, specific binding of *P. aeruginosa* to asialoGM-1 receptors and persistent inflammation probably play a role in decreased clearing capacity for *P. aeruginosa* in CF patients (6). Chronic infection of the airways with *P. aeruginosa* accelerates the progression to irreversible lung damage (7).

Although CF is a genetic disorder and although bacterial colonisation is persistent, the clinical course is typically characterised by exacerbations and remissions of pulmonary complaints. This suggests that external factors influence this course, for example viral infections. Viral infections are the most common cause of acute respiratory symptoms in otherwise healthy children. There is increasing evidence that respiratory viral infections play an important role in the morbidity of chronic respiratory diseases. In asthma and chronic obstructive pulmonary disease (COPD), viral infections are responsible for 60-85% of acute exacerbations in both children and adults (8). In addition, patients with asthma seem more susceptible to respiratory viral infections, with a higher impact on pulmonary morbidity, than

healthy controls (9). In COPD synergism between bacteria and viruses has been suggested to induce infections (10). Several studies demonstrate that viral infections in vitro (11-13) and in vivo (14) enhance adherence of bacteria, like *Haemophilus influenzae* and *Streptococcus pneumoniae*, to epithelial cells. This way viral infection might facilitate bacterial infections.
In contrast to other chronic lung diseases like asthma and COPD, in patients with CF data on the role of respiratory viral infections and its interaction with bacteria are almost lacking.

SCOPE AND OUTLINE OF THIS THESIS

In this thesis we hypothesize that patients with CF have an increased susceptibility to viral infections compared to healthy controls. Derived from this hypothesis we focused on clinical and pathophysiological aspects of respiratory viral infections in children with CF, especially on the possible synergism between viruses and bacterial infections. We encountered the following questions:

1. Do children with CF have an increased prevalence or a different distribution of respiratory viral infections compared to healthy controls?
2. Is the clinical impact respiratory viral infections in CF patients increased compared to healthy controls?
3 Are respiratory viral infections associated with bacterial acquisition in CF?
4. Can we find mechanisms explaining possible synergism between respiratory viruses and bacteria?

At the start of the study we reviewed the actual knowledge about the role of respiratory viral infections in CF (Chapter 2). Chapter 3 focuses on the prevalence and clinical impact of respiratory viral infections in young children with CF compared to healthy controls. In chapter 4 we study whether young children with CF have an increased susceptibility to one of the most prevalent respiratory viruses, i.e. human rhinovirus. Chapter 5 describes the differences in prevalence of respiratory viruses between symptomatic and a-symptomatic episodes in healthy children and children with CF. In chapters 6 and 7 we focus on prevalence and diagnosis of bacterial acquisition associated with respiratory viral infections. In chapter 8 we investigate

binding of *P. aeruginosa* to normal and CF epithelial cells after respiratory syncytial virus (RSV) infection in vitro, and try to find mechanisms that explain increased binding of bacteria.

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Chapter 2

Respiratory viral infections in cystic fibrosis: a review

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ABSTRACT

Respiratory viral infections in CF patients are associated with an increase in morbidity at short and long term. Viral infections have a greater impact on CF patients compared to non CF controls. They result in increased respiratory symptoms, deterioration of Shwachman and radiological scores, prolonged hospitalizations, a persistent decrease of pulmonary function, increased use of antibiotics and a higher frequency of exacerbations at follow up. In addition, interaction between viruses and bacteria in CF is suggested. Some studies observe increased new bacterial colonisation and raised anti-pseudomonal antibodies in episodes of respiratory viral infections. Experimental data suggest that increased virus replication, impaired specific anti-bacterial defense and increased adherence of bacteria play a role in the pathogenesis of respiratory viral infections in CF. Further knowledge about the role of viruses and interaction with bacteria in CF lung disease might result in new therapeutic strategies to improve prognosis of CF patients.

1. INTRODUCTION

CF is characterized by chronic inflammation, bacterial colonisation and recurrent infections of the lung, resulting in irreversible deterioration of lung function and early death. *Pseudomonas aeruginosa* is one of the most important pathogens. Chronic colonisation of the airways with *P. aeruginosa* is associated with progressive and irreversible lung disease. Besides a constitutive inflammatory state CF typically has a course of exacerbations and remissions of pulmonary complaints, suggesting that external factors influence this course. Respiratory viruses are known to cause significant morbidity in infants, elderly and the immuno-compromised. In asthma up to 80% of exacerbations in children are associated with viral infections (1).
In this review, we summarize current knowledge of epidemiology and clinical impact of viral infections in CF. Secondary, we describe experimental data concerning the pathogenetic role of respiratory viruses in CF pulmonary disease.

2. CLINICAL STUDIES ON VIRAL INFECTIONS IN CF

2.1 Viral epidemiology in CF:

Viral infections are often associated with worse respiratory symptoms in CF patients. In 13-52% of patients with an increase in respiratory lower tract symptoms a viral pathogen is detected (2-10), with higher percentages in younger than in older patients (7). In 40 % of cases with a pulmonary exacerbation a virus was detected, compared to only 9% in those with a stable clinical condition (2).
Many different viruses are reported (Table 1). Frequencies are influenced by the detection method used by the individual investigator. Respiratory syncytial virus (RSV) represents 9-58% of all reported viruses, with the highest incidence in young children (2,3). Influenza A and B take 12-27%, or even up to 77% in one small study (5). Parainfluenzavirus is found in lower frequencies, only one study showing a contribution of 43% (7). Adenovirus is reported in 8-15% of all viruses. Most studies failed to detect rhinovirus, probably because the use of serology or culture. Serology is difficult due to the large number of rhinovirus subtypes and culture is relatively insensitive. Polymerase chain reaction (PCR) is up to eleven times more sensitive to detect rhinoviruses in children (11). The only study using PCR demonstrated rhinoviruses in 58% of cases with respiratory complaints (10). Other viral agents like enteroviruses, Epstein Barr virus and cytomegalovirus have been

shown to play a role in CF respiratory symptoms in low frequencies. Coronavirus was reported only once with a frequency of 28% (8).

2.2 Clinical impact of viral infections:

Respiratory viral infections occur in equal frequency in CF patients and healthy controls or sibs (4,9,12). Also, the frequency of virus infections seems not to be associated with pre-existing clinical condition (12), although one study reported a possible association (4). However, the clinical impact in CF patients is far beyond the virus related morbidity in healthy controls (4,9,12).

In CF patients viral upper respiratory tract infections are associated with lower respiratory tract symptoms in 31% - 76% (5,13). Respiratory viruses were detected in 40% of patients who needed hospitalization (2,3). CF patients were hospitalised for 10-22 days (2,3,12,14), while none of a matched non-CF control group needed hospital care (4,12).

Viral infections also cause increased long term respiratory morbidity in CF patients. RSV infection resulted in a prolonged oxygen need (mean 2.9 months) in 5/7 hospitalised young CF children (2). Several studies demonstrated a deterioration of Shwachman scores (10,12-14) or radiological scores (2,3,14) at long term follow up after respiratory viral infections. The severity of deterioration was related to the frequency of respiratory viral infections (12,13) or hospitalizations (2). Patients with virus associated lower respiratory tract infections had more frequently pulmonary exacerbations or hospitalizations (6,12,14) and a higher use of antibiotics (9,10,13) at long term follow-up, compared to CF patients without virus associated lower respiratory tract infections.

2.3 Impact on pulmonary function:

Respiratory viral infections in CF patients are associated with a decline in pulmonary function. Pulmonary function testing was performed in 9 of 12 clinical studies (Table 2).

FEV1 and FVC declined after virus associated lower respiratory tract infection in CF patients > 6 years (6,10,12,13). A high frequency of viral upper respiratory tract infections was associated with a large decline of FEV1 and FVC (13). Only one study showed no effect on FVC or RV/TLC (9).

A persistent decline of pulmonary function after viral infections was also found in CF patients < 6 years (4). There was a decline in V'maxFRC and a rise in FRC, especially after RSV infection. V'maxFRC increased during follow up in healthy

controls and CF children with upper respiratory tract symptoms, but deteriorated in CF children with lower respiratory tract infections.

2.4 Conclusion clinical studies:

Viruses are frequently isolated in CF patients with respiratory symptoms. The frequency and distribution of respiratory viruses is similar in CF patients and controls. RSV, influenza virus, parainfluenzavirus and adenovirus are the most frequently reported, but other viruses may be underreported for technical reasons. Respiratory viral infections in CF patients are associated with an increase of morbidity at short and long term. They result in increased respiratory symptoms, deterioration of Shwachman and radiological scores, prolonged hospitalizations, increased use of antibiotics and a higher frequency of exacerbations at follow up. In contrast to healthy controls, viral infections often result in a persistent decrease of pulmonary function in CF patients.

3. BACTERIAL-VIRAL INTERACTION IN CF

3.1 Clinical studies:

The airways of most young children with CF are colonised with *Haemophilus influenzae* and *Staphylococcus aureus*. With increasing age pseudomonal colonisation varies from 42% to 100% (4,6,8-10,13,14), with additional *Burkholderia cepacia* colonisation in older patients (6,8,10). In just a few patients no bacteria are detectable. Several data from clinical studies suggest interaction between viruses and bacteria in CF.

In 60-68% of cases new bacterial colonisation is found during the viral season (15). New bacterial colonisation predominantly occurs within three weeks after a viral upper respiratory tract infection (13). More specifically, 85% of new pseudomonal colonisation followed a viral upper respiratory tract infection within 3 weeks (13). In 35% of patients who were hospitalised for a viral lower respiratory tract infection pseudomonal colonisation was noticed within 12-60 months (3). In 11-47% of patients with intermittent or chronic colonisation a viral infection is followed by a rise in anti-pseudomonal antibodies (7). The strongest association between a viral infection and rise in anti-pseudomonal antibodies was found after RSV infection. Twenty percent of all patients who developed chronic pseudomonal colonisation had a previous RSV infection, compared to 7% of those who did not (7).

Table 1. Distribution of respiratory viruses in case of an increase of respiratory symptoms in CF patients

Author	Positive samples	Detection method	RSV	Infl.virus AB	Parainfl.virus	Adenovirus	Picornavirus	Other
Hiatt PW ('99)	26/150	C,S	21%	26%	15%	15%	23%	*
Armstrong D ('98)	14/26	I,C	40%	27%	13%	*	20% (13% RV)	*
Collinson J ('96)	51/119	P	*	*	*	*	100% (41% RV)	*
Smyth AR ('95)	44/157	I,C,S,P	9%	12%	12%	9%	58 % RV	*
Pribble CG ('90)	23/80	C,S	14%	41%	5%	*	14 % (9% RV)	26% CV
Hordvik NL ('89)	13/35	C,S	15%	77%	0%	*	8% RV	*
Ong ELC ('89)	11/92	I,C,S,P	0%	27%	*	9%	55 % (45% RV)	9% CMV
Ramsey BW ('89)	98/398	C,S	21%	12%	29%	7%	29 % (26%RV)	2% HSV
Abman SH ('88)	12/30	I,C	58%	0%	18%	8%	8% EV	8% CMV
Wang EL ('84)	105	C,S	23%	25%	40%	12%	*	*
Petersen NT ('81)	63/332	S	19%	24%	43%	14%	*	*

*I = immunofluorescence; RV = rhinovirus; C = culture; CV = coronavirus 229E; S = serology; EV = enterovirus; P = PCR; CMV = cytomegalovirus; * = not mentioned; HSV = herpes implex virus*

Table 2. Outcome data in clinical studies relating viral infections in CF patients

Author	Pulmonary function testing	URTI	Shwachman score	Radiologic score	Hospitalization	Antibiotics
Hiatt PW	FRC, V'maxFRC	Yes	Yes	Brasfield	Yes	No
Armstrong D	No	No	No	No	Yes	Yes
Collinson J	FVC, FEV1 (>6 yr)	Yes	Yes	Chrispin-Norman	Yes	Yes
Smyth AR	FVC, FEV1	Yes	Yes	Chrispin-Norman	No	Yes
Winnie GB	FVC, FEV1	No	Yes	Brasfield	Yes	No
Pribble CG	FVC, FEV1, FEF25, PEF	Yes	Yes	Brasfield	Yes	No
Hordvik NL	PEF (daily), FVC, FEV1, FEF25-75	No	No	No	No	No
Ong ELC	No	Yes	No	No	No	No
Ramsey BW	FVC, FEV1, FEF25-75, TLC, RV, RV/TLC	No	Yes	No	No	Yes
Abman SH	No	Yes	No	Brasfield	Yes	No
Wang EL	FVC, FEV1, FEF25-75, TLC, RV, RV/TLC	Yes	Yes	Brasfield	Yes	Yes
Petersen NT	No	Yes	No	No	Yes	No

*URTI = upper respiratory tract infection; * = not mentioned*

3.2 Experimental data on mechanisms:

Experimental data on the effects of viral infections in CF are scarce.

Some studies suggest higher virus replication and impairment of the innate host defense in CF. Intrapulmonary influenza virus titres were significantly increased in mice with chronic *P. aeruginosa* infection, compared to control mice (16). Increased virus replication was also found after parainfluenzavirus infection of CF human airway epithelial cells, compared to controls (17). Increased virus replication might explain the more severe and prolonged symptoms during viral infections in CF patients compared to healthy controls.

One of the possible causes of increased virus replication and of virus persistence might be a reduced production of respiratory nitric oxide (NO), which is an important part of the innate anti-viral defense. Increased production of NO protects against viral infections (18). In CF-patients expression of the NO producing enzyme NO synthase type 2 (NOS2) is considerably decreased, while the IFN-γ dependant antiviral host defense is intact.

An aberrant immune response to viral infections has also been found in other chronic respiratory illnesses, like asthma (19,20). It might be suggested that repeated viral infections lead, in the presence of an aberrant immune response, to an increased virus replication and hyper-inflammation with increasing symptoms and pulmonary damage.

Viral infections might also facilitate infections with colonizing bacteria in patients with CF. Viral infections cause destruction of the epithelial barrier with loss of cilia and loss of tight junctions. This results in increased permeability and exposure of the respiratory basement membrane, leading to increased possibilities for bacteria to bind (21). Bacterial adherence to virus infected cells is enhanced because bacteria can use viral glycoproteins and other virus induced receptors on the host cell membrane as bacterial receptors (22-24).

A few studies suggest that viral infection leads to modulation of the immune response, impairing specific anti-bacterial defense (25-27). In a chronic *P. aeruginosa* infected mouse model fatal pneumonia was induced by pneumococcal infection following influenza virus infection. In the absence of the virus the mice survived the pneumococcal super-infection (16). Influenza infection caused a significant increase in inflammatory cells and cytokine release, and suppressed neutrophil function.

Earlier data suggest interaction between specific viruses and bacteria in CF. Influenza virus seems to play a role in the adherence of *P. aeruginosa* to respiratory

epithelial cells. Uninfected and influenza-infected murine tracheas were exposed to six different strains of *P. aeruginosa*. All of the strains adhered to desquamating cells of the infected trachea, but not to normal mucosa or regenerating epithelium (28).

In summary, experimental data suggest increased virus replication in CF which can result in severe pulmonary damage and which can facilitate infections with bacteria colonizing the CF lungs. More data, especially in the human model, are necessary to further elucidate the role of viruses in CF lung disease.

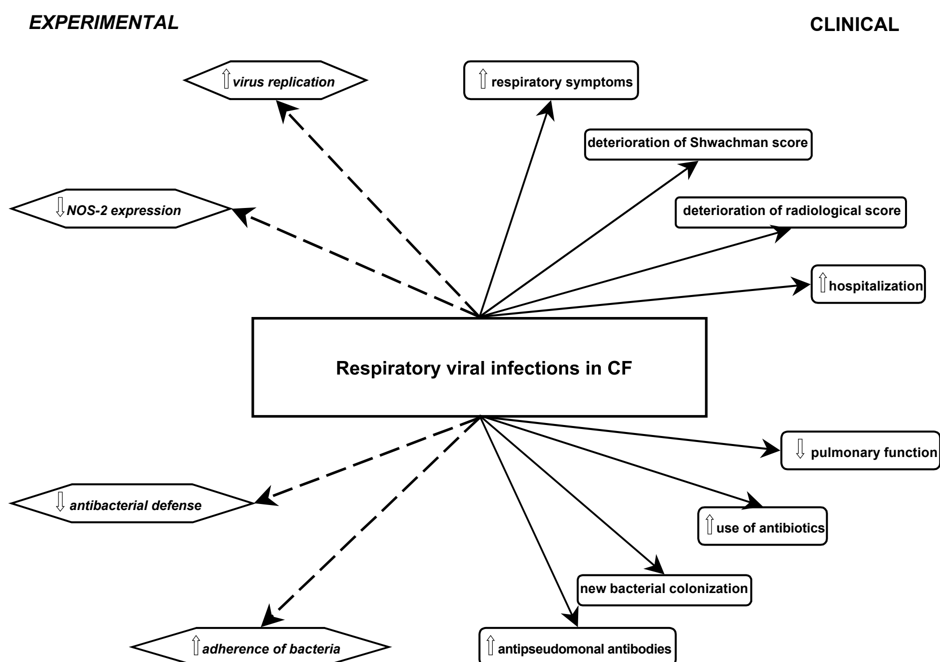

Figure 1. Overview of clinical and experimental data on respiratory viral infections in CF

4. IMPLICATIONS

Further knowledge about the role of viruses in CF lung disease might result in new therapeutic strategies to improve prognosis of patients with CF.

Prevention of viral infections in patients with CF might be reached by active or passive immunization. At present, annual influenza vaccination is advised to all

CF patients, although there are some doubts about its effectiveness (4,9,29,30). Passive immunization against RSV can be performed with paluvizumab, but data on effectiveness in CF patients are lacking. Active vaccination against RSV in CF patients is in development (31).

Another strategy might be inhibition of virus replication. Effective antiviral agents are available. Amantadine (32), zanamivir (33) and oseltamavir (34) can be used against influenza virus infection. Treatment with oseltamavir improved survival from 0% to 75% in a mouse model of secondary pneumococcal pneumonia after influenza virus infection, even when therapy was delayed for up to 5 days after influenza virus infection (35). Ribavirine can possibly be used against RSV (36) and plecoranil against picornaviruses (37). Beside these specific virus inhibitors, there is some indication that interferon (17) and statins (38) can interfere with virus replication.

Influencing the interaction between viruses and bacteria could be a next pathway to diminish respiratory morbidity in CF patients. Low-threshold use of antibiotics during viral infections in patients with CF might prevent secondary bacterial infections, but data are lacking. Further knowledge about specific interaction between viruses and bacteria might lead to development of new therapeutic options.

Up till now, there is no evidence for most suggested options in general, or more specifically in CF. Efforts should be made to design studies exploring these options.

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Chapter 3

Prevalence and impact of respiratory viral infections in young children with cystic fibrosis: a prospective cohort study

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ABSTRACT

Objective

The prevalence and impact of acute respiratory viral infections in young children with cystic fibrosis (CF) still needs to be elucidated. We aimed to investigate differences in upper and lower respiratory tract symptoms in relation to respiratory viral infections detected with polymerase chain reaction between young children with CF and healthy controls.

Methods

In a 6-month winter period 20 young children with CF and 18 age-matched healthy controls were contacted twice a week to detect symptoms of an acute respiratory illness (ARI). If any symptom was present a home visit was made for physical examination and nasopharyngeal swab for viral analysis. In addition parents were instructed to take nasopharyngeal swabs every two weeks.

Results

Children with CF and healthy controls had a similar frequency of ARI, 3.8 ± 1.0 SD and 4.2 ± 1.7 SD episodes, respectively. Although there were no significant differences in upper respiratory tract symptoms the children with CF had longer periods of lower respiratory tract symptoms (22.4 ± 22.2 SD versus 12.8 ± 13.8 SD days, $p=0.002$) and a higher mean severity score per episode (2.35 ± 0.64 SD versus 1.92 ± 0.46 SD, $p=0.02$). In addition, a similar increase in upper respiratory tract symptom score was associated with a significantly higher increase in lower respiratory tract symptom score in children with CF. No differences in the seasonal occurrence and distribution of respiratory viruses were observed, with picornaviruses and coronaviruses being the most prevalent.

Conclusions

Although there were no differences in seasonal occurrence and distribution of PCR detected respiratory viruses, ARI are frequently associated with increased lower respiratory tract morbidity in young children with CF. This might suggest new therapeutic strategies to improve prognosis of patients with CF.

INTRODUCTION

Viral infections are the most common cause of acute respiratory symptoms in otherwise healthy children. The relationship between viral infections and chronic respiratory diseases such as asthma has long been recognised. It has been estimated that up to 85% of asthma attacks in children in the community are associated with respiratory viral infections(1). The role of respiratory virus infections in patients with cystic fibrosis (CF) is, however, less clear.

A few prospective studies suggest that the clinical impact of respiratory viral infections on patients with CF is more severe than the virus-related morbidity in healthy controls. Viral respiratory tract infections in children and adults with CF appear to be associated with a significant risk for pulmonary exacerbation, hospitalisation, and a decline in lung function (2-6). In addition, respiratory viral infections seem to predispose to secondary bacterial colonisation and infection (7,8). The differences in clinical impact of respiratory viral infections between patients with CF and healthy controls might be due to differences in frequency or distribution of viruses, or due to patient factors related to CF. The few available studies until now yielded no evidence that viral infections are more common in individuals with CF (3,6,9).
However, several methodological characteristics of these studies need to be addressed. Most studies report data only from hospitalised patients, they lack the use of healthy controls, or failed to present longitudinal data. In addition, earlier reports may have underestimated the prevalence of viral infections because relatively insensitive viral detection methods were used, like serology and tissue cultures (2,5,6,9). More recently, polymerase chain reaction (PCR) has improved the detection of respiratory viruses (10). Finally only sparse data about the role of newly-detected viruses implicated in lower respiratory tract infections in young children (e.g. human metapneumovirus and coronavirus NL63) are available in relation to CF.

This study was designed to investigate differences in the prevalence and clinical impact of virus-associated acute respiratory illnesses (ARI) between young children with CF and healthy controls, using sensitive PCR detection methods.

METHODS

Study design and subjects

A prospective longitudinal study was conducted during a 6-month respiratory virus season, from November until May. A total of 20 young children with CF aged 0-7 years were enrolled and completed the study. Diagnosis of CF was based on the presence of two or more of the following criteria: sweat chloride > 60 mEq/L, positive genetic testing and clinical features consistent with CF. All children had been negative for *Pseudomonas aeruginosa* until start of the study. A total of 19 age-matched healthy controls were enrolled; 1 failed to complete the study. All had a negative history of respiratory or other major disease. The study was approved by the local medical ethics review committee (UMC Utrecht) and the parents gave written informed consent.

Study Protocol

At enrollment, demographic data, the child's medical history, and environmental risk factors were documented using questionnaires. Physical examination was performed and throat or cough cultures were obtained to determine bacterial colonisation. Parents were instructed to take nasopharyngeal swabs for viral PCR from their child every two weeks. This swab was sent within 24 hours by mail to the study coordinator. Throughout the study period, one of two physicians as study coordinators (BE or MZ) contacted the families twice a week by telephone or e-mail. Any symptom of a respiratory illness was assessed with a standard questionnaire and directly tabulated. When an ARI was identified, the study coordinator made a home visit within 48 hours for physical examination and a nasopharyngeal swab for viral PCR. CF patients were treated according the guidelines of the CF Centre Utrecht. Antibiotics were prescribed by the treating pediatrician or family doctor. No specific anti-viral treatment or prophylaxis was prescribed to CF patients or healthy controls.

MEASURES

Respiratory illness questionnaire

The twice-weekly respiratory illness questionnaire concerned both upper

respiratory tract symptoms (URTS) and lower respiratory tract symptoms (LRTS), as similarly defined in prior studies (3,9). Recorded parental reported URTS were: coryza (rhinorrhea or nasal congestion); sore throat; ear ache; ear discharge and temperature > 38°C. A score of 0 was given if absent, 1 if present, giving a maximum score of 5 points. Recorded parental reported LRTS were: (increase in) cough; (increase in) sputum production or productive cough; shortness of breath; reduced exercise tolerance, decreased appetite and malaise. Based on the patient diary information a score of 0 was given if absent, a score of 1 if there was a moderate increase compared to baseline, and a score of 2 if severe, giving a maximum score of 12. An ARI was diagnosed if the child had coryza, or one of the other URTS in combination with temperature > 38°C, or a total score of LRTS ≥ 2. To diagnose a new ARI episode the total symptom score had to be returned to 0 for at least 1 week.

Physical examination

Physical examination was performed at the beginning and at the end of the study (baseline if no respiratory symptoms), and at each home visit (with respiratory symptoms) after an ARI was diagnosed. It comprised standardized measurements of weight, respiratory rate per minute and transcutaneous O2 saturation. Assessment of retractions, ear-throat-nose examination and auscultation of the lungs were made. Signs of rhinitis, pharyngitis, otitis, and abnormal auscultation (rales or crackles) were assessed.

Virus detection

Respiratory virus infection was established by detection of virus from nasopharyngeal swabs using standard PCR techniques as earlier described (11). Specimens were collected with a cotton-tipped swab from both the nose and the posterior oropharynx. Both swabs were inserted into a single vial containing Gly-medium with pimaricine 0.1 mg/ml as viral transport medium. Samples were stored at -20°C until analysis. PCR was performed at the National Institute of Public Health and the Environment (Bilthoven, The Netherlands). Both nasopharyngeal swabs were tested for rhinovirus, enterovirus, coronavirus 229E, coronavirus OC43, coronavirus NL63, human metapneumovirus, respiratory syncytial virus A and B, influenzavirus A and B, adenovirus, as well for *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* by using standard PCR techniques.

Statistical analysis

Comparisons of the distributions of categorical variables between groups were examined using Chi-square or Fisher's exact test and the means of continuous variables using the two-tailed Student's *t* test of independent variables or the nonparametric Mann Whitney U test.

Correlation between total (seasonal) URTS and total (seasonal) LRTS score per individual for CF patients and healthy controls was assessed with Pearson's standard regression analysis. Multiple linear regression analysis was used to analyse differences in increment for total LRTS per total URTS between CF and healthy controls. A significance level of $p<0.05$ was used throughout. The analyses were performed using the Statistical Package for the Social Sciences (SPSS, version 12.0, Chicago, IL USA).

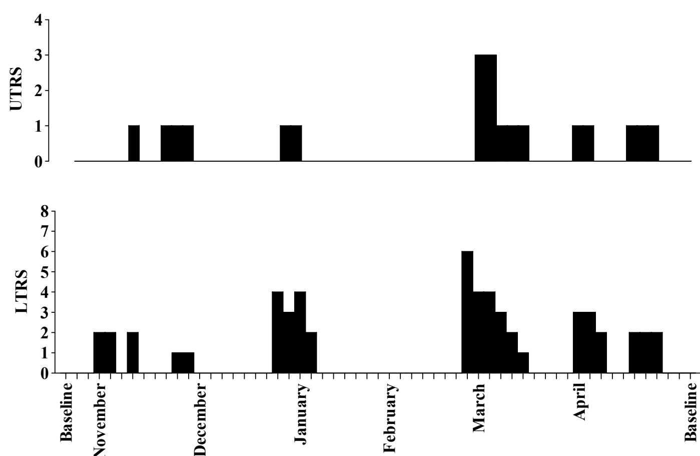

Figure 1. Example of a chart of respiratory symptom scores for one patient with CF taking part in the study. The horizontal axis represents the 6-month study period, the vertical axis presents the symptom scores. The arrows represent home visits.

RESULTS

Baseline

There were no significant differences between the children with CF and the healthy controls for age, weight, respiratory rate, number of siblings, day-care or school attendance at baseline (Table 1). The children with CF had lower baseline SpO2 values ($p<0.01$), 65% had been vaccinated for influenza and 70% used antibiotic

prophylaxis throughout the study period versus none of the healthy controls (both p<0.001). Children with CF had more often a positive bacterial throat or cough swab culture than healthy controls at start of the study (p<0.05), mainly for *Staphylococcus Aureus* and *Haemophilus influenzae*.

Table 1. Characteristics of the study group

	CF (n=20)	Controls (n=18)	p-value
Age (yr)	3.5 ± 2.1	3.5 ± 1.7	0.96
Homozygous ΔF508	10 (50%)	NA	
Weight (kg)	15.5 ± 5.8	16.2 ± 4.3	0.67
Respiratory rate/min[b]	25.9 ± 6.6	23.9 ± 6.4	0.23
O2-saturation (%)[c]	97.5 ± 1.0	98.2 ± 1.0	0.009[a]
Number of siblings	1.4 ± 0.9	1.4 ± 1.0	0.95
Day-care / school (days/week)	2.4 ± 1.8	2.8 ± 1.7	0.49
Influenza vaccination	13 (65%)	0	<0.001[a]
Antibiotic prophylaxis	14 (70%)	0	<0.001[a]
Home visits (per individual)	3.8 ± 1.99	3.6 ± 1.98	0.25
Missed visits (total number per group)	12	16	
Positive culture (start of the study)	16 (80.0%)	8 (44.4%)	0.023[a]
Staphylococcus aureus	12 (50.0%)	2 (20%)	0.002[a]
Haemophilus influenzae	6 (25.0%)	0	0.021[a]
E. Coli	2 (8.3%)	1 (10%)	1.0
Other gram negative bacteria	4 (16.7%)	5 (50%)	0.71
Streptococcus pneumoniae	0	0	
Streptococcus hemolyticus	0	2 (20%)	0.22

Data are expressed as mean value per individual ± SD or absolute values with percentages. NA = not applicable. [a]statistically significant, p < 0.05; [b]measured if no complaints at beginning and/or end of the study, n=33 in CF and n=30 in healthy controls; [c]measured if no complaints at beginning and/or end of the study, n=29 in CF and n=30 in healthy controls.

Respiratory illnesses

Data from the 6-month period were plotted for each child to allow visual inspection of respiratory illness patterns (Figure 1). The coded data were analysed. Based on data from patient diary records controls and children with CF had a similar number of ARI, resulting in a similar number of home visits. No differences for mean URTS score per episode were found (Table 2). Children with CF tended to have longer periods of URTS, however the difference did not reach statistical significance (p=0.1). For LRTS there were significant differences between children with CF and healthy controls in both duration and severity of the episodes.

Children with CF had longer periods of LRTS ($p<0.01$) and had higher mean LRTS scores per episode ($p<0.05$). Physical examination during ARI periods showed a significantly higher increase of respiratory rate ($p<0.001$) and a stronger decrease of SpO2 ($p<0.01$) in children with CF, both compared to baseline measurements. In addition, retractions ($p<0.001$) and abnormal auscultation ($p<0.01$) were observed more frequently in children with CF. Antibiotic courses were prescribed by a family physician or treating specialist 24 times to 13 children with CF, compared with 2 courses to 2 healthy controls ($p<0.01$).

Table 2. Clinical data of the study group

	CF (n=20)	Controls (n=18)	p-value
ARI episodes	3.8 ± 1.0	4.2 ± 1.8	0.36
Upper respiratory tract symptoms			
Duration of episodes (days)	22.2 ± 21.3	16.4 ± 19.5	0.1
Severity score per episode	1.11 ± 0.14	1.12 ± 0.32	0.89
Lower respiratory tract symptoms			
Duration of episodes (days)	22.4 ± 22.2	12.8 ± 13.8	0.002[a]
Severity score per episode	2.35 ± 0.64	1.92 ± 0.46	0.022[a]
Physical examination			
Δ Respiratory rate/min[b]	6.6 ± 8.0	2.1 ± 5.6	<0.001[a]
Δ O2 saturation (%)[b]	2.0 ± 2.1	0.8 ± 1.7	0.001[a]
Retractions	13/77 (16.9%)	0/65 (0%)	<0.001[a]
Abnormal auscultation	25/77 (32.5%)	8/65 (12.3%)	0.005[a]
Antibiotic courses	24	2	0.001[a]

Data are expressed as mean values per individual ± SD or absolute values with percentages. [a]statistically significant, $p < 0.05$; [b]differences between measurements with and without complaints.

No differences in separate URTS score items were found between CF patients and healthy controls (Table 3). Temperature > 38°C was reported more frequently in healthy controls, while the LRTS score items (increase in) cough ($p<0.05$), (increase in) sputum or productive cough ($p<0.01$) and shortness of breath ($p<0.05$) were reported more often in children with CF. To study the relationship between URTS and LRTS the sum of all URTS scores was plotted versus the sum of all LRTS scores of the whole period per individual (Figure 2). High URTS scores were associated with high LRTS scores for each group (R Sq Linear = 0.40 for healthy controls and R Sq linear = 0.55 for children with CF). In children with CF an increase in URTS score was associated with a significantly higher increase in LRTS score compared to healthy controls ($p<0.01$).

Table 3. Frequency of separately scored upper and lower respiratory tract symptoms

	CF (n=20)	Controls (n=18)	p-value
Upper respiratory tract symptoms			
Ear pain	13 (0)	12 (0.5)	0.21
Ear discharge	14 (0)	9 (0)	1
Sore throat	14 (0)	40 (1)	0.9
Coryza	409 (22)	335 (16)	0.47
Temperature > 38°C	25 (1)	35 (2)	0.046[a]
Lower respiratory tract symptoms			
Increase cough	400 (21)	233 (10)	0.025[a]
Increase sputum/productive cough	297 (15)	106 (2.5)	0.004[a]
Shortness of breath	80 (2)	18 (0)	0.025[a]
Reduced exercise tolerance	58 (1)	34 (0.5)	0.51
Reduced appetite	132 (5)	81 (4)	0.41
Malaise	69 (2)	63 (3)	0.63

Data are expressed as total reported frequencies of the group with median per patient between parentheses. [a]statistically significant, $p < 0.05$ (Mann Whitney U test)

Detection of respiratory pathogens

PCR analysis for viral and atypical pathogens was performed in a comparable number of specimens in both groups, with a mean number of samples per patient of 17.6 ± 2.64 SD in CF versus 16.3 ± 2.16 SD in controls (p=0.1).

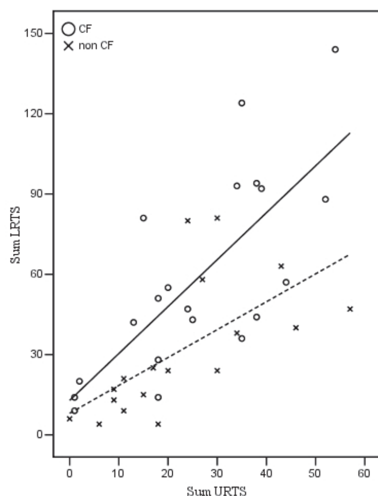

Figure 2. Relationship between the sum of all URTS scores and LRTS scores of the 6-month study period per individual in 20 children with CF (solid line, R Sq linear = 0.55) and in 18 healthy controls (dashed line, R Sq Linear = 0.40). A similar increase in URTS score gives a significantly ($p < 0.01$) higher increase in LRTS score in children with CF compared to healthy controls (multiple linear regression analysis).

Table 4 presents the seasonal occurrence of the different pathogens during the study period. No significant differences were found between the two groups. All children in both groups had an infection with rhinovirus during the study period, followed by enterovirus and coronavirus as the most prevalent viruses.

Table 4. Seasonal documented incidences of viral and atypical pathogens

	CF (n=20)	Controls (n=18)	p-value
Rhinovirus	20 (100%)	18 (100%)	NA
Enterovirus	7 (35%)	10 (55.6%)	0.20
Corona 229E	18 (90%)	13 (72.2%)	0.22
Corona OC43	15 (75%)	13 (72.2%)	1
Mycoplasma pneumoniae	12 (60%)	7 (38.9%)	0.19
Chlamydia pneumoniae	12 (60%)	9 (50%)	0.54
Respiratory syncytial virus A	2 (10%)	2 (11.1%)	1
Respiratory syncytial virus B	7 (35%)	4 (22.2%)	0.39
Corona NL-63	4 (20%)	4 (22.2%)	1
hMetapneumovirus	3 (15%)	4 (22.2%)	0.69
Adenovirus	1 (5%)	1 (5.6%)	1
Influenza A	0	1 (5.6%)	0.47
Influenza B	1 (5%)	1 (5.6%)	1

Data are expressed as absolute number and percentages of individuals with at least one PCR for the tested pathogens. NA = not applicable.

DISCUSSION

Our study shows that acute respiratory illnesses are associated with increased lower respiratory tract symptoms in children with CF, compared to healthy controls. While there is no difference in the frequency of ARI and mean URTS scores, children with CF have longer and more severe periods of LRTS. In addition, a similar increase in URTS score is associated with a significantly higher increase in LRTS score in children with CF. No differences in the occurrence and distribution of respiratory viruses were observed between the two groups. These findings argue that patient factors related to CF play an important role in the differences in clinical course.

Our findings underline that CF is a disease that involves primarily the lower airways. During ARI patients with CF more frequently present LRTS as cough, sputum production and shortness of breath. In our study physical examination confirmed

the reported symptoms. In children with CF an ARI causes a significantly higher increase of respiratory rate, a stronger decrease of SpO2, more retractions and abnormal auscultation compared to non CF controls. Earlier data from different study settings showed that URTS are associated with LRTS in 31-76% of patients with CF (2,12). Upper respiratory tract infections are related to a decline in lung function (2,3,5,6), a higher use of antibiotics (5), and a high hospitalisation rate (3,6,12,13). Surprisingly, in our study none of the children in either group were hospitalised during the study period. This might be due to the young age of the children; probably most of them had not developed important structural lung abnormalities before this study. Also, the extensive use of antibiotic prophylaxis and low threshold prescription of antibiotic courses in the children with CF in our centre might have prevented hospital admissions.

In this study we used upper and lower respiratory tract symptom scores, as described by others (3,6,9,14,15). A lack of validated symptom scores in young children with CF might result in reporter- and observer bias. However, in our study both symptoms (as reported by the parents), clinical findings of the investigators and objective parameters (respiratory rate and oxygen saturation) all pointed into the same direction. This argues for a reasonable internal validity of our symptom scores. In our study population the diagnosis ARI was never made on non-respiratory symptoms only.

Several patient factors in CF might be suggested to cause the differences in clinical impact of a viral infection on LRTS between children with CF and healthy controls. Children with CF were more frequently colonised with potential pathogenic bacteria at enrolment. The higher prevalence of *Staphylococcus aureus* in children with CF is in line with expectations and is known to cause pulmonary morbidity (16). Apparently, the relatively high rate of antibiotic prophylaxis targeting *S. Aureus* in the children with CF can not prevent these differences.
Secondly, the higher impact of a viral infection on LRTS might be the manifestation of a diminished specific anti-viral defense. Zheng et al. found a higher viral replication in CF airway epithelium and increased production of pro-inflammatory cytokines due to an impaired NOS-2 signalling pathway, which is an important part of the innate anti-viral defense (17). More recently Colasurdo et al. showed that CF mice have an exaggerated inflammatory response, but impaired ability to clear respiratory syncytial virus (18). It might be speculated that this higher

viral replication and exaggerated inflammation causes more local damage in the airways. In our study virus associated ARI occurred equally in children with CF and healthy controls; however, we did not quantify viral loads which could have further supported these findings. Furthermore, it might be very interesting to investigate the differential influence of the individual viruses on the level of lower respiratory tract symptoms in CF patients compared to healthy controls in a larger study. Due to the relatively small numbers of patients our data lack sufficient power to adequately perform such analysis.
Thirdly, synergism between bacteria and viruses might contribute to the higher impact of ARI. Several studies suggest that respiratory viral infections facilitate *P aeruginosa* acquisition and colonisation (2,3,7,8,13,19). In patients with intermittent or chronic *P. aeruginosa* infection a viral infection is often followed by a rise in anti-pseudomonal antibodies (8). It might also be speculated that structural abnormalities of the lower airways in patients with CF could be responsible for the increased lower respiratory tract morbidity. In contrast to earlier studies (4,6,9,12,20) in our study we focussed on young children with, as yet, few structural abnormalities in their lower airways. In older patients with more advanced lower airways abnormalities the differences may be even more pronounced.

The differences in clinical impact do not appear to be related to virus-specific characteristics. The seasonal frequency and distribution of the different viruses and atypical pathogens as presented was similar in children with CF and healthy controls. Our results are comparable to earlier findings in children in the general population (21). In CF equal numbers of viral infections were observed in CF patients and healthy controls (6,9), only one study described an increased number of viral infections in healthy controls (3). However, earlier studies may have underestimated the prevalence of viral infections because they used relatively insensitive detection methods, like serology and tissue cultures (2,5,6,12). We used the more sensitive PCR to detect viruses and atypical pathogens. Only a few studies in CF have used PCR for this aim. Two studies used PCR for the detection of picornaviruses; they reported 43% positive samples for picornaviruses in individuals with a cold (2), and 16% rhinovirus in case of a pulmonary exacerbation (5). A third study used PCR for detection of several viral and atypical pathogens; they found a low rate of 16% positive nasopharyngeal aspirates at regular outpatient visits, irrespective of symptoms and without comparison to healthy controls (22). Our data are to our

knowledge the first results concerning the newly-detected coronavirus NL63 in relation to CF, without differences between children with CF and healthy controls. This virus is known to be implicated in lower respiratory tract infections in young children in general (23,24).

Our findings underline the importance of respiratory viral infections in lower respiratory tract morbidity in young children with CF. Modern treatment strategies of pulmonary complaints are mainly aimed at treating bacterial infections with antibiotics and sputum evacuation. With an improving survival in young CF patients due to better anti-bacterial treatment, antiviral therapy might become a new therapeutic goal in the future. Increasing evidence that respiratory viral infections, possibly in synergism with bacteria, play an important role in irreversible local damage emphasizes the possible importance of prevention of viral infections, for example by active or passive immunization. However, influenza vaccination could not prevent an influenza infection in one patient with CF in our study. Viral inhibitors might be used in an early phase of a viral infection, antibiotic treatment or prophylaxis during a viral infection, and development of specific viral–bacterial interaction blockers might be interesting options (25).

In summary, this study clearly shows that acute respiratory illnesses are associated with increased LRTS in young children with CF, compared to healthy children. These first results of extensive viral analysis by PCR in CF demonstrate no major differences in seasonal frequency or distribution of respiratory viral infections. Patients factors related to CF seem to play an important role in these differences in clinical course. Our findings confirm the suggestion that respiratory viral infections play an important role in pulmonary morbidity in young children with CF. It might suggest new therapeutic strategies to further improve prognosis of patients with CF.

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Chapter 4

Frequency and duration of rhinovirus infections in children with cystic fibrosis and healthy controls: a longitudinal cohort study

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ABSTRACT

Background

Respiratory viral infections are an important cause of morbidity in patients with chronic respiratory diseases, such as cystic fibrosis (CF). We hypothesised that patients with CF are more susceptible to human rhinovirus (HRV) infections than healthy controls.

Methods

In a 6-month winter period 20 young children with CF (0-7 years) and 18 age-matched healthy controls were sampled bi-weekly for HRV-PCR using nasopharyngeal swabs, irrespective of respiratory symptoms. Respiratory symptoms were scored twice a week. If any symptom was present an additional sample was obtained. All HRV positive samples were genotyped to distinguish HRV subtypes.

Results

We analyzed 645 samples, with comparable total numbers of samples in both groups. HRV was detected in 40.8% of all analyzed samples. Children with CF had significantly more HRV positive samples compared to healthy controls, with a mean number (± SD) of 8.1 ± 2.3 versus 5.7 ± 2.9 samples per individual ($p<0.01$). Prolonged detection (> 2 weeks) with the same HRV subtype occurred more frequently in the CF patients ($p<0.01$). The genetic distribution and pattern of phylogenetic diversity of the different HRV subtypes was similar in both groups.

Conclusion

These are the first in vivo longitudinal data showing that HRV is detected more frequently and persists for longer periods in CF patients compared to healthy controls. This might indicate increased viral replication due to decreased anti-viral defense in patients with CF.

INTRODUCTION

There is increasing evidence that respiratory viral infections play an important role in the morbidity of patients with chronic respiratory diseases. In asthma and chronic obstructive pulmonary disease (COPD), viral infections trigger 60-85% of acute exacerbations in both children and adults(1). In patients with cystic fibrosis (CF) virus infections appear to be associated with increased respiratory symptoms and hospitalizations, a persistent decrease in pulmonary function, and increased or new bacterial colonisation (2).

Human rhinoviruses (HRV) are the most frequent cause of acute upper respiratory tract infections in humans and are usually associated with common cold (3). Moreover, there is evidence that HRV infections may not always be mild or restricted to the upper respiratory tract; they are also associated with more severe lower respiratory tract morbidity in young children, in the elderly, and in immuno-compromised patients (4). In CF, HRV infections are shown to be involved in respiratory lower tract symptoms (5-7). However, this research has been hampered by lack of healthy controls, by studies in which analyses were only done in case of symptoms, and by the insensitivity of conventional virus-detection techniques. More recently, techniques using PCR have resulted in an up to ten-fold increase in the sensitivity of HRV detection (8).

Patients with asthma have an increased susceptibility to HRV infection in vivo (9,10), which could play an important role in the frequent association between viral infections and exacerbations in asthma. Studies suggest an increased viral replication caused by an impaired innate immune defense in CF (11,12). However, earlier clinical data indicate that viral respiratory infections occur in equal frequency in CF patients and healthy control (6,13,14). Elucidating whether patients with CF have an increased susceptibility to viral infections could improve appropriate targeting of new treatments to further improve the prognosis in CF lung disease.

This study was designed to examine whether CF patients have an increased susceptibility for HRV infections compared to healthy controls. It is the first study to use sensitive PCR and sequencing techniques to detect and discriminate between different HRV subtypes. In a 6-month longitudinal cohort study we investigated

whether young children with CF have a higher rate of HRV acquisition, and whether the duration of HRV episodes differs between CF patients and healthy controls.

METHODS

Study design and subjects

A prospective longitudinal study was conducted during a 6-month winter season (from November through April). A total of 20 young children with CF aged 0-7 years were enrolled and completed the study. Diagnosis of CF was based on the presence of two or more of the following criteria: sweat chloride > 60 mEq/L, positive genetic testing, and clinical features consistent with CF. All children had been negative for *Pseudomonas aeruginosa* until start of the study. A total of 19 age-matched healthy controls were enrolled; one of these failed to complete the study. All had a negative history of respiratory or other major disease. The study was approved by the local medical ethics review committee (UMC Utrecht) and the parents gave written informed consent.

Study protocol

At enrolment demographic data, the child's medical history, and environmental risk factors were documented using questionnaires. Parents were instructed to take nasopharyngeal swabs for viral PCR from their child every two weeks, irrespective of respiratory symptoms (regular samples). The swabs were sent as soon as possible by mail to the study coordinator. Throughout the study period, one of two physicians (BE or MZ) contacted the families twice a week by telephone or e-mail. Any symptom of a respiratory illness was assessed with a standard questionnaire, concerning both upper and lower respiratory tract symptoms. When respiratory symptoms were identified an additional nasopharyngeal swab for viral detection was collected (i.e. sick samples).

Viral sampling

Specimens were collected with a cotton-tipped swab from both the nose and the posterior oropharynx. Both swabs were inserted into a single vial containing GLY medium with pimaricine 0.1 mg/ml as viral transport medium. Samples were stored at -20°C until analysis. PCR was performed at the National Institute of Public

Health and the Environment (Bilthoven, the Netherlands). Both nasopharyngeal swabs were tested for HRV.

PCR, sequencing and phylogenetic analysis

Viral RNA was isolated from 200 µl of the original sample using the High pure RNA isolation kit (Roche, Basel, Switzerland). cDNA synthesis, nested PCR and Southern blotting were carried out to detect HRV as described previously(15). In case of a positive PCR for HRV, the sample was sequenced. PCR 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 Maths, Gent, Belgium) with a maximum parsimony algorithm performing 100 bootstraps. The HRV sequences were defined as different from each other when in the analysis > 1% of the nucleotides was different. Subtypes were defined as different when sequence homologies were < 90%.

Statistical analysis

Comparisons of the means of continuous variables between groups were examined using the two-tailed Student's *t* test of independent variables or the nonparametric Mann-Whitney U test, the distributions of categorical variables using Pearson's Chi-square. A significance level of $p<0.05$ was used throughout. The analyses were performed using the Statistical Package for the Social Sciences (SPSS, version 12.0, Chicago, IL, USA).

RESULTS

Baseline

There were no significant differences between the children with CF and the healthy controls for age, number of siblings, day-care or school attendance at baseline (Table 1). Children with CF significantly more often used antibiotic prophylaxis than healthy controls.

Table 1. Characteristics of the study group

Parameter	CF (n=20)	Controls (n=18)	p-value
Age (yr)	3.5 ± 2.1	3.5 ± 1.7	0.96
Homozygous ΔF508	10 (50%)	NA†	NA†
Number of siblings	1.4 ± 0.9	1.4 ± 1.0	0.95
Day-care/school attendance (days/week)	2.4 ± 1.8	2.8 ± 1.7	0.49
Antibiotic prophylaxis	14 (70%)	0	<0.001*

Data are expressed as mean ± SD values or absolute values with percentages.
**Statistically significant, p<0.05 (t-test or Chi-square).*
†Not applicable.

Sampling

A comparable number of samples for viral analysis (regular samples and sick samples together) were obtained in both groups during the season (p=0.1, Table 2). There was no significant difference between groups in missed regular samples, with a mean per individual of 1.1 ± 1.0 SD samples in CF versus 1.2 ± 1.1 SD in non CF (p=0.95). A similar number of sick samples was collected in both groups, with a mean number of 3.8 ± 2.0 SD samples per individual in CF versus 3.6 ± 2.0 SD in controls (p=0.25).

Table 2. Sampling, rhinovirus PCR and sequencing

Parameter	CF (n=20)	Controls (n=18)	p-value
Total number of samples per individual	17.6 ± 2.6	16.3 ± 2.2	0.1
Number of HRV-positive samples per individual	8.1 ± 2.3	5.7 ± 2.9	0.007*
Number of HRV-subtypes per individual	4.9 ± 1.5	3.8 ± 1.7	0.04*
Number of children with prolonged HRV-episodes†	13 (65%)	4 (22%)	0.008*
Total number of prolonged HRV-episodes†	20	6	0.046*

Data are expressed as mean numbers ± SD or absolute values with or without percentages.
**Statistically significant, p<0.05 (t-test or Chi-square).*
†Episode = defined as same HRV subtype in 2 subsequent samples in the regular frequency of 14 days.

Rhinovirus PCR and sequencing

We analysed 645 samples. HRV was detected in 45.7% (161/352) of nasopharyngeal samples from children with CF and in 34.8% (102/293) of samples from healthy controls, which results in significantly more HRV positive samples in children with CF than in healthy controls (p=0.007, Table 2). To study whether this higher

frequency of HRV positive samples in CF patients is the result of an increased acquisition of different HRV subtypes or of prolonged detection of the same subtype, we performed sequencing of all HRV-PCR positive samples. Sequencing of HRV positive samples was successful in similarly high percentages, 82.7% ± 13.1 SD in patients with CF and 80.7% ± 20.3 SD in controls, respectively ($p=0.97$). Children with CF had a higher mean number of different HRV subtypes per individual compared to healthy controls ($p<0.05$). In addition, more children with CF had at least one prolonged episode defined as the same HRV subtype in 2 subsequent samples in the regular frequency of 14 days ($p=0.008$), and a higher absolute number of prolonged episodes ($p=0.046$) compared to healthy controls. The distribution and pattern of phylogenetic diversity of the several HRV subtypes was similar in both groups (Figure 1 and 2).

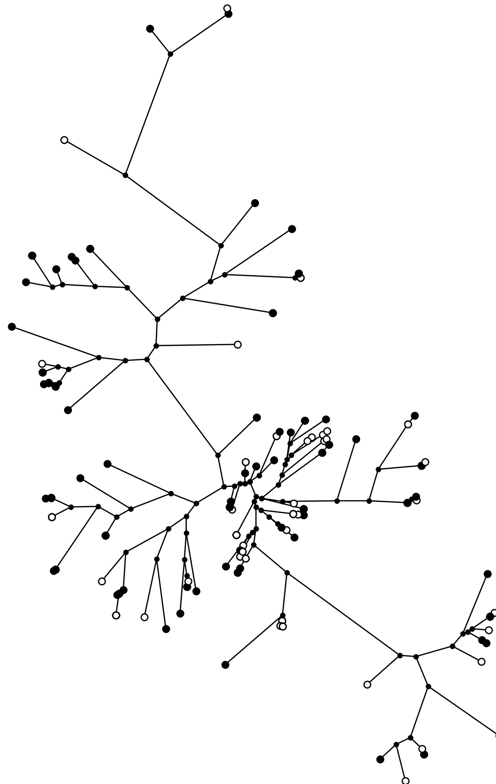

Figure 1. Phylogenetic analysis of HRV positive samples. Closed symbols indicate CF patients and open symbols healthy controls. Sequence data were analyzed with maximum parsimony algorithm performing 100 bootstraps. Subtypes were defined as different when sequence homologies were < 90%.

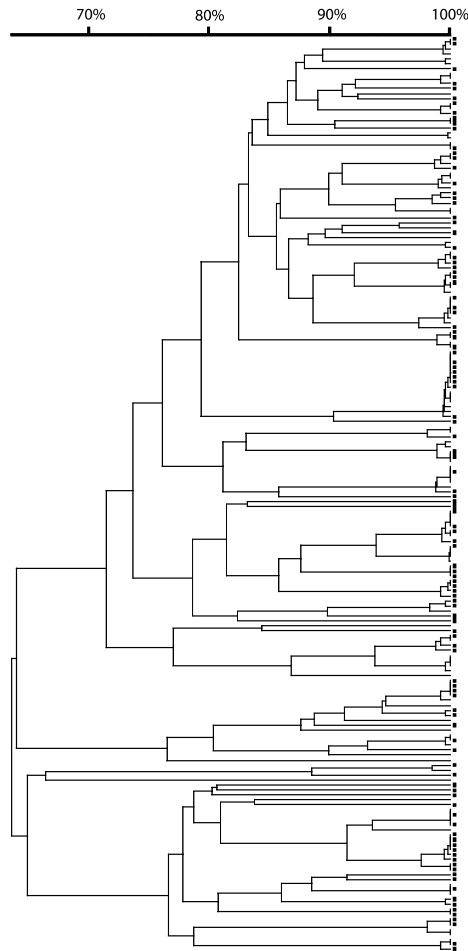

Figure 2. Phylogenetic analysis of HRV positive samples. Closed symbols indicate CF patients and open symbols healthy controls. Sequence data were analyzed with maximum parsimony algorithm performing 100 bootstraps. Subtypes were defined as different when sequence homologies were < 90%.

DISCUSSION

This study has shown that HRV is a common virus which is more frequently detected and persists for longer periods in young children with CF compared to matched healthy controls, without differences in distribution and diversity of the several HRV subtypes. The results suggest that children with CF have the same exposure to HRV compared to healthy children, but have an increased risk of

acquisition. The more frequent and prolonged detection might indicate increased viral replication of HRV in children with CF.

These are the first in vivo data showing that CF patients have more frequent HRV infections, compared to healthy controls. A higher frequency of HRV positive samples in children with CF could be due to prolonged detection of the same HRV subtypes, or due to more frequent acquisition of different rhinoviruses from the environment. In this study we observed a higher mean number of HRV subtypes per individual as well as prolonged duration of detection of the same HRV subtypes. Earlier studies showed no differences in frequency of virus-related acute respiratory illnesses between CF patients and healthy controls (6,13,14); however, these studies only used culture or serology to prove viral infections including HRV. A few studies have used PCR to detect HRV infections in CF patients, but they failed to sample during symptom-free episodes and lacked adequate non-CF control groups (5,7,16). Our study adds unique longitudinal data from healthy and diseased children irrespective of complaints, using genotyping of HRV subtypes to distinguish prolonged detection of the same HRV strain from increased infections with different HRV subtypes.

Several factors might have caused the increased susceptibility of CF patients for HRV infections. First, patients with CF might be more susceptible to specific HRV subtypes due to virus-related factors; however, sequencing of HRV showed no differences in pattern and phylogenetic distribution of the several HRV subtypes in CF patients compared to healthy controls. Second, patient-related factors could cause differences in susceptibility. Our findings of more frequent and prolonged HRV detection might suggest a higher viral replication in patients with CF, which is in line with earlier data suggesting a diminished specific anti-viral defense. Zheng et al. found higher viral replication in CF airway epithelium due to an impaired NOS-2 signalling pathway, which is an important part of the innate anti-viral defense(12). More recently Colasurdo et al. showed that CF mice have an impaired ability to clear other respiratory viruses like respiratory syncytial virus (11).
Our data in CF patients are in line with findings in other chronic respiratory diseases such as asthma and COPD. In patients with COPD, respiratory viruses are more likely to be isolated in patients with a history of frequent exacerbations, suggesting that these patients are more susceptible to viral infection (17). In addition, it has been

postulated that bacterial colonisation in COPD patients contributes to increased susceptibility to viral infection by up-regulation of ICAM-1 expression in bronchial epithelial cells, either directly or through induced inflammation (18). In vitro and in vivo studies in asthma have shown a higher susceptibility for HRV. Persistent HRV-RNA was detectable in asthmatic children with more severe exacerbations, but data on genotyping of HRV subtypes are lacking in these patients (19). It has been shown that an increased viral load in asthma patients compared to controls is associated with a deficient production of several types of interferon (9,20,21). These data in asthma, COPD and CF suggest that an increased susceptibility to HRV infection in these chronic respiratory diseases is possibly associated with an impaired anti-viral defense, increased viral replication and bacterial colonisation.

Some potential limitations in our study design need to be addressed. 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 over 80% without differences between both groups seems acceptable and comparable to results in earlier studies. Furthermore, it might have been interesting to quantify viral load to confirm higher viral replication, and to study viruses other than HRV. Our results are in line with earlier CF studies that showed more and prolonged lower respiratory symptoms in relation to viral infections including HRV in patients with CF compared to healthy controls (6,14).

Our findings of increased susceptibility for HRV in CF might be interesting in a clinical perspective. Modern treatment strategies in CF are mainly aimed at treating bacterial infections with antibiotics and sputum evacuation. With an improving survival in young CF patients due to better anti-bacterial treatment, antiviral therapy might become a new therapeutic goal to further improve prognosis in the future. Development of anti-rhinovirus immunization or viral inhibitors in an early phase of an HRV infection could be valuable if a higher viral load in relation to respiratory morbidity in CF patients is confirmed in future studies.

In summary, these are the first in vivo and longitudinal data showing that CF patients have more frequent HRV infections compared to healthy controls. Genotyping of HRV subtypes shows that mainly patient-related factors cause the increased

susceptibility of CF patients for HRV infections. We suggest that the more frequent and prolonged detection of HRV in CF patients, without differences in distribution and phylogenetic diversity of the several HRV subtypes, might indicate increased viral replication of HRV in children with CF. If confirmed, this finding of increased susceptibility to HRV infections in young CF patients could aid appropriate targeting of new treatments to further improve prognosis in CF lung disease.

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Chapter 5a

Respiratory pathogens in children with and without respiratory symptoms

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ABSTRACT

Background

Respiratory viruses are thought to be responsible for the majority of the respiratory illnesses in infancy and childhood. Recent literature shows that respiratory pathogens are also found in asymptomatic children.

Objectives

To investigate the prevalence of respiratory pathogens in the presence or absence of respiratory symptoms in young children and to identify whether age and/ or co-infections modify the impact of respiratory pathogens on symptoms.

Methods

In a prospective longitudinal study 18 healthy children aged 0-7 years were followed for respiratory symptoms during 13 subsequent episodes of two weeks, covering a six-month winter period. Halfway each episode nose and throat swabs were collected regardless of any symptoms. Polymerase chain reaction (PCR) was performed on thirteen common respiratory pathogens. Episodes were defined 'asymptomatic' if no symptoms of any respiratory tract illness were present one week prior to one week after sampling.

Results

A total of 230 episodes were observed. In 56% of the symptomatic episodes a pathogen was detected, compared to 40% of the asymptomatic episodes ($p=0.03$). In children aged 0-2 years only 9% of the pathogen positive episodes were asymptomatic, compared to 26% in children aged 3-4 years, and 36% in children aged 5-7 years ($p=0.04$ and $p=0.01$, respectively). In 17% of the symptomatic episodes multiple pathogens were found, compared to 3% of the asymptomatic episodes ($p=0.02$).

Conclusions

Respiratory pathogens are frequently detected in children without any respiratory symptoms. Younger age and coincidence of multiple pathogens are associated with an increase of symptomatic respiratory episodes.

INTRODUCTION

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

Many studies have investigated the prevalence of respiratory viruses during respiratory illnesses, but little is known about the prevalence of viruses in non-symptomatic children. It remains unclear whether viruses are actually the cause of the respiratory symptoms or just simply colonize the respiratory tract during symptomatic episodes. It can be speculated that not every virus infection leads to respiratory symptoms and that the pathogenicity might depend on host or environmental factors. The respiratory and immune system of young children is still immature and might be more susceptible for respiratory viruses (5). We hypothesised that viral infections are likely to have the most serious effect on young children with a developing respiratory and immunological system. Furthermore, we hypothesised that the infection with multiple respiratory pathogens will more often lead to respiratory symptoms compared to infection with one single pathogen (6-9). Prospective scheduled viral sampling with frequent follow-up of respiratory symptoms in a cohort of young children can reveal the association between the presence of respiratory pathogens and respiratory symptoms. However, such studies are lacking in the literature until now.

The aim of our study was to determine the prevalence of respiratory pathogens in the presence or absence of respiratory tract symptoms in young children, and to identify whether age and co-infections modify the impact of a pathogen on the respiratory system. In this study we use sensitive polymerase chain reaction (PCR) techniques for detection of thirteen common respiratory pathogens.

MATERIAL AND METHODS

Study design and subjects

Nineteen healthy children aged 0-7 years were prospectively followed from November to May. None of the children had a history of asthma or recurrent respiratory complaints. Parents were contacted twice a week via telephone or e-mail by one of the two study coordinators to determine the presence of any symptoms of a 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 dyspnoea, all with or without a temperature above 38°C.
Samples were collected every two weeks regardless of any respiratory symptoms. The two-weekly sampling frequency during the study resulted in about 13 subsequent observation episodes per child. An episode was defined as 'asymptomatic' if there were no respiratory symptoms during a complete period of one week prior to and one week after sampling. An episode was defined as 'symptomatic' if there were any respiratory symptoms during the period of one week prior to and one week after sampling.
The study was approved by the local medical ethics committee (UMC Utrecht) and the parents gave written informed consent.

Pathogen detection

Respiratory pathogens were detected from nose and throat swabs 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 two swabs were collected into a single vial containing GLY medium with pimaricine 0.1 mg/ml as viral transport medium and sent to our laboratory via regular mail. Samples were stored at –20°C until further analysis. Feasibility of virus sampling by the parents is described earlier (10).
PCRs were 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 and *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* were analyzed by PCR essentially as described earlier (3).

The PCR for adenovirus consisted of 40 cycles 1 min 94°C, 1,5 min 45°C, 1 min 72°C with a final extension of 10 min at 72°C (PE 9700) with primers GCCGCAGTGGTCTTACATGCACAT, ARCACNCCNCGRATGTCAAAG and CAGCACGCCGCGGATGTCAAAGT and amplicons were analyzed by gel electrophoresis.

The real-time PCR for human coronavirus NL63, influenza viruses A and B and RSV A and B was performed using the Lightcycler 2.0 format with Lightcycler ®Taqman Mastermix (Roche). A separated RT step with AMV reverse transcriptase was used, for NL63 60 min 42°C and for influenza and RSV 60 min 50°.

The reaction for NL63 consisted of 10 min 95°C, 1 cycle, 5 sec 50°C, 10 sec 72°C, 45 cycles 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 (11).

The reaction for influenza consisted of 10 min 95°C, 1 cycle, 10 sec 95°C,20 sec 50°C, 10 sec 72°C, 45 cycles with primers 5-'AAGACCAATCCTGTCACCTCTGA-3'and 5'-CAAAGCGTCTACGCTGCAGTCC -3' with probe 5'-6Fam-TTTGTGTTCACGCTCACCGTGCC-BHQ1-3' for influenza A targeting the MP gene and for influenza B:5'-TGAAGGACATTCAAAGC-3' and 5'-ACCAGTCTAATTGTCTC-3' with probe 5'-YY-AGCACCGATTACACCAG-BHQ1-3' targeting the NS gene.

The reaction for RSV consisted of 10 min 95°C, 1 cycle; 15 sec 95°C, 47 sec 60°C, 45 cycles with primers 5'-TGAACAACCCAAAAGCATCA-3' and 5'-CCTAGGCCAGCA GCATTG-3' with probe 5'-6Fam-AATTTCCTCACTTCTCCAGTGTAGTATTAGG –BHQ1-3' for RSV A and for RSV B 5'-TGTCAATATTATCTCCTGTACTACGTTGAA-3' and 5-GATGGCTCTTAGCAAAGTCAAGTTAA-3' with probe 5'-YY-TGATACATTAAATAAGGATCAGCTGCTGTCATCCA-BHQ1-3' both targeting the nucleocapsid gene.

Statistical analysis

Statistical analysis was performed using SPSS Inc., 2001, Chicago USA, version 12.0. The Chi-square test was used to compare differences between groups. A p-value <0.05 was considered significant.

RESULTS

Of the 19 children, 18 were followed during the entire 6-month period; one was lost to follow-up after the ninth week of the study and was excluded from the analysis. Other characteristics of the group are presented in Table 1.

Table 1. Characteristics of the study group.

	N=18
Age (yr)	3.4 (0-7)
Gender (male/female)	3/15
Number of siblings/ child	1.4 (0-4)
Number of symptomatic episodes/ child	9.2 (4-13)
Number of asymptomatic episodes/ child	3.6 (0-9)

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

Symptomatic episodes occurred more often than asymptomatic episodes. A total of 230 episodes were observed; in 119 episodes (52%) samples were positive for one or more respiratory pathogens. Table 2 gives the results of the PCRs during asymptomatic and symptomatic episodes.

Table 2. Respiratory pathogens in asymptomatic and symptomatic episodes.

	Asymptomatic episodes N=65	Symptomatic episodes N=165	p-value*
Any pathogen	26 (40)	93 (56)	0.03
Single pathogen			
Picornaviruses	16 (25)	45 (27)	0.82
Coronaviruses	5 (8)	15 (9)	0.38
Respiratory syncytial virus	0 (0)	1 (1)	NA
Influenza virus	0 (0)	1 (1)	NA
Human metapneumovirus†	0 (0)	0 (0)	NA
Adenovirus	0 (0)	1 (1)	NA
Chlamydia Pneumoniae	3 (5)	2 (1)	NA
Mycoplasma pneumoniae†	0 (0)	0 (0)	NA
Multiple pathogens	2 (3)	28 (17)	0.02

Data are expressed as absolute values (with percentages)
Abbreviations: NA = not applicable.
* *p-value is calculated with chi-square.*
† *hMPV and MP were never found as a mono-infection, only as a co-infection with another pathogen.*

In symptomatic episodes a significantly higher number of pathogens were found compared to asymptomatic episodes. In 56% of the symptomatic episodes a pathogen was detected, compared to 40% of the asymptomatic episodes (p=0.03). Picornavirus was the most prevalent virus; it was found in 27% of the symptomatic episodes and in 25% of the asymptomatic episodes; this difference was not significant (p= 0.82). The second most prevalent detected pathogen was coronavirus, which was detected in equal numbers of symptomatic and asymptomatic episodes (p=0.38). Human metapneumovirus (hMPV) and *Mycoplasma pneumoniae* (MP) were never detected as a mono-infection but only as a co-infection with another pathogen.

We analysed the influence of age on the occurrence of symptoms during an episode. Figure 1 shows the distribution of symptomatic and asymptomatic episodes in the different age categories when one or more pathogens were detected. With increasing age, virus-positive children are increasingly asymptomatic. In children aged 0-2 years only 9% of the virus positive episodes was asymptomatic, compared to 26% in children aged 3-4 years, and 36% in children aged 5-7 years (Chi-square p=0.04 and p=0.01, respectively).

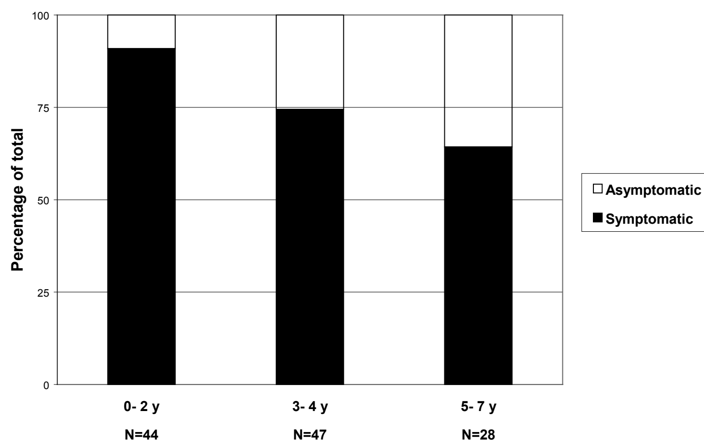

Figure 1. Percentages of the symptomatic and asymptomatic episodes in each age category when one or more pathogens were detected.

In 17% of the symptomatic episodes multiple pathogens were seen, compared to 3% of the asymptomatic episodes. This difference in prevalence of infections with multiple pathogens between the symptomatic and asymptomatic episodes was significant (p=0.02). 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 35% of the episodes were asymptomatic compared to 7% of the episodes with multiple pathogens ($p<0.01$).

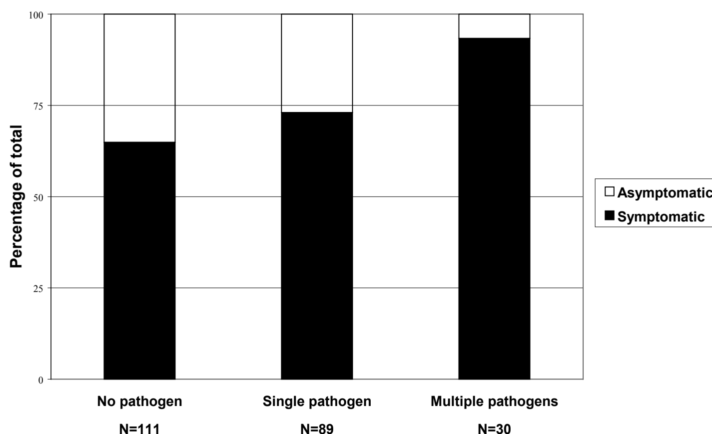

Figure 2. Percentages of the symptomatic and asymptomatic episodes related to the number of found pathogens.

DISCUSSION

Our data show that respiratory pathogens are frequently found in children without any respiratory symptoms (~40%). Younger age and coincidence of multiple pathogens result more often in symptomatic episodes. The uniqueness of our study is the longitudinal sampling of healthy children at home and the use of a broad panel of thirteen respiratory pathogens for detection. In this study we sampled only during the winter season, which limits the general applicability of our results. Although we sampled a relatively small number of children, the number of samples taken is large and the longitudinal design 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 the picornavirus, it was found in both symptomatic as well as in asymptomatic episodes. The fact that picornaviruses are often found in asymptomatic children is not surprising as it is generally seen as a relatively mild pathogen which can colonize the nasal mucosa without causing

symptoms (4). On the other hand, recent studies attribute a more important role for picornaviruses in both upper respiratory tract infections as well as in lower respiratory tract infections (2;12). Our study shows that children with a picornavirus can be both asymptomatic and symptomatic, indicating that other, presumably host and environmental determinants, play a role in the pathogenicity of this virus.
Coronaviruses were found as the second most prevalent single virus in asymptomatic as well as in symptomatic episodes. This is in line with literature where coronaviruses account for approximately one third of the common colds in children (13). It was also often found in multiple infections (13-15), which both point to a relatively mild pathogenicity of coronaviruses.
Because of the small number of detections with RSV, influenza virus and adenovirus in our study, it is difficult to draw conclusions about the association of these viruses with respiratory illness. Nevertheless it is striking that, in contrast to picorna- and coronaviruses, these viruses were only detected in symptomatic and never in asymptomatic episodes. The majority of the infections with *C. pneumoniae* were found in asymptomatic episodes (~60%). The other remarkable finding was that *Mycoplasma pneumoniae* was never seen as single infection; however, *Mycoplasma* was found in 20% of the samples in which multiple pathogens were detected. These results might indicate that the pathogenicity of atypical pathogens in healthy children is probably limited, possibly responsible for silent transmission (16). HMPV was also never seen as a single infection.

Of the asymptomatic children, 40% carried one or more pathogens. In literature the prevalence in asymptomatic children ranges from 5 to 68% (3;17-20). This wide range might be explained by differences in study population, definition of asymptomatic episodes, and the difference in sampling methods and virus detection. The majority of the studies were done in older children, usually hospitalised for elective surgery. Furthermore, most studies have 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 person during a certain period. Winther et al. (19) also sampled longitudinally and found a prevalence of picornavirus of 9% in asymptomatic children. The difference with our findings could be explained by the fact that they used a period of 4 weeks around the onset of a respiratory illness for symptomatic episodes.

In our study, in 44% of the symptomatic episodes no pathogen was found; this is a relatively high percentage. One explanation might be that our healthy population did not have a viral load that was high enough to detect the pathogen. In 71% of these symptomatic episodes a pathogen was found in the samples two weeks before or after these symptomatic episodes (data not shown). Perhaps the pathogen was already cleared from the respiratory tract and the post-viral symptoms remained without the pathogen (20) or the symptoms preceded the detection of a pathogen. Another explanation could be that the diagnostic panel missed some additional known or unknown pathogens.

In this study we questioned whether age is important in the development of symptomatic disease after entry of a pathogen. The underdeveloped respiratory and immunological system in young children can result in a different immune response and an increased susceptibility for viral infections (21). The children aged 0 to 3 were more often symptomatic when a pathogen was present. The symptomatic episodes decreased with age (Figure 2). It is possible that most children, at a certain age, have come across most of the different respiratory pathogens or developed cross-immunity and therefore react without any noticeable respiratory symptoms.
We found a higher prevalence of multiple pathogens in symptomatic children compared to asymptomatic children. There is some debate about the role of infections with multiple pathogens and disease severity. Some argue that multiple pathogens cause more severe disease (6-9), whereas others report no difference between single and multiple infections in disease severity (8;22;23). Our findings support the former and indicate that there is an association between infections with multiple pathogens and illness. Further studies are needed to elucidate the exact mechanism behind infections with multiple pathogens and disease severity.

In conclusion, our study shows that respiratory pathogens are often detected in children without any respiratory tract symptoms. In the youngest age group detection of respiratory pathogens is more often associated with clinical disease. In addition, detection of multiple pathogens is also more often associated with disease. The clinical importance of these findings is that not every pathogen detection can explain respiratory illness; other factors, like age and co-infections should also be taken into account.

ACKNOWLEDGEMENTS

We thank all the parents and children who were willing to participate in our study. We also thank B. van der Zwan and T. Yimam (National Institute of Public Health and the Environment, Bilthoven, The Netherlands) for the laboratory assistance.

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Chapter 5b Addendum

Respiratory pathogens in children with cystic fibrosis with and without respiratory symptoms

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Tom F.W. Wolfs
Berry Wilbrink
Cornelis K. van der Ent

INTRODUCTION

Respiratory pathogens are frequently detected in the general population in children without any respiratory symptoms. Younger age and co-incidence of multiple viruses are associated with an increase of symptomatic respiratory episodes in these children (chapter a). We aimed to study if these relationships could also be observed in young children with CF. We used methods as described previously in chapter a.

RESULTS

All 20 children with CF were followed during the entire 6-month period. Other characteristics of the group are comparable to the group in chapter a (Table 1).

Table 1. Characteristics of the study group

CF children	N=20
Age (yr)	3.5 (0-7)
Gender (male/female)	10/20
Number of siblings	1.4 (0-3)
Number of symptomatic episodes	9.3 (4-13)
Number of asymptomatic episodes	2.1 (0-8)

Data are expressed as mean values (with range)

A total of 228 episodes were observed. Symptomatic episodes occurred more often than asymptomatic episodes, 186 (82%) versus 42 (18%) respectively. In contrast to healthy controls no significant difference in number of any detected pathogens was found between symptomatic and asymptomatic episodes in children with CF (Table 2). In 63% of the symptomatic episodes a pathogen was detected, compared to 57% of the asymptomatic episodes ($p=0.47$). Similarly to the healthy controls picornavirus was the most prevalent virus; it was found in 49% of the symptomatic episodes versus 43% of the asymptomatic episodes respectively ($p= 0.50$). The second most prevalent detected pathogen was coronavirus, which was detected in equal numbers of symptomatic and asymptomatic episodes ($p=0.51$).

Table 2. Respiratory pathogens in asymptomatic and symptomatic episodes

	Asymptomatic episodes N=42	Symptomatic episodes N=186	p-value
Any pathogen	24 (57)	118 (63)	0.47
Single pathogen			
Picornavirus	18 (43)	91 (49)	0.50
Coronavirus	17 (40)	84 (45)	0.60
Respiratory syncytial virus	1 (2)	7 (4)	NA
Influenza virus	8 (19)	28 (15)	0.51
Human metapneumovirus	1 (2)	6 (3)	NA
Adenovirus	0 (0)	1 (1)	NA
Chlamydia Pneumoniae	0 (0)	2 (1)	NA
Mycoplasma pneumoniae	0 (0)	1 (1)	NA
Multiple pathogens	4 (10)	8 (4)	0.24

Data are expressed as absolute values (with percentages). Abbreviations: NA = not applicable. Statistically significant p<0.05 (Chi square).

We analysed the influence of age on the occurrence of symptoms during an episode of 2 weeks. Figure 1 shows the distribution of symptomatic and asymptomatic episodes in the different age categories when one or more pathogens were detected. In contrast to healthy controls increasing age has no consistent influence on being asymptomatic in case of a positive PCR. In children with CF aged 0-2 years 12% of the PCR positive episodes was asymptomatic, compared to 27% in children aged 3-4 years, but 13% in children aged 5-7 years.

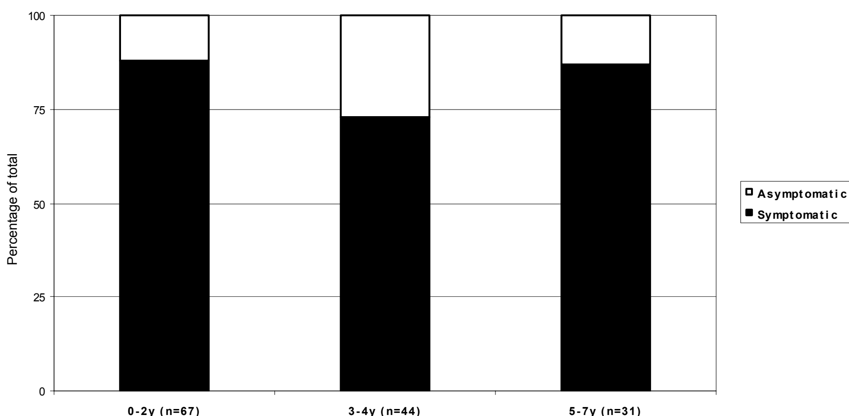

Figure 1. Influence of age on symptoms: percentages of symptomatic and asymptomatic episodes in each age category when at least one pathogen was detected.

In addition, we studied the influence of multiple pathogens in one episode on being symptomatic. In 14% of the symptomatic episodes multiple pathogens were seen in children with CF, compared to 17% of the asymptomatic episodes. This difference in prevalence of multiple pathogens between the symptomatic and asymptomatic episodes was non-significant, in contrast to the findings in healthy controls. Figure 2 shows similarly to the healthy controls the distribution of symptomatic and asymptomatic episodes when no pathogen, a single pathogen or multiple pathogens were detected. In children with CF there was no consistent trend or significant difference in the percentage of asymptomatic episodes between the groups, while in healthy controls such a significant difference was found between the no pathogen and multiple pathogens groups.

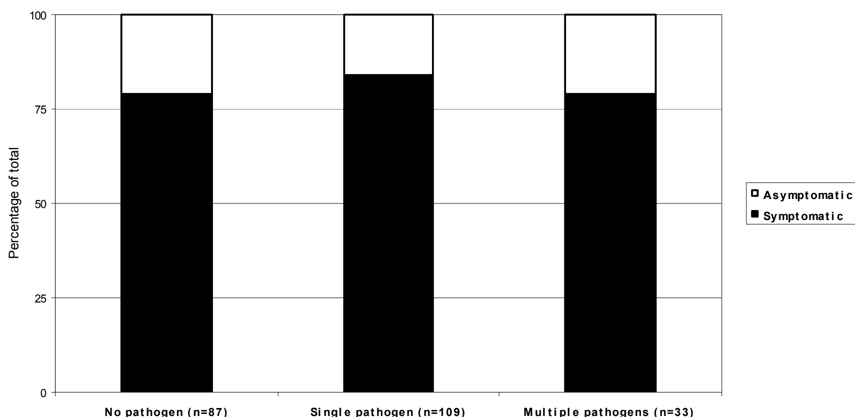

Figure 2. Influence of co-infection on symptoms: percentages of symptomatic and asymptomatic episodes related to number of detected pathogens

CONCLUSION

Young children with CF have few asymptomatic episodes during a winter-period. In these asymptomatic periods viral pathogens can frequently be detected, similar to healthy children from the general population. However, the effects of age and co-incidence of viruses, as observed in children from the general population, could not be detected in children with CF. Probably this is due to the higher level of baseline symptoms in CF-patients.

Chapter 6

High *Pseudomonas aeruginosa* acquisition rate during acute respiratory infection in healthy and cystic fibrosis children

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Andre Fleer
Jan L.L. Kimpen
Cornelis K. van der Ent

Thorax 2006;61;641-642

INTRODUCTION

Chronic colonisation of the lungs with *Pseudomonas aeruginosa* in patients with cystic fibrosis (CF) is associated with reduced lung function and life expectancy. Prevention of chronic colonisation might be achieved by avoidance of, or early and aggressive treatment of primary *P. aeruginosa* acquisition (1). Segregation of uninfected individuals from chronically *P. aeruginosa* colonised CF patients is advocated to prevent cross infection (2). As surveillance studies suggest that the airways of healthy children are rarely colonised with *P. aeruginosa* (3), healthy individuals are not regarded as a potential source of *P. aeruginosa* acquisition. In addition, it has been shown that acquisition of *P. aeruginosa* in CF patients is often preceded by a viral respiratory infection (4). We hypothesised that the incidence of *P. aeruginosa* acquisition during periods of acute respiratory infections (ARI) is equal in both healthy and CF individuals, and considerably exceeds the prevalence in asymptomatic children shown in surveillance studies.

METHODS

We performed systematic oropharyngeal cultures during periods of ARI between November and May in 20 young children with CF of mean (SD) age 3.6 (2.0) years (range 0.1 to 7.4) and 19 unrelated age matched healthy controls of mean (SD) age 3.6 (1.7) years. All children were negative for *P. aeruginosa* at the start of the study. Subjects were contacted twice a week with a standard questionnaire regarding symptoms of ARI. If any symptom was present a physician performed an oropharyngeal culture. Cultures from CF patients were also taken at routine visits. The study was approved by the local ethics review committee and all parents of the children gave written informed consent.

RESULTS

A mean (SD) number of 7.5 (2.7) (range 2-13) and 5.1 (1.8) (range 2-9) oropharyngeal cultures were taken from CF patients and healthy controls, respectively. During the study period 6 children with CF (30%) had at least one *P. aeruginosa* positive

culture, compared to 7 (37%) healthy controls. Cultures following a positive culture in healthy children were always negative for *P. aeruginosa*, while in 4 of 6 (67%) CF children short-term follow-up cultures remained positive for *P. aeruginosa* and anti-pseudomonal treatment was started.

DISCUSSION

This study showed that *P. aeruginosa* acquisition frequently occurs in periods of ARI in both children with CF and healthy controls. While healthy individuals easily clear *P. aeruginosa*, most CF patients remain positive and require anti-pseudomonal treatment. In the present study we sampled during periods of ARI, which are highly related to respiratory viruses in otherwise healthy children (5). In line with former data in CF, these results suggest that respiratory viral infections facilitate *P. aeruginosa* acquisition and colonisation (4). The high prevalence of *P. aeruginosa* in the airways of healthy children during ARI is in contrast with earlier findings which suggest that *P. aeruginosa* colonisation rarely occurs in the airways of healthy individuals (3). Our data could suggest that even healthy individuals with ARI are a potential source for *P. aeruginosa* acquisition in CF patients. If confirmed, it could have major consequences for current segregation policies which simply avoid contacts between CF patients. It might imply limiting contacts between both CF and non CF individuals in periods of ARI. Or should we conclude that prevention of *P. aeruginosa* acquisition is practically unrealistic?
Our data urge for studies on the relationship between respiratory viral infections and bacteria in CF, and on the transmission of *P. aeruginosa* between healthy individuals and CF patients. New insights might change current prevention rules and might open new approaches to effective prevention of *P. aeruginosa* acquisition in patients with CF. Prophylactic treatment with anti-pseudomonal antibiotics in periods of ARI might be an interesting option.

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Chapter 7

Oropharyngeal cultures in children with cystic fibrosis: more frequently at home?

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Submitted

SUMMARY

Background

Oropharyngeal cultures (OPC) are often used to identify potential respiratory pathogens and determine antibiotic treatment in young patients with cystic fibrosis (CF). Increasing the culture frequency in children with CF might be helpful for early detection of potential bacterial pathogens, but would result in a major burden in CF care as currently organized. We explored the feasibility of OPC collected at home.

Methods

Samples were collected in children with CF during routine outpatient clinic visits and the same day at home by parents and sent in by mail.

Results

From 105 children with CF who agreed to participate 98 paired samples were collected. No clinically relevant differences, irrespective of age, in diagnostic yield between hospital and home cultures were found. For most microbes the diagnostic yield of two OPC samples together was considerably higher compared to the yield of one OPC sample. The rate of one or both *P. aeruginosa* positive cultures was significantly higher in the chronically infected children than in intermittently colonised and previously negative children.

Conclusions

It is feasible to obtain OPC samples in children with CF by parents at home, with similar diagnostic yield compared to routine clinical OPC. In addition, our findings suggest that increasing the culture frequency with OPC samples taken at home may improve the diagnostic yield of potential pathogens and depends for *P. aeruginosa* on the colonisation status of the child.

INTRODUCTION

Cystic fibrosis (CF) is characterized by chronic inflammation, bacterial colonisation and recurrent infections of the lung, which lead to permanent lung damage and early death. Aggressive treatment of pulmonary infections is considered to be one of the major reasons for the recent improvement in life expectancy. In addition, there is some evidence that early recognition and eradication of airway pathogens may further delay the progression of lung disease. Especially early detection and eradication of *Pseudomonas aeruginosa* might prevent the establishment of chronic *P. aeruginosa* infection and its associated accelerated decline in lung function and well being (1-4).

The challenge for clinicians is making an early diagnosis of lower airway infection. Due to young age or mild disease many patients are unable to expectorate sputum. Since bronchoalveolar lavage (BAL) is invasive and requires general anesthesia, oropharyngeal cultures (OPC) are often used in clinical practice to identify potential respiratory pathogens and determine treatment. It has been shown that these cultures have lower predictive values compared to BAL or sputum (5,6), but predictive values might be improved by repeating OPC (7). Current guidelines in clinical CF care advise to obtain a culture for potential bacterial respiratory pathogens at every outpatient clinic visit and at the onset of exacerbations of respiratory illness (8). As in most CF centers patients are routinely seen once every 3 months, this is also the frequency that respiratory bacterial cultures are performed in stable CF patients. Increasing the culture frequency in a hospital setting would result in an increase of disease burden for patients and a major increase of workload for CF centers. We prospectively determined in an observational study whether parents or caregivers could reliably collect OPC specimens for culture in a domiciliary setting.

METHODS

Study design and subjects

A prospective, observational study of OPC sampling was conducted between January and April in CF patients aged 0-18 years, attending the pediatric outpatient clinic of the CF-Center Utrecht, The Netherlands. Diagnosis of CF was based on

the presence of two or more of the following criteria: sweat chloride > 60 mEq/L, positive genetic testing and clinical features consistent with CF. All children were treated according to the treatment protocol of our CF Center. Prior to the study patients were classified for *P. aeruginosa* status according to the criteria of Lee et al (9) (chronic colonisation: >50% of all cultures positive; intermittent colonisation: <50% of all cultures positive with at least one positive culture; no colonisation: all cultures negative for *P.aeruginosa* in the recent 2 years).

Study Protocol

OPC samples were collected during routine outpatient clinic visits, irrespective of clinical status. One of two investigators (BE or GR) swabbed the posterior oropharyngeal wall and tonsillar pillars with a dry cotton tipped swab (until the patient coughed). Subsequently, the swab was placed in culture medium, labeled, and sent to the microbiology laboratory for analysis. In addition parents and children were asked to take an oropharyngeal swab the same day at home. When agreeing parents were thoroughly instructed and demonstrated by one of two investigators how to take an OPC from their child, in the same way as described above. After taking the OPC at home the swab (sterile transport swab, Copan Innovation, Italy) was placed in its culture medium, the date was marked and parents sent it within 24 hours at room temperature by mail to the study coordinator, who registered date of receipt. All swabs were processed in the Microbiology Laboratory of the University Medical Center Utrecht. Swabs were inoculated onto 5% blood agar (BA), chocolate agar (Choc, for isolation of *Haemophilus influenzae*), mannitol salt agar (MSA, for *Staphylococcus aureus*), Mac Conkey agar (McC, for gram negative bacteria), *Burkholderia cepacia* selective agar (Bur-cep), Malt extract agar (ME, for yeasts) and Sabouraud dextrose agar (SDA, for fungi). Agar plates were incubated for either 48 hours (BA, Choc, MSA, McC, ME) or 72 hours (Bur-Cep) at 37°C aerobically, with (BA, Choc) or without 5% carbon dioxide. For recovery of fungi, SD plates were incubated for 10 days aerobically at both 22°C and 37°C.

Analysis

Data analysis included descriptive measures (i.e., numbers and percentages for categorical data, means ± SD for continuous variables). Mc Nemar's Chi-square test for paired samples was used to test for significant differences in culture results from hospital and home. The Chi-square test or Fisher's exact test tables were used when age (<8 and >8 years) and colonisation status were compared between

groups. $P < 0.05$ was considered significant in all analyses. The analyses were performed using the Statistical Package for the Social Sciences (SPSS, version 12.0, Chicago, IL, USA).

RESULTS

During the study period a total of 98 paired (hospital-home) samples were obtained from 94 children (45 boys, 49 girls). The mean age was 9.5 years (SD 4.8, range 1-17). A total of 11 patients and their parents agreed to participate but didn't return the home OPC by mail, for unknown reasons. According to Lee's criteria 29 samples were from children with chronic colonisation with *P. aeruginosa*, 8 from intermittently colonized, and 61 from children who were not colonised prior to the study. The mean time between sampling by the parents at home and inoculation at the microbiology laboratory, including postal delivery, was 4.6 days (SD 1.9, range 2-9 days).

In all hospital OPC one or more different microbes were detected, 4 home OPC were completely negative. Thirty-four sets of samples (35%) were identical for all cultured organisms. In 54% of all children *P. aeruginosa* was found in at least one of the two samples, in 60% *S. aureus*, in 14% *H. influenzae*, in 83% *Candida species*, in 16% *Aspergillus fumigatus*. *B. cepacia* was not detected in any of the patients.

Table 1. Comparison of hospital and home oropharyngeal cultures

N=98	Hospital *or* home OPC positive (=ref)	Hospital OPC positive	Home OPC positive	Hospital *and* home OPC positive	p-value
P. aeruginosa	53	41 (77%)	45 (85%)	33 (62%)	0.5
S. aureus	59	50 (85%)	51 (86%)	42 (71%)	1
H. influenzae	13	9 (69%)	5 (39%)	1 (8%)	0.39
S. maltophilia	8	3 (38%)	5 (63%)	0 (0%)	0.73
E. coli	5	4 (80%)	5 (100%)	4 (80%)	1
Other gram negative	14	6 (43%)	14 (100%)	6 (43%)	0.008[a]
Streptococcus spp	9	4 (44%)	6 (67%)	1 (11%)	0.73
Candida spp	81	66 (82%)	76 (94%)	61 (75%)	0.041[a]
A. fumigatus	16	14 (88%)	10 (63%)	8 (50%)	0.29

Data are expressed as absolute values with percentages compared to reference. Abbreviations: OPC = oropharyngeal culture; ref = reference. [a]Statistically significant, p<0.05 (Mc Nemar's paired samples test hospital versus home).

Table 1 shows the diagnostic yield of hospital and home OPC if microbial positivity in one of the two samples is used as gold standard. With exception for *Candida spp* and 'other Gram negative bacteria' no differences in diagnostic yield between hospital and home cultures was found. Home cultures revealed significantly more *Candida spp* and 'other Gram negative bacteria' than hospital OPC (76 versus 66, and 14 versus 6, respectively). For most micro-organisms the diagnostic yield of two OPC samples together was considerably higher compared to the yield of one OPC sample (hospital or home). The highest percentage of concordance between results from hospital and home OPC was seen for *E. coli* (80%), *Candida spp* (75%) and *S. aureus* (71%).
Presuming that OPC sampling in young children could be more difficult than in older children, we evaluated whether age had an effect on the results. No differences in diagnostic yield of hospital and home OPC could be detected between children ≤ 8 years (n=44) and children > 8 years (n=54).

Additionally, we studied whether the diagnostic yield for *P. aeruginosa* was influenced by colonisation status of the children. Table 2 shows the diagnostic yield of hospital and home OPC related to colonisation status, if *P. aeruginosa* positivity in one of the two samples is used as gold standard. In 89.7% of chronically infected children *P. aeruginosa* was detected in both cultures. The rate of *P. aeruginosa* positive cultures was significantly higher in the chronically infected children than in the combined groups of intermittently colonised and previously negative children (p<0.001 for hospital, home, and hospital and home positive, respectively).

Table 2. Influence of colonisation status on diagnostic yield of *P. aeruginosa*

Colonisation status	Hospital *or* home OPC positive (=ref)	Hospital OPC positive	Home OPC positive	Hospital *and* home OPC positive
Chronically positive (n=29)	29	28 (97%)[a]	27 (93%)[a]	26 (90%)[a]
Intermittently positive (n=8)	8	4 (50%)	6 (75%)	2 (25%)
Negative (n=61)	16	9 (56%)	12 (75%)	5 (31%)

Data are expressed as absolute values with percentages compared to reference. Abbreviations: OPC = oropharyngeal culture; ref = reference. [a]Statistically significant, p<0.001 (Chi-square chronically colonised versus combined intermittently and non-colonised).

DISCUSSION

Our observational study clearly shows that it is feasible to collect OPC in children with CF at home by their parents, with a similar diagnostic yield compared to samples obtained during outpatient clinic visits. To our knowledge, this is the first study comparing home and hospital sampling of OPC. In addition, increasing the culture frequency by taking OPC samples at home may improve the detection rate of potential bacterial pathogens in children with CF, for *P. aeruginosa* especially in previously non- or intermittently *P. aeruginosa* colonised children.

Strikingly, without significant differences per bacterial strain or fungi between hospital and home OPC we found total concordance of all cultured microbes together between the paired OPC in only 34%. First, this might be explained by local differences in bacterial distribution or density in the oropharynx. It is known from other studies that even the results of repeated samples of BAL or sputum in CF patients can be different with respect to the detected species or genotypes recovered from these samples (7,10,11). Second, parental technique in taking OPC might have influenced results. However, in only 4 home OPC we found no microbes at all, which suggests that parents adequately took OPC at home. In addition, in a small subset two OPC were taken at the same time during a visit by the investigators, with even in this case different results per OPC (data not shown). The diagnostic deficit due to sampling errors stresses the importance of frequently repeating OPC in CF patients. Third, sending the home swabs per mail might have influenced the results of the bacterial cultures. For example, the decreased rate of recovery of *H. influenza*e found in home OPC, although non-significant, may have been related either to bacterial loss because of temperature or to bacterial overgrowth by *P. aeruginosa*. In addition, the higher rate of other gram negative bacteria in home OPC might be explained by possible contamination and overgrowth with oral flora.

Our results are in line with earlier studies suggesting that combining results of more OPC can partially overcome the relatively low sensitivity of OPC compared to BAL in children with CF. Several studies have shown a relatively low sensitivity (ranging from 44-82%) for *P. aeruginosa* in OPC in young children with CF, compared to BAL as a gold standard (5,6,12). Specificity (or negative predictive value) was rather high

(>90%) in these studies. Burns et al (7) showed that a second OPC within 3 months could improve positive predictive value from 69% to 83% and negative predictive value from 85 to 97% for *P. aeruginosa* compared to BAL, respectively. Although these results have been discussed (13), we found in our study some indications that combining results of two OPC may improve the detection rate of several potential pathogens for the respiratory tract in CF, independent of age.

A low threshold for detection of potential pathogens is desirable for reasons of early detection and possible eradication, but could imply some over-treatment. It has the risk of unnecessary antibiotic treatment and increasing the risk of selecting resistant organisms. In addition, the workload for diagnostic laboratories could be substantial. In our study, 16 of 52 previously *P. aeruginosa* negative patients had new colonisation with *P. aeruginosa.* At further analysis 5 of these 16 children had *P. aeruginosa* in both cultures. In 11 previously negative patients *P. aeruginosa* was found in only one of the paired OPC samples. Short-term follow-up culture (within a month and without treatment) in 8 of these children showed a negative result in 6 children, and again *P. aeruginosa* in 2 for which they were subsequently treated. Increasing sampling frequency potentially improves sensitivity, but is associated with decreased specificity and chance of detecting transient upper airway colonisation rather than genuine lower airway infection. However, in the view of preventing chronic colonisation it might still be an interesting option to further explore in daily clinical practice. In the mean time, to prevent over-treatment with a higher frequency of OPC, we suggest to have at least two cultures positive for *P. aeruginosa* before eradication therapy could be considered.

Our data also show that the diagnostic yield for *P. aeruginosa* depends on colonisation status of the child. In previously negative or intermittently-colonised children a single OPC detected *P. aeruginosa* in only 50-75% of the *P. aeruginosa* positive patients, while only 25-31% of *P. aeruginosa* positive patients revealed *P. aeruginosa* in both cultures (Table 2). In chronically *P. aeruginosa* colonised children almost all culture pairs were both positive. We suggest that a higher bacterial density in chronically colonised patients results in a higher chance of a positive culture. Rosenfeld et al (6) similarly showed a higher sensitivity for *P. aeruginosa* in OPC when growth of *P. aeruginosa* $> 10^5$ cfu / ml was compared to any growth in BAL. Although our results might propose that a higher culture frequency in chronically

colonised *P. aeruginosa* patients is less meaningful, it still might be suggested being useful for determination of resistance, new strains or other species.
In our study the diagnostic yield of *Candida spp* in OPC at home was higher compared to clinical OPC. This might be the result of sending the oropharyngeal swab by mail, with the risk of contamination and overgrowth with oral flora. The rather high rate of *Candida spp* (81% and 93% respectively) in our study is somewhat higher than the reported 47-75% prevalence reported in previous studies (14-17). Possibly there has been under-reporting in these previous studies, or our high rate could be the consequence of relatively high numbers of children with CF receiving antibiotic prophylaxis in our CF centre.

There are some potential weaknesses in our study design. Culture results are not related to the gold standard of BAL. However, this is an invasive method and it was not the direct goal of this study. Our study represents daily clinical practice as it is, and primarily answers the question if taking additional OPC samples by parents at home is feasible. In the view of the results it might have been interesting to assess the value of two clinic cultures, e.g. at arrival and departure. Another option could be to shift the second culture at home to the midpoint between two clinic visits and to assess additional diagnostic value in this case.

In conclusion, our study shows that it is feasible to involve parents in taking OPC samples at home in children with CF and to send it in by mail for culture. We suggest that sampling of additional OPC at home may increase the diagnostic yield of potential pathogens in CF. If confirmed, it could enable early eradication therapy which might be helpful in further improvement of the prognosis of CF patients.

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Chapter 8

RSV mediates *Pseudomonas aeruginosa* binding to cystic fibrosis and normal epithelial cells

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ABSTRACT

Background

Cystic fibrosis lung disease typically has a course of exacerbations and remissions, suggesting that external factors like viral infections can influence this course. Clinical data suggest synergism between respiratory syncytial virus (RSV) infections and *Pseudomonas aeruginosa* in cystic fibrosis (CF) lung disease.

Methods

We studied the influence of RSV infection on adherence of *P. aeruginosa* to IB3-1, HEp-2 and A549 epithelial cell monolayers in vitro.

Results

RSV infection of epithelial cells as well as simultaneous addition of RSV and *P. aeruginosa* to non-infected cells both strongly enhanced the pseudomonal adherence to epithelial cells. The increased adherence varied from 1.2 to 8.2-fold in case of previous RSV infection, and from 1.7 to 16.1-fold in case of simultaneous addition. We observed direct binding of RSV to *P. aeruginosa*, and blocking of RSV with heparin eliminated the effect on increased adherence. It suggests that RSV possibly acts as a coupling agent between *P. aeruginosa* and epithelial cells.

Conclusions

RSV enhances *P. aeruginosa* infection of respiratory epithelial cells. It suggests a role of specific viral-bacterial interactions in exacerbations of CF lung disease, which could have important implications on prevention and treatment.

INTRODUCTION

Cystic fibrosis (CF) is characterized by chronic inflammation, bacterial colonisation and recurrent infections of the lung, which results in irreversible deterioration of lung function and early death. *Pseudomonas aeruginosa* is one of the most important bacterial pathogens in CF. Chronic infection of the airways with *P. aeruginosa* accelerates the progression to irreversible lung damage (1). Impaired mucociliary transport and anti-pseudomonal defense, specific binding to asialoGM-1 receptors and persistent inflammation probably plays a role in chronic pseudomonal colonisation in CF patients (2). Besides a constitutive inflammatory state CF lung disease typically has a course of exacerbations and remissions. This suggests that external factors influence this course, for example viral infections. Although the prevalence of viral infections seems to be equal in CF patients and healthy controls (3, 4), CF patients are more likely to develop a lower respiratory tract infection with more and prolonged symptoms (3, 5, 6). The mechanisms behind these differences are not elucidated.

Synergism between viruses and bacteria in inducing infections of respiratory epithelium has been described both in vitro (7-10) and in vivo (11-13). Circumstantial evidence suggests that initial pseudomonal colonisation and persistent infection in CF might be facilitated by respiratory viral infections, especially by RSV (14-16). We speculate that synergism between respiratory viral infections and pseudomonal colonisation plays an important role in the pathogenesis of CF pulmonary disease. Therefore we studied if RSV infection enhances the adherence of *P. aeruginosa* to human respiratory epithelial cells and we tried to elucidate some of the underlying mechanism.

METHODS

Cell cultures

IB3-1 human cystic fibrosis bronchial cells (JHU-52), HEp-2 human nasopharyngeal carcinoma cells (CCL-23) and A549 human pneumocyte type II carcinoma cells (CCL-185) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Stocks of cellular suspensions were stored at -180°C in liquid nitrogen. Cells in 24 or 96 well microtiterplates (Costar, Cambridge, MA, USA)

were cultured in a CO2 incubator to confluence at 37°C in Isocove's Modified Dulbecco's Medium (IMDM, Gibco, Paisley, UK) containing 5% foetal calf serum (FCS, HyClone, Utah, USA) and gentamicin 0.01 mg/ml (Gibco, Paisley, UK). Cells used for experiments were between 10-25 passages.

Bacteria and labelling

Two mucoid (Pa01 and Pa02) and two non-mucoid (Pa03 and Pa04) clinical *P. aeruginosa* strains were collected from different CF patients. Two other strains were obtained from ATCC, 39342 (mucoid) and 15692 (non-mucoid). Bacteria were stored in microbanks (Pro-Lab Diagnostics, Austin, Texas, USA) at -70°C. Prior to testing, bacteria were grown on blood agar plates (Trypticase Soy agar with 5% sheep blood, Becton Dickinson, Heidelberg, Germany) at 37°C, inoculated in Todd-Hewitt broth (Difco, Detroit, USA) supplemented with 0.5% yeast extract and grown shaking overnight at 37°C.

Bacteria were harvested by centrifugation at 3270 X g. Pelleted bacteria were washed three times with phosphate-buffered saline (PBS, Cambrex, Verviers, Belgium) and pelleted by centrifugation at 9300 X g during 5 min. For cytometric experiments, bacteria were suspended in 0.1M sodium carbonate buffer pH 9.0 and adjusted in a spectrophotometer to a concentration of 10^9 bacteria/ml (OD_{660nm} = 1.0). The bacteria were freshly labelled with a saturated fluorescein isothiocyanate solution (FITC, 10 mg/ml dimethylsulfoxide (DMSO), Merck, Germany), to a final concentration of 0.5 mg/ml, and incubated for 1h at 4°C. Efficiency of FITC-coupling as measured in a fluorescence activated cell sorter (FACS) varied between 76-92% labelled bacteria, depending on the bacterial strain. After washing thrice with PBS, bacteria were suspended in PBS^{2+}/ BSA 2.5% (Ca^{2+} 0.15 mM, Mg^{2+} 0.5 mM enriched with 2.5% bovine serum albumin (Instruchemie, Hilversum, Netherlands) to a final concentration of 2.0 X 10^8 CFU/ml (OD_{660nm} = 0.25) for direct use, or 4.0 X 10^8 CFU/ml (OD_{660nm} = 0.5) for simultaneous addition with RSV.

Viral stocks

RSV serotype A (ATCC VR 1302, Manassas, VA, USA) was grown on HEp-2 monolayers as described previously (8). Aliquots containing 3.0 X 10^6 PFU/ml were stored at –180°C in liquid nitrogen.

Adherence assay

Adherence experiments were performed as described previously (8). For RSV infection experiments monolayers were grown to confluence in 3-4 days, for experiments with simultaneous addition of RSV and *P. aeruginosa* including heparin blocking and filtration monolayers were grown to confluence in 2 days. Medium was removed from the cellular monolayers and 50μl of FITC-labelled bacteria were added. After centrifugation at 160 X g (IEC Centra 3C, IEC, Milford, USA) for 10 min the plates were incubated at room temperature for 30 min. Total fluorescence (TF) was measured by a fluorescence multi-well plate reader (CytoFluor II, PerSeptive Biosystems, Framingham, MA, USA), with excitation at 485 nm and emission at 530 nm, three readings per well. Subsequently, wells were washed four times with PBS^{2+} to remove non adhering *P. aeruginosa* and after addition of 50μl PBS^{2+} measured again to determine the fluorescence of adhering bacteria (adherence fluorescence = AF). The final adherence was calculated, as adherence = AF / TF x 100 (%).

Viral infection of monolayers

RSV infection of IB3-1, HEp-2 and A549 monolayers was induced as described previously (8). Cells and serial dilutions (log 3.0 - 4.8) of 50 μl of an RSV suspension or IMDM as a control were incubated in 96 well microtiter plates at 37°C in a CO2 incubator. Cytopathological effect (CPE) and percentage of remaining monolayer for the different RSV dilutions were scored under light microscope after each adherence assay.

*Binding of RSV to P. aeruginosa_*

Equal volumes of non-labelled bacteria (4.0 X 10^8 CFU/ml) and RSV suspension (3.0 X 10^6 PFU/ml and 5.0 X 10^7 PFU/ml respectively) or PBS/BSA 0.5% as control were co-incubated for 30 min at 37°C in an incubator. After washing and centrifugation twice for 5 min at 9300 X g, 25 μl of FITC labelled monoclonal antibodies against RSV-glycoprotein F (Imagen Kit for RSV, Glostrop, Denmark) was added and incubated for 30 min at 4°C, followed by two washes and suspension in PBS. Fluorescence of 10000 bacteria was analysed in a FACScan flowcytometer, expressed as % positive bacteria.

Simultaneous addition of RSV and P. aeruginosa

Equal volumes of FITC-labelled bacteria (4.0 X 10^8 CFU/ml) and RSV suspension (3.0 X 10^6 PFU/ml) or PBS/BSA 0.5% as control were co-incubated for 30 min at 37°C to form a complex. This mixture (50 µl) was added to a 2 days confluent and non-RSV infected monolayer in a 96 wells microtiter plate. These were incubated at room temperature for 30 min after centrifugation at 160 X g for 10 min. Adherence was measured as described before. To study the effect of heparin, a solution of 100 IE/ml sodium heparin was added to the monolayers before adding the bacteria. Heparin-treated monolayers were incubated for 30 min at 37°C in a CO2 incubator. Then the heparin solution was removed, bacteria were added and adherence was measured as described above.

Filtration and purification of RSV suspension

RSV suspension as originally cultured was spun through a 100 kD nitrocellulose filter (Microcon 100 kD filter, Millipor, Bedford, MA, USA) and the filtrate without RSV virions was used to repeat the simultaneous addition experiments. Purified RSV was prepared by polyethylene glycol (PEG) precipitation.

Scanning electron microscopy

RSV infected and non-infected HEp-2 cells were grown to confluent monolayers on fibrinogen-coated glass cover slips in 24-well microtiter plates. We added 50 µl unlabeled *P. aeruginosa* (Pa01) = 2.0 X 10^8 bacteria/ml (OD_{660nm} = 0.25) to the infected and control monolayers, or co-incubated *P. aeruginosa* and RSV suspension to the non-infected monolayers, and incubated for 30 min after centrifugation (10 min at 160 X g). Plates were washed four times with PBS and fixated during 10 min at room temperature with glutaraldehyde 2% (Merck, Darmstadt, Germany) in 0.1 M phosphate buffer, pH 7.4. Monolayers were dehydrated stepwise with ethanol 80% for 10 min at room temperature, followed by ethanol 99. 9% for 10 min at room temperature. Then hexamethyldisilazane 15 µl (Fluka Chemical Corp., Ronkonkoma, USA) was added for 10 min to each specimen. Samples were glued on a specimen mount with carbon-based glue (CCC-adhesive, Electron Microscopy Sciences, Hatfield, USA) after drying. Samples were titanium-sputtered (Sputter Coater 208HR, Cressington Scientific Instruments Ltd., Watford, U.K.) and examined with a scanning electron microscope FEI XL30SFEG (Royal Philips Electronics, Eindhoven, The Netherlands).

Statistics

Cytometric fluorescence adherence experiments were performed three times, and in each experiment 7 wells per same treatment were used. Results are expressed as mean +/- SEM. Data of RSV – *P. aeruginosa* coupling and RSV filtration experiments are from one of two representative experiment and expressed as mean ± SD. Each of these experiments was performed in duplicate. All comparisons were done by Student's t test with statistical program SPSS for Windows 12.0.2 (SPSS Inc, Chicago, USA). P<0.05 is considered significant for these comparisons.

RESULTS

Effect of RSV infection on P. aeruginosa adherence

The in vitro adherence of the different *P. aeruginosa* strains to human respiratory epithelial cells with and without RSV infection was tested to evaluate the effect of virus infection on pseudomonal adherence. Adherence of *P. aeruginosa* to non-infected respiratory epithelial cells varied for each strain and cell type. Maximum adherence to IB3-1 cells was found at an RSV infecting dose of log 3.35 on the third day of RSV infection, to HEp-2 and A549 cells on the fourth day at RSV infecting doses of log 3.70 and 3.18 respectively (data not shown).

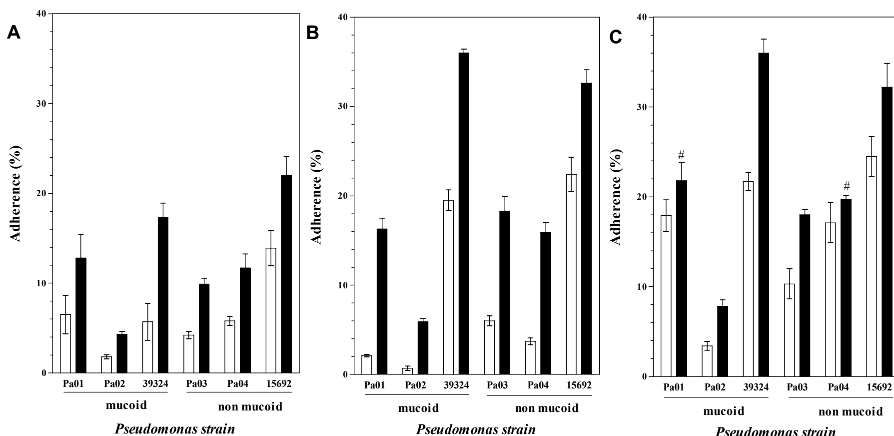

Figure 1. Adherence of P. aeruginosa strains to RSV infected IB3-1 (A), HEp-2 (B) and A549 (C) monolayers (solid bars) compared to non-infected monolayers (open bars), as measured by cytometric fluorescence assay. Data are represented as mean ± SEM of 3 experiments; in each experiment 7 wells per treatment were used. T-test (p<0.05), # = non significant.

This RSV infection of respiratory epithelial cells consistently resulted in an increased maximum adherence of all *P. aeruginosa* strains after 30 minutes incubation to these cells (IB3-1 day 3, HEp-2 and A549 day 4), compared to non-infected epithelial cells (Figure 1). It varied between the different strains and cell types from 1.2 to 8.2-fold, compared to no RSV. This maximum increased adherence of each pseudomonas strain after RSV infection, compared to no RSV, was significant for all strains and all cell types ($p<0.05$), except for strains Pa01 and Pa04 on A549 cells. RSV concentrations above log 3.35 for IB3-1, log 3.7 for HEp-2 and log 3.18 for A549 (Figure 2) resulted in loss of monolayers due to cytopathological effect on day 3 (IB3-1) and day 4 (HEp-2 and A549), with a decrease of total measured pseudomonas adherence. In addition, we found the enhanced adherence of *P. aeruginosa* to be RSV-dose dependent in all three cell types (Figure 2), on day 3 (IB3-1) and day 4 (HEp-2 and A549) respectively.

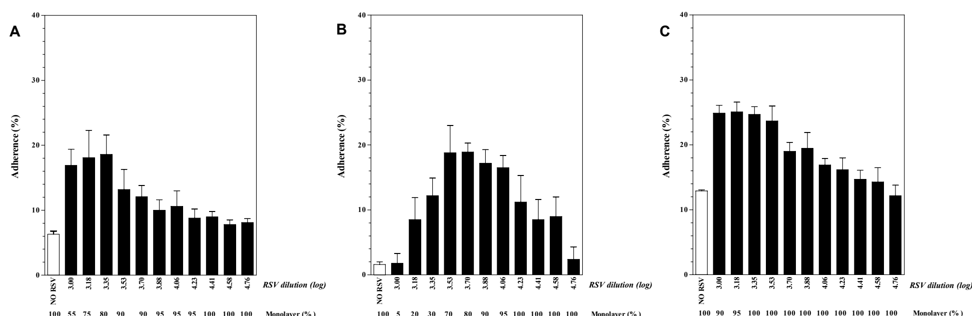

Figure 2. RSV dose dependent adherence of P. aeruginosa strain Pa01 to non-infected (open bars) and RSV infected IB3-1(A), HEp-2 (B) and A549 (C) monolayers (solid bars), as measured by cytometric fluorescence assay. Data from one representative experiment ± SD performed in triplicate are shown, 7 wells per treatment were used.

Binding of RSV to P. aeruginosa

We hypothesized that the increased pseudomonal adherence could be due to binding to virus-induced up regulated cellular membrane proteins or to viral glycoproteins expressed on the cell membrane. We first tested if RSV and pseudomonas bacteria can bind directly to each other. Pseudomonal suspension was compared with a suspension of RSV and *P. aeruginosa*. RSV with a dose of 3.0×10^6 PFU/ml bound directly to all *P. aeruginosa* strains, varying from 0.34 to 1.67 % (mean 0.94 ± 0.58 SD) RSV glycoprotein F positive bacteria. In addition, an increased RSV dose of 5.0×10^7 PFU/ml resulted in a dose dependent and increased binding of RSV and *P. aeruginosa*. It varied from 4.7 to 9.97%, mean 6.58 ± 2.09 SD (data not shown).

Effect of simultaneous addition of RSV and P. aeruginosa

To further explore the role of RSV in increased pseudomonal adherence we compared the adherence of *P. aeruginosa* alone with *P. aeruginosa* co-incubated with RSV to non-infected epithelial cells. Adherence of *P. aeruginosa* alone to non-infected respiratory epithelial cells grown to confluence in 2 days varied for each strain and cell type. The co-incubated RSV - *P. aeruginosa* complex consistently led to an increased adherence of *P. aeruginosa* to the respiratory epithelial cells, compared to *P. aeruginosa* alone (Figure 3). This increased adherence varied between the different strains and cell types from 1.7 to 16.1-fold and was significant for all strains and in all cell types ($p<0.05$).

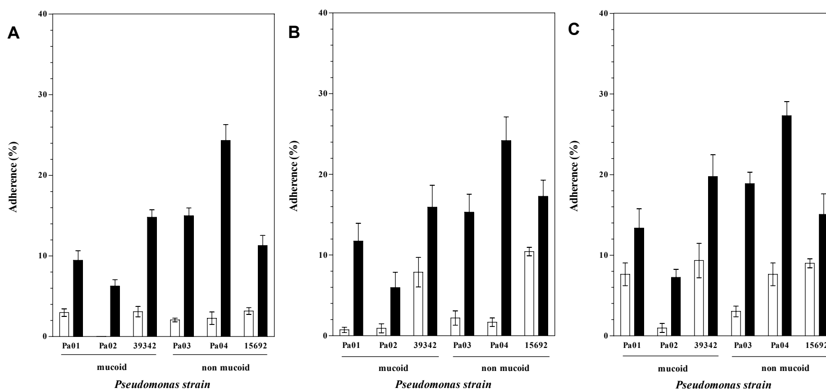

Figure 3. Adherence of P. aeruginosa simultaneously added with RSV to non-infected IB3-1 (A), HEp-2 (B) and A549 (C) monolayers (solid bars) compared to P. aeruginosa alone (open bars), as measured by cytometric fluorescence assay. Data are represented as mean ± SEM of 3 experiments; in each experiment 7 wells per treatment were used. Significant difference in all cell lines and with all strains (t-test, $p<0.05$).

Effect of RSV filtrate and purified RSV suspension

The increased adherence of *P. aeruginosa* to non-infected epithelial cells when added simultaneously with RSV could be due to RSV acting as a coupling agent between bacteria and epithelial cells or alternatively due to inflammatory mediators in the RSV suspension inducing up regulation of cellular membrane proteins involved in pseudomonal adherence. To investigate these two possibilities we incubated *P. aeruginosa* with either unfiltered RSV suspension or RSV filtrate. The RSV filtrate contained all components including inflammatory mediators from the originally RSV culture except the RSV virions by spinning through a filter. Similar adherence experiments were repeated with a purified RSV suspension without inflammatory mediators. Figure 4 shows that both original and purified RSV suspensions yielded

similarly increased pseudomonal adherence of 30.7% ± 0.8 SD and 33.0% ± 1.12 SD respectively. The filtrate induced no increase at all (3.8% ± 0.5 SD), compared to no RSV (5.6 %± 0.4 SD).

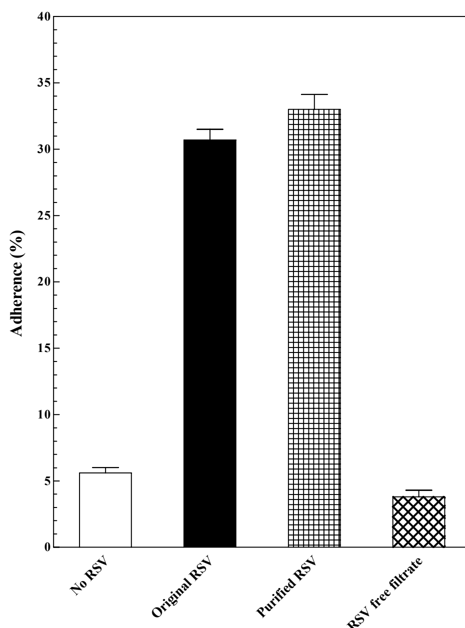

Figure 4. Originally used and PEG-precipitated, purified RSV suspension gave similar increased adherence of strain Pa01 to IB3-1 monolayer compared to control, while there was no increase of adherence with RSV free filtrate. Data from one of two representative experiments ± SD performed in duplicate are shown.

Effect of glycoprotein G blocking by heparin on binding

RSV binding to host cells is mediated by glycoprotein G, which can be specifically blocked by heparin. To investigate whether the enhanced binding of the co-incubated RSV - *P. aeruginosa* complex to non-infected epithelial cells was mediated by RSV glycoprotein G we pre-treated cells with heparin. Heparin had no effect on the adherence of *P. aeruginosa* to non-infected cells itself (data not shown). Figure 5 shows that pre-treatment of the non-infected cells with heparin led to nearly complete blocking of the enhanced adherence of co-incubated RSV - *P. aeruginosa* complex, which was significant ($P<0.05$) for all tested strains. This blocking was heparin dose-dependent and similar blocking of enhanced adherence with heparin was found in HEp-2- and A549-cells (data not shown).

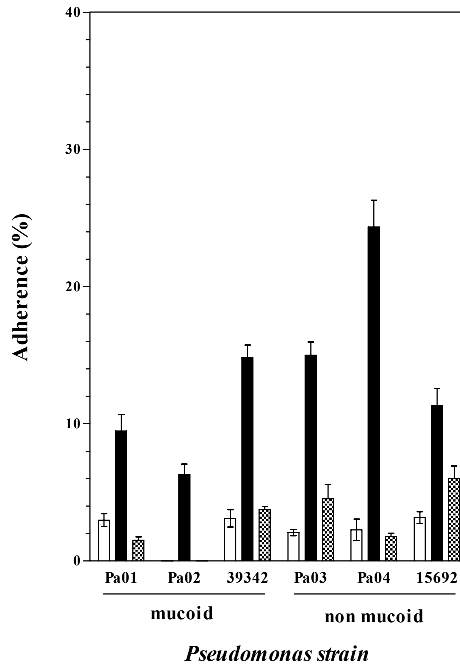

Figure 5. Pre-treatment of IB3-1 cells with heparin (blocked bars) gave nearly complete blocking of the enhanced adherence of co-incubated RSV - P. aeruginosa complex (solid bars), when compared to P. aeruginosa alone (open bars) and as measured by cytometric fluorescence assay. This increase is significant for all strains (t-test, $p<0.05$). Data are represented as mean ± SEM of 3 experiments; in each experiment 7 wells per treatment were used.

Visualization by scanning electron microscopy

Because adherence measured by fluorescence of FITC-labelled bacteria is an indirect and possibly rather insensitive method, we visualized the effect of previous RSV infection and simultaneous addition of RSV with *P. aeruginosa* on adherence by performing scanning electron microscopy studies. Incubation of *P. aeruginosa* without RSV showed only a few bacteria adhering to epithelial cells (Figure 6b). A major increase in adherence was observed when *P. aeruginosa* was added to RSV infected epithelial cells, adhering mainly to syncytia induced by the RSV infection (Figure 6c + d). In contrast, a more even distribution was observed when *P. aeruginosa* and RSV were added simultaneously to uninfected epithelial cells, again considerably increased compared to adherence in the absence of RSV (Figure 6e + f).

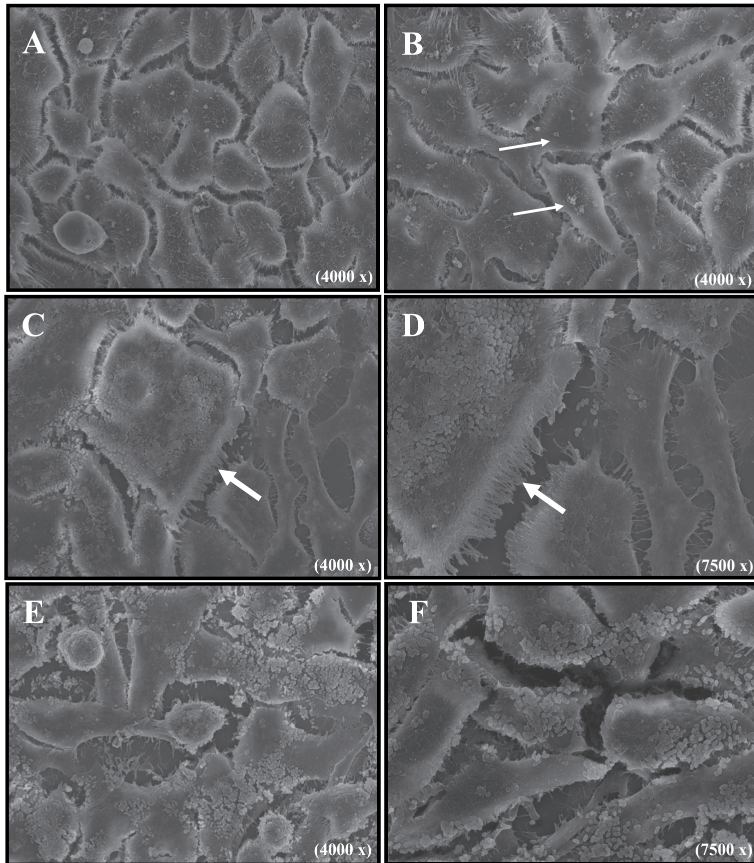

Figure 6. Visualization of pseudomonal adherence to HEp-2 cells by scanning electron microscopy (x4000/7500) shows non-infected cells without P. aeruginosa (A x4000), non-infected cells with little adherence (arrows) of P. aeruginosa (B x4000), large amounts of P. aeruginosa mainly adhering to syncytia (arrows) of RSV infected cells (C x4000 and D x7500) and large amounts of P. aeruginosa adhering more even after simultaneous addition of co-incubated RSV and P. aeruginosa to non-infected cells (E x4000 and F x7500).

DISCUSSION

Our study shows that both RSV infection of human respiratory epithelial cells and simultaneous addition of co-incubated RSV and *P. aeruginosa* to non-infected epithelial cells strongly enhance the adherence of *P. aeruginosa* to these cells. This was shown with both laboratory and clinical bacterial strains and in multiple cell types. In addition, we observe direct binding of RSV to *P. aeruginosa,* suggesting that RSV possibly acts as a coupling agent between *P. aeruginosa* and epithelial cells.

Specific blocking with heparin eliminates the increased adherence of *P. aeruginosa*, suggesting that this coupling of *P. aeruginosa* to the cells is mediated through RSV glycoprotein G. Scanning electron microscopy shows a striking difference in pattern of pseudomonal adherence to RSV-infected epithelial cells compared with simultaneous addition of co-incubated RSV and *P. aeruginosa* to non-infected cells, suggesting that different mechanisms of increased adherence are possible.

There are several potential mechanisms to explain our findings of RSV-induced enhancement of pseudomonal adherence to respiratory epithelial cells. Bacteria can bind to virus-induced and up-regulated cellular membrane proteins, and to induced viral glycoproteins like glycoprotein F, G and neuraminidase on the surface of infected cells (17). We show in addition that RSV possibly acts as a coupling agent for pseudomonal binding to respiratory epithelial cells.
Our data offer several arguments to suggest such a direct coupling mechanism. Simultaneous addition of co-incubated RSV and *P. aeruginosa* results in increased binding of bacteria to RSV uninfected monolayers. During the simultaneous addition of RSV and *P. aeruginosa* expression or up-regulation of cellular or viral proteins on the epithelial cell membranes, or up-regulation of inflammatory mediators is less likely because of the relatively short time interval between adding of co-incubated bacteria and virions and the assessment of adherence. In addition, filtration experiments show that the enhancing effect of RSV on pseudomonas adherence is due only to RSV virions and not to any other products in the RSV suspension, such as inflammatory mediators produced during RSV culture. We demonstrate furthermore a direct binding between RSV and *P. aeruginosa*, in a dose dependent manner. We suggest that this coupling mechanism can be mediated through binding of *P. aeruginosa* to glycoprotein G, known to be expressed on the surface of RSV (17). Pre-treatment of respiratory cells with heparin, which interacts with glycoprotein G (18), gives a nearly complete blocking of the enhanced adherence of co-incubated RSV - *P. aeruginosa* complex. Non specific blocking by heparin, for example inhibition of inflammatory mediators, is less probable in view of the short time-interval.
Scanning electron microscopy confirms the findings of the fluorescence assays by showing only little adherence of *P. aeruginosa* to respiratory epithelial cells in the absence of RSV, and a strongly enhanced pseudomonal adherence in the presence of the virus. It shows furthermore a striking difference in pattern of bacterial

adherence. While *P. aeruginosa* mainly adheres to syncytia after RSV infection of epithelial cells, the strongly increased adherence is more evenly distributed in case of simultaneous addition of RSV and *P. aeruginosa* to non-infected cells. This could suggest that the RSV-*P. aeruginosa* complex can bind to any epithelial cell, while adherence of *P. aeruginosa* alone is enhanced by binding presumably to virus induced glycoproteins expressed on syncytia after RSV infection.

The present study is the first to report that RSV infection can increase the adherence of *P. aeruginosa* to respiratory epithelial cells in vitro. This corresponds to earlier clinical observations that initial pseudomonas colonisation might be facilitated by respiratory viral infections, especially by RSV (14-16). In addition, a viral infection can be associated with a rise in anti-pseudomonal antibodies in CF patients with intermittent or chronic pseudomonal colonisation, especially in case of RSV infection (16). These findings are furthermore consistent with several studies that demonstrate enhanced adherence of bacteria, like *Haemophilus influenzae* and *Staphylococcus aureus*, to epithelial cells after viral infection in vitro (10, 19, 20) and in vivo (21, 22). However, there are only few experimental data about synergism between viruses and *P. aeruginosa* in inducing infections. In a study of Ramphal et al *P. aeruginosa* only adheres to murine tracheas when injured by influenza infection (23). Seki et al showed influenza infection to play an important role in inducing fatal pneumococcal pneumonia in chronic *P. aeruginosa* infected mice (24). Recently Stark et al found a decreased clearance of *P. aeruginosa* from the lungs after a RSV infection in non CF mice (25). Although *P. aeruginosa* is the most important bacterial pathogen in CF, the synergism between RSV and *P. aeruginosa* in infecting respiratory epithelial cells seemed not to be CF-specific in the present study. We observed no consistent differences between IB3-1 (CF) cells and HEp-2 or A549 (non-CF) cells, however not having used isogenic cell lines.

There are limitations of our study. Our in vitro data do not necessarily reflect in vivo situations and the role of other viruses on adherence of *P. aeruginosa* still has to be determined. However, we used multiple cell types and both clinical and laboratory bacterial strains with consistent findings of enhanced adherence. Adherence of *P. aeruginosa* without RSV varies in similar cell types between the different experiments, but monolayers were depending on the experiment grown to confluence in 2 or 3-4 days respectively. Induction of specific bacterial factors

might be another mechanism influencing adherence, but was outside the scope of the present study.

Our findings of RSV-induced enhancement of pseudomonal adherence in general might be interesting in a clinical perspective. *P. aeruginosa* is a selective and important pathogen in CF. Recent data in CF mice underline the relevance of RSV infections in pulmonary morbidity in CF (26). It might be suggested that viral infections like RSV facilitate bacterial colonisation, for example with *P. aeruginosa.* Further knowledge about synergism between viruses and bacteria in CF lung disease might result in new therapeutic strategies to improve prognosis of patients with CF. If our in vitro results can be confirmed in vivo, the suggestion that viral infections can play a role in bacterial colonisation and pulmonary CF exacerbations might implicate adaptation of current therapeutic strategies and development of new options. For example nebulization with heparin might possibly reduce the risk of bacterial colonisation and infection in case of an RSV infection. More in general, prevention of viral infections will be emphasized, for example by active or passive immunization. Viral inhibitors might be used in an early phase of a viral infection, antibiotic treatment or prophylaxis during a viral infection and development of other specific viral – bacterial interaction blockers might be interesting options.

In summary, this is the first report that RSV infection of respiratory epithelial cells and simultaneous addition of RSV and *P. aeruginosa* to non-infected epithelial cells both strongly enhance the in vitro adherence of *P. aeruginosa*. In this process RSV possibly acts as a coupling agent. Heparin seems able to block RSV and to eliminate this enhanced bacterial adherence. It confirms the suggestion that viral infections possibly play a role in bacterial colonisation and pulmonary CF exacerbations. If these results can be confirmed in vivo, it could have important implications on treatment of CF pulmonary disease.

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Chapter 9

Summary and discussion

SUMMARY AND DISCUSSION

Aggressive treatment of bacterial infections has been one of the most important factors in the dramatic improvement of life expectancy in patients with cystic fibrosis (CF). Until now, the role of respiratory viral infections was elucidated only partially. Viruses are known to play an important role in the morbidity of other chronic respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD). We speculated that respiratory viruses can play an important role in CF lung disease. Therefore, this thesis focuses on clinical and pathophysiological aspects of respiratory viral infections in children with CF, especially on the possible synergism between viruses and bacteria.

The main findings of this thesis are:

1. Children with CF and healthy controls have a similar frequency of virus-associated acute respiratory illnesses, without differences in seasonal exposure or distribution of respiratory viruses between these children.
2. Respiratory viral infections have a higher impact on lower respiratory tract symptoms in children with CF than healthy controls.
3. Compared to healthy controls, young children with CF are more susceptible to human rhinovirus (HRV), which is the most prevalent respiratory virus. HRV is detected more frequently and persists for longer periods in patients with CF.
4. Virus-related respiratory illnesses are frequently associated with *Pseudomonas aeruginosa* acquisition in both children with CF and healthy controls, but lead to persistent colonisation only in CF. It is feasible to detect *P. aeruginosa* colonisation by oropharyngeal cultures at home.
5. Respiratory syncytial virus (RSV) infection of human epithelial cells and simultaneous addition of RSV and *P. aeruginosa* to non-infected epithelial cells both strongly enhance *P. aeruginosa* adhesion. RSV possibly acts as a coupling agent and the enhancement can be effectively blocked by heparin.

These findings confirm that respiratory viruses play an important role in pulmonary morbidity in patients with CF. Although the exposure to viruses is equal in CF patients and controls, patients with CF react differently to respiratory viruses. Patients with CF have more symptoms, which might at least partially be caused by increased viral loads and increased bacterial colonisation. These aspects are

summarized in Figure 1. The different aspects and implications of this model will be discussed in this chapter.

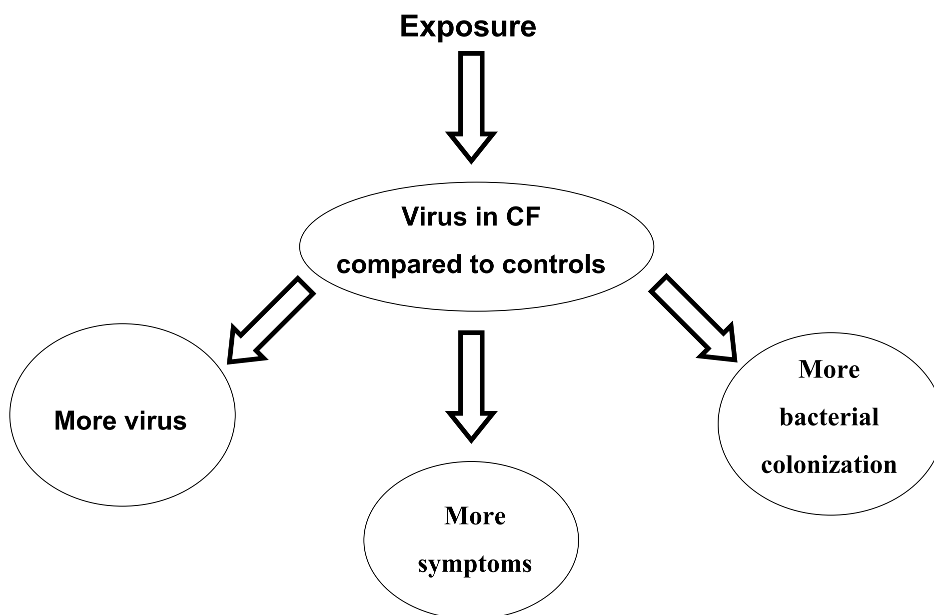

Figure 1

Exposure

Differences in exposure to viruses between children with CF and healthy controls might engage differences in handling of viruses and in symptoms due to viral infections. However, virus-associated acute respiratory infections and upper respiratory symptoms are found in equal frequency in children with CF and healthy controls **(chapter 3)**. There are no differences in seasonal frequency and distribution of the different viruses **(chapter 3)**. Furthermore, focusing on HRV reveals no specific HRV-subtype in patients with CF compared to healthy controls. The genetic distribution and pattern of phylogenetic diversity of the different rhinovirus subtypes was similar in both groups **(chapter 4)**. These data together imply that not virus-specific characteristics are responsible for differences in reaction on respiratory viral infections between children with CF and healthy controls. Exposure to respiratory viruses has to be similar. Only a few studies previously compared viral infections between patients with CF and healthy controls. Our data are in line with findings that equal numbers of viral infections

were observed in CF patients and healthy controls. One study described a higher rate of proven viral infections in healthy controls, but a similar rate of reported acute respiratory illnesses compared to children with CF (1). The interpretation of these studies might be limited by the use of relative insensitive detection methods, such as serology and tissue cultures. To our knowledge, our data concerning genetic sequencing of rhinovirus-subtypes in a longitudinal and clinical setting are unique.

More symptoms

Without differences in exposure to respiratory viruses, several data in this thesis argue that CF patients are more susceptible to respiratory viruses than healthy controls. Viral infections have a higher impact on lower respiratory tract symptoms in children with CF, compared to healthy controls **(chapter 3)**. Children with CF have longer and more severe periods of lower respiratory tract symptoms (LRTS). In contrast to other studies we performed close follow-up through twice weekly contact; and in case of new symptoms suggesting an acute respiratory illness (ARI) we performed home visits for evaluation, including physical examination and measurement of oxygen saturations. Children with CF not only have more increase in cough, increase in sputum or productive cough, and more shortness of breath in case of an virus-associated ARI, but symptoms were confirmed with objective measurements during home visits. They have more work of breathing (increase in respiratory rate and more retractions), a stronger decrease in oxygen saturation and more abnormal findings at lung auscultation than healthy controls. These clinical relevant findings resulted in more prescriptions of antibiotics by the treating doctor to children with CF. Our findings are in line with earlier data which suggested that viral respiratory infections in CF patients are associated with an increased morbidity; increased respiratory symptoms (1-3), deterioration of Shwachman (3-5) and radiological scores (4;6;7), prolonged hospitalizations (3;7;8) and increased use of antibiotics (2;4;5) have been shown. Our studies lack long-term follow-up, but previous studies showed a persistent decrease in lung function (1;4), and a higher frequency of exacerbations at long-term follow-up (3;8).
To explain these increased symptoms compared to healthy controls several arguments might be used. First of all, a difference in direct effect of respiratory viruses on CF patients could be explained from patient-related factors such as pre-existent condition. Young children with CF have few asymptomatic episodes during a winter-period. In these asymptomatic periods viral pathogens can frequently be

detected, similar to healthy children from the general population. However, the effects of age and co-incidence of viruses, as observed in children from the general population, could not be detected in children with CF **(Chapter 5)**. It might be suggested that this can be explained by the pre-existent clinical condition in CF, with a higher level of baseline symptoms in CF-patients. Structural damage of the lower airways, even in young CF patients (9;10), and an exaggerated inflammatory state causing a permanently triggered and active immune-system might overrule factors which play an important role in healthy children such as age and co-infections. However, this was not studied in this thesis. Previous data show that the frequency of viral infections seems not to be associated with pre-existing clinical condition (3), although one study reported a possible association (1). It could be interesting to investigate the role of pre-existent local damage and new damage in relation to symptoms. Older patients with CF, with presumably more pulmonary damage, might be studied. In long-term follow-up lung function and CT scans of the lungs might be useful to measure local damage and long-term consequences.

More virus

Another factor that seems different in CF patients compared to healthy controls is the virus quantity in CF. Without differences in exposure we observed that children with CF harbour more often HRV and that detection of HRV is prolonged compared to healthy controls **(chapter 4)**. These are the first in vivo data showing that in CF patients HRV is more frequently detected. A higher HRV detection frequency could be due to prolonged detection of the same rhinovirus subtype, or due to more frequent acquisition of different rhinoviruses; we observed both. We speculate that more frequent HRV detection and prolonged HRV detection is associated with a higher viral replication in patients with CF. A diminished anti-viral defense might cause a higher viral replication in case of exposure to a rhinovirus, provoking a higher viral load. This higher viral load would give more often a level high enough to detect with PCR, and as it will take longer to clear all the virus prolonged periods with detectable viral loads will be found. This would be in line with some earlier data in CF. It has been shown that CF mice have an exaggerated inflammatory response, but impaired ability to clear respiratory syncytial virus with higher viral loads (11). In addition, increased virus replication and increased production of pro-inflammatory cytokines were found after in vitro parainfluenza infection of CF human airway cells, compared to controls (12) . This might be

explained by a reduced production of respiratory nitric oxide (NO), which is an important part of the innate antiviral defense. Increased production of NO protects against viral infections. In CF patients expression of the NO producing enzyme NO synthase type 2 is considerably decreased, while the IFN-gamma dependent antiviral host defense is intact (13). An aberrant immune response to viral infections has also been found in other chronic respiratory diseases, like asthma. However, an increased viral load compared to controls seems in asthma patients associated with a deficient production of several types of interferon (14).

The link from more virus in CF to more symptoms is not very clear yet. In this thesis there are no data which link more virus directly to more symptoms. It might be speculated that a higher viral replication and associated exaggerated inflammation cause more local damage in the airways, which could explain the more severe and prolonged symptoms during viral infections in CF patients compared to healthy controls as in chapter 3. On the other hand, viruses can also be detected up to 57% in asymptomatic periods **(chapter 5)**, although these periods were very sparse in the young CF patients compared to healthy controls. It might be suggested that prolonged detection in CF is associated with a relatively low viral load just detectable with PCR, but without directly provoking symptoms in such sparse asymptomatic episodes. But data on this subject are lacking. There are some data from studies in non-CF populations indicating that the level of viral load is associated with the level of symptoms. In young infants hospitalised for lower airway infections measurements of viral loads, by real-time quantitative real-time PCR, are helpful in determining the role of RSV role in respiratory infections (15). Another study revealed a significant association between disease severity and nasopharyngeal RSV viral load (16). In addition, quantification of human metapneumovirus (hMPV) RNA allows correlating high viral load in nasopharyngeal secretions with acute respiratory symptoms in infants with acute lower respiratory tract infection. Conversely, hMPV aetiology was questioned in infants with low viral load (17). To further study the role of increased susceptibility for viral infections it might be interesting to quantify viral load, to relate viral load to symptomatic episodes and to study other viruses than rhinovirus. Investigating inflammation and immune-response could help to elaborate the role of possible diminished anti-viral defense and associated hyper-inflammation.

More bacterial colonisation

A third factor possibly explaining the higher impact of viral infections in CF is interaction between viruses and bacteria. Virus-related respiratory illnesses are frequently associated with *P. aeruginosa* acquisition in both children with CF and healthy controls, but with persistent colonisation in CF **(chapter 6)**. The high prevalence of *P. aeruginosa* acquisition in healthy children is in contrast with earlier findings, which suggest that *P. aeruginosa* seldom can be found in healthy controls (18). It might be suggested that as cultures were not specifically obtained in case of an acute respiratory illness, transient colonisation in healthy individuals has been missed in those studies. In CF, several studies confirm our finding that respiratory viral infections facilitate *P aeruginosa* acquisition and colonisation. Increased rates of new and persistent colonisation with *P. aeruginosa* have been found in association with the viral season (19), with viral upper respiratory tract infections (4) and with viral lower respiratory tract infections (20). One of the possible mechanisms explaining more bacterial persistence in CF is increased binding of bacteria to respiratory epithelium under influence of viral infection. RSV is able to enhance *P. aeruginosa* binding to respiratory epithelial cells in vitro **(Chapter 8)**. RSV and *P. aeruginosa* are able to bind to each other, which suggests that RSV possibly acts as a coupling agent between epithelial cells and bacteria **(Chapter 8)**. Other mechanisms explaining increased binding in the presence of a virus have earlier been described in non CF studies, such as increased binding of bacteria to damaged epithelium (21), or to up-regulated specific receptors (22;23). Further in vitro and in vivo studies in a CF mouse model might elaborate the importance of these mechanisms in CF.

Bacterial infections of the airways result in respiratory symptoms. In CF the inflammatory response to bacterial infections is exaggerated (24;25), leading to a severe and sustained inflammation in the lungs. Both in vivo and in vitro studies have shown that viral infections can influence bacterial driven inflammation. It has been shown that the severity of pneumonia in patients co-infected with influenza virus and bacteria was significantly higher than in those infected with bacteria alone (26). Mortality in a *S. pneumoniae* and influenzavirus co-infection mouse model correlated with the development of severe lung inflammation and pneumonia, but not with bacteraemia (27). Some other studies suggest that viral infection leads to modulation of the immune response, impairing specific anti-bacterial defense (28-

31). More specifically for CF, pneumonia was fatal in a chronic *P. aeruginosa* mouse model when pneumococcal infection was combined with influenza virus, not without the virus. Influenzavirus caused a significant increase in inflammatory cells and cytokine release, and suppressed neutrophil function In addition, intrapulmonary influenza titres were significantly increased in these mice with chronic *P. aeruginosa* infection, compared to control mice (32). And in CF patients with intermittent or chronic *P. aeruginosa* infection a viral infection is often followed by a rise in anti-pseudomonal antibodies, especially in case of an RSV infection (33). We speculate that already present bacteria and / or new bacteria in synergism with a respiratory viral infection worsen inflammation and symptoms, compared to bacterial or viral infection alone. In a mouse model, the role of pre-existent colonisation with bacteria and associated inflammation in the severity of respiratory viral infections might be explored. In CF patients, it could be interesting to study the relationship between symptoms, infections with specific viruses and the acquisition of bacteria such as *S. aureus* and *P. aeruginosa*, and to compare these findings to patients already colonised with these bacteria. Investigating inflammation, immune-response and local damage in relation to respiratory symptoms could help to further understand synergism between viruses and bacteria in patients with CF

Clinical implications

The findings of this thesis underline the importance of viral respiratory infections, even common colds, and its interaction with bacteria in lower respiratory tract morbidity in young children with CF. Current treatment strategies of pulmonary symptoms in CF are mainly aimed at treating bacterial infections with antibiotics and sputum evacuation. Our findings might suggest new therapeutic strategies to further improve prognosis of patients with CF.

It seems obvious that complete prevention of colds would only be possible by total long-term isolation from the community (34). More realistically, prevention of viral infections in patients with CF might be reached by active or passive immunization. According to many national recommendations and the clinical practice of many CF centres, influenza vaccination is regularly performed in individuals with CF (35), but evidence from randomized controlled trials is lacking (36). Some data indicate that such practice may yet play a role in preventing its subsequent acquisition (37). Several types of vaccines against RSV and parainfluenza infections are being developed and are in early clinical trials (38;39). A very recent retrospective study

was not conclusive about the usefulness of paluvizumab for prevention of RSV infections in infants with CF (40), passive immunization against other viruses are currently not available.

Seasonal prophylaxis and post-exposure prophylaxis with antiviral drugs is another interesting option. Amantadine, zanamivir and oseltamavir can be used against influenza virus infection (30;41;42). Treatment with oseltamavir improved survival from 0% to 75% in a mouse model of secondary pneumococcal pneumonia after influenza virus infection, even when therapy was delayed for up to 5 days after influenza virus infection (43). Recent advances in antirhinoviral drugs include the development of pleconaril (44) as a viral capsid binder, and ruprintivir (45) as a human rhinovirus 3C protease inhibitor. The role of intercellular adhesion molecule 1 (ICAM-1) as the main cellular receptor for rhinoviruses has led to attempts to block the attachment of the virus to the receptor, using a recombinant soluble ICAM-1. In clinical trials it reduced severity of experimental rhinovirus infections, but the effect was modest (46). Another area that has yet to be tested is nuclear factor-kappaB (NF-kB) inhibition, as many RV-induced inflammatory mediators are NF-kB dependent. Besides these specific virus inhibitors, there is some indication that interferon (12) and statins (47) can interfere with virus replication in general. Finally, newer anti-viral agents such as arbidol have the ability to elicit protective broad-spectrum anti-viral activity against for example influenza A, rhinovirus, coxsackievirus and RSV.
Furthermore, there is evidence that some antiviral agents even have antibacterial properties. Many respiratory pathogens, including *P. aeruginosa*, express neuraminidases that modify epithelial cells by exposing potential bacterial receptors. *P. aeruginosa* neuraminidase is crucial for the initial colonisation of the respiratory tract and contributes to the formation of biofilm. Viral neuraminidase inhibitors in clinical use are able to block *P. aeruginosa* biofilm production in vitro (48). In addition, scarce evidence exists for the efficacy of zanamivir in the prevention of bacterial complications, especially in high-risk patients (49).

Influencing the interaction between viruses and bacteria could be a next pathway to diminish respiratory morbidity in CF patients, although no clinical data are currently available. Blocking of RSV with heparin eliminated the effect on increased adhesion, suggesting that this coupling of *P. aeruginosa* to the cells is mediated

through RSV glycoprotein G **(chapter 8)**, which could suggest to explore the use of nebulization with heparin. Low-threshold use of antibiotics during viral infections in patients with CF might prevent colonisation or secondary bacterial infections, but data are lacking. Increasing knowledge about the central role of the host inflammatory response in producing symptoms of the common cold has led to attempts to treat colds with combinations of antiviral and anti-inflammatory agents (50). Combined corticosteroids and beta2 agonists suppress rhinovirus-induced chemokines through synergistic and additive mechanisms (51).
And finally, macrolides seem to have anti-inflammatory properties. They decrease production of mucus by epithelial cells and biosynthesis of pro-inflammatory cytokines from monocytes and epithelial cells by inhibiting NF-kB (52).

In summary, as complete prevention seems unrealistic, prevention by active and passive immunization could be further studied specifically in CF patients, although options are at this time sparse and not fully protective. Studies of anti-viral agents are at present hampered by difficulties in fast diagnostic distinction between the several viruses to use specific anti-viral agents, or the lack of available broad-spectrum agents. Currently, it could be interesting to study the use of antibiotics to influence the synergism between viruses and bacteria. In the view of the moderate correlation between detection of viruses and concurrent respiratory symptoms in children with CF, and the frequency of viral infections at young age it might be appealing to compare the use of continuous or intermittent antibiotic prophylaxis with as needed antibiotics.

CONCLUSIONS

This thesis shows that respiratory viruses play an important role in CF lung disease, either directly or through interaction with bacteria. It adds new data to limited earlier findings concerning the role of respiratory viral infections in CF. In Figure 2 the most important new results are underlined and added to the summary of previous knowledge.
Seasonal exposure to respiratory viruses and the frequency of virus-associated acute respiratory illnesses is similar between children with CF and healthy controls. However, the impact of respiratory viruses, even of "common colds",

on clinical symptoms is in CF patients far beyond the virus-related morbidity in healthy controls. We add detailed data that children with CF have prolonged and more severe episodes of lower respiratory tract complaints. Mainly patient-related factors seem to explain the higher susceptibility for respiratory viral infections. In this thesis new clinical evidence is presented that rhinovirus is detected more frequently and for longer periods in patients with CF. We suggest that a higher viral replication due to an impaired anti-viral defense causes increased viral loads in CF patients. Furthermore, this thesis confirms the suggestion that CF patients have virus-associated bacterial colonisation. Virus-related respiratory illnesses are frequently associated with *P. aeruginosa* acquisition in both children with CF and healthy controls, but lead to persistent infection only in CF. Increased binding of bacteria under influence of viral infection could be one of the mechanisms explaining this phenomenon. We describe that *P. aeruginosa* binding to epithelial cells is strongly enhanced by RSV infection. In addition, RSV possibly acts as a coupling agent between bacteria and cells and the increased binding can be effectively blocked by heparin. Finally, we suggest that pre-existent clinical condition, including an increased inflammatory state, might explain differences in reaction to respiratory viruses between healthy and CF children. Taking our data together, we propose that in CF patients viral infections may lead, in the presence of new or persistent bacterial colonisation, to a vicious circle with increased virus replication and exaggerated inflammatory response, with increasing symptoms and pulmonary damage. Further studies are needed to confirm these suggestions.
The findings of this thesis might have important clinical implications. New data in this thesis indicate that it is feasible to detect *P. aeruginosa* colonisation by oropharyngeal cultures at home. This might be helpful in early detection of new bacterial colonisation, for example in preventing chronic colonization. Current treatment strategies of pulmonary symptoms in CF are mainly aimed at treating bacterial infections with antibiotics and sputum evacuation. Our results which underline the importance of respiratory viral infections might suggest new therapeutic strategies to further improve prognosis of patients with CF.

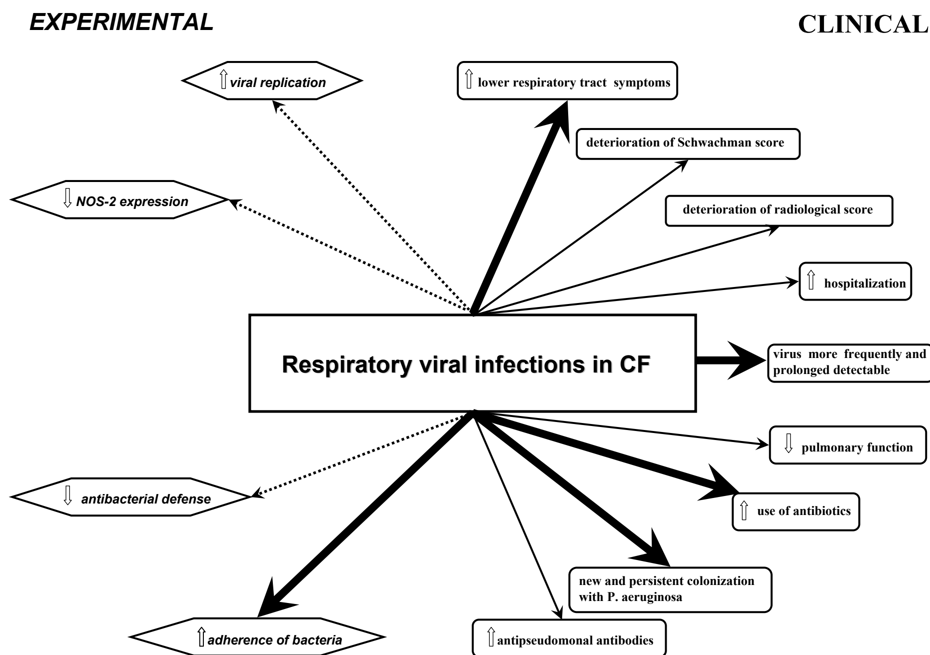

Figure 2

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SAMENVATTING

Cystic fibrosis (CF) is een van de meest voorkomende letale erfelijke ziektes in de Kaukasische bevolking, veroorzaakt door afwijkingen in het Cystic Fibrosis Transmembrane Regulator (CFTR) gen. Door afwijkend chloortransport over de celmembraan ontstaat taai slijm, dat zich ophoopt in diverse organen. Dit resulteert in de longen in obstructie van de luchtwegen geassocieerd met chronische inflammatie, kolonisatie met bacteriën en recidiverende longinfecties. Dit leidt uiteindelijk tot onomkeerbare schade aan longstructuur, met verslechtering van de longfunctie en vervroegd overlijden.
Agressieve behandeling van longinfecties met antibiotica, met name gericht tegen voor CF specifieke bacteriën zoals *Pseudomonas aeruginosa*, hebben de afgelopen decennia geleid tot flinke verbetering van de gemiddelde overlevingsduur. Verdere verbetering van de overlevingsduur zou mogelijk bereikt kunnen worden door ook te focussen op andere verwekkers van luchtweginfecties bij kinderen, zoals virussen. Bij andere chronische luchtwegaandoeningen zoals astma en COPD is bekend dat respiratoire virussen, ook in interactie met bacteriën, een belangrijke rol spelen in klachten. Bij patiënten met CF is dat minder duidelijk.

In dit proefschrift beantwoorden we daarom de volgende vragen:
1. Maken kinderen met CF vaker of andere virusinfecties in de luchtwegen door dan gezonde kinderen?
2. Zijn de klinische gevolgen van virusinfecties in de luchtwegen groter bij kinderen met CF dan gezonde kinderen?
3. Geeft het doormaken van een virusinfectie in de luchtwegen een grotere kans op kolonisatie met nieuwe bacteriën bij kinderen met CF?
4. Kunnen we mechanismen vinden die mogelijke interactie tussen respiratoire virussen en bacteriën verklaren?

Uit de resultaten van dit proefschrift blijkt dat bij kinderen met CF virussen in de luchtwegen inderdaad een belangrijke, en tot nu toe misschien onderschatte rol spelen in luchtwegklachten. Blootstelling aan respiratoire virussen en de frequentie van virus geassocieerde acute luchtweginfecties is gelijk bij kinderen met en zonder CF. Maar de impact van respiratoire virussen op klinische symptomen, zelfs van gewone “verkoudheidsvirussen”, is veel groter bij kinderen met CF dan

bij gezonde controles. We hebben gedetailleerd aangetoond dat kinderen met CF langere en ernstigere periodes van lage luchtwegklachten hebben. Met name patiënt gerelateerde factoren lijken de hogere gevoeligheid voor virusinfecties in de luchtwegen te verklaren. We leveren nieuw klinisch bewijs dat rhinovirus, een van de meest voorkomende verkoudheidsvirussen, vaker en in langere periodes bij patiënten met CF te vinden is. Er zijn aanwijzingen dat een hogere virusreplicatie, door een verminderde afweer tegen virussen, leidt tot de aanwezigheid van grotere hoeveelheden virus bij de CF patiënt. Verder wordt in dit proefschrift bevestigd dat, bij kinderen met CF, het doormaken van een virusinfectie in de luchtwegen een grotere kans op kolonisatie met nieuwe bacteriën geeft. Virus geassocieerde luchtweginfecties gaan vaak gepaard met acquisitie van *P. aeruginosa* bij zowel kinderen met als zonder CF, maar leiden alleen bij kinderen met CF tot persisterende infectie. Toegenomen hechting van bacteriën aan luchtwegepitheel onder invloed van virus is één van de mogelijk verklarende mechanismen voor interactie tussen bacteriën en respiratoire virussen. Wij tonen aan dat P. aeruginosa binding aan luchtwegepitheel in vitro sterk toeneemt onder invloed van respiratoir syncitieel virus (RSV) infectie. Daarnaast lijkt RSV hierin op te treden als "koppelaar" tussen bacteriën en cellen, en kan deze toegenomen hechting in vitro effectief worden afgeremd met heparine.

De resultaten beschreven in dit proefschrift kunnen interessante klinische implicaties hebben. Wij laten voor de eerste keer zien dat het goed mogelijk is om thuis in plaats van in het ziekenhuis keelkweken af te nemen om bacteriële kolonisatie te inventariseren. Dit zou behulpzaam kunnen zijn in het vroeg opsporen en daarna behandelen van nieuwe bacteriële kolonisatie, om chronische infectie te voorkomen. Bovendien is huidige longtherapie bij patiënten met CF met name gebaseerd op behandelen van infecties met antibiotica en op sputumevacuatie. Onze resultaten suggereren dat behandelingen gericht op virale infecties en de interactie tussen bacteriën en virussen ook een interessante optie zouden kunnen zijn om de prognose van CF patiënten verder te verbeteren.

CURRICULUM VITAE

Bart van Ewijk werd op 4 september 1971 geboren te Utrecht. Zijn middelbare schoolperiode bracht hij door op het Gymnasium Celeanum te Zwolle, waar hij in 1989 zijn Gymnasium-β diploma behaalde. Daarna startte hij met de opleiding Geneeskunde in Maastricht, waarbij hij tevens een jaar in Straatsburg (Frankrijk) heeft gestudeerd. Tijdens zijn studie heeft hij een student-assistentschap verricht bij Dr. J.E.E. Hendriks, kinderarts-pulmonoloog in Maastricht. Het betrof o.a. onderzoek naar de invloed van inhalatiesteroïden op de groei van kinderen. Daarnaast volgde hij keuze- en wetenschapsstages bij de afdelingen fysiologie (rol van α1-agonisten op het hart) en kindergeneeskunde. Keuzeco-schappen werden ingevuld binnen de kinderlongziekten, kinder-intensive care en anesthesiologie. In 1997 behaalde zijn artsendiploma en begon als AGNIO kindergeneeskunde in Zwolle. In 1998 werd hij AGNIO kindergeneeskunde in het Wilhelmina Kinderziekenhuis in Utrecht, en begin 1999 begon hij aan zijn opleiding tot kinderarts. Hij volgde zijn opleiding in het Academisch Ziekenhuis Maastricht met als opleider prof.dr. R.A. Donckerwolcke, en in het Atrium Heerlen met als opleider Dr. S.B. van der Meer. Tijdens zijn opleiding tot kinderarts heeft hij binnen de vakgroep kinderlongziekten onderzoek opgezet naar de rol van astmaverpleegkundige en online thuismonitoring in zorg voor kinderen met astma. Eind 2003 begon hij als fellow kinderlongziekten in het Wilhelmina Kinderziekenhuis, met prof.dr. C. K. Van der Ent als opleider. Vanaf januari 2004 is hij kinderarts. Zijn fellowship kinderlongziekten heeft hij gecombineerd met promotieonderzoek, dat geresulteerd heeft in dit proefschrift. Vanaf april 2006 is hij geregistreerd als kinderarts-pulmonoloog. Tot september 2007 heeft hij gewerkt bij de vakgroep kinderlongziekten in het Wilhelmina Kinderziekenhuis te Utrecht, sindsdien is hij werkzaam als lid van de vakgroep kindergeneeskunde van de Tergooiziekenhuizen, locaties Blaricum en Hilversum.
Bart heeft zijn studietijd en opleidingen gecombineerd met vele nevenactiviteiten, zoals diverse functies bij zijn studentenvereniging Circumflex, het opzetten en begeleiden van kindervakantieweken en het opzetten van jeugdtechnisch beleid bij hockeyclub Hockeer. Hij is fanatiek amateur-violist, en is trainer-coach van jeugdhockeyteams bij Kampong. Bart woont samen met Karen de Vooght, die ook op 21 mei haar proefschrift verdedigt.

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van Ewijk BE, van der Zalm MM, Wolfs TFW, Fleer A, Kimpen JLL, Wilbrink B, van der Ent CK. Prevalence and impact of respiratory viral infections in young children with cystic fibrosis: a prospective cohort study. *Accepted Pediatrics 2008.*

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van der Zalm MM, **van Ewijk BE**, Wilbrink B, Uiterwaal SPM, Fleer A, Wolfs TFW, van der Ent CK. Respiratory pathogens in children without respiratory symptoms. *Submitted.*

van Ewijk BE, Ruiter G, Wolfs TFW, Fleer A, Kimpen JLL, van der Ent CK. Oropharyngeal cultures in children with cystic fibrosis: more frequently at home? *Submitted.*

NAWOORD

Een bijzondere periode ga ik met het afronden van dit boekje min of meer afsluiten. En dat geeft een dubbel gevoel. Dubbel omdat ik aan de ene kant van het bedrijven van wetenschap erg genoten heb, aan de andere kant was het afronden van dit proefschrift in combinatie met een nieuwe baan toch een zware bevalling. Het feit dat met en voor mij, na een spannende periode, beslissingen over mijn eventuele wetenschappelijke carrière uitgekristalliseerd zijn maakte het er soms niet makkelijker op. Onderzoek doen maakt nieuwsgierig, en uit het beantwoorden van een hypothese of onderzoeksvraag komt bijna altijd weer een nieuwe onderzoeksvraag voort. Het lijkt me prachtig om dat ingezette proces te continueren. Aan de andere kant zie ik enorme kansen en uitdagingen in de directe patiëntenzorg en het opleiden van bevlogen jonge mensen die in de gezondheidszorg komen werken. Het maken van keuzes is soms lastig als je breed geïnteresseerd bent, maar het is zeker ook een voorrecht keuzes te kunnen maken.

Het was een bijzondere periode omdat het verrichten van wetenschappelijk onderzoek een vrijwel continu creatief proces is, uitdagend en inspirerend. Het dwingt je tot kritisch nadenken, analyseren en het beste in je boven halen. Ik heb er een hoop nuttige zaken van geleerd. Aan de andere kant heeft ook de onderzoekswereld helaas een schaduwzijde, en ben ik een paar illusies armer. Het meest bijzondere aan deze periode vind ik de creatieve, inspirerende en betrokken mensen met wie ik op de een of andere manier in deze periode heb mogen samenwerken. Ik heb ervan genoten en sommigen wil ik in het bijzonder even bedanken of benoemen.

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